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Biologie Evolutive et Ecologie

Dynamique évolutive de la gynodioécie chez *Silene nutans* et conditions de son maintien en populations

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Je dédie cette thèse à mes très chers parents, ma moitié et Boulbaba

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Emna Lahiani, Pascal Touzet, Cécile Godé, Mathilde Dufay
Submitted to Plant biology

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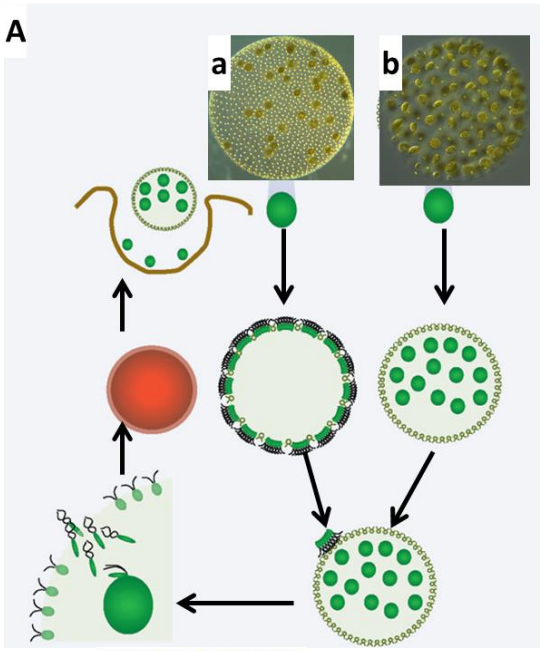
Mathilde Dufay, Emna Lahiani, Benjamin Brachi, B
International Journal of Plant Sciences. 2010. 171: 53-62.

L'important dans la recherche c'est l'imprévisible

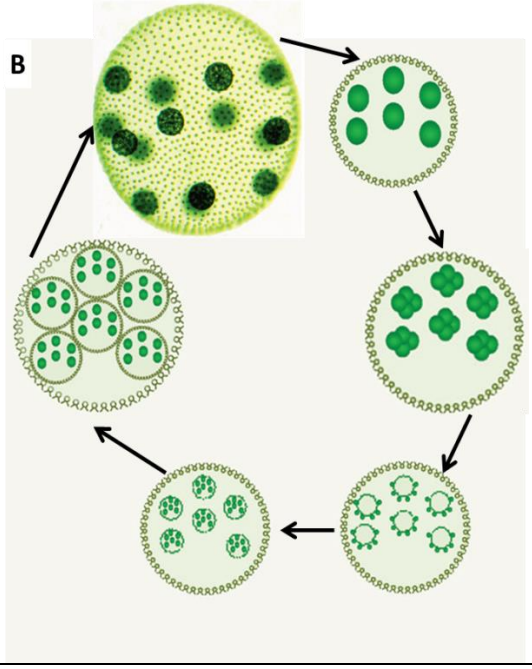
François Gros et François Jacob

Encadré I. Exemples de reproduction asexuée et sexuée dans le genre *Volvox* adapté et modifié de Umen (2011)



A. Reproduction sexuée chez des algues vertes du genre *Volvox* (Volvocaceae)

a) Sphère sexuelle mâle, b) Sphère sexuelle femelle
Reproduction sexuée chez des algues vertes du genre *Volvox* (*Volvocaceae).* Les colonies forment des gamètes mâles (spermatozoïdes) et des gamètes femelles (oosphères) qui sont libérés dans l'eau. Quand un spermatozoïde se colle à une oosphère et la féconde, il se forme un zygote diploïde qui se développe en une nouvelle colonie et qui donne naissance à des nouvelles sphères sexuelles haploïde par méiose.



B. Reproduction asexuée chez des algues vertes du genre *Volvox* (Volvocaceae)

Au cours de sa multiplication végétative la colonie végétative mère peut se diviser et former des colonies filles à l'intérieur de la sphère. Les colonies filles se trouvent libres quand la colonie mère meurt.

Introduction générale

La reproduction est l'ensemble des processus par lesquels une espèce se perpétue, en créant de nouveaux individus (Mottier, 1910). C'est une des activités fondamentales, partagée par tous les êtres vivants (avec la nutrition et la croissance). En effet, toute espèce doit posséder un système de reproduction efficace, sans quoi elle est menacée d'extinction. Si la reproduction permet une perpétuation de l'espèce dans le temps, elle est souvent couplée à un système de dispersion dans l'espace. Il s'agit des systèmes permettant de coloniser de nouveaux milieux, et d'augmenter les chances de survie de l'espèce. Il existe un coût de la reproduction, correspondant grossièrement aux ressources, énergétiques notamment, que l'individu ou l'espèce alloue à la reproduction (Reznick, 1985) qui peut affecter leur succès reproducteur et leur survie par la suite.

Il existe deux types de reproduction dans la nature. La reproduction sexuée est assurée par la fécondation, c'est-à-dire par fusion des gamètes mâle et femelle donnant un œuf (ou zygote) (Encadré I). Cette reproduction permet le maintien d'une diversité génétique au sein des populations, car elle assure le brassage génétique (Mottier, 1910). Le deuxième type de reproduction est la reproduction asexuée, appelée aussi multiplication végétative. Elle désigne tous les autres moyens de multiplication où n'interviennent ni gamète ni fécondation (Encadré I). Dans certains cas, le patrimoine génétique des parents est strictement identique aux descendants, aux erreurs près dues aux petites possibilités de mutation lors de réplication de l'ADN. Le terme reproduction est réservé essentiellement à la reproduction sexuée.

1. Diversité et évolution des systèmes de reproduction chez les plantes à fleurs

L'organe assurant la reproduction sexuée chez les angiospermes (les plantes à fleurs) est la fleur. Les fleurs constituent une importante innovation de l'évolution, qui a permis au monde des plantes d'accéder à des méthodes de reproduction entièrement nouvelles, et a amené à une importante coévolution avec les pollinisateurs . Les fleurs présentent une grande diversité morphologique qui génère une diversité des systèmes de reproduction des angiospermes.

La majorité des angiospermes produisent exclusivement des fleurs bisexuelles ou hermaphrodites, portant des organes femelles et mâles au sein de la même fleur (Yampolsky & Yampolsky, 1922 ; Torices *et al.*, 2011). L'hermaphrodisme est généralement considéré comme le système de reproduction ancestral chez les angiospermes. Cependant, une bonne partie des espèces des plantes à fleurs arrangent leurs organes femelles et mâles dans des fleurs différentes et/ou dans des individus différents (Yampolsky & Yampolsky, 1922 ; Barrett, 2002; Charlesworth, 2006; Torices *et al.*, 2011). On obtient ainsi deux classes de systèmes de reproduction au sein de ce groupe : les espèces monomorphes, qui ne contiennent qu'un seul phénotype sexuel et les espèces polymorphes qui contiennent généralement deux phénotypes sexuels et parfois plus. Parmi les systèmes de reproduction monomorphes, on compte l'hermaphrodisme, la monoécie, gynomonoécie, l'andromonoécie et la trimonoécie et parmi les polymorphes on peut citer la dioécie, la gynodioécie et l'androdioécie (figure 1.1) (Bawa & Beach, 1981 ; Torices *et al.*, 2011).

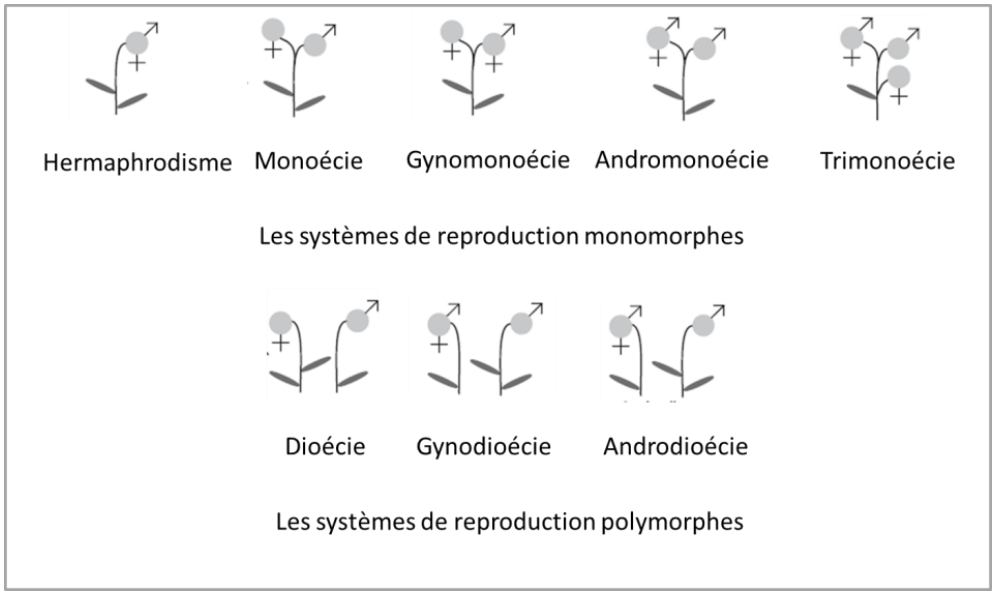


Figure 1.1. Les différents systèmes de reproduction observés chez les angiospermes adapté et modifié de Torices *et al.*, (2011)

Les systèmes de reproduction des angiospermes peuvent être classés selon d'autres traits que le type de gamètes produit au sein des fleurs et des individus, comme le mode de pollinisation (généralement soit anémophile, soit entomophile) ou le taux d'allogamie qui varie de façon quasi continue entre espèces. L'ancêtre des angiospermes est considéré comme étant hermaphrodite, entomophile et plutôt allogame (Barrett, 2002). Cet ancêtre a subi plusieurs réversions indépendantes au cours de l'évolution pour générer des espèces avec différents systèmes de reproduction, de mode de pollinisation et des taux d'allogamie (Barrett, 2010; Torices *et al.*, 2011).

Comprendre l'origine, le maintien et l'évolution de la diversité des systèmes de reproduction observés chez les angiospermes est un vrai défi pour les biologistes. Une des transitions communément étudiées est la transition vers la dioécie (un système de reproduction polymorphe au sein duquel les fleurs femelles et mâles sont portées par des plantes distinctes) (*p.e.* Renner & Ricklefs, 1995; Barrett, 2002; Marais *et al.*, 2011). La transition de l'hermaphrodisme vers la dioécie doit se passer en deux étapes minimum et doit impliquer un passage par

un système de reproduction transitoire (figure 1.2, synthétisée dans Barrett, 2002). Les deux voies possibles impliquent soit le passage par la monoécie (un système de reproduction monomorphe au sein duquel les plantes portent des fleurs femelles et mâles) ou par la gynodioécie (un système de reproduction polymorphe caractérisé par la présence dans les populations naturelles des plantes femelles et des plantes hermaphrodites).

La première voie possible de transition vers la dioécie *via* la monoécie a été explorée par des études phylogénétiques. Il n'existe pas de modèle théorique qui explore cette transition vu que le déterminisme génétique de la monoécie n'est pas connu, ce qui ne permet pas de considérer des scénarios génétiques. Une étude de l'ensemble des Angiospermes a montré que la dioécie a été fortement associée à la monoécie. Les auteurs prédisent que la seule possibilité pour un groupe d'espèces d'acquérir la dioécie est la présence de la monoécie chez ces groupes d'espèces mais ils suggèrent de confirmer ces résultats avec des études phylogénétiques (Renner & Ricklefs, 1995). Certaines études phylogénétiques ont essayé d'étudier cette voie de transition. Ces travaux documentent une possible évolution de la dioécie via la monoécie dans certains genres de plantes (*p.e.* Renner & Won, 2001 dans le genre *Siparunaceae*; Renner *et al.*, 2007 dans le genre *Acer*; Volz & Renner, 2008 dans le genre *Bryonia*). En outre, chez *Sagittaria latifolia*, l'existence des populations polymorphes (plantes monoïques et d'autres dioïques) soutient l'évolution de la dioécie *via* la monoécie (Dorken *et al.*, 2002).

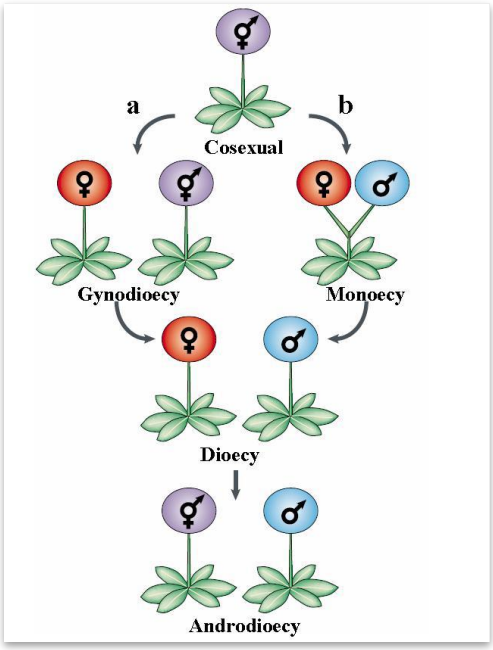


Figure 1.2. Evolution des systèmes de reproduction chez les angiospermes (d'après Barrett, 2002). a et b sont les deux voies possibles d'évolution de l'hermaphrodisme vers la dioécie.

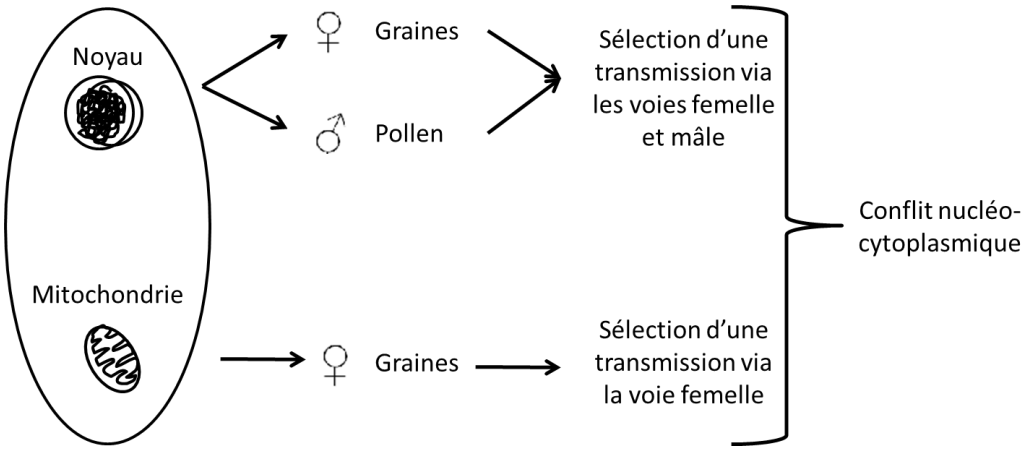
La deuxième voie possible de l'évolution de l'hermaphrodisme vers la dioécie implique un passage par la gynodioécie. Cette voie a été modélisée théoriquement et nécessite l'invasion de mutants mâles stériles dans les populations monomorphes suivie par une sélection contre la fonction femelle chez les hermaphrodites restants (vu qu'ils vont consacrer plus de ressources à leur fonction mâle au détriment de leur fonction femelle) (Charlesworth & Charlesworth, 1987 ; Maurice *et al.*, 1994; Schultz, 1994). L'analyse phylogénétique des systèmes de reproduction des espèces de *Lepechinia* sect. *Parviflorae* et *Salviifoliae* (Lamiaceae) et de leurs patrons de distribution par Hart (1985), a montré que la dioécie a vraisemblablement évolué deux fois à partir de la gynodioécie chez ces espèces. C'était aussi le cas d'autres genres d'espèces d'angiospermes étudiés comme le genre *Silene* (Desfeux *et al.*, 1996; Mrackova *et al.*, 2008; Marais *et al.*, 2011).

La transition de l'hermaphrodisme vers la dioécie en passant par la gynodioécie pose des questions sur la stabilité et le maintien de la gynodioécie dans les populations des plantes.

2. La gynodioécie

Au cours de cette thèse je me suis intéressée à un polymorphisme sexuel particulier qui est la gynodioécie. Je m'intéresse plus spécifiquement à la dynamique évolutive de ce système de reproduction. La gynodioécie est un système de reproduction qui couvre entre 5 et 10 % des espèces des angiospermes (Darwin, 1877). Les femelles dans les populations gynodioïques sont des mâles stériles. Bien que des cas de stérilité mâle nucléaire soient connus (*p.e. Fragaria virginiana* par Ashman, 1999), la stérilité mâle est généralement induite par des gènes mitochondriaux (Saumitou-Laprade *et al.*, 1994). Le déterminisme génétique de la gynodioécie est en effet souvent le résultat de l'interaction des génomes nucléaires et mitochondriaux (*p.e.* McCauley *et al.*, 2000; Delph *et al.*, 2007). Le génome mitochondrial code pour des facteurs qui bloquent la production de pollen viable, d'où le terme de stérilité mâle cytoplasmique *(CMS* pour *Cytoplasmic Male Sterility)*. Le génome nucléaire contrôle la restauration de la fonction mâle en réprimant l'action de facteurs de stérilité par le biais d'allèles dits de restauration. Ainsi les individus qui portent une *CMS* mais sans le ou les restaurateurs associés seront des femelles et les individus qui portent en plus le ou les restaurateurs seront des hermaphrodites dits restaurés. Enfin, les individus qui portent un cytoplasme non stérilisant seront des hermaphrodites dits normaux. Ces deux génomes n'ont pas le même mode d'hérédité. Le génome mitochondrial est d'hérédité maternelle (Cosmides & Tooby, 1981; McCauley, 1994; Petit *et al.*, 2005), alors que le génome nucléaire est d'hérédité biparentale. Le polymorphisme sexuel observé au sein de ces populations gynodioïques peut ainsi être considéré comme le fruit d'un conflit génétique entre ces deux génomes, chacun essayant de maximiser sa propre transmission aux dépens de l'autre (Cosmides & Tooby, 1981; Saumitou-Laprade *et al.*, 1994) (Encadré II).

Encadré II. Le conflit nucléo-cytoplasmique



Le conflit génomique peut être défini comme une situation où la sélection a des effets antagonistes sur les compartiments génétiques. La différence d'hérédité entre les compartiments nucléaire et cytoplasmique peut provoquer un conflit génomique qui s'exprime chez les plantes gynodioïques par la stérilité mâle nucléo-cytoplasmique. L'hérédité des facteurs cytoplasmiques est maternelle alors que l'hérédité des gènes nucléaires est biparentale. Cela génère une situation où les intérêts des génomes sont en opposition. Les gènes mitochondriaux transmis par la voie femelle uniquement ont intérêt à bloquer la voie mâle si cela augmente leur transmission. En revanche, les gènes nucléaires transmis pareillement par la voie femelle ou mâle ont intérêt à maintenir ces deux voies. Ainsi quand il s'agit de production de pollen : les gènes mitochondriaux ont intérêt à favoriser l'apparition de la stérilité mâle alors que les gènes nucléaires ont intérêt à favoriser les fertilité mâle et femelle (Gouyon *et al.*, 1991)

3. Les prédictions théoriques du maintien de la gynodioécie

3.1. Invasion des CMS et l'avantage femelle

La question est comment les gènes de stérilité mâle, une fois apparus dans les populations naturelles, peuvent envahir une population ?

Ces gènes pourront être sélectionnés du moment que le nombre de graines produites par les individus femelles est supérieur à celui des hermaphrodites ou que ces graines sont de meilleure qualité que celles produites par les hermaphrodites (Darwin, 1877 ; Dufay & Billard, 2012). Ceci confère aux individus porteurs de ces gènes un avantage qui permettra l'invasion de ces gènes dans les populations naturelles végétales. Cet avantage, appelé "avantage femelle", a été démontré expérimentalement chez de nombreuses espèces gynodioïques (synthétisé dans Shykoff *et al.*, 2003 et dans Dufay & Billard, 2012) et peut être expliqué par les deux phénomènes suivants :

- une réallocation des ressources qui étaient destinées à la production de pollen par les individus femelles à la production d'ovules ;
- un évitement de la dépression de consanguinité des individus femelles qui sont obligatoirement allogames, et donc la possibilité de produire des descendances de meilleure qualité que les hermaphrodites s'ils s'autofécondent.

Un petit avantage en terme de succès reproducteur femelle suffit théoriquement pour permettre aux individus femelles d'envahir les populations dans le cas d'un polymorphisme sexuel nucléo-cytoplasmique (Lewis, 1941). Dans le cas où ce polymorphisme est purement nucléaire, les mutations de stérilité mâle ne peuvent théoriquement envahir que si les individus porteurs de ces mutations compensent la perte de fonction mâle en produisant deux fois plus de descendants par la fonction femelle (Lewis, 1941).

Le gène de stérilité mâle peut se fixer après avoir envahi la population provoquant son extinction si aucun autre phénomène n'intervient comme par exemple la limitation pollinique ou la restauration de la fertilité mâle par des gènes nucléaires qui peuvent contrecarrer l'effet de ce gène. Dans le cas contraire,

la gynodioécie ne persiste que le temps de l'invasion du gène de stérilité mâle. Il est alors primordial de pouvoir comprendre les conditions qui permettent le maintien de ces gènes dans la population. Autrement dit : quels mécanismes permettent à ces gènes de se maintenir dans les populations gynodioïques sans se fixer ?

3.2. Maintien de la gynodioécie dans les populations naturelles

Plusieurs modèles théoriques ont essayé de déterminer les conditions de maintien de ce polymorphisme. Du fait qu'on suspecte que la majorité des espèces gynodioïques ont un déterminisme sexuel nucléo-cytoplasmique, le maintien de la gynodioécie dépend donc du maintien de ce polymorphisme nucléo-cytoplasmique. Ainsi aucune des deux entités (cytoplasmique *vs* nucléaire) ne doit théoriquement gagner le conflit et aucune des deux types de mutations ne doit se fixer. Ces études ont engendré deux classes de modèles. Dans la première classe, la gynodioécie peut se maintenir *via* la sélection seule dans une population panmictique de taille infinie et dans la deuxième classe, la dérive et/ou la migration viennent s'ajouter à la sélection pour maintenir la gynodioécie dans une population structurée.

3.2.1. Le maintien via la sélection seule

Dans le cas du modèle reposant sur une population panmictique de taille infinie, il est nécessaire d'introduire un deuxième paramètre qui est le coût des allèles restaurateurs silencieux (*i.e.* quand ils sont associés à un cytoplasme différent de celui qu'ils restaurent) (Charlesworth, 1981; Gouyon *et al.*, 1991; Dufay *et al.*, 2007). C'est l'équivalent du coût d'une résistance en absence du pathogène (Gouyon *et al.*, 1991).

Ces modèles suggèrent que les apparitions des *CMS* sont des évènements rares qui seront sélectionnés grâce à l'avantage femelle. Les *CMS* seront ainsi fréquentes dans la population faisant augmenter les fréquences des restaurateurs de fertilités associés. Les restaurateurs devenus fréquents dans la population vont faire baisser la fréquence des *CMS* et vont par la suite subir un coût silencieux, ce

qui engendre la diminution de leur fréquence. Enfin, les *CMS* qui sont devenues rares seront de nouveaux sélectionnées et vont augmenter en fréquence. Ce type de dynamique engendre ainsi des oscillations de fréquences de *CMS* et de restaurateurs de la fertilité mâle. Cette dynamique est générée par une forme particulière de sélection : la sélection fréquence-dépendante négative (Figure 1.3). Certains auteurs ont suggéré que les oscillations de fréquence de *CMS* et de restaurateurs de la fertilité mâle dans le temps vont engendrer des variations de sexe ratio entre populations qui ne se trouvent pas au même moment de la dynamique. Certaines études empiriques chez quelques espèces gynodioïques qui documentent des variations de sexe ratio ont trouvé des résultats cohérents avec cette dynamique pour expliquer les variations de sexe ratio en populations naturelles (*p.e. Thymus vulgaris* par Manicacci *et al.*, 1996 ; *Silene vulgaris* par Olson & McCauley, 2002 ; *Daphne laureola* par Alonso, 2005 ; *Plantago maritima* par Nilsson & Agren, 2006 et *Beta vulgaris* par Dufay *et al.*, 2009).

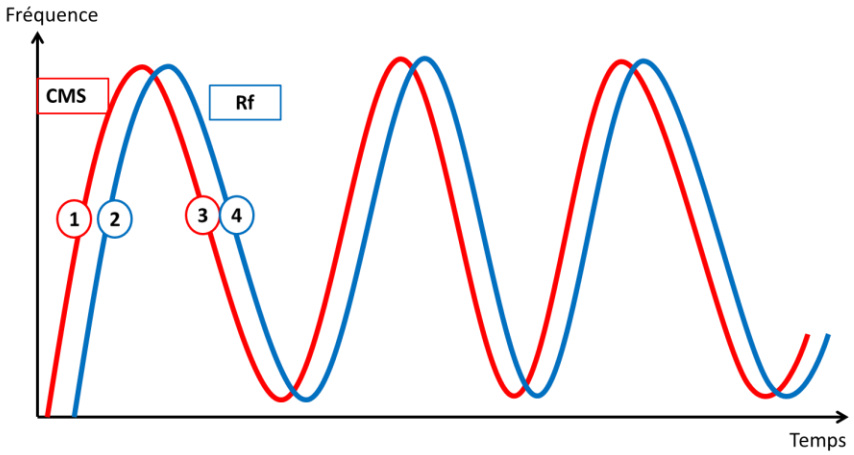


Figure 1.3. Sélection Fréquence-dépendante négative. Des cycles répétés au cours du temps de : 1) La *CMS* est sélectionnée grâce à l'avantage femelle et donc augmente en fréquence, 2) Le restaurateur associé devenu rare augmente alors en fréquence, 3) Le restaurateur devenue fréquent fait baisser la fréquence de la *CMS* et 4) Le restaurateur subit un coût silencieux qui fait baisser sa fréquence.

3.2.2. Le modèle épidémique

La limite des modèles de sélection seule est qu'ils prennent en compte une population de très grande taille, isolée et panmictique, négligeant ainsi des processus tels que la dérive génétique pouvant agir dans les populations de taille finie, ainsi que les flux de gènes existant entre ces populations. La migration par le pollen et les graines peut être limités dans l'espace (Irwin *et al.*, 2003; Robledo-Arnuncio *et al.*, 2004; Krauss *et al.*, 2009), ce qui peut engendrer des variations des fréquences alléliques dans les populations. La dérive peut encore accentuer ce phénomène en modifiant encore ces fréquences en fixant ou provoquant l'extinction de certains allèles (Nilsson & Agren, 2006 ; Dufay & Pannell, 2010). Quand le phénotype sexuel est déterminé génétiquement comme dans le cas de la gynodioécie, une variation des fréquences alléliques peut engendrer des variations de fréquences phénotypiques. Chez les espèces gynodioïques, le succès reproducteur d'un phénotype sexuel dépend de la disponibilité des partenaires sexuels dans la population. La structure et la répartition des sexes dans l'espace jouent sur la disponibilité en partenaires dans l'espace, Ainsi, la structure génétique et l'agrégation des sexes dans l'espace peuvent avoir un effet sur le succès reproducteur de chaque phénotype sexuel et par la suite sur le maintien de la gynodioécie. Ces aspects sont abordés en détails dans une population naturelle de *Silene nutans* dans le chapitre 3.

Dans la deuxième classe de modèles expliquant le maintien de la gynodioécie, une *CMS* associée à un avantage sélectif va augmenter en fréquence. De ce fait, les restaurateurs associés vont aussi augmenter en fréquence. Si ces derniers ne sont pas coûteux, ils se fixent dans la population conduisant à la perte du polymorphisme sexuel jusqu'à ce qu'une nouvelle *CMS* envahisse la population grâce à la migration de gènes entre un ensemble de populations interconnectées (une métapopulation), provoquant des invasions récurrentes de *CMS*. Ceci entraînera une stérilité mâle transitoire dans les populations, encore une fois grâce à un avantage femelle. Dans cette classe de modèles le maintien de la gynodioécie

est sous une dynamique épidémique (Frank, 1989; Couvet *et al.*, 1998) (Figure 1.4).

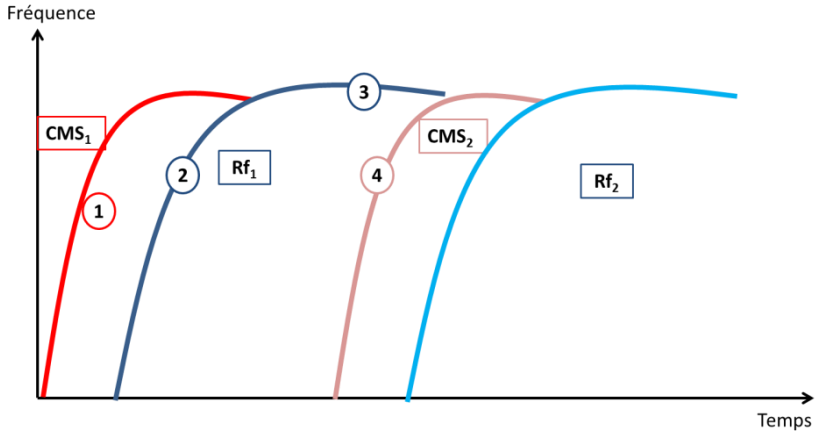


Figure 1.4. La dynamique épidémique. 1) La *CMS* est sélectionnée grâce à l'avantage femelle et donc augmente en fréquence, 2) le restaurateur associé devenu rare augmente en fréquence et fait baisser la fréquence de femelles, 3) Le restaurateur n'étant pas coûteux se fixe dans la population et 4) Une autre *CMS* envahit la population provenant d'autres populations par migration.

3.2.3. Conséquence sur la diversité cytoplasmique

Les deux classes de modèles prédisent des patrons opposés de diversité cytoplasmique au sein des espèces gynodioïques. La dynamique épidémique devrait réduire la diversité cytoplasmique, étant donné que les nouveaux cytoplasmes stérilisants vont balayer les populations, ce qui conduit à une homogénéisation du génotype cytoplasmique au sein et entre les populations (Charlesworth, 2002; Ingvarsson & Taylor, 2002). En revanche, la sélection fréquence-dépendante négative impliquée dans le modèle de polymorphisme nucléo-cytoplasmique stable devrait entraîner une forte diversité nucléotidique des génomes cytoplasmiques chez les espèces gynodioïques comparant aux espèces hermaphrodites ou dioïques, parce que les haplotypes non recombinants sont

maintenues, potentiellement sur de longues périodes de temps, et peuvent ainsi accumuler de nombreuse mutations (Charlesworth, 2002; Stadler & Delph, 2002; Touzet & Delph, 2009). Parce qu'il existe une diversité importante des systèmes de reproduction au sein du genre *Silene* (espèces hermaphrodites, gynodioïques et dioïques, figure 1.5), plusieurs études comparatives ont été conduites au sein de ce genre (*p.e.* Desfeux *et al.*, 1996 ; Marais *et al.*, 2011). Les conclusions de ces études étaient contradictoires. Certaines études supportent l'hypothèse d'une dynamique épidémique (*p.e.* Ingvarsson & Taylor, 2002 ; une diversité chloroplastique plus faible chez *Silene vulgaris* (espèce gynodioïque) par rapport à *Silene latifolia* (espèce dioïque)). D'autres études soutiennent l'hypothèse d'une sélection fréquence-dépendante négative (une diversité mitochondriale importante chez *Silene acaulis* (Stadler & Delph, 2002) et chez *Silene vulgaris* (Houliston & Olson, 2006) ou une diversité mitochondriale plus importante chez les espèces gynodioïques *S. acaulis*, *S. vulgaris* et *S. nutans* que chez d'autres espèces non-gynodioïques (Touzet & Delph, 2009)). Néanmoins, certains auteurs ont montré que les taux de mutations mitochondriaux dans le genre *Silene* sont très variables (Mower *et al.*, 2007; Barr *et al.*, 2007; Sloan *et al.*, 2008; Sloan *et al.*, 2009). Par conséquent, la forte diversité des gènes mitochondriaux chez les espèces gynodioïques dans les études précédentes peut aussi être expliquée par ce phénomène. Ainsi un des objectifs de ma thèse est de distinguer entre ces deux types de scénarios parce qu'ils ne sont pas supposé laisser le même patron de polymorphisme et ainsi déterminer la dynamique responsable du maintien de la gynodioécie chez *Silene nutans* (espèce étudiée) en utilisant une approche comparative avec une espèce dioïque appartenant au même clade génétique tout en contrôlant les variations des taux de mutations mitochondriaux entre les deux espèces (Chapitre 1).

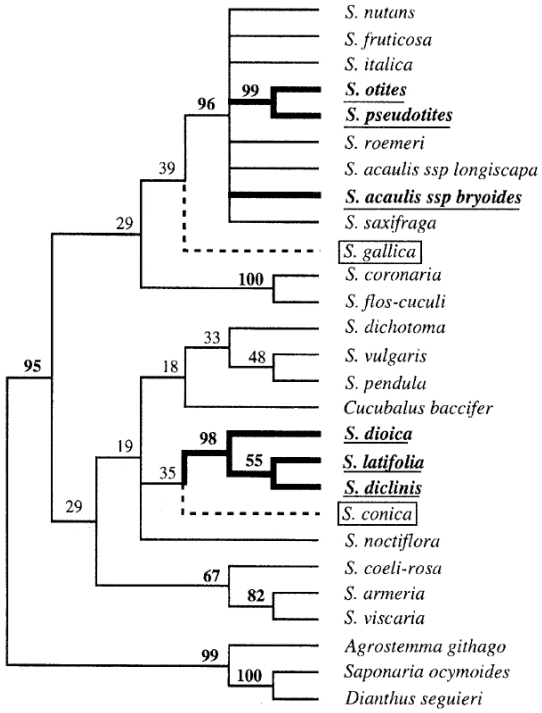


Figure 1.5. Phylogénie de quelques espèces du genre *Silene*, d'après Desfeux *et al.*(1996). Les espèces dioïques sont indiquées en gras et soulignées, et les espèces hermaphrodites sont encadrées. Toutes les autres espèces peuvent être considérées comme étant gynodioïques. Les valeurs indiquées sur les nœuds sont les valeurs de bootstrap

4. L'avantage femelle

L'avantage femelle peut théoriquement résulter *(i)* de la réallocation des ressources qui étaient destinées à la production de pollen vers la fonction femelle, *(ii)* de l'évitement de la dépression de consanguinité par les individus femelles qui sont forcées d'avoir une fécondation croisée ce qui n'est pas le cas pour les individus hermaphrodites. Comme expliqué précédemment, l'avantage femelle est un paramètre crucial pour la dynamique de la gynodioécie. Ainsi, plusieurs études se sont intéressées à l'occurrence, l'amplitude et la variation de ce paramètre.

4.1. L'occurrence, l'amplitude et les causes de l'avantage femelle

Plusieurs études ont montré l'existence d'un avantage femelle dans les populations gynodioïques (synthétisé dans Shykoff *et al.*, 2003 et dans Dufay &

Billard, 2012). Théoriquement, l'amplitude de cet avantage doit être supérieure à 2 si le déterminisme de la gynodioécie est purement nucléaire. En revanche, un avantage femelle entre 1 et 2 est suffisant au maintien de ce polymorphisme sexuel si le déterminisme de la gynodioécie est nucléo-cytoplasmique (Lewis, 1941). Dufay & Billard (2012), ont constaté que le plus souvent l'ampleur de l'avantage femelle détecté chez les espèces gynodioïques était comprise entre 1 et 2.

Cet avantage se résumant en une meilleure performance des femelles peut concerner plusieurs traits. Parmi les traits mesurés dans les comparaisons femelles et hermaphrodites : le nombre d'ovules, la production des fruits, le nombre, la taille et le poids des graines, la germination des graines et la survie des plantules (synthétisé dans Shykoff *et al.*, 2003 et dans Dufay & Billard, 2012). Les différences de succès reproducteur entre femelles et hermaphrodites peuvent résulter de la réallocation de ressources qui étaient destinées à la production du pollen (Darwin, 1877), de la limitation en pollen et de la capacité des hermaphrodites à s'autoféconder quand ils sont limités en pollen (suggérer chez *Glechoma longituba* par Zhang *et al.*, 2008) et de l'évitement de la dépression de consanguinité (Darwin, 1877) (Figure 1.6).

Premièrement, les femelles peuvent détourner les ressources qui étaient destinées à la production de pollen pour produire plus d'ovules que les hermaphrodites (Darwin, 1877). Chez certaines espèces gynodioïques, il s'avère que les femelles produisent plus d'ovules que les hermaphrodites (synthétisé dans Shykoff *et al.*, 2003) résultant en un plus grand nombre de graines. Aussi les femelles peuvent réallouer ces ressources pour produire des graines de meilleure qualité que celles des hermaphrodites (Darwin, 1877). En deuxième lieu, l'efficacité de pollinisation peut causer des différences de succès reproducteur entre hermaphrodites et femelles (Widen & Widen, 1990 chez *Glechoma hederacea*). La limitation pollinique est un phénomène observé chez plusieurs

espèces gynodioïques (*p.e.* Widen & Widen, 1990 ; Asikainen & Mutikainen, 2005a ; Zhang *et al.*, 2008 ; De Cauwer *et al.*, 2010).

Une faible quantité de pollen disponible dans la population peut provoquer une baisse du succès reproducteur des femelles plus marquée que les hermaphrodites. La limitation pollinique peut aussi résulter d'une différence d'attraction des pollinisateurs chez les espèces entomophiles. Plusieurs auteurs ont documenté une différence d'attraction des plantes femelles et hermaphrodites aux polinisateurs : les pollinisateurs préféraient visiter les hermaphrodites (*p.e.* Asikainen & Mutikainen, 2005b; Griffin & Byers, 2012) ce qui peut engendrer une limitation en pollen importante chez les individus femelles. Cette différence d'attraction peut être liée à un nombre plus important de fleurs produites par les hermaphrodites ou à une différence de tailles de fleurs. La différence d'attraction des pollinisateurs peut ainsi affecter le succès reproducteur de chaque phénotype sexuel. Il semble ainsi nécessaire d'étudier la biologie de la pollinisation et les relations entre les pollinisateurs et les plantes ainsi que la contribution des différents groupes de pollinisateurs dans le succès reproducteur des espèces entomophiles. J'ai essayé de comparer la contribution des pollinisateurs nocturnes et diurnes au succès reproducteur chez *Silene nutans* dans mon dernier chapitre de thèse (Chapitre 4). En outre, quelques études ont montré que les hermaphrodites, quand ils se trouvent dans des patches avec des fréquences élevées de femelles, souffrent moins de la limitation pollinique que ces dernières. Certains auteurs ont suggéré que ceci est peut être dû à la capacité des individus hermaphrodites à s'autoféconder (*p.e.* Zhang *et al.*, 2008 chez *Glechoma longituba*). Enfin, l'avantage femelle peut résulter de l'évitement de la dépression de consanguinité subi par les hermaphrodites et donc une baisse de la qualité des graines produites par autofécondation des hermaphrodites ce qui fait diminuer leur valeur sélective (Darwin, 1877) (figure 1.6).

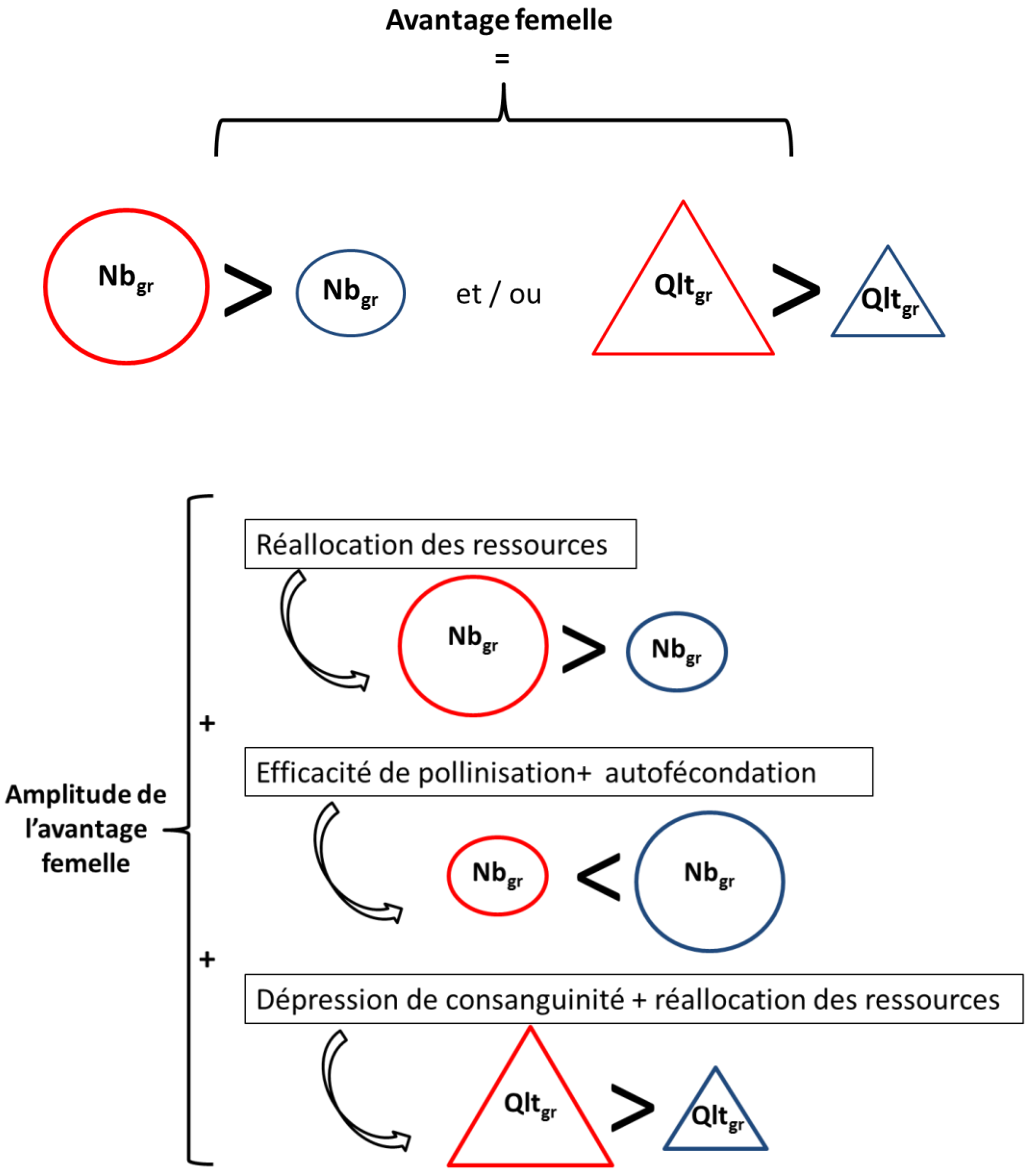


Figure 1.6. Définition et amplitude de l'avantage femelle. Nb_{gr} : nombre de graines, Qlt_{gr} : qualité des graines. En rouge les femelles et en bleu les hermaphrodites. Encadrés : les processus qui peuvent déterminer l'amplitude de l'avantage femelle

4.2. Variation de l'avantage femelle

Certains modèles théoriques ont jusqu'ici considéré l'avantage femelle comme un paramètre constant, même ceux qui ont étudié les espèces dans un contexte de métapopulation (Frank, 1989 ; Pannell, 1997; Couvet *et al.*, 1998 mais voir Dornier & Dufay, sous presse). Cependant cet avantage est potentiellement variable entre les populations étant donné que les causes principales de cet avantage à savoir les ressources disponibles dans les populations, la limitation pollinique, l'autofécondation et la dépression de consanguinité peuvent varier entre les populations.

Les ressources disponibles dans les populations comme la quantité d'eau disponible peuvent varier entre populations. Une étude chez *Nemophila menziesii* (Hydrophyllaceae) a montré que le succès reproducteur des différents phénotypes sexuels varie avec la disponibilité de l'eau avec des femelles plus résistantes au manque d'eau, ce qui peut engendrer une variation de l'amplitude de l'avantage femelle entre les population selon la disponibilité de l'eau (Barr, 2004). Un autre paramètre qui semble varier entre les populations gynodioïques, est le sexe ratio (*p.e.* Manicacci *et al.*, 1996 ; Laporte *et al.*, 2001 ; Olson *et al.*, 2006 ; Dufay *et al.*, 2009). Les variations du sexe ratio peuvent avoir un impact sur la limitation pollinique. D'abord parce que la quantité de pollen disponible peut varier entre les populations selon la fréquence des producteurs de pollen. Ensuite, par ce que les populations contenant beaucoup de femelles qui semblent attirer moins les pollinisateurs peuvent souffrir d'une attractivité globale moindre. Ces deux phénomènes peuvent engendrer des variations d'amplitude de limitation pollinique entre les populations. Certaines études en populations naturelles ont documenté une limitation pollinique plus importante quand les hermaphrodites sont rares (*p.e.* McCauley & Brock, 1998 chez *Silene vulgaris*; Widen & Widen, 1990 chez *Glechoma hederacea).* Quand les femelles et les hermaphrodites ne subissent pas de la même façon la limitation pollinique (avec des hermaphrodites qui s'autofécondent quand ils sont limités en pollen), la variation de la limitation pollinique selon le sexe ratio pourrait engendrer une variation des taux

d'autofécondation entre les populations. Une seule étude chez une autre espèce gynodioïque *Silene vulgaris*, a montré que les taux d'autofécondation sont plus élevés quand les hermaphrodites sont rares (Miyake & Olson, 2009)mais sans documenter l'impact sur l'avantage femelle. Un fort taux d'autofécondation pourrait donc baisser l'avantage femelle en procurant une assurance reproductive aux hermaphrodites. Néanmoins, ces mêmes taux élevés d'autofécondation peuvent contrebalancer cet effet en faisant subir aux hermaphrodites une réduction de la qualité de leurs descendances en cas de dépression de consanguinité. En tenant compte en plus de la variation potentielle de la dépression de consanguinité entre les populations qui peuvent contenir plus ou moins de mutations délétères, la combinaison de l'effet de la variation de l'autofécondation et de la dépression de consanguinité entre les populations peut engendrer des variations de l'avantage femelle entre ces populations. Cependant, il n'y a pas eu d'étude expérimentale visant à étudier l'impact de la variation du taux d'autofécondation et de la dépression de consanguinité sur l'avantage sélectif des femelles. L'occurrence, l'amplitude et la variation de l'avantage femelle selon le sexe ratio ont fait l'objet de mon deuxième chapitre de thèse.

