

The Effect of Breeding System on Polymorphism in Mitochondrial Genes of *Silene*

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ABSTRACT

Gynodioecy is a breeding system characterized by the co-occurrence of hermaphrodite and female individuals, generally as the result of nuclear–cytoplasmic interactions. The question remains whether the genetic factors controlling gynodioecy are maintained in species over long evolutionary timescales by balancing selection or are continually arising and being replaced in epidemic sweeps. If balancing selection maintains these factors, then neutral cytoplasmic diversity should be greater in gynodioecious than hermaphroditic species. In contrast, epidemic sweeps of factors controlling gynodioecy should decrease cytoplasmic diversity in gynodioecious relative to hermaphroditic species. We took a comparative approach in which we sequenced two mitochondrial genes, cytochrome b (*cob*) and cytochrome oxidase (*coxI*), for multiple populations of several hermaphroditic, gynodioecious, and dioecious species in the genus *Silene*. Breeding system was predictive of polymorphism. Gynodioecious species harbor many old haplotypes while hermaphroditic and dioecious species have little to no nucleotide diversity. The genealogical structure of neither gene departed from neutral expectations. Taken together, our results suggest that balancing selection acts on cytoplasmic male-sterility factors in several gynodioecious species in the genus.

ALTHOUGH most flowering plants have a hermaphroditic breeding system, 5–10% of species exhibit gynodioecy, in which female and hermaphrodite plants coexist in populations (DARWIN 1877; RICHARDS 1997). In the majority of gynodioecious species, gender is determined by the interaction of cytoplasmic determinants of male-sterility (mitochondrial genes) with nuclear genes: the male-sterility genes prevent successful pollen production, while the nuclear genes restore male fertility (CHASE and GABAY-LAUGHNAN 2004). The dynamics of this nuclear–cytoplasmic interaction can be understood in light of the theory of genomic conflict (COSMIDES and TOOBY 1981; SAUMITOU-LAPRADE *et al.* 1994; FRANK 2000), because the inheritance mode of cytoplasmic factors (uniparental) and nuclear genes (biparental) generates a situation where selective interests are in opposition, as follows. Any mitochondrial genetic variant that reduces allocation to pollen in exchange for increased seed production or seed quality will be favored, since mitochondrial genes are transmitted only through seeds. In contrast, reallocation of resources from pollen to seeds will reduce the transmission of nuclear genes, because biparental transmission depends on success through both seeds and

pollen. Consistent with this idea of conflict, nuclear genes often restore male fertility by overcoming the male-sterility effects of the cytoplasm by altering transcription or translation (CHASE 2007; DELPH *et al.* 2007).

One major question that remains is whether the genetic factors controlling nuclear–cytoplasmic gynodioecy are maintained in species over long evolutionary timescales (via balancing selection) or are continually arising and being replaced either locally or globally (epidemic dynamics). The two alternative scenarios will have different effects on any cytoplasmic neutral-locus diversity, as these loci will be linked to the male-sterility genes given uniparental inheritance and the absence of recombination. In the case of haplotypes being maintained over a long period of time through balancing selection, diversity is expected to be high, because of the accumulation of mutations (HUDSON and KAPLAN 1988; STÄDLER and DELPH 2002), with a genealogical structure of cytoplasmic loci close to neutral expectation (TAKAHATA 1990). Conversely, epidemic dynamics would induce a lower diversity, since under this scenario new sterilizing cytoplasms will continuously arise and sweep through populations, replacing the former cytoplasms (CHARLESWORTH 2002; INGVARSSON and TAYLOR 2002).

The genus *Silene* contains species with a variety of breeding systems, including ones that are hermaphroditic, gynodioecious, and dioecious (DESFEUX *et al.* 1996). This diversity of breeding systems allows application of the comparative method to test the question of whether balancing selection or epidemics have a pre-

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dominant effect on the evolutionary dynamics of sex determination in gynodioecious species. Previous studies on cytoplasmic diversity in *Silene* have led to contradictory conclusions. Sequences of the mitochondrial gene *cob* in the gynodioecious species *Silene acaulis* revealed unexpectedly high nucleotide polymorphism, and highly divergent haplotypes within the species, suggesting that balancing selection maintains polymorphism over a long period of time (STÄDLER and DELPH 2002). In contrast, a comparison of chloroplast and nuclear gene diversity in gynodioecious *S. vulgaris* and dioecious *S. latifolia* revealed a lower chloroplastic diversity in *S. vulgaris*, suggesting recurrent selective sweeps caused by epidemic dynamics (INGVARSSON and TAYLOR 2002).

Either balancing selection or episodic sweeps acting on genetic factors underlying gynodioecy should alter patterns of nucleotide diversity at loci linked to these factors in gynodioecious relative to non-gynodioecious species. Thus, we predicted that the level and pattern of polymorphism in mitochondrial genes would vary by breeding system. We took a comparative approach in which we sequenced two mitochondrial genes, cytochrome b (*cob*) and cytochrome oxidase (*coxI*), for multiple populations of several hermaphroditic, gynodioecious, and dioecious species. From these sequences we evaluated the number of haplotypes per gene for each species, how these haplotypes clustered by species, and whether recombination events were likely to have contributed to haplotype diversity, and we estimated the coalescent time of haplotypes and performed neutrality tests. Taken together, our results support the hypothesis that balancing selection, rather than episodic selective sweeps, largely drives the evolutionary dynamics of cytoplasmic male-sterility factors in this genus.

MATERIALS AND METHODS

We sampled *Silene* species to include four hermaphroditic, three gynodioecious, and three dioecious species (Table 1) and to include different clades from within the genus. The gynodioecious species used belonged to two different clades that were estimated to have differentiated 8.21 million years ago (MYA), with *S. nutans*/*S. acaulis* divergence time estimated to be 1.30 MYA. Non-gynodioecious species belonged to three different clades, with divergence time from 4.66 to 4.81 MYA (MOWER *et al.* 2007; Figure 1). When possible, several populations per species from different geographical regions were sampled (with an average of five populations per species), and several individuals per population were sequenced (with an average of 5 individuals per population), for a total of 241 individuals. One accession of *Gypsophila repens* was also sequenced as a Caryophyllaceae outgroup species.

Molecular analysis: Two mitochondrial genes were sequenced, the cytochrome b gene (*cob*) and the gene coding for the first subunit of cytochrome oxidase (*coxI*). We chose these genes because they are described as being exclusively mitochondrial, with no known transfer to the nuclear genome (GRAY *et al.* 1999; ADAMS *et al.* 2002).

