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Evidence for paternal transmission and heteroplasmy in the mitochondrial genome of *Silene vulgaris*, a gynodioecious plant

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Gynodioecy refers to the co-occurrence of females and hermaphrodites in the same population. In many gynodioecious plants, sex is determined by an epistatic interaction between mitochondrial and nuclear genes, resulting in intragenomic evolutionary conflict, should the mitochondrial genome be maternally inherited. While maternal inheritance of the mitochondrial genome is common in angiosperms, few gynodioecious species have been studied. Here, the inheritance of the mitochondrial genes *atpA* and *coxI* was studied in 318 *Silene vulgaris* individuals distributed among 23 crosses. While maternal inheritance was indicated in 96% of the individuals studied, one or more individuals from each of four sib groups displayed a genotype that was identical to the father, or that did not match either parent. Given evidence that inheritance is not strictly maternal, it was hypothesized that some individuals could carry a mixture of maternally and paternally derived copies of the mitochondrial genome, a condition known as heteroplasmy. Since heteroplasmy might be difficult to detect should multiple versions of the mitochondrial genome co-occur in highly unequal copy number, a method was devised to amplify low-copy number forms of *atpA* differentially. Evidence for heteroplasmy was found in 23 of the 99 individuals studied, including cases in which the otherwise cryptic form of *atpA* matched the paternal genotype. The distribution of shared nucleotide sequence polymorphism among *atpA* haplotypes and the results of a population survey of the joint distribution of *atpA* and *coxI* haplotypes across individuals supports the hypothesis that heteroplasmy facilitates formation of novel mitochondrial genotypes by recombination.
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Introduction

Maternal inheritance of the mitochondrial genome has been shown in most species of angiosperms that have been studied, and thus the fitness of mitochondrial genes is usually considered to be a function of seed production (Reboud and Zeyl, 1994; Mogensen, 1996). However, maternal transmission of the mitochondrial genome in angiosperms is not universal, with paternal or biparental cytoplasmic inheritance having been observed in a few species (Nagata *et al*, 1999). Since the inheritance of the mitochondrial genome has only been studied in a small fraction of all wild species of angiosperms (Zhang *et al*, 2003), it is not clear how often nonmaternal inheritance might be a feature of the genetics of natural plant populations or influence the fitness of mitochondrial genes.

One consequence of biparental inheritance is that transmission of copies of mitochondrial genes through both pollen and ovules could result in seeds that carry a heterogeneous collection of mitochondrial genomes inherited from both the mother and the father. That is, biparental inheritance could lead to heteroplasmy. In such cases, intraindividual genetic drift during organellar replication, cell division, and growth (Birky, 2001; Rand, 2001) could result in very unequal representation of different copies of mitochondrial genes in different modules of the plant or among the offspring of a heteroplasmic parent, greatly complicating the interpretation of crossing studies. Further, heteroplasmy could facilitate the creation of novel mitochondrial recombinants (Saville *et al*, 1998; Städler and Delph, 2002). The extent of mitochondrial heteroplasmy in natural populations of plants, and its biological significance, also remains largely unexplored. Further studies of mitochondrial DNA (mtDNA) inheritance and heteroplasmy, encompassing a wide diversity of plant taxa and life histories, would seem warranted.

Knowledge of the inheritance of the mitochondrial genome would seem particularly relevant in gynodioecious plants. Gynodioecious species are those in which hermaphroditic and female individuals co-occur in the same population. In gynodioecious systems, sex determination is often under cyto-nuclear genetic control, resulting from an epistatic interaction between cytoplasmic male sterility (CMS) elements found in the mitochondrial genome and nuclear loci that restore male fertility (Saumitou-Laprade *et al*, 1994; Budar *et al*, 2003). CMS-based gynodioecy has been cited as one of the most widely studied examples of intragenomic conflict, owing to the difference between CMS elements and nuclear restorer loci in mode of transmission, and in the currency of fitness (eg Cosmides and Tooby, 1981; Hurst *et al*, 1996;

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Werren and Beukeboom, 1998). A pleiotropic effect of male sterility is often an increase in the quantity or quality of seeds produced by females, compared to hermaphrodites, resulting in a higher cytoplasmic fitness for those individuals carrying unrestored CMS elements (Frank, 1989; Couvet *et al*, 1990; Budar *et al*, 2003).

Strict maternal inheritance of the mitochondrial genome is assumed in most mathematical models of sex ratio evolution in gynodioecious systems with cytonuclear sex determination (Jacobs and Wade, 2003). With paternal or biparental inheritance, the high seed production of females can occasionally contribute to the cytoplasmic fitness of their mates. Occasional transmission of the mitochondrial genome through pollen also raises the possibility that some individuals could be heteroplasmic for two or more forms of CMS, each requiring different mechanisms of restoration.