5. L'espèce étudiée : *Silene nutans*

5.1. Le genre Silene

Le genre *Silene* est un genre propice à l'étude des systèmes de reproduction puisqu'il comprend des espèces hermaphrodites, gynodioïques et dioïques (Desfeux *et al.*, 1996). Cette diversité au sein du groupe permet de différencier les traits liés à la phylogénie et ceux associés au système de reproduction. La gynodioécie est commune dans ce genre et il semble que la gynodioécie ou l'hermaphrodisme sont les systèmes de reproduction ancestraux les plus probables dans ce groupe (Desfeux *et al.*, 1996; Marais *et al.*, 2011). La gynodioécie évolué vers la dioécie au moins deux fois de façons indépendantes (Desfeux *et al.*, 1996; Mrackova *et al.*, 2008; Marais *et al.*, 2011). En outre, la gynodioécie a été très largement étudiée au sein de ce groupe : déterminisme génétique , composantes

du succès reproducteur des différents phénotypes sexuels, structuration de population, les pollinisateurs etc. (Tableau 1.1), faisant de ce genre le cas de gynodioécie le mieux connu après les espèces agricoles même si les gènes de stérilité et les restaurateurs n'ont pas encore été identifiés.

Tableau 1.1. Quelques aspects étudiés chez les espèces gynodioïques du genre *Silene*

	Silene nutans	***Silene vulgaris***	***Silene acaulis***	***Silene italica***	***Silene stockenii***	***Silene littorea***
Déterminisme génétique	NC*[1]	NC*[4]	NC*[7]	NC*[8]	NC*[9]	NC*[10]
Avantage Femelle	Oui[2]	Oui[5]	Oui[5]	Oui[5]	Non[5]	Oui [11]
Structuration des populations	-------	Les cytoplasmes sont structurés localement au niveau des populations (56 populations dans 9 régions dans l'est de l'Amérique du nord)[6]	Structuration des cytoplasmes à une toute petite échelle (<=2m)(5 populations en Alaska)[7]	-----	-----	-----
Polinisateurs	Diurnes et nocturnes[3]		Lépidoptère[3]	Diurnes et nocturnes [3]	-----	-----

* NC : Nucléo-cytoplasmique ; 1) Garraud *et al.*, 2011 ; 2) Garraud, Thèse de doctorat 2011 ; 3) Jürgens *et al.*, 2002a ; 4) Taylor *et al.*, 2001 ; 5) synthétisé dans Dufay & Billard, 2012; 6) McCauley *et al.*, 2003 ; 7) Klaas & Olson, 2006 ; 8) Lafuma & Maurice, 2006 ; 9) Talavera *et al.*, 1996 ; 10) Vilas & Garcia, 2006 ; 11) Guitian & Medrano, 2000

5.2. Silene nutans

5.2.1. Distribution géographique

S. nutans possède une aire de distribution continentale qui s'étend sur toute l'Europe jusqu'en Sibérie et au Caucase. C'est une espèce caractéristique des paysages de steppe, que l'on retrouve sur une large gamme écologique de substrats (figure 1.7). A sa frontière occidentale (Grande-Bretagne, NW France, la Belgique et les Pays-Bas), *S. nutans* est rare, montrant une distribution dispersée en petit patchs, et souvent des populations de petite taille (Hepper, 1956; Van Rossum *et al.*, 2003).

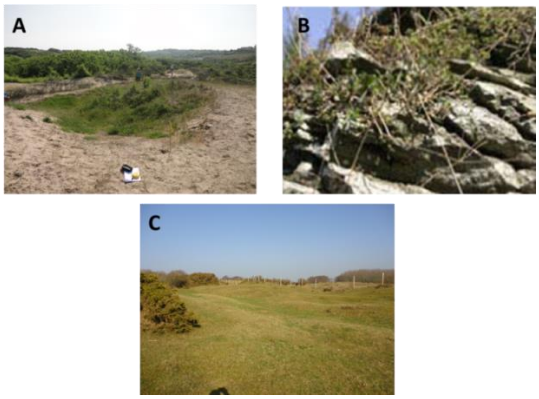


Figure 1.7. Différents habitats de *Silene nutans* dans le nord de la France A) *Silene nutans* sur substrat siliceux à Ecault, B) *Silene nutans* sur substrat calcaire à Tiennes et C) *Silene nutans* sur substrat siliceux à Ambleteuse

5.2.2. Caractéristiques biologiques

Silene nutans est une espèce diploïde de la famille des Caryophyllacées. C'est une plante vivace à rosette (Hauser & Weidema, 2000). C'est une plante herbacée ayant un port ligneux. La couleur dominante des fleurs est le blanc mais on trouve aussi le rose et d'autres couleurs selon les populations (Figure 1.8). Elle est décrite comme une espèce gynodioïque- gynomonoïque en populations naturelles avec des plantes femelles (mâles stériles), hermaphrodites et des plantes intermédiaires dites gynomonoïques (porteurs d'un mélange de fleurs femelles et hermaphrodites, dont le déterminisme est encore inconnu) (Figure 1.9 ; Jürgens *et al.*, 2002a; Dufay *et al.*, 2010). Garraud *et al.* (2011) ont montré que le déterminisme sexuel de la gynodioécie chez *S. nutans* est un déterminisme nucléo-cytoplasmique avec 2 à 3 *CMS* différentes et jusqu'à 4 restaurateurs associés à une seule *CMS* et que deux de ces *CMS* ont une distribution géographique large à l'échelle de l'Europe présentant des niveaux de restauration très différents.

Figure 1.8. Différentes déclinaisons de couleurs de fleurs de *Silene nutans*

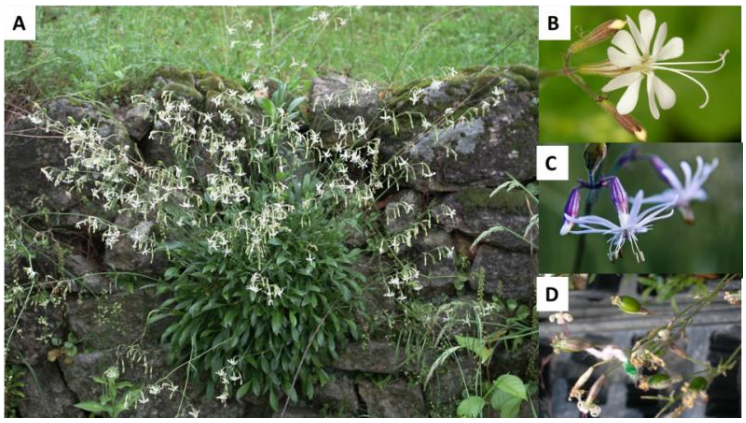


Figure 1.9. A *Silene nutans*, B fleur femelle, C fleur hermaphrodite et D Fruits (capsules) de *Silene nutans*

S. nutans est une espèce entomophile et auto-compatible, ce qui fait d'elle une espèce gynodioïque type. Les fleurs parfaites de cette espèce sont protandres, mais l'autofécondation peut se produire par geitonogamie puisque les plantes portent souvent plusieurs fleurs à une date donnée. Les composés chimiques de l'odeur florale identifiés chez *S. nutans* (Jürgens *et al.*, 2002) sont des éléments communs d'une large gamme de composés identifiés dans les fleurs parfumées des angiospermes (Knudsen *et al.*, 1993). Ces composés sont caractéristiques des fleurs pollinisées par des papillons (Jürgens *et al.*, 2002; Knudsen & Tollsten, 1993). Toutefois, la longueur du calice des fleurs de *S. nutans* les rendent moins restreintes aux sphingidés (Sphingidae), ce qui suggère que la pollinisation peut

être effectuée par un différents groupes de pollinisateurs (Jürgens *et al.*, 2002). Cette espèce présente un syndrome de pollinisation nocturne comme les pétales de couleur pâle (Jürgens *et al.*, 2002a). La biologie de la pollinisation de cette espèce reste tout de même assez peu connue mais on sait que les fleurs sont visitées par des insectes nocturnes (principalement des noctuidés (Noctuidae) et des sphingidés (Sphingidae)) et diurnes comme des abeilles (Apidae) et des syrphes (Syrphidae) (Jürgens *et al.*, 1996).

Le sexe ratio observé en populations naturelles varie selon les populations de 0% à 60% des plantes femelles (Mathilde Dufaÿ, HDR.). La taille des populations est fortement variable et peut être extrêmement faible (Hauser & Weidema, 2000; Van Rossum & Prentice, 2004)

6. Objectifs et démarche de la thèse

Au cours de ma thèse, j'ai étudié l'évolution et le maintien de la gynodioécie chez *Silene nutans*. Dans ce manuscrit, j'aborderai pour commencer les patrons de diversité cytoplasmique afin de déterminer le type de dynamique qui permet le maintien du polymorphisme nucléo-cytoplasmique chez *S. nutans* (Chapitre 1). Ensuite dans le deuxième chapitre j'essayerai d'estimer empiriquement l'impact de deux paramètres qui sont l'autofécondation et la limitation pollinique sur l'avantage femelle en conditions expérimentales, dans deux situations contrastées de sexe ratio local. Dans le troisième chapitre, je me place dans une population naturelle de *S. nutans* afin de tenter de valider les résultats observés en populations expérimentales, en particulier la limitation pollinique et la variation du taux d'autofécondation. Dans cette même étude, j'ai étudié la structure génétique de cette population et j'ai tenté de déterminer les flux de gènes qui se produisent au sein de cette population, puisqu'une structuration en patches peut générer des biais de visites de pollinisateurs entre les taches de plantes, provoquant potentiellement des différences de succès reproducteur entre les

plantes. Enfin, dans le dernier chapitre, je mettrai l'accent sur la relation de *S. nutans* avec ses pollinisateurs et nous comparerons la contribution des différents groupes de pollinisateurs (diurnes et nocturnes) au succès reproducteur de *S. nutans*.

Chapitre 1

Disentangling the effects of mating systems and mutation rates on cytoplasmic diversity in gynodioecious Silene nutans and dioecious Silene otites

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ORIGINAL ARTICLE

Disentangling the effects of mating systems and mutation rates on cytoplamic diversity in gynodioecious *Silene nutans* and dioecious *Silene otites*

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Many flowering plant species exhibit a variety of distinct sexual morphs, the two most common cases being the co-occurrence of females and males (dioecy) or the co-occurrence of hermaphrodites and females (gynodioecy). In this study, we compared DNA sequence variability of the three genomes (nuclear, mitochondrial and chloroplastic) of a gynodioecious species, *Silene nutans,* with that of a closely related dioecious species, *Silene otites*. In the light of theoretical models, we expect cytoplasmic diversity to differ between the two species due to the selective dynamics that acts on cytoplasmic genomes in gynodioecious species: under an epidemic scenario, the gynodioecious species is expected to exhibit lower cytoplasmic diversity than the dioecious species, while the opposite is expected in the case of balancing selection maintaining sterility cytoplasms in the gynodioecious species. We found no difference between the species for nuclear gene diversity, but, for the cytoplasmic loci, the gynodioecious *S. nutans* had more haplotypes, and higher nucleotide diversity, than the dioecious relative, *S. otites*, even though the latter has a relatively high rate of mitochondrial synonymous substitutions, and therefore presumably a higher mutation rate. Therefore, as the mitochondrial mutation rate cannot account for the higher cytoplasmic diversity found in *S. nutans*, our findings support the hypothesis that gynodioecy in *S. nutans* has been maintained by balancing selection rather than by epidemic-like dynamics.
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Keywords: balancing selection; mitochondrial mutation rate; dioecy; gynodioecy; *Silene nutans*; *Silene otites*

INTRODUCTION

Mating systems are major factors affecting species' genetic and genomic diversity (Charlesworth and Wright, 2001; Glémin *et al.*, 2006). Mating system differences are particularly striking in flowering plants, including a variety of sexual polymorphisms, that is, the co-occurrence of morphologically distinct sex phenotypes (reviewed by Barrett (2010)). Among these are dioecy, the co-occurrence of females and males within a given species, and gynodioecy, females co-occurring with hermaphrodites (Darwin, 1877; Renner and Ricklefs, 1995). Gynodioecy has been considered either as a stable mating system or as a transient state during the evolution of dioecy. The maintenance of gynodioecy has long been considered an evolutionary puzzle. It often involves a genomic conflict between the nuclear and cytoplasmic genomes, which differ in their mode of transmission (Lewis, 1941; Cosmides and Tooby, 1981; Saumitou-Laprade *et al.*, 1994). Specifically, female (that is, male-sterile) individuals in gynodioecious species result from factors in the maternally inherited mitochondrial genome (called cytoplasmic male sterility or CMS factors). Hermaphroditic individuals can result either when male-sterility factors are absent, or from the presence of bi-parentally transmitted nuclear restorer factors that counteract the action of the male-sterility factors and allow normal pollen development (reviewed by Chase (2007) and Delph *et al.* (2007)). Hermaphrodites in gynodioecious species reproduce via both their female and male functions, while females reproduce only via female functions, so females might be expected to be at a selective disadvantage and quickly be eliminated, resulting in a monomorphic hermaphroditic population (Valdeyron *et al.*, 1973). Two classes of theoretical models have been proposed to account for the maintenance of sterility factors in populations.

In the first class of models, females must have a selective advantage in female functions (that is, higher seed fitness of females than hermaphrodites, due to resource reallocation to female function or avoidance of inbreeding depression). This female advantage combined with a cost of restorer alleles, at least when they are associated with cytoplasms different from the one they restore, can allow the maintenance of a nuclear-cytoplasmic polymorphism. This is a form of balancing selection involving negative frequency-dependent selection (Charlesworth, 1981; Gouyon *et al.*, 1991; Dufay *et al.*, 2007). Under such assumptions, CMS factors are advantageous only when restorer alleles are rare (when they are mainly carried by females), while restorer alleles are selected for only when CMS factors are frequent.

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The second class of models posits gene flow between a set of interconnected populations (a metapopulation), causing recurrent invasions of CMS factors, which results in transient male sterility in the populations, again through a female fertility advantage. The increase in frequency of CMS factors within a local population provides a selective advantage for restorer factors, which may invade from other populations, and ultimately become fixed in the local population, leading to loss of its sexual polymorphism until a new CMS invades. Under this class of models, the maintenance of gynodioecy results from epidemic-like dynamics (Frank, 1989; Couvet *et al.,* 1998).

The two classes of models make opposite predictions for cytoplasmic diversity. Epidemic dynamics should reduce nucleotide diversity, because new sterilizing cytoplasms repeatedly sweep through local populations, leading to homogenization of the cytoplasmic genotype within and across populations (Ingvarsson and Taylor, 2002). In contrast, the balancing selection involved in the stable nucleo-cytoplasmic polymorphism model should lead to higher nucleotide diversity of the mitochondrial genome in gynodioecious species compared with hermaphroditic or dioecious species, because non-recombining haplotypes are maintained, potentially over long periods of time, and can accumulate different mutations (Hudson and Kaplan, 1988; Charlesworth, 2002; Städler and Delph, 2002; Touzet and Delph, 2009).

These assumptions have been tested in the genus *Silene*, which includes a diversity of mating systems, including hermaphroditic, gynodioecious and dioecious species (for example, Desfeux *et al.*, 1996; Jürgens *et al.*, 2002) and thus allows the use of comparative tests of whether balancing selection or epidemic dynamics have predominantly affected the evolutionary dynamics of gynodioecy. However, previous studies comparing cytoplasmic diversity among *Silene* species with different reproductive systems have led to contradictory conclusions. Ingvarsson and Taylor (2002) showed that sequence variation at chloroplast loci within the gynodioecious species *Silene vulgaris* is low relative to that in *Silene latifolia*, a closely related dioecious species, whereas the two species did not differ in diversity at a nuclear gene studied, tending to support epidemic dynamics. Conversely, Städler and Delph (2002) studying the nucleotide diversity of a mitochondrial gene in gynodioecious *S. acaulis*, described a large number of divergent haplotypes, which they attributed to the signature of balancing selection. Moreover, Houliston and Olson (2006) showed also high mitochondrial gene diversity in *S. vulgaris* contradicting Ingvarsson and Taylor's conclusion. Finally, a comparative study of mitochondrial gene diversity on a sample of three gynodioecious (*S. acaulis*, *S. vulgaris* and *S. nutans*) and seven non-gynodioecious *Silene* species showed that mitochondrial gene diversity was high in gynodioecious species when compared with non-gynodioecious ones, favouring again the 'balancing selection' model (Touzet and Delph, 2009). One major problem, unresolved by previous studies, is that the difference of mitochondrial diversity between species can be explained not only by the mating system but also by the mitochondrial mutation rate, which has been found to be extremely variable among genes and among species in the *Silene* genus (Barr *et al.*, 2007; Mower *et al.*, 2007; Sloan *et al.*, 2008; Sloan *et al.*, 2009). It is thus necessary in comparative studies to control this effect to disentangle the confounding effects of balancing selection and an elevated mitochondrial mutation rate. In the current study, we therefore compared two closely related *Silene* species belonging to the same subgenus, the gynodioecious *S. nutans*, with nucleo-cytoplasmic gynodioecy (Garraud *et al.*, 2011), and the dioecious *S. otites*. To assess the most likely evolutionary scenario involved in the maintenance of gynodioecy in *S. nutans*, we compared diversity in the two species, using loci sampled from all three genomes, mitochondrial, chloroplastic and nuclear. Nuclear genes help us to control for possible demographic differences between the two species, such as recent bottlenecks reducing diversity. We then used HKA tests (Hudson *et al.*, 1987) to control for mutation rate differences, and also used chloroplast loci (after testing for molecular clock rate differences for the chloroplast genome) as a way to test whether the observed differences could be due to mitochondrial mutation rate variation. Owing to their predominant uniparental inheritance, linkage disequilibrium (LD) is expected between the chloroplast genome and the targets of selection in the mitochondrial genome, and therefore both cytoplasmic genomes should exhibit the same signature of selection (whether epidemics or balancing selection). However, paternal leakage has been documented in other *Silene* species, disrupting complete LD between the cytoplasmic genomes (McCauley *et al.*, 2005), so we also tested for recombination between and within the mitochondrial and chloroplast genomes of both species.

MATERIALS AND METHODS

Species and plant material

S. nutans (Caryophyllaceae) is a diploid, long-lived perennial rosette plant growing in dry, open grass communities of hillsides. It is a gynomonoecious–gynodioecious (gynodioecious, but with some individuals having flowers of both sex types) self-compatible species (Desfeux *et al.,* 1996; Dufay *et al.*, 2010). It has a wide distribution range, extending from North-Western Europe to Siberia and the Caucasus (Hegi, 1979; Van Rossum *et al.*, 1996; Van Rossum *et al.*, 1999). *S. otites* (Caryophyllaceae) is a dioecious perennial plant common in low-altitude rocks and arid slopes (Desfeux *et al.*, 1996). It is distributed across Europe, extending from the centre of Spain, eastwards to Lithuania and Bulgaria (*Flora Europaea*).

We sampled a single individual per population of both species, in a paired sampling scheme with geographically 'co-located' accessions, on a wide geographic scale (Figure 1). We obtained a total of 47 accessions per species, and sequenced 20–37 accessions per gene/species. The *S. nutans* plants were collected from natural populations (Table 1), whereas those of *S. otites* were obtained from the herbarium of the Meise Botanical Garden, Belgium (F. Van Rossum), except for four populations for which seeds were grown in the greenhouse (Supplementary Table 1). We used one plant of the dioecious species *S. latifolia* as an outgroup.

Molecular analyses

To assess mitochondrial diversity, we sequenced two genes, coding for cytochrome *b* (cob) and for the first sub-unit of cytochrome oxidase (*cox1*). There have been no known transfer of either of these genes to the nuclear genome among angiosperms, that is, they are exclusively mitochondrial (Gray *et al.*, 1999; Adams *et al.*, 2002; Touzet and Delph, 2009). Four nuclear autosomal genes were also sequenced, the ATP-binding-cassette transporter gene (*ABCtrp*), the gene coding for the α sub-unit of the eukaryotic elongation factor-1 (*ELF*), the α tubulin gene (*ATUB*) and *X4*, putatively coding for fructose-2,6-bisphosphatase protein (Atanassov *et al.*, 2001; Marais *et al.*, 2011). Note that *X4* is not sex-linked in *S. otites* (Mrackova *et al.*, 2008). Finally, we sequenced four chloroplast fragments: three intergenic spacer sequences *trnG-trnS* (*GS*), *trnL-trnF* (*LF*) and *psbA-trnH* (*psbA*), and the fragment of the *matK* gene, that is believed to code for a maturase based on structural similarities to other such gene (Neuhaus and Link, 1987; Mohr *et al.*, 1993; Hilu *et al.*, 2003) and is the only maturase of higher plant plastids (Vogel *et al.*, 1997).

Total genomic DNA was extracted and purified from leaves using the NucleoSpin 96 Plant kit (Macherey-Nagel, Düren, Germany). PCR reactions were performed using 40 cycles of 30 s at 94 °C, 45 s at annealing temperature (Supplementary Table 2) and 1 min at 72 °C, with an initial step of 1 min at 94 °C and a final step of 10 min at 72 °C. Each mitochondrial gene was amplified with two pairs of primers, generating overlapping fragments (Supplementary Table 2).

PCR products were purified using Millipore MultiScreen-PCRμ96 filter plates (PCR filter plates) (Millipore Corporation, Billerica, MA, USA). Using

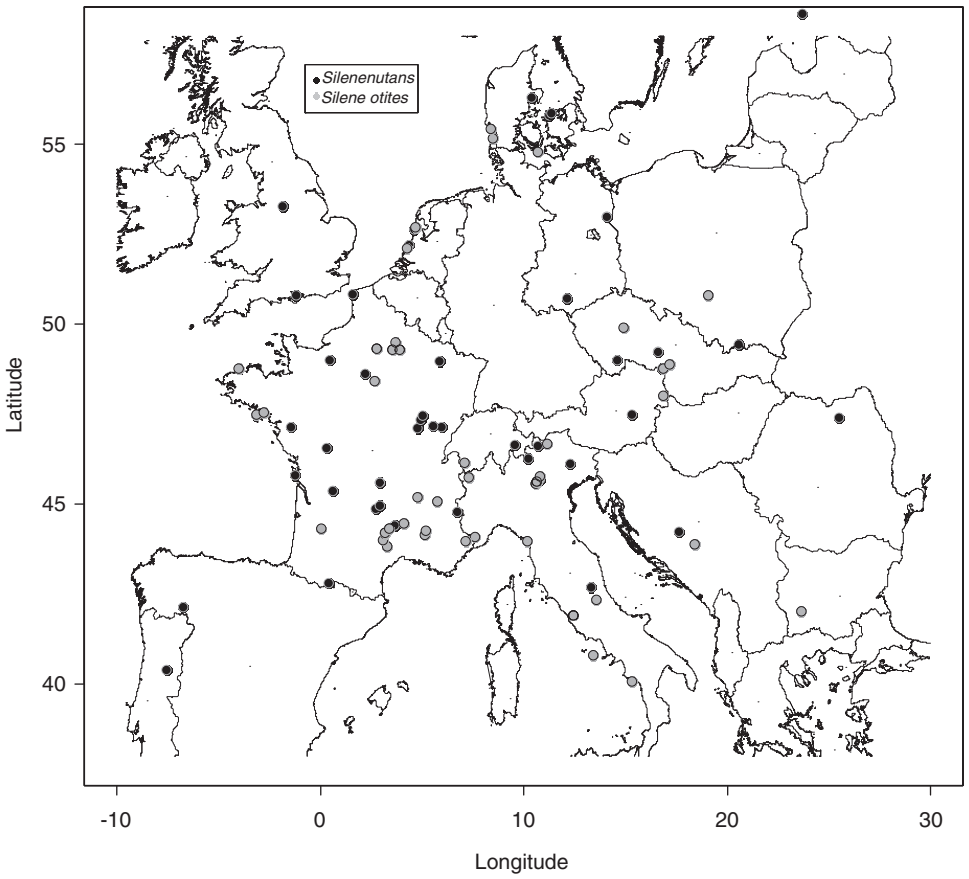

Figure 1 Geographical locations of the *S. nutans* and *S. otites* samples studied.

the Big Dye Terminatorv3.1 Cycle Sequencing Kit and an ABI 3130 (Applied Biosystems, Carlsbad, CA, USA), we directly sequenced both strands of the purified PCR products except for the two nuclear genes *ATUB* and *ELF*; these two genes were cloned using the TA Cloning Kit with pCR 2.1 vector (Invitrogen, Carlsbad, CA, USA). Positive colonies were then screened for presence of the appropriate-sized insert by direct PCR, using the conditions described by the manufacturer, with the primers M13-F (5′-CACGACGTTG-TAAAACGAC-3′) and M13-R (5′-GGATAACAATTTCACACAGG-3′). When a haplotype was found only once, it was confirmed by sequencing from an independent PCR reaction. All sequences were deposited in EMBL (accessions KC211324 to KC211517).

Statistical analyses

Sequences were aligned manually using Bioedit version 7.0.5.3 (Hall, 1999).

Plant mitochondrial transcripts are known to undergo post-transcriptional C–U editing at non-synonymous sites (Gray and Covello, 1993; Maier *et al.*, 1996; Brennicke *et al.*, 1999). Such editing may result in C–T DNA polymorphism not being reflected as a polymorphism in the mRNA. Consequently, while the site would be predicted to be non-synonymous from the DNA, with editing, the mutation would not alter the amino-acid sequence. Edited sites were predicted using the online resource PREP-Mt (http://www.prep-mt.net; Mower, 2005), with a cutoff value of 0.2.

We estimated nucleotide diversity both as π, the average number of nucleotide differences per site between a pair of randomly chosen sequences (Nei, 1987), and as Watterson's θ_w (Watterson, 1975). We also estimated the average numbers of nucleotide substitutions per site, K, between the species studied and the outgroup *S. latifolia* and K_s, the value for synonymous site. To compare the numbers of haplotypes and numbers of segregating sites of nuclear and cytoplasmic sequences between the two species, one-sided paired Wilcoxon signed-rank tests were performed using R version 2.11.1. The minimum numbers of recombination events R_m, were estimated by the four-gametes test of Hudson and Kaplan (1985) and LD between cytoplasmic polymorphic sites was estimated by |D'| (Lewontin, 1964). All parameters were estimated with DnaSP version 5 (Librado and Rozas, 2009). A permutation procedure was used to test whether LD observed within genomes (between polymorphic sites located within either the chloroplastic or the mitochondrial genomes) was significantly different from that observed between genomes (between polymorphic sites, one located on the chloroplast and the other in the mitochondrion).

Mitochondrial synonymous substitution rates vary greatly between different *Silene* species, potentially confounding mutation rate differences affecting diversity with diversity differences due to different selection regimes. We took account of potential mutation rate differences in two different ways. First, we compared synonymous divergence from the outgroup *S. latifolia* of the mitochondrial genes with that of the chloroplast genes (for which no variation in mutation rate has been documented).

Second, we tested for neutrality of the observed polymorphisms by computing Tajima's D (Tajima, 1989), which is based on the difference between π and θ_w, and Fu and Li's D (Fu and Li, 1993), which is based on differences between the total number of mutations in the external branches of the genealogy (with *S. latifolia* as an outgroup) and the overall number of mutations. These two tests were performed using DnaSP version 5 (Librado and Rozas, 2009). We then used a maximum-likelihood-ratio test of the standard neutral model, using multilocus data on polymorphism within species and divergence between species. This model (MLHKA) is based on the HKA test, which evaluates the fit of polymorphism and divergence to expectations under the neutral theory, even if the mutation rates differ between two species (Hudson *et al.*, 1987), but allows for an explicit test of selection at individual loci in a multilocus framework. Under the neutral theory,

Table 1 Diversity measures (number of haplotypes and of segregating sites, θ_w and π) of the three genomes in *S. nutans* and *S. otites* and results of the neutrality tests (Tajima's *D* between π and θ_w and Fu and Li's *D* with *S. latifolia* as an outgroup)

Genome	*Genes*	*Species*	*Length (pb)*	*Pop/ seq*	*Number of haplotypes*	*Segregating sites*	$\theta_w \pm$ *s.d.* ($\times 10^{-3}$)	$\pi \pm$ *s.d.* ($\times 10^{-3}$)	*Tajima's* D	*Fu and Li's* D *(S. latifolia)*
Nuclear	*X4*	*S. nutans*	578	22/44	4	23	9.15 ± 1.08	4.70 ± 2.05	−1.6062	1.7216
		S. otites	578	22/44	4	6	2.39 ± 1.15	4.66 ± 0.46	2.4906*	1.1846
	ELF	*S. nutans*	210	27/54	10	6	6.27 ± 2.99	6.45 ± 0.86	−0.3085	−0.4465
		S. otites	210	27/54	10	7	7.31 ± 3.31	10.27 ± 0.78	1.0516	1.2367
	ATUB	*S. nutans*	389	19/38	38	42	25.70 ± 8.36	45.08 ± 1.60	0.9779	0.8380
		S. otites	389	19/38	38	39	23.36 ± 7.82	41.51 ± 1.46	0.8380	1.0604*
	ABCtrp	*S. nutans*	352	35/70	5	6	3.54 ± 1.66	1.90 ± 0.36	−1.1045	1.1483
		S. otites	352	35/70	5	6	3.54 ± 1.66	3.59 ± 0.44	0.0325	1.1483
Mitochondrial	*Cob*	*S. nutans*	980	26/26	11	9	2.41 ± 1.07	2.21 ± 0.33	−0.2663	1.4610
		S. otites	980	26/26	4	4	1.07 ± 0.61	1.97 ± 0.20	2.2611*	1.0941
	Cox1	*S. nutans*	1037	22/22	16	18	4.76 ± 1.89	3.76 ± 1.89	−0.7760	−1.0939
		S. otites	1037	22/22	8	9	2.38 ± 1.08	2.74 ± 0.28	0.5095	1.4774
Chloroplast	*GS*	*S. nutans*	533	37/37	7	7	3.75 ± 1.74	2.75 ± 0.17	−0.7533	0.4829
		S. otites	533	37/37	3	5	2.88 ± 1.49	5.57 ± 0.49	2.2884	0.9282
	psbA	*S. nutans*	299	37/37	9	12	10.69 ± 4.29	13.73 ± 0.80	0.8813	0.5275
		S. otites	299	37/37	3	9	8.52 ± 3.68	10.86 ± 2.61	0.4298	0.9282
	LF	*S. nutans*	505	37/37	6	6	3.27 ± 1.60	1.74 ± 0.38	−1.4607	0.2110
		S. otites	505	37/37	3	10	5.61 ± 2.36	8.85 ± 1.11	1.7480	1.4035
	matK	*S. nutans*	684	37/37	7	6	2.10 ± 1.03	3.2 ± 0.22	1.4091	1.0488
		S. otites	684	37/37	3	4	1.40 ± 0.78	1.94 ± 0.45	0.9297	0.9282
Chloroplast concatenated	*Cp*	*S. nutans*	2021	37/37	11	31	4.04 ± 1.37	4.28 ± 0.31	0.1400	0.6943
		S. otites	2021	37/37	6	28	3.77 ± 1.29	5.71 ± 0.76	1.7257	1.6834

*$P<0.05$.

within-species diversity should correlate with between-species divergence (Kimura, 1983); an unexpectedly high divergence can therefore suggest positive selection, whereas an excess level of within-species polymorphism can detect balancing selection (Hudson *et al.*, 1987). The MLHKA approach compares the relative extents of polymorphism and divergence across loci, and assesses the overall fit of the data to a neutral model that assumes the same ratios of polymorphism and divergence at all loci. We used this approach to compare the polymorphism to divergence ratio between *S. nutans* and the outgroup species *S. latifolia* with that between *S. otites* and the same outgroup, combining likelihood across all gene sequences of *S. nutans* and *S. otites* for a given genome. The version used was developed by Wright and Charlesworth (2004) and is available from http://labs.eeb.utoronto.ca/wright/Stephen_I._-Wright/Programs.html. The program was run under a strictly neutral model for a total of one million chains, followed by a 'selection' model in which the *S. nutans* loci were designated candidates to test for the action of selection, again for a total of one million chains. Significance was assessed using the likelihood-ratio test where minus twice the difference in log-likelihood between the nested models is approximately chi-squared distributed with a number of degrees of freedom equal to the number of genes tested.

Neighbour-Joining (NJ) trees were built using the software MEGA version 4.1 (Kumar *et al.*, 2004) with Kimura's two parameters model (Kimura, 1980) and a uniform gamma value, including transitions and transversions.

RESULTS

Editing assessment

To accurately evaluate the non-synonymous polymorphism in our data set, we used the online resource PREP-Mt (Mower, 2005) (with a cutoff value of 0.2) to detect potential edited sites on non-synonymous variants. Only one site was predicted to be edited: site 747 of *cox1* (but that still remains non-synonymous after editing: $G_{747}C_{748}G_{749}/G_{747}T_{748}G_{749}$ translated (A_{249}/V_{249}) becomes after editing $G_{747}T_{748}G_{749}/G_{747}T_{748}T_{749}$ translated (A_{249}/V_{249})]. The amino-acid sequences of both genes were thus deduced and revealed several variable sites, generating, after editing, four different *cob* and seven different *cox1* amino-acid sequences (Supplementary Table 3). Two peptide sequences from the sequences of the *cob* gene were shared by both species, which was not the case for the peptide sequences of *cox1*.

Neutrality tests

With only three exceptions, all in *S. otites*, the frequency spectra suggested no strong departures from neutrality in either species (Table 1). However, for *S. otites*, significantly positive Tajima's *D* was found for the mitochondrial *cob* gene, and the nuclear *X4* gene, and significantly positive Fu and Li's *D* value for the nuclear *ATUB* gene. Overall, across the different loci studied, Tajima's *D* tended to be more negative in *S. nutans* than in *S. otites*, suggesting possible recent population growth in the former, and/ or a recent bottleneck in the latter.

Phylogenetic relationships between the two species

We built NJ trees of haplotypes using *S. latifolia* as an outgroup. As the two species were closed, we used the same outgroup for them. The NJ trees revealed that *ABCtrp, X4, cob* and the chloroplastic sequences, clustered according to the species (Supplementary Figure 1). For *ATUB, ELF* and *cox*, the NJ trees exhibited an incomplete lineage sorting of haplotypes. For *ABCtrp, X4* and *ELF* sequences, the haplotypes of *S. otites* were a subset of those seen in *S. nutans*. Therefore, we evaluated the level of shared polymorphism between the two species.

One shared mutation between *S. nutans* and *S. otites* was found in *matK*, in *LF* and in *cob* gene (Supplementary Table 4). We found no fixed sites between the two species for the *cox1* sequences, but detected two shared polymorphisms. The concatenated chloroplast sequences showed one shared mutation. These observations suggested either that the two species have recently diverged, or that introgression had occurred between them.

Similar nuclear diversity in both species

For the nuclear genes, the numbers of haplotypes were identical in *S. nutans* and *S. otites* for every locus analyzed, and ranged from 4 to 38 (Table 1); a one-tailed paired Wilcoxon signed-rank test revealed no significant difference. There was also no difference in the number of segregating sites (S) ($V=3$; P-value $=0.18$). θ_w was also very similar between the two species, except for the *X4* gene, with more variable sites in *S. nutans* than *S. otites* (9.15 ± 1.08 vs 2.39 ± 1.15, respectively), mostly due to the presence in *S. nutans* of two singleton haplotypes that contributed 22 out of a total of 23 polymorphic sites. MLHKA tests did not detect any diversity difference between the two species for the nuclear genes ($-2.\text{deltaL} = 6.2482$, $\text{df} = 4$, P-value $= 0.1813$; Table 3). Taken together, the results from the nuclear genes suggest that any difference in cytoplasmic diversity between the two species should not be ascribed to a difference in their demographic history.

Table 2 The ratios of polymorphism (π) and divergence (K) between the two species and *S. latifolia* on mitochondrial and chloroplastic genes/fragments

Genes	*Species*	π	K	π/K	$(\pi/K)_{nu}/(\pi/K)_{ot}$
cob	*S. nutans*	0.00221	0.00992	0.22278226	1.34907
cob	*S. otites*	0.00198	0.01199	0.16513761	—
cox1	*S. nutans*	0.00377	0.00537	0.70204842	1.99126
cox1	*S. otites*	0.00275	0.00780	0.35256410	—
GS	*S. nutans*	0.00314	0.76129	0.00412458	0.83249
GS	*S. otites*	0.00390	0.78716	0.00495452	—
Psba	*S. nutans*	0.05306	0.34419	0.15415904	4.57477
Psba	*S. otites*	0.00632	0.18755	0.03369768	—
LF	*S. nutans*	0.00125	0.05256	0.02378234	0.18625
LF	*S. otites*	0.00925	0.07244	0.12769188	—
Matk	*S. nutans*	0.00323	0.04470	0.07225951	1.92151
Matk	*S. otites*	0.00196	0.05212	0.03760553	—

Test for mitochondrial mutation rate differences

The *S. nutans* and *S. otites* chloroplast sequences showed similar silent site divergence from *S. latifolia* ($K_s = 97.8 \times 10^{-3}$ and 92.4×10^{-3}, respectively). In contrast, both *S. nutans* mitochondrial genes were less diverged from *S. latifolia* than those of *S. otites* (at synonymous sites $K_s = 19.8 \times 10^{-3}$ and 16.8×10^{-3} for *S. nutans cob* and *cox1*, respectively, vs *S. otites* values of $K_s = 36.1 \times 10^{-3}$ and 31.3×10^{-3} for *cob* and *cox1*, respectively), suggesting neutral substitution rate in *S. nutans* half that in *S. otites*, and therefore a lower mutation rate. Thus, higher diversity in the *S. nutans* mitochondrial genome (see next section) is unlikely to be caused by a higher mutation rate.

Comparison of cytoplasmic diversity between the gynodioecious and dioecious species

The level of diversity for the cytoplasmic genes was strikingly different between the two species. The number of haplotypes was higher in *S. nutans* than in *S. otites* for both mitochondrial loci (Table 1). The *cob* gene had 11 distinct haplotypes in *S. nutans*, vs only 4 in *S. otites* (Supplementary Table 3; Table 2). For *cox1*, *S. nutans* had 16 haplotypes, twice the number in *S. otites* (8). The number of polymorphic sites was also twice as high for *S. nutans* as *S. otite*s for both genes (9 vs 4 and 18 vs 9, for *cob* and *cox1*, respectively). In line with the mitochondrial results, the concatenated chloroplast sequences also had more haplotypes in *S. nutans* than *S. otites* (11 vs 6) (Table 1). Across all the cytoplasmic loci, one-sided paired Wilcoxon signed-rank tests revealed a significant difference in the number of haplotypes ($V=21$; P-value $=0.018$), but not for the number of segregating sites ($V=17$; P-value $=0.104$). However the latter result is due mainly to a single chloroplast gene (*LF*), and excluding this gene resulted in a significant difference ($V=15$; P-value $=0.028$) between the two species.

Interestingly, the elevated diversity observed in *S. nutans* as compared to *S. otites* was much more pronounced for the mitochondrial genes than the chloroplast genes. Indeed, for the mitochondrial genes studied, *cob* and *cox1*, both the nucleotide diversity measures, θ_w and π, were higher in *S. nutans* than in *S. otites*, as were the polymorphism/divergence ratios (Table 2). The MLHKA program estimated a 3.88-fold elevation of diversity in *S. nutans* compared

Table 3 Comparison of genome diversity (nuclear, mitochondrial, chloroplastic) between *S. nutans* and *S. otites* by the MLHKA test

Genome	*Gene*	*S. nutans*		*S. otites*		*Maximum likelihood*		*P*-value
		θ	k	θ	k	*Neutral model*	*Selection model*	
Nuclear	*X4*	0.01135	0.9124	0.00828	1			
	ELF	0.00795	0.9431	0.00758	1			
	ATUB	0.00988	2.8905	0.01218	1			
	ABCtrp	0.00468	0.8238	0.00569	1			
	Average		1.39245			−52.0885	−48.9644	0.181
Mitochondrial	*cob*	0.00156	1.6115	0.00166	1			
	cox1	0.00083	6.149	0.00167	1			
	Average		3.88025			−21.4399	−18.5157	0.054
Chloroplast	*psbA*	0.01014	1.1345	0.005335	1			
	LF	0.00155	2.0271	0.002458	1			
	matK	0.00119	2.0597	0.001941	1			
	Average		1.34242			−64.7002	−59.3524	0.030

k measures the degree to which diversity increases or decreases by the action of selection: $k>1$ (balancing selection), $k<1$ (purifying selection).

with *S. otites*, which was close to significance, for these two mitochondrial genes ($-2.\text{deltaL} = 58.5$, $df = 2$, $P\text{-value} = 0.053$; Table 3). Chloroplast diversity was also higher in *S. nutans* than in *S. otites*, but there was only a 1.34-fold estimated difference, and only three of the four chloroplast fragments showed higher π in the gynodioecious species, and only two had a larger θ_w. Nevertheless, the MLHKA test using all four sequences still indicated a significant difference ($-2.\text{deltaL} = 10.70$, $df = 4$, $P\text{-value} = 0.030$; Table 3).

The lesser elevation in diversity in *S. nutans* for the chloroplast than the mitochondrial genes is consistent with incomplete LD between variants in the cytoplasmic genomes, which could result through occasional paternal leakage leading to heteroplasmy. Although mitochondrial inheritance is probably largely uniparental, there is evidence of heteroplasmy in *S. vulgaris* (McCauley *et al.*, 2005; McCauley and Ellis, 2008; Pearl *et al.*, 2009) and recombination in mitochondrial genes of several gynodioecious *Silene* species (Städler and Delph, 2002; Houliston and Olson, 2006; Touzet and Delph, 2009). Four-gamete tests (Hudson and Kaplan, 1985) indeed revealed clear evidence for recombination within as well as between mitochondrial and chloroplast genomes for both species. The minimum number of recombination events R_m detected between the mitochondrial gene *cob* and the concatenated chloroplast sequences was 1 for both *S. nutans* and *S. otites.* No recombination was detected between *cox1* and the chloroplast sequences in either species. Recombination was also apparent within mitochondrial genes, with at least two and one recombination events for *S. nutans* and *S. otites* within *cob*, respectively, and even more for *cox1,* with at least five and two recombination events for *S. nutans* and *S. otites,* respectively.

In line with this observation, significant breakdown of LD was observed between chloroplastic and mitochondrial genomes in *S. nutans* (mean LD within genomes = 0.947 vs mean LD between genomes = 0.853, $P < 0.01$). No such difference was observed in *S. otites* (0.979 vs 0.964, respectively, $P > 0.05$).

DISCUSSION

What can we conclude about the evolutionary processes maintaining gynodioecy in *S nutans*? This gynodioecious species exhibits higher diversity in its cytoplasmic genes, compared with the dioecious *S. otites.* Interestingly, this diversity difference is the opposite of the mitochondrial mutation rate difference, as the rate is lower in *S. nutans*. Altogether, these results are consistent with the 'balancing selection' scenario, in which natural selection maintains cytoplasmic haplotypes over long periods of time specifically in the gynodioecious species.

Previous studies on *Silene* species suggested balancing selection as the most probable dynamics maintaining nuclear-cytoplasmic gynodioecy. In particular, Touzet and Delph (2009) showed that gynodioecious species exhibited more mitochondrial haplotypes and more divergent ones when compared with hermaphroditic or dioecious species. However, the question remained whether the result could not be explained by a variation in the mitochondrial mutation rate, which can be high among *Silene* species, as pointed out later by several studies (Barr *et al.*, 2007; Mower *et al.*, 2007; Sloan *et al.*, 2008; Sloan *et al.*, 2009). This is particularly critical when one considers that the species that exhibited the highest diversity (*S. acaulis* and *S. nutans*) belong to the same subgenus clade, while the non-gynodioecious species belong to another clade. For the current study, we chose a pair of phylogenetically closely related species, gynodioecious *S. nutans* and dioecious *S. otites*, to limit this phenomenon. Using a sample representative of both species, we assessed the nucleotide diversity of multiple genes in the three genomes, to control any demographic effect with the nuclear data and any variation of mitochondrial mutation rate with the chloroplastic data. Convincingly, thanks to the chosen methodology, we showed that mutation rate is not the proximal cause of the higher cytoplasmic diversity found in *S. nutans* and therefore that balancing selection maintains gynodioecy in populations. Our results apparently exhibit some discrepancy with a former study conducted by Sloan *et al.* (2009) that found, by using a phylogenetic approach, that the mitochondrial mutation rate was higher in *S. nutans* compared with *S. otites*. However, this higher rate in *S. nutans* was mainly due to an increased rate specific to *atp1,* illustrating, as pointed out by the authors, the large variation in the estimated mutation rate among the genes studied (*nad9*, *cox3*, *atp1* and *atp9* in this case). Because none of these genes were included in the current study, these two sets of results are not necessarily contradictory.