Total genomic DNA was extracted and purified from fresh leaves using the DNeasy Plant Mini Kit (QIAGEN, Valencia, CA) according to manufacturer's instructions (QIAGEN). Each reaction was performed using 35 cycles of 45 sec at 94°, 45 sec at 53°, and 1 min at 72°, with an initial step of 5 min at 95° and a final step of 10 min at 72°. Each gene was amplified with two pairs of primers generating overlapping fragments. For *cob*, for all *Silene* species except for *S. noctiflora* (for a sequence length of 1041 bp), the primers were CobF1, AGCATTGATAGATTATCCAACC/CobR717, GATGCCCCA AAACATTAGGA and CobF362, TTGGGGTCAGATGAGC TTTT/CobR1084, ATTCTTCTTCCAACCTCGTCC. For *coxI*, for all *Silene* species except for *S. noctiflora* (for a sequence length of 1037 bp) the primers were Cox1F1, GGAGCAGTT GATTTAGCCAT/Cox1R663, CCCAGAATTTGCCAGGACTA and Cox1F473, TTGATACCCGCGCTTACTTC/Cox1R1077, CCATTCCAGTGTGGGTGAAT. Because of its high divergence from the other species, specific primers were developed for *S. noctiflora*. For *cob* (for a sequence length of 883 bp) the primers were SnCob1F11, TGAGTTATTGGTGGGGCTTC/SnCobR694, GATGCCCCAAAAGATTAGGA and SnCobF348, GATGAGCTTCTGGGGAGCAAC/SnCobR933, ACAATCTG CAAAAGCAACC. For *coxI* (for a sequence length of 1003 bp) the primers were SnCox1nF27, TCTTCATCTCTCT GGTGTTTCATC/SnCox1R629, CCGACGGTGAACAAAAA GAT and SnCox1F473, TTGATACCCGCGCTTACTTC/SnCox1R1073, TCCAGTGTGGGGCAATTAGA.

PCR products were purified using a QIAquick PCR purification kit (QIAGEN) and directly sequenced on both strands, using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Norwalk, CT) and an ABI 3730 (Applied Biosystems, Foster City, CA) at the Indiana Molecular Biology Institute. When a haplotype was found only once, it was confirmed by sequencing from an independent PCR reaction. Sequences newly determined in this study were deposited in GenBank under accessions EU883246–EU883282 for *cob* sequences and EU883283–EU883309 for *coxI* sequences. Additional nucleotide sequences of another caryophyllid outgroup, *Beta vulgaris* (Chenopodiaceae), were obtained from GenBank (*cob* and *coxI*, accession BA000009). Additional *cob* and *coxI* amino acid sequences were obtained from the alignment of the online resource PREP-Mt (<http://www.prep-mt.net>; MOWER 2005).

Statistical analyses: Sequence alignment was performed manually using Bioedit version 7.0.5.3 (HALL 1999). Plant mitochondrial and chloroplast transcripts are known to undergo post-transcriptional C–U editing at nonsynonymous 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 nonsynonymous 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 0.5. The following summary statistics were calculated using DnaSP version 4.10.9 (ROZAS *et al.* 2003): Θ_{ws} , diversity per site estimated from the total number of mutations (WATTERSON 1975); π , diversity as the average number of nucleotide differences per site between a pair of randomly chosen sequences (NEI 1987); K_s , the average number of pairwise synonymous substitutions per synonymous site between (different) haplotypes; and K_a , the average number of pairwise nonsynonymous substitutions per nonsynonymous site between (different) haplotypes. We also used estimates of absolute synonymous substitution rates (referred to as Rs) from a previous study (MOWER *et al.* 2007).

TABLE 1
Description of samples sequenced for *cob* and *coxI*

Species	Breeding syst	Location	Code	<i>N</i>	<i>N</i> _{pop}	Total <i>N</i>				
<i>S. acaulis</i>	GD	Iceland B	ISLB	5	4	19				
		Iceland V	ISLV	5						
		Greenland	GRL	6						
		Pas de Cheville, Switzerland	Pas	3						
<i>S. vulgaris</i>	GD	Prague, Czech Republic	Pra	5	10	46				
		Seefeld, Austria	See	9						
		Bennington, Vermont	Ben	9						
		Ontario, Canada	Ont	4						
		Stamford, New York	Sta	7						
		Torla, Spain	Tor	1						
		Puerto de Cotefablo, Spain	Pue	3						
		Virginia	Va	6						
		Dijon, France	Dij	1						
		Sussex, United Kingdom	Sus	1						
		<i>S. nutans</i>	GD	Queyras, France			Que	9	5	23
				Auvergne, France			Auv	6		
Dordogne, France	Dor			6						
Jura, France	Jur			1						
Finland	FIN			1						
<i>S. latifolia</i>	D	Alençon, France	Ale	10	8	59				
		Larche, France	Lare	10						
		Prague, Czech Republic	Pra	10						
		San Valentin, Austria	San	10						
		Vieira do Minho, Portugal	Vie	10						
		Authie, France	Aut	8						
		Villeneuve d'Ascq, France	Vil	1						
		Toulouse, France	Tou	1						
<i>S. dioica</i>	D	Edinburgh, United Kingdom	Edi	3	3	5				
		Cumbria, United Kingdom	Cum	1						
		Switzerland	CHE	1						
<i>S. diclinis</i>	D	Xativa A, Spain	XatA	8	5	37				
		Xativa B, Spain	XatB	9						
		Buixcano, Spain	Bui	9						
		Playa de Mora A, Spain	MorA	7						
		Playa de Mora C, Spain	MorC	4						
<i>S. virginica</i>	H	Yellowwood, Indiana	Yel	6	4	24				
		Morgan Monroe, Indiana	Mor	6						
		Giles A, Virginia	GilA	6						
		Giles B, Virginia	GilB	6						
<i>S. douglasii</i>	H	Var. <i>douglasii</i> I11	douI	2	4	5				
		Var. <i>douglasii</i> B13	douB	1						
		Var. <i>ovaria</i>	ova	1						
		Var. <i>rupinae</i> WF7.3	rup	1						
<i>S. scouleri</i>	H	Fairplay, Colorado	Col	10	1	10				
<i>S. noctiflora</i>	H	Virginia	Va	7	4	13				
		Lund, Sweden	Lun	2						
		The Netherlands	NLD	1						
		Romania	ROU	3						

For each species, the breeding system (Breed syst: GD, gynodioecy; D, dioecy; H, hermaphroditic), the geographical location of populations, the location code, the number of individuals sampled per population (*N*), the number of populations per species (or varieties for *douglasii*) (*N*_{pop}), and the total number of individuals analyzed (Total *N*) are listed.

