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ISABELLE DE CAUWER

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Importance relative des flux de gènes et de la valeur sélective individuelle dans les variations de sex ratio chez une espèce gynodioïque, *Beta vulgaris* ssp. *maritima*

Soutenue le 28 juin 2010, devant un Jury composé de :

Isabelle OLIVIERI, Professeur, Université de Montpellier II	Rapporteur
John PANNELL, Professeur, Université d'Oxford, UK	Rapporteur
Frédéric AUSTERLITZ, Chargé de Recherche CNRS, Université de Paris XI	Examinateur
Joël CUGUEN, Professeur, Université de Lille 1	Examinateur
Oscar GAGGIOTTI, Professeur, Université Joseph Fourier, Grenoble	Examinateur
Christophe THÉBAUD, Professeur, Université Paul Sabatier, Toulouse	Examinateur
Jean-François ARNAUD, Maître de Conférences, Université de Lille 1	Directeur de thèse
Mathilde DUFAŸ, Maître de Conférences, Université de Lille 1	Directeur de thèse

U.F.R. de Biologie

École doctorale « Sciences de la Matière, du Rayonnement et de l'Environnement »

Laboratoire de Génétique et Évolution des Populations Végétales, UMR CNRS 8016

RÉGION Nord-Pas de Calais

cnrs

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INTRODUCTION GENERALE

I - EVOLUTION DES SEX RATIOS

"Let us now take the case of a species producing […] an excess of one sex – we will say males – these being superfluous and useless. Could the sexes be equalized through natural selection?"
Darwin (1871)

Chez les espèces où la reproduction repose sur l'existence de sexes séparés, les différents sexes sont généralement produits en fréquences équivalentes, et ceci quelques soient les mécanismes génétiques déterminant l'expression du sexe. Pourquoi en est-il ainsi ? Comme l'avait déjà deviné Darwin (1871), cette observation est directement liée à une action stabilisatrice de la sélection naturelle. L'idée, formalisée plus tard par Fisher (1930), est simple. Imaginons une espèce chez laquelle la reproduction repose sur la rencontre entre des individus mâles et femelles. Au cours de chaque évènement reproducteur, les deux sexes transmettent donc obligatoirement un nombre égal de gamètes à la génération suivante (un descendant étant toujours issu de la combinaison d'un gamète mâle et d'un gamète femelle). Imaginons également que, chez cette espèce, les mâles sont produits en excès par rapport aux femelles. Dans ce cas, les femelles seront fortement avantagées : il est probable qu'elles accèdent toutes à la reproduction, ce qui ne sera vraisemblablement pas le cas pour l'ensemble des mâles. Les couples de parents produisant plus de descendants femelles que la moyenne seront alors fortement avantagés : leurs filles accédant toutes à la reproduction, le nombre de descendants obtenus à la génération suivante sera supérieur à ce qui est observé en moyenne pour les autres couples. En conséquence, si cette capacité à produire plus de femelles est héritable, elle se répandra rapidement, et la production d'individus femelles augmentera au cours du temps. Une fois que les fréquences de mâles et de femelles seront rééquilibrées, l'avantage lié à une surproduction de femelles disparaitra. Ce raisonnement est bien évidemment valable si on remplace les femelles par les mâles.

Ainsi, dans une population panmictique de taille infinie, le sex ratio (proportion de mâles et de femelles) est à l'équilibre lorsque l'effort total déployé pour produire des mâles est égal à l'effort total déployé pour produire des femelles. Un sex ratio équilibré constitue une « stratégie évolutivement stable » (Maynard Smith, 1982). N'importe quelle mutation favorisant la production de l'un ou l'autre des sexes sera théoriquement rapidement éliminée après son apparition dans une population. Il s'agit du principe de Fisher.

ENCADRÉ 1 : QUELQUES LIMITES DU PRINCIPE DE FISHER

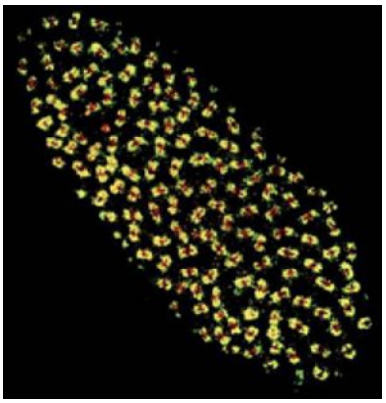
Cas 1 – Écart à la panmixie et sex-ratio

Les gamètes femelles étant généralement limitants chez les espèces à sexes séparés (ceux-ci étant plus coûteux à produire que des gamètes mâle, Bateman, 1948), le sexe ratio optimal d'une population serait d'être constitué d'une majorité de femelles et de quelques mâles en nombre suffisant pour féconder toutes les femelles. Selon le principe de Fisher, ce sex ratio optimal est une stratégie instable : un mutant capable de produire plus de descendants mâles serait en effet avantagé et une telle mutation se répandrait rapidement dans la population. L'une des hypothèses sous jacentes du principe de Fisher est que la reproduction est panmictique, c'est-à-dire que les couples s'unissent au hasard. Cette hypothèse peut être violée lorsque la reproduction ne se fait pas au hasard mais dans des unités de petite taille. Le sex ratio à l'équilibre peut alors se rapprocher alors du sex ratio "optimal" (Hamilton, 1967).

Pour illustrer le propos, prenons l'exemple du couple figuier / agaonide. Chez les agaonides (pollinisateurs du figuier), les accouplements ne se font pas au hasard. En effet, un nombre limité de femelles (X) pondent dans une figue. Les descendants mâles nés dans la figue fécondent alors les descendants femelles nés dans cette même figue, avant que les descendants femelles ne dispersent. Aucune fécondation n'a lieu en dehors du fruit. Dans ce cas, pour une femelle donnée, il peut être intéressant de produire une descendance où le sex ratio est biaisé en faveur des femelles. Plus le nombre X de femelles fondatrices est faible, plus les descendants femelles d'une fondatrice donnée ont de chances d'être fécondées par leurs frères, qui leur sont apparentés et transmettent donc eux aussi une partie des gènes de la femelle fondatrice considérée. Lorsque le nombre de fondatrices tend vers 1, le nombre de mâles produit devrait alors tendre vers 0 (Hamilton, 1967). Des travaux empiriques montrent ainsi que les femelles peuvent effectivement ajuster le sex ratio de leur ponte en fonction du nombre d'autres femelles pondant dans la figue (Herre, 1985).

Cas 2 – Distorsion cytoplasmique des sex-ratios: le cas de Wolbachia chez les arthropodes

Les *Wolbachia* sont des bactéries parasites endosymbiotiques, transmises uniquement par la voie femelle, qui infectent un grand nombre d'espèces d'arthropodes, en particulier des insectes (environ 60% des espèces d'insectes seraient concernées). Cette bactérie est capable de manipuler la reproduction de son hôte, augmentant par la même occasion sa propre transmission (*p.e.* Rigaud & Juchault, 1993). En raison de son mode de transmission uniquement maternel, cette bactérie présente dans le cytoplasme peut ainsi augmenter sa valeur sélective en biaisant la sex ratio en faveur des femelles. Plusieurs mécanismes de manipulation à l'origine d'un biais de sex ratio dans les populations infectées tels que le « male-killing » ou la parthénogénèse ont été identifiés au sein de différents ordres (Stouthamer *et al.*, 1999).



La règle des sex ratios équilibré est fréquemment observée dans la nature, mais il existe des situations où certaines hypothèses de Fisher ne sont pas respectées (Hamilton, 1967). C'est le cas, par exemple, des populations à l'intérieur desquelles il existe une structure spatiale : les évènements de reproduction ne sont alors pas aléatoires, ce qui peut modifier les attendus théoriques d'un sex ratio équilibré comme le montre l'exemple des Agaonides pollinisateurs du figuier présenté dans l'Encadré I. De façon alternative, certains types de mutations peuvent échapper à l'action stabilisatrice de la sélection. Chez les espèces où l'expression du sexe est liée à des chromosomes sexuels, une mutation sur ces chromosomes peut dans certains cas entrainer des écarts importants au ratio 1 : 1 (Hamilton, 1967). Par ailleurs, des éléments cytoplasmiques engendrant des distorsions de sex ratio peuvent modifier les attendus théoriques d'un sex ratio équilibré (il existe de nombreux exemples chez les arthropodes, *cf.* Encadré I).

Par ailleurs, si le principe de Fisher a été initialement développé en pensant à des modèles animaux, et plus spécifiquement aux espèces gonochores (*i.e.* les espèces au sein desquelles les sexes sont séparés) qui représentent la majorité des espèces animales, les systèmes de reproduction (*i.e*. manière dont se répartissent les fonctions mâles et femelles au sein d'une population) sont nettement plus variés au sein des végétaux (Barrett, 2002). La question de l'évolution des sex ratios peut alors être posée pour une grande variété de situations différentes. Un bref aperçu de cette diversité sexuelle est proposé dans la partie suivante.

II - DIVERSITE SEXUELLE DANS LE MONDE VEGETAL

La diversité des organes reproducteurs chez les plantes à fleurs (aussi appelées « Angiospermes ») surpasse largement celle observée dans tous les autres groupes d'organismes vivants (Barrett, 2002 ; Barrett, 2010). La Figure 1 illustre bien les importantes différences morphologiques qui peuvent être observées entre espèces. Etant donné que les organes reproducteurs n'ont qu'une seule et unique fonction, celle de transmettre les gènes aux générations suivantes, l'immense variété de stratégies de reproduction observée chez les végétaux peut paraître particulièrement étonnante. Parmi les diverses contraintes qui peuvent être invoquées pour expliquer cette diversité, l'évitement de l'autofécondation a probablement joué un rôle déterminant dans l'apparition des polymorphismes sexuels.

Chez les Angiospermes, l'hermaphrodisme (*i.e*. le cas de figure où chaque individu est capable de se reproduire à la fois par la voie mâle et par la voie femelle) est largement majoritaire (Richards, 1997) et est généralement considéré comme le système de reproduction ancestral (Encadré II). Une plante donnée peut donc théoriquement se reproduire sans avoir besoin de l'intervention d'un autre individu. Dans les faits, il existe des plantes essentiellement allogames, pour lesquelles la reproduction se fait entre individus distincts, et des espèces autogames, pour lesquelles la reproduction peut se faire par autofécondation (le père et la mère d'un descendant donné sont alors le même individu). Si l'autofécondation permet théoriquement une plus grande assurance reproductive, elle peut aussi augmenter fortement les chances que des allèles délétères récessifs soient exprimés dans les descendants. On parle alors de dépression de consanguinité, qui se traduit par une diminution de la valeur sélective de

descendants issus d'autofécondation relativement à des descendants produits par fécondation croisée (Charlesworth & Charlesworth, 1987; Husband & Schemske, 1996 ; Hedrick & Kalinowski, 2000). Pour pallier à cet effet néfaste de la consanguinité, il existe de nombreuses stratégies pour favoriser la fécondation croisée chez les plantes. Dans le but d'empêcher des grains de pollen de germer sur des fleurs apparentées à la fleur qui les a produit, certaines espèces ont développé des barrières morphologiques (*p.e*. l'hétérostylie, voir Fig. 1f) ou temporelles (avec des pièces florales mâles et femelles qui ne sont pas matures au même moment). Un grand nombre d'espèces possèdent des systèmes d'auto-incompatibilité

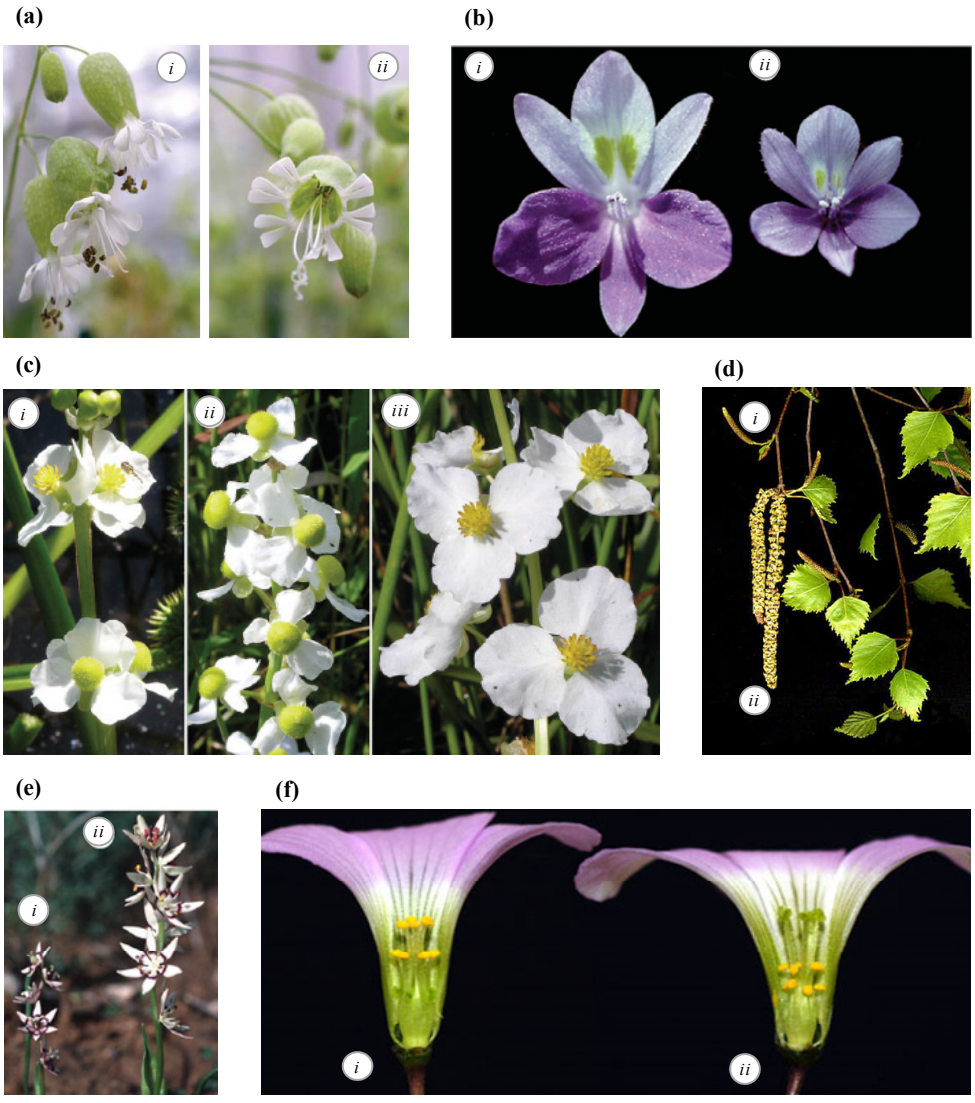


Fig. 1 Échantillon de la diversité de morphologies florales chez les Angiospermes. (**a**) fleurs d'un individu hermaphrodite (*i*) et fleurs d'un individu femelle (*ii*) chez l'espèce gynodioïque *Silene vulgaris*, (**b**) fleur spécialisée dans l'allofécondation (*i*) et dans l'autofécondation (*ii*) chez *Eichhornia paniculata,* (**c**) inflorescence d'un individu monoïque (*i*), inflorescence d'un individu femelle (*ii*) et inflorescence d'un individu mâle (*iii*) chez l'espèce trioïque *Sagittaria latifolia*, (**d**) inflorescence femelle (*i*) et mâle (*ii*) chez une entomophile monoïque, *Betula verrucosa* , (**e**) dimorphisme de taille entre un individu femelle (*i*) et un individu mâle (*ii*) chez une espèce dioïque *Wurmbea dioica*, (**f**) fleurs brévistyle (*i*) et longistyle (*ii*) chez une espèce hétérostyle, *Oxalis alpina*

chimiques (le grain de pollen ne peut alors pas germer sur une plante possédant le même allèle d'auto-incompatibilité que lui, Castric & Vekemans, 2004). De ce point de vue, l'apparition d'individus unisexués (mâles ou femelles) dans des populations hermaphrodites constitue également une façon possible de favoriser les fécondations croisées (même si d'autres mécanismes peuvent être évoqués pour expliquer l'apparition d'individus unisexués, voir par exemple les gènes CMS décrits plus loin). Ainsi, chez les Angiospermes, une grande diversité d'intermédiaires existe en effet entre l'hermaphrodisme et la dioécie, où chaque individu est soit mâle, soit femelle. De façon schématique, on peut distinguer trois genres distincts : les femelles, les mâles et les hermaphrodites, mais de nombreuses combinaisons de ces genres sont possibles dans les populations naturelles (Gouyon, 2009). La complexité est encore accrue du fait que certains individus peuvent produire un mélange de fleurs de ces différents genres (voir Figure 1).

De manière plus générale, chez les espèces végétales qui présentent un polymorphisme sexuel au sens large (sexes différents, hétérostylie, systèmes d'auto-incompatibilité…), les individus reproducteurs sont subdivisés en plusieurs groupes reproducteurs distincts (Barrett, 2002). Chez ces espèces, le succès reproducteur d'un phénotype sexuel ou d'un morphe floral particulier ne dépendra pas seulement des caractéristiques intrinsèques du groupe auquel il appartient, mais il sera également fonction de la fréquence locale des individus de ce groupe (*p.e.* : Carlsson-Granér *et al.*, 1998; Jesson & Barrett, 2002; Stehlik *et al.*, 2006; Van Rossum *et al.*, 2006). De la même façon que pour le cas simple de deux sexes séparés, le succès reproducteur d'un phénotype sexuel ou d'un morphe floral particulier peut alors être soumis à une sélection fréquence-dépendante. Ce type de sélection est décrit depuis longtemps comme l'une des composantes principales de l'évolution des sex-ratios chez les plantes (Fisher, 1930).

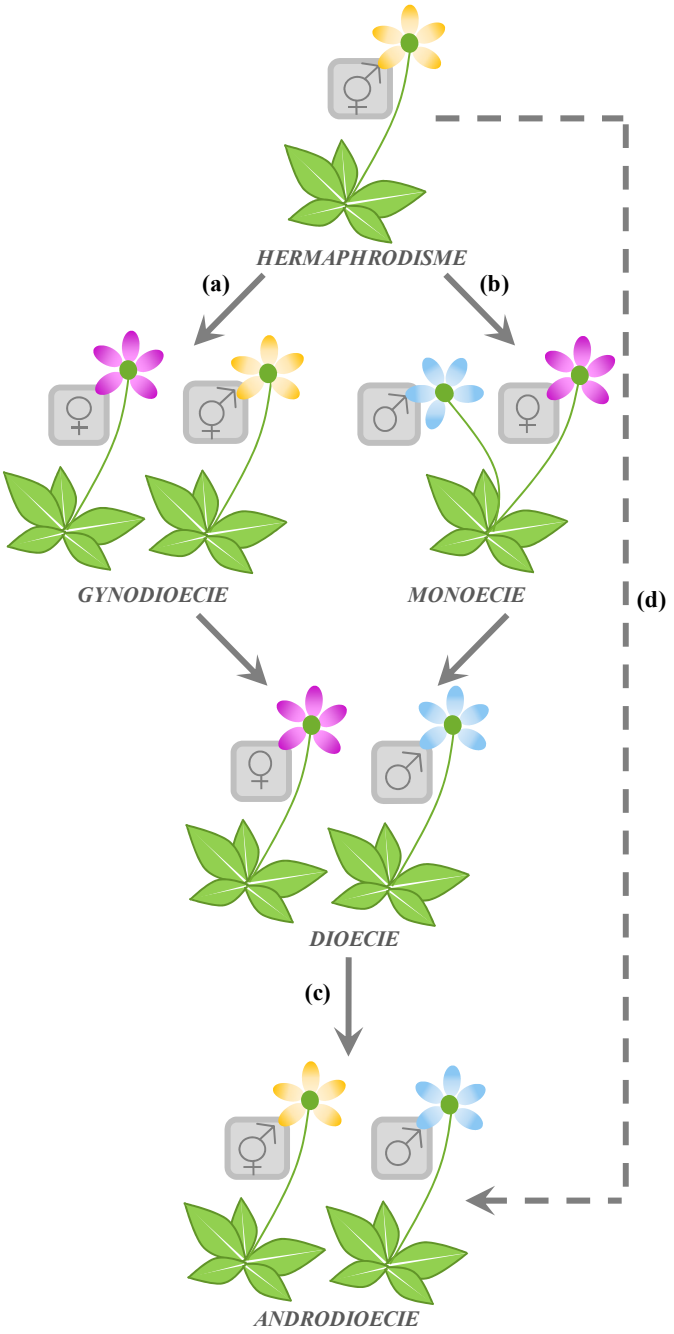
Cette thèse ayant pour objet l'étude de la variation spatiale de sex ratio chez une espèce présentant un polymorphisme sexuel particulier, la gynodioécie (coexistence d'individus hermaphrodites et femelles dans les populations naturelles), une description plus détaillée de ce système de reproduction est nécessaire avant d'aller plus loin.

ENCADRÉ II : POLYMORPHISMES SEXUELS ET TRANSITIONS EVOLUTIVES

Il existe théoriquement deux voies possibles pour réaliser la transition évolutive entre l'hermaphroditisme, considéré comme l'état ancestral, et la dioécie, c'est-à-dire la coexistence de deux sexes séparés.

La première voie (**a**) implique dans un premier temps l'invasion des populations hermaphrodites par des gènes cytoplasmiques de stérilité-mâle (*CMS*). Les populations sont alors gynodioïques, comprenant à la fois des individus femelles et des individus hermaphrodites. Dans un second temps, des gènes modifiant la fertilité femelle transforment graduellement les individus hermaphrodites en mâles, résultant en l'apparition de populations dioïques. L'existence de cette première voie est bien établie, tant par des études théoriques (Maurice *et al.*, 1994; Pannell, 1997) que par des études empiriques (Barrett, 1992; Delph & Carroll, 2001).

La seconde voie (**b**) est moins étudiée. Elle fait également appel à l'existence d'une étape intermédiaire, où les populations sont monoïques, c'est-à-dire que les individus expriment les deux fonctions sexuelles, mais les fleurs mâles et femelles sont séparées sur une même plante. Dans ce cas, l'action d'une sélection disruptive sur l'allocation des individus à la fonction mâle et à la fonction femelle pourrait engendrer l'émergence de sexes séparés.



Finalement, l'androdioécie, qui est un système de reproduction très rare où coexistent des individus hermaphrodites et des individus mâles, est considéré comme étant un système dérivé de la dioécie (voie (**c**), *p.e.* Wolfe *et al.*, 2001). Jusqu'à très récemment, il était communément admis que l'androdioécie ne pouvait pas apparaître directement dans des populations hermaphrodites, mais cette vision a été modifiée par une étude récente (Saumitou-Laprade *et al.*, 2010) qui montre que l'existence de relations d'incompatibilité particulières entre les hermaphrodites peut faciliter l'apparition et le maintien d'individus mâles dans certains systèmes hermaphrodites (**d**).

Adapté et modifié de Barrett , 2002

III – UN SYSTEME DE REPRODUCTION ORIGINAL : LA GYNODIOÉCIE

Définition

La gynodioécie correspond à un système de reproduction particulier, caractérisé par la coexistence de plantes femelles et de plantes hermaphrodites au sein de populations naturelles. Classiquement, les individus fonctionnellement femelles produisent des fleurs à étamines atrophiées, voire absentes, et accèdent donc à la reproduction uniquement *via* la voie femelle, c'est-à-dire par la production de graines. Les individus hermaphrodites, quant à eux, portent des fleurs leur permettant de se reproduire à la fois par la voie femelle (production de graines) et par la voie mâle (production de pollen). Ce système de reproduction particulier est relativement commun dans la nature : on estime qu'il est présent chez 7% des Angiospermes, ce qui fait de lui le deuxième système de reproduction le plus commun, après l'hermaphrodisme (Richards, 1997). Ce système de reproduction est classiquement considéré comme l'une des étapes possibles dans la transition entre des systèmes de reproduction strictement hermaphrodites et des systèmes de reproduction dioïques (voir Encadré I et Lloyd, 1976; Charlesworth & Charlesworth, 1978; Maurice *et al.*, 1994). En plus de son rôle probable de transition évolutive, ce type de système de reproduction suscite un intérêt particulier depuis des décennies, du fait qu'il semble intuitivement difficile de maintenir des individus mâles-stériles dans des populations hermaphrodites (Lewis, 1941; Lloyd, 1975). Les conditions du maintien de femelles dans les populations naturelles vont alors dépendre grandement du déterminisme génétique impliqué dans l'expression du sexe, qui peut être purement nucléaire, ou alors impliquer à la fois des gènes cytoplasmiques et de gènes nucléaires.

Gynodioécie nucléaire

Dans le cas d'un déterminisme purement nucléaire du sexe, un individu exprimant un phénotype femelle doit compenser la perte de la fonction mâle par une augmentation proportionnelle de sa production de graines. Ainsi, pour que des plantes femelles soient maintenues dans les populations naturelles, elles doivent nécessairement produire au moins deux fois plus de graines que les individus hermaphrodites (Lewis, 1941 ; Charlesworth & Charlesworth, 1978). Cette quantité plus importante de graines produites par les individus femelles relativement aux individus hermaphrodites est appelée avantage femelle. La fréquence de femelles dans une population varie alors en fonction de l'intensité de cet avantage femelle et peut théoriquement atteindre 50% dans le cas d'un avantage femelle infini (ce qui correspond en réalité à une population dioïque, Lewis, 1941). Plusieurs études empiriques confirment effectivement ces prédictions et des avantages femelles extrêmement élevés ont été décrits chez les espèces présentant un déterminisme purement nucléaire du sexe (*p.e.* Ashman, 1999). Cet avantage femelle peut découler de deux mécanismes distincts. Premièrement, les ressources non allouées à la production de pollen peuvent être redirigées vers une production de graines accrue et/ou vers une production de graines de meilleure qualité (*p.e.* Couvet *et al.*, 1986; Asikainen & Mutikainen, 2003; Olson *et al.*, 2006). Le deuxième avantage possible pour les femelles est lié au fait qu'une majorité des espèces gynodioïques sont auto-compatibles (Charlesworth, 1981). En conséquence, une certaine proportion de la descendance de chaque hermaphrodite pourra être issue d'autofécondation et être sujette à des phénomènes de dépression de consanguinité. Les femelles, qui ne peuvent pas avoir recours à l'autofécondation pour leur reproduction, auront alors en moyenne des descendants de meilleure qualité

(*p.e*. Thompson & Tarayre, 2000; Delph, 2004; Chang, 2007). Le déterminisme nucléaire reste toutefois relativement peu commun parmi les espèces gynodioïques. Pour une majorité des plantes présentant ce système de reproduction, les avantages femelles mesurés ne sont pas aussi prononcés, et des données de croisement ont permis d'éliminer rapidement les déterminismes les plus simples au profit d'un modèle plus complexe : le déterminisme cytonucléaire.

Gynodioécie cytonucléaire

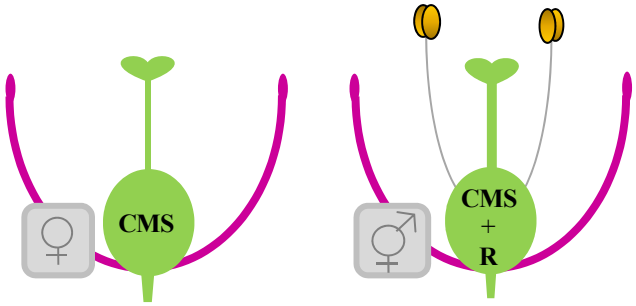
Chez les espèces gynodioïques, le déterminisme sexuel implique généralement deux catégories distinctes de gènes : des gènes cytoplasmiques et des gènes nucléaires (*p.e*. Dommée *et al.*, 1987; Boutin-Stadler *et al.*, 1989; Koelewijn & van Damme, 1995; Ronfort *et al.*, 1995; McCauley *et al.*, 2000; Delph *et al.*, 2007). Les gènes cytoplasmiques, nommés gènes *CMS* (pour « Cytoplasmic Male Sterility »), sont généralement présents dans le génome mitochondrial et induisent chez les individus qui les portent une annulation de la fonction mâle, à moins que leur action ne soit contrecarrée par des gènes nucléaires, appelés gènes de restauration de la fonction mâle. Comme illustré dans l'Encadré III, un individu sera donc fonctionnellement femelle s'il porte une CMS en l'absence du, ou des, allèle(s) de restauration de cette CMS. Le phénotype hermaphrodite sera quant à lui exprimé par des individus qui portent soit un cytoplasme CMS en combinaison avec les allèles de restauration nucléaires appropriés (on parlera alors d'hermaphrodite restauré), soit un cytoplasme non stérilisant (on parlera alors d'hermaphrodite « normal »). L'existence de ces cytoplasmes non-stérilisants, qui ne sont jamais associés à des individus exprimant un phénotype femelles, semble cependant assez marginale chez les espèces gynodioïques : ils ont été décrits uniquement chez *Raphanus sativus* (Yamagishi & Terachi, 1994; Murayama *et al.*, 2004), chez *Beta vulgaris* (Cuguen *et al.*, 1994; Fénart *et al.*, 2006) et chez *Plantago lanceolata* (de Haan *et al.,* 1997b).

Gynodioécie cytonucléaire et conflit génétique

Ce déterminisme génétique particulier du sexe fait de la gynodioécie cytonucléaire un modèle classique de conflit génétique (Frank, 1989 ; Saumitou-Laprade *et al.*, 1994). Ce conflit est lié au fait que les gènes cytoplasmiques et nucléaires diffèrent en termes de mode de transmission. En effet, chez les Angiospermes, les gènes cytoplasmiques présentent une transmission quasi-exclusivement maternelle (McCauley, 1994; Birky, 2001; Petit *et al.*, 2005), alors que les gènes nucléaires présentent une transmission biparentale. Dés lors, les gènes CMS n'étant jamais transmis par le pollen, il devient avantageux pour un gène CMS d'être porté par un phénotype produisant plus de graines et/ou des graines de meilleure qualité. Les exemples empiriques de cet avantage femelle chez des espèces présentant une gynodioécie cytonucléaire sont abondants (*p.e*. Assouad *et al.*, 1978; Poot, 1997; Graff, 1999; Olson *et al.*, 2006). De ce point de vue, les CMS sont assez proches des éléments cytoplasmiques engendrant des distorsions de sex ratio chez les arthropodes évoqués précédemment. Un gène CMS non-restauré (*i.e*. porté par une femelle) peut donc directement augmenter sa propre valeur sélective *via* une production de graines accrue et/ou de meilleure qualité, ceci au détriment du génome nucléaire du fait de l'absence de production de pollen. A l'inverse, un restaurateur nucléaire pourra augmenter sa propre valeur sélective en diminuant la fonction femelle (production de graine) au profit de la fonction mâle (production de pollen).

ENCADRÉ III : DÉTERMINISME SEXUEL ET GYNODIOECIE CYTONUCLÉAIRE

Les gènes cytoplasmiques de stérilité mâle (*CMS*), présents dans le génome mitochondrial, induisent chez les individus qui les portent une annulation de la fonction mâle, à moins que leur action ne soit contrecarrée par la présence d'allèles nucléaires restaurant la fonction mâle (*R*).



Cas 1 Déterminisme sexuel dans le cas où tous les cytoplasmes sont des *CMS* (*i.e.* les différents cytoplasmes peuvent tous être associés avec un phénotype femelle, F). Dans l'exemple choisi, deux *CMS* coexistent, (*CMS1* et C*MS2*) chacune étant restaurée par un allèle nucléaire dominant (*R1* et *R2*). Les individus restaurés expriment un phénotype hermaphrodite (H). Les locus responsables de la restauration de la *CMS1* et de la *CMS2* sont indépendants.

Locus de restauration 1		Locus de restauration 2		CMS1	CMS2
Allèle 1	Allèle 2	Allèle 1	Allèle 2		
R1	r1	R2	r2	H	H
R1	R1	R2	r2	H	H
r1	r1	R2	r2	F	H
R1	r1	R2	R2	H	H
R1	R1	R2	R2	H	H
r1	r1	R2	R2	F	H
R1	r1	r2	r2	H	F
R1	R1	r2	r2	H	F
r1	r1	r2	r2	F	F

Cas 2 Déterminisme sexuel dans le cas où il existe un cytoplasme non-stérilisant, nommé *NCMS* (les individus porteurs du cytoplasme *NCMS* n'expriment jamais de phénotype femelle). Dans l'exemple choisi, un cytoplasme stérile (*CMS1*), restauré par un allèle nucléaire dominant (*R1*), coexiste avec un cytoplasme non-stérilisant *NCMS*. On peut alors distinguer trois type sexuels: (i) les femelles, F, (ii) les hermaphrodites porteurs de la *CMS* et restaurés pour la fonction mâle, HR, et (iii) les hermaphrodites *NCMS* , H.

Locus de restauration		CMS1	NCMS
Allèle 1	Allèle 2		
R1	r1	HR	H
R1	R1	HR	H
r1	r1	F	H

Les deux exemples choisis ici sont des simplifications délibérées. Dans les faits, les espèces gynodioïques sont souvent caractérisées par l'existence de multiples *CMS* (de Haan *et al.*, 1997b; Charlesworth & Laporte, 1998; Dudle *et al.*, 2001; van Damme *et al.*, 2004; Fénart *et al.*, 2006), chaque CMS étant associée à des allèles nucléaires de restauration qui lui sont propres. De plus, plusieurs études empiriques suggèrent que le déterminisme de la restauration implique plusieurs locus à effets additifs (l'intensité de la production de pollen étant alors proportionnel au nombre d'allèles de restauration porté par l'individu, Koelewijn & van Damme, 1996; Dufay *et al.*, 2008) ou plusieurs locus avec un effet de seuil (l'individu sera alors soit complètement restauré pour la fonction mâle, soit femelle, en fonction du nombre d'allèles présents, *cf.* Ehlers *et al.*, 2005).

Comment maintenir un polymorphisme cytonucléaire ?

Plusieurs modèles théoriques ont montré que, dans le cas d'un déterminisme cytonucléaire de la gynodioécie, les femelles ne peuvent être maintenues à l'équilibre que lorsqu'une une force s'oppose à la fixation des allèles de restauration (Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufay *et al.*, 2007). Une possibilité pour expliquer le fait que les allèles de restauration n'arrivent pas à fixation en population naturelle est l'existence d'un coût de la restauration (voir Encadré IV). De nombreuses études ont tenté de mettre en évidence un tel coût, mais ces efforts ont souvent été infructueux du fait d'une faible connaissance des bases génétiques de la restauration chez une majorité d'espèces gynodioïques. Au final, seulement deux études empiriques ont démontré formellement l'existence d'un coût de la restauration, chez *Plantago lanceolata* et chez *Lobelia siphilitica* (de Haan *et al.*, 1997a; Bailey, 2002), même s'il existe par ailleurs quelques preuves indirectes chez *Beta vulgaris* et chez *Phacelia dubia* (Dufay *et al.*, 2008; del Castillo & Trujillo, 2009).

Le coût de la restauration n'est pas la seule force qui pourrait éviter la fixation des allèles de restauration dans les populations gynodioïques. Une approche théorique développée par Couvet *et al.* (1998) suggère que dans le cas où les populations sont soumises à une dynamique en métapopulation, avec des extinctions et des recolonisations récurrentes de populations locales, on peut maintenir à la fois le polymorphisme cytonucléaire nécessaire à l'existence de la gynodioécie et obtenir des fréquences importantes (*i.e*. supérieures à 50%) de femelles au sein des populations, ceci sans invoquer un coût de la restauration. Ce modèle prédit ainsi qu'au sein d'un ensemble dynamique de populations, les colonisateurs ont plus de chance d'être des femelles (les femelles produisant des graines en plus grande quantité et/ou qualité) et, grâce à cet avantage femelle, les fréquences de CMS peuvent alors connaitre une croissance « épidémique » avant l'arrivée des allèles nucléaires de restauration. Les proportions de femelles les plus élevées sont alors obtenues dans les populations les plus récemment fondées, comme suggéré chez le thym (Belhassen *et al.*, 1989).

Dans les cas particuliers où des cytoplasmes stérilisants coexistent avec les gènes CMS, comme chez *Raphanus sativus*, *Beta vulgaris* et *Plantago lanceolata* (de Haan *et al.,* 1997b; Murayama *et al.*, 2004; Fénart *et al.*, 2006), les hermaphrodites porteurs de CMS et restaurés pour la fonction mâle doivent théoriquement avoir une valeur sélective plus faible que les hermaphrodites porteurs de cytoplasmes non-stériles (Dufay *et al.*, 2007). Ce « coût de la CMS » permet de maintenir les hermaphrodites porteurs de cytoplasmes non-stériles. En effet, dans le cas contraire, les cytoplasmes non-stériles seraient rapidement éliminés du fait qu'ils ne sont jamais associés à un avantage femelle, contrairement aux cytoplasmes CMS.

Sex ratios et variations spatiales

De nombreuses espèces gynodioïques sont caractérisées par des variations considérables de la proportion relative de femelles et d'hermaphrodites d'une localité à une autre (*p.e.* Tarayre & Thompson, 1997; Olson & McCauley, 2002; Asikainen & Mutikainen, 2003; Alonso, 2005; Nilsson & Agren, 2006; Cuevas *et al.*, 2008; Dufay *et al.*, 2009). Ces variations géographiques de sex ratio résultent à la fois de processus non sélectifs et de l'action différentielle de la sélection sur les différents génotypes et phénotypes sexuels en présence.

ENCADRÉ IV : LE COÛT DE LA RESTAURATION

L'existence d'un coût de la restauration a souvent été évoquée pour expliquer le maintien du polymorphisme cytonucléaire classiquement associé à la gynodioécie. Dans les modèles théoriques, un coût de la restauration permet en effet d'éviter la fixation des allèles de restauration quand l'avantage femelle est faible (inférieur à deux) et de maintenir un polymorphisme sexuel. Ce coût de la restauration peut être modélisé en faisant appel à différentes hypothèses :

(i) le coût peut être dominant (*i.e.* le coût s'exprime dés qu'une copie du restaurateur est présente chez un individu) ou récessif (*i.e.* le coût s'exprime uniquement quand l'individu est homozygote pour l'allèle de restauration)

(ii) le coût peut affecter la fonction mâle (production de pollen) ou la fonction femelle (production de graines). Pour des raisons physiologiques, on s'attend à ce que le coût de la restauration porte plutôt sur la fonction mâle (Delph *et al.*, 2007). Dans les faits, les effets négatifs de la présence du restaurateur ont été montrés aussi bien sur la fonction mâle (Bailey, 2002; Dufay *et al.*, 2008) que sur la fonction femelle (de Haan *et al.*, 1997; del Castillo & Trujillo, 2009)

(iii) le coût peut être exprimé selon trois modalités distinctes (Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufay *et al.*, 2007). Premièrement, le restaurateur peut avoir un effet négatif indépendant du cytoplasme avec lequel il est associé. On parle alors de **coût constitutif**. Deuxièmement, ce coût peut n'être exprimé que dans le cas de figure où le restaurateur est associé avec le cytoplasme stérilisant qui lui est spécifique (*i.e.* le cytoplasme qu'il restaure). Il s'agit alors d'un **coût exprimé**. Troisièmement, l'effet négatif du restaurateur peut n'être exprimé que dans les cytoplasmes qui ne lui sont pas spécifiques. On parle alors de **coût silencieux**.

L'effet de ces différents modes de restauration sur le maintien du polymorphisme cytonucléaire a été testé à l'aide de plusieurs modèles théoriques. Il n'est pas possible de maintenir un polymorphisme cytonucléaire stable dans tous les cas (voir Tableau 1, ci-dessous). De façon générale, il semble qu'un coût récessif permette de maintenir un polymorphisme cytonucléaire pour une plus grande gamme de valeurs de l'avantage femelle et du coût de la restauration que dans le cas d'un coût dominant (Bailey *et al.*, 2003; Dufay *et al.*, 2007). De plus, il apparait plus difficile de maintenir le polymorphisme cytonucléaire lorsque le coût de la restauration affecte la fonction femelle, en regard de ce qui est observé quand le coût de la restauration affecte la fonction mâle (Bailey *et al.*, 2003; Dufay *et al.*, 2007). Finalement, il semble difficile (voir impossible, selon les modèles, ex : Dufay *et al.* 2007) de maintenir un polymorphisme cytonucléaire dans le cas d'un coût exprimé, contrairement à ce qui est observé pour des coûts constitutifs ou silencieux.

Tableau 1 : Synthèse des résultats obtenus par plusieurs études théoriques concernant la perte ou le maintien du polymorphisme cytonucléaire, en fonction (i) du déterminisme du coût de la restauration (dominant ou récessif), (ii) de la fonction reproductive affectée par le coût de la restauration (mâle ou femelle) et (iii) de la modalité d'action du coût (constitutif, exprimé ou silencieux)

Dominance du coût	Modalité du coût	Coût sur la fonction mâle ou sur la fonction femelle	Resultats	Références
Dominant	Silencieux	Mâle	Maintien	Gouyon et al. (1991), Dufaÿ et al. (2007)
		Femelle	Maintien	Gouyon et al. (1991), Dufaÿ et al. (2007)
	Exprimé	Mâle	Perte	Bailey et al. (2003) , Dufaÿ et al. (2007)
		Femelle	Perte	Bailey et al. (2003), Dufaÿ et al. (2007)
	Constitutif	Mâle	Maintien	Frank (1989), Bailey et al. (2003), Dufaÿ et al. (2007)
		Femelle	Maintien	Charlesworth (1981), Delannay (1981), Bailey et al. (2003), Dufaÿ et al. (2007)
Récessif	Silencieux	Mâle	Maintien	Gouyon et al. (1991), Dufaÿ et al. (2007)
		Femelle	Maintien	Gouyon et al. (1991), Dufaÿ et al. (2007)
	Exprimé	Mâle	Maintien	Bailey et al. (2003)
		Mâle	Perte	Dufay et al (2007)
		Femelle	Perte	Bailey et al. (2003) , Dufaÿ et al. (2007)
	Constitutif	Mâle	Maintien	Bailey et al. (2003), Dufaÿ et al. (2007)
		Femelle	Maintien	Bailey et al. (2003), Dufaÿ et al. (2007)

Tableau adapté de Delph *et al.* (2007)

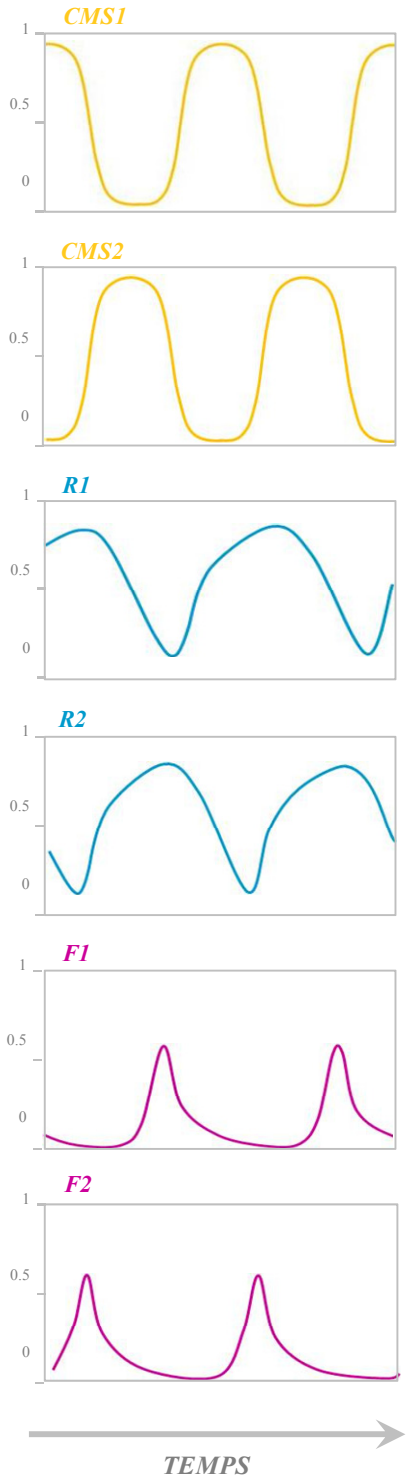
Les cas des processus non-sélectifs

Chez la plupart des espèces végétales, les individus ne sont pas distribués de façon homogène dans l'espace mais forment plutôt des agrégats géographiques. De plus, les flux de gènes *via* la dispersion du pollen ou des graines sont classiquement limités dans l'espace, ce qui a pour résultat des évènements de reproduction non-aléatoires dans l'espace (*p.e*. Smouse *et al.*, 1999; Burczyk *et al.*, 2002; Robledo-Arnuncio *et al.*, 2004; Krauss *et al.*, 2009). En conséquence, les fréquences alléliques peuvent varier d'une population à une autre. Chez les espèces gynodioïques, étant donné que le phénotype sexuel est déterminé génétiquement (gènes cytoplasmiques de stérilité mâle et allèles nucléaires de restauration), ces variations de fréquences alléliques peuvent se traduire par des variations importantes de la proportion relative d'hermaphrodites et de femelles dans l'espace. Au sein d'une population structurée, la dérive génétique peut alors renforcer les effets d'une migration limitée, en modifiant localement les fréquences des allèles liés à l'expression du sexe, voire en provoquant la fixation ou la perte de ces allèles (Nilsson & Agren, 2006). De la même façon, des évènements de fondation peuvent renforcer la structure spatiale des sexes en diminuant les chances qu'une CMS donnée soit associée avec les allèles de restauration appropriés, ce qui peut avoir un impact important sur la fréquence relative des deux phénotypes sexuels (Manicacci *et al.*, 1996).

Effets de la sélection

En parallèle des effets de la migration, de la dérive génétique et des évènements de fondation, plusieurs études suggèrent que les sex ratios locaux peuvent aussi être modifiés par la sélection naturelle, lorsque les valeurs sélectives des différents phénotypes sexuels sont affectées différemment par les caractéristiques de l'environnement. Une étude récente de Nilsson et Agren (2006) sur *Plantago maritima* montre par exemple (i) que la fécondité relative des femelles et des hermaphrodites varie fortement d'une localité à une autre et (ii) que les femelles sont plus fréquentes dans les localités où leur fécondité est élevée. Par ailleurs, il semble que les femelles soient classiquement plus performantes dans les milieux les plus difficiles, par exemple dans les sites les plus secs ou les moins riches en nutriments (*p.e*. Vaughton & Ramsey, 2004; Caruso & Case, 2007). La sélection peut donc agir différemment sur les phénotypes sexuels en fonction de l'environnement dans lequel on se place. A cet effet particulier de la sélection dans des environnements variables se surajoutent les attendus de plusieurs modèles théoriques qui suggèrent que le sex ratio à l'équilibre ne correspond pas forcément à un équilibre ponctuel, même en considérant des environnements équivalents. Ces modèles montrent que, sous l'effet de la sélection, les fréquences des gènes CMS et des restaurateurs nucléaires associés peuvent présenter des variations cycliques au cours du temps, entrainant en retour des variations cycliques de sex ratio (*p.e*. Gouyon *et al.*, 1991; Dufay *et al.*, 2007). Ces modèles invoquent des mécanismes de sélection fréquence-dépendante affectant les différents gènes impliqués dans le déterminisme du sexe et permettent d'expliquer à la fois les variations de sex ratio entre populations ainsi que les très fortes fréquences de femelles qui sont parfois observées dans la nature (*p.e*. Dommée *et al.*, 1987 ; Nilsson & Agren, 2006; Caruso & Case, 2007). Ce résultat, conséquence directe du conflit génétique évoqué précédemment, est expliqué plus en détail dans l'Encadré V. Il n'existe pas encore de démonstrations empiriques de ce phénomène, et l'obtention de preuves demanderait un suivi fin de nombreuses localités différentes sur de longues fenêtres de temps (voir toutefois Dufay *et al.*, 2009).

ENCADRÉ V : SEX RATIOS A L'EQUILIBRE



En fonction de l'intensité de l'avantage femelle et du coût de la restauration, le sex ratio théorique à l'équilibre peut correspondre soit à un équilibre ponctuel, soit à un équilibre dynamique avec des oscillations cycliques de la proportion de femelles (Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufay *et al.*, 2007).

La figure V-1 illustre la façon dont la fréquence des gènes de stérilité mâle et des allèles de restauration qui leur sont associés peuvent fluctuer au cours du temps, provoquant des oscillations de la proportion de femelles dans les populations. Dans l'exemple proposé, deux *CMS* coexistent (*CMS1* et *CMS2*), chacune étant restaurée par un allèle nucléaire dominant (*R1* et *R2*). Les individus femelles (*F1* et *F2*) bénéficient d'un avantage femelle et les restaurateurs sont associés à un coût silencieux sur la fonction mâle. La population est au départ composée essentiellement d'individus *CMS1* restaurés pour la fonction mâle et de quelques individus *CMS2* non restaurés. Les oscillations observées correspondent alors aux étapes suivantes :

1 – Les individus porteurs de la *CMS2* exprimant majoritairement un phénotype femelle du fait de la faible fréquence du restaurateur *R2*, la *CMS2* est généralement associée à l'avantage femelle. En conséquence, la fréquence de cette *CMS* croît rapidement, au détriment de la *CMS1*.

2 – Lorsque la fréquence de la *CMS2* approche l'unité, une sélection positive forte s'exerce sur le restaurateur de cette *CMS* (*R2*). Dans cette étape, contrairement à l'étape précédente, les processus en jeux sont quasi-exclusivement « intra-cytoplasme » (entre individus porteurs de la *CMS2*) : les valeurs sélectives « mâle » et « femelle » entrent toutes les deux en jeu.

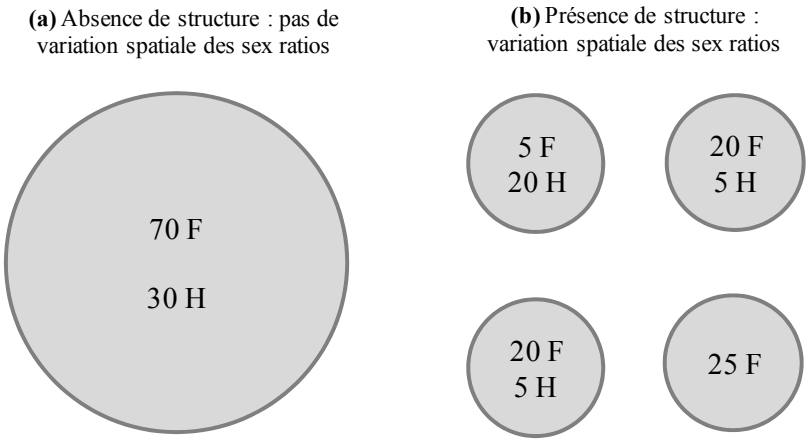
3 – Dans la phase suivante, la majorité des individus portent la *CMS2* et sont restaurés pour la fonction mâle. Du fait du coût silencieux de la restauration, les allèles *R1* sont alors fortement contre-sélectionnés (ces allèles induisent en effet un coût quand ils sont associés au cytoplasme *CMS2*)

4 – Quand la fréquence de *R1* approche de 0, la situation obtenue équivaut à la situation initiale inversée : la population est composée essentiellement d'individus *CMS2* restaurés pour la fonction mâle et de quelques individus *CMS1* non restaurés. Le processus décrit ci-dessus se répète alors (avec la *CMS1* au lieu de la *CMS2*), et ainsi de suite.

Fig. V-1 Evolution au cours du temps de la fréquence des gènes cytoplasmiques de stérilité mâle (*CMS1* et *CMS2*), de leurs restaurateurs nucléaires spécifiques (*R1* et *R2*) et de la proportion de femelles associées à chaque *CMS* (*F1* et *F2*) dans une population panmictique de taille infinie.

D'après Gouyon *et al.* (1991)

Finalement, chacun des processus décrit ci-dessus (ainsi que diverses combinaisons des ces processus) peuvent aboutir à l'établissement d'une structuration très prononcée des phénotypes sexuels dans l'espace. La disponibilité en partenaires de chaque phénotype sexuel va alors varier entre localités (Fig. 2). Les deux phénotypes sexuels coexistant chez les espèces gynodioïques peuvent alors être soumis à une sélection de type fréquence-dépendante, si la valeur sélective des phénotypes sexuels dépend de leur fréquence relative dans un voisinage donné (Lloyd, 1975). Chez les espèces gynodioïques, un exemple bien documenté de ce type de phénomène porte sur la diminution du succès reproducteur des femelles lorsque celles-ci sont en forte fréquence et que le pollen devient un facteur limitant (*p.e*. Widen & Widen, 1990; McCauley, 1997; McCauley & Brock, 1998; Graff, 1999). Les femelles sont donc soumises à une sélection fréquence-dépendante négative. En revanche, pour les hermaphrodites situés à l'intérieur de voisinages biaisés en faveur des femelles, la situation devient plus complexe : ceux-ci bénéficient de l'abondance de partenaires du fait de la diminution de la compétition entre pères potentiels. Il a néanmoins été montré que les descendants d'hermaphrodites pouvaient souffrir des effets néfastes de la consanguinité chez les espèces auto-compatibles (McCauley & Brock, 1998; Miyake & Olson, 2009).



Fréquence absolue d'hermaphrodites	$p_H = 0.30$	$p_H = 0.30$
Fréquence relative d'hermaphrodites	$p_{HH} = 0.30$ $p_{HF} = 0.30$	$p_{HH} = 0.60$ $p_{HF} = 0.17$

Fig. 2 Illustration de l'influence de la structure et de la répartion des sexes dans l'espace sur la disponibilité locale en partenaires de chaque type sexuel (F : femelle et H : hermaphrodite). Dans les deux cas, 100 individus (70 femelles et 30 hermaphrodites) sont répartis (**a**) dans une unique population panmictique ou (**b**) dans un ensemble de sous-populations structurées. Dans la population panmictique, la fréquence absolue d'hermaphrodites (p_H) est égale à la fréquence relative d'hermaphrodites du point de vue des hermaphrodites (p_{HH}) et à la fréquence relative d'hermaphrodites du point de vue des femelles (p_{HF}). Dans l'ensemble structuré, la fréquence relative d'hermaphrodites du point de vue des femelles (p_{HF}) est plus faible que la fréquence absolue d'hermaphrodites (p_H), qui est elle même plus faible que que la fréquence relative d'hermaphrodites du point de vue des hermaphrodites (p_{HH}). Si le pollen est un facteur limitant le succès reproducteur femelle, ce désavantage affectera les deux phénotypes sexuels avec une intensité équivalente dans une population panmictique, mais aura un effet plus prononcé sur les femelles que sur les hermaphrodites quand les phénotypes sexuels sont structurés dans l'espace. Le même type de raisonnement peut être appliqué à la disponibilité en femelles.
Voir détails des calculs dans McCauley & Taylor, 1997

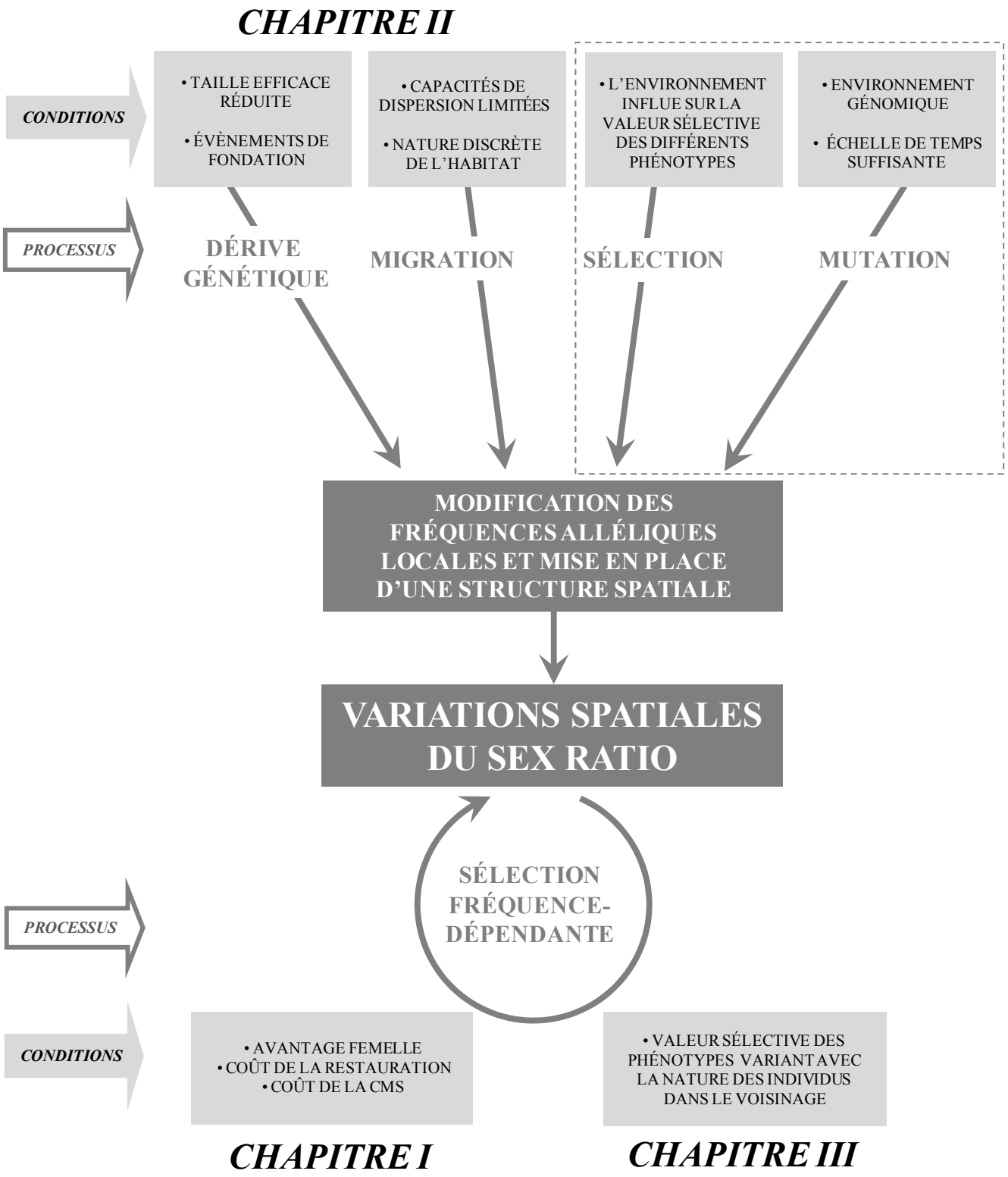


Fig. 3 Schéma synthétique des différents processus qui peuvent avoir un impact sur la répartion des différents phénotypes sexuels dans l'espace, et description des différents objectifs de ce travail de thèse. Chacun des trois chapitres sera dédié à l'étude d'une partie des processus potentiellement responsables de la variation spatiale des sex ratio. La thèse étant exclusivement focalisée sur des processus à petites échelles spatiales, les aspects inclus dans le cadre en tirets ne seront pas traités.

Au final, les variations spatiales de la proportion relative d'hermaphrodites et de femelles dans l'espace peuvent être induites par l'action de la sélection ainsi que par des processus non sélectifs. L'ensemble des processus détaillés ci-dessus constituent le fil conducteur de ce travail de thèse et sont schématisés dans la Figure 3.

IV - LE MODELE BIOLOGIQUE : BETA VULGARIS *SSP.* MARITIMA

Systématique

Beta vulgaris ssp. *maritima* appartient à la famille des *Amaranthaceae*. Le genre *Beta* est subdivisé en quatre sections (*Beta*, *Nanae*, *Corollinae* et *Procumbentes*). La section *Beta* contient 3 espèces distinctes : *Beta macrocarpa, Beta patula* et *Beta vulgaris* (Letschert, 1993). Les différentes variétés de betteraves cultivées appartiennent toutes à l'espèce *Beta vulgaris* et y sont désignées sous le nom de *Beta vulgaris* ssp. *vulgaris*. Les diverses formes sauvages sont quant à elles rassemblées au sein de deux sous espèces : une sous espèce restreinte aux îles et au littoral grec ainsi qu'au Proche Orient, *Beta vulgaris* ssp. *adanensis* et l'espèce étudiée dans ce travail de thèse, *Beta vulgaris* ssp. *maritima.* Les formes cultivées et sauvages de *Beta vulgaris* sont totalement interfertiles (Letschert, 1993; Bartsch *et al.*, 2003).

Distribution géographique

Beta vulgaris ssp. *maritima* est présente sur tout le pourtour méditerranéen, le long des côtes atlantiques de l'Europe, au sud de la Suède et autour des îles britanniques, ainsi que sur des îles éloignées telles que les Açores (Letschert, 1993). Cette espèce est strictement inféodée au littoral dans la partie septentrionale de sa distribution. On la rencontre alors essentiellement dans une étroite bande côtière, au niveau des estuaires, sur des plages de galets ou de sable, sur des falaises ou dans des milieux plus anthropisés en bord de mer (Fig. 4). Plus au sud (région méditerranéenne), les populations colonisent ponctuellement l'intérieur des terres et leur niche écologique se diversifie, incluant des sites rudéraux tels que les bords de route, les friches ou les fossés. On peut rencontrer des populations à plusieurs dizaines de kilomètres de la côte. Dans le sud-ouest de la France, ces populations « rudérales » se trouvent alors parfois en contact avec les zones de production de semences de betteraves cultivées (Van Dijk, 2004). C'est à l'intérieur de cette région que des hybridations entre le compartiment sauvage et le compartiment cultivé génèrent une forme dite « mauvaise herbe », qui est invasive dans les cultures de betteraves dans le nord de la France (Desplanque, 1999; Cuguen *et al.*, 2004; Fénart *et al.*, 2008 ; Arnaud *et al.*, 2009).

Caractéristiques biologiques

Cycle de vie

Beta vulgaris ssp. *maritima* est une espèce pérenne et itéropare. L'espèce présente une grande variabilité de durée de vie pouvant aller de deux à dix ans, si les conditions environnementales sont suffisamment stables (Hautekèete *et al.*, 2002).

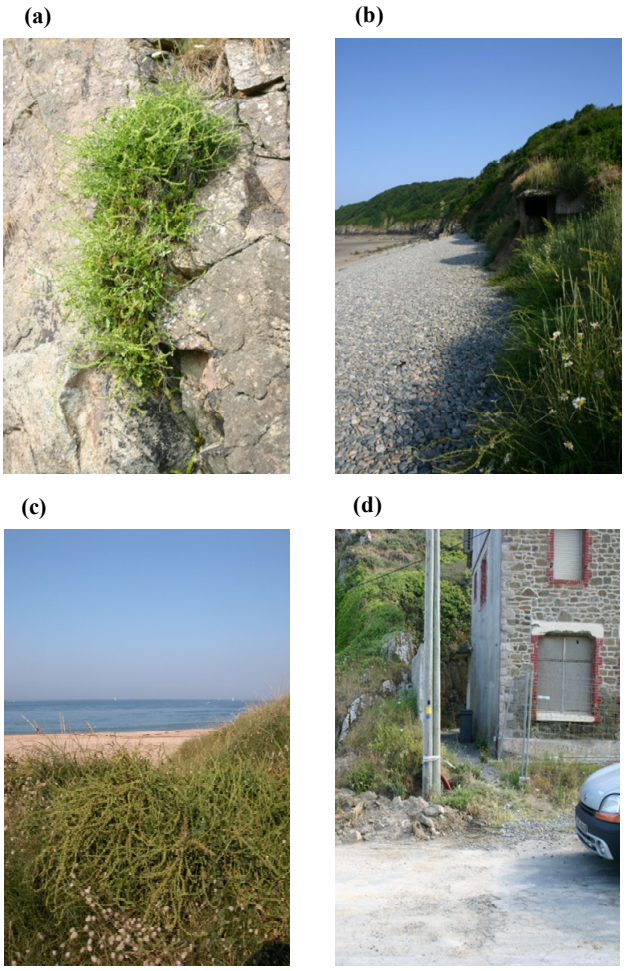


Fig. 4 Différents types d'habitats classiquement colonisés par la betterave maritime (*B. vulgaris* ssp. *maritima)* dans les régions étudiées dans ce manuscript (nord de la France et Bretagne): (**a**) rochers et falaises, (**b**) et (**c**) laisses de mer sur des plages de galets ou de sable, (**d**) milieux anthropisés en bord de mer.

L'âge des plantes au moment de la première floraison varie : au nord de l'aire de répartition, la première floraison a généralement lieu la deuxième année tandis qu'au sud elle peut intervenir dès la première année (Van Dijk *et al.*, 1997). Ce gradient latitudinal est associé au polymorphisme d'un gène particulier, le gène *B* (pour « bolting »), qui détermine le besoin de vernalisation (Boudry *et al.*, 1994). A ce locus, un allèle dominant *B* annule le besoin de vernalisation et permet la floraison dés la première année, tandis que l'induction de la floraison chez les individus homozygotes pour l'allèle *b* nécessite une certaine quantité de froid. Au nord de l'aire de répartition, l'allèle *b* est généralement fixé ce qui explique pourquoi la floraison n'a lieu qu'au cours de la deuxième année dans ces régions.

Les graines produites peuvent être dormantes et constituent potentiellement des banques de graines pérennes dans les populations naturelles ou dans les cultures de betteraves sucrières (Sester *et al.*, 2006 ; Wagmann, 2008; Arnaud *et al.*, 2010).

Biologie de la reproduction

B. vulgaris est une plante anémophile. L'espèce est auto-incompatible (Owen, 1942; Larsen, 1977), contrairement aux autres espèces de la section *Beta*. Les fleurs sont disposées le long d'axes floraux et sont usuellement groupées en paquets de 2 à 8. Le nombre d'axes floraux porté par les plantes

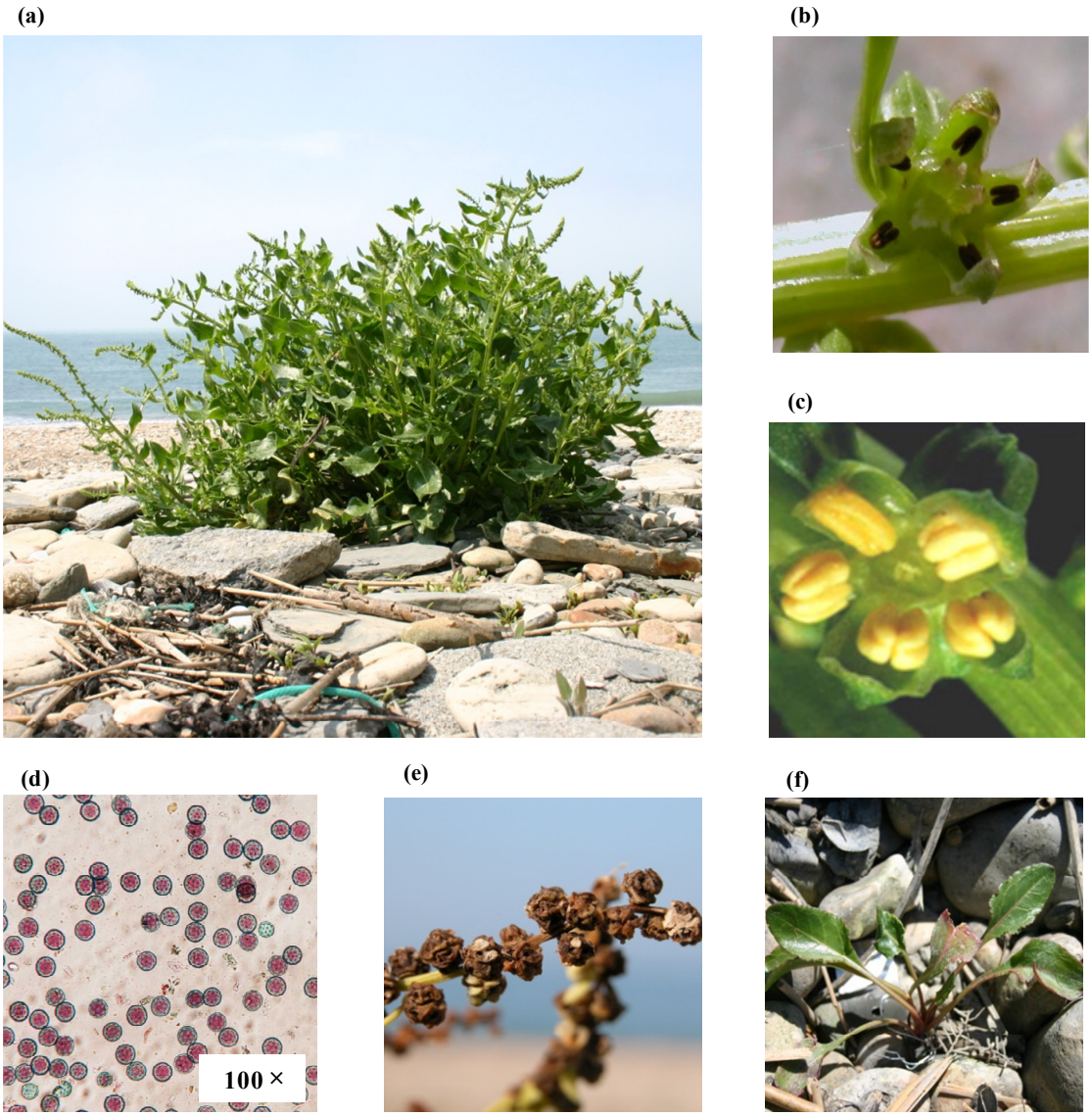


Fig 5 Caractéristiques biologiques de la betterave maritime (*B. vulgaris* ssp. *maritima)* : morphologie de l'individu adulte en début de floraison (**a**), fleur d'un individu femelle, présentant des étamines réduites et vides (**b**), fleur d'un individu hermaphrodite, avec des étamines jaunes et remplies de pollen (**c**), grains de pollen observés au microscope après une coloration alexander, qui permet la distinction entre les grains non-viables, colorés en vert, et les grains viables, colorés en violet (**d**), les fruits (glomérules), qui résultent de la fusion de plusieurs fleurs uniovulées, et contiennent entre 1 et 8 graines (**e**), et plantule en population naturelle (**f**).

varie beaucoup entre les individus (de quelques uns à plusieurs centaines) et plusieurs dizaines de milliers de fleurs peuvent être produites par individu. Le fruit, appelé glomérule, résulte de la fusion des fleurs au sein de chaque paquets de fleurs. Chaque glomérule est donc un fruit composé, constitué de plusieurs fleurs distinctes. Chaque fleur étant uniovulée, le nombre potentiel de graines par glomérule est égal au nombre de fleurs initialement présentes. Lors de sa maturation, le glomérule prend l'aspect d'un fruit sec non charnu, les pièces périanthaires entourant les graines formant du liège (Fig. 5e). Après la maturation, toutes les structures reproductives se dessèchent et la plante passe l'hiver sous forme d'une rosette de feuilles.

Les populations gynodioïques de *B. vulgaris*, comprenant à la fois des individus hermaphrodites et femelles, sont relativement fréquentes le long de côtes françaises (Cuguen *et al.*, 1994; Forcioli *et al.*,

1998 ; Dufay *et al.*, 2009). Le déterminisme impliqué dans l'expression du sexe est bien connu et implique des interactions entre des gènes cytoplasmiques de stérilité mâle (CMS) et des allèles nucléaires de restauration de la fonction mâle. Quatre CMS distinctes peuvent induire un phénotype femelle (CMS *E*, CMS *G*, CMS *H*, CMS *Svulg*), à moins que leur action ne soit contrée par des allèles nucléaires de restauration (Cuguen *et al.*, 1994; Fénart *et al.*, 2006). Ces quatre CMS diffèrent en termes de fréquence dans les populations naturelles : si les CMS *E* et *G* sont relativement fréquentes, les deux autres montrent en revanche des distributions plus sporadiques (voir Fig. 6). La CMS *H*, par exemple, est complètement absente dans le nord et dans l'ouest de la France (*i.e.* la région concernée par ce travail de thèse). La CMS *Svulg* est également peu fréquente. Cette CMS étant largement utilisée dans les variétés cultivées, sa présence dans les populations naturelles peut être considérée comme une preuve d'échanges génétiques entre les compartiments cultivés et sauvages, notamment dans le nord de la France, où la betterave sucrière est cultivée de façon extensive (Arnaud *et al.*, 2003; Viard *et al.*, 2004). Les taux de restauration sont également variables entre CMS : dans les populations naturelles, la CMS *G* est en moyenne significativement moins restaurée que les CMS *E* et *Svulg* (Dufay *et al.*, 2009). Ceci pourrait être lié au fait que cette stérilité soit physiologiquement plus difficile à restaurer ou, de façon alternative, à une apparition plus récente de cette CMS.

En plus de ces quatre cytoplasmes stérilisants, une grande variété d'autres cytoplasmes ne sont jamais associés avec le phénotype femelle (Cuguen *et al.*, 1994; Fénart *et al.*, 2006). Trois types sexuels peuvent donc être distingués : les femelles (qui portent une CMS sans les allèles de restauration appropriés), les hermaphrodites restaurés (qui portent une CMS ainsi que les allèles de restauration appropriés) et les hermaphrodites « normaux » (qui portent un cytoplasme mâle-fertile). Cette distinction est importante pour étudier et comprendre le maintien de gynodioécie dans cette espèce. Comme expliqué ci-dessus, pour maintenir ce type de polymorphisme (cytoplasmes fertiles, cytoplasmes stérilisants et restaurateurs nucléaires de la fertilité mâle), trois conditions doivent théoriquement être réunies : (i) un avantage femelle, (ii) un coût de la restauration et (iii) un coût de la CMS pour les hermaphrodites restaurés.

Un avantage femelle faible et non-significatif a été détecté précédemment chez *B. vulgaris* ssp. *maritima* (de l'ordre de 1.2, Boutin, 1984). Cette étude n'était cependant focalisée que sur un nombre restreint de traits potentiellement associés à la valeur sélective et d'autres travaux sont donc nécessaires pour quantifier l'avantage femelle chez cette espèce. De plus, sur la base d'une étude portant sur deux populations correspondant à deux situations très contrastées en termes de sex ratio et de taux de restauration, une preuve indirecte d'un coût silencieux de la restauration a pu être obtenue chez *B. vulgaris* (Dufay *et al.*, 2008). L'analyse de la production et de la viabilité du pollen des deux catégories d'hermaphrodites (hermaphrodites « normaux » et restaurés) a en effet permis de dévoiler : (i) une moindre qualité du pollen des hermaphrodites restaurés, vraisemblablement due à une restauration parfois incomplète de la fertilité mâle et (ii) une moindre qualité du pollen des hermaphrodites « normaux » les plus susceptibles de porter le restaurateur, ce qui pourrait correspondre à un coût silencieux des allèles de restauration.

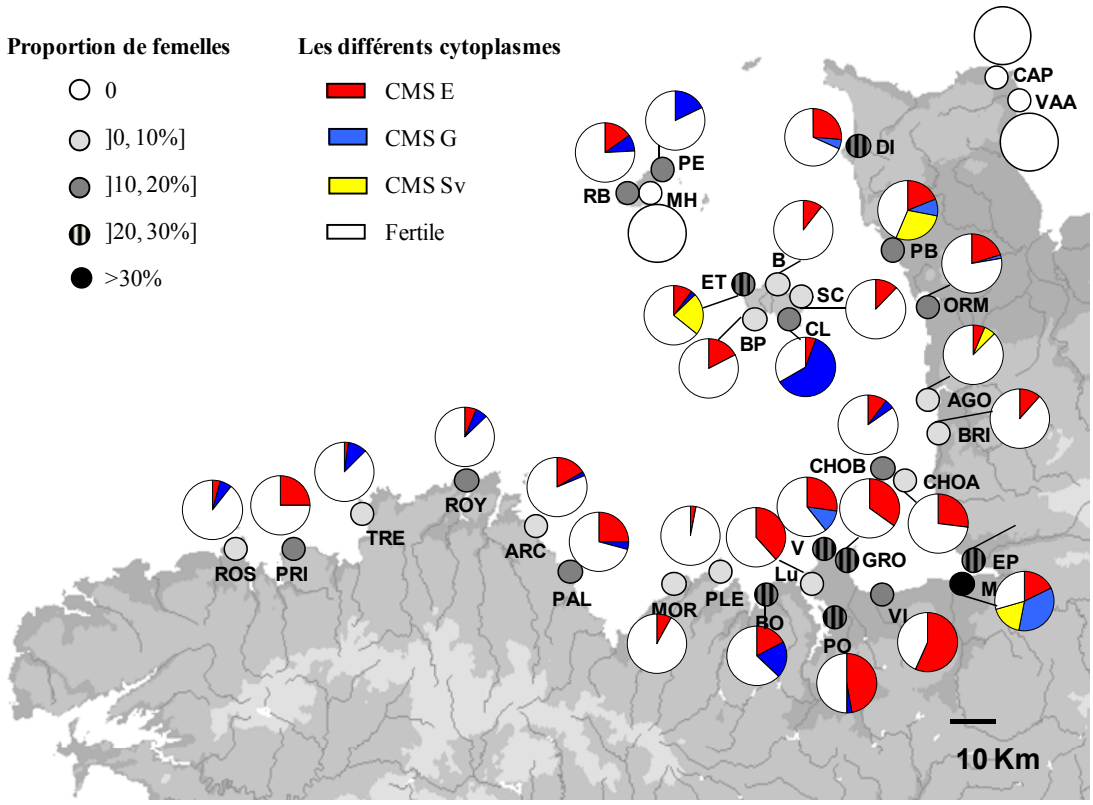


Fig. 6 Localisation de 33 populations de *Beta vulgaris* ssp. *maritima* étudiées dans la baie Anglo-Normande. Pour chaque population, le sex ratio (proportion de femelles) est indiqué, ainsi que la fréquence des différents cytoplasmes (fertiles et stériles).

Tiré de Dufay *et al.* (2009)

V - PLAN DE LA THESE

Chez les espèces gynodioïques, il est fréquent d'observer d'importantes variations spatiales de sex ratio, ceci même sur des échelles spatiales aussi fines que la centaine de mètres (*p.e.* Graff, 1999; Laporte *et al.*, 2001; Olson *et al.*, 2006). L'objectif de ce travail de thèse est d'identifier, dans la mesure du possible, parmi les différents processus précédemment évoqués et pouvant être impliqués dans ces variations, lesquels entrent en jeu dans les populations naturelles de *B. vulgaris* ssp. *maritima* (Fig. 3). Cette étude s'articule ainsi autour de trois axes principaux de recherches :

(1) Nous avons vu que des différences d'investissement reproducteur entre les différents phénotypes sexuels peuvent avoir un effet crucial sur le maintien du polymorphisme cytonucléaire associé à la gynodioécie et sur les sex ratios observés dans les populations naturelles. A ce jour, un avantage femelle marginal a été suggéré chez *B. vulgaris* ssp. *maritima* sur la base d'un nombre limité de traits, et les différences de qualité de pollen entre les différents types d'hermaphrodites précédemment décrits n'ont pas été vérifiées en conditions contrôlées (Boutin *et al.* 1984 ; Dufay *et al.* 2008). Le *CHAPITRE I* vise donc à examiner et à quantifier les éventuelles différences d'investissement reproducteur au travers d'un ensemble de mesures portant sur la fonction mâle, en termes de quantité et de qualité du pollen

produit, ainsi que dans la fonction femelle, en termes de nombre d'ovules, de taux de mise à fruits et de taux de germination. L'ensemble de ces mesures ont été menées en conditions contrôlées en serres expérimentales et sont discutées à la lumière des attendus théoriques pour le maintien d'un polymorphisme sexuel.

(2) La dérive génétique, les évènements de fondation et la migration jouent un rôle important dans la distribution de la diversité génétique et la mise en place de structure génétique spatiale. Il est possible d'obtenir des informations à fine échelle sur l'importance relative de ces différents processus au travers de leurs signatures imprimées dans la structure génétique spatiale des populations, ceci au moyen de marqueurs moléculaires hautement polymorphes. Dans le *CHAPITRE II*, des outils de génétique des populations sont ainsi utilisés pour obtenir des informations sur l'intensité des processus stochastiques associés à l'action de la dérive génétique et des effets de fondations (Partie 1). L'importance des flux de gènes historiques cumulés au cours du temps est également évaluée. La comparaison de l'arrangement spatial de la diversité génétique associée à des marqueurs cytoplasmiques et à des marqueurs nucléaires permet en plus d'obtenir une image de la part relative de la dispersion effectuée au moyen des graines et de celle effectuée au travers des flux polliniques (Partie 1). Les effets possibles de l'existence d'une banque de graines pérennes sur l'évolution temporelle de la diversité génétique et son impact sur la taille efficace des populations est également abordée (Partie 2). Enfin, les effets cumulés de l'ensemble de ces processus sur la répartition de gènes associés au déterminisme du sexe, et donc sur la distribution des phénotypes sexuels dans l'espace, sont discutés en détail.