More generally, our results complement and partly confirm conclusions drawn by studies that used other methodological approaches to investigate the evolutionary dynamics driving the evolution of gynodioecy and found variation in sex ratio among populations that fits expectations under balancing selection (for example, Dufay *et al.* (2009) in *Beta vulgaris*) and empirical evidence for frequency-dependent individual reproductive success, that is a necessary condition for such dynamics to occur (for example, Graff (1999) in *Sidalcea malviflora*; Williams *et al.* (2000) in *Geranium richardsonii*; McCauley and Brock (1998) and Miyake and Olson (2009) in *Silene vulgaris* and De Cauwer *et al.* (2010a, b) in *Beta vulgaris*).

The non-gynodioecious sister species to which the nucleotide diversity of *S. nutans* was compared in this study is dioecious (with males and females). This reproductive system has evolved many times independently in flowering plants (reviewed by Renner and Ricklefs (1995)). Gynodioecy may sometimes be a step in the evolutionary route from hermaphroditism to dioecy (reviewed by Barrett (2002)) and several theoretical studies have shown that nucleo-cytoplasmic gynodioecy (as in *S. nutans*) can evolve towards dioecy, through the replacement of hermaphrodites by males (Maurice *et al.*, 1994; Schultz, 1994). Although this evolutionary transition has received little empirical support (Spigler and Ashman, 2012), it could have occurred in the genus *Silene*. Gynodioecy is the ancestral mating system in the genus, and at least two independent transitions from gynodioecy towards dioecy have probably occurred: one leading to the *S. latifolia* group, and one to the *S. otites* one (Desfeux *et al.*, 1996; Mrackova *et al.*, 2008; Marais *et al.*, 2011). Dioecy in *S. otites*, is thought to have evolved from gynodioecy only recently, because (i) intermediate stages between the two mating systems have been reported, with occasional hermaphroditic individuals being found (Desfeux *et al.*, 1996) and (ii) the *S. otites* sex-determining homomorphic chromosomes seem to be at an evolutionarily much younger stage than those of dioecious *S. latifolia* (Mrackova *et al.*, 2008). Käfer *et al.* (2012) tested recently whether dioecious species suffered from a less efficient purifying selection in comparison with non-dioecious ones in *Silene* due to an expected reduction of their effective population size. Contrarily to *Silene latifolia*, which exhibited the expected effect, they did not find any trace of it in *S. otites*, suggesting also a recent transition to dioecy in the species. This view is consistent with several of our results, such as the fact that *S. otites* haplotypes are often a subset of the *S. nutans* ones and the shared polymorphism for most of the genes studied between the two species.

If the hypothesis of evolution of dioecy in *S. otites* from nuclear-cytoplasmic gynodioecy has not been formally established in the

literature at this point, this does not affect our conclusion that balancing selection is probably involved in *S. nutans*. One should note, however, that when dioecy evolves in such models, balancing selection on the mitochondrial genome should not continue in the dioecious species, which usually becomes fixed for the genotype. Consistently with our findings, such transition from gynodioecy to dioecy should thus lead to loss of diversity in the cytoplasmic genome, even in a newly evolved dioecious species. For a better understanding of the transition from gynodioecy to dioecy, it would be interesting to investigate *S. acaulis* genetic diversity, as a recent study by Marais *et al.* (2011) suggests that *S. acaulis* is indeed the closest relative to dioecious *S. otites*.

DATA ARCHIVING

Data deposited in the Dryad repository: doi:10.5061/dryad.gd93s and in Genbank: accession numbers: KC211324 to KC211517.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on Heredity website (http://www.nature.com/hdy)

Supplementary Table 1.

Description of sequenced accessions of *S. nutans* and *S. otites*: geographical coordinates, location and origin.

Population code	Latitude	Longitude	Country	Location	Collector / Herbarium bar code
S. nutans					
N1	40°24'N	7°32'W	Portugal	Manteigas, Serrada Estrêla	BR - S.P. 974 148
N2	42°07'N	6°45'W	Spain	Ribadelago, Zamora	BR - S.P. 671 830
N3	42°40'N	13°19'E	Italy	Amatrice, Rieti	BR - S.P. 903 214
N4	42°49'N	0°25'E	France	Aranvielle, Hautes Pyrénées	F. Van Rossum
N5	44°13'N	17°40'E	Bosnia-Herzegovina	Travnik, central Bosnia	BR - S.P. 671 841
N6	44°23'N	3°41'E	France	Runes, Cévennes	F. Van Rossum
N7	44°47'N	6°44'E	France	Arvieux, Queyras	F. Van Rossum
N8	44°52'N	2°46'E	France	Devèze, Auvergne	F. Van Rossum
N9	44°57'N	2°56'E	France	Pierrefort, Auvergne	F. Van Rossum
N10	45°21'N	0°38'E	France	Bourdeilles, Dordogne	F. Van Rossum
N11	45°35'N	2°56'E	France	Murol, Auvergne	F. Van Rossum
N12	45°40'N	10°49E	Italy	Creste di Naole, Prada, Veneto	BR - S.P. 671 832
N13	45°47'N	1°12'W	France	Royan, Charente maritime	O. Raspé
N14	46°06'N	12°17'E	Italy	Monte Faverghera, Belluno	I.S. Botanic Garden, Padova University
N15	46°15'N	10°15'E	Italy	Sondrio, Lombardy	H.C. Hauffe
N16	46°33'N	0°20'E	France	Saint Benoit, Poitou	F. Van Rosum
N17	46°37'N	10°42'E	Italy	Laas, Bolzano, Südtirol	I.S. Botanical Garden, Berlin University
N18	46°38'N	9°35'E	Switzerland	Salouf ob Alp Muntér, Grisons	I.S. Botanical Garden St-Gallen
N19	47°06'N	4°48'E	France	Savigny-les-Beaune, Bourgogne	E. Schmidt
N20	47°08'N	5°58'E	France	Les Granges, Jura	F. Van Rossum
N21	47°08'N	1°25'W	France	Château-Thébaud, Loire-Atlantique	S. Le Cadre

N22	47°10'N	5°33'E	France	Serre forest, Jura	F. Van Rossum
N23	47°20'N	4°56'E	France	Plombières-les-Dijon, Bourgogne	E. Schmidt
N24	47°23'N	25°32'E	Romania	Pietrosul Broştenilor, Eastern Carpathians	I.S. Botanical Garden, Iaşi University
N25	47°27'N	5°03'E	France	Epagny, Bourgogne	E. Schmidt
N26	47°29'N	15°19'E	Austria	Sonnenalpe Naßfeld E, Kärnten	I.S. Botanical Garden, Salzburg University
N27	48°24'N	2°42'E	France	Fontainebleau forest, Île-de-France	Herbarium
N28	48°37'N	2°14'W	France	Saint-Cast-le-Guildo, Côtes d'Armor	S. Le Cadre
N29	48°46'N	16°51'E	Czech Republic	Charvátská Nová Ves, South Moravia	I.S. Botanic Gardens and Arboretum, Mendel University of Agriculture and Forestry Brno
N30	48°58'N	5°54'E	France	Jaulny, Lorraine	S. Le Cadre, B. Brachi
N31	48°59'N	14°36'E	Czech Republic	Ceske Budejovice, South Bohemia	P. Šmilauer
N32	49°00'N	0°29'W	France	Thury-Harcourt, Normandie	F. Van Rossum
N33	49°14'N	16°39'E	Czech Republic	Brno-Obřany, Moravia	BR - S.P. 671 849
N34	49°25'N	20°34'E	Poland	Małe Pieniny, Jaworki, Beskids	I.S. Botanical Garden, Berlin University
N35	50°43'N	12°09'E	Germany	Hellingen, Landkreis Hildburghausen	S. Le Cadre, B. Brachi
N36	50°47'N	1°10'W	United Kingdom	Gosport, South Hampshire	F. Van Rossum, S. Le Cadre
N37	50°49'N	1°38'E	France	Ambleteuse, Boulonnais	B. Brachi
N38	52°12'N	4°23'E	The Netherlands	Katwijk aan Zee, Zuid-Holland	S. Le Cadre, B. Brachi
N39	52°37'N	4°38'E	The Netherlands	Egmond aan Zee, Noord-Holland	S. Le Cadre, B. Brachi
N40	52°58'N	14°06'E	Germany	Gellmersdorf, Landkreis Uckermark	S. Le Cadre, B. Brachi
N41	53°15'N	1°49'W	United Kingdom	Buxton, Derbyshire	F. Van Rossum, S. Le Cadre
N42	55°47'N	11°17'E	Denmark	Nekselø, NW Zealand	H.H. Bruun
N43	55°50'N	11°23'E	Denmark	Ordrup Næs, NW Zealand	H.H. Bruun
N44	56°17'N	10°23'E	Denmark	Vosnæs pynt, N of Århus, Jutland	BR - S.P. 671 825
N45	58°36'N	23°42'E	Estonia	Lôo, Saaremaa	H.C. Prentice
N46	60°28'N	22°15'E	Finland	Turku, Varsinais Suomi	M. Christenhusz
N47	64°44'N	20°39'E	Sweden	Skellefteå, Västerbotten	F. Van Rossum

S. otites

O1	40°47′N	13°27′E	Italy	San Stefano,	M. Hood
O2	40°03′N	15°22′E	Italy	Licusati, Capri, Campania	BR - S.P. 843 755
O3	41°53′N	12°29′E	Italy	None	L. Delph, I.S. Royal Botanical Garden Kew
O4	41° 53′N	12° 29′E	Italy	none	D. Charlesworth
O5	42°01′N	23°40′E	Bulgaria	Jakoruda, Rila Mts, Blagoevgrad	BR - S.P. 903 215
O6	42°20′N	13°35′E	Italy	Barisciano, Abruzzo	BR - S.P. 974 150
O7	43°48′N	3°19′E	France	Pegairolles-de-l'Escalette, Hérault	BR - S.P. 974 155
O8	43°52′N	18°25′E	Bosnia-Herzegovina	None	L. Delph, I.S. Royal Botanical Garden Kew
O9	43°57′N	10°11′E	Italy	Marmi, Lucca	BR - S.P. 974 153
O10	43°59′N	3°04′E	France	Devèze de Lapanouse de Cernon, Aveyron	BR - S.P. 698 268
O11	44°04′N	7°37′E	France	La Brigue, Alpes maritimes	BR - S.P. 698 248
O12	44°04'N	7°10'E	France	La Bolline, Valdeblore, Alpes maritimes	BR - S.P. 900 562
O13	44°07′N	5°11′E	France	Bédoin, Vaucluse	BR - S.P. 698 254
O14	44°09′N	3°12′E	France	Le Maubert, Aveyron	BR - S.P. 698 264
O15	44°12′N	3°13′E	France	Le Rozier, Lozère	BR - S.P. 698 246
O16	44°13′N	3°13′E	France	Saint Marcellin, Mostuéjouls, Aveyron	BR - S.P. 698 262
O17	44°14′N	5°11′E	France	Mollans-sur-Ouvèze, Drôme	BR - S.P. 698 251
O18	44°19′N	0°05′E	France	Casteljaloux, Lot et Garonne	BR - S.P. 698 279
O19	44°19′N	3°24′E	France	Gorges du Tarn, Aveyron	BR - S.P. 974 199
O20	44°19′N	3°24′E	France	Chely du Tarn, Aveyron	BR - S.P. 600 916
O21	44°28′N	4°06′E	France	Saint-Jean-de-Pourcharesse, Ardèche	BR - S.P. 698 252
O22	45°33′N	10°37′E	Italy	Albisano, Lago di Garda, Veneto	BR - S.P. 698 271
O23	45°37′N	10°40′E	Italy	Torri del Benaco, Lago di Garda, Veneto	BR - S.P. 698 258
O24	45°03′N	5°45′E	France	Saint-Pierre-de-Mésage, Isère	BR - S.P. 928 800
O25	45°11′N	4°49′E	France	Saint-Vallier, Var	BR - S.P. 600 905
O26	45°38′N	7°21′E	Italy	Arpisson, Valle di Cogne, Aosta	BR - S.P. 698 278

O27	45°46′N	10°49′E	Italy	Malcesine, Veneto	BR - S.P. 698 257
O28	46°07′N	7°08′E	Switzerland	Charrat, Valais	BR - S.P. 698 267
O29	46°40′N	11°10′E	Italy	Merano, Trentine-Alto Adige	BR - S.P. 698 277
O30	47°29′N	3°07′W	France	Penthièvre, Morbihan	BR - S.P. 807 238
O31	47°32′N	2°46′W	France	Sarzeau, Morbihan	BR - S.P. 698 280
O32	48°00'N	16°52′E	Austria	Parndorf, Burgenland	BR - S.P. 698 259
O33	48°25′N	2°42′E	France	Fontainebleau, Île-de-France	BR - S.P. 698 260
O34	48°45′N	4°01′W	France	Batz, Bretagne	BR - S.P. 698 281
O35	48°53′N	16°39′E	Czech Republic	Dolní Věstonice, Břeclav, South Moravia	BR - S.P. 698 274
O36	48°53′N	17°11′E	Czech Republic	Rohatec, South Moravia	BR - S.P. 974 154
O37	49°16′N	3°34′E	France	Bruys, Aisne	BR - S.P. 698 272
O38	49°17′N	3°55′E	France	Châlons-sur-Vesle, Champagne	BR - S.P. 698 269
O39	49°18′N	2°48′E	France	Béthisy-Saint-Pierre, Picardie	BR - S.P. 949 618
O40	49°28′N	3°42′E	France	Neuville-sur-Ailette, Laonnois	BR - S.P. 698 273
O41	50°08′N	14°24′E	Czech Republic	Bohnice, Praha, Central Bohemia	BR - S.P. 698 270
O42	50°48'N	19°17'E	Poland	Olsztyn, Częstochowa	BR - S.P. 698 245
O43	52°06'N	4°16′E	The Netherlands	Scheveningen, Zuid-Holland	BR - S.P. 698 282
O44	52°40'N	4°42′E	The Netherlands	Het Woud, Bergen, Noord-Holland	BR - S.P. 974 151
O45	54°46'N	10°43′E	Denmark	Kirkeby, Rømø, Jutland	BR - S.P. 698 275
O46	55°08'N	8°30′E	Denmark	Lakolk, Rømø, Jutland	BR - S.P. 698 276
O47	55°25'N	8°23'E	Denmark	Fanø, Jutland	BR - S.P. 698 244

Supplementary Table 2.

Sequence, size and Temperature of annealing of primers (Ta)

Genome	Gene	Primer	Sequence	Size (pb)	Ta (°C)
Nuclear	*X4*	*X4-F*	AATGGGATTTCCAGAGGAAC	20	60.2
	X4	*X4-R*	CACCCAGTTTTCCAAGAATG	20	60.2
	ELF	*ELF-F*	TAACGGTTATGCCCCAGTTC	20	56
	ELF	*ELF-R*	GACTCCAACAGCAACGGTCT	20	56
	ATUB	*ATUB-F*	TGCCCCCGTCATCTCTG	17	56
	ATUB	*ATUB-R*	ACCTTCCTCCATACCCTCAC	20	56
	ABCtrp	*ABCtrp-F*	CGACTCCATCCTGACC	16	52
	ABCtrp	*ABCtrp-R*	GCTCCTCCTTGTATTCC	17	52
Mitochondrial	*cob*	*SaCobFM1*	CACGACGTTGTAAAACGACAGCATTTGATAGATTATCCAACC	42	53
	cob	*SaCobRM717*	GGATAACAATTTCACACAGGGATGCCCCAAAACATTAGGA	40	53
	cob	*SaCobFM362*	CACGACGTTGTAAAACGACTTGGGGTCAGATGAGCTTTT	39	53
	cob	*SaCobRM1084*	GGATAACAATTTCACACAGGATTCTTCTTCCAACTCGTCC	40	53
	cox1	*SaCox1FM1*	CACGACGTTGTAAAACGACGGAGCAGTTGATTTAGCCAT	39	53
	cox1	*SaCox1RM663*	GGATAACAATTTCACACAGGCCCAGAATTTGCCAGGACTA	40	53
	cox1	*SaCox1FM473*	CACGACGTTGTAAAACGACTTGATACCCGCGCTTACTTC	39	53
	cox1	*SaCox1RM1077*	GGATAACAATTTCACACAGGCCATTCCAGTGTGGGTGAAT	40	53
Chloroplast	*trnG-trnS*	*TrnS (GCU)*	GATAACAATTTCACACAGGGCCGCTTTAGTCCACTCAGC	39	53
	trnG-trnS	*TrnG (UCC)*	ACGACGTTGTAAAACGACGAACGAATCACACTTTTACCAC	40	53
	psbA-trnH	*TrnH-FM*	CACGACGTTGTAAAACGACACTGCCTTGATCCACTTGGC	39	53
	psbA-trnH	*psbA-RM*	GGATAACAATTTCACACAGGCGAAGCTCCATCTACAAATGG	41	53
	trnL-trnF	*TrnL-FM*	CACGACGTTGTAAAACGACGGTTCAAGTCCCTCTATCCC	39	53
	trnL-trnF	*TrnF-RM*	GGATAACAATTTCACACAGGATTTGAACTGGTGACACGAG	40	53
	matK	*MatK-F1M*	CACGACGTTGTAAAACGACATCTTGGTTCAAATCCTTCGGTA	42	53
	matK	*MatK-R1M*	GGATAACAATTTCACACAGGATCAATAATATCCGAATCCGATAA	44	53

Supplementary Table3A.

Haplotype structure of *ABCtrp*

Haplotypes	8	50	59	80	104	110	125	128	203	206	215	233	239	275	278	350
ABCtrp_nutans_1(46)	G	C	G	G	G	T	A	G	T	G	A	T	C	C	G	C
*ABCtrp_nutans*_2(18)	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
*ABCtrp_nutans*_3(2)	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.
*ABCtrp_nutans*_4(2)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
*ABCtrp_nutans*_5(2)	.	.	.	T	.	.	.	.	.	T	.	.	.	T	.	.
*ABCtrp_otites*_1(36)	A	.	.	.	A	C	C	A	.	.	T	.	T	.	.	.
*ABCtrp_otites*_2(20)	A	T	.	.	A	C	.	A	.	.	T	.	T	.	.	.
*ABCtrp_otites*_3(6)	A	.	.	.	A	C	C	A	.	.	.	.	T	.	.	.
*ABCtrp_otites*_4(2)	A	.	.	.	A	C	C	A	.	.	.	C	.	.	T	.
*ABCtrp_otites*_5(6)	A	T	.	.	A	C	C	A	.	.	T	.	T	.	.	.
*ABCtrp_latifolia*_1(1)	.	.	.	.	.	C	.	.	.	T	.	.	.	.	T	.

Supplementary Table3B.

Haplotype structure of *ELF*

Haplotypes	17	26	92	107	110	152	155	161
ELF_nutans_1(24)	C	T	C	C	A	T	G	T
ELF_nutans_2(7)	.	.	.	.	.	.	.	C
ELF_nutans_3(7)	.	.	.	.	.	.	C	.
ELF_nutans_ 4(1)	.	.	.	.	.	G	.	.
ELF_nutans_5(8)	.	.	.	.	.	.	C	C
*ELF_nutans*_6(2)	.	.	T	T	T	.	C	C
ELF_nutans_7(2)	.	.	.	.	.	.	T	C
ELF_nutans_8(1)	.	.	T	.	T	.	.	.
ELF_nutans_9(1)	.	.	.	.	T	.	.	.
ELF_nutans_10(1)	.	.	T	.	.	.	C	.
ELF_otites_1(11)	.	.	.	.	.	.	C	C
ELF_otites_2(15)	.	.	.	T	.	.	C	C
ELF_otites_3(6)	.	.	.	T	.	.	.	.
ELF_otites_4(9)	.	.	T	T	T	.	C	C
ELF_otites_5(2)	.	.	T	T	.	.	.	.
ELF otites6(1)	T	.	.	T	.	.	C	C
ELF otites7(2)	.	.	.	T	T	.	.	.
ELF_otites_8(1)	T	.	.	.	.	.	C	C
ELF_otites_9(2)	T	G	.	.	.	.	C	C
ELF_otites_10(5)	.	.	.	.	.	.	.	.
ELF_latifolia_1(1)	.	.	.	.	.	.	C	C

Supplementary Table3C.

Haplotype structure of *X4*

Haplotypes	5	56	62	86	89	116	149	161	164	170	217	248	305	314	326	333	356	359	360	361
X4_nutans_1(14)	T	A	T	T	C	C	T	T	C	G	T	C	G	A	G	A	T	G	G	C
X4_ nutans_2(26)	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
X4_ nutans_3(2)	C	T	.	.	.	A	.	G	.	.	.	T	.	.	A	.	.	.	.	.
X4_nutans_4(2)	C	T	C	C	T	A	C	G	T	A	G	T	A	G	.	C	C	C	.	T
X4_otites_1(12)	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
X4_otites_2(18)	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
X4_otites_3(10)	C	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	C	.
X4_otites_4(4)	C	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	C	.
X4_latifolia_1(1)	C	T	.	C	.	G	C	G	T	A	G	T	A	.	A	.	C	.	.	T

Haplotypes	389	400	409	418	428	455	476	512	530
X4_nutans_1(7)	A	C	A	T	A	G	C	T	G
X4_ nutans_2(13)	.	.	.	.	.	.	.	.	.
X4_ nutans_3(1)	.	.	.	.	.	.	.	.	.
X4_nutans_4(1)	G	.	G	.	G	.	.	.	A
X4_otites_1(6)	.	.	.	.	.	.	T	C	.
X4_otites_2(9)	.	.	.	.	.	.	.	C	.
X4_otites_3(5)	.	T	.	C	.	A	.	C	.
X4_otites_4(2)	.	T	.	C	.	A	T	C	.
X4_latifolia_1(1)	C	.	G	.	G	.	.	C	A

Supplementary Table3D.

Haplotype structure of *ATUB*

Haplotypes	35	44	46	47	62	68	98	128	131	137	140	152	168	176	179	184	185	186	194	197	203	207	212
ATUB_nutans_1(1)	T	C	C	A	G	C	A	C	T	G	A	C	C	T	A	C	G	A	A	G	C	T	T
ATUB_nutans_2(1)	A	T	A	C	.	.	G	.	.	T	.	G	G	.	.	T	A	.	.	.	T	.	.
ATUB_nutans_3(1)	A	T	A	C	.	.	G	.	.	T	.	G	G	.	.	T	A	.	.	.	T	.	.
ATUB_nutans_4(1)	.	.	.	C	A	.	C	.	.	T	.	.	.	.	.	.	A	C	.	.	T	.	.
ATUB nutans *5*(1)	A	.	A	C	.	.	G	.	.	T	.	G	G	.	.	T	A	.	.	.	T	.	.
ATUB_nutans_6(1)	G	.	.	.	T	.	G	.	G	.	.	.	.	.	G	.	C	C	.	.	T	C	.
ATUB_nutans_7(1)	G	.	.	C	.	.	G	.	.	T	.	G	.	.	.	.	A	.	.	.	T	.	.
ATUB_nutans_8(1)	.	.	.	C	.	.	G	.	.	T	.	.	G	.	.	.	A	.	.	.	T	.	.
ATUB_nutans_ 9(1)	.	.	.	.	T	.	G	.	G	.	.	.	G	C	G	.	.	.	.	.	T	C	.
ATUB_nutans_10(1)	.	T	.	C	.	T	G	.	.	T	.	G	G	.	.	.	A	.	.	.	T	C	.
*ATUB_nutans*_11(1)	A	T	.	C	T	T	G	.	.	T	.	.	G	.	.	T	.	C	.	.	.	.	.
*ATUB_nutans*_12(1)	.	.	.	C	C	.	C	.	.	T	.	.	.	A	G	.	A	C	.	.	T	C	.
ATUB_nutans_13(1)	.	.	.	C	A	.	.	.	.	T	.	G	.	C	G	.	A	C	.	.	.	C	.
ATUB_nutans_14(1)	.	T	.	C	A	T	G	.	.	T	.	.	G	.	.	.	A	.	.	.	T	C	.
ATUB_nutans_15(1)	.	T	.	C	A	.	G	.	G	.	.	G	G	C	.	.	.	C	.	.	T	.	.
ATUB_nutans_16(1)	.	T	.	C	A	.	C	.	.	T	.	.	G	A	G	.	A	C	.	.	T	.	.
ATUB_nutans_17(1)	A	T	.	C	T	.	G	.	G	.	.	.	.	.	.	.	A	C	.	.	.	C	.
ATUB_nutans_18(1)	.	.	.	C	T	.	G	.	.	T	.	G	G	.	.	.	A	.	.	.	T	.	.
ATUB_nutans_ 19(1)	A	.	.	C	A	.	.	.	G	.	.	.	.	C	G	.	A	C	.	.	T	C	.
ATUB_nutans_20(1)	A	T	A	C	.	T	G	.	.	T	.	.	G	.	.	.	A	.	.	.	T	.	.
ATUB_nutans_21(1)	G	T	.	C	T	T	G	.	G	.	.	.	.	C	G	.	.	C	.	.	.	.	G
ATUB_nutans_22(1)	.	.	.	T	A	.	G	.	.	T	.	.	G	.	.	T	A	C	.	.	T	.	.
ATUB_nutans_23(1)	.	.	.	C	A	.	G	.	.	T	.	.	.	A	G	.	.	C	.	.	T	C	.

ATUB_nutans_24(1)	.	.	.	C	A	.	G	.	.	T	.	.	.	A	G	.	C	C	.	.	.	C	.
ATUB_nutans_25(1)	.	.	.	C	A	.	G	.	G	.	.	G	.	.	.	.	A	.	.	.	T	C	.
ATUB_nutans_26(1)	A	T	A	C	A	.	G	.	.	T	G	G	.	.	.	T	A	.	.	.	T	.	.
ATUB_nutans_27(1)	A	.	A	C	.	.	G	.	.	T	.	.	.	.	.	.	A	.	.	.	T	.	.
ATUB_nutans_28(1)	A	T	A	C	.	.	G	.	.	T	G	G	G	.	.	T	A	.	.	.	T	.	.
ATUB_nutans_29(1)	G	.	.	.	T	.	G	.	.	T	.	.	.	.	G	.	C	C	.	.	T	C	.
ATUB_nutans_30(1)	G	.	.	C	A	.	G	.	.	T	.	G	.	.	.	.	A	.	.	.	T	.	.
ATUB_nutans_31(1)	.	.	A	C	A	.	.	.	.	T	.	.	.	.	G	.	C	C	.	.	T	C	.
ATUB_nutans_32(1)	A	T	A	C	A	.	G	.	.	T	.	.	.	.	.	.	A	.	.	.	T	.	.
ATUB_nutans_33(1)	G	T	.	C	T	.	G	.	G	.	.	.	.	C	.	.	.	.	.	.	T	.	.
ATUB_nutans_34(1)	G	.	.	C	A	.	C	.	.	T	.	.	.	A	.	.	A	.	.	.	T	C	.
ATUB_nutans_35(1)	.	.	.	C	A	.	G	.	.	T	.	.	.	A	G	.	C	C	.	.	.	C	.
ATUB_nutans_36(1)	.	.	.	C	A	.	G	.	.	T	.	.	.	A	G	.	C	C	.	.	.	C	.
ATUB_nutans_37(1)	.	.	.	C	T	.	G	.	G	.	.	.	.	C	.	.	.	.	.	.	T	.	.
ATUB_nutans_38(1)	.	.	.	C	T	.	G	.	.	T	.	.	.	.	.	T	A	.	.	.	T	.	.
ATUB_otites_1(1)	.	T	A	C	T	.	G	.	.	T	.	.	G	.	.	.	A	C	.	.	T	C	.
ATUB_otites_2(1)	.	T	A	C	.	.	G	.	.	T	.	.	G	.	.	.	A	C	.	.	T	.	.
ATUB_otites_3(1)	.	T	A	C	T	.	C	T	G	.	.	.	G	.	.	.	C	.	.	.	T	.	.
ATUB_otites_4(1)	.	T	A	C	C	.	G	.	.	T	.	.	G	.	.	T	A	.	.	.	T	.	.
ATUB*_otites_ 5*(1)	G	T	A	C	A	.	C	.	.	T	.	.	G	C	G	.	.	C	.	.	T	C	.
ATUB_otites_6(1)	G	T	A	C	T	.	G	.	G	T	.	.	G	A	.	.	A	C	C	.	T	.	G
ATUB_otites_7(1)	G	T	A	C	A	.	G	.	G	.	.	.	G	C	.	.	A	C	.	.	T	C	.
ATUB_otites_8(1)	G	T	A	C	A	.	G	.	.	T	.	.	G	.	.	.	A	.	.	.	T	.	.
ATUB_otites_ 9(1)	G	T	A	C	T	.	G	.	G	.	.	.	G	C	.	.	.	C	.	T	T	.	.
ATUB_otites_10(1)	G	T	A	C	C	.	G	.	.	T	.	.	G	C	.	.	A	.	.	.	T	C	.
*ATUB_otites*_11(1)	G	T	A	C	T	A	G	T	G	.	.	.	G	C	.	.	.	.	.	.	T	.	.
*ATUB_otites*_12(1)	G	T	A	C	C	.	C	.	.	T	.	.	G	.	.	.	A	.	.	.	T	.	.
ATUB_otites_13(1)	G	T	A	C	.	.	G	.	G	.	.	.	G	C	G	.	.	C	.	T	T	C	G
ATUB_otites_14(1)	G	T	A	C	T	.	C	.	.	T	.	.	G	.	.	.	A	C	.	.	T	.	.
ATUB_otites_15(1)	G	T	A	C	T	.	.	.	G	.	.	.	G	C	.	T	.	G	.	T	T	C	G
ATUB_otites_16(1)	G	T	A	C	T	.	G	.	.	T	.	.	G	.	.	T	A	G	.	.	T	.	.

ATUB_otites_17(1)	G	T	A	C	T	.	C	.	G	.	.	.	G	C	.	.	.	C	C	T	T	C	.
ATUB_otites_18(1)	G	T	A	C	.	.	G	.	.	T	.	.	G	.	.	T	A	.	.	.	T	.	.
ATUB_otites_ 19(1)	G	T	A	C	T	.	.	T	G	.	.	.	G	C	.	.	A	C	C	.	T	.	G
ATUB_otites_20(1)	G	T	A	C	.	T	G	.	.	T	G	.	G	.	.	.	.	.	.	.	T	.	.
ATUB_otites_21(1)	.	T	A	C	A	.	C	.	.	.	.	.	G	C	G	.	.	C	.	.	T	C	.
ATUB_otites_22(1)	.	T	A	C	A	.	C	.	.	T	.	.	G	.	G	.	A	C	.	.	T	.	.
ATUB_otites_23(1)	G	T	A	C	T	.	G	T	G	.	.	G	G	C	G	.	.	C	C	T	T	C	G
ATUB_otites_24(1)	G	T	A	C	T	.	G	.	.	T	G	.	G	.	.	T	A	.	.	.	T	.	.
ATUB_otites_25(1)	.	T	A	C	T	T	C	.	G	T	G	.	G	.	.	T	.	.	.	T	T	.	.
ATUB_otites_26(1)	.	T	A	C	A	.	C	.	.	T	.	.	G	C	.	.	C	C	.	.	T	C	.
ATUB_otites_27(1)	G	T	A	C	T	.	C	.	.	.	.	.	G	C	.	.	.	C	C	T	T	C	.
ATUB_otites_28(1)	G	T	A	C	.	.	G	.	.	T	.	.	G	C	.	.	A	.	.	.	T	.	.
ATUB_otites_29(1)	G	T	A	C	T	.	.	T	G	.	.	.	G	C	.	.	.	C	C	T	T	.	G
ATUB_otites_30(1)	G	T	A	C	T	.	.	.	.	T	.	.	G	A	G	.	C	C	.	.	T	C	.
ATUB_otites_31(1)	.	T	A	C	A	.	.	.	.	T	.	.	G	C	G	.	.	C	.	.	T	C	G
ATUB_otites_32(1)	.	T	A	C	.	.	G	.	.	T	.	.	G	A	.	.	C	C	.	.	T	.	.
ATUB_otites_33(1)	G	T	A	C	T	.	C	.	G	.	.	.	G	C	G	.	.	C	.	.	T	C	G
ATUB_otites_34(1)	G	T	A	C	T	.	G	.	.	T	.	.	G	.	.	.	C	C	.	.	T	.	.
ATUB_otites_35(1)	.	T	A	C	T	.	.	.	G	.	.	.	G	C	G	.	.	C	.	.	T	C	G
ATUB_otites_36(1)	.	T	A	C	A	T	.	.	.	T	.	.	G	.	.	T	A	.	.	.	T	.	.
ATUB_otites_37(1)	G	T	A	C	T	.	G	.	G	.	.	.	G	C	G	.	.	C	.	.	T	C	G
ATUB_otites_38(1)	G	T	A	C	A	T	G	.	.	T	G	.	G	A	G	T	A	.	.	.	T	.	.
ATUB_latifolia_1(1)	A	T	A	C	.	T	G	.	.	T	.	A	G	.	.	T	A	.	.	.	T	.	.

Haplotypes	231	236	243	245	248	250	257	263	266	272	274	281	284	290	293	294	310	311	314	317	323	326
ATUB_nutans_1(1)	C	A	C	C	C	C	G	C	G	G	T	C	T	A	C	T	C	A	C	T	G	G
ATUB_nutans_2(1)	.	.	.	G	A	.	C	T	T	C	.	.	.	.	.	G	.	.	.	.	.	A
ATUB_nutans_3(1)	A	.	.	G	A	.	C	T	T	C	.	.	.	.	.	G	.	.	.	.	.	A
ATUB_nutans_4(1)	.	.	.	.	T	.	.	.	T	.	A	.	G	.	.	C	.	G	.	.	T	A
ATUB nutans *5*(1)	.	.	.	G	A	.	C	T	T	C	.	.	.	.	.	G	.	.	.	.	.	A
ATUB_nutans_6(1)	A	T	.	.	.	A	.	.	.	.	A	T	G	G	T	.	G	G	T	G	T	.
ATUB_nutans_7(1)	A	.	.	.	A	A	.	T	T	C	.	.	.	.	.	C	.	G	.	.	.	.
ATUB_nutans_8(1)	.	.	.	G	.	.	.	T	T	.	.	.	.	.	.	G	.	.	.	.	T	A
ATUB_nutans_ 9(1)	A	T	.	.	.	.	C	.	.	.	.	.	G	.	.	G	.	.	.	.	T	A
ATUB_nutans_10(1)	.	.	.	G	A	.	C	.	T	C	.	.	.	.	.	G	.	.	.	.	.	A
*ATUB_nutans*_11(1)	.	.	.	.	.	A	.	T	T	.	.	.	.	.	.	C	G	.	T	.	T	.
*ATUB_nutans*_12(1)	A	.	.	.	.	A	.	.	T	T	C	.	G	.	G	G	.	G	.	.	.	.
ATUB_nutans_13(1)	A	.	.	.	T	A	.	.	T	.	A	.	G	G	.	.	G	G	.	.	T	.
ATUB_nutans_14(1)	A	.	.	.	T	.	.	T	T	T	A	.	.	.	T	G	.	G	.	.	.	.
ATUB_nutans_15(1)	.	.	.	G	T	.	C	T	.	.	.	T	.	.	T	.	.	.	.	.	T	.
ATUB_nutans_16(1)	A	.	.	.	T	A	.	.	T	T	A	.	.	.	T	C	.	G	.	.	T	.
ATUB_nutans_17(1)	A	.	.	.	.	.	.	.	.	.	A	T	G	G	T	G	.	G	.	.	T	.
ATUB_nutans_18(1)	.	.	.	G	A	.	C	T	T	T	.	.	.	.	.	G	.	.	.	.	.	.
*ATUB_nutans_* 19(1)	.	.	.	.	.	A	.	.	.	.	.	.	G	.	T	.	.	G	.	.	T	.
ATUB_nutans_20(1)	.	.	.	.	A	.	C	T	T	T	.	.	.	.	.	G	.	.	.	.	.	.
ATUB_nutans_21(1)	A	.	.	.	.	A	.	.	.	.	G	G	G	G	T	.	G	G	.	G	.	.
ATUB_nutans_22(1)	A	.	.	.	.	.	.	.	T	C	.	.	.	.	T	G	.	G	.	.	.	.
ATUB_nutans_23(1)	A	.	.	.	T	A	.	.	T	.	A	.	.	G	T	.	.	.	T	.	T	.
ATUB_nutans_24(1)	A	.	.	.	T	A	.	.	T	T	A	.	G	G	T	C	.	G	T	.	T	.
ATUB_nutans_25(1)	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	G	.	.	.	.	T	A

ATUB_nutans_26(1)	.	.	.	G	.	.	C	T	T	C	.	.	.	.	.	G	.	.	.	.	.	A
ATUB_nutans_27(1)	.	.	.	.	.	.	C	T	T	.	.	.	.	G	T	G	.	G	.	.	T	A
ATUB_nutans_28(1)	.	.	.	G	A	.	C	T	T	C	.	.	.	.	.	G	.	.	.	.	.	A
ATUB_nutans_29(1)	A	.	.	.	.	A	.	.	.	.	A	T	.	.	T	.	G	G	T	G	T	.
ATUB_nutans_30(1)	A	.	.	.	A	A	.	T	T	T	.	.	.	.	.	G	.	G	.	.	.	.
ATUB_nutans_31(1)	.	.	.	.	.	.	.	.	T	T	A	.	G	G	T	C	.	G	T	.	T	.
ATUB_nutans_32(1)	.	.	.	.	.	.	.	T	T	T	.	.	.	.	.	G	.	.	.	.	.	A
ATUB_nutans_33(1)	A	.	.	.	A	.	.	.	.	.	A	.	C	.	T	G	G	G	T	G	T	.
ATUB_nutans_34(1)	A	.	.	.	.	G	.	.	T	T	.	.	G	.	.	G	.	.	.	.	.	.
ATUB_nutans_35(1)	A	.	.	.	T	A	.	.	T	.	.	.	G	G	.	C	.	G	T	.	T	.
ATUB_nutans_36(1)	A	.	.	.	T	A	.	.	T	C	C	.	G	G	T	C	.	G	T	.	T	.
ATUB_nutans_37(1)	A	.	.	.	.	A	.	T	.	.	.	.	.	.	.	.	G	G	.	.	.	.
ATUB_nutans_38(1)	.	.	.	G	.	.	C	T	T	C	.	.	.	.	.	G	.	G	.	.	.	.
ATUB_otites_1(1)	.	.	.	.	A	A	.	.	.	.	.	.	.	.	G	G	.	G	.	.	T	.
ATUB_otites_2(1)	.	.	.	G	A	.	.	.	T	T	.	.	G	G	T	G	.	.	.	.	.	.
ATUB_otites_3(1)	A	T	.	.	A	A	.	.	.	.	.	.	G	.	G	G	.	G	.	.	T	.
ATUB_otites_4(1)	.	.	.	G	A	.	C	T	T	C	.	.	.	.	.	G	.	.	.	.	.	A
ATUB nutans *5*(1)	A	.	.	.	A	A	.	T	T	.	.	G	.	.	.	G	.	G	.	.	T	.
ATUB_otites_6(1)	.	.	.	.	A	A	.	.	.	T	.	.	G	G	T	G	.	G	.	.	.	.
ATUB_otites_7(1)	A	T	.	.	A	A	.	.	.	T	.	.	G	.	T	G	G	.	.	.	T	.
ATUB_otites_8(1)	.	.	.	.	A	.	C	T	T	C	.	.	.	.	.	G	.	.	.	.	.	A
ATUB_otites_ 9(1)	.	T	.	.	A	A	C	T	.	.	.	.	.	.	G	G	G	.	.	.	.	A
ATUB_otites_10(1)	A	.	.	.	A	.	.	T	T	T	.	.	G	.	G	G	.	.	.	.	T	.
*ATUB_otites*_11(1)	.	T	T	G	A	.	C	T	.	.	.	.	.	.	G	G	G	.	.	.	.	A
*ATUB_otites*_12(1)	A	.	.	.	A	A	.	T	T	C	.	.	G	.	T	G	.	G	.	.	T	.
ATUB_otites_13(1)	A	T	.	.	A	A	.	.	.	.	.	G	G	G	.	G	.	G	.	.	T	.
ATUB_otites_14(1)	A	.	.	.	A	A	.	T	T	C	.	.	.	.	T	G	.	G	.	.	T	.
ATUB_otites_15(1)	A	T	T	.	A	A	.	.	.	.	.	.	G	.	G	G	G	G	.	.	T	.
ATUB_otites_16(1)	.	.	.	.	A	A	C	T	T	T	.	.	.	.	T	G	.	.	.	.	.	.
ATUB_otites_17(1)	A	T	T	.	A	A	.	.	.	.	.	.	.	.	G	G	.	G	.	.	T	.
ATUB_otites_18(1)	A	.	.	G	A	.	C	T	T	C	.	.	.	.	T	G	.	.	.	.	.	A

ATUB_otites_ 19(1)	A	T	T	.	A	A	.	T	.	.	.	.	C	.	G	G	G	G	.	.	T	.
ATUB_otites_20(1)	.	.	.	G	A	.	C	T	T	C	.	.	.	.	T	G	.	.	.	.	.	A
ATUB_otites_21(1)	A	.	.	.	A	A	.	.	T	.	.	G	G	G	T	G	.	G	T	.	T	.
ATUB_otites_22(1)	.	.	.	.	A	A	.	T	T	C	.	.	.	.	T	G	.	G	.	.	T	.
ATUB_otites_23(1)	A	T	T	.	A	A	.	.	.	.	.	.	G	G	G	G	G	G	.	G	T	.
ATUB_otites_24(1)	A	.	.	.	A	.	.	T	T	C	.	.	.	.	T	G	.	G	.	.	.	.
ATUB_otites_25(1)	A	.	.	.	A	A	.	.	.	.	.	.	.	.	T	G	G	G	T	.	C	.
ATUB_otites_26(1)	.	.	.	G	A	.	C	T	T	C	.	.	.	.	.	G	.	.	T	.	.	.
ATUB_otites_27(1)	A	T	T	.	A	A	.	T	.	.	A	.	G	.	.	G	.	G	.	.	T	.
ATUB_otites_28(1)	.	.	.	G	A	.	C	T	T	C	C	.	.	.	.	G	.	.	.	.	.	A
ATUB_otites_29(1)	A	.	T	.	A	A	.	.	.	.	G	G	.	.	T	G	G	G	.	.	.	.
ATUB_otites_30(1)	A	.	.	.	A	A	.	.	T	T	A	.	G	G	T	G	.	G	T	.	T	.
ATUB_otites_31(1)	A	.	.	.	A	A	.	.	.	.	A	G	G	.	T	G	G	G	T	G	T	.
ATUB_otites_32(1)	A	.	.	.	A	A	.	.	T	T	A	.	G	.	T	G	.	G	.	.	.	.
ATUB_otites_33(1)	A	.	T	.	A	A	.	.	.	.	G	G	G	G	T	G	.	G	T	.	T	.
ATUB_otites_34(1)	.	.	.	.	A	A	.	T	T	T	A	.	.	.	.	G	.	G	.	.	.	.
ATUB_otites_35(1)	A	.	.	.	A	A	.	.	.	.	.	.	G	G	.	G	G	G	.	.	T	.
ATUB_otites_36(1)	.	.	.	G	A	.	C	T	T	C	C	.	.	.	T	G	.	G	.	.	.	A
ATUB_otites_37(1)	A	.	.	.	.	A	.	.	.	.	C	.	C	G	G	G	G	G	.	G	C	.
ATUB_otites_38(1)	A	.	.	.	.	.	.	T	T	T	.	.	.	.	.	G	.	G	.	.	.	A
ATUB_latifolia_1(1)	.	.	.	.	A	.	C	T	T	C	.	.	.	.	.	G	.	.	.	.	C	A

Supplementary Table3E.

Haplotype structure of *cob*

Haplotypes	112	126	144	223	289	363	408	444	510	570	735	768	841	858	927	Fhap	75	97	286
Cob_ nutans_1(1)	C	C	A	C	G	A	A	C	A	A	G	C	T	A	A	1	L	V	L
Cob_ nutans_2(1)	.	.	.	A	.	.	.	.	.	.	T	.	.	.	G	2	I	.	.
Cob_ nutans_3(2)	.	.	.	.	.	.	G	.	.	.	.	.	.	.	G	1	.	.	.
Cob_ nutans_4(2)	.	.	.	.	T	.	.	T	.	.	T	.	.	.	.	3	.	L	.
Cob_ nutans_5(2)	.	.	.	.	.	.	.	T	C	.	T	.	.	.	.	1	.	.	.
Cob _nutans_6(10)	.	.	.	.	.	.	.	T	.	.	T	.	.	.	.	1	.	.	.
Cob_ nutans_7(1)	.	.	.	.	.	.	.	T	.	.	T	.	.	.	G	1	.	.	.
Cob _nutans_8(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	1	.	.	.
Cob _nutans_9(4)	.	.	.	A	.	.	.	T	.	.	T	.	C	.	.	2	I	.	.
Cob_ nutans_10(1)	.	.	.	A	.	.	.	T	.	.	T	.	.	.	.	2	I	.	.
Cob_ nutans_11(1)	.	.	.	A	.	.	.	T	.	.	T	.	.	T	G	4	I	.	F
Cob _otites_1(8)	T	T	G	A	.	G	.	.	C	C	.	A	.	T	.	4	I	.	F
*Cob _otites_2(*2)	T	T	G	A	.	.	.	.	C	C	.	A	.	.	.	2	I	.	.
Cob _otites_3(13)	.	T	G	A	.	.	.	.	C	.	.	A	.	.	.	2	I	.	.
Cob _otites_4(3)	.	T	G	A	.	.	.	.	C	.	.	A	.	T	.	2	I	.	F
Cob_latifolia_1(1)	.	.	.	A	.	.	.	.	C	.	T	.	.	T	G	4	I	.	F

Fhap indicate functional haplotype number (different amino acid sequences are considered as potentially different functional haplotypes)

Supplementary Table3F.