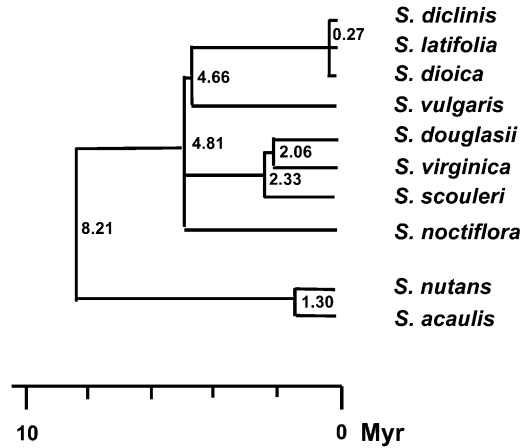


FIGURE 1.—Chronogram of *Silene* species analyzed in the study, based on chloroplastic sequences (adapted from MOWER *et al.* 2007). Estimated node ages are indicated.

We performed the following neutrality tests: Tajima's D (TAJIMA 1989), based on the difference between estimators of diversity using the average pairwise nucleotide difference (π) and the number of segregating sites (Θ_w); Fu and Li's D with an outgroup (*B. vulgaris*) (FU and LI 1993), the test statistic being based on the differences between the total number of mutations in the external branches of the genealogy and the total number of mutations; the K -test (DEPAULIS and VEUILLE 1998), which compares the observed number of haplotypes *vs.* the range of the expected number of haplotypes (calculated using 1000 neutral coalescent simulations); and the four-gamete test for recombination (HUDSON and KAPLAN 1985). Recombination was not included in the confidence interval simulations for the K -test to generate a conservative test for the minimum number of haplotypes.

The effect of breeding system (gynodioecious, dioecious, hermaphrodite) as well as the position in the phylogenetic tree (four branches from Figure 1: branch 1, *S. diclinis*, *S. latifolia*, *S. dioica*, and *S. vulgaris*; branch 2, *S. douglasii*, *S. virginica*, and *S. scouleri*; branch 3, *S. noctiflora*; and branch 4, *S. nutans* and *S. acaulis*) was estimated by a Kruskal–Wallis test using Minitab version 13.20 (Minitab).

Neighbor-joining (NJ) trees were built on *cob* and *cox1* sequences, using the software MEGA version 3.1 (KUMAR *et al.* 2004) with Kimura's two-parameter model (KIMURA 1980). Then the NJ trees were analyzed using Tree-puzzle (SCHMIDT *et al.* 2002) at the Mobyle portal (<http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py>), with a maximum-likelihood (ML) method using the Hasegawa–Kishino–Yano nucleotide model (HKY85) (HASEGAWA *et al.* 1985) and the different rate-heterogeneity models proposed by Tree-puzzle (uniform rate, eight discrete gamma-distributed rates, one invariable and one variable rate, a mixed model with one invariable rate and eight gamma-distributed rates). Then, for each rate-heterogeneity model tested, the likelihood-ratio test (LRT) of the molecular clock (FELSENSTEIN 1981) was conducted. Note that while only the results on the mixed model with one invariable rate and eight gamma-distributed rates are given (with the highest likelihood), the other models generated similar outputs. The test was applied on three data sets per gene. First we tested the hypothesis using the whole sequence data set on *cob* or *cox1* (only on a set of unique sequences), with *B. vulgaris* as the outgroup. Second, using DnaSP version 4.10.9 (ROZAS *et al.* 2003) we generated sequence alignments composed of only fourfold degenerated sites to keep only neutral sites. Third, we ran the same test, "fixing" the polymorphic sites that were

suspected to have recombined by the four-gamete test (changed into N in the whole alignment).

For *cob* and *cox1*, we compared the topologies of the NJ trees with the tree where *S. nutans* and *S. acaulis* haplotypes were constrained to be clustered by species, using the Kishino–Hagesawa test (KISHINO and HAGESAWA 1989). Trees were drawn using the software MEGA version 3.1 (KUMAR *et al.* 2004).

RESULTS

The sequencing of near full-length *cob* and *cox1* for a large sample composed of four hermaphrodite, three gynodioecious, and three dioecious *Silene* species revealed striking differences in nucleotide polymorphism, with gynodioecious species exhibiting more segregating sites and more haplotypes than non-gynodioecious species (Table 2). *Cob* and *cox1* haplotype structures are given in Tables 3 and 4, respectively. As *cob* and *cox1* from *S. noctiflora* exhibited high divergence relative to the other species in the genus, the haplotypes for *S. noctiflora* were not included in Tables 3 and 4. Briefly, *S. noctiflora* exhibited only one haplotype in *cob* and two haplotypes in *cox1* with only one segregating site. When compared to *S. latifolia*'s most common haplotypes, 91 of 883 sites were found to differ between *S. noctiflora* and *S. latifolia* for *cob*, and 94 of 1003 sites differed for *cox1*.

***Cob* structure in *Silene*:** Over the nine remaining *Silene* species, sequencing of the near-full-length *cob* gene (1041 bp) revealed 30 segregating sites, resulting in 32 *cob* haplotypes (Table 3). While hermaphroditic and dioecious species were fixed for a single haplotype (with the exception of *S. diclinis*), all three gynodioecious species were found to be polymorphic for *cob*.

The dioecious species *S. latifolia* and *S. dioica* were fixed for the same haplotypes for *cob*_1, which was also shared with the dioecious species *S. diclinis*. *Silene diclinis*, a species endemic to the Valencia region in Spain (PRENTICE 1976), was the only dioecious species to exhibit multiple haplotypes: one nonsynonymous mutation separated *cob*_1 from *cob*_2 (T/G₅₉₂), and the latter haplotype was found only in the Buixcano population where it was fixed. The hermaphroditic species *S. virginica*, *S. scouleri*, and *S. douglasii* exhibited only 1 *cob* haplotype, with *S. scouleri* and *S. douglasii* sharing the same haplotype (*cob*_3).

For the gynodioecious species *S. vulgaris*, four haplotypes were revealed from 46 individuals from 10 populations from the European and North American continents (Table 1). The number of haplotypes per population varied from one [in Puerto de Cotefablo, Spain (Pue); Ontario, Canada (Ont); and Seefeld, Austria (See)] to three [in Stamford, NY (Sta)]. Haplotypes *cob*_1 (shared with the three dioecious species), *cob*_5, and *cob*_7 were found in more than two populations (5, 7, and 2, respectively), without clear geographical structuring.

