Nonneutral Evolution of Organelle Genes in *Silene vulgaris*

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ABSTRACT

Knowledge of mitochondrial gene evolution in angiosperms has taken a dramatic shift within the past decade, from universal slow rates of nucleotide change to a growing realization of high variation in rates among lineages. Additionally, evidence of paternal inheritance of plant mitochondria and recombination among mitochondrial genomes within heteroplasmic individuals has led to speculation about the potential for independent evolution of organellar genes. We report intraspecific mitochondrial and chloroplast sequence variation in a cosmopolitan sample of 42 *Silene vulgaris* individuals. There was remarkably high variation in two mitochondrial genes (*atp1*, *atp9*) and additional variation within a third gene (*cob*). Tests for patterns of nonneutral evolution were significant for *atp1* and *atp9*, indicative of the maintenance of balanced polymorphisms. Two chloroplast genes (*matK*, *ndhF*) possessed less, but still high, variation and no divergence from neutral expectations. Phylogenetic patterns of organelle genes in both the chloroplast and mitochondria were incongruent, indicating the potential for independent evolutionary trajectories. Evidence indicated reassociation among cytoplasmic genomes and recombination between mitochondrial genes and within *atp1*, implying transient heteroplasmy in ancestral lineages. Although the mechanisms for long-term maintenance of mitochondrial polymorphism are currently unknown, frequency-dependent selection on linked cytoplasmic male sterility genes is a potential candidate.

UNTIL recently (STÄDLER and DELPH 2002; CHO *et al.* 2004; PARKINSON *et al.* 2005), the coding regions of the organelle genomes of angiosperms were viewed as highly constrained, with low rates of mutation and strict maternal inheritance (WOLFE *et al.* 1987). This has resulted in the assumption that intraspecific variation in organelle genes should be low or even absent. Contradictory patterns of extremely high mtDNA variation have recently been observed in *Pelargonium* (PARKINSON *et al.* 2005), *Plantago* (CHO *et al.* 2004), and *Silene* (STÄDLER and DELPH 2002). Two of these genera (*Plantago* and *Silene*) are model systems for studies of the evolution of gynodioecy in plants (DE HAAN *et al.* 1997; CHARLESWORTH and LAPORTE 1998). The elevated rate of synonymous substitutions in *Pelargonium* has been attributed to an increase in mutation rate or a reduction in the efficacy of DNA repair mechanisms (PARKINSON *et al.* 2005). In *Plantago*, high diversity at the mitochondrial *cytochrome oxidase I* (*cox1*), *adenosine 5'-triphosphate* (*atp1*), and small subunit RNA-encoding DNA (*SSU rDNA*) loci has been attributed to similar causes (CHO *et al.* 2004). *Silene acaulis* (L.) Jacq. possessed high diversity of mitochondrial *apocytochrome b* (*cob*) haplotypes, but this was attributed to ancient

coalescence due to long-term maintenance of a balanced polymorphism (STÄDLER and DELPH 2002).

If selection maintains a balanced polymorphism in mitochondrial types for long periods, one candidate for the target of selection is cytoplasmic male sterility (CMS) genes that influence sex expression. Sex expression in many gynodioecious species (those with females and hermaphrodites in natural populations) is controlled by a complex joint cytonuclear polymorphism between CMS genes and their associated restoration of male fertility (*Rf*) genes (SAUMITOU-LAPRADE *et al.* 1994; SCHNABLE and WISE 1998). Theoretical studies predict that through time CMS and *Rf* frequency cycles are generated from negative frequency-dependent selection assuming both female compensation (females produce more seeds than hermaphrodites; DARWIN 1887) and a cost to carrying *Rf* genes (cost of restoration, CHARLESWORTH 1981; DELANNEY *et al.* 1981). CMS/*Rf* cycles are stabilized by specific relationships between the fitnesses of particular CMS types, by the costs of carrying *Rf* genes (FRANK 1989; GOUYON *et al.* 1991), and by low levels of paternal leakage of CMS genes (WADE and McCAULEY 2005), potentially providing mechanisms whereby the joint CMS/*Rf* polymorphism is maintained over long periods. CMS genes are ubiquitous in plants and have utility within breeding programs of nearly every major crop species. For reasons that are yet to be discovered, the male-sterile phenotype associated with the expression of CMS is uncommon in natural populations of the majority of

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plant species, with gynodioecy found in only 7% of angiosperm taxa (RICHARDS 1997).

Even if some form of balancing selection maintains CMS/*Rf* polymorphism on an ecological timescale, there remains considerable contention over whether polymorphism in CMS genes is maintained over longer time periods (FRANK 1989; GOUYON *et al.* 1991) or whether newly arisen types more likely spread as epidemics within gynodioecious populations (INGVARSSON and TAYLOR 2002). For instance, CMS and their associated *Rf* genes may spread to fixation in the absence of costs of restoration (CHARLESWORTH 1981; FRANK 1989; GOUYON *et al.* 1991), or demographic effects and drift may cause the loss/fixation of a particular CMS type when CMS genes are at a low/high frequency during a cycle. Both of these outcomes are consistent with an epidemic model.