(3) Par ailleurs, au sein des espèces sexuellement polymorphes, il existe de nombreux exemples de sélection fréquence-dépendante impliquant, par exemple, la morphologie florale chez les espèces hétérostyles et énantiostyles (Jesson & Barrett, 2002; Van Rossum *et al.*, 2006), le polymorphisme de couleur florale chez les espèces d'orchidées qui ne synthétisent pas de nectar (Gigord *et al.*, 2001), ou la fréquence des différents allèles au locus *S* chez les espèces auto-incompatibles (Wagenius *et al.*, 2007). Le *CHAPITRE III* de ce document s'attache tout particulièrement à décrire les interactions particulières qui peuvent exister entre la structure spatiale des sexes à très fine échelle spatiale (décrite dans le Chapitre II) et le succès reproducteur mâle et femelle des différents phénotypes sexuels (décrit dans le Chapitre I). Dans un premier temps (Partie 1), nous nous demanderons si le succès reproducteur des femelles dépend de la quantité de partenaires sexuels disponibles, autrement dit, de la présence et de la fréquence d'hermaphrodites fonctionnels au voisinage immédiat. La réponse à cette question est d'autant plus intéressante qu'à ce jour très peu d'études ont révélé l'existence de phénomènes de limitation pollinique chez des espèces à pollinisation anémophile, le vent étant potentiellement un vecteur de dispersion très performant. Dans un second temps, nous nous intéresserons aux effets possibles de la structure des phénotypes sexuels sur succès reproducteur mâle des hermaphrodites. En particulier, sous l'hypothèse d'une compétition entre hermaphrodites pour l'accès à la reproduction, est-il possible que la transmission des gènes au travers du pollen soit accrue dans des voisinages où les femelles sont majoritaires ? En d'autres termes, les hermaphrodites bénéficient-ils de l'avantage du rare quand ils sont en faibles fréquences ? Nous examinerons en particulier le sort des allèles de restauration en populations structurées. Les hermaphrodites porteurs de CMS et restaurés pour la fonction mâle sont en moyenne de mauvais producteurs de pollen par rapport aux hermaphrodites non-porteurs de CMS (Dufay *et al*. 2008), mais du fait de la forte structure génétique spatiale des gènes cytoplasmiques, ils sont fréquemment trouvés à

proximité des femelles. On peut alors s'interroger sur leur succès reproducteur (i) lorsqu'ils sont effectivement localisés dans des dèmes biaisés en faveur des femelles (Partie 2) et (ii) lorsqu'ils sont en compétition avec les hermaphrodites non-porteurs de CMS (Partie 3).

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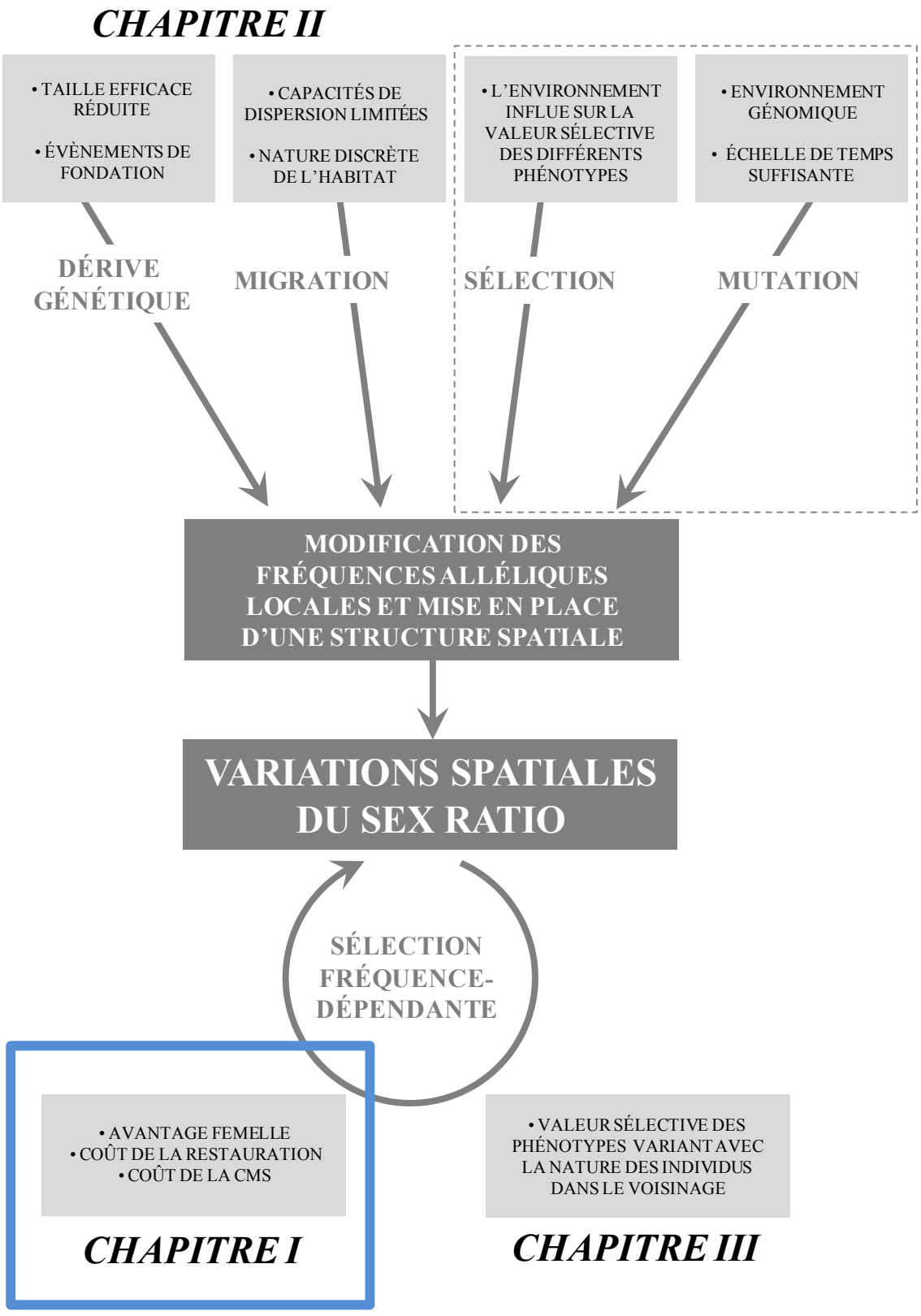
CHAPITRE I

PHENOTYPE SEXUEL ET SUCCES REPRODUCTEUR INDIVIDUEL

Problématique : Les différents génotypes et les différents phénotypes sexuels différent-ils en termes de valeur sélective ?

Le maintien d'individus femelles au sein de populations gynodioïques dépend de l'existence d'un polymorphisme au niveau des gènes contrôlant l'expression du sexe, c'est-à-dire les gènes CMS (« Cytoplasmic Male Sterility ») et les restaurateurs nucléaires de fertilité mâle. En théorie, ce type de polymorphisme peut être maintenu si les femelles compensent la perte de la fonction mâle par une meilleure valeur sélective (avantage femelle) et si une force s'oppose à la fixation des allèles de restauration (coût de la restauration, voir Gouyon *et al*. 1991). Chez les espèces où des cytoplasmes non-stérilisants coexistent avec les cytoplasmes CMS, une condition additionnelle doit être remplie : les hermaphrodites porteurs de la CMS et restaurés pour la fonction mâle doivent être désavantagés en termes de fonction femelle par rapport aux hermaphrodites non porteurs de CMS (coût de la CMS, voir Dufay *et al*. 2007). Chez ces espèces, on peut également s'interroger sur la valeur sélective mâle des deux catégories d'hermaphrodites. Si un coût de la restauration a déjà été suggéré chez *B. vulgaris* (Dufay *et al*. 2008), les autres facteurs pouvant influencer le maintien de la gynodioécie, ainsi que la fréquence des femelles, restent à explorer. En caractérisant la valeur sélective des femelles, des hermaphrodites porteurs de CMS et restaurés pour la fonction mâle et des hermaphrodites non porteurs de CMS, nous avons essayé (i) de détecter et de quantifier l'avantage femelle et le coût de la CMS, ainsi que de (ii) comparer la production de pollen entre les deux types d'hermaphrodites.

Photo : Terrain expérimental où ont eu lieu les mesures décrites dans ce chapitre.



Sex-specific fitness variation in gynodioecious *Beta vulgaris* ssp. *maritima*: do empirical observations fit theoretical predictions?

I. DE CAUWER, J.-F. ARNAUD, A. COURSEAUX & M. DUFAY

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ABSTRACT

In gynodioecious species (hermaphrodites and females), the maintenance of sex polymorphism relies on sex determination and on the relative fitness of the different phenotypes. Components of male (pollen production and quality) and female (flower production, fruit and seed set) fitness were measured in gynodioecious *Beta vulgaris* spp. *maritima,* in which sex is determined by interactions between cytoplasmic male sterility (CMS) genes and nuclear restorers of male fertility. We found that (i) female had a relatively low advantage over hermaphrodites, (ii) restored hermaphrodites (carrying both CMS genes and nuclear restorers) suffered a slight decrease in female function compared to non-CMS hermaphrodites and (iii) restored CMS hermaphrodites were poor pollen producers compared to non-CMS hermaphrodites, probably as a consequence of complex determination of restoration. These observations potentially have important consequences on the conditions of maintenance of sexual polymorphism in *B. vulgaris* and are discussed in the light of existing theory.

INTRODUCTION

Flowering plant species exhibit a large range of reproductive strategies. After hermaphroditism, gynodioecy is the most common breeding system (about 7% of angiosperms, Richards, 1997). In gynodioecious species, two sexual phenotypes coexist in natural populations: individuals can be classified as females or hermaphrodites, depending on their ability to produce pollen. Since females reproduce only through seeds, they apparently transmit their genes only half as frequently as hermaphrodites, which possess both sexual functions. The maintenance of females in gynodioecious species has intrigued evolutionary biologists for decades and numerous theoretical models have been developed (e.g. Charlesworth & Ganders, 1979; Frank, 1989; Gouyon *et al.*, 1991; Couvet *et al.*, 1998; Bailey *et al.*, 2003; Dufay *et al.*, 2007). All of these models share one common condition for the maintenance of gynodioecy: females must compensate for their gametic disadvantage relative to hermaphrodites via higher female reproductive fitness (Lewis, 1941; Lloyd, 1974). A number of empirical studies have suggested that female plants outperform hermaphrodites in one or several aspects of female reproduction. This female advantage can result from two distinct mechanisms: females produce more seeds or seeds of better quality than hermaphrodites, due to reallocation of resources that are not used to produce male gametes (e.g. Avila-Sakar & Domínguez, 2000) and/or females benefit from inbreeding avoidance and produce, on average, higher quality seeds than hermaphrodites that partly self-pollinate (e.g. Chang, 2007).

The magnitude of female advantage needed to maintain females in natural populations critically depends on how sex is genetically determined. In simple nuclear determination, females need to produce at least twice as many offspring through seeds as hermaphrodites to compensate for their loss of pollen production (Lewis, 1941). However, in most gynodioecious species, sex determination involves cytonuclear interactions, leading to a more complex mode of inheritance (Saumitou-Laprade *et al.*, 1994; Delph *et al.*, 2007), and sexual phenotype depends on interactions between cytoplasmic (maternally inherited) male sterility genes (CMS genes) and nuclear (biparentally inherited) male fertility restorers. To develop as a female, an individual must carry a non-restored CMS gene. To develop as a hermaphrodite, an individual must either carry a CMS gene in combination with the matching restoration allele (restored hermaphrodite), or carry a non-CMS cytoplasm (although the existence of non-sterilising cytoplasms has not been verified in most gynodioecious species). As in nuclear determination, females must have some fecundity advantage compared to hermaphrodites. However, the magnitude of the female advantage required to maintain gynodioecy has been shown to be lower than in the case of strict nuclear determination (Gouyon *et al.*, 1991; Dufay *et al.*, 2007). This is because the absence of male gamete production does not affect the cytoplasmic fitness of an individual and a moderate increase of seed quantity or quality thus confers a selective advantage to CMS genes (Cosmides & Tooby, 1981). On the other hand, since nuclear genes are biparentally transmitted, the loss of pollen production directly

reduces their transmission. Consequently, when CMS genes become frequent in a population, nuclear restorers of male fertility should be selected for. Theoretical models suggest that there must be some forces opposing to the fixation of restorer alleles to maintain cytonuclear polymorphism within populations. This can be achieved through two distinct processes. First, restorer alleles are frequently thought to be associated with a cost (acting on either male or female reproductive success) that prevents their fixation and allows the maintenance of cytonuclear polymorphism (Charlesworth & Ganders, 1979; Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufay *et al.*, 2007). Alternatively, metapopulation dynamics, with recurrent extinctions and recolonisations of demes, have been shown to maintain cytonuclear polymorphism without requiring any cost of restoration, by preventing local populations from reaching equilibrium (Couvet *et al.*, 1998). In addition, in the particular case of species containing non-sterilising cytotypes, hermaphrodites that do carry a male-sterility mutation (restored CMS hermaphrodites) must have a disadvantage in female fitness compared to those that carry a non-sterilising cytotype (Dufay *et al.*, 2007). This cost of CMS is theoretically necessary to maintain non-CMS hermaphrodites that would otherwise be eliminated because they are never associated with female advantage. Altogether, female advantage, cost of restoration and cost of CMS genes theoretically allow frequency-dependent selection to maintain cytonuclear polymorphism, often through large oscillations of sex ratios, with CMS genes being selected for when restorer alleles are rare and restorers being selected for when CMS genes are frequent (Dufay *et al.*, 2007).

Theoretical models generally assume that the restoration of male fertility is achieved through one allele (but see Frank, 1989; Bailey & Delph, 2007). However, empirical work suggests that genetic determination of restoration may be more complex (Charlesworth & Laporte, 1998; Koelewijn, 2003; Ehlers *et al.*, 2005). In case of polygenic determination of restoration, male-sterile individuals may rarely be fully restored in natural populations and male fitness may vary quantitatively among restored CMS hermaphrodites (e.g. Dufay *et al.*, 2008). This may slow down the selection of restoration and ultimately modify the conditions of maintenance of cytonuclear polymorphism, thereby favouring the maintenance of females in gynodioecious populations (Bailey & Delph, 2007).

Female advantage, cost of restoration, cost of CMS genes and incomplete restoration of male fertility are all important parameters that directly influence the reproductive output of individuals in gynodioecious species. Knowing the relative male and female fitness of the different possible genotypes is of crucial importance when attempting to understand the maintenance of sexual polymorphism associated with cytonuclear gynodioecy. From this point of view, gynodioecious *Beta vulgaris* ssp. *maritima* is a relevant model for investigating sex-specific fitness variations because the genetic basis of sex is well known: male sterility is associated with four particular mitochondrial types, called CMS *E*, *G*, *Svulg* and *H*. These sterilising cytoplasms coexist with male-fertile cytoplasms, and these different cytotypes can be identified with molecular markers (Cuguen *et al.*, 1994; Forcioli *et al.*, 1998; Desplanque *et al.*, 2000; Fénart *et al.*, 2006). By coupling data on genotypes and sexual phenotypes, it is possible to directly identify

females, non-CMS hermaphrodites and restored CMS hermaphrodites. This paper reports the results of an investigation designed to evaluate the reproductive differences between females and the two hermaphroditic types existing within *B. vulgaris* ssp. *maritima* populations. Because this species is self-incompatible, inbreeding avoidance cannot account for potential reproductive advantage of females compared to hermaphrodites. Therefore, if any female advantage is detected, it should be exclusively due to resource reallocation. The general effects of male sterility on flower production, fruit set and seed set were examined to gain insight into patterns of resource allocation from male to female function. Specifically, by investigating sex-related differences in fitness among females, restored CMS hermaphrodites and non-CMS hermaphrodites, we tested the following hypotheses: (i) Do female individuals have a reproductive advantage compared to conspecific hermaphroditic plants? (ii) Is there any evidence for a cost of CMS genes when examining the female reproductive output of restored CMS hermaphrodites? (iii) Because previous studies have suggested somewhat complex patterns of restoration determination and an incomplete restoration of male fertility in some restored CMS hermaphrodites in the wild (Dufay *et al.*, 2008), we also attempted to evaluate possible differences in pollen production between the two hermaphroditic types in controlled conditions. Our results are discussed in light of existing theoretical models.

Study species

Sea beet, *B. vulgaris* ssp. *maritima,* is a diploid species (2n=18) widely distributed along the western European coast and around the Mediterranean basin where it colonises coastal habitats just at the upper level of high tides (Laporte *et al.*, 2001; Arnaud *et al.*, 2003; Viard *et al.*, 2004; Fievet *et al.*, 2007). It is a short-lived perennial and wind-pollinated species (Letschert, 1993). *B. vulgaris* is known to be self-incompatible, with up to four gametophytic S loci (Owen, 1942; Larsen, 1977). Each individual bears one to several hundred floral stems carrying a long, dense racemose inflorescence at their apex. In addition to the main inflorescence, each floral stem also commonly develops secondary flowering axes. Fruits are the product of the clustered, joint development of several flowers that mature into a single, hard, woody seed ball. Each cluster of flowers contains one to eight flowers and, because the flowers are uniovulate, each fruit contains one to eight seeds. These aggregated fruits have no particular dispersal mechanism: seed dispersal is thought to be mainly local (Arnaud *et al.*, 2009; De Cauwer *et al.*, 2010b), although hydrochory may lead to occasional long distance dispersal events (Fievet *et al.*, 2007). An individual plant can bear few to several thousand flowers. Only some of the flowers open simultaneously along the floral stems within an individual plant. Plants flower from mid-May to mid-July.

In contrast to some other gynodioecious species, a large part of cytoplasmic diversity in *B. vulgaris* is associated with non-sterilising factors. Only four out of 20 mitochondrial haplotypes described in wild populations are clearly associated with male sterility: CMS *E*, CMS *G*, CMS *H* and CMS *Svulg* (Cuguen *et al.*, 1994; Forcioli *et al.*, 1998; Desplanque *et al.*, 2000; Fénart *et al.*, 2006). Historical relationships between CMS and non-CMS haplotypes suggest that CMS haplotypes have arisen independently, with sterilising cytoplasms belonging to different lineages derived from an ancestral non-sterilising cytoplasm (Fénart *et al.*, 2006).

Plant material

Plants surveyed in this study were derived from seeds that were collected during summer 2007 from two study sites located in Brittany (western France) and named *MOR* (N 48°34,168, E -2°34,831) and *PAL* (N 48°40,497, E -2°52,911). Individuals growing in these two sites have been exhaustively sampled, phenotyped and genotyped for another study (De Cauwer *et al.*, in prep-a). Based on these previous investigations, the cytotypes and sexual phenotypes of mother plants used in the present study were known. We chose to focus on one particular CMS cytotype: CMS *E*, which is also the most common male-sterile cytotype in natural populations (see Dufay *et al.*, 2009). Seeds used in this study came from 29 mother plants from *MOR* (9 females, 8 restored CMS hermaphrodites and 12 non-CMS hermaphrodites) and 28 mother plants from *PAL* (3 females, 7 restored CMS hermaphrodites and 18 non-CMS hermaphrodites). For each mother plant, 10 fruits were sown in October 2007 to obtain at least five offspring. These 57 progenies

were grown in a greenhouse until each individual reached a 6-leaves stage. At the end of December 2007 individuals were vernalised at 6°C for 9 weeks to induce flowering. The same vernalisation protocol was used before the second year of the study. At the end of March, pots were randomly set out in an experimental garden.

Plant characterization

The 57 progenies (mean number of offspring per progeny ± SD = 5.12 ± 1.56) were surveyed for two consecutive flowering seasons (2008 and 2009), allowing us to verify the consistency of results across years and to investigate potential sexual phenotype lability. At the onset of flowering, sexual phenotype was determined (*i.e.* female or hermaphrodite) by examining several flowers on different parts of each surveyed individual. Plants with brown or white reduced anthers were considered to be females and plants with yellow anthers with obvious pollen production were considered to be hermaphrodites. Some individuals showed intermediate phenotypes (light-coloured yellow anthers with little pollen production) and were also classified as hermaphrodites.

The cytotype of each mother plant was known (CMS *E* or non-CMS). Among individuals that were expressing a hermaphroditic phenotype, it thus was possible to discriminate restored CMS-hermaphrodites and non-CMS hermaphrodites. Among all the individuals that flowered at least once time (2008 and/or 2009) we obtained 37 females, 103 restored CMS hermaphrodites and 134 non-CMS hermaphrodites (N_{TOT} = 274 flowering individuals). Restoration rates (*i.e.* proportion of individuals exhibiting a hermaphroditic phenotype among plants carrying a CMS cytotype) were calculated within CMS progenies. In addition, for each of the 57 progenies, the number of flowering individuals was counted to calculate a progeny flowering rate. Survivorship was also determined by a census in May 2009, two years after planting, allowing us to calculate a survival rate for each progeny.

Temporal survey and flower production

All plants were monitored during the entire flowering season in 2008 and 2009. This survey started on 13 May 2008 and lasted 62 days the first year of the study. In the second study year, flowering started on 18 May 2009 and lasted 63 days. For each individual, the dates of the onset and end of flowering were noted and the duration of flowering was calculated. Additionally, the total number of flowers as well as the total number of flower clusters (potential fruits) produced during the whole flowering season were counted on a subsample of plants in 2009.

For each individual, the largest floral stem was chosen for temporal measurements of male and female fitness. Every 8 days, starting from the onset of flowering, the number of flowers and the number of flower clusters that opened along the main floral stem were counted. Each assessed stem section was marked. For each individual, one to three consecutive sections were obtained, depending on the duration of flowering. Monitoring was standardised for all individuals,

allowing us to compare male and female traits among plants (see below) and providing some temporal information for the analysis of these traits.

Fruit and seed production

Mid-August, several weeks after the end of flowering, ripe fruits were selectively collected between the marks along the main floral stem. We then calculated fruit set as the number of fruits relative to the number of flower clusters that were initially produced, for each plant and each temporal section. Given the structure of fruits in *B. vulgaris*, it was not possible to directly count the number of seeds per fruit to assess seed set. Measuring the germination rates for the collected fruits — by counting the number of seedlings that emerged from fruits — was the only way to estimate the number of viable seeds per fruit. To compare germination of seeds produced by the three different sexual phenotypes, 1 to 20 fruits from the first temporal section and 1 to 10 fruits for the second and third sections were sown, according to individual fruit production. For mother plants that produced more than 20 fruits in the first temporal section or more than 10 fruits in the following sections on the main inflorescence, fruits surveyed for germination were chosen randomly. Overall, we sowed 1483 fruits in 2008 (203 produced by females, 400 produced by restored CMS hermaphrodites and 880 produced by non-CMS hermaphrodites) and 4360 fruits in 2009 (681 produced by females, 1941 produced by restored CMS hermaphrodites and 1738 produced by non-CMS hermaphrodites). After monitoring for 2 months and removing all seedlings that germinated, all fruits were stored in dry conditions at room temperature for 4 weeks, to break dormancy. Final germination rates were determined after a second 2 months survey. We then estimated seed set as the ratio between the number of seedlings and the estimated number of available ovules, for each plant and each temporal section. The initially number of available ovules was estimated by multiplying the number of sown fruits by the mean number of flowers per flower cluster in the temporal section (each flower being uniovulate). This estimator of seed set not only describes seed production, but also germination ability.

Finally, a combined estimator of female fitness was computed for the data generated in 2009, by multiplying the total number of flower clusters (potential fruits produced during the whole flowering season), fruit set in the first temporal section along the main floral stem and seed set in the first temporal section along the main floral stem for a subsample of individuals (11 females, 40 restored CMS hermaphrodite and 30 non-CMS hermaphrodites).

Pollen production

Pollen production was determined for all flowering individuals. For each plant and for each temporal section, two nearly opened buds localised on the main floral stem were collected and dissected to obtain two anthers per bud. Anthers were stored separately in 95% ethanol until pollen counts were performed. Details on the counting procedure and the utilisation of the particle counter [CASY® model TT (Innovatis, Bielefeld)] are described in Dufay *et al*. (2008). The number of detected particles was determined for 400 size classes ranging from 0.125 to 50 µm. Prior observations showed that non-viable pollen grains in *B. vulgaris* ssp. *maritima* are smaller

than viable pollen grains (Dufay *et al.*, 2008). Typical peaks of particles with sizes ranging from 10 to 24 µm were observed in samples collected from hermaphrodites. These peaks did not occur in blank samples. As a result, we only considered this 10-24 µm zone for pollen counts. Two sub-peaks were recognisable (10-13.5 µm and 13.5-24 µm), with some individuals specifically producing one of the two peaks and others producing both. We categorized particles within these two size classes, and considered three variables for subsequent analyses: (i) total pollen quantity, (ii) proportion of large pollen grains (13.5-24 µm) and (iii) quantity of large pollen grains. The latter has been shown to affect the number of sired seedlings in the natural populations of *Beta vulgaris* (De Cauwer *et al.*, in prep-b). The total number of pollen grains and the number of large pollen grains were obtained from the values provided by the particle counter after correcting for the dilution ratio. Pollen production was assessed for 9 females, 26 restored CMS hermaphrodites and 49 non-CMS hermaphrodites in 2008 and for 36 females, 98 restored CMS hermaphrodites and 126 non-CMS hermaphrodites in 2009. Four different counts (one per anther) were obtained for each individual within each temporal section, yielding a total number of 1089 samples for the 2008 flowering season and 3463 for the 2009 flowering season. Within each of the three temporal sections, the mean and the coefficient of variation (ratio of the standard deviation to the mean) of the four counts were calculated prior to statistical analyses. Mean proportion of viable pollen grains was arcsine-root square transformed to improve the normality of the residuals. Finally, to compare the overall investment in male function between the two hermaphroditic types, a combined estimator of male fitness was computed for the data generated in 2009 by multiplying the total number of flowers produced during the whole flowering season and the number of viable pollen grains produced per flower during the first temporal section along the main floral stem.

In order to verify that the proportion of large pollen grains was a reliable estimator of the proportion of viable pollen grains, pollen viability was also estimated using Alexander staining (Alexander, 1969) for the first temporal section on a subsample of individuals. To do so, we used anthers that were not collected for pollen counts. Within three hours of collection, pollen was removed from the anthers and placed on a glass slide. One drop of Alexander solution (10 mL 95% ethanol, 1 mL 1% malachite green in 95% ethanol, 5 g of phenol, 5 mL 1% acid fuschin in H_2O, 0.5 mL 1% orange G in H_2O, 2 mL glacial acetic acid, 25 mL glycerol and 50 mL water), which stains viable pollen purple, was added to each pollen sample. The pollen samples were then examined under a light microscope at x100 magnification. Two hundred pollen grains per sample, when available, were scored as either purple or green, and the proportion of viable pollen grains was calculated as the ratio of purple-stained pollen grains to the total number of pollen grains.

Data analyses

Data generated in 2008 and 2009 were analysed separately. For variables describing the families (survival rates, flowering rates and restoration rates for CMS families, see table 1), we tested for an effect of the sexual phenotype of the mother plant and population using a general linear model. Survival rates, flowering rates (measured in 2008 and 2009) and restoration rates within CMS families were arcsine-root square transformed to improve normality of data.

For all variables describing individual fitness for the reproductive traits listed above (fruit set, seed set and pollen production descriptors, see table 1), the purpose of the study was to compare sexual phenotypes, while controlling for possible population and mother plant effects. However, because of the particular determination of sex in gynodioecious *B. vulgaris*, it was not possible to build complete statistical models that could simultaneously test the effect of population (*MOR* and *PAL*), identity of the mother plant (57 mother plants) and the sexual phenotype of the individual (female, restored CMS hermaphrodite and non-CMS hermaphrodite). This is because male sterility is maternally inherited, and therefore the sex of an individual is not independent of the identity of the mother plant: non-CMS mother plants produce only non-CMS hermaphrodites, while CMS mother plants produce both females and restored CMS hermaphrodites. As a consequence, instead of using a single test, the effect of sexual phenotype was assessed by working on subsamples of the data sets including only two sexual phenotypes. For each study year, three different data sets were thus generated: (i) a data set including non-CMS hermaphrodites and restored CMS hermaphrodites, (ii) a data set including non-CMS hermaphrodites and females and (iii) a data set including females and restored CMS individuals.

Two different statistical models were then used: one for comparisons involving individuals carrying different cytotypes (non-CMS hermaphrodites *vs.* restored CMS hermaphrodites and non-CMS hermaphrodites *vs.* females) and another one for comparisons of individuals carrying the same cytotype (females *vs.* restored CMS individuals). In the first case, we tested for effect of population of origin, mother plant cytotype and mother plant identity (nested within the interaction between population and mother plant cytotype). As we used truncated data sets including only two sexual phenotypes, testing for mother plant cytotype (CMS *vs*. non-CMS) also directly tested for an effect of the individual sexual phenotype. In the second case (when comparing individuals carrying the same cytotype, *i.e.* females and restored CMS hermaphrodites), it was possible to test directly for an effect of population, mother plant identity (nested within population) and sexual phenotype of the individual. In these models, plant sexual phenotype (or mother plant cytotype, depending on the model) and mother plant population were treated as fixed factors, while mother plant identity was treated as a random factor.

Procedures used for statistical comparisons between the three sexual phenotypes also depended on whether there were some replicate measurements per plant. For variables consisting of single data points per plant (Table 1) comparisons between sexual phenotypes were performed using general linear models. When within-plant replicates were available (Table 1), we used repeated-measurement general linear models. In the particular case of variables describing pollen production (*i.e.* total pollen quantity, quantity of large pollen grains and ratio of large pollen grains, as well as the associated coefficients of variation), comparisons only involved non-CMS hermaphrodites and restored CMS hermaphrodites. All analyses were performed using SAS (PROC GLM).

Table 1: Summary of all the variables used to characterize phenology, female fitness and male fitness in *B. vulgaris* in the current study, along with the number of progenies (**a**) and the number of individuals (**b**) that were used in statistical analyses for the two consecutive study years (2008 and 2009). CV: coefficient of variation. [a]: variables consisting of single data points per plant. [b]: variables for witch three within-plant replicates were available along the main floral stem.

(a) Number of progenies

Variable	Number of progenies derived from non-CMS mother plants	Number of progenies derived from restored-CMS mother plants	Number of progenies derived from female mother plants
Flowering rate (2008)	27	15	11
Flowering rate (2009)	30	15	12
Restoration rate	30	15	12
Survival rate	30	15	12

(b) Number of individuals

Variable	Number of non-CMS hermaphrodites	Number of restored CMS hermaphrodites	Number of females
Flowering onset (2008)[a]	49	26	9
Flowering onset (2009)[a]	127	100	36
Flowering duration (2008)[a]	13	10	4
Flowering duration (2009)[a]	127	100	36
Total number of flowers (2009)[a]	40	30	11
Total number of flower clusters (2009)[a]	40	30	11
Number of flowers along the main floral stem (2008)[b]	27	18	7
Number of flowers along the main floral stem (2009)[b]	67	66	23
Mean number of flowers per cluster along the main floral stem (2008)[b]	27	18	7
Mean number of flowers per cluster along the main floral stem (2009)[b]	67	66	23
Fruit set (2008)[b]	35	22	7
Fruit set (2009)[b]	67	66	23
Seed set (2008)[b]	30	14	6
Seed set (2009)[b]	30	39	10
Estimation of overall female fitness (2009)[a]	40	29	11
Mean number of pollen grains (2008)[b]	35	21	7
Mean number of pollen grains (2009)[b]	107	82	34
CV of the number of pollen grains (2008)[b]	35	21	7
CV of the number of pollen grains (2009)[b]	107	82	34
Mean proportion of large pollen grains (2008)[b]	35	21	7
Mean proportion of large pollen grains (2009)[b]	107	82	34
CV of the proportion of large pollen grains (2008)[b]	35	21	7
CV of the proportion of large pollen grains (2009)[b]	107	82	34
Mean number of large pollen grains (2008) [b]	35	21	7
Mean number of large pollen grains (2009) [b]	107	82	34
CV of the number of large pollen grains (2008)[b]	35	21	7
CV of the number of large pollen grains (2009)[b]	107	82	34
Proportion of viable pollen grains - Alexander stain (2008)[a]	47	23	-
Proportion of viable pollen grains - Alexander stain (2009)[a]	120	84	-
Estimation of overall male fitness (2009)[a]	40	30	-

RESULTS

Among the total number of available plants (N = 293, mean number of offspring per progeny ± SD = 5.12 ± 1.56), nearly all flowered during at least one flowering season (N = 274). The proportion of flowering individuals was quite low in 2008 compared to 2009 (0.57 in 2008 and 0.96 in 2009). In both years, progeny flowering rates did not depend on the population of origin or on the sexual phenotype of the mother plant (Table 2). Similarly, progeny survivorship, determined by a census two years after sowing, was not influenced by the population of origin or by the sexual phenotype of the mother plant (Table 2). All flowering individuals were phenotyped in the experimental garden and measurements of pollen production were used to verify the assigned sexual phenotypes (see below). The 274 flowering individuals included 37 females, 103 restored CMS hermaphrodites and 134 non-CMS hermaphrodites. Therefore, 74% of individuals carrying a CMS gene expressed a hermaphroditic phenotype. Progeny restoration rates were calculated as the proportion of restored CMS hermaphrodites within progenies from females and restored CMS hermaphrodites and varied from 0.2 to 1. Interestingly, the sexual phenotype of the mother plant had a significant effect on family restoration rates (Table 2): female mother plants produced progenies with lower restoration rates than CMS hermaphrodite mother plants (mean ± SD: 0.61 ± 0.29 for female mother plants and 0.83 ± 0.23 for restored CMS hermaphroditic mother plants).

Table 2: Results of general linear models testing for the effect of population and sexual phenotype of the mother plant on flowering rate, restoration rate and survival rate calculated within the 57 studied progenies. All three rates were arcsine-root square transformed to improve the normality of the residuals. Flowering rates were calculated for the two flowering seasons (2008 and 2009). Survivorship was determined by a census two years after planting. All interactions were non-significant and were dropped from statistical analyses. Significant P-values ($P < 0.05$) are shown in bold.

Variable	**Source of variation**	df	MS	F	*P*
Flowering rate (2008)	Mother Plant Sex	2	0.1525	0.75	0.4776
	Population	1	0.0336	0.17	0.6861
	Error	53	0.2035		
Flowering rate (2009)	Mother Plant Sex	2	0.0180	1.47	0.2392
	Population	1	0.0330	2.70	0.1064
	Error	53	0.0122		
Restoration rate	Mother Plant Sex	2	0.8534	7.32	**0.0123**
	Population	1	0.4166	3.57	0.0708
	Error	53	0.1166		
Survival rate	Mother Plant Sex	2	0.0215	0.39	0.6815
	Population	1	0.0002	0	0.9469
	Error	53	0.0555		

Temporal survey and flower production

Because flowering duration and flowering synchrony potentially have important effects on individual fitness, flowering phenology was investigated in the three sexual phenotypes (females, restored CMS hermaphrodites and non-CMS hermaphrodites). On average, individuals flowered for 35.54 days in 2008 and for 35.84 days in 2009. The three sexual phenotypes were statistically indistinguishable with regard to the date of flowering onset and total flowering duration (Table 3).

The number of flowers and the number of flower clusters that opened along the main floral stem were counted every 8 days for all flowering individuals. Overall, the mean number of flowers produced per day along the main floral stem (± SD) was 5.39 ± 3.15 in 2008 and 5.26 ± 3.30 in 2009. The mean number of flower clusters produced per day along the main floral stem (± SD) was 2.55 ± 1.34 in 2008 and 3.22 ± 1.56 in 2009. Repeated-measurement analyses were conducted on all individuals for which three temporal measurements were available (see Table 1). Females appeared to produce significantly more flowers than the two hermaphroditic types in 2008 and more flowers than non-CMS hermaphrodites in 2009 (Table 4 and Figure 1). In both study years, the two hermaphroditic types could not be differentiated with regard to flower production. Given that *B. vulgaris* flowers are uniovulate, flower production is directly correlated with ovule production. Our results thus suggested that females may have some advantage over hermaphrodites (regardless of their cytotype) in terms of ovule production. However, the three sexual phenotypes were statistically similar in terms of mean number of flowers per flower cluster in both flowering seasons (Table 4 and Figure 1). Both the number of flowers and the mean number of flowers per flower cluster decreased over time, with a continuous decrease of both variables from the first to the third temporal section along the main floral stem (Figure 1).

The total number of flower clusters and total number of flowers (produced during the whole 2009 flowering season and assessed for a subsample of 81 individuals) was slightly greater in females compared to hermaphroditic plants, although non-significant (see Table 3). The mean number (± SD) of flower clusters was 819 ± 410 for females, 789 ± 406 for restored CMS hermaphrodites and 718 ± 437 for non-CMS hermaphrodites. The mean number (± SD) of flowers was 1181 ± 470 for females, 1136 ± 480 for restored CMS hermaphrodites and 1076 ± 568 for non-CMS hermaphrodites.

Table 3: Results of general linear models carried on two descriptors of flowering phenology (flowering onset and flowering duration), the total number of flowers and total number of flower clusters (produced during the entire 2009 flowering season). Results are presented for the two successive years of survey (except for flower counts that were performed only in 2009). Significant *P*-values ($P < 0.05$) are shown in bold.

		2008				2009			
Variable	**Source of variation**	**df**	**MS**	**F**	***P***	**df**	**MS**	**F**	***P***
(a) Comparison between non-CMS hermaphrodites and CMS hermaphrodites									
Flowering onset	Population	1	72.40	0.27	0.6033	1	368.78	3.97	**0.0496**
	Cytotype	1	187.66	0.71	0.4030	1	39.06	0.42	0.5180
	Mother Plant (Population * Cytotype)	49	289.87	1.52	**0.0407**	54	99.53	1.35	0.0778
	Error	98	190.94			171	73.86		
Flowering duration	Population	1	1.48	0.01	0.9068	1	15.85	0.19	0.6673
	Cytotype	1	119.84	1.13	0.2968	1	1.55	0.02	0.8929
	Mother Plant (Population * Cytotype)	25	113.42	2.06	0.0960	54	86.23	1.05	0.4023
	Error	12	55.13			174	82.37		
Total number of flowers	Population					1	297279	0.94	0.3373
	Cytotype					1	11428	0.04	0.8505
	Mother Plant (Population * Cytotype)					35	341116	1.77	0.0532
	Error					32	192768		
Total number of flower clusters	Population					1	77235	2.62	0.1134
	Cytotype					1	6004	0.20	0.6541
	Mother Plant (Population * Cytotype)					34	33224	3.02	**0.0011**
	Error					32	11009		
(b) Comparison between non-CMS hermaphrodites and females									
Flowering onset	Population	1	46.88	0.17	0.6779	1	269.66	2.82	0.0972
	Cytotype	1	268.60	1.00	0.3203	1	17.16	0.18	0.6685
	Mother Plant (Population * Cytotype)	48	283.92	1.42	0.0809	45	102.99	1.28	0.1463
	Error	82	199.95			116	80.31		
Flowering duration	Population	1	94.34	0.85	0.3673	1	154.28	1.61	0.2090
	Cytotype	1	57.53	0.52	0.4793	1	18.41	0.20	0.6564
	Mother Plant (Population * Cytotype)	21	117.13	2.18	0.1466	46	106.64	1.46	0.0537
	Error	7	53.67			118	73.05		
Total number of flowers	Population					1	912385	2.90	0.0996
	Cytotype					1	27282	0.09	0.7705
	Mother Plant (Population * Cytotype)					25	337742	2.02	0.0928
	Error					13	166951		
Total number of flower clusters	Population					1	70736	2.39	0.1337
	Cytotype					1	9247	0.31	0.5804
	Mother Plant (Population * Cytotype)					24	31662	1.88	0.1181
	Error					13	16827		
(c) Comparison between CMS hermaphrodites and females									
Flowering onset	Population	1	7.60	0.04	0.8464	1	5.65	0.07	0.7966
	Sexual Phenotype	1	283.02	0.82	0.3799	1	103.37	1.50	0.2230
	Mother Plant (Population)	17	145.98	0.42	0.9549	25	85.59	1.24	0.2197
	Error	15	345.71			108	68.81		
Flowering duration	Population	1	108.25	4.42	0.1703	1	34.61	0.65	0.4275
	Sexual Phenotype	1	120.05	4.90	0.1573	1	15.57	0.28	0.5963
	Mother Plant (Population)	6	59.21	2.42	0.3213	25	53.36	0.97	0.5159
	Error	2	24.50			108	55.17		
Total number of flowers	Population					1	15663	0.06	0.8016
	Sexual Phenotype					1	371472	1.91	0.1783
	Mother Plant (Population)					21	276332	1.42	0.1934
	Error					27	194527		
Total number of flower clusters	Population					1	24259	1.30	0.2611
	Sexual Phenotype					1	1855	0.13	0.7258
	Mother Plant (Population)					21	21099	1.43	0.1899
	Error					27	14775		

Table 4: Results of repeated-measures analyses carried on the number of flowers and the mean number of flowers per flower cluster produced along the main floral stem. Results are presented for the two consecutive years of study: 2008 and 2009. Significant *P*-values ($P < 0.05$) are shown in bold.

			2008				2009			
Variable		**Source of variation**	**df**	**MS**	**F**	***P***	**df**	**MS**	**F**	***P***
(a) Comparison between non-CMS hermaphrodites and CMS hermaphrodites										
Number of flowers	Between	Population	1	54.8	0.14	0.7088	1	1387	1.91	0.1710
		Cytotype	1	26.4	0.07	0.7953	1	1343	1.85	0.1778
		Mother Plant (Population*Cytotype)	31	684.9	1.77	0.0526	49	835.0	1.15	0.2867
		Error	34	386.2			81	727.0		
	Within	Time	1	1607.5	6.57	**0.0149**	2	22256	69.07	**<.0001**
		Time*Population	1	1.9	0.01	0.9294	2	927.3	2.88	0.0591
		Time* Cytotype	1	39.4	0.16	0.6905	2	926.4	2.88	0.0593
		Time*Mother Plant (Population*Cytotype)	31	250.7	1.03	0.4700	98	458.1	1.42	**0.0239**
Mean number of flowers per cluster	Between	Population	1	0.506	1.59	0.2154	1	1.042	1.97	0.1641
		Cytotype	1	0.668	2.10	0.1563	1	0.093	0.18	0.6756
		Mother Plant (Population*Cytotype)	31	0.615	1.94	**0.0311**	49	0.843	1.59	**0.0313**
		Error	34	0.318			81	0.529		
	Within	Time	1	4.021	63	**<.0001**	2	21.823	208.25	**<.0001**
		Time*Population	1	0.067	1.05	0.3120	2	0.254	2.42	0.0922
		Time* Cytotype	1	0.003	0.04	0.8418	2	0.217	2.07	0.1292
		Time*Mother Plant (Population*Cytotype)	31	0.060	0.94	0.5618	98	0.134	1.28	0.0812
(b) Comparison between non-CMS hermaphrodites and females										
Number of flowers	Between	Population	1	757.4	2.48	0.1299	1	508.8	0.72	0.3999
		Cytotype	1	3937.0	12.87	**0.0016**	1	3174	4.50	**0.0389**
		Mother Plant (Population*Cytotype)	25	776.6	2.54	**0.0153**	37	714.2	1.01	0.4785
		Error	22	306.0			50	705.7		
	Within	Time	1	1933.9	6.21	**0.0207**	2	21242	69.83	**<.0001**
		Time*Population	1	219.5	0.71	0.4101	2	213.8	0.70	0.4976
		Time* Cytotype	1	346.7	1.11	0.3027	2	400.2	1.32	0.2729
		Time*Mother Plant (Population*Cytotype)	25	157.8	0.51	0.9486	74	466.9	1.53	**0.0231**
Mean number of flowers per cluster	Between	Population	1	0.196	0.59	0.4511	1	0.8174	1.53	0.2215
		Cytotype	1	0.037	0.11	0.7423	1	0.3646	0.68	0.4122
		Mother Plant (Population*Cytotype)	25	0.287	0.86	0.6411	37	0.856	1.61	0.0593
		Error	22	0.333			50	0.5332		
	Within	Time	1	4.388	68.06	**<.0001**	2	13.266	136.63	**<.0001**
		Time*Population	1	0.335	5.2	**0.0327**	2	0.0574	0.59	0.5557
		Time* Cytotype	1	0.322	5	**0.0359**	2	0.048	0.49	0.6116
		Time*Mother Plant (Population*Cytotype)	25	0.084	1.3	0.2692	74	0.1206	1.24	0.1555
(c) Comparison between CMS hermaphrodites and females										
Number of flowers	Between	Population	1	642.2	0.89	0.3628	1	1558	2.46	0.1220
		Sexual Phenotype	1	4298.7	5.99	**0.0307**	1	40.2	0.06	0.8020
		Mother Plant (Population)	17	664.7	0.93	0.5684	24	747.8	1.18	0.2946
		Error	12	717.5			62	633.6		
	Within	Time	1	803.3	7.23	**0.0197**	2	16547	42.01	**<.0001**
		Time*Population	1	102.9	0.93	0.3549	2	216.0	0.55	0.5792
		Time* Sexual Phenotype	1	4.9	0.04	0.8372	2	1821	4.62	**0.0116**
		Time*Mother Plant (Population)	17	298.0	2.68	**0.0439**	48	524.5	1.33	0.1062
Mean number of flowers per cluster	Between	Population	1	0.329	1.16	0.3036	1	1.9661	4.02	**0.0493**
		Sexual Phenotype	1	0.051	0.18	0.6803	1	1.6892	3.46	0.0678
		Mother Plant (Population)	17	0.767	2.69	**0.0432**	24	0.656	1.34	0.1768
		Error	12	0.285			62	0.4888		
	Within	Time	1	2.509	40.66	**<.0001**	2	11.193	107.42	**<.0001**
		Time*Population	1	0.183	2.97	0.1106	2	0.1888	1.81	0.1675
		Time* Sexual Phenotype	1	0.022	0.36	0.5585	2	0.0345	0.33	0.7186
		Time*Mother Plant (Population)	17	0.051	0.82	0.6525	48	0.1728	1.66	**0.0137**

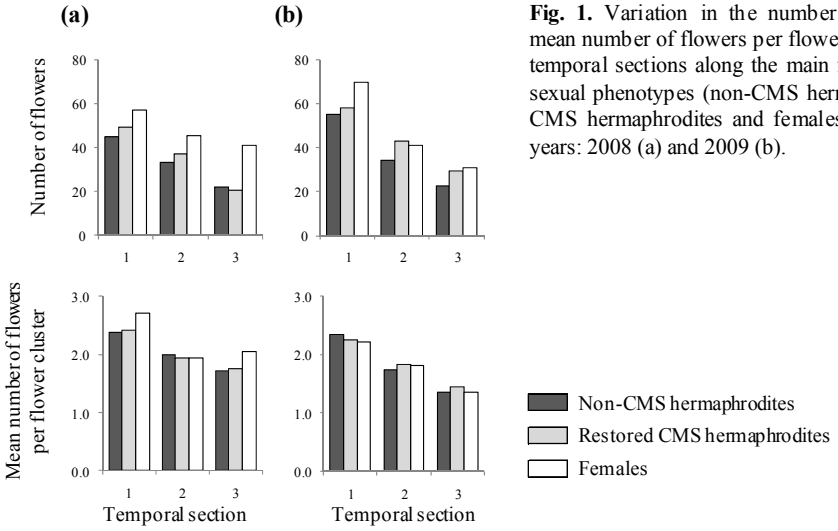

Fig. 1. Variation in the number of flowers and the mean number of flowers per flower cluster for the three temporal sections along the main floral stem, the three sexual phenotypes (non-CMS hermaphrodites, restored CMS hermaphrodites and females) and the two study years: 2008 (a) and 2009 (b).

Fruit and seed production

In *B. vulgaris*, each fruit is derived from the joint development of all flowers belonging to the same flower cluster. Based on the number of initially available flower clusters, it was thus possible to assess fruit set for each temporal section by calculating the ratio between the number of flower clusters and the number of fruits that were obtained at the end of the flowering season. Overall, 52.49% of the 3010 flower clusters observed in 2008 produced a fruit. In 2009, fruit set was also limited, with 51.01% of the 10 957 flower clusters observed producing a fruit. When investigating the effect of sexual phenotype on fruit set, non-CMS hermaphrodites appeared to have an advantage over restored CMS hermaphrodites in 2008 (Table 5 and Figure 2), while other comparisons were non-significant. This may suggest a cost of CMS genes for restored CMS hermaphrodites, although none of the three comparisons among sexual phenotypes were significant in 2009. Similar to what was observed for flower production, fruit set decreased significantly with time (Table 5 and Figure 2). Seed set, calculated as the ratio between the number of emerging seedlings and the estimated number of available ovules, was quite low for both study years (mean ± SD: 49.63% ± 19.83 in 2008 and 40.31% ± 23.40 in 2009). The vast majority of fruits germinated before dry-storage (79.84% in 2008 and 85.81% 2009), suggesting low seed dormancy. Germination rates between seeds from females, restored CMS hermaphrodites and non-CMS hermaphrodites were not statistically significant (Table 5 and Figure 2). Seed set was relatively constant across temporal sections, contrary to what was observed for fruit set (Table 5 and Figure 2).

The overall investment in female function was estimated for a subsample of individuals by multiplying the total number of flower clusters (potential fruits produced during the whole 2009

flowering season), fruit set in the first temporal section along the main floral stem and seed set in the first temporal section along the main floral stem. Although the value estimated in female plants was higher than those estimated in hermaphroditic plants, no statistical differences were detected among the three sexual phenotypes (mean ± SD; 223 ± 137 for female individuals, 192 ± 135 for restored CMS hermaphrodites and 194 ± 178 for non-CMS hermaphrodites, $P > 0.05$ for the three comparisons). The ratio of the average value observed in females to the average value observed in hermaphrodites (regardless of their cytotype) was 1.15, providing an estimate of the level of female advantage in *B. vulgaris*.

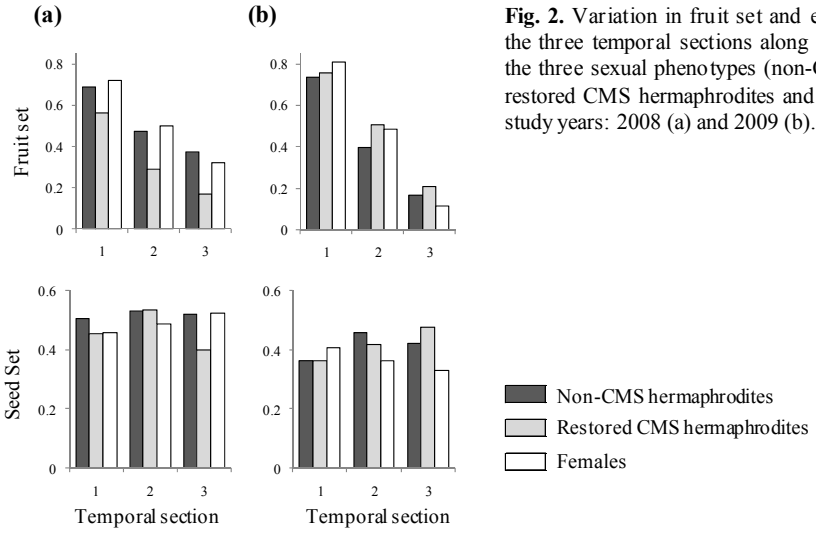

Fig. 2. Variation in fruit set and estimated seed set for the three temporal sections along the main floral stem, the three sexual phenotypes (non-CMS hermaphrodites, restored CMS hermaphrodites and females) and the two study years: 2008 (a) and 2009 (b).

Table 5: Results of repeated-measures analyses carried on seed set and fruit set measured along the main floral stem. Fruit set and seed set were arcsine-square root transformed before statistical tests. Results are presented for the two consecutive years of study: 2008 and 2009. Significant *P*-values ($P < 0.05$) are shown in bold.

			2008				2009			
Variable		**Source of variation**	**df**	**MS**	**F**	***P***	**df**	**MS**	**F**	***P***
(a) Comparison between non-CMS hermaphrodites and CMS hermaphrodites										
Fruit set	Between	Population	1	0.010	0.04	0.8392	1	0.183	0.67	0.4147
		Cytotype	1	2.807	11.74	**0.0020**	1	0.793	2.92	0.0915
		Mother Plant (Population*Cytotype)	30	0.278	1.16	0.3498	49	0.290	1.07	0.3917
		Error	27	0.239			81	0.272		
	Within	Time	1	1.952	39.16	**<.0001**	2	17.133	231.67	**<.0001**
		Time*Population	1	0.054	1.09	0.3067	2	0.009	0.12	0.8905
		Time* Cytotype	1	0.020	0.41	0.5290	2	0.147	1.98	0.1408
		Time*Mother Plant (Population*Cytotype)	30	0.038	0.77	0.7597	98	0.071	0.97	0.5709
Seed set	Between	Population	1	0.075	1.00	0.3304	1	0.175	1.88	0.1809
		Cytotype	1	0.004	0.05	0.8300	1	0.012	0.13	0.7231
		Mother Plant (Population*Cytotype)	25	0.043	0.57	0.9048	36	0.104	1.11	0.3861
		Error	18	0.075			30	0.093		
	Within	Time	1	0.063	3.96	0.0621	2	0.110	4.73	**0.0123**
		Time*Population	1	0.002	0.14	0.7116	2	0.020	0.86	0.4270
		Time* Cytotype	1	0.009	0.54	0.4705	2	0.016	0.67	0.5168
		Time*Mother Plant (Population*Cytotype)	25	0.014	0.85	0.6556	72	0.023	1.01	0.4891
(b) Comparison between non-CMS hermaphrodites and females										
Fruit set	Between	Population	1	0.063	0.23	0.6346	1	0.044	0.18	0.6769
		Cytotype	1	0.177	0.66	0.4275	1	0.011	0.04	0.8331
		Mother Plant (Population*Cytotype)	25	0.224	0.84	0.6658	37	0.263	1.05	0.4294
		Error	17	0.268			50	0.250		
	Within	Time	1	1.134	32.20	**<.0001**	2	11.538	135.58	**<.0001**
		Time*Population	1	0.040	1.14	0.3015	2	0.087	1.02	0.3626
		Time* Cytotype	1	0.008	0.22	0.6448	2	0.004	0.04	0.9574
		Time*Mother Plant (Population*Cytotype)	25	0.034	0.95	0.5544	74	0.069	0.81	0.8326
Seed set	Between	Population	1	0.091	2.70	0.1265	1	0.154	2.89	0.1113
		Cytotype	1	0.039	1.14	0.3065	1	0.011	0.21	0.6551
		Mother Plant (Population*Cytotype)	23	0.049	1.44	0.2579	23	0.114	2.13	0.0723
		Error	12	0.034			14	0.053		
	Within	Time	1	0.031	1.51	0.2423	2	0.013	0.55	0.5850
		Time*Population	1	0.000	0.00	0.9603	2	0.024	1.05	0.3621
		Time* Cytotype	1	0.002	0.10	0.7624	2	0.001	0.04	0.9592
		Time*Mother Plant (Population*Cytotype)	23	0.014	0.69	0.7890	46	0.041	1.80	0.0509
(c) Comparison between CMS hermaphrodites and females										
Fruit set	Between	Population	1	0	0	0.9781	1	0.551	2.34	0.1313
		Sexual Phenotype	1	0.714	3	0.1140	1	0.178	0.76	0.3875
		Mother Plant (Population)	16	0.238	1	0.5173	24	0.269	1.14	0.3300
		Error	10	0.238			62	0.236		
	Within	Time	1	0.612	8.25	**0.0166**	2	9.702	183.77	**<.0001**
		Time*Population	1	0.005	0.07	0.7938	2	0.023	0.44	0.6467
		Time* Sexual Phenotype	1	0.002	0.03	0.8692	2	0.101	1.91	0.1522
		Time*Mother Plant (Population)	16	0.053	0.72	0.7307	48	0.065	1.24	0.1738
Seed set	Between	Population	1	0.014	0.08	0.7947	1	0.105	1.14	0.2955
		Sexual Phenotype	1	0	0	0.9944	1	0.012	0.13	0.7234
		Mother Plant (Population)	12	0.034	0.19	0.9909	21	0.107	1.16	0.3606
		Error	5	0.179			25	0.092		
	Within	Time	1	0	0.04	0.8511	2	0.030	1.43	0.2493
		Time*Population	1	0.043	8.20	**0.0353**	2	0.028	1.34	0.2722
		Time* Sexual Phenotype	1	0.037	7.07	**0.0449**	2	0.008	0.39	0.6785
		Time*Mother Plant (Population)	12	0.010	1.90	0.2478	42	0.043	2.05	**0.0076**

Pollen production

The frequency of viable pollen grains estimated using Alexander staining ranged from 0 to 1 for both study years, with important variation among individuals (mean proportion of viable pollen grains ± SD: 0.77 ± 0.75 in 2008 and 0.81 ± 0.75 in 2009). The proportions of large pollen grains (estimated with the particle counter) and of viable pollen grains (estimated with the staining method) were statistically correlated ($R_{PEARSON}$ = 0.660 in 2008 and $R_{PEARSON}$ = 0.816 in 2009, $P < 10^{-4}$ in both cases), although the correlation values were not as high as in other studies performed on the same species (see Dufay *et al.*, 2008).

Four anthers were collected along the main floral stem for each of the three temporal sections and for each flowering individual. Within each temporal section, the mean and the coefficient of variation of the four counts was calculated for three descriptors of pollen production: (i) total pollen quantity, (ii) proportion of large pollen grains and (iii) quantity of large pollen grains. Overall, six different response variables were thus used in our study (mean and coefficient of variation for each descriptor). The average quantity of pollen grains per anther varied greatly among plants, particularly in individuals carrying a CMS gene (Figure 3). Visual inspection of graphs suggest the occurrence of a threshold in pollen quantity within CMS individuals, with some plants producing very little pollen and other individuals which were at least partially restored for male fertility produced more than 7000 pollen grains per anther (Figure 3). Furthermore, these data were consistent with the sexual phenotypes that were assigned in the common garden (Figure 3). According to these results, we considered that individuals producing less than 7000 pollen grains per anther were females, as in a previous study (Dufay *et al*. 2008), and female plants were excluded from the following analysis of pollen production. The two hermaphroditic types were statistically indistinguishable with regard to the quantity of pollen (average for the four anthers collected within each temporal section) in both study years (Table 6). However, non-CMS hermaphrodites showed significantly lower coefficients of variation (among anthers, for each date) for the quantity of pollen compared to restored CMS hermaphrodites in both flowering seasons (Table 6 and Figure 5). Our results also show that the quantity of pollen in 2009 varied over time, with a decrease from the beginning to the end of the flowering season.

Different results were found in terms of pollen size. Sexual phenotype was found to have a significant effect on the proportion of large pollen grains (estimator of pollen viability), with restored hermaphrodites producing a lower proportion of large pollen grains than non-CMS ones (81% ± 15 and 65% ± 19 in 2008 and 87% ± 10 and 69% ± 22 in 2009 for non-CMS hermaphrodites and restored CMS hermaphrodites, respectively). Furthermore, as shown in Figure 4, 70% of non-CMS hermaphrodites produced more than 80% of large pollen grains, whereas only 31% of restored CMS hermaphrodites produced pollen above this threshold in 2008. A similar trend was observed the following year (84% and 44%, see Figure 4). Contrary to what was observed for the total number of pollen grains, no difference in the coefficient of variation (among anthers, for each date) of the proportion of large pollen grains was detected between the two types

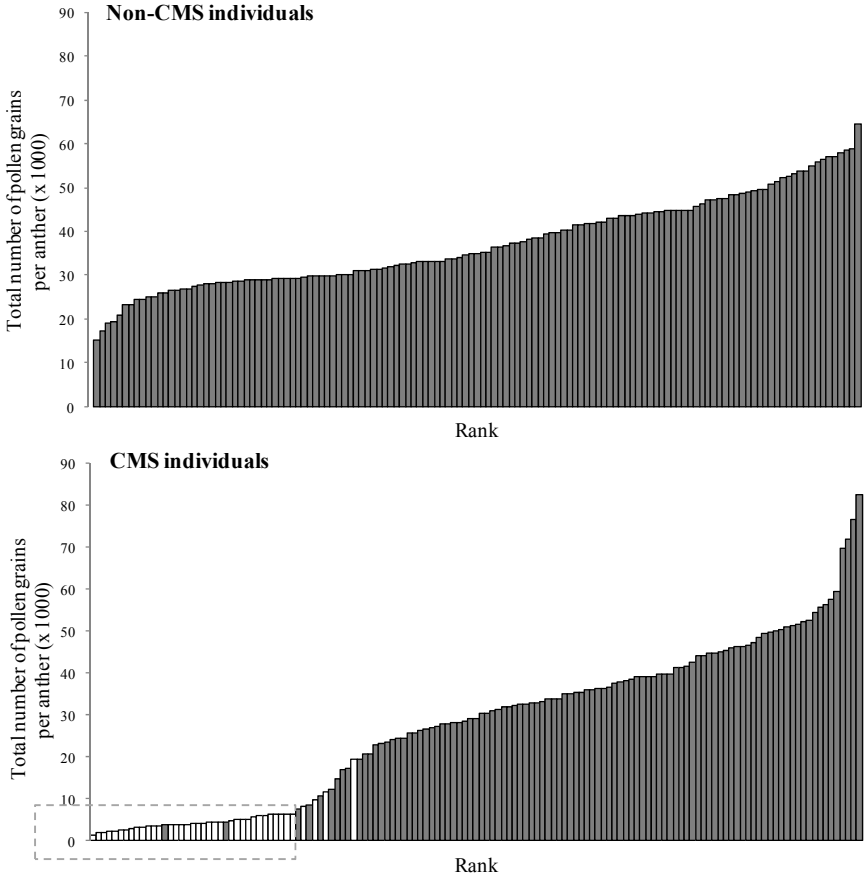

Fig. 3. Quantity of pollen (average over the four measured anthers) produced by the first flowers that opened along the main floral stem, for all studied *Beta vulgaris* ssp. *maritima* individuals. For individuals that flowered both in 2008 and 2009, the mean value over the both study years was calculated. Individuals are ranked according to the quantity of pollen per anther. Plants that were initially typed as females are represented in white, as hermaphrodites in grey. The dashed box contains all plants that were considered as females in statistical analyses (mean pollen quantity produced per anther in the first flowering week <7000 pollen grains).

of hermaphrodites. Time had no effect on the quality of pollen or on intra-individual variation of pollen quality in either study year (Table 6 and Figure 5).

The same results were found when considering pollen viability estimations obtained using the staining method on the first temporal section: non-CMS hermaphrodites produced a higher proportion of viable pollen grains than restored CMS hermaphrodites, although this effect was only marginal in 2008 ($P = 0.083$ in 2008 and $P < 10^{-4}$ in 2009). This observation, along with the significant correlation between the proportion of large pollen grains (estimated with the particle counter) and that of viable pollen grains (estimated with the staining method), confirms that the proportion of large pollen grains (size >13.5 µm) is a satisfactory estimator of viability.

Altogether, our results suggest that pollen quantity is independent of sexual phenotype and decreases during the flowering season, whereas pollen quality is constant during the flowering

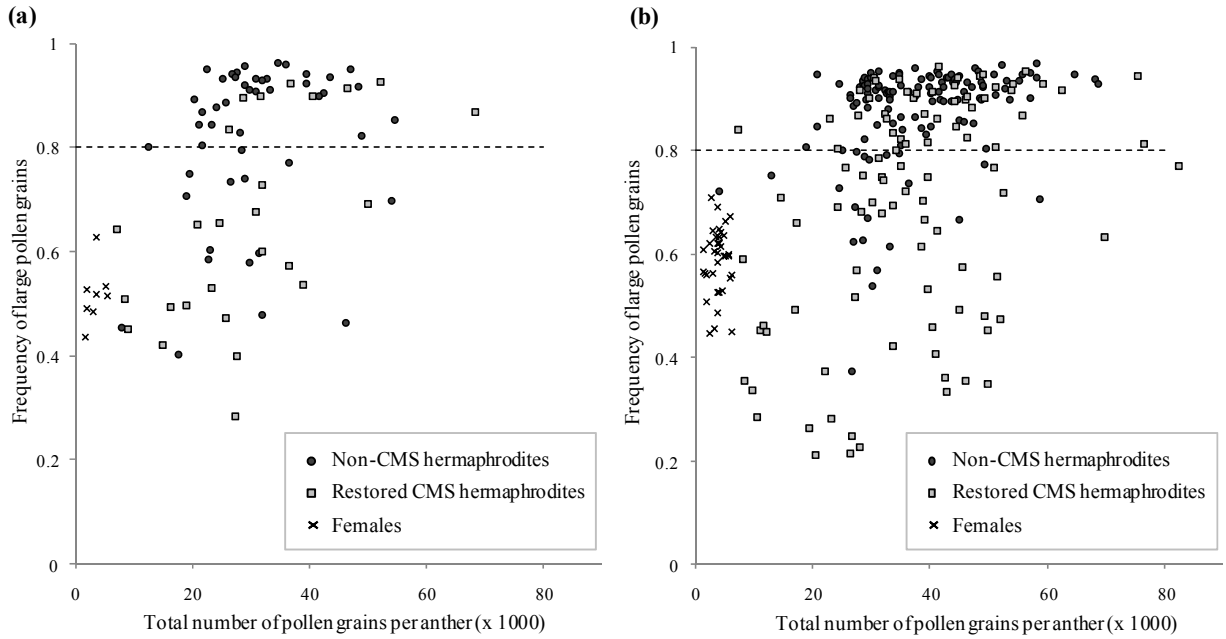

Fig. 4. Pollen quantity (mean value over the four anthers collected in the first temporal section) and pollen viability (mean frequency of large pollen grains over the four anthers collected in the first temporal section) for all studied individuals in 2008 (a) and 2009 (b). The dashed lines separate individuals producing more than 80% of viable pollen from those producing less than 80% of viable pollen.

season but varies with sexual phenotype. When combining both variables, an estimator of the number of large (viable) pollen grains per anther was obtained. As shown in Table 6, there was a significant effect of sexual phenotype on this variable as well as on its coefficient of variation. Non-CMS hermaphrodites produced a significantly higher number of large pollen grains per anther and were characterised by lower levels of intra-individual variance in both study years (Figure 5). We found that the number of pollen grains significantly decreased with time only in 2009. The coefficient of variation was positively affected by time, with an increase of intra-individual variation during the 2009 flowering season.

The overall investment in male function was estimated for a subsample of individuals by multiplying the total number of flowers produced during the whole 2009 flowering season by the mean number of viable pollen grains produced per flower (each flower containing five anthers) during the first temporal section along the main floral stem. Based on this global estimator of male fitness, restored CMS hermaphrodites produced marginally lower amounts of viable pollen than non-CMS hermaphrodites (mean ± SD; $6.36\times10^6 \pm 4.24\times10^6$ viable pollen grains for restored CMS hermaphrodites and $8.63\times10^6 \pm 5.23\times10^6$ viable pollen grains for non-CMS hermaphrodites, $P = 0.057$).

Table 6: Results of repeated-measures analyses carried on three different descriptors of pollen production for hermaphroditic plants: (i) the number of pollen grains, (ii) the proportion of large pollen grains (estimating pollen viability) and (iii) the number of large pollen grains. For each of these descriptors, the mean value and coefficient of variation (CV) were calculated over the four measured anthers. Proportion of non-viable pollen grains were arcsine-square root transformed before statistical tests. Results are presented for the two consecutive years of study. Significant *P*-values ($P < 0.05$) are shown in bold.

			2008				2009			
Variable		**Source of variation**	**df**	**MS**	**F**	***P***	**df**	**MS**	**F**	***P***
Mean number of pollen grains	Between	Population	1	1.19E+09	4.72	**0.0400**	1	1.52E+09	4.39	**0.0381**
		Cytotype	1	6.80E+08	2.69	0.1138	1	3.38E+07	0.10	0.7552
		Mother Plant (Population*Cytotype)	28	3.32E+08	1.32	0.2490	53	3.49E+08	1.01	0.4735
		Error	24	2.53E+08			128	3.46E+08		
	Within	Time	2	5.79E+07	1.20	0.3104	2	1.71E+09	21.65	**<.0001**
		Time*Population	2	9.12E+06	0.19	0.8285	2	6.21E+07	0.79	0.4562
		Time* Cytotype	2	2.01E+07	0.42	0.6623	2	1.00E+07	0.13	0.8807
		Time*Mother Plant (Population*Cytotype)	56	3.18E+07	0.66	0.9337	106	5.57E+07	0.71	0.9798
CV of the number of pollen grains	Between	Population	1	0.0002	0.01	0.9211	1	0.0160	0.52	0.4708
		Cytotype	1	0.1544	9.60	**0.0049**	1	0.6200	20.27	**<.0001**
		Mother Plant (Population*Cytotype)	28	0.0283	1.76	0.0814	53	0.0273	0.89	0.6752
		Error	24	0.0161			128	0.0306		
	Within	Time	2	0.0542	3.69	**0.0324**	2	0.0540	2.72	0.0675
		Time*Population	2	0.0083	0.57	0.5714	2	0.0099	0.50	0.6077
		Time* Cytotype	2	0.0181	1.24	0.2998	2	0.0229	1.15	0.3170
		Time*Mother Plant (Population*Cytotype)	56	0.0126	0.86	0.7078	106	0.0212	1.07	0.3336
Mean proportion of large pollen grains	Between	Population	1	0.0897	1.05	0.3167	1	0.4928	8.18	**0.0049**
		Cytotype	1	1.3281	15.48	**0.0006**	1	4.0082	66.57	**<.0001**
		Mother Plant (Population*Cytotype)	28	0.0953	1.11	0.3999	53	0.1630	2.71	**<.0001**
		Error	24	0.0858			128	0.0602		
	Within	Time	2	0.0237	1.30	0.2809	2	0.0349	2.84	0.0602
		Time*Population	2	0.0117	0.65	0.5287	2	0.0626	5.09	**0.0068**
		Time* Cytotype	2	0.0182	1.00	0.3736	2	0.0080	0.65	0.5216
		Time*Mother Plant (Population*Cytotype)	56	0.0118	0.65	0.9392	106	0.0189	1.53	**0.0034**
CV of the proportion of large pollen grains	Between	Population	1	0.0161	0.27	0.6069	1	0.3115	9.06	**0.0032**
		Cytotype	1	0.0387	0.66	0.4260	1	0.0077	0.22	0.6373
		Mother Plant (Population*Cytotype)	28	0.0878	1.49	0.1639	53	0.0518	1.51	**0.0323**
		Error	24	0.0591			128	0.0344		
	Within	Time	2	0.0242	0.51	0.6024	2	0.0448	1.58	0.2088
		Time*Population	2	0.0637	1.35	0.2691	2	0.0541	1.91	0.1509
		Time* Cytotype	2	0.1511	3.20	**0.0496**	2	0.0572	2.01	0.1358
		Time*Mother Plant (Population*Cytotype)	56	0.0470	1.00	0.5091	106	0.0332	1.17	0.1605
Mean number of large pollen grains	Between	Population	1	1.28E+09	4.61	**0.0421**	1	3.00E+09	9.63	**0.0024**
		Cytotype	1	1.80E+09	6.49	**0.0177**	1	2.71E+09	8.69	**0.0038**
		Mother Plant (Population*Cytotype)	28	3.32E+08	1.20	0.3295	53	4.37E+08	1.40	0.064
		Error	24	2.77E+08			128	3.12E+08		
	Within	Time	2	7.14E+07	1.54	0.2251	2	1.27E+09	21.46	**<.0001**
		Time*Population	2	1.05E+07	0.23	0.7991	2	1.35E+08	2.28	0.1041
		Time* Cytotype	2	2.26E+07	0.49	0.6181	2	2.42E+06	0.04	0.9598
		Time*Mother Plant (Population*Cytotype)	56	3.06E+07	0.66	0.9335	106	6.20E+07	1.05	0.3688
CV of the number of large pollen grains	Between	Population	1	0.0072	0.28	0.6046	1	0.0160	0.45	0.5021
		Cytotype	1	0.3716	14.30	**0.0009**	1	1.0717	30.27	**<.0001**
		Mother Plant (Population*Cytotype)	28	0.0525	2.02	**0.0419**	53	0.0468	1.32	0.1046
		Error	24	0.0260			128	0.0354		
	Within	Time	2	0.0763	2.62	0.0830	2	0.0772	3.29	**0.0388**
		Time*Population	2	0.0354	1.22	0.3045	2	0.0341	1.45	0.2355
		Time* Cytotype	2	0.0411	1.42	0.2529	2	0.0396	1.69	0.1868
		Time*Mother Plant (Population*Cytotype)	56	0.0169	0.58	0.9739	106	0.0294	1.25	0.0784

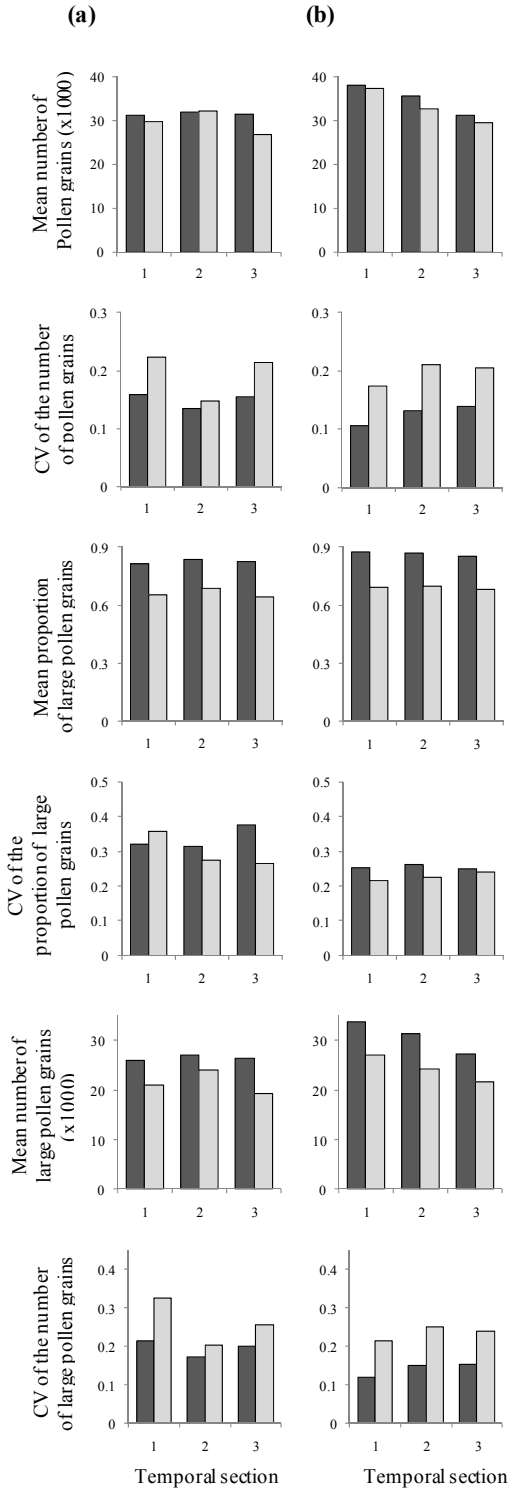

Fig. 5. Variation in the number of pollen grains, the proportion of large pollen grains and the number of large pollen grains [mean and coefficient of variation (CV) over the four measured anthers] for the three temporal sections along the main floral stem, the two sexual phenotypes (non-CMS hermaphrodites and restored CMS hermaphrodites) and the two study years: 2008 (a) and 2009 (b).

Sex lability

Because pollen counts were available for two consecutive flowering years for some plants, it was possible to investigate sex lability across time. Sexual phenotype was relatively stable across flowering seasons. All individuals that flowered during the two consecutive study years exhibited the same sexual phenotype based on their pollen production, except two individuals that were considered as restored CMS hermaphrodites in 2008 (*i.e.* individuals producing more than 7000 pollen grains per anther) and classified as females the following year. However, these two individuals were quite inefficient pollen producers in 2008 (mean numbers of pollen grains per anther for the first flowering section were 7128 and 8363), thus the difference in assigned sexual phenotypes can be attributed to a small difference in pollen production between years. Individual pollen production in the two consecutive study years were significantly correlated ($R_{PEARSON} = 0.781$, $P < 10^{-4}$ for the total number of pollen grains per anther in the first temporal section; $R_{PEARSON} = 0.707$, $P < 10^{-4}$ for the proportion of viable pollen grains in the first temporal section, $R_{PEARSON} = 0.447$, F = 17.21, $P < 10^{-4}$ for total number of viable pollen grains per anther in the first temporal section).

DISCUSSION

The maintenance of females within species exhibiting cytonuclear gynodioecy is regulated by the existence of polymorphism at cytoplasmic and nuclear sex-determining genes (CMS genes and nuclear restorers of male fertility). Theoretical models suggest that this type of polymorphism can be maintained if female plants outperform hermaphrodites in one or several aspects of female reproduction (female advantage) and if there are selective or stochastic pressures opposing the fixation of nuclear restorers (a cost of restoration, see Gouyon *et al.,* 1991 and/or metapopulation dynamics, see Couvet *et al.* 1998). In the particular case of species in which non-sterilising cytoplasms coexist with CMSs, an additional condition must be fulfilled: hermaphrodites that carry a restored CMS must have a disadvantage in female fitness compared to those that carry a non-sterilising cytotype (cost of CMS genes, see Dufay *et al*., 2007). Finally, in species in which male-fertile cytotypes occur, such as *B. vulgaris*, an additional issue can be raised: do the two hermaphroditic types differ in terms of male fitness? While a silent cost of restoration has already been suggested in *B. vulgaris* ssp. *maritima* (Dufay *et al.*, 2008), other conditions for the maintenance of cytonuclear gynodioecy have not yet been explored. By investigating sex-related differences in fitness among females, restored CMS hermaphrodites and non-CMS hermaphrodites, our study was designed (i) to explore the possible existence of a female advantage and a cost of CMS genes for several components of female reproductive output and (ii) to confirm the differences in pollen production between the two hermaphroditic types that had been suggested by previous studies.

A female advantage in B. vulgaris*?*

Female individuals are known to be common in natural populations of *B. vulgaris* ssp. *maritima*. In a previous study investigating the occurrence of gynodioecy among 33 natural populations in Brittany (western France), Dufay *et al.* (2009) found that females were present in 91% of the studied sites, with frequencies ranging from 2 to 43%. Among all theoretical models devoted to assessing the conditions of maintenance of females in gynodioecious species, regardless of how sex is genetically determined in the model, one common feature is that females must compensate for their gametic disadvantage relative to hermaphrodites through higher female fitness (e.g. Charlesworth & Ganders, 1979; Frank, 1989; Gouyon *et al.*, 1991; Couvet *et al.*, 1998; Bailey *et al.*, 2003; Dufay *et al.*, 2007). In the present study, four traits associated with female reproductive output were measured: fruit set (ratio between the number of flower clusters and the number of fruits), seed set (ratio between the number of emerging seedlings and the estimated number of available ovules), mean number of flowers per flower cluster and the number of flowers, and all these traits were measured for three consecutive temporal sections along the main floral stem. The two first traits are directly related to female fitness, whereas flower counts also describe potential differences in terms of male function (see below). Our results suggest that differences in fruit set and seed set probably do not account for the maintenance of females in

natural populations of *B. vulgaris*, because females did not perform better than hermaphrodites. The same trend was observed for the mean number of flowers per flower cluster, suggesting that fruits produced by females contain, on average, the same number of potential seeds as hermaphrodites. Nonetheless, when considering the number of flowers produced along the main floral stem, females were found to outperform both hermaphroditic types in 2008 and non-CMS hermaphrodites in 2009. As flowers of *Beta vulgaris* are uniovulate, an increase of the number of flowers directly translates into an increase of the number of available ovules, which may represent an advantage for females. The same trend was observed for the total number of flowers produced by individuals during the whole 2009 flowering season, as well as for the estimator of overall investment in female function, although not significant in either case. The latter was used to quantify the level of female advantage in *B. vulgaris* (ratio of the average value observed in females to the average value observed in hermaphrodites) providing an estimate of 1.15. Compared to other species with cytonuclear gynodioecy, in which female advantage is often significant for several different reproductive traits (reviewed in Shykoff *et al.*, 2003; Dufay & Billard, submitted), the evidence for female advantage may seem somewhat equivocal in *B. vulgaris.* Although quite uncommon in gynodioecious species, there are a number of other species in which no clear female advantage has been documented (e.g. Alonso & Herrera, 2001; Miyake *et al.*, 2009).