TABLE 2
Diversity measures of *cob* and *cox1* in *Silene* and results from neutrality tests

Species	BS	Pop	<i>N</i>	<i>H</i>	<i>S</i>	Θ_w ($\times 10^3$)	$\pi \pm$ SD ($\times 10^3$)	K_s ($\times 10^3$)	K_a ($\times 10^3$)	K_a/K_s	Tajima's <i>D</i>	Fu and Li's <i>D</i> (beet)	<i>K</i> -test
<i>cob</i>													
<i>S. latifolia</i>	D	7	59	1	0	0	0	0.00	0.00				
<i>S. dioica</i>	D	3	5	1	0	0	0	0.00	0.00				
<i>S. diclinis</i>	D	5	37	2	1	0.23	0.36 \pm 0.07	0.00	1.26		0.85	0.56	2_2
<i>S. douglasii</i>	H	4	5	1	0	0	0	0.00	0.00				
<i>S. scouleri</i>	H	1	10	1	0	0	0	0.00	0.00				
<i>S. virginica</i>	H	4	24	1	0	0	0	0.00	0.00				
<i>S. noctiflora</i>	H	4	13	1	0	0	0	0.00	0.00				
<i>S. vulgaris</i>	GD	10	46	4	4	0.87	1.46 \pm 0.09	7.50 (4.02)	0.84	0.11	1.55	-0.42	3_5
<i>S. acaulis</i>	GD	4	19	13	21	6.05	5.19 \pm 0.52	11.81 (3.8)	3.67	0.31	-0.55	-1.03	5_10*
<i>S. nutans</i>	GD	5	23	12	15	4.16	2.82 \pm 0.54	10.36 (7.09)	2.11	0.20	-1.16	-0.39	5_13
<i>cox1</i>													
<i>S. latifolia</i>	D	8	20	3	2	0.54	0.60 \pm 0.09	5.29 (5.36)	0.00	0.00	0.24	-0.66	2_3
<i>S. dioica</i>	D	2	2	1	0	0	0	0	0.00				
<i>S. diclinis</i>	D	2	3	1	0	0	0	0	0.00				
<i>S. douglasii</i>	H	2	2	1	0	0	0	0	0.00				
<i>S. scouleri</i>	H	1	2	1	0	0	0	0	0.00				
<i>S. virginica</i>	H	4	4	1	0	0	0	0	0.00				
<i>S. noctiflora</i>	H	4	10	2	1	0.35	0.35 \pm 0.16	4.10	0.00	0.00	0.01		2_2
<i>S. vulgaris</i>	GD	8	16	2	1	0.29	0.12 \pm 0.10	4.00 (4.02)	0.00	0.00	-1.16	-1.52	2_2
<i>S. acaulis</i>	GD	4	16	6	8	2.32	1.38 \pm 0.35	9.26 (9.37)	0.43	0.05	-1.46	-0.97	3_8
<i>S. nutans</i>	GD	5	21	10	14	3.75	3.42 \pm 0.31	12.11 (6.42)	0.97	0.08	-0.32	0.73	4_11

For each species, breeding system (BS, with GD, gynodioecy; D, dioecy; H, hermaphroditic), number of populations per species (Pop), number of individuals analyzed (*N*), number of haplotypes (*H*), number of segregating sites (*S*), diversity per site estimated from the total number of mutations (Θ_w), diversity as the average number of nucleotide differences per site between a pair of randomly chosen sequences (π), interhaplotype average number of pairwise synonymous substitutions per synonymous site (K_s) and in parentheses K_s estimated on the "nonrecombination" data set, and interhaplotype average number of pairwise non-synonymous substitutions per nonsynonymous site (K_a) are listed. Significance for *K*-test: **P* < 0.05.

For the gynodioecious species *S. acaulis*, the sampling revealed a total of 13 *cob* haplotypes from 19 individuals from 4 European populations (Table 1). The number of haplotypes per population varied between 1 [in Switzerland (CHE)] and 5 [in Greenland (GRL) and Iceland B (ISLB)]. Two haplotypes (*cob*_9 and *cob*_13) were shared by 2 populations from Greenland and Iceland (ISLB). Among the 12 haplotypes found in the present study, 6 haplotypes had been found in a previous study (STÄDLER and DELPH 2002). In total, from both studies, there are 16 different haplotypes among 78 individuals studied from a total of 10 populations (4 from North America, 6 from Europe). At least 6 haplotypes are found in >1 population, 3 of which are found in Europe and North America: *cob*_9 (ex AK-3 from STÄDLER and DELPH 2002) found in Alaska, Iceland, and Greenland; *cob*_19 (ex AK-2) found in Alaska and Greenland; and *cob*_20 (ex CO-2) found in Colorado, Alaska, and Greenland.

For the gynodioecious species *S. nutans*, despite a geographically limited sampling (four populations in France, separated from each another by an average of 500 km, and one in Finland), a total of 12 haplotypes from 23 individuals were observed. The number of haplotypes per population varied between 1 [in Auvergne, France (Auv) and Dordogne, France (Dor)] and 9 [in Queyras, France (Que)]. Haplotype *cob*_22 was shared among three populations [Dor; Jura, France (Jur); and Que].

From the observed diversity in *cob*, a subsample was selected to assess *cox1* diversity (selecting individuals that did not share the same *cob* haplotype), except for the non-gynodioecious species and *S. vulgaris*, where at least one individual per population was sequenced.

Cox1 structure in *Silene*: The sequencing of *cox1* (1037 bp) revealed 24 segregating sites, resulting in 21 haplotypes among the nine analyzed *Silene* species

(Table 4). Here again, we found the same trends as with *cob*: the gynodioecious species *S. acaulis* and *S. nutans* showed the greatest diversity, with 6 and 10 haplotypes, respectively, while the dioecious species *S. dioica* and *S. diclinis* and the hermaphroditic species *S. virginica*, *S. douglasii*, and *S. scouleri* were fixed. A notable difference between the two genes was the limited number of haplotypes of the gynodioecious species *S. vulgaris* (2), which was slightly lower than that in *S. latifolia* (3). Among the 3 haplotypes found in *S. latifolia*, 1 was common with *S. vulgaris* (*cox1_1*) and 1 was common with *S. dioica* and *S. diclinis* (*cox1_2*). Hermaphroditic species *S. douglasii*, *S. scouleri*, and *S. virginica* were fixed for the same haplotype (*cox1_4*).

Phylogenetic trees of *cob* and *cox1* sequences of *Silene*: NJ trees of *cob* and *cox1* sequences were built, using *B. vulgaris* as the outgroup (Figure 2). The *cob* and *cox1* NJ trees did not exhibit a complete clustering of haplotypes by species, in particular in the case of *S. nutans* and *S. acaulis* haplotypes. The observed topology is suggestive of ancestral polymorphism or hybridization. We compared the topologies of these NJ trees and a tree constrained such that *S. nutans* and *acaulis* haplotypes were clustered by species. The nonconstrained *cob* and *cox1* trees had the highest likelihood regardless of which model-rate heterogeneity was tested and the difference was significant using the Kishino–Hagesawa test (KISHINO and HAGESAWA 1989) [for mixed model, one invariable site/eight gamma-distributed rates (model with the highest likelihood), one-sided Kishino–Hagesawa test, $P = 0.001$ for *cob*, $P = 0.027$ for *cox1*].