Despite its significance for the evolution and maintenance of gynodioecy, our knowledge of patterns of inheritance of the mitochondrial genome in gynodioecious species is limited. Similarly, few studies of gynodioecious species have been designed to detect mitochondrial heteroplasmy or its consequences. Here, we report evidence that the inheritance of the mitochondrial genome in *Silene vulgaris*, a gynodioecious plant, is not strictly maternal, that heteroplasmy may be a common feature of this species, and that one population-level consequence of this heteroplasmy is a reduction of gametic disequilibrium among alleles at two mitochondrial loci. While the results presented here are based on properties of the *atpA* and *coxI* mitochondrial loci, genes not known to be involved in CMS in *S. vulgaris*, it is hoped that the information learned from the study of these two genes will reflect more general properties of the *S. vulgaris* mitochondrial genome, including those specific regions of the genome responsible for CMS that are yet to be identified.

Materials and methods

Study system

S. vulgaris is a weedy gynodioecious species native to Europe and introduced to North America over 250 years ago. It has been the subject of numerous studies designed to investigate the interaction between its gynodioecious breeding system and other features of its population biology (eg Jolls and Chenier, 1989; Pettersson, 1992; McCauley, 1998; McCauley and Brock, 1998; McCauley *et al*, 2000; Ingvarsson and Taylor, 2002; Emery and McCauley, 2002; Olson and McCauley, 2002). Previous crossing studies have indicated that genetic control of sex expression is cytonuclear, with evidence that multiple forms of CMS can be found in natural populations and that these CMS factors interact epistatically with multiple nuclear restorer loci (Charlesworth and Laporte, 1998; Taylor *et al*, 2001). Indirect evidence for occasional heteroplasmy of CMS elements also exists (Andersson, 1999), based on studies of gynomonoecious plants (ie those in which female and hermaphrodite flowers co-occur on the same individual).

Genetic markers

The investigations of mitochondrial variation at the individual, family, and population level reported here all make use of restriction fragment length polymorphism (RFLP) obtained by digesting the amplification products of polymerase chain reactions (PCR) with the appropriate restriction enzymes. In order to develop PCR/RFLP mtDNA markers portions of two mitochondrial genes, *atpA* and *coxI*, were each amplified from a number of *S. vulgaris* individuals using primers developed by CW dePamphilis of Pennsylvania State University (Barkman *et al*, 2000; Bowe *et al*, 2000). Observed differences between individual *atpA* sequences included the gain or loss of several *Alu*I restriction sites and several *Msp*I restriction sites. All of the sequenced individuals could be placed into one of six categories (referred to as haplotypes A→F) based on the presence or absence of *Alu*I and *Msp*I restriction sites within the 1083 bp region of *atpA* amplified by the PCR. This information was used to develop a PCR/RFLP-based protocol for the rapid genotyping of individuals used in this study. For each individual, DNA was extracted from a single leaf, or two paired leaves from the same branch, using either Qiagen Dneasy plant DNA extraction kits or CTAB extraction buffer (Doyle and Doyle, 1987). This genomic DNA was used as a template for PCR using the *atpA* primers mentioned above. A 10 µl aliquot of the PCR product was digested with *Alu*I, and the resulting fragments visualized by electrophoresis in a 4% Metaphor agarose gel stained with ethidium bromide. A separate 10 µl aliquot of the PCR product was digested with *Msp*I, with the digestion products again visualized by electrophoresis and ethidium bromide staining. The *Alu*I and *Msp*I fragment profiles obtained for each individual determined which of the six *atpA* PCR/RFLP haplotypes it carried. A similar protocol was developed for determining *coxI* haplotypes, except that the restriction profile was based on double digestion of a single 10 µl aliquot of the 1437 bp *coxI* PCR product with *Msp*I and *Dde*I. Three *coxI* haplotypes could be detected by this method, which will be referred to as haplotypes 1→3.

Family study

The first study to be reported consists of a series of parent–offspring comparisons with regard to *atpA* or *coxI* PCR/RFLP haplotype. If the inheritance of the mitochondrial genome is strictly maternal, and if all ovule donors are homoplasmic with regard to the mitochondrial genome, mother and offspring genotypes should be identical (barring mutation), regardless of the mitochondrial genotype of the pollen donor. With occasional paternal transmission, some offspring genotypes could match the father rather than the mother, or reflect a mixture of maternal and paternal genotypes (ie be heteroplasmic). Detection of heteroplasmy with PCR-based markers might depend on the ability of the PCR to amplify both versions of the gene simultaneously, which could be difficult if they are present in very unequal copy number. Undetected, or cryptic, heteroplasmy in one or both parents could be particularly problematic in family studies, because haplotypes too rare to be detected in a parent could still be transmitted to offspring, and then by chance increase in relative frequency during the successive episodes of replication and cell division associated with offspring growth. Thus, dissimilarity between an offspring and both of its parents could be an indication of heteroplasmy in one or both parents.