Haplotype structure of *cox1*

Haplotypes	46	67	169	199	283	355	358	383	469	568	640	763	768	775	788	880	889	928	940	954	957	1000	1015
Cox1_ nutans_1(2)	C	C	G	A	A	G	C	G	T	C	T	T	C	G	C	A	A	A	C	G	C	G	A
Cox1 _nutans_2(1)	.	.	.	.	.	.	.	T	C	.	.	.	.	.	.	.	.	.	.	C	.	.	G
Cox1 _nutans_3(2)	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	T	.	G	.	.
Cox1 _nutans_4(1)	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	G	.	T	.	G	.	.
Cox1 _nutans_5(2)	.	.	.	.	.	.	.	.	C	.	.	.	.	T	.	.	.	C	T	.	G	.	.
Cox1 _nutans_6(2)	.	.	.	.	.	.	.	.	C	.	.	G	.	T	.	.	.	.	.	.	.	C	.
Cox1 _nutans_7(1)	.	.	.	C	.	.	.	.	C	.	.	.	.	.	.	.	.	.	T	.	G	.	.
Cox1 _nutans_8(1)	.	.	.	.	.	T	T	.	C	.	.	.	.	.	.	.	.	.	T	.	.	.	.
Cox1 _nutans_9(2)	.	.	.	.	.	.	.	.	C	.	.	.	.	T	.	.	.	.	.	.	.	.	G
Cox1 _nutans_10(1)	.	T	.	.	.	.	.	.	C	.	.	.	.	T	.	.	.	.	.	.	.	.	.
Cox1 _nutans_11(2)	.	.	.	.	.	.	.	.	C	.	.	.	.	T	.	.	G	.	T	.	G	.	.
Cox1 _nutans_12(1)	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	G	.	.	.	G	.	.
Cox1 _nutans_13(1)	.	.	.	C	.	.	.	.	C	.	.	G	.	.	.	.	.	.	T	.	.	.	.
Cox1 _nutans_14(1)	.	.	.	.	.	.	.	.	C	.	.	G	.	.	A	.	.	.	T	.	G	.	.
Cox1 _nutans_15(1)	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	G	.	.
Cox1 _nutans_16(1)	.	.	.	.	.	.	.	.	C	.	.	G	.	.	.	.	.	.	T	.	.	.	.
Cox1 _otites_1(1)	T	.	T	.	.	.	.	.	C	T	C	.	G	.	.	G	G	.	.	.	G	C	.
Cox1 _otites_2(3)	.	.	.	.	.	.	.	T	C	.	C	.	.	.	.	G	G	.	.	.	G	C	.
Cox1 _otites_3(7)	T	.	T	.	C	.	.	.	C	T	C	.	.	.	.	G	G	.	.	.	G	C	.
Cox1 _otites_4(7)	.	.	.	.	.	.	.	.	C	.	C	.	.	.	.	G	G	.	.	.	G	C	.
Cox1 _otites_5(1)	T	.	T	.	C	.	.	T	C	T	C	.	.	.	.	G	G	.	.	.	G	C	.
Cox1 _otites_6(1)	T	.	T	.	C	.	.	.	C	.	C	.	.	.	.	G	G	.	.	.	G	C	.
Cox1 _otites_7(1)	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	G	C	.
Cox1_ otites_8(1)	T	.	T	.	C	.	.	.	C	T	C	.	G	.	.	G	G	.	.	.	G	C	.
*Cox1_latifolia*_1(1)	.	.	.	.	.	.	.	.	C	.	.	.	.	T	.	.	.	C	.	.	G	C	.

Haplotypes	F_{hap}	128	249	256	263	318	319	333
Cox1_ nutans_1(2)	1	A	V	T	Q	C	S	K
Cox1 _nutans_2(1)	2	S	.	.	.	S	.	.
Cox1 _nutans_3(2)	3	.	.	.	.	.	C	.
Cox1 _nutans_4(1)	3	.	.	.	.	.	C	.
Cox1 _nutans_5(2)	3	.	.	.	.	.	C	.
Cox1 _nutans_6(2)	4	.	.	.	.	.	.	N
Cox1 _nutans_7(1)	3	.	.	.	.	.	C	.
Cox1 _nutans_8(1)	1	.	.	.	.	.	.	.
Cox1 _nutans_9(2)	1	.	.	.	.	.	.	.
Cox1 _nutans_10(1)	1	.	.	.	.	.	.	.
Cox1 _nutans_11(2)	3	.	.	.	.	.	C	.
Cox1 _nutans_12(1)	3	.	.	.	.	.	C	.
Cox1 _nutans_13(1)	1	.	.	.	.	.	.	.
Cox1 _nutans_14(1)	5	.	.	.	K	.	C	.
Cox1 _nutans_15(1)	3	.	.	.	.	.	C	.
Cox1 _nutans_16(1)	1	.	.	.	.	.	.	.
Cox1 _otites_1(1)	6	.	.	R	.	.	C	N
Cox1 _otites_2(3)	7	S	.	.	.	.	C	N
Cox1 _otites_3(7)	8	.	.	.	.	.	C	N
Cox1 _otites_4(7)	8	.	.	.	.	.	C	N
Cox1 _otites_5(1)	7	S	.	.	.	.	C	N
Cox1 _otites_6(1)	8	.	.	.	.	.	C	N
Cox1 _otites_7(1)	8	.	.	.	.	.	C	N
Cox1_ otites_8(1)	6	.	.	R	.	.	C	N
*Cox1_latifolia_*1(1)	8	.	.	.	.	.	C	N

Fhap indicate functional haplotype number (different amino acid sequences are considered as potentially different functional haplotypes)

Supplementary Table3G.

Haplotypes structure of *Cp*

Haplotypes	25	26	29	38	42	63	64	65	66	67	68	71	72	74	77	80	82
*Cp_nutans*_1(3)	T	C	C	A	T	C	A	T	A	T	C	C	C	C	T	A	A
Cp_nutans_2(14)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
*Cp_nutans*_3(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
*Cp_nutans*_4(9)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
*Cp_nutans*_**5**(4)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cp_nutans_6(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
*Cp_nutans*_7(1)	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
*Cp_nutans*_ 8(1)	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
Cp_nutans_9(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cp_nutans_10(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cp_nutans_11(1)	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
Cp_otites_1(1)	C	.	G	C	.	0	0	0	T	G	A	T	A	T	A	0	T
Cp_otites_2(1)	C	.	G	.	.	0	0	0	T	G	A	T	A	T	A	0	T
*Cp_otites_3(*17)	C	.	G	C	.	0	0	0	T	G	A	T	A	T	A	0	T
*Cp_otites_4(*6)	A	G	.	.	.	0	0	0	T	G	A	T	A	T	A	0	T
*Cp_otites_5(*11)	C	.	G	.	.	0	0	0	T	G	A	T	A	T	A	0	T
*Cp_otites*_6(1)	C	.	G	.	.	0	0	0	T	G	A	T	A	T	A	0	T
Cp_latifolia_1(1)	0	0	0	0	.	0	0	0	T	G	A	T	A	T	A	0	T

0 indicates a deletion

Haplotypes	83	84	85	86	87	88	89	91	94	97	98	99	100	101	102	103	104	107
*Cp_nutans*_1(3)	G	A	C	G	T	T	G	A	A	A	C	G	C	T	T	C	A	C
Cp_nutans_2(14)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
*Cp_nutans*_3(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
*Cp_nutans*_4(9)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
*Cp_nutans*_**5***(*4)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cp_nutans_6(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cp_nutans_7(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
*Cp_nutans_* 8(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cp_nutans_9(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cp_nutans_10(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cp_nutans_11(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cp_otites_1(1)	T	T	T	C	A	A	A	G	C	T	T	A	T	A	A	G	G	0
Cp_otites_2(1)	T	T	T	C	A	A	A	G	C	T	T	A	T	A	A	G	G	0
*Cp_otites_3(*17)	T	T	T	C	A	A	A	G	C	T	T	A	T	A	A	G	G	0
*Cp_otites_4(*6)	T	T	T	A	A	A	A	G	C	T	T	A	T	A	A	G	G	0
*Cp_otites_5(*11)	T	T	T	C	A	A	A	G	C	T	T	A	T	A	A	G	G	0
*Cp_otites*_6(1)	T	T	T	C	A	A	A	G	C	T	T	A	T	A	A	G	G	0
Cp_latifolia_1(1)	T	T	T	.	.	A	A	G	G	T	T	A	T	A	.	T	T	0

Haplotypes	110	115	116	119	120	121	122	123	124	125	126	127	128	129	135	138	140
*Cp_nutans*_1(3)	G	T	C	T	T	C	C	A	C	C	T	C	T	T	G	A	G
Cp_nutans_2(14)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
*Cp_nutans*_3(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
*Cp_nutans*_4(9)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
*Cp_nutans*_**5***(4)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cp_nutans_6(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cp_nutans_7(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
*Cp_nutans_* 8(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cp_nutans_9(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cp_nutans_10(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cp_nutans_11(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cp_otites_1(1)	A	A	G	0	0	0	0	0	0	0	0	0	0	A	T	G	A
Cp_otites_2(1)	A	A	G	0	0	0	0	0	0	0	0	0	0	A	T	G	A
*Cp_otites_3(*17)	A	A	G	0	0	0	0	0	0	0	0	0	0	A	T	G	A
*Cp_otites_4(*6)	A	A	G	0	0	0	0	0	0	0	0	0	0	A	T	C	A
*Cp_otites_5(*11)	A	A	G	0	0	0	0	0	0	0	0	0	0	A	T	G	A
*Cp_otites*_6(1)	A	A	G	0	0	0	0	0	0	0	0	0	0	A	T	G	A
Cp_latifolia_1(1)	A	A	G	0	0	0	0	0	0	0	0	0	0	A	A	.	A

Haplotypes	141	143	144	146									161	162	
*Cp_nutans*_1(3)	A	A	T	C	0	0	0	0	0	0	0	0	G	T	0
Cp_nutans_2(14)	.	.	.	.	0	0	0	0	0	0	0	0	T	A	C
*Cp_nutans*_3(1)	.	.	.	.	0	0	0	0	0	0	0	0	.	.	0
*Cp_nutans*_4(9)	.	.	.	.	0	0	0	0	0	0	0	0	.	.	0
*Cp_nutans*_**5***(*4)	.	.	.	.	0	0	0	0	0	0	0	0	.	.	0
Cp_nutans_6(1)	.	.	.	.	0	0	0	0	0	0	0	0	T	A	C
*Cp_nutans*_7(1)	.	.	.	.	0	0	0	0	0	0	0	0	.	.	0
*Cp_nutans*_ 8(1)	.	.	.	.	0	0	0	0	0	0	0	0	.	.	0
Cp_nutans_9(1)	.	.	.	.	0	0	0	0	0	0	0	0	.	.	0
Cp_nutans_10(1)	.	.	.	.	0	0	0	0	0	0	0	0	.	.	0
Cp_nutans_11(1)	.	.	.	.	0	0	0	0	0	0	0	0	.	.	A
Cp_otites_1(1)	G	T	G	T	A	A	G	T	A	T	A	T	.	.	0
Cp_otites_2(1)	G	T	G	T	A	A	G	T	A	T	A	T	.	.	0
*Cp_otites_3(*17)	G	T	G	T	A	A	G	T	A	T	A	T	.	.	0
*Cp_otites_4(*6)	.	T	G	T	A	A	T	T	A	T	A	T	.	.	0
*Cp_otites_5(*11)	G	T	G	T	A	A	G	T	A	T	A	T	.	.	0
*Cp_otites*_6(1)	G	T	G	T	A	A	G	T	A	T	A	T	.	.	0
Cp_latifolia_1(1)	.	.	A	T	A	A	T	T	A	T	A	T	.	.	0

Haplotypes	182	190	191	192	193	194	195	196	197	198	208
*Cp_nutans*_1(3)	T	G	T	A	A	T	T	T	T	T	A
Cp_nutans_2(14)	.	.	.	.	.	.	.	.	.	.	.
*Cp_nutans*_3(1)	.	.	.	C	C	.	.	.	.	.	.
*Cp_nutans*_4(9)	.	.	.	C	.	.	.	.	.	.	.
*Cp_nutans*_**5***(*4)	.	.	.	C	T	.	.	.	.	.	.
Cp_nutans_6(1)	.	.	.	.	.	.	.	.	.	.	.
Cp_nutans_7(1)	.	.	.	C	.	.	.	.	.	G	.
*Cp_nutans_* 8(1)	.	.	.	C	C	.	.	.	.	G	.
Cp_nutans_9(1)	.	.	.	C	T	.	.	.	.	.	.
Cp_nutans_10(1)	.	.	.	C	.	.	.	.	.	G	.
Cp_nutans_11(1)	.	.	.	.	.	.	.	.	.	.	0
Cp_otites_1(1)	.	T	0	0	0	0	0	0	0	.	.
Cp_otites_2(1)	.	T	0	0	0	0	0	0	0	.	.
*Cp_otites_3(*17)	.	T	0	0	0	0	0	0	0	.	.
*Cp_otites_4(*6)	G	T	0	0	0	0	0	0	0	.	.
*Cp_otites_5(*11)	.	T	0	0	0	0	0	0	0	.	.
*Cp_otites*_6(1)	.	T	0	0	0	0	0	0	0	.	.
Cp_latifolia_1(1)	.	T	0	0	0	0	0	0	G	C	.

Haplotypes	232	238	239	240	249	253	255	262	263	278		339	367	398	399	441	463	510	534	546	606
*Cp_nutans*_1(3)	A	C	T	G	A	C	T	A	A	G	0	G	A	C	A	G	C	C	A	A	T
Cp_nutans_2(14)	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.
*Cp_nutans*_3(1)	.	T	.	.	.	.	.	.	.	.	0	.	C	.	.	.	.	.	.	.	.
*Cp_nutans*_4(9)	.	.	G	.	.	T	.	C	.	.	0	.	C	.	.	.	.	.	.	.	.
*Cp_nutans_5(*4)	.	.	G	.	.	.	.	C	.	.	0	.	C	.	.	.	.	T	.	.	.
Cp_nutans_6(1)	.	.	.	.	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.
Cp_nutans_7(1)	.	.	G	.	.	.	.	C	.	.	0	.	C	.	.	.	.	.	.	.	.
*Cp_nutans_* 8(1)	.	.	G	.	.	.	.	C	.	.	0	.	C	.	.	.	.	.	.	.	.
Cp_nutans_9(1)	.	.	G	.	.	.	.	C	.	.	0	.	C	.	.	.	.	T	.	.	.
Cp_nutans_10(1)	.	T	.	.	.	.	.	.	.	.	0	.	C	.	.	.	.	.	.	.	.
Cp_nutans_11(1)	.	.	.	T	.	.	.	.	.	.	0	.	.	.	.	.	.	.	.	.	.
Cp_otites_1(1)	0	.	.	.	C	.	A	.	C	A	A	C	.	T	.	T	.	.	C	C	.
Cp_otites_2(1)	0	.	.	.	C	.	A	.	C	A	A	C	.	T	.	T	G	.	C	C	G
*Cp_otites_3(*17)	0	.	.	.	C	.	A	.	C	A	A	C	.	T	C	T	.	.	C	C	.
*Cp_otites_4(*6)	0	.	.	.	C	.	A	.	C	A	A	C	.	T	.	T	G	.	C	C	G
*Cp_otites_5(*11)	0	.	.	.	C	.	A	.	C	A	A	C	.	T	C	T	.	.	C	C	.
*Cp_otites*_6(1)	0	.	.	.	C	.	A	.	C	A	A	C	.	T	.	T	.	.	C	C	.
Cp_latifolia_1(1)	0	.	.	.	T	.	A	T	.	A	G	.	.	A	.	.	.	.	.	.	.

Haplotypes	626	700	736	774	892	900	926				983	995		1020				1041	1054
*Cp_nutans*_1(3)	G	C	A	T	A	G	A	0	0	0	A	A	0	T	0	0	0	C	C
Cp_nutans_2(14)	T	.	.	.	.	.	.	0	0	0	.	.	0	.	0	0	0	.	A
*Cp_nutans*_3(1)	.	.	.	G	.	.	.	0	0	0	.	.	0	.	0	0	0	.	A
*Cp_nutans*_4(9)	T	.	.	G	.	A	.	0	0	0	.	.	0	.	T	0	0	.	A
*Cp_nutans*_5*(*4)	.	.	.	G	.	.	.	0	0	0	.	.	0	.	0	0	0	.	A
Cp_nutans_6(1)	T	.	.	.	.	A	.	0	0	0	.	.	0	.	0	0	0	.	A
Cp_nutans_7(1)	T	T	.	G	.	.	.	0	0	0	.	.	0	.	T	T	0	.	A
*Cp_nutans_* 8(1)	T	T	.	G	.	.	.	0	0	0	.	.	0	.	T	T	0	0	A
Cp_nutans_9(1)	.	.	.	G	.	.	.	0	0	0	.	.	0	.	0	0	0	0	A
Cp_nutans_10(1)	.	.	.	G	.	.	.	0	0	0	.	.	0	.	0	0	0	.	A
Cp_nutans_11(1)	T	.	.	.	.	.	.	0	0	0	.	.	0	A	T	T	0	.	A
Cp_otites_1(1)	T	.	C	.	T	.	G	T	T	0	T	T	0	.	T	T	T	.	A
Cp_otites_2(1)	.	.	C	.	T	.	G	0	0	0	T	T	0	.	T	0	0	.	A
*Cp_otites_3(*17)	T	.	C	.	T	.	G	T	0	0	T	T	0	.	T	T	0	.	A
*Cp_otites_4(*6)	.	.	C	.	T	.	G	T	0	0	.	T	0	.	t	0	0	.	A
*Cp_otites_5(*11)	T	.	C	.	T	.	G	T	T	T	.	T	T	.	T	T	T	.	A
*Cp_otites*_6(1)	T	.	C	.	T	.	G	T	0	0	.	T	0	.	T	0	0	.	A
Cp_latifolia_1(1)	C	A	.	.	.	.	G	0	0	0	.	.	0	.	0	0	0	.	A

Haplotypes	1077							1087	1101	1107	1147	1148	1171	1172	1173	1174	1175	1176	
*Cp_nutans*_1(3)	A	0	0	0	0	0	0	T	T	A	A	A	T	A	T	T	A	T	0
Cp_nutans_2(14)	.	0	0	0	0	0	0	.	.	.	.	.	.	.	.	.	.	.	T
*Cp_nutans*_3(1)	.	0	0	0	0	0	0	.	.	C	.	.	.	.	.	.	.	.	0
*Cp_nutans*_4(9)	.	0	0	0	0	0	0	.	.	.	.	.	.	.	.	.	.	.	0
*Cp_nutans*_5*(*4)	.	0	0	0	0	0	0	.	.	.	.	.	.	.	.	.	.	.	0
Cp_nutans_6(1)	.	0	0	0	0	0	0	.	.	.	.	.	.	.	.	.	.	.	0
Cp_nutans_7(1)	.	0	0	0	0	0	0	.	.	.	C	C	.	.	.	.	.	.	0
*Cp_nutans_* 8(1)	.	0	0	0	0	0	0	.	.	.	C	C	.	.	.	.	.	.	T
Cp_nutans_9(1)	.	0	0	0	0	0	0	.	.	.	.	.	.	.	.	.	.	.	0
Cp_nutans_10(1)	.	0	0	0	0	0	0	.	.	C	.	.	.	.	.	.	.	.	0
Cp_nutans_11(1)	.	0	0	0	0	0	0	.	.	.	.	.	.	.	.	.	.	.	0
Cp_otites_1(1)	C	A	T	A	A	T	A	.	.	.	.	.	0	.	.	.	.	.	0
Cp_otites_2(1)	C	A	T	A	A	T	A	.	.	.	.	.	0	.	.	.	.	.	0
*Cp_otites_3(*17)	C	A	T	A	A	T	A	.	.	.	.	.	0	.	.	.	.	.	0
*Cp_otites_4(*6)	.	A	T	A	A	T	A	G	A	C	.	.	0	.	.	.	.	.	0
*Cp_otites_5(*11)	C	A	T	A	A	T	A	.	.	.	.	.	0	0	0	0	0	0	0
*Cp_otites*_6(1)	.	A	T	A	A	T	A	G	A	C	.	.	0	.	.	.	.	.	0
Cp_latifolia_1(1)	.	0	0	0	0	0	0	.	.	.	.	.	0	0	0	0	0	0	0

Haplotypes							1177	1178	1179	1180	1181	1182	1187	1204	1237									
*Cp_nutans*_1(3)		0	0	0	0	0	A	T	T	A	T	T	A	T	T	0	0	0	0	0	0	0	0	0
Cp_nutans_2(14)	A		T	T	A	T	.	.	.	.	.	.	.	.	.	0	0	0	0	0	0	0	0	0
*Cp_nutans*_3(1)		0	0	0	0	0	.	.	.	.	.	.	.	.	.	0	0	0	0	0	0	0	0	0
*Cp_nutans*_4(9)		0	0	0	0	0	.	.	.	.	.	.	.	.	.	0	0	0	0	0	0	0	0	0
*Cp_nutans_***5***(*4)		0	0	0	0	0	.	A	.	.	.	.	.	.	.	0	0	0	0	0	0	0	0	0
Cp_nutans_6(1)		0	0	0	0	0	.	.	.	.	.	.	.	.	.	0	0	0	0	0	0	0	0	0
Cp_nutans_7(1)		0	0	0	0	0	.	.	.	.	.	.	.	.	.	0	0	0	0	0	0	0	0	0
*Cp_nutans_* 8(1)	A		T	T	A	T	.	.	.	.	.	.	.	.	.	0	0	0	0	0	0	0	0	0
Cp_nutans_9(1)		0	0	0	0	0	.	A	.	.	.	.	.	.	.	0	0	0	0	0	0	0	0	0
Cp_nutans_10(1)		0	0	0	0	0	.	.	.	.	.	.	.	.	.	0	0	0	0	0	0	0	0	0
Cp_nutans_11(1)		0	0	0	0	0	.	.	.	.	.	.	.	.	.	0	0	0	0	0	0	0	0	0
Cp_otites_1(1)		0	0	0	0	0	0	0	0	0	0	.	.	.	.	0	0	0	0	0	0	0	0	0
Cp_otites_2(1)		0	0	0	0	0	0	0	0	0	0	.	.	.	.	0	0	0	0	0	0	0	0	0
*Cp_otites_3(*17)		0	0	0	0	0	0	0	0	0	0	.	.	.	.	0	0	0	0	0	0	0	0	0
*Cp_otites_4(*6)		0	0	0	0	0	.	.	.	.	.	.	.	G	.	A	T	A	T	A	A	T	T	G
*Cp_otites_5(*11)		0	0	0	0	0	0	0	0	0	0	.	.	.	G	0	0	0	0	0	0	0	0	0
*Cp_otites*_6(1)		0	0	0	0	0	.	A	.	.	.	0	0	G	.	0	0	0	0	0	0	0	0	0
Cp_latifolia_1(1)		0	0	0	0	0	0	0	0	0	0	.	.	.	.	0	0	0	0	0	0	0	0	0

Haplotypes																																1262
*Cp_nutans*_1(3)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	A
Cp_nutans_2(14)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.
*Cp_nutans*_3(1)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.
*Cp_nutans*_4(9)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.
*Cp_nutans_***5***(*4)		0	0	0	0	0	T	C	C	G	G	T	A	C	C	T	A	T	A	T	A	A	T	T	G	T	A	A	T	.	
Cp_nutans_6(1)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.
Cp_nutans_7(1)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.
*Cp_nutans_* 8(1)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.
Cp_nutans_9(1)		0	0	0	0	0	T	C	C	G	G	T	A	C	C	T	A	T	A	T	A	A	T	T	G	T	A	A	T	.	
Cp_nutans_10(1)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.
Cp_nutans_11(1)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.
Cp_otites_1(1)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.
Cp_otites_2(1)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.
*Cp_otites_3(*17)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.
*Cp_otites_4(*6)	T		A	A	C	T	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	G
*Cp_otites_5(*11)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.
*Cp_otites*_6(1)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	G
Cp_latifolia_1(1)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	T

Haplotypes	1265	1290	1297	1298	1299	1300	1301	1302	1303	1322	1375	1380	1410	1411	1412	1413
*Cp_nutans*_1(3)	A	T	T	T	G	A	C	A	C	A	G	G	A	A	A	T
Cp_nutans_2(14)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
*Cp_nutans*_3(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
*Cp_nutans*_4(9)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
*Cp_nutans*_5*(*4)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cp_nutans_6(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cp_nutans_7(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
*Cp_nutans_* 8(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cp_nutans_9(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cp_nutans_10(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cp_nutans_11(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cp_otites_1(1)	G	G	0	0	0	0	0	0	0	.	A	A	0	0	0	0
Cp_otites_2(1)	G	G	0	0	0	0	0	0	0	.	A	A	0	0	0	0
*Cp_otites_3(*17)	G	G	0	0	0	0	0	0	0	.	A	A	0	0	0	0
*Cp_otites_4(*6)	.	G	.	.	.	.	.	.	.	.	A	A	0	0	0	0
*Cp_otites_5(*11)	.	G	.	.	.	.	.	.	.	C	A	A	0	0	0	0
*Cp_otites*_6(1)	.	G	.	.	.	.	.	.	.	.	A	A	0	0	0	0
Cp_latifolia_1(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	T

Haplotypes	1414	1415	1416	1417	1418	1419	1420	1421	1427	1454	1465	1469	1471							
*Cp_nutans*_1(3)	A	A	A	A	T	A	A	A	0 T	C	T	T	A	0	0	0	0	0	0	
Cp_nutans_2(14)	.	.	.	.	.	.	.	.	0 .	.	.	.	.	0	0	0	0	0	0	
*Cp_nutans*_3(1)	.	.	.	.	.	.	.	.	0 .	.	.	.	C	0	0	0	0	0	0	
*Cp_nutans*_4(9)	.	.	.	.	.	.	.	.	0 .	.	.	.	C	0	0	0	0	0	0	
*Cp_nutans*_5*(*4)	.	.	.	.	.	.	.	.	0 G	.	.	.	C	0	0	0	0	0	0	
Cp_nutans_6(1)	.	.	.	.	.	.	.	.	0 .	.	.	.	.	0	0	0	0	0	0	
*Cp_nutans*_7(1)	.	.	.	.	.	.	.	.	0 .	.	.	.	C	0	0	0	0	0	0	
*Cp_nutans*_ 8(1)	.	.	.	.	.	.	.	.	0 .	.	G	.	C	0	0	0	0	0	0	
Cp_nutans_9(1)	.	.	.	.	.	.	.	.	0 .	.	.	.	.	0	0	0	0	0	0	
Cp_nutans_10(1)	.	.	.	.	.	.	.	.	0 .	.	.	.	C	0	0	0	0	0	0	
Cp_nutans_11(1)	.	.	.	.	.	.	.	.	0 .	.	.	.	.	0	0	0	0	0	0	
Cp_otites_1(1)	0	0	0	0	0	0	0	0	0 A	A	.	A	.	0	0	0	0	0	0	
Cp_otites_2(1)	0	0	0	0	0	0	0	0	0 A	A	.	A	.	0	0	0	0	0	0	
*Cp_otites_3(*17)	0	0	0	0	0	0	0	0	0 A	A	.	A	.	0	0	0	0	0	0	
*Cp_otites_4(*6)	0	0	0	0	0	0	0	0	A A	A	.	.	.	0	0	0	0	0	0	
*Cp_otites_5(*11)	0	0	0	0	0	0	0	0	0 A	.	.	.	.	A	T	A	T	A	A	
*Cp_otites*_6(1)	0	0	0	0	0	0	0	0	A A	A	.	.	.	0	0	0	0	0	0	
Cp_latifolia_1(1)	.	.	.	.	.	.	.	T	T G	A	.	.	.	0	0	0	0	0	0	

Haplotypes											1497	1498	1499	1500	1501	1502	1503	1504
*Cp_nutans*_1(3)	0	0	0	0	0	0	0	0	0	T		A	T	A	A	A	T	A
Cp_nutans_2(14)	0	0	0	0	0	0	0	0	0	.		.	.	.	.	.	.	.
*Cp_nutans*_3(1)	0	0	0	0	0	0	0	0	0	.		.	.	.	.	.	.	.
*Cp_nutans*_4(9)	0	0	0	0	0	0	0	0	0	.		.	.	.	.	.	.	.
*Cp_nutans*_**5***(*4)	0	0	0	0	0	0	0	0	0	.		.	.	.	.	.	.	.
Cp_nutans_6(1)	0	0	0	0	0	0	0	0	0	.		.	.	.	.	.	.	.
Cp_nutans_7(1)	0	0	0	0	0	0	0	0	0	.		.	.	.	.	.	.	.
*Cp_nutans_* 8(1)	0	0	0	0	0	0	0	0	0	.		.	.	.	.	.	.	.
Cp_nutans_9(1)	0	0	0	0	0	0	0	0	0	.		.	.	.	.	.	.	.
Cp_nutans_10(1)	0	0	0	0	0	0	0	0	0	.		.	.	.	.	.	.	.
Cp_nutans_11(1)	0	0	0	0	0	0	0	0	0	.		.	.	.	.	.	.	.
Cp_otites_1(1)	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0
Cp_otites_2(1)	0	0	0	0	0	0	0	0	0	.		.	.	.	.	.	.	.
*Cp_otites_3(*17)	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0
*Cp_otites_4(*6)	0	0	0	0	0	0	0	0	0	.		.	.	.	.	.	.	.
*Cp_otites_5(*11)	A	T	A	A	A	T	A	A	C	.		.	.	.	.	.	.	.
*Cp_otites*_6(1)	0	0	0	0	0	0	0	0	0	.		.	.	.	.	.	.	.
Cp_latifolia_1(1)	0	0	0	0	0	0	0	0	0	.		.	A	0	0	0	0	0

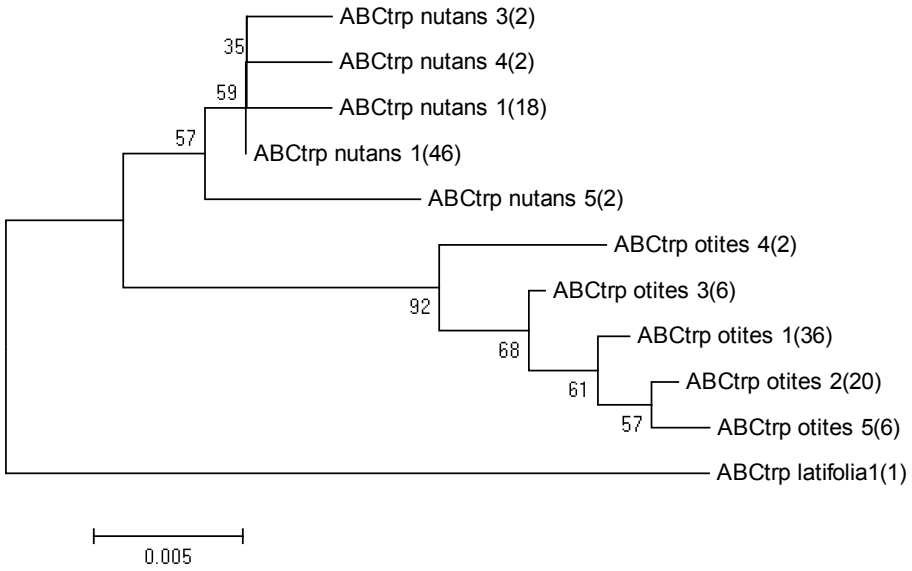
Haplotypes	1505	1506	1507	1508	1509	1510	1511	1526	1527	1528	1529	1530	1531	1532	1533	1537	1538	1541	1581
*Cp_nutans_*1(3)	A	A	T	A	A	C	A	A	T	A	T	A	A	T	A	T	A	G	T
Cp_nutans_2(14)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
*Cp_nutans_*3(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
*Cp_nutans_*4(9)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
*Cp_nutans_***5***(*4)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cp_nutans_6(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
*Cp_nutans_*7(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
*Cp_nutans_* 8(1)	.	.	.	.	.	.	.	.	.	.	0	0	0	0	0	.	.	.	.
Cp_nutans_9(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cp_nutans_10(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cp_nutans_11(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cp_otites_1(1)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.	.	.	G
Cp_otites_2(1)	.	.	.	.	.	.	.	0	0	0	0	0	0	0	0	.	.	.	G
*Cp_otites_3(*17)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.	.	.	G
*Cp_otites_4(*6)	.	.	.	.	.	.	.	0	0	0	0	0	0	0	0	.	.	T	G
*Cp_otites_5(*11)	.	.	.	.	.	.	.	0	0	0	.	.	0	.	.	A	C	T	G
*Cp_otites_*6(1)	.	.	.	.	.	.	.	0	0	0	0	0	0	0	0	.	.	T	G
Cp_latifolia_1(1)	0	0	0	0	0	.	.	0	0	0	0	0	0	0	0	0	0	.	.

Haplotypes	1581	1587								1656	1661	1687	1716	1717	1728	1746	1747	1764		1802	1842
*Cp_nutans*_1(3)	T	A	0	0	0	0	0	0	0	A	A	C	A	T	C	G	G	C	T	A	T
Cp_nutans_2(14)	.	0	0	0	0	0	0	0	0	.	.	A	.	.	.	.	.	.	G	.	.
*Cp_nutans*_3(1)	.	0	0	0	0	0	0	0	0	.	.	A	.	.	.	.	.	.	G	.	G
*Cp_nutans*_4(9)	.	0	0	0	0	0	0	0	0	.	.	A	.	.	.	.	.	.	G	.	.
*Cp_nutans*_**5***(*4)	.	0	0	0	0	0	0	0	0	.	.	A	.	.	.	.	.	.	G	.	.
Cp_nutans_6(1)	.	0	0	0	0	0	0	0	0	.	.	A	.	.	.	.	.	.	G	.	.
Cp_nutans_7(1)	.	0	0	0	0	0	0	0	0	.	.	A	.	.	.	.	.	.	G	.	.
*Cp_nutans_* 8(1)	.	0	0	0	0	0	0	0	0	.	.	A	.	.	.	.	.	.	G	.	.
Cp_nutans_9(1)	.	0	0	0	0	0	0	0	0	.	.	A	.	.	.	.	.	.	G	.	.
Cp_nutans_10(1)	.	0	0	0	0	0	0	0	0	.	.	A	.	.	.	.	.	.	G	.	G
Cp_nutans_11(1)	.	0	0	0	0	0	0	0	0	.	.	A	.	G	.	.	.	.	G	.	.
Cp_otites_1(1)	G	0	0	0	0	0	0	0	0	G	0	A	C	.	T	A	A	T	.	.	.
Cp_otites_2(1)	G	0	0	0	0	0	0	0	0	G	0	A	C	.	T	A	A	T	.	.	.
*Cp_otites_3(*17)	G	0	0	0	0	0	0	0	0	G	0	A	C	.	T	A	A	T	.	.	.
*Cp_otites_4(*6)	G	0	0	0	0	0	0	0	0	G	0	A	C	.	T	A	A	T	.	.	.
*Cp_otites_5(*11)	G	0	0	0	0	0	0	0	0	G	0	A	C	.	T	A	A	T	.	.	.
*Cp_otites*_6(1)	G	0	0	0	0	0	0	0	0	G	0	A	C	.	T	A	A	T	.	.	.
Cp_latifolia_1(1)	.	A	A	C	A	A	C	A	G	.	.	A	.	.	.	T	T	.	A	C	G

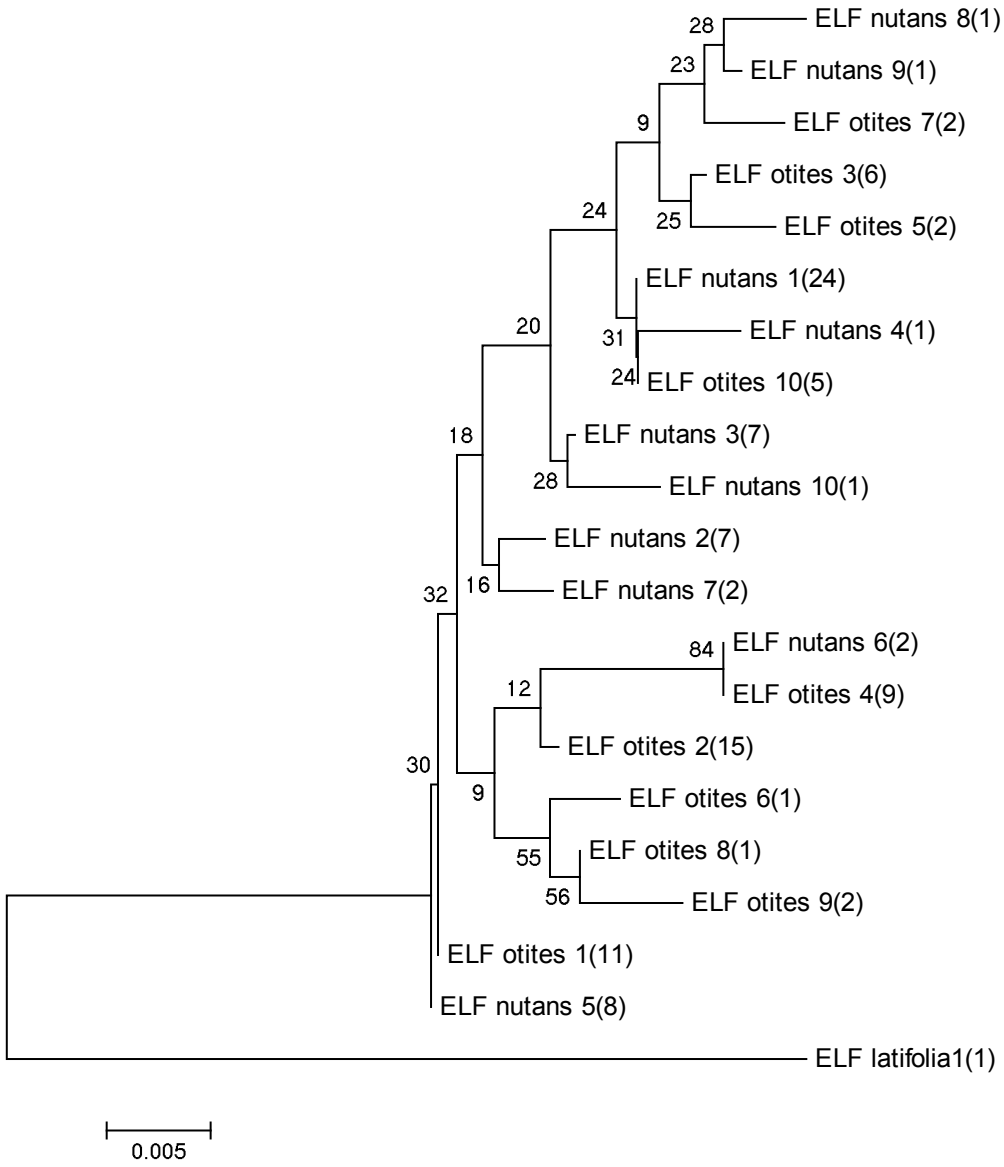
Supplementary Table 4.

Fixed sites, shared and exclusive polymorphic sites between *S. nutans* and *S. otites* for the three genomes.

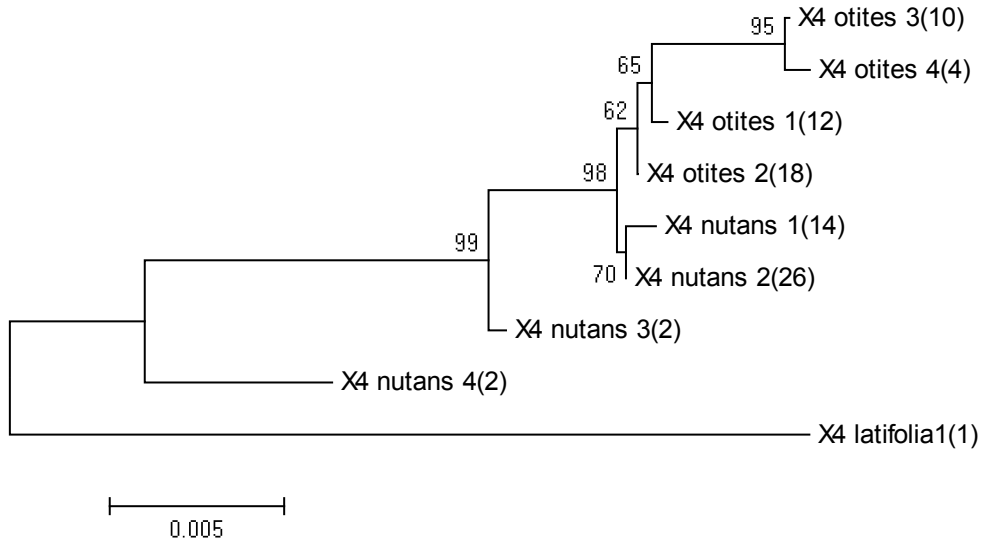
Genome	Genes	Fixed mutation	Shared mutations	Exclusive polymorphic mutation of	
				S. otites	***S. nutans***
Nuclear	*X4*	1	0	6	23
	ELF	0	5	2	2
	ATUB	0	45	7	13
	ABCtrp	4	0	6	6
Mitochondrial	*cob*	3	1	3	8
	cox1	0	2	7	16
Chloroplast	*GS*	8	0	5	7
	psbA	40	0	8	9
	LF	4	1	9	4
	MatK	9	1	3	5



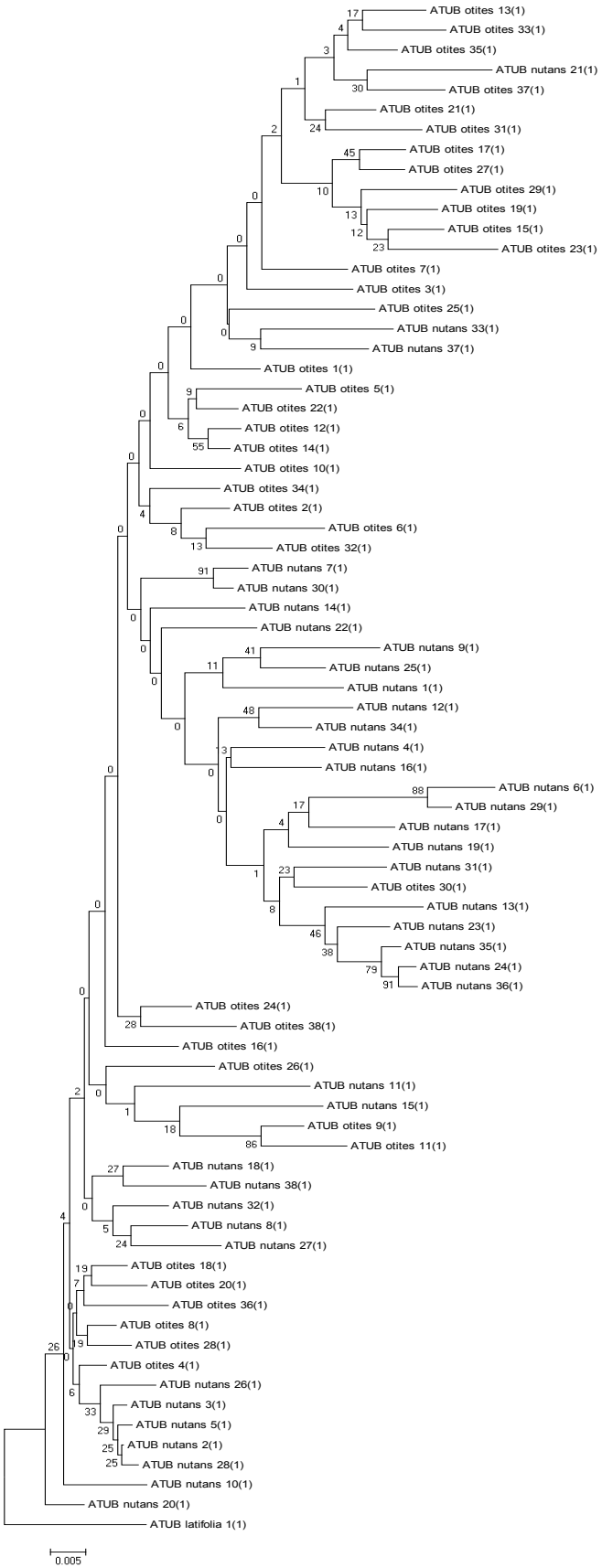
Sup_figure 1A. Phylogenetic tree generated by Neighbor-Joining for sequences of nuclear gene *ABCtrp* for *S. nutans* and *S. otites*, with *S. latifolia* as outgroup. Haplotype numbers per species are indicated as well as their frequency in parentheses.



