Mitochondrial Heteroplasmy and Paternal Leakage in Natural Populations of Silene vulgaris, a Gynodioecious Plant

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It is currently thought that most angiosperms transmit their mitochondrial genomes maternally. Maternal transmission limits opportunities for genetic heterogeneity (heteroplasmy) of the mitochondrial genome within individuals. Recent studies of the gynodioecious species Silene vulgaris and Silene acaulis, however, document both direct and indirect evidence of mitochondrial heteroplasmy, suggesting that the mitochondrial genome is at times transmitted via paternal leakage. This heteroplasmy allows the generation of multi-locus recombinants, as documented in recent studies of both species. A prior study that employed quantitative PCR (q-PCR) on a limited sample provided direct evidence of heteroplasmy in the mitochondrial gene atp1 in S. vulgaris. Here, we apply the q-PCR methods to a much larger sample and extend them to incorporate the study of an additional atp1 haplotype along with two other haplotypes of the mitochondrial gene coxl to evaluate the origin, extent, and transmission of mitochondrial genome heteroplasmy in S. vulgaris. We first calibrate our q-PCR methods experimentally and then use them to quantify heteroplasmy in 408 S. vulgaris individuals sampled from 22 natural populations located in Virginia, New York, and Tennessee. Sixty-one individuals exhibit heteroplasmy, including five that exhibited the joint heteroplasmy at both loci that is a prerequisite for effective recombination. The heteroplasmic individuals were distributed among 18 of the populations studied, demonstrating that heteroplasmy is a widespread phenomenon in this species. Further, we compare mother and offspring from 71 families to determine the rate of heteroplasmy gained and lost via paternal leakage and vegetative sorting across generations. Of 17 sibships exhibiting coxl heteroplasmy and 14 sibships exhibiting atp1 heteroplasmy, more than half of the observations of heteroplasmy are generated via paternal leakage at the time of fertilization, with the rest being inherited from a heteroplasmic mother. Moreover, we show that the average paternal contribution during paternal leakage is about 12%. These findings are surprising, given that the current understanding of gynodioecy assumes that mitochondrial cytoplasmic male sterility elements are strictly maternally inherited. Knowledge of the dynamics of mitochondrial populations within individuals plays an important role in understanding the evolution of gynodioecy, and we discuss our findings within this context.

Introduction

It is widely believed that the mitochondrial genome is strictly maternally inherited in most angiosperm species (e.g., Reboud and Zeyl 1994; Mogensen 1996; Birky 2001). If so, maternal inheritance would tend to enforce homoplasmy (within individual homogeneity) of the mitochondrial genome, in that mutation would then be the primary source of sequence variation between copies of the genome found within the same individual, and bottlenecks in within-cell mitochondrial population size associated with cell division would limit the accumulation of such variation (Birky 2001). However, several recent studies of two members of the plant genus Silene challenge this view and present direct and indirect evidence that heteroplasmity, and perhaps nonmaternal inheritance, of the mitochondrial genome may not be all that uncommon. Indirect evidence comes from the observation of collections of individuals of both Silene acaulis and Silene vulgaris that together meet the “four gamete” criterion for the detection of intragenic or intergenic recombination within the mitochondrial genome (Städlér and Delph 2002; McCauley et al. 2005; Houlston and Olson 2006; Barr et al. 2007; McCauley and Ellis 2008). That is, more sequence or multi-locus genotype combinations are present than could be accounted for by mutations within nonrecombining lineages, implying recombination (Hudson and Kaplan 1985). Because recombination can create novel genotypes most easily when the participating molecules differ in sequence, evidence for recombination is indirect evidence for heteroplasmy. Direct evidence comes from a study of S. vulgaris by Welch et al. (2006) that used quantitative PCR (q-PCR) methodology to document and quantify heteroplasmy in the mitochondrial gene atp1 (atpA) in a number of S. vulgaris individuals sampled from several natural populations.