The individuals used for this study were selected from a large number of sibships maintained in the Vanderbilt University greenhouse for other purposes (see Bailey and McCauley, 2005). The origin of the sibships is as follows. Hermaphroditic individuals collected as seeds from various North American populations were used as parents in one of two types of crosses, self-fertilization or outcrossing with another unrelated hermaphrodite. Seeds produced by these crosses (up to 50 seeds/cross) were planted in the greenhouse and raised to flowering. Following the crosses, the parental plants were discarded, but only after a DNA sample was taken from each parent, or a full sibling of that parent, using DNA extraction methods described above.

The PCR/RFLP *atpA* genotype of each parental line was determined by methods described above. Using this information, 23 groups of offspring were selected for study. Included were four sibships resulting from self-fertilization, five sibships resulting from an outcross between two hermaphrodites carrying the same *atpA* haplotype, and 14 sibships resulting from crosses between two hermaphrodites carrying different *atpA* haplotypes. DNA was extracted from 10 to 15 members of each of the 23 sibships and used as a template in the PCR reaction necessary for determining the *atpA* haplotype of that individual. Thus, the final data set allowed for 23 comparisons of the *atpA* haplotypes of 10–15 full siblings with each other and with that of the maternal and paternal lines that created them.

A similar tactic was taken with regard to the *coxI* PCR/RFLP haplotypes for the members of one family group showing strong evidence for paternal transmission (see below).

Intraindividual study

The possibility has been raised above that heteroplasmy might not be detected by a PCR-based marker if copies of one form of that marker are numerically dominant in the sample of DNA used as a template in the amplification reaction. In an effort to develop a method of detecting intraindividual variation in our mitochondrial markers that is otherwise cryptic to our standard protocol, the following technique was devised. Inspection of the original *atpA* sequence data revealed that *atpA* haplotypes C, D, E, and F share a single *Sma*I restriction site, whereas no *Sma*I sites are found in haplotype A or B sequences. Thus, digestion of the genomic DNA of individuals known to carry the C, D, E, or F *atpA* haplotype with *Sma*I should inhibit subsequent amplification of that DNA with the *atpA* primers. However, if the mitochondrial genome also carried a relatively few copies of the A or B forms of *atpA*, these should still be suitable for amplification, and perhaps would amplify more efficiently under these conditions if the *Sma*I treatment knocked down any copies of the C, D, E, or F forms that would otherwise be competitors during the reaction. Thus, the *Sma*I knockdown treatment might reveal cryptic heteroplasmy in individuals in which a minority of A and/or B *atpA* molecules coexist with a majority of C, D, E, or F molecules.

This approach was applied to 99 offspring individuals selected from the Family Study (and to their respective parental lines) using the following protocol. For each individual a 10 μl aliquot of genomic DNA, taken from the same DNA extraction used as a template in the Family Study, was digested with *Sma*I. The digested DNA was then used as a template in the standard *atpA* PCR amplification. As a control, an additional 10 μl aliquot of genomic DNA was subjected to the *Sma*I digestion protocol, except that water was substituted for the restriction enzyme. This was also used as a template in a separate PCR using the *atpA* primers. The expectation is that the control reactions should generate the same *atpA* haplotype scored for that individual in the Family Study. For those individuals whose original haplotype was scored as C, D, E, or F, the *Sma*I knockdown treatment should yield either haplotype A or B, if that individual carried copies of those molecules at a low level, or no product if it was homoplasmic for C, D, E, or F molecules. Those parents whose original genotype was A or B should not be affected by the *Sma*I treatment.

Evidence for recombination

The presence of direct repeats within the plant mitochondrial genome facilitates the occurrence of intra- and intermolecular recombination events (Mackenzie and McIntosh, 1999). However, recombination within or between loci would be most likely to generate novel mitochondrial genotypes if intraindividual variation occurred at the locus or loci in question. Thus, evidence for intra- or interlocus recombination within the mitochondrial genome could also be indirect evidence for heteroplasmy (Städler and Delph, 2002).

Evidence for past intralocus recombination can be obtained from DNA sequences by examining the pattern of shared nucleotide polymorphism and conducting statistical tests designed to test the null hypothesis that patterns indicative of recombination are, in fact, due to homoplasy (Städler and Delph, 2002). To that end, *atpA* sequences representing one of each of the six *S. vulgaris* PCR/RFLP haplotypes were aligned manually and examined for shared nucleotide polymorphism. In addition, the *atpA* sequence obtained from one *S. latifolia* individual was used as an out-group.