Several possible explanations could account for levels of female advantage as low as what was observed here in *B. vulgaris*. First, our study may have ignored the proper variables to measure in order to clearly demonstrate the occurrence of a female advantage. Observations in controlled conditions may not necessarily reflect behaviour in the wild, because fitness measures can differ greatly between laboratory and natural conditions (e.g. Dudash, 1990). Because females have been shown to perform better than hermaphrodites in harsher environments for several gynodioecious species (reviewed in Ashman, 2006), further studies of female reproductive output in *B. vulgaris* should be conducted in varying natural conditions. Additionally, female advantage may occur in traits that were not studied here. Even if female fitness was investigated in several traits, the quality of offspring produced by the different sexual phenotypes was not directly examined (although our estimator of seed set also included germination ability). Further studies measuring seedling survival and seedling size are thus needed to compare the female fitness in the three different sexual phenotypes. Moreover, adult survivorship was measured two years after sowing, while *B. vulgaris* can live at least 10 years in stable habitats (Hautekèete *et al.*, 2002). Because adult survival is a key fitness parameter in perennial species, long-term surveys of individuals should also be performed to define the lifetime reproductive output of the different sexual phenotypes.

Second, theoretical models predict that very low levels of female advantage are sufficient to maintain females in the case of cytonuclear gynodioecy (slightly > 1, see Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufay *et al.*, 2007). A slight selective advantage may therefore be difficult to detect statistically. However, all these models consider infinite populations and do not account for

the potential effects of sex-ratio structure. In natural populations of *B. vulgaris,* sexual phenotypes are classically strongly structured in space (Laporte *et al.*, 2001; De Cauwer *et al.*, 2010b). Because female reproductive output has been shown to decrease in female-biased environments in *B. vulgaris* (De Cauwer *et al.*, 2010a), as well as in various other gynodioecious species (Widen & Widen, 1990; Graff, 1999; Alonso, 2005; Zhang *et al.*, 2008), low levels of female advantage could easily be close to nil in cases where sexual phenotypes are structured in space.

Another possible explanation of our results derives from the characteristics of the mating system in our study species. Whereas females are obligate outcrossers and depend entirely upon pollen produced by hermaphrodites for reproduction, hermaphrodites in most gynodioecious species are self-compatible (Charlesworth, 1981). Avoidance of selfing and inbreeding depression has been shown to favour females over hermaphrodites in some self-compatible plant species (e.g. Chang, 2007), which could ultimately promote selection for male sterility. As *B. vulgaris* is known to be self-incompatible in the wild (Owen, 1942, Larsen, 1977, but see Arnaud et al. 2010 for cultivated accessions), inbreeding avoidance cannot confer an advantage to females, which may limit the magnitude of female advantage (Dufay & Billard, submitted).

Finally, non-equilibrium processes can have important effects on the spatial distribution and the maintenance of sex-determining genes, both through recurrent introduction of new CMS cytotypes *via* mutation (e.g. Belhassen *et al.*, 1993) and founder events (Couvet *et al.*, 1998). Since previous studies performed on *B. vulgaris* on large geographical scales have shown that females are always associated with the four known sterilizing cytoplasms (Dufay *et al.*, 2009), these CMS genes probably did not arise recently. *B. vulgaris* is typically found along the coast, where populations are potentially subject to high tides and storm disturbances. Several empirical studies have indeed suggested that recurrent extinctions and founder events may be common within natural *B. vulgaris* population (Laporte *et al.*, 2001; De Cauwer *et al.*, 2010b), which may result in frequent mismatches between CMS genes and restorer alleles. Whether females could be maintained in recurrently disturbed populations without benefitting from a fitness advantage (or benefitting from a very low advantage) remains an open question, and needs to be investigated theoretically.

A cost of CMS genes?

In gynodioecious species where non-sterilising cytotypes coexist with CMS genes, restored CMS hermaphrodites are theoretically expected to show a decrease in female reproductive output compared to non-CMS hermaphrodites. This condition allows non-CMS cytotypes to be maintained in the population even if they are never associated with a female advantage (Dufay *et al.*, 2007). This difference in female fitness can have at least two proximal (not easily distinguishable) causes: a cost of expressed restorer alleles acting on female fitness and/or a cost of CMS genes that is not compensated by female advantage in restored CMS hermaphrodites. Because very few gynodioecious species have been found to contain both CMS and non-CMS cytotypes, this condition can only be verified in a restricted number of cases. To date, *Raphanus*

sativus is the only documented case in which non-CMS hermaphrodites outperform restored CMS hermaphrodites (Miyake *et al.*, 2009).

In the current study, restored CMS hermaphrodites did not outperform non-CMS hermaphrodites for any of the measured traits associated with female fitness, except for fruit set in the 2008 flowering season. However, as it was not detected in the second year of the study, this trend is inconclusive. As for female advantage, the low performance of CMS hermaphrodites could also have been overlooked because the fitness parameters were measured in non-natural conditions or because differences occur through other components of female fitness. However, because the female advantage, if present, is apparently very low in *B. vulgaris*, only a small difference between the two types of hermaphrodites is theoretically necessary to maintain the polymorphism within a population (Dufay *et al.*, 2007), and may be, again, particularly difficult to detect experimentally.

Restoration of male fertility and the dynamics of cytonuclear gynodioecy

Although theoretical models generally consider very simple genetic determination for restoration of male fertility (but see Frank, 1989; Bailey & Delph, 2007), empirical studies of the genetics of restoration have repeatedly rejected this assumption (e.g. Charlesworth & Laporte, 1998; Koelewijn, 2003; Ehlers *et al.*, 2005). A complex determination of restoration means that some restored CMS hermaphrodites may be only partially restored. Partial restoration probably could decrease pollen production and affect the degree of selection for restorer alleles in natural populations. In a previous study, Dufay *et al.* (2008) showed that pollen quality varies quantitatively among restored CMS hermaphrodites in *B. vulgaris* and that restored CMS hermaphrodites are inferior to non-CMS hermaphrodites in terms of pollen quality. However, because this previous study was conducted in natural populations, it was not possible to determine whether a part of these results were due to some micro-environmental variation or to age differences among the surveyed plants.

The present study characterised pollen production in a standardised environment, using plants of the same age and controlling for the potential effect of flowering time on male function. Our results confirmed the previous observations made by Dufay *et al.* (2008): pollen quality varied importantly among restored CMS hermaphrodites and restored CMS hermaphrodites were inferior to non-CMS hermaphrodites in terms of pollen quality. Interestingly, these trends remained when taking into account the total flower production, suggesting that pollen production differences probably directly translate into differences in male reproductive output. Indeed, a previous study using a paternity analysis approach in *B. vulgaris* strongly suggested that pollen viability affects the number of sired seedlings in the natural populations (De Cauwer *et al.*, in prep-b). In addition, we found that sexual phenotype not only affected the mean quality (number of large pollen grains), but also the intra-individual variance in the number of large pollen grains. The number of large pollen grains produced by restored CMS hermaphrodites during a given temporal section was, on average, not only lower than what was observed for non-CMS hermaphrodites, but also more

variable. The existence of intermediate phenotypes has been documented in other species, with individuals carrying flowers with non-dehiscent and/or less numerous anthers, producing lower quantity and/or quality of pollen (e.g. Koelewijn & van Damme, 1996; Poot, 1997) or carrying a mixture of female and perfect flowers (e.g. Shykoff, 1992; Lopez-Villavicencio *et al.*, 2005). Similar to female performance, the efficiency of restorer alleles may also vary from locality to locality as a consequence of environmental variation. A decrease in the efficiency of restorer alleles in harsh environments could further favour the maintenance of females.

The differences in pollen quality between the two types of hermaphrodites directly resulted from a high inter-individual variance among restored CMS hermaphrodites, with some of these individuals producing pollen of very low quality while others produced pollen equivalent to non-CMS hermaphrodites. This was true in both study years, as plant sexual phenotype did not vary across flowering seasons. This suggests that restoration of male fertility is not as simple as usually supposed in theoretical studies. Crossing experiments are clearly needed to elucidate the exact nature of genetic determination of restoration in *B. vulgaris*, but some general considerations can be drawn from our results.

First, even though this study was conducted on relatively small progenies and on individuals derived from two study sites where restoration rates were relatively important (79% and 50% of CMS individuals being restored hermaphrodites in *MOR* and in *PAL* respectively), the hypothesis of a recessive restorer allele fails to explain the lack of completely unrestored progenies, particularly for the progenies for which the mother plant was a female. Second, the high variation in pollen quantity and quality among CMS hermaphrodites is consistent with a polygenic determination of restoration, and the overall pollen performance of a given CMS hermaphrodite may be related to the number of restorer alleles carried by the plant. Restorer alleles may then not only offset male sterility by counteracting the action of CMS genes, but also determine the quality of pollen produced by restored hermaphrodites. Along with the results of Dufay *et al.* (2008) found in the wild, our observations suggested that some of the restored CMS hermaphrodites potentially contribute poorly to pollination in natural populations, probably as a result of such complex genetic determination of restoration.

It is not clear how continuous variation in viable pollen production would change the prediction of existing models. Dufay *et al.* (2007) showed that it is not possible to maintain male-fertile and CMS cytotypes if non-CMS hermaphrodites are systematically better pollen producers than restored CMS hermaphrodites (*i.e.* in the case of an expressed cost of restoration). However, in our case study, incompletely restored hermaphrodites may constitute a reservoir of restorers in the population until the appearance of fully restored hermaphrodites, which would efficiently produce pollen and change the population sex ratio. Such complex determination may then slow down the positive selection of restorer factors and help maintain females in populations, as recently showed by Bailey & Delph (2007).

However, the effects of population structure could potentially modify the effects of selection on restoration alleles. Sex-determining genes are classically strongly structured in space (e.g. Tarayre & Thompson, 1997; Olson & McCauley, 2002; Asikainen & Mutikainen, 2003; Alonso, 2005; Nilsson & Agren, 2006; Cuevas *et al.*, 2008; Dufay *et al.*, 2009), and this could modify the effects of selection in different ways. On the one hand, population structure at sex-determining genes should greatly limit the probability of appearance of fully restored hermaphrodites in local demes, particularly in species growing in disturbed environment, such as *B. vulgaris*, because of the increased risk of stochastic loss of restoration alleles. On the other hand, population structure may also favour restored CMS hermaphrodites over non-CMS ones, by clustering restored CMS hermaphrodites with females, as observed in a particular population of *B. vulgaris*, where restored hermaphrodites, although being poor pollen producers, sired significantly more seedlings than non-CMS hermaphrodites (De Cauwer *et al.*, 2010b). Because population structure can modify the effects selection in two very different ways, theoretical models taking into account the effects of both population structure and complex genetic determination are clearly needed to further explore the conditions of maintenance of sex polymorphism.

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CHAPITRE II

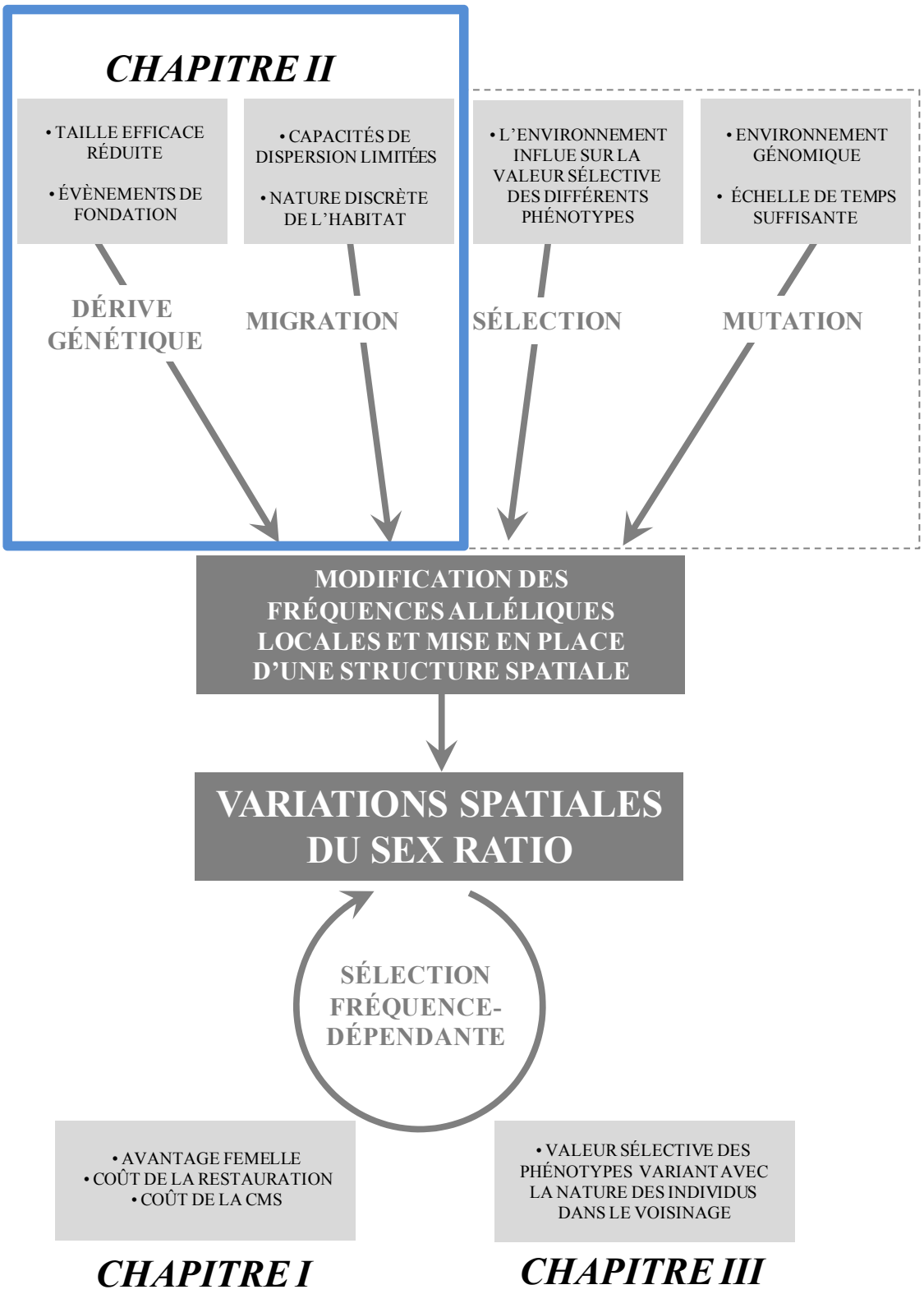
STRUCTURE GENETIQUE SPATIALE ET TEMPORELLE

Problématique : Dans quelle mesure la dérive génétique, les évènements de fondation et la migration peuvent-ils influencer la répartition spatiale de la diversité génétique neutre et des gènes associés au sexe chez* B. vulgaris *?

Par leurs actions combinées, la dérive génétique, les évènements de fondation et la migration jouent un rôle important dans la distribution de la diversité génétique et la mise en place de structure génétique spatiale. Au sein des populations gynodioïques, ces processus pourraient donc être partiellement responsables des fortes variations spatiales de sex ratio communément observées dans la nature. Les objectifs de ce chapitre sont (i) d'identifier l'échelle spatiale adéquate pour étudier la répartition de la diversité génétique en population structurée et (ii) d'obtenir des informations sur l'importance relative de ces différents processus dans la mise en place de cette structure génétique.

Dans une première partie, la répartition de la diversité nucléaire et cytoplasmique dans l'espace a été étudiée au moyen de marqueurs moléculaires hautement polymorphes, permettant d'obtenir des informations sur l'impact de la migration et des évènements de fondation dans plusieurs sites d'étude de *B. vulgaris*. La comparaison de l'arrangement spatial de la diversité génétique neutre associée à des marqueurs cytoplasmiques et à des marqueurs nucléaires à fine échelle nous a notamment permis d'obtenir une image de la part relative de la dispersion effectuée au moyen des graines et au travers des flux polliniques. Dans une deuxième partie, l'analyse de données génétiques sur plusieurs cohortes différentes nous a fourni des informations sur les conséquences de l'existence de banques de graines pérennes sur l'évolution temporelle de la diversité génétique. Finalement, cet échantillonnage temporel nous a permis d'avoir une estimation de la taille efficace des populations étudiées et donc de l'effet potentiel de la dérive génétique sur la diversité génétique.

Photo : Disposition des individus le long du trait côte au sein de l'un des sites d'étude considéré dans ce chapitre (Audresselles, Nord Pas de Calais, France).



Gynodioecy in structured populations: (I) understanding fine-scale sex ratio variation in *Beta vulgaris* ssp. *maritima*

I. DE CAUWER, M. DUFAY, B. HORNOY, A. COURSEAUX & J.-F. ARNAUD

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ABSTRACT

Among sexually polymorphic plant populations, natural selection, random processes, and gene flow are known to generate sex ratio variations. In gynodioecious species, in which hermaphrodites and females coexist, the relative effect of these processes on the maintenance of sex polymorphism is still up for debate. The aim of this study was to document sex ratio and cytonuclear genetic variation at a very local scale in wind-pollinated gynodioecious *Beta vulgaris* ssp. *maritima*, and attempt to elucidate which processes explained the observed variation. Our two study sites were characterized by geographically distinct patches of individuals and appeared to be dynamic entities, with recurrent establishment of distinct haplotypes through independent founder events. Along with substantial variation in sex ratio and unexpectedly low gene flow, our results showed a high genetic differentiation among a mosaic of genetically distinct demes, with isolation by distance or abrupt genetic discontinuities taking place within a few tens of meters. Overall, random founder events with restricted gene flow appeared to be primary determinants of sex structure, by promoting the clumping of sex-determining genes. High levels of sex structure provide a landscape for differential selection acting on sex-determining genes, which could modify the conditions of maintenance of gynodioecy in structured populations.

INTRODUCTION

Populations of sexually polymorphic plants species typically vary in sex ratio. In the particular case of gynodioecious plants, in which both hermaphroditic and male-sterile (female) individuals can be observed in natural populations, sex ratio can differ considerably among localities (e.g. Medrano *et al.*, 2005; Nilsson & Agren, 2006; Dufay *et al.*, 2009). It is generally acknowledged that variation in sex ratio among sexually polymorphic plant populations arises from (1) differential selection on the phenotypes in varying environments, (2) deterministic oscillations due to frequency-dependent selection and/or (3) non-selective processes, such as random founder events or spatially restricted migration (reviewed in McCauley & Bailey, 2009). Understanding the causes of variation in the relative frequencies of the co-occurring sexual phenotypes thus helps gain insight into the mechanisms behind the maintenance of females and hermaphrodites in natural populations.

Although poorly understood in most sexually polymorphic species, the genetic basis of sex determination is well known for several gynodioecious species. It has been shown that sex determination generally involves epistatic interactions between cytoplasmic genes for male sterility (CMS) and nuclear genes that restore male fertility (e.g. Dommée *et al.*, 1987; Boutin-Stadler *et al.*, 1989; Koelewijn & van Damme, 1995; Delph *et al.*, 2007). To develop as a female, an individual must carry an unrestored CMS gene. To develop as a hermaphrodite, an individual must either carry a CMS gene in combination with the matching restoration alleles (restored hermaphrodite), or carry a non-CMS cytoplasm (although the existence of non-sterilizing cytoplasms has not been verified in most gynodioecious species, but see Fénart *et al.*, 2006). The knowledge of the genetic basis of sex determination is an essential prerequisite for understanding the mechanisms that are responsible for sex-ratio variation.

Several studies have suggested that sex ratio can vary from locality to locality as a local response to natural selection (Vaughton & Ramsey, 2004; Nilsson & Agren, 2006; Caruso & Case, 2007). Typically, female plants are more frequent in sites subject to harsh conditions, probably due to their better seed production under stressful conditions compared to hermaphrodites (reviewed in Ashman, 2006). In addition to the effect of natural selection in spatially varying environments, theoretical models also suggest that interactions between nuclear restorers and CMS genes can lead to cyclic variations in population sex ratio, independently of ecological factors (see Gouyon *et al.*, 1991; Dufay *et al.*, 2007). In this case, a direct relationship between the relative fitness of sexual phenotypes and population sex ratio may be weak and difficult to detect (Dufay *et al.*, 2009).

In addition to the effects of natural selection, three non-selective mechanisms can also contribute to variation in sex ratio, by altering local frequencies of CMS genes and restorer alleles. First, it is well acknowledged that random founder events can cause variation in sex ratio. After a founder event, local allele frequencies, including frequencies of sex-determining genes, depend on

the number of founding seeds colonizing an unoccupied habitat as well as on the origin of these seeds (Wade & McCauley, 1988; Whitlock & McCauley, 1990). The effects of founder events are expected to be even more important in species that are subject to important extinction/recolonization dynamics (e.g. Manicacci *et al.*, 1996), because new populations may not persist long enough to allow substantial changes of founding allele frequencies through gene flow or selection. Founder effects are also expected to increase female frequencies rather than hermaphrodite frequencies for three reasons. First, because females often produce more seeds than hermaphrodites (reviewed in Shykoff *et al.*, 2003), population founders may be more likely to have female mothers. Second, due to dominance effects among nuclear restorer alleles, mating between related individuals or self-fertilization of restored CMS hermaphrodites that are heterozygous at restorer loci are expected to produce some homozygous recessive female offspring, when a new population is composed of few restored CMS hermaphrodites (Emery & McCauley, 2002; Bailey & McCauley, 2005). Finally, newly established populations are unlikely to contain all restorer alleles for the CMS types that are present in the founders, even if there are several founders coming from diverse sources (Nilsson & Agren, 2006). The second non-selective mechanism that can play a fundamental role in sex-ratio variation is dispersal, because it directly acts on the partitioning of genetic diversity within and among structured demes (Loveless & Hamrick, 1984; Hamrick & Nason, 1996; Ennos, 2001). The magnitude and spatial patterns of gene flow through pollen and seed can thus have crucial effects on the distribution of sexes in space. Indeed, dispersal rates among established populations determine the probability of sex-determining genes (CMS genes and restorer alleles) to establish in populations and theoretical models suggest that pollen and seed dispersal among structured populations can modify the dynamics expected under selection only (Dufay & Pannell, 2010). The third non-selective mechanism is genetic drift. In small populations, genetic drift can override selective processes, as suggested by studies showing that population size can have an effect on sex ratio through the fixation or loss of either CMS genes or restorer alleles (Nilsson & Agren, 2006; Caruso & Case, 2007). All three of these processes can strongly affect the spatial distribution of nuclear and cytoplasmic genetic diversity.

In most cases, the effects of natural selection, founder events, dispersal and genetic drift on sex-ratio variation have been studied at important geographical scales, by comparing populations separated by several kilometers (e.g. McCauley, 1998; Medrano *et al.*, 2005) or even working at regional scales (e.g. Nilsson & Agren, 2006; Caruso & Case, 2007; Dufay *et al.*, 2009). However, because several studies also document sex-ratio variation at very local scales (within populations, e.g. Manicacci *et al.*, 1996; Laporte *et al.*, 2001; Olson *et al.*, 2006), these processes could also act at local scales.

The aim of this study was to document sex-ratio variation at very local scale in wind-pollinated gynodioecious *Beta vulgaris* ssp. *maritima* and attempt to understand the processes underlying the observed variation. At local geographical scales, no variation is expected in climatic conditions or herbivore pressure. In addition, in a wind-pollinated species, reproductive

output does not rely on any variation in pollinator abundance. Consequently, we focused on the other effects likely to affect sex ratio, in particular founder events through colonization processes and migration through seed and pollen dispersal, using genetic information, within two study sites of *B. vulgaris*. This species constitutes a good model for understanding the processes responsible for sex-ratio variation because the genetic basis of sex determination is well known: male sterility is associated with four particular mitochondrial types, called CMS *E*, *G*, *Svulg* and *H*. These sterilizing cytoplasms coexist with male-fertile cytoplasms, and these different cytotypes can be identified with molecular markers (Cuguen *et al.*, 1994; Forcioli *et al.*, 1998; Desplanque *et al.*, 2000; Fénart *et al.*, 2006). When coupling data on genotypes and sexual phenotypes, it is possible to directly measure female frequencies, the frequencies of the distinct sterilizing cytoplasms and the rates of restoration per sterilizing cytoplasm (by recording the proportion of hermaphroditic plants for each sterilizing cytoplasm). Using information on phenotypes, sex-determining genes and neutral nuclear markers, we addressed the following questions: Is there any sex structure within the study sites? Is there any genetic structure within the study sites? If so, how is spatial genetic variation partitioned and to what extent is this genetic structure due to limited migration (through seeds and pollen) and/or random founder events?

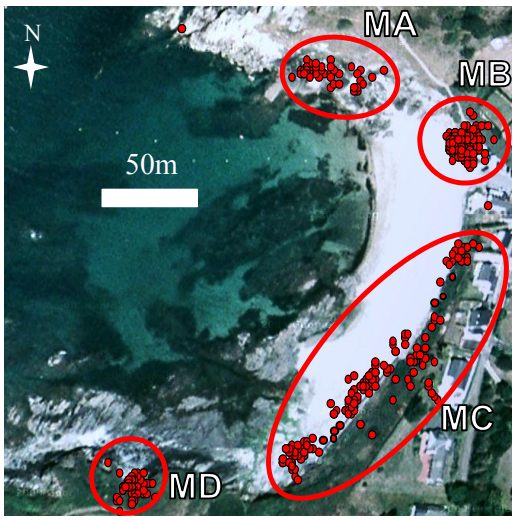
The species

Wild sea beet, *Beta vulgaris* ssp. *maritima* is a diploid species (2n = 18) widely distributed along the western coasts of Europe and around the Mediterranean Sea. It is a short-lived perennial and wind-pollinated species (Letschert, 1993). *B. vulgaris* has a gametophytic self-incompatibility system, with up to four gametophytic S loci (Owen, 1942; Larsen, 1977). Estimates of selfing rates in natural conditions have confirmed a pure outcrossing system (De Cauwer *et al.*, 2010). There is no vegetative reproduction, and dispersal thus only occurs through seeds and/or pollen movement. Seeds are aggregated in an irregular, dry body that contains 1-8 seeds. These aggregated fruits have no particular dispersal mechanism: seed dispersal is thought to be mainly local (Arnaud *et al.*, 2009; De Cauwer *et al.*, 2010), although hydrochory may lead to occasional long-distance dispersal events (Fievet *et al.*, 2007). This study was carried out in Brittany (western France), where sea beets colonize areas located along estuaries, just above the upper tide level, on cliffs overhanging the sea and in other coastal habitats (Letschert, 1993; Laporte *et al.*, 2001; Arnaud *et al.*, 2003; Viard *et al.*, 2004).

Study sites, sampling and laboratory procedures

Exhaustive sampling was carried out in two study sites located in Brittany (France), within two isolated coves separated by 30 km. The first one, called *MOR*, is located near Planguenoual (N 48°34,168; E -2°34,831), stretches over approximately 300 m and comprised 1098 flowering individuals in 2007. The second one, named *PAL*, is located near Plouha (N 48°40,497; E -2°52,911), stretches over approximately 500 m and comprised 615 flowering individuals in 2007. At both sites, individuals were clustered in geographically distinct patches, with few isolated individuals (Figure 1). As pollen and seed flow are known to be mainly local in this species (De Cauwer *et al.*, 2010), considering these geographical clusters of individuals as a proxy of genetic units for all subsequent statistical analyses may be a good prerequisite: four and five geographical groups of individuals were therefore considered throughout this study for *MOR* and *PAL*, respectively (see Figure 1). The cytoplasmic and nuclear genotypic structure, the genetic differentiation among geographical clusters, and the search of hidden genetic structure (see below) will allow us to validate the biological relevance of this geographical criterion. Within these sites, leaves were collected for genotyping on all individuals that flowered during the study year (*i.e.* all adult individuals). The location of all flowering plants was mapped. Additionally, the sexual phenotype was determined (female or hermaphrodite) for almost all individuals (98.5% in *MOR* and 97.4% in *PAL*).

(a) *MOR*



(b) *PAL*

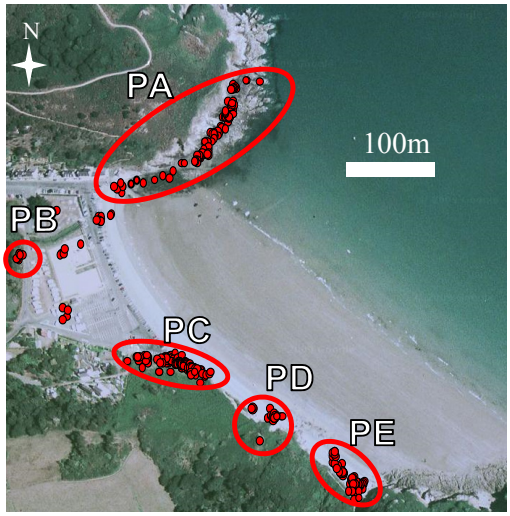


Fig. 1 Spatial distribution of *Beta vulgaris* ssp. *maritima* individuals (red dots) at the two study sites, *MOR* (a) and *PAL* (b), located in Brittany (western France). The locations of the geographical patches (*MA*, *MB*, *MC* and *MD* in *MOR* and *PA, PB, PC, PD* and *PE* in *PAL*) are circled in red.

DNA was extracted from dried leaves and purified using the NucleoSpin®96 Plant Kit (Macherey-Nagel). All sampled individuals were genotyped using three different marker types. First, nine nuclear microsatellite loci chosen for high polymorphism were used to describe the neutral diversity in both sites: *Bmb6* (Cureton *et al.*, 2002); *Bvm3* (Mörchen *et al.*, 1996); *GTT1, CAA1* (Viard *et al.*, 2002); *SB04*, *SB06*, *SB07*, *SB15* (Richards *et al.*, 2004); and *FDSB1027* (McGrath *et al.*, 2007). Second, cytoplasmic polymorphism was characterized using four mitochondrial minisatellite loci: *TR1*, *TR2*, *TR3* and *TR4* (Nishizawa *et al.*, 2000). Each genotype combination for these four minisatellite loci will be analyzed as a single haplotype, as the entire mitochondrial genome is generally inherited as a single linkage unit. Finally, diagnostic

cytoplasmic PCR-RFLP markers were used to distinguish among the three main CMS types: two mitochondrial markers that correspond to the CMSs *Svulg* and *G*, and a chloroplast marker for CMS *E* (Ran & Michaelis, 1995; Dufay *et al.*, 2008). In this study, we did not attempt to detect the occurrence of the fourth sterilizing cytoplasm (CMS *H*) because this cytotype is known to be absent from Brittany (Cuguen *et al.*, 1994). Amplification procedures and detection of polymorphism for these three marker types have been detailed in previous studies (Fénart *et al.*, 2007; Fénart *et al.*, 2008; Dufay *et al.*, 2009; De Cauwer *et al.*, 2010). A total of 1713 samples were genotyped and missing data rates were 2.3%, 1.6% and 0.6% for nuclear microsatellites, mitochondrial minisatellite haplotypes and cytoplasmic male sterility PCR-RFLP markers, respectively.

Statistical analyses

Nuclear and cytoplasmic diversity

The total number of sampled alleles at each locus for nuclear microsatellites ($A_{N\ Nuc}$) and the number of haplotypes obtained with cytoplasmic minisatellites ($A_{N\ Cyto}$) were counted within both study sites, as well as within each distinct geographical patch of individuals. We used the rarefaction method described in El Mousadik & Petit (1996) to calculate allelic richness ($A_{R\ Nuc}$ and $A_{R\ Cyto}$), which is a measure of the number of alleles (or haplotypes) that is independent of sample size (*i.e.* patch size in our study, as the sampling was exhaustive). To be able to compare allelic richness, values were standardized to a common sample size (N=18 individuals, *i.e.* the size of the smallest geographical patch in this study), using FSTAT version 2.9.3 (Goudet, 1995). Expected heterozygosities (H_e) as well as intra-population fixation indexes (F_{IS}) were also calculated for nuclear microsatellite loci using FSTAT version 2.9.3 (Goudet, 1995). Departure from Hardy–Weinberg equilibrium within each site and each geographical patch was tested by comparing the distribution of F_{IS} values for the observed data set with its distribution for a randomized data set obtained after 10 000 permutations of alleles among individuals within geographical patches. Additionally, to compare the level of individual genetic diversity among the three sexual phenotypes (female, restored CMS hermaphrodites and non-CMS hermaphrodites), mean individual heterozygosity (HL) was computed following Aparicio *et al.* (2006) for each individual, using GENHET (an R function developed by Coulon, 2009).

Finally, data were tested for linkage disequilibrium for all locus pairs at both sites, as well as within each geographical patch, using exact tests based on a Markov-chain method as implemented in GENEPOP version 4.0 (Raymond & Rousset, 1995). Tests were conducted with dememorization number set to 10 000, for 1000 batches and 10 000 iterations. Significance of *P*-values was assessed after Bonferroni correction to eliminate significance by chance (Rice, 1989).

Historical estimates of population structure and gene flow

We estimated pairwise population differentiation (F_{ST}) among the two study sites and among the distinct geographical patches within each site with 10 000 permutations of individuals

between patches, using a G test for significance of results (Goudet *et al.*, 1996). Significance of *P*-values for pairwise F_{ST} values was assessed after Bonferroni correction (Rice, 1989). We further attempted to compare levels of gene flow through seed and pollen dispersal. Under the assumptions of the Wright's island model, Ennos (1994) demonstrated that, assuming migration-drift equilibrium, a ratio (r) of the amount of pollen migration (m_p) over the amount of seed migration (m_s) can be inferred from *F*-statistics estimated by both nuclear biparentally inherited markers (F_{STN}) and maternally inherited markers (F_{STC}) from the following equation:

$$r = \frac{m_p}{m_s} = \frac{\left(\frac{1}{F_{STN}} - 1\right)(1 + F_{IS}) - 2\left(\frac{1}{F_{STC}} - 1\right)}{\left(\frac{1}{F_{STC}} - 1\right)}$$

In this equation, *F*-statistics are computed according to the Weir & Cockerham (1984) procedure and F_{IS} refers to the mean multilocus estimates of F_{IS} calculated over the nine microsatellite loci.

Finally, the hierarchical approach developed by Yang (1998) and implemented in HIERFSTAT (R function developed by Goudet, 2005) was used to carry out the estimation of variance components following a three-level hierarchical method: we estimated variance components within individuals, among individuals within haplotypes, among haplotypes within geographical patches and among geographical patches. We thus computed F_{IT} (genetic differentiation among individuals within each site), F_{IH} (non-random mating within haplotypes), F_{HP} (differentiation among haplotypes within geographical patches) and F_{PT} (differentiation among geographical patches). The effect of haplotype was assessed by permuting individuals among haplotypes within geographical patches (10 000 permutations). The effect of geographical patches was assessed by carrying out 10 000 permutations of haplotypes among geographical patches.

Bayesian estimate of population structure

Hidden population structure may confound estimates of genetic structure using classical *F*-statistics (Weir, 1996). In our study, using geographical patches of individuals as the predefined population units may not necessarily accurately reflect true population structure. To test the assumption that distinct geographical patches represent well-defined genetic demes, we used a Bayesian model-based clustering algorithm to infer population structure and to probabilistically assign individuals to subpopulations within both study sites. We used no prior information on the geographical location in which the individuals were sampled. Prichard *et al.*'s procedure (2000), implemented in STRUCTURE version 2.3.2., was used to cluster individuals in *K* subpopulations, using the multi-locus genotypes of the individuals and minimizing departures from Hardy-Weinberg expectations and linkage disequilibria. A series of 10 independent runs were conducted, with different proposals for *K*, testing all values from 1 to 15. Each run was conducted assuming population admixture and correlation of allele frequencies (Falush *et al.*, 2003) and included 100

000 iterations after a burn-in period of 10 000 iterations. To check for the convergence of the Markov chain Monte Carlo (MCMC), the consistency of results was checked for the ten replicates performed for each values of *K*. Finally, the most probable number of clusters (*K*) was determined using the *ad hoc* statistic *ΔK,* based on the rate of change in the log probability of data between successive *K* values, as described in Evanno *et al.* (2005).

Estimates of recent migration rates among geographical patches

Evidence of recent migration events among geographical patches was assessed using a Bayesian multilocus procedure described in Wilson & Rannala (2003). Relative to indirect estimators of long-term gene flow, this method requires few assumptions and can be applied to populations that are far from equilibrium. This method uses individual multilocus genotypes to estimate rates of recent migration (*i.e.* within the last few generations) among populations, along with the posterior probability distribution of individual immigrant ancestries, population allele frequencies and population inbreeding coefficient (Wilson & Rannala, 2003). The MCMC was run for 2×10^6 iterations after a burn-in period of 10^6 iterations using BAYESASS (Wilson & Rannala, 2003). Samples were collected every 2000 iterations to infer posterior probability distribution of parameters. Both data sets were independently run five times with different random seed values to verify the consistency of the results across runs. To examine the strength of the information in both data sets, 95% confidence intervals were computed for migration rates and compared to a scenario where all proposed changes along the Markov chain are accepted (simulating a situation when there is no information in the data set).

Testing for spatial genetic structure within geographical patches

The levels of spatial genetic structure within geographical patches were assessed by performing statistical correlation analyses between a genetic kinship estimate and pairwise geographical distance, for both nuclear data (microsatellites loci) and cytoplasmic data (minisatellite haplotypes) using SPAGeDi version 1.2. (Hardy & Vekemans, 2002). Nason's kinship coefficient F_{ij} (Loiselle *et al.*, 1995) was chosen as a pairwise estimator of genetic relatedness, as it has robust statistical properties (Vekemans & Hardy, 2004). Kinship coefficients (F_{ij}) were regressed on the natural logarithm of geographical distance ($\ln(d_{ij})$), thereby providing the regression slopes *b*. Standard errors were assessed by jackknifing over loci (for nuclear microsatellites) and the significance of the regression slopes were calculated by 10 000 permutations of individual locations (for nuclear microsatellite and cytoplasmic haplotypes). To visualize spatial genetic structure, F_{ij} values were averaged over a set of distance classes (10 distance classes, defined to obtain approximately the same number of individual pairs within each distance class) and plotted against geographical distances. Finally, to compare the strength of spatial genetic structure between nuclear and cytoplasmic data, as well as between the different geographical patches, we used the statistic S_p described Vekemans & Hardy (2004), and defined as $S_p = - b / (1-F_N)$, where F_N is the mean F_{ij} between neighboring individuals ($d_{ij} < 2m$).

Sex polymorphism and spatial sex ratio variation

In *MOR*, individuals were found in four geographical patches called *MA*, *MB*, *MC* and *MD* (mean number of individuals was 273, ranging from $N_{MIN} = 69$ to $N_{MAX} = 635$, see Table 1), with four isolated individuals. In *PAL*, individuals were clustered in five patches called *PA*, *PB*, *PC*, *PD* and *PE* (mean number of individuals was 118, ranging from $N_{MIN} = 18$ to $N_{MAX} = 300$, see Table 1), with 23 isolated individuals (Figure 1). Overall sex ratio (proportion of females) was 1.6% in *MOR* and 12.3% in *PAL*, with important variation in sex ratios among patches (Table 1). At both study sites, we found gynodioecious patches (*MC, PB, PC* and *PE*) and patches where there were no females (*MA, MB, MD, PA* and *PD*). Among the nine studied geographical patches, the sex ratio (*i.e.* proportion of females) varied between 0 and 42.9%.

Male-sterilizing cytoplasms were detected at moderate frequencies, with 13% of the genotyped individuals carrying a CMS (8.4% and 25.7% in *MOR* and *PAL*, see Table 1). The most frequent CMS cytotype was CMS *E*, which was present at both sites and in five out of the nine geographical patches. Local (*i.e.* within patch) CMS *E* frequencies ranged from 0 to 89% and local restoration rates for this particular CMS gene ranged from 45 to 100%. CMS *G* occurred only sporadically, with 11 individuals found in one geographical patch in the *PAL* site (Figure 4). Among those 11 individuals carrying CMS *G,* two expressed a hermaphroditic phenotype, suggesting that nuclear restorers of male fertility for that particular CMS gene were present in *PAL*. The last CMS type, CMS *Svulg,* was absent from our data set. In all gynodioecious patches, the genetic determination of sexual phenotypes was cytonuclear, with the coexistence of different cytotypes (associated with male sterility or not) and some individuals carrying CMS cytotypes expressing a hermaphroditic phenotype (and thus carrying nuclear restorers of male fertility). Among the geographical patches where females were absent, individuals carrying CMS genes were either restored for male function (*MA*) or completely absent (*MB, MD, PA* and *PD*).

Cytonuclear diversity

Over the whole data set, nuclear microsatellite loci exhibited moderate to high levels of polymorphism, with the number of alleles ranging from 4 (*Gtt1*) to 19 (*Bmb6*) in *MOR* and from 3 (*Gtt1*) to 21 (*Bmb6*) in *PAL*. The total number of alleles was 124 (97 in *MOR* and 99 in *PAL*) and the mean number of alleles per locus (±SD) was 10.78 (±5.43) in *MOR* and 11 (±6.10) in *PAL*. The number of sampled alleles ($A_{N\ Nuc}$), allelic richness ($A_{R\ Nuc}$), expected heterozygosity (H_E) and estimated intra-population fixation index (F_{IS}) per study site and per geographical patch are given in Table 1.

Table 1: Major characteristics of the different geographical patches, occurrence of gynodioecy and measures of genetic diversity on nuclear and cytoplasmic data within the two study sites (*MOR* and *PAL*) and within the different geographical patches of *Beta vulgaris* ssp. *maritima*. $N_{TOT\ is}$ the total number of flowering individuals (with the numbers in parentheses corresponding to isolated individuals, growing outside the geographical patches). *D* corresponds to the mean density of individuals. The frequency of CMS genes corresponds to the proportion of individuals carrying a cytoplasmic male sterility and the sex-ratio is the proportion of females. NF, NH_{CMS} and NH_{NCMS} are the number of females, restored CMS hermaphrodites non-CMS hermaphrodites (with the number between brackets corresponding to non-phenotyped individuals). The frequency of restoration is the proportion of restored hermaphrodites in CMS individuals. Allelic richness values ($A_{R\ Nuc}$ and $A_{R\ Cyto}$) were standardized to a common sample size (N=18 individuals, *i.e.* the size of the smallest geographical patch in this study). Significance of multilocus F_{IS} estimates per site and per geographical patch were tested using 10 000 random permutations of alleles among individuals within geographical patches. S_p values estimate the intensity of spatial genetic structure in each geographical patch. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; NS: non-significant

		MOR				
		MA	*MB*	*MC*	*MD*	***Overall***
Patch description	N_{TOT}	69	635	281	109	**1094 (4)**
	D (ind/m²)	0.057	0.843	0.081	0.138	**0.529**
	Habitat type	Cliff	Sand/Pebbles	Sand/Pebbles	Cliff	-
Male sterility and restoration	Frequency of CMS genes	0.088	0	0.296	0	**0.084**
	Sex ratio	0	0	0.063	0	**0.016**
	NF / NH_{CMS} / NH_{NCMS}	0 / 3 / 62 (4)	0 / 0 / 633 (2)	17 / 60 / 195 (9)	0 / 0 / 106 (3)	**17 / 63 / 996 (18)**
	Frequency of restoration	1.000	-	0.779	-	**0.788**
Multilocus estimates of nuclear diversity	Mean number of alleles per locus, $A_{N\ Nuc}$	5.444	6.889	9.222	5.111	**10.778**
	Mean allelic richness per locus, $A_{R\ Nuc}$	4.542	4.500	6.153	3.911	**5.896**
	Expected heterozygosity, H_E	0.512	0.562	0.614	0.487	**0.544**
	Fixation index, F_{IS}	0.057**	0.038***	0.091***	0.014*	**0.052*** **
	*S*p statistic and significance of the slope (b)	0.0086**	0.0133***	0.0420***	0.0041 ^{NS}	-
Cytoplasmic diversity	Number of haplotypes, $A_{N\ Cyto}$	3	4	6	1	**8**
	Haplotype richness, $A_{R\ Cyto}$	2.98	2.759	5.199	1	**6.271**
	*S*p statistic and significance of the slope (b)	0.4864***	0.1336***	1.1357***	-	-

		PAL					
		PA	*PB*	*PC*	*PD*	*PE*	***Overall***
Patch description	N_{TOT}	146	18	300	33	95	**592 (23)**
	D (ind/m²)	0.034	0.019	0.176	0.028	0.053	**0.109**
	Habitat type	Cliff	Sand/Pebbles	Sand/Pebbles	Cliff	Sand/Pebbles	-
Male sterility and restoration	Frequency of CMS genes	0	0.889	0.383	0	0.228	**0.257**
	Sex ratio	0	0.429	0.210	0	0.033	**0.123**
	NF / NH_{CMS} / NH_{NCMS}	0 / 0 / 142 (4)	6 / 6 / 2 (4)	61 / 45 / 185 (9)	0 / 0 / 32 (1)	3 / 18 / 71 (3)	**70 / 69 / 432 (21)**
	Frequency of restoration	-	0.500	0.425	-	0.857	**0.496**
Multilocus estimates of nuclear diversity	Mean number of alleles per locus, $A_{N\ Nuc}$	5.889	4.333	8.778	4.667	6.111	**11**
	Mean allelic richness per locus, $A_{R\ Nuc}$	4.231	4.333	5.129	4.114	5.220	**5.704**
	Expected heterozygosity, H_E	0.497	0.566	0.604	0.511	0.589	**0.554**
	Fixation index, F_{IS}	0.072***	-0.090*	0.008**	-0.060*	0.028^{NS}	**0.019*** **
	*S*p statistic and significance of the slope (b)	0.0266***	0.0158 ^{NS}	0.0185***	0.0202***	0.0695***	-
Cytoplasmic diversity	Number of haplotypes, $A_{N\ Cyto}$	3	2	6	1	2	**6**
	Haplotype richness, $A_{R\ Cyto}$	2.187	2	4.535	1	2	**4.345**
	*S*p statistic and significance of the slope (b)	0.4318***	0.0640 ^{NS}	0.0314**	-	2.8462***	-

At both study sites, nuclear microsatellites showed a significant deficit of heterozygotes compared to Hardy-Weinberg expectations (F_{IS} = 0.051 in *MOR* and F_{IS} = 0.019 in *PAL*; $P < 0.001$ in both cases). As significant single-locus F_{IS} values were not specifically associated with one locus, heterozygote deficiency could not be attributed to the presence of null alleles (data not shown). When subdividing each study site into its geographical patches, fixation indexes remained significantly positive for all four geographical patches in *MOR* but for only two geographical patches in *PAL* (Table 1). These departures from Hardy-Weinberg expectations may be due to the presence of distinct genetic clusters and/or isolation by distance within geographical patches (see below). Similarly, at the level of the study site, an important proportion of the 36 pairs of nuclear loci deviated from linkage equilibrium at $P < 0.05$ after Bonferroni correction (18 in *MOR* and 14 in *PAL*). As for fixation indexes, the proportion of locus pairs showing linkage disequilibrium decreased when subdividing the data sets into geographical patches. Interestingly, this decrease was even greater when dividing each geographical patch into the distinct haplotypes present within each patch (see Figure 2). At the haplotype level, the few pairs of loci that were still in linkage disequilibrium involved different locus combinations.

Mean individual heterozygosity (Mean HL ± SD) was 0.399 (± 0.174) in *MOR* and 0.358 (± 0.178) in *PAL*. Using a general linear model, we simultaneously tested the effects of the study site (two levels, *MOR* and *PAL*), geographical patch (nine levels: *PA, PB, PC, PD, PE, MA, MB, MC, MD*), local density (number of individuals within a radius of 10 m) and sexual phenotype (three levels, female, restored CMS hermaphrodite and non-CMS hermaphrodite) on the level of individual heterozygosity. Study site, geographical patch and phenotype all had a significant effect (see Table 2). Interestingly, post-hoc Tukey pairwise comparisons showed that non-CMS hermaphrodites had lower individual heterozygosity compared to females and restored CMS

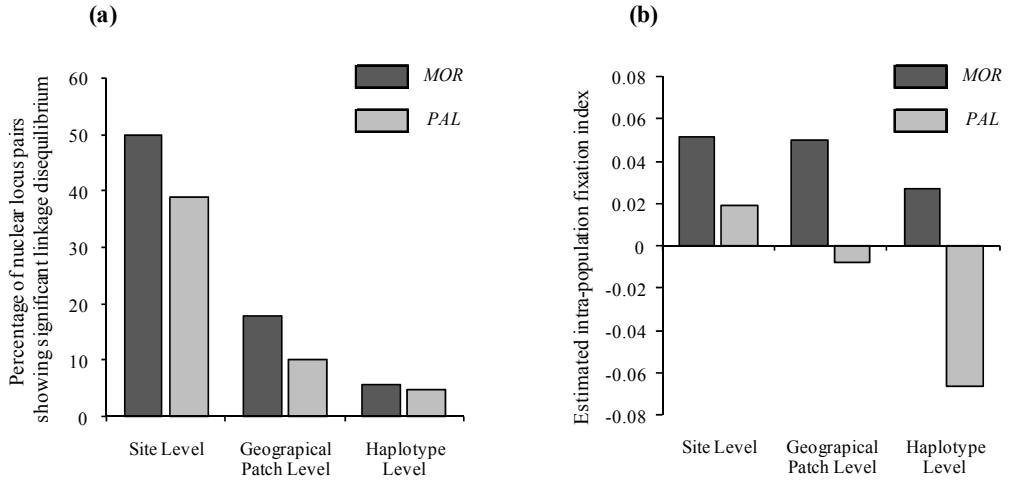


Fig. 2 Linkage disequilibrium and multilocus F_{IS} estimates for different levels of population subdivision. (a) Percentage of nuclear locus pairs showing significant linkage disequilibrium ($P < 0.05$ after Bonferroni correction) and (b) estimated intra-population fixation indices (F_{IS}) at the site level, the geographical patch level (mean values) and the haplotype level (mean values). *MOR* and *PAL* refer to the two study sites.

hermaphrodites ($P < 0.05$). Females and restored CMS hermaphrodites showed similar levels of individual heterozygosity ($P = 0.6869$). When reducing the data set to gynodioecious geographical patches (*i.e.* only patches containing females), we obtained similar results except for the patch effect, which was not significant (data not shown). Statistical differences between sexual phenotypes, explored using Tukey pairwise comparisons, were the same as the complete statistical model that included all geographical patches.

Table 2: Results of the general linear model testing simultaneously for the effects of study site (*MOR* and *PAL*), geographical patch (*MA, MB, MC, MD, PA, PB, PC, PD* and *PE*), local density (number of individuals within a radius of 10 m) and sexual phenotype (female, restored CMS hermaphrodite or non-CMS hermaphrodite) on the level of individual heterozygosity (HL) in *Beta vulgaris* ssp. *maritima*. All the interactions between main factors were non-significant and were dropped from the analysis

Source of variation	*df*	*MS*	*F*	*P*
Population	1	0.133	4.58	0.0325
Geographical Patch (Population)	7	0.182	6.25	<.0001
Density	1	0.006	0.19	0.6630
Sexual phenotype	2	0.308	10.57	<.0001
Error	1610	46.839		

In contrast to nuclear loci, cytoplasmic mitochondrial minisatellites displayed low levels of polymorphism and yielded a total of 10 distinct haplotypes of which eight were present in *MOR* and six were present in *PAL*. The number of sampled haplotypes ($A_{N\ Cyto}$) and the haplotypic richness ($A_{R\ Cyto}$) per geographical patch of individuals are given in Table 1. As expected, each male-sterilizing cytoplasm detected in our study sites (CMS *E* and CMS *G*) was exclusively associated with a unique minisatellite haplotype. We found a positive association between nuclear allelic richness ($A_{R\ Nuc}$) and haplotypic richness ($A_{R\ Cyto}$) found within geographical patches (Spearman's Rho=0.75; $P_{two\text{-}tailed}= 0.002$) as well as a positive association between nuclear allelic richness ($A_{R\ Nuc}$) and expected heterozygosity (H_E) (Spearman's Rho=0.9; $P_{two\text{-}tailed} = 0.002$).

Patterns of genetic differentiation

Strong spatial differentiation between geographical patches was found using mitochondrial minisatellite markers (overall $F_{STC} = 0.606$ in *MOR* and 0.429 in *PAL*; $P < 0.001$ in both cases, see Table 3). The spatial genetic structure was less pronounced for the nine nuclear microsatellites but remained highly significant (overall $F_{STN} = 0.107$ in *MOR* and 0.082 in *PAL*; $P < 0.001$ in both cases, see Table 2). Pairwise F_{ST} among patches were all significant and ranged from 0.154 to 0.914 for cytoplasmic data and from 0.039 to 0.217 for nuclear data (Table 3). The levels of differentiation calculated between the two study sites were lower than overall intra-site estimations ($F_{STC} = 0.278$ and $F_{STN} = 0.068$).

Based on these contrasted levels of genetic differentiation between cytoplasmic and nuclear markers, the *r*-value (estimating the ratio between pollen and seed gene flow) was equal to 11.5 in *MOR* and 6.6 in *PAL*. Under the assumptions of Wright's island model at equilibrium, this result suggests that gene flow occurs predominantly through pollen dispersal at both study sites.

Table 3: Genetic differentiation (F_{ST}) estimated for all the pairs of geographical patches of *Beta vulgaris* ssp. *maritima*, using nuclear data (F_{STN}, upper half of the matrices) and cytoplasmic data (F_{STC} , lower half of the matrices), as well as overall F_{ST} estimates within the two study sites (MOR and PAL). Significance of genetic differentiation was tested with 10 000 random permutations of individuals between geographical patches, using a G test for significance of results (Goudet *et al*. 1996). *: $P < 0.05$, **: $P < 0.01$; ***: $P < 0.001$; NS: non-significant

MOR					***PAL***					
	MA	*MB*	*MC*	*MD*		*PA*	*PB*	*PC*	*PD*	*PE*
MA	-	0.072***	0.112***	0.217***	*PA*	-	0.116***	0.090***	0.137***	0.122***
MB	0.683***	-	0.076***	0.180***	*PB*	0.842***	-	0.039***	0.163***	0.060***
MC	0.348***	0.547***	-	0.130***	*PC*	0.338***	0.219**	-	0.091***	0.043***
MD	0.832***	0.817***	0.356***	-	*PD*	0.884***	0.914***	0.373***	-	0.073***
					PE	0.762***	0.554***	0.229***	0.154*	-
Overall F_{STN}		0.107***			*Overall* F_{STN}		0.082***			
Overall F_{STC}		0.606***			*Overall* F_{STC}		0.429***			

Finally, multilocus hierarchical *F*-statistics showed evidence for hierarchical structuring of nuclear genetic variation within both study sites. In *MOR*, F_{HP} (differentiation among haplotypes within geographical patches) was 0.0472 and F_{PT} (differentiation among geographical patches) was 0.0808 (both with $P < 0.001$). In *PAL*, F_{HP} was 0.0285 and F_{PT} was 0.0666 (both with $P < 0.001$). Overall, these results confirmed the fact that geographical patches were differentiated and further suggested that the distribution of nuclear diversity depended on a mosaic of different haplotypes within patches.

Bayesian analysis of population structure

The results of the Bayesian analysis suggest that populations were hierarchically structured at both study sites, as the *K* versus *ΔK* distribution was multi-modal, with one mode at *K*=2 for both data sets and another mode at *K*=5 in *PAL* and at *K*=6 in *MOR* (see Figure 3). The obtained results appeared to be geographically meaningful, as the inferred genetic clusters corresponded very closely to the existing geographical patches (see Figure 4). When $K = 2$ at *MOR*, the algorithm clustered individuals growing in the northern part of the site in the first group (*MA* and *MB*) and individuals growing in the southern part of the site in the second group (*MC* and *MD*). Neighboring geographical patches were thus clustered together. Similarly, for $K = 2$ at PAL, one group was comprised of individuals growing in the southern part of the study site (*PB, PC, PD* and *PE*) and the other one included individuals located in the northern part of the site (*PA*). The second clustering solution (*K*=5 in *PAL* and *K*=6 in *MOR*) also showed geographically consistent results (see Figure 4). Some of the predefined geographical patches of individuals corresponded to homogeneous genetic clusters (*MA, MD*, *PA* and *PD*). Accordingly, individuals growing in these patches were mainly assigned to one particular genetic cluster. In contrast, a mosaic structure was found within *MC* and *PE*, with contiguous groups of genetically distinct individuals. Finally, individuals growing in MB and PC were mainly admixed individuals, with a trend for clinal

variation at the latter. Including spatial information in the prior distribution on individual admixture coefficients, as suggested by Durand et al. (2009), did not improve the resolution of Bayesian assignment and yielded very similar results, which strengthened the biological relevance of the depicted geographical partitioning.

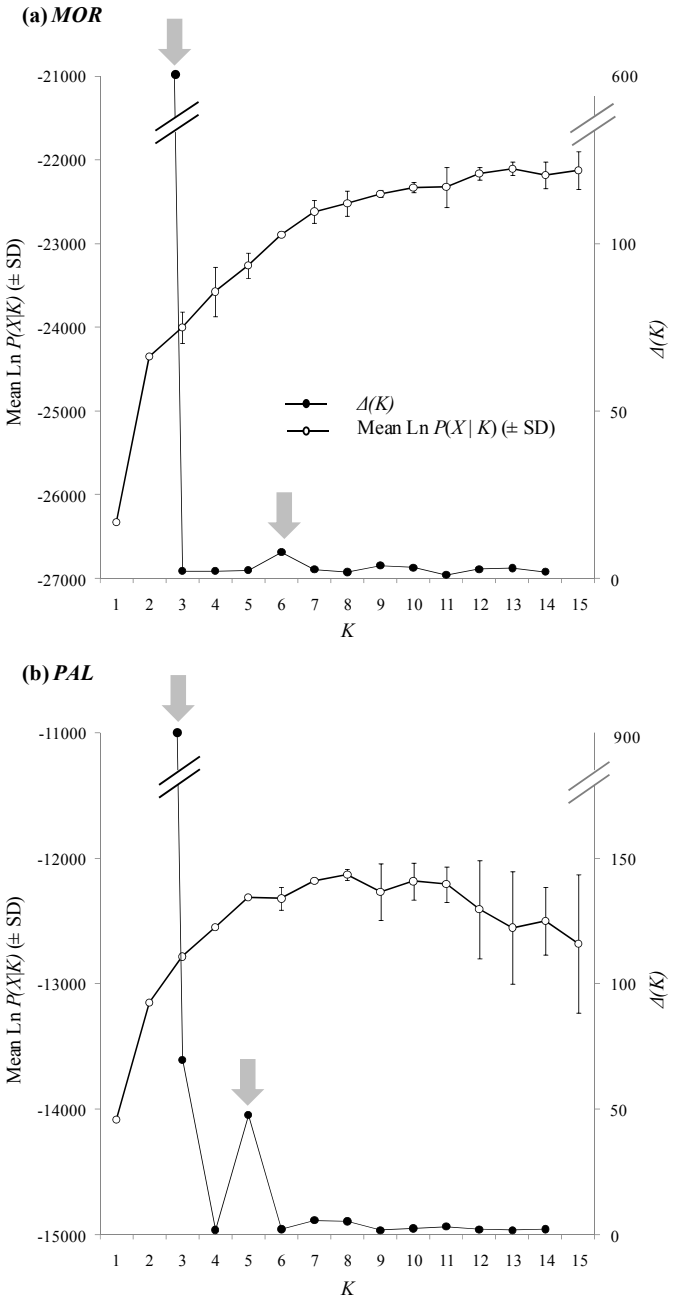


Fig. 3 Estimated number of populations assessed with the clustering method described in Pritchard *et al.* (2000), with mean (± SD) probabilities of the data Ln *P(X|K)* over 10 replicated runs plotted against the putative number of clusters *K* (ranking from *K*=1 to *K*=15 clusters) and standardized second order rate of change of Ln *P(X|K)*, *ΔK*, plotted against the putative number of clusters *K*, for the two study sites, *MOR* (a) and *PAL* (b).

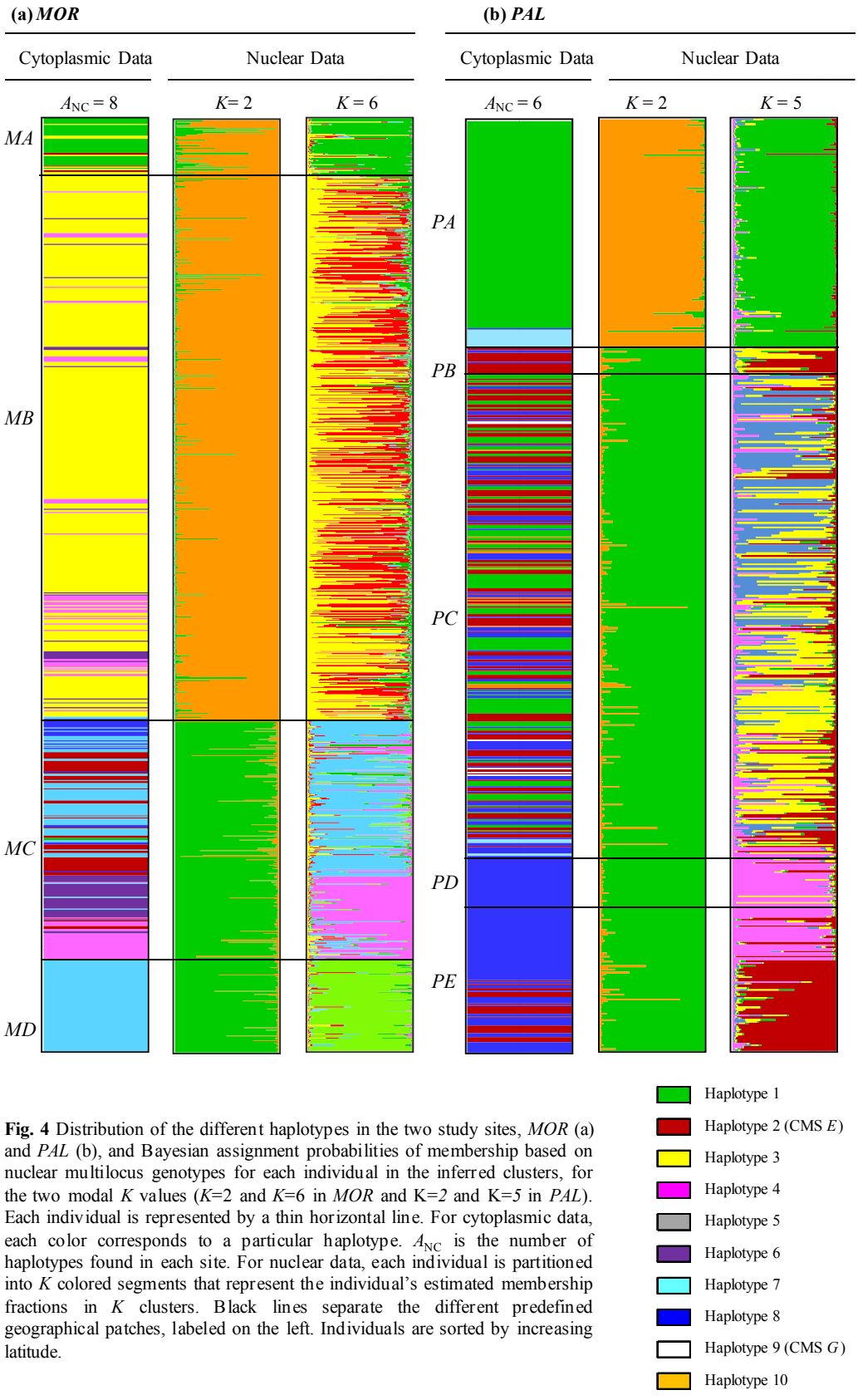


Fig. 4 Distribution of the different haplotypes in the two study sites, *MOR* (a) and *PAL* (b), and Bayesian assignment probabilities of membership based on nuclear multilocus genotypes for each individual in the inferred clusters, for the two modal *K* values (*K*=2 and *K*=6 in *MOR* and K=*2* and K=*5* in *PAL*). Each individual is represented by a thin horizontal line. For cytoplasmic data, each color corresponds to a particular haplotype. A_{NC} is the number of haplotypes found in each site. For nuclear data, each individual is partitioned into *K* colored segments that represent the individual's estimated membership fractions in *K* clusters. Black lines separate the different predefined geographical patches, labeled on the left. Individuals are sorted by increasing latitude.

Estimates of recent migration rates among geographical patches

Recent migration rates (*i.e.* within the last few generations) were estimated among the distinct geographical patches within both study sites using Wilson and Rannala's method (2003). Confidence intervals (CIs) obtained from both data sets were considerably smaller than those obtained from the null hypothesis (when simulating the effect of having no information in the data from which to estimate migration rates), suggesting that the data sets contained a sufficient amount of information to support the results. Within all geographical patches, more than 90% of individuals were identified as originating from their own source patch (non-migrants individuals), except for *PB* and *PE* , where slightly more important levels of incoming gene flow were detected (Table 4). *PAL* showed higher migration rates between patches compared to *MOR* (Table 4). We also detected asymmetrical migration rates for the pairs *MA – MB*, *PD – PE* and *PB – PC*. However, in the latter patch-pair, given the large overlap in CIs, it was not possible to conclude as to the statistical significance of the observed asymmetry in gene flow. Interestingly, those pairs corresponded to neighboring geographical patches, suggesting that dispersal events occurred preferentially between adjacent patches, but not necessarily in strict symmetry.

Table 4: Mean (95% CI) posterior distribution for contemporary migration rates among *Beta vulgaris* ssp. *maritima* geographical patches within the two study sites (*MOR* and *PAL*). Values along the diagonal (bold) are the percentage of individuals derived from the source patch (*i.e*. non migrants). Migration occurs from the geographical patch listed at the top of the column into geographical patches listed on the left.

MOR					
	MA	*MB*	*MC*	*MD*	
MA	**90.835 (86.386 - 94.878)**	8.409 (4.431 - 12.834)	0.445 (0.004 - 1.889)	0.311 (0.002 - 1.313)	
MB	0.064 (0 - 0.218)	**99.883 (99.604 - 99.997)**	0.027 (0 - 0.141)	0.026 (0 - 0.125)	
MC	0.308 (0.002 - 1.125)	0.127 (0.001 - 0.571)	**99.222 (97.931 - 99.938)**	0.342 (0.003 - 1.211)	
MD	0.203 (0.001 - 0.989)	0.273 (0.001 - 1.185)	0.351 (0.004 - 1.353)	**99.172 (97.717 - 99.919)**	
PAL					
	PA	*PB*	*PC*	*PD*	*PE*
PA	**99.485 (98.538 - 99.941)**	0.130 (0.001 - 0.603)	0.145 (0 - 0.707)	0.126 (0 - 0.611)	0.113 (0 - 0.574)
PB	2.768 (0.013 - 10.067)	**75.777 (67.510 - 90.464)**	14.178 (2.598 - 24.080)	1.145 (0.004 - 5.257)	6.132 (0.044 - 19.592)
PC	0.192 (0.002 - 0.713)	5.561 (3.683 - 11.078)	**92.004 (86.142 - 94.792)**	2.037 (0.694 - 3.853)	0.204 (0.001 - 0.850)
PD	0.557 (0.001 - 2.769)	1.515 (0.123 - 5.264)	2.240 (0.139 - 6.385)	**94.915 (89.003 - 98.776)**	0.774 (0.001 - 3.735)
PE	0.381 (0.001 - 1.597)	0.283 (0.001 - 1.347)	0.397 (0.001 - 1.846)	13.214 (9.550 - 17.241)	**85.725 (81.673 - 89.445)**

Testing for isolation by distance

Several general observations can be made from the spatial genetic structure observed within the geographical patches. First, as shown by correlograms (Figure 5), our results suggest a significant decline in genetic similarity with geographical distance regardless of the type of genetic marker (cytoplasmic or nuclear) within all geographical patches, except *MD* (nuclear data) and *PB* (nuclear and cytoplasmic data, see S_P statistics in Table 1). These two patches were also the smallest: the maximum distance between individuals is 35 meters in *MD* and 10 meters in *PB* and these spatial scales are probably too small for the establishment of isolation by distance patterns.

Second, there was considerable difference in the extent of spatial genetic structure between cytoplasmic and nuclear genetic variation. The S_P statistic for nuclear markers was on average 20-fold lower than for cytoplasmic haplotypes. These results confirmed the fact gene flow occurred predominantly through pollen dispersal within both study sites.

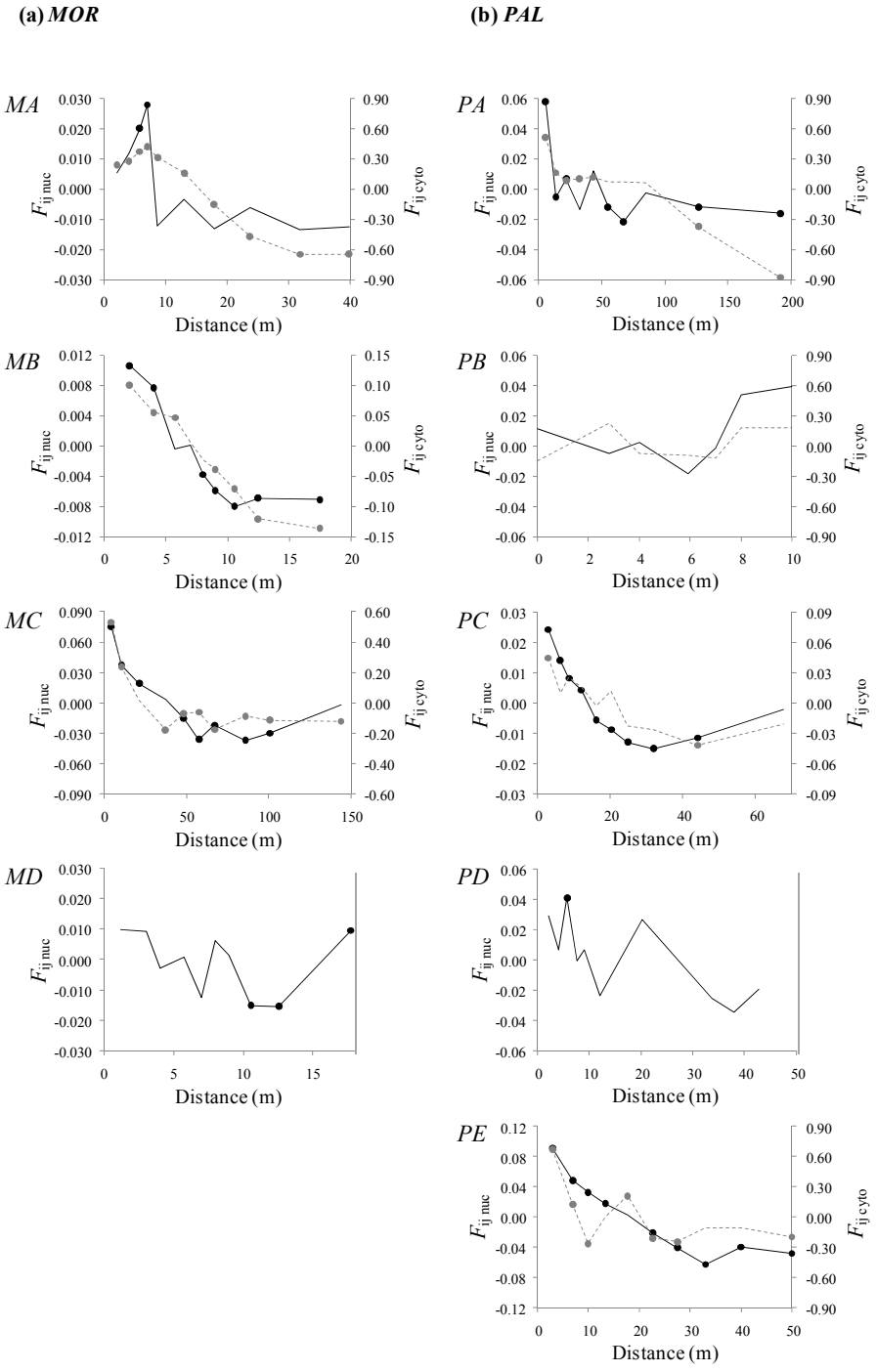


Fig. 5 Average pairwise kinship coefficient (F_{ij}, Loiselle *et al.* 1995) between individuals for nuclear microsatellites (black lines) and cytoplasmic haplotypes (gray dashed lines) in the two study sites (*MOR* and *PAL*), plotted against the geographical distance within each predefined geographical patch. Dots indicate significant F_{ij} values ($P < 0.05$ or less). *MD* and *PD* were monomorphic for cytoplasmic data.

DISCUSSION

The aim of this study was to examine fine-scale spatial structure of sexual phenotypes in conjunction with partitioning of neutral genetic diversity, so as to draw inferences on the processes underlying the observed spatial sex-ratio variation at two study sites of the gynodioecious *Beta vulgaris* ssp. *maritima.* More precisely, we investigated to what extent sex structure can be caused by random founder events and limited migration.

The development of sex structure in gynodioecious populations

Sex ratio in a population is the combined result of CMS frequency and restoration rate for each CMS type. In this study, we found that both CMS frequency and restoration rate varied greatly from one geographical patch to another (0 to 88.9% of individuals carrying a CMS gene and 42.5 to 100% restoration in CMS individuals). As a result, the sex ratio also showed pronounced variation among geographical patches (0 to 42.9% females). Although the spatial structure of sexual phenotypes has rarely been studied at fine geographical scales, our results confirm that local-scale sex-ratio structure is a common feature in gynodioecious species (e.g. Manicacci *et al.*, 1996; Laporte *et al.*, 2001; Olson *et al.*, 2006). Such striking spatial variation can be explained by the effects of natural selection, founder events, dispersal and genetic drift.

Sex structure can arise as a result of recurrent selection within relatively persistent and well-established populations. In our study, geographical patches may have reached a stable equilibrium sex -ratio and among patch variation may be linked to environmental variation. In general, the proportion of female plants is high in harsh environments, probably due to their better seed production under stressful conditions as compared to hermaphrodites (e.g. Delph, 1990; Ashman, 1999; Asikainen & Mutikainen, 2003). Given that our study was conducted at a very local scale (i.e. over only a few 100s of m), the large variation in sex ratio cannot be attributed to strong environmental gradients. However, within both study sites, *B. vulgaris* patches were localized in two distinct environments: plants were growing either on sea cliffs (patches *MA, MD, PA* and *PD*) or in a substratum formed by sand and pebbles, at the upper high-tide level (patches *MB, MC, PB, PC* and *PE*). Although there is no information on the actual ecological characteristics in the different geographical patches (soil moisture, nutrients, light exposure), cliff habitats are probably harsher, because individual growth is strongly constrained by its ability to develop roots in small crevices. If cliffs are effectively the most unfavorable environments, females may be expected to have a better reproductive output compared to hermaphrodites and to be particularly frequent in such habitats. However, the opposite trend was observed in our study sites, because no females were found in the geographical patches located on cliffs. Alternatively, the absence of CMS on the cliffs may be a consequence of the fact that these environments are more difficult to colonize, as clearly illustrated by the limited number of haplotypes found in the patches growing on cliffs (mean haplotype richness for geographical patches located on cliffs was nearly half of that for geographical patches growing in the other environments; $A_{\mathrm{R\ Cyto}}$ 1.79 and

3.30, respectively). If so, the absence of CMS genes on cliffs may be a simple consequence of the limited probability of finding rare cytotypes in habitats that are intrinsically difficult to colonize. This trend was also observed by Laporte (2001), who compared two populations: the first population, growing on a cliff, showed a very low cytoplasmic diversity compared with another one located in an estuary. Further studies, including more sites and more detailed characterization of ecological features of the different habitats are needed to investigate whether the observed trend is a common feature in *B. vulgaris.*

Population structure and the mode of colonization have long been recognized as major factors influencing the maintenance and the loss of genetic variation (Slatkin, 1977; Wade & McCauley, 1988). In the case of gynodioecious species, non-equilibrium processes can have important effects on spatial distribution of sex-determining genes, both through recurrent introduction of new CMS cytotypes (*via* mutation or migration) and founder events (Couvet *et al.*, 1998). All female individuals in our study sites were carrying CMS genes that are also found at regional scales (Dufay *et al.*, 2009), suggesting that these CMS genes have not arisen recently. This implies rather slow mutational dynamics of the mitochondrial genome in *B. vulgaris*, contrary to what has been suggested for gynodioecious *Thymus vulgaris* (Belhassen *et al.*, 1993). If mutation does not seem to be an important factor driving sex ratio variation in *B. vulgaris*, the high levels of neutral genetic structure suggested that founder events, along with restricted gene flow, might be responsible for the observed sex structure. *B. vulgaris* is generally patchily distributed along the sea shore, where seasonal storms cause frequent disturbance, sometimes leading to complete extinction of local patches. Even if we have no information about the actual extinction rates, we observed localized extinctions in the *PAL* site, as well as one colonization event in *MOR* (personal observation), between 2006 and 2007 (year of the study). In this case, the levels of genetic variation among patches (and thus sex structure) are theoretically expected to increase compared with the case of an island model with no extinction/recolonization dynamics and with founders drawn at random from source populations (Wade & McCauley, 1988; Whitlock & McCauley, 1990). In our study, highly significant genetic differentiation levels among geographical patches (as measured by pairwise F_{ST} estimates for nuclear and cytoplasmic data) and the results of assignment tests showed that predefined geographical patches corresponded very closely to genetically distinct groups that have probably originated from different founder events. Additionally, although founder events and drift could cause the loss of CMS genes (and thus decrease the probability of female phenotypes), these processes could also increase the probability of local CMS/restorer mismatches, increasing the frequency of females in newly founded populations (Manicacci *et al.*, 1996; Couvet *et al.*, 1998).

Interestingly, when comparing our results on patches separated by a few hundred meters or less with the results of a previous study performed at regional scale (33 sites sampled over several hundreds of kilometers, see Fievet *et al.*, 2007), we found similar levels of nuclear genetic differentiation (0.107 in *MOR*, 0.082 in *PAL* and 0.089 at the regional scale). Regarding cytoplasmic data, the levels of differentiation were even higher at local scale than at regional scale

(0.606 in MOR, 0.429 in PAL and 0.278 at the regional scale). This may be due to metapopulation dynamics: if seeds colonizing a new site originate from several source sites or if there are multiple independent colonization events, a site, taken as a whole, can show high levels of genetic diversity (Slatkin, 1977). Conversely, at smaller scales (within sites), genetic drift will tend to randomly fix different alleles in different local patches, leading to an increase in the genetic differentiation among local patches within sites (Ingvarsson & Giles, 1999), which can ultimately generate fine-scale sex-ratio variation in the case of gynodioecious species.

Overall, the observed levels of genetic structure suggested that our study sites are subject to frequent disturbance, which could partly explain the strong sex-ratio variation within both study sites. However, the consequences of such dynamics on sex structure depend largely on the degree of persistence of local patches. With low extinction rates, natural selection can substantially modify the initial allele frequencies within local patches, whereas important extinction rates can render the effects of natural selection ephemeral. Additionally, the levels of structure not only depend on the number and the origin of seeds colonizing an unoccupied patch, but also on the magnitude of migration between patches (Whitlock & McCauley, 1990).

Gene flow through seed and pollen dispersal

The effect of random founder events can be countered by migration events between established populations. Our results showed evidence of very restricted gene flow within both study sites. Indeed, Bayesian estimates of contemporary gene flow between geographical patches were exceptionally low considering the spatial scale at which our study was conducted. Additionally, patterns of spatial genetic structure within each geographical patch, synthetically quantified by S_p statistics, suggested that isolation by distance or abrupt genetic discontinuities can take place within a few tens of meters. Even if *B. vulgaris* is a wind-pollinated and self-incompatible plant, features that are likely to decrease genetic variance among demes (Loveless & Hamrick, 1984; Vekemans & Hardy, 2004), the patterns of gene flow within our study sites seemed limited enough to maintain the strong genetic structure initially generated by founder events. In addition, the spatial distribution of pollination events can also have direct consequences on the sex ratio in the next generations. If gene flow between individuals is mainly local, as in our study sites, inbreeding or biparental inbreeding may enhance the production of females within progenies (Emery & McCauley, 2002; Bailey & McCauley, 2005).