One source of heteroplasmy would be the mixing of mitochondrial genomes that would occur if mitochondrial inheritance is not strictly maternal, but rather is occasionally biparental owing to episodes of paternal leakage through pollen. Birky (2001) discusses how occasional leakage combined with stochastic events during organelle transmission and subsequent cell division could result in a continuum of levels of homoplasy/heteroplasy among individuals. Limited evidence of mitochondrial paternal leakage in S. vulgaris was found in the results of a crossing study described by McCauley et al. (2005). Evidence of heteroplasmy and paternal leakage in the two species of Silene is particularly interesting because each species is gynodioecious (co-occurrence of female and hermaphrodite individuals) with likely cyto-nuclear sex determination involving interactions between cytoplasmic male sterility (CMS) elements in the mitochondrial genome and nuclear genes that restore male fertility (Charlesworth and Laporte 1998; Taylor et al. 2001). Thus, understanding the population biology of the mitochondrial genome may be one key to understanding the evolutionary dynamics of gynodioecy in these species (McCauley and Olson 2008). More broadly, such information should be useful when
considering the dynamics of plant mitochondrial gene and genome evolution, especially given recent interest in this area (e.g., Cho et al. 2004; Mower et al. 2007; Sedge et al. 2007; Sloan et al. 2008).

Though heteroplasmy can arise from occasional biparental inheritance of the mitochondrial genome, McCauley and Ellis (2008) point out that biparental inheritance would only result in mitochondrial genome heterogeneity when the egg and pollen donors transmit different mitochondrial genotypes. Because the local population structure of cytoplasmic genomes is very high in many angiosperms (Petit et al. 2005), including *S. vulgaris* (Olson and McCauley 2002; McCauley and Ellis 2008), it may be that the opportunity for mating between individuals carrying different mitochondrial genotypes is limited. This problem is exacerbated in species in which self-fertilization is common, such as in the hermaphrodite morph of *S. vulgaris* (Pettersson 1992; Emery 2001). McCauley and Ellis (2008) made two observations in a study of *S. vulgaris* that support this premise. First, despite providing evidence for intergenic recombination between the mitochondrial gene pairs *atp1/cob*, *atp1/cob*, and *cox1/cob*, a moderately high level of linkage disequilibrium between the genes was also found, indicating limited opportunity for recombination between them. Further, McCauley and Ellis (2008) point out that, in order for recombination to produce novel two-locus genotypes, an individual would have to be heteroplasmic for both loci in question. Biparental inheritance would only generate joint heteroplasmy when the egg and pollen donor genotypes differed at both loci.

Given the observations of Welch et al. (2006) and McCauley and Ellis (2008) and the known population biology of *S. vulgaris* several questions emerge. First, how widespread is mitochondrial heteroplasmy in *S. vulgaris* and how evenly is it distributed among populations and individuals? Welch et al. (2006) found *atp1* heteroplasmy to be fairly common in one of the three populations studied and rather rare in the other two, though the overall number of individuals sampled was limited. Among the majority of those individuals deemed heteroplasmic by Welch et al. (2006) one or the other of the *atp1* markers was found to be greatly numerically predominant. Second, how common is the joint heteroplasmy necessary for effective recombination between the two mitochondrial loci in question? The *atp1* locus was the only mitochondrial gene assayed for heteroplasmy by Welch et al. (2006). If an additional mitochondrial gene were to be included in the assay one could also investigate the frequency at which the joint heteroplasmy necessary for recombination to generate novelty occurs in natural populations. Finally, at what rate is mitochondrial heteroplasmy incorporated into natural populations by paternal leakage, and at what rate is it lost? By comparing heteroplasmy levels in mothers with those of their offspring, one could begin to understand the transmission dynamics of the mitochondrial genome in open pollinated natural populations. Specifically, one could ask whether the heteroplasmy seen in an individual was more likely to have arisen through paternal leakage occurring during the fertilization event that created that individual or have been inherited from a heteroplasmic mother and traced to leakage during some prior generation. In those cases in which immediate paternal leakage was inferred, the evenness of biparental inheritance could be quantified.

In this study, we combine more comprehensive q-PCR based measures of heteroplasmy at the *atp1* locus with a newly developed assay of *cox1* to investigate levels of mitochondrial heteroplasmy in 408 *S. vulgaris* individuals sampled from 22 natural populations in eastern North America not previously studied with regard to heteroplasmy. We first document the distribution of detectable heteroplasmy within and among populations for each gene individually, and then investigate the frequency of the joint *atp1/cob* heteroplasmy necessary for generation of novel recombinants of the loci in question. We then compare levels of heteroplasmy found in a subset of these individuals with those of their offspring, collected as seed at the time of the field sampling, in order to estimate the frequency and magnitude of paternal leakage in these natural populations. The consequences of the results of these studies for the evolution and maintenance of gynodioecy are then discussed.