At the population level, recombination between two polymorphic loci can be inferred from the presence of all possible two-locus genotypes (Saville *et al*, 1998). In order to evaluate the diversity of joint *atpA*/*coxI* haplotypes occurring in natural populations of *S. vulgaris*, seeds originating from a number of European populations of *S. vulgaris* were planted in the Vanderbilt University greenhouse and raised until enough foliage was produced for DNA extraction. Aliquots of this genomic DNA were used as a template for two PCR amplifications per individual, one using the *atpA* primers and one using the *coxI* primers. The resulting amplification products were used to determine an *atpA* and a *coxI* PCR/RFLP haplotype for 89 individuals using methods outlined for the Family Study. Statistical independence of *atpA* and *coxI* haplotype identities across individuals was tested using a *G*-test of Independence (Sokal and Rohlf, 1995).

Results

Family study

The results of the crossing study are presented in Table 1. The *atpA* PCR/RFLP haplotype was determined for 318

Table 1 *atpA* haplotypes of 23 full sib families created by greenhouse crosses of *S. vulgaris* lineages of known *atpA* haplotype

Family	*Maternal haplotype*	*Paternal haplotype*	*#Offspring/ offspring haplotype*
1	D	Self	10 D
2	B	Self	10 B
3	A	Self	10 A
4	C	Self	10 C
5	A	A	15 A
6	E	E	15 E
7	D	D	15 D
8	D	D	14 D, 1 A
9	D	D	15 D
10	D	B	15 D
11	B	A	15 B
12	B	A	15 B
13	A	D	14 A, 1 E
14	A	B	15 A
15	E	C	15 E
16	B	C	6 B, 8 C, 1 B/C[a]
17	C	B	15 C
18	C	E	15 C
19	C	A	15 C
20	B	A	14 B
21	A	B	14 A, 1 C
22	D	C	12 D
23	D	A	12 D

[a]B/C refers to an individual whose restriction pattern resembles an overlay of the B and C haplotypes.

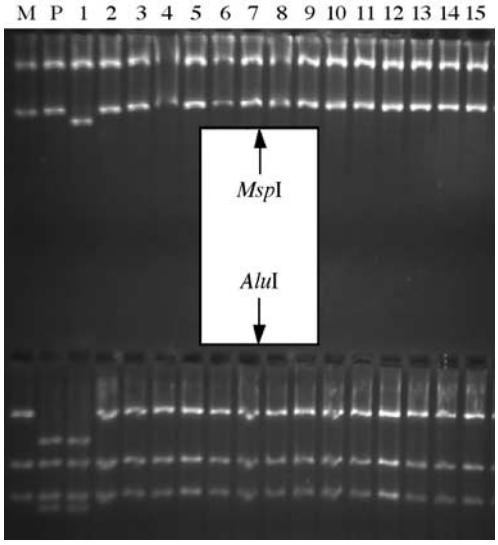

Figure 1 *Msp*I and *Alu*I restriction fragments observed following digestion of *atpA* PCR product obtained from maternal (M) or paternal (P) *S. vulgaris* individuals, along with 15 of their offspring. Note that offspring 2–15 carry haplotypes that match the maternal line (haplotype A), whereas offspring 1 carries a haplotype (C) that is not carried by either parent. The father is haplotype B.

offspring distributed among 23 families. Of these, 306 individuals (96%) carried the expected maternal haplotype. Strict maternal inheritance was shown for at least one family carrying each of the five *atpA* haplotypes (A→E) used in the study (no representatives of haplotype F were discovered), demonstrating that the results were not haplotype dependent. Four of the 23 sibships (17%) contain at least one individual whose *atpA* genotype does not resemble the mother (see Figure 1 for an example), including three sibships in which the offspring genotype does not resemble either the maternal or the paternal line. In the fourth of these sibships (family 16), eight of 15 offspring resemble the paternal, rather than the maternal line. This group also contains an individual whose *atpA* restriction profile resembles what would be expected if the maternal and paternal profiles were superimposed over one another, much as would be expected in a heteroplasmic individual in which the maternal and paternal contributions were each amplified. The *coxI* PCR/RFLP genotype was also determined for every individual in family 16. The paternal and maternal lines used to create the family 16 sibship also carried different *coxI* PCR/RFLP haplotypes. Only the eight individuals that carried the paternal *atpA* haplotype in family 16 also carried the paternal *coxI* haplotype.