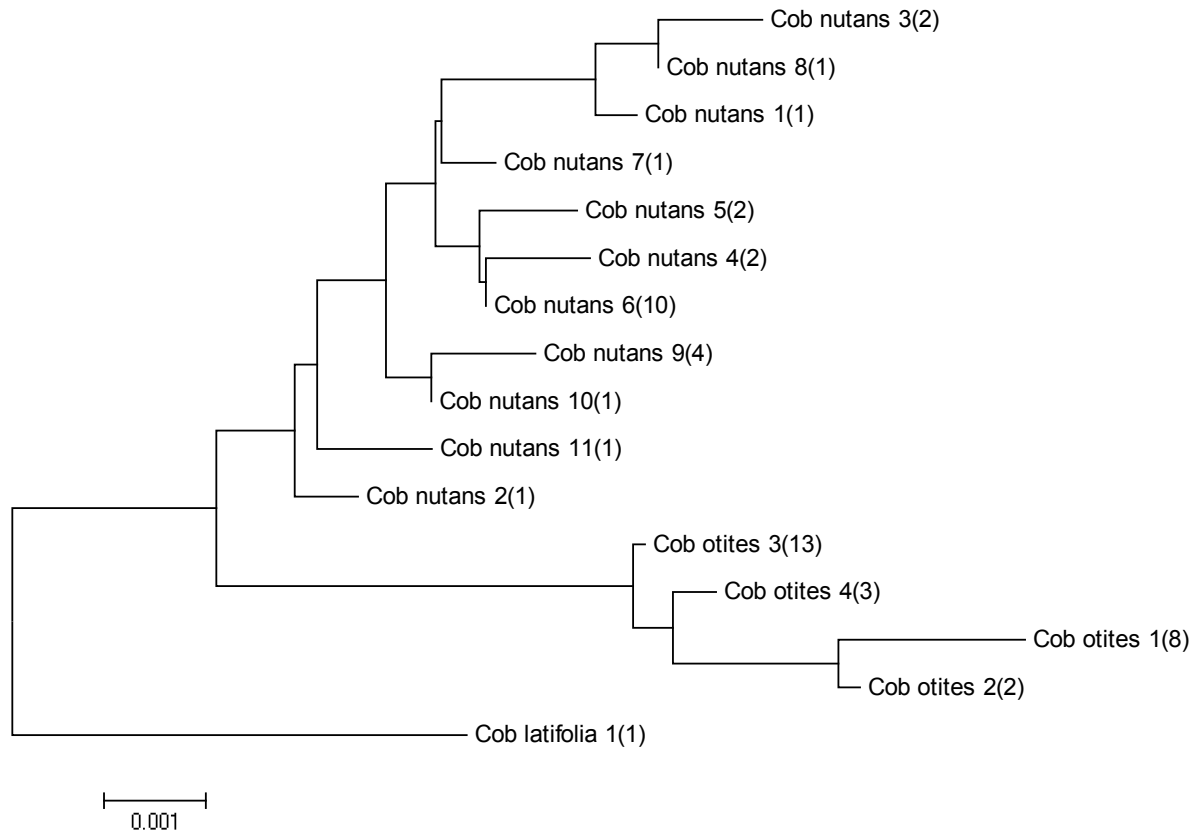
Sup_figure 1B. Phylogenetic tree generated by Neighbor-Joining for sequences of nuclear gene *ELF* for *S. nutans* and *S. otites*, with *S. latifolia* as outgroup. Haplotype numbers per species are indicated as well as their frequency in parentheses.



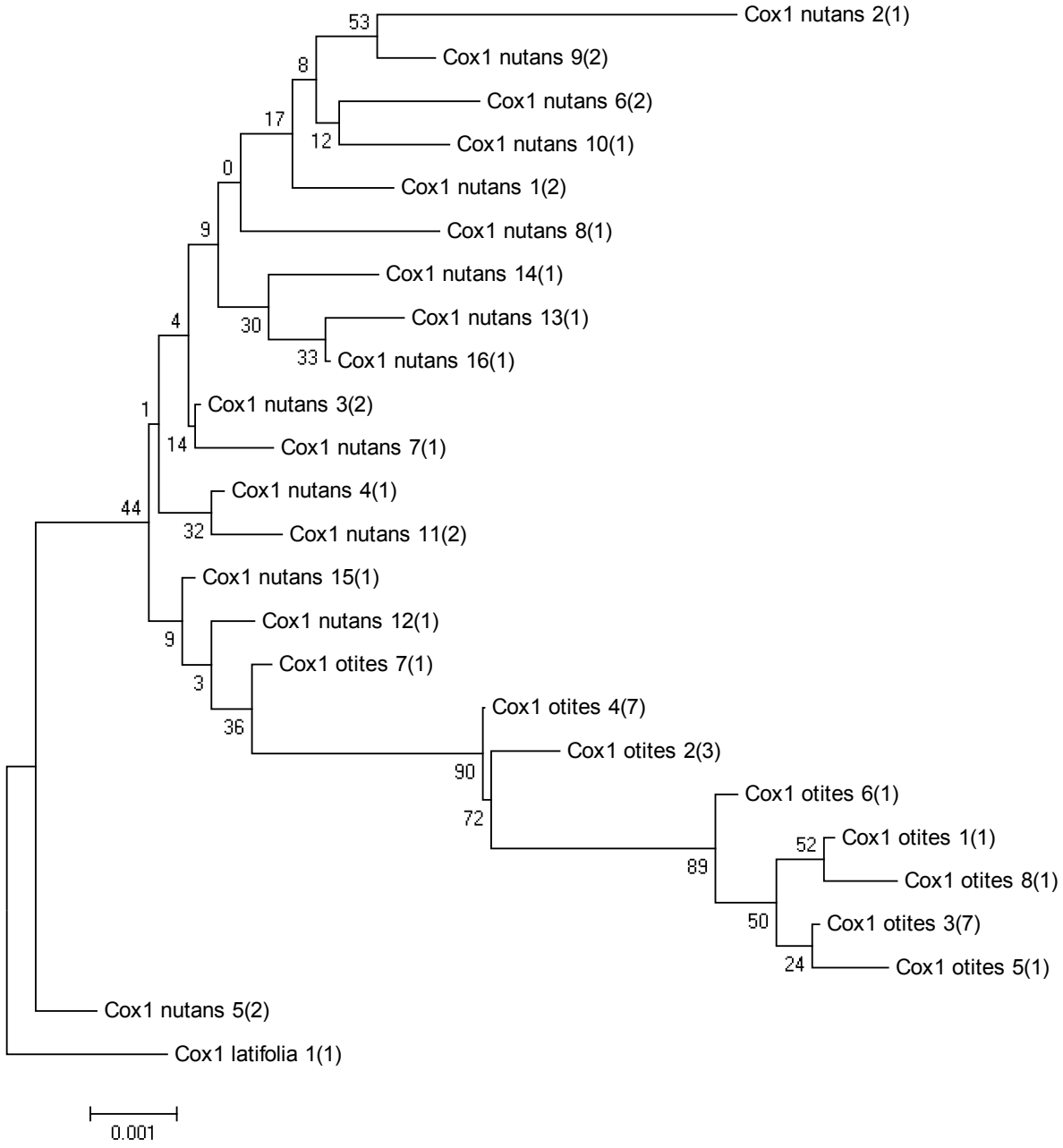
Sup_figure 1C. Phylogenetic tree generated by Neighbor-Joining for sequences of nuclear gene *X4* for *S. nutans* and *S. otites*, with *S. latifolia* as outgroup. Haplotype numbers per species are indicated as well as their frequency in parentheses.



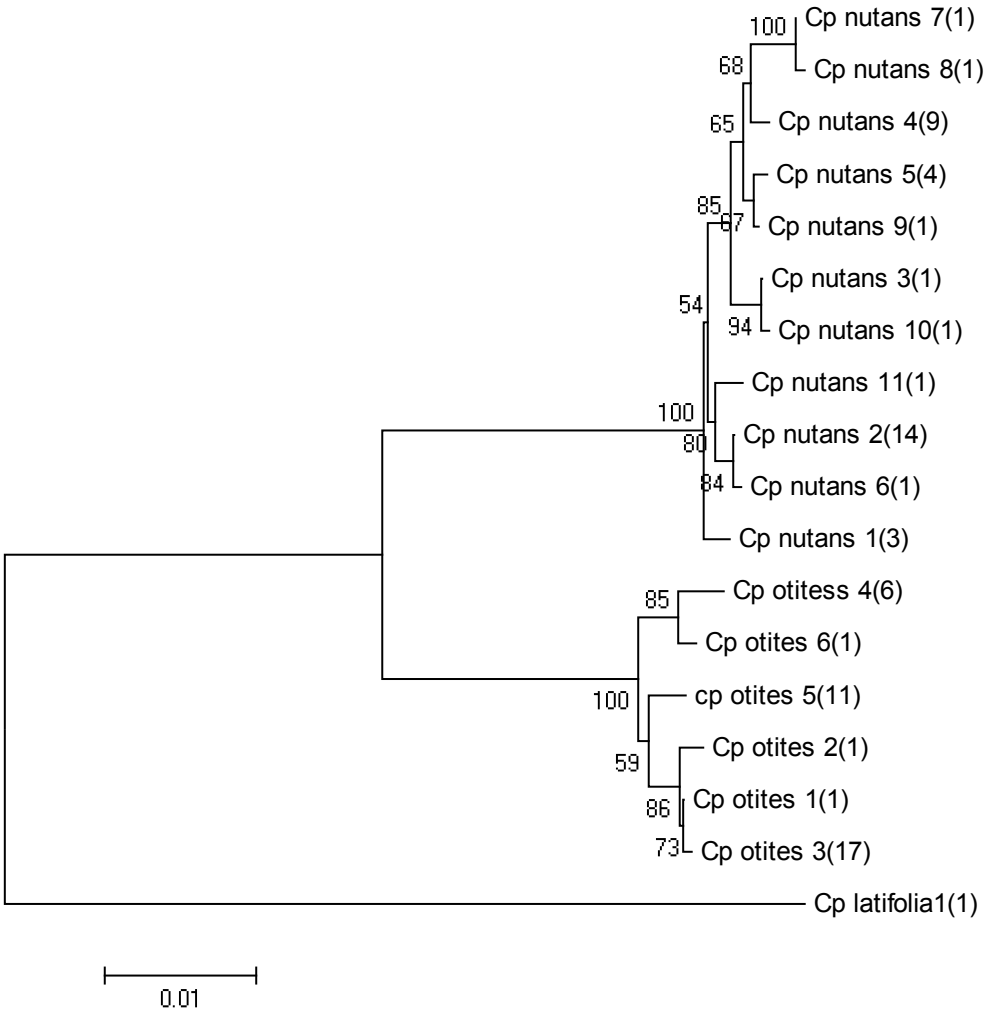
Sup_figure 1D. Phylogenetic tree generated by Neighbor-Joining for sequences of nuclear gene *ATUB* for *S. nutans* and *S. otites*, with *S. latifolia* as outgroup. Haplotype numbers per species are indicated as well as their frequency in parentheses.



Sup_figure 1E. Phylogenetic tree generated by Neighbor-Joining for sequences of mitochondrial gene *cob* for *S. nutans* and *S. otites*, with *S. latifolia* as outgroup. Haplotype numbers per species are indicated as well as their frequency in parentheses.



Sup_figure 1F. Phylogenetic tree generated by Neighbor-Joining for sequences of mitochondrial gene *cox1* for *S. nutans* and *S. otites*, with *S. latifolia* as outgroup. Haplotype numbers per species are indicated as well as their frequency in parentheses.



Sup_Figure 1G. Phylogenetic tree generated by Neighbor-Joining for sequences of concatenated chloroplastic sequences of *S. nutans* and *S. otites*, with *S. latifolia* as outgroup. Haplotype numbers per species are indicated as well as their frequency in parentheses.

Chapitre 2

When is it worth to be an hermaphrodite? Contradictory effects of self-pollination in gynodioecious Silene nutans, as a function of local sex-ratio.

Emna Lahiani, Pascal Touzet, Emmanuelle Billard, Mathilde Dufay

En révision

Photo d'un des deux patches expérimentaux

Abstract:

In gynodioecious species, females and hermaphrodites plants coexist, with common variation in sex-ratio among populations. Sex-ratio may impact the occurrence and the magnitude of the female advantage, an important condition for females to be maintained, as it should affect the occurrence of pollen limitation and the magnitude of self-pollination in hermaphrodites. However, the overall impact of sex-ratio, and in particular the impact of self-pollination on gynodioecy dynamics is far from being intuitive and was never investigated in details. In the current study, we measured pollination efficiency in both females and hermaphrodites and the selfing rate in hermaphrodites in two experimental populations showing contrasted sex-ratio, in gynodioecious *Silene nutans*. We found an impact of plant gender, population, and their interaction on pollination efficiency, with females suffering from stronger pollen limitation when being locally frequent. In such biased sex-ratio situation, the selfing rate of hermaphrodites was increased and provided hermaphrodites with a reproductive assurance over females. Finally, by integrating both beneficial (reproductive assurance) and costly effects (through inbreeding depression) of self-pollination, we showed in which situation females should be favoured by natural selection.

Running head: Effect of selfing on female advantage

Key words: Gynodioecy, pollen limitation, self-pollination, sex-ratio, female advantage, *Silene nutans*.

Introduction

Gynodioecy is an intriguing reproductive system with females and hermaphrodites co-occurring within the same species, which has been documented in many different families in Angiosperms. Theoretically, the only way for females to compensate for the loss of gene transmission through pollen production is to benefit from a higher female fitness compared to hermaphrodites. When male-sterility is caused by a nuclear allele, the invasion of females requires at least a twofold advantage, and a gender polymorphism can be then maintained through Fisherian selection (Lewis, 1941). In the common case of a cytoplasmic allele causing male sterility, because cytoplasmic genes are only transmitted through ovules, a limited female advantage is sufficient for females to invade the population (Lewis, 1941). As soon as such cytoplasmic male sterility occurs in a population, nuclear alleles restoring pollen production are strongly selected for (Saumitou-Laprade *et al.*, 1994). Nuclear-cytoplasmic gynodioecy can theoretically be maintained through frequency-dependent selection under a large set of conditions (Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufay *et al.*, 2007). Because this type of frequency-dependent selection can produce strong oscillations of sex-ratios within populations through time, and because different populations of a given species may thus be at different phases of the cycling dynamics at a given date, this has been sometimes put forward as an explanation for the typical variation in sex-ratio among populations or among patches within the same population, in many gynodioecious species (Dufay *et al.*, 2009; McCauley *et al.*, 2000; Laporte *et al.*, 2001; Olson *et al.*, 2005; Olson *et al.*, 2006). The impacts of founder effect, genetic drift and variation in habitat quality have also been proposed to explain such common variation in sex-ratio (Nilsson & Agren, 2006; Caruso & Case, 2007; Dufay & Pannell, 2010).

According to theoretical predictions, the occurrence of a female advantage, *i.e.* higher female fitness of females compared to hermaphrodites, is thus one important condition of gynodioecy maintenance for both nuclear and nuclear-cytoplasmic gynodioecy. This difference between females and hermaphrodites has been empirically

verified in several gynodioecious species (reviewed in Shykoff *et al.*, 2003) and, according to the case, this advantage could be explained by resource reallocation from male to female function and/or to the fact that obligatory outcrossed females avoid self-pollination and the associated inbreeding depression (reviewed in Dufay & Billard, 2012). The magnitude of female advantage has been shown to influence the dynamics of gynodioecy (e.g. Bailey *et al.*, 2003; Dufay *et al.*, 2007) but all theoretical models have so far considered female advantage as a fixed parameter, with no possible variation though space and/or time. This probably does not fit with what happens in many gynodioecious species, since the magnitude of female advantage may depend on various components of habitat quality in particular when females are more stress-resistant than hermaphrodites (e.g. an effect of altitude in *Gingidia flabellate* in Webb (1981) and of water availability in Barr (2004)). In addition, the magnitude and even the occurrence of the female advantage seems to vary with sex-ratio of the patch or the population, but with no clear pattern, since both positive and negative correlations were found between female frequency and the magnitude of female advantage (reviewed in Dufay & Billard, 2012). Several explanations hold for these contradictory results. On the one hand, high female frequency is sometimes expected to increase the magnitude of pollen limitation (Maurice & Fleming, 1995; McCauley & Brock, 1998; Case & Ashman, 2009). If females and hermaphrodites respond differently to pollen limitation, this could thus impact the magnitude of female advantage. On the other hand, self-compatible hermaphrodites may express a selfing rate that depends on population sex-ratio, thereby impacting female advantage in two contradictory ways. In case of a strong inbreeding depression, high selfing rate should provide females with a fitness advantage (and thus increase the female advantage), while in case of pollen limitation, self-pollination may provide hermaphrodites with reproductive assurance (and thus decrease the female advantage). Thus, while variation in sex-ratio is extremely common in gynodioecious species (e.g. Manicacci *et al.*, 1996; Asikainen & Mutikainen, 2003; Alonso *et al.*, 2007; Caruso & Case, 2007; Dufay *et al.*, 2009; Van Etten & Chang, 2009), there is no comprehensive prediction of how sex-ratio variation should impact female advantage, and ultimately, the dynamics of gynodioecy. No theoretical predictions have been made

on the topic, and empirical studies have only addressed partially this question. On the one hand, some empirical studies have documented a negative correlation between female frequency and the reproductive success of females, but with no explicit investigation of the possible role of pollen limitation and self-pollination (e.g. McCauley & Brock, 1998; Zhang *et al.*, 2008). On the other hand, only Miyake & Olson (2009) have documented the positive correlation between local female frequency and selfing rate in hermaphrodites, but with no empirical investigation of how such variation impacts female advantage.

The aim of this study is to empirically investigate female advantage in a gynodioecious species, by disentangling the impact of pollination efficiency (in both females and hermaphrodites) and selfing rate (in hermaphrodites) in two contrasted situations of local sex-ratio. Our study focuses on the gynodioecious, entomophilous and self-compatible *Silene nutans* (Caryophyllaceae). The genetic determination of sex is nuclear-cytoplasmic in this species (Garraud *et al.*, 2011) and, possibly for this reason, sex-ratio has been found to strongly vary among populations and among patches within populations (Dufay, unpublished data). In this species, as well as in many others, how sex-ratio affects female advantage is thus an important issue. Our study is carried out in semi-controlled conditions, to control for other parameters that may affect female reproductive success, such as habitat quality or pollinator availability, and to allow the two experimental populations to differ only for the local sex-ratio. This allow us to compare the magnitude of female advantage between populations with female-biased versus hermaphrodite-biased sex-ratio, and investigate how this may influence the dynamics of gynodioecy.

Materials and methods

Study species

Silene nutans (Caryophyllaceae) is a diploid, long-lived perennial rosette plant growing in dry, open grass communities of hillsides. It is described as gynomonoecious-gynodioecious, with female, gynomonoecious (plants bearing both perfect and pistillate flowers), and hermaphroditic individuals found in natural populations (Jurgens *et al.*,

2002; Dufay *et al.*, 2010). Sex-ratio varies among populations from 0% to 60% of female plants (Dufay, unpublished data). Population size is strongly variable and can be extremely small (Hauser & Weidema, 2000; Van Rossum & Prentice, 2004). Flowers are visited by a number of different insect species, including Noctuidae, Sphingidae, Hymenoptera and nectar robbing Hymenoptera (Jurgens *et al.*, 1996). Perfect flowers are protandrous, but self-pollination can occur by geitonogamy.

Plant material

Individual plants used in the experiment were sampled in 2008 from natural populations. All plants were cloned from plantlets grown under greenhouse and over-wintered for ten weeks during winter 2010. Plants were then potted in a soil mix (3/4 compost; 1/4 perlite) and placed in greenhouse at a temperature of 20°C for seven weeks until the population reached the peak of flowering. Plants mainly originated from a single wild population in Olloy-sur-Viroin in Belgium (Van Rossum *et al.*, 1997). To complete our experimental populations in order to reach our objectives in terms of number of individuals and sex-ratio (see below), we also used a few genotypes from other localities in Germany, France and United Kingdom (Supplementary Table 1).

DNA extraction and genotyping

All hermaphroditic plants (i.e. potential fathers of seeds produced in each experimental population, see below) were genotyped in order to estimate the magnitude of self-pollination. For this purpose, DNA was extracted from 10-15 mg of leaf tissue using MACHEREY-NAGEL NucleoSpin® 96 Plant II Kits. DNA sample from each plant was assayed for five scored microsatellite loci (for primers see Supplementary Table2). Amplification reactions were carried out by polymerase chain reaction (PCR) with 20 ng of DNA in 10µl reaction volume containing 5µl of Qiagen multiplex Kit 2x, 1µl of primer mix (10x) (0.75µM of Scored forward primer and 3.75µM of reverse primer) and 1µl of sterile water. Cycling conditions for PCR amplification were 95°C for 15min, five cycles of 45s at 95°C, 5min at 68°C with a step-down of 2°C per cycle, 1min at 72°C, fives cycles of 45s at 95°C, 5min at 58°C with a step-down of 2°C per

cycle, 1min at 72°C, 27 cycles of 45s at 95°C, 30s at 47°C, 1min at 72°C and finally 72°C for 10 minutes. Amplification products were separated on Applied Biosystems 3130 capillary sequencer. Raw data were analysed using GENMAPPER version 3.5 (Applied Biosystems). Individuals with doubtful or missing peaks or for which mismatches occurred between mothers and progeny were genotyped a second time.

Experimental populations with contrasted sex-ratios

A total of 144 plants (72 females and 72 hermaphrodites) were used in the experiment. In a common garden, we created two experimental populations, separated by more than 40m, each population containing 72 individual plants. The two populations had contrasting local sex-ratio, the first one being female-biased (hereafter *FB* population, with 85% of individual plants - 60 females out of 72 plants - and 78% of flowers being female), while the second one was hermaphroditic-biased (hereafter *HB* population, with 85% of individual plants - 60 hermaphrodites out of 72 plants - and 87% of flowers being hermaphroditic). In each of the two populations, 36 individual plants, all from the same natural population of Olloy, were followed (see below). These focal plants were placed in the center of the experimental population and were randomly moved every two days during the whole experiment. The other 36 individual plants were placed around the focal individuals, in order to obtain similar plant densities, but contrasted sex-ratios in the two populations. All plants were watered every day.

To constitute the two experimental populations, individual plants were chosen according to two criteria. First, we avoided as much as possible gynomonoecious plants, in which the proportion of female flowers varies from 0.03 to 0.9 and also varies through time. Second, we also used the microsatellite genotypes of hermaphrodites as a criterion, in order to improve our estimation of selfing rate. We ensured that, in each experimental population, every potential father (i.e. hermaphroditic individual) had a specific genotype, different from the genotypes of all other potential fathers.

Pollen limitation and pollination efficiency

From 20th May 2010, 72 focal individual plants (12 hermaphrodites and 24 females in the *FB* population; 12 females and 24 hermaphrodites in the *HB* population) were surveyed for their pollination for one entire week. The first day of the experiment,

three flowers were marked at bud stage on each focal plant. These flowers were left untreated, thus receiving only natural levels of pollination (hereafter open-pollinated flowers). On a subsample of the focal plants (12 hermaphroditic plants and 12 female plants in each population), we additionally marked three other flowers at bud stage. These flowers were hand-pollinated once a day, by gently brushing receptive stigmas with fresh ripe pollen collected every day on thirteen hermaphrodites, which were growing in a greenhouse and were different from the plants located in the two experimental populations. We thus had two categories of focal plants: manipulated plants bearing 3 open-pollinated and 3 hand-pollinated flowers (12 hermaphroditic plants and 12 female plants in both populations) and control plants on which only 3 open-pollinated flowers were followed (12 female plants in the *FB* population and 12 hermaphroditic plants in the *HB* population). On the two types of marked flowers (open-pollinated and hand-pollinated), we estimated the fruit set as the proportion of marked flowers setting fruit two weeks later. At that time, to prevent seed loss, fruits were covered with mesh bags before they opened. Fruits were then collected as they matured, four weeks after pollination; seeds were counted and the weight of all seeds per fruit was recorded. For each focal plant and for each treatment, we thus obtained one average value of seed number per fruit, and one average value for the weight of one seed. We estimated the pollination success as the product: fruit set x average number of seeds per fruit. This was calculated for each treatment (open/ hand-pollinated), for each plant.

Plants are considered as pollen limited if additional pollen increases fruit or seed production (Schemske, 1980; Willson & Schemske, 1980; Bierzychudek, 1981; Burd, 1994; Larson & Barrett, 2000). We thus analyzed the occurrence of pollen limitation by comparing open-pollinated and hand-pollinated within each manipulated plant for their fruit set, their average seed number and their pollination success, by performing paired t-tests. We performed four different analyses, one per plant gender and per population. One must note, however, that when additional pollen is applied to only some flowers on a plant, resources may be shunted away from untreated flowers. Using the immediate increase in seed production due to supplemental pollination as a measure pollen

limitation is thus potentially confounded with this compensation among flowers (Ashman *et al.*, 2004). To assess this possible mechanism, we compared open-pollinated flowers of control plants and open-pollinated flowers of manipulated plants for their fruit set and their seed production, in each population and for each plant gender. If there was some compensation, one should record a higher seed production in open-pollinated flowers of control plants compared to open-pollinated flowers of manipulated plants. Finally, we analyzed the effect of population, plant gender, and their interaction on fruit set, average seed number per fruit and resulting pollination efficiency for all surveyed open-pollinated flowers. Analyses of fruit set were performed by using a logistic regression (binomial distribution, log link function, proc GENMOD, SAS), correcting for overdispersion (dscale option, proc GENMOD, SAS). Average seed weight, average seed number per fruit and pollination success were performed by using an ANOVA (proc GLM, SAS). In order to reach a normal distribution of residuals, we log-transformed the average seed number and the pollination success (Kolmogorov-Smirnov test of normality: $P>0.15$ for all shown analyses). All statistical analyses were conducted using SAS (SAS version 9.1.3, 2002).

Estimation of selfing rates

We estimated the selfing rate of the 36 focal hermaphrodites (24 individuals in the *HB* population and 12 individuals in the *FB* population). When it was possible, 25 seeds per fruit of open-pollinated flowers of these plants were sown in Petri dishes on Whatman paper. Filter papers were kept moistened during germination. The location of dishes was regularly changed. After 12 days, up to 50 seedlings from each plant were randomly selected, transplanted into a soil mix (3/4 compost; 1/4 perlite), and placed at 20°C with daily moistening to minimize any stress of transplantation. Six weeks later, plantlets were collected in order to extract their DNA by using MACHEREY-NAGEL NucleoSpin® 96 Plant II Kits. 1100 plantlets from the 36 families were genotyped by the five scored microsatellites loci described previously. Outcrossing rates were determined using Ritland (2002) multilocus maximum likelihood estimation program (MLTR Version 3.4, accessible at http://genetics.forestry.ubc.ca/ritland/programs.html).

Standard deviations were determined based on 1000 bootstrap analyses; maternal genotypes were estimated as part of the maximum likelihood procedure. Within each population, population estimates allowed us to calculate overall multilocus (t_m) and mean single locus (t_s) outcrossing rates, bootstraps using families as units of observation. Family estimates gave outcrossing rates per family, bootstraps using individual offspring as units of observation. We considered selfing rate as $s = 1- t_m$.

Differences in selfing rate between the two populations were analyzed by using a logistic regression (binomial distribution, log link function, proc GENMOD, SAS), correcting for overdispersion (dscale option, proc GENMOD, SAS). Moreover, we used these results to estimate the number of seeds produced by outcrossing in each focal plant. To do so, we multiplied the average seed number per fruit by the outcrossing rate for each hermaphroditic plant. We performed the same calculation for female plants, by considering the outcrossing rate to be 1. On these new data, we tested the effect of population, plant gender and their interaction by using proc GLM (SAS), as explained previously. One must note that log transformation did not help in reaching normal distribution of residuals in that case. We thus present the analyses for non transformed data, but with a non-normal distribution of residuals (Kolmogorov-Smirnov, $P < 0.01$).

Results

Fruit set of open-pollinated flowers did not significantly differ between control and manipulated plants neither in the *FB* population (12 manipulated females vs. 12 control females: $\chi^2_{1,22} = 1.98$, $P = 0.159$) nor in the *HB* population (12 manipulated hermaphrodites vs. 12 control hermaphrodites: $\chi^2_{1,22} = 1.39$, $P = 0.238$). The same result was found for the average seed number per fruit (*FB* population: $F_{1,22} = 3.39$, $P = 0.079$; *HB* population: $F_{1,22} = 1.69$, $P = 0.206$). It is thus unlikely that open-pollinated flowers to which hand-pollinated flowers were compared within manipulated plants suffered from lower resources allocation. This suggests that any difference between these two types of flowers can be attributed to pollen limitation. Focusing on manipulated plants, we found a significantly larger fruit set in hand-pollinated flowers compared to open-

pollinated flowers carried by the same plant, for focal female plants in both patches and for focal hermaphrodites in the *FB* patch only, indicating some pollen limitation affecting fruit set in these three categories of plants (Table 1). Evidence for pollen limitation was also found on the number of seeds per fruit and on the pollination success (fruit set x seed number) for both plant genders in both patches (Table 1). However, one must note that seed number per fruit was extremely variable among hand-pollinated flowers (from 6 seeds to 128 seeds per fruit), even for a given plant gender in one particular patch, which could be due to potential failure in hand pollination. Thus, the magnitude of pollen limitation may have been underestimated and the difference between the number of seeds produced by hand-pollinated and open-pollinated flowers could not be analyzed further, as it was not a reliable estimation of the intensity of pollen limitation. Thus, to compare pollination efficiency among genders and populations, we then focused on open-pollinated flowers (that were, on average, limited in pollen supply, according to our results).

Table 1: Results of paired t-tests comparing pollination efficiency between hand-pollinated and open-pollinated flowers within manipulated plants. D is the difference for all variables between the two pollination treatments (hand pollinated – open pollinated) and the test indicates whether this difference is significantly different from 0. For each variable, four analyses were run, one per plant gender and experimental population.

Variable	D	t	*P*
Fruit set			
Female plants in the FB patch	0.21	2.90	0.014
Hermaphroditic plants in the FB patch	0.20	2.52	0.02
Female plants in the HB patch	0.11	2.33	0.04
Hermaphroditic plants in the HB patch	0.01	0.16	0.87
Average seed number per fruit			
Female plants in the FB patch	58.25	5.74	0.0001
Hermaphroditic plants in the FB patch	51.26	8.41	$< 10^{-4}$
Female plants in the HB patch	54.55	8.41	$< 10^{-4}$
Hermaphroditic plants in the HB patch	56.97	8.20	$< 10^{-4}$
Pollination success (fruit set x average seed number)			
Female plants in the FB patch	55.44	5.90	0.0001
Hermaphroditic plants in the FB patch	52.98	7.04	$< 10^{-4}$
Female plants in the HB patch	55.04	8.45	$< 10^{-4}$
Hermaphroditic plants in the HB patch	49.05	9.08	$< 10^{-4}$

Regarding open-pollinated flowers, fruit set was significantly lower in the *FB* population compared to the *HB* population ($\chi^2_{1,68} = 5.80$, $P = 0.016$) but was not affected by plant gender ($\chi^2_{1,68} = 0.04$, $P > 0.1$) nor by the interaction between the two factors ($\chi^2_{1,68} = 0.77$, $P > 0.1$). Average seed number and pollination success were both significantly lower in female plants and in the *FB* population (Table 2). These two variables were also affected by the interaction between the two factors (Table 2), with post-hoc pairwise comparisons showing significantly lower values on female plants in the *FB* population compared to three other plant categories (Fig. 1). Finally, the average weight of one seed did not significantly differ according the population nor plant gender

(Table 2), suggesting no resource reallocation increasing seed quality in plants experiencing stronger pollen limitation.

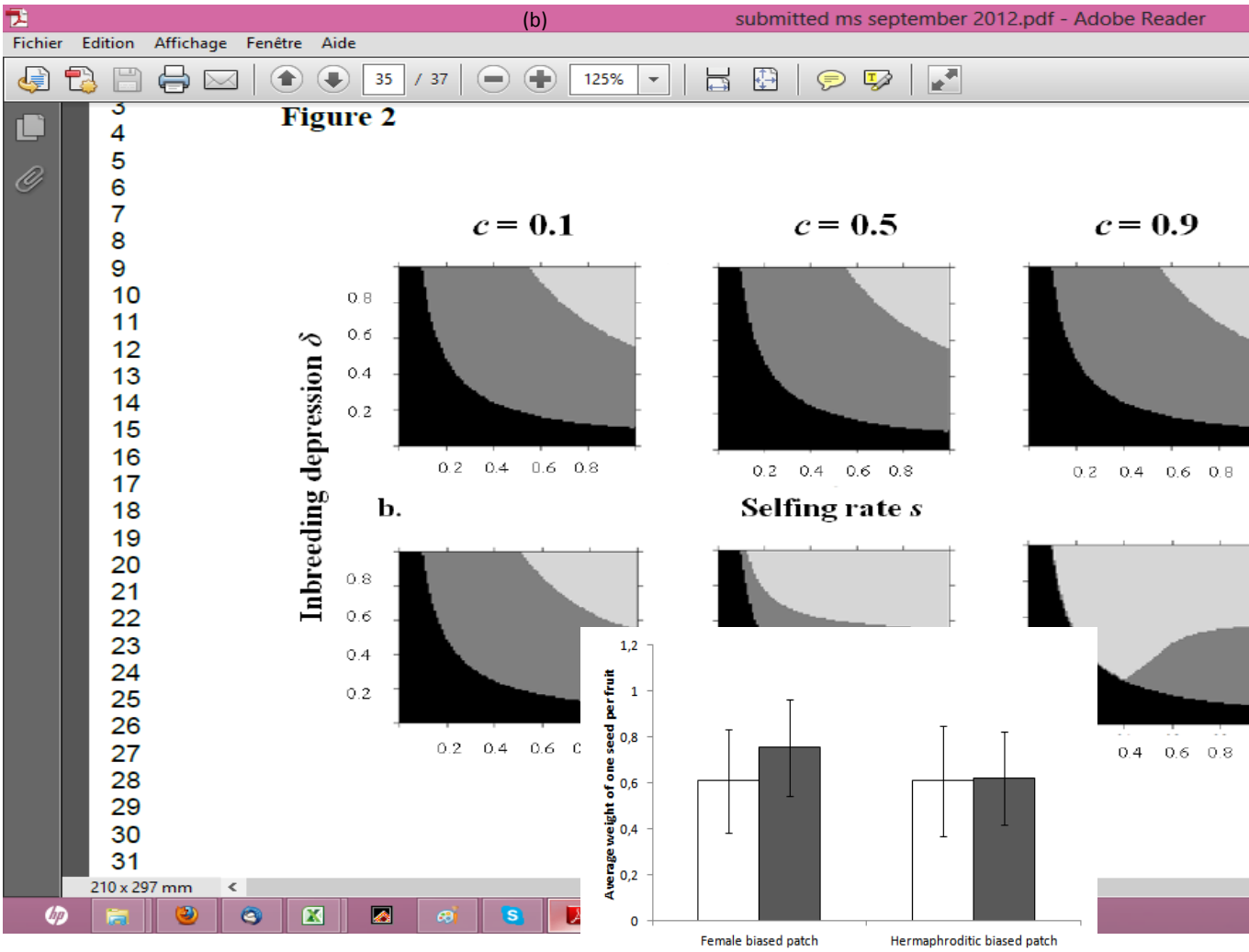
Table 2 : Results of the analyses of variance on the various estimates of pollination efficiency on open-pollinated flowers.

Variable and sources of variation	**DF**	**MS**	**F**	***P***
Average seed number per fruit*:				
Plant gender	1	0.799	9.95	0.0024
Patch	1	0.563	7.02	0.01
Plant gender x patch	1	0.485	6.05	0.0165
Error	68	0.080		
Average seed number produced by outcrossing:				
Plant gender	1	307.76	3.04	0.085
Patch	1	1736.14	17.15	$< 10^{-4}$
Plant gender x patch	1	68.19	0.67	0.414
Error	68	101.24		
Average weight of one seed:				
Plant gender	1	0.06	1.44	0.23
Patch	1	0.09	2.08	0.15
Plant gender x patch	1	0.07	1.5	0.22
Error	68	0.04		
Pollination success (fruit set x average seed number)*				
Plant gender	1	0.948	8.44	0.005
Patch	1	1.259	11.21	0.0013
Plant gender x patch	1	0.747	6.65	0.0121
Error	68	0.112		

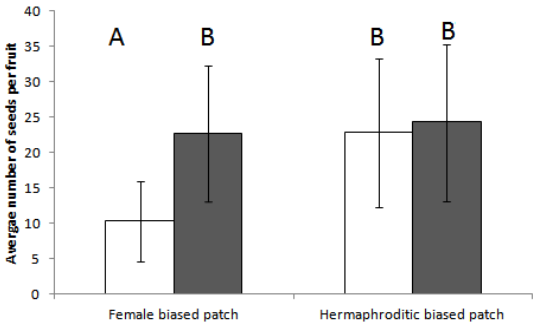
* log transformed

(a)

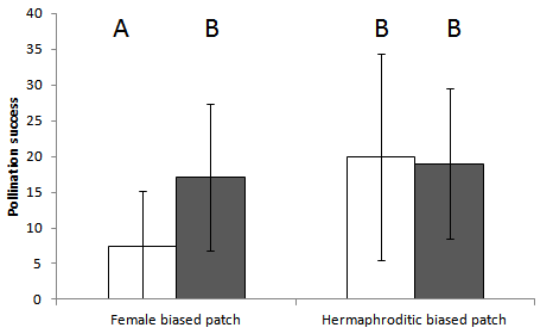
(b)



(c)



(d)



(e)

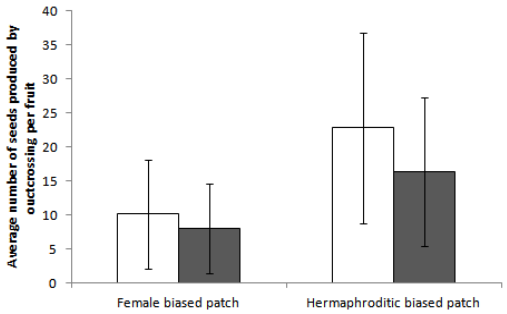


Figure 1.(a) fruit set, (b) average weight of one seed per fruit, (c) average number of seeds per fruit, (d) pollination success (fruit set x average seed number) and (e) average number of outcrossed seeds per fruit, of open-pollinated flowers, as a function of plant gender and population. White: female plants, grey: hermaphroditic plants. Bars indicate standard deviation. Letters A and B indicate significant differences among levels, according to the post-hoc Tuckey tests, when the interaction between population and plant gender was significant.

Selfing rate of hermaphroditic plants varied from 0 to 1, according to the family. We found that hermaphrodites experiencing a high local female frequency self-pollinated at a significantly higher rate that the other group (s = 0.508 ± 0.071 in the *FB* population; s = 0.347 ± 0.07 in the *HB* population; $\chi^2_{1,34}$ = 8.18, P = 0.0042; Fig. 2). Finally, the number of outcrossed seeds (estimated on the basis of the selfing rate in each family) was significantly higher in the *HB* compared to the *FB* population, but it was only marginally affected by plant gender and it did not depend on the interaction between the two factors (Table 3, Fig. 1).

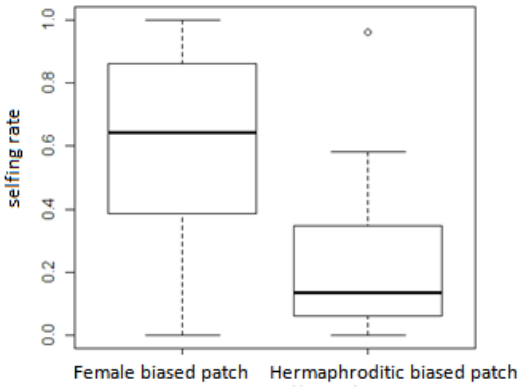


Figure 2. Distribution of selfing rates in open-pollinated hermaphroditic plants from both experimental populations.

Table 3 : Multilocus (t_m) and mean single locus (t_s) outcrossing rates and biparental inbreeding (t_m-t_s) estimated in the two populations (*HB*: hermaphroditic-biased; *FB*: female-biased).

	HB patch	**FB patch**
Families (progenies)	24 (733)	12 (306)
t_m (SD)	0.653 (0.070)	0.492 (0.071)
t_s (SD)	0.477 (0.053)	0.446 (0.079)
t_m-t_s (SD)	0.176 (0.025)	0.046 (0.019)
Family t_m range (N)	0.041-1 (10)	0-1(8)

Discussion

The aim of this study was to investigate how pollination efficiency and self-pollination vary between two experimental patches, characterized by the same

population size and density, the same soil quality but showing very contrasted sex-ratios. Our experimental patches were of a moderate size, with relatively high plant and flower density, thus mimicking some of the very diverse situations that have been observed in natural populations of *Silene nutans* (Dufay, unpublished data). This experiment compared two very contrasted values of sex-ratio, with one experimental patches exhibiting a female frequency higher than values that have been observed in nature. However, natural populations of *Silene nutans* often exhibit a patchy distribution with local sex-ratios that strongly vary among patches, and some patches sometimes exhibit extremely high female frequencies (up to 100%, Dufay and Lahiani, unpublished data). According to our results, the plants located in our experimental populations experienced some pollen limitation. Pollen limitation has been documented in several species (reviewed in Knight *et al.*, 2005), including gynodioecious ones (e.g. Graff, 1999; Alonso, 2005; De Cauwer *et al.*, 2010) and it thus very likely that our experiment reflects some natural situations in *Silene nutans*, in particular for populations of the species that show small size and / or some degree of isolation (Hauser & Weidema, 2000; Van Rossum & Prentice, 2004).

Our study documents some pollen limitation on both fruit set and seed number per fruit, the first variable being likely to depend on the number of pollinator visits, while the second one may reflect the time spent on flowers by pollinators, and the quantity of pollen grains they carry. According to our results, the intensity of pollen limitation seems to vary according to the experimental population, plant gender and their interaction, as fruit set and/or seed number per fruit recorded on open-pollinated flowers significantly depended on these factors. One must note that in all cases, the average seed weight did not differ between genders nor between experimental populations, suggesting that plants experiencing the highest level of pollen limitation (i.e. females in the female-biased population, see below) did not increase the quantity of resources within seeds produced. Thus, recorded differences in terms of fruit set, seed number and ultimately in terms of pollination success, are likely to represent an actual decrease in female reproductive success.

An effect of plant gender was detected, with female plants exhibiting an overall lower seed number per fruit and a lower pollination success compared to hermaphrodites. Such result could be attributed to the smaller flower size in female individuals, which has been reported in this species (Dufay *et al.*, 2010) as in many other gynodioecious species (reviewed in Shykoff *et al.*, 2003). The effect on pollinator attraction can rely on a lower visual attractivity of small flowers and/or on the fact that female flowers may produce lower quantities of nectar or attractive volatile compounds (as found, for instance, in dioecious *Silene latifolia* : Waelti *et al.*, 2009). Pollinator preference for hermaphroditic flowers is a common result in gynodioecious species (e.g. Williams *et al.*, 2000; Asikainen & Mutikainen, 2005; Griffin & Byers, 2012) and it sometimes translates into stronger pollen limitation in female individuals (e.g. Alonso, 2005 but see Shykoff *et al.*, 2003). A difference between the two experimental populations was also detected on all pollination measurements (*i.e.* fruit set, seed number and resulting pollination success), suggesting an effect of the local sex-ratio on the intensity of pollen limitation. Such result could be explained by an overall lower attractivity of the female-biased population and/or by a lower local availability of pollen in this population. To our knowledge, no studies have been able to discriminate between these two hypotheses, except De Cauwer *et al.* (2010) that showed a stronger pollen limitation in patches containing many female plants in wind-pollinated *Beta vulgaris*. Obviously, in such species, only a variation in local pollen load can explain the sex-ratio effect on pollination efficiency.

Interestingly, a strong interaction between the population and plant gender was detected for seed number per fruit and for pollination success, suggesting that pollen limitation driven by the local sex-ratio affects the two genders differently. In other words, hermaphrodites seem to suffer less than females from the negative effect of a high local female frequency on pollination efficiency. A few other studies have reported similar results - Widen & Widen, (1990) in *Glechoma hederacea*, McCauley & Brock (1998) in *Silene vulgaris* and Zhang *et al.* (2008) in *Glechoma longituba*- and sometimes postulated that this pattern may be explained by the ability to self-pollinate, that would provide hermaphrodites with reproductive assurance in pollen-limited

situations. With the current study, we have the opportunity to directly test for this hypothesis. Indeed, we found that hermaphroditic plants were significantly more likely to self-pollinate in the female-biased population. To our knowledge, this is only the second demonstration, after Miyake & Olson (2009) in *Silene vulgaris,* for such correlation between sex-ratio and selfing rate of hermaphrodites in a gynodioecious species. Importantly, our results further show that such frequent self-pollination in the female biased population at least partly explains why hermaphrodites suffer less from pollen limitation ensuring seed production via selfing when outcross pollen is rare.