**Materials and Methods**

**Collection of Genetic Material**

*Silene vulgaris* is a gynodioecious, short-lived perennial species native to Eurasia but now rather widespread in disturbed habitats such as roadsides in northeastern North America. It is insect pollinated with the hermaphrodite morph capable of geitenogamous (between flower) self-fertilization. Individual plants often produce numerous seed capsules, each holding up to 100+ seeds.

Individuals surveyed for mitochondrial heteroplasmy fell into two categories, those sampled as adults from natural populations and their offspring. Four hundred and eight adult individuals were sampled from 22 natural populations in the eastern United States during the summers of 2006 and 2007 (table 1). An individual was sampled by removing a single leaf, which was returned to Vanderbilt University for DNA extraction. Seventy-one of these 408 individuals (from 14 populations) were sampled further by removing the ripe seed capsule nearest to the point from which the leaf was removed. In all cases, the seed capsule and the leaf were found on the same stem, usually less than 10 cm from one another. Seeds from that capsule were planted in the Vanderbilt University greenhouse and grown until sufficient leaf material was produced for DNA extraction. Most of the maternal and some of the offspring DNA were extracted from leaf material using the method of Doyle and Doyle (1987). All additional DNA was extracted using an Applied Biosystems 6100 Nucleic Acid PrepStation and associated protocols (Foster City, CA).

**Q-PCR Methodology**

The q-PCR method used by Welch et al. (2006) for the detection of *atp1* heteroplasmy was modified as follows: McCauley et al. (2005) describe five PCR/RFLP based *atp1* alleles designated A-E. The TaqMan probes developed by Welch et al. (2006) could distinguish either haplotype A or B from either haplotype D or E via the q-PCR
Table 1

<table>
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<th>Population</th>
<th># Adults</th>
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<td>VT-2</td>
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Table 2

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<th>Oligonucleotide Sequences</th>
<th>Table 2 Probes Used in the atp1 A/D and A/B and the cox1 1/3 Taqman q-PCR Assays</th>
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<td>Primers</td>
<td>Q-PCR primers</td>
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<tr>
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<td>Probe A2</td>
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<td>Probes</td>
<td>Probe B</td>
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</tr>
<tr>
<td>Probes</td>
<td>Probe 3</td>
</tr>
<tr>
<td>Notel: Numbers in parentheses refer to the subset of samples assayed for atp1 only.</td>
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GenBank (accession nos. DQ422872–DQ422877) and Applied Biosystem’s software Primer Express v. 3.0. Information concerning PCR primers and TaqMan probes used in this study can be found in table 2.

In preparation for each of the three q-PCR assays, each DNA sample was digested by different restriction enzymes in two separate reactions to create so-called reciprocal knock backs, as described by Welch et al. (2006). Template digest was done with each enzyme could then be used for q-PCR. This should prevent or reduce interference in amplification of one allele in a heteroplasmic sample (Welch et al. 2006; Wolff and Gemmell 2008). For each digest, 5 µl of genomic DNA template was used in 10 µl reaction mixture. The restriction sites in each of these digests were specific to each of the two haplotypes detectable in each of the three assays. In the atp1 A/B reciprocal knock backs, for example, a restriction enzyme capable of cutting the target sequence of haplotype A of atp1, but not the other atp1 haplotypes, was paired with an enzyme that could cut the target sequence of all other atp1 haplotypes except haplotype A. Each digestion led to the reduction in the number of copies of intact sequence of a given haplotype in the template DNA, therefore enhancing the chances of detection of any rarer competing haplotype, or “cryptic heteroplasmacy,” during q-PCR analysis (Welch et al. 2006; see also Wolff and Gemmell 2008). Specifically, the restriction enzymes

“A/D assay.” However, McCauley and Ellis (2008) have shown that A and B are the two most common haplotypes in eastern North America, and it would be most informative to be able to distinguish between them in another q-PCR assay. Here, new probes were designed for use in the A/D assay that should enhance their specificity, and an additional TaqMan genotyping assay that targets a second variable site within the atp1 gene was designed and utilized to distinguish atp1 haplotype A from all other haplotypes in