Intraindividual study

The *Sma*I restriction knockdown method, as well as the control treatment, was applied to the genomic DNA of 99 offspring and their parents, distributed among 15 families. The results are summarized in Table 2. The control treatment of the parents and offspring yielded the same *atpA* haplotype seen for that individual in the Family Study. The *Sma*I treatment yielded the same *atpA* haplotype seen in the Family Study in the eight parents whose haplotype was A or B. The *Sma*I treatment resulted in a different *atpA* haplotype in one paternal line of the remaining 20 parents, and in 23 of the 99 offspring (see Figure 2 for an example). In the remaining individuals, the *Sma*I-treated DNA template did not yield any PCR product.

Table 2 *atpA* haplotype revealed when *S. vulgaris* genomic DNA is digested with *Sma*I prior to PCR with *atpA* primers

Family/(cross)	*Original haplotype*	*Haplotype after* SmaI *treatment*
1 (D self)	6 D	6 blank
4 (C self)	6 C	6 blank
6 (E × E)	8 E	8 blank
7 (D × D)	8 D	8 blank
8 (D × D[d])	7 D	7 A
10 (D × B)	8 D	8 blank
13 (A × D)	1 E	1 A[a]
15 (E × C)	8 E	8 blank
16 (B × C)	4 C	2 B[a], 2 blank
17 (C × B)	8 C	7 A[c], 1 blank
18 (C × E)	8 C	8 blank
19 (C × A)	8 C	4 A[b], 4 blank
21 (A × B)	1 C	1 A[a]
22 (D × C)	10 D	9 blank, 1 A[c]
23 (D × A)	8 D	8 blank

The original haplotype column presents the number of individuals from each sibship that were studied and their respective haplotypes in the Family Study. Blank refers to no PCR product after *Sma*I treatment. Superscripts a, b, and c refer to haplotypes that resemble the maternal haplotype, the paternal haplotype, and neither, respectively. Superscript d indicates that *Sma*I treatment of the paternal line also revealed haplotype A.

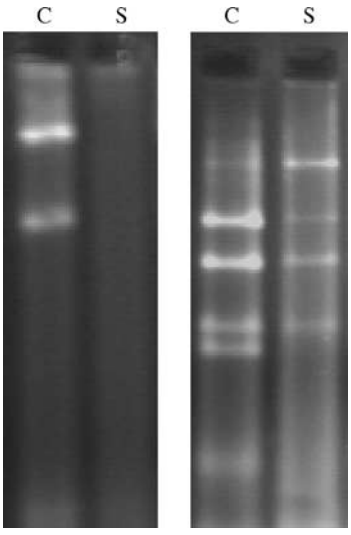

Figure 2 *Alu*I restriction fragments observed for two *S. vulgaris* individuals when an *Sma*I digestion treatment (S) or a control treatment (C) was applied to genomic DNA prior to PCR amplification using *atpA* primers. For the individual on the left, haplotype E is seen in the control, whereas no PCR product was obtained in the *Sma*I treatment. For the individual on the right, haplotype C is replaced by haplotype A following the *Sma*I treatment, an example of cryptic heteroplasmy.

In four of the offspring whose haplotype did not resemble the mother in the Family Study, treatment with *Sma*I prior to PCR yielded the maternal type. This included two individuals from Family 16 whose Family Study haplotype resembled the father. In seven individuals from Family 17, the *Sma*I haplotype does not match either parental line, whereas the Family Study haplotypes indicate maternal inheritance. In the one family in which the *Sma*I treatment differed from the control in the paternal line (Family 8), this same difference between treatments was also seen in seven of his offspring. It should be noted that the haplotype seen after *Sma*I treatment of this family (haplotype A) was identical to the haplotype seen in one member of this sibship that did not resemble the mother in the Family Study.

Evidence for recombination

In all, 11 shared polymorphic nucleotide sites were found within the *S. vulgaris atpA* sequences (Table 3). One observation of such data that is expected with recombination would be the occurrence of all recombinatorial pairs of nucleotides at some paired polymorphic positions, as is the case at paired positions 3–4 (AG, AC, CG, and CC) and 8–9 (AA, AG, CA, and CG) (Table 3). There are a number of published statistical tests that can be used to evaluate evidence for recombination of this sort against a null model in which the apparent recombination is due to homoplasy. Each of these tests requires particular assumptions about the model of evolution and the sampling strategy (eg Saville *et al*, 1998; Städler and Delph, 2002). Inspection of Table 3 reveals a striking pattern that allows for a very simple statistical test conservative to these assumptions. The first four nucleotide polymorphisms found in haplotype C are shared with haplotype A and *S. latifolia*, whereas the last seven polymorphisms found in haplotype C are shared with haplotype F. This pattern could be viewed as evidence for either intragenic recombination or homoplasy. A simple statistical test to distinguish these two possibilities first assumes that haplotype C is derived from haplotype F without recombination, and that the TTAA motif it shares with haplotype A is due to homoplasy. Assuming that each of the four nucleotide positions has undergone a mutation, and that all nucleotide–nucleotide transitions are equally likely, yields a probability of a ACGC→TTAA transition of $(1/3)^4$ or 0.012. (Assuming that haplotype F is derived from haplotype A without recombination yields a probability of a GTGAATA→CGAAGTG transition of $(1/3)^5$ or 0.004.) Although more realistic assumptions incorporating a much lower mutation rate and unequal nucleotide usage would undoubtedly yield a lower probability of homoplasy, the simple conservative test used here provides strong statistical evidence for a recombination event.