Recombination test: Four-gamete tests (HUDSON and KAPLAN 1985) revealed that recombination events were suspected for *cob* for the three gynodioecious species, with R_m (minimum number of recombination events) = 4, 5, and 1 for *S. acaulis*, *S. nutans*, and *S. vulgaris*, respectively, and for *cox1* $R_m = 2$ for *S. nutans*. In addition, in *S. nutans*, one intergenic recombination event between sites *cob*₉₈₁ and *cox1*₇₆₃ was detected (Table 5).

***S. noctiflora* polymorphism:** In the course of our sequencing we observed a high divergence in *cob* and *cox1* of the hermaphrodite species *S. noctiflora* compared with the other *Silene* species. We recently showed that the divergence observed in numerous mitochondrial genes, and in *cob* and *cox1* in particular, was a result of a recent burst in the rate of synonymous mutation that was restricted to the *S. noctiflora* lineage (MOWER *et al.* 2007) and that was comparable to what has been observed in *Plantago* (CHO *et al.* 2004) or *Pelargonium* (PARKINSON *et al.* 2005).

By building a chronogram to follow the evolution of the absolute rate of mitochondrial synonymous substitution (R_s), R_s was estimated for three terminal branches: *S. noctiflora*, *S. latifolia/vulgaris*, and *S. acaulis/nutans* (MOWER *et al.* 2007). It was shown that while the

R_s in the *S. noctiflora* lineage had a value of 90 substitutions per site per billion years (SSB), *S. latifolia* and *S. acaulis* terminal branches had average R_s 's of 0.54 and 1.55 SSB, respectively, which are not significantly different ($Z = 1.5682$, $P = 0.117$) and were more in line with what is known for mitochondrial genes.

We assessed polymorphism of *cob* and *cox1* in *S. noctiflora*, to see whether the effect of the recent burst of the mitochondrial synonymous substitution rate was still observable in this hermaphroditic species. Thirteen individuals from one American and three European populations (The Netherlands, Romania, and Sweden) were sequenced for *cob* and 10 for *cox1*. These individuals were fixed for *cob* and exhibited only one synonymous variant on *cox1*. This suggests that the recent burst of mutation has most likely been selected against as suggested in other high mitochondrial mutation rate lineages (PARKINSON *et al.* 2005), but also (and more interestingly) that the species has not maintained haplotype diversity. Hence, R_s variation among species appears unlikely to be a major contributing factor to the observed pattern of polymorphism.

Age estimation of *cob* and *cox1* haplotypes: The molecular-clock hypothesis was tested on *cob* and *cox1* NJ trees, to tentatively estimate divergence times of observed haplotypes using the LRT. For *cob*, the molecular-clock hypothesis was rejected on the whole sequence alignment regardless of the model of rate heterogeneity used [for mixed model, one invariable site/eight gamma-distributed rates (model with the highest likelihood), LRT = 93.74, d.f. = 32, $P = 5.6 \times 10^{-8}$]. This was not the case when the test was run on fourfold degenerate sites, supposed to be neutral [for mixed model, one invariable site/eight gamma-distributed rates (model with the highest likelihood), LRT = 27.66, d.f. = 20, $P = 0.118$]. We also ran the molecular-clock test on a modified sequence alignment, fixing sites that were detected to be recombining sites, since recombination could also be a possible cause of the hypothesis being rejected. Nevertheless, the hypothesis was still rejected regardless of the type of substitution rate used but not as significantly as with the original data set [for mixed model, one invariable site/eight gamma-distributed rates (model with the highest likelihood), LRT = 40.95, d.f. = 24, $P = 0.017$].

Using the same methodology on *cox1*, the molecular-clock test was rejected on the original sequence alignment [for mixed model, one invariable site/eight gamma-distributed rates (model with the highest likelihood), LRT = 45.7, d.f. = 21, $P = 0.001$] but conserved on the fourfold degenerate sites [for mixed model, one invariable site/eight gamma-distributed rates (model with the highest likelihood), LRT = 0.32, d.f. = 21, $P = 1$] and on the “nonrecombination” data set (for mixed model, one invariable site/eight gamma-distributed rates (model with the highest likelihood), LRT = 27.64, d.f. = 18, $P = 0.066$). Therefore, besides