One important question however remains: is the ability to self-pollinate an overall benefit or a handicap for hermaphrodites? Indeed, even though self-pollination increases seed production and apparently provides hermaphrodites with a fitness advantage compared to females, one must remember that inbreeding depression may decrease offspring quality and lead to the opposite effect. Because the occurrence and the magnitude of the female advantage potentially depends on both effects, one cannot intuitively predict how self-pollination can influence the dynamics of gynodioecy. A previous study carrying on *S. nutans* found strong inbreeding depression with a magnitude of $\partial = 0.3$, by comparing seed weight, germination rate, and seedling growth between progenies produced by self-pollination *vs*. outcrossing (Dufay *et al.*, 2010). We used this value to quantitatively estimate the overall effect of self-pollination on the female fitness of hermaphrodites and therefore to calculate the magnitude of the female advantage as the following: $FA = \frac{number\ of\ seeds_{(F)}}{number\ of\ seeds_{(H)} \times (1 - s\partial)}$. The number of seeds produced by females (F) and hermaphrodites (H) was estimated through the pollination success measured in our experiment; regarding hermaphrodites, we took into account that a $s\partial$ portion of seeds would die due to inbreeding depression (s: selfing rate, δ: inbreeding depression). By using the parameter values estimated in each experimental population, we thus obtained two values of female advantage: 0.51 and 1.17 in the female-biased population and in the hermaphroditic-biased population, respectively. As long as one keeps in mind that our conclusions remain for this particular value of inbreeding depression, our estimation of female advantage allows us

to predict the evolutionary dynamics of gynodioecy in the two situations investigated by our experiment. First, one can conclude that in conditions mimicked by the hermaphroditic-biased population, females benefit from a female advantage > 1, which is a sufficient condition for a cytoplasmic gene of male sterility to invade (Lewis, 1941). In such case, although self-pollination may provide hermaphrodites with an advantage in terms of seed production, the overall effect of self-pollination seems to benefit females, through the impact of a strong inbreeding depression. On the contrary, in the female-biased population, pollen limitation of females is too strong and their higher offspring quality is not sufficient to provide them a fitness advantage over hermaphrodites. In this case, the ability to self-pollinate thus benefits hermaphrodites. This suggests that even in situations with some pollen limitation, females should be favoured by natural selection when they are not rare. In contrast, as soon as females occur at high frequency as a result of founder effect, genetic drift or deterministic sex-ratio oscillations, they should be counter-selected and their frequency should thus decrease, generating a negative frequency-dependent effect. Interestingly, in their theoretical study, McCauley and Taylor (1997) have found that such process could contribute to the maintenance of gynodioecy in a meta-population, when spatial distribution of the two genders is not uniform among populations.

By simultaneously measuring pollination efficiency in females and hermaphrodites and selfing rate in hermaphrodites, and by integrating all contradictory effects of selfing rate on the female reproductive success, we were able to give a quantitative estimation of the effect of self-pollination on gynodioecy dynamics, underlying that the mating system of a plant species should strongly influence the fate of male sterility mutations. Our results also directly document that the strength and the direction of effects linked with self-pollination strongly depend on local sex-ratio, which thus must be recognized as an important parameter for the evolutionary dynamics of gynodioecious species.

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Supplementary Tables

Supplementary Table 1. List of populations from which individual plants were collected

Supplementary Table 2. Primer list of microsatellites loci

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Locality/Region	Country	Number of plants used
Olloy-sur-Viroin	Belgium	136
Loffenau / Kreis Rastatt	Germany	1
Saint-Cast-le-Guildo/ Côtes d'Armor/Bretagne	France	1
Vireux / Champagne-Ardenne	France	1

Supplementary table 1. List of populations from which individual plants were collected

Moëlan-sur-mer / Finistère/ Bretagne	France	1
Devèze / Cantal / Auvergne	France	1
Savigny-les-Beaune/ Côte d'Or/ Bourgogne	France	1
Littlehampton/ West Sussex	United Kingdom	1
Swanage / Dorset	United Kingdom	1

Supplementary table 2. Primer list of microsatellites loci

Loci	Forward sequence	Reverse sequence
B09	AAGGGCACAAAATTGAGAAGG	GTGTCTTCCAAAGGTGAAGCTCATATAAACC
E08	GTTGGTCGTTGGTAGTTCACAG	GTGTCTTAATGCGAATCGGTCAATTTTAC
G01	CCCTACCTCATAGCAACAAGC	GTGTCTTCCTTCTCCTCCTTCCTTTAACC
H07	AAGCAAAACCCCTTATAAGCATC	GTGTCTTACCTTTCCCCTTCCTCCTTT
D10	CGGGCTAAGTTTACAGCATCA	GTGTCTTTGCCGTTATGCCATTCATTA

Chapitre 3

Genetic structure and reproductive success in a natural population of gynodioecious Silene nutans (Caryophyllaceae)

En préparation

Photo des plantes protégées au cours de l'étude en population naturelle à Ecault

Chapitre 3

Introduction

Gynodioecy, the co-occurrence of females and hermaphrodites, is a relatively common sexual system in flowering plants (reviewed in Webb *et al.*, 1999). Nuclear-cytoplasmic gynodioecy is the result of a complex interaction between maternally inherited male-sterility genes in the mitochondrial genome and biparentally inherited male-fertility restorers in the nucleus. The polymorphism poses the intriguing question as to the conditions under which male-sterile individuals can be maintained with hermaphrodites. Nuclear-cytoplasmic gynodioecy can theoretically be maintained through frequency-dependent selection if females benefit from a female advantage and the carriers of nuclear restorer genes pay a fitness cost relative to those that do not (Bailey *et al.*, 2003; Dufay *et al.*, 2007; Gouyon *et al.*, 1991). The female advantage could occur through the production of either more seeds (by reallocating resources from pollen to seed production; e.g. (Ashman, 2003), or of better progeny (obligatory outcrossed progeny that do not show the effects of inbreeding depression , as opposed to the progeny of partially selfing hermaphrodites; e.g. (Kohn, 1988; Kohn and Biardi, 1995; Weller and Sakai, 2005).

Female advantage could be variable between populations. For example, female and hermaphrodites respond differently to pollen limitation and the selfing rate of hermaphrodites vary both depending on local sex-ratios (Lahiani *et al.* in revision). The two sexes are thus expected to have different relative reproductive success. This could have an impact on the magnitude of female advantage. Female advantage is an important component of gynodioecy dynamics (Darwin, 1877), therefore when studying gynodioecious populations it is important to estimate pollination efficiency and the selfing rates which could have an impact on this parameter and thereafter on the dynamics of gynodioecy.

Although the effect of frequency-dependent selection on gynodioecy is clearly acknowledged, most theoretical models rely on infinite panmictic populations and do not take into account the fact that population structure could strongly modify the expected results of frequency-dependent selection (but see Couvet *et al.*, 1998; Dufay and Pannell, 2010; Pannell, 1997). Metapopulation models enable us to understand the

evolution of traits that do not experience the same selection pressures in each local population, as well as to consider the effect of different levels of selection (Olivieri *et al.*, 1990). In this regard, stochastic founder effects (Couvet *et al.*, 1985), locally resulting in increased chances of losing nuclear or cytoplasmic polymorphism (Byers *et al.*, 2005; Caruso and Case, 2007; Nilsson and Ägren, 2006; Olivieri *et al.*, 1990), are likely to affect the evolutionary dynamics of gynodioecy in a metapopulation. Dufay and Pannell (2010) have found that in structured populations the combination of the effect of selection and drift without integrating the effect of gene flow, could cause the loss of gynodioecy in conditions that would maintain it in an infinite panmictic population. However, this polymorphism could be maintained when they tested the combination of effect of selection, drift and seed and pollen flow. It is therefore crucial to estimate gene flow among and within gynodioecious populations, especially because in several natural populations, fine-scale structure has been shown or suspected (e.g. *Psychotria officinalis* in (Loiselle *et al.*, 1995); *Silene vulgaris* in (Olson *et al.*, 2006); *Beta vulgaris* in (De Cauwer a *et al.*, 2010) and in (De Cauwer b *et al.*, 2010)).

Frequency-dependent selection can produce strong oscillations of sex ratios within populations through time (Dufay *et al.*, 2009; Laporte *et al.*, 2001; McCauley *et al.*, 2000; Olson *et al.*, 2006; Olson *et al.*, 2005), generating large and small scale heterogeneity of sex-ratio among and within populations among different demes. Sex-ratio variation associated with population structure can influence the fitness of the two sexes differently, especially when integrating effects of pollen limitation and selfing rate because the reproductive success of a given plants should be affected by the availability of mates within local neighborhood (Alonso, 2005; Graff, 1999; McCauley *et al.*, 2000; Oddou-Muratorio *et al.*, 2006; Olson *et al.*, 2005). For example, pollen limitation could increase when females are aggregated together, (Graff, 1999; Lahiani *et al.*, in revision; Olson *et al.*, 2006) or selfing rates of hermaphrodites could increase with decreased hermaphrodite frequency (Lahiani *et al.*, in revision; Miyake, Olson, 2009). This could alter reproductive success of the different sexual phenotypes and probably the maintenance of gynodioecy (McCauley, 1998; McCauley and Taylor, 1997; Pannell, 1997). Therefore, it is important to estimate the efficiency of pollination and the selfing

rate in natural population and to study the effect of sex-ratio variation in structured population on such parameters.

Our study focuses on the gynodioecious, entomophilous and self-compatible *Silene nutans* (Caryophyllaceae). We aimed at evaluating the sex structure and assessing the genetic structure of a natural population at nuclear and cytoplasmic loci in order to estimate gene flow at a fine scale level, evaluate the level of selfing among focal plants as well as their reproductive success.

Materials and Methods

Study species

Silene nutans (Caryophyllaceae) is a diploid, long-lived perennial rosette plant growing in dry, open grass communities of hillsides. It is described as gynomonoecious-gynodioecious, with female, gynomonoecious (plants bearing both perfect and pistillate flowers), and hermaphroditic individuals found in natural populations (Jürgens *et al.*, 2002). It shows a wide continental distribution range, extending from north-western Europe to central Siberia and South-Caucasus (Van Rossum *et al.*, 1996; Van Rossum *et al.*, 1999; Van Rossum, Prentice, 2004). At its western border (Great Britain, NW France, Belgium and the Netherlands), *S. nutans* is locally rare, showing a patchy distribution with scattered, often small, populations (Hepper, 1956; Van Rossum *et al.*, 2003). Flowers are visited by a number of different insect species, including Noctuidae, Sphingidae, Hymenoptera and nectar robbing Hymenoptera (Jürgens *et al.*, 1996). Perfect flowers are protandrous, but self-pollination can probably occur by geitonogamy, since plants often carry several open flowers simultaneously. Gene dispersal could occur through seeds and/or pollen movement or by vegetative reproduction (vegetative cloning). Seeds are dispersed from an aperture at the top of the capsule by vibrations of the flower stalk (Hauser, Weidema, 2000).

Study area and sampling procedure

In 2012, we studied a population of *S. nutans* located on sand costal dunes in Ecault (North of France) (50° 40′ 00″ North; 1° 36′ 15″ East). We conducted an

exhaustive sampling of individuals in this population extended in over approximately 20 m, with a total of 144 individuals. We recorded the global positioning system coordinates of all ramets using a GARMIN GPS map60CS (accuracy of 2–5 m), which enabled us to map the location of all sampled individual within geographical patches. Sampled individuals were grouped in five geographically patches (Ec_1, Ec_2, Ec_3, Ec_4 and Ec_5, Fig. 1). We noted the sexual phenotype for all flowering individuals (female, hermaphroditic or gynomonoecious) and we collected leaves for all sampled individuals. We also noted the individuals that were infested by the fungus *Microbotryum violaceum,* causing anther smut in several species of the Caryophyllaceae family (Giraud *et al.*, 2008).

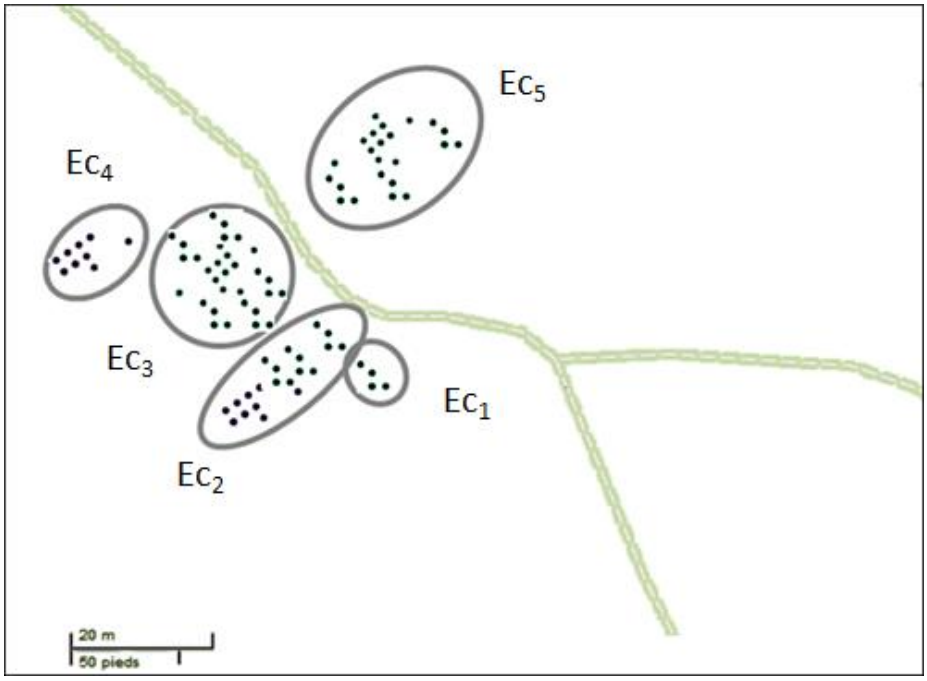

Figure 1. Spatial distribution of *Silene nutans* individuals at study site Ecault (North of France). The locations of the geographical patches (Ec1, Ec2, Ec3, Ec4 and Ec5) are circled in grey, and individuals are represented by black dots. Continuous line between the patch Ec5 and the others others is a path dunes

Nuclear and cytoplasmic markers

All sampled individuals were genotyped thanks to eight nuclear microsatellite loci (for the first four loci -*B09, E08, G01, H07*- see (Lahiani *et al.*, in revision) and for the four other loci -*Sil19, Sil24, Sil35, Sil37*- see Supplementary Table 1). DNA was extracted from 10-15 mg of leaf tissue using MACHEREY-NAGEL NucleoSpin® 96 Plant II Kits. Amplification reactions were carried out by polymerase chain reaction (PCR) with 20 ng of DNA in 10µl reaction volume containing 5µl of Qiagen multiplex Kit 2x, 1µl of primer mix (10x) (0.75µM of Scored forward primer and 3.75µM of reverse primer) and 1µl of sterile water. We used the same cycling conditions as in described in Lahiani *et al.* (in revision) for the first four nuclear microsatellite loci. For the other ones, cycling conditions for PCR amplification were: 95°C for 15 min, 32 cycles of 45s at 94°C, 1 min at 55°C, 1.15 min at 72°C and finally 60°C for 30 minutes. Amplification products were separated on Applied Biosystems 3130 capillary sequencer. Raw data were analyzed using GENEMAPPER version 3.5 (Applied Biosystems). Individuals with doubtful or missing peaks were genotyped a second time.

To investigate the cytoplasmic diversity, SNPs were developed from sequenced haplotypes of *Silene nutans* (Lahiani *et al.*, 2013). Three nucleotides of the mitochondrial gene *cob*: *cob 223*, *cob 444*, *cob 735* and 6 nucleotides of the chloroplastic genome corresponding to two chloroplastic fragments, the intergenic spacer sequences *psbA-trnH: Cp 42 and* the fragment of the gene *matK*: *Cp 397, Cp 540, Cp 656, Cp 730, Cp 804* were selected for the presence of neutral Single Nucleotide Polymorphism (SNP). The selected set of 9 SNPs was used for developing KASPar assay . The detection of SNP is based on "allele specific" PCR, using the KASPar ® method. Amplification reactions were carried out by polymerase chain reaction (PCR) with 30 ng of DNA in 8.11µl reaction volume containing 4 µl of KASPAR (2x) reaction mix and 0.11 µl of assay (mix of three primer). Cycling conditions for PCR amplification were 94°C for 15min, ten cycles of 20s at 94°C , 60s at 65°C with a step-down of 0.8°C per cycle, twenty six cycles of 20s at 94°C , 60s at 57°C, and finally 12°C for 10 minutes. The fluorescence was detected by Roche LightCycler ® 480. Individual haplotypes were defined as a combination of allelic states for all these SNPs.

Estimation of nuclear genetic diversity

Within each distinct geographical patch, we estimated standard genetic diversity parameters : the number of alleles of each locus (N_A), the expected heterozygosities (H_e) using FSTAT version 2.9.3 (Goudet, 1995). To evaluate the extent of differences within studied population, the fixation index (F_{IS}) was calculated (Weir, Cockerham, 1984). The significance levels were adjusted for multiple tests using the sequential Bonferroni correction (Rice, 1989). By comparing the F_{IS} values obtained with the distribution within for a randomized data set obtained after 10,000 permutations of alleles among individuals geographical patches, we then tested within each geographical patch the departure from Hardy–Weinberg equilibrium. Then, we checked for all loci, as well as within each geographical patch, for linkage disequilibrium using implementation in FSTAT. The significance levels were adjusted for multiple tests using the sequential Bonferroni correction (Rice, 1989).

Traditional test for population differentiation were performed by calculating (F_{ST}) based on allelic identity estimated among geographical patches with 10 000 permutations of individuals between patches, using a G test for significance of results (Goudet *et al.*, 1996). The significance levels were adjusted for multiple tests using the sequential Bonferroni correction (Rice, 1989).

Bayesian investigation of the observed spatial population structure

The patterns of the population structure were further investigated using the model-based Bayesian clustering procedure in STRUCTURE version 2.3.2 (Pritchard *et al.*, 2000), which assigns individuals to *K* subpopulations based on their Multilocus genotype with no prior information on the geographical location in which the individuals were sampled. Note that the analysis should be interpreted cautiously since the studied species self-pollinates and this could have an impact on the assignation procedure. STRUCTURE was run for K = 1–15; in each run we assume that geographical patches admixture and correlation of allele frequencies (Falush *et al.*, 2003) and included 1 000 000 iterations after a burn-in period of 10 000 iterations. All

runs were repeated 5 times for each K, in order to check for the convergence of the Markov chain Monte Carlo (MCMC). Finally, the most probable number of clusters (*K*) was determined using the *ad hoc* statistic Δ*K*, based on the rate of change in the log probability of data between successive *K* values, as described in Evanno *et al.* (2005). We used STRUCTURE HARVESTER website and program for visualizing STRUCTURE output and implementing the Evanno method (Earl, Vonholdt, 2012).

Pollination efficiency and selfing rate

Five patches (Ec_1, Ec_2, Ec_3, Ec_4 and Ec_5) containing respectively 4, 41, 59, 14 and 26 individuals were studied. We selected a sample of individuals for a more detailed survey, choosing flowering, non-infested individuals and avoiding as much as possible gynomonoecious, which can vary in proportion of female flowers through time (Garraud *et al.*, 2011). A total of 34 plants in the five patches were thus marked and protected from rabbits attacks by fence cages in June 2012 (N = 3 plants in Ec_1, N = 8 plants in Ec_2, N = 11 plants in Ec_3, N = 6 plants in Ec_4, N = 6 plants in Ec_5, Table 1). All plants surveyed in Ec_2, Ec_3, Ec_4 and Ec_5 patches were hermaphroditic and plants in Ec_1 were all female plants.

We surveyed marked individuals throughout two flowering periods during the flowering episode of the studied year (from 21st to 28th June 2012 and from 28th to 5th July 2012) and marked three receptive flowers on each plant at the beginning of both periods. In the beginning of each flowering period, we counted the number of open flowers on the surveyed plants as well as the total number of flowers within each patch, by counting female and perfect flowers separately. To prevent seed loss, marked fruits were covered by mesh bags before they opened. On July 18th 2012, we calculated fruit set as the number of fruits at the end of the experiment for all flowers produced by the whole plants, relative to the number of flowers produced by each surveyed plant during both flowering periods. Then, marked fruits were collected and seed set of each flowering period was calculated as the number of seeds relative to the number of ovules produced per marked fruit.

Selfing rate was estimated for each surveyed hermaphroditic plant from patches Ec_2, Ec_3, Ec_4 and Ec_5 (Ec_1 patch contained only female plants). Seeds were sown in a germination soil mix, and placed at 20°C with daily moistening. Ten weeks later, plantlets were collected in order to extract their DNA using MACHEREY-NAGEL NucleoSpin® 96 Plant II Kits. 979 plantlets from 21 hermaphroditic families (on average 34 ± 15 plantlets per family) were genotyped by the eight scored microsatellites loci described previously (we did not obtain seedlings from all followed hermaphrodites as sometimes they did not set fruits or because of seedling mortality). Outcrossing rates were determined using Ritland multilocus maximum likelihood estimation program (MLTR Version 3.4, accessible at http://genetics.forestry.ubc.ca/ritland/programs.html) (Ritland, 2002). Standard deviations were determined based on 1000 bootstrap analyses. Two kinds of analyses were performed: population estimates allowed us to calculate overall multilocus (t_m) and mean single locus (t_s) outcrossing rates, with bootstraps using families as units of observation. Family estimates gave outcrossing rates per family, bootstraps using individual offspring as units of observation. We considered selfing rates as $s = 1- t_m$. Different analyses were run, one for each flowering period.

We analyzed fruit set as a function of the patch by using a logistic regression (binomial distribution, log link function, proc GENMOD, SAS), correcting for overdispersion (dscale option, proc GENMOD, SAS). The seed set of each flowering period (SS_1 and SS_2) was analyzed as a function of the patch and the number of flowers produced by each surveyed plant for each flowering period, also by using a logistic regression (binomial distribution, log link function, proc GENMOD, SAS), correcting for overdispersion (dscale option, proc GENMOD, SAS). Then, we performed paired *t-tests* to compare the difference of seed set between the two flowering periods. Variation in individual selfing rates (estimated by MLTR program) among patches and between flowering periods were analyzed using a logistic regression (binomial distribution, log link function, proc GENMOD, SAS), correcting for overdispersion (dscale option, proc GENMOD, SAS). All statistical analyses performed were conducted using SAS (SAS version 9.1.3, 2002).

We then calculated a reproductive success (RS) value for each surveyed plant at each flowering period as the following: RS = FN * FS * SS * [(1-s) + $s\,\partial$], where FN was the flower number, FS was the fruit set, SS was the seed set, s was the selfing rate (estimated by MLTR program) and $s\partial$ the portion of seeds that would die due to inbreeding depression, with $\text{inbreeding depression}\ \partial = 0.3$ *as* estimated in *S. nutans* by (Dufay *et al.*, 2010).

Paternity analysis

Paternity analysis was carried out for 1200 seedlings (using seedling produced by female and hermaphroditic plants) using CERVUS 3.0.3 (Kalinowski *et al.*, 2007; Marshall *et al.*, 1998). CERVUS uses a likelihood-based approach and assigns paternity according to the highest logarithm of the likelihood (LOD score). LOD scores are calculated by determining the likelihood of assignment of a parent relative to the likelihood of arbitrary parents. We applied the following simulation parameters to find the confidence level of paternity analysis assignment: 10,000 simulated mating events; 137 candidate father plants; five as the minimum number of loci; 0.003 as genotyping error rate (estimated as the proportion of error by dividing the number of mismatch between mothers and offspring by the total number of comparison between mothers and offspring for the eight nuclear loci we assumed that plants could self-pollinate thus all adult plants were treated as candidate father plants. In the paternity analysis, we used 95% as strict and 80% as relaxed confidence levels as recommended by (Marshall *et al.*1998). Thus for all offsprings we could have three possible alternative outcomes: (i) paternity was significantly attributed to another sampled adult or to the mother, (ii) paternity could not be significantly assigned to one of the sampled adults, it is the case 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 could not be attributed because the male parent was outside the study area.

Results

Sex ratio variation, plants and flowers density

Individuals were clustered in five geographical patches called Ec_1, Ec_2, Ec_3, Ec_4 and Ec_5 patches (mean number of individuals was 29, ranging from N_{MIN} = 4 to N_{Max} = 59, see Table 1 and Fig.1). Female plants were observed in only one patch (patch Ec_1 that only contained female individuals). Three patches contained a mixture of gynomonoecious and hermaphroditic plants (patch Ec_2, Ec_3 and Ec_5) and one patch contained only hermaphroditic plants (patch Ec_4) (Table 1, Fig. 1).

The number of flowers produced in each patch during the two flowering periods was variable among patches (First flowering period: from 216 to 1143, mean number = 692, second flowering period: from 213 to 3572, mean number = 1301, Table 2). In both flowering periods the number of female flowers in Ec_2, Ec_3 and Ec_4 patches (Ec_5 did not contain female flower) was very small, and the ratio of female flower was overall very low (Table 2). The frequency of plants infested by the *Microbotryum violaceum* was high essentially in the patch Ec_3 (56% of flowering plants were infested in this patch) and was null in the patch Ec_1 (Table 1).

Table1. Number of plants and flowering plants of each plant gender per patch

Patch	Total Number of plants	Number of surveyed plants	Number of flowering plants of each gender				Number of infested plants
			H plants*	F plants*	GM plants*	NF plants*	
Ec_1	4	3	0	3	0	1	0
Ec_2	41	8	32	0	2	7	16
Ec_3	59	11	48	0	9	2	33
Ec_4	14	6	10	0	0	4	2
Ec_5	26	6	20	0	4	2	6

* H plants: hermaphroditic plants; F plants: female plants; GM plants: gynomonoecious plants; NF plants: non flowering plants

Table2. Number of female and perfect flowers produced in each patch for during both flowering period

* FF: Female Flowers; PF: Perfect Flowers

Genetic diversity and patterns of genetic differentiation on nuclear data

The obtained results of genetic diversity of nuclear microsatellite loci, showed that they exhibited moderate to high levels of polymorphism, with a number of alleles ranging from 2 (*H07* and *Sil19*) to 20 (*Sil24*). Overall, the population fixation index (F_{IS}) was -0.013 ; The table 3 summarizes the value obtained of the number of sampled alleles (N_A), expected heterozygosity (H_E) and estimated intra-population fixation index (F_{IS}) for each geographical patch and for all the population.

Table3. Measures of genetic diversity on nuclear data within the geographical patches

Patch	Number of plants	Number of flowers produced in the first flowering period		Number of flowers produced in the second flowering period	
		FF*	PF*	FF*	PF*
Ec_1	4	216	0	213	0
Ec_2	41	3	1140	7	1777
Ec_3	59	0	1135	77	3495
Ec_4	14	0	102	0	408
Ec_5	26	8	852	17	503

of the study population of *Silene nutans*

Multilocus estimates of nuclear diversity	Ec_1	Ec_2	Ec_3	Ec_4	Ec_5	All
Mean number of allelles per locus N_A	2.375	5.37	5.12	3.87	4	6.5
Expected heterozygosity, H_E	0.56	0.49	0.50	0.54	0.46	0.497
Fixation index, F_{IS}	0.326*	0.033*	-0.034*	-0.067*	-0.066*	-0.013

* Not significant

Nuclear microsatellites did not show any deficit of heterozygotes compared to Hardy–Weinberg expectations in any patches (Pairwise F_{st} among patches ranged from 0.038 to 0.172 for nuclear loci and were all significant except one between Ec_1 and E_{c2} (Table 4), overall the F_{st} of the population was 0.069).

Table4. Genetic differentiation (F_{ST}) estimated for all pairs of geographical patches of *Silene nutans*, using nuclear data

	Ec2	Ec3	Ec4	Ec5
Ec1	0.0387^{NS}	0.0788**	0.1729***	0.1374***
Ec2		0.0223***	0.0478**	0.0965***
Ec3			0.0579**	0.1083***
Ec4				0.1349***

*P<0.05, **P<0.01, ***P<0.001

Bayesian analysis of population structure

We obtained two genotypic clusters in the studied population by the Bayesian analysis (Fig.2 and Fig.3). Patches Ec_1 to Ec_4 corresponded to a mix of both clusters while Ec_5, the most remote patch, was constituted by only one cluster.

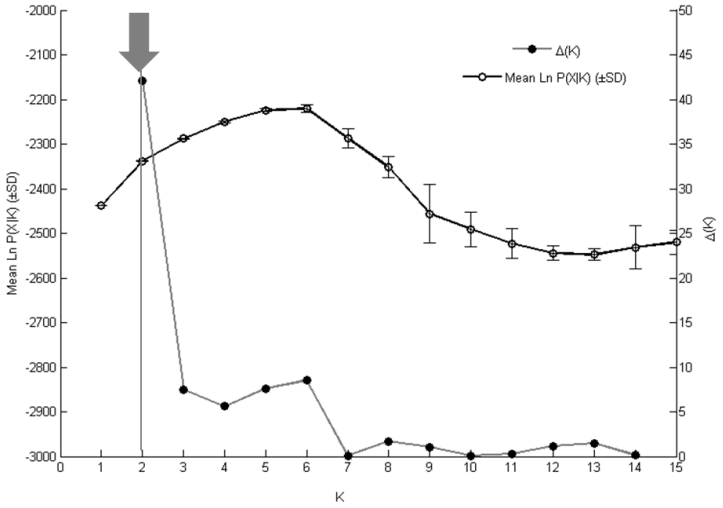

Figure 2. Estimated number of genotypic group in the studied population by the Bayesian analysis for *K*(ranging from 1 to 15) clusters with mean(±SD) of the ln of probabilities of data over 5 replicated runs and standardized and in the second order the rate of change in the ln probability of data between successive *K* values, *ΔK*

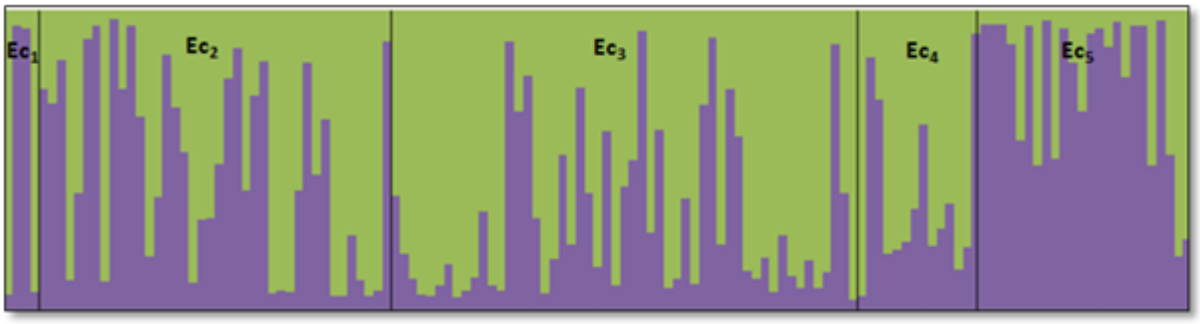

Figure 3. Inferred population structure obtained using STRUCTURE software, for the modal *K* values (*K* = 2. Each vertical line represents one individual multilocus genotype. Genetic clusters are represented by different colors, with cluster 1 in green and cluster 2 in purple. Geographical patches are separated by a vertical black line

Genetic Cytoplasmic diversity

In contrast to nuclear loci, mitochondrial and chloroplastic SNPs were not polymorphic among studied individuals and yielded only one haplotype for all individuals of the population (Table 5).

Table5. Cytoplasmic haplotype obtained

SNPs	*cob 223*	*cob 444*	*cob 735*	*Cp 42*	*Cp 397*	*Cp 540*	*Cp 656*	*Cp 730*	*Cp 804*
Alleles	C/A	C/T	G/T	T/G	C/A	C/T	T/G	C/T	T/G
Ecault_haplotype	A	T	T	T	C	C	T	C	G

Pollination efficiency and selfing rate

Overall, fruit set varied from 0.02 to 0.44 among surveyed individuals. Average fruit set at the patch level varied from 0.25 to 0.34 with no significant difference among patches ($\chi^2_{4,29}$ = 5.89, P = 0.2) (Fig. 4). In particular, the only patch containing female plants did not differ from the others, suggesting no effect of plant gender in our dataset.

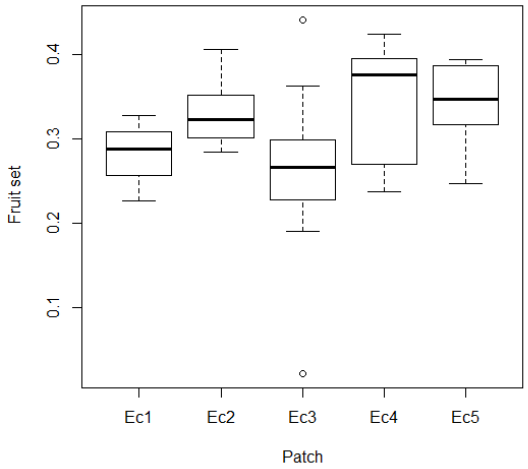

Figure 4. Distribution of fruit set of surveyed plants in each patch

Because not all marked flowers set fruits, seed set was obtained on only 20 surveyed plants during the first flowering period and on 26 followed plants during the second flowering period. Seed set of the first (SS_1) and the second flowering period (SS_2) varied from 0 to 0.88 and from 0.13 to 0.9 respectively (Fig.5) and did not vary significantly among patches (SS_1: $\chi^2_{4,15}$ = 0.56, P = 0.96; SS_2: $\chi^2_{4,21}$ = 0.05, P = 0.99).

Nor did vary with the number of flowers produced by the surveyed plants (SS_1: $\chi^2_{14,5}$ = 6.12, P = 0.96; SS_2: $\chi^2_{24,1}$ = 7.28, P = 0.99).

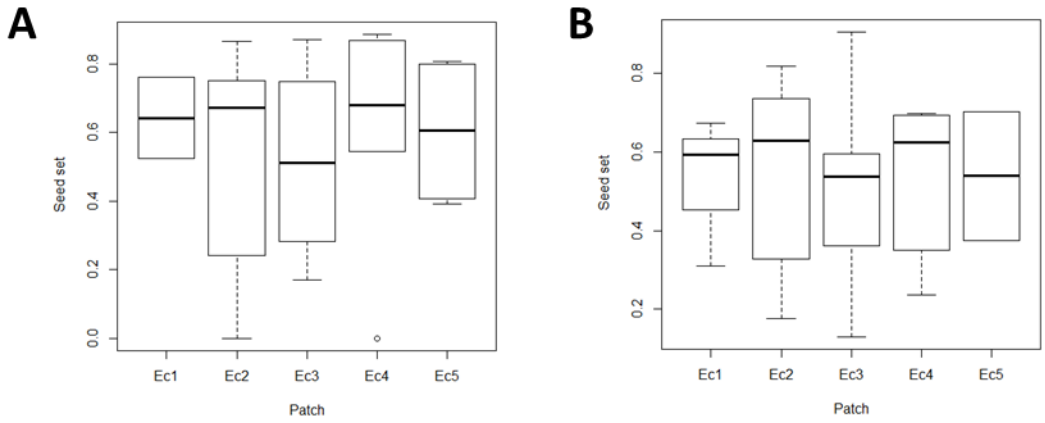

Figure 5. Distribution of seed set of surveyed plants in each patch for each flowering period. A: first flowering period; B: second flowering period

Selfing rate was estimated for 327 seedlings from 12 hermaphroditic families for the first flowering period and for 652 seedlings from 17 hermaphroditic families for the second one. Selfing rate varied from 0 to 1 according to the family (Fig. 6). The selfing rates did not differ significantly among patches (first flowering period: $\chi^2_{8,3}$ = 1.86, P = 0.6; second flowering period: $\chi^2_{13,3}$ = 2.27, P = 0.51) between flowering periods (mean s ± SD = 0.395 ± 0.118 and mean s ± SD = 0.395 ± 0.118 in the first and the second flowering periods respectively ; $\chi^2_{27,1.}$ = 0.8, P = 0.3). Also, for neither flowering period was selfing rate correlated with the number of flowers carried by the plant, with the proportion of flowers of the patch that were carried by the focal plant (P > 0.05 for all analyses).

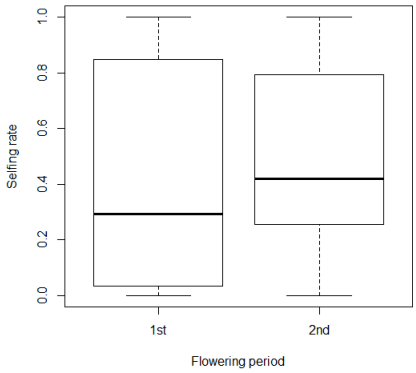

Figure 6. Distribution of selfing rate of hermaphroditic plants for each flowering period

Reproductive success (RS) ranged from 0.36 to 24.7. Although values obtained for female plants were in the upper half of the distribution, we found no clear signal of a strong fitness advantage of females compared to hermaphrodites (Fig. 7).

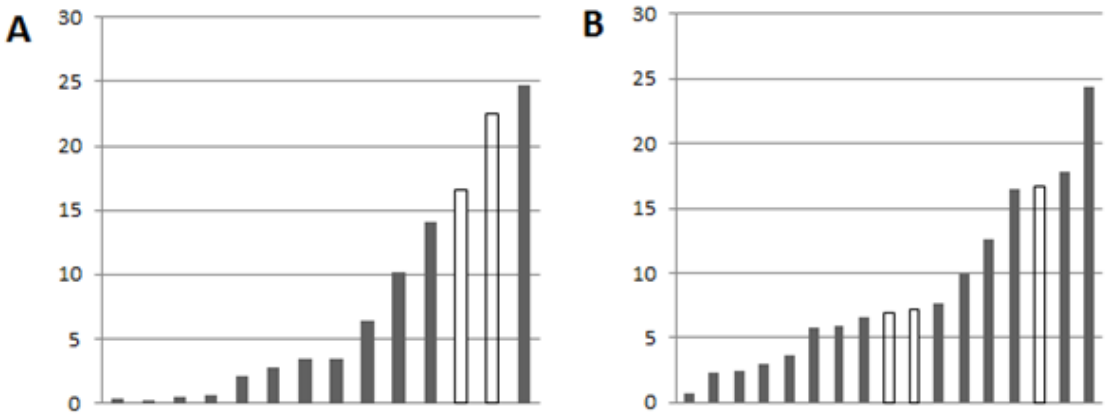

Figure 7. Distributions of reproductive success amplitude of surveyed individuals in both flowering periods. A: first flowering period; B: second flowering period. Grey bars: hermaphroditic individuals; White bars: female individuals

Contemporary pollen flow

From the 1200 seedling treated for the paternity analysis, 310 seedlings had only one compatible father (confidence level of 80% or more). For the other 890 seedlings

(74.16 %), it was not possible to assign the most likely father, because two hermaphrodites showed equal probability to be assigned as the father. For this reason we could not estimate the selfing rate based on the assignment analysis as we did not have information for all individuals and thus our estimation could be biased given the fact that the selfing rate could be highly variable in *S. nutans* (Lahiani *et al.*, in revision).

Among the 310 seedlings for which a paternity assignment could be performed, we found that a majority of seedlings produced in the patches Ec_2, Ec_3 and Ec_4 were sired by a father located in the same patch (Table 6). In the patch Ec_5, the assigned fathers belonged equally to the same patch or other patches and in the patch Ec_1, all fathers were assigned with high probability from other patches, as expected since all plants from this patch were females (Table 6).

Table6. The number (percentage) of the potential fathers from each patch for seedlings in each given patch

Mother patch\ Father patch	Ec_1	Ec_2	Ec_3	Ec_4	Ec_5	Selfed progenies	Outcrossed progenies
Ec_1	0	4(10.5%)	16(42.1%)	1(2.6%)	17(44.8%)	0	38
Ec_2	0	67(80.72%)	24(28.91%)	1(1.2%)	1(1.2%)	28	65
Ec_3	0	14(20.58%)	48(70.58%)	2(2.94%)	4(5.88%)	22	46
Ec_4	0	1(1.06%)	16(17.02%)	72(76.6%)	5(5.31%)	61	33
Ec_5	0	5(29.41%)	5(29.41%)	0	7(41.17%)	0	17

* Selfed progenies: number of seedling produced by self-pollination among those assigned to a potential father in each patch; outcrossed progenies: number of seedling produced by outcross-pollination among those assigned to a potential father in each patch

Discussion

In this study we aimed to survey a natural population of gynodioecious *Silene nutans*, in order to evaluate the structure in terms of nuclear and cytoplasmic genetic diversity, and in terms of sex phenotype distribution. It also aimed to evaluate pollination efficiency, selfing rate and the reproductive success of each sexual phenotype, in order to gain some insights into the dynamics of gynodioecy in that species. It must be kept in mind that the studied population is found at the western margin of its distribution, when populations are rare and isolated. This could explain the overall low nuclear diversity found in the population.

Sex ratio variation

Local-scale sex ratio structure is a relatively common feature in gynodioecious species (e.g. De Cauwer *et al.*, 2012; Olson *et al.*, 2006). In our study, overall sex ratio was low but the very few female plants were all clustered together. Low female frequency seems to be common in *S. nutans* although high frequencies were found in some natural populations (up to 0.6, but with more than half of investigated populations containing less than 0.1 of female plants, Dufay, unpublished data). This apparently contrasts with gynodioecious *Silene vulgaris*, which exhibits variable but on average, larger female frequencies in natural populations which could reach 75% of female plants (McCauley *et al.*, 2000).

Population genetic structure and gene flow

In natural populations, the density of plants and the abilities of dispersal though seeds or/ and pollen and the mating systems may have an impact in the gene flow, selection and genetic drift in natural populations. The combination of such factors could shape the local structure of plant population (Loveless and Hamrick, 1984). Since population structure could influence the reproductive success of different sexual phenotype in gynodioecious populations by privileging mating with neighbors, it is important to evaluate the occurrence and the magnitude of fine scale spatial structure in such populations (De Cauwer *et al.*, 2012).

In our study population, nuclear markers showed a moderate but significant genetic differentiation level among geographical patches (as measured by pairwise F_{ST} estimates). The levels of nuclear genetic differentiation obtained in our study on a small scale were smaller than the ones measured at a much larger geographical scale in the same species (study of 67 populations in Europe F_{ST} = 0.27, (Delalande *et al.*, 2012). The results obtained by the Bayesian analysis of population structure suggest that our population is not probably structured in five genetic patches; this could be due to insufficient polymorphic nuclear microsatellites loci essentially for low number of markers, - or to a non-negligible gene flow detected between patches. The results of paternity analysis all showed that the pollen dispersal events were geographically restricted and mainly occurred intra-patch, although the distances between neighboring patches did not exceed three meters. In a study conducted on gynodioecious and entomophilous *Silene vulgaris,* (Olson and McCauley) (2002) found the same tendency of limited gene flow where most seeds disperse close to parents within populations but still having some long distances disperse at random with respect to distance from the source. The tendency of gene flow to be restricted in our study could be explained by foraging behavior of some pollinators that tend to forage primarily within rather than between flower patches (Altizer *et al.*, 1998), to spend more time foraging in the same flowers patches (Waddington, 1983) and have short distance movement (Faheem *et al.*, 2004). Such patterns could also generate high levels of selfing rates.

Reproductive success of plants

Another study conducted in semi-controlled conditions on the same species showed that both local sex ratio and plant gender strongly affected pollination efficiency (Lahiani *et al.*, in revision). However, because our study population did not exhibit any gradient in female frequencies among patches, we could not test for such an effect in natural conditions. The fruit set measured in our studied population was low compared to the results obtained by the study cited above: the obtained fruit set indeed represented half of the fruit set measured in the experimental patch associated with a moderate pollen limitation and the two-third of the fruit set measured in another patch associated with strong pollen limitation. This may suggest that plants of the studied population

suffered from very strong pollen limitation. This could also be due to the high level of infestation caused by the fungus *Microbotryum violaceum*, resulting in lower pollen load. Fruit set and seed set were highly variable among surveyed plants during both flowering periods, but we found no effect of local plant density nor of plant gender. Although our genetic results strongly suggest that pollinator behavior primarily generates pollen flow within patches, the fact that these patches are extremely close from each other may have buffered the effect of patch characteristics on the attraction of the (rare) pollinators.

Selfing rate should impact the occurrence and the magnitude of female advantage, an important condition of gynodioecy maintenance for both nuclear and nuclear-cytoplasmic gynodioecy and for this reason, needs to be estimated in self-compatible gynodioecious species. In our study, selfing rates were extremely variable between plants and flowering periods, which is consistent with the strong inter-individual variation documented by (Lahiani *et al.*) (in revision) in experimental conditions. In the current study, selfing rates did not seem to increase with the number of flowers of given plant, nor with the ratio of number of flowers within a patch that belong to the focal individual. Thus, although the selfing rate is known to vary according to local conditions (e.g. local sex ratio in (Lahiani *et al.*, in revision)), our study suggests that it could not be easily predicted in natural populations, and should be considered as highly variable at least in the conditions experimented by the surveyed population.

Values of individual reproductive success based on flower production, pollination efficiency and selfing rate showed strong variation among surveyed plants. Although female plants showed relatively high values of reproductive success, they did not seem to benefit from a clear female advantage. Lahiani *et al.* (in revision) showed that females could lose their fitness advantage in pollen-limited conditions, which could actually explain the results obtained in this particular study population. Others studies, focusing on different ecological conditions, are thus clearly needed to investigate this question.