A comparative molecular population genetic approach may show promise for discriminating among the three alternatives proposed to shape mitochondrial nucleotide diversity. For instance, elevated mutation rates would be expected to increase diversity within lineages in all genes in the genome, whereas selection may target only specific genes or genomic regions. Furthermore, elevated mutation rates are not expected to influence either the relative lengths of internal and external branches or the expected numbers of haplotypes, whereas selection will (FU and LI 1993; DEPAULIS and VEUILLE 1998). An example of the influence of long-term balancing selection is found in plant self-incompatibility alleles wherein negative frequency-dependent selection may have preserved variants for 27–50 million years, leading to *trans*-specific polymorphism (IOERGER *et al.* 1990; UYENOYAMA 1995). Finally, epidemics generate short internal branches in gene genealogies as all alleles coalesce to the allele responsible for the epidemic (HATCHER 2000; INGVARSSON and TAYLOR 2002). If CMS genes either were maintained over long periods or were characterized by spreading as epidemics, a naive prediction might be for similar patterns of long or short time to coalescence, respectively, for CMS alleles. However, studies of species wherein multiple CMS genes have been identified have shown that CMS variants are not allelic, but rather each is constructed from different sets of genes to create a novel chimeric open reading frame (SCHNABLE and WISE 1998). Nonetheless, some researchers have posited that since the mitochondrial genome is inherited as a single linkage unit and the chloroplast and mitochondria are co-inherited, these genomes may harbor signals of past balancing selection or epidemics via hitchhiking (OLSON and McCAULEY 2000; INGVARSSON and TAYLOR 2002; CHO *et al.* 2004).

Here we assess patterns of nucleotide diversity in a cosmopolitan sample of *S. vulgaris* (Moench.) Garcke individuals, a well-studied gynodioecious plant species (MARSDEN-JONES and TURRILL 1957; PETTERSSON 1992; OLSON *et al.* 2005). We estimate diversity in three

mitochondrial and two chloroplast genes to determine whether genomewide hitchhiking can indeed influence genetic diversity throughout the mitochondrial genome and potentially extend to the chloroplast genome, which is often co-inherited. The presence of intraspecific diversity in the mitochondrial and chloroplast genomes also offers an opportunity to explore the transmission dynamics and role of recombination on the cytoplasmic genomic compartments. With rare exceptions (REBOUD and ZEYL 1994), the mitochondria and chloroplast in angiosperms are thought to be primarily maternally inherited (BIRKY 2001; McCAULEY *et al.* 2005). Phylogenetic patterns among co-inherited genes or genomes are expected to be congruent, due to a process akin to linkage disequilibrium (OLSON and McCAULEY 2000). The absence of such congruence implies independent transmission histories perhaps resulting from horizontal transfer (BERGTHORSSON *et al.* 2003) or paternal leakage (McCAULEY *et al.* 2005), producing heteroplasmic individuals. Over generations, heteroplasmy is expected to be reduced through random genetic drift (ALBERT *et al.* 1996; ROZE *et al.* 2005). As heteroplasmy is a necessary precondition for recombination among divergent genes or genomes, the presence of recombination within or among cytoplasmic genomes not only indicates that cytoplasmic genes may have the potential to evolve as independent units, but also indicates that transmission dynamics may be much less constrained than previous studies have indicated. *S. vulgaris* is an excellent candidate for better understanding the mitochondrial and chloroplast evolution as previous studies have identified high levels of mitochondrial polymorphism within small populations (OLSON and McCAULEY 2000; ŠTORCHOVÁ and OLSON 2004), and small samples have indicated that sequence diversity at coding regions may also be elevated relative to that in other taxa (McCAULEY *et al.* 2005).

MATERIALS AND METHODS

S. vulgaris (Moench.) Garcke (Caryophyllaceae) seeds were collected from four regions (Virginia and New York/Vermont; central Europe, Czech Republic/Germany; and Siberia, Russia; Figure 1) and grown in the Institute of Arctic Biology greenhouse at the University of Alaska (Fairbanks, AK). Eight populations were located in Giles and Craig counties, Virginia (628, GR, CO, AJ, MV, AR, WR, and EB, Figure 1; listed as populations 3, 4, 5, 6, 11, 12, 14, and 15, respectively, in OLSON and McCAULEY 2002), and four populations were located in New York (NY) and Vermont (Vt) (Vt17b, Vt100, and VtLnG, Figure 1; McCAULEY *et al.* 2003). Of the central European populations, three were located near Prague, Czech Republic (Kovary Meadow, KM; Institute Garden, InG; Sedlac railway station, SD), two were in northern Moravia, Czech Republic (Mount Keprník, MK4; Rychleby Mountains, RM), two were from southern Moravia (Klentnice, KL; Lednice, Ld), and the eighth population was located near Gemsteltal (GM) in the Allgauer Alps, Austria (Figure 1; ŠTORCHOVÁ and OLSON 2004). One population was collected from Krasnojarsk (Krn)

FIGURE 1.—Map of the locations of *Silene vulgaris* samples collected for this study. The large dots represent multiple populations from within the same locality, whereas the small dots represent the locations of single populations.

in south central Russia near Lake Baikal (Figure 1). One plant was arbitrarily selected for study from each of one to five maternal families from each of these 21 populations of *S. vulgaris*. Two additional *Silene* species were included as potential outgroups and to determine the extent of interspecific divergence. Seeds from *S. uniflora* Roth (also called *S. maritima*), a species closely related to and not entirely reproductively isolated from *S. vulgaris* (MARSDEN-JONES and TURRILL 1957; RUNYEON-LAGER and PRENTICE 2000), originated in Saint Catherine's Bay, United Kingdom. Seeds from *S. latifolia* Poir. were collected in the courtyard at the Abbey of St. Thomas, Brno, Czech Republic; *S. vulgaris* and *S. latifolia* are not interfertile.