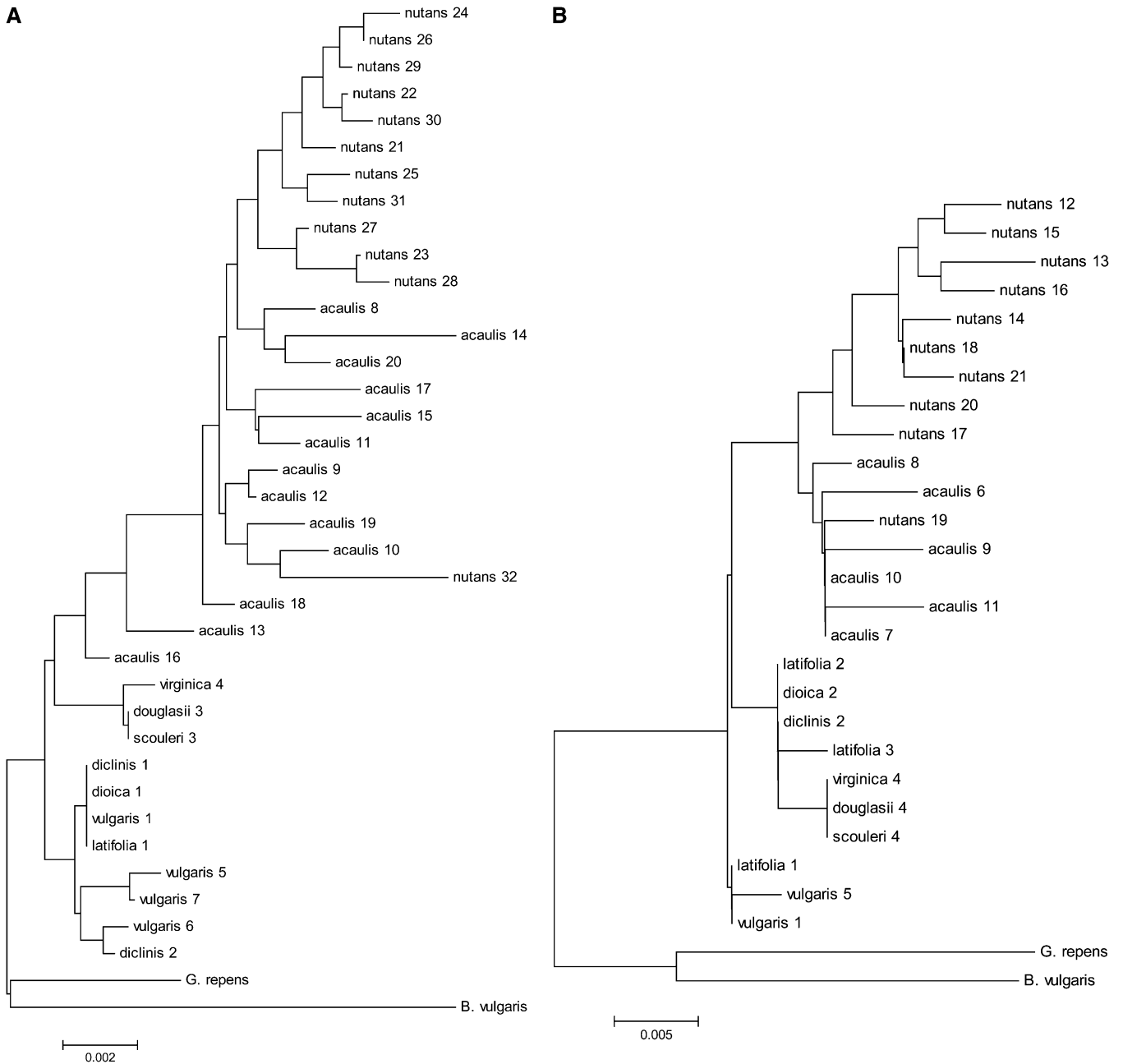


FIGURE 2.—Neighbor-Joining trees of (A) *cob* and (B) *cox1* sequences using Kimura's two-parameter model. For each haplotype the species in which it was found and the number (as in Tables 3 and 4) are indicated. Haplotypes found in Caryophyllid outgroups, *Gypsophila repens* (*Gr*) (Caryophyllaceae) and *Beta vulgaris* (*Bv*) (Chenopodiaceae) are also indicated. Scales of substitution per site are indicated.

mutation-rate variation, selection and recombination could be at least partly responsible for the rejection of the molecular-clock hypothesis on *cob* and *cox1*.

To have a conservative estimate of divergence times among haplotypes, we thus used the nonrecombination data sets. The estimation of the average R_s in the *S. acaulis/nutans* lineage and the *S. vulgaris/latifolia* lineages, 1.55 and 0.54 SSB, respectively (MOWER *et al.* 2007), enabled us to calculate an average estimated coalescent time of haplotypes within species using the average modified K_s among the different haplotypes

from the nonrecombination data set for *cob* and *cox1* (Table 5). For *cob* haplotypes, the average divergence time was 9.1 MYA for *S. nutans*, 4.9 MYA for *S. acaulis*, and 15.6 MYA for *S. vulgaris*. These haplotype divergence times all predate the species divergence times, with, for example, a divergence time between *S. nutans* and *S. acaulis* estimated to be >1 MYA (MOWER *et al.* 2007). The same was true for *cox1* haplotypes, where it was estimated to be 8.3 MYA for *S. nutans*, 12.1 MYA for *S. acaulis*, 14.9 MYA for *S. vulgaris*, and 19.9 MYA for *S. latifolia*.

TABLE 5

Segregating sites from *cob* and *cox1* showing evidence of four gametes between 10 pairs of sites

Species	Ind	<i>cob</i>					<i>cox1</i>						
		223	246	510	592	735	858	927	954	981	763	775	940
<i>S. vulgaris</i>	Va_1				G					C			
<i>S. vulgaris</i>	Va_2				T					A			
<i>S. vulgaris</i>	Sta_2				G					A			
<i>S. vulgaris</i>	Tor_1				T					C			
<i>S. acaulis</i>	Pas_1			A		T		A					T
<i>S. acaulis</i>	ISLB_1			A		G		T					T
<i>S. acaulis</i>	ISLB_2							T					G
<i>S. acaulis</i>	ISLB_3		T	C		G		A					
<i>S. acaulis</i>	ISLB_4		T	A									
<i>S. acaulis</i>	ISLB_5		G	A									
<i>S. acaulis</i>	ISV_4		G	C		T		T					
<i>S. acaulis</i>	GRL_3							A					G
<i>S. nutans</i>	Que_1	C						T	G		T	G	C
<i>S. nutans</i>	Que_2	C						A	A		T	T	T
<i>S. nutans</i>	Que_6												T
<i>S. nutans</i>	Auv_1							A		G	G	T	C
<i>S. nutans</i>	Dor_3	T						A			T	T	
<i>S. nutans</i>	FIN	T						T	G		G	T	

Ind, individual for which the sequence was found (Tables 2 and 3). Boxes between pairs of sites indicate each gametic type.

Predicted editing sites in *cob* and *cox1*: To accurately evaluate the nonsynonymous polymorphism in our data set, we used the online resource PREP-Mt (MOWER 2005) (with a cutoff value of 0.5), to detect potential edited sites on nonsynonymous variants. Only two sites were predicted to be edited: site 241 in *cob* and site 954 in *cox1* [but that still remains nonsynonymous with the editing: $T_{953}G_{954}T_{955}/T_{953}C_{954}T_{955}$ translated C_{318}/S_{318} becomes after editing $T_{953}G_{954}T_{955}/T_{953}T_{954}T_{955}$ (C_{318}/F_{318})]. The amino acid sequences of both genes were thus deduced and revealed several variable sites, generating potentially different functional haplotypes (Tables 3 and 4).

Overall diversity for *cob* and *cox1* among *Silene* species: Globally, nucleotide diversity in *cob* and *cox1* was higher in gynodioecious species when compared with non-gynodioecious species (Table 2). The same trend was observed when diversity in *cob* or *cox1* was estimated on segregating sites (θ_w , WATTERSON 1975).

Given that breeding system is not independent of phylogeny, we conducted a Kruskal–Wallis test to evaluate the effect of breeding system and phylogenetic position on nucleotide diversity. Both factors had a strong effect, with breeding system being slightly more significant than phylogenetic position ($H = 12.85$, $DL = 2$, $P = 0.002$; $H = 12.47$, $DL = 3$, $P = 0.006$, respectively). This illustrates the difficulty of separating the individual effects of these two factors.