McCauley and Ellis (2008). Because haplotype 4 also differs by 3 bp from all other known cox1 haplotypes at the consensus sequence used to design q-PCR primers, it was felt that any cox1 assay of an individual likely to carry haplotype 4 could prove unreliable owing to reduced efficiency of amplification. Because McCauley and Ellis (2008) found an absolute association between individuals carrying the D or E atp1 haplotype and the cox1 haplotype 4, no cox1 data were taken from the 25 individuals deemed to be atp1 D or E, or their offspring. All Taqman genotyping probes and primers were designed using sequences available in
mspl and taqzl were used in the A/D assay to knock-back haplotypes D and A, respectively; hsm1 and alu were used in the A/B assay to knock-back haplotypes A and B; and mspl and bstNI were used in the 1/3 assay to knock-back haplotypes 1 and 3. Following these knock-back digestions, a total of six q-PCR trials were performed for most individuals considering the A/D, A/B, and 1/3 assays.

For the q-PCR analysis of each individual, 25-μl reactions were used. Each reaction consisted of ~10-ng whole genomic DNA, 12.5-μl TaqMan Universal PCR Master Mix (Applied Biosystems), 2.25 μl of both forward and reverse primers (10 μM), and 2 μl of each of the two 2.5-μM probes (Applied Biosystems) specific to a given assay. The six probes each have a 5’ fluorophore and a 3’ quencher. Upon hybridization with the amplified mitochondrial DNA, the exonucleolytic activity of the Taq polymerase separates the probe’s fluorophore and quencher, allowing fluorescence to escape and be detected by the ABI Prism 7300 Real-Time PCR System used in this study. The number of thermal cycles required to generate a significant increase in fluorescent signal relative to that of a passive reference dye is denoted the Ct value. Ct values were calculated using the ABI Prism 7300 SDS v. 1.3.1 software (Applied Biosystems). Reactions were run for 40 cycles of 15 s at 95 °C followed by 1 min at 60 °C (see Welch et al. 2006 for additional details).

Heteroplasmy Calibration Experiment

The q-PCR protocol yields a Ct value for each probe. The Ct value is inversely proportionate to the number of target copies in the original sample. When two probes are used, the difference between the probe-specific Ct values is a function of the relative copy number of their respective targets. In a homoplasmic individual, one of the probes should never produce enough fluorescence to generate a Ct value. In an individual heteroplasmic for the two haplotypes detectable by a given pair of probes, the Ct value for the more common, or primary, haplotype should be the smaller of the two. In order to establish the relationship between differences in Ct value and relative copy numbers for the A/B, A/D, and 1/3 probe combinations, the following preliminary experiment was performed.

Based on an initial assay, DNA from individuals found to be homoplasmic for haplotypes A, B, D, 1, and 3 were selected for use in three types of dilution series. The A/D dilution series utilized eight homoplasmic individuals, four each for haplotypes A and D, the A/B dilution series incorporated another eight homoplasmic individuals, four each for haplotypes A and B, and the 1/3 dilution series contained an additional eight homoplasmic individuals, four each for coxl haplotypes 1 and 3. The homoplasmic individuals chosen for the A/D assay were selected through a screening of a total of 365 individuals. Similarly, the homoplasmic individuals chosen for the A/B assay were selected through a screening of 162 individuals, and the individuals chosen for the 1/3 assay were selected through a screening of a total of 89 individuals.

Six sequential 1:4 dilutions were made from each of the homoplasmic individuals used in each assay. These diluted samples were then mixed so as to result in ratios of 1:1024, 1:64, 1:4, 4:1, 64:1, and 1024:1 that mimicked various levels of A/D, A/B, or 1/3 heteroplasmy. These mixtures were each replicated eight times. Each mixture was then subjected to the appropriate reciprocal knock back in the manner described previously. The subsequent q-PCR analyses yielded probe-specific Ct values that reflected our “artificial heteroplasmy.” For each set of probes, the difference between the probe-specific Ct values should be a function of the known relative concentrations of the two target haplotypes. In our data set, we found that a log transformation of the known initial relative concentrations resulted in a linear relationship between the variables, most likely because PCR initially results in exponential growth in amplicon number. This linear function was established by regression using data from the Calibration Experiment and then rearranged algebraically and used to estimate the unknown relative copy number (i.e., the haplotype score—see below) from the difference in Ct values determined when each of the three probe combinations are used to assay DNA samples taken from individuals from natural populations.