Total genomic DNA was isolated and purified from leaf tissue using a QIAGEN (Valencia, CA) DNeasy plant mini kit. We PCR amplified three mitochondrial (*adenosine 5'-triphosphate* subunit 1, *atp1*; *adenosine 5'-triphosphate* subunit 9, *atp9*; *apocytochrome b*, *cob*) and two chloroplast genes (*maturase K*, *matK*; *NADH dehydrogenase F*, *ndhF*) from each individual. PCRs were performed in 35- μ l reactions, with 15–40 ng of genomic DNA template and a 50° annealing temperature using standard protocols (ŠTORCHOVÁ and OLSON 2004). Sequencing reactions were carried out using the ABI Big-Dye terminator sequencing kit and sequenced directly on an ABI 3100 automated sequencer. Sequences had no indications of heterozygosity (e.g., overlapping chromatograms), suggesting that if heteroplasmy was present, we were sequencing the most common allele. PCR primer sequences and aligned data sets are available upon request.

As ancient gene transfers from the mitochondrial to the nuclear genome or nuclear pseudogenes are possible in plants (MOWER *et al.* 2004), we confirmed maternal inheritance of the *atp1* and *atp9* regions to ensure that they were indeed of cytoplasmic origin. For *atp1*, a reciprocal cross was conducted between two plants with types C and F (see Table 1) polymorphic for *Sma*I restriction sites in *atp1*. *Atp1* was amplified from 16 progeny from each mother (32 total) and allelic inheritance was determined after separation of digested PCR products on a 3% agarose gel stained with ethidium bromide. All progeny possessed the same haplotype as their mother. Under a null hypothesis of biparental inheritance as expected for nuclear copies, the probability of finding only the single haplotype in the progeny from each maternal parent was 0.00002, with a power of 99%. For *atp9*, a reciprocal cross was conducted between 62812 and a plant with type C (see Table 1) that differed in a *Mva*I restriction site. Ten progeny from the maternal parent with type C and 7 progeny from 62812 as a maternal parent were genotyped. *Atp9* restriction polymorphisms exhibited strict maternal inheritance; the probability of all 17 progeny inheriting the maternal haplotype under a null hypothesis of biparental inheritance and nuclear localization was 0.001, with a power of 91%. As it is known that low levels of biparental inheritance occur in *S. vulgaris*, one might also be interested in the probability of detecting biparental inheritance given our sample sizes. Using the 95% confidence interval of the zero term of the Poisson distribution to estimate the minimum detectable frequency of biparental inheritance, a total sample size of 49 (combined across *atp1* and *atp9*) allows detection of not less than 6.08% (2.98/49) biparental progeny. In addition to verifying the maternal inheritance of these subunits, Southern blots with *atp1*-specific probes hybridize strongly, indicating that *atp1* is present in high copy number as is expected for genes located in the mitochondria. *Atp1*, *atp9*, and *cob*, like most other respiratory genes, have never been found to be lost in the mitochondrial genome and transferred to the nuclear genome in plants, making this very unlikely in this taxon (ADAMS *et al.* 2002). Finally, a phylogeny of the *atp1* data set from *S. vulgaris* and that from a much larger study

(BERGTHORSSON *et al.* 2003) were combined to rule out the possibility of horizontal transfer via parasitic plants. In this analysis, *atp1* haplotypes from *S. vulgaris* formed their own clade with high bootstrap support and were not associated with any other genera; thus, there is no evidence for horizontal transfer of organelles or genes as has been shown in other groups with high organelle genomic variation (MOWER *et al.* 2004).

We reamplified and resequenced a subset of samples (SD6, NY006, Ld5, EB3AG, 62812, NY007, Vt17b, and Gm7) to reconfirm the patterns of diversity and recombination within and between *atp1* and *atp9*. Although we feel it is unlikely that cross-sample contamination could have produced the results reported here, it cannot be discounted with complete certainty.