Additionally, our results showed strong differences in the extent of spatial genetic structure between nuclear and cytoplasmic loci, with the genetic structure at maternally inherited cytoplasmic markers being stronger than what was observed for nuclear loci, suggesting a predominance of pollen migration over seed migration within the two study sites. This was quantified through the estimates of the *r*-ratio (ratio of the amount of pollen migration (m_p) over the amount of seed migration (m_s) inferred from *F*-statistics) that was high within both study sites, especially compared to other studies reporting wide-range cytonuclear spatial genetic structure (see Petit *et al.*, 2005). Additionally, the S_p statistics (measuring the strength of spatial genetic

structure within geographical patches) was also on average 20-fold lower for nuclear markers than for cytoplasmic haplotypes. These observations are in agreement with other studies that have compared the spatial structure of cytoplasmic and nuclear markers at local scales in angiosperms, including some gynodioecious species (Tarayre *et al.*, 1997; McCauley, 1998). The relative magnitude of gene flow through seed and pollen dispersal should be taken into account in theoretical models, as both processes are expected to have different consequences on the maintenance of cytonuclear gynodioecy. In their theoretical model exploring the conditions of maintenance of cytonuclear polymorphism in a subdivided population, Dufay & Pannell (2010) showed that, while gynodioecy is systematically lost under drift alone, seed or pollen dispersal could maintain cytonuclear gynodioecy. More precisely, seed dispersal was shown to promote the maintenance of cytonuclear polymorphism at the level of the whole population as well as at the level of local demes, while pollen dispersal could not counter the loss of cytonuclear polymorphism at the level of the local deme, but promoted its maintenance at the level of subdivided population taken as a whole.

In plant species that are often subject to catastrophic forms of disturbance, long-distance seed dispersal is essential for a metapopulation to persist (Cain *et al.*, 2000). In our study, in addition to the fact that geographical patches showed significant levels of differentiation, our results also suggested some hierarchical sub-structuring due to the presence of different haplotypes within geographical patches. Along with the observation of decreasing values of genetic linkage disequilibrium and F_{IS} estimators when dividing geographical patches according to haplotype identities, this strongly suggested that our study sites are dynamic entities, with recurrent and independent establishment of new haplotypes through seed migration (Olson & McCauley, 2002). The fact that this signal was detected within both study sites suggests that (i) the arrival of different haplotypes is quite recent and/or (ii) reproduction may preferentially occur between individuals sharing the same haplotype, probably as a consequence of the patchiness of haplotypes within geographical patches and spatially restricted pollen flow. Overall, even if seed migration is low compared to pollen migration in *B. vulgaris* (see above), geographical patches where several haplotypes were found are probably the result of independent seed immigration events. As several seeds can be contained in one fruit, the opportunity for kin-structured foundation by groups of sibs is enhanced (e.g. Torimaru *et al.*, 2007). Our results then suggest the following scenario: when one or multiple fruits of common origin (each fruit containing several seeds sharing the same haplotype) arrive in a patch, several seedlings carrying the same haplotype are likely to become established. In a second phase, the haplotype frequency increases and, because pollen flow is mainly local, groups of individuals carrying the same haplotype can remain genetically differentiated from neighboring clusters carrying different haplotypes. In addition to the partitioning of cytonuclear diversity within geographical patches when several different haplotypes coexist, the recurrent arrival of new haplotypes also increases the level of nuclear allelic richness within geographical patches (as shown by the significant correlation between $A_{R\,Nuc}$ and $A_{R\,Cyto}$).

Finally, this particular functioning could also affect individual heterozygosity (HL) of CMS and non-CMS individuals differently. Female and restored CMS hermaphrodites showed significantly higher levels of individual heterozygosity compared to non-CMS hermaphrodites in both study sites. This may be a consequence of the fact that CMS and non-CMS seeds do not have the same mating opportunities when they become established in a patch: (i) when non-CMS seeds establish in a patch, they produce only hermaphrodites, and given the spatially limited pollen dispersal, a large part of mating events probably occurs between relatives, generating some biparental inbreeding; (ii) when CMS seeds establish in a patch, they produce either females or restored hermaphrodites, which have been shown to produce lower pollen quality than non-CMS individuals (Dufay *et al.*, 2008) and, as a consequence, a larger proportion of mating events must involve individuals that were already occupying the patch.

Perspectives: the effects of sex structure on individual fitness

Our results suggest that random founder events and limited gene flow between patches can be responsible for fine-scale sex-ratio variation. Furthermore, such sex-ratio variation can also directly influence the mating success of the different sexual phenotypes, since the fitness of the different coexisting sexes can be frequency-dependent (McCauley & Bailey, 2009). For instance, in female-biased patches, pollen limitation of seed production has been shown to decrease the reproductive output of female individuals in several gynodioecious species (e.g Widen & Widen, 1990; McCauley & Brock, 1998; Graff, 1999; Alonso, 2005; Zhang *et al.*, 2008; De Cauwer *et al.*, submitted). Additionally, high cytoplasmic differentiation provides a landscape for differential selection, since restorer alleles are selected for only in the presence of their specific haplotype, but are either neutral or even selected against (under the hypothesis of a cost of restoration) in its absence. In addition, genetic structure of cytoplasmic genes, by promoting the clumping of CMS genes, may favor restored hermaphrodites that are the only close potential functional males able to pollinate local females (De Cauwer *et al.*, 2010). However, the fate of restorer alleles remains equivocal when restored CMS-hermaphrodites are clustered together with non-CMS ones. Ongoing paternity analyses in the same study sites aim at resolving this issue.

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Short-term evolution of genetic structure in populations of gynodioecious *Beta vulgaris* ssp. *maritima*, a species with perennial seed banks

I. DE CAUWER, M. DUFAY, B. HORNOY & J.-F. ARNAUD

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ABSTRACT

A fundamental issue in plant biology is the ability of seeds to disperse in time, provided that seeds can remain dormant and stored in the soil, giving rise to a seed bank. In gynodioecious species where both females and hermaphrodites coexist, perennial seed bank could provide opportunities to store and reintroduce the genes determining the sexual phenotype, despite the action of selection or genetic drift. This study focuses on the potential effect of seed bank resurgence and contrasting sex-ratios on the short-term (three generations) evolution of cytonuclear genetic diversity in structured populations of the gynodioecious *Beta vulgaris* ssp. *maritima.* We observed a negative effect of census population size on temporal genetic differentiation among adults and seedling cohorts. However, very narrow changes in allelic frequencies and in the levels of nuclear and cytoplasmic spatial genetic structure were depicted across time and the local sex-ratio did not appear to affect genetic features of populations. Despite a general trend for stable genetic structure, the estimated effective population sizes were moderate and suggested a low number of successful breeders. Altogether, our results suggested that, at least over short-time scales, large seed pools generated by only a moderate fraction of the flowering individuals can sustain both neutral genetic diversity and diversity at sex-determining genes, which could modify the expected effects of gene flow, genetic drift and selection on the maintenance of sexual polymorphisms in structured populations.

INTRODUCTION

Within a species range, gene flow among populations creates patterns of neutral genetic variation that depend on various ecological and biological features like, among other things, the dispersal capabilities of the species, the landscape configuration, the existence of geographical barriers to gene flow, the history of the populations, or the breeding system (Wright, 1946; Slatkin, 1985; Broquet & Petit, 2009). Genetic signatures associated with the cumulative effect of dispersal over time can reveal the scale over which populations are prone to the action of local selective forces and indicate the relative opportunities for gene flow and genetic drift to modify the evolutionary trajectories of populations. In plants, contrarily to many animal taxa, gene flow only occurs through dispersal of propagules that can be of two distinct types, pollen and seeds (male gametophytes and young sporophytes, respectively), and which differ in a number of ways. Plants thus have complex and asymmetrical patterns of gene transmission, with nuclear genes transmitted both by seed and pollen through the female and male function, and cytoplasmic genes only inherited in seeds through the female function. Because pollen grains typically move further than seeds, contrasting levels of genetic differentiation are generally described for nuclear and cytoplasmic genetic diversity, depending on the mating system, which can range from pure outcrossing to pure selfing (Petit *et al.*, 2005). Overall, both pollen and seed movements govern the set up of fine-scale genetic distinctiveness and the size of local genetic neighborhoods within which the interplay of genetic drift or selective processes can operate (Loveless & Hamrick, 1984; Heywood, 1991; Knowles *et al.*, 1992; Vekemans & Hardy, 2004).

Beside the effects of gene flow on spatial genetic structure, an additional fundamental issue in plant biology is the ability of seeds to disperse in time, provided that seeds can remain dormant and stored in the soil, giving rise to a seed bank. A long-lived seed bank is often advocated as a main evolutionary determinant, generating important reservoirs of genetic variation and buffering against the effects of local extinction of maternal lineages in adult plant populations, caused either by selection or drift due to demographic and environmental stochasticities (Epling *et al.*, 1960; Templeton & Levin, 1979; Mahy *et al.*, 1999; Shimono *et al.*, 2006; Honnay *et al.*, 2008). Such banks of sleeping genes not only provides the opportunity to increase the effective population size by counteracting the eroding effect of genetic drift (Gottlieb, 1974; Vitalis *et al.*, 2004), but also may be a crucial factor in maintaining sexual polymorphism, in particular cytonuclear gynodioecy, in which females and hermaphrodites coexist in natural populations (e.g. Boutin-Stadler *et al.*, 1989; Del Castillo, 1994).

In gynodioecious species, the sex polymorphism is commonly a consequence of a genomic conflict between biparentally inherited nuclear genes and maternally inherited cytoplasmic genes. Indeed, sex determination generally involves epistatic interactions between cytoplasmic genes for male sterility (CMS) and nuclear genes that restore male fertility (e.g. Boutin *et al.*, 1987; Dommée *et al.*, 1987; Koelewijn & van Damme, 1995; Delph *et al.*, 2007). To

develop as a female, an individual must carry an unrestored CMS gene. To develop as a hermaphrodite, an individual must either carry a CMS gene in combination with the matching restoration alleles (restored hermaphrodite), or carry a non-CMS cytoplasm (non-CMS-hermaphrodite). In their theoretical model exploring the conditions of maintenance of cytonuclear polymorphism in a subdivided population, Dufay & Pannell (2010) showed that, while gynodioecy is systematically lost under drift alone, seed dispersal could maintain cytonuclear gynodioecy. Similarly, the existence of long-lived seed bank may thus provide opportunities to change the evolutionary trajectories of CMS and nuclear restorers of male fertility by storing and reintroducing recurrently the genes determining the sexual phenotype.

This study focuses on the gynodioecious sea beet (*Beta vulgaris* ssp. *maritima*) which provides a model of choice for studying the effect of seed bank resurgence on the genetic structure and on the local sex-ratio in structured populations for the following reasons: (i) the genetic basis of sex determination is well-known: male sterility is associated with four particular mitochondrial types, called CMS *E*, *G*, *Svulg* and *H*. These sterilizing cytoplasms coexist with male-fertile cytoplasms, and the different cytotypes can be identified with molecular markers (Cuguen *et al.*, 1994; Fénart *et al.*, 2006). When coupling data on genotypes and sexual phenotypes, it is possible to measure directly the frequencies of the distinct sterilizing cytoplasms and the rates of restoration per sterilizing cytoplasm (by recording the proportion of hermaphroditic plants for each sterilizing cytoplasm); (ii) pronounced spatial genetic structure and important variations in local sex ratios are often found at very local scale (i.e. a few tens of meters, e.g. Laporte *et al.*, 2001; De Cauwer *et al.*, 2010b); (iii) because the species forms discrete populations in costal habitats where environmental disturbances frequently occur during important high tides or winter storms, populations can be subject to large fluctuations in population size (Fievet *et al.*, 2007); (iv) this species is characterized by heritable seed dormancy, with seeds that can remain stored in the soil during several years, possibly leading to the establishment of long-lived seed banks that could buffer against environmental disturbances (Sester *et al.*, 2006; Wagmann, 2008; Arnaud *et al.*, 2010a).

In this study, we took advantage of the knowledge of study sites previously characterized for cytoplasmic and nuclear genetic distinctiveness, local sex-ratio and nature of sterilizing genes (De Cauwer *et al.*, in prep-a), to address the following issues: (1) Do the levels of genetic diversity and spatial genetic structure in seedlings vary with the actual adult population size and the local density? The expectation would be to find higher levels of genetic diversity in patches with important population size (Honnay *et al.*, 2008) and weaker spatial genetic structure for dense patches (Vekemans & Hardy, 2004). (2) Is there some genetic differentiation over time and does the magnitude of differentiation vary with the actual adult population size? Differentiation among consecutive cohorts could be stronger in small populations for at least two different reasons. First, the action of genetic drift on allelic frequencies is expected to stronger in small populations. Second, because dense clustering of individuals promotes intra-deme mating events, incoming gene flow may be higher in small populations (García *et al.*, 2005; Fénart *et al.*, 2007). (3) How

does the within-population genotypic structure vary through time? This may depend on the levels of intra-population gene flow, on the magnitude of competition between newly established seedlings, which can potentially modify the distribution of diversity through the action of thinning, as well as on the local sex ratio, which may modify the opportunity for mating events between non-relatives (Del Castillo, 1994; García *et al.*, 2005; Shimono *et al.*, 2006) (4) Finally, we attempted to describe the variation in effective population size among study sites and among generations, in the light of information about the census population size and the local sex-ratio. Cytonuclear gynodioecy has been theoretically shown to reduce the effective population size as compared to hermaphroditism (Laporte *et al.*, 2000). Nonetheless, to the best of our knowledge, only one empirical study dealt with the interplay of seed bank and gynodioecious breeding system on the genetic effective population size (Del Castillo, 1994). In the present work, we used adult and seedlings genetic characterization to estimate the effective population size using both single-sample and temporal estimators (see Jorde & Ryman, 2007; Waples & Do, 2010).

The species

Wild sea beet, *Beta vulgaris* ssp. *maritima* is a diploid species (2n = 18) widely distributed along the western coasts of Europe and around the Mediterranean Sea. It is a short-lived perennial and wind-pollinated species (Letschert, 1993). *B. vulgaris* has a gametophytic self-incompatibility system, with up to four gametophytic S loci (Owen, 1942; Larsen, 1977) that, jointly with inbreeding depression, allow a pure outcrossing mating system in the wild (Arnaud *et al.*, 2010b; De Cauwer *et al.*, 2010b). There is no vegetative reproduction, and dispersal thus only occurs through seeds and/or pollen movement. Seeds are aggregated in an irregular, dry body that contains 1-8 seeds. These aggregated fruits have no particular dispersal mechanism and no obvious morphological adaptation for active transport. Because of simple gravity-driven seed fall, seed dispersal is thought to be mainly local (Arnaud *et al.*, 2009; De Cauwer *et al.*, 2010b), although hydrochory may lead to occasional long-distance dispersal events (Fievet *et al.*, 2007). Germination timing is mediated by dormancy which can be released by cold or dry periods. Previous studies suggested that over 40% of total germination result from dormant seed (Wagmann, 2008). This study was carried out in Brittany and in northern France, where sea beets colonize areas located along estuaries, just above the upper tide level, on cliffs overhanging the sea and in other coastal habitats (Laporte *et al.*, 2001; Viard *et al.*, 2004). In this area, most individuals bolt and flower only the second year after seedling emergence because of a strong vernalization requirement, *i.e.* the process by which the exposure to cold temperatures is necessary for the plant to switch from the vegetative to the reproductive stage (Van Dijk, 2009).

Study sites and sampling

Three sites were used in the current study (Fig. 1). Two of them are located in Brittany (western France), within two isolated coves separated by 30 km and will be named *MOR* and *PAL* throughout this study. *MOR*, is situated near Planguenoual (N 48°34,168; E -2°34,831), stretches over approximately 300 m and comprised 1098 flowering individuals in 2007. *PAL* is located near Plouha (N 48°40,497; E -2°52,911), stretches over approximately 500 m and comprised 615 flowering individuals in 2007. These two site were previously studied and analyzed for historical patterns of gene flow and gender-specific fitness differences in male reproductive success in other studies (De Cauwer *et al.*, in prep-a; De Cauwer *et al.*, in prep-b). The last study site is located in Northern France, on a beach near Audresselles (*AUD*; N 50°49.101, E 1°35.676), stretches over 500 m and comprised approximately 400 flowering individuals in 2007. Within these sites, individuals were clustered in distinct geographical patches: four, five and two distinct geographical sub-units could be defined in *MOR*, *PAL* and *AUD*, respectively (Fig. 1). As pollen and seed flow are known to be mainly local in this species (De Cauwer *et al.*, 2010b), considering these geographical clusters of individuals as a proxy for genetic units for all subsequent statistical analyses may be a good prerequisite. An exhaustive sampling was carried out on adult individuals

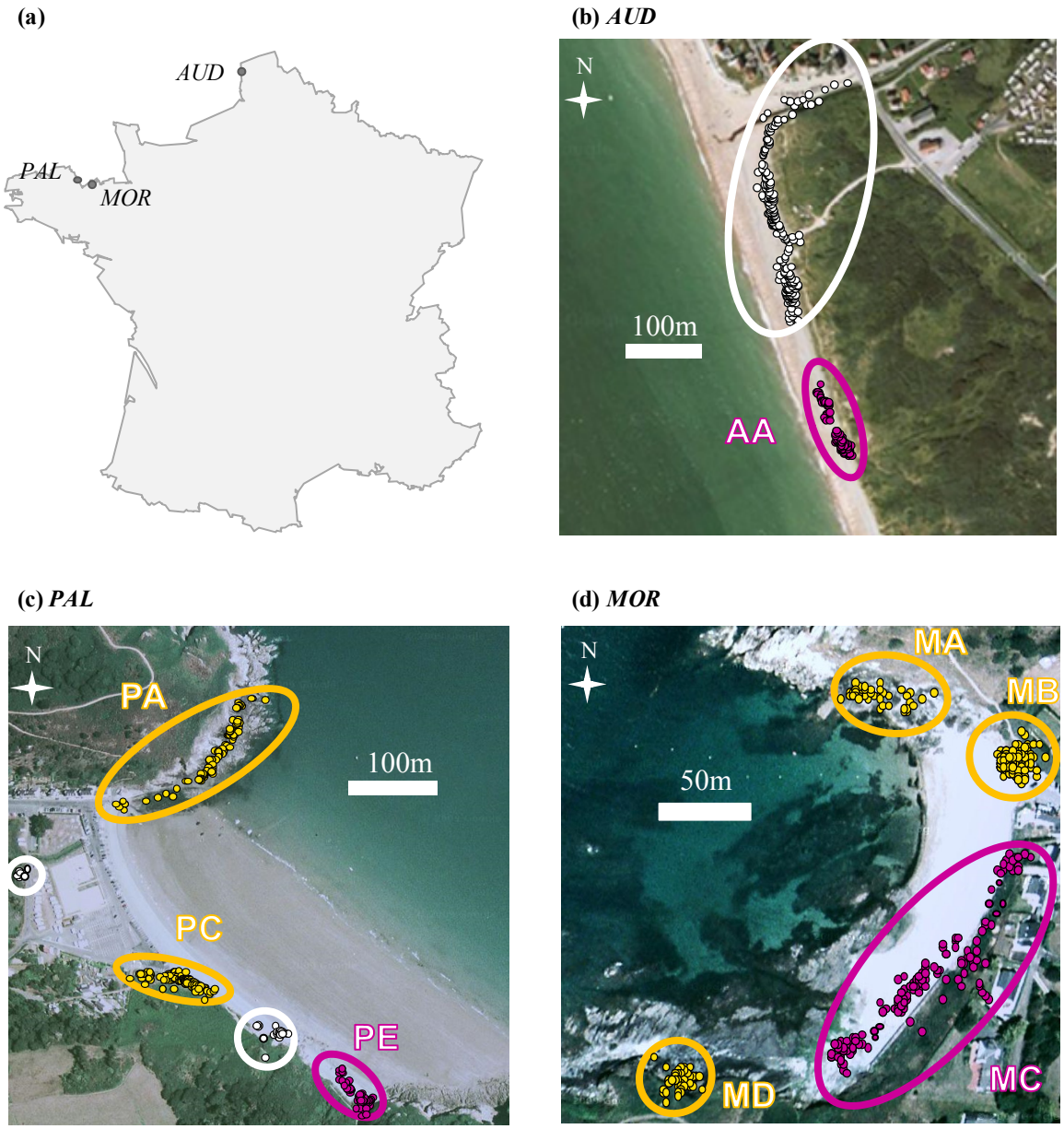

Fig. 1 Location of the three study sites (a) and spatial distribution of *Beta vulgaris* ssp. *maritima* adult individuals in 2007 (colored dots) at the three study sites, *AUD* (b), *PAL* (c) and *MOR* (d). The locations of the studied geographical patches (*AA* in *AUD, PA, PC* and *PE* in *PAL* and *MA*, *MB*, *MC* and *MD* in *MOR*) are circled. Geographical patches where seedlings were sampled during the two consecutive years of the study (2007 and 2008) are circled in pink. Geographical patches where seedlings were only sampled during the second year of the study (2008) are circled in orange. White circles represent geographical patches were no seedlings were sampled .

(*i.e.* flowering individuals) for the two sites located in Brittany (*MOR* and *PAL*). At *AUD*, exhaustive sampling of adult individuals was carried only in one particular geographical patch, located in the southern part of the population (Fig. 1). Seedlings were collected during two consecutive years (2007 and 2008). The first year of the study, seedlings were sampled in one

geographical patch in each site (*PE* at *PAL, MC* at *MOR* and *AA* at *AUD*, see Fig. 1). The following year, the same sampling was conducted in *AUD* and extended to other geographical patches in *PAL* and *MOR,* provided more than 20 seedlings were found. Overall, seedlings were collected in three patches of individuals in 2007 and in eight patches of individuals in 2008 (Fig. 1). Contrary to adults, sampling of seedlings was not exhaustive. All seedlings were collected in late spring (May – June) several weeks before seed production, which occurs from July to September. These seedlings were thus derived from dormant seed banks.

For all the sampled individuals (adults and seedlings collected during the two consecutive years of the study), leaves were collected for genotyping and the location of all flowering plants was mapped. Overall, 1869 adult individuals and 769 seedlings were used in this study (see Table 1 for repartition among study sites and geographical patches). In addition, the sexual phenotype was determined (female or hermaphrodite) for almost all adult individuals within each study site (98.5% in *MOR,* 97.4% in *PAL* and 63% in *AUD*). Plants with brown or white and reduced anthers were considered to be females and plants with yellow anthers with obvious pollen production were considered to be hermaphrodites (Dufay *et al.*, 2008). Some individuals showed intermediate phenotypes (light-coloured yellow anthers with little pollen production) and were also classified as hermaphrodites. Because of vernalization requirement, seedlings only flower in the second year after emerging. As a consequence, the phenotype of collected seedlings could not be scored.

Table 1: Number of individuals sampled, local densities and occurrence of gynodioecy within each geographical patch and within the different cohorts in the three study sites of *Beta vulgaris* ssp. *maritima* (*AUD*, *MOR* and *PAL*). N_{TOT} is the total number of sampled individuals (adult sampling was exhaustive, contrary to seedling sampling). *D* is the average density of adult individuals (ind/m²). $Freq_{CMS}$ is the frequency of CMS genes, *i.e.* the proportion of individuals carrying a cytoplasmic male sterility. The sex-ratio (measured on adults only) is the proportion of females. NF, NH_{CMS} and NH_{NCMS} are the number of females, restored CMS hermaphrodites and non-CMS hermaphrodites (with the number between brackets corresponding to non-phenotyped individuals).

Site	Geographical patch and cohort	N_{IND}	*D* (ind/m²)	$Freq_{CMS}$	Sex-Ratio	NF / NH_{CMS} / NH_{NCMS}
AUD	*AA* Adults	241	0.168	0.988	0.763	116 / 33 / 3 (89)
	AA Seedlings (2007/2008)	192 / 120	-	0.984 / 0.975	-	-
MOR	*MA* Adults	69	0.057	0.088	0.000	0 / 3 / 62 (4)
	MA Seedlings (2008)	25	-	0.080	-	-
	MB Adults	635	0.843	0.000	0.000	0 / 0 / 633 (2)
	MB Seedlings (2008)	24	-	0.000	-	-
	MC Adults	280	0.081	0.296	0.063	17 / 60 / 195 (9)
	MC Seedlings (2007/2008)	77 / 115	-	0.286 / 0.235	-	-
	MD Adults	107	0.138	0.000	0.000	0 / 0 / 106 (3)
	MD Seedlings (2008)	20	-	0.000	-	-
PAL	*PA* Adults	144	0.034	0.000	0.000	0 / 0 / 142 (4)
	PA Seedlings (2008)	20	-	0.000	-	-
	PC Adults	300	0.176	0.383	0.210	61 / 45 / 185 (9)
	PC Seedlings (2008)	88	-	0.455	-	-
	PE Adults	93	0.053	0.228	0.033	3 / 18 / 71 (3)
	PE Seedlings (2007/2008)	70 / 45	-	0.200 / 0.156	-	-

Laboratory procedures

DNA was extracted from leaves and purified using the NucleoSpin®96 Plant Kit (Macherey-Nagel). All sampled adults and seedlings were genotyped using three different marker types. First, nine nuclear microsatellite loci chosen for high polymorphism were used to describe the neutral genetic diversity: *Bmb6* (Cureton *et al.*, 2002); *Bvm3* (Mörchen *et al.*, 1996); *GTT1, CAA1* (Viard *et al.*, 2002); *SB04*, *SB06*, *SB07*, *SB15* (Richards *et al.*, 2004); and *FDSB1027* (McGrath *et al.*, 2007). Second, cytoplasmic polymorphism was characterized using four mitochondrial minisatellite loci: *TR1*, *TR2*, *TR3* and *TR4* (Nishizawa *et al.*, 2000). Each genotype combination for these four minisatellite loci will be analyzed as a single haplotype, as the entire mitochondrial genome is generally inherited as a single linkage unit. Finally, diagnostic cytoplasmic PCR-RFLP markers were used to distinguish among the three main CMS types: two mitochondrial markers that correspond to the CMSs *Svulg* and *G*, and a chloroplast marker for CMS *E* (Ran & Michaelis, 1995; Dufay *et al.*, 2008). In this study, we did not attempt to detect the occurrence of the fourth sterilizing cytoplasm (CMS *H*) because this cytotype is known to be absent in the studied area (Cuguen *et al.*, 1994). Amplification procedures and detection of polymorphism for these three marker types have been detailed in previous studies (Fénart *et al.*, 2008; Dufay *et al.*, 2009; De Cauwer *et al.*, 2010b). A total of 2665 samples were genotyped and missing data rates were 0.92%, 1.61% and 0.53% for nuclear microsatellites, mitochondrial minisatellite haplotypes and cytoplasmic male sterility PCR-RFLP markers, respectively.

Statistical analyses

Nuclear and cytoplasmic diversity

We used the rarefaction method described in El Mousadik & Petit (1996) to calculate allelic richness ($A_{R\ Nuc}$ and $A_{R\ Cyto}$), which is a measure of the number of alleles (or haplotypes) that is independent of sample size. To be able to compare allelic richness among the different cohorts and among the different locations, values were standardized to a common sample size (N=20 individuals, *i.e.* the size of the smallest sample in this study), using FSTAT version 2.9.3 (Goudet, 1995). Intra-population fixation indexes (F_{IS}) were also calculated for nuclear microsatellite loci using FSTAT version 2.9.3 (Goudet, 1995). Departures from Hardy–Weinberg equilibrium within each cohort and each geographical patch was tested by comparing F_{IS} values for the observed data set with their distribution for a randomized data set obtained after 10 000 permutations of alleles among individuals within geographical patches. Finally, we estimated pairwise population differentiation (F_{STN} for nuclear data and F_{STC} for cytoplasmic haplotypes) between (i) the adults and seedlings of the 2007-cohort, (ii) the adults and seedlings of the 2008-cohort and (iii) the seedlings of the 2007-cohort and the seedlings of the 2008-cohort. Analyses included 10 000 permutations of individuals among the groups of individuals, using a G test for significance of results (Goudet *et al.*, 1996). Significance of *P*-values for pairwise F_{ST} values was assessed after Bonferroni correction (Rice, 1989).

Testing for spatial genetic structure within geographical patches

The levels of spatial genetic structure within geographical patches were assessed by performing statistical correlation analyses between pairwise kinship estimates and pairwise geographical distances, for both nuclear data (microsatellites loci) and cytoplasmic data (minisatellite haplotypes) using SPAGeDi version 1.2. (Hardy & Vekemans, 2002). Nason's kinship coefficient F_{ij} (Loiselle *et al.*, 1995) was chosen as a pairwise estimator of genetic relatedness, as it has robust statistical properties (Vekemans & Hardy, 2004). Kinship coefficients (F_{ij}) were regressed on the natural logarithm of geographical distance ($\ln(d_{ij})$), thereby providing the regression slopes b. Standard errors were assessed by jackknifing over loci (for nuclear microsatellites) and the significance of the regression slopes were calculated by 10 000 permutations of individual locations (for nuclear microsatellite and cytoplasmic haplotypes). Finally, to compare the strength of spatial genetic structure between nuclear and cytoplasmic data, as well as between the different geographical patches and different cohorts, we used the statistic S_p described Vekemans & Hardy (2004), and defined as $S_p = - b / (1-F_N)$, where b is the slope calculated over 30 m and F_N is the mean F_{ij} between neighboring individuals ($d_{ij} < 2$m).

Estimation of the effective population size

The effective population size, N_e, is defined as the size of an ideal population (Fisher, 1930) that has the same rate of change of allele frequencies or heterozygosity as the observed population. N_e is typically smaller than the real population because the latter rarely behaves in an ideal manner (*i.e.* having equal sex ratios, constant population size, discrete generations and an equal contribution of individuals to reproduction). Because of the difficulty of collecting sufficient amounts of demographic data to measure N_e, various indirect estimators based on genetic data have been developed. Genetic estimates of contemporary effective population size can be based either on a single sample or two temporal samples (Luikart *et al.*, 2010; Saarinen *et al.*, 2010). Both approaches were used in this study.

The first estimator used in this study (N_{eLD}) is based on the single-sample method, which uses linkage disequilibrium (LD) information. The principle is that a decreasing N_e (*i.e.* fewer parents) will generate non-random associations among alleles at different loci (Waples, 1991). We used the Burrows' Δ as a point estimator based on LD and implemented in the LDNE software (Waples & Do, 2010). This method corrects for biases associated with small sample sizes, does not depend on the assumption of random mating and yields unbiased estimates of LD ($\hat{r}^2$) from which *Ne* can be derived. To ensure no bias due to low frequency alleles, *Ne* estimates were calculated after excluding alleles with frequencies less than 5%. N_{eLD} estimators were computed for the adult cohort in each geographical patch, as well as for one (*MA*, *MB*, *MD*, *PA* and *PC*) or two (*AA*, *MC* and *PE*) samples of seedlings, depending on whether seedlings were collected only in 2008 or both in 2007 and 2008.

The second estimator used in this study (N_{eTEMP}) is based on the temporal method, which uses the magnitude of random changes in allele frequencies over time. We used the moment-based

temporal estimator of *Ne* described in Jorde and Ryman (2007) and implemented in the TEMPOFS software, which limits the bias associated with small sample sizes and skewed allele frequencies. Temporal estimates of *Ne* were calculated using the number of adults as census size and according to sample plan 1, *i.e.* when individuals are either sampled after reproduction or non-destructively sampled and returned back into the population before reproduction (Waples, 1989). In *AA*, *MC* and *PE* (where seedlings were sampled both in 2007 and 2008) N_{eTEMP} was estimated for two distinct data sets (adults – seedlings 2007 and adults-seedlings 2008). For the other geographical patches (*MA*, *MB*, *MD*, *PA* and *PC*), N_{eTEMP} was estimated using adults and seedlings collected in 2008.

Distribution of sex determining genes and sex ratios

Cytoplasmic male sterility genes (CMSs) were present in the three study sites and in five of the eight studied geographical patches. Overall, 33.1% of sampled individuals (adults and seedlings) were carrying a CMS gene. The frequency of CMS genes varied importantly from one location to another, ranging from 8.8% to 98.8% in adults and from 8.0% to 98.4% in seedlings, and was relatively stable across sampling years (see Table 1). Nearly all individuals carrying a CMS gene were carrying CMS *E* (99.5%). CMS *G* only occurred sporadically. In the first cohort (adults), it was only found in 11 plants, all located in one particular geographical patch (*PC*). CMS *G* was absent in the seedlings collected in *PC* but was detected in two geographical patches where it was absent in the first cohort: *PE* (one seedling collected in 2007) and in *MC* (two seedlings collected in 2008). None of the sampled individuals was carrying CMS *Svulg*.

Adult individuals were also phenotyped. It was thus possible to discriminate females and restored hermaphrodites among individuals carrying CMS genes. Both CMS genes detected in our study sites were partly restored for male fertility. The restoration rates (proportion of CMS individuals expressing a hermaphroditic phenotype) varied importantly from one location to another (22% to 100%). As a result of both frequency of CMS genes and restoration rates varying among the geographical patches, local sex ratio (proportion of females) also showed pronounced spatial variation (ranging from 0 to 76.3%). Females were completely absent in *MB*, *MD* and *PA*, where CMS genes were missing, as well as in *MA*, where the few individuals carrying a CMS gene were all restored for male fertility (see Table 1).

Genetic diversity

Over the whole data set, nuclear microsatellite loci exhibited moderate to high levels of polymorphism, with the number of alleles ranging from 4 (*Gtt1*) to 29 (*Bmb6*). The total number of alleles (cumulated on adults and seedlings) was 137 (83 in *AUD*, 119 in *MOR* and 104 in *PAL*) and the mean number of alleles per locus (±SD) was 13.44 (±7.16). The variation of allelic richness ($A_{R\ Nuc}$) and estimated intra-population fixation index (F_{IS}) among geographical patches and among cohorts are illustrated in Figure 2. Average allelic richness per locus ranged from 3.88 (for the seedlings collected in *MA* in 2008) to 7.07 (for the seedlings collected in *MC* in 2008) and appeared to be quite stable across sampling years (Figure 2). Intra-population fixation indexes were variable among geographical patches as well as across time (ranging from -0.078 for the adults sampled in *AA* to 0.119 for the seedlings collected in *MC* in 2008). Considering adult individuals, nuclear microsatellites showed a significant deficit of heterozygotes compared to Hardy-Weinberg expectations in almost all patches, excepted for *PE*, where the observed deviation was not significant, and for *AA*, where the opposite tendency was detected, with a significant excess of heterozygotes (see Figure 2). As significant single-locus F_{IS} values were not specifically

associated with one locus, this overall trend towards a heterozygote deficiency could not be attributed to the presence of null alleles (data not shown). In the geographical patches where a significant deficit of heterozygotes was observed for adults, the same tendency remained for seedlings, although it was significant only in three locations (*MC*, *PA* and *PC*). In *PE*, the two cohorts of seedlings showed an important increase in F_{IS} values compared to what was observed in the adult cohort. In *AA*, the excess of heterozygotes detected for the adults decreased for the seedlings sampled in 2007 (becoming non-significant) and disappeared for the seedlings sampled in 2008 (see Figure 2).

In contrast to nuclear loci, cytoplasmic mitochondrial minisatellites displayed low levels of polymorphism and yielded a total of 11 distinct haplotypes of which three were present in *AUD,* nine were present in *MOR* and six were present in *PAL*. Haplotypic richness ($A_{R\ Cyto}$) varied importantly among geographical patches, as illustrated in Figure 2. As expected, each male-sterilizing cytoplasm detected in our study sites (CMS *E* and CMS *G*) was exclusively associated with a unique minisatellite haplotype. Whereas most patches exhibited a relatively constant cytoplasmic diversity over time, substantial changes in allelic richness were found in *PE*, where $A_{R\ Cyto}$ is two-fold higher in seedlings collected in 2007 compared to both adult individuals and seedlings collected in 2008, suggesting an introduction of new haplotypes by spatial migration or by a resurgence of seed bank.

In a study conducted in tandem with the current one (De Cauwer *et al.*, in prep-a), the magnitude of genetic differentiation among geographical patches was estimated in the two study sites located in Brittany (*MOR* and *PAL*). Pairwise F_{ST} estimates were all significant, regardless of the type of genetic marker that was considered, with F_{STC} values (calculated on cytoplasmic data) ranging from 0.154 to 0.914 and F_{STN} values (calculated on nuclear data) ranging from 0.039 to 0.217. In the current study, the levels of genetic differentiation were calculated among distinct cohorts within each studied geographical patch. The observed levels of temporal differentiation appeared to be relatively low, with F_{STC} values ranging from slightly negative values (*i.e.* no differentiation) to 0.114 and F_{STN} values ranging from slightly negative values to 0.031 (Table 2). Among the eight studied patches, significant differentiation among distinct cohorts only occurred in three geographical patches for nuclear data (*AA*, *MC* and *PE*) and in two geographical patches for cytoplasmic data (*MC* and *PE*). When comparing temporal and spatial levels of differentiation within the two study sites where these two estimates were available (i.e. in the sites where several patches were studied: PAL and MOR, see De Cauwer *et al.*, in prep-a), F_{ST} values among successive cohorts were on average 13 and 23-fold lower than F_{ST} values among geographical patches, for nuclear and cytoplasmic data respectively. We found a negative association between the observed values of temporal differentiation and the total number of adult individuals located within the studied geographical patches for both marker types (Spearman's Rho = 0.71; $P = 0.028$ for nuclear data and Spearman's Rho = 0.75; $P = 0.033$ for cytoplasmic data, see Figure 3). Finally, we found no significant effect of local sex ratios on the levels of genetic diversity, as measured by A_R or F_{IS} (Spearman's Rho, all at $P > 0.05$).

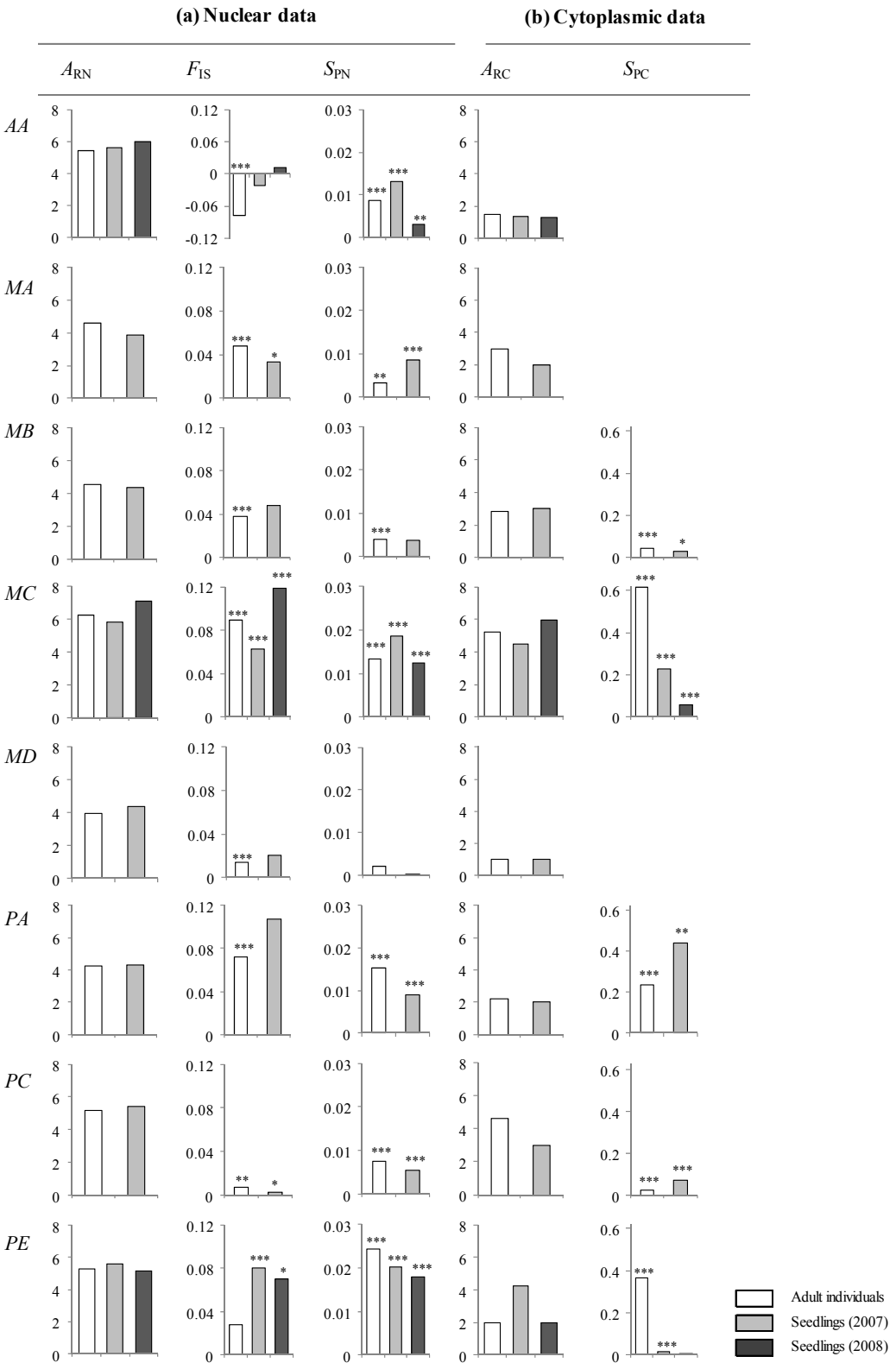

Fig. 2 Measures of genetic diversity on nuclear data (a) and on cytoplasmic data (b) within the different geographical patches of *Beta vulgaris* ssp. *maritima (AA* in the *AUD* site, *MA, MB, MC* and *MD* in the *MOR* site, *PA, PC* and *PE* in the *PAL* site*)*. Allelic richness values (A_{RN} and A_{RC}, for nuclear and cytoplasmic data, respectively) were standardized to a common sample size (N=20 individuals, *i.e.* the size of the smallest sample in this study). Significance of multilocus F_{IS} estimates per geographical patch were tested using 10 000 random permutations of alleles among individuals. S_p values estimate the intensity of spatial genetic structure in each geographical patch. Spatial genetic structure was considered to be significant when the regression slopes were significant (tested using 10 000 permutations of individual locations) *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

Table 2: Levels of pairwise genetic differentiation (F_{ST}) estimated between (i) the adults and seedlings of the 2007-cohort (A – S_{2007}), (ii) the adults and seedlings of the 2008-cohort (A – S_{2008}) and (iii) the seedlings of the 2007-cohort and the seedlings of the 2008-cohort (S_{2007} - S_{2008}), for nuclear data (a) and cytoplasmic data (b) for all studied patches of *Beta vulgaris* ssp. *maritima.* Significance of genetic differentiation was tested with 10 000 random permutations of individuals between geographical patches, using a G test for significance of results (Goudet *et al*. 1996). *: $P < 0.05$

		(a) F_{STN}			**(b)** F_{STC}		
Site	Patch	A - S_{2007}	A - S_{2008}	S_{2007} - S_{2008}	A - S_{2007}	A - S_{2008}	S_{2007} - S_{2008}
AUD	*AA*	0.0054	0.0048*	0.0045	-0.0041	-0.0056	-0.0067
MOR	*MA*	-	0.0308	-	-	0.0749	-
	MB	-	-0.0046	-	-	-0.0137	-
	MC	-0.0011	0.0086*	0.0105*	0.0008	0.0145*	0.0201
	MD	-	0.0012	-	-	-	-
PAL	*PA*	-	0.0116	-	-	0.0125	-
	PC	-	0.0031	-	-	0.0269	-
	PE	0.0161*	0.0042	0.0241*	0.0998*	-0.0062	0.1141

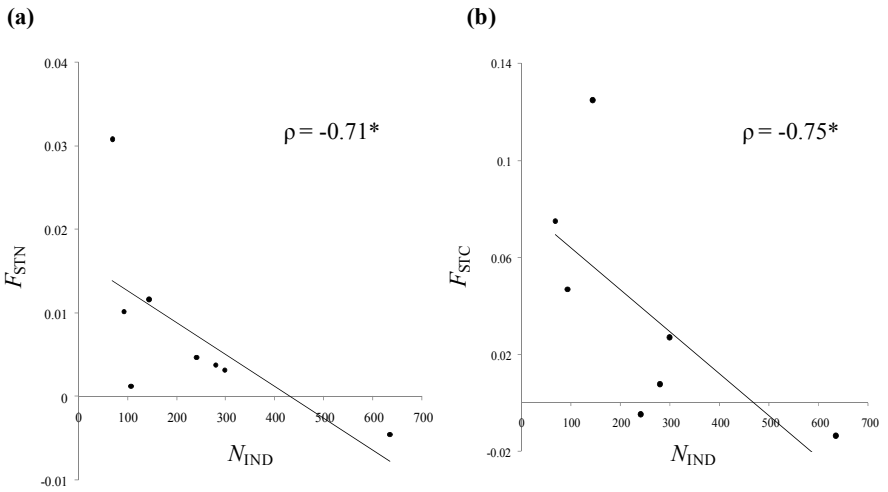

Fig. 3 Levels of genetic differentiation between adult individuals and seedlings (F_{ST}) within geographical patches plotted against patch size (*i.e.* number of adult individuals, N_{IND}), for nuclear data (a) and for cytoplasmic data (b), and line of best fit. When seedlings were available for the two consecutive years of the study (2007 and 2008), the average value was considered. ρ : Spearman's rank correlation coefficient. *: $P < 0.05$

Testing for spatial genetic structure within geographical patches

B. vulgaris natural populations classically display strong spatial structure at very restricted spatial scales (a few tens of meters, see Laporte *et al.*, 2001; De Cauwer *et al.*, 2010b). This was also the case in our study sites: nuclear spatial genetic structure within geographical patches was shown to be significant for all patches and for the successive cohorts, except *MD* for adult individuals and *MD* and *MB* for seedlings. Spatial genetic structure was also characterized using cytoplasmic data (minisatellite haplotypes), except for *MD,* where all individuals were carrying the same haplotype and for *AA* and *MA,* where more than 95% of individuals were carrying the same haplotype. Similarly to what was observed for nuclear data, cytoplasmic spatial

genetic structure was found to be significant in all geographical patches and for all cohorts (see Figure 2). However, there was considerable difference in the magnitude of spatial genetic structure between cytoplasmic and nuclear variation, as quantified by the S_p statistic. On average, the levels of spatial genetic structure for nuclear data were 11-fold lower than what was observed for cytoplasmic data (see Figure 2). The levels of spatial genetic structure (S_p statistics) measured in each geographical patch for adult individuals was significantly correlated with what was observed in the following cohorts for nuclear data (Spearman's Rho = 0.83; P = 0.008) but not for cytoplasmic data (Spearman's Rho = 0.10; P = 0.475).

Local density had a negative effect on the levels of spatial genetic structure for nuclear data although this effect was significant only in seedlings (Spearman's Rho = -0.525; P = 0.098 and Spearman's Rho = -0.738; P = 0.023 for adults and seedlings, respectively). The same trend remained for cytoplasmic data but was non-significant in both cases (Spearman's Rho = -0.500; P = 0.225 and Spearman's Rho = -0.400; P = 0.258 for adults and seedlings). No conclusive effect of sex-ratio on the strength of spatial genetic structure was depicted, neither in adults or seedlings.

Estimates of effective population size (N_e)

Single-sample *Ne* estimated based on linkage disequilibrium (N_{eLD}) and temporal-*Ne* estimates based on the changes of allele frequencies over sampling years (N_{eTEMP}) are presented in Table 3. Both estimators were generally in good concordance, yielding *Ne* estimates of the same order of magnitude that ranged from 8 to 236, depending on the geographical patch and excluding infinite estimates. N_e/N values were low: almost all estimates were below 0.3 (see Table 3), regardless of the method that was used. We found no correlation between local sex-ratios in adults and the two estimates of effective population size (N_{eLD} and N_{eTEMP}). N_{eLD} estimates (calculated on the consecutive cohorts: adults, seedlings collected in 2007 and seedlings collected in 2008) appeared to be quite stable across time, with largely overlapping CIs for different cohorts located in the same patch, except for *AA*, were the effective population size estimate obtained in 2008 was larger than what was observed in the previous samples. Overall, no conclusive trends could be drawn from the effects of sex-ratio on the values of N_e/N, as gynodioecious patches (*i.e*. patches where females were found) were either characterized by moderate or high effective population size relatively to the census size measured on adults (see Table 3).

Table 3: Estimations of the effective population size (95% CI) for the different geographical patches according to the single-sample method based on linkage disequilibrium (a) and on the temporal method based on the magnitude of random changes in allele frequencies over time (b). N_{eLD} estimates were computed separately for the adult and seedling cohorts in each geographical patch (A: adults, S_{2007}: seedlings collected in 2007 and S_{2008}: seedlings collected in 2008). N_{eTEMP} estimates were based on the changes in allele frequencies between adults and seedlings collected in 2007 (A - S_{2007}) and between adults and seedlings collected in 2008 (A - S_{2008}). N_e/N ratios were then calculated using the number of adult individuals within each geographical patch as census size (N).

		(a) N_{eLD}						**(b)** N_{eTEMP}			
Site	Patch	A	N_e/N	S_{2007}	N_e/N	S_{2008}	N_e/N	A - S_{2007}	N_e/N	A - S_{2008}	N_e/N
AUD	*AA*	23 (11 - 94)	0.095	13 (6 - 38)	0.054	123 (26 - ∞)	0.510	34 (21 - 82)	0.141	37 (19 - 530)	0.154
MOR	*MA*	9 (5 - 16)	0.130	-	-	8 (3 - 20)	0.116	-	-	6 (4 - 19)	0.087
	MB	16 (8 - 42)	0.025	-	-	28 (12 - 348)	0.044	-	-	∞	∞
	MC	50 (20 - ∞)	0.179	40 (16 - ∞)	0.143	17 (10 - 34)	0.061	236 (101 - ∞)	0.843	23 (13 - 129)	0.082
	MD	22 (9 - ∞)	0.206	-	-	19 (9 - 75)	0.178	-	-	42 (12 - ∞)	0.393
PAL	*PA*	15 (7 - 43)	0.104	-	-	12 (6 - 29)	0.083	-	-	15 (9 - 60)	0.104
	PC	11 (6 - 27)	0.037	-	-	20 (10 - 68)	0.067	-	-	52 (33 - 120)	0.173
	PE	11 (6 - 20)	0.118	22 (11 - 19)	0.237	14 (8 - 28)	0.151	11 (8 - 23)	0.118	25 (16 - 60)	0.269

B. vulgaris ssp. *maritima* forms discrete populations in coastal habitats where environmental disturbances frequently occur during high tides or winter storms, and natural populations are known to undergo large fluctuations in population size, sometimes leading to local extinctions. In the face of important environmental disturbances, seed storage is a common adaptation in plants (Epling *et al.*, 1960). As in many species growing in disturbed and unpredictable habitats, *B. vulgaris* seeds are known to exhibit dormancy (Sester *et al.*, 2006; Wagmann, 2008; Arnaud *et al.*, 2010a). Because a single individual can produce up to several thousands seeds, dormancy could result in the built up of very large seed banks in natural populations. Theory predicts that the seed banks may play an important role in the evolutionary dynamics of plant populations, by buffering against demographic fluctuations and genetic bottlenecks caused by intermittent disturbance and habitat fragmentation (Templeton & Levin, 1979; Cabin, 1996; Vitalis *et al.*, 2004). By comparing the levels of genetic diversity and spatial genetic structure among adults and seedlings emerging from seed banks in eight different locations, we attempted to gain some insights into the genetic composition and the functioning of seed banks in *B. vulgaris,* as well as in the potential impact of seed banks on the evolution of local sex ratios.

Short-term evolution of genetic diversity

Numerous empirical studies investigating the genetic composition in adults and in seedlings emerging from seed banks showed similar genetic composition in the different cohorts (Gottlieb, 1974; Mahy *et al.*, 1999; Barrett *et al.*, 2005; Ayre *et al.*, 2009). This was also the case in the current study: within each geographical patch, the observed levels of allelic richness for nuclear and cytoplasmic data displayed limited variation among the different temporal samples. Similarly, the observed departures from Hardy-Weinberg equilibrium were relatively constant over time, excepted in *AA* and *PE* (see below), and the estimates of genetic differentiation between successive cohorts were low and non-significant in most cases. Altogether, our results thus suggested very narrow changes in genetic composition across time. There are two possible explanations for the observed similarity between consecutive cohorts. First, in the case of long-lived seed banks, both individuals from the adult cohort and seedlings from the following cohorts may represent a random sample of the same seed bank comprising non-germinated seeds produced over numerous generations, which may prevent any substantial genetic change over time (e.g. Gottlieb, 1974). Alternatively, a majority of seedlings emerging from the seed bank in spring may derive from seeds deposited during the previous flowering season. Because *B. vulgaris* is a perennial species (Hautekèete *et al.*, 2002), seedlings collected in 2007 and 2008 may thus be the product of the reproduction of the adult plants that were present in 2007. Although there is no information about the actual viability duration of buried seeds in *B. vulgaris,* germination rates in *B. vulgaris* are known to decrease sharply with time and seed depth, while the presence of light

stimulates germination (Sester *et al.*, 2006). The observed genetic similarity between consecutive cohorts is thus likely to be due to the fact that the studied seedlings were produced by the adults collected in the different localities in 2007.

Although the allelic richness was similar in adults and seedlings, some nuclear alleles and some cytoplasmic haplotypes that were locally absent in the first cohort were detected in the following ones. For instance, CMS *G*, that was quite rare in all our study sites, was found in seedlings but not in adults in two distinct geographical patches (*MC* and *PE*). Because adults were exhaustively sampled, this observation corresponds to the establishment of a new CMS in localities where it was previously missing. When a pool of persistent dormant seed exists, it is not clear whether a newly established haplotype arrived following spatial migration or whether the haplotype was derived from plants occupying the patch in previous years. Both mechanisms could indeed account for the presence of seedlings carrying CMS *G* in geographical patches were this particular male sterility was previously lacking. While the cytonuclear polymorphism associated with gynodioecy is expected to be lost in structured populations under drift alone, seed dispersal, in space and/or in time could contribute to maintain sex polymorphism (Dufay & Pannell, 2010).

Finally, although the levels of genetic differentiation between different cohorts were low and non-significant in almost all geographical patches, we found a negative association between the values of temporal genetic differentiation (F_{ST}) and the adult census population size, suggesting that small populations may be more prone to variation in allelic frequencies. This result is good illustration of the effect of local population size on the establishment of genetic variation, through variation in the intensity of genetic drift (Honnay *et al.*, 2008). It may further suggest that small populations could be more permeable to incoming gene flow, which could also lead to temporal changes in allele frequencies. Indeed, in large populations, local pollen clouds are likely to be saturated by neighboring conspecifics, impeding long-distance gene flow from outside, in contrast to low-density patches that are more prone to receive external gene flow (Fénart *et al.*, 2007; De Cauwer *et al.*, 2010b).

Spatial genetic structure in the different cohorts

Despite the fact that wind-mediated pollination and strict outcrossing breeding system are expected to weaken the spatial genetic structure (Loveless & Hamrick, 1984; Vekemans & Hardy, 2004), we found evidences of a significant fine-scale genetic structure in both adult individuals and the following cohorts. The levels of genetic structure, quantified by the S_p statistic, were relatively stable across time for nuclear data, but not for cytoplasmic data. No clear interpretations can be put forward to explain this discrepancy. One possibility would be that changes in cytoplasmic genetic structure may reflect a non-equilibrium state (e.g. Olson & McCauley, 2002). In *B. vulgaris* populations, while nuclear diversity often exhibits clear trends of isolation by distance, cytoplasms are typically clustered in space and form mosaics, with each haplotype probably tracing back to the original colonists belonging to different maternal lineages (De Cauwer *et al.*, in prep-a). Differential resurgences of haplotypes may then allow for temporal

changes in S_p statistics, in contrast to nuclear diversity that would have achieved an equilibrium state of spatial genetic structure across time because of more efficient local pollen flow.

The levels of spatial genetic structure observed in the studied patches showed a trend to decrease with local density. Previous studies also documented such negative association in various species (e.g. Murawski & Hamrick, 1991; Gonzales *et al.*, 2010). This tendency is thought to result from the fact that when levels of density are important, the local number of available mates is much higher. In addition, in *B. vulgaris* populations, under high densities, the floral stems of neighboring individuals tend to be intertwined, which may result in the overlapping in seed shadows. Under isolation by distance, the strength of spatial genetic structuring is then expected to be inversely proportional to the density (Heywood, 1991; Vekemans & Hardy, 2004).

Departures from Hardy-Weinberg expectations

In addition to the lack of clear temporal patterns of variation in the genetic composition, another general observation was the overall trend for heterozygote deficits. Several biological processes can be advocated to explain the fact that positive F_{IS} values were found in almost all studied patches and in the different consecutive cohorts (although not significant in all cases, see Fig. 2). First, these departures from Hardy-Weinberg expectations may be due to the occurrence of distinct genetic clusters and/or isolation by distance within geographical patches, such local variation in allelic frequencies leading to a spatial Wahlund effect. Genetic discontinuities have indeed been documented in the adults located *MC* and *PE* (De Cauwer *et al.*, in prep-a) and the present study suggests significant spatial genetic structure due to isolation by distance for both nuclear and cytoplasmic data within all studied patches, regardless of the cohort that is considered (except *MD* for adult individuals and *MD* and *MB* for seedlings). Second, mixtures of different cohorts cannot be ruled out and this could result in a temporal Wahlund effect. However, in the particular case of seed bank–induced age structure, Vitalis *et al*. (2004) theoretically showed that a delayed germination does not generate a temporal Wahlund effect unless the population sizes are very small (≤10 individuals), which is not the case in our study.

Two exceptions remained regarding the general trend for a stable genotypic composition between adults and seedlings: patches *AA* and *PE*. In contrast with what was observed in the other study patches, adult individuals located in *AA* showed a significant excess in heterozygotes. This trend disappeared in the following cohorts. This result may be related to sex ratio variation across time. The proportion of females observed in this particular patch was previously estimated in 2005 (De Cauwer *et al.*, 2010b) and was slightly higher than what is reported here (0.867 in 2005 and 0.763 in 2007). As the proportion of CMS individuals was similar, the observed variation in sex ratio was mainly due to an increase in the restoration rate through time (0.098 in 2005 compared to 0.221 in 2007). A possible consequence of this increase in the proportion of local pollen donors might be a decrease of the amounts of pollen coming from outside the geographical patch. Indeed, the occurrence of females is likely to promote long-distance mating events (e.g. García *et al.*, 2005). As a consequence of past female predominance within this particular geographical patch,

pollen exchanges maybe took place among geographically and genetically distinct individuals earlier in the patch's history, probably involving hermaphrodites located northward along the coastline (see Fig. 1). Mating events between genetically differentiated pools of genes may then have given rise to an adult cohort characterized by significant excess in heterozygotes. The subsequent increase of the level of inbreeding may stem from an increased siring success of the rare restored hermaphrodites in the vicinity of females (see De Cauwer *et al.*, 2010b), leading to a mixture of full or half-sibs in the collected seedlings and explaining the observed increase in F_{IS} values across time.

The second exception involves the study patch *PE* where some variations were observed in the genotypic composition, along with substantial changes in cytoplasmic diversity and spatial structure. The increase in F_{IS} values and the cytoplasmic *Sp* statistics that dropped to zero for seedlings sampled in 2007 and 2008 are likely to involve the resurgence and spatial mixing of genetically differentiated cohorts. These changes in genetic features are presumably related to winter storms during 2006 and 2007 years, that strongly disturbed this part of the beach, which is close to upper level of high tides (IDC, MD and JFA, pers. obs.)

Sex ratio variation

As what was observed for nuclear microsatellites and cytoplasmic haplotypes, the frequency of CMS genes within geographical patches was relatively stable across time. In gynodioecious populations, one condition thought to be necessary to maintain females is the existence of a female advantage. In other words, females must compensate for their gametic disadvantage relative to hermaphrodites *via* higher female reproductive fitness (Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufay *et al.*, 2007). As a consequence, in the localities were CMS genes are at least partially associated with female phenotypes (*i.e.* when the restoration genes are not fixed), one could expect an increase of CMS frequencies over time. However, the evidences for female advantage are somewhat equivocal in *B. vulgaris,* as previous studies documented very low and non-significant levels of female advantage (Boutin *et al.*, 1987; De Cauwer *et al.*, in prep.). Female advantage in *B. vulgaris* is probably too restricted to observe significant changes in CMS frequencies, especially over the time-scale considered in the current study. Additionally, as females are strictly seed dispersers, the proportion of females could affect the levels of spatial genetic structure. This was not the case in our study, possibly because of the limited number of populations studied and because of the important number of other factors that potentially impact spatial genetic structure, like for instance the age of the population, the local density or the rates of incoming gene flow. Long-term studies with additional locations, contrasted in terms of sex ratio, are required to gain some insight in the impact of gynodioecy on the patterns of spatial genetic structure.

Beyond cytoplasmic identity, sexual phenotypes were also scored for adults. Both CMS frequency and restoration rate varied greatly from one geographical patch to another (0 to 98.8% of adults carrying a CMS gene and 22.1 to 100% restoration rate in CMS adults). As a result, the

sex ratio of adult individuals also showed pronounced variation among geographical patches (0 to 76.3% females). Such striking spatial variation can be explained by the effects of selection, founder events, dispersal and genetic drift (Olson & McCauley, 2002; McCauley & Bailey, 2009). Given that the CMS frequencies were stable across time, the sexual phenotype in seedlings only depends on temporal variation in restorer frequencies. Although the genetic composition of the studied geographical patches appeared to be relatively stable across time for neutral markers, variation in restorer frequencies may result from selective processes. As in our study species, sex ratios are often strongly structured in space in gynodioecious species (e.g. Medrano *et al.*, 2005; Nilsson & Agren, 2006; Dufay *et al.*, 2009) and, as a consequence, pollen-limited seed production can occur in female-biased localities (see Widen & Widen, 1990; Graff, 1999; De Cauwer *et al.*, 2010a). In such case, individuals expressing a hermaphroditic phenotype are expected to be strongly advantaged, because they are the only potential pollen donors in the vicinity (De Cauwer *et al.*, 2010b). Accordingly, Boutin-Stadler *et al.* (1989) showed that whereas frequency of CMS remained stable across two generations in two populations of *B. vulgaris*, substantial differences in female frequencies were observed in one population, presumably because of a rapid invasion of restorer genes.

Temporal genetic differentiation and effective population sizes

The genetic effective population size (N_e) depends on the patterns of gene dispersal, the nature of the mating system, the heterogeneity in reproductive success among individuals and various demographic factors, such as population density (Waples, 1991; Waples & Do, 2010). N_e is a key parameter in population genetics because it influences the levels of genetic drift, and thus predicts the degree of inbreeding, the rate of fixation of selectively advantageous and deleterious genes, as well as the amount of genetic differentiation between populations (Frankham, 1995; Luikart *et al.*, 2010). The existence of seed banks has been shown to increase N_e in some plant species, sometimes leading to N_e/N estimates superior to unity (see Lundemo *et al.*, 2009). Nonetheless, in our study, estimated N_e/N ratios were low in all patches (almost always inferior to 0.3). This confirms the general view that effective population sizes are often smaller than census sizes (Frankham, 1995). We recently showed that a strong variance in male fertility among hermaphrodites, associated with spatially restricted pollen flow, are likely to be responsible for a low number of effective pollen donors within some of the studied patches (De Cauwer *et al.*, in prep-b). Skewed reproductive success among hermaphroditic individuals may then result in the restricted effective population sizes we observed.

Altogether, a general trend emerged from this study: while the opportunity for genetic drift to act is suggested by the small *Ne* estimates we found, possibly implying a high reproductive variance among individuals both in terms of male and female function, no substantial temporal changes in allelic frequencies were depicted across the studied patches. Along with allelic frequencies, allelic richness fluctuated with very narrow amplitude. Similarly, no striking changes in genetic composition and similar *Ne* estimates, ranging from 20 to 30, were found in an annual gynodioecious species that widely fluctuate in census size (Del Castillo, 1994). This could suggest

that, at least over short-time scale, only a moderate number of successful breeders, along with large seed banks, are sufficient to protect against the loss of genetic diversity in structured populations. In gynodioecious populations, this could also modify importantly the expected effects of selection (Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufay *et al.*, 2007), and genetic drift and migration (Couvet *et al.*, 1998; Dufay & Pannell, 2010) on the frequency of sex determining genes.

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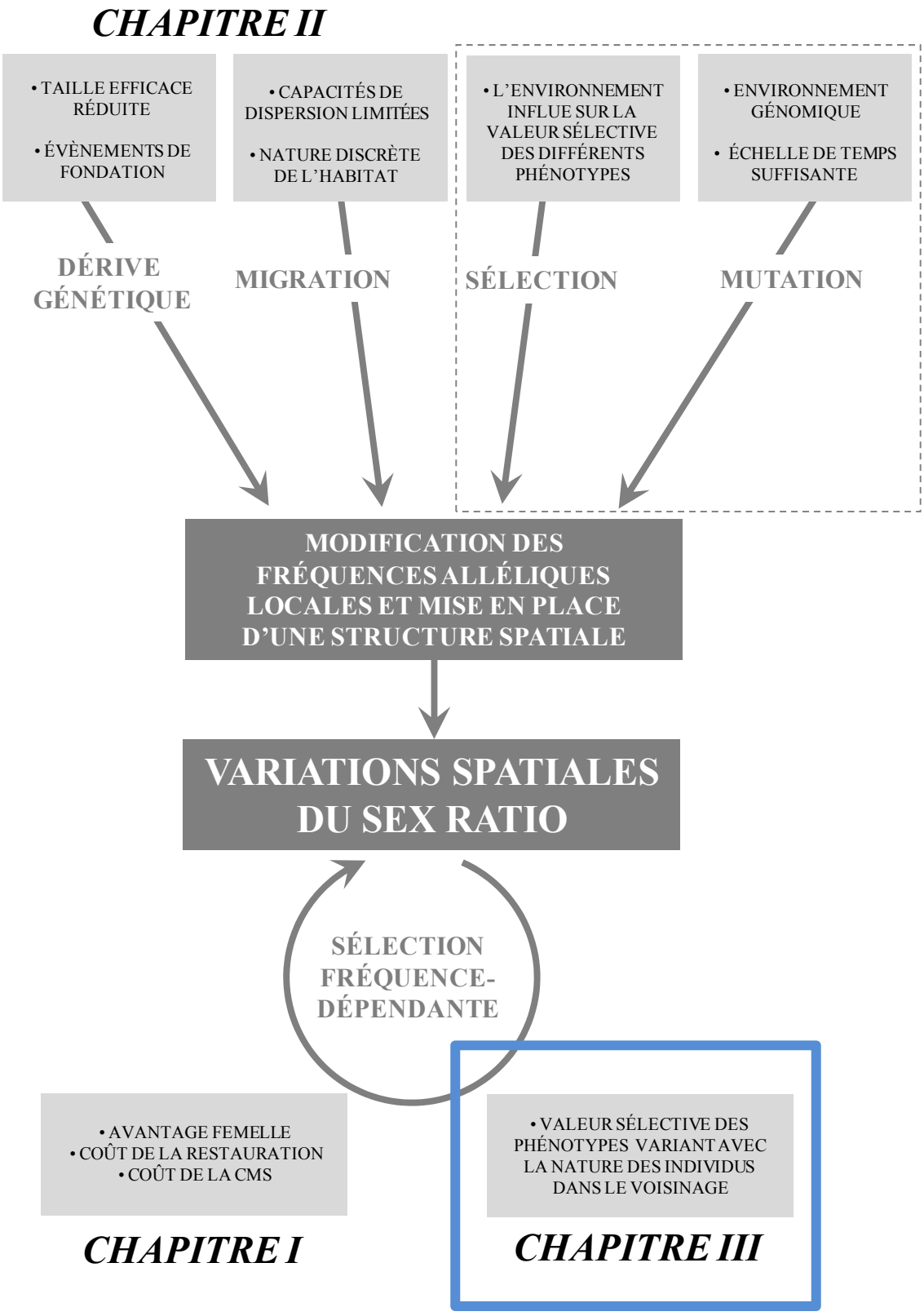
CHAPITRE III

INTERACTIONS ENTRE LA STRUCTURE GENETIQUE SPATIALE ET LE SUCCES REPRODUCTEUR

Problématique : Le succès reproducteur des différents phénotypes sexuels varie-t-il avec le sex ratio local ?

Dans les chapitres précédents, nous nous sommes attachés (i) à décrire les différences de valeur sélective mâle et femelle entre les différents phénotypes sexuels (femelles, hermaphrodites porteur de CMS et restaurés pour la fonction mâle, et hermaphrodites non porteurs de CMS) et (ii) à étudier la structure spatiale de la diversité génétique neutre et celle des gènes liés au déterminisme du sexe chez *B. vulgaris*. Dans le chapitre 3, nous allons essayer d'explorer les interactions particulières entre la forte structure spatiale observée pour les phénotypes sexuels et le succès reproducteur des individus. Dans un premier temps (Partie 1), nous nous demanderons si le succès reproducteur des femelles dépend de la quantité de partenaires sexuels disponibles, autrement dit, de la présence et de la fréquence d'hermaphrodites fonctionnels au voisinage immédiat. Dans un second temps, nous nous intéresserons aux effets possibles de la structure des phénotypes sexuels sur le succès reproducteur mâle des hermaphrodites. En particulier, sous l'hypothèse d'une compétition entre hermaphrodites pour l'accès à la reproduction, est-il possible que la transmission des gènes au travers du pollen soit accrue dans des voisinages où les femelles sont majoritaires ? Nous nous intéresserons en particulier au sort des allèles de restauration en populations structurées. Si l'on se rappelle que les hermaphrodites porteurs de CMS et restaurés pour la fonction mâle sont de mauvais producteurs de pollen par rapport aux hermaphrodites non-porteurs de CMS (*cf*. Chapitre I) mais que, du fait de la forte structure génétique spatiale, ils sont fréquemment trouvés à proximité des femelles (*cf*. Chapitre II), on peut s'interroger sur leur succès reproducteur (i) lorsqu'ils sont effectivement localisés dans des dèmes biaisés en faveur des femelles (Partie 2) et (ii) lorsqu'ils sont en compétition avec les hermaphrodites non-porteurs de CMS (Partie 3).

Photo : Population de *B. vulgaris* ssp. *maritima* en haut de l'estran, dans la zone de laisses de mer (site du Palus, Côtes d'Armor, France)



Pollen limitation of female reproductive success at fine spatial scale in a gynodioecious and wind-pollinated species, *Beta vulgaris* ssp. *maritima*

I. DE CAUWER, J.-F. ARNAUD, E. SCHMITT & M. DUFAY

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ABSTRACT

In sexually polymorphic plants, the spatial distribution of sexes is generally not random. Local variation in phenotype frequencies are expected to affect individual fitness of the different phenotypes in a frequency-dependent way. In gynodioecious species (co-occurrence of hermaphrodites and females), if sexual phenotypes are structured in space and if pollen flow is geographically restricted, local pollen availability should vary among demes. Female fitness may thus be restricted when hermaphrodites are locally rare. To test this hypothesis, we analysed how the reproductive output of females varied among demes, within two natural populations of the gynodioecious wind-pollinated *Beta vulgaris* ssp. *maritima*. Plants growing in female-biased areas were found to have low fruit and seed sets, due to pollen limitation, but did not reallocate resources towards better offspring. Our results highlight the important effects that fine-scale processes can have on individual fitness and on the evolution of sex ratio in sexually polymorphic plants.

INTRODUCTION

In sexually polymorphic flowering plants, natural populations are reproductively sub-divided into separate sexes or into distinct mating groups that differ in floral morphology (Barrett, 2002). In these species, the reproductive output of a given sexual morph may not only depend on its own sex-category, but also on the local frequency of the sexual phenotypes with which it can mate (e.g. Carlsson-Granér *et al.*, 1998; Jesson & Barrett, 2002; Stehlik *et al.*, 2006; Van Rossum *et al.*, 2006). The frequency-dependent nature of reproductive output in sexually polymorphic plant populations has long been recognized as an important component of the evolution of sexual morph ratios (Fisher, 1930).

These effects are expected to be of crucial importance in the case of gynodioecious species, in which females and hermaphrodites co-occur in natural populations. The genetics underlying sex expression in gynodioecious plants commonly involves cytonuclear epistatic interactions, with cytoplasmic male sterility (CMS) genes located in the mitochondria that confer a female phenotype, unless their action is counteracted by nuclear male fertility restorers (Saumitou-Laprade *et al.*, 1994; Chase, 2007). In such species, the frequencies of the genes that determine sexual phenotype and the sex ratios often vary considerably among populations (e.g. Tarayre & Thompson, 1997; Olson & McCauley, 2002; Asikainen & Mutikainen, 2003; Alonso, 2005; Nilsson & Agren, 2006; Cuevas *et al.*, 2008; Dufay *et al.*, 2009), suggesting that pollen availability should vary among populations as well. Consequently, one expects limited pollination to occur when the pollen donors are locally rare. Besides, when the sex ratio shows pronounced variation among populations, each gender clusters with its own gender rather than with the other. Hermaphrodites are thus likely to grow in hermaphrodite-biased populations and to experience less pollen limitation, whereas clustered females are likely to undergo pollen limitation. As a result, the negative effect of pollen limitation on fitness is theoretically expected to mainly affect female individuals (McCauley & Taylor, 1997; Pannell, 1997). Because any difference in seed production between females and hermaphrodites is of crucial importance for the maintenance of this particular sexual polymorphism (Charlesworth & Charlesworth, 1978; Gouyon *et al.*, 1991), pollen limitation, especially in females, should be investigated carefully. Indeed, several studies have demonstrated that seed production of female plants was limited by pollen availability in populations showing high female frequencies (e.g. Alonso, 2005; Zhang *et al.*, 2008).

Moreover, sex ratio in gynodioecious species is also known to vary at very local scales (Laporte *et al.*, 2001; Olson *et al.*, 2006; De Cauwer *et al.*, in press). This suggests that pollen-limited seed production may also occur in female-biased neighbourhoods within populations, although this has been shown in a restricted number of species only (see Widen & Widen, 1990; Graff, 1999). In addition, because pollen limitation should be found at small scale only when pollen flow is spatially restricted within populations, one may wonder to what extent such process could occur in wind-pollinated species. Indeed, pollen from wind-pollinated plants has

traditionally been assumed to be abundant (Cruden, 1977) and to travel long distances (Loveless & Hamrick, 1984), and to date, pollen limitation has been found in many different animal-pollinated species (Burd, 1994; Larson & Barrett, 2000; Ghazoul, 2005; Knight *et al.*, 2005) while this seems to be extremely rare in wind-pollinated plants (Friedman & Barrett, 2009, but see Knapp *et al.*, 2001; Koenig & Ashley, 2003; Davis *et al.*, 2004).

This work was carried out on wind-pollinated, gynodioecious *Beta vulgaris* ssp. *maritima* (L.) Arcangeli. In this species, to develop as a female, an individual must carry an unrestored CMS gene. To develop as a hermaphrodite, an individual must either carry a CMS gene in combination with the appropriate restoration alleles (restored hermaphrodite), or carry a non-CMS cytoplasm (Cuguen *et al.*, 1994; Forcioli *et al.*, 1998; Fénart *et al.*, 2006). Previous studies have suggested that the fitness of female plants could be frequency-dependent because of pollen limitation in *Beta vulgaris,* even if it is a wind-pollinated species. First, population structure in terms of sexual phenotypes generally seems to be very pronounced at small spatial scales (Laporte *et al.*, 2001; De Cauwer *et al.*, in press), probably resulting from limited seed dispersal and random founder events (Fievet *et al.*, 2007). Second, Dufay *et al*. (2008) showed that pollen viability is highly variable among pollen donors and significantly lower in restored (CMS) hermaphrodites compared to non-CMS hermaphrodites. As restored hermaphrodites are often clustered with females in natural populations due to the strong genetic structure observed for cytoplasmic genes (Laporte *et al.*, 2001; De Cauwer *et al.*, in press), only a restricted proportion of pollen reaching stigmas on females may be viable. Finally, the distribution of pollen dispersal events appears to be leptokurtic, with both a non-negligible proportion of long-distance dispersal events and more than 40% of mating events occurring at less than 15 meters (De Cauwer *et al.*, in press, see also Fénart *et al.* 2007). In *B. vulgaris* ssp. *maritima,* within-population pollen movement may be thus sufficiently restricted to cause female reproductive failure when hermaphrodites are locally rare.

In this study, we investigated whether pollen limitation causes a variation in female fitness within natural populations. More precisely, we searched for a difference in seed production among demes of plants that contained either many, few or no hermaphrodites. Because only female individuals could be found in all of these contrasted situations, our work focused on the reproductive output of females only. Moreover, since processes occurring at a very fine-scale have been acknowledged to play an important role in the dynamics of gynodioecy, we decided to compare the effects of pollen availability among several demes within two populations, rather than among many different populations. Besides, this fine-scale experimental design allowed us to extensively survey the reproductive output of plants throughout the whole flowering season, thus providing data representative of the global plant reproductive success. Finally, to ensure that variable resource levels or differences in the age of plants among study patches did not confound our attempts to assess the effects of sex ratio on female reproductive output, we complemented our observations on natural individuals with transplanted individuals. We specifically addressed the following questions: (1) Is pollen flow sufficiently restricted to cause pollen limitation in wind-pollinated *B. vulgaris*? (2) Is fine-scale variation of sexual polymorphism responsible for spatial

variation in fruit and/or seed production of female plants? (3) If so, do pollen-limited plants reallocate resources that were not used for fruit and/or seed production towards better offspring quality?

Study species

Sea beet, *Beta vulgaris* ssp. *maritima,* is a diploid species (2n=18) widely distributed along the western European coast and around the Mediterranean basin where it colonises coastal habitats just at the upper level of high tides (Laporte *et al.*, 2001; Arnaud *et al.*, 2003; Viard *et al.*, 2004; Fievet *et al.*, 2007). It is a short-lived perennial, wind-pollinated and self-incompatible species (Letschert, 1993). Each individual bears one to several hundred floral stems carrying long, dense racemose inflorescences at their apex. Wild sea beet fruits are the product of the conjoint development of several flowers that mature into a single hard and woody fruit aggregate (hereafter fruits). Each cluster of flowers contains 1 to 8 flowers and, because the flowers are uniovulate, each fruit contains 1 to 8 seeds. An individual plant bears from a few to several thousand flowers (I. De Cauwer, unpublished data). Only some of the flowers open simultaneously along the floral stems within an individual plant. Plants flower from the end of May through mid-July and are widely synchronous.

Sexual phenotype in *B. vulgaris* is determined by interactions between maternally inherited cytoplasmic male sterility genes (CMS genes) and biparentally inherited nuclear-male fertility restorers. In contrast to some other gynodioecious species, a large part of cytoplasmic diversity in *B. vulgaris* is associated with non-sterilising factors. Only four of 20 mitochondrial haplotypes described in wild populations are associated with sexual polymorphism, meaning that two types of hermaphrodites co-exist: restored hermaphrodites (carrying CMS genes) and non-CMS hermaphrodites (Cuguen *et al.*, 1994; Forcioli *et al.*, 1998; Fénart *et al.*, 2006). Populations of *B. vulgaris* are known to be highly structured for nuclear and cytoplasmic genes involved in gender polymorphism (Laporte *et al.*, 2001; De Cauwer *et al.*, in press), allowing us to examine the effect of population structure and variation in local sex ratio on female reproductive output.