A case of nuclear gynodioecy at the population level?

Surprisingly, in our studied population, all individuals (including female plants) carried the same CMS cytoplasm, most likely identical to the AMB cytoplasm described by Garraud *et al.* (2011) found in another population from North of France (Ambleteuse) meaning that only variation in their nuclear genotypes could explain the difference of phenotypes between females and hermaphrodites. This corresponds to a case of nuclear gynodioecy. By screening a small sample of individuals in 67 European populations, Delalande *et al.* (2012) also documented an absence of cytoplasmic diversity in most of these populations. Our study thus confirms that, although being nuclear-cytoplasmic at the species level (Garraud *et al.*, 2011), gynodioecy can be nuclear at a local scale. Theoretically, a transition from nuclear-cytoplasmic to nuclear gynodioecy can occur either because of a very large female advantage (>2) that leads to a fast increase of CMS frequency until fixation before the nuclear restorer is sufficiently common to stop it, or because of random processes (Dufay and Pannell, 2010). In our studied population, female plants do not seem to benefit from a large female advantage, which suggests that either female advantage used to be high but then decreased in magnitude, possibly due to strong pollen limitation or that the loss of cytoplasmic diversity occurred through founder effect or drift. In both cases, females should decrease in frequency and gynodioecy could ultimately be lost from the population.

Supplementary Tables

Supplementary Table 1. Primer list of microsatellites loci

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Supplementary table 1. Primer list of microsatellites loci

Loci	Forward sequence	Reverse sequence
Sil19	TTCTGAGAATTTGCACTTGAATC	ACAAGTAACAATCTTATCCTCCATACT
Sil24	AATGGGTGTTGGAGAGGGA	AAAGAACGGGAAGAAGGAGG
Sil35	TCTGTGAATCTGTGATACTAACTGC	ACCTCTATCCCACCATGTCA
Sil37	AAAGATGATTCATGTCAGGCG	TGATGTTGGCCTGTACATTTC

Chapitre 4

Do diurnal vs. nocturnal pollinators differ in their impact on pollination efficiency and self-pollination in Silene nutans (Caryophyllaceae)?

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Soumis à Plant biology

Photo d'une fleur couverte le jour à gauche et d'une fleur couverte la nuit à droite

ABSTRACT

Pollination syndromes open the question of the impact of different pollinator communities on plant pollination success. Gynodioecious *Silene nutans* exhibits floral traits associated with a nocturnal pollination syndrome, but because flowers remain open during the day, it may be pollinated by diurnal pollinators also. We compared pollination efficiency between diurnal and nocturnal pollination in an experimental population, by measuring fruit set and seed production. We also compared the selfing rate, which should have a strong impact on the reproductive success of the plants, although such issue has never been investigated so far. Flowers exposed to nocturnal pollinators produced significantly more fruits and seeds than that those exposed to diurnal pollinators. However diurnal pollinators still contribute to 20 % of the overall seed production ensured by the plants. This suggests that *S. nutans* may have mixed pollination strategy. Additionally, selfing rate exhibited by day-pollinated flowers tended to be larger (although non significant). Because seedlings produced through self-pollination often suffer from inbreeding depression, we suggest that such difference between the two pollinator communities should be taken into account by further studies.

Running head: Nocturnal *vs* diurnal pollination in *Silene nutans*

Keywords : pollination; selfing-rate; *Silene nutans*; nocturnal syndrome

INTRODUCTION

Animal pollination is a critical ecosystem service, as the majority of angiosperms rely on pollinating insects for their sexual reproduction (Albrecht et al., 2012; Kearns et al., 1998; Ollerton et al., 2011). Although some entomophilous plant species have evolved highly specialized flowers and are pollinated by a single specialist visitor, generalist pollination systems are more common and widely distributed than pairwise species interactions (Fenster et al., 2004; Freitas and Sazima, 2006; Johnson and Steiner, 2000; Waser et al., 1996). Because floral visitors of a given species potentially vary in their behavior, their morphology and their local frequency, they usually also vary in their contribution to seed production through their pollinating activity (Ortega-Baes et al., 2011; Young et al., 2007). It is usually thought that such variation in pollination efficiency among floral visitors has led to the selection of floral traits that maximize the interaction with the most efficient pollinators, constituting the principal source in the evolution of angiosperm flower (reviewed in Stebbins, 1970). The suite of floral traits, including floral rewards, exhibited by a plant species, and associated with the attraction and the utilization of a specific group of pollinators defines its pollination syndrome (Fenster et al., 2004).

One commonly studied pollination syndrome is linked with the phenology of the pollinators, and opposes nocturnal *vs*. diurnal pollination (Fenster et al., 2004). Although some plant species are susceptible to pollinator activity during both day and night, especially those with flowers that remain opened and functional for a long period of time (Amorim et al., 2012), many species have developed specialized syndromes of nocturnal or diurnal pollination, such as the emission of particular volatile compounds, specific temporal dynamics of scent release or nectar production, floral shape, floral color and individual flower phenology (Jürgens et al., 2002; Ortega-Baes et al., 2011). Many *Silene* species (Caryophyllaceae) exhibit a nocturnal pollination syndrome, with pale flowers that open at dusk and emit an intense scent at night, which suggests some degree of specialization (Gimenez-Benavides et al., 2007; Kephart et al., 2006). However, Jürgens et al., (1996) found that several species from this genera were visited

by both nocturnal (mainly Noctuidae and Sphingidae) and diurnal insects (Apidae and Syrphidae), which opens the question of the consistency between the described pollination syndrome and the effective night *vs*. day pollination. Consistently, several studies have compared pollination efficiency between diurnal and nocturnal visitors in several *Silene* species (Barthelmess et al., 2006; Gimenez-Benavides et al., 2007; Young, 2002) and species from other genera (Amorim et al., 2012; McMullen, 2011; Ortega-Baes et al., 2011; Ortiz et al., 2000). Additionally, because nocturnal and diurnal insects potentially vary in their behavior (Faheem et al., 2004), these two pollinator communities may possibly lead to different rates of self-pollination. In case of inbreeding depression, differences in selfing rate appear crucial for estimating the reproductive success of the plants. However, to our knowledge, no study has investigated such difference between diurnal and nocturnal pollination.

In this study, we focused on *Silene nutans*, a gynodioecious species (co-occurrence of females and hermaphrodites), which exhibits a nocturnal pollination syndrome but is visited by both nocturnal and diurnal pollinators (Jürgens et al., 2002). Our objective was to test whether *S. nutans* obtains comparatively a higher reproductive success by nocturnal pollinators compared to diurnal pollinators, as we may expect based on its flowering syndrome. Therefore, we compared (1) fruit set and seed production between flowers pollinated by diurnal *vs*. nocturnal pollinators and (2) selfing rates generated by diurnal and nocturnal pollinators. Both pollination efficiency and selfing rates are important parameters, since they both influence the evolutionary dynamics of gynodioecy as they should affect the difference in seed production between females and hermaphrodites (Dornier and Dufay, in press).

MATERIAL AND METHODS

Study species

Silene nutans (Caryophyllaceae) is a diploid, long-lived perennial rosette plant growing in dry, open grass communities of hillsides. It is described as gynomonoecious-gynodioecious, with female, gynomonoecious (plants bearing both perfect and pistillate flowers), and hermaphroditic individuals found in natural populations (Dufay et al.,

2010; Jürgens et al., 2002). Flowers scent compounds identified in *S. nutans* (Jürgens et al., 2002) are common components of a wide array of scented angiosperm flowers (see Knudsen et al., 1993), following the general trend of floral scent compounds typical for moth-pollinated flowers (Jürgens et al., 2002; Knudsen and Tollsten, 1993). However, calyx length of *S. nutans* flowers makes them less restricted to Sphingids and suggests that pollination may rely on a more diverse group of pollinators (Jürgens et al., 2002). Flowers are visited by both nocturnal (mainly Noctuidae and Sphingidae) and diurnal insects such as Apidae and Syrphidae (Jürgens et al., 1996) but no comparison in their efficiency has been performed to date. Perfect flowers are protandrous, but self-pollination occurs by geitonogamy as plants often carry several to many flowers at a given date. Selfing rate varies among individual plants and possibly among population, as a function of sex ratio (Lahiani, unpublished data).

Plant material

Individual plants used in the experiment were sampled in 2008 from natural populations. All plants were cloned from plantlets grown under greenhouse and over-wintered for ten weeks during winter 2010. Plants were then potted in a soil mix (3/4 compost; 1/4 perlite) and placed in greenhouse at a temperature of 20°C for seven weeks until the population reached the peak of flowering (Table 1). Plants originated from a mixture of wild populations located in Belgium, Germany and France (Table 1). Plants were followed from bud stage until they produced fruits.

Table 1. List of populations from which individual plants were collected

Locality/Region	Country	Number of plants used
Olloy-sur-Viroin	Belgium	10
Loffenau / Kreis Rastatt	Germany	1
Chaptoceaux / Maine-et-Loire /Bretagne	France	3
Vireux / Champagne-Ardenne	France	3
Epagny /Côte d'Or/ Bourgogne	France	1
Mülheim am Donau/ Kreis Tuttlingen	Germany	1
Corcelles-les-Monts/Côte d'Or/ Bourgogne	France	1

DNA extraction and genotyping

All plants (i.e. potential fathers of seeds produced) were genotyped in order to estimate the magnitude of self-pollination. For this purpose, DNA was extracted from 100-150 mg of leaf tissue using MACHEREY-NAGEL NucleoSpin® 96 Plant II Kits. DNA sample from each plant was assayed for four scored microsatellite loci (for primers see Table 2). Amplification reactions were carried out by polymerase chain reaction (PCR) with 20 ng of DNA in 10 µl reaction volume containing 5 µl of Qiagen multiplex Kit 2x, 1 µl of primer mix (10x) (0.75 µM of Scored forward primer and 3.75 µM of reverse primer) and 1 µl of sterile water. Cycling conditions for PCR amplification were 95°C for 15 min, five cycles of 45 s at 95°C, 5 min at 68°C with a step-down of 2°C per cycle, 1 min at 72°C, fives cycles of 45 s at 95°C, 5 min at 58°C with a step-down of 2°C per cycle, 1 min at 72°C, 27 cycles of 45 s at 95°C, 30 s at 47°C, 1 min at 72°C and finally 72°C for 10 minutes. Amplification products were separated on Applied Biosystems 3130 capillary sequencer. Raw data were analysed using GENMAPPER version 3.5 (Applied Biosystems). Individuals with doubtful or missing peaks or for which mismatches occurred between mothers and progeny were genotyped a second time.

Table 2. Primer list of microsatellites loci

Locis	Forward sequence	Reverse sequence
B09	AAGGGCACAAAATTGAGAAGG	GTGTCTTCCAAAGGTGAAGCTCATATAAACC
E08	GTTGGTCGTTGGTAGTTCACAG	GTGTCTTAATGCGAATCGGTCAATTTTAC
G01	CCCTACCTCATAGCAACAAGC	GTGTCTTCCTTCTCCTCCTTCCTTTAACC
H07	AAGCAAAACCCCTTATAAGCATC	GTGTCTTACCTTTCCCCTTCCTCCTTT

Comparison of efficiency of diurnal vs. nocturnal pollination

To compare pollination efficiency between diurnal and nocturnal pollination, we created an experimental patch composed by 20 hermaphroditic plants in a common garden. Eleven focal plants were followed for their pollination. All plants were watered every day. Survey was performed from 2nd to 9th June 2010. The first day of the experiment, six flowers were marked at the beginning of their stigma receptivity on each focal plant. Marked flowers were randomly assigned to one of the two treatments: nocturnal pollination (3 flowers per plant), or diurnal pollination (3 flowers per plant). Flowers were covered by a mesh bag to prevent pollination from 4.30h GMT (dawn) to 20.30 h GMT (dusk) for flowers pollinated during the night, and from 20.30 h GMT (dusk) to 4.30h GMT (dawn) for flowers pollinated during the day. We interrupted the experiment when flowers were no longer receptive. We estimated fruit set as the proportion of marked flowers setting fruit two weeks later. To prevent seed loss, fruits were covered by mesh bags before they opened. Fruits were collected as they matured, four weeks after pollination, and seeds were counted. For each focal plant and for each treatment, we thus obtained an average value of seed number per fruit. We estimated the pollination success as the product: fruit set x average number of seeds per fruit. This was calculated for each treatment (day / night-pollinated), for each focal plant. We performed paired *t-tests* to compare the difference in fruit set, seed number per fruit and in pollination success between treatments, within each focal plant.

Estimation of selfing rates

We measured the selfing rate on flowers pollinated during night or day for the 11 focal plants. For this purpose, 60 seeds per fruit of marked flowers were sown in Petri dishes on Whatman paper. After 12 days, 60 seedlings of each plant (when it was possible) were randomly selected, transplanted into a soil mix (3/4 compost; 1/4 perlite), and placed at 20°C with daily moistening to minimize stress of transplantation. Six weeks later plantlets were collected in order to extract their DNAs using MACHEREY-NAGEL NucleoSpin® 96 Plant II Kits. A total of 882 plantlets from the 11 families were genotyped by the 4 scored microsatellites loci described previously. Outcrossing rates were determined using Ritland (2002) multilocus maximum likelihood estimation program (MLTR Version 3.4, accessible at http://genetics.forestry.ubc.ca/ritland/programs.html). Standard deviations were determined based on 1000 bootstrap analyses; maternal genotypes were estimated as part of the maximum likelihood procedure. Two kinds of analyses were performed: population estimates allowed us to calculate overall multilocus (t_m) and mean single locus (t_s) outcrossing rates, bootstraps using families as units of observation. Family estimates gave outcrossing rates per family, bootstraps using individual offspring as units of observation. We considered selfing rates as $s = 1- t_m$. We performed paired *t-tests* to compare selfing rates between day and night pollination within each focal plant.

RESULTS

Efficiency of diurnal *vs.* nocturnal pollination

Pollination occurred during both day and night, since at least a portion of flowers from both treatments set fruit. Some diurnal pollinators such as bumblebee, bees and hoverflies, were observed visiting the flowers of the patch during the experiment. We found a significantly larger fruit set in night-pollinated flowers compared to day- pollinated ones (0.81 *vs*. 0.51 on average respectively, Table 3). Similarly, both the average number of seeds per fruit and the pollination success (fruit set x average seed number) were significantly larger for flowers pollinated during night

compared to those that were pollinated during the day (51 *vs*. 17 seeds on average per fruit; average pollination success of 37.4 *vs*. 8.6, Table 3). Similar results were found when performing paired Wilcoxon signed-rank tests to compare the two treatments (fruit set $N = 11$, $T = 3.62$, $P = 0.01$; average seed number $N = 11$, $T = 2.45$, $P = 0.04$; pollination success $N = 11$, $T = 3.69$, $P < 0.001$).

Table 3. Results of paired t-tests comparing pollination efficiency between nocturnal and diurnal pollination. D is the difference between the two pollination treatments. The test indicates whether this difference is significantly different from 0.

Variable	**N**	**D**	**t**	***P***
Fruit set	11	0.303	3.63	0.0046
Average seed number per fruit	11	33.31	2.46	0.0337
Pollination success (fruit set x seed number)	11	28.742	3.69	0.004
Selfing rate	7	-0.044	1.68	0.1436

Selfing rate during nocturnal *vs.* diurnal pollination

Selfing rate varied from 0 to 0.7, according to the family. Overall, selfing rate measured in flowers pollinated during night was $s = 0.132 \pm 0.062$ versus $s = 0.215 \pm 0.054$ for flowers pollinated during day. Paired *t-tests* were performed for this analysis on only 7 individuals out of the 11 surveyed plants because we did not obtained fruits or seedling from both treatments for all individual plants. In 5 out of the 7 cases, selfing rate was higher for day-pollinated flowers compared to night-pollinated ones; in one case selfing rate was similar between treatments and it was higher for the nocturnal treatment only in one plant. However, no significant difference was found neither from the paired *t-tests* (Table3) nor from Wilcoxon signed-rank tests ($N = 7$, $T = -1.68$, $P = 0.15$) probably because of a lack of statistical power.

DISCUSSION

This study aimed at comparing nocturnal and diurnal pollination in a species described with a nocturnal pollination syndrome. The main novelty of the current study was to not only compare pollination efficiency between the two phases of pollination but also the selfing rate, which in case of inbreeding depression, should have a strong impact on the reproductive success of the plants. Moreover, because *Silene nutans* is gynodioecious, and because the conditions of maintenance of this reproductive system depends on the relative female fitness of females and hermaphrodites (Gouyon et al., 1991), which in turn depends on pollination efficiency and self-pollination (Dornier and Dufay, in press), it is necessary to get an accurate understanding of its pollination biology. One must note that in case of a widely distributed species such as *S. nutans*, pollinator assemblages as well as the relative contribution of diurnal and nocturnal pollinators should vary among natural populations. Our study was conducted in semi-controlled conditions, thus on a possibly different pollinator assemblage than those found in some natural populations, but it was performed within the natural distribution area of the species and should thus constitute a representative point of the conditions ensured by the species in this portion of its distribution.

Our results showed that both fruit set and seed number per fruit were higher in flowers pollinated by nocturnal pollinators compared to flowers pollinated during day, which was expected according to the apparent nocturnal pollination syndrome of the species. The fact that nocturnal and diurnal visitors that have been described on *S. nutans* (Jürgens et al., 1996) seem to exhibit some differences in their body shape and their foraging behavior (Faheem et al., 2004) could explain our results. Several other studies have found that pollination efficiency differed between day and night (*e.g.* Amorim et al., 2012; Ortiz et al., 2000) and they usually found a consistent pattern with the pollination syndrome of the plant, when it had been described (*e.g.* Luo et al., 2011; Ortega-Baes et al., 2011). However, the difference in the contribution of diurnal *vs.* nocturnal pollinators is not always straightforward, with some studies showing no significant difference between the two phases (*e.g.* McMullen, 2011) or contrasted

results according to the trait measured (*e.g*. Gimenez-Benavides et al., 2007). One must note however, that even if diurnal pollination was less efficient, it still constituted a non negligible part of the overall seed production, with an average pollination success of 8.6, which thus represents 20 % of the overall seed production ensured by the plants). Keeping in mind that such pattern is likely to vary among natural populations (that show different pollinator assemblages and different weather conditions that may affect flower phenology), it suggests that *S. nutans* may have a mixed pollination strategy. Given the fact that the main described nocturnal visitors belonging to the *Hadena* genus (Gimenez-Benavides et al., 2007 and Lahiani, pers. obs.) also acts as a seed parasite, a non-negligible complementary contribution of -non-parasitic- diurnal pollinators may contribute to increase seed production in some populations.

To complete the results in terms of pollination efficiency, we also investigated how selfing rate varied between seeds produced during day and night. Although no significant difference was detected, we found an interesting tendency, with selfing rates being higher in flowers pollinated during day compared to those pollinated during night. Further studies should be conducted on a larger sample, in order to verify this tendency. This result could be explained by a difference in the foraging behavior between nocturnal and diurnal insects, with nocturnal pollinators, such as large moths, usually being long distance fliers (Linhart and Mendenhall, 1977; Shykoff and Bucheli, 1995; Young, 2002), whereas many diurnal pollinators tend to forage primarily within rather than between flower patches (Altizer et al., 1998), spend more time foraging in the same flowers patches (Waddington, 1983) and have short distance movement (Faheem et al., 2004). Together with the results of Barthelmess et al. (2006) who found stronger inter-population gene flow ensured by nocturnal pollination in dioecious *S. alba*, our study suggests that pollinator phenology can have some important impact on the genetic diversity of the progeny they contribute to produce. Indeed, in the case of *S. nutans* that exhibits a strong inbreeding depression (Dufay et al., 2010), even though diurnal pollinators substantially contributed to pollination in our experiment, their overall impact must be decreased, because the seeds they contributed to produce should have a

lower quality. We thus suggest that similar measurements should be conducted by studies that compare day and night pollination, in order to take this type of phenomenon into account.

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Conclusion et perspectives

L'objectif de cette thèse était d'identifier la dynamique évolutive responsable du maintien de la gynodioécie et d'étudier certaines conditions nécessaires à l'occurrence d'une meilleure performance des individus femelles et à la variation de l'ampleur de cet avantage, condition nécessaire au maintien de la gynodioécie. Nous avons opté pour deux approches différentes qui sont : une approche de génomique des populations et une approche de biologie des populations. Plus précisément, nous avons essayé de répondre aux questions suivantes : Quelles sont les forces évolutives intervenant dans le maintien des facteurs de stérilité mâle chez *Silene nutans* ? Quels sont les différences de succès reproducteurs des différents phénotypes sexuels ? Quel est l'impact des variations du sexe ratio sur leur succès reproducteur ? Et enfin, quel est l'impact de ce polymorphisme sexuel sur la structuration des populations naturelles ? Cette conclusion sera donc l'occasion d'évoquer les réponses partielles à ces questions ainsi que les perspectives de ce travail.

1. Dynamique de la gynodioécie chez *Silene nutans*

Selon les prédictions des modèles théoriques, la gynodioécie peut se maintenir soit par une sélection fréquence-dépendante (Gouyon *et al.*, 1991) soit par une dynamique épidémique (Couvet *et al.*, 1998). Les deux catégories d'hypothèses ne devraient pas laisser le même patron de diversité cytoplasique. Une espèce gynodioïque devrait avoir une diversité cytoplasmique plus importante qu'une espèce non gynodioïque si la gynodioécie est maintenue par une sélection fréquence dépendante et devrait avoir une diversité plus faible si la gynodioécie est maintenue grâce à une dynamique épidémique.

En se basant sur une étude comparative de la diversité nucléotidique dans les trois génomes (nucléaire, mitochondrial et chloroplastique), nous avons pu montrer que la diversité cytoplasmique chez *Silene nutans* est plus importante que chez *Silene otites*, une espèce dioïque proche de *Silene nutans* appartenant au même clade génétique (Fig.1.5). Cette différence aurait pu être due à un taux de substitutions synonymes

mitochondriales plus importants chez *S. nutans*. Néanmoins, notre étude a montré que ce taux semble être plus élevé chez *S. otites.* Par conséquent, nous avons pu conclure que le patron observé chez *S. nutans* est bien la signature d'une sélection fréquence-dépendante négative, engendrant le maintien d'haplotypes pendant de longues périodes de temps, et permettant ainsi une accumulation de mutations au sein de ces haplotypes (Chapitre 1). Un autre argument qui irait dans le sens d'un maintien de la gynodioécie par la sélection fréquence-dépendante négative chez *S. nutans* est l'existence d'un coût de la restauration. Ce coût a été mis en suggéré chez au moins trois espèces gynodioïques (*Plantago lanceolata* par de Haan *et al.*, 1997;chez *Lobelia siphilitica* par Bailey, 2002 et chez Beta vulgaris par Dufay *et al.*, 2008 et par De Cauwer *et al.*, 2012). Ce coût n'est pas encore mis en évidence chez *S. nutans* mais il semble nécessaire d'en vérifier l'existence pour compléter nos travaux.

La gynodioécie peut aussi être considérée comme un état transitoire dans l'évolution de l'hermaphrodisme vers la dioécie (synthétisé dans Barrett, 2002). Dans le genre *Silene*, elle semble être un état ancestral (Fig.1.5) qui aurait évolué vers la dioécie deux fois, une fois dans le clade *Otites* et une fois dans le clade *Melandrium.* La transition vers la dioécie dans le clade *Otites* semble être plus récente que dans le clade *Melandrium.* Premièrement, dans le clade *Otites* la plupart des espèces sont des espèces dioïques mais on trouve aussi des espèces non-dioïques ce qui n'est pas le cas dans le clade *Melandrium* (Käfer *et al.*, 2012). En plus, les chromosomes sexuels chez les espèces dioïques de ce clade semblent être plus récents que ceux de l'espèce dioïque *S. latifolia* (Mrackova *et al.*, 2008). En outre, certaines études ont décrit des stades intermédiaires entre la gynodioécie et la dioécie dans les populations naturelles de *S. otites* et même parfois la présence d'individus hermaphrodites (Desfeux *et al.*, 1996). Egalement, dans notre étude nous avons montré que les haplotypes de *S. otites* semblent être un sous-ensemble des haplotypes de *S. nutans* et que pour certains gènes (gènes nucléaires) les deux espèces montrent la même diversité (Chapitre 1). Enfin Käfer *et al.* (2012) n'ont pas détecté de différence de taille efficace des populations entre les espèces du clade *Otites* et les espèces non-dioïques étudiées (S. *nutans* en particulier)

alors qu'ils ont trouvé une réduction de taille efficace des populations chez les espèces du clade *Melandrium*. Ils suggèrent ainsi que la dioécie est récente dans le clade *Otites* au point qu'elle n'a pas encore eu le temps de laisser son empreinte (une réduction de la taille efficace des populations) dans les génomes de ces espèces. Nos résultats ainsi que ceux de Käfer *et al.* (2012), suggèrent que ces espèces ont évolué récemment vers la dioécie. Ces résultats nous montrent encore une fois que le genre *Silene* est un genre propice pour l'étude de l'évolution des systèmes de reproduction mais nous amènent à se poser des questions concernant les conditions et les pressions de sélection impliquées dans la transition de la gynodioécie vers la dioécie. Une étude est en cours au sein de l'équipe visant à déterminer les gènes qui peuvent être impliqués dans la transition de la gynodioécie à la dioécie.

2. Occurrence et variation de l'avantage femelle

Une des conditions nécessaires au maintien de la gynodioécie quel qu'en soient la dynamique et le déterminisme, est une différence de succès reproducteurs entre les phénotypes sexuels (femelle et hermaphrodite). Notamment, si les plantes femelles ont un succès reproducteur femelle plus important que les plantes hermaphrodites, cela compense théoriquement le fait qu'elles ne produisent pas de pollen et cela permet le maintien du polymorphisme. Dans le cas d'un maintien *via* une sélection fréquence-dépendante négative, deux conditions sont nécessaires pour que se polymorphisme see maintienne dans la population : l'occurrence d'un avantage femelle et l'existence d'un coût des restaurateurs de fertilité de la fonction mâle (Gouyon *et al.*, 1991) (figure 1.3). Dans le cadre de cette thèse, nous nous sommes intéressés uniquement à l'avantage femelle : son occurrence, son ampleur et sa variation.

2.1. Avantage femelle en terme de nombre de graines

Dans le deuxième et le troisième chapitre de la thèse, plusieurs traits liés à deux paramètres essentiels (la limitation pollinique et le taux d'autofécondation) pouvant affecter l'avantage femelle ont ainsi été mesurés en conditions semi-contrôlées et dans une population naturelle. Nous avons montré que l'avantage femelle est inexistant

quand les femelles souffrent d'une limitation pollinique forte (patch biaisé femelle dans le chapitre 2) et que cet avantage est moyen de l'ordre de 1,17 quand la limitation pollinique était modérée (patch biaisé hermaphrodite dans le chapitre 2). Dans une étude précédente chez *S. nutans*, Claire Garraud a montré que les femelles avaient un avantage femelle assez élevé en termes de production de graines dans des conditions optimales. Les femelles dans cette étude produisaient 3,7 (en 2008) à 6,9 (en 2009) plus de graines que les hermaphrodites (Garraud, Thèse de doctorat 2011). La production de graines dépend de la production d'ovules par fleur mais aussi du nombre de fleurs total qui a été produit par la plante. Dans l'étude menée par Claire Garraud, le nombre de fleurs produites a été inclus dans l'estimation de l'avantage femelle vu que ce paramètre était variable avec des femelles produisant plus de fleurs que les hermaphrodites. Au cours de notre étude nous n'avons pas observé de différences de nombre de fleurs produits par les hermaphrodites et les femelles.

Nous n'avons pas détecté d'avantage femelle en terme de production de graines dans les deux populations expérimentales (Chapitre 2) contrairement aux résultats de Claire Garraud (Thèse de doctorat, 2011). Les femelles produisent autant de graines que les hermaphrodites quand elles souffrent d'une limitation pollinique modérée et moins de graines que les hermaphrodites quand cette limitation en pollen est forte. Il semble ainsi que la limitation en pollen subit par les femelles dans notre étude baisse le succès reproducteur au point que l'avantage femelle en termes de production de graines disparait (Chapitre 2). La limitation pollinique est un phénomène observé chez plusieurs espèces gynodioïques (*p.e.* Graff, 1999; Alonso, 2005; De Cauwer *et al.*, 2010). Quel est le processus qui permet aux hermaphrodites de ne pas subir de la même façon que les femelles la limitation en pollen et par la suite faire baisser l'amplitude de l'avantage femelle ?

Une étude chez *Glechoma longituba*, a montré que les hermaphrodites ne souffraient pas de la limitation en pollen comme était le cas pour les femelles dans cette étude (Zhang *et al.*, 2008). Les auteurs suggèrent que ceci peut être dû à la possibilité des hermaphrodites de s'autoféconder quand ils sont limités en pollen. Cette capacité peut en effet conférer à ces individus une assurance reproductrice dans ces conditions

mais cette hypothèse n'avait, à notre connaissance, jamais été vérifiée. Chez *Silene nutans,* nous avons détecté des taux d'autofécondation plus élevés quand les hermaphrodites sont rares et donc très limités en pollen (Chapitre 2). Nos résultats suggèrent très fortement que les hermaphrodites produisent plus de graines que les femelles grâce à ces taux élevés d'autofécondation. Quand la limitation en pollen est modérée, les taux d'autofécondation, que nous avons mesurés étaient moyens (s= 0.34). Ceci peut expliquer que le nombre de graines produites par les femelles et les hermaphrodites était similaire. Les taux élevés d'autofécondation que nous avons observés quand les femelles sont fréquentes peuvent être dû à une préférence des pollinisateurs pour visiter les hermaphrodites (phénomène montré chez un certain nombre d'espèces gynodioïques par *p.e.* (Williams *et al.*, 2000; Griffin & Byers, 2012)). En d'autres termes, des différences d'attractivité des plantes femelles et hermaphrodites, dues à une plus petite taille des fleurs femelles chez *S. nutans,* (Dufay *et al.*, 2010), peut engendrer des séquences de visites répétées du même individu hermaphrodite et donc une plus grande probabilité de recevoir son propre pollen, et ce, d'autant plus que les producteurs de pollen sont rares. Une seule autre étude chez une autre espèce gynodioïque, *Silene vulgaris*, a montré que les hermaphrodites exhibent des taux d'autofécondation plus élevés quand ils sont rares (Miyake & Olson, 2009). De ce fait, le fort avantage femelle détecté par Garraud (Thèse de doctorat 2011) dans des conditions optimales avec des femelles produisant plus de graines suggère que le taux d'autofécondation des hermaphrodites dans ces conditions est faible. Cette hypothèse est à vérifier dans le futur.

Les taux d'autofécondation individuels que nous avons détectés sont très variables que ce soit en conditions expérimentales ou naturelles (Chapitre 2 et 3). Ainsi, il est indispensable, pour estimer les taux d'autofécondation en populations naturelles, de prendre en compte un grand nombre de plantes dans ces populations. Nous avons également trouvé une tendance de variation des taux d'autofécondation selon le groupe de pollinisateurs (diurnes et nocturnes). Les pollinisateurs nocturnes avaient tendance à engendrer des taux d'autofécondation plus faible (Chapitre 4). Ces variations peuvent avoir un impact sur le succès reproducteur. Il est ainsi primordial, de vérifier en

populations naturelles et avec un nombre plus important d'individus si cette tendance se confirme.

2.2. Avantage femelle en terme de qualité des graines

Dans une étude précédente chez *S. nutans* à laquelle j'ai participé, nous avons montré que les graines issues d'autofécondation subissaient une réduction de leur qualité de l'ordre de 30% suite à une forte dépression de consanguinité par rapport aux graines issues d'allofécondation . Les graines issues d'autofécondation avaient un poids plus petit et un taux de germination plus faible que les graines allo-fécondées. Les plantules issues de ces graines avaient aussi des rosettes plus petites, avec une biomasse plus faible que celles qui étaient issues de graines allo-fécondées (Dufay *et al.*, 2010, Annexe 2). Ainsi, nous avons considéré que les graines issues d'autofécondation dans notre étude devaient subir une réduction de leur qualité équivalente à celle mesurée dans cette étude.

Nous avons montré dans cette étude que les femelles ne bénéficient pas d'un avantage sélectif en termes de production de graines (chapitre 2) mais semblent avoir des graines potentiellement de meilleure qualité dans certaines conditions. Les femelles produisent le même nombre ou plus de graines allo-fécondées que les hermaphrodites quand elles sont respectivement très ou moyennement limitées en pollen. L'évitement de l'autofécondation et par la suite de la dépression de consanguinité semblent augmenter ainsi la qualité des graines des femelles. L'effet combiné de ces trois paramètres qui sont l'autofécondation, la dépression de consanguinité et la limitation pollinique a fait l'objet de certaines études théoriques (Dornier & Dufaÿ, sous presse). Nos résultats vont dans le même sens que ces prédictions. Quand les femelles souffraient beaucoup plus que les hermaphrodites d'une importante limitation pollinique, même si les hermaphrodites manifestaient un fort taux d'autofécondation (*s*) et donc subissaient une forte dépression de consanguinité (δ), ceci n'était pas suffisant pour faire baisser la performance des hermaphrodites pour que les femelles profitent d'un avantage sélectif (Chapitre 2). Mais quand les femelles souffraient d'une limitation

en pollen modérée, elles bénéficient d'un avantage femelle avec des graines de meilleure qualité (Chapitre 2). Il semble ainsi que la fréquence des femelles peut être critique à leur maintien à partir d'un certain seuil vu que l'avantage femelle diminue et devient inférieur à 1 quand les femelles sont fréquentes.

2.3. Les femelles peuvent-elles se maintenir en populations chez *S.nutans ?*

La question est comment les femelles qui étaient très rares dans la population naturelle étudiée peuvent-elles se maintenir (3 femelles : Chapitre 3) ? De plus, la gynodioécie dans cette population semble être localement nucléaire (un même cytoplasme de tous les individus indépendamment du phénotype sexuel : Chapitre 3). Une autre étude à l'échelle de l'Europe chez *S. nutans* suggère qu'il existe beaucoup de populations présentant un déterminisme purement nucléaire localement (Delalande *et al.*, 2012). Dans ce cas les femelles doivent bénéficier d'un avantage femelle assez élevé (supérieur à 2) pour se maintenir dans ces populations (Lewis, 1941). Nous n'avons pas pu vérifier l'occurrence d'un avantage femelle dans la population naturelle étudiée du fait du faible nombre de femelles dans cette population. En revanche, nous avons montré que les femelles et les hermaphrodites dans cette population souffraient d'une limitation pollinique très importante ce qui confirme la forte limitation pollinique que nous avons détectée en populations expérimentales (Chapitre 3). De plus, il semble que la limitation pollinique en population naturelle était plus importante (une plus mauvaise production de fruits et de graines : chapitre 3) que celles détectées dans les deux populations expérimentales étudiée (Chapitre 2), ce qui pourraient fortement diminuer voire annuler totalement l'avantage femelle dans cette population. De ce fait, d'autres études incluant d'autres populations de plus grande taille et avec plus de femelles sont nécessaires pour pouvoir estimer l'amplitude de l'avantage femelle dans ces populations et comprendre comment les femelles arrivent à se maintenir dans de tels conditions ?

Nous avons montré que l'avantage femelle était modéré quand les femelles étaient rares et inexistant quand les femelles étaient fréquentes. Ainsi, les variations du sexe ratio local peuvent affecter l'amplitude de l'avantage femelle (Chapitre 2). Des

flux de pollen restreint dans l'espace peuvent engendrer des variations des fréquences alléliques et par la suite des variations du sexe ratio local dans les populations. Nous avons montré que chez *S. nutans*, les flux de pollen semblent être limités dans l'espace, au cours de notre étude en population naturelle (Chapitre 3). Mais nous n'avons pas pu vérifier si l'avantage femelle varie selon le sexe ratio local. Cette hypothèse est à confirmer dans d'autres populations naturelles contenant plus de femelles et avec des gradients de fréquences des femelles. En revanche, tenant compte des flux de pollen majoritairement restreints dans l'espace et de la différenciation significative entre les différents dèmes dans la population naturelle étudiée (Chapitre 3), ceci suggère que nous devons considérer les populations naturelles chez *S. nutans* comme des populations structurées tout en gardant une migration non négligeable entre les différents dèmes.

Nous avons aussi montré que *S. nutans* a une pollinisation mixte (pollinisateurs nocturnes et diurnes) malgré son syndrome de pollinisation de nuit. Mais le succès reproducteur varie selon le groupe de pollinisateurs, avec des pollinisateurs nocturnes plus efficaces dans la production des fruits et des graines chez les individus hermaphrodites (Chapitre 4). Cette étude a été effectuée en population expérimentale dans l'aire de distribution de *S. nutans* uniquement sur des individus hermaphrodites. Le succès reproducteur d'un individu donné est un paramètre clé dans le maintien de la gynodioécie. Par conséquent, il est nécessaire de valider ces résultats en populations naturelles en incluant des individus femelles et hermaphrodites et d'évaluer l'abondance de ces deux groupes de pollinisateurs en populations naturelles afin d'évaluer leur contribution respective dans le succès reproducteur des plantes de *Silene nutans*.

En conclusion, étant donné que les plantes femelles chez *S. nutans* ne semblent pas produire plus d'ovules par fleurs que les hermaphrodites (E.lahiani, pers. obs), la production d'un nombre plus important d'ovules fécondés par les femelles ne semble pas être due à la réallocation des ressources (qui étaient destinées à la production de pollen) pour une production plus importante d'ovules par ces individus mais plutôt à l'efficacité de pollinisation. Par contre, une meilleure production de graines des femelles chez *S. nutans* semble dépendre des ressources qu'elles peuvent réallouer à la

transformation de ces ovules fécondés en graines. D'autre part, la qualité des graines produites peut être affectée par les ressources réallouées par les femelles ainsi que par l'amplitude des taux d'autofécondation du fait de la dépression de consanguinité qui peut diminuer le succès reproducteur des hermaphrodites (figure 1.6).

3. Les individus gynomonoïques

Une autre catégorie de plantes a été observée chez *Silene nutans*, il s'agit des plantes gynomonoïques. Ces plantes ont toujours été écartées ou négligées du fait qu'on ne connait pas bien leur déterminisme génétique. On ne sait pas comment varie leur succès reproducteur. Nous nous sommes intéressés à étudier le maintien des femelles dans les populations naturelles, mais il semble que les gynomonoïques sont plus fréquents dans ces populations (Chapitre3, Mathilde Dufaÿ, HDR). Chez *S. nutans*, ces individus semblent produire plus de graines par rapport aux hermaphrodites mais moins que les femelles (Garraud, Thèse de doctorat 2011). De plus, Garraud a montré que les gynomonoïques, chez *S. nutans*, produisent moins de pollen que les hermaphrodites étant donné qu'ils portent des fleurs femelles qui ne produisent pas de pollen mais que ce pollen est de qualité comparable à celle du pollen produit par les hermaphrodites (Garraud, Thèse de doctorat 2011). Néanmoins, nous avons montré dans une autre étude que la proportion de pollen viable par fleur produit par les individus gynomonoïques est corrélée négativement avec la proportion des fleurs femelles produits par ces individus (Dufay *et al.*, 2010). Par ailleurs chez *Dianthus sylvestris*, Collin & Shykoff (2003) ont montré que les fleurs femelles des individus gynomonoïques s'autofécondaient (par geitonogamie) autant que les fleurs hermaphrodites des plantes hermaphrodites et moins que les fleurs hermaphrodites des plantes gynomonoïques.

Les individus gynomonoïques en populations naturelles sont parfois plus fréquents que les femelles, il apparaît donc essentiel de déterminer leur succès reproducteur par rapport à celui des femelles et des hermaphrodites. En particulier , il serait intéressant de connaitre comment, , varie le succès reproducteur des deux types de fleurs de ce troisième phénotype sexuel chez *S. nutans* et ainsi le succès reproducteur

femelle de ces individus (nombre et qualité de graines) ? Pour répondre à cette question nous avons conduit une expérimentation en conditions semi-contrôlées. Nous n'avons pas encore terminé ce travail et les analyses sont en cours (les détails de l'expérimentation sont dans l'Annexe 2). Au cours de cette étude nous avons essayé de comparer les tailles de fleurs, les tailles des fruits et les productions de fruits et de graines entre les fleurs hermaphrodites des individus hermaphrodites et gynomonoïques ainsi que les fleurs femelles de ces derniers en fonction du sexe ratio local. De plus, nous avons essayé de comparer les différents taux d'autofécondation de ces trois types de fleurs selon le sexe ratio (au niveau de la plante et au niveau du patch). Les analyses des différents paramètres sont en cours.

Cette étude permettra de poser un premier jalon pour comprendre le rôle des gynomonoïques dans la dynamique de la gynodioécie.

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Annexe 1

Le succès reproducteur des individus gynomonoïques chez *Silene nutans*

Questions :

Parmi les questions qu'on se pose sont, quel est le succès reproducteur femelle des individus gynomonoïques ? Est-ce que cet avantage varie selon le sexe ratio? A sex-ratio équivalent est ce que les différentes catégories de fleurs (fleurs femelles et hermaphrodites des plantes gynomonoïques et fleurs hermaphrodites des fleurs hermaphrodites et fleurs femelles des plantes femelles) ont les mêmes taux d'autofécondation? Et est-ce que le taux d'autofécondation est variable selon le type des gynomonoïques (différents types selon le pourcentage des fleurs femelles par plantes) ?

Expérimentation :

Deux blocs contenant bloc1 : 5 Hermaphrodites et 5 gynomonoïques et bloc2 : 6 Hermaphrodites et 8 gynomonoïques, ont été construit afin d'étudier le succès reproducteur des individus gynomonoïques chez *Silene nutans*. Les plantes étaient issues de différentes populations naturelles de l'Europe. A chaque vague de floraison le sexe ratio de chaque plante en termes de fleur femelles et hermaphrodites et le sexe ratio fonctionnel (le nombre de fleur femelles, le nombre de fleurs hermaphrodites au stade mâle et au stade femelle) ont été estimés. Les plantes étaient suivies jusqu'à la fin de leur floraison, il y avait ainsi quatre vagues de floraison dans le premier bloc et cinq vagues de floraison dans le deuxième bloc.

A chaque vague de floraison, trois fleurs (quand c'était possible) de chaque type de fleurs (femelle et hermaphrodite) sur les individus gynomonoïques et trois fleurs hermaphrodites sur les individus hermaphrodites ont été marquées. Les fleurs marquées étaient des fleurs réceptives. La longueur d'un pétale au hasard de chaque fleur marquée a été mesurée. Il y avait ainsi trois types de fleurs : les fleurs femelles des individus gynomonoïques, les fleurs hermaphrodites des individus gynomonoïques et les fleurs hermaphrodites des individus hermaphrodites.

Quand les fruits marqués arrivaient à maturité, ils été couvert pour empêcher la perte des graines et des ovules. Le taux de mise à fruits de chaque type de fleurs marquées à chaque vague de floraison ont été estimé. Ensuite, la taille des fruits et le

taux de mises à graines des fruits marqués ont été estimés. Ensuite, 35 graines de chaque fruit ont été mises à germer quand c'était possible. Douze jours après le début de la germination, les plantules ont été repiquées dans un mélange de terreau et perlite. Six semaines plus tard, les plantules ont été récoltées et séchées afin d'extraire leur ADN.

Les analyses statistiques ainsi que le génotypage des descendances grâce à 8 locus microsatellites sont en cours afin de déterminer les taux d'autofécondation des trois types de fleurs et la variation de ses taux selon le sexe ratio fonctionnel de la plante ainsi que le sexe ratio du bloc correspondant à la vague de floraison durant laquelle le fruit été produit.

Annexe 2

Gender variation and inbreeding depression in gynodioecious-gynomonoecious *Silene nutans* Caryophyllaceae).
Mathilde Dufay, Emna Lahiani, Benjamin Brachi, B
International Journal of Plant Sciences. 2010. 171: 53-62.

Cet article concerne ma participation aux recherches au sein du laboratoire GEPV dans le cadre de mon stage de projet de fin des études.

Annexe 2

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GENDER VARIATION AND INBREEDING DEPRESSION IN GYNODIOECIOUS-GYNOMONOECIOUS *SILENE NUTANS* (CARYOPHYLLACEAE)

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Gynodioecy involves the stable co-occurrence of females and hermaphrodites. Its maintenance theoretically depends on differences in female and male reproductive success among gender morphs. Although many gynodioecious species also include gynomonoecious individuals that carry a mixture of female and perfect flowers, little is known about the male and female fitness of this third morph. Here, we present the first study of the reproductive system of *Silene nutans*, including females, gynomonoecious plants, and hermaphrodites. By measuring 10 floral traits in controlled conditions, we showed that females bear smaller and lighter flowers than hermaphrodites, with female and perfect flowers of gynomonoecious plants being intermediates. By measuring pollen quantity and quality, we showed that gynomonoecious plants had a lower potential male fitness than hermaphrodites at the level of both flowers and individuals. In addition, gynomonoecious plants were shown to widely vary their proportion of female flowers (0.03–0.9) and their floral traits, suggesting a quantitative restoration of male fertility. Finally, controlled pollinations showed evidence for inbreeding depression ($\delta = 0.3$) in progeny of hermaphrodites and gynomonoecious individuals, affecting both pre- and postdispersal traits; this could provide a selective advantage for females.