Sequences lacked indels, easing manual alignment. The following summary statistics were calculated using DNAsp version 4.0 (ROZAS *et al.* 2003): θ , per site estimated from the total number of mutations (WATTERSON 1975); θ_s , per site, including only synonymous nucleotide differences; π , diversity, the average number of nucleotide differences per site between a pair of randomly chosen sequences (NEI 1987); K_s , average number of pairwise synonymous substitutions per synonymous site; K_a , average number of pairwise nonsynonymous substitutions per nonsynonymous site; Tajima's *D* (TAJIMA 1989), based on the differences between the number of segregating sites (θ) and the average number of nucleotide differences per sequence (π); Fu and Li's *D* with an outgroup (*Nicotiana tabacum*) (FU and LI 1993), the test statistic is based on the differences between the total number of mutations in the external branches of the genealogy and the total number of mutations; *K*-test (DEPAULIS and VEUILLE 1998), 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. Specific hierarchical hypotheses concerning differences between chloroplast and mitochondrial genes and differences among mitochondrial genes were constructed and evaluated using log-likelihood tests as implemented in MLHKA (WRIGHT and CHARLESWORTH 2004). *N. tabacum* was chosen as an outgroup because its shared ancestor with *S. vulgaris* has little chance of also sharing *Silene* mitochondrial haplotypes; also it was the closest relative of *Silene* for which all five genes were available in GenBank. It has been demonstrated that the presence of population subdivision can lead to overestimates of deviations from neutrality using the HKA test (INGVARSSON 2004). Population subdivision within our study was minor; the same alleles were present in different sample regions. Additionally, all loci are organellar, and no comparisons are made with nuclear data that are under a different population dynamic (*cf.* INGVARSSON and TAYLOR 2002).

Phylogenetic hypotheses were constructed using PAUP* with an HKY model of substitution (SWOFFORD 2003). The best-fit substitution model was chosen using Modeltest (POSADA and CRANDALL 1998). Partition homogeneity tests, to assess the null hypothesis of congruence between the phylogenies of each gene, were carried out using PAUP* (SWOFFORD 2003). RDP version 2.0 was used to detect potential recombination, using the default settings (MARTIN and RYBICKI 2000). RDP implements both phylogenetic and substitution methods to test for recombination. Phylogenetic methods (RDP, Bootscan) compare phylogenies constructed from different parts of the genomes—if the phylogenies have different topologies then it is assumed that recombination has occurred. Substitution methods (GENECONV, Chimaera, Max-Chi, SiScan) look for clustering within sites with substitutions or whether

TABLE 1
Polymorphic sites in the three mitochondrial (*atp1*, *atp9*, *cob*) and two chloroplast (*matK*, *ndhF*) coding regions of *S. vulgaris*

	Gene				
	<i>atp1</i>	<i>atp9</i>	<i>cob</i>	<i>matK</i>	<i>ndhF</i>
SD6	11223334444555666790	1111111111111111	557	11111223333444455	11133455666778
Type A	112249575790159022899071	2355688991122333344556888	7119	13347914789095593579925	68900556588013
Type B	291275561592502756103284	048952519581726958149127	7676	96966510066591881105624	80416147522630
MV22h	GAGCATCTAGACACCGTAGTAACC	CTAGATCGACGCTACTTGTGTCTC	ATCT	CATGAATCCTGATCCAGCGGACT	TCGGTCCTTTCTCC
SD3				T.....G.....	
VtLnG		A.....	...C		...A.....
Type C		T.....			
Type D		G...AT...C...ACT		G.T.....	
NY006		A..GC.T.TAA..AC.T.AC...	..G..	T.....	...A.....
NY007		A..GC.T.TAA..AC.T.AC...	..G.C	T.....	
Ld5		AG...T...T...ATTG...			
Vt17b	AG...T...T...ATTG...			T...T...G.....	
EB3AG	AG...T...T...ATTG...			T.....	
GM7	AG...T...T...ATTG...			A.....	
MK4	AG...T...T...ATTG...				...T.....
KL1	AG...T...T...ATTG...		..G..	T.....	
KL2	AG...T...T...ATTG...				
Ld4	AG...T...T...ATTG...			T...A...G...A.....	A.....
RM4	AG...T...T...ATTG...			T...A...G...A.....	
RM8	AG...T...T...ATTG...				
Vt100	AGT...CTCG.GTCG...CGTAT				
InG	AGT...CTCG.GTCG...CGTAT				
Type E	...TG...				
KM5	...TG...				
EB12A	.G...T...T...ATTG...				
Km	AGT...CT.G...T...AT				
Type F	...TGCTCG...TCG...T.GA...AT		G...		
Substitution type	ssnssssssssssssssssssss	ns nssssssssssssssss	ssnn	n nn nn nn s n	ssnn
<i>S. latifolia</i>	AG...T...T...ATTG...	.A..GCTT.TAA..AC.T.AC...		..CTT.A..C.GC.TG.TTACAG	...CTACGCTGTA
<i>S. uniflora</i>	AG...T...T...ATTG...	..GT.....		.T..T..T...G.....	

Dots indicate that the base at that site is identical to the one found in the reference sequence, SD6. Position of the base in the gene is indicated above the alignment. Type A, Ld3, CO-9F, 62812; type B, AJ8-E, CO-5F MVh17, GRf6c, GR6-6, CO-50; type C, 6282B, 6283B, 6283b, 62876, 6283F; type D, AR-34, AR-9H; type E, KM3, KM4; type F, WR-05, WR-07, AR-22.