Study sites and local sex ratio

It is important to examine the frequency-dependence of female fitness in populations that show normal variation in hermaphrodite frequency. Previous studies on *B. vulgaris* have shown that, at the population level, hermaphrodite frequencies vary from 57% to 100% (Dufay *et al.*, 2009) and that within-population sex ratio variation can be even more pronounced (De Cauwer *et al.*, in press). In this study, we took advantage of the natural within-population variation in sex ratio in two natural populations: (i) a population at Roscoff (N 48° 43.268, E - 4° 00.548, Brittany - France) comprising several thousand flowering individuals clustered in large individual patches along the coast, surveyed in 2007 and (ii) a population at Audresselles (N 50° 49.101, E 1° 35.676, Northern France) comprising approximately 400 flowering individuals, surveyed in 2008. Within each study population, we defined two patch types: (i) potentially pollen-limited patches (PPL patches), characterised by hermaphrodite frequencies lower than 15% and (ii) patches where

hermaphrodites were frequent and where no pollen limitation was expected (NPL patches), characterised by hermaphrodite frequencies greater than 70%. In Roscoff, we studied one PPL patch and one NPL patch. These two patches were separated by 600 m with only a few isolated plants growing in between ($N < 50$). In Audresselles, we chose two PPL patches and one NPL patch (See Fig. 1). As in Roscoff, a few isolated individuals were growing between the study patches ($N < 10$). Sexual phenotype was determined for all flowering individuals in the study patches during the peak flowering period and the local sex ratio (*i.e.* local hermaphrodite frequency) was determined for each patch in both populations (Fig. 1).

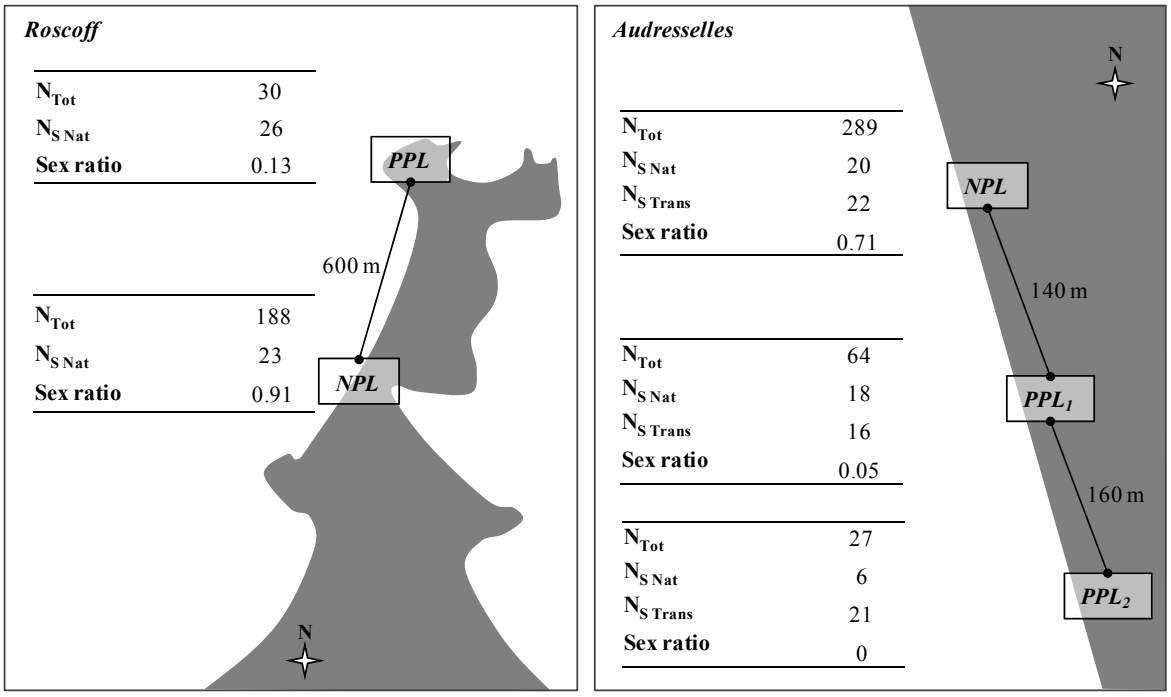

Fig. 1: Schematic diagram of the patches studied within the two populations along the shore line, showing the total number of individuals per patch (N_{tot}), the number of surveyed individuals (N_S, with N_{SNat} for natural individuals and N_{STrans} for transplanted individuals), local sex ratio (*i.e.* hermaphrodite frequency within each patch) and geographical distances between patches. PPL patches are the study patches comprised of potentially pollen-limited individuals (characterised by hermaphrodite frequencies of lower than 15%) and NPL patches are the study patches where hermaphrodites are frequent and where no limitation is expected (characterised by hermaphrodite frequencies higher than 70%).

Female fitness

Surveyed females

To examine the effect of pollen limitation on female fitness within the two study populations, we randomly marked individuals growing in the patches at the beginning of the flowering season. Because of the very low numbers (or even the absence) of hermaphrodites in some of the study patches, only female individuals were used in our study. If pollen limitation

effectively constrains female fitness in *B. vulgaris* ssp. *maritima,* the same results are expected for both sexes because self-incompatible hermaphroditic individuals cannot compensate for a lack of local pollen donors by selfing. At Roscoff, 23 females growing in the NPL patch and 26 females growing in the PPL patch were surveyed. At Audresselles, we studied 20 females located in the NPL patch, 18 females located in the PPL_1 patch and 6 females located in the PPL_2 patch (See Fig. 1). To prevent confounding effects, we selected plants that were flowering nearly at the same time.

Additionally, to ensure that variable resource levels among study patches did not confound our attempts to assess the effects of sex ratio on female reproductive output, we complemented our survey in natural conditions with transplanted individuals. These plants were half-sibs, sharing the same female mother plant originating from Roscoff population. They were sown in autumn 2007 and grown together in the same greenhouse conditions. In 2008, we used pots to transplant 90 individuals in the Audresselles population before flowering had begun (30 individuals per studied patch). Among these transplanted individuals, three plants expressing a hermaphroditic phenotype were removed and 28 plants did not bloom. Finally, 59 out of the 90 transplanted females were effectively surveyed: 22, 16 and 21 female plants in the NPL patch, PPL_1 patch and PPL_2 patch respectively (Fig. 1).

Field methods

A large majority of experimental studies looking for evidences of pollen limitation in plant populations employ supplemental hand pollination methods. Basically, if the reproductive output of an individual plant increases after supplemental hand pollination, the plant is assumed to be pollen limited. However, this approach has at least two important caveats. First, supplemental pollination may involve higher quality pollen than natural pollen (Ashman *et al.*, 2004), yielding false interpretations. Second, several authors have pointed out that resource reallocation may influence estimates of pollen limitation (Stephenson, 1981; Zimmerman & Pyke, 1988). This issue should be taken seriously, especially when the supplemental pollination treatment is applied to a fraction of the flowers of the studied individuals (Knight *et al.*, 2006). A simple solution to the problem of resource reallocation would be to apply pollen supplementation to whole plants (Ashman *et al.*, 2004), but this was impossible in our study species, given that each individual produces several hundreds if not several thousands of flowers in a single flowering season. For all these reasons, we chose to compare open pollinated females located in areas characterized with highly contrasted sex ratios, without using classical supplementations.

For each surveyed female, one to five floral stems of similar size were tagged. Each week, starting from the onset of flowering, the number of flowers and the number of flower clusters that opened along the tagged stems were counted. Each newly opened stem section was delimited with a mark. This temporal survey started on 19 May 2007 and lasted 5 weeks in the Roscoff population. In Audresselles, flowering started on 15 May 2008 and lasted 6 weeks. This temporal survey allowed us to assess when each ovule was receptive and potentially fertilised, providing temporal information for the analysis of female reproductive output.

In mid-August, several weeks after the end of flowering, fruits from each surveyed stem were selectively collected between the marks. We then calculated fruit set as the number of fruits relative to the number of flower clusters for each plant and each temporal section. Given the structure of fruits in *B. vulgaris*, it was not possible to directly count the number of seeds per fruit to assess seed set. Measuring the germination rates for the collected fruits – by counting the number of seedlings that emerged from fruits – was the only way to estimate the number of viable seeds per fruit. To compare germination of seeds produced in the different pollination environments, one to 50 fruits per mother plant were sown (according to individual fruit production) and seedling emergence was monitored for 2 months. For mother plants that produced more than 50 fruits on the surveyed stems, the 50 sown fruits were randomly chosen. As a result, we sowed 1179 fruits from the Roscoff population (873 for the NPL patch and 306 for the PPL patch), 1635 fruits produced by the natural individuals of the Audresselles population (783 for the NPL patch, 752 and 100 for the PPL_1 and PPL_2 patch, respectively) and 927 fruits produced by the transplanted individuals of the Audresselles population (668 for the NPL patch, 180 and 79 for the PPL_1 and PPL_2 patch, respectively). Each seedling was collected 15 days after emergence, dried in an oven (48 h at 56°C) and weighed to the nearest 0.01 mg. After this first step, all fruits from both populations were stored in dry conditions at room temperature for four weeks, to remove dormancy. Final germination rates were determined after a second 2 month survey. We then estimated seed set as the ratio between the number of seedlings and the number of initially available ovules (*i.e.* the number of flowers, as flowers are uniovulate), for each plant and each temporal section. Because nutrients in potting soil probably decrease with repeated watering, only the seedlings emerging from the first survey were weighed. Similarly, a few seedlings presenting signs of fungal infection were not used to measure seedling weight. In total, we weighed 853 seedlings from the Roscoff population (629 for the NPL patch and 224 for the PPL patch), 1022 seedlings produced by the natural individuals of the Audresselles population (509 for the NPL patch, 493 and 20 for the PPL_1 and PPL_2 patch, respectively) and 469 seedlings produced by the transplanted individuals of the Audresselles population (356 for the NPL patch, 80 and 33 for the PPL_1 and PPL_2 patch, respectively).

Data analysis

Female reproductive output partly depends on the probability that viable pollen reaches a receptive flower. In our study, if female fertility was limited by pollen availability in a frequency-dependent way, fruit set and seed set should be lower in the PPL patches than in the NPL patches for the three data sets (natural individuals in the Roscoff population, natural individuals in the Audresselles population and transplanted individuals in the Audresselles population). Because flowering duration was variable among surveyed females, we could not use repeated-measures statistical tests: these tests can only be used to analyse a data set with the same number of temporal measures for all plants and this would have greatly reduced the sample size. Logistic regressions were used to analyse two variables: fruit set (proportion of flower clusters giving a fruit during a given temporal section) and seed set (proportion of seedlings emerging from fruits, relative to the

number of flowers produced, during a given temporal section). Two explanatory factors were tested throughout these analyses: the flowering date (coded as a quantitative factor: week one to five, starting on 19 May 2007 in Roscoff and week one to six, starting on 15 May 2008 in Audresselles) and the patch type (coded as a qualitative factor: PPL and NPL in Roscoff, PPL_1, PPL_2 and NPL in Audresselles). These regressions were performed using PROC GENMOD (binomial distribution, log link function) in SAS (version 9.1) with a correction for over-dispersion. Throughout this paper, all non-significant interaction terms between variables were dropped from the analyses. Differences between individual patches were explored using contrast analyses.

If fruit set and seed set depend on the neighbourhood, flower production and seed quality may also depend on the local sex ratio. Indeed, under favourable pollination conditions, energy may be diverted from future flower production as earlier flowers clusters set fruit, whereas under low pollination, individuals might reallocate resources to increase flower production or seed quality. We thus compared the average number of flowers produced per day and per stem and average seedling dry mass between the PPL and NPL patches using general linear models (PROC GLM in SAS version 9.1). These analyses tested for an effect of flowering date (week one to five in Roscoff and week one to six in Audresselles) and patch type (PPL and NPL in Roscoff, PPL_1, PPL_2 and NPL in Audresselles). Differences between individual patches were explored using post-hoc Tukey pairwise comparisons.

Pollination neighbourhood and female reproductive output

The observed local sex ratios were highly variable within both study populations. At Roscoff, we found 13% and 91% of hermaphrodites for the PPL patch and the NPL patch, respectively. At Audresselles, we observed 0%, 5% and 71% of hermaphrodites in the PPL_2 patch, the PPL_1 patch and the NPL patch, respectively (see Fig. 1).

In the Roscoff population, a total of 12 924 flowers were observed, forming 4982 flower clusters (mean number of flowers per cluster ± SD: 2.62 ± 0.44) of which 2130 were still present at the end of the flowering season. Among these flower clusters, 1621 (76%) set fruit. On natural individuals of the Audresselles population, we observed 24 836 flowers and 10 196 flower clusters (mean number of flowers per cluster ± SD: 2.38 ± 0.58). Of these clusters, 7532 were still present in the summer and 2371 (31%) set fruit. The transplanted individuals in Audresselles produced 12 081 flowers distributed across 5417 flower clusters (mean number of flowers per cluster ± SD: 2.29 ± 0.42). Among these flower clusters, 4613 were collected at the end of the flowering season and 1100 (24%) set fruit. Using logistic regressions, we tested for an effect of the patch (PPL and NPL in Roscoff, PPL_1, PPL_2 and NPL in Audresselles) and of the flowering date on the proportion of flower clusters that were lost (because of bad weather conditions or passers-by accidentally damaging stems) between flowering and fruit collection. Neither of these two factors had an effect on the proportion of lost flower clusters ($P > 0.05$ in all cases).

On average, the surveyed floral stems that were used in this study (one to five for each surveyed female, according to the size of the individual) flowered for 18 days (SD ± 6 days) in Roscoff and for 26 days (SD ± 5 days) and 22 days (SD ± 9 days) for natural and transplanted individuals in Audresselles, respectively. As determined from general linear models, flowering length was not significantly affected by patch type ($P > 0.05$ in all cases). Likewise, the size of the surveyed individuals, estimated by the number of flowering stems, did not vary among the study patches (general linear models, $P > 0.05$ in all cases).

Local sex ratio and fruit set

Overall, the proportion of flower clusters setting fruit was 20% for plants located in PPL patches (*i.e.* the potentially pollen-limited patches, with less than 15% of hermaphrodites) compared to 57% in NPL patches (*i.e.* patches where no pollen limitation was expected, characterised by hermaphrodite frequencies higher than 70%.). The effects of patch type (PPL and NPL in Roscoff, PPL_1, PPL_2 and NPL in Audresselles) and flowering date were tested on fruit set for the three different data sets (natural individuals from Roscoff, natural and transplanted individuals from Audresselles) and are presented in Table 1. Patch type appeared to have a highly significant effect in all cases. As illustrated in Figure 2, individuals located in NPL patches always set more fruit than individuals growing in low local hermaphrodite frequencies, except for the

NPL/PPL_1 comparison involving the natural individuals of Audresselles for which no significant difference was found. For both transplanted and natural individuals of the Audresselles population, fruit set was significantly higher for females located in the PPL_1 patch (comprising 5% of hermaphrodites and located at 140 m from the NPL patch) compared with females in the PPL_2 patch (comprising only females and located at 300 m from the NPL patch). Flowering date had an overall negative effect on fruit set in the Roscoff population, illustrating that fruit set decreased throughout the flowering season. As illustrated by the significant interactions between flowering date and patch type (Table 1), time had an even stronger negative effect on fruit set in NPL and PPL_1 patches for the natural females of Audresselles as well as in the NPL patch for the transplanted individuals of Audresselles (data not shown).

When reducing our data sets to the first flowering week for all studied plants to simplify the analyses, we obtained the same results concerning the effect of patch type (significant effect in the three cases: $\chi^2_1 = 29.25$; $P < 0.0001$ in Roscoff, $\chi^2_2 = 38.08$; $P < 0.0001$ for the natural individuals of Audresselles and $\chi^2_2 = 98.32$; $P < 0.0001$ for the transplanted individuals of Audresselles). Statistical differences between patches, explored using contrast analyses, were the same as in the complete statistical model that included all flowering weeks.

Table 1. Results of the log-linear models testing for the effect of patch type and flowering date on the fruit set and the seed set (both following a binomial distribution), for the two gynodioecious populations of sea beet (*Beta vulgaris* ssp. *maritima*). Two different types of individual were used at Audresselles: natural individuals and transplanted individuals. For quantitative factors that were found to have a significant effect, the symbol between brackets shows the sign of the regression slope. All non-significant interaction terms between variables were dropped from the statistical analyses.

	Roscoff			**Audresselles**			**Audresselles**		
	Natural individuals			Natural individuals			Transplanted individuals		
Variable and source of variation	*df*	χ^2	*P*	*df*	χ^2	*P*	*df*	χ^2	*P*
Fruit Set									
Patch type	1	57.93	$<10^{-4}$	2	16.52	0.0003	2	54.58	$<10^{-4}$
Flowering date	1	8.27	0.004 (-)	1	0.27	0.6026	1	1.97	0.1602
*Patch type*Flowering date*	-			2	14.79	0.0006	2	16.22	0.0003
Seed Set									
Patch type	1	5.83	0.0158	2	6.03	0.0490	2	6.13	0.0465
Flowering date	1	1.82	0.1776	1	0.31	0.5765	1	6.74	0.0094 (+)
*Patch type*Flowering date*	-			-			2	5.17	0.0752

Local sex ratio and seed set

The results for seed set estimated *via* germination rates mirrored the patterns found for fruit set, with a significant effect of the patch type for the three data sets (Table 1). The ratio between the number of seedlings and the number of available ovules was greater for mother plants located in the NPL patches compared with what was observed in PPL patches (Fig. 2). As for fruit set, individuals located in NPL patches always had significantly higher seed set than individuals

growing in PPL patches, except for the NPL/PPL_1 comparison for the natural individuals of Audresselles, where no difference was observed. In addition to the effect of patch type, flowering date had a positive effect on seed set only for the transplanted individuals of the Audresselles population (Table 1). This positive effect of flowering time was stronger in both PPL patches than in the NPL patch (data not shown), as illustrated by the significant interaction between the patch type and the flowering date (Table 1).

Overall, the effect of patch type on seed set seemed to be weaker than what was observed for fruit set (Table 1) and when our data sets were reduced to the first flowering week for each individual, the effect of patch type could no longer be detected.

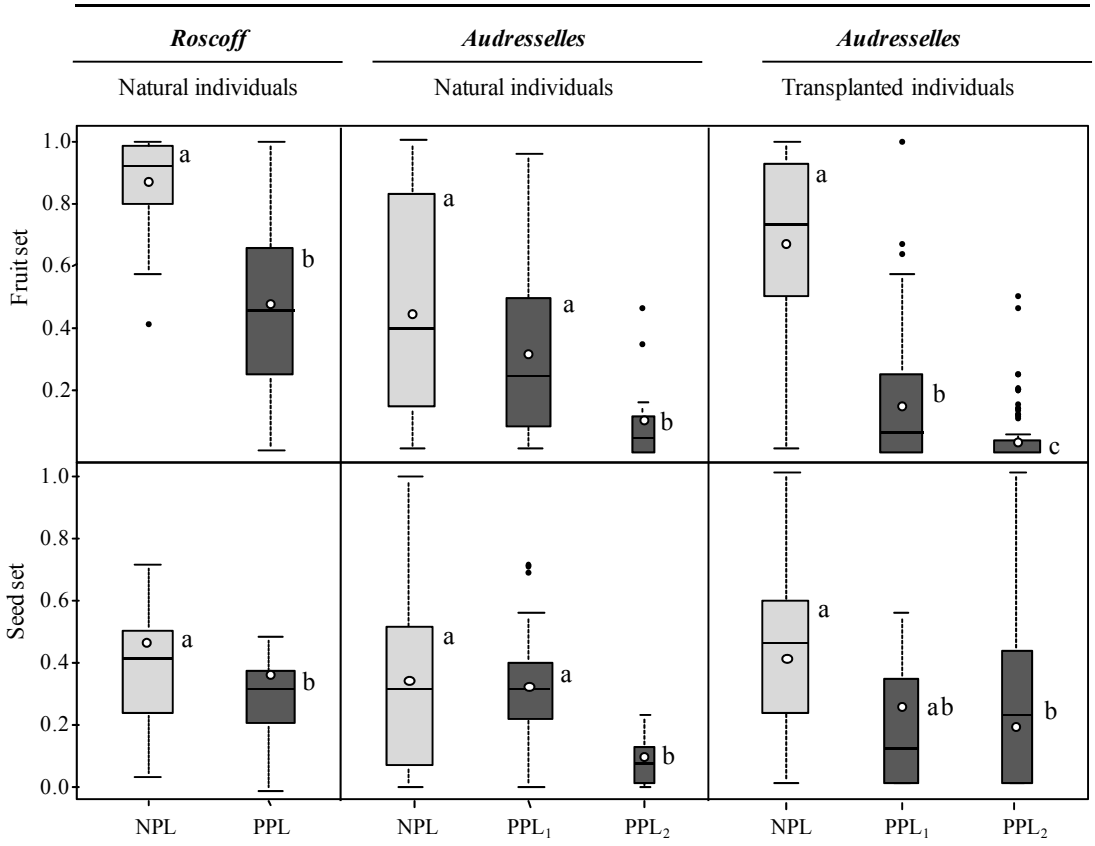

Fig. 2: Box plots of fruit set and seed set by patch type for the three data sets (natural individuals at Roscoff, natural individuals at Audresselles and transplanted individuals at Audresselles). PPL (potentially pollen limited) patches are represented by dark grey boxes and NPL (no pollen limitation expected) patches are represented by light grey boxes. White circles indicate the mean observed values and the horizontal line within the box indicates the median. The box contains the observed values from the lower quartile (25%) to the upper quartile (75%) of the distribution and includes 50% of the observed values. The box whiskers encompass 98% of the observed values (1% to 99% of the distribution - the black circle are outliers). Different letters indicate significant pairwise comparisons (contrast analyses, $P < 0.05$).

Maternal fitness and offspring quality

Altogether, the results presented above strongly suggest that different levels of pollen availability, resulting from local differences in sex ratio, affect at least two important components of female reproductive output: fruit set and seed set. However, to draw clear conclusions, it was necessary to assess whether differences in pollination neighbourhoods could influence the quality of offspring and/or maternal fitness *via* resource reallocations. In other words, we investigated whether pollen-limited females produced more ovules and/or better seedlings than females growing in more favourable pollen conditions, due to reallocation of resources that were not used for setting seeds and fruits. Thus, the effects of patch type and flowering date were tested on the average number of flowers produced per day and per stem and average seedling weight.

Ovule production

The average number of flowers produced per day and per stem was 6.90 (SD ± 2.95) at Roscoff, 6.24 (SD ± 2.63) and 3.95 (SD ± 1.37) for natural and transplanted individuals at Audresselles, respectively. If resource reallocation effectively occurs, greater ovule production is expected for individuals located in areas where pollen producers are rare. This was indeed the case in two data sets: females growing in PPL environments produced more ovules than those located in NPL environments in the Roscoff population as well as for the natural individuals in the Audresselles population (Table 2). In the Roscoff population, we found no significant effect of flowering date. The significant interaction between patch type and flowering date can be explained by a positive effect of time on ovule production for PPL individuals and a negative effect of the time on ovule production for NPL individuals. For the natural individuals in Audresselles, there was no statistically significant difference between NPL and PPL_1 individuals, which all produced significantly less ovules than PPL_2 individuals. In this data set, flowering date had an overall negative effect and there was no significant interaction between patch type and flowering date. Finally, no flowering date or patch type effects were detected on the ovule production in transplanted individuals at Audresselles.

Offspring quality

Average seedling dry weight 15 days after emergence was 58 mg (SD ± 12 mg) at Roscoff, 52 mg (SD ± 17 mg) and 47 mg (SD ± 20 mg) for natural and transplanted individuals at Audresselles, respectively. If pollen-limited females reallocate resources that were not used for fruit and/or seed production toward better offspring, females growing in PPL environments should produce heavier seedlings compared to females located in NPL environments. Patch type influenced seedling weight only in transplanted individuals at Audresselles, with PPL_1 individuals producing significantly lighter offspring than NPL and PPL_2 individuals, and seedling weight was not affected by flowering date in any of the three data sets (Table 2). Overall, there was no clear trend suggesting resource reallocation towards better offspring for females growing in potentially pollen limited environments, at least for the estimator of offspring quality that was used in this study.

Table 2. Results of the general linear models testing the effect of patch type and flowering date on the mean number of flowers produced per day and per stem and the mean seedling weight for the two gynodioecious populations of sea beet (*Beta vulgaris* ssp. *maritima*). Two different types of individual were used at Audresselles: natural individuals and transplanted individuals. For quantitative factors that were found to have a significant effect, the symbol between brackets shows the sign of the regression slope. All non-significant interaction terms between variables were dropped from the statistical analyses.

Variable and source of variation	**Roscoff** Natural individuals				**Audresselles** Natural individuals				**Audresselles** Transplanted individuals			
	df	*MS*	*F*	*P*	*df*	*MS*	*F*	*P*	*df*	*MS*	*F*	*P*
Mean Ovule Number												
Patch type	1	46.61	5.70	0.0186	2	140.53	30.30	$<10^{-4}$	2	0.39	0.21	0.8145
Flowering date	1	4.72	0.58	0.4489	1	55.83	12.04	0.0007 (-)	1	2.97	1.56	0.2130
*Patch type*Flowering date*	1	76.96	9.42	0.0027	-				-			
Error	112	8.17			160	4.63			176	1.90		
Seedling weight												
Patch type	1	9.21E-05	0.69	0.4106	2	8.47E-05	0.29	0.7488	2	3.61E-03	10.93	$<10^{-4}$
Flowering date	1	2.45E-04	1.83	0.1812	1	1.74E-04	0.6	0.4415	1	5.18E-04	1.57	0.2140
Error	59	7.9E-03			109	3.18E-02			73	3.3E-04		

DISCUSSION

This study showed evidence for pollen limitation of reproduction in *Beta vulgaris* spp. *maritima*. Such result was unexpected in a wind-pollinated plant species at such a small spatial scale (a few hundred meters). Indeed, as wind pollination is thought to have primarily evolved as a mechanism of reproductive assurance, pollen limitation in wind-pollinated species is probably not as frequent as in animal-pollinated species (Friedman & Barrett, 2009). The results presented here provide, to the best of our knowledge, the first account that pollen abundance and high pollen dispersal capabilities, both prime features of wind-pollinated plants, may not always be sufficient to ensure optimal female reproductive output in sexually polymorphic species, even at a small spatial scale. Below, we discuss the relation between population sex structure and female fitness, possible patterns of resource reallocation and the outcomes for the dynamics of polymorphic mating systems.

Population structure and female reproductive output

In *B. vulgaris*, as in many other gynodioecious species, both nuclear and cytoplasmic genes are involved in sex expression (Fénart *et al.*, 2006). In this study, sexual phenotypes were strongly structured in both populations, which is probably the consequence of both (i) limited seed dispersal, resulting in limited dispersal of the CMS genes (cytoplasmic male sterility genes) and (ii) founding events that limit associations between particular CMS genes and their associated restorers in some local patches (e.g. Manicacci *et al.*, 1996; Tarayre & Thompson, 1997; De Cauwer *et al.*, in press). As expected by theory (McCauley & Taylor, 1997), female reproductive output of *Beta vulgaris* was found to decrease in female-biased patches compared with more favourable pollen environments, probably because of insufficient pollen receipt when hermaphrodites are locally rare. Indeed, both fruit set and seed set were lower in female-biased patches (*i.e*. PPL patches) for natural individuals of both populations as well as for transplanted individuals that had been grown in standardised conditions.

Pollen limitation appeared to affect seed set and fruit set with varying intensity: plants located in the most favourable pollen environments (NPL patches) set on average three times more fruits and 1.4 times more seeds than plants growing in pollen-limited areas. The effect of patch type on seed set thus seemed to be weaker than what was observed for fruit set. This is a common trend: experimental studies measuring fruit set frequently report a stronger effect of pollen limitation than those measuring seed set, perhaps because many plants species will not produce fruit unless adequate pollen receipt occurs to fertilise most ovules (Mitchell, 1997; Knight *et al.*, 2006). In addition to the effect of variable pollen environments, our results also suggest that fruit set tends to decrease during the flowering season at the Roscoff population. This trend was also observed in both natural and transplanted individuals of Audresselles, but only for the individuals growing in the most favourable pollen environments. This overall negative effect of flowering time can result from (i) a decrease in available pollen during the flowering season (Burd, 1994)

and/or (ii) a decrease in individual resources with time (Wesselingh, 2007). Given our experimental design, we could not quantify the relative impact of these two processes on female reproductive output.

Interestingly, for both transplanted and natural individuals of the Audresselles population, fruit set and seed set were higher for females located in the PPL_1 patch, compared with females of the PPL_2 patch. This better fruit set in PPL_1 can be explained by (i) the occurrence of a few hermaphrodites in that specific patch (whereas they were completely lacking within the PPL_2 patch) and/or (ii) the geographical proximity of the NPL patch (140 meters). These results suggest that even if pollen dispersal in *B. vulgaris* shows a leptokurtic distribution, with a relatively large proportion of long distance dispersal events (De Cauwer *et al.*, in press; Fénart *et al.*, 2007), female reproductive output in female-biased patches may depend on the geographical proximity of hermaphrodite patches.

Pollen limitation and resource reallocation

Altogether, our results suggest that within-population structure of sexual phenotypes can affect at least two important components of female reproductive success. Sex structure is thus likely to cause a decrease in the overall female contribution to the next generation compared to a panmictic population and could ultimately result in a negative frequency-dependent selection against females. However, this trend could be countered if pollen-limited females reallocate resources that were not used for seed or fruit production towards increased offspring quality (Ashman *et al.*, 2004). In this study, we found no clear evidence of such reallocation in pollen-limited environments. In addition to offspring quality, females in pollen-limited environments tended to significantly increase their ovule production in the two data sets including natural individuals, but not for the transplanted individuals. These differences may be related to the fact that root development, and thus available resource quantity, was limited for transplanted females because they were maintained in pots during the flowering survey. In natural individuals, reallocating resources towards increased ovule production may not increase female reproductive output. Flowering is largely synchronous within populations and local sex ratio is thus expected to be quite stable during a given flowering season. As a consequence, enhanced ovule production in pollen-limited areas would probably not result in higher female fitness. However, as we studied only one flowering season in both populations, we cannot exclude resource allocation trade-offs between seed or fruit production and other components of maternal fitness in perennial species (such as growth, survival and probability of flowering in the next years, see Ashman *et al.*, 2004; Knight *et al.*, 2006). Nevertheless, the pollen limitation of seed and fruit set that was measured in the current study seems to be strong enough to result in a global reduction of female fitness, at least within a given flowering season.

Implications for the dynamics of gynodioecy

In structured populations, when the sex ratio varies among sub-populations, similar sexual phenotypes tend to be clustered together. In gynodioecious species, sex structure is thus likely to

result in reproductive assurance for hermaphrodites, which are – on average – located in favourable pollen environments, whereas females are more likely to undergo pollen limitation, because they are clustered together. As a consequence, the negative effect of pollen limitation on fitness mainly affects female individuals (e.g. Widen & Widen, 1990; Sugawara, 1993; Graff, 1999; Alonso, 2005; Zhang *et al.*, 2008). In that context, pollen limitation is likely to have an effect on the growth of female-biased patches, if growth rates are sensitive to changes in fruit and seed production (Ashman *et al.*, 2004). As a result, overall female frequencies and thus CMS frequencies in structured populations are likely to be lower compared with an ideal panmictic population (McCauley & Taylor, 1997; Pannell, 1997). The perennial life cycle and the long-lived seed bank in *B. vulgaris* probably buffer this negative effect of pollen limitation on female reproductive output. However, since gene flow through seed and pollen dispersal is thought to be limited both among and within *B. vulgaris* populations (Fievet *et al.*, 2007; Arnaud *et al.*, 2009; De Cauwer *et al.*, in press), seed banks in female-biased patches are likely to be female-biased too and pollen limitation will persist as long as non-CMS cytoplasms and/or restorer alleles associated with the local CMS are lacking. According to Dufay *et al.* (2009), the three main CMSs that occur in *B. vulgaris* are characterised by significantly different average restoration rates. CMSs that are poorly restored may show an increased probability for being pollen limited, which would slow down their invasion.

Whereas females are obligate outcrossers and depend entirely upon hermaphrodite pollen for reproduction, hermaphrodites in most gynodioecious species are self-compatible (Charlesworth, 1981). If pollen limitation occurs within a population, selfing is likely to favour reproduction in hermaphrodites, although this positive effect can be modified by inbreeding depression (Maurice & Fleming, 1995), and females are generally thought to undergo stronger reproductive limitation (e.g. Widen & Widen, 1990; Sugawara, 1993; McCauley & Brock, 1998; Graff, 1999; Alonso, 2005; Zhang *et al.*, 2008, but see Shykoff et al., 2003). However, this situation probably does not hold true in *B. vulgaris*, as this species is largely self-incompatible (Owen, 1942; Larsen, 1977). In species where selfing cannot compensate the lack of local pollen donors, hermaphrodites and females may undergo similar levels of pollen limitation of fruit and seed production. Seed and fruit set of hermaphrodites are thus also probably lower in female-biased patches compared to hermaphrodites located in more favourable pollen environments. Our study aimed to compare extremely contrasted situations in terms of sex-ratio, and this particular experimental design did not allow us to investigate pollen limitation in hermaphrodites. However, this constitutes a very interesting perspective of this work, which could be carried out through a controlled experimental study. Thus, if self-incompatible hermaphrodites are effectively affected by pollen limitation, frequency-dependent selection could act on different levels depending on the mating system: in self-compatible species, selection should act both at the individual level and at the deme level, whereas in self-incompatible species, selection is likely to operate only at the deme level (Dufay & Pannell, in press). As in our study sites, *B. vulgaris* populations are frequently a mosaic of demes where CMS genes are frequent and demes where CMS genes are rare (Laporte *et*

al., 2001; De Cauwer *et al.*, in press). Hermaphrodites bearing a CMS gene and the appropriate restorer allele are thus likely to be located in female-biased patches and should on average suffer more pollen limitation than non-CMS hermaphrodites. In addition to this effect of structure on female fitness, male reproductive output can be affected too because hermaphrodites located in female-biased patches may sire more seedlings compared to hermaphrodites in hermaphrodite-biased patches, which undergo stronger pollen competition (De Cauwer *et al.*, in press). As a result, sex structure is likely to affect the three sexual phenotypes co-existing in natural population in different ways: (i) female fitness of non-CMS hermaphrodites may be favoured because being clustered together provides them reproductive assurance; however, their male fitness is likely to be affected because of local conspecific pollen competition; (ii) females suffer a global fitness reduction because of pollen limitation and (iii) restored CMS hermaphrodites are affected by pollen limitation for their female fitness but have enhanced male fitness because they are often clustered with females and are the only available local pollen donors. Population structure can thus have contrasting effects on different fitness components in hermaphrodites and the consequences of the combination of such processes should be worth to investigate by theoretical studies of gynodioecy

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CONCLUSIONS AND PERSPECTIVES

Our results clearly illustrate how fine-scale population structure of sexual phenotypes resulting from random founder events affects individual female fitness through inadequate pollen receipt when hermaphrodites are locally rare. This frequency-dependent feature of female reproductive output was unexpected at such geographically restricted scales, especially in a wind-pollinated species. Pollen abundance and long-distance dispersal, which are characteristics of wind pollinated plants, may thus not always be sufficient to ensure optimal female reproductive output in sexually polymorphic species. In our study species, fine-scale population structure of sexual phenotypes is likely to result in lower female frequencies and lower CMS frequencies compared with a panmictic population. Empirical studies on the maintenance of gynodioecy, the evolution of sex ratios or, more generally, the dynamics of any polymorphic trait under frequency-dependent selection should probably not consider gene or morph frequencies at the scale of the whole population, but rather investigate how these frequencies vary at restricted geographical scales.

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Effects of fine-scale genetic structure on male mating success in gynodioecious *Beta vulgaris* ssp. *maritima*

I. DE CAUWER, M. DUFAY, J. CUGUEN & J.-F. ARNAUD

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ABSTRACT

Plant mating systems are known to influence population genetic structure because pollen and seed dispersal are often spatially restricted. However, the reciprocal outcomes of population structure on the dynamics of polymorphic mating systems have received little attention. In gynodioecious sea beet (*Beta vulgaris* ssp. *maritima*), three sexual types co-occur: females carrying a cytoplasmic male sterility (CMS) gene, hermaphrodites carrying a non-CMS cytoplasm and restored hermaphrodites that carry CMS genes and nuclear restorer alleles. This study aimed at investigating the effects of fine-scale genetic structure on male reproductive success of the two hermaphroditic forms. Our study population was strongly structured and characterized by contrasting local sex-ratios. Pollen flow was constrained over short distances and depended on local plant density. Interestingly, restored hermaphrodites sired significantly more seedlings than non-CMS hermaphrodites, despite the previous observation that the former produce pollen of lower quality than the latter. This result was explained by the higher frequency of females in the local vicinity of restored (CMS) hermaphrodites as compared to non-CMS hermaphrodites. Population structure thus strongly influences individual fitness and may locally counteract the expected effects of selection, suggesting that understanding fine scale population processes is central to predicting the evolution of gender polymorphism in angiosperms.

INTRODUCTION

Dispersal events play a fundamental role in the evolutionary dynamics of populations by connecting distant demes in a metapopulation network and thereby have a strong impact on the partitioning of genetic diversity within and among structured demes (Loveless & Hamrick, 1984; Hamrick & Nason, 1996; Ennos, 2001; Pannell & Dorken, 2006; Sork & Smouse, 2006). Beyond the magnitude of gene flow, the strength of the spatial genetic structure also varies with local adaptation processes and with the intrinsic characteristics of the species, including the mating system (*e.g.* unequal reproductive success). In return, fine-scale population subdivision may affect key evolutionary parameters such as the effective population size which determines the intensity of genetic drift (Whitlock & Barton, 1997). In addition, when the reproductive success of a given genotype depends on its local frequency, population structure, by clustering similar genotypes, may also have a profound effect on individual fitness (Olson *et al.*, 2006). In this respect, a better understanding of the interactions between individual fitness and fine-scale population structure requires the study of traits that are under frequency-dependent selection. Different examples of reproductive traits being under frequency dependent selection have been studied in plants, including flower morphology in heterostylous and enantiostylous plants (Jesson & Barrett, 2002; Van Rossum *et al.*, 2006), color polymorphism in rewardless orchids (Gigord *et al.*, 2001), S-locus alleles in self-incompatible species (Wagenius *et al.*, 2007) or sex-expression in dioecious and gynodioecious species (Fisher, 1930; Lewis, 1941).

Gynodioecy is a gender polymorphism that refers to plant species in which females and hermaphrodites coexist in natural populations, making this breeding system particularly relevant to address questions about the long-lasting evolutionary consequences of population structuring through local variation in sex ratio (Frank & Barr, 2001; Olson *et al.*, 2005). The genetics underlying sex expression in gynodioecious species commonly involves interactions between cytoplasmic male sterility (CMS) genes located in mitochondria, and nuclear male fertility restorers (Dommée *et al.*, 1987; Boutin-Stadler *et al.*, 1989; Saumitou-Laprade *et al.*, 1994; Koelewijn & Van-Damme, 1995; Ronfort *et al.*, 1995; McCauley *et al.*, 2000b; Dudle *et al.*, 2001; Delph *et al.*, 2007). The existence of such a cytonuclear polymorphism has attracted interest for several decades and a number of empirical and theoretical studies have attempted to determine the conditions for a stable maintenance of gynodioecy. Typically, CMS genes prevent production of fertile pollen in hermaphrodites, inducing a male sterile (female) phenotype. Theory predicts that a CMS gene should spread in a population as soon as fitness through the female function is higher in females than in hermaphrodites (Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufay *et al.*, 2007). This female advantage, due to the reallocation of resources from pollen to ovules or to the avoidance of inbreeding depression, has been measured in several gynodioecious species (*e.g.*(Couvet *et al.*, 1986; Asikainen & Mutikainen, 2003; Olson *et al.*, 2006). As soon as CMS becomes frequent within a population, nuclear (biparentally inherited) alleles which are able to restore male function

are likely to be selected for. This is because, when CMS is frequent, genotypes that carry nuclear restorer alleles are the only hermaphrodites that are able to sire hermaphroditic offspring that will keep efficiently transmitting their genes, through both pollen and ovules. Theoretical models, based on the effect of selection only, postulate that the maintenance of cytonuclear polymorphism also requires a cost of restoration of moderate magnitude, so that restorers can increase in frequency when CMS is frequent but cannot reach fixation (Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufay *et al.*, 2007). Overall, sex-ratio should evolve according to negative frequency-dependent selection, with CMS genes being selected for when restorers are rare and nuclear restorers being selected for when CMS occurs at high frequency. Although the effect of frequency-dependent selection on gynodioecy is clearly acknowledged, most of the theoretical studies model infinite panmictic populations and do not take into account the fact that population structure and metapopulation dynamics could strongly modify the expected results of frequency-dependent selection (Couvet *et al.*, 1998; Dufay & Pannell, 2010).

In gynodioecious species sex ratio often varies considerably among populations as well as at a local scale within populations (McCauley *et al.*, 2000a; Laporte *et al.*, 2001; Olson *et al.*, 2005; Olson *et al.*, 2006; Dufay *et al.*, 2009). When the spatial distribution of genders is not uniform, the fitness of a given sexual phenotype may depend on the mate availability in the subset of the population with which it interacts, rather than the composition of the entire population (Graff, 1999; McCauley *et al.*, 2000a; Alonso, 2005; Olson *et al.*, 2005; Oddou-Muratorio *et al.*, 2006; Isagi *et al.*, 2007). The interaction between female fitness and local sex-ratio has been investigated in a number of studies and it is now well established that population structure can reduce the fitness of females through pollen limitation when females are spatially clustered, compared with the case of panmixia (e.g. McCauley *et al.*, 2000a). However, little is known about the effect of spatial structure of genders on male reproductive success. Intuitively, male reproductive output is expected to increase in female biased patches, when only a few pollen donors are available to sire all the neighboring females. Once a restorer allele is established in a population, through mutation or migration, the maintenance of gynodioecy in a population will depend on the fate of this restorer, which is influenced by the fitness of individuals carrying the restorer and the intensity of the cost of restoration, but probably also by the intensity of drift and the identity of surrounding individuals in the case of fine scale spatial gender variation. Therefore, studying fine-scale spatial genetic structure and male reproductive success together should provide a more comprehensive insight into the key evolutionary processes that maintain gynodioecy in natural populations.

Here, we study how fine-scale spatial structure and differential male reproductive success among individuals interact to shape the evolution of gender polymorphism in the gynodioecious sea beet, *Beta vulgaris* ssp. *maritima.* Gender expression in this plant species is determined by interactions between maternally inherited CMS genes and biparentally inherited nuclear male fertility restorers. In contrast to some other gynodioecious species, a large part of cytoplasmic diversity in *Beta vulgaris* is not associated with sterilizing factors. Indeed, only four out of the

twenty mitochondrial haplotypes described in wild populations are associated with gender polymorphism, meaning that two types of hermaphrodites coexist: restored hermaphrodites (carrying both CMS genes and nuclear restorer alleles) and non-CMS hermaphrodites (Cuguen *et al.*, 1994; Forcioli *et al.*, 1998; Laporte *et al.*, 2001; Fénart *et al.*, 2006). A recent study showed that both pollen quantity and quality vary quantitatively among restored hermaphrodites, suggesting a complex genetic determination of nuclear restoration (Dufay *et al.*, 2008). This study also showed that pollen viability was significantly lower in restored (CMS) hermaphrodites than in non-CMS hermaphrodite. These results suggest that restoration of male fertility might be incomplete in some of the restored hermaphrodites. An overall lower pollen quality in restored hermaphrodites may have long-lasting consequences in terms of spread of restoration genes: if restored hermaphrodites do not efficiently compete with non-CMS hermaphrodites, selection of restoration could be slower than predicted by classical models (Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufay *et al.*, 2007). Therefore, the sea beet provides a unique opportunity to study the fate of restorer genes by comparing the effective male fitness between restored and non-CMS hermaphrodites.

Additionally, because populations of *B. vulgaris* are known to be highly structured for genes involved in gender polymorphism (Laporte *et al.* 2001), we wanted to assess whether population structure and variation of local sex ratio could affect male reproductive success. In other words, is the fate of restorer alleles only influenced by the poor pollen quality produced by hermaphrodites that carry them, or is it also influenced by the spatial distribution of genders within populations? One way to answer this question is to compare the male reproductive success of the two hermaphroditic forms when (i) restored hermaphrodites are known to produce pollen of lower quality and (ii) fine-scale variation of gender polymorphism is sufficiently pronounced to allow the experimental detection of any effect of population structure on male reproductive success.

We focused on a natural population of *Beta vulgaris* which provided such opportunity. First, we will show that local sex ratio largely varies within our study site because of a pronounced fine-scale cytonuclear structure. Second, a detailed paternity analysis allowed us to characterize the distribution of pollen dispersal events within the studied population. We will show that (i) besides the occurrence of long-distance pollen flow, as usually expected for wind pollinated species, a large part of mating events occur at a very restricted geographical scale, and that (ii) neighborhood features, such as local conspecific density, affect the patterns of pollen flow. Finally, because quantity and quality of pollen produced by the two hermaphroditic forms were recorded in this particular population, the same year, by a study in tandem with ours (Dufay *et al.* 2008), we could use the results of our paternity analysis to answer the following questions: (i) does the lower quality of pollen of restored hermaphrodites cause a reduction of male reproductive success and (ii) does local sex ratio affect the reproductive success of hermaphrodites? This is, to the best of our knowledge, the first study dealing with the effect of gender polymorphism and population structuring on the effective male reproductive success in a gynodioecious species.

The species

Wild sea beet, *Beta vulgaris* ssp. *maritima* is a diploid species (2n = 18) widely distributed along the western coast of Europe and around the Mediterranean basin. It is a short lived perennial and wind-pollinated species (Letschert, 1993). *Beta vulgaris* is largely self incompatible, with up to four gametophytic S loci (Owen, 1942; Larsen, 1977), but up to now, there is no clear knowledge on the levels of outcrossing in natural conditions. There is no vegetative reproduction, and thus dispersal can only occur through seeds and/or pollen movement. Seeds are aggregated in an irregular dry body that contains 1-8 seeds. This seedball has no particular dispersal mechanism and is primarily dispersed by gravity or by water movements during high tide (Fievet *et al.*, 2007). This study was carried out in northern France, where sea beets colonize areas located along estuaries, just at the upper level of the tide, cliffs overhanging the sea and other coastal habitats (Letschert, 1993; Laporte *et al.*, 2001; Arnaud *et al.*, 2003b; Viard *et al.*, 2004).

Male sterility in *Beta vulgaris* ssp. *maritima* is associated with four particular mitochondrial types, called CMS *E*, *G*, *Svulg* and *H*, the other mitotypes being associated with male fertile phenotypes (Cuguen *et al.*, 1994; Forcioli *et al.*, 1998; Desplanque *et al.*, 2000; Fénart *et al.*, 2006). In our study species, historical relationships between CMS and non-CMS haplotypes suggest independent apparitions of CMS haplotypes with sterilizing cytoplasms belonging to different lineages derived from an ancestral non-sterilizing cytoplasm (Fénart *et al.*, 2006).

Study site, phenotyping and sampling

We focused on a population located near Audresselles (N 50° 49.101, E 1° 35.676) in Northern France. The focal population extended over 500m and consisted of approximately 400 flowering individuals. Within this population, a total of 280 flowering plants were uniformly sampled in order to cover the whole area of the population (Figure 1). The locations of each sampled plant was mapped, sexual phenotype was determined (female or hermaphrodite), and leaves were collected for molecular studies (Figure 1A). Among the 206 individuals identified as hermaphrodites, 195 were classified into three groups based on relative size, measured by the number of floral stems: group 1 (1 to 15 floral stems, N = 59 individuals), group 2 (15 to 30 floral stems, N = 87 individuals) and group 3 (more than 30 floral stems, N = 49 individuals). We also described the local neighborhood around each sampled individual by counting the number of flowering hermaphroditic and female individuals within 15 meters.

Following cytoplasmic genotyping (see below), we were able to discriminate between normal (carrying a male-fertile cytoplasm) and restored (carrying a CMS-associated cytoplasm) hermaphrodites, and then selected eight females, eleven normal hermaphrodites and two restored hermaphrodites as maternal plants for the paternity analysis (see Figure 1B). These mother plants

were chosen in order to mirror the frequencies of the different genders in the study site. Seeds were randomly collected on five stems for each maternal plant in mid-august. Seeds were then germinated in a greenhouse and grown until each seedling had several leaves. A total of 1019 seedlings were collected for molecular studies (48 ± 12 per seed parent).

In wind-pollinated species, pollen flow often includes substantial amounts of long distance dispersal (Dow & Ashley, 1998; Streiff *et al.*, 1999; Burczyk *et al.*, 2004; Robledo-Arnuncio & Gil, 2005; Fénart *et al.*, 2007). Accounting for the paternal contribution of plants growing outside the studied area is important for assessing the connectivity between populations and to understand processes shaping population structure at a larger spatial scale (Sork & Smouse, 2006; Slavov *et al.*, 2009). In order to study the possibility of external gene flow, the two closest neighboring populations, situated on both sides of Audresselles, were additionally sampled (20 individuals per population) and mapped. These populations were located 1.2 and 3.5 km from the study site and comprised very few individuals (approximately 30 and 50 individuals respectively).

Genetic data collection

We used a NucleoSpin®96 Plant Kit (Macherey-Nagel) to extract and purify total DNA from dried leaf tissue as described in Fénart *et al.* (2007). This procedure yielded a total of 320 sampled adults and 1019 offspring.

Nuclear diversity

Sampled individuals, including mother plants and seedlings, were genotyped at 10 microsatellite loci: *GAA1*, *GTT1*, *GCC1*, *BVM3*, *CAA1* (Mörchen *et al.*, 1996; Viard *et al.*, 2002); *SB04*, *SB06*, *SB07*, *SB15* (Richards *et al.*, 2004); and *FDSB1027* (McGrath *et al.*, 2007). Loci were amplified in two multiplexed PCR. The first multiplex was performed in a 10.5 μL reactions consisting of: 25 ng of DNA template, 1 μL of Buffer 10X (Perkin-Elmer, Norwalk, CT, USA), 2.9 mM $MgCl_2$, 0.2 μg/μL of BSA, 2% of DMSO, either 0.1 μM (for loci *GTT1*, *BVM3*, *CAA1* and *FDSB1027*) or 0.05 μM of each primer (for locus *GCC1*), 290 μM of each dNTP and 0.9 U/μL of hot start *Taq* polymerase (*AmpliTaq* Gold, Perkin-Elmer, Norwalk, CT, USA). The second multiplex was performed in the same conditions, with 0.1 μM (for loci *GAA1* and *SB04*) or 0.05 μM of each primer (for loci *SB06*, *SB07* and *SB15*). PCR was conducted on a 9700 thermal cycler (Perkin-Elmer, Norwalk, CT, USA) under the following conditions: 5 min denaturing at 94°C, 45 sec denaturing at 94°C, 45 sec annealing at 54°C and 45 sec extension at 72°C and a final extension step at 72°C for 10 min, after 40 cycles.

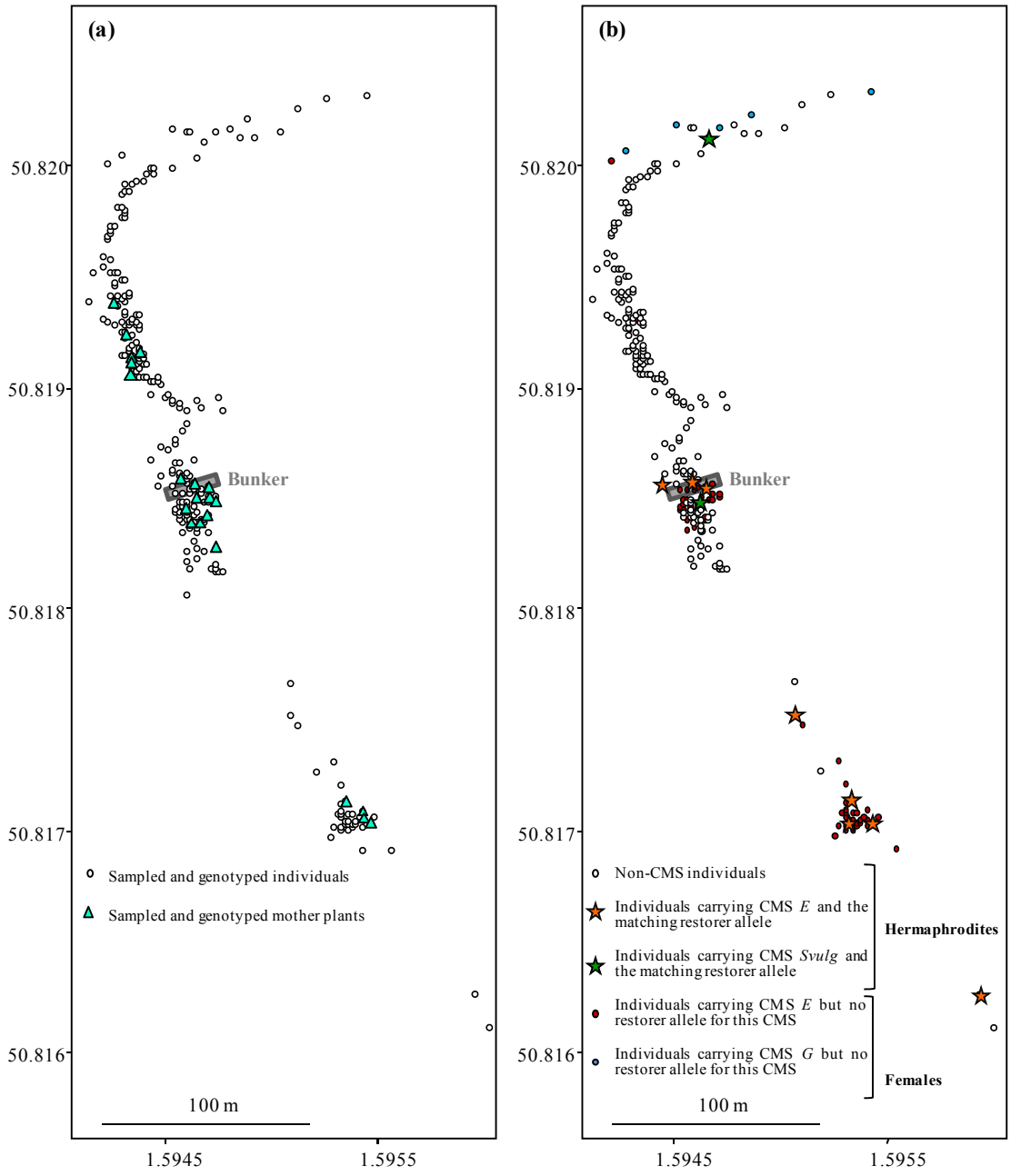

Fig. 1 Spatial locations for sampled individual sea beet (*Beta vulgaris* ssp. *maritima*) plants. (a) Individuals sampled for genotyping and mother plants used for paternity analysis; (b) associated sexual phenotypes and CMSs within the study population.

Cytoplasmic diversity

Several cytoplasmic male sterilities (CMSs) have been described in sea beets, of which three are most common: *Owen* CMS (also called *Svulg*), which has been widely used in plant breeding of the sugar beet (Owen, 1945 ; Arnaud *et al.*, 2003b; Viard *et al.*, 2004), and two others, *E* and *G*, found exclusively in wild beet populations (Cuguen *et al.*, 1994; Desplanque *et al.*, 2000;

Laporte *et al.*, 2001; Fénart *et al.*, 2006; Dufay *et al.*, 2009). Diagnostic cytoplasmic PCR-RFLP markers were used to distinguish among the three main CMSs: two mitochondrial markers that enable to identify the CMSs *Svulg* and *G*, and a chloroplast marker for CMS *E*. Primers for detection of CMS *Svulg* are described in Ran & Mickaelis (1995), and those for CMS *G* and CMS *E* in Dufay *et al.* (2008).

We conducted standard PCR procedures in 15 μl reactions consisting of: 25 ng of DNA, 3.5 mM $MgCl_2$, 200 μg/mL of BSA, 200 μM of each dNTP, 0.2 μM of forward and reverse primers, 0.375 U of *Taq* polymerase (Perkin-Elmer). Amplifications were performed with the following method: 3 min denaturing at 95°C, followed by 35 cycles of 45 sec denaturing at 94°C, 45 sec annealing at 55°C and 1 min extension at 72°C, with a final extension step of 72°C for 10 min. Allele sizing could be directly performed for CMS *G* by separating DNA products using a 2% agarose gel and visualizing after ethidium bromide staining under UV light. Polymorphism detection for both CMS *Svulg* and *E* needed a supplementary restriction step before visualization:

(i) for CMS *Svulg*: 5 μL of the PCR product digested in a 10 μL reaction with 0.2 mM of spermidin and 2 U of *Taq*I, conducted at 65°C overnight, as described in Arnaud *et al.* (2003b).

(ii) for CMS *E*: 5 μL of the PCR product digested in a 10-μL reaction with 0.2 mM of spermidin and 1.5 U of *Alu*I, conducted at 37°C overnight.

We characterized mitochondrial polymorphism by genotyping individuals at four mitochondrial minisatellite loci: *TR1*, *TR2*, *TR3* and *TR4* (Nishizawa *et al.*, 2000). PCRs were carried out in 10.5 μl volumes containing 3 mM $MgCl_2$, 200 μM of each dNTP, 0.2 mg/ml of BSA, 120 μM of each forward and reverse primer, 0.625 U of *Taq* polymerase (Perkin Elmer) and ≈ 50 ng of template DNA. Cycling conditions consisted of an initial denaturation step of 5 min at 94°C followed by 30 cycles of 30 sec at 94°C, 60 sec annealing at 62°C, 30 sec at 72°C, and ending with a final extension for 10 min at 72°C. As the entire mitochondrial genome is generally inherited as a single linkage unit, each genotype combination for these 4 minisatellite loci will be analysed as an haplotype, as in Fievet *et al.* (2007).

Detection and analysis of PCR products

Allele sizing of both minisatellite and microsatellite multiplex amplified products was performed using an ABI Prism® 3100 Genetic Analyzer 16-capillary array system (Applied Biosystems) as described in Fénart *et al.* (2007) for nuclear data and Fénart *et al.* (2008) for cytoplasmic data.

Statistical analyses

Nuclear and cytoplasmic diversity

Genotypic disequilibrium was tested for all locus pairs in GENEPOP version 3.4 (Raymond & Rousset, 1995). Exact tests used the Markov-chain method based on the contingency

tables for all pairs of loci in each population (Raymond & Rousset, 1995). Tests were conducted with the dememorization number set to 10 000, for 1000 batches and 10 000 iterations. Significance of *P*-values were assessed after Bonferroni correction, as suggested by Rice (1989).

Standard population genetic indexes were calculated (number of alleles A_n, observed heterozygosity H_o, gene diversity H_e and unbiased fixation index F_{IS}) in FSTAT version 2.9.3 (Goudet, 1995). Significance of single locus F_{IS} as well as mean overall F_{IS} estimate were tested using 10 000 random permutations of alleles among individuals. We also used FSTAT to estimate pairwise population differentiation (F_{ST}) among distinct patches (see below) with 10 000 permutations of the data, using a G test for significance of results (Goudet *et al.* (1996). F_{IS} and F_{IT} estimates were also estimated, and significance was tested by permuting alleles within patches and over the total population with 10000 permutations.

We estimated inbreeding coefficients to determine whether inbreeding could be responsible for variation in male reproductive success. We estimated individual inbreeding coefficients using a multilocus estimator for each adult individual as well as for progenies, following the procedure described in Ritland & Travis (2004).

Bayesian analysis of population structure

Recent studies indicate that Bayesian clustering methods can accurately describe genetic population clusters, and even in cases of weak spatial genetic structure, for example in newly founded populations likely to be far from migration-drift equilibrium (e.g. Coulon *et al.*, 2006; Rowe & Beebee, 2007). To assess the number of genetic populations within our study site, we used GENELAND version 1.0.5 (Guillot *et al.*, 2005) to visualize genetically distinct groups and to detect genetic discontinuities across the focal area. We analyzed the data first by allowing *K* to vary from one to 10, with 10 independent runs under the following settings: 200 000 MCMC iterations, maximum rate of Poisson process fixed to 100, maximum number of nuclei in the Poisson–Voronoi tessellation fixed to 250, and the Dirichlet model for allelic frequencies. We then inferred the number of groups in our sample from the modal *K* of these 10 runs and ran the MCMC with *K* fixed to this number. Other parameters remained similar to those of the runs with variable *K*. The posterior probability of group membership for each pixel of the spatial domain was then computed (using a burn-in of 10000 iterations).

Spatial autocorrelation

Once group membership was established for each individual of the studied population, the second step was to determine whether spatially restricted gene flow may produce the rise of spatial genetic structure, even within groups of genetically related individuals. We used spatial autocorrelation analyses to examine the spatial arrangement of genetic variability across subpopulations and to compare the strength of spatial structuring from nuclear and cytoplasmic data. These analyses make no assumptions about the scale of spatial patterns or population genetic parameters (Sokal & Oden, 1978; Barbujani, 1987) and provide a detailed description of gene

frequency variation in space that allows inference of micro-evolutionary processes shaping the distribution of gene frequencies (Arnaud *et al.*, 2003a; Storfer *et al.*, 2007). In this study, we used the kinship coefficient ρ_{ij} developed by Loiselle *et al.* (1995) as autocorrelation index. This kinship coefficient allows the integration of multi-allelic data, or even multiple loci for nuclear data, and is relatively unbiased in the presence of low frequency alleles (Loiselle *et al.*, 1995; Aspi *et al.*, 2006; Born *et al.*, 2008). Ten distance classes were defined in order to obtain approximately the same number of individual pairs within each distance class. We calculated confidence intervals over sampling coordinates (under the null hypothesis of no spatial genetic structure) with 10 000 permutations of haplotypes (mitochondrial minisatellites) and multilocus nuclear microsatellite genotypes using SPAGeDI version 1.2 (Hardy & Vekemans, 2002). To compare the strength of spatial genetic structure between nuclear and cytoplasmic data, as well as between genetically differentiated groups of individuals, we used the statistic S_p introduced by Vekemans & Hardy (2004), which is independent on the sampling scheme used, *i.e.* independent of arbitrarily set distance intervals. S_p statistics were calculated using linear distances and the slope b of regressions analyses was tested for significance by randomly permuting individuals over spatial locations, as described above.

Paternity analysis, outcrossing rate, pollen dispersal and male mating success

Paternity exclusion probabilities (*EP*) were computed following Jamieson and Taylor (1997), using the program CERVUS version 2.0 (Marshall *et al.*, 1998). For the 1019 seedlings, paternity assignment was performed using the maximum likelihood-based method described in Marshall *et al.* (1998) and implemented in the program CERVUS version 2.0. All parental individuals (except females) were considered as potential fathers for each offspring (N = 206 hermaphrodites). Paternity likelihood was estimated using the ratio of probabilities (the LOD–score) defined in Meagher *et al.* (1986). To determine whether the paternity of offspring can be assigned to the hermaphrodite with the highest paternity likelihood, we used the difference in likelihood score between the most likely parental hermaphrodites (ΔLOD). The critical value (Δ_C) of Δ below which paternity cannot be attributed at 80% was determined using a distribution of Δ obtained from 20000 simulated mating events. This distribution was generated assuming the following parameters: (i) observed allele frequencies at each locus (calculated for the entire data set including the genotypes of all the reproductive adults and offspring) (ii) estimated male breeding population size (number of candidate parents) and the proportion of candidate parents sampled, respectively set to 400 and 0.7, (iii) the proportion of loci mistyped, set to 0. As advised by Oddou-Muratorio *et al*. (2003), it is better to introduce a null scoring error into CERVUS, because a non-null error rate always results in an augmentation of type I errors (*i.e.* assignment of a wrong father to a given offspring, while the true father is either another sampled individual or a non sampled individual) and type II errors (*i.e*. non assignment of paternity to the father of the offspring while this father is among the sampled individuals). For each offspring, the likelihood-based parentage analysis produced three possible alternative outcomes. (i) Paternity could not be significantly assigned to one of the sampled adults, either because the male parent was outside the

study area or was one of the non-sampled adults within the population. Also included within this group are the cases where two or more adults were compatible with the offspring but with a difference (Δ) in LOD-score too low to attribute paternity to the most likely parent. (ii) Paternity was attributed to the mother, allowing us to estimate the selfing rate of each mother. (iii) Paternity was significantly attributed to another sampled adult and it was then possible, knowing the position of both mother and assigned father, to calculate their pairwise geographical distance. Deviation of the observed dispersal pattern from the distribution of pollen dispersal events expected under panmixia (*i.e.* distribution of mate pairs sampled in each distance class) were tested using a χ^2 test.

We also investigated the effect of several phenotypical and ecological parameters on male reproductive success. For this we analyzed the variance in the number of seedlings sired by each potential father with a logistic regression (Poisson distribution, log-link function, PROC GENMOD, SAS) and corrected for overdispersion (dscale option, PROC GENMOD, SAS). Four factors, describing individual characteristics of the potential fathers were tested: (i) cytoplasmic identity (restored hermaphrodite versus non-CMS hermaphrodites), (ii) size (three levels, according to the number of flowering stems, as above), (iii) geographical position (two levels, defined from the probability of population membership, see results) and (iv) inbreeding coefficient. In addition, four factors describing the local neighborhood of each potential father within a radius of 15 meters were tested: (i) number of flowering plants, (ii) number of flowering females, (iii) number of flowering hermaphrodites, and (iv) the local sex ratio (determined by the frequency of females in the focal area). Finally, we tested whether the factors listed above statistically depended on each other, by comparing all four variables that described local neighborhoods between (i) restored hermaphrodites and non-CMS hermaphrodites and (ii) plants located in the southern and the northern subpopulation, using non-parametric Wilcoxon Mann-Whitney tests.

As supplementary information, correlation of outcrossed paternity (*i.e.* the proportion of full sibs within an outcrossed progeny array) was estimated using the maximum-likelihood approach under a mixed-mating system model implemented in the MLTR v3.2 software package (Ritland 2002).

Cytonuclear diversity and sex polymorphism

Male sterilizing cytoplasms were detected at relatively high frequency, particularly the CMS *E,* with 84 individuals out of 280 sampled plants (30%). Two other CMS types were also present but at lower frequencies: 6 individuals (2.14%) for *G*, and 2 individuals (0.71%) for *Svulg* (Figure 1B). The proportion of females was high (29%), suggesting very low frequencies of restorer alleles in this population. We observed only eight hermaphrodites carrying the haplotype associated with CMS *E* (restoration rate of 9.52%). Almost all females and all individuals carrying CMS *E* clustered within patches located in the southern part of the population (see Figure 1B). We did not observe restored hermaphrodites for CMS *G*, whereas the two individuals carrying CMS *Svulg* were restored for male fertility and were thus expressing a hermaphroditic phenotype. Given the observed rarity of *Svulg* male sterility, we excluded these two hermaphrodites from statistical comparisons of male reproductive success.

Mitochondrial minisatellite loci displayed moderate levels of polymorphism, with 1 to 7 alleles per locus, for a total of 12 alleles giving 8 different haplotypes (*cf.* Table 1). There was a strong linkage disequilibrium between these haplotypes and the CMS genes (all at $P < 0.05$), each of the three CMSs being exclusively associated with a unique minisatellite haplotypes. The levels of diversity exhibited by nuclear microsatellites and cytoplasmic minisatellites were of the same order of magnitude than those that are generally observed in natural sea beet populations (e.g. Fievet *et al.*, 2007; Fénart *et al.*, 2008). Nuclear microsatellite loci were highly polymorphic with an overall total of 83 alleles, the number of alleles ranging from 2 (*Gcc1*) to 19 (*Caa1*). The number of sampled alleles (A_e), expected heterozygosity (H_e) and estimated intra-population fixation index (F_{IS}) are presented in Table 1. The overall average F_{IS} was non-significant (F_{IS} = 0.009). A significant departure from HW genotypic proportions was observed for only two loci (*SB06* and *SB15*). Expected heterozygosity (H_e) was relatively high across all loci (0.157 to 0.846), except for *Gtt1* and *Gaa1*, which showed the lowest values for diversity and expected heterozygosity, as already reported in previous studies (Arnaud *et al.*, 2003b; Fievet *et al.*, 2007). Significant linkage disequilibrium was detected in 15 of the 45 pairwise comparisons for microsatellite loci (after Bonferroni correction, $P < 0.05$). We also investigated cytonuclear associations between the minisatellite haplotypes and the microsatellite loci: 8 of the 10 pairs showed significant linkage disequilibrium (*1027*, *Bvm3*, *Caa1*, *Gtt1*, *Gaa1*, *SB04*, *SB06* and *SB07*, after Bonferroni correction, $P < 0.05$).

Within the population, individual inbreeding coefficient values varied widely from 0 to 0.966 for the adults, the mean value being 0.0753. Estimates of inbreeding coefficient varied between 0 and 1 for studied seedlings, with a mean value of 0.0832, which was significantly higher than what was observed for adults (Mann-Whitney, $N_{seedlings} = 1019$ $N_{adults} = 280$, $Z = -2.43$, P[two-tailed] = 0.014). The calculation of individual inbreeding coefficient is characterized by a

large variance (Ritland & Travis, 2004) and there is no direct relationship between the inbreeding coefficient and the intrapopulation fixation index as measured by multilocus level of heterozygosity (Slate *et al.*, 2004), which may explain why mean values of inbreeding coefficient differed from mean F_{IS} values.

Table 1 Genetic diversity estimated for each locus and over all loci in a wild population of sea beet (*Beta vulgaris* ssp. *maritima*). Listed for each locus are the number of sampled alleles per locus (A_n), expected heterozygosities (H_E), intrapopulation fixation indexes (F_{IS}), and the observed allele size ranges. **:$P < 0.01$; ***:$P < 0.001$; NS : non-significant

Microsatellite nuclear loci					Minisatellite cytoplasmic loci		
	A_n	H_e	F_{IS}	Allele size range		A_n	Allele size range
1027	7	0.556	0.069^{NS}	175-201	*TR*1	7	439-794
Bvm3	14	0.846	-0.012 ^{NS}	96-123	*TR*2	1	404-404
Caa1	19	0.82	0.032 ^{NS}	139-194	*TR*3	2	420-482
Gcc1	2	0.474	-0.107 ^{NS}	96-99	*TR*4	2	410-438
Gtt1	3	0.23	0.047 ^{NS}	112-118			
Gaa1	3	0.157	0.025 ^{NS}	182-191			
SB04	10	0.332	-0.03 ^{NS}	170-193			
SB06	5	0.682	0.078**	145-165			
SB07	11	0.778	0.044 ^{NS}	245-272			
SB15	9	0.69	-0.066***	140-176			
Mean	8.3	0.557	0.009 ^{NS}				

Within population structure

Individual population membership

First, we ran the MCMC 10 times, allowing K to vary, in order to verify the consistency of the results. All these trial runs in GENELAND identified $K = 2$ as the modal value for the number of genetic populations, which we then fixed to compute the posterior probability of individual membership to one of the clusters, and the population membership for each pixel of the spatial domain (Figure 2). The transition between the two genetically distinct clusters occurred over a few meters. The southern part of the population appeared to be strikingly separated from the northern part by a thin boundary. The location of this boundary corresponds to that of an ancient bunker, which may be a physical barrier to pollen and seed dispersal. Three groups of plants (19 individuals) located in the northern part of the study area were assigned to the southern group with a very high probability ($P > 0.9$), suggesting seed dispersal over several hundred meters (see Figure 2). Cytoplasmic identity of these 19 individuals was either non-sterilizing or sterilizing.

The two subpopulations did not differ in gene diversity, allelic richness ($P > 0.05$ in both cases, *t*-test using loci as resampling units), or inbreeding levels estimated following Ritland and Travis method (Mann-Whitney using individuals as resampling units, $N_{north} = 160$, $N_{south} = 120$, $Z = -0.1739$, P[two-tailed] = 0.431). F_{IS} fixation indexes were also estimated within each of the two

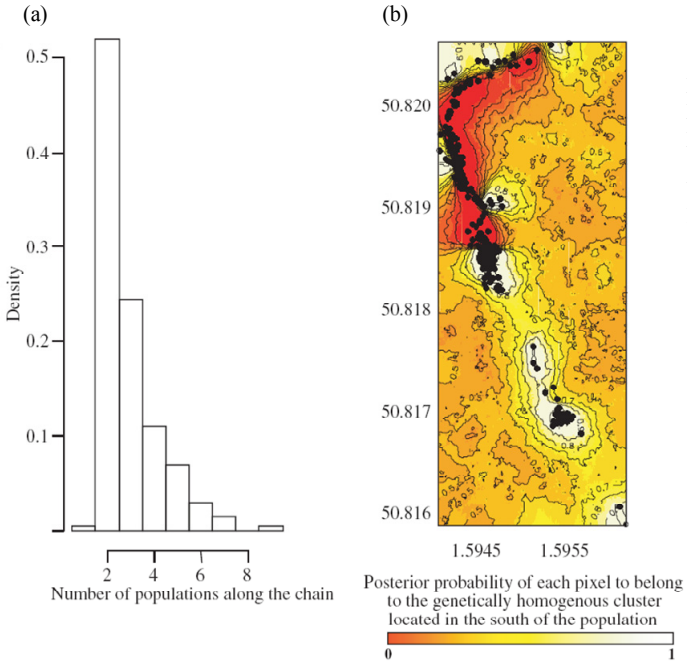

Fig. 2 (a) Posterior distribution of probability density of the number *K* of populations (b) Map of the posterior probability of population membership of each pixel of the spatial domain to belong to one of the two genetically homogenous clusters, using the Bayesian analyses of population structure described in Guillot *et al.* (2005). Scale of X and Y axis designed geographical coordinates in decimal degrees.

genetically distinct clusters: no significant departure from Hardy Weinberg expectations within these subpopulations was observed (F_{IS} = 0.007 and -0.017 for the southern and the northern part of the population, respectively). Genetic differentiation between the two groups was highly significant, based on both nuclear (F_{ST} = 0.03, $P < 0.001$) and cytoplasmic data (F_{ST} = 0.36, $P <$ 0.001). Furthermore, we investigated the cytonuclear associations at the subpopulation level for the eight loci that displayed significant linkage disequilibrium over the whole population. Only three comparisons remained significant: *Bvm3* in both subpopulations, and *Caa1* and *Gaa1* in the northern subpopulation only. As the disequilibrium at *Bvm3* implies different alleles and haplotypes, depending on the subpopulation, it is most probably due to a genetic structuring effect within the study site.

Frequencies of male sterile cytoplasms and females differed between the two genetically distinct clusters. In the southern cluster, 65% of the individuals had a sterilizing cytoplasm and 60% had a female phenotype. In the northern cluster, only 5% of individuals possessed a sterilizing cytoplasm and 4% a female phenotype. Additionally, 75% of restored hermaphrodites were located in the southern cluster.

Spatial genetic structure

Results of the spatial autocorrelation analysis showed that the southern cluster was highly structured relative to the northern cluster (Figure 3). As shown by correlograms, a continuous decline in genetic similarity with physical distance was only observed for the southern cluster (Figure 3a & 3c). In this cluster, there was also a considerable difference in the extent of spatial genetic structure between cytoplasmic and nuclear genetic variation: the S_P statistic for nuclear markers was 18-fold lower than for cytoplasmic haplotypes, whereas in the northern cluster there was very low spatial genetic structure for either marker type (S_P = 0.0168 and 0.005 respectively,

Figure 3b & 3d). These observations suggested contrasted patterns of spatial genetic structure between the two genetically differentiated clusters of individuals as well as a general trend for isolation by distance processes for both cytoplasmic and nuclear data over the whole population.

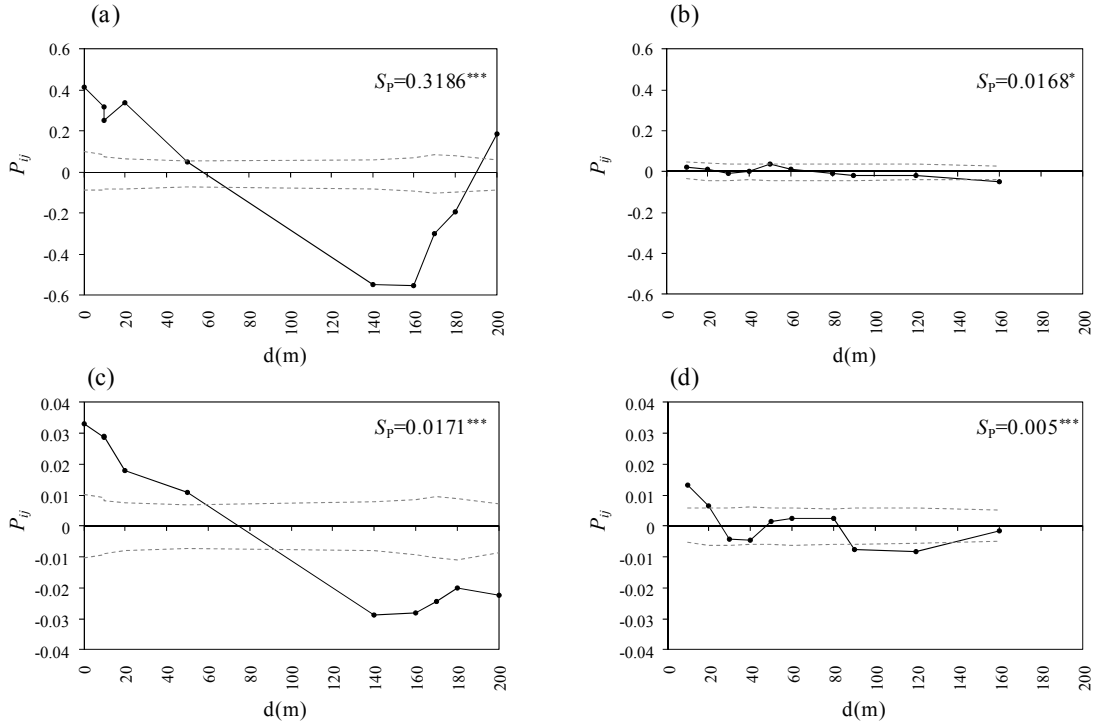

Fig. 3 Average pairwise kinship coefficient (p_{ij}, Loiselle *et al.* 1995) between individuals as a function of the distance (meters). Patterns of relatedness based on cytoplasmic haplotypes for individuals in the southern cluster (a) and the northern cluster (b), and patterns of relatedness based on nuclear data for individuals in the southern cluster (c) and the northern cluster (d). The dashed lines represent upper and lower 95% confidence intervals for the null hypothesis of no relatedness between individuals. *: $P < 0.05$; ***: $P < 0.001$.

Contemporary pollen flow

The cumulative exclusion probability was high ($EP = 0.993$), suggesting that the loci were highly suitable for paternity analysis. Out of 1019 seedlings, 492 (48%) had no compatible father among the reproducing individuals collected (either because the male parent was outside the study area or was among the non-sampled adults within the population), 526 (51%) had only one compatible father (confidence level of 80% or more), and one showed a mismatch with the genotype of the mother at one locus (this seedling was excluded in further analyses). None of the studied seedlings showed equal probability to be assigned to two or more fathers.

Among the 526 seedlings with paternity assignments, 13 (3%) were sired with high probability by a father situated in one of the two additional neighboring populations sampled to gauge rate of external pollen flow. This suggests that pollen flow can occur over several kilometers (at least up to 3.5 km) along the coastline. Within the studied population, paternity was assigned to the mother of 14 seedlings, providing an overall selfing rate estimate of 3%. Among

the 13 hermaphroditic mother plants, only 5 showed partial selfing, often at very low rates (1 to 3 seedlings per mother) with the exception of one individual (7 out of 19 seedlings assigned to the mother plant).

Based on the outcrossed mating events within the population, pollen dispersal distances ranged up to 355 meters, with an average of 96 meters and median of 24 meters. The resulting frequency distributions of pollen dispersal distances departed from the expected distribution under random mating and clearly indicated a pollen flow constrained over relatively short distances (Figure 4a). Twenty-nine percent of the pollination events occurred between the two clusters defined above. By accounting for densities of conspecifics near the assessed fathers, we found that the pattern of local density significantly affected pollen dispersal distance. Pollen flow was limited to short distances for fathers situated in patchy areas (Figure 4c), with an average of 27 meters and a median of 9 meters, whereas the pollen dispersal was significantly higher for spatially isolated fathers (Figure 4b), with an average of 104 meters and median of 80 meters (Mann-Whitney, $N_{isolated} = 96$, $N_{non\text{-}isolated} = 96$, $Z = 9.46$, P[two-tailed] $< 10^{-4}$).