Overall, the different neutrality tests, either based on frequency spectra (Tajima's D and Fu and Li's D with *B. vulgaris* as the outgroup) or based on the number of haplotypes (K -test), did not reveal any departure from

neutral expectations. The only exception was the 13 observed *cob* haplotypes in *S. acaulis*, which was more than the upper bound of the 95% confidence interval [5–10] and is most likely caused by recombination. Indeed, when intermediate recombination is allowed (as estimated from the data), the observed number of haplotypes falls within the 95% confidence interval [12–19].

Nonsynonymous polymorphism in gynodioecious species: Haplotypes of *cob* or *cox1* found in our samples exhibited nonsynonymous mutations, resulting, after editing, in 18 different *cob* and 5 different *cox1* amino acid sequences (Tables 3 and 4). For *cob*, except for the case of *S. diclinis*, all the non-gynodioecious species were fixed for one of the two amino acid sequences found among the non-gynodioecious species (considered as different functional haplotypes, FHap I and II). Both sequences were segregating in *S. vulgaris*, while *S. acaulis* and *S. nutans* exhibited 12 and 6 additional amino acid sequences (1 being shared by both species), respectively. For *cox1*, of the 5 different amino acid sequences we found, all non-gynodioecious species and gynodioecious *S. vulgaris* were fixed on a single amino acid sequence, while *S. acaulis* shared the same amino acid sequence and exhibited an additional one, and *S. nutans* had three additional ones.

In both genes, codons appeared to be fixed among a sample of Angiosperm species and globally highly conserved across a broader phylogenetic mitochondrial range (from *Arabidopsis thaliana* to the unicellular green algae *Chaetopharidium globosum*). Codons were nevertheless polymorphic within our samples (from the reference

alignment available on the online resource PREP-Mt, MOWER 2005; Tables 3 and 4). In particular in *cob*, codon 198 was highly polymorphic in *S. acaulis* and exhibited amino acids not found in the reference alignment (Y, F instead of A or G); in *cox1*, highly conserved codons 319 and 333 segregated only in *S. nutans*.

For the calculation of nonsynonymous diversity, predicted edited sites were taken into account. The average K_a/K_s ratios for *cob* and *cox1* were <1.0 , indicating that both protein-coding genes are selectively constrained. In *Silene* species, *cox1* appears to be more constrained with a ratio two magnitudes lower than *cob*. However, we observed heterogeneity in the ratio of K_a/K_s among genes when gynodioecious species were compared. The ratio of K_a/K_s for *cob* was twice as high for *S. acaulis* as for *S. vulgaris* or *S. nutans*, while for *cox1* the K_a/K_s for *S. acaulis* was only 60% that observed for *S. nutans*. The higher ratio could be indicative of relaxed selection (through the accumulation of mildly deleterious mutations) or positive selection occurring at the species level on *cob* in *S. acaulis* and on *cox1* in *S. nutans* (RAND and KANN 1996).

DISCUSSION

We found differences in the pattern of polymorphism of two mitochondrial genes, *cob* and *cox1*, that were associated with the breeding system of the *Silene* species we investigated. Gynodioecious species exhibited strikingly more polymorphism than either hermaphroditic or dioecious species. The observed differences could also be a result of variation in mutation rate; however, such rates were shown to be comparable (not significantly different) among studied species, with one notable exception, the hermaphroditic species *S. noctiflora*. This species had a recent spectacular burst in synonymous mutation rate (MOWER *et al.* 2007), but as we showed here, did not exhibit a concomitantly high level of polymorphism. Moreover, haplotypes found in gynodioecious species had coalescent times that preceded speciation, suggesting that demographic or selective processes must be maintaining these ancient haplotypes. Not only was the number of segregating sites or haplotypes higher in gynodioecious species for both mitochondrial genes, but also polymorphic haplotypes could be translated into different amino acid sequences.

Overall polymorphism and breeding systems in *Silene*: Two alternative evolutionary scenarios have been suggested regarding cytoplasmic haplotypes in gynodioecious species: balancing selection *vs.* epidemic sweeps. These two scenarios are expected to have contrasting effects on levels of polymorphism at cytoplasmic loci linked to male-sterilizing factors. In the case of haplotypes being maintained over a long period of time through balancing selection, neutral nucleotide

polymorphism is expected to be high as a consequence of the accumulation of silent mutations within and among haplotypes (STÄDLER and DELPH 2002). Conversely, epidemic dynamics are expected to induce lower polymorphism since under this scenario newly arisen sterilizing cytoplasms spread and replace the former cytoplasms (CHARLESWORTH 2002).

We conducted this study with the hypothesis that gynodioecious species should exhibit either more or less nucleotide diversity compared with non-gynodioecious species. We took the approach of sampling several species per type of breeding system, tentatively representing their current geographical distribution. However, such a study can hardly avoid biases at different levels. For example, a geographical bias might exist in cases for which the limited sampling is not representative of the species distribution. This could apply to *S. nutans*, an herbaceous species having a continental distribution range extending from northwestern Europe to central Siberia and the South Caucasus, for which mostly French populations were analyzed. However, such a bias would tend to underestimate the level of diversity; and on the contrary, we found relatively high levels of diversity for this species.

In addition, a phylogenetic bias could occur when the various species representing a given breeding system share an ancestor, as is the case for the three dioecious species in our study. The ideal case would be to assess sister species that were different in their breeding system, but this is not possible as breeding systems are not uniformly distributed in *Silene* (DESFEUX *et al.* 1996). Nevertheless, in the present study we were able to assess the diversity of non-gynodioecious species that represent multiple sections of the genus, with the three gynodioecious species belonging to two ancient separated lineages (node separating the two clusters estimated as being ~ 8 million years old; MOWER *et al.* 2007).

We observed a large effect of breeding system on the two mitochondrial genes analyzed, with gynodioecious species exhibiting more haplotypes, which had accumulated more segregating sites, than non-gynodioecious ones. Except for dioecious *S. latifolia* in *cox1* and dioecious *S. diclinis* in *cob*, all non-gynodioecious species were fixed for both genes; the nucleotide diversity (π) of *S. diclinis* was an order of magnitude lower than that of *S. acaulis* and *S. nutans*, and the diversity of *S. latifolia* was two to five times lower. *S. vulgaris*, while systematically exhibiting polymorphism, was the least polymorphic gynodioecious species in our study. Recent studies on several mitochondrial genes have revealed that *cob* (*cox1* was not sequenced) was not the most polymorphic locus in *S. vulgaris*, potentially illustrating variation in mutation rate among mitochondrial genes (HOULISTON and OLSON 2006; BARR *et al.* 2007).