TABLE 2
Diversity measures in organelle genes of *Silene vulgaris*

Gene	θ	θ_s	π	Tajima's <i>D</i>	Fu and Li's <i>D</i>	<i>K</i> -test
<i>atp1</i>	0.005	0.018	0.006	0.33	1.47*	7, 9–19*
<i>atp9</i>	0.021	0.070	0.021	−0.33	1.69*	8, 7–18
<i>cob</i>	0.001	0.001	0.001	−0.30	1.02	6, 3–5*
<i>matK</i>	0.004	0.002	0.003	−0.80	−0.54	8, 4–10
<i>ndhF</i>	0.001	0.002	0.0002	−1.76	−2.20	5, 3–5

θ per site is estimated from the total number of mutations (WATTERSON 1975); θ_s per site includes only synonymous nucleotide differences; π , nucleotide diversity, is the average number of nucleotide differences per site between a pair of randomly chosen sequences (NEI 1987); Tajima's *D* (TAJIMA 1989) is based on the differences between the number of segregating sites and the average number of nucleotide differences per sequence; Fu and Li's *D* with an outgroup (FU and LI 1993) is based on the differences between the total number of mutations in the external branches of the genealogy and the total number of mutations; *K*-test (DEPAULIS and VEUILLÉ 1998), actual no. of haplotypes, range of expected no. of haplotypes, significance. **P* < 0.05.

substitutions occur within the window of analysis more frequently than a calculated statistical distribution. In addition, the four-gamete test (HUDSON and KAPLAN 1985) was applied to the data. This test relies on the infinite-sites model and deems that where we find a combination of all four possible combinations of a pair of variable sites the most parsimonious explanation is recombination. It also is possible that such a pattern can be generated by recurrent mutations (homoplasy) at an individual site.

RESULTS

Patterns of inter- and intraspecific diversity and divergence: Considerable diversity was found within the three mitochondrial and two chloroplast genes in *S. vulgaris* (Tables 1 and 2), with remarkably high levels of mitochondrial intraspecific variation for angiosperms (Table 2). The mean pairwise numbers of synonymous substitutions per total synonymous sites (K_s) for the eight *atp9* haplotypes found in *S. vulgaris* were similar to those found in comparisons of *atp9* haplotypes from rice and maize (Table 3), two species that are believed to have diverged 50 MYA (GAUT 1998). K_s for the seven *atp1* haplotypes in *S. vulgaris* also was high, approximately half the value found in comparisons of *atp1* haplotypes from rice and maize (Table 3). K_s for the six apocytocytome b (*cob*) haplotypes was not as high as that for *atp1* and *atp9* and was an order of magnitude lower than that found both between rice and maize and the strikingly high value found among *S. acaulis* haplotypes (Table 3).

Plant mitochondrial and chloroplast transcripts are known to undergo post-transcriptional C–U editing at nonsynonymous sites (GRAY 1996). Such editing may result in C–T DNA polymorphism not being reflected as a polymorphism in the mRNA; thus although the site is predicted to be nonsynonymous from the DNA, with editing the mutation will not alter the amino acid. In our data set, four nonsynonymous variable sites are potentially influenced by C–U RNA editing: site 21 in *atp9*, site 796 in *cob*, and sites 19 and 140 in *matK*. Many of the analyses presented herein do not utilize in-

formation on the coding state of the site; however, we have presented adjusted K_s/K_a -values for these three genes by scoring these sites as synonymous rather than nonsynonymous substitutions, as well as the nonadjusted values (Table 3).

TABLE 3
Intraspecific average interhaplotype pairwise synonymous and nonsynonymous substitutions per site in organelle genes of *Silene vulgaris* relative to other taxa and the rice–maize divergence

Gene	Length	Variable sites	K_s	K_a
<i>Silene vulgaris</i>				
<i>atp1</i>	1067	25	0.0373	0.0022
<i>atp9</i>	213	21	0.0961 (0.1142)	0.0076 (0.0053)
<i>cob</i>	817	4	0.0018 (0.0035)	0.0022 (0.0014)
<i>matK</i>	572	9	0.0020 (0.0061)	0.0059 (0.0048)
<i>ndhF</i>	1067	4	0.0033	0.0010
<i>Silene acaulis</i> ^a				
<i>cob</i>	1041	22	0.0118	0.0038
<i>Plantago</i> spp. ^b				
<i>atp1</i>	615	183	1.1249	0.0206
<i>ndhF</i>	1624	213	0.0795	0.0078
<i>cox1</i>	1344	363	0.1908	0.1331
<i>rbcl</i>	1317	181	0.0753	0.0067
Rice–maize ^a				
<i>atp1</i>	1521	—	0.0598	0.0146
<i>atp9</i>	216	—	0.0748	0.0126
<i>cob</i>	1155	—	0.0241	0.0028
<i>matK</i>	1558	133	0.1993	0.0613
<i>ndhF</i>	1697	185	0.1460	0.0243

K_s , interhaplotype average number of pairwise synonymous substitutions per synonymous site; K_a , interhaplotype average number of pairwise nonsynonymous substitutions per nonsynonymous sites. Values in parentheses are adjusted to reflect potential RNA editing.

^a STÄDLER and DELPH (2002) following GAUT (1998).

^b CHO *et al.* (2004)—GenBank accession nos. AJ389588–AJ389621 and AY818897–AY818951.