Male reproductive success

For each sampled hermaphrodite, we analyzed the number of assigned seedlings as a function of several parameters: cytoplasmic identity (CMS *versus* non-CMS hermaphrodites), inbreeding coefficient, individual size, geographical location (south *versus* north), as well as quantitative descriptors of the neighborhood of each focal plant (number of flowering individuals, number of females, number of hermaphrodites and local sex ratio, *i.e.* female frequency around each focal plant). Because some of the factors listed above were dependent on each other (see below), we chose to test each factor separately in a first step. The results are summarized in Table 2.

Table 2 Results of eight log linear models carried out on the number of assigned seedlings (following a Poisson distribution) for all potential fathers in the study population of sea beet (*Beta vulgaris* ssp. *maritima*). In each of the eight models, the effect of one factor was tested (either related to the individual characteristics or to the neighborhood of each plant). These analyses were performed with 195 potential fathers, except for the effect of local female frequency, which could not be calculated for four potential fathers that had no other plants in their vicinity. North and south refer to the two clusters inferred with Bayesian clustering methods. CMS and non-CMS refer to sterilizing and non-sterilizing cytoplasms respectively.

Source of variation	*dF*	χ^2	*P*	Effect
Individual characteristics				
Cytoplasmic identity	1	5.88	0.0153	CMS > Non CMS
Geographical location	1	10.13	0.0015	South > North
Size of the individual	2	0.47	0.7907	-
Inbreeding coefficient	1	0.25	0.6198	-
Neighborhood characteristics (within a radius of 15 meters around each focal plant)				
Number of plants	1	17.32	$<10^{-4}$	Positive
Number of females	1	20.98	$<10^{-4}$	Positive
Number of hermaphrodites	1	2	0.1589	-
Female frequency	1	15.30	$<10^{-4}$	Positive

Neither individual size nor inbreeding coefficient significantly affected the number of sired seedlings. However, we did record an effect of cytoplasmic identity, with restored hermaphrodites that appeared to sire a higher number of seedlings than non-CMS hermaphrodites. There was also a geographical effect, with hermaphrodites located in the southern cluster being associated with a higher number of seedlings than plants located in the northern cluster. We also found a significant effect of the neighborhood of each potential father: the number of flowering

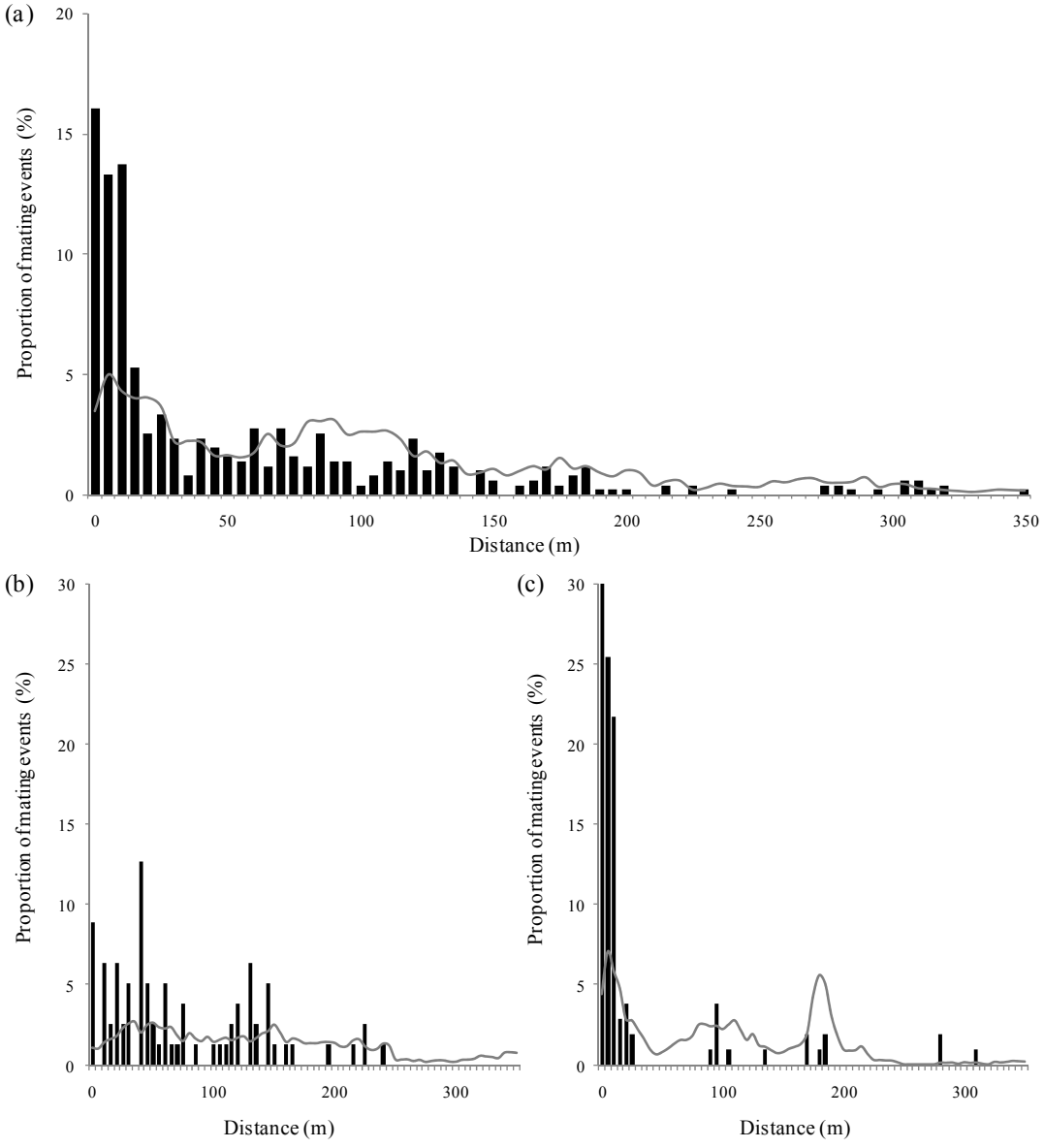

Fig. 4 Proportion of mating events as a function of geographical distance between mates within the studied population for (a) all the outcrossing pollination events (N = 526 seedlings), (b) spatially isolated fathers (< 15 individuals in a 15 m neighborhood, N = 96 seedlings), and (c) fathers situated in patchy areas (> 45 individuals in a 15 neighborhood, N = 96 seedlings). The grey line represents the expected numbers of mating events under the null hypothesis of random mating (*i.e.* the proportion of mate pairs sampled in each distance class).

plants, the number of females and the local female ratio within a radius of 15 meters all had a strong positive effect on their estimated number of seedlings; only the number of hermaphrodites around plants had no significant effect (Table 2). One must notice that we obtained similar results with descriptors of local neighborhood based on other values of radiuses around each hermaphrodite (data not shown).

In all, three major types of factors seemed to affect male fitness of the hermaphrodites: cytoplasmic identity, geographical location and local neighborhood. These factors appeared to be strongly inter-dependent; plants in the southern cluster had more locally available mates (number of flowering plants, number and frequency of females; Table 3). More importantly, number and frequency of females in neighborhoods was significantly higher for restored (CMS) hermaphrodites (20.30 ± 14.68 females, *i.e.* 62.47% ± 27.20% of females) than non-CMS hermaphrodites (3.02 ± 6.33 females, *i.e.* 8.59% ± 15.93% of females). Thus, restored hermaphrodites probably experienced less pollen competition than hermaphrodites carrying a non-sterilizing cytoplasm. For example, for each of the four mother plants situated in the extreme south, more than 50% of the assigned seedlings were sired by one of the three directly neighboring restored hermaphrodites (Figure 5). As a result of the reduced number of potential fathers in the areas were the sex ratio was female biased, the estimated paternity correlation (*i.e.* fraction of seedlings that share the same father) is significantly higher in the southern part of the population (0.161 ± 0.036), compared to what was observed in the northern cluster (0.084 ± 0.016).

Table 3 Results of non-parametric tests (Wilcoxon Mann-Whitney) on four variables describing the neighborhood of each potential father ($N = 195$) as a function of either cytoplasmic identity of the plant or its geographical location.

Variable	Source of variation	*N*	*Z*	*P*	Effect
Number of plants	Cytoplasmic identity	195	2.17	0.03	CMS > Non CMS
	Geographical location	195	4.09	$<10^{-4}$	South > North
Number of females	Cytoplasmic identity	195	3.82	$<10^{-4}$	CMS > Non CMS
	Geographical location	195	7.06	$<10^{-4}$	South > North
Number of hermaphrodites	Cytoplasmic identity	195	-2.41	0.02	Non CMS > CMS
	Geographical location	195	-1.46	0.14	-
Female frequency	Cytoplasmic identity	191	4.99	$<10^{-4}$	CMS > Non CMS
	Geographical location	191	7.39	$<10^{-4}$	South > North

When all factors were tested simultaneously on the number of seedlings per potential father, only cytoplasmic identity and the number of flowering plants in the neighborhood remained significant, as well as the interaction between cytoplasmic identity and female frequency in the vicinity of each focal plant (Table 4). Interestingly, in this model, restored (CMS) hermaphrodites had a marginally lower male fitness than non-CMS ones. In other words, when correcting for the variability in the neighborhood of each potential father (that favors restored hermaphrodites, cf. Table 3), restored (CMS) hermaphrodites apparently lose their reproductive advantage. This suggests that the better male fitness of restored hermaphrodites highlighted by the one factor model (cf. Table 2) is not due to their intrinsic capability of siring seeds, but rather only to their geographical location. Finally, the significant interaction between cytoplasmic identity and female frequency in the neighborhood was due to a positive effect of female frequency for restored (CMS) hermaphrodites only. Indeed, while local female frequency strongly varied across the population for restored hermaphrodites, with a particularly beneficial neighborhood in the southern sub-population, local sex-ratio was virtually constant among non-CMS hermaphrodites (see Figure 5).

Table 4 Results of the complete log linear model testing for all the main factors simultaneously, carried out on the number of assigned seedlings (following a Poisson distribution) for all potential fathers in the study population of sea beet (*Beta vulgaris* ssp. *maritima*). All the interactions between main factors were tested, and only Cytoplasmic identity * Female frequency had a significant effect. CMS and non-CMS refer to sterilizing and non-sterilizing cytoplasms respectively.

Source of variation	*dF*	χ^2	*P*	Effect
Cytoplasmic identity	1	3.55	0.0597	CMS < Non CMS
Geographical location	1	2.40	0.1217	-
Size of the individual	2	1.17	0.5572	-
Inbreeding coefficient	1	0.06	0.8012	-
Number of plants	1	8.52	0.0035	Positive
Female frequency	1	1.71	0.1911	-
Cytoplasmic identity * Female frequency	1	6.89	0.0087	Positive effect for CMS plants only

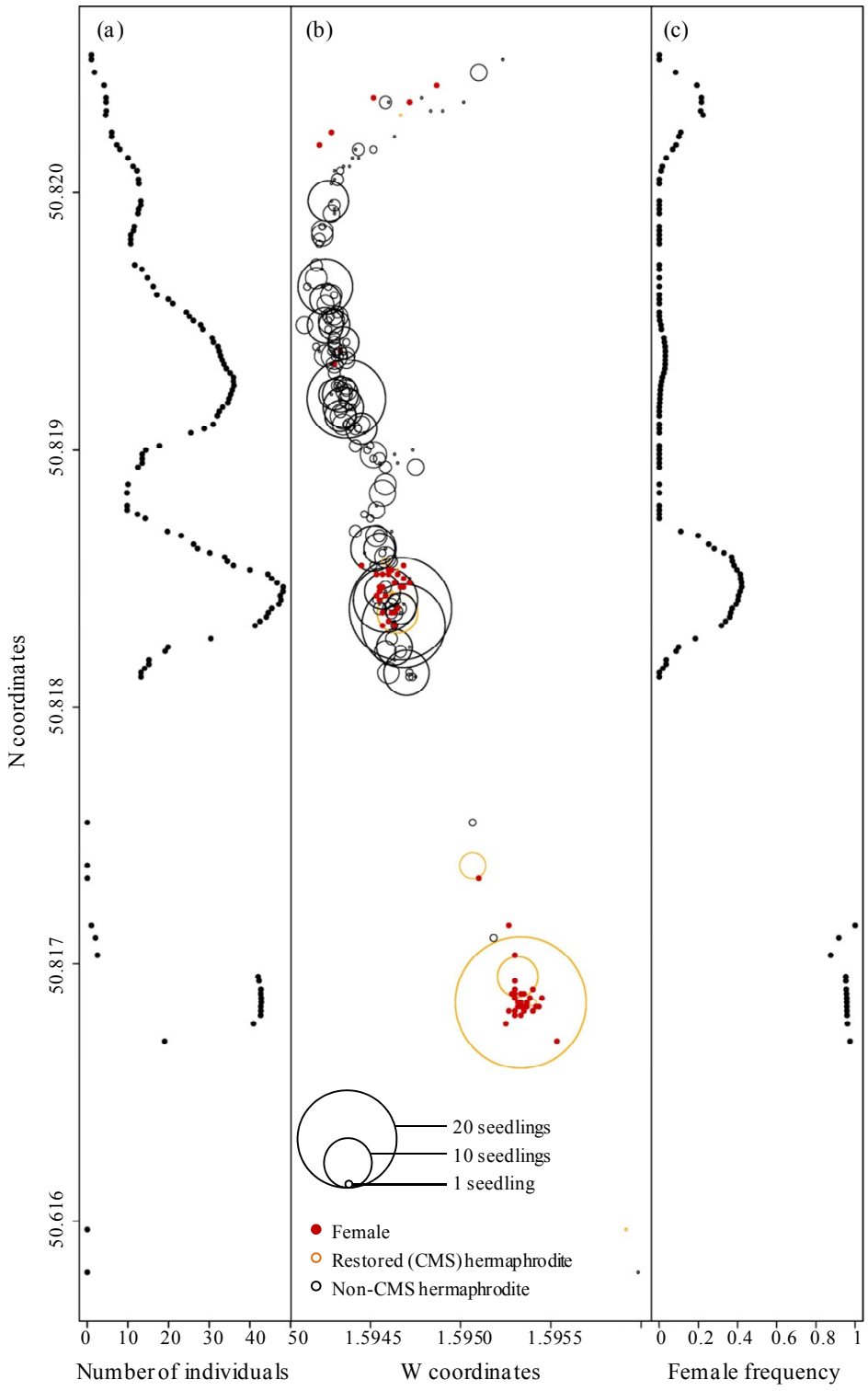

Fig. 5 Spatial variation in male reproductive success within the study site, for non-CMS hermaphrodites and restored hermaphrodites (b), along with spatial variation in the number of individuals calculated within a radius of 15m (a) and spatial variation in the female frequency within the same radius (c). The bunker marks the boundary between the northern and the southern subpopulations defined by the Bayesian assignment test.

DISCUSSION

Many theoretical and empirical studies of gynodioecy have focused on the question of how a joint cytonuclear polymorphism can be maintained at the sex-determining loci. Theory has shown that the maintenance of sexual polymorphism is affected by (i) selection on both male and female fitness components (Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufay *et al.*, 2007), and (ii) population structure and gene flow (Frank, 1989; Pannell, 1997; Couvet *et al.*, 1998; McCauley *et al.*, 2000a). This study investigates both processes by characterizing fine-scale genetic structure and contemporary gene flow along with male reproductive success, to understand the evolutionary dynamics of gynodioecy in the sea beet.

The rise of population genetic structure and variation of local sex ratio

Local genetic structure in plant populations is shaped by a combination of factors involving gene flow, selection and genetic drift, which in turn are influenced by mating system, and ecological factors, such as density or dispersal capabilities of seed and pollen (Loveless & Hamrick, 1984). In this study, we observed strong differences in the extent of spatial genetic structure between nuclear and cytoplasmic loci, and found that at a small spatial scale, genetic structure of maternally inherited cytoplasmic markers was stronger than at nuclear loci. This is likely due to a difference in the mode of inheritance: nuclear genes are dispersed via seed and pollen, whereas mitochondrial genes are only dispersed via seeds. This has been demonstrated in other studies, in which maternally inherited cpDNA or mtDNA were more structured than nuclear DNA, both at the local, within-population scale and at the larger metapopulation scale (McCauley, 1998; Oddou-Muratorio *et al.*, 2001; Olson & McCauley, 2002; Fievet *et al.*, 2007).

Using Bayesian clustering analyses based on nuclear multilocus genotypes, we observed a strong genetic partition in the study population between two distinct north and south clusters. The partitioning of the clusters corresponds with the location of a potential landscape barrier (a bunker), which may limit pollen and seed dispersal between the south and north. However, we also detected some first generation migration events involving 19 individuals located in the northern cluster that were assigned to the southern. This suggests that seeds may periodically travel several hundred meters, probably following water movements during high tide (e.g. Fievet *et al.*, 2007). Similarly, paternity analyses (see below) indicated that a substantial proportion of pollen dispersal occurs between the two clusters. This striking genetic discontinuity of the studied population goes along with marked differences in CMS genes and sex ratios. Ninety percent of the plants carrying male sterilizing cytoplasms were found in the southern part of the population. Consequently, sex ratios were also markedly different between the two sub populations (60% and 4% of females in the southern and the northern cluster, respectively). This structure in sex ratio is expected to persist into future generations because offspring sex-ratio from females is usually female biased whereas offspring from hermaphrodites is hermaphrodite biased. Local structure of cytoplasmic types and sexual phenotypes seems to be a general trend in many gynodioecious species (McCauley *et al.*,

2000a; Laporte *et al.*, 2001; Olson *et al.*, 2005; Olson *et al.*, 2006; Dufay *et al.*, 2009) and it has been shown to affect individual fitness, in terms of female fertility (McCauley *et al.*, 1996; Graff, 1999; Taylor *et al.*, 1999; Olson *et al.*, 2006), but also in terms of male fertility, as we show in this study. In the particular case of *Beta vulgaris*, two types of hermaphrodites are found in natural populations because of the co-occurrence of male fertile and male sterile cytoplasms. In this regard, we found a similar spatial trend in the geographical occurrence of restorer alleles with most of the restored hermaphrodites located in the southern cluster.

Interestingly, within-population structure appeared to be stronger in the southern cluster compared to the northern cluster, especially for cytoplasmic haplotypes. Variation in cytoplasmic diversity within and among populations can occur from either stochastic or selective factors influencing the spread of advantageous alleles (Olson & McCauley, 2002). If seed dispersal between populations is a rare event, a newly founded population should have only a few cytoplasmic haplotypes. Hence, this stronger genetic structure could be the consequence of a recent founder event involving genetically related individuals that shared the same sterilizing cytoplasm (see Manicacci *et al.*, 1996). This being the case, even pollen flow between subpopulations would not have had enough time to homogenize the family structure that arises after a founder event. This hypothesis is supported by significant genetic differentiation between the north and south clusters, both for nuclear and for cytoplasmic data. Additionally, the fact that the southern cluster was mainly composed of females, that are strictly seed dispersers, could reinforce this observed strong genetic structure. The observed bias in sex ratio in the southern cluster also restricts the number of neighboring potential fathers, which reinforces the correlated paternity within a progeny array. As a consequence, a larger part of the seedlings of each progeny are full sibs, which results in local accumulation of related individuals in family clusters (see Torimaru *et al.*, 2007).

Pollen flow within and between subpopulations

Beyond historical estimates of gene flow, we used progeny arrays and paternity analysis to gain some insights on the pattern of real-time pollen flow. Pollen dispersal followed a leptokurtic distribution with most dispersal events occurring at short distances and a long tail of low level pollen dispersal over larger distances. Indeed, more than 40 % of mating events occurred at spatial scales not exceeding 15 meters, which was unexpected for a weedy anemophilous species. Beyond a simple spatial-dependent pattern of pollen dispersal, the density of conspecific individuals is a major determinant of pollination distance and effective number of pollen donors (e.g. Oddou-Muratorio *et al.*, 2006; Fénart *et al.*, 2007). In this study, we found that the pattern of local plant density strongly affected pollen dispersal distance. Pollen flow was limited to short distances for fathers situated within dense patches of individuals, whereas the distance between mates was significantly higher for spatially isolated fathers. The individuals located within dense patches of flowering beets compete in a pollen cloud saturated by the closest neighboring conspecifics (see Fénart *et al.*, 2007). Therefore, strong clustering of individuals should counteract the long distance pollination events expected in a wind-pollinated species, and

may accentuate patterns of isolation-by-distance (e.g. García *et al.*, 2005; Ishihama *et al.*, 2006, but see Isagi *et al.* 2007; Byrne *et al.*, 2007).

To understand the processes occurring at larger scale, pollen flow at longer distances must be taken into account. In our study, 29% of the pollination events occurred between the north and south clusters. This result may seem contradictory with the significant differentiation between the two sub-populations. However, this measurement of gene flow only represents real time events that do not necessarily result immediately in a genetic homogenization over the whole population. In other words, the strong genetic structure observed in our study site, probably resulting from distinct founder events, persists despite the currently high rates of pollen flow because the study species is perennial. Furthermore, 2.5% of the seedlings were apparently sired by fathers located in two neighboring populations sampled outside the study site, suggesting the possibility of long distance pollen flow in sea beet (over several kilometers). Other studies based on paternity analysis have also reported long distance immigration events (e.g. Goto *et al.*, 2006; Bittencourt & Sebbenn, 2007; Bacles & Ennos, 2008; Slavov *et al.*, 2009). It should be noted, however, that the vast majority of studies reporting patterns of contemporary pollen dispersal focused on either entomophilous species or wind-pollinated trees. To the best of our knowledge this study is the first to investigate pollen flow in a weedy wind-pollinated species, meaning that any generalization or comparison is difficult to assess (but see Fénart *et al.*, 2007 for a study of the same species in an agronomical context). These results are especially important because rare long distance pollen-mediated gene flow can have dramatic consequences by introducing locally novel alleles into a breeding neighborhood (Frank & Barr, 2001; Sork & Smouse, 2006). In a general context, the introduction of alleles in a population has obvious implications in terms of genetic diversity. In the particular case of gynodioecy, long distance pollen dispersal is likely to affect the evolutionary dynamics of sex ratios by introducing restorers of male fertility.

Male reproductive success and evolutionary dynamics of gynodioecy

As already outlined, two types of hermaphrodites coexist in natural populations of gynodioecious sea beets: those carrying a sterilizing cytoplasm (restored hermaphrodites) and those with a non sterilizing cytoplasm. Another study conducted in tandem with ours, the same year, estimated pollen viability on the same individual plants that were used in the current study and showed that non-CMS hermaphrodites produced better pollen than restored hermaphrodites (Dufay *et al.*, 2008). The paternity analysis aimed to investigate whether these phenotypic differences actually resulted in differences in male reproductive success. Quite surprisingly our results showed that the opposite may be the case: restored hermaphrodites (CMS hermaphrodites) appeared to sire more seedlings than normal hermaphrodites. This unexpected observation is a likely consequence of the spatial structure, since CMSs and sexes tended to be clustered and restored hermaphrodites experienced a stronger availability of females in their direct vicinity. This particular structure probably causes spatial variation in pollen competition and the restored hermaphrodites were the only available fathers in the areas where the sex ratio was female biased. As a consequence, as soon as it benefits of the advantage of being rare, even a bad pollen producer

can efficiently transmit its genes. The interplay between female fitness and local sex ratio has been studied in several gynodioecious species, all of which found that individual fitness cannot be predicted without taking into account the local sex ratio (Graff, 1999; Taylor *et al.*, 1999; Alonso, 2005). The present study shows that the same is true for male fitness. In this particular case, clustering of females and restored hermaphrodites apparently counteracts the expected disadvantage of restored hermaphrodites in terms of mean number of pollination events. This particular relationship between local structure and fitness is similar to what is observed in self-incompatible species, where reproduction can be negatively affected by identity of neighboring individuals, in cases of local low diversity of self-incompatibility alleles (Wagenius *et al.*, 2007). Such idea could be generalized to any system in which individual fitness is likely to depend on the identity of the neighboring individuals.

The frequencies of sexual phenotypes and the results of paternity analyses actually fit theoretical predictions of selection models: in the first phase, CMS genes increase in frequency by benefitting from a female advantage. After a few generations, the population is then mainly composed by females (Gouyon *et al.*, 1991; Couvet *et al.*, 1998; Dufay *et al.*, 2007). At this stage, selection is expected to favor restorers of male fertility because of pollen limitation occurring in female biased populations (Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufay *et al.*, 2007). In other words, restorers are expected to increase in frequency because of their association with the rare gamete type, pollen. In our empirical study, pollen flow is mainly local, making this fitness advantage inherently dependent on the composition of the direct neighborhood. Our results thus suggest that frequency-dependent selection occurs at a very restricted spatial scale (*i.e.* within a genetically distinct cluster).

What remains unclear is what occurs in the following stages of the evolutionary dynamics of gynodioecy. There are probably two advantages of being a hermaphrodite in an area where females are common, in terms of male reproductive success: (i) there are more potential mates per capita to receive pollen and (ii) female are expected to be better mates, due to the female reproductive advantage that is expected in gynodioecious species. Such female advantage has not been found yet in our study species, but it has been clearly demonstrated in many other gynodioecious species (e.g. Avila-Sakar & Domínguez, 2000; Thompson & Tarayre, 2000; Marshall & Ganders, 2001; Lopez-Villavicencio *et al.*, 2005). Nonetheless, in the first stages of the selection of restorers, female advantage may be lowered by pollen limitation. These considerations point out that population structure can affect the nuclear and cytoplasmic genomes in a different way, even in the same plant. In terms of more general evolutionary biology, strong spatial genetic structure is likely to amplify the expected effects of frequency dependent selection by clustering together similar genotypes.

CONCLUSIONS & PERSPECTIVES

This study demonstrates that spatially-restricted gene dispersal can enhance fine-scale structuring of both genetic diversity and sexual phenotypes. Because this study was carried out on a population which was characterized by extremely contrasted local sex ratios and genotype frequencies, it clearly illustrates how fine scale population structure strongly affects the individual male fitness, and may even counteract the expected effects of natural selection. This result was quite unexpected over such a geographically restricted scale, especially in a wind-pollinated species. Empirical studies that look at the maintenance of gynodioecy, the evolution of sex-ratio, or more generally the dynamics of any polymorphic trait under frequency-dependent selection should probably not considerer gene or morph frequencies at the scale of the whole population, but rather investigate how these frequencies vary at a fine geographical scale. A better understanding of the interplay between neighborhood characteristics and individual fitness within populations is indeed required to identify the relevant spatial scale at which frequency dependent selection is likely to drive polymorphism dynamics. This also underlies the need for spatially explicit models, which should integrate the effect of local genotype frequencies together with gene flow among demes on the evolution of polymorphism.

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Gynodioecy in structured populations: (II) effects on male mating success in *Beta vulgaris* ssp. *maritima*

I. DE CAUWER, M. DUFAY, E.K. KLEIN & J.-F. ARNAUD

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ABSTRACT

Variation among individuals in fitness traits is advocated as a major process driving the adaptive evolution in sexually polymorphic plants. In gynodioecious species, where females and hermaphrodites coexist, gender-specific differences in fitness are expected to explain the maintenance of females. In gynodioecious *Beta vulgaris* ssp. *maritima*, sex determination involves cytoplasmic male sterility genes (CMS) and nuclear restorers of male fertility, and both restored CMS hermaphrodites and non-CMS hermaphrodites occur. Using genotypic information on seedlings and on all flowering adults within structured populations, we investigated whether male fertility was influenced by genotypic and phenotypic differences, while taking into account the shape and scale of pollen dispersal and the geographical position of individuals. Along with spatially restricted pollen flow, we showed that male fecundity was significantly affected by individual size, pollen quality and cytoplasmic identity. Siring success of non-CMS hermaphrodites was significantly higher than restored CMS hermaphrodites, but strongly depended on the genetic neighborhood: male fertility of non-CMS hermaphrodites was significantly reduced by the occurrence of restored CMS hermaphrodites in their vicinity. Our results demonstrate a silent cost of restoration on male function, a condition theoretically expected to maintain a stable sexual polymorphism in gynodioecious species.

INTRODUCTION

From an ecological and an evolutionary perspective, variation among individuals in fitness traits associated with survival and reproduction is often advocated as a major process driving the adaptive evolution of populations, their range extension and the probability of species extinction. The magnitude of fitness heterogeneity among individuals is a fundamental issue because it directly influences the effective population size and thus the opportunity for genetic drift to decrease the efficiency of natural selection. Within natural plant populations, the relative reproductive output of individuals depends on a combination of their own intrinsic characteristics, the ability of their propagules to disperse in space and the availability of potential mates, which often depends on their geographical proximity. Over the last decades, the development of highly informative genetic markers and important methodological advances allowed exploring the variation in reproductive success among plants, using parentage/paternity analyses. Numerous empirical studies have shown that pollen and seed dispersal is commonly skewed toward short geographical distances (e.g. Smouse *et al.*, 1999; Burczyk *et al.*, 2002; Robledo-Arnuncio *et al.*, 2004) and that the male and female contributions to mating events are typically highly uneven among individuals (e.g. García *et al.*, 2005; Nishizawa *et al.*, 2005; Oddou-Muratorio *et al.*, 2005). Observed heterogeneity in individual contributions to reproduction can be explained both by the geographical position of individuals and by a number of phenotypic and ecological characteristics of potential mates. Indeed, the spatial localization of pollen producers relative to mother plants can affect male reproductive output as much as phenotypic and ecological determinants of male fertility (Burczyk *et al.*, 2002; Oddou-Muratorio *et al.*, 2005). Empirical evaluations of fitness heterogeneity among individual thus requires taking into account explicitly the localization of individual along with the shape and scale of the distribution of dispersal events. Among the various existing paternity analyses methods, the neighborhood model, initially developed by Adams & Birkes (1991) and modified by Burczyk *et al.* (2002) and Oddou-Muratorio *et al.* (2005) allows such conjoint estimation of the pollen dispersal kernel and the heterogeneity in male fertility. Such approach could be very useful in studying the evolution of sexually polymorphic flowering plants (*i.e.* in which natural populations are sub-divided into distinct mating groups that differ in floral morphology or in sexual phenotype) because it allows (i) to assess whether individuals belonging to different morphs have different male and female fitnesses and (ii) to understand how sex determining genes travel across the landscape.

Although poorly understood in most sexually polymorphic species, the genetic basis of sex determination is well known for several gynodioecious species (*i.e.* coexistence of females and hermaphrodites in natural populations). It commonly involves interactions between cytoplasmic male sterility genes (CMS genes) and nuclear genes that counteract the action of CMS genes and restore male function (e.g. Boutin *et al.*, 1987 ; Dommée *et al.*, 1987; Koelewijn & van Damme, 1995; Delph *et al.*, 2007). To develop as a female, an individual must carry an unrestored CMS

gene. To develop as a hermaphrodite, an individual must carry a CMS gene in combination with the matching restorer alleles (restored hermaphrodite). Gynodioecious species often contain multiple different CMS genes, each requiring its own mode of restoration (e.g. de Haan *et al.*, 1997b; Charlesworth & Laporte, 1998; Dudle *et al.*, 2001; van Damme *et al.*, 2004). Numerous empirical and theoretical studies have attempted to determine the conditions for a stable maintenance of gynodioecy. Because cytoplasmic genes are only transmitted through seeds, theory predicts that a CMS gene should spread in a population as soon as there is a seed-fertility advantage for females compared to hermaphroditic conspecifics (Charlesworth, 1981; Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufay *et al.*, 2007). On the other hand, since nuclear genes are biparentally transmitted, the loss of pollen production directly causes a loss of their transmission. Therefore, nuclear restorers of male fertility should be selected for when CMS genes become frequent in a population. Theoretical models suggest that there must be some forces opposing to the fixation of restorer alleles to maintain cytonuclear polymorphism within populations. This can be achieved through (i) metapopulation dynamics, with recurrent extinctions and recolonization of demes, that prevent local populations to reach equilibrium (Couvet *et al.*, 1998) and / or the existence of negative pleiotropic effects of nuclear sex-determining genes on fitness, *i.e.* a cost of restoration, that prevents the fixation of restorers and allows the maintenance of cytonuclear polymorphism (Charlesworth & Ganders, 1979, Bailey, 2003; Frank, 1989; Gouyon *et al.*, 1991; Dufay *et al.*, 2007). Various types of cost of restoration are considered in theoretical studies: constitutive cost (independent of the cytoplasm), silent cost (only expressed when the restorer is associated with another cytoplasm than the one it restores) and expressed cost (only expressed when the restorer is associated with the cytoplasm that it restores). However, it is generally acknowledged that an expressed cost is not a biologically realistic component of gynodioecious systems (Delph *et al.*, 2007) and theoretical studies showed that it does not allow the maintenance of cytonuclear polymorphism associated with gynodioecy (p.e. Dufay *et al.*, 2007).

In addition, non sterilizing cytotypes (*i.e.* never associated with a female phenotype) are also described in a few gynodioecious species, including sea beet, *Beta vulgaris* ssp. *maritima* (Fénart *et al.*, 2006). In this case, an individual exhibiting a hermaphroditic phenotype can either be a restored CMS hermaphrodite or a non-CMS hermaphrodite. In *B. vulgaris*, restored CMS hermaphrodites have been shown to produce low quality pollen compared to non-CMS hermaphrodites, both in the wild (Dufay *et al.*, 2008) and in controlled conditions (De Cauwer *et al.*, in prep.-a). These differences in pollen quality between the two categories of hermaphrodites directly resulted from a high inter-individual variance among CMS restored hermaphrodites, with some of these individuals producing pollen of very low quality while some others produced pollen equivalent to non-CMS hermaphrodites. Such a quantitative variation of pollen quality produced by restored CMS-hermaphrodites was hypothesized to be the result of a polygenic determination of restoration. Indeed, complex restoration patterns are likely to decrease the probability to find fully restored hermaphrodites in natural populations (Charlesworth & Laporte, 1998; Koelewijn, 2003; Ehlers *et al.*, 2005). In addition, Dufay *et al*. (2008) showed contrasted situations that

depended on the level of restoration rate within populations: non-CMS hermaphrodites located in a population where restorer alleles were frequent were poor pollen producers compared to non-CMS hermaphrodites located in another population characterized by a lower restoration rate. This preliminary finding provided an indirect evidence for a silent cost of restoration. Overall, male fertility of *Beta vulgaris* hermaphroditic individuals thus seems to depend critically on the genotype of individuals. Pollen production in restored CMS hermaphrodites is likely to increase with the number of restorer alleles. In contrast, in the case of a silent cost of restoration, pollen production in non-CMS hermaphrodites could be negatively affected by the presence of restorer alleles. Although no genetic markers of restoration are currently available, the local frequency of restored hermaphrodites could be a relevant estimator of the probability of a given non-CMS hermaphrodite to carry restorer alleles because pollen flow is known to be spatially restricted in this species (De Cauwer *et al.*, 2010b).

Both overall heterogeneity in male reproductive success and cytoplasm-related differences in male fitness are expected to profoundly modify the conditions of maintenance of the cytonuclear polymorphism associated with gynodioecy. Indeed, important differences in male contributions among hermaphrodites will decrease the effective population size, and thus amplify the levels of genetic drift, which has been shown to increase the risk of loss of cytonuclear polymorphism (Dufay & Pannell, 2010). In addition, cytoplasm-related differences in male fitness, with restored CMS hermaphrodites producing low quality pollen compared to non-CMS hermaphrodites, could possibly slow down the selection of restoration and ultimately modify the conditions of maintenance of cytonuclear polymorphism, favoring the maintenance of females in gynodioecious populations (Bailey *et al.*, 2003). Empirical studies often compare reproductive traits among sexual phenotypes and use these observations as a proxy for reproductive output. In the current study, using genotypic information about seeds and about all the potential fathers in two study sites, we investigate whether phenotypic and ecological differences actually translate in male reproductive success differences in *Beta vulgaris* ssp. *maritima*. To do so, we use an approach related to the neighborhood model (Adams & Birkes, 1991; Burczyk *et al.*, 2004; Oddou-Muratorio *et al.*, 2005) that allows to estimate conjointly the level of selfing, the magnitude of gene flow from pollen sources outside the local population and the variance in fertility among males within each study sites. Specifically, we investigate to what extend male fertility in *Beta vulgaris* hermaphroditic plants is influenced by pollen quality, plant size, flowering phenology, sexual phenotype and the probability to carry restorer alleles, while taking into account the shape and scale of pollen dispersal and the geographical position of individuals. Finally, we discuss how these results can have profound consequences on the dynamics of sexual polymorphisms, and we stress the importance of accurately determining the individual reproductive variance because it directly impacts the effective population size of local neighborhoods.

The species

Sea beet, *Beta vulgaris* ssp. *maritima* is a diploid species (2n = 18) widely distributed along the western coast of Europe and around the Mediterranean basin. It is a short lived perennial and wind-pollinated species (Letschert, 1993). In the wild, *B. vulgaris* is a outcrossing species characterized by a gametophytic self incompatibility system, with up to four gametophytic S loci (Owen, 1942; Larsen, 1977). However, a self-fertility factor that offsets the self-incompatibility can be found in crop and weed beet lineages (Owen, 1942; Arnaud *et al.*, 2010). There is no vegetative reproduction, and dispersal can thus only occur through seeds and/or pollen movement. Seeds are aggregated in an irregular dry body that contains 1-8 seeds. These aggregated fruits have no particular dispersal mechanism: seeds are primarily dispersed by gravity, mostly involving short-range dispersal events (Arnaud *et al.*, 2009; De Cauwer *et al.*, 2010b), although hydrochory may lead to occasional long distance dispersal events during very high tides (Fievet *et al.*, 2007). This study was carried out in Brittany (western France), where sea beets colonize areas located along estuaries, just at the upper level of the tide, cliffs overhanging the sea and other coastal habitats (Letschert, 1993; Laporte *et al.*, 2001; Viard *et al.*, 2004).

This species constitutes a relevant model for studying the relative male reproductive output of the two hermaphroditic types (*i.e.* non-CMS hermaphrodites and restored CMS hermaphrodites) because the genetic basis of sexes is well known: male sterility is associated with four particular mitochondrial cytotypes, called CMS *E*, *G*, *Svulg* and *H*. These sterilizing cytoplasms coexist with male fertile cytoplasms, and all the different cytotypes can be identified with molecular markers (Cuguen *et al.*, 1994; Desplanque *et al.*, 2000). Historical relationships between CMS and non-CMS haplotypes suggested independent apparitions of CMS haplotypes with sterilizing cytoplasms belonging to different lineages derived from an ancestral non-sterilizing cytoplasm (Fénart *et al.*, 2006).

Study sites

An exhaustive sampling was carried out in two study sites located in Brittany (western France), within two isolated coves separated by 30 km. The first one, called *MOR*, is located near Planguenoual (N 48°34,168; E -2°34,831), extends over approximately 300 m and consisted of 1098 flowering individuals in 2007. The second one, named *PAL*, is located near Plouha (N 48°40,497; E -2°52,911), extends over approximately 550 m and comprised 615 flowering individuals in 2007. In both sites, individuals appeared to be strongly clustered in space: in *MOR*, individuals were growing in four geographical patches called *MA*, *MB*, *MC* and *MD* (mean number of individuals was 273, ranging from N_{MIN} = 69 to N_{MAX} = 635, see Table 1), and in *PAL*, individuals were clustered within five patches called *PA*, *PB*, *PC*, *PD* and *PE* (mean number of individuals was 118, ranging from N_{MIN} = 18 to N_{MAX} =300, see Table 1). Leaves were collected

for genotyping on all individuals that flowered during the study year (*i.e.* all adult individuals) and the location of all flowering plants was mapped. Genetic data concerning adult individuals were analyzed in a companion study (De Cauwer *et al.*, in prep). The main result of this study was the existence of a very pronounced spatial genetic structure within both study sites, with high levels of nuclear and cytoplasmic differentiation among geographical clusters of genetically related individuals, probably as a result of the action of recurrent extinction and recolonization events along with restricted gene flow.

Table 1: Major characteristics of the different geographical patches within the two study sites (*MOR* and *PAL*), including the total number of flowering individuals (N_{TOT}, with the number between brackets corresponding to isolated individuals, growing outside the geographical patches), the mean density of individuals (*D*), the habitat type (geographical patches were growing on cliffs or in soils composed of a mixture of sand and shingles), the proportion of individuals carrying a cytoplasmic male sterility (frequency of CMS genes), the proportion of female individuals (sex ratio), the number of individuals in each phenotype category (NF: number of females, NH_{CMS}: number of restored CMS hermaphrodites and NH_{NCMS}: number of non-CMS hermaphrodites, with the number between brackets corresponding to non-phenotyped individuals) and the proportion of restored hermaphrodites in CMS individuals (frequency of restoration).

Study Sites	Geographical Patches	N_{TOT}	D (ind/m²)	Habitat type	Frequency of CMS genes	Sex ratio	NF / NH_{CMS} / NH_{NCMS}	Frequency of restoration
MOR	*MA*	69	0.057	Cliff	0.088	0	0 / 3 / 62 (4)	1
	MB	635	0.843	Sand/Shingle	0	0	0 / 0 / 633 (2)	-
	MC	281	0.081	Sand/Shingle	0.296	0.063	17 / 60 / 195 (9)	0.779
	MD	109	0.138	Cliff	0	0	0 / 0 / 106 (3)	-
	Overall	**1094 (4)**	**0.529**	**-**	**0.084**	**0.016**	**17 / 63 / 996 (18)**	**0.788**
PAL	*PA*	146	0.034	Cliff	0	0	0 / 0 / 142 (4)	-
	PB	18	0.019	Sand/Shingle	0.889	0.429	6 / 6 / 2 (4)	0.5
	PC	300	0.176	Sand/Shingle	0.383	0.21	61 / 45 / 185 (9)	0.425
	PD	33	0.028	Cliff	0	0	0 / 0 / 32 (1)	-
	PE	95	0.053	Sand/Shingle	0.228	0.033	3 / 18 / 71 (3)	0.857
	Overall	**592 (23)**	**0.109**	**-**	**0.257**	**0.123**	**70 / 69 / 432 (21)**	**0.496**

Characterization of adult individuals

Sexual phenotypes

Sexual phenotype was determined (female or hermaphrodite) for almost all adult individuals within each study site (98.5% in *MOR* and 97.4% in *PAL*). Plants with brown or white and reduced anthers were considered to be females and plants with yellow anthers with obvious pollen production were considered to be hermaphrodites. Some individuals showed intermediate phenotypes (light-coloured yellow anthers with little pollen production) and were also classified as hermaphrodites. Overall sex ratio (proportion of females) was 1.6% in *MOR* and 12.3% in *PAL*, with important variation of sex ratios from one patch to another (Table 1). Given that the cytotype of each mother plant was known (CMS or non-CMS), it was possible to discriminate the two hermaphroditic types: restored CMS-hermaphrodites and non-CMS hermaphrodites (Table 1). The

local neighborhood around each individual was described by counting the total number of flowering individuals, the number of restored CMS-hermaphrodites, the number of non-CMS hermaphrodites and the number of female individuals within a radius of 10 meters.

Characterization of pollen production

Pollen production was determined for a subsample of flowering individuals, including female plants. For each individual, the day of the flowering onset, two nearly opened buds localised on one of the main floral stems were collected and dissected to obtain two anthers per bud. For each individual, these four anthers were stored separately in 95% ethanol until pollen counts were performed. Details on the counting procedure and the utilisation of the particle counter [CASY® model TT (Innovatis, Bielefeld)] are described in Dufay *et al*. (2008). The number of detected particles was determined for 400 size classes ranging from 0.125 to 50 µm. Pollen size has been shown to be a reliable estimator of pollen viability in various plant species (see Kelly *et al.*, 2002). In *B. vulgaris,* previous studies have also showed that non-viable pollen grains were smaller than viable pollen grains (Dufay *et al.*, 2008; De Cauwer *et al.*, in prep.-a), with pollen grains being typically distributed in two different size categories, the first one (10 to 13.5 µm) corresponding to non-viable pollen grains whereas the second one (13.5 to 24 µm) includes viable pollen grains. Usually, some individuals specifically produce one of the pollen types and some others produce both. Particles were categorized within these two size classes and the total number of large pollen grains (larger than 13.5 µm) was assessed for the four anthers collected on each studied individual. The mean number of large (*i.e*. viable) pollen grains was then calculated over the four anthers, for 12 females, 30 restored CMS hermaphrodites and 224 non-CMS hermaphrodites in *MOR* and for 25 females, 31 restored CMS hermaphrodites and 162 non-CMS hermaphrodites in *PAL* (see also Table 2 for repartition among geographical patches). Using general linear models, we tested for an effect of sexual phenotype on the mean number of viable pollen grains, while controlling for potential effects of the date of flowering onset and of the geographical patch. Differences between individual sexual phenotypes were then explored using pairwise Tukey comparisons. Data generated in *MOR* and in *PAL* were analyzed separately.

Estimating total investment in reproduction

At the end of the flowering season, the length of inflorescences was measured on three randomly chosen floral stems for a subsample of individuals located within the main gynodioecious patches (*MC* in *MOR*, *PC* and *PE* in *PAL*, see Table 2). An estimator of individual investment in reproduction was then obtained by multiplying the mean inflorescence length (over the three measures) by the total number of floral stems. This estimator was calculated for 14 females, 30 restored CMS-hermaphrodite and 147 non-CMS hermaphrodites in *MOR* and for 47 females, 52 restored CMS-hermaphrodite and 214 non-CMS hermaphrodites in *PAL* (see also Table 2 for repartition among geographical patches). Data generated in *MOR* and in *PAL* were analyzed separately. As for the pollen counts, general linear models were used to test for an effect of sexual phenotype on the estimator of investment in reproduction, while controlling for potential

effects of the date of flowering onset and of the geographical patch (in *PAL* only, as measures were carried out in only one patch in *MOR*). Differences between individual sexual phenotypes were explored using pairwise Tukey comparisons.

Table 2 : Number of individuals used to characterize pollen production (N_{POLL}) and total investment in reproduction (N_{INVEST}), as well as number of mother plants (N_{MP}), number of offspring (N_O) and mean number of offspring per progeny (Mean $_{PROG}$ ± SD) for the different geographical patches within the two study sites (*MOR* and *PAL*).

Study Sites	Geographical Patches	N_{POLL}	N_{INVEST}	N_{MP}	N_O	Mean $_{PROG}$
MOR	*MA*	16	0	6	143	23.83 (± 1.33)
	MB	45	0	6	133	22.17 (± 6.94)
	MC	191	191	32	773	24.16 (± 2.85)
	MD	14	0	6	150	25 (±0)
	Overall	**266**	**191**	**50**	**1199**	**23.98 (± 2.29)**
PAL	*PA*	12	0	12	297	24.75 (± 0.87)
	PB	0	0	0	0	-
	PC	120	221	15	357	23.80 (± 4.13)
	PD	8	0	8	197	24.63 (± 1.06)
	PE	78	92	15	368	24.53 (± 1.81)
	Overall	**218**	**313**	**50**	**1219**	24.38 (± 2.51)

Offspring genotyping

A total of 50 mother plants were randomly selected in each study site for seed sampling (six females, five restored CMS hermaphrodites and 39 non-CMS hermaphrodites in *MOR*, three females, seven restored CMS hermaphrodites and 40 non-CMS hermaphrodites in *PAL*). Seeds were collected in all the geographical patches except in *PB* because of very low levels of fruit set. The repartition of mother plants among geographical patches is given in Table 2. The distance between the chosen mother plants ranged from less than a meter to 278 meters, with a mean of 111 meters in *MOR* and from less than a meter to 542 meters, with a mean of 232 meters in *PAL*. Seeds were randomly collected on each of these maternal plants in mid-august 2007, sowed in a greenhouse in November 2007 and grown for two months, until each seedling had several leaves. A total number of 2418 seedlings were then collected for molecular studies (1199 originating from *MOR* and 1219 originating from *PAL*, see Table 2 for mean ± SD number of progeny analyzed per mother plant). For the 2418 studied seedlings, DNA was extracted from dried leaf tissue and purified using the NucleoSpin®96 Plant Kit (Macherey-Nagel). Nine nuclear microsatellite loci chosen for high polymorphism and previously used to genotype all adult individuals in both study sites were screened for genetic diversity: *Bmb6* (Cureton *et al.*, 2002); *Bvm3* (Mörchen *et al.*, 1996); *GTT1, CAA1* (Viard *et al.*, 2002); *SB04*, *SB06*, *SB07*, *SB15* (Richards *et al.*, 2004); and *FDSB1027* (McGrath *et al.*, 2007). Amplification procedures and detection of polymorphism can be found in previous studies (Fénart *et al.*, 2008; De Cauwer *et al.*, 2010b). Among the 2418 studied seedlings, 3.67% of genotypes included missing data.

Mating system analyses

Within each geographical patch, the mating parameters were estimated using a maximum-likelihood approach based on mixed mating models (Ritland, 2002). Average single locus outcrossing rates (t_s), multilocus outcrossing rates (t_m), correlation of outcrossed paternity (r_p, describing the probability that two randomly chosen offspring within an open pollinated family are full sibs) and correlation of selfing (r_s, estimating the normalized variance in selfing rates among families within a given population) were computed using the numeric Newton-Raphson algorithm and population gene frequencies. All mating system parameters were estimated with MLTR 3.2 (Ritland, 2002). For all parameters, standard errors were calculated based on 1000 bootstraps, re-sampling families within the geographical patches. The difference between multilocus and single locus outcrossing rates (t_m - t_s) provides an estimation of the proportion of apparent selfing that is due to biparental inbreeding. The number of effective pollen donors (N_{ep}) can be approximated by 1/ r_p (Sampson & Byrne, 2008; Mimura *et al.*, 2009).

Modeling the dispersal kernel and heterogeneity in male fecundity

In each study site, spatially explicit mating models were used to investigate how the mating probability depended on (i) sexual phenotype (*i.e.* restored CMS hermaphrodite or non-CMS hermaphrodite), (ii) pollen quality, estimated has the mean number of viable pollen grains produced per anther, (iii) investment in reproduction, estimated as the cumulated size of floral stems, (iv) neighborhood type, which corresponds to the presence or absence of restored hermaphrodites in a radius of 10 meters, (v) flowering synchrony, *i.e.* difference in the date of flowering onset between mothers and fathers and (vi) physical distance between individuals.

Following Burczyk *et al.* (2002) and Oddou-Muratorio *et al.* (2005), we considered that each offspring could be the result of either (i) self-pollination (with probability *s*), (ii) pollen coming from outside the study sites (with probability *m*) or (iii) pollen coming from one of the sampled individuals (with probability 1 - *m* - *s*). A seed *o* sampled on a mother-plant j_o with genotype g_{jo} is then expected to have the genotype g_o with probability:

$$P\left(g_o \middle| g_{j_o}\right) = sT(g_o \middle| g_{j_o}, g_{j_o}) + (1-s-m)\sum_{k:\,father} \pi_{j_o k} T\left(g_o \middle| g_{j_o}, g_k\right) + mT\left(g_o \middle| g_{j_o}, AF\right)$$

(eqn 1)

with *s* the selfing rate, *m* the rate of incoming pollen flow, (1 - *m* - *s*) the probability that the pollen donor is inside the plot and π_{jk} the composition of the pollen pools described below (eqn 3). The allele frequencies (*AF*) in the background pollen pool (*i.e.* incoming pollen) were measured independently in *MOR* and *PAL* from the estimated contribution of individuals located outside the study sites (*i.e.* through offspring that have no compatible father inside). The transition probabilities $T(.|.,.)$ are the Mendelian likelihoods to observe a genotype for a seed conditional to

the genotype of the parents (Meagher, 1986). The information carried by the genotypes of all seeds sampled in one site is gathered in the log-likelihood function assuming that all fecundation events are independent,

$$\log L = \sum_{o:offspring} \log\left[sT(g_o | g_{j_o}, g_{j_o}) + (1-s-m) \sum_{k:father} \pi_{j_o k} T\left(g_o | g_{j_o}, g_k\right) + mT\left(g_o | g_{j_o}, AF\right) \right]$$

(eqn 2)

The proportion of pollen from each father k in the pollen pool of each mother j originating from all known fathers, π_{jk}, is assumed to follow the mass-action law,

$$\pi_{jk} = \frac{Pop_k Sex_k Fec_k Pheno_{jk} Disp_{jk}}{\sum_{l:father} Pop_l Sex_l Fec_l Pheno_{jl} Disp_{jl}}$$

(eqn 3)

where Pop_k in $(0,+\infty)$ account for differences in individual male fecundities among the different geographical patches.

The effects of the sexual phenotype (*i.e.* female, restored CMS hermaphrodite or non-CMS hermaphrodite) and neighborhood type (*i.e.* the presence or absence of restored hermaphrodites in a radius of 10 meters) are given by a matrix of parameters to estimate:

(i) for plants k with no restored CMS hermaphrodites in the neighborhood, we set $Sex_k = F = 0$ for females, $Sex_k = H_{CMS}$ for restored CMS hermaphrodites, $Sex_k = H_{NCMS0} = 1$ for non-CMS hermaphrodites and $Sex_k = NT$ for non-typed individuals (*i.e.* individuals for which the sexual phenotype was not scored)

(ii) for plants k with restored CMS hermaphrodites in the neighborhood, we set $Sex_k = F = 0$ for females, $Sex_k = H_{CMS}$ for restored CMS hermaphrodites, $Sex_k = H_{NCMS1}$ for non-CMS hermaphrodites and $Sex_k = NT$ for non-typed individuals.

The effects of reproductive investment (RI_k) and pollen production (PP_k) are accounted for through an exponential relation,

$$Fec_k = \exp\left[b_{RI}\left(RI_k - \overline{RI}\right)\right] \exp\left[b_{PP}\left(PP_k - \overline{PP}\right)\right]$$

(eqn 4)

For the individuals that were not scored for *RI* or *PP*, the corresponding exponential term was replaced by a parameter NS_{RI} (independent on the patch) or NS_{PP} (different in the different patches)

The effect of phenology is given by a Gaussian curve,

$$Pheno_{jk} = \exp\left[-\frac{\left(\Delta_{jk} - \Delta_{opt}\right)^2}{2\sigma_{\Delta}^2}\right]$$

(eqn 5)

where Δ_{jk} is the difference in phenology between mother *j* and father *k* and Δ_{opt} is the optimal difference in phenology .

The effect of physical distance between individuals was modelled using a dispersal kernel, describing the probability density that a pollen grain lands at a given position away from the source. As suggested in previous studies (Oddou-Muratorio *et al.*, 2005; Fénart *et al.*, 2007), we investigated several shapes for the dispersal kernel including the exponential-power function and the logistic function (reviewed in Austerlitz *et al.*, 2004).

The exponential-power kernel used to model the effect of distance on mating probability is given by,

$$Disp_{jk} = \frac{b}{2\pi a^2 \Gamma(2/b)} \exp\left[-\frac{d_{jk}^{\,b}}{a^b}\right]$$

(eqn 6)

where d_{jk} is the distance between mother *j* and father *k,* Γ is a gamma function, *a* is a scale parameter for distance and *b* is a shape parameter (Clark, 1998). The case of $b = 2$ corresponds to a bivariate Gaussian distribution, whereas $b = 1$ corresponds to a bivariate exponential distribution. The average pollen dispersal distance (δ) is then given by $\delta = [a\,\Gamma(3/b)\,/\Gamma(2/b)]$.

The logistic function is given by,

$$Disp_{jk} = \frac{b}{2\pi a^2 \Gamma(2/b)\Gamma(1-2/b)}\left(1+\frac{d_{jk}^{b}}{a^b}\right)^{-1}$$

(eqn 7)

(7)

By maximizing the log-likelihood log$L$ (eqn 2) in Mathematica 7.1 (Wolfram Research), we jointly estimated the parameters s, m, (Pop_1, Pop_2… Pop_{max}), b_{RI}, NS_{RI} , b_{PP}, (NS_{PP1} , …NS_{PPmax}), H_{CMS}, H_{NCMS1}, NT, Δ_{opt}, σ_{Δ}, a and b, independently in the two sites (*MOR* and *PAL*).

The significance of each effect was tested with a Type III likelihood-ratio test (LRT). For each test, the log-likelihood of a model without the tested effect was computed. The deviance (*i.e.* twice the difference between the log-likelihood obtained for the complete model and the log-likelihood obtained for the model without the tested effect) was then compared to a chi-square distribution, with the number of degrees of freedom equal to the difference in the number of parameters between these two models.

Pollen production

Our results confirmed the previous observations that (i) overall pollen quantity in *B. vulgaris* (regardless of the size of pollen grains) shows important variation among individuals carrying CMS genes and that (ii) there is a threshold in pollen quantity, with some plants producing very few pollen grains and some other individuals that are at least partially restored for male fertility and produce more than 7000 pollen grains per anther (data not shown, see Dufay *et al.*, 2008; De Cauwer *et al.*, in prep.-a). As in these earlier studies, all plants that were categorized as females during flowering produced less than 7000 pollen grains per anther.

The size of pollen grains has repeatedly been shown to be a relevant estimator of pollen viability in *Beta vulgaris* (Dufay *et al.*, 2008; De Cauwer *et al.*, in prep.-a). Pollen grains are typically distributed in two different size categories, the first one (< 13.5 µm) corresponding to non-viable pollen grains whereas the second one (> 13.5 µm) includes viable pollen grains. In our study, sexual phenotype had a significant effect on the number of large (viable) pollen grains, estimated for a subsample of individuals in both study sites (Table 3). As expected, individuals categorized as females during flowering produced negligible quantities of large pollen grains (on average 19 fold less large pollen grains than hermaphroditic conspecifics). Interestingly, pairwise comparisons confirmed the previous observation that restored CMS hermaphrodites are poor pollen producers compared to non-CMS hermaphrodites (Dufay *et al.*, 2008; De Cauwer *et al.*, in prep.-a). Indeed, within both study sites, the number of large pollen grains was significantly lower in restored CMS hermaphrodites than in non-CMS hermaphrodites (see Fig. 1). In addition to the effects of sexual phenotype, we also detected a strong negative effect of the date of flowering onset, with the best pollen producers being the individuals that started flowering early in spring 2007. Finally, the significant effect of the interaction between sexual phenotype and date of flowering onset (detected in *PAL* only, see Table 3) was due to a stronger negative effect of time on non-CMS hermaphrodites than on restored CMS hermaphrodites.

Estimation of total investment in reproduction

In our study, an estimator of the overall investment in reproduction was obtained by multiplying the mean inflorescence length (calculated over three floral stems) by the total number of floral stems for a subsample of individuals in both study sites. The three sexual phenotypes types appeared to be statistically indistinguishable with regard to this estimation of cumulated inflorescence length in both study sites (Table 3 and Fig. 1). Only the date of flowering onset had a significant effect, with small individuals flowering, on average, later than individuals showing a more important investment in reproduction.

Table 3: Results of the general linear models testing simultaneously for the effects of sexual phenotype (female, restored CMS hermaphrodites or non-CMS hermaphrodites), geographical patch and date of the flowering onset on (a) the number of large (viable) pollen grains and (b) the estimator of total investment in reproduction, in *Beta vulgaris* ssp. *maritima*. All non-significant interactions between main factors were dropped from the statistical analyses. Bold numbers correspond to significant *P*-value ($P < 0.05$).

(a) Number of large pollen grains					
Study Sites	Source of variation	df	MS	F	*P*
MOR	*Sexual Phenotype*	2	3559217	19.67	**<0.0001**
	Geographical Patch	3	124256.84	0.67	0.5719
	Date	1	12361701.77	66.54	**<0.0001**
	Error	257	185775.18		
PAL	*Sexual Phenotype*	2	5341450.31	20.47	**<0.0001**
	Geographical Patch	3	527320.43	2.02	0.1122
	Date	1	3138428.78	12.03	**0.0006**
	*Date * Sexual Phenotype*	2	1095064.63	4.20	**0.0164**
	Error	213	260988.38		
(b) Estimator of the total investment in reproduction					
Study Sites	Source of variation	df	MS	F	*P*
MOR	*Sexual Phenotype*	2	176441.35	1.86	0.1594
	Date	1	1444507.33	15.20	**<0.0001**
	Error	165	95020.06		
PAL	*Sexual Phenotype*	2	197756.67	1.22	0.2970
	Geographical Patch	1	364171.94	2.25	0.1353
	Date	1	6882860.02	42.54	**<0.0001**
	Error	179	161807.02		

Mating system analyses

Results from the progeny arrays analyses are reported in Table 4. The estimated levels of selfing rate s (= 1-t_m) were considered significant when the standard errors obtained with the bootstrapping procedure did not overlap 1. Despite the fact that *B. vulgaris* ssp. *maritima* is a self-incompatible species, some geographical patches (*MC*, *PA*, *PC* and *PD*) exhibited slight but significant departures from complete outcrossing with multilocus estimates of selfing rate ranging from 0.7% (in *PD*) to 7.1% (in *PA*) (Table 4). The estimation of the proportion of apparent selfing that is due to biparental inbreeding (t_m - t_s) ranged from slightly negative values (*i.e.* no biparental inbreeding, in *MD*) to 19.1% (in *PA*). The estimated levels of biparental inbreeding decreased significantly with local density (Spearman's Rho = -0.64, $P = 0.048$). Based on the estimation of the levels of correlation of paternity (r_p), the number of males contributing to the paternal mating pool ($N_{ep} = 1/r_p$) was estimated within each geographical patch, providing values ranging from 2.8 (in *PA*) to 9.8 (in *MB*) (Table 4). We found a significant positive association between local density and the N_{ep} values (Spearman's Rho = 0.64, $P = 0.048$), suggesting that mother plants located in dense patches were on average pollinated by a higher number of fathers than more isolated mother plants.

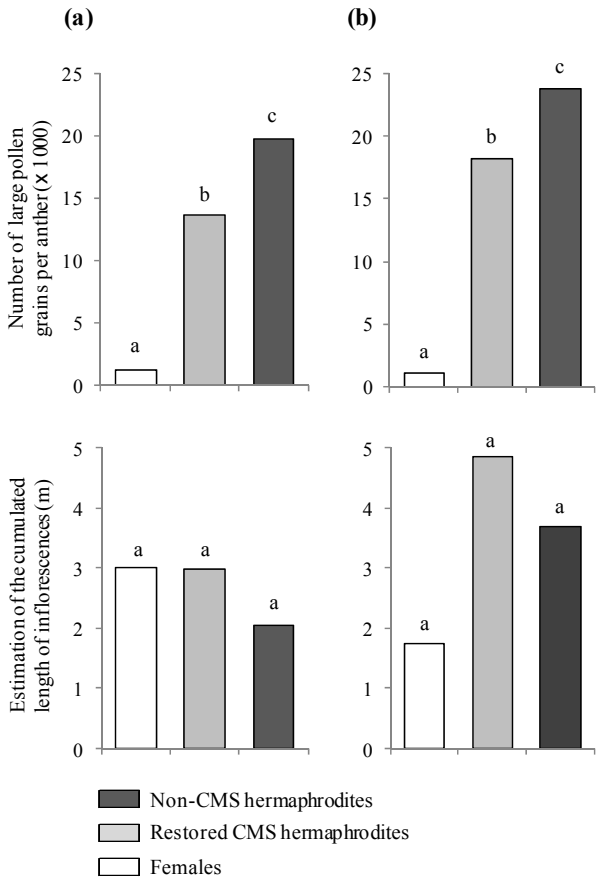

Fig. 1 Variation of the number of large (viable) pollen grains per anther (mean value over the four measured anthers) and estimator of the total investment in reproduction (estimation of the cumulated length of floral stems) for the three sexual phenotypes (non-CMS hermaphrodites, restored CMS hermaphrodites and females) and the two surveyed study sites: *MOR* (a) and *PAL* (b). Upward letters show the results of Tukey pairwise comparisons

Table 4: Estimates of mating system parameters for the different geographical patches within each study site (*MOR* and *PAL*).t_s and t_m are the average single locus and multilocus outcrossing rates. The difference between multilocus and single locus outcrossing rates (t_m - t_s) provides an estimation of the proportion of apparent selfing that is due to biparental inbreeding. r_p is the correlation of outcrossed paternity The number of effective pollen donors (N_{ep}) was approximated by 1/ r_p.

Study Sites	Geographical Patches	t_m	t_s	t_m-t_s	r_p	N_{ep}
MOR	*MA*	1.000 (0.029)	0.932 (0.028)	0.068 (0.039)	0.116 (0.033)	8.621
	MB	0.975 (0.083)	0.954 (0.034)	0.021 (0.071)	0.102 (0.025)	9.804
	MC	0.961 (0.012)	0.820 (0.025)	0.141 (0.023)	0.300 (0.042)	3.333
	MD	0.997 (0.036)	1.044 (0.055)	-0.047 (0.064)	0.204 (0.037)	4.902
PAL	*PA*	0.844 (0.085)	0.653 (0.098)	0.191 (0.031)	0.348 (0.076)	2.874
	PC	0.981 (0.010)	0.930 (0.031)	0.050 (0.029)	0.151 (0.030)	6.623
	PD	0.963 (0.030)	0.905 (0.057)	0.057 (0.047)	0.256 (0.053)	3.906
	PE	0.988 (0.029)	0.932 (0.031)	0.056 (0.037)	0.288 (0.053)	3.472

Dispersal kernel and heterogeneity in male fecundity

For each study site, a neighborhood model was used to estimate jointly the characteristics of the pollen dispersal kernel and the heterogeneity in male fertility. This allowed us to explore how male mating probabilities in the two study sites (*MOR* and *PAL*) were affected by the distance between reproducing plants, as well as by several factors that are potentially related to male fertility: (i) sexual phenotype, (ii) pollen quality, (iii) investment in reproduction, (iv) neighborhood type (*i.e.* presence or absence of restored hermaphrodites in a radius of 10 meters) and (v) flowering synchrony. The significance of the different tested effects is summarized in Table 5.

Effect of distance on mating probability

As expected, the effect of the physical distance between individuals on the mating probability was strong and highly significant in both study sites (Table 5). The best fit was obtained with the exponential power function in *MOR* and with a logistic function in *PAL*, leading to contrasted probabilities of long distance dispersal with a rather thin tailed kernel in *PAL* and a fat-tailed kernel in *MOR* (see Fig. 2b). The models yielded low estimates of mean pollen dispersal distances (δ = 22.52 m and 6.55 m in *MOR* and in *PAL*, respectively).

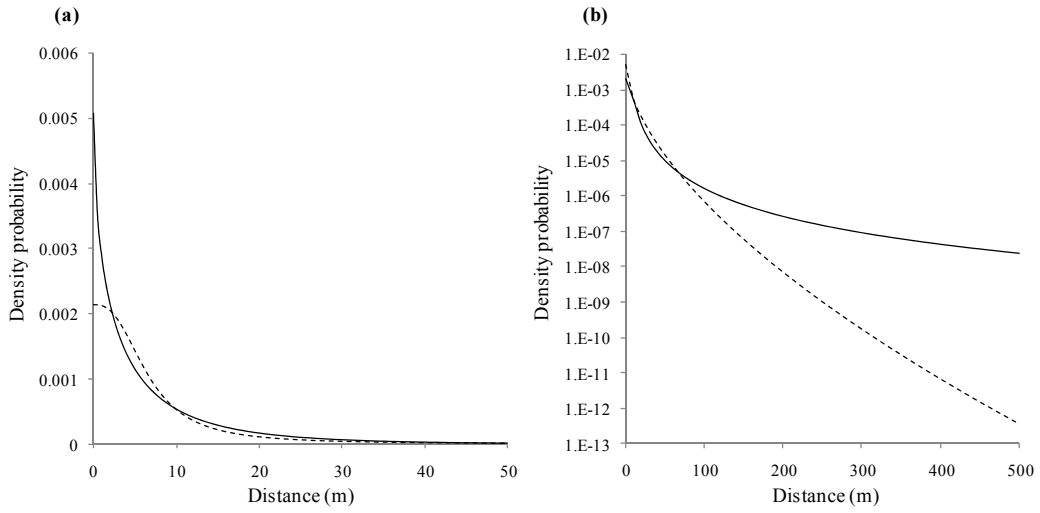

Fig. 2 Dispersal kernels estimated using the spatially explicit mating models in *MOR* (black line) and in *PAL* (dashed line). (a) density probability plotted against distance and (b) log-plot version of the same distributions, providing a better representation of their tails

Male fecundity

Geographical patch, investment in reproduction, pollen production, flowering phenology and interaction between sexual phenotype and neighborhood type all had a significant effect on male mating probabilities (Table 5). As expected, the estimated male fecundity increased strongly with the investment in reproduction (*i.e.* cumulative inflorescence length) and pollen production

(*i.e.* number of viable pollen grains per anther). The effect of investment in reproduction was similar in both study sites, whereas pollen production had a markedly stronger effect in *MOR* (Fig. 3b and 3c). As illustrated in Fig. 3a, the effect of phenology was also different between the study sites, with different values of optimal asynchrony (*i.e.* optimal difference in the flowering onset between mother and father). In *MOR*, male fecundity was maximal for fathers starting to flower 0.36 days before mother plants, whereas in *PAL*, the estimated optimal asynchrony was 7.81 days. Non-CMS hermaphrodites were shown to perform better than restored CMS-hermaphrodites in

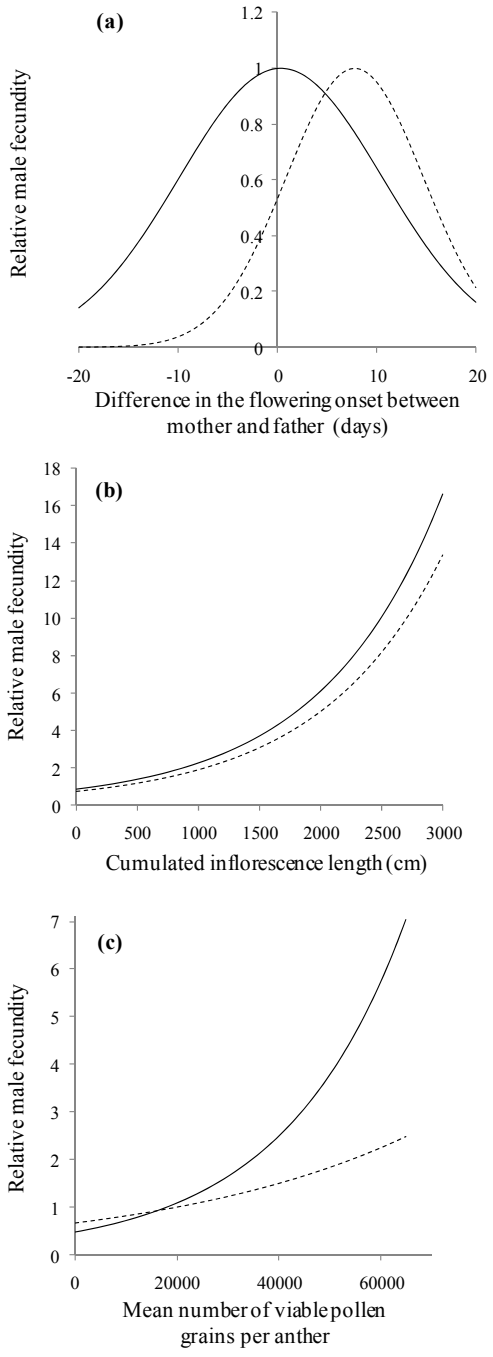

Fig. 3 Effects of flowering phenology (a), investment in reproduction (b) and pollen production (c) on the relative male fecundity, estimated using the spatially explicit mating models, in *MOR* (black line) and in *PAL* (dashed line)

both study sites. However, male fecundity of non-CMS hermaphrodites was significantly affected by the neighborhood type: non-CMS hermaphrodites that were growing in the vicinity of restored CMS-hermaphrodites (and thus potentially carrying silent restorer alleles) suffered a disadvantage compared to non-CMS hermaphrodites that had no restored CMS-hermaphrodites in their neighborhood (Table 5 and Fig. 4).

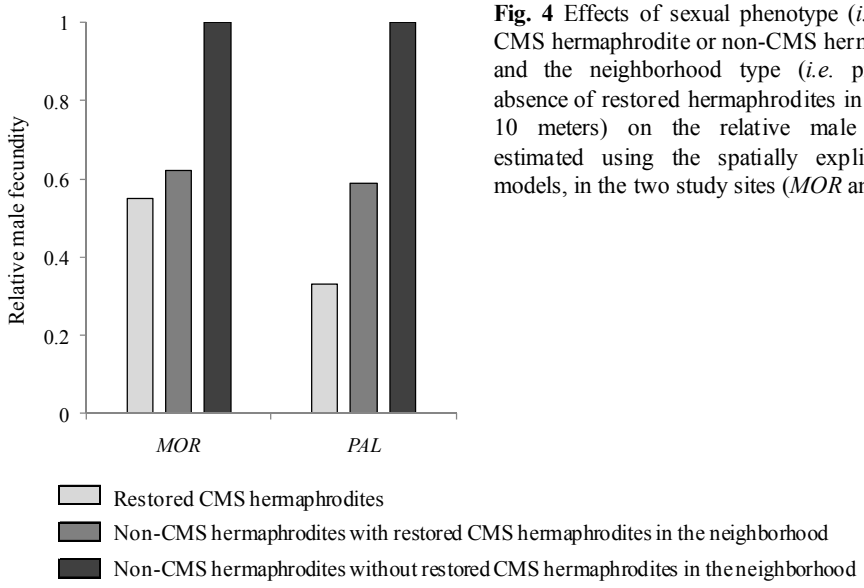

Fig. 4 Effects of sexual phenotype (*i.e.* restored CMS hermaphrodite or non-CMS hermaphrodite) and the neighborhood type (*i.e.* presence or absence of restored hermaphrodites in a radius of 10 meters) on the relative male fecundity, estimated using the spatially explicit mating models, in the two study sites (*MOR* and in *PAL*)

Selfing and immigration

The estimated immigration rate (*i.e*. proportion of seeds fathered by foreign pollen) was similar in both study sites (m = 0.12 and 0.08 in *MOR* and in *PAL*, respectively), suggesting low levels of incoming pollen flow at both sites. The estimated self pollination rates were very low and equivalent in both study sites (s = 0.02 in *MOR* and 0.03 in *PAL*). Despite the gametophytic self-incompatibility system, this confirmed that occasional selfing events in *B. vulgaris* natural populations, as also showed by patch-level estimates of selfing rate following the method of Ritland (2002), see above.

Table 5: Significance of the different tested effects taken into account in the spatially explicit models on mating probability, for the two study sites (*MOR* and *PAL*). logL are the log-likelihoods obtained for the complete model and for the models without each tested effect. ΔAIC (Akaike Information Criterion) describe the relative strength of the different tested effects. *P*-values were obtained with type III likelihood-ratio tests.

	(a) MOR				**(b) PAL**			
	df	-logL	ΔAIC	*P*	*df*	-logL	ΔAIC	*P*
Complete model	**19**	**13901**	-	-	**19**	**12774**	-	-
Distance	17	15530	3254	0.0000	17	14702	3852	0.0000
Geographical patch	16	13907	6	0.0074	16	12794	34	0.0000
Sexual phenotype * Neighborhood type	15	13907	4	0.0174	15	12793	30	0.0000
Investment in reproduction	17	13958	110	0.0000	17	12852	152	0.0000
Pollen production	12	13965	114	0.0000	12	12807	52	0.0000
Phenology	17	13906	6	0.0067	17	12821	90	0.0000

DISCUSSION

The characterization of mating patterns is a central issue in population genetics. Indeed, the evolutionary dynamics of natural populations depend critically on the relative proportion of short and long distance dispersal, as well as on the level of reproductive variance among individuals, which both affect the effective population size and the intensity of genetic drift. Using an approach related to the neighborhood model (Adams & Birkes, 1991; Burczyk *et al.*, 2004; Oddou-Muratorio *et al.*, 2005) in two study sites of the gynodioecious *B. vulgaris* ssp. *maritima*, we investigated whether genotypic and phenotypic differences among hermaphrodites translate into male reproductive success differences, while taking into account the shape and scale of pollen dispersal and the geographical position of individuals. This allowed us (i) to gauge the probability of long distance pollen dispersal, and thus the potential ability of restorer alleles to travel across the landscape, (ii) to explore whether the genotypic differences (restored CMS hermaphrodites *vs*. non-CMS hermaphrodites) and phenotypic differences (pollen production, investment in reproduction and phenology) among potential fathers translate into effective differential male reproductive output and (iii), to verify the existence of a cost of restoration and to assess its magnitude, by comparing non-CMS hermaphrodites that are likely to carry silent restorers and non-CMS hermaphrodites that are unlikely to carry them.

Dispersal capabilities and mating system

Gene dispersal, through seed and pollen flow, is a key parameter in shaping the dynamics and evolution of plant populations, as they affect genetic exchanges among individuals and among demes (Levin, 1981; Loveless & Hamrick, 1984; Hamrick & Nason, 1996; Sork & Smouse, 2006). Using the genotypes of all potential fathers and of a set of progeny arrays, we assessed the effect of physical distance on mating probabilities by estimating the shape and scale of pollen dispersal in *B. vulgaris*. Our results suggest that mating distances are highly variable in natural population of *B. vulgaris*, with most pollination events occurring at restricted distances, but a non-negligible proportion of long distance pollen flow. Consistently with this predominance of short distance reproduction, the models yielded low estimates of mean pollen dispersal distances (δ = 22.52 m and 6.55 m in *MOR* and in *PAL*, respectively) and suggested limited levels of pollen flow coming from outside the study sites (m = 0.12 and 0.08 in *MOR* and in *PAL*, respectively). This result was quite unexpected in a wind-pollinated species. The slightly lower levels of incoming pollen flow in *PAL* could be due to a higher spatial isolation from surrounding populations. Indeed, both sides of the *PAL* site were constituted of unfavorable cliff habitats on several kilometers, whereas we found three small populations (< 50 individuals) located between 1 and 1.7 kilometers away from *MOR*. Our results also suggest different patterns of long distance pollen flow. In *MOR* the best fit was obtained for the fat tailed exponential power kernel, whereas in *PAL*, the data were best explained by the logistic kernel, with a rapid decrease of the dispersal function, implying less long-distance dispersal events than what was observed in *MOR*. Although there were no obvious

physical barriers to pollen flow in the two study sites, these differences may arise from contrasted levels of exposure to the pollen dispersal vector, the wind. Although fat tailed dispersal kernels are increasingly considered as a common feature in natural plant populations (e.g. Hardy *et al.*, 2004; Devaux *et al.*, 2005; Oddou-Muratorio *et al.*, 2005; Gérard *et al.*, 2006; Goto *et al.*, 2006), this study illustrates that the shape of pollen dispersal can vary among localities.

Overall, despite the fact that *B. vulgaris* is a self incompatible, wind-pollinated species found in open and windy habitats, features that are all expected to favor extensive pollen flow, pollen dispersal thus appeared to be strongly skewed toward short distances, confirming the results of previous studies (Fénart *et al.*, 2007; De Cauwer *et al.*, 2010b). Wind pollination is thought to have evolved primarily as a mechanism of reproductive assurance (Culley *et al.*, 2002), and pollen from wind-pollinated plants has traditionally been assumed to be abundant (Cruden, 2000) and to travel long distances (Loveless & Hamrick, 1984). Although the first statement is validated in *B. vulgaris* (see Dufay *et al.*, 2008; De Cauwer *et al.*, in prep.-a), with pollen-ovule ratios that are largely superior to what is observed on average in entomophilous species (Cruden, 2000), the second assumption does not seem to be true in our study species. This could be due to the plant morphology and to the spatial arrangement of individuals in *B. vulgaris* populations. Indeed, each individual can carry up to several hundred floral stems, and, because individuals form dense patches along the shore, the floral stems of neighboring individuals are often intertwined, which may promote spatially restricted dispersal distances. In addition, high densities were shown to decrease the levels of biparental inbreeding and to increase the number of fathers participating to a local pollen pool in our study sites, which confirms that spatial arrangement of flowering plants strongly impacts pollen dispersal. High population densities seem to restrict pollination distances and to promote reproductive events between diverse mates.

Altogether, this results help understanding the important levels of spatial genetic structure that are classically observed in *B. vulgaris* at restricted spatial scales (e.g. Laporte *et al.*, 2001), as well as the low estimates of effective population sizes relative to census sizes that were previously reported in the same sites that are used in the current study (De Cauwer *et al.*, in prep.-b).