Keywords: gynodioecy, gynomonoecy, sex polymorphism, pollen viability, inbreeding depression, floral traits.

Introduction

Gynodioecy, one of the most common sexual polymorphisms in plants (Richards 1997), involves the stable maintenance of male sterile plants that have lost one of their sexual functions with hermaphrodites that possess both; this polymorphism has puzzled evolutionary biologists for decades. Commonly, this mating system is due to the conflicting interactions of cytoplasmic and nuclear genomes, with male sterility genes in the mitochondria, the effect of which is counteracted by nuclear alleles that restore male fertility (Saumitou-Laprade et al. 1994). Basically, sex polymorphism remains in a population as soon as a nuclear-cytoplasmic polymorphism is maintained. Theoretical studies have indicated that frequency-dependent selection could maintain such polymorphism if (1) females produce, at least marginally, more (or better) seeds than hermaphrodites and (2) carriers of nuclear restorer genes pay a fitness cost relative to those that do not (Gouyon et al. 1991; Bailey et al. 2003; Dufay et al. 2007). Understanding how such sexual polymorphism can be maintained in a given plant species thus requires a careful comparison of reproductive success among sex morphs.

A better fitness of females compared with female fitness of hermaphrodites has been reported in many gynodioecious species, matching with theoretical predictions. According to the species, such "female compensation" can be expressed as a difference in fruit set (Asikainen and Mutikainen 2003), number of seeds (Kohn 1989), seed quality (Delph and Mutikainen 2003), or offspring survival or growth (Chang 2006). Although it has been suggested that female compensation results from reallocating resources no longer used for pollen production to other (female) fitness parameters (Barr 2004), in many species, female compensation may partially result from an avoidance of inbreeding depression for female (obligatory outcrossed) progenies (Agren and Wilson 1991; Sakai et al. 1997; Ramsey et al. 2006). Investigations of variance of seed and fruit production between females and hermaphrodites, with attention to the possible role of inbreeding depression, is thus necessary for understanding gynodioecy. To this end, many studies carried out on insect-pollinated gynodioecious species have also included a comparison of floral traits among sexual phenotypes, since differences in attraction of pollinators could give rise to a higher seed set in one of the sex categories (Talavera et al. 1996; Ramsey and Vaughton 2002).

However, the study of female reproductive success within gynodioecious species is not sufficient, since male fitness is also expected to vary among gender morphs, for several reasons. First, the cost of restorer alleles, put forward by theoretical studies, is likely to affect male reproductive success (Gouyon et al. 1991; Dufay et al. 2007). Second, many gynodioecious species include a third intermediate morph either (1) carrying flowers with nondehiscent/less numerous anthers or producing lower quantity or quality of pollen (Koelewijn and Van Damme 1996; Poot 1997; Dufay et al. 2008) or (2) carrying a mixture of female and perfect flowers (gynomonoecious individuals, as frequently described in Caryophyllaceae; Shykoff 1992; Talavera et al. 1996; Maurice 1999;

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Guitian and Medrano 2000; Lopez-Villavicencio et al. 2005). Such intermediate morphs are sometimes thought to be the result of partial restoration of male sterility (Ehlers et al. 2005) but are rarely taken into account in experimental or theoretical studies (but see Koelewijn 1996; Collin and Shykoff 2003; Lafuma and Maurice 2006; Bailey and Delph 2007). Because the dynamics of gynodioecy also depend on the fertility of intermediates relative to females and hermaphrodites, measurements of their reproductive success through both male and female function are needed, with attention to the consequence of selfing and inbreeding depression.

We present the first study of the mating system of *Silene nutans*, which has been indicated in several studies to be gynodioecious-gynomonoecious (Desfeux et al. 1996; Jürgens et al. 1996, 2002) but for which no precise comparative data on females, gynomonoecious plants, and hermaphrodites have been collected. We performed measurements of potential male and female fitness in plants grown in controlled conditions, and we address the following questions. (1) How does the proportion of female flowers vary among gynomonoecious individuals? Does gender vary quantitatively from hermaphrodites to females in *S. nutans*? (2) How do floral traits vary with gender? In particular, do female flowers of female and gynomonoecious plants differ for some floral traits, while perfect flowers of hermaphroditic and gynomonoecious plants differ for others? (3) Do gynomonoecious and hermaphroditic plants differ in their pollen production and pollen quality at the level of either the flower or the individual? (4) How does seed quality vary with gender? (5) Do selfed offspring suffer from inbreeding depression? If so, could the avoidance of such inbreeding depression provide a fitness advantage for females? Finally, does inbreeding depression vary between hermaphroditic and gynomonoecious lineages?

Material and Methods

Study Species

Silene nutans (Caryophyllaceae) is a diploid, long-lived perennial rosette plant growing in dry, open grass communities of hillsides. It has been described as gynomonoecious-gynodioecious, with female, gynomonoecious, and hermaphroditic individuals found in natural populations (Jürgens et al. 2002). Flowers are visited by a number of different insect species (Jürgens et al. 1996). Perfect flowers are protandrous, but self-pollination can occur by geitonogamy. The seeds are dispersed from an aperture at the top of the capsule by vibrations of the flower stalk.

Sex Ratio and Floral Traits

This study was carried out on a collection of *S. nutans* individuals from seven populations, four of these from Belgium and three from central France (table 1). During spring 2007, plants were placed in a greenhouse during their whole flowering period. Of these, 58 produced sufficient flowers for further study. Newly opened flowers on each of these plants were checked twice weekly so that at the end of the flowering period, each individual plant was assigned to one of these three sex categories: female (F), hermaphrodite (H), or gynomonoecious (G). Additionally, a quantitative measure of gender was performed on plants from Belgian populations ($n = 44$) by recording the exact number of female and perfect flowers produced throughout the whole flowering season. This provided the proportion of female flowers for each individual plant (reaching 1 for females, 0 for hermaphrodites, and intermediate values for gynomonoecious).

On each of the 58 test plants, a sample of floral buds was marked and then collected 3 d after flower opening. On females and hermaphrodites, two to three flowers were sampled; on gynomonoecious plants, two to three flowers per sex category (female and perfect) were sampled for each individual plant. In this way, a total of 142 flowers were collected and measured for the following traits: flower total mass, calyx length and width, length and width of one randomly selected petal, stigma length, ovary length and width, gynoecium mass, and ovary mass.

Average floral trait values per individual plant were analyzed with a general linear model (proc GLM, SAS). For gynomonoecious plants, two values were analyzed, one average

Table 1

List of Populations from Which Individual Plants Were Collected

Population	Latitude N	Longitude E	Sample size	Collected material	No. plants followed	Measures performed for this study
Central France:						
Queyras	44°46′	6°44′	65	Seeds	8	FT, CROSS
Dordogne	45°19′	0°35′	16	Seeds	2	FT, CROSS
Auvergne	44°43′	2°21′	43	Seeds	4	FT, CROSS
Belgian:						
Leffe	50°15′	4°54′	29	Rosette	11	FT, QS, POL, CROSS
Tienne	50°05′	4°40′	14	Rosette	4	FT, QS, POL
Olloye	50°04′	4°36′	30	Rosette	7	FT, QS, POL
Vireux	50°05′	4°43′	36	Rosette	22	FT, QS, POL, CROSS

Note. Data listed for each population are name and geographical coordinates, the number of individual plants either collected as seeds and grown in greenhouse or collected as rosettes and transplanted to the greenhouse, the number of individual plants that produced enough flowers for inclusion in the study, and the types of measurements performed on plants from each population: measurements of floral traits (FT), quantitative estimation of sex (QS), by recording the proportion of female flowers, measurements of pollen production (POL), and control pollination (CROSS).

value for female flowers and one for perfect flowers. Two explanatory variables were tested, population and sex, with sex being a combination of plant sex and flower sex (the sex factor thus had four levels: female flowers of female plants [FF], female flowers of gynomonoecious plants [FG], perfect flowers of gynomonoecious plants [PG], perfect flowers of hermaphroditic plants [PH]). Post hoc pairwise comparisons were performed with Tukey's tests. For GLM analyses, normality of residuals was checked (Kolmogorov-Smirnov: $P > 0.1$ for all analyses). The same analysis was run on plants from only the Belgian populations by testing for an effect of population and sex as a quantitative factor (i.e., the proportion of female flowers).

Pollen Quantity and Pollen Viability

Pollen production was analyzed on plants from the Belgian populations ($n = 42$; 11 gynomonoecious and 31 hermaphrodites). For each plant, two to three floral buds were chosen, and all anthers from each bud were collected and stored in ethanol at 95%. Ethanol was then evaporated and samples were placed at 56°C for 24–48 h to force anther dehiscence. One milliliter of distilled water was then added to each pollen sample and sonicated to separate pollen grains and remove them from the anthers. Tubes were then vortexed, and the number of pollen grains was estimated in 200 μL of suspension. A particle counter CASY model TT (Innovatis, Bielefeld) 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 before counting. The particle counter then sampled three volumes of 400 μL from the suspension and provided the result for the total 1200 μL analyzed. The number of detected particles was determined for 400 size classes ranging from 0.125 to 150 μm using the software CASY Excel 2.1. Prior observation had shown that nonviable pollen grains in *S. nutans* were of smaller size than viable pollen grains. These counts were then used to estimate both total pollen production and fraction of viable pollen grains. The total number of pollen grains was obtained from the values provided by the particle counter after correcting for the dilution ratio, that is, by multiplying all values by $5 \times 5200/1200$. The 5200/1200 factor allows estimation of the quantity of pollen grains in the 5-mL solution in which the particle counter sampled, and the 5 factor allows estimation of total pollen per flower, since only 200 μL over a total of 1 mL were used.

We analyzed the proportion of viable pollen grains, testing for two explanatory variables, population and sex (coded either as a qualitative variable [i.e., hermaphrodite vs. gynomonoecious individuals] or as a quantitative variable [i.e., the proportion of female flowers carried by each plant]), by using a logistic regression (binomial distribution, log link function, proc GENMOD, SAS) and correcting for overdispersion (dscale option, proc GENMOD, SAS). The average quantity of pollen grains per flower as well as an estimation of plant male fitness (defined as the product: number of viable pollen grains per flower $\times$ number of perfect flowers carried by the plant) were analyzed with a general linear model (proc GLM, SAS).

Pollen viability was also directly estimated with an Alexander stain on a subsample of plants ($n = 30$) to assess the correlation between pollen grain size and viability. To cover the largest variance in pollen viability, we sampled both gynomonoecious plants ($n = 7$) and hermaphrodites ($n = 23$) from all four populations. On these plants, two additional freshly opened flowers were collected the same day as the floral buds used for particle counter analysis. Within 3 h of collection, pollen was removed from the flowers 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 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 H_2O; Alexander 1969), which stains pollen cytoplasm in purple and exine in green, 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. Pollen samples were then examined under LM at $\times$100 magnification. Two hundred pollen grains per sample 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.

Crossing Design and Measure of Inbreeding Depression

On 42 individual plants from the collection (6 females, 5 gynomonoecious plants, and 31 hermaphrodites), six flowers were marked at the bud stage and enclosed in a mesh bag to avoid accidental pollination events. As soon as flowers opened, their stamens were cut; hand-pollinations were performed once stigmas became receptive. On gynomonoecious and hermaphroditic plants, three flowers were self-pollinated and three others were cross-pollinated; on female plants, all flowers were cross-pollinated. Self-pollinations were performed with pollen collected from other flowers of the same plant; cross-pollinations were made using a mixture of pollen from three hermaphrodites from the same population. Few gynomonoecious plants were included in this experiment, since the sexual phenotype could be assigned with certainty only at the end of the flowering season, once the crossing experiment had already been performed.

At fruit maturity, seeds were collected and counted. A sample of 100 seeds per mother plant and cross type (inbred vs. outcrossed) was constituted and weighted. Each group of 100 seeds was then randomly split into two lots of 50 seeds, from which the total mass was also measured. Each lot of 50 seeds was randomly assigned to group 1 or 2 (defining different growth conditions at seedling stage). Seeds were then sown in Petri dishes on Wattman paper; germination rate and mortality at early stage were monitored for each lot of 50 seeds. Average seed mass was analyzed with an ANOVA (proc GLM, SAS); rate of germination was analyzed using a logistic regression (binomial distribution, log link function, proc GENMOD, SAS), correcting for overdispersion (dscale option, proc GENMOD, SAS).

After 12 d, 10 seedlings per lot were randomly selected, transplanted into a soil mix (3/4 compost; 1/4 perlite), and placed at 20°C with daily moistening to minimize stress of transplantation. After 2 wk, seedlings were spread into two growth conditions and followed for growth and survival in the green-

house; for each mother plant and cross type, two lots of seedlings were followed, one for each growth condition. In condition 1, temperature was set between 21° and 25°C with daily watering. In condition 2, temperature mostly followed natural conditions, and daily temperature ranged between 15° and 25°C; watering occurred every 5 d only (allowing time for the soil to dry between two consecutive watering events). At 8 wk, seedlings were collected, and their dry mass was measured and analyzed with an ANOVA, testing for an effect of the mother plant, its gender, its population, growth conditions, and cross type (proc GLM, SAS).

Finally, for each lot of seeds (per mother plant, cross type, and growth conditions), a multiplicative measure of cumulative offspring quality was computed as proportion of germination × proportion of seedling survival × mean dry mass. For each growth condition, we thus calculated the relative performance of inbred versus outcrossed offspring as follows: $\delta = (W_O - W_I)/W_{max}$, with W_O and W_I being the cumulative offspring quality of outcrossed and inbred offspring, respectively, and W_{max} being the larger of the two first values.

Results

Sex Ratio and Comparison of Floral Traits among Sex Types

Among the 58 individual plants followed for their floral traits, 7 were purely females, 17 were gynomonoecious, and 34 were hermaphrodites. Among all floral traits, only flower mass, petal length, and petal width significantly depended on sex. All other variables depended either on population only or on neither of these factors (table 2). Flower mass, petal length, and petal width of hermaphrodites (PH) were significantly higher than for flowers carried by females (FF), with female and perfect flowers from gynomonoecious individuals being statistically intermediate (table 3).

To better understand the status of flowers carried by gynomonoecious plants, we then focused on the Belgian populations on which the exact number of flowers as well as the proportion of female flowers per plant had been monitored. Flower number varied from 7 to 48; it differed only marginally among populations ($F_{3,42} = 2.27$, $P = 0.09$) but did not differ among sex morphs ($F_{2,43} = 0.03$, $P > 0.1$). Among plants from Belgian populations, 14 were gynomonoecious, with the proportion of female flowers varying from 0.03 to 0.9 (mean proportion = 0.33, SD = 0.28; fig. 1). Using the proportion of female flowers per plant, we could thus test for an effect of quantitative estimate of gender on floral traits. For these analyses, because very few female flowers could be measured for their floral traits, we chose to reduce our data set to perfect flowers ($n = 41$, 14 gynomonoecious and 27 hermaphrodites). We found a significantly negative effect of the proportion of female flowers on calyx length, petal length, and petal width (table 4). Similar results hold when our analysis was focused on gynomonoecious plants: a significantly negative effect of the proportion of female flowers was found for calyx length ($F_{1,13} = 8.03$, $P = 0.017$) and petal length ($F_{1,13} = 11.76$, $P = 0.006$). These results suggest that perfect flowers carried by "female-biased" gynomonoecious plants tend to be smaller.

Table 2

ANOVA of Floral Traits on Flowers Carried by Female, Gynomonoecious, and Hermaphroditic Individual Plants

Variable and source of variation	df	MS	*F*	*P*
Flower mass:				
Population	6	.00031	1.89	.09
Sex	3	.00049	3.01	.038
Error	52	.00016		
Calyx length:				
Population	6	.6351	.70	.6495
Sex	3	1.0945	1.21	.3156
Error	52	.9051		
Calyx width:				
Population	6	.4549	4.43	.0011
Sex	3	.0718	.70	.5564
Error	52	.1026		
Petal length:				
Population	6	1.7962	1.50	.1954
Sex	3	18.1495	15.19	<.0001
Error	52	1.1946		
Petal width:				
Population	6	.2481	1.86	.1051
Sex	3	.7422	5.57	.0022
Error	52	.13324		
Stigma length:				
Population	6	19.0979	1.21	.3149
Sex	3	3.0796	.20	.8990
Error	52	15.7501		
Ovary length:				
Population	6	2.7534	12.33	<.0001
Sex	3	.3828	1.71	.1754
Error	52	.2233		
Ovary width:				
Population	6	.0671	1.10	.3764
Sex	3	.1056	1.73	.1730
Error	52	.0612		
Ovary mass:				
Population	6	.000016	1.78	.1215
Sex	3	.000003	.36	.7808
Error	52	.000009		
Gynoecium mass:				
Population	6	.000007	.59	.7370
Sex	3	.000004	.36	.7793
Error	52	.000012		

Note. Sex is a combination of flower sex and plant sex (with four levels because gynomonoecious plants carry both female and hermaphroditic flowers).

Pollen Quantity and Viability

Pollen grain size, as estimated by the particle counter, was highly variable both within and among individual plants. Two major size classes could be defined: from 30 to 40 μm (small pollen grains) and from 40 to 60 μm (large pollen grains). On 30 individual plants, both the proportion of large pollen grains, estimated with the particle counter, and the proportion of viable pollen grains, estimated with Alexander stain, were recorded. These two variables were positively correlated ($R^2 = 0.8$; fig. 2). Thus, we assumed that the proportion of large pollen grains was a reliable estimation of the proportion of viable pollen grains.

Table 3

Results of Multiple Pairwise Comparisons of Floral Traits among Sex Categories for Analyses That Found a Significant Sex Effect

Variable	FF	FG	PG	PH
Flower mass (g)	$.0484^{A}$	$.0539^{AB}$	$.0613^{AB}$	$.0645^{B}$
Petal length (cm)	7.10^{A}	9.19^{B}	10.14^{BC}	10.58^{C}
Petal width (cm)	1.397^{A}	1.530^{AB}	1.763^{AB}	1.916^{B}

Note. FF = female flowers of female plants; FG = female flowers of gynomonoecious plants; PG = perfect flowers of gynomonoecious plants; PH = perfect flowers of hermaphrodites. Numbers are average values (least squares means, PROC GLM) for each sex category. Letters indicate categories significantly different from one another (results of post hoc Tukey's test; $P < 0.05$).

In the 42 plants that were monitored for their pollen production, neither population nor plant sex had an effect on pollen quantity produced per flower (proc GLM, $P > 0.1$). However, the proportion of viable pollen grains did vary according to both population ($\chi^2_{4,36} = 14.24$, $P = 0.0066$) and plant sex (hermaphrodites vs. gynomonoecious individuals: $\chi^2_{1,36} = 6.28$, $P = 0.0122$), with hermaphroditic plants producing a higher proportion of viable pollen grains than gynomonoecious ones. Similarly, when coding plant sex as a quantitative variable (the proportion of female flowers carried by the plant), we found a significant effect of both population ($\chi^2_{4,36} = 11.76$, $P = 0.0192$) and sex ($\chi^2_{1,36} = 6.79$, $P = 0.0091$), with the proportion of female flowers being negatively correlated with the proportion of viable pollen grains at the flower level. Finally, we calculated an estimate of plant potential male fitness by multiplying the number of viable pollen grains produced per flower by the number of perfect flowers and found a marginally higher estimate of male fitness for hermaphrodites compared with gynomonoecious plants ($25,082 \pm 20,086$ vs. $12,715 \pm 10,836$ viable pollen grains; proc GLM; $F_{1,36} = 3.68$, $P = 0.0629$).

Seed and Offspring Quality

Average seed mass was calculated for each lot of 100 seeds. On this data set, we tested for an effect of (1) population of the mother plant and (2) cross type (with five levels) that included both sex of the mother plant and the breeding treatment (self- vs. cross-pollination). We found an effect of population ($F_{5,68} = 16.47$, $P < 0.0001$) but no cross type effect ($P > 0.1$). On the contrary, the rate of germination did not depend on population (proc GENMOD: $\chi^2_{5,68} = 8.47$, $P > 0.1$) but did depend on cross type ($\chi^2_{4,68} = 11.41$, $P = 0.023$). Contrast analyses revealed that crossed seeds of hermaphrodites had a higher germination rate than selfed seeds of both gynomonoecious plants and hermaphrodites (proc GENMOD, $P < 0.05$). No other cross type effect was found to affect growth of offspring (proc GENMOD, $P > 0.1$). In particular, no difference was found between seeds produced by females and those produced by other sex morphs. Therefore, we reduced our data set to include only progenies produced by gynomonoecious plants and hermaphrodites and focused on the comparison between outcrossed and inbred offspring.

Focusing on gynomonoecious and hermaphroditic plants, paired *t*-tests were performed to compare average seed mass and germination rate between inbred and outcrossed seeds within each maternal offspring. This revealed a larger seed mass for outcrossed seeds compared with inbred seeds (0.45 vs. 0.41 g, respectively; $t = 2.47$, $P = 0.018$, $n = 36$). Seeds produced by cross-pollination also showed a higher germination rate (0.91 vs. 0.81 for cross and selfed, respectively; $t = 4.02$, $P = 0.0003$, $n = 36$). Germination rate was also analyzed with a logistic regression (without performing pairwise comparison within each maternal offspring): while neither

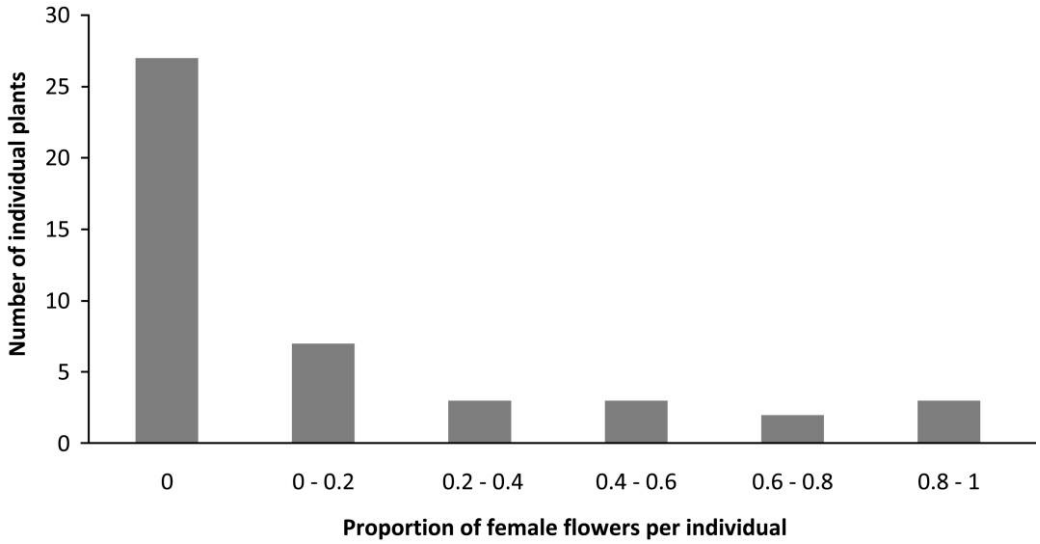

Fig. 1 Gender variation among individual plants from Belgian populations. Plants that carried no female flowers were purely hermaphroditic. Other individual plants were either gynomonoecious or females. Three individual plants had from 80% to 100% female flowers; they included two females (100% female flowers) and one gynomonoecious plant that carried 90% of female flowers.

Table 4

ANOVA of Floral Traits Measured on Hermaphroditic Flowers Carried by Both Hermaphrodites and Gynomonoecious Plants from Belgian Populations

Variable and source of variation	df	MS	*F*	*P*
Flower mass:				
Population	3	.00014	1.03	.3890
Female flowers (%)	1	.00013	.96	.3343
Error	36	.00013		
Calyx length:				
Population	3	.1889	.25	.8605
Female flowers (%)	1	4.0376	5.35	.0265
Error	36	.7546		
Calyx width:				
Population	3	.0988	.90	.4519
Female flowers (%)	1	.0197	.18	.6740
Error	36	.1101		
Petal length:				
Population	3	2.3260	2.10	.1172
Female flowers (%)	1	12.0039	10.84	.0022
Error	36	1.1071		
Petal width:				
Population	3	.0309	.33	.8027
Female flowers (%)	1	.3703	3.97	.0540
Error	36	.0933		
Stigma length:				
Population	3	10.2064	.57	.6145
Female flowers (%)	1	16.4119	.91	.3468
Error	36	18.0595		
Ovary length:				
Population	3	3.3116	12.87	<.0001
Female flowers (%)	1	.0433	.17	.6848
Error	36	.2582		
Ovary width:				
Population	3	.0840	1.32	.2821
Female flowers (%)	1	.0467	.74	.3968
Error	36	.0635		
Ovary mass:				
Population	3	.000029	3.35	.0297
Female flowers (%)	1	.000002	.19	.6633
Error	36	.000009		
Gynoecium mass:				
Population	3	.000015	1.22	.3161
Female flowers (%)	1	.000003	.24	.6285
Error	36	.000012		

Note. Both population and quantitative gender (proportion of female flowers) were tested on each trait.

population nor sex affected germination rate, both breeding treatment (self vs. cross) and average seed mass simultaneously affected this variable (proc GENMOD, cross type effect: $\chi^2_{1,69} = 7.92$, $P = 0.0049$; average seed mass: $\chi^2_{1,69} = 8.18$, $P = 0.0042$). When average seed mass was included in the model, breeding treatment still significantly affected germination rate, suggesting that differences in germination rate between cross types were not only because of a difference in seed mass.

Dry biomass of seedlings after 8 wk did not depend on the sex of their mother (gynomonoecious plant vs. hermaphrodite) but did depend on the mother's identity (nested in population × maternal sex), population, breeding treatment, and growth conditions (table 5). Overall, seedling from selfed seeds averaged 76% of the mass of the average for the outcrossed treatments, and seedlings that grew in environment 1 (warmer temperature and regular water supply) produced 25% more biomass than those in environment 2. No significant interaction was found between the main factors.

The relative performance of inbred versus outcrossed offspring δ, based on the cumulative offspring quality for each maternal plant, was 0.31 on average, with strong variation among families (SD = 0.41), and was significantly different from 0 ($t = 5.18$, $P < 0.0001$, $n = 48$). It did not depend on growth conditions, population, or gender of the mother plant (gynomonoecious individual vs. hermaphrodite: $P > 0.1$).

Discussion

To our knowledge, this is the first study of sex polymorphism in *Silene nutans*. *Silene nutans* had been described as gynodioecious in studies carrying out pollination biology within the *Silene* genus and was sometimes presented as gynodioecious-gynomonoecious (Desfeux et al. 1996; Jürgens et al. 1996, 2002). However, other studies that focused on maternal choice (Hauser and Siegismund 2000) and flowering phenology (Hauser and Weidema 2000) of *S. nutans* populations in Denmark and Sweden did not mention any gender variation within the species. When sex polymorphism is not the primary aim of the survey, many studies do not necessarily report the occurrence of females in populations and even less likely of gynomonoecious plants. Future studies should thus investigate whether sex polymorphism effectively varies across the species' range, as it has been observed in many other gynodioecious species (Thompson and Tarayre 2000; Asikainen and Mutikainen 2003; Nilsson and Agren 2006; Dufay et al. 2009). This work carried out on plants from Belgian and French populations revealed that a sex polymorphism occurred within both regions and that gynomonoecious individuals were always more frequent than females (29% vs. 12% in this study). These results are similar to those found in other *Silene* species or other so-called gynodioecious species belonging to the Caryophyllaceae family (Shykoff 1992; Maurice 1999; Guitian and Medrano 2000).

Comparison of Females and Hermaphrodites

As a result of low female frequency in populations, our data set included very few females. Several floral traits were nevertheless compared between females and hermaphrodites, revealing smaller petals and lower mass in flowers of female plants. This is similar to results found in many insect-pollinated gynodioecious species (Puterbaugh et al. 1997; Williams et al. 2000; Ramsey and Vaughton 2002; Caruso et al. 2003; Chang 2006), including several *Silene* species and other Caryophyllaceae (*Silene stockenii*: Talavera et al. 1996; *Gypsophila repens*: Lopez-Villavenciado et al. 2003; *Silene italica*: Lafuma and Maurice 2006). Petal size is thought to be an important trait for pollinator attraction and is consequently often considered as a typically "male trait" strongly selected for in hermaphrodites (or males in dioecious species) compared with females (Queller 1983). Thus, future studies should measure

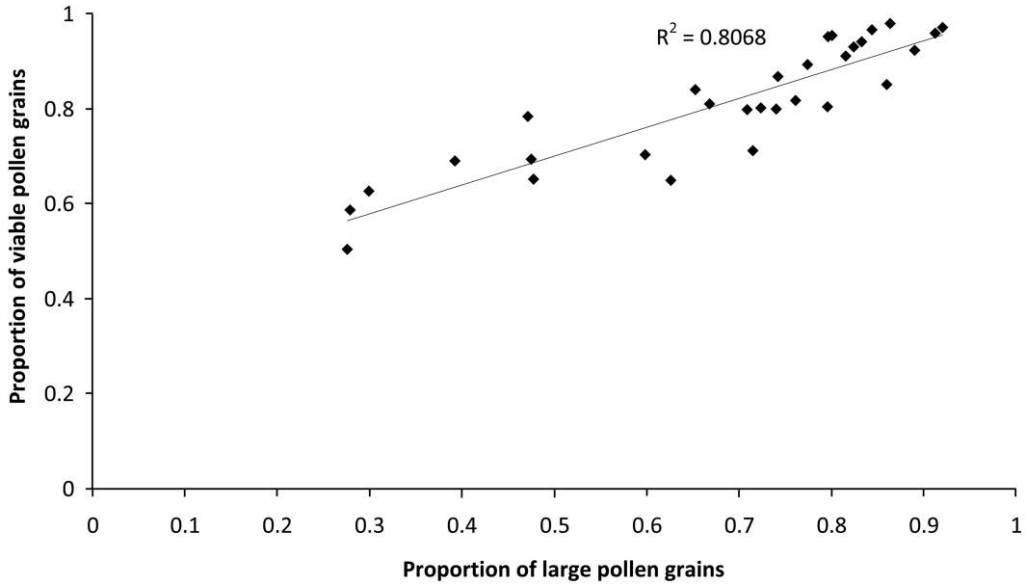

Fig. 2 Correlation between the proportion of large pollen grains, obtained with the particle counter (*X*-axis), and the proportion of viable pollen grains, from observation with Alexander stain (*Y*-axis). This analysis was carried out on 30 individual plants (comprising both hermaphrodites and gynomonoecious individuals), from which both measures were taken from flowers that opened at the beginning of the flowering season.

fruit set and seed set in natural populations of *S. nutans* in order to investigate whether these differences in flower size lead to a decrease in pollinator attraction and in seed set for female individuals.

Nonetheless, in many gynodioecious species, females have been found to compensate for the loss of their male reproductive function by increasing their female reproductive success commonly in terms of seed set, fruit set, or seed quality (Kohn 1989; Asikainen and Mutikainen 2003; Delph and Mutikainen 2003; Chang 2006). Such female advantage can result either from resource allocation from male to female function or from an avoidance of self-pollination and associated inbreeding depression (Agren and Wilson 1991; Sakai et al. 1997; Barr 2004; Ramsey et al. 2006). In *S. nutans*, although females produced smaller and lighter flowers and could subsequently benefit from higher resources to invest in seed production, no such female advantage was detected in terms of seed mass, germination rate, or offspring quality. It is, however, difficult to interpret these results because of the reduced statistical power of our analyses due to the small number of included females. Future works will attempt to enlarge the sample size and investigate the possible differences in both seed number and seed quality between females and other sex morphs, with special attention on the effect of the breeding treatment. Because this study showed evidence for strong inbreeding depression, one should expect a female advantage to be found when comparing obligatory outcrossed female progenies and inbred offspring from hermaphrodites and gynomonoecious individuals. Moreover, because theory predicts sex ratio in populations to be correlated with the magnitude of female advantage (Bailey et al. 2003; Dufay et al. 2007), the overall low frequency of females in *S. nutans* could

Table 5

Results of ANOVA (PROC GLM) of Dry Mass on 8-wk-old Offspring Produced by Hermaphrodites and Gynomonoecious Plants in Two Different Growth Conditions

Source of variation	df	MS	*F*	*P*	Effect
Mother (population × gender)	25	.31	10.89	<.0001	
Growth conditions	1	1.40	49.60	<.0001	Condition 1 > condition 2
Breeding treatment	1	1.21	42.75	<.0001	Cross > self
Population	4	.24	1.08	.3858	
Maternal gender	1	.02	.10	.7542	
Population × maternal gender	1	.34	1.89	.1798	
Error	747	.02			

Note. Condition 1: temperature between 21° and 25°C with regular water supply. Condition 2: temperature between 15° and 25°C with water supply every 5 d only. Both the identity of the mother plant, nested in population × maternal gender, and population were coded as random factors. Interactions between main factors were not found significant.

actually be due to a low magnitude female advantage. If so, this would have reduced the probability of detection of any statistical difference between females and hermaphrodites, in particular on such a restricted data set.

Inbreeding Depression and Consequences for the Reproductive System

We found evidence for severe inbreeding depression in gynomonoecious and hermaphroditic lineages in controlled conditions for both pre- and postdispersal traits. Selfing resulted in a decrease of both seed mass and seed germination when compared within each maternal progeny. While seed mass affected seed germination, it was not the only factor that explained the difference in germination between selfed and outcrossed seeds, suggesting that other mechanisms than seed provision are involved in the effect of the breeding treatment. Inbreeding depression was also found when measuring offspring vegetative growth in both optimal and more restrictive growth conditions. In both environments, outcrossed progeny reached larger vegetative size and dry mass than selfed progeny. Because one of the environmental conditions tested in this study was closer to natural conditions in terms of temperature and water supply, this suggests that such inbreeding depression should also be found in natural populations. Furthermore, although an effect of the environmental conditions was consistently shown on vegetative growth, we found no interaction between the environment and the breeding treatment, indicating that outcrossed progeny did not show a better resistance to stressful conditions compared with selfed progeny.

Overall, the value of inbreeding depression, based on the inbred/outbred differences in cumulative fitness for early stages of life cycle, was found to be quite severe (0.3), similar to results found in other gynodioecious species (Mutikainen and Delph 1998; Delph 2004; Chang 2007). Furthermore, the measurement of offspring dry mass prevented the observation of selfed and outcrossed progeny in the later steps of their life cycle. Hence, inbreeding depression in *S. nutans* may be stronger than estimated by this study, particularly if vegetative growth at later stages and flowering probability are also affected, as shown in other *Silene* species (Mutikainen and Delph 1998; Emery and McCauley 2002; Glaettli and Goudet 2006).

In self-compatible gynodioecious species, inbreeding depression is an important parameter to consider because female fitness of female plants should be increased compared with hermaphrodites, providing cytoplasmic male sterility genes with a selective advantage. To evaluate the role of inbreeding depression for sex polymorphism in *S. nutans*, future studies will have to measure the actual selfing rate in natural populations. Although the large floral display in *S. nutans* (up to more than 200 flowers; M. Dufay, personal observations) is expected to increase the probability of geitonogamy, the natural rate of self-pollination could be limited by the ability of maternal choice between self- and cross-pollen, as shown in the same species by Hauser and Siegismund (2000).

Gynomonoecious Plants in Silene nutans

Gynomonoecious plants were found to be frequent in *S. nutans* and to form a heterogeneous category according to the proportion of female flowers. These results are similar to results found for other gynodioecious-gynomonoecious *Silene* species (Shykoff 1992; Maurice 1999; Guitian and Medrano 2000). Overall, the few studies that have attempted to compare gynomonoecious individuals with other sex morphs have found that they were intermediate between females and hermaphrodites either for the number of flowers (Poot 1997; Lafuma and Maurice 2006), seed set, or fruit set (Agren and Willson 1991; Lafuma and Maurice 2006) or for offspring quality (Delph and Mutikainen 2003). In *S. nutans*, we found female and perfect flowers of gynomonoecious plants to be statistically intermediate for flower mass and petal size, compared with flowers of females and hermaphrodites. More interestingly perhaps, we found the gynomonoecious category to be heterogeneous not only for their relative proportion of female and perfect flowers but also for their floral traits: gynomonoecious plants that carried a high proportion of perfect flowers had perfect flowers that resemble more those of hermaphrodites, with larger petals and heavier flowers. To our knowledge, such correlation has not been investigated in other gynodioecious-gynomonoecious species. Because it stresses a high variance within the gynomonoecious category, this could indeed partially explain why gynomonoecious plants are often described as statistical intermediates for their floral traits or for plant fitness.

In this study, we also found a difference between gynomonoecious plants and hermaphrodites for pollen production. Even without considering male fitness at the level of the individual, which was consistently found to be in favor of hermaphrodites that produce pollen from all flowers, we found pollen quality to be higher in hermaphrodites compared with gynomonoecious plants. In *S. italica*, Lafuma and Maurice (2006) found similar results, although this difference was not found in all plant families. These results raise the question of both the conditions of maintenance of gynomonoecious plants in populations and of the determination of their gender morph.

Several hypotheses have been proposed for the determination of the gynomonoecious morph, including the effect of environmental factors (e.g., gynomonoecious-gynodioecious *S. italica*: Maurice 1999; gynomonoecious *Silene noctiflora*: Folke and Delph 1997), and partial restoration of male fertility (Koloewijn and Van Damme 1996). These two hypotheses are nonexclusive: if multiple nuclear genes are involved in the restoration of male fertility, sex is a quantitative trait, the expression of which may be affected by environmental factors (Koelewijn and Van Damme 1996). In this study, half of gynomonoecious plants carried few female flowers (from 3% to 19%), which generally opened at the beginning of the flowering period; these plants resembled hermaphrodites in terms of both pollen quality and flower size. In the other half, the proportion of female flowers quantitatively varied from 30% to 90%, with this proportion being inversely correlated with flower size and pollen quality. These results suggest that restoration of male fertility could be quantitative and involve multiple nuclear loci. In some other gynodioecious species, pollen viability and/or anther development and dehiscence vary quantitatively among individual plants while being apparently constant within each plant (*Plantago coronopus*: Koelewijn and Van Damme 1996; *Thymus vulgaris*: Thompson et al. 2002; *Beta vulgaris*: Dufay et al. 2008). Ehlers et al.

(2005) suggested that such interindividual variation was the likely result of a polygenic restoration of male fertility. In *S. nutans*, such quantitative determination of restoration could affect both the development of anthers within some of the flowers (perfect vs. female) and the quality of pollen produced within perfect flowers in individuals that do not carry all restorer alleles at the different loci.

To date, only Bailey and Delph (2007) investigated the consequences of polygenic restoration of male fertility. However, because their study considered restoration as a threshold trait, it could not apply to species in which male fitness quantitatively varies among individual plants. Theoretical studies are thus needed to investigate whether gynomonoecious plants should be found at equilibrium in natural populations. Indeed, gynomonoecious plants could be a simple by-product of a quantitative determination of sex; during the phase of selection of restorer alleles, one expects to find genotypes carrying only a fraction of the restorer alleles. Gynomonoecious individuals should thus be found only during transitory phases, which are then replaced by fully restored hermaphrodites as long as restorer alleles increase in frequency. Alternatively, to explain the occurrence (and sometimes the large frequencies) of gynomonoecious plants in gynodioecious plant species, Desfeux (1996) postulated that gynomonoecy could be a bet-hedging strategy. Under this hypothesis, gynomonoecious individuals would gain some advantage in female fitness (saving resources by producing less pollen than hermaphrodites) while being protected against strong pollen limitation. This is at least partially consistent with the findings of Davis and Delph (2005). Carrying out on gynomonoecious *S. noctiflora*, this study showed that perfect flowers were capable of autonomous selfing, providing reproductive assurance when pollination is low, whereas female flowers produced only outcrossed seeds that avoided the cost of inbreeding depression but depended on pollinator availability. Even if such bet-hedging advantage has not been found in gynomonoecious plants within gynodioecious species, one can imagine similar processes to occur.

The dynamics of sex ratios within populations as well as the occurrence of gynomonoecious plants at equilibrium should strongly depend on male and female fitness components of these intermediates compared with females and hermaphrodites. In *S. nutans*, we found that gynomonoecious plants should experience a reduction in male fitness compared with hermaphrodites by producing less attractive flowers and lower pollen quantity and quality. On the other hand, no advantage in female fitness was found for gynomonoecious plants compared with hermaphrodites in terms of either seed quality or the magnitude of inbreeding depression. As mentioned, the consequences of inbreeding depression strongly depend on the value of selfing rates in natural populations. This holds to explain the maintenance of both females (thereby avoiding the severe inbreeding depression found in this study and consequently benefiting from fitness advantages compared with other sex morphs) and gynomonoecious plants as they compete with hermaphrodites. Whether hermaphroditic and gynomonoecious individuals experience the same rate of self-pollination should therefore be crucial to maintain the polymorphism. At this point, no clear predictions can be made; while one could have guessed that selfing through geitonogamy is less likely in gynomonoecious plants (that carry less perfect flowers), Collin and Shykoff (2003) found no such difference in *Dianthus sylvestris*. Much additional information is therefore needed to understand both the determination and the conditions of maintenance of intermediate sex morphs in both *S. nutans* and other gynodioecious species.

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Résumé

Les angiospermes présentent une diversité importante de leurs systèmes de reproduction. La gynodioécie – la coexistence d'individus mâles stériles dits femelles et des individus hermaphrodites- est un des systèmes les plus communs après l'hermaphrodisme. L'objectif de ma thèse était de déterminer les forces évolutives impliquées dans le maintien d'un tel polymorphisme sexuel et d'étudier certaines conditions nécessaires à l'occurrence d'une meilleure performance des individus femelles condition nécessaire au maintien de la gynodioécie dans les populations de *Silene nutans*. Par une approche de génomique des populations j'ai comparé le polymorphisme des trois génomes (nucléaire, mitochondrial et chloroplastique) d'une espèce gynodioïque, *Silene nutans* et une espèce dioïque proche, *Silene otites*. J'ai montré que *S. nutans* avaient plus d'haplotypes cytoplasmiques et que la diversité cytoplasmique était plus élevée chez *S. nutans* que chez l'espèce dioïque, *S. otites*, suggérant que le maintien des facteurs génétiques de la gynodioécie est sous l'action d'une sélection fréquence-dépendante négative Un deuxième volet de ma thèse concerne l'occurrence et la variation de l'amplitude de l'avantage femelle. Grâce à une approche de biologie et de génétique des populations, utilisées en populations expérimentales et naturelles, j'ai comparé le succès reproducteurs des femelles et hermaphrodites. J'ai montré qu'en conditions expérimentales, les femelles bénéficient d'un avantage sélectif modéré quand elles sont rares et que cet avantage disparait a quand les femelles sont fréquentes. D'autre part, cet avantage semble dépendre de l'amplitude de la limitation pollinique, des taux d'autofécondation des hermaphrodites et de la dépression de consanguinité. J'ai également montré que les taux d'autofécondation étaient très variables entre les individus en populations expérimentaleet naturelle. Par ailleurs, j'ai montré qu'en population naturelle les plantes souffrent d'une forte limitation pollinique et que les flux de pollen dans cette population était majoritairement restreint dans l'espace. J'ai aussi montré que le déterminisme de la gynodioécie dans cette population naturelle est purement nucléaire. Enfin, j'ai mis en évidence une meilleure contribution des pollinisateurs nocturnes au succès reproducteur chez *S. nutans* par rapport aux pollinisateurs diurnes de cette espèce dans une population expérimentale.

Mots clés : gynodioécie, *Silene nutans*, sélection fréquence-dépendante, avantage femelle, sexe ratio, limitation pollinique, autofécondation, flux de gènes, syndrome de pollinisation nocturne

Abstract

Angiosperm presents an important diversity of their sexual systems. The gynodioecy- the coexistence of females and hermaphrodite individuals- is one of the most common breeding systems after hermaphroditism. The objective of my thesis was to determine the evolutionary processes maintaining gynodioecy in *Silene nutans* and to study essential conditions for the occurrence of a better performance of female plants, important condition in the dynamics of gynodioecy. By a population genomic approach, I explored the polymorphism of three genomes (nuclear, mitochondrial and chloroplastic) of one gynodioecious species, *Silene nutans* and a dioecious species, *Silene otites*. I showed that *S. nutans* had more cytoplasmic haplotypes, and higher cytoplasmic diversity, than the dioecious relative, *S. otites* suggesting that the maintenance of gynodioecy is under negative frequency-dependent selection. A second part of my thesis concerns the occurrence and variation of the magnitude of female advantage. By population biology and population genetics approaches, used in experimental and natural populations, I compared the reproductive success of females and hermaphrodites. I showed that in experimental conditions, female individuals had moderate female advantage when they were rare and that this advantage was lost when females were common. On the other hand, this advantage seemed to depend on the magnitude of pollen limitation, selfing rate and inbreeding depression. I have also showed that self-fertilization rates were highly variable between individuals in both experimental and natural populations. Moreover, I have showed that plants in natural population suffered from severe pollen limitation and that pollen flow in this population was mostly limited in space. I also showed that the determinism of gynodioecy in this natural population is purely nuclear. Finally, I found a greater contribution of nocturnal pollinators to reproductive success in *S. nutans* in experimental population compared to diurnal ones.

Keywords: gynodioecy, *Silene nutans*, frequency-dependent selection, female advantage, sex-ratio, pollen limitation, selfing, genes flow, nocturnal pollination syndrome