For both *atp1* and *atp9*, one *S. vulgaris* haplotype (represented by Vt17b and Ld5, Table 1) was identical to the haplotypes found in *S. latifolia* and *S. uniflora*, whereas other haplotypes were extremely diverged, for *atp1*, for *atp9*, or for both (Figure 2, Table 1). The majority of mitochondrial variation occurred at synonymous codon positions. Four of the 25 variable sites in *atp1* resulted in amino acid changes, with $K_a/K_s = 0.0590$ (ROZAS *et al.* 2003 following NEI and GOJOBORI 1986). For *atp9*, 4 of the 21 variable sites in *S. vulgaris* resulted in amino acid changes, with $K_a/K_s = 0.0791$. Three of the four variable sites in *cob* resulted in amino acid changes ($K_a/K_s = 1.2222$). K_a/K_s -values presented for *atp9* and *cob* are unadjusted for potential RNA editing; adjusted values can be seen in Table 3.

Multivariate HKA tests indicated that intraspecific and interspecific divergence was not correlated among all five genes. Specifically, intraspecific diversity of mitochondrial genes was significantly greater than that of chloroplast genes (likelihood-ratio test = 24.22, d.f. = 2, $P < 0.0001$; using *N. tabacum* as an outgroup). Moreover, among mitochondrial genes intraspecific diversity for *atp1* and *atp9* was greater than that of *cob* (likelihood-ratio test = 9.655, d.f. = 1, $P < 0.002$).

The seven *atp1* haplotypes observed were significantly fewer than expected assuming 25 segregating sites as determined by neutral coalescent simulations of the expected distribution of haplotypes (*K*-test, Table 2). For *atp9*, haplotype numbers were within the 95% confidence intervals for the expected distribution given the observed numbers of segregating sites, whereas in *cob* there were more haplotypes than expected (Table 2). For these tests we assumed no recombination, which leads to conservative estimates of the expected number of haplotypes. If recombination is allowed, then the number of haplotypes present in *cob* is not significantly different from neutral expectations (expected number of haplotypes is four to seven). Because recombination was detected in *cob* using the four-gamete test (see below), we suspect that the elevated number of haplotypes results from recombination. Fu and Li's *D*-statistic detected a significant departure from neutral expectations for *atp1* and *atp9* (Table 2) as can be seen via relatively long internal branches for these genes (Figure 2). No other tests of nonneutrality using Tajima's *D* and Fu and Li's *D* were significant (Table 2).

Interspecific divergence in *matK* and *ndhF* was substantial between *S. vulgaris* and *S. latifolia* (Table 1). Intraspecific variations in synonymous sites among the distinct haplotypes of these two *S. vulgaris* chloroplast genes were both two orders of magnitude lower than that found between rice and maize (Table 3). In *matK*, there was a bias toward nonsynonymous substitutions, with unadjusted $K_a/K_s = 2.9500$. Two of the four variable sites in *ndhF* were nonsynonymous, with $K_a/K_s = 0.3030$. No tests of nonneutrality (*K*-test, Tajima's *D*, Fu and Li's *D*) for either chloroplast gene were significant.

Reassociation and recombination: Evidence for reassociation between genomes and recombination within genomes was detected at three levels of organization: between the chloroplast and mitochondrial genomes, between genes within the mitochondrial genome, and between sites within genes in the mitochondria. Strict co-inheritance of genomes is expected to generate congruent phylogenetic histories between genes in different genomes, whereas the absence of congruence implies at least somewhat independent histories (DESPLANQUE *et al.* 2000; OLSON and MCCAULEY 2000). Pairwise partition-homogeneity tests (FARRIS *et al.* 1994) indicated incongruence between the pairwise histories of *matK* and *atp1* ($P = 0.003$), *matK* and *atp9* ($P = 0.001$), and *atp1* and *atp9* ($P = 0.001$). No additional pairwise comparisons detected incongruence using a Bonferroni-adjusted critical value of 0.005 ($\alpha = 0.05/10$ comparisons), although *cob* and *ndhF* phylogenies did not contain sufficient structure to allow for a powerful test.

Recombination between mitochondrial genes can be inferred from some particularly striking patterns wherein all four possible combinations of genes were observed in different individuals (Table 1). Examination of Table 1 reveals that the most common combined *atp1*–*atp9* haplotype in our sample (represented by SD6) is differentiated from the *atp1*–*atp9* haplotype carried by NY006 and NY007 in 12 changes localized in *atp9*. Ld5 and Vt17b possess the *atp9* haplotype of NY006 and NY007, but differ from SD6, NY006, and NY007 by 8 changes in *atp1*. Finally, individuals such as EB3AG possess the *atp1* haplotype of Ld5 and Vt17b, but have the *atp9* type of SD6. Given the large numbers of differences between the two *atp1* haplotypes (8) and the two *atp9* haplotypes (12), it is highly unlikely that all four combinations of *atp1*–*atp9* haplotypes arose via independent mutational events. This conclusion is supported by the detection of a recombination event between *atp1* and *atp9* haplotypes by all three non-phylogenetic substitution models implemented in RDP (Chimaera, Max-Chi, and SiScan; RDP version 2.0). Finally, the four-gamete test was applied to all pairwise comparisons between segregating sites within each gene and detected three potential intragenic recombination events within *atp1* (between positions 22 and 147, 415 and 507, and 507 and 978; Table 1), one event within *atp9* (between 34 and 148; Table 1), and one event within *cob* (sites 516 and 796; Table 1). The event between sites 22 and 147 of *atp1* was the only recombination event that was detected using all six recombination detection models implemented in RDP (RDP, GENECONV, Bootscan, Chimaera, Max-Chi, and SiScan).