The joint *atpA*/*coxI* haplotype was determined for 89 *S. vulgaris* individuals collected from natural populations in Europe. Several additional individuals carrying rare *atpA* haplotypes not detected previously were excluded from the analysis. Note that *atpA* haplotypes C, D, E, and F were not found in the European sample. The distribution of two-locus genotypes is presented in Table 4. It can be seen that one or more individuals carrying each of the possible two-locus genotypes were found. However, the two loci are not in gametic equilibrium, in that alleles at the two loci are not in random association with one another across individuals ($G_{\text{independence}} = 8.69$, $\text{df} = 2$, $P = 0.013$).

Table 3 Shared polymorphic nucleotides occurring within an 850 bp region of the mitochondrial *atpA* gene sequenced in one *S. latifolia* individual and six *S. vulgaris* individuals previously distinguished by *atpA* PCR/RFLP's

Haplotype	*Polymorphic nucleotides (relative position)*										
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
S. latifolia	T	T	A	A	G	T	G	A	A	T	A
A	**T**	**T**	**A**	**A**	G	T	G	A	A	T	A
C	**T**	**T**	**A**	**A**	**C**	**G**	**A**	**A**	**G**	**T**	**G**
F	A	C	G	C	**C**	**G**	**A**	**A**	**G**	**T**	**G**
B	T	T	A	C	G	T	G	C	A	T	A
D and E	A	C	G	C	C	G	A	C	G	C	A

Table 4 Number of joint *atpA*/*coxI* haplotypes found in a sample of 89 *S. vulgaris* individuals

	CoxI *haplotype*		
	1	*2*	*3*
atpA haplotype			
A	24 (30.2)	28 (24.0)	9 (6.9)
B	20 (13.8)	7 (11.0)	1 (3.1)

Expected values under a null hypothesis of random association of haplotypes between loci are given in parenthesis.
Note: Several individuals with rare *atpA* haplotypes are not included.

Discussion

The main results of the studies of *S. vulgaris* presented here are: (1) mother and offspring mtDNA haplotypes do not always match when PCR products derived from coding regions are used as mitochondrial genetic markers in controlled crosses, (2) more than one form of these mitochondrial genetic markers can coexist within the same individual, and (3) there is evidence of both intra- and interlocus recombination. These results are consistent with the hypotheses that (1) the inheritance of the mitochondrial genome in *S. vulgaris* is not strictly maternal, (2) an individual can inherit copies of the mitochondrial genome from both parents simultaneously, resulting in a heteroplasmic state, and (3) heteroplasmy facilitates the creation of novel mitochondrial genotypes through intra- and intergenic recombination. It would be useful to consider how more specific details of the results support these hypotheses, how the results might be interpreted in light of competing alternate hypotheses, and how the results presented here compare to results from previous studies of *S. vulgaris*.

When considering evidence for transmission of the mitochondrial genome through pollen, one should probably assume that copies of the mitochondrial genome are likely to be transmitted to the embryo through the ovule as well. Further, it is possible that the relative frequencies of the maternally and paternally derived copies could change by chance during growth and development (Birky, 2001; Rand, 2001). Documentation of paternal transmission in the Family Study would occur when copies of the mitochondrial genome transmitted through pollen occur at a number sufficient to be detectable by PCR, perhaps by increasing in relative frequency by intraindividual drift during growth. Should that happen, and if the maternal line of molecules is lost by chance, or remains at a level not easily detected by PCR, the inheritance would appear to be paternal. The best evidence for paternal transmission of this sort comes from family 16, in which eight of 15 offspring match the paternal, rather than the maternal line, for both the *atpA* and *coxI* markers.