Heteroplasmy in Natural Populations

A total of 408 adults from 22 natural populations and 572 offspring from 71 of these adults were screened for atpl and coxl heteroplasmy using the reciprocal knockback method and the q-PCR A/D, A/B, and 1/3 assays. Each individual sampled from the field was assigned atpl and coxl haplotype scores based on these assays. The coxl haplotype score is the proportion of coxl copies determined to be haplotype 3 based on the 1/3 assay. In this assay, a haplotype score of 1 signifies an individual homoplasmic for coxl haplotype 3, and a score of 0 indicates that no haplotype 3 is present. An intermediate score would indicate a heteroplasmic mixture of haplotype 3 and at least one of the other haplotypes, most likely haplotype 1.

The overall atpl haplotype score is contingent on the results of both the A/B and A/D assays. By considering scores generated by both assays for a given individual, one can determine the proportion of atpl copies deemed to be haplotype A, B, or D/E. Specifically, a haplotype score of 1 in the A/B assay indicates that the individual is homoplasmic for atpl haplotype A. A score of 0 for that assay indicates that no copies of haplotype A are present and that the individual must contain haplotype B, D/E or be heteroplasmic for some mixture of these haplotypes. A score between 0 and 1 would indicate “A—other” heteroplasmy. A score of 1 in the A/D assay indicates that the individual contains only haplotypes A, B, or an A/B mixture. A score of 0 indicates only haplotypes D, E, or a D/E mixture. Thus, a score of 0 (0% A) in the A/B assay and 1 (100% A or B) in the A/D assay would indicate an individual homoplasmic for atpl haplotype B. A score of 0 on both the A/B and A/D assays would indicate either homoplasmic for haplotype D or E or D/E heteroplasm, which cannot be resolved by our assays. An intermediate score for the A/B assay and a score of 1 for the A/D assay would indicate A/B heteroplasm. A score of 0 on the A/B assay and an intermediate score on the A/D assay would indicate heteroplasm involving a mixture of B and D/E. Finally,
intermediate scores for both the A/B and A/D assays would indicate either a mixture of A and D/E or a three-way mixture of A, B, and D/E.

For both genes, an individual was arbitrarily designated as heteroplasmic for a locus if a minority haplotype represented at least 0.5% of the mitochondrial population (0.995 > locus-specific haplotype score > 0.005). Although we feel that our method is capable of detecting minority haplotypes at levels as low as 0.001 (see Results), we question the biological significance of these rare copies and opt for the more conservative 0.005 cutoff (see also Wolff and Gemmell 2008). All individuals heteroplasmic for a given locus were also assigned a heteroplasmy score consisting of \[\frac{\left[2 \times \text{haplotype score} \times (1 - \text{haplotype score})\right]}{0.5}\]. That is, the probability that two random copies of the gene picked from that individual differed in a way detectable by our probes, standardized by the maximum probability obtained when two haplotypes are in equal copy number in a sample.

**Results**

**Heteroplasmy Calibration Experiment**

The relationships between the difference in \(C_t\) values for the \(\text{cox1}\) haplotype 3 and other competing \(\text{cox1}\) haplotypes, the \(\text{atp1}\) haplotype A and \(\text{atp1}\) haplotype B, and the \(\text{atp1}\) haplotype A and \(\text{atp1}\) haplotype D estimated by the three q-PCR assays, and the known relative proportions of these haplotypes created by our experimental mixtures, are presented in figure 1. It can be seen that all three q-PCR assays are very consistent at estimating the level of heteroplasmy over a broad range of relative copy numbers, based on the difference in probe-specific \(C_t\) values found in a given assay (\(r^2 > 0.99\) in all three cases). The linear regression equations generated by these three experiments and included in figure 1 can be rearranged to estimate the unknown amount of heteroplasmy within the individuals sampled from natural populations. Those individuals not yielding a measurable \(C_t\) value for one member of a given pair of probes were arbitrarily assigned a \(C_t\) value of 50 for that probe, which always resulted in that individual being assigned as homoplasmic for the haplotype corresponding to the alternate probe within that pair.