Heterogeneity in male fertility

In addition to the strong effect of physical distance between plants, we were also able to identify several phenotypic and genotypic factors influencing male mating success in gynodioecious *B. vulgaris*. Our results were consistent among the two study sites and suggested that (i) hermaphrodites with the higher levels of investment in reproduction (*i.e.* cumulated inflorescence length) performed significantly better than hermaphrodites exhibiting moderate flowering, (ii) male fertility increased significantly with the mean number of viable pollen grains per anther, (iii) male fertility was maximal for fathers starting to flower before mother plants, (iv) non-CMS hermaphrodites performed significantly better than restored CMS hermaphrodites and (v) non-CMS hermaphrodites found in the close vicinity of restored CMS individuals were

disadvantaged compared to non-CMS hermaphrodites found in neighborhoods where restored CMS individuals were absent.

Sexual phenotype is known to affect pollen production in *B. vulgaris*. Indeed, previous studies of pollen production suggested that restored CMS individuals were on average poor pollen producers compared to non-CMS hermaphrodites (Dufay *et al.*, 2008; De Cauwer *et al.*, in prep.-a). This was also the case in the present study, as illustrated by the significant differences in terms of number of viable pollen grains that were produced by the two distinct hermaphroditic types. In all of these studies, differences in pollen quality between the two categories of hermaphrodites resulted from a higher variance among CMS restored hermaphrodites, with some of these individuals producing pollen of very low quality while some others produced pollen equivalent to non-CMS hermaphrodites. Such a quantitative variation of pollen quality produced by restored CMS-hermaphrodites is thought to be the result of a polygenic determination of restoration. Indeed, complex restoration patterns are likely to decrease the probability to find fully restored hermaphrodites in natural populations (Charlesworth & Laporte, 1998; Koelewijn, 2003; Ehlers *et al.*, 2005). As shown by paternity analyses in the current study, pollen quality directly affected the relative fertility of individuals. The fact that we found a significant effect of sexual phenotype (*i.e.* non-CMS hermaphrodite or restored CMS hermaphrodites) while pollen quality was specifically taken into account in the models suggests that the significant difference in terms of pollen quality is not the only factor explaining the differences between the two hermaphroditic types. Further experiments, testing for example for the germination ability of large (viable) pollen grains, are needed to complete the comparison of the two hermaphroditic types.

While female advantage in seed fitness has been confirmed in numerous studies (reviewed in Shykoff *et al.*, 2003), empirical evidences of a cost of restoration on individual fitness remain scarce (see however de Haan *et al.*, 1997a; Bailey, 2002; Dufay *et al.*, 2008; del Castillo & Trujillo, 2009). Theoretical models suggest that the cost of restoration may affect either female or male fitness, but, due to physiological constraints, the most likely source of cost is probably related to male fitness components in hermaphrodites (Bailey, 2002). Because pollen dispersal is clearly spatially restricted in *B. vulgaris* (see also Fénart *et al.*, 2007; De Cauwer *et al.*, 2010a), the presence or absence of restored CMS hermaphrodites may be a good estimator of the probability to find restorer alleles in a given neighborhood. In our study, male fertility of non-CMS hermaphrodites was clearly affected by the presence of restored CMS hermaphrodites in the vicinity. Non-CMS hermaphrodites that were likely to carry silent restorers were significantly disadvantaged compared to non-CMS hermaphrodites that had no restored CMS hermaphrodites in their vicinity, with a male fertility loss that was on average 38% in *MOR* and 41% in *PAL*. As this trend was observed in both study sites, these results clearly indicate a silent cost of restorers. The various genotypes occurring in gynodioecious populations thus vary in their ability to transmit their genes to the next generation, which must directly affect the maintenance and the dynamics of gynodioecy.

Dynamics of gynodioecy

Evolutionary biologists have long been interested in explaining how, in gynodioecious species, females which lack the pollen production function can successfully be maintained in natural populations containing hermaphrodites that gain fitness through both seed and pollen production (Lewis, 1941). In gynodioecious species, sex determination generally results from interactions between cytoplasmic male sterility genes (CMS genes) and nuclear genes that counteract the action of CMS genes and restore male function (Saumitou-Laprade *et al.*, 1994). The maintenance of females in natural populations thus relies on the maintenance of a polymorphism at both cytoplasmic and nuclear sex determining genes. Theory has shown that this can only be achieved when females show higher seed fitness compared to hermaphrodites (a female advantage) and when there is a force opposing to the fixation of restorer alleles (like a cost of restoration, see Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufay *et al.*, 2007). The effect of the presence of females on the effective population size N_e (i.e. the size of an ideal population that has the same rate of change of allele frequencies or heterozygosity as the observed population, Fisher, 1930) can have various consequences depending on the nature of mating system. In self-compatible species, the occurrence of females could increase N_e because their presence promotes outcrossing (Gouyon & Couvet, 1987). In self-incompatible species, like *B. vulgaris*, high frequencies of females could result in a reduction of N_e because of an increase of inbreeding, due to the reduced amount of pollen donors (McCauley & Brock, 1998) and because the maintenance of females implies a variance of fitness between sexual phenotypes, considering either cytoplasmic genes (female advantage) or nuclear genes (cost of restoration) (Laporte *et al.*, 2001). Although female advantage is known to by very low in *B. vulgaris* (De Cauwer *et al.*, in prep.-a), the results of the current study suggest that male reproductive output is highly variable, and these important variations are partly explained by (i) the sexual phenotype of hermaphrodites (*i.e.* non CMS hermaphrodite and restored CMS hermaphrodite) and (ii) the possible existence of a silent cost of restoration. This variance in male fertility, associated with spatially restricted pollen flow, could be responsible for the low numbers of effective pollen donors (N_{ep}) observed within the different geographical patches and could result in restricted effective population sizes.

Altogether, local dispersion and uneven male fertilities among hermaphroditic individuals are thus expected to result in a reduction of effective population size, which could in turn affect the dynamics of gynodioecy through the effects of genetic drift. In their theoretical model exploring the conditions of maintenance of cytonuclear polymorphism in a subdivided population, Dufay & Pannell (2010) showed that, while gynodioecy is systematically lost under drift alone, seed and pollen dispersal could maintain cytonuclear gynodioecy. Although our study demonstrates that pollen dispersal is strongly skewed towards short distances, we also found a non-negligible proportion of long distance pollen flow (over several hundred meters). This could allow the spread of restorer alleles between adjacent geographical patches. The fate of a restorer allele in one particular location will then depend critically on the genetic composition in the neighborhood. On one hand, if the restorer arrives in a locality where the matching CMS genes are absent,

individuals carrying the restorer will probably suffer a silent cost of restoration, as suggested by the current study. In this situation, restorer alleles should then be rapidly eliminated by selective processes. On the other hand, if the restorer arrives in a deme where females carrying the matching CMS gene predominate, restored CMS hermaphrodites will be associated with the limiting gamete, pollen. In this case, restorer alleles should be strongly selected for because of reduced competition among hermaphrodites, as showed by De Cauwer *et al.* (2010b). This situation exemplifies a frequency-dependent selection process, a general selective mechanism driving the evolution of various breeding systems in flowering plants (Barrett, 2002; Castric & Vekemans, 2004). Finally, the occurrence of a silent cost of restoration on male fertility along with the differential reproductive success among CMS and non-CMS hermaphrodites supports the theoretical predictions that, although being a peculiar sexual polymorphism, females can be maintained and gynodioecy can be a stable polymorphic sexual system in wild populations.

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SYNTHESE GENERALE

Photo : Hampe florale de *Beta vulgaris* ssp. *maritima*, marquée et suivie pour l'analyse de paternité effectuée sur le site d'Audresselles (Nord-Pas-de-Calais, France), *cf.* Chapitre III, partie 2.

Tableau 1 Synthèse des principaux résultats obtenus au cours de ce travail de thèse. F : individu exprimant un phénotype femelle (*i.e.* individu portant un cytoplasme stérilisant (CMS) sans les allèles nucléaires de restauration de la fonction mâle), H_{CMS} : individu hermaphrodite porteur d'un cytoplasme CMS et restauré pour la fonction mâle, H_{NCMS} : individu hermaphrodite et non-porteur de CMS.

		Principaux résultats
CHAPITRE I Phénotype sexuel et succès reproducteur individuel		• F, H_{CMS} et H_{NCMS} ne diffèrent pas en termes de survie (à l'échelle de l'étude, *i.e.* deux ans) • F, H_{CMS} et H_{NCMS} ne diffèrent pas en termes de phénologie de floraison (début de floraison et durée totale de la floraison) • Existence d'un avantage femelle faible, essentiellement lié à une plus grande production de fleurs • Existence d'un coût de la CMS faible, associé au taux de mise à fruit et uniquement sur l'une des deux années de l'étude • Le nombre de grains de pollen viables produit est plus variable entre H_{CMS} qu'entre H_{NCMS} • Les H_{CMS} produisent, en moyenne, un moins grand nombre de grains de pollen viables que les H_{NCMS} • La variabilité intra individuelle du nombre de grains de pollen viables produits est plus forte chez les H_{CMS} que chez les H_{NCMS}
CHAPITRE II Structure génétique spatiale et temporelle	*Partie 1*	• Le niveau de différentiation entre dèmes séparés de quelques dizaines de mètres est similaire à ce qui est classiquement observé sur des échelles régionales • La fréquence des gènes liés au déterminisme du sexe varie de façon importante à une échelle très locale, ce qui engendre une forte structure des sexes dans l'espace • Les flux de gènes entre dèmes adjacents sont très limités, avec des flux de pollen qui prédominent largement sur les flux de graines • La très forte structure spatiale observée semble être le résultat d'une migration limitée et d'évènements de fondations indépendants
	Partie 2	• Les niveaux de diversité nucléaire et cytoplasmique sont similaires entre adultes et descendants issus de la banque de graine • La fréquence des CMS est également relativement stable au cours du temps, mais de nouvelles CMS peuvent apparaître de façon sporadique • Les estimations de la taille efficace des populations sont systématiquement faibles par rapport aux tailles de population mesurées
CHAPITRE III Interactions entre la structure génétique spatiale et le succès reproducteur	*Partie 1*	• Les taux de mise à fruits et de mise à graines dans les voisinages biaisés en faveur des femelles sont plus faibles que dans les voisinages biaisés en faveur des hermaphrodites • Ces résultats, obtenus sur deux populations naturelles, sont aussi valables pour des individus transplantés en population naturelle • Les individus qui souffrent de limitation pollinique ne semblent pas réallouer de ressources vers la production de descendants de meilleure qualité
	Partie 2	• Du fait de la forte structure des cytoplasmes dans l'espace, les H_{CMS} peuvent se retrouver plus souvent au voisinage de femelles que les H_{NCMS} • Malgré leur moins bonne production de pollen, les H_{CMS} montrent un meilleur succès reproducteur effectif que les H_{NCMS} quand ils profitent, comme dans cette étude, d'un avantage spatial, du fait de la proximité immédiate de femelles
	Partie 3	• Les distances moyennes de pollinisation sont faibles, le succès reproducteur mâle dépend donc fortement de la distance physique entre individus • En plus de cette composante spatiale, le succès reproducteur mâle dépend aussi de (i) la production de pollen, (ii) la taille des individus, (iii) la phénologie de floraison et (iv) du phénotype sexuel (H_{NCMS} *vs.* H_{CMS}) • Les H_{CMS} sont désavantagés quand ils ne bénéficient plus de l'avantage du rare mis en évidence dans la Partie 2 • Les allèles de restauration induisent un coût silencieux sur la fonction mâle chez les H_{NCMS}

L'objectif de ce travail de thèse était d'identifier quels processus pouvaient expliquer le maintien du polymorphisme cytonucléaire et les variations de sex ratio à fine échelle dans les populations gynodioïques de *Beta vulgaris* ssp. *maritima*. Pour cela, nous avons utilisé conjointement des outils de biologie des populations et de génétique des populations pour (i) quantifier les variations de valeur sélective entre les différents phénotypes / génotypes qui coexistent dans les populations gynodioïques de *B. vulgaris* ssp. *maritima*, (ii) comprendre le fonctionnement des populations naturelles en étudiant la structure génétique spatiale et temporelle pour des marqueurs neutres ainsi que pour les gènes impliqués dans le déterminisme du sexe chez cette espèce et (iii) explorer comment la forte structure spatiale du sexe peut, à son tour, affecter la valeur sélective des différents phénotypes sexuels. L'ensemble des résultats obtenus dans les trois chapitres de ce manuscrit sont résumés dans le tableau 1, et discutés plus en détail dans cette synthèse générale.

I - VALEURS SELECTIVES DES DIFFERENTS GENOTYPES ET MAINTIEN DE LA GYNODIOECIE

L'avantage femelle

Un nombre important de modèles théoriques ont été construits pour comprendre les conditions du maintien des femelles dans les populations gynodioïques. Différentes hypothèses ont ainsi été testées, impliquant entre autres (i) le type de déterminisme génétique, qui peut être purement nucléaire, (Valdeyron *et al.*, 1973; Lloyd, 1975) ou cytonucléaire, avec des interactions entre gènes CMS et allèles nucléaires de restauration (Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufay *et al.*, 2007), (ii) le type de population considéré, avec soit une population panmictique de taille infinie (Gouyon *et al.*, 1991), soit l'existence de sous-unités structurées dans l'espace (Couvet *et al.*, 1998; Dufay & Pannell, 2010), (iii) le fait que tous les cytoplasmes peuvent être stérilisants (Gouyon *et al.*, 1991; Bailey *et al.*, 2003), ou une partie de la diversité cytoplasmique peut correspondre à des cytoplasmes mâle-fertiles (Dufay *et al.*, 2007), (iv) le mode d'expression du coût de la restauration, qui peut être constitutif, silencieux ou exprimé (Bailey *et al.*, 2003; Dufay *et al.*, 2007) et (v) la possibilité d'avoir une limitation pollinique du succès reproducteur femelle dans les dèmes où le sex ratio est biaisé en faveur des femelles (McCauley & Taylor, 1997).

Malgré la grande variété de possibilités testées, une condition apparait strictement nécessaire pour maintenir des femelles en populations naturelles dans toutes les situations

biologiques : l'existence d'un avantage femelle. En d'autres termes, il faut que la valeur sélective des femelles *via* la fonction femelle soit plus importante que la valeur sélective des hermaphrodites *via* cette même fonction femelle. Cet avantage est alors quantifié de la façon suivante :

$$AF = \frac{\omega_{S\ Femelle}}{\omega_{S\ Hermaphrodites}}$$

où ω_S représente la valeur sélective *via* la fonction femelle.

Dans le premier chapitre de cette thèse, plusieurs paramètres potentiellement liés à la fonction femelle ont ainsi été mesurés en conditions contrôlées sur des individus femelles et des individus hermaphrodites (nombre de fleurs, nombre de paquets de fleurs, taux de mise à fruits, taux de germination), ce qui a permis de trouver un avantage femelle global restreint et non-significatif (de l'ordre de 1.15, Chapitre I). En outre, l'avantage femelle détecté reposait principalement sur une production de fleurs légèrement plus élevée chez les femelles que chez les hermaphrodites, les autres traits n'étant pas différents entre phénotypes sexuels. Ces résultats posent la question du maintien des femelles en populations naturelles chez *B. vulgaris* ; en effet, parmi les autres espèces présentant une gynodioécie cytonucléaire, il est fréquent de trouver un avantage significatif sur plusieurs traits liés à la valeur sélective femelle (revue dans Shykoff *et al.*, 2003; Dufay & Billard, soumis). Toutefois, un avantage femelle de l'ordre de celui que nous avons mesuré peut théoriquement permettre le maintien du polymorphisme cytonucléaire associé à la gynodioécie (Encadré I).

Le coût de la restauration

En plus d'un avantage femelle, le maintien du polymorphisme cytonucléaire nécessite l'existence d'une force qui empêche la fixation des allèles de restauration dans les populations, telle qu'un coût de la restauration (*p.e.* Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufay *et al.*, 2007). Dans ce contexte, l'un des résultats les plus intéressants de cette thèse est la confirmation de l'existence d'un tel coût sur la fonction mâle des hermaphrodites chez *B. vulgaris*. En effet, les H_{NCMS} localisés dans des dèmes où les H_{CMS} étaient présents (et donc potentiellement porteurs de restaurateurs) montrent, en moyenne, une fertilité mâle réduite par rapport aux H_{NCMS} localisés dans des dèmes où les H_{CMS} étaient absents (Chapitre III – Partie 3). Ceci nous a permis de quantifier grossièrement l'amplitude de ce coût silencieux chez les H_{NCMS}, en comparant l'estimation de la fertilité mâle obtenue pour les H_{NCMS} trouvés dans les deux types de voisinage (présence ou absence de H_{CMS}). Le coût silencieux serait alors de l'ordre de 0.4 (*i.e.* les H_{NCMS} vraisemblablement porteurs de restaurateur(s) ont une fertilité mâle réduite de 40% par rapport aux H_{NCMS} qui ne portent pas de restaurateurs). Cette estimation reste à nuancer car elle représente probablement une sous évaluation du coût réel. En effet, les H_{NCMS} localisés au voisinage de H_{CMS}

ne portent pas tous le, ou les, gène(s) de restauration de façon certaine. De plus, si cette observation exclut d'emblée l'hypothèse d'un coût exprimé de la restauration (*i.e.* s'exprimant uniquement chez les hermaphrodites porteurs de la CMS restaurée), elle ne permet pas encore de trancher entre coût silencieux et coût constitutif. Par ailleurs, la forte variation de qualité de pollen entre les H_{CMS} (Chapitre I), sans doute expliquée par un déterminisme polygénique de la restauration aboutissant à la coexistence de H_{CMS} plus ou moins bien restaurés, laisse penser que le coût pourrait fonctionner comme un coût constitutif. Des analyses complémentaires sur les mêmes jeux de données sont actuellement en cours pour examiner le comportement des H_{CMS} dans les deux types d'environnements. Il serait notamment intéressant de voir si la fréquence d'H_{CMS} au voisinage d'un H_{CMS} donné affecte son succès reproducteur : sous l'hypothèse d'une restauration de type polygénique, on peut en effet s'attendre à ce que la capacité à produire du pollen soit dépendante du nombre d'allèles de restauration porté par un H_{CMS}, et donc associée à la fréquence d'H_{CMS} dans le voisinage immédiat. Par ailleurs, si le coût de la restauration semble maintenant avéré sur la fonction mâle, d'autres études sont nécessaires pour évaluer l'existence d'une action délétère des allèles de restauration sur la fonction femelle, même si cette voie semble être moins probable (*cf.* Bailey, 2002).

Au final, l'avantage femelle serait donc de l'ordre de 1.15 et le coût de la restauration de l'ordre de 0.4. Lorsque l'on se réfère aux résultats théoriques présentés dans l'Encadré I, ces valeurs de paramètres permettent un maintien du polymorphisme cytonucléaire, dans le cas d'une population panmictique de taille infinie. Dans le chapitre II, dédié à la description de la distribution de la diversité génétique dans l'espace, nos résultats suggèrent au contraire l'existence d'une forte structure génétique, chacun des sites d'étude correspondant à une mosaïque de dèmes génétiquement différenciés. De plus, les estimations de la taille efficace des dèmes étudiés sont systématiquement beaucoup plus faibles que les tailles de population mesurées (*i.e.* nombre d'individus observés). Dans l'ensemble, le fonctionnement des populations naturelles de *B. vulgaris* semble être très éloigné de la panmixie, ce qui devrait favoriser l'action de la dérive génétique et modifier les conditions du maintien du polymorphisme cytonucléaire. L'approche théorique développée par Dufay & Pannell (2010) suggère par exemple que dans une population structurée, l'association entre un avantage femelle limité (comme celui décrit chez *B. vulgaris*) et la dérive génétique peut empêcher la dynamique d'invasion des CMS dans les dèmes.

ENCADRÉ I : LE MAINTIEN DU POLYMORPHISME CYTONUCLÉAIRE

En termes de déterminisme de la gynodioécie, le modèle théorique proposé par Dufay *et al.* (2007) est celui qui se rapproche le plus ce qui est observé chez l'espèce étudiée, *B vulgaris* ssp. *maritima* : des cytoplasmes non-stérilisants coexistent avec des gènes cytoplasmiques de stérilité mâle (CMS) et les individus porteurs de CMS peuvent être restaurés pour la fonction mâle s'ils portent les allèles nucléaires de restauration adéquats.

Si l'on se reporte aux valeurs d'avantage femelle et de coût de la restauration obtenus dans ce travail de thèse pour *B. vulgaris*, il semble théoriquement possible de maintenir le polymorphisme cytonucléaire associé à la gynodioécie (voir Fig. I-1). Toutefois, ce modèle examine les conditions de maintien des femelles dans le cas d'une population panmictique de taille infinie, et cette hypothèse est loin d'être respectée en population naturelle. Les effets potentiel de la structure génétique sur le maintien de la gynodioécie sont discutés plus en détails dans la suite de la synthèse générale.

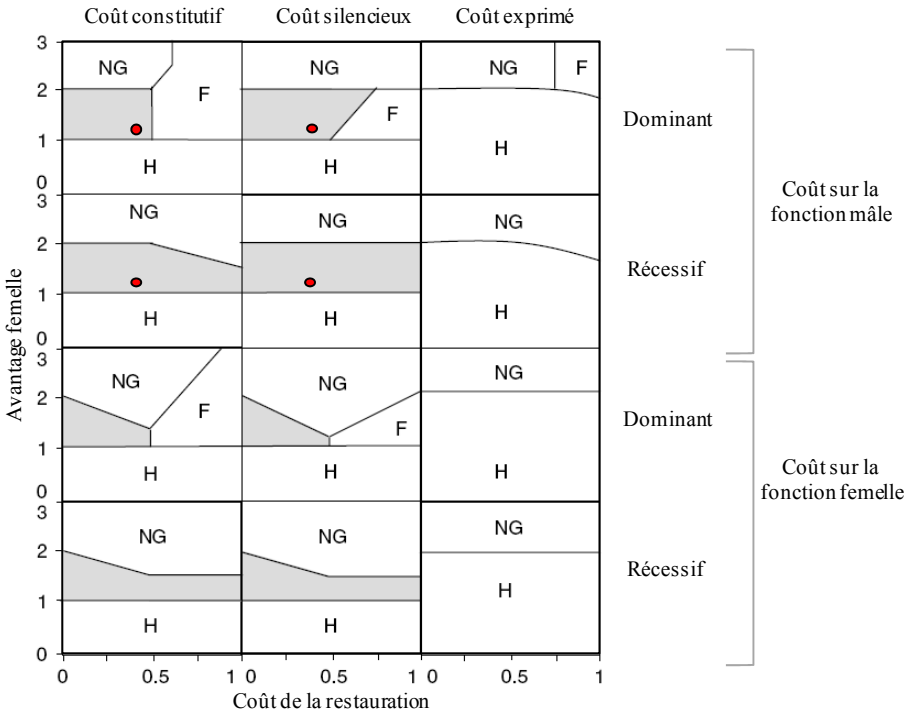

Fig. I-1 Etat de la population en fonction des valeurs de paramètres choisies pour l'avantage femelle et le coût de la restauration. Le coût de la restauration peut (i) être constitutif, silencieux ou exprimé, (ii) porter soit sur la fonction mâle, soit sur la fonction femelle et (iii) être dominant ou récessif. Quatre résultats distincts peuvent être observés :
• *H* : réversion vers l'hermaphrodisme (*i.e* les cytoplasmes CMS ne sont pas maintenus),
• *NG* : gynodioécie nucléaire (*i.e.* les cytoplasmes CMS arrivent à fixation et le polymorphisme est maintenu au locus nucléaire de restauration),
• *F* : la population finale n'est composée que de femelles,
• le polymorphisme cytonucléaire est maintenu (zones grisées)
Les points rouges indiquent les valeurs obtenues dans ce travail de thèse (avantage femelle, $AF = 1.15$, coût de la restauration, $c = 0.40$).

Figure tirée de Dufaÿ *et al.* (2007)

II - STRUCTURE GENETIQUE ET FONCTIONNEMENT DES POPULATIONS NATURELLES DE B. VULGARIS

Quelques rappels théoriques…

Dans la nature, les espèces végétales sont généralement divisées en populations spatialement discrètes, échangeant de gènes *via* des flux de pollen et de graines (*e.g.* Giles & Goudet, 1997; Tero *et al.*, 2003; Honnay *et al.*, 2009). Si ces flux de gènes sont suffisamment restreints, la dérive génétique pourra modifier les fréquences alléliques locales, ce qui engendrera la mise en place d'une structure génétique spatiale (Encadré II). L'échelle spatiale à laquelle ce phénomène sera observé dépendra alors des capacités de dispersion de l'espèce considérée (Slatkin, 1985), ainsi que des particularités de l'habitat (Storfer *et al.*, 2007). Chez les angiospermes, deux vecteurs principaux permettent ainsi la dispersion des gènes dans l'espace : le pollen et les graines. Ces deux vecteurs n'étant classiquement pas dispersés de la même façon, une asymétrie importante a souvent été documentée entre les patrons de migration observés pour les gènes cytoplasmiques, véhiculés uniquement par les graines du fait de leur transmission maternelle, et les patrons de migration observés pour les gènes nucléaires, dispersés par les graines et par le pollen (Petit *et al.*, 2005).

Au sein d'un ensemble de populations structurées, la répartition de la diversité génétique et les niveaux de différentiation génétique entre populations pourront également être modifiés par un fonctionnement en métapopulation, avec des évènements récurrents d'extinction et de recolonisation des taches d'habitat favorables (Wade & McCauley, 1988; Whitlock & McCauley, 1990 ; Pannell & Charlesworth, 1999). La métapopulation dans son ensemble pourra alors perdurer sur des fenêtres de temps beaucoup plus importantes que chaque population locale prise séparément, pourvu que les taux de colonisation soient suffisants (Hanski, 1999). L'impact de ce type de fonctionnement sur la structure des populations dépend de façon critique des propriétés des colonisateurs (*i.e.* des graines qui fondent une nouvelle population), en particulier de leur nombre et de leur origine, ainsi que du nombre relatif de colonisateurs et de migrants échangés entre populations. Selon Whitlock & McCauley (1990), la différentiation entre populations locales augmentera par rapport à un modèle en île classique si :

$$k < 2N_m / (1-\varphi) + 0.5$$

où k représente le nombre de colonisateurs, N_m le nombre de migrants et φ la probabilité que deux colonisateurs proviennent de la même population. Lorsque $\varphi = 1$ (tous les colonisateurs proviennent de la même population, modèle dit de « propagule pool », Slatkin, 1977), les évènements de colonisation augmenteront alors systématiquement le niveau de différentiation entre population locales. En revanche, lorsque $\varphi = 0$ (tous les colonisateurs proviennent de populations différentes, modèle dit de « migrant pool »), le niveau de différentiation entre populations locales augmentera par rapport à un modèle sans extinction et recolonisation uniquement si le nombre de colonisateurs est faible par rapport au nombre de migrants. A

l'inverse, si les colonisateurs sont nombreux, le niveau de différentiation entre populations locales diminuera par rapport à un modèle sans extinction et recolonisation (Wade & McCauley, 1988; Whitlock & McCauley, 1990).

Les différents modèles présentés dans le paragraphe précédent sont nécessairement des caricatures du fonctionnement réel des populations naturelles, la réalité correspondant probablement le plus souvent à des situations intermédiaires. De plus, du fait de la dynamique extrêmement lente de colonisation et d'extinction chez la plupart des espèces végétales, la mise en évidence concrète d'un fonctionnement en métapopulation demanderait le suivi de nombreuses localités sur des fenêtres de temps importantes, et de fait, les exemples de métapopulations végétales restent rares dans la littérature (synthétisé dans Freckleton & Watkinson, 2002). Ce cadre théorique permet toutefois de discuter les résultats obtenus au cours de cette thèse et de faire des hypothèses sur le fonctionnement des populations naturelles de *B. vulgaris*.

Fonctionnement des populations chez *B. vulgaris*

Une caractéristique commune à tous les sites d'études considérés au cours de ce travail de thèse était la très forte structure à échelle locale (Chapitre II – Partie 1). Des dèmes adjacents (*i.e.* séparés par quelques dizaines de mètres) présentaient des niveaux de différenciation nucléaires très similaires à ce qui avait été observé récemment sur une échelle régionale (*i.e.* sur plusieurs centaines de kilomètres, Fievet *et al.*, 2007). En ce qui concerne les marqueurs cytoplasmiques, les niveaux de différentiation observés à échelle locale étaient même notablement supérieurs à ce qui avait été décrit à échelle régionale. Outre le fait de confirmer la pertinence de l'échelle extrêmement locale considérée tout au long de cette étude, cette observation permet d'emblée d'exclure un fonctionnement panmictique au sein des sites considérés (Encadré II, Fig. II-1a).

Cette observation, qui suggère des échanges de gènes limités entre dèmes séparés par quelques dizaines de mètres, pouvait sembler particulièrement surprenante au premier abord. Nos résultats suggèrent cependant (i) une prédominance forte des évènements de pollinisation à faible distance, (ii) des flux de graines encore bien plus restreints et (iii) que les rares flux de gènes inter-dèmes se font essentiellement entre dèmes adjacents. Les échanges sont donc possibles, mais restent limités (excluant donc l'hypothèse b de l'Encadré II). De plus, au sein de chacun des sites d'étude, le contenu en haplotypes cytoplasmiques était extrêmement variable d'un dème à l'autre. Le fait que les niveaux de différenciation cytoplasmique entre dèmes d'un même site d'étude étaient plus forts à petite échelle qu'à échelle régionale (Fievet *et al.*, 2007) suggère que les évènements de fondations ont un impact plus important que la migration entre dèmes établis, au moins pour les gènes cytoplasmiques.

L'estimation précise des taux d'extinctions et de recolonisations demanderait le suivi de nombreuses populations pendant de longues fenêtres de temps, mais ce type d'évènement semble assez fréquent et a souvent été observé en populations naturelles, du fait de la nature extrêmement

perturbée de l'habitat. Dans le cadre de cette thèse, les deux sites localisés au Palus et au Morvan ont été sujets à de fortes tempêtes hivernales en 2006-2007. Une mortalité importante des adultes, entrainant l'extinction de l'un des dèmes, a été observée lors de la campagne de terrain qui a eu lieu le printemps suivant. Les dèmes au sein des sites étudiés semblent donc bien soumis à des extinctions locales.

Dans le cas des populations naturelles de *B. vulgaris*, à une échelle locale comme celle considérée tout au long de cette étude, la dynamique de populations s'apparenterait donc à un fonctionnement intermédiaire entre (i) des échanges de proches en proches, comme attendus sous un modèle en stepping-stone (Encadré II, Fig. II-1c), et (ii) un système en métapopulation classique, où des extinctions locales de dèmes peuvent survenir (Encadré II, Fig. II-1e). Toutefois, du fait de l'existence de banques de graines, l'observation d'un évènement de recolonisation peut correspondre à deux situations distinctes, qui auront potentiellement des effets très différents sur la répartition de la diversité au sein de la métapopulation : (i) les nouveaux arrivants sont issus d'évènement de migration, ou (ii) les nouveaux arrivants sont issus de graines stockées dans le sol. Étant donné que chaque individu peut produire plusieurs dizaines de milliers de graines, la question de la durée de vie d'une graine dans le sol devient alors cruciale, et ce paramètre reste méconnu chez la *B. vulgaris.*

Sur une échelle plus régionale, il semble que les échanges de gènes entre populations s'établissent principalement au travers de flux de pollen suivant un modèle classique d'isolement par la distance, où la dispersion du pollen est contrainte par le trait de côte (Fievet *et al*., 2007). La diversité cytoplasmique observée suggère en revanche des événements de colonisation n'impliquant pas forcément de contrainte spatiale dans la dispersion, la répartition des haplotypes ne suivant pas un arrangement géographique clair.

Au final, les effets additionnés de la dérive génétique, liée aux évènements de fondation et à la taille efficace réduite des dèmes établis (Chapitre II – Partie 2), et des flux de gènes restreints dans l'espace ont clairement un impact important sur les fréquences locales des gènes associés au déterminisme sexuel chez *B. vulgaris*. Dans cette thèse, l'importante structure spatiale observée à la fois pour des marqueurs cytoplasmiques et pour des marqueurs nucléaires s'accompagnait en effet de fortes variations spatiales du sex ratio. Ces variations peuvent alors affecter en retour la valeur sélective des différents phénotypes sexuels. La dernière partie de cette synthèse résume nos observations sur l'impact de la structure spatiale des sexes sur le succès reproducteur individuel.

ENCADRÉ II : LES POPULATIONS STRUCTURÉES

Dans le cas particulier de populations arrangées de façon linéaire dans l'espace, comme c'est le cas chez *B. vulgaris* ssp. *maritima*, on peut, de façon très schématique, considérer cinq modèles de fonctionnement distincts (Fig.II-1). Dans les trois premiers modèles les populations sont pérennes (Fig.II-1a, b et c), alors que dans les deux derniers cas de figure, les populations peuvent être soumises de façon récurrente à des extinctions et à des recolonisations (Fig.II-1d et e).

Dans le premier cas (Fig.II-1a), malgré la nature apparemment discrète de l'habitat et des populations dans l'espace, les populations sont toutes connectées par des flux de gènes importants et aléatoires, formant un ensemble panmictique. Dans le deuxième cas (Fig.II-1b), l'habitat et les populations présentent également une distribution discrète dans l'espace, en revanche, les flux de gènes entre populations sont nuls. Le troisième modèle (Fig.II-1c) correspond à un fonctionnement en "stepping-stone", avec des flux de gènes qui se font préférentiellement entre populations adjacentes (Kimura & Weiss, 1964).

Du fait de la nature éphémère des populations, les deux derniers modèles correspondent à des fonctionnements en métapopulation. Dans le quatrième modèle (Fig.II-1d), des populations "sources", relativement pérennes et localisées dans des habitats de bonne qualité, exportent des migrants, qui sont reçus par des populations périphériques localisées dans des habitats moins favorables. Ces populations "puits" arriveraient à extinction définitive sans l'apport récurrent de migrants de la population "source". Enfin, le dernier modèle (Fig.II-1e) correspond à un modèle "classique" de métapopulation : toutes les populations sont éphémères et les migrants peuvent avoir des origines diverses.

On considère qu'un ensemble de populations structurées fonctionne en métapopulation « classique » si quatre conditions distinctes sont remplies : (i) les habitats adaptés sont spatialement discrets, (ii) toutes les populations peuvent être soumises à extinction, (iii) après une extinction locale, le site libéré peut être recolonisé et (iv) les populations ne s'éteignent pas toutes de façon synchrone (Hanski, 1999).

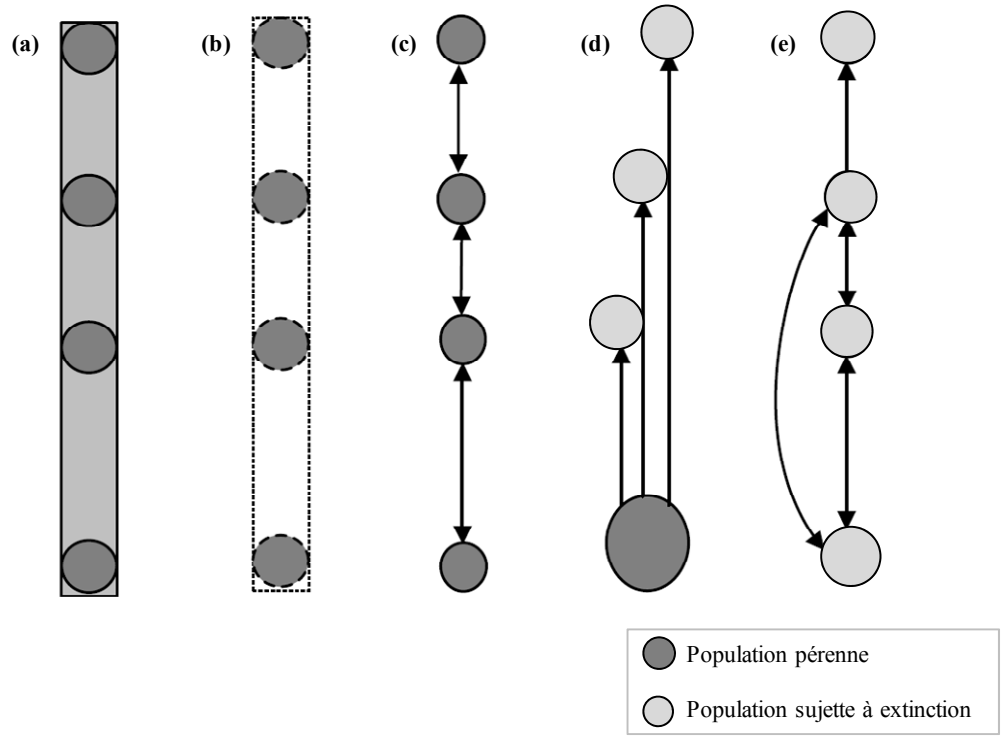

Fig. II-1 Représentation schématique des différents modèles de fonctionnement possibles dans le cas d'un arrangement linéaire des populations: (a) l'ensemble est une population panmictique (b) les populations sont génétiquement isolées, (c) fonctionnement en "stepping-stone", (d) métapopulation de type "source-puit", (e) fonctionnement en métapopulation classique

Figure inspirée de Tero *et al.* (2003)

III - LA GYNODIOECIE EN POPULATIONS STRUCTUREES

Une structure spatiale à fine échelle, comme celle mise en lumière dans ce travail de thèse, peut potentiellement avoir des effets variés sur la valeur sélective des individus. Par exemple, si les évènements de reproduction sont plus courants entre individus voisins qu'entre individus tirés au hasard, la structure génétique peut favoriser les phénomènes de dépression de consanguinité. Par ailleurs, le niveau de structure génétique spatiale et l'amplitude relative de la dispersion peuvent aussi influencer la dynamique de coévolution entre les plantes et leur pathogènes (Thrall & Burdon, 2002) ou entre les plantes et leurs mycorhizes mutualistes (Taylor *et al.*, 2004). Finalement, la structure peut aussi affecter la valeur sélective des individus, quand cette valeur sélective dépend du phénotype / génotype sexuel des individus dans le voisinage (*p.e.* McCauley & Brock, 1998; Wagenius *et al.*, 2007). Les espèces gynodioïques présentant typiquement de fortes variations de sex ratio à échelle locale, elles constituent de bons candidats pour examiner les effets de la sélection fréquence-dépendante sur les différents phénotypes en populations structurées.

Du point de vue des femelles

Classiquement, on considère que la valeur sélective femelle n'est pas limitée par la disponibilité des gamètes mâles, mais plutôt par la quantité de ressources et la qualité de l'environnement (Bateman, 1948). Dans les populations végétales, où les individus sont sessiles et ne peuvent par définition pas rechercher leurs partenaires de façon active, la disponibilité locale de pollen peut cependant varier avec le nombre et le type sexuel des individus dans un voisinage donné. Les exemples de limitation pollinique de la production de graines et de fruits abondent chez les espèces entomophiles (revue dans Burd, 1994; Larson & Barrett, 2000; Knight *et al.*, 2005) mais restent plutôt rares chez les anémophiles (voir toutefois Knapp *et al.*, 2001; Davis *et al.*, 2004). Chez les espèces à pollinisation entomophile, le ratio pollen sur ovules est généralement de quelques milliers de grains de pollen pour un ovule, tandis que chez les espèces à pollinisation anémophile, ce ratio atteint fréquemment plusieurs milliers pour un (Cruden, 1977; Cruden, 2000). Nos travaux montrent que ceci est effectivement le cas chez *B. vulgaris*. Les transitions évolutives vers l'anémophilie, associées à cette production importante de pollen, sont souvent présentées comme une adaptation permettant une certaine assurance reproductive (Barrett, 2010). Nos résultats montrent cependant que le succès reproducteur des individus femelles dépend de façon importante du sex ratio dans le voisinage immédiat et suggèrent que, du fait de la forte structure des sexes dans l'espace, des phénomènes de limitation pollinique sont possibles chez cette espèce (Chapitre III – Partie 1). Le faible avantage femelle décrit chez *B. vulgaris* pourrait donc rapidement être annulé en populations structurées (Tableau 2), dans les dèmes où celles-ci sont très fréquentes.

Pour étudier ce phénomène, nous avons délibérément choisi des dèmes où le sex ratio était soit très biaisé en faveur des femelles, soit très biaisé en faveur des hermaphrodites. Pour aller plus loin, il faudrait multiplier les points pour mettre en relief les processus intervenant dans des situations intermédiaires. Dans le cas le plus simple, la relation entre sex ratio local et succès reproducteur femelle pourrait être linéaire (*p.e.* McCauley, 1998). Il est toutefois également possible d'envisager des réponses plus complexes : la probabilité de mise à fruit peut par exemple dépendre de la quantité de pollen reçu (Mitchell, 1997), la limitation pollinique n'ayant alors lieu qu'à partir d'une valeur seuil de sex ratio. Par ailleurs, notre étude était fondée sur une dichotomie hermaphrodites / femelles qui reste sans doute très simplificatrice. Chez *B. vulgaris*, comme mentionné précédemment, on peut distinguer deux catégories d'hermaphrodites selon la nature du cytoplasme (H_{CMS} et H_{NCMS}). Étant donné qu'il est maintenant bien établi que ces deux catégories diffèrent en termes de qualité du pollen produit (*p.e*. Dufay *et al.*, 2008), il faudrait aussi explorer l'effet de la nature des hermaphrodites localement disponibles sur les phénomènes de limitation pollinique. L'idée sous-jacente est que, pour des sex ratio équivalents, les phénomènes de limitation polliniques pourraient être plus importants lorsque les pères potentiels dans le voisinage sont des H_{CMS}, contrairement au cas où ces pères potentiels seraient des H_{NCMS}. La question se complique encore si l'on se rappelle que plusieurs CMS différentes coexistent (et qu'elles ne sont potentiellement pas toutes restaurées avec la même efficacité), et que les H_{NCMS} peuvent aussi souffrir d'un coût de la restauration lorsqu'ils portent des restaurateurs silencieux.

Tableau 2 : Synthèse des valeurs sélectives associées au phénotype femelle et au phénotype hermaphrodite, du point de vue de gènes cytoplasmiques et de gènes nucléaires. Le nombre de signes « + » est proportionnel à la valeur sélective attendue. ω_{CF} : succès reproducteur d'une femelle, du point de vue des gènes cytoplasmiques, ω_{NF} : succès reproducteur d'une femelle, du point de vue des gènes nucléaires, ω_{CH} : succès reproducteur d'un hermaphrodite, du point de vue des gènes cytoplasmiques et ω_{NH} : succès reproducteur d'un hermaphrodite, du point de vue des gènes nucléaires.

	ω_{CF} (=ω_{NF})	ω_{CH}[1]	ω_{NH}[2]
Dème biaisé en faveur des hermaphrodites	++++ (Pas de limitation pollinique - Avantage femelle)	+++ (Pas d'avantage femelle)	++ (Compétition pollinique)
Dème biaisé en faveur des femelles	++ (Avantage femelle, mais limitation pollinique)	+ (Limitation pollinique)	++++ (Avantage du rare)

[1] : Indépendemment du voisinage considéré, ω_{CH} dépendra aussi du cytoplasme de l'hermaphrodite (sous l'hypothèse d'un coût de la CMS).

[2] : Indépendemment du voisinage considéré, ω_{NH} dépendra aussi du cytoplasme ($\omega_{NHNCM} > \omega_{NHCMS}$), ainsi que de la présence d'allèle de restauration silencieux chez les H_{NCMS}.

Du point de vue des hermaphrodites

Dans le cas d'une structure spatiale des phénotypes sexuels dans l'espace, les femelles se retrouvent le plus souvent regroupées entre elles et les hermaphrodites sont le plus souvent regroupés avec d'autres hermaphrodites. Lorsque l'on considère l'ensemble des dèmes, les effets néfastes de la limitation polliniques affecteront donc majoritairement les individus femelles (McCauley, 1997). Les espèces gynodioïques étant fréquemment auto-compatibles (Charlesworth, 1981), les hermaphrodites localisés dans les dèmes biaisés en faveur des femelles devraient en plus partiellement échapper à la limitation pollinique, ceci grâce à l'autofécondation (si on néglige les effets de dépression de consanguinité). Toutefois, *B. vulgaris* est une espèce autoincompatible et, en dépit du fait que nous n'ayons pas réalisé de mesures de limitation pollinique sur les hermaphrodites, on peut s'attendre à ce que la production de fruits / graines diminue pour les hermaphrodites localisés dans les dèmes biaisés en faveur des femelles (Tableau 2), bien que cette hypothèse reste à tester.

La reproduction *via* la voie femelle chez les hermaphrodites dépend donc aussi probablement du sex ratio local. En ce qui concerne la reproduction par la voie mâle chez les hermaphrodites, nos résultats montrent que le sex ratio local peut aussi avoir un impact important (Chapitre III – Partie 2). Ainsi, des fréquences localement importantes de femelles sont avantageuses pour les hermaphrodites présents, qui sont alors les seuls donneurs de pollen potentiels et bénéficient de « l'avantage du rare ». Du fait de la forte structuration spatiale des cytoplasmes, et donc des CMSs, cet avantage pourrait de surcroit bénéficier plus souvent aux H_{CMS} qu'aux H_{NCMS}. Par ailleurs, il est intéressant de noter que, pour un hermaphrodite donné, de fortes fréquences de femelles peuvent avoir des effets divergents : un effet négatif du point de vue de la valeur sélective femelle, et un effet positif du point de vue de la valeur sélective mâle.

Dans les situations où les hermaphrodites prédominent, le succès de pollinisation des hermaphrodites pourrait en revanche baisser du fait de la compétition pour l'accès aux ovules (Tableau 2). Dans ce cas de figure, le succès de pollinisation reposera certainement de façon importante sur le génotype des individus, qui, comme nous l'avons montré, peut directement affecter la fécondité réalisée des hermaphrodites. Ainsi, en cas de compétition pollinique liée à de fortes densités locales d'hermaphrodites, les H_{NCMS} non-porteurs de restaurateurs silencieux sont plus performants que les H_{NCMS} affectés par un coût silencieux de la restauration, qui sont eux même meilleurs que les H_{CMS} (Chapitre III – Partie 3).

Au final, nos résultats illustrent bien le fait que l'évolution des sex ratios en populations structurées chez les plantes gynodioïques est un processus très complexe, répondant à la fois à des forces sélectives s'exerçant à l'échelle des gènes (impliquant un conflit cytonucléaire), de l'individu / du génotype (*p.e.* coût silencieux de la restauration) et à l'échelle du dème (*p.e.* limitation pollinique). Ces processus sélectifs, fortement dépendants de la nature des individus au voisinage, sont probablement des phénomènes très généraux dans l'évolution des systèmes de

reproduction, et laissent bien évidemment une place à des processus non déterministes tel que l'effet du hasard au travers de la dérive génétique.

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ANNEXE I

S. FENART, J.-F. ARNAUD, I. DE CAUWER & J. CUGUEN (2008) Nuclear and cytoplasmic genetic diversity in weed beet and sugar beet accessions compared to wild relatives: new insights into the genetic relationships within the *Beta vulgaris* complex species. *Theoretical and Applied Genetics*, **116**: 1063-1077.

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ORIGINAL PAPER

Nuclear and cytoplasmic genetic diversity in weed beet and sugar beet accessions compared to wild relatives: new insights into the genetic relationships within the *Beta vulgaris* complex species

Stéphane Fénart · Jean-François Arnaud · Isabelle De Cauwer · Joël Cuguen

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Abstract Hybridization between cultivated species and their wild relatives is now widely considered to be common. In the *Beta vulgaris* complex, the sugar beet seed multiplication areas have been the scene of inadvertent pollination of sugar beet seed bearers by wild ruderal pollen donors, generating a weedy form of beet which infests sugar beet fields in European countries. Up to now, investigations of evolutionary dynamics of genetic diversity within the *B. vulgaris* complex were addressed using few genetical markers and few accessions. In this study, we tackled this issue using a panel of complementary markers: five nuclear microsatellite loci, four mitochondrial minisatellite loci and one chloroplastic PCR-RFLP marker. We sampled 1,640 individuals that illustrate the actual distribution of inland ruderal beets of South Western France, weed beets and wild sea beets of northern France as well as the diversity of 35 contemporary European diploid cultivars. Nuclear genetic diversity in weed beets appeared to be as high as those of ruderal beets and sea beets, whereas the narrowness of cultivar accessions was confirmed. This genetic bottleneck in cultivars is even more important in the cytoplasmic genome as only one haplotype was found among all sugar beet cultivars. The large majority of weed beet populations also presented this unique cytoplasmic haplotype, as expected owing to their maternal cultivated origin. Nonetheless, various cytoplasmic haplotypes were found within three populations of weed beets, implying wild-to-weed seed flows. Finally, our findings gave new insights into the genetical relationships between the components of the *B. vulgaris* complex: (1) we found a very strong genetic divergence between wild sea beet and other relatives, which was unexpected given the recent evolutionary history and the full cross-compatibility of all taxa and (2) we definitely confirmed that the classification into cultivated, wild, ruderal and weed forms according to their geographical location, phenotype or their domesticated status is clearly in accordance with genetic clustering despite the very recent domestication process of sugar beet.

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S. Fénart · J.-F. Arnaud · I. De Cauwer · J. Cuguen (✉)
Laboratoire de Génétique et Évolution des Populations Végétales, UMR CNRS 8016, Université des Sciences et Technologies de Lille, Lille 1, Bâtiment SN2, 59655 Villeneuve d'Ascq Cedex, France
e-mail: joel.cuguen@univ-lille1.fr

Introduction

Species complexes consist of clusters of closely related species or subspecies that are able to exchange genetic material in natural conditions (e.g. Pernès 1984; Coyne and Orr 2004). Species complexes are thus of great interest to understand hybridization, introgression or speciation processes related to different environmental features, spatial isolation or variations in life-history traits (e.g. Bowen et al. 2001; Jørgensen et al. 2002; Shaffer et al. 2004). The knowledge of the extent of genetic diversity and relationships within and among crop species and their wild relatives is also essential for the efficient use of plant genetic resource collections, in order to prevent wild populations from the introgression of characters from cultivated accessions, or to improve crop quality (Soleimani et al. 2002; Fernie et al. 2006; Mariac et al. 2006; Tani et al. 2006).

The major crop species have been generated by the assortment of characters selected from their wild relatives

during domestication processes (Olsen and Schaal 1999; Zohary and Hopf 2000; Goodrich and Wiener 2005; Zeder et al. 2006; Ross-Ibarra et al. 2007). Gene movements in crop–wild species complexes are not limited to the movements of genes from wild to crops through human selection, but introgression of cultivated traits into their wild relatives has clearly been documented in 12 of the 13 most important food crops in some parts of their agricultural distribution (Ellstrand et al. 1999; see also Stewart et al. 2003). Moreover, in seven cases out of 13, introgression of domesticated traits has had consequences on weed species by increasing their competitiveness (Ellstrand et al. 1999; Ellstrand and Schierenbeck 2000).

A recent and extensive essay on crop–wild interactions was devoted for a large part to the special case of the *Beta vulgaris* complex (Ellstrand 2003). This species complex is of particular interest since crop, wild and weed forms of *B. vulgaris* can be found in sympatric situations in Europe and are all interfertile (Santoni and Berville 1992; Boudry et al. 1993; Bartsch et al. 1999). According to their habitat, four types of beets can be distinguished within the *B. vulgaris* complex: (1) sea beets [*B. vulgaris* subsp. *maritima* (L.) Arcangeli] mostly found along the western European Mediterranean coastlines (Letschert 1993) and colonising areas located along estuaries, just at the upper level of the tide and, more rarely, cliffs overhanging the sea (Raybould et al. 1996; Laporte et al. 2001; Fievet et al. 2007); (2) cultivated beets which have been known for more than 2,000 years in the eastern Mediterranean regions (Ford-Lloyd and Williams 1975) and now including sugar beet, fodder beet, table beet and leaf beet. While sugar beet production fields are principally found in northern European countries, sugar beet seeds are mainly produced in South-Western France or Northern Italy (Bartsch et al. 1999). In seed production fields, two rows of pollen donors (classically tetraploid but increasingly diploid) frame four rows of diploid male-sterile seed bearers that are characterised by a particular cytoplasmic male sterility (CMS) only found in cultivars (the "OwenCMS", see Owen 1945), (3) weed beets, infesting some sugar-beet fields and leading to severe agronomic problems since the seventies (Horsney and Arnold 1979); (4) inland ruderal beets that are associated with disturbed man-made habitats and whose status is not clear, being either viewed as feral beets escaped from private gardens or cultivated fields and running wild, or typical wild beets originating from Mediterranean coast (Desplanque et al. 1999).

Gene flow between cultivated and wild relatives is likely to occur and has already been demonstrated by local studies either from wild-to-crop (Boudry et al. 1993; Desplanque et al. 1999) or from crop-to-wild (Bartsch et al. 1999), mainly in seed production areas. In the main European seed production area in south-western France, sugar beet seed bearers can be accidentally pollinated by ruderal pollen donors (Boudry et al. 1993; Desplanque et al. 1999). The ensuing crop–wild F1 hybrids are mixed with sugar beet seeds that are sown in growing fields in Northern France as well as in other European countries. While sugar beets are biennial, crop–wild F1 hybrids, considered as weeds, have inherited from their wild parents their early bolting ability and as a consequence can flower and reproduce during the crop season (Boudry et al. 1993, 1994; Van Dijk 2004). This unwanted recurrent hybridization is thus at the origin of weed beets, identified as the cause of serious agronomic problems for the last 30 years in Europe, with significant effects on sugar beet yield and quality (Longden 1989; Brants and Hermann 1998; Bartsch et al. 1999; Desplanque et al. 2002; Bartsch et al. 2003). This hybrid origin of weed beet has been firstly attested indirectly (i.e. not genetically) by their capacity to bolt without vernalization, revealing the presence of the dominant *B* allele, inherited from the ruderal parent and cancelling any cold requirement to bolt (Santoni and Berville 1992; Boudry et al. 1993). It has been shown that weed beets could act as an escape route for cultivated traits to wild beet populations (Arnaud et al. 2003; Cuguen et al. 2004; Viard et al. 2004) and that pollen flow between weed beet populations can occur over large distances (Fénart et al. 2007).

Despite the documented knowledge of each form of the *B. vulgaris* complex, only fragmented information is available about their genetical relationships and, as far as we are aware, only one study tackled this problem but with only few accessions and few genetic PCR–RFLP markers (Desplanque et al. 1999). In fact, comparison of the genetic diversity and its evolutionary dynamics among the different forms has never been performed over a large sample set with a sufficient number of highly polymorphic loci. In this respect, this study aimed at filling this lack and was based on the assessment of the nuclear and cytoplasmic genetic diversity found within the four forms of beet, each represented by a very large sample dataset. We thus investigated the genetic polymorphism of a large sample of (1) weed beet populations from different locations in the French and Belgian sugar beet production areas, (2) major accessions of the contemporary sugar beet cultivars, (3) representative populations of inland ruderal beets found within the French seed production area, in south-western France and (4) wild sea beet populations collected along 1,000 km coasts, from south Brittany to the North of France. All these individuals were characterized both at the cytoplasmic and nuclear level using highly polymorphic markers. The nuclear genetic diversity of each accession was assessed using five highly polymorphic microsatellites and we traced back the maternal genome using both maternally inherited mitochondrial minisatellites and one chloroplastic PCR–RFLP marker specific of the cytoplasmic male sterility used in cultivars (i.e. "OwenCMS", see Owen 1945).

The large sample set as well as the diversity and complementarity of the molecular tools used in this study allowed us to precisely investigate and compare the amount of genetic diversity found within each forms of *B. vulgaris* and to assess their genetical relationships and, in particular, to clarify the taxonomic position of weed and inland ruderal beets.

Materials and methods

Sampling

Beta vulgaris is a diploid short-lived perennial species ($2n = 18$) widely distributed along the European coastline and around the Mediterranean Basin (Letschert 1993), where the domestication process of beets occurred more than 2000 years ago (Ford-Lloyd and Williams 1975; Zohary and Hopf 2000). To investigate the genetic diversity and relationships within the *B. vulgaris* complex, we relied on a large sample set of representative populations of wild, cultivated and weed beets:

A total of 11 populations of wild sea beets (38 ± 11 individuals per population for a total of 416 individuals) were sampled along the Channel French coastline and labelled with acronyms S_{01}–S_{12} (Table 1, Fig. 1).

In the French seed production area, 12 populations of inland ruderal beets (50 ± 2 individuals per populations for a total of 596 individuals) were collected and labelled from R_{01} to R_{12} (Table 1, Fig. 1). These inland ruderal populations were found to be associated with past and present road works (roadsides, car parks), rubble deposits or garden edges.

Weed beets' genetic diversity has been assessed on a total of 481 weed beet individuals (40 ± 14 individuals per population), collected among 12 cultivated fields from Northern France and Southern Belgium. These 12 populations of weed beets were labelled from W_{01} to W_{12} (Table 1, Fig. 1).

Sugar beet genetic diversity was studied from a panel of 35 cultivars kindly provided by the 'Institut Technique de la Betterave industrielle' (ITB, the French Institute that studies and promotes specific and new agronomic qualities of beet cultivars). These 35 diploid cultivars, launched on the European market between 1999 and 2003, have been released by 13 of the major European seed companies. As only few individuals were available for each cultivar (4–5 individuals per cultivar, for a total number of 147 individuals) and for the sake of statistical robustness, cultivars were grouped into 13 "populations" according to seed companies and are labelled from C_{01} to C_{13} (Table 1).

This large sample set of 1,640 individuals allowed us (1) to precisely characterise the genetic diversity of the contemporary cultivars as well as those of inland ruderal beets of south western France, weed beets and wild sea beets of northern France and (2) to assess the genetic relationships within the *B. vulgaris* complex.

Genetic data collection

DNA extraction

Extraction and purification of total DNA were performed using a DNeasy 96 Plant Kit (Qiagen Inc.) according to manufacturer's protocol.

Cytoplasmic diversity

We checked for the occurrence of OwenCMS cytotype using a diagnostic chloroplastic PCR–RFLP marker related to a polymorphic *Hin*dIII site mapped in the *petG-psbE* chloroplast fragment (Ran and Michaelis 1995). Primers used, PCR conditions and DNA digestion of this PCR–RFLP method were applied as described by Ran and Michaelis (1995). This polymorphism allows to distinguish OwenCMS from non-OwenCMS lines (Desplanque et al. 2000; Viard et al. 2002, 2004; Arnaud et al. 2003). In order to obtain more precise information about the cytoplasmic diversity, we characterised the mitochondrial polymorphism by genotyping individuals at four mitochondrial minisatellite loci named Tr1, Tr2, Tr3 and Tr4 (Nishizawa et al. 2000). Multiplex PCR amplifications were carried in a 10.5 µl volume containing 3 mM $MgCl_2$, 200 µM of each dNTP, 0.2 µg/µL of BSA, 0.2 µM of each forward and reverse primer, 0.625 U of *Taq* polymerase (Applied Biosystems) and ≈50 ng of template DNA. Cycling condition included an initial denaturation step of 5 min at 94°C followed by 30 cycles of 30 s at 94°C, 60 s annealing at 62°C, 30 s at 72°C. Final extension was conducted 10 min at 72°C, using a 9700 thermal cycler (Applied Biosystems).

Among wild and cultivated accessions, *B. vulgaris* carries about 20 different mitotypes, previously described in Forcioli et al. (1998) and Desplanque et al. (2000). In order to assign a mitotype name to each wild, weed and ruderal individual genotyped in the present study, we identified the association between mitotypes and minisatellite haplotypes within a reference database recently used to examine the genealogical relationship between the different mitotypes depicted in Fénart et al. (2006).

Nuclear diversity

Individuals were genotyped at five nuclear microsatellite loci named GAA1, GTT1, GCC1, BVM3, CAA1 (Mörchen et al. 1996; Viard et al. 2002). Loci GTT1, GCC1 and

Table 1 Population acronym, sample location (name of the nearest town), sample size (*N*), GPS coordinates (WGS84 system) and taxon (wild sea beets, wild ruderal beets, weed beets or cultivar) of each population sampled for this study

Population acronym	Sample location	*N*	GPS coordinates	Taxon
S_{01}	Nieuwpoort	37	N 51°09.000′ –E 2°43.000’	Wild sea beets
S_{02}	Grand-Fort Philippe	41	N 51°00.334′–E 2°05.802′	Wild sea beets
S_{03}	Hourdel	38	N 50°12.900′–E 1°33.900′	Wild sea beets
S_{04}	Pourville-sur-mer	16	N 49°55.083′–E 1°01.944′	Wild sea beets
S_{05}	Cabourg	46	N 49°17.509′–E −0°07.651′	Wild sea beets
S_{06}	Cap Lévi	47	N 49°41.303′–E −1°28.397′	Wild sea beets
S_{07}	Pointe de l’Agon	18	N 49°00.093′–E −1°34.497′	Wild sea beets
S_{08}	Trébeurden	50	N 48°46.442′–E −3°34.950′	Wild sea beets
S_{09}	Argenton	48	N 48°31.391′–E −4°45.610′	Wild sea beets
S_{10}	Guidel	35	N 47°46.092′–E −3°31.528′	Wild sea beets
S_{11}	Lihou (Gernsey)	40	N 49°27.298′–E −2°39.196′	Wild sea beets
R_{01}	Saint Clar	49	N 43°53.508′–E 0°46.381′	Wild ruderal beets
R_{02}	Laplume	51	N 44°06.613′–E 0°31.867′	Wild ruderal beets
R_{03}	Pargan-Taillac	51	N 44°03.459′–E 0°35.361′	Wild ruderal beets
R_{04}	Cuq	50	N 44°04.979′–E 0°41.953′	Wild ruderal beets
R_{05}	Flamarens	50	N 44°01.055′–E 0°47.653′	Wild ruderal beets
R_{06}	Nérac	50	N 44°08.104′–E 0°20.520′	Wild ruderal beets
R_{07}	Vic-Fézensac	50	N 43°45.292′–E 0°17.730′	Wild ruderal beets
R_{08}	Plieux	50	N 43°57.019′–E 0°43.925′	Wild ruderal beets
R_{09}	Port-sainte Marie	45	N 44°14.999′–E 0°23.567′	Wild ruderal beets
R_{10}	Galapian	50	N 44°17.981′–E 0°24.831′	Wild ruderal beets
R_{11}	Montpézat	50	N 44°20.759′–E 0°31.493′	Wild ruderal beets
R_{12}	Prayssas	50	N 44°17.218′–E 0°30.494′	Wild ruderal beets
W_{01}	Illies	39	N 50°32.603′–E 2°49.365′	Weed beets
W_{02}	Illies	39	N 50°32.506′–E 2°49.823′	Weed beets
W_{03}	Fournes	40	N 50°34.972′–E 2°54.372′	Weed beets
W_{04}	Beaucamps-Ligny	24	N 50°35.822′–E 2°55.906′	Weed beets
W_{05}	Herlies	75	N 50°34.799′–E 2°52.631′	Weed beets
W_{06}	Ault	45	N 50°09.627′–E 1°29.631′	Weed beets
W_{07}	Vieille-Chapelle	45	N 50°35.945′–E 2°44.032′	Weed beets
W_{08}	Wissant	20	N 50°53.937′–E 1°41.891′	Weed beets
W_{09}	Wissant	20	N 50°53.057′–E 1°41.523′	Weed beets
W_{10}	La Goudinière	45	N 50°40.453′–E 3°23.782′	Weed beets
W_{11}	Merkem	45	N 50°57.574′–E 2°52.864′	Weed beets
W_{12}	Ingelmunster	44	N 50°56.323′–E 3°16.684′	Weed beets
C_{01}	2 Cultivars	6	–	Cultivars
C_{02}	1 Cultivar	4	–	Cultivars
C_{03}	2 Cultivars	9	–	Cultivars
C_{04}	2 Cultivars	9	–	Cultivars
C_{05}	2 Cultivars	10	–	Cultivars
C_{06}	3 Cultivars	14	–	Cultivars
C_{07}	2 Cultivars	6	–	Cultivars
C_{08}	1 Cultivar	5	–	Cultivars
C_{09}	9 Cultivars	36	–	Cultivars
C_{10}	1 Cultivar	5	–	Cultivars
C_{11}	3 Cultivars	14	–	Cultivars
C_{12}	5 Cultivars	21	–	Cultivars
C_{13}	2 Cultivars	8	–	Cultivars

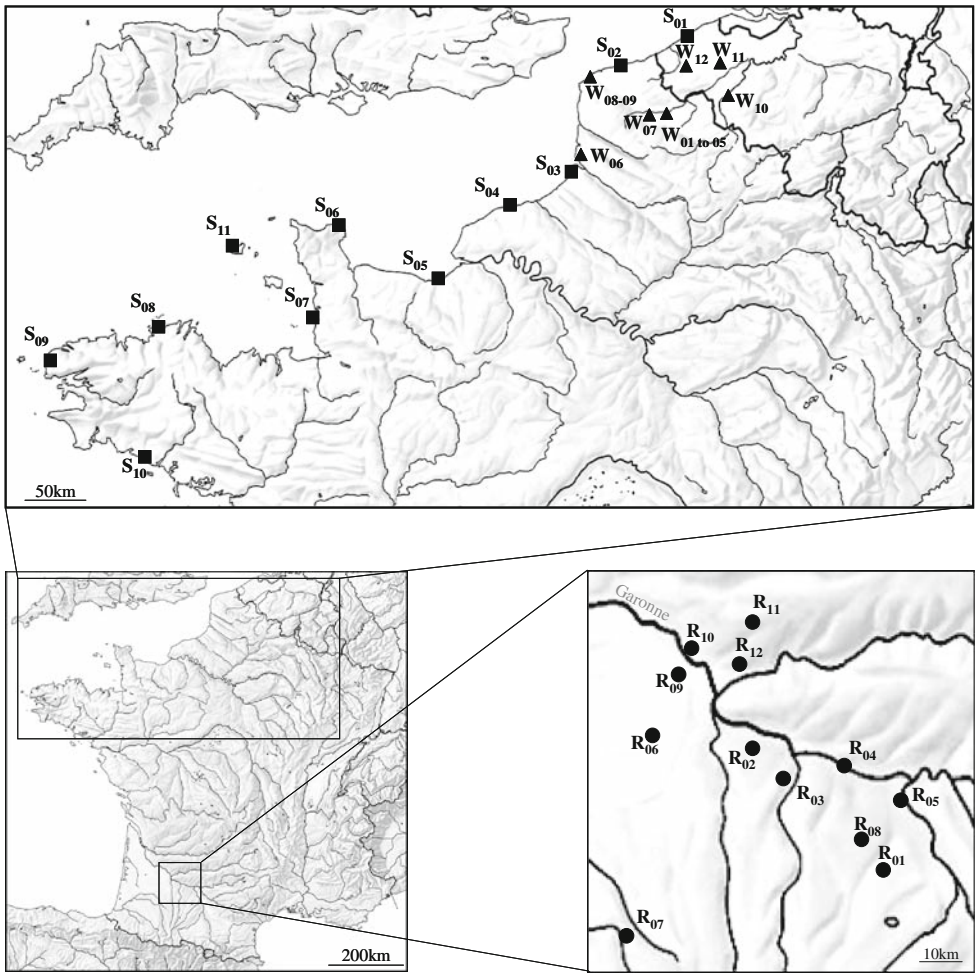

Fig. 1 Spatial location of sampled populations of wild and weed populations of *Beta vulgaris*. Wild inland ruderal beet populations (labelled with an "R") are visualised by *black circles*; wild sea beet populations ("S") are visualised by *black triangles* and weed beet populations ("W") are visualised by *black squares*

BVM3 have been recently mapped on chromosomes VI, II and IX, respectively (Laurent et al. 2007). These five microsatellite loci were amplified into a multiplex polymerase chain reactions (PCR) performed in a 10.5 µL volume mix as follows: 2.5 µL of DNA template (corresponding to a quantity of 25 ng), 1 µL of PCR Buffer 10× (Applied Biosystems), 2.9 mM $MgCl_2$, 0.2 µg/µL of BSA, 2% of DMSO (Dimethyl sulfoxid), 0.1 µM of each forward and reverse primer for loci GTT1, BVM3, CAA1 and GAA1 and 0.05 µM of each primer for loci GCC1, 290 µM of each dNTP and 0.9 U/µL of hot start *Taq* polymerase (*AmpliTaq* Gold, Applied Biosystems). PCR was carried out on a 9700 thermal cycler (Applied Biosystems) under the following conditions: 10 min denaturing at 94°C followed by 40 cycles of 45 s denaturing at 94°C, 45 s annealing at 54°C and 45 s extension at 72°C and a final extension step at 72°C for 10 min.

Detection and analysis of PCR products

To check for the presence of OwenCMS, *petG–psbE* cpDNA *Hind*III-digested products were separated using 0.8% agarose gel electrophoresis and visualized after ethidium bromide staining under UV light. Individuals carrying the OwenCMS are visualized by a two-bands pattern (454 base pairs (bp) and 109 bp), while non-OwenCMS individuals are characterised by an undigested 563 bp fragment (Ran and Michaelis 1995).

Detection of both minisatellite and microsatellite fragments was performed with an ABI Prism® 3100 Genetic Analyzer 16-capillary array system (Applied Biosystems) following manufacturer's protocols. For each individual, 2.5 µL of PCR product were mixed with 9.6 µL of Hi-Di™ formamide (Applied Biosystems) and 0.4 µL of GeneScan™–1000ROX™ size standard (Applied Biosystems) for the mitochondrial DNA minisatellites or 0.4 µL of GeneScan™-500LIZ size standard (Applied Biosystems) for microsatellites loci. The ABI Prism® 3100 Genetic Analyzer was set with the D matrix filter to detect the four dyes VIC™ (green), PET™ (red), NED™ (yellow) and 6-FAM™ (blue) used to label the forward primers of the four minisatellite markers Tr1, Tr2, Tr3 and Tr4 respectively. For nuclear microsatellites, the G5 matrix filter was used to detect alleles of GCC1, GTT1, BVM3, CAA1 and GAA1,

forward primers of which were labelled with dyes PET™ (red), NED™ (yellow), VIC™ (green), 6-FAM™ (blue) and NED™ (yellow), respectively. Raw data of electrophoresis obtained were read using GENEMAPPER v3.7 (Applied Biosystems). Individuals with doubtful genotypes (i.e. with missing data or presenting new alleles) were genotyped a second time at all loci.

Nuclear genetic data analysis

For each population, nuclear genetic diversity was examined by calculating allelic frequencies, allelic richness (A_r) following the rarefaction procedure of El Mousadik and Petit (1996), the genetic diversity (*He*) sensu Nei (1978) and the unbiased intra-population fixation index (F_{IS}) for each microsatellite locus and over all loci using GENEPOP version 3.3 (Raymond and Rousset 1995). Genotypic linkage disequilibrium was estimated prior to other analyses using GENEPOP version 3.3 (Raymond and Rousset 1995). Heterozygote deficiencies and significance of deviations from Hardy–Weinberg equilibrium within each population of weed, wild and inland ruderal beets were tested using a score test (Raymond and Rousset 1995). Permutation tests, implemented in the software FSTAT version 2.9.3.2. (Goudet 1995), were used to compare allelic richness, genetic diversity and F_{IS} between wild, ruderal and weed groups and also between French and Belgian weed beets populations (10,000 permutations).

Assuming that it should be useless and erroneous to compute genetic diversity (*He*) and intra-population fixation index (F_{IS}) in the 13 composite cultivar samples, we only compared allelic richness of sugar beet cultivars with either weed, wild, or inland ruderal beet groups using permutation tests as described above.

Genetic relationships among the *B. vulgaris* complex

Genetic relationships among the *B. vulgaris* complex were described in an unrooted neighbour-joining tree based on Cavalli-Sforza and Edwards' (1967) chord distance (D_{CE}) and using the software POPULATIONS v1.1.24 (Olivier Langella, available at http://www.pge.cnrs-gif.fr/bioinfo/). Bootstrap values were obtained based on 10,000 replications over populations. The ensuing populations' tree was visualised using TREEVIEW (Rod Page, available at http://www.taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Genetic differentiation between populations was assessed by pairwise F_{ST} estimates between populations following the ANOVA procedure of Weir and Cockerham (1984) using FSTAT version 2.9.3.2. Significance of pairwise F_{ST} was tested by randomly permuting multilocus genotypes among population samples (log-likelihood statistics *G*, 10,000 permutations) as suggested by Goudet et al. (1996). We also performed a hierarchical analysis of molecular variance (AMOVA) using Arlequin v3.1 (Excoffier et al. 2005, available at http://www.cmpg.unibe.ch/software/arlequin3/) to analyse the partition of the genetic variance within (F_{SC}) and between (F_{CT}) wilds, weed and cultivar groups. Bonferroni adjustments for simultaneous statistical tests were applied following Rice (1989).

We further tested the correspondence of taxonomic origin with genetic clusters by applying a model-based clustering algorithm, using the STRUCTURE software (Pritchard et al. 2000). This Bayesian method identifies clusters of genetically related individuals from multilocus genotypes, using or not prior knowledge of their group affiliation. This approach assumes that there are *K* groups contributing to the gene pool of the sampled populations. Individuals can have membership in multiple clusters, membership coefficients summing to 1 across clusters. In our case study, the membership of each individual was tested for a range of genetic clusters from $K = 2$ to $K = 5$, without prior information on their taxonomical affiliation. Each run consisted of a burn-in period of 1,000 steps followed by 10^6 MCMC (Monte Carlo Markov Chain) replicates, assuming that allele frequencies are uncorrelated across clusters. Repeated runs of STRUCTURE produced identical results to those shown.