Retention of both paternally derived and maternally derived copies of the mitochondrial genome at relatively high copy number during development would result in a heteroplasmic individual whose PCR/RFLP profile resembles an overlay of the two parental types. This was seen in one member of the family 16 sibship in the Family Study. With biparental inheritance, there must also be cases in which a paternal lineage of molecules is transmitted to the embryo during fertilization but does not rise to a frequency high enough to dominate the PCR. This hypothesis suggests that there should exist a number of cryptically heteroplasmic individuals in which either the maternal or paternal lineage of molecules exists at levels that are difficult to detect by PCR, when in competition with a majority lineage traced to the other parent. Support for this possibility comes from the knockdown experiment in which treatment with *Sma*I caused two of the members of family 16 displaying paternal inheritance in the Family Study to display the maternal genotype, and several members of family 19 displaying maternal inheritance in the Family Study to display the paternal genotype.

Given that an individual is heteroplasmic, a heterogeneous collection of molecules could be passed to its offspring, even when inheritance is uniparental. A haplotype that is at low frequency in a cryptically heteroplasmic parent could, by chance, drift to high frequency during maturation of a minority of its offspring, resulting in the occasional appearance of an unexpected genotype in a PCR survey. Remember that two of the individuals that did not resemble either parent in the Family Study did display the maternal haplotype after *Sma*I treatment. In addition, cases of father–offspring haplotype matches were revealed in family 8 after the *Sma*I treatment revealed the father to be cryptically heteroplasmic.

An alternate hypothesis that must be considered is that there could exist two or more paralogous copies of *atpA* in the mitochondrial genome of *S. vulgaris* that can be amplified by the PCR primers used in this study. Perhaps one or the other of the paralogs amplifies differentially, depending on the family being studied. One reason for the differential PCR amplification might be large differences between paralogs in copy number. Studies of the mitochondrial genomes of several plant species have documented a phenomenon known as substoichiometric shifting in which subgenomic mtDNA molecules, normally maintained in low abundance relative to the rest of the mitochondrial genome, can shift in copy number abruptly between generations (Mackenzie and McIntosh, 1999). This process is most likely under nuclear control and has been implicated in CMS in some species (Mackenzie and McIntosh, 1999; Arrieta-Montiel *et al*, 2001). If paralogous copies of *atpA* are involved in such a process, shifting events could result in dissimilarity between mother and offspring in the *atpA* haplotype scored by PCR, of the sort seen in the Family Study. The cryptic heteroplasmy revealed by the *Sma*I knockdown experiment could be a consequence of the effects of the inequity in copy number on the outcome of PCR. The key difference between the paternal transmission hypothesis and the substoichiometric shifting hypothesis is that, in the latter, mother–offspring dissimilarity and apparent heteroplasmy could be observed even if the mitochondrial genome is strictly maternally inherited. A secondary difference is that under the shifting hypothesis deterministic forces, rather than intraindividual genetic drift, determine relative copy number.

While paternal transmission and substoichiometric shifting would be expected to generate several similar empirical observations, given the experiments described here, some specific features of the data set seem best explained by the paternal transmission hypothesis. Foremost is the observation that eight members of family 16 carry the paternal haplotype for both *coxI* and *atpA*. Under the substoichiometric shifting hypothesis, one would have to imagine that there were paralogous copies of both loci whose copy number shifted simultaneously, and that the observed father–offspring similarity was coincidental for both loci. In addition, there are a number of examples in the Intraindividual Study in which *Sma*I treatment of offspring DNA exposed a cryptic *atpA* haplotype also seen in the paternal, but not the maternal line.

The results of several previous studies of the genus *Silene* are relevant to the interpretation of those presented

here. Two other studies of *S. vulgaris* have used genetic markers to investigate the inheritance of the mitochondrial genome in this species (Olson and McCauley, 2000; Andersson-Ceplitis and Bengtsson, 2002). Strict maternal inheritance was found in both cases. However, given the large sample sizes needed to detect rare paternal inheritance with a high degree of probability (Milligan, 1992), we do not regard the low rate of nonmaternal inheritance reported here and the results of the previous studies as necessarily contradictory. Milligan (1992) presents an equation based on the binomial distribution that is often used to set an upper bound on the probability of nonmaternal inheritance, given a sample size (n) and an arbitrary degree of certainty. For example, Olson and McCauley (2000) state that given their results there is a 95% probability that the actual rate of nonmaternal inheritance is less than 19.3%. Thus, while they showed convincingly that the inheritance of the mitochondrial genome is predominantly maternal, their power to detect rare nonmaternal inheritance events at a frequency comparable to that reported here was relatively weak. Given their much larger sample sizes, Andersson-Ceplitis and Bengtsson (2002) had the statistical power to detect nonmaternal inheritance with 95% probability if its true rate of occurrence is greater than about 1%. However, the equation suggested by Milligan (1992) assumes that each observation is independent, which may not always be the case. Typically, the Milligan calculations are based on a sample size n that is obtained by pooling some number of offspring from each of a relatively small number of crosses (as is the case in Olson and McCauley 2000; Andersson-Ceplitis and Bengtsson, 2002 and this study). There is evidence from studies of chloroplast DNA (cpDNA) (Birky, 1995; Mogensen, 1996) that the probability of nonmaternal inheritance can vary among crosses or among genotypes. If this is the case, the statistical power to detect nonmaternal inheritance decreases as the total number of observations is apportioned among fewer and fewer crosses, since observations within families are not independent. Thus, the results presented here demonstrate that nonmaternal inheritance can occur upon occasion, but do not allow for estimation of its rate with any degree of precision, whereas the previous studies demonstrated that nonmaternal inheritance is rare, but do not eliminate the possibility that it could occur at a frequency comparable to that reported here.