**Heteroplasmy in Natural Populations**

The haplotype scores obtained from the \(\text{atp1}\) A/B, \(\text{atp1}\) A/D, and \(\text{cox1}\) 1/3 assays of the 408 adults collected from natural populations are tabulated in Supplementary File One, Supplementary Material online. In summary, 36 of the individuals assayed for \(\text{atp1}\) (8.8%) were heteroplasmic, including 32 heteroplasmic for the A/B \(\text{atp1}\) haplotypes and four heteroplasmic for \(\text{atp1}\) B/D. No individuals were found to be heteroplasmic for \(\text{atp1}\) A/D. These 30 individuals were distributed among 11 populations. The individuals found to be heteroplasmic for \(\text{atp1}\) were distributed among 14 populations. Of these heteroplasmic individuals, the mean \(\text{atp1}\) heteroplasmy score was 0.07, with the distribution of \(\text{atp1}\) haplotype scores presented in figure 2a. Thirty of the 383 individuals sampled as adults for \(\text{cox1}\) (7.8%) were heteroplasmic. These 30 individuals were distributed among 14 of the 22 populations sampled. The distribution of \(\text{cox1}\) haplotype scores for these 30 individuals is illustrated in figure 2b. This distribution of haplotype scores yields an average \(\text{cox1}\) heteroplasmy score of 0.10. Although \(\text{cox1}\) and \(\text{atp1}\) heteroplasmy co-occurred in seven populations,
only five of the 383 individuals assayed for both genes (1.3%), from three of those populations, exhibited joint ATP1–COX1 heteroplasmy. Overall, a total of 61 individuals (30 ATP1þ36 COX1) displayed mitochondrial heteroplasmy that was detectable by our assays at the 0.005 or greater level. These 61 individuals were distributed among 18 of the 22 populations studied. The distribution of two-locus genotypes among the remaining individuals deemed homoplasmic in assays of both loci is presented in table 3.

The haplotype scores obtained from the ATP1 A/B, ATP1 A/D, and COX1 1/3 assays of the 572 individuals collected as seed are tabulated in Supplementary File Two, Supplementary Material online, along with the scores of their respective mothers. In summary, 28 of the offspring individuals (4.9%) were heteroplasmic for ATP1 (24 A/B mixtures, 3 B/D mixtures, and 1 A/D mixture), distributed among 17 of 71 families. Thirty-two of the subset of 518 of these individuals (6.2%) who were also assayed for COX1 were heteroplasmic for that gene. These 32 individuals were distributed among 14 of the 63 families assayed for COX1. One offspring individual was simultaneously heteroplasmic for ATP1 and COX1.

Some information on transmission dynamics can be gained from these field samples by comparing mother–offspring haplotype or heteroplasmy scores, first in the subset of cases in which the mother is homoplasmic for a given gene and separately for those cases in which the mother is heteroplasmic. When the mother is homoplasmic, mother–offspring differences most likely arise due to paternal leakage at the time the offspring zygote is formed. Thus, examining the offspring of homoplasmic mothers is particularly instructive. In two instances, both involving ATP1, a mother homoplasmic for one ATP1 haplotype produced an offspring homoplasmic for another, suggesting complete paternal inheritance. More common were instances in which a mother homoplasmic for a given gene produced heteroplasmic offspring, suggesting biparental inheritance. For example, 18 of the 471 offspring (3.8%) produced by mothers homoplasmic for ATP1 were themselves heteroplasmic for ATP1. Similarly, 24 of 521 offspring (4.6%) of mothers homoplasmic for ATP1 produced offspring that were heteroplasmic for that gene. Thus, over 50% of the heteroplasmic offspring found in this study were produced by homoplasmic mothers. Of course in the great majority of cases, the offspring of a mother homoplasmic for a given ATP1 or COX1 haplotype was scored as homoplasmic for that same haplotype, as expected with maternal inheritance.