DISCUSSION

Many aspects of cytoplasmic diversity in *S. vulgaris*, from the range of nucleotide diversity to the nonneutral

FIGURE 2.—Maximum-likelihood phylogenies of the three mitochondrial (*atp9*, *cob*, *atp1*) and two chloroplast (*matK*, *ndhF*) genes. Bootstrap support values >50 are shown above the branches. Individuals with five-character-long names are *Silene vulgaris* from North America, and those with three-character names are from Europe. Outgroup sequences from *Nicotiana tabacum* and *Beta vulgaris* were downloaded from GenBank (accession nos. BA00042, NC001879, BA000009, and AY514832).

patterns of diversity in *atp1* and *atp9*, to the absence of complete linkage disequilibrium, indicate that the cytoplasmic genomes are evolving in a manner unlike those of most previously studied taxa. Our results bolster the findings of LAROCHE *et al.* (1997), CHO *et al.* (2004), and PARKINSON *et al.* (2005), which showed highly variable rates of mitochondrial gene evolution among different lineages and genes in interspecific and intergeneric comparisons, and extend these observations to include the presence of high diversity within species. Here we offer several possible explanations for these widespread and enigmatic patterns in *Silene*.

Diversity: We observed extremely high intraspecific nucleotide diversity at *atp9*, perhaps the highest average number of pairwise differences among intraspecific mitochondrial haplotypes observed for any mitochondrial gene to date (Table 2). The most divergent *atp9* haplotypes (*e.g.*, Vt17b *vs.* 6283B; $K_s = 0.37$) exhibited five times the level of divergence found between rice and maize and one-third the level found for *atp1* within the entire genus *Plantago*, which has the highest documented rate of intrageneric mitochondrial evolution (CHO *et al.* 2004; MOWER *et al.* 2004). A comparable study of intraspecific mitochondrial nucleotide diversity showed that *S. acaulis* also exhibited high intraspecific variation at the *cob* mitochondrial coding region (STÄDLER and DELPH 2002). The common ancestor of *S. acaulis* and *S. vulgaris* is represented by the earliest members of the genus (OXELMAN *et al.* 1997), indicating that high mitochondrial variation may be a characteristic of the entire genus, although the amount of variation in particular genes such as *cob* is not consistent across taxa (Table 3).

Three potential mechanisms may be responsible for the high rates of variation within plants: (1) lineage-specific increased mutation rates of mitochondrial genes throughout the genome (CHO *et al.* 2004; PARKINSON *et al.* 2005); (2) divergence in allopatry or in separate species and recent hybridization or introgression; and (3) the maintenance of ancient mitotypes resulting from long-term balancing selection, possibly due to the presence of cytoplasmic male-sterility genes (STÄDLER and DELPH 2002). For the first hypothesis to be correct for *Silene*, different mitochondrial lineages within *S. vulgaris* would be predicted to have different mutation rates, an explanation that is possible but perhaps unlikely at the intraspecific scale (but see CHO *et al.* 2004). Also, we found that different genes within the *S. vulgaris* mitochondrial genome evolve at different rates. Such a pattern is unlikely if elevated diversity results from a genomewide increase in mutation rate, but could result if the mutation rate is elevated for particular genes and not for others.

Population structure, hybridization, and introgression can generate high levels of diversity such as were exhibited in *S. vulgaris*. Although *S. vulgaris* exhibits population structure at an ecological timescale

(McCAULEY 1998; OLSON and McCAULEY 2002), populations in the United States and Europe have been isolated for only a few hundred years (McCAULEY *et al.* 2003) and are found in disturbed areas that are not conducive to long-term population persistence. The lack of allopatric isolation and the presence of diverged haplotypes in similar geographic locations (Figure 1, Table 1) suggest that recent divergence in allopatry was not likely to generate the observed patterns of diversity. Moreover, if more ancient allopatric isolation among European and/or Asian populations is responsible for high contemporary variation, a genomewide influence would be expected; so under this hypothesis it is puzzling why elevated diversity was not found among all mitochondrial and chloroplast genes. *S. vulgaris* is widespread throughout Europe and known to hybridize with some closely related sympatric *Silene* species such as *S. uniflora* (RUNYEON-LAGER and PRENTICE 2000); thus, hybridization and introgression from different species is a potential mechanism generating some diversity. *S. vulgaris* does not hybridize with highly divergent species such as *S. latifolia* and *S. acaulis*, however, so introgression is unlikely to explain the presence and persistence of the extremely divergent haplotypes in *S. vulgaris*.

Haplotype diversity in *atp1* and *atp9* is characterized by long internal and short external branches, a pattern consistent with the maintenance of divergent haplotypes over long time periods due to either balancing selection or population structure. As discussed above, it is unlikely that this pattern was generated by population structure. Although *atp1* and *atp9* genealogies exhibited nonneutral evolutionary patterns, it seems unlikely that the target of selection was either *atp1* or *atp9* because most polymorphisms in these genes occurred at synonymous sites (Table 1). Instead, allelic variation in mitochondrial genes may be maintained via hitchhiking effects with selection on nearby linked loci. The most likely candidates are CMS genes that theoretical models suggest may be maintained via negative frequency-dependent selection (FRANK 1989; GOUYON *et al.* 1991).