Evidence for mitochondrial heteroplasmy in *S. vulgaris* was presented in a study by Andersson (1999) based on differences in the offspring sex ratios produced by female and hermaphrodite flowers found on the same gynomonoecious individual, and on the spatial distribution of the two flower morphs across the plant. A plausible interpretation of these results is that gynomonoecious individuals inherit more than one form of CMS, and that these forms segregate spatially within the plant during growth and development (Andersson, 1999). In a study of *S. acaulis*, a related gynodioecious species, Städler and Delph (2002) suggested that their evidence for mitochondrial intragenic recombination implied the existence of heteroplasmy.

Two previous observations of the population genetics of *S. vulgaris* would not be expected, given the results presented here. A perfect association between mitochondrial *nad4* restriction haplotype and the presence or absence of mitochondrial plasmids was found in a study of Swedish populations of *S. vulgaris* by Andersson-Ceplitis (2002). Occasional paternal transmission or heteroplasmy in the mitochondrial genome would be expected to break up these associations. Olson and McCauley (2000) studied the joint distribution of cpDNA and mtDNA haplotypes among *S. vulgaris* individuals collected from a number of local populations in southwestern Virginia. They found a perfect association between cpDNA and mtDNA haplotypes across individuals. No mtDNA haplotype was associated with more than one cpDNA haplotype. Multiple mtDNA haplotypes were nested within some cpDNA types. This is what one might expect if cpDNA and mtDNA were both strictly maternally inherited, but evolved at different rates. However, a recent study of cpDNA and mtDNA variation in *S. vulgaris* populations in Europe by Storchova and Olson (2004) found cases of mtDNA haplotypes nested within cpDNA types and other cases in which cpDNA haplotypes were nested within mtDNA types.

It remains to be seen how the paternal transmission and heteroplasmy documented here by greenhouse crosses influence the genetics of natural populations. Several features of the population biology of *S. vulgaris* would seem to reduce the opportunity for paternal transmission to impact the population genetics of the mitochondrial genome. Most importantly, a study of *S. vulgaris* populations in Virginia by Emery (2001) has shown that hermaphrodites have a self-fertilization rate of about 40%. Paternal transmission with self-fertilization would not introduce additional heterogeneity to a mitochondrial genome. Similarly, mitochondrial genes display modest to high local population structure (Andersson-Ceplitis, 2002; Olson and McCauley, 2002; Storchova and Olson, 2004), and so a significant fraction of outcross events would be between genetically similar individuals. Recall, however, that one case of cryptic heteroplasmy was discovered in a paternal individual that was collected as a seed from a natural population. Further, the evidence for mitochondrial recombination in our study of European populations suggests the signature of biparental inheritance and heteroplasmy, as does the evidence for heteroplasmy presented by Andersson (1999). Further studies of natural populations are clearly indicated.

The evidence presented here for paternal transmission and heteroplasmy in the mitochondrial genome of *S. vulgaris* comes from studies of two mitochondrial genes not known to be involved in CMS in this species. The relevance of these results for the study of gynodioecy depends on the likelihood that mitochondrial CMS elements behave similarly. Until the CMS elements are identified molecularly, it will be difficult to study their inheritance directly. Still, the possibility of occasional paternal transmission of mitochondrial CMS elements, and that this paternal transmission can result in heteroplasmy, is intriguing. When transmission of CMS is not strictly maternal, any advantage enjoyed by females in seed production does not translate directly into a fitness advantage for the cytoplasmic genes that she carries, perhaps influencing the dynamics of sex ratio evolution. How heteroplasmy for CMS affects sex expression is unclear, but must depend, in part, on how the different CMS factors become distributed spatially across the

plant. Full restoration of a plant heteroplasmic for CMS would be difficult if it requires distinct restorer alleles for each type of CMS carried by that plant. Given the results presented here for *S. vulgaris*, it would seem important to consider the possibility of nonmaternal inheritance and heteroplasmy when studying the population biology of any gynodioecious plant species for which cytonuclear sex determination is suspected, but in which the inheritance of the mitochondrial genome is not known.

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