It must be noted that an alternative scenario, although not exclusive, can be invoked in light of a recent study on *S. vulgaris* (SLOAN *et al.* 2008). In this study, it

was shown that the substitution rate estimated from seven mitochondrial loci was variable among *Silene* species and potentially among lineages at the species level as well. Even though the biological explanation of this lineage-specific phenomenon is not known, the rejection of the molecular-clock hypothesis for *cob* and *cox1* trees is suggestive of mutation-rate variation occurring at least among species (in addition to selection and recombination). However, the difference in absolute mutation rate (R_s) between lineages cannot be the sole explanation for the absence of polymorphism in the non-gynodioecious species. *S. noctiflora* exemplifies the lack of a clear link between polymorphism and synonymous mutation rate, as this hermaphroditic species does not exhibit any polymorphism in *cob* and exhibits just 1 segregating site in *cox1* (of four geographically distant populations), despite a recent burst of high mutation rate. Consequently, factors other than mutation-rate variation must be invoked to explain the observed pattern of polymorphism.

This study confirms previous results found for *S. acaulis* on *cob* from STÄDLER and DELPH (2002) and shows that the same is true of the *cox1* gene for *S. acaulis* but also in two additional gynodioecious species, *S. nutans* and *S. vulgaris*. For *S. vulgaris*, we did not observe a reduction in diversity as previously suggested by INGVARSSON and TAYLOR (2002), and this is in accord with what was found in a more recent study (HOULISTON and OLSON 2006). Although we do not present the data here because full sequences were not obtained (most likely because of primer mispairing), we also observed polymorphism in partial sequences of *cob* and/or *cox1* in another gynodioecious *Silene* species, *S. italica* and in another gynodioecious species in the Caryophyllaceae, *G. repens*. This suggests that the evolutionary dynamics underlying gynodioecy—in this case long-term maintenance of haplotypes—are quite general in this plant family.

Using estimates of absolute synonymous substitution rates from a former study (MOWER *et al.* 2007), the average age of *cob* and *cox1* haplotypes found within a species predated the estimated time of speciation. The ancestral nature of the observed polymorphism is also suggested by the fact that most polymorphic sites are shared among *Silene* species. In addition, phylogenetic analysis showed that clustering of haplotypes by species did not always occur, in particular for *S. acaulis* and *S. nutans*. This pattern could be explained by hybridization events or ancestral polymorphism. However, to our knowledge, no hybridization between these two species has ever been reported. Moreover, they do not share similar habitats and occur only in allopatry or parapatry. Thus, we believe that the observed pattern is reminiscent of the ancestral polymorphism observed in loci under balancing selection such as self incompatibility (SI) loci (reviewed in CHARLESWORTH *et al.* 2005). Interestingly, in the multiallelic SI system it has been shown that the genealogical structure of SI loci is

expected to be close to neutral expectation (VEKEMANS and SLATKIN 1994). This was also observed in our study, given that neutrality could not be rejected by Tajima's test. Finally, we also showed that haplotypes are old enough to be shared among geographically distant populations (see also STÄDLER and DELPH 2002).

In addition to the accumulation of point mutations through long periods of time, data from the three gynodioecious species in our study suggest that recombination events seem to be partially responsible for the current haplotype diversity. Intragenic recombination in plant mitochondria was first suggested for *cob* in *S. acaulis* (STÄDLER and DELPH 2002). Further evidence of intragenic as well as intergenic recombination in *S. vulgaris* has recently been shown for several mitochondrial genes (HOULISTON and OLSON 2006; McCAULEY and ELLIS 2008). In addition, data have been shown that are congruent with the idea that heteroplasmy can occur through occasional paternal leakage in *S. vulgaris* (McCAULEY *et al.* 2005; WELCH *et al.* 2006), one of the conditions that can yield recombination between variant mitochondrial genomes. Without overestimating the role of recombination in shaping intragenic diversity in *Silene* mitochondria (see discussion in BARR *et al.* 2007 and McCAULEY and ELLIS 2008), the accumulation of evidence of four-gamete types for several species and genes suggests a common occurrence of this phenomenon in the genus, most likely through past heteroplasmy events. Interestingly, a recent theoretical study has shown that occasional paternal transmission of mitochondria could facilitate the maintenance of gynodioecy (WADE and McCAULEY 2005).

Nonsynonymous mutations at sites that are highly conserved in plants: Our data revealed that our gynodioecious study species not only have more haplotypes and segregating sites at the species level than the other species, but also contain a large number of nonsynonymous polymorphic sites that result in several coded proteins. For example, over all *Silene* species we studied, taking into account potentially edited sites, we found in *cob* a total of 12 replacement sites resulting in 18 different amino acid sequences, and in *cox1* a total of 4 replacement sites generating 5 different amino acid sequences. A total of 12 different *cob* amino acid sequences were found in *S. acaulis*, 6 in *S. nutans*, with 1 being shared between both species. In addition, *S. nutans* exhibited 3 of the 5 detected *cox1* amino acid sequences. In contrast, some of these polymorphic sites are fixed in a representative of Angiosperms and highly conserved when we extend the comparison to the liverwort *Marchantia polymorpha*, the stonewort *Chlora vulgaris*, and the unicellular green algae *C. globosum*. Similarly, most of our non-gynodioecious study species have the conserved amino acid at these positions.

Considering the ratio between nonsynonymous and synonymous substitution rates for *cob* and *cox1* at the

species level (K_a/K_s), it appears that different selective constraints could be acting on these protein-coding genes across the studied species. Indeed, in *S. acaulis*, *cox* appears to be either less selectively constrained than in *S. nutans* or *S. vulgaris* or under positive selection. Conversely, *cox1* might be more relaxed (or positively selected) in *S. nutans* than in *S. vulgaris* or *S. acaulis*.

The question remains whether these genes could be directly involved in male sterility in *S. acaulis* or *S. nutans*. Molecular studies on cytoplasmic sterility in crop species revealed that male-sterility genes are usually of a chimeric nature, as the result of intragenomic recombination (HANSON and BENTOLILA 2004; CHASE 2007). Nevertheless, a study in gynodioecious *B. vulgaris* ssp. *maritima* suggested variant mitochondrial subunits of the respiratory complexes as potential male-sterility factors (DUCOS *et al.* 2001). Reciprocal crosses, to determine the number of CMS genes segregating in *S. acaulis* and *S. nutans*, and physiological studies to assess the *in vitro* and *in vivo* activities of the corresponding protein complexes will be needed to disentangle the effect of the observed nonsynonymous polymorphisms.

In conclusion, the polymorphism observed for two mitochondrial genes strikingly contrasted gynodioecious species with non-gynodioecious species. The occurrence of many different haplotypes in gynodioecious species, old enough to have accumulated numerous mutations and to be found across a large geographical distribution, is in favor of the balancing-selection hypothesis as a major force in the maintenance of gynodioecy in the *Silene* genus.

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