In contrast to our results, studies of chloroplast intergenic spacer regions in *S. vulgaris* found patterns consistent with epidemic-like selective sweeps (INGVARSSON and TAYLOR 2002). Specifically, intraspecific variation at chloroplast intergenic spacer regions in *S. vulgaris* was depauperate relative to that in *S. latifolia* (a dioecious congener) after calibration against variation at a nuclear locus, consistent with epidemic-like selective sweeps (INGVARSSON and TAYLOR 2002). This is in opposition to patterns consistent with the long-term maintenance of high diversity in two of the mitochondrial genes that we studied (*atp1* and *atp9*) in *S. vulgaris*. One explanation for these contrasting results is that chloroplast and mitochondrial genes may have sufficiently independent inheritance that selection in one genome does not influence patterns of diversity in the other.

Congruence, heteroplasmy, and recombination: If organelles are strictly maternally inherited, we would expect the mitochondria and chloroplast genomes to contain congruent phylogenetic signals (OLSON and McCAULEY 2000). We found an absence of congruence between mitochondrial and chloroplast genes and also between mitochondrial genes. The only pairs of coding regions for which congruence could not be rejected included *ndhF* or *cob*, where there was little phylogenetic signal. The absence of congruence illustrates the departure from the current paradigm of organelle gene evolution in *S. vulgaris* and indicates the presence of at least transient heteroplasmy, non-co-inheritance of organelles, and recombination.

The data reported here contrast with those by OLSON and McCAULEY (2000), which found no evidence of incongruence between chloroplast and mitochondrial markers in *S. vulgaris* from Virginia. Both studies included individuals from some of the same populations in Virginia and in the current study, Virginia samples exhibited chloroplast-mitochondrial incongruence; thus, the difference in outcomes of the studies did not result from the smaller geographic scale sampled in OLSON and McCAULEY (2000). The other major difference between the studies is that the current study included DNA sequences from coding regions only, whereas OLSON and McCAULEY (2000) included cpDNA noncoding sequences and mtDNA RFLP Southern blots associated with regions flanking the *cytochrome oxidase I* locus. The RFLP flanking regions and intergenic spacers evolve relatively quickly compared to coding regions, and thus we suspect that each data set is capable of detecting reassociation among the chloroplast and mitochondria at different timescales. Thus, there may be no evidence for recent chloroplast-mitochondria reassociation as shown by OLSON and McCAULEY (2000), but strong evidence for reassociation of the genomes over a longer timescale (shown here).

Our data exhibit strong evidence for the exchange of genetic information among mitochondrial genomes occurring within the timescale of species. Nonetheless, reciprocal recombination between different alleles from mitochondrial homologs must still be a relatively rare event; otherwise recombination would have generated more intermediate allelic variation between the divergent alleles we observed. If the *atp1* and *atp9* haplotypes are ancient, even low rates of recombination would be preserved in present-day haplotypes. The absence of high diversity and ancient haplotypes in *cob* is puzzling in light of the high diversity found in *S. acaulis* (STÄDLER and DELPH 2002), but possibly explained by the often-found close proximity of CMS genes and *atp* subunits in species where CMS genes are well characterized (HANSON and BENTOLILA 2004). We conjecture that *atp1* and *atp9* may be more tightly linked than *cob* to putative CMS genes. The influence of

hitchhiking selection decreases rapidly with distance from the target of selection (HUDSON and KAPLAN 1988; SCHIERUP *et al.* 2000) and thus may have only weak influence on diversity at *cob*. This linkage conjecture must be tempered, however, by our incomplete knowledge of the stability of the physical proximity of plant mitochondrial genes over time. For hitchhiking to be an explanatory mechanism, the physical linkage between genes must be maintained in the face of frequent rearrangements to which mitochondrial genomes are prone (PALMER and HERBON 1988).

The larger question of why few angiosperm species that carry CMS have high frequencies of females in populations (which characterizes gynodioecy) remains unresolved. The presence of CMS and associated male-fertility restorers in several agricultural crop species such as maize and rice, despite the absence of gynodioecy in their wild relatives, suggests that the rate of mutation of new CMS genes may not differ considerably among gynodioecious and nongynodioecious species. There appear to be two quite different fates of CMS genes after they arise within a species. In most species, such as the wild relatives of crops, CMS and their associated male-fertility restorer genes probably spread as epidemics, eventually homogenizing the mitochondrial genome within the ultimately hermaphroditic populations. However, in many of the species that we recognize as gynodioecious, a confluence of associations between the fitnesses of CMS types and their restorers, and perhaps even paternal inheritance, allows the long-term maintenance of CMS polymorphism within a species. A comprehensive theory of the complex evolutionary forces that lead to gynodioecy may one day unite our understanding of the evolution of CMS and mitochondrial molecular evolution within all angiosperms and promises to continue as one of the major themes in modern studies of plant breeding system evolution.

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