In summary, there are 44 offspring in our sample whose haplotype scores differ from those of their respective homoplasmic mothers (considering both ATP1 and COX1). The average absolute value of the difference between the haplotype score of each of these 44 individuals and their respective mothers is 0.121, with a maximum value of 1 (see fig. 3 for the distribution of differences). That is, when leakage occurs, it typically results in biparental inheritance with a fairly small contribution from the father, though it is possible for the pollen donor to contribute the great majority of the copies of the mitochondrial genome inherited by an offspring.

When the mother is heteroplasmic, there can be mother–offspring differences in haplotype scores owing to vegetative sorting of mitochondria during the cell division events leading to the formation of gametes (intraindividual genetic drift). On average, this random process would be expected to cause a decline in the level of
heteroplasmy. Sixty offspring were produced by mothers who were themselves heteroplasmic for \( \text{atp1} \). Of these 60 offspring, four were heteroplasmic for \( \text{atp1} \). In all 60 cases, the heteroplasmy score of the offspring was less than that of the mother, with an average change of \(-0.094\) between generations. Fifty-one offspring were produced by mothers heteroplasmic for \( \text{cox1} \). Of these 51 offspring, 14 were heteroplasmic. The level of heteroplasmy declined in 41 of these 51 offspring, relative to their respective mothers. Heteroplasmy increased in 10 individuals. Overall, the average change for \( \text{cox1} \) was \(-0.003\).

**Discussion**

In a previous study by Welch et al. (2006), mitochondrial heteroplasmy was quantified in \( S. \) vulgaris for the first time, but the number of populations and individuals sampled was far too small to evaluate accurately the distribution of mitochondrial heteroplasm within and among both individuals and natural populations or to begin to understand the transmission dynamics that determine that distribution. Further, by considering only one gene (\( \text{atp1} \)), the previous study could not evaluate the frequency of the joint heteroplasmy required for effective intergenic recombination. By incorporating a broader array of mitochondrial markers and applying them to a much larger sample, the results presented here allow one to address these issues, as outlined below.

Overall, the pattern that emerges from the results presented here is in keeping with the “quantitative” model of organelle transmission proposed by Birky (2001) in that inheritance is usually maternal but with varying degrees of biparental inheritance resulting in a continuum of heteroplasmy levels among individuals within populations. The survey of mature plants from natural populations reveals that heteroplasmy in \( S. \) vulgaris is widespread geographically, in that it was found in 18 of 22 populations surveyed. It is only moderately common, however, in that about 15% of individuals in the total sample were heteroplasmic at one or both of the marker genes, given our criteria. Further, the level of heteroplasmy within those individuals deemed “heteroplasmic” is modest. For example, the average heteroplasmy score of about 0.10 for \( \text{cox1} \) would result when an individual carries a mixture consisting of about 2.5% of one haplotype and 97.5% of another (\( (0.025 \times 0.975)/0.5 \)). The joint \( \text{atp1/cox1} \) heteroplasmy required for effective recombination between the two genes is quite rare, having been detected in less than 1.5% of these individuals. As predicted by McCauley and Ellis (2008) the opportunity for intergenic recombination to create novel two-locus haplotypes appears to be limited.

Examination of the offspring of homoplasmic mothers yields inferences about paternal leakage that are in keeping with these results. About 4% of the offspring of these mothers were heteroplasmic, a state assumed to arise via paternal leakage of mitochondria during fertilization. In these heteroplasmic offspring, the distribution of haplotypes was uneven, with the contribution of the mother usually, but not always, numerically dominant. This suggests that paternal leakage of mitochondrial DNA is a relatively rare event in \( S. \) vulgaris, and when it occurs, the contribution of the pollen donor to biparental inheritance is often limited. The consistent between-generation decline in heteroplasmy seen when mother–offspring comparisons are made for heteroplasmic mothers must be due at least in part to vegetative sorting, given that leakage is not all that common. Taken together, the counteracting processes causing an increase in heteroplasm in seen in the offspring of homoplasmic mothers and the decrease in heteroplasmy seen in the offspring of heteroplasmic mothers must account for the standing level of heteroplasmy seen in natural populations (“leakage equilibrium,” sensu McCauley and Olson 2008).