Results

Cytoplasmic diversity

The presence of the *Hin*dIII restriction site in the *petG–psbE* chloroplast fragment revealed the occurrence of OwenCMS cytotype in all sugar beet individuals as well as in all individuals sampled within nine out of the 12 weed beet populations (Table 2). The association between mitotypes and mitochondrial minisatellite haplotypes obtained from the analysis of the collection data set of Fénart et al. (2006) are presented into brackets in the legend of Fig. 2 and a total of 10 mitotypes were represented according to their association with minisatellite haplotypes. A clear dichotomy appeared between, on the first hand, cultivars and weed beet populations where the OwenCMS is majoritary and, on the other hand, wild ruderal and sea beet populations characterized respectively by eight and nine mitotypes and a quasi-absence of the OwenCMS. All individuals carrying the OwenCMS cytotype also exhibited a unique combination of alleles (haplotype) from the four mitochondrial minisatellites loci: 500, 404, 420 and 438 bp, for Tr1, Tr2, Tr3 and Tr4, respectively (see Fig. 2). These results confirmed the maternal cultivated origin of the large majority of weed beet individuals and highlighted the lack of cytoplasmic diversity among cultivars. Nonetheless,

Table 2 Cytoplasmic and nuclear diversity within each sampled population and mean values over all samples for each taxon

	Cytoplasmic diversity		Nuclear diversity					
	Rate of OwenCMS cytotype	Number of minisatellite haplotypes	BVM3	CAA1	GCC1	GTT1	GAA1	All
Wild sea beet populations: (mean nuclear F_{ST}: 0.147, $P < 10^{-3}$; mean cytoplasmic F_{ST}: 0.359, $P < 10^{-3}$)								
S_{01}	0%	1	A_r: 4.33/H_e: 0.769 F_{IS}: 0.016*	2.57/0.443 0.025^{NS}	1.99/0.485 0.276^{NS}	2.06/0.372 0.345*	2.72/0.520 0.117**	2.73/0.518 0.134^{NS}
S_{02}	0%	5	4.60/0.773 −0.072^{NS}	3.97/0.695 0.439***	2.03/0.357 0.043^{NS}	2.66/0.548 −0.113^{NS}	2.35/0.460 −0.06^{NS}	3.12/0.567 0.062***
S_{03}	0%	2	3.35/0.567 −0.161^{NS}	3.86/0.742 0.078^{NS}	2.28/0.534 0.064^{NS}	1.97/0.454 −0.274^{NS}	1.61/0.191 −0.104^{NS}	2.61/0.498 −0.058^{NS}
S_{04}	0%	1	3.91/0.746 0.330**	3.58/0.694 0.279^{NS}	1.25/0.063 0.000^{NS}	2.00/0.517 0.153^{NS}	1.00/0.000 NC[a]	2.35/0.404 0.257*
S_{05}	2.38%	6	4.60/0.818 −0.009^{NS}	4.28/0.791 −0.017^{NS}	1.41/0.105 −0.032^{NS}	3.08/0.649 −0.224^{NS}	3.05/0.589 −0.246^{NS}	3.28/0.590 −0.106**
S_{06}	0%	3	4.37/0.777 0.124^{NS}	4.33/0.779 0.178*	2.57/0.511 0.101^{NS}	1.99/0.491 −0.140^{NS}	1.70/0.217 0.446**	2.99/0.555 0.113**
S_{07}	5.56%	4	3.69/0.722 0.154^{NS}	4.26/0.785 0.026^{NS}	1.40/0.108 −0.030^{NS}	1.98/0.458 0.029^{NS}	2.42/0.384 −0.157^{NS}	2.75/0.491 0.033^{NS}
S_{08}	0%	6	4.54/0.812 −0.010^{NS}	4.51/0.784 0.031^{NS}	1.35/0.096 −0.043^{NS}	1.98/0.479 −0.294^{NS}	1.88/0.264 −0.134^{NS}	2.85/0.487 −0.067^{NS}
S_{09}	0%	5	4.61/0.822 0.138**	3.76/0.687 0.040^{NS}	2.08/0.420 0.057^{NS}	2.60/0.549 −0.441^{NS}	1.64/0.179 0.166^{NS}	2.94/0.531 −0.018**
S_{10}	0%	5	5.41/0.880 0.091^{NS}	4.36/0.738 0.071*	1.22/0.056 −0.015^{NS}	2.47/0.479 −0.014^{NS}	2.22/0.395 −0.014^{NS}	3.14/0.510 0.047^{NS}
S_{11}	0%	6	3.88/0.725 0.000^{NS}	5.31/0.872 −0.007^{NS}	1.20/0.051 −0.013^{NS}	1.95/0.418 −0.166^{NS}	1.68/0.183 −0.068^{NS}	2.80/0.450 −0.040^{NS}
Mean	0.72%	4	4.30/0.765 0.061^{NS}	4.07/0.728 0.092*	1.71/0.253 0.009^{NS}	2.25/0.492 −0.102^{NS}	2.03/0.307 −0.016^{NS}	2.87/0.509 0.025*
Wild ruderal beet populations: (mean nuclear F_{ST}: 0.067, $P < 10^{-3}$; mean cytoplasmic F_{ST}: 0.449, $P < 10^{-3}$)								
R_{01}	0%	4	4.94/0.843 −0.065^{NS}	4.23/0.760 0.113^{NS}	2.41/0.466 0.124^{NS}	3.03/0.680 0.159^{NS}	2.08/0.376 0.132^{NS}	3.34/0.625 0.079^{NS}
R_{02}	0%	3	5.05/0.838 0.181^{NS}	4.22/0.786 0.053^{NS}	2.56/0.580 0.324*	3.27/0.707 −0.137^{NS}	2.26/0.428 0.038^{NS}	3.47/0.668 0.090***
R_{03}	0%	2	5.06/0.857 0.405***	4.10/0.757 0.120^{NS}	3.03/0.659 0.227*	3.38/0.714 0.011^{NS}	1.99/0.266 0.043^{NS}	3.51/0.651 0.187***
R_{04}	0%	3	4.01/0.730 0.096^{NS}	3.66/0.681 0.148^{NS}	1.94/0.416 −0.107^{NS}	2.90/0.636 0.150^{NS}	1.89/0.252 0.049^{NS}	2.88/0.543 0.086^{NS}
R_{05}	0%	4	5.18/0.868 0.055^{NS}	2.81/0.570 0.264^{NS}	3.05/0.598 0.096^{NS}	3.33/0.705 0.064^{NS}	1.95/0.361 −0.273^{NS}	3.27/0.620 0.065^{NS}
R_{06}	0%	1	3.68/0.744 0.194*	3.98/0.759 0.157^{NS}	1.91/0.380 0.210^{NS}	3.60/0.750 −0.067^{NS}	2.37/0.547 −0.024^{NS}	3.11/0.636 0.088^{NS}
R_{07}	0%	3	4.49/0.810 −0.061^{NS}	3.21/0.580 −0.103^{NS}	2.80/0.642 0.222*	2.75/0.617 −0.168^{NS}	2.08/0.314 −0.018^{NS}	3.07/0.593 −0.026**
R_{08}	0%	2	4.73/0.829 0.083^{NS}	3.40/0.668 0.132*	2.79/0.589 −0.053^{NS}	3.20/0.679 0.204*	1.50/0.149 −0.077^{NS}	3.12/0.583 0.087^{NS}
R_{09}	0%	2	3.17/0.655 0.017^{NS}	3.78/0.758 0.237*	2.21/0.492 0.052^{NS}	3.13/0.663 −0.039^{NS}	1.78/0.281 0.131^{NS}	2.81/0.570 0.080^{NS}
R_{10}	0%	3	4.48/0.785 0.006^{NS}	2.63/0.437 0.359***	1.97/0.332 0.398**	2.83/0.551 0.165*	2.25/0.414 0.227^{NS}	2.83/0.504 0.190***
R_{11}	0%	4	5.25/0.871 0.104^{NS}	3.21/0.651 −0.044**	2.61/0.578 0.308***	3.06/0.653 −0.010^{NS}	1.69/0.187 0.252*	3.16/0.588 0.095***
R_{12}	0%	5	4.24/0.775 0.278**	3.82/0.720 0.348**	2.39/0.541 0.056^{NS}	3.03/0.659 0.083^{NS}	1.99/0.394 0.086^{NS}	3.09/0.618 0.189***
Mean	0%	3	4.52/0.800 0.108**	3.59//0.677 0.149***	2.47/0.523 0.155***	3.13/0.668 0.035^{NS}	1.99/0.331 0.047^{NS}	3.14/0.600 0.101***

Table 2 continued

	Cytoplasmic diversity		Nuclear diversity					
	Rate of OwenCMS cytotype	Number of minisatellite haplotypes	BVM3	CAA1	GCC1	GTT1	GAA1	All
Weed beet populations: (mean nuclear F_{ST}: 0.056, $P < 10^{-3}$; mean cytoplasmic F_{ST}: 0.380, $P < 10^{-3}$)								
W_{01}	100%	1	4.46/0.799 0.165**	3.72/0.675 −0.025^{NS}	2.10/0.514 −0.198^{NS}	2.79/0.580 −0.106^{NS}	1.53/0.145 −0.058^{NS}	2.92/0.543 −0.021^{NS}
W_{02}	100%	1	4.56/0.787 0.023^{NS}	3.65/0.671 0.083^{NS}	2.43/0.537 0.045^{NS}	2.40/0.420 −0.161^{NS}	1.38/0.100 −0.031^{NS}	2.88/0.503 0.011^{NS}
W_{03}	100%	1	4.36/0.789 0.050^{NS}	3.56/0.694 0.243***	2.47/0.564 −0.152*	2.82/0.572 −0.135^{NS}	1.57/0.145 0.310*	2.96/0.553 0.032**
W_{04}	100%	1	3.97/0.699 0.047^{NS}	4.57/0.814 0.079*	2.04/0.371 0.215^{NS}	2.76/0.620 0.260^{NS}	2.16/0.330 0.115^{NS}	3.10/0.567 0.133***
W_{05}	100%	1	3.63/0.688 0.147**	2.83/0.601 −0.220^{NS}	2.23/0.519 0.050^{NS}	2.86/0.527 −0.087^{NS}	1.34/0.090 0.261^{NS}	2.58/0.485 −0.011^{NS}
W_{06}	75.56%	2	4.70/0.810 0.204**	3.64/0.694 0.199**	2.16/0.523 −0.105^{NS}	2.74/0.599 0.295*	1.63/0.200 0.111^{NS}	2.97/0.565 0.158**
W_{07}	100%	1	5.40/0.880 0.102^{NS}	3.22/0.672 0.308***	2.72/0.607 −0.035^{NS}	2.49/0.563 0.050^{NS}	1.00/0.000 NCa	2.97/0.544 0.111***
W_{08}	60%	5	6.04/0.922 0.187*	2.58/0.559 0.016^{NS}	2.18/0.497 −0.106^{NS}	2.42/0.491 0.185*	1.69/0.226 0.337***	2.98/0.539 0.110***
W_{09}	100%	1	5.63/0.889 −0.066^{NS}	3.45/0.693 0.089^{NS}	2.17/0.461 0.086^{NS}	2.74/0.607 0.306***	1.21/0.053 NC[a]	3.04/0.541 0.084*
W_{10}	100%	1	4.80/0.828 0.034^{NS}	3.72/0.738 0.308**	2.07/0.489 −0.045^{NS}	2.76/0.564 −0.065^{NS}	1.49/0.128 −0.043^{NS}	2.97/0.549 0.070*
W_{11}	100%	1	4.06/0.780 0.202**	3.63/0.752 0.498***	1.99/0.489 −0.044^{NS}	2.07/0.489 0.046^{NS}	1.89/0.258 0.053^{NS}	2.73/0.554 0.197***
W_{12}	33.33%	3	4.76/0.821 0.004*	2.92/0.619 −0.137^{NS}	1.99/0.503 −0.039^{NS}	2.81/0.641 0.113^{NS}	1.55/0.148 0.249^{NS}	2.81/0.546 0.003^{NS}
Mean	89.07%	1.58	4.70/0.808 0.100***	3.46/0.682 0.120***	2.21/0.506 −0.027^{NS}	2.64/0.556 0.058**	1.54/0.152 0.130***	2.91/0.541 0.073**
Cultivar accessions: (mean nuclear F_{ST}: 0.082, $P < 10^{-3}$; mean cytoplasmic F_{ST}: not calculable (monomorphic))								
C_{01}	100%	1	2.58[b]	1.98	2.00	1.98	1.00	1.91
C_{02}	100%	1	3.00	3.00	2.00	2.00	1.00	2.20
C_{03}	100%	1	2.40	2.00	1.71	1.99	1.00	1.82
C_{04}	100%	1	3.34	1.71	2.42	2.00	1.00	2.09
C_{05}	100%	1	2.05	2.00	1.90	2.21	1.65	1.96
C_{06}	100%	1	1.94	2.31	1.92	2.00	1.00	1.83
C_{07}	100%	1	2.67	2.00	2.00	2.65	1.00	2.06
C_{08}	100%	1	1.98	2.00	2.00	2.00	1.00	1.80
C_{09}	100%	1	2.88	2.76	1.99	1.99	1.21	2.17
C_{10}	100%	1	3.60	1.98	1.98	2.00	1.00	2.11
C_{11}	100%	1	2.47	1.50	1.98	2.00	1.00	1.79
C_{12}	100%	1	2.06	1.99	1.96	1.94	1.00	1.79
C_{13}	100%	1	2.80	2.00	2.00	1.99	1.00	1.96
Mean	100%	1	2.60	2.09	1.99	2.06	1.07	1.96

For cytoplasmic diversity, we estimated the rate of OwenCMS cytotype and the number of mitochondrial minisatellite haplotypes found within each population. For each nuclear microsatellite loci (BVM3, CAA1, GCC1, GTT1 and GAA1), we estimated the allelic richness (A_r), the expected heterozygosity (H_e) and the intrapopulation fixation index (F_{IS}). Mean genetic differentiation (F_{ST}) values within each taxon are also presented for nuclear and cytoplasmic diversity

NS non significant

[a] NC: not calculable. locus GAA1 showed only one allele in S_{04} and W_{09} or a second allele present in only one individual in W_{12}, making it impossible to estimate F_{IS} value

[b] Cultivar samples presented in this study are made artificially of different cultivars pooled together according to their producers and then computing either H_e or F_{IS} for these composite populations is not relevant

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, for significance of heterozygotes deficiency

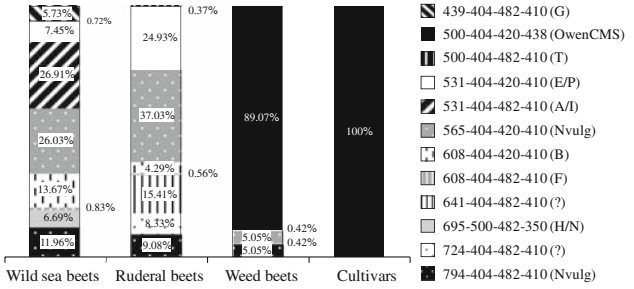

Fig. 2 Proportions of each minisatellite haplotype found within wild sea beets, inland ruderal beets, weed beets and cultivars. Each haplotype is based on the association of alleles of Tr1, Tr2, Tr3 and Tr4 loci, respectively, indicated by their size in base pairs. Associations of minisatellites haplotypes and mitotypes defined from the collection data set used in Fénart et al. (2006) are presented into brackets. The question mark indicates that the concerned minisatellite haplotype was not represented in the core collection

non-OwenCMS individuals were also found within the weed beet populations at a rate of 10.94% (Fig. 2).

The occurrence of non-OwenCMS individuals concerns the weed populations W_{06}, W_{08} and W_{12} (see Table 2) where four different minisatellite haplotypes also found in ruderal and wild populations (Fig. 2) were detected. Two of these haplotypes (565-404-420-410 and 794-404-482-410) are commonly found in association with a non-OwenCMS mitotype called *Nvulg* widely distributed in wild populations (Cuguen et al. 1994; Desplanque et al. 2000; Fénart et al. 2006). The two others (531-404-420-410 and 695-500-420-350) were, respectively found in association with mitotypes H/N and E/P following the reference database nomenclature defined in Desplanque et al. (2000) and Fénart et al. (2006) (see Fig. 2).

Nuclear diversity

Exact tests for genotypic linkage disequilibria between microsatellite loci within each population (except the composite samples of cultivars) showed five significant P values out of 360 comparisons (1.39%), 18 being expected from type I error. Multiple tests across all populations yielded no significant adjusted P values, also attesting that the five detected linkage disequilibria may be artifactual, either imputed to a small number of alleles and/or to population substructuring.

Statistics of population genetic diversity (A_r, H_e and F_{IS}) are presented in Table 2 for each locus and overall loci in each sample as well as their mean values overall sample for each form of *B. vulgaris*. Allelic richness (A_r) ranged from 1.00 (locus GAA1; population W_{07} and S_{04}) to 6.04 (Locus BVM3; population W_{08}) and amount of expected heterozygosity (H_e) were relatively high across all loci and samples, except for locus GAA1, which showed the lowest values for allelic richness and expected heterozygosity. A_r, H_e and F_{IS} did not significantly differ between wild ruderal, weed and wild sea beet populations. Nonetheless, a general trend toward higher F_{IS} values for weed and inland ruderal beet populations can be visualised in Table 2 (mean F_{IS} of 0.073 and 0.101, respectively) compared to wild sea beet populations (0.025). Finally, permutation tests confirmed that allelic richness was significantly lower ($P < 10^{-3}$) for cultivated beet (mean A_r of 1.960) compared to ruderal, weed and sea beets groups (mean A_r of 3.14, 2.91 and 2.87, respectively, see Table 2).

Genetic relationships within the *B. vulgaris* complex

Within each taxon, a very clear genetic differentiation between populations was found at the cytoplasmic level (F_{ST} of 0.359, 0.449 and 0.380; all at $P < 10^{-3}$ for wild sea beet, inland ruderal beet and weed beet populations, respectively), except between the cultivar accessions exhibiting only the OwenCMS mitotype (Table 2). Results of the AMOVA analysis indicates that the level of genetic differentiation is high either between or within weed beet, wild sea beet and inland ruderal beet groups ($F_{SC} = 0.416$ and $F_{CT} = 0.479$; both at $P < 10^{-3}$).

Mean nuclear population differentiation estimated over all studied populations—including cultivars—highlighted a significant genetic differentiation ($F_{ST} = 0.285$; $P < 10^{-3}$). Within each form of beets, mean population differentiation was low: mean F_{ST} values were of 0.056, 0.082 and 0.067; all at $P < 10^{-3}$, for weed, cultivated and ruderal beets respectively but were more pronounced between the wild sea beet populations ($F_{ST} = 0.147$; $P < 10^{-3}$). Pairwise nuclear F_{ST} estimates over all populations can be visualised in the Electronic supplementary material S1. Highly significant pairwise differentiation between all ruderal and wild populations and all other populations was detected. In contrast, only few differentiation estimates appeared to be significant between cultivars and weed beet populations, suggesting evidence for close genetical affinities between these two forms. It should also be emphasised that no significant genetic differentiation was observed among cultivar samples, except for 3 pairwise comparisons (see Electronic supplementary material S1).

The neighbour-joining tree of populations presented in Fig. 3 showed a clear genetic distinctiveness between the four forms, each corresponding to a single cluster of wild sea beet populations, wild ruderal beet populations, weed beet populations and cultivar accessions. Despite this clear genetic clustering by taxons, we can nonetheless depict a relative proximity of weed beet and cultivar samples populations with ruderal population, compared to wild sea beet populations. This highlighted the evolutionary divergence between wild sea beet and wild ruderal beet populations. In addition, the congruence between taxonomic and genetic dis-

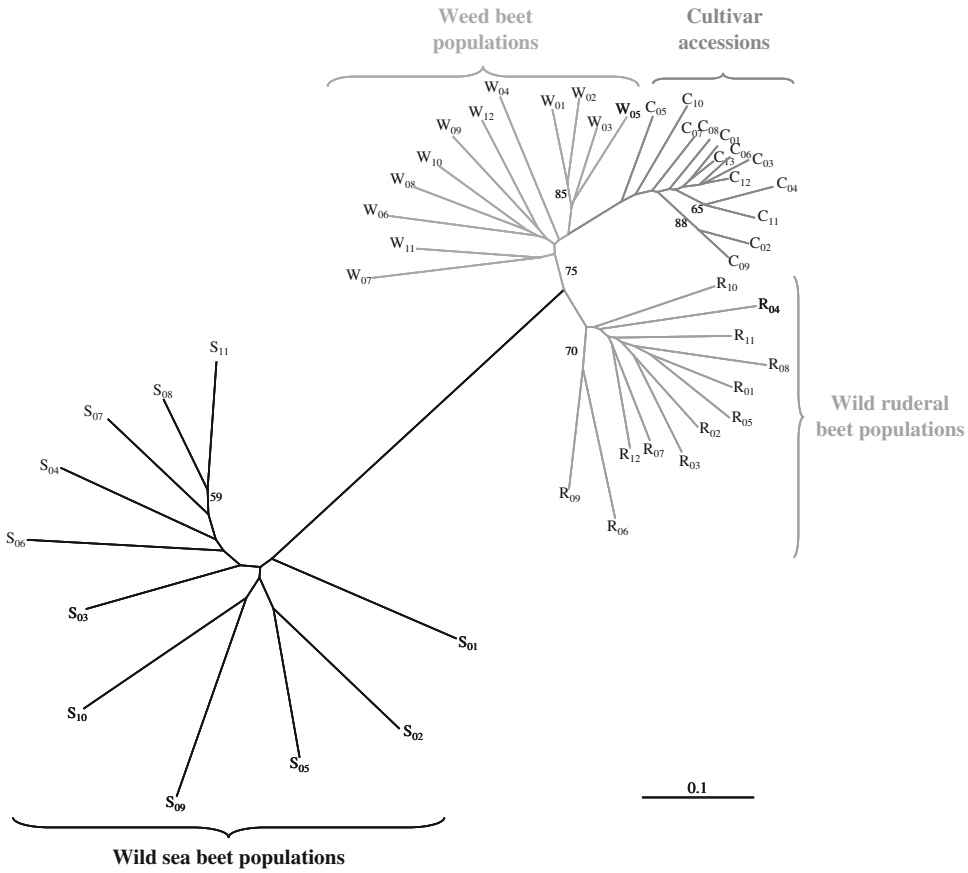

Fig. 3 Neighbour-joining tree describing genetic relationships among the *Beta vulgaris* complex based on the Cavalli-Sforza and Edwards' (1967) chord distance (D_{CE}). Wild inland ruderal beet populations are labelled with an "R"; wild sea beet populations with an "S"; weed beet populations with a "W" and cultivar accessions with a "C". Significance of each node was tested with 10,000 permutations over populations and only bootstrap values >50% are reported on the figure

tinctiveness was also well supported by results of the Bayesian analysis. Indeed, the most likelihood number of genetic clusters was $K = 4$, each corresponding to a taxon. Figure 4 showed that wild sea beets, inland ruderal beets and cultivars clearly constituted different genetic clusters. For weed beets, most individuals belonged to a single and distinct genetic cluster. However, a large part of individuals (23%) presented high values of individual memberships ($q > 0.75$) assigning them into the genetic cluster of cultivars. Moreover, but to a lesser extent, weed beets have a large proportion of their nuclear genome that come from the inland ruderal beet group, result suggestive of a genetic admixture between cultivated and inland ruderal beets. These differences of membership of weed beet individuals into the cultivar cluster may reflect that the weed beet populations are continuously replenished by new crop–wild F1 hybrids or cultivar bolters.

In the same way, results of the AMOVA analysis were in complete agreement with the topology of the neighbour-joining tree and showed a significant genetic differentiation between sea beet, ruderal beet, weed beet and cultivar groups with an overall F_{CT} value (i.e. fixation index corresponding to the genetic variance among groups over total) of 0.266, whereas the mean genetic variance among populations within each group (F_{SC}) was equal to 0.084, meaning that genetic differences were significantly more pronounced between forms than within each form. Pairwise estimates of F_{CT} were also computed. Comparisons of "cultivar-weed" and "weed-ruderal" produced the weakest F_{CT} values (0.054 and 0.057; all at $P < 10^{-4}$, respectively) whereas medium values were found for comparisons of "sea-ruderal" and "cultivar-ruderal" (0.104 and 0.146; all at $P < 10^{-4}$, respectively) and comparisons of "sea-cultivar" and "sea-weed" presented the highest F_{CT} values (0.434 and 0.386; all at $P < 10^{-4}$, respectively).

Discussion

Bottleneck in sugar beet cultivars

In this study we compared genetic diversity at both nuclear and mitochondrial level of a large set of accessions from the four forms of the *B. vulgaris* complex. Results of nuclear polymorphism analysis using five microsatellite loci clearly show a deficit in allelic richness in sugar beet cultivars, compared to wild, ruderal and weeds beet populations. This low level of genetic diversity is likely to result from the bottleneck associated to domestication and the ensuing breeding process and has been widely documented in both plant and animal domesticated species (e.g. Eyre-Walker et al. 1998; Clark et al. 2004; Otero-Arnaiz et al. 2005; Vasemagi et al. 2005; Zhu et al. 2007).

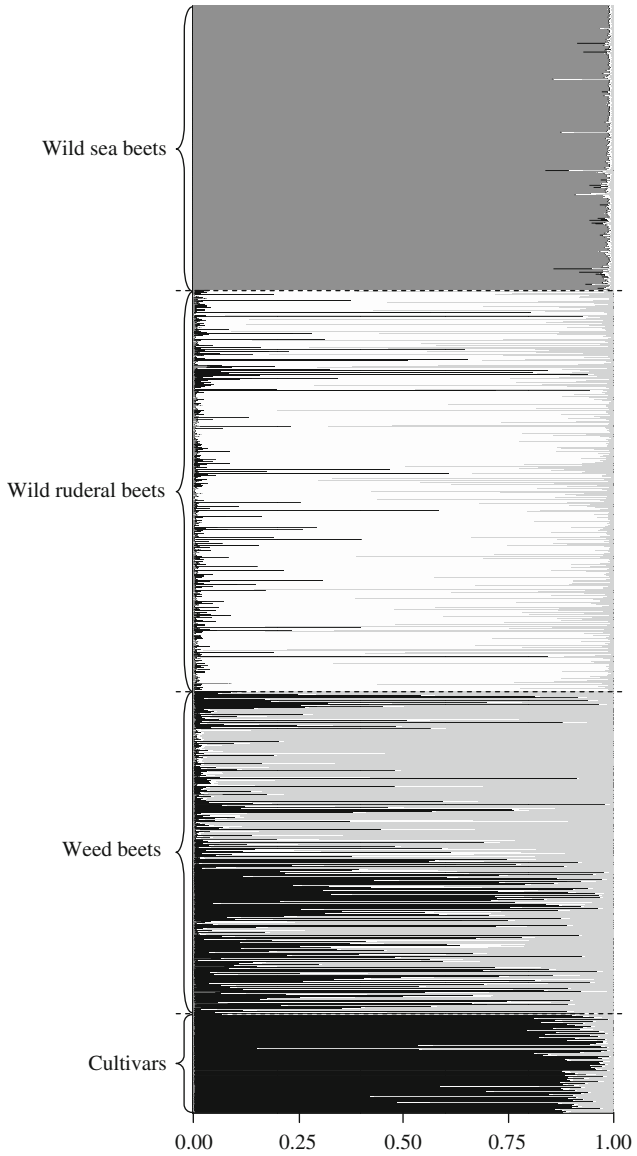

Fig. 4 Bayesian analysis of the nuclear genetic structure within the *Beta vulgaris* species complex. Individual membership was estimated assuming a number of $K = 4$ clusters and using no prior information on taxon membership (see text for explanations). Each individual was represented by a thin vertical line, which was partitioned into four coloured segments that indicated the individual's membership fractions into the four clusters (*dark grey* wild sea beets, *white* wild ruderal beets, *light grey* weed beets and *black* cultivars)

The genetic bottleneck related to the breeding process is expected to be higher in cytoplasmic genomes, especially when a particular character such as cytoplasmic male sterility is used (Provan et al. 1999). This is currently the case in sugar beet since, using four mitochondrial minisatellites, we only found a single haplotype over the 35 analysed cultivars coming from 13 European seed companies, compared to a total of ten mitochondrial haplotypes found within the wild sea beet and ruderal beet populations (Fig. 2). This unique haplotype found in cultivars corresponds to the combination of alleles 500, 404, 420 and 438, for minisatellite loci Tr1, Tr2, Tr3 and Tr4, respectively and is strictly associated with the OwenCMS cytoplasm (see also Fénart et al. 2006). While important efforts have been made to improve sugar beet using wild relatives (reviewed in Panella and Lewellen 2007), only one CMS germplasm has been used in sugar beet cultivars for decades (Owen 1945). Several different CMS types have been described in *B. vulgaris* ssp. *maritima* (Cuguen et al. 1994; Desplanque et al. 2000) and for exemple Touzet et al. (2004) proposed that *G* CMS could be a valuable alternative in sugar beet breeding.

Interestingly, a very low genetic differentiation (based on pairwise F_{ST} estimates, see Electronic supplementary material S1) was observed among the 13 cultivar samples each corresponding to a single sugar beet seed company. Together, these findings confirm the wide use of a restricted set of related maternal lines in cultivars (Owen 1945) as well as the use of more differentiated paternal lines which may result from a choice of the breeders to maintain a sufficient level of diversity as a baseline to future selection programs (McGrath et al. 1999).

Weed beet genetic diversity

Nuclear genetic diversity appeared to be high in weed beet populations, compared to sugar beet cultivars. Allelic richness and gene diversity for weed beets are of the same magnitude as in wild sea beet and wild ruderal beet populations and are significantly higher than in the cultivars. This is consistent with a wild paternal origin of weed beets and, as a consequence, confirms the hybrid origin of weed beets, characterised by a high level of genetic diversity owing to mixing of different gene pools. These high values of A_r and H_e observed in all weed beet populations highlight the large polymorphism introduced by the ruderal pollen donors as well as the recurrent introduction of new hybrids within these populations (see also Viard et al. 2002).

Furthermore, global F_{ST} value over weed beet populations (0.056; $P < 10^{-3}$) as well as pairwise genetic differentiation among weed beet populations (see Electronic supplementary material S1) underlined the genetic isolation of weed beet populations even when populations are in the vicinity of each other (e.g. W_{08} and W_{09}), implying independent founding events and distinct demographic histories, even if pollen of weed beet can disperse over large distances (Fénart et al. 2007). Additionally, no significant correlation between pairwise F_{ST} estimates and geographical distances between weed beet populations was observed ($r^2 = 0.0318$; $P = 0.78$, Mantel test after 10,000 permutations), that is no genetic isolation by distance was detected.

In addition, strong significant heterozygote deficiencies were found within eight populations of weed beets out of 12 with a mean F_{IS} estimate of 0.073 ($P < 10^{-3}$). This find-

ing, almost unexpected in a self incompatible species such as *B. vulgaris* (Larsen 1977; Bruun et al. 1995), is nevertheless in accordance with previous studies that already revealed such departures from Hardy–Weinberg equilibrium within weed beet populations (Viard et al. 2002; Arnaud et al. 2003; Cuguen et al. 2004; Viard et al. 2004) compared to wild sea beet populations (Fievet et al. 2007). These significant heterozygote deficiencies can be due either to population spatial substructuring or to a mixture of cohorts, related to recurrent infestation events and to differential recruitments of the soil seed bank; both events producing either a spatial or temporal Wahlund effect (Wahlund 1928; Hattemer 1982). Moreover, besides the intrinsic self-incompatibility of *B. vulgaris* taxa, a dominant mendelian self-fertility factor has been identified by Owen (1942). It was then widely introduced in the cultivated germplasm to produce inbred lines (Mackay et al. 1999). Some weed beets could have inherited this self-fertility factor from their cultivated maternal parent within the seed production area and, therefore introduced it into the weed populations present in sugar beet fields, with in an associated way, significant heterozygote deficiencies.

As weed beets result from accidental pollination events of seed bearers by ruderal beets, they are expected to carry the same cytoplasm as their cultivated maternal parent, i.e. OwenCMS, and thus to present a uniformity of mitochondrial DNA (Boudry et al. 1993). The signature of a cultivated maternal origin of weed beets has been revealed in 9 weed beets populations out of 12 (89.07% of the sampled individuals). However, three different cases of discrepancy between sugar beet and weed beet cytoplasm were revealed by our study and concerned three populations (W_{06}, W_{08} and W_{12}, see Table 2). In those populations, besides the OwenCMS mitotype, the most frequent minisatellite haplotypes are associated with the mitotype *Nvulg* (Fig. 2), the most widely found mitotype in the wild (Cuguen et al. 1994; Forcioli et al. 1998; Desplanque et al. 2000) and considered to be the mitochondrial ancestral state in *B. vulgaris* (Fénart et al. 2006). The presence of this cytotype could be related to the cultivation of ancient cultivars that did not carried the OwenCMS cytoplasm and that could have been conserved in the seed bank. The two remaining non-OwenCMS minisatellite haplotypes concerned two individuals of the coastal population of Wissant A (W_{08}). The first one, 531-404-420-410, was described in a large panel of wild sea beet populations of the French Channel Coasts where it was found in a strict association with mitotype *E*, a wild CMS mitotype (Fievet et al. 2007). Its occurrence in weed beets can be related to the presence of wild populations in the neighbourhood of this field (J.-F. Arnaud and S. Fénart, personal observations), highlighting a possible wild-to-weed seed movement. Arnaud et al. (2003) illustrated the possibility of human mediated seed movement from sugar beet fields to a wild population 1.5 km away. Our results show that the opposite movement can also be detected, underlining locally a possible influence of wild sea beet populations on the genetic diversity of weed beet populations. The last minisatellite haplotype, 695-500-482-350, has been found in association with two rare mitotypes *H* and *N* (Fig. 2, see also Fénart et al. 2006), both observed only in wild ruderal populations within the cultivar seed multiplication area (Desplanque et al. 2000). Their presence in weed beet populations suggests the possibility of accidental contamination by wild ruderal plants during the seed multiplication process.

Relationship within the *B. vulgaris* complex

The status of weed beets as the result of accidental and recurrent hybridisation between ruderal beets and cultivated seed bearers in the seed production area has been widely discussed (Boudry et al. 1993; Cuguen et al. 1994). However, since the preliminary study of Desplanque et al. (1999) based on few polymorphic markers, no further genetical investigations have been performed to finely assess genetic relationships within the *B. vulgaris* complex, i.e. cultivated, weed, ruderal and sea beets.

To address this issue, we investigated the genetic divergence within the *B. vulgaris* complex by focusing on a large panel of populations including the 35 sugar beet cultivars, 12 weed beet populations, 12 populations of ruderal beets sampled in the sugar beet seed production area in south-western France and 11 populations of sea beet from the French Channel coasts. The unrooted neighbour-joining tree, presented in Fig. 2, revealed a striking monophyletic clustering of accessions as a function of their origin, showing that the classification into cultivated, wild, ruderal and weed forms according to their geographical location, phenotype or their domesticated status is clearly supported by genetic data. Results of the analyses of molecular variance are also in complete agreement with a strict clustering of wild, weed and cultivated forms of beet. To gain further insights, pairwise F_{CT} were computed for each possible pair of groups and clearly documented: (1) the close intermediate taxonomical position of weed beets between cultivars and wild ruderal beets, reinforcing their hybrid status between these two forms; (2) closer genetic affinities of wild ruderal beets with weed beets lineages and cultivars compared to wild sea beet populations sampled along the Channel coastline (3) the clear genetic divergence of wild sea beets compared either to cultivars and weed beets. In particular, nuclear genetic diversity was strongly pronounced when wild sea beet populations were compared with cultivated and weed populations (F_{CT} of 0.434 and 0.386; both at $P < 10^{-4}$, respectively).

This striking genetic distinctiveness was not completely expected given (1) the recent evolutionary divergence of

the different groups through domestication process and (2) the full cross-compatibility within the *B. vulgaris* complex (Letschert 1993). The topology of the tree is also in agreement with the one presented in Desplanque et al. (1999) but highlights the intermediate position of weed beets between cultivars and wild populations. Furthermore, the results of the Bayesian analysis of individual clustering also indicated a clear genetic distinctiveness between wild sea beet, wild ruderal beets and cultivars. A genetic proximity between weed beets and cultivars was also revealed as a significant part of weed beet individuals clustered into the group of cultivars. Such individuals may be cultivar bolters with a low level of vernalization requirement.

Cytoplasmic data revealed an even more important genetic distinctiveness, not only between taxa but also within populations of the same group ($F_{CT} = 0.479$ and $F_{SC} = 0.416$ and see Table 2 for F_{ST} values within each taxa). Indeed, cytoplasmic effective population size is at least two fold lower than nuclear one in a gynodioecious species (Laporte et al. 2000), and seeds of *B. vulgaris* have no particular mechanism for long distance dispersal and, except accidental seed movements previously invoked, seed dispersal is expected to be restricted, leading to a stronger cytoplasmic differentiation compared to nuclear markers (see Fievet et al. 2007, for similar results on wild sea beet populations).

The origin of inland ruderal beet has been subject to debate, being presented either as natural forms of non-coastal wild beets (De Bock 1986) or as originated from feral forms and as a consequence related to cultivated beets (Bartsch et al. 1993, 1999). Our finding of high cytoplasmic and nuclear genetic diversities is consistent with the results of Desplanque et al. (1999) considering ruderal form as a wild relative of *B. vulgaris*. However, even if ruderal beets belong to the wild compartment, as revealed by their genetic diversity both at the nuclear and cytoplasmic level, their genetic distinctiveness from wild sea beet populations sampled on the Channel coast suggests distinct evolutionary histories and may support a common ancestry of French South Western ruderal beets with Mediterranean sea beet populations. Nevertheless, the topology of the neighbour-joining tree as well as the results of the AMOVA also suggests a genetic proximity between ruderal populations and sugar beet cultivars. It should also be noted that few individuals of inland ruderal beet (2.3%) exhibited high values of individuals clustering (>0.75) to the cultivar cluster (Fig. 4). This may reflect a possible gene flow from sugar beet cultivars to wild ruderal beet populations, due to the spatial proximity of ruderal populations with seed multiplication fields that produce high amounts of pollen. As a consequence, these populations have to be taken into account with extreme care for the risk assessment of (trans)gene escape in the wild within the seed production areas (Bartsch et al. 1999).

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ANNEXE II

M. DUFAY, V. VAUDEY, I. DE CAUWER, P. TOUZET, J. CUGUEN & J.-F. ARNAUD (2008) Variation in pollen production and pollen viability in natural populations of gynodioecious Beta vulgaris spp. maritima: evidence for a cost of restoration? *Journal of Evolutionary Biology*, **21**: 202-212.

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Variation in pollen production and pollen viability in natural populations of gynodioecious *Beta vulgaris* ssp. *maritima*: evidence for a cost of restoration of male function?

M. DUFAŸ, V. VAUDEY,[1] I. DE CAUWER, P. TOUZET, J. CUGUEN & J.-F. ARNAUD

Laboratoire de Génétique et Évolution des Population Végétales, UMR-CNRS 8016, Université des Sciences et Technologies de Lille – Lille 1, Villeneuve d'Ascq Cedex, France

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cost of restoration;
cytoplasmic male sterility;
gynodioecy;
partial male sterile;
pollen quantity;
pollen viability.

Abstract

Gynodioecious species are defined by the co-occurrence of two clearly separated categories of plants: females and hermaphrodites. The hermaphroditic category may, however, not be homogeneous, as male fitness may vary among hermaphrodites as a result of many biological factors. In this study, we analysed estimates of pollen quantity and viability in the gynodioecious *Beta vulgaris* ssp. *maritima*, comparing hermaphrodites bearing a male-fertile cytotype and hermaphrodites bearing cytoplasmic male sterility (CMS) genes, which are counteracted by nuclear restoration factors. We show that: (i) pollen quantity continuously varies among restored hermaphrodites, suggesting a complex genetic determination of nuclear restoration; (ii) pollen viability was lower in restored (CMS) hermaphrodites than in non-CMS hermaphrodites, probably because of incomplete restoration in some of these plants; and (iii) pollen quantity and viability also varied among hermaphrodites with male-fertile cytotypes, possibly a result of a silent cost of restoration. Finally, we discuss the consequences of these results for pollen flow and the dynamics of gynodioecy.

Introduction

Gynodioecy is a plant breeding system where females and hermaphrodites coexist in populations. This breeding system is the second most common in the European Flora (Richards, 1997). For decades, this system has been a topic of interest for evolutionary biologists for several reasons. First, it can be considered as a possible evolutionary step from hermaphroditism to dioecy and may then constitute a key system to understand the evolution of plant reproductive systems (Charlesworth & Charlesworth, 1978; Desfeux *et al.*, 1996; Barrett, 2002). Second, sex expression in gynodioecious species often results from complex genetic interactions between cytoplasmic male sterility (CMS) genes and nuclear restorer genes (Chase, 2007); this provides the basis for a genetic conflict between cytoplasmic and nuclear genes due to the effect of opposite selective pressures on the sexual phenotype of plants (Cosmides & Tooby, 1981; Saumitou-Laprade *et al.*, 1994; Hurst *et al.*, 1996; Werren & Beukeboom, 1998; Jacobs & Wade, 2003; Delph *et al.*, 2007).

According to evolutionary theory, CMS can spread in a population as soon as fitness through female function is higher in females than in hermaphrodites (female advantage; Charlesworth & Ganders, 1979; Frank, 1989; Gouyon *et al.*, 1991). As cytoplasmic genes are only transmitted through seeds, the loss of the male function (i.e. pollen production) in females is not associated with a cost for CMS genes. Indeed, either only a moderately larger number of seeds or a slightly better quality of seeds is likely to provide CMS with a selective advantage. On the other hand, the loss of pollen production entails a loss of transmission for nuclear genes. As soon as CMS becomes frequent in a population, nuclear alleles that are able to restore pollen production may be selected. Two main factors have been proposed to explain why restorer genes are not driven to fixation in gynodioecious species: (i) gene flow and metapopulation processes that regularly introduce new CMSs in populations (Frank, 1989; Couvet

Correspondence: Mathilde Dufaÿ, Laboratoire de Génétique et Évolution des Populations Végétales, UMR CNRS 8016, Bâtiment SN2, Université des Sciences et Technologies de Lille – Lille 1, 59655 Villeneuve d'Ascq Cedex, France. Tel: +33 3 20 33 59 23; fax: +33 3 20 43 69 79; e-mail: mathilde.dufay@univ-lille1.fr
[1]*Present address*: Centre d'Ecologie Fonctionnelle et Evolutive, UMR-CNRS 5175, Montpellier, France.

et al., 1998); or (ii) a cost induced by restorer alleles: when the corresponding CMS becomes less frequent, restoring male fertility provides no or a small selective advantage, so that the cost causes a decrease in restorer frequency (Charlesworth & Ganders, 1979; Frank, 1989; Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufaÿ *et al.*, 2007). In the latter case, frequency-dependent selection can lead either to limit-cycle dynamics (i.e. undamped oscillations) or to stable equilibrium, that can be reached after a prolonged period of dampened oscillations (Dufaÿ *et al.*, 2007). Thus, both these dynamics may explain the high variations in female frequencies that are sometimes observed among populations in gynodioecious species (Frank, 1989; Gouyon *et al.*, 1991).

Whereas several empirical studies have found a female fitness advantage in gynodioecious species (Alonso & Herrera, 2001; Shykoff *et al.*, 2003 for reviews), little is known about restorer alleles. In particular, although the cost of restoration is a central theme in theoretical studies, only two studies have empirically suggested that restorer genes could negatively affect plant fitness, either through female (de Haan *et al.*, 1997a) or male function (Bailey, 2002). Moreover, although models often assume that restoration of male fertility is achieved through one allele (but see Frank, 1989), empirical work suggests that genetic determination of restoration may be more complex (Charlesworth & Laporte, 1998; Koelewijn, 2003; Touzet *et al.*, 2004). Indeed, a recent study, based on three of the best studied gynodioecious species, pointed out that restoration may be often polygenic (Ehlers *et al.*, 2005). If more than one gene of restoration is involved, male fitness is then expected to quantitatively vary among plants, which could slow down the selection of restorers and thus strongly influence the dynamics of gynodioecy (Charlesworth & Laporte, 1998). To our knowledge, however, few studies have quantified variation in pollen production and quality among gynodioecious populations (but see Glaettli & Goudet, 2006).

In some cases, variation in male fitness among plants is further increased by the presence of cytoplasmic polymorphism in populations. Indeed, although only the most well-known gynodioecious species contain CMS cytoplasms (*Silene vulgaris*: Olson & McCauley, 2002; *Silene acaulis*: Städler & Delph, 2002; *Thymus vulgaris*: Belhassen *et al.*, 1991), this does not seem to be the rule for all species. In particular, non-CMS cytotypes have been reported in at least two gynodioecious species (*Plantago lanceolata*: de Haan *et al.*, 1997b; *Beta vulgaris* ssp. *maritima*: Cuguen *et al.*, 1994). It should be noted that male-fertile cytoplasm in populations includes 'normal' cytoplasm that never produced any sterilizing factors or sterilizing cytoplasm for which the nuclear restorer genes are currently fixed. Because of the lack of CMS markers and data from reciprocal crosses (e.g. Van Damme *et al.*, 2004), the real occurrence of male-fertile cytotypes is usually unknown. Thus, for many gynodioecious species, we do not know whether all hermaphrodites are in fact restored hermaphrodites (CMS) or whether both restored CMS and non-CMS hermaphrodites co-occur in populations. In the latter case, these hermaphrodites may also differ in their pollen production and male fitness abilities.

The cost of restoration, complexity of genetic determination of restoration and co-occurrence of hermaphrodites having different cytoplasms are all possible sources of variation in male fitness and may strongly affect pollen flow, selection of restorer genes and ultimately, the dynamics of gynodioecy. The aim of this study was to assess variation in hermaphrodite potential male fitness in terms of pollen quantity and quality estimated in two natural populations of the gynodioecious wild beet, *B. vulgaris* ssp. *maritima*, including normal hermaphrodites and hermaphrodites bearing one of the two most frequent CMS cytotypes found in wild beet populations (CMS *E*, see Fénart *et al.*, 2006). First, we will examine how pollen production varies among CMS plants, which will give us some clues about the genetic determination of restoration. Second, we will compare pollen quantity and quality in non-CMS and CMS hermaphrodites and discuss the possible consequences on selection of restorer alleles. Third, because frequency-dependent selection is supposed to play an important role in the maintenance of the system, we will compare pollen quantity and quality between two populations that are at two difference stages of gynodioecy dynamics, in terms of CMS and restorer frequencies.

Material and methods

The species

Beta vulgaris ssp. *maritima* is a gynodioecious short-lived perennial species, which is widely distributed along the western coasts of Europe and around the Mediterranean Basin. In northern Europe, wild beets mainly colonize areas located along estuaries, just at the upper level of the tide and, more rarely, cliffs overhanging the sea (Letschert, 1993; Laporte *et al.*, 2001; Fievet *et al.*, 2007). When plants bolt to flowering, stems develop with a main axis bearing flowering branches and sometimes secondary flowering axes. Consequently, each plant can produce up to several thousands of flowers. Plants are self-incompatible and pollinated by wind. In wild beet populations, hermaphrodites often coexist with male-sterile plants that do not produce pollen. Among the 20 mitochondrial haplotypes described in beet, four haplotypes are clearly associated with male sterility: *E*, *G*, *H* and *Svulg* (Saumitou-Laprade *et al.*, 1993; Cuguen *et al.*, 1994; Laporte *et al.*, 1998; Desplanque *et al.*, 2000; Touzet *et al.*, 2004). In contrast with other gynodioecious species, nonsterilizing cytotypes constitute a large part of the mitochondrial diversity in wild beet (Cuguen *et al.*, 1994; Forcioli *et al.*, 1998; Laporte *et al.*, 2001). Moreover, the most frequent cytoplasm is male fertile and because the four

different CMS are derived from it, this male-fertile cytotype is likely to be ancestral (Fénart *et al.*, 2006).

Study populations and sampling procedures

This work was carried out in two natural gynodioecious populations of wild beet, located on the northern coast of France, 30 km apart. Both populations were linear and comprised approximately 400 plants. One population (Audresselles, 50°49.101′N; 1°35.676′E) was located on a seaside resort; the other population (Canche, 50°32.231′N; 1°35.597′E) was located along the estuary of the river Canche. In April 2005, a total of 398 plants were continuously sampled along the populations: 280 plants were collected in Audresselles and 118 plants were collected in Canche. Sampling consisted in leaf collection and plant labelling and was not affected by plant sex as it took place before flowering. During flowering in June 2005, we recorded the sex ratio by assigning a sexual phenotype to each labelled flowering plant. Plants with brown or white and much reduced anthers were considered to be male sterile. Plants with yellow anthers that produced pollen were considered to be hermaphrodites. Some of the plants had an intermediate phenotype that produced more light-coloured yellow anthers with no obvious pollen grains and, as such, these plants were categorized as intermediate phenotypes. In total, 273 and 96 plants were assigned to a sexual phenotype in Audresselles and Canche respectively; not all labelled plants flowered and some plant could not be retrieved during the second survey in June.

Molecular analyses

In wild beet, among the four CMSs that have been described, the three most widely distributed are the Owen CMS (also called *Svulg*), which is widely used in plant breeding of sugar beet (Owen, 1945; Arnaud *et al.*, 2003), and two additional ones called *E*, and *G*, which are exclusively found in wild beet populations (Cuguen *et al.*, 1994; Desplanque *et al.*, 2000; Laporte *et al.*, 2001). Extraction and purification of total DNA from the 398 sampled plants were performed using a NucleoSpin®96 Plant Kit, and following the standard protocol for isolation of DNA from dried plant leaf tissue outlined in the NucleoSpin®96 Plant Kit protocol handbook (Macherey-Nagel, Düren, Germany). To characterize the three main CMSs, we used diagnostic cytoplasmic PCR markers. The PCR step for each CMS was conducted using standard procedures. The primers used to amplify the chloroplastic marker associated with CMS *Svulg* are described in Ran & Michaelis (1995). To detect CMS *G*, one unpublished pair of primers was used to amplify a specific fragment (forward primer: TTTCTCTTTATGGATAACCAATTCA – reverse primer: AGGATTCCTTTTGTAACCCAAT). CMS *E* was detected by PCR-RFLP (forward primer: GTTCCC ACTCACGACCCATA – Reverse primer: CCGACTAGTT CCGGGTTC) using the following procedure: 5 μL of the PCR product was digested in a 10-μL reaction with 0.2 mM of spermidin and 1.5 U of *Alu*I, conducted at 37 °C overnight.

Pollen sample and analysis

Pollen was sampled on plants at the beginning of the flowering period during June 2005. In both populations, we selected genotyped plants, such that both CMS and non-CMS plants were sampled. We focused on CMS type *E*, which was the most frequent CMS cytotype in Audresselles and the only CMS cytotype in Canche (see results below). Moreover, we selected plants that were at the beginning of their flowering phase to obtain results for pollen production that could be compared among plants. In total, pollen was sampled on 115 plants: 43 non-CMS and 23 CMS plants in Audresselles, and 13 non-CMS and 36 CMS plants in Canche. For each plant, two floral buds localized on two different stems of equal size were chosen and two anthers per bud were collected and stored separately in ethanol at 95 °C. Ethanol was then evaporated and samples were placed in oven at 56 °C for 24–48 h to force anther dehiscence. One millilitre of distilled water was then added to each pollen sample and sonicated to separate pollen grains from the anther and each other. Tubes were then vortexed and the number of pollen grains was estimated in 200 μL of solution (this number multiplied by five gives the total pollen count). A particle counter CASY® model TT (Innovatis, Bielefeld, Germany) was used to estimate the number of pollen grains in a solution of 5 mL of pure water CASY® ton for cell counter, in which the 200 μL of distilled water and pollen were diluted. Each sample was shaken to equally distribute pollen in the solution immediately prior to counting. The particle counter then sampled three volumes of 400 μL from the solution and provided the result for the total 1200 μL analysed. The number of detected particles was determined for 400 size classes ranging from 0.125 to 50 μm using the software CASY® excel 2.1. Prior observations had shown that nonviable pollen grains in *B. vulgaris* ssp. *maritima* were of smaller size than viable pollen grains (Boutin *et al.*, 1987). These counts were then used to estimate both total pollen production and fraction of viable pollen grains. Every 20 tubes a blank solution was analysed to estimate the size classes corresponding to pollen grains, by comparing pollen samples with empty solutions.

Pollen viability

Pollen viability was also estimated with Alexander stain on a subsample of plants to assess the correlation between pollen grain size and viability. To cover the largest variance in pollen viability, we sampled both non-CMS hermaphrodites and plants bearing CMS *E* cytotype, including (restored) hermaphrodites and

intermediate phenotypes. On these plants, an additional freshly opened flower was collected the same day than the floral buds used for particle counter analysis. Within 3 h of collection, pollen was removed from the flower and placed on a glass slide. One drop of Alexander solution (10 mL of 95% ethanol, 1 mL of 1% malachite green in 95% ethanol, 5 g of phenol, 5 mL of 1% acid fuschin in H_2O, 0.5 mL of 1% orange G in H_2O, 2 mL of glacial acetic acid, 25 mL of glycerol and 50 mL of H_2O; Alexander, 1969), which stains viable pollen purple, was added to each pollen sample. A coverslip was used to mix and cover the pollen and Alexander mixture, after which the coverslip was sealed using clear nail varnish. The pollen samples were then examined under a light microscope at ×100 magnification. More than 200 pollen grains per sample, when available, were scored as either purple or green, and the viable proportion of pollen grains was calculated as the ratio of purple-stained pollen grains to the total number of pollen grains. We estimated pollen viability for, respectively, eight and 17 plants in Audresselles and Canche, among which 12 carried a CMS *E* cytotype. It should be noted that Alexander stain is only an estimation and not an absolute measure of viability. Keeping this in mind, we will consider thereafter that it will be a convenient estimate of viability.

Data analyses

Typical peaks of particles with size ranging from 10 to 24 μm were observed on samples collected from hermaphrodites, which clearly did not appear on blank samples; thus, we only considered this 10–24 μm zone for pollen counting. Two subpeaks were recognizable within this size range: 10–13 and 16–24 μm, with some individuals specifically producing one of the two peaks and some others producing both (Fig. 1). We categorized particles within these two size classes, and considered three variables for subsequent analyses: total pollen quantity, quantity of large pollen grains (16–24 μm) and ratio of large pollen grains. For each of these three variables, the average value over the four collected anthers per plant was calculated and assigned to each studied plant. The total number of pollen grains and the number of large pollen grains were obtained from the values provided by the particle counter after correcting for the dilution ratio, i.e. by multiplying all values by $5 \times 5200/1200$. The factor 5200/1200 allows to estimate the quantity of pollen grains in the 5-mL solution in which the particle counter sampled, and the five factor allows to estimate the quantity of pollen in the whole anther, as only 200 μL over a total of 1 mL was used.

Because the quantity of pollen grains appeared to continuously vary among CMS plants (see below), we used the total number of pollen grains (small and large) produced per anther to objectively distinguish between the females and restored hermaphrodites: individuals that produced a small number of pollen grains were considered as females (see below). On this basis, the effects of sexual phenotype (three levels: females (CMS), restored hermaphrodites (CMS) and non-CMS hermaphrodites) and of population (two levels) were tested on

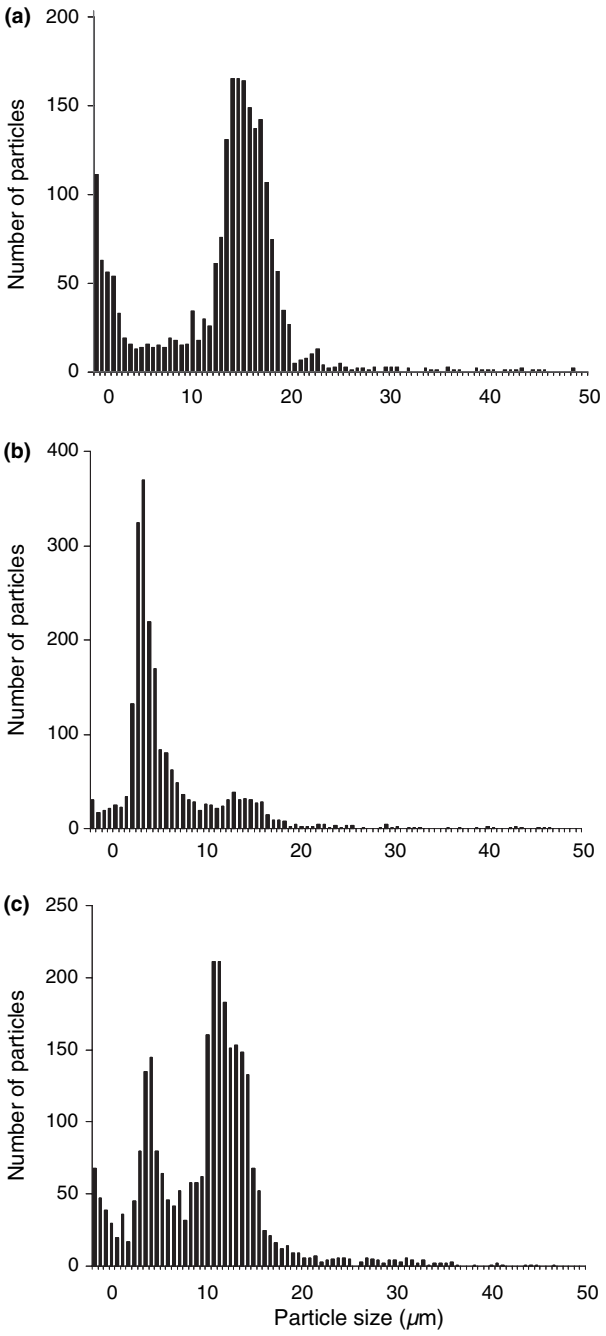

Fig. 1 Examples of particle counting: number of particles as a function of particle size (μm). These depict the pollen zone (10–24 μm) for three different hermaphroditic plants. Plant (a) had one major peak of large pollen grains (16–24 μm), plant (b) produced mainly small pollen grains (10–13 μm) and plant (c) produced both.

the frequency and the number of large pollen grains, using an ANOVA (proc GLM, SAS, SAS Institute Inc., Cary, NC, USA). The effects of population and sexual phenotype were also tested on the total number of pollen grains per anther; however, because the number of pollen grains was used to distinguish between females and restored hermaphrodites, females were not included in the data set and this variable was compared only between restored hermaphrodites and non-CMS hermaphrodites. Data were either log transformed (number of pollen grains and number of large pollen grains) or arcsine-square root transformed (frequency of large pollen grains) to obtain normally distributed residuals.

Results

CMS cytoplasm frequency, sex ratio and restoration rate in populations

Three PCR markers specific to CMSs enabled us to identify all females. Number of CMS plants, sex ratios and restoration rate (i.e. for a given CMS, the ratio of the number of restored hermaphrodites to the total number of individuals bearing the CMS) in each population are given in Table 1. Whilst sex ratios were similar in both populations (29% females in Audresselles and 31% in Canche), CMS frequencies and restoration rates differed among the study populations. Three different CMSs (*E*, *G* and *Svulg*) were found in Audresselles, with CMS *E* being the most frequent (30% of sampled plants vs. 2% and 0.7% for CMSs *G* and *Svulg* respectively). The rates of restoration for the different CMSs were 0.12, 0 and 1, for CMSs E, G and *Svulg* respectively. These different rates of restoration among the three CMSs are consistent with another study conducted on a larger geographical scale (P. Touzet, unpublished data). In contrast to the Audresselles population, only the CMS *E* was found in Canche. This CMS occurred at a higher proportion than in Audresselles: 79.6% of the 118 sampled plants had the CMS *E*. In this population, the rate of restoration was much higher than in Audresselles: 60% or 42%, depending on whether intermediate phenotypes were included in the estimation or not.

Pollen size vs. pollen viability

The frequency of viable pollen, estimated as the frequency of purple grains with Alexander stain, ranged from 0.09 to 0.99 among the 25 studied plants. For each of these plants, the value of pollen viability was analysed together with the average frequency of large pollen grains obtained with the particle counter: the Pearson's correlation coefficient between the two variables was 0.9 (Fig. 2). In agreement with the results obtained by Kelly *et al.* (2002) on *Mimulus guttatus* and *Collinsia verna*, estimating pollen size is robust method to estimate pollen viability in *B. vulgaris* spp. *maritima*. We will thus use the frequency of large pollen grains as a measure of pollen viability.

Pollen production in CMS *E* plants

As CMS *E* was the only CMS found in Canche, we focused on this cytotype. The average quantity of pollen per anther (including both large and small grains) was highly variable among CMS *E* plants, ranging from 781 to

Table 1 Number of plants described as females, intermediates and hermaphrodites for each cytotype (CMSs *E*, *G* and *Svulg* and male fertile cytotype *Nvulg*) in both populations.

Cytotype	Females	Intermediates	Hermaphrodites	Unknown	Total	Restoration rate
Audresselles						
CMS *E*	73	0	10	1	84	0.12
CMS *G*	6	0	0	0	6	0
CMS *Svulg*	0	0	2	0	2	1
Non-CMS	0	0	182	6	188	–
Total	79	0	194	7	280	–
Sex ratio (%)	29	0	71	–	–	–
Canche						
CMS *E*	30	24	22	18	94	0.6 (0.42)
CMS *G*	0	0	0	0	0	–
CMS *Svulg*	0	0	0	0	0	–
Non-CMS	0	0	20	4	24	–
Total	30	24	42	22	118	–
Sex ratio (%)	31	25	44	–	–	–

Cases for which sex was unknown were plants that had been sampled before flowering, and which either were not flowering or were misled in June. The rate of restoration for a given CMS is the frequency of restored hermaphrodites divided by the total number of individuals, for which a sexual phenotype had been assigned and carrying the CMS under consideration. In the Canche population, two rates of restoration were calculated, taking into account either only restored hermaphrodites or both restored hermaphrodites and intermediates (within brackets).

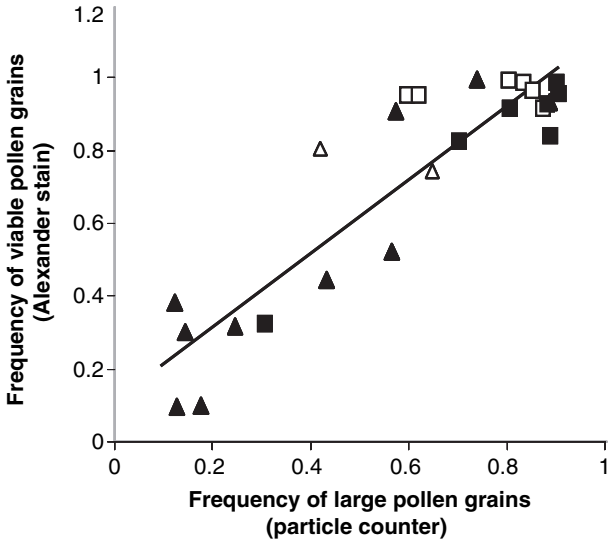

Fig. 2 Correlation between pollen size and pollen viability in *Beta vulgaris*, measured on 25 plants from two wild populations of Canche (black symbols) and Audresselles (open symbols), comprising both CMS (triangles) and male-fertile (squares) hermaphrodites.

45 520 pollen grains per anther (Fig. 3). Plants that were categorized as females during flowering (i.e. having brown or white and reduced anthers) produced less than 7000 pollen grains. All plants categorized as hermaphrodites (i.e. having yellow anthers and obvious pollen production), thus being restored hermaphrodites, produced more than 13 000 pollen grains. Finally, plants categorized as intermediates in the field, i.e. with more light-coloured yellow anthers and no obvious pollen grains (labelled with an asterisk in Fig. 3) were also intermediates in terms of pollen quantity, and slightly overlapped with females. This suggests that the quantity of pollen varies continuously among CMS *E* plants.

For following analyses, we needed to distinguish among CMS plants, females from plants that express one of several nuclear restoration alleles. CMS plants were thus categorized in two groups, based on their average pollen quantity per anther. The first group (within boxes in Fig. 3) included all plants that had been categorized as females and two plants that had been categorized as intermediates but that produced average quantities of pollen comparable with females. Thereafter, we will consider these plants as females, with little or no pollen production. The second group (outside boxes in Fig. 3) consisted of plants that had been considered as potential pollen producers (categorized in the field as intermediates or hermaphrodites). For the rest of the study, we will consider them as CMS plants bearing one or several restoration alleles of male fertility and we will refer to them as restored hermaphrodites.

The proportion of viable pollen was also highly variable among CMS *E* plants, ranging from 12% to 90%, but was not correlated with the number of pollen

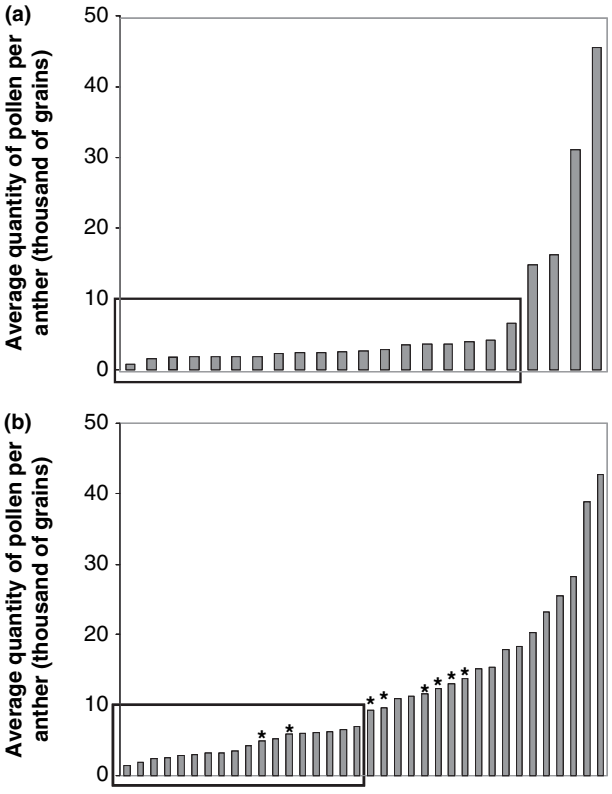

Fig. 3 Plants carrying the CMS *E* cytotype, from Audresselles (a) and Canche (b) populations, ranked for their quantity of pollen per anther (average value over the four measured anthers). Symbols (*) indicate the plants that had been categorized as intermediates in the field, with lightly coloured yellow anthers and no obvious pollen production. The boxes contain all plants that will be considered as females in further analyses.

grains, irrespective of whether females were included in the data set or not [effect of the ratio of viable pollen on the total number of pollen grains: $P > 0.05$, Proc GLM; Pearson's coefficients $r = 0.13$ ($n = 59$) and -0.31 ($n = 22$) for analyses on data set with and without females respectively].

Variation of pollen quantity and viability among sexual phenotypes and populations

The total number of pollen grains (viable and nonviable pollen grains) did not significantly differ between CMS-restored hermaphrodites and non-CMS hermaphrodites ($F_{1,77} = 0.03$; $P = 0.85$); only the population had an effect, individuals from Audresselles producing significantly more pollen grains than individuals from Canche ($F_{1,77} = 14.5$; $P = 0.003$). The ratio of viable pollen grains differed significantly among populations and sexual phenotypes, with a marginally significant effect of their interaction (Table 2). Overall, plants from Audresselles produced a higher proportion of viable pollen than plants

Table 2 ANOVA of size of pollen grains, as a measure of pollen viability in *Beta vulgaris* on a subsample of plants of Audresselles and Canche populations (n = 115).

Source	d.f.	Type III SS	F	P
Population	1	0.86	17.53	<0.0001
Sexual phenotype	2	2.10	21.38	<0.0001
Population × sexual phenotype	2	0.26	2.61	0.08
Error	109	5.36		

The frequency of viable (large) pollen grains per anther (arcsine-square root transformed) depended on the sexual phenotypes (females, restored hermaphrodites and true hermaphrodite individuals), the population and their interaction.

from Canche. Concerning the effect of sexual phenotype, non-CMS hermaphrodites produced a significantly (Tukey test, $P < 0.0005$) higher proportion of viable pollen grains (0.7) than females and restored hermaphrodites (respectively 0.41 and 0.47) with no significant difference between females and restored hermaphrodites (Tukey pairwise comparison, $P > 0.1$). The same sex effect holds when individuals classified as intermediates in the field were removed from the data set: restored hermaphrodites produced a lower proportion of viable pollen grains than non-CMS hermaphrodites (sex effect: $F_{1,107} = 20.30$; $P < 0.0001$). Regarding the marginally significant interaction between both factors, the pairwise comparisons indicated that non-CMS hermaphrodites from Audresselles produced a significantly higher proportion of viable pollen than non-CMS hermaphrodites from Canche (Tukey pairwise comparison, $P < 0.05$). By contrast, restored CMS hermaphrodites of the two populations were not significantly different from each other ($P > 0.1$).

When combining both variables (i.e. number of pollen grains and frequency of viable pollen grains), an estimation of the number of viable pollen grains could be obtained. Using this response variable, a significant effect was found for population, sexual phenotype and their interaction (Table 3). Overall, plants from Audresselles produced a significantly higher quantity of viable pollen grains than plants from Canche. Non-CMS hermaphrodites, restored hermaphrodites and females produced on average, respectively, 25 081 ± 2078 (n = 56), 9069 ± 2358 (n = 22) and 1305 ± 82 (n = 37) viable pollen grains per anther, with all values being significantly different from each other (Tukey pairwise comparisons, $P < 0.05$). The significant effect of the interaction between population and sexual phenotype was due to a difference between non-CMS hermaphrodites of Canche and Audresselles ($P < 0.05$), whereas the restored hermaphrodites of the two populations belonged to the same statistical category.

Table 3 ANOVA of the number of viable (large) pollen grains per anther (log transformed) in *Beta vulgaris*, on a subsample of plants of Audresselles and Canche populations (n = 115).

Source	d.f.	Type III SS	F	P
Population	1	6.59	12.69	0.0005
Sexual phenotype	2	118.70	114.31	<0.0001
Population × sexual phenotype	2	10.57	10.18	<0.0001
Error	109	56.59		

The number of viable pollen grains depended on the sexual phenotype (females, restored hermaphrodites and true hermaphrodites individuals), the population and their interaction.

Focus on non-CMS hermaphrodites

The same statistical analyses were conducted using the data set containing only non-CMS hermaphrodites. For all variables, a population effect was found (quantity of pollen grains: $F_{1,55} = 12.34$, $P = 0.0009$; frequency of viable pollen grains: $F_{1,55} = 10.66$, $P = 0.0019$; quantity of viable pollen grains: $F_{1,55} = 17.96$, $P < 0.0001$), non-CMS hermaphrodites from Audresselles producing higher quantity and quality pollen than non-CMS hermaphrodites from Canche. Regarding the ratio of viable pollen grains, it should be noted that population differences were not due to an overall lower pollen viability of all non-CMS hermaphrodites from Canche, but rather to a high proportion of non-CMS hermaphrodites from Canche that produced extremely low frequencies of viable pollen grains, whereas some others produced viable pollen in frequencies comparable with non-CMS hermaphrodites from Audresselles: 74% of non-CMS hermaphrodites from Audresselles showed a ratio of pollen viability higher than 0.8, against only 38% of non-CMS hermaphrodites from Canche (Fig. 4).

Discussion

Variation of pollen among CMS plants: some clues about the genetics of restoration?

The study on pollen quantity revealed a continuous variation of this trait among CMS plants, partly due to the occurrence of intermediate phenotypes (as already noticed by Boutin *et al.*, 1987). These intermediate phenotypes, visually recognizable with anthers being lighter coloured and with no obvious pollen production are also called partial male steriles and have been reported in *B. vulgaris* ssp. *maritima* in other CMS systems (Owen, 1942, 1945; Hjerdin-Panagopoulos *et al.*, 2002; Touzet *et al.*, 2004), as well as in other gynodioecious species (Van Damme & Van Delden, 1982; Koelewijn & Van Damme, 1996; Charlesworth & Laporte, 1998; Glaettli & Goudet, 2006). The presence of intermediate phenotypes usually suggests that at least two different loci or co-dominant alleles at the same locus are involved in the process of restoration.

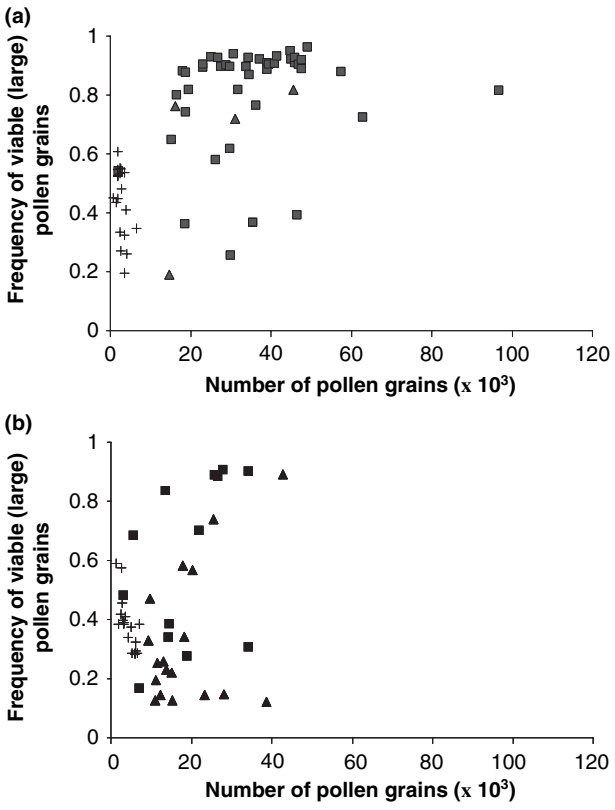

Fig. 4 Pollen viability and quantity in all plants in Audresselles (a) and Canche (b). Crosses picture females, triangles picture restored hermaphrodites and squares picture male fertile (non-CMS) hermaphrodites.

Moreover, in our study, the quantity of pollen per anther was highly variable, even among fully restored hermaphrodites (excluding intermediate phenotypes) (Fig. 3). This is probably at least partly due to environment: even though pollen production was measured for all plants at the beginning of flowering and in the same geographical region, one cannot exclude micro-environmental variation among plants. Future studies conducted in controlled conditions will help to understand whether the variation in pollen quantity among restored hermaphrodites is only due to environmental variations or whether polygenic determination of restoration can also be invoked in *B. vulgaris* ssp. *maritima*, as recently hypothesized for several gynodioecious species (Ehlers *et al.*, 2005).

We estimated variation in pollen viability among plants, using pollen size, a good estimator of pollen grains viability. Some plants had high pollen production but suffered from very low quality. As a matter of fact, pollen viability did not correlate with pollen quantity in restored CMS hermaphrodites, suggesting that another factor, independent of the process controlling pollen quantity, may act on viability. Viability of pollen also exhibited large variation among restored CMS hermaphrodites, but was not continuously distributed (Fig. 4). This suggests that pollen viability may have a simpler mechanism of genetic determination than the number of pollen grains or may be less sensitive to environmental variation. Interestingly, the same phenomenon was observed in an experiment in controlled conditions on a maternal progeny segregating for male fertility on another CMS (M. Dufaÿ, unpublished results).

Are restored hermaphrodites good pollen producers?

In most studied gynodioecious species, only two types of plants are considered: females and restored hermaphrodites, both bearing CMS genes. In such case, dynamics and maintenance of gynodioecy rely on differences in fitness between these two types of plants. In the case of *B. vulgaris* ssp. *maritima*, an additional question must be raised: are restored CMS hermaphrodites as fit as non-CMS ones? In other words, does the presence of male sterility genes, although counteracted by restorers, affect the performance of these plants, compared with genotypes that do not bear any active male sterility gene? Restorers have been shown in several crop species to modify the transcripts of the CMS genes (Hanson & Bentolila, 2004; Chase, 2007). However, if restorers cannot efficiently modify the transcripts or if this modification drives some negative effects on the expression of other genes, one might expect a lower fitness for restored hermaphrodites (discussed in Delph *et al.*, 2007).

In this study, we found that restored CMS hermaphrodites did not produce lower quantity of pollen but did produce pollen of lower viability than non-CMS hermaphrodites. This lower quality of pollen may be partly due to an incomplete restoration of male fertility: indeed, a group of restored CMS hermaphrodites, possibly lacking one allele of restoration, exhibited very low levels of pollen viability and consequently strongly decreased the average quality of pollen in restored hermaphrodites (Fig. 4). Some other restored hermaphrodites were demonstrated to reach very high frequencies of viable pollen, comparable with the non-CMS hermaphrodites that produced the highest proportion of viable pollen (Fig. 4). This suggests that a complete restoration of CMS is possible to achieve.

Because pollen viability and quantity were not correlated, the difference between non-CMS and restored CMS hermaphrodites in pollen viability remained even when excluding intermediate phenotypes from the analyses. Consequently, even CMS plants that appeared to be well restored for their quantity of pollen can have low male performance. We also compared the number of viable pollen grains between non-CMS and CMS hermaphrodites, using the combined measures of pollen quantity and viability and thus providing an estimate of potential male fitness. Restored CMS hermaphrodites that produced a very high number of pollen grains but of

low viability had consequently a lower potential male fitness than non-CMS hermaphrodites.

Consequences for selection of restorer alleles

Our results suggest that some of the restored CMS hermaphrodites potentially contribute poorly to pollination in natural populations. Because restoration alleles can at least be transmitted through seeds, incompletely restored hermaphrodites may constitute a reservoir of restorers in the population until the appearance of fully restored hermaphrodites, which would efficiently produce pollen and change the population sex ratio. Such complex determination may then slow down the positive selection of restorer factors and help to maintain females in populations, as recently showed by Bailey & Delph (2007). Moreover, restorer alleles can also be eliminated by drift before the apparition of an efficient combination of restorer factors in the population (Charlesworth & Laporte, 1998). This would consequently increase the frequency of females, especially in isolated and/or small populations. A complex determination of the restoration of male fertility may thus constitute an alternative explanation to the correlation between female frequencies and population size that have been observed in some gynodioecious species (e.g. Caruso & Case, 2007).

Differences among populations: the result of a silent cost of restoration?

The observed differences in pollen viability between populations could be due to environmental variation between these populations. However, an effect of the interaction between population and sexual phenotype suggested that the various sexual phenotypes were not equally affected by this population effect. When restored hermaphrodites were compared between populations, no statistical difference was found. By contrast, a significant difference was shown among non-CMS hermaphrodites, plants from Canche having lower pollen viability than plants from Audresselles. Besides the global population effect, non-CMS hermaphrodites from Canche seem to have a lower potential male fitness than those from Audresselles.

Interestingly enough, these two populations had strongly contrasting ratios of CMS and of restoration of male fertility. The Audresselles population exhibited low frequencies of both CMS and restorers, whereas we found high frequencies of CMS and restorers in the Canche population, which suggests that they are at two different stages of the gynodioecy dynamics. In Canche, restored hermaphrodites represented half of the hermaphrodites of the population. Thus, as a result of pollen flow within the population, many non-CMS hermaphrodites were likely to carry the restorer alleles as well. Consequently, a cost of restoration could explain why non-CMS hermaphrodites in Canche had lower pollen viability, and ultimately a lower potential male fitness than non-CMS hermaphrodites from Audresselles. Interestingly, the lower performance by hermaphrodites in Canche was due to a high frequency of individuals suffering from very low pollen viability, whereas the other non-CMS hermaphrodites produced good levels of pollen viability (Fig. 4). Conversely, the frequency of non-CMS hermaphrodites in Audresselles, exhibiting this low pollen quality was lower than 10%. This shows that low pollen viability possibly occurred in non-CMS hermaphrodites in Audresselles, but at a frequency that is consistent with a lower restorer frequency in this population.

This study may provide a third case of a cost of restoration in a gynodioecious species, after having been suggested in *P lanceolata*, (de Haan *et al.*, 1997a) and *Lobelia siphilitica* (Bailey, 2002). The restoration cost suggested by our study may imply a 'silent' (also called 'alien') cost of restoration, as they occur in the presence of the 'other' cytoplasm (Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Delph *et al.*, 2007). In a species containing both CMS and non-CMS cytotypes, such an alien cost has been shown to be necessary to maintain gynodioecy (Dufaÿ *et al.*, 2007). Ideally, to measure a cost associated with restoration alleles, controlled crosses assisted with genetically linked molecular markers could be conducted.

Conclusion

We have demonstrated that pollen quantity and viability can exhibit large variation within and among gynodioecious populations, which could be partly attributed to the action of the restorer genes and their possible associated cost. Therefore, we would expect highly variable contributions to pollination among diverse types of hermaphrodites in gynodioecious species. Future studies should attempt to understand the consequences of this variation in pollen on the effective male fitness of the various types of hermaphrodites and on selection of restorer alleles. In other word, how do the pollen traits we measured effectively relate to male fitness and real gender functionality? This question is currently addressed through a paternity analysis conducted within the Audresselles population by comparing restored and non-CMS hermaphrodites in their success to sire a seedling.

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ANNEXE III

Liste des communications orales ou affichées (posters) à des congrès nationaux et internationaux avec résumés publiés.

Communications orales :

DE CAUWER, I., ARNAUD, J.-F. & DUFAŸ, M. (2009) Effects of fine-scale genetic structure on female mating success in a gynodioecious species *The Evolution Conference 2009*, 12-16 juin, University of Idaho, Moscow, Idaho, USA.

HORNOY, B., **DE CAUWER, I.**, DUFAŸ, M. & ARNAUD, J.-F. (2008) Variation spatio-temporelle de la diversité nucléo-cytoplasmique chez une espèce gynodioïque. *XXème Réunion annuelle du Groupe de Biologie et Génétique des Populations*. Agrocampus Ouest - Rennes.

DE CAUWER, I., ARNAUD, J.-F., DUFAŸ, M. & CUGUEN, J. (2007) Spatial genetic structure and male mating success in the gynodioecious species *Beta vulgaris* ssp. *maritima*: does restoration of male fertility matter? *40th Population Genetics Group Meeting* 10-12 January, University of Manchester, UK.

DE CAUWER, I., DUFAŸ, M., CUGUEN, J. & ARNAUD, J.-F. (2007) Dispersion pollinique et succès reproducteur mâle chez une espèce gynodioique : importance relative de la restauration de la fertilité mâle. *XXIXème Réunion du Groupe de Biologie et Génétique des Populations*, Université de Poitier.

Communications affichées :

DE CAUWER, I., ARNAUD, J.-F., DUFAŸ, M. & CUGUEN, J. (2006) Étude de la structuration génétique en liaison avec la gynodioécie dans une population naturelle de betteraves maritimes, *Beta vulgaris* ssp. *maritima*. I – Organisation spatiale de la diversité génétique. *XXVIIIème Réunion du Groupe de Biologie et Génétique des Populations*, Université de Lille 1.

DE CAUWER, I., ARNAUD, J.-F., DUFAŸ, M. & CUGUEN, J. (2006) Étude de la structuration génétique en liaison avec la gynodioécie dans une population naturelle de betteraves maritimes, *Beta vulgaris* ssp. *maritima*. II – Dispersion du pollen et succès reproducteur mâle. *XXVIIIème Réunion du Groupe de Biologie et Génétique des Populations*, Université de Lille 1.

RÉSUMÉ

Chez les plantes à fleurs, où une immense variété de stratégies de reproduction est rencontrée, la dispersion des gènes s'opère classiquement *via* des flux de pollen pour la voie mâle, et *via* des flux de graines pour la voie femelle. La gynodioécie correspond à un système de reproduction original, caractérisé par la coexistence de plantes femelles et de plantes hermaphrodites au sein de populations naturelles. Ce système de reproduction suscite depuis longtemps un intérêt particulier, du fait d'un paradoxe évolutif apparent : les individus femelles, ayant perdu une voie de transmission de leur information génétique, devraient être désavantagés par rapport aux hermaphrodites, dotés des deux voies de transmission des gènes. L'objet de ce travail thèse était d'expliquer le maintien des femelles et les importantes variations spatiales de sex ratio fréquemment observées dans les populations naturelles de betterave maritime (*Beta vulgaris* ssp. *maritima*), chez laquelle le déterminisme du sexe implique des interactions entre des gènes cytoplasmiques induisant la stérilité mâle (CMS) et des allèles nucléaires de restauration de la fonction mâle. En mêlant des approches de biologie et de génétique des populations, trois thématiques ont ainsi été abordées. (i) La valeur sélective des différents types sexuels a été mesurée, en faisant appel à des mesures phénotypiques en conditions contrôlées et à des analyses de paternité en populations naturelles. Ceci a permis de révéler un avantage femelle extrêmement restreint ainsi que des variations importantes du succès reproducteur mâle chez les hermaphrodites, liées notamment au génotype des individus et à l'existence d'un coût de la restauration. (ii) La caractérisation moléculaire de la diversité génétique au sein de plusieurs populations naturelles et entre plusieurs cohortes successives nous a permis de mettre en lumière l'importance relative des effets de la migration, des évènements de fondation et de la dérive génétique sur la structure spatiale des phénotypes sexuels. Les variations observées de sex ratio à très fine échelle semblent ainsi expliquées par des effets de fondation multiples couplés à des flux de pollen et de graines restreints dans l'espace. (iii) Finalement, nous mettons en évidence l'effet important que cette forte structure spatiale des sexes peut avoir, à la fois sur les individus hermaphrodites et sur les individus femelles. L'ensemble de nos résultats montre qu'une structure spatiale à fine échelle, générée par des évènements de fondation et des flux de gènes limités, peut affecter de façon importante la dynamique de la gynodioécie dans la nature.

ABSTRACT

In flowering plants, which exhibit a spectacular diversity of reproductive strategies, gene dispersal generally occurs through two distinct pathways: pollen for the male function, and seed for the female function. Among sexually polymorphic flowering plants, gynodioecy refers to a particular breeding system in which females and hermaphrodites co-occur in natural populations. Since females reproduce only through seeds, they apparently transmit their genes only half as frequently as hermaphrodites, which gain fitness through both seed and pollen production. This apparent evolutionary paradox has attracted the attention of evolutionary biologists as far as the mid-nineteenth century. The aim of this PhD thesis was to understand the successful maintenance of female individuals and the important spatial variations in sex ratio that are often observed in natural populations of gynodioecious *Beta vulgaris* ssp. *maritima*, in which sex is determined by interactions between cytoplasmic male sterility (CMS) genes and nuclear restorers of male fertility. Using population biology and population genetics approaches, three distinct themes were considered. (i) First, male and female fitness of the different sexual types were compared, using both measures in controlled conditions and paternity analyses in the wild. While our results only suggest a very restricted female advantage, we detected strong male fitness differences among hermaphrodites that were partially explained by the genotype of individuals and by the occurrence of a cost of restoration. (ii) The study of the distribution of genetic diversity in several natural populations and in several consecutive cohorts allowed us to quantify the relative impact of migration, founder events and genetic drift on the spatial distribution of sexes. Overall, random founder effects with spatially restricted pollen and seed flow appeared to be the primary determinants of sex ratio variations. (iii) Finally, we explore how such sex ratio variation can affect the reproductive output of hermaphrodites and females. Altogether, we show that fine-scale spatial structure, resulting from the joint action of founder events and limited gene flow, can notably modify the dynamics of gynodioecy in natural populations.