It is important to distinguish between the heteroplasmy specifically detected by our current \( \text{cox1/atp1} \) marker system and heteroplasmy more broadly defined as any mixture of mitochondrial genomes within an individual not identical by maternal descent. Similarly, the paternal leakage detected by our markers is a subset of leakage defined as the transmission of any copies of the mitochondrial genotype from the pollen donor, regardless of haplotype. The detectable cases of heteroplasmy and leakage are a subset of the actual cases owing to the possibility of transmission of copies of the mitochondrial genome from a pollen donor identical in marker genotype to the pollen recipient. With random mating and two haplotypes at frequencies \( p \) and \( q \), respectively, only the fraction \( 2pq \) would consist of pollen donor and recipient pairings that differ in genotype. So, at best (i.e., \( p = q \)), only about half of leakage events would involve transmission of a marker type different from the pollen recipient. In \( S. \) vulgaris, however, the probability that pollen donor and recipient carry similar marker genotypes is influenced by both the possibility that the hermaphrodite morph undergoes self-fertilization and the fact that there is considerable local population structure when cytoplasmic genes are considered. For example, the average
of the population that is the hermaphrodite morph is on the order of 70%, though this varies considerably from population to population (McCauley et al. 2000), and on average, hermaphrodites self-fertilize about 35% of the time (Emery 2001). Further, \( F_{ST} \) for mitochondrial genes is approximately 0.65, even at the local population level (Olson and McCauley 2002; McCauley and Ellis 2008). Assuming most mating is local, this considerably reduces the probability that outcrosses would be between individuals carrying different mitochondrial genotypes. Further, because \( atp1 \) and \( cox1 \) are in some degree of linkage disequilibrium (McCauley and Ellis 2008), adding an additional marker locus does not greatly increase the power of detection of heteroplasmy or paternal leakage. Thus, even if our \( cox1 \) and \( atp1 \) haplotypes are relatively equal in overall frequency when considering the sample pooled across study populations (McCauley and Ellis 2008), the combination of self-fertilization by some hermaphrodites, local population structure, and linkage disequilibrium between marker loci should limit considerably the statistical efficacy of our marker system to detect paternal leakage and heteroplasmy broadly defined.

Given these limitations, it is our opinion that even by combining information from \( atp1 \) and \( cox1 \) markers, we are capable of detecting somewhat fewer than half of the actual cases of paternal leakage. Thus, the true frequency of paternal leakage is thus probably considerably greater than that documented by counting offspring whose haplotype scores differ from their respective homoplastic mothers. This also raises the question of how much of the decline in heteroplasmy seen in the offspring of heteroplasmic mothers is due to vegetative sorting and how much is due to leakage of the majority haplotype. The contribution of vegetative sorting to the decline depends on the number of mitochondria transmitted from mother to daughter cell and the number of cell divisions separating the leaf sampled from the mother and the leaf sampled from the offspring for DNA extraction (Rand 2001). This information is unknown.

On the other hand, in those cases in which paternal leakage is detected by comparing the haplotype of a homoplastic mother with those of her offspring, we feel that our estimate of the relative magnitude of the contribution of a pollen donor to a given heteroplasmic offspring is relatively accurate. The only likely circumstances in which we might underestimate the contribution of the pollen donor to the heteroplasmic offspring of a homoplastic mother might occur when the pollen donor is itself heteroplasmic for the marker loci. In this case, only the fraction of the contribution of the pollen donor that differed from the pollen recipient would be detectable.

What of the significance of these results for the evolution of gynodioecy? McCauley and Olson (2008) discuss how the effects of heteroplasmy on recombination and the effects of paternal leakage on fitness could influence the evolution of gynodioecy in systems with CMS/restorer sex determination. It is known that some gynodioecious species can have two or more forms of CMS, each requiring different nuclear restorers (de Haan et al. 1997; Charlesworth and Laporte 1998; van Damme et al. 2004). Recombination within the mitochondrial genome could enhance the rate at which novel multi-locus CMS genotypes are generated and influence the nature of the CMS restorer coevolution that drives the evolution of gynodioecy. The results presented here suggest that paternal leakage of mitochondrial CMS could create the heteroplasmy needed for this to occur but at a rate limited by the impact of population structure and self-fertilization. A second possible impact of paternal leakage of CMS discussed by McCauley and Olson (2008) concerns the impact of leakage on the fitness of hermaphrodites from the cytoplasmic perspective. With strict maternal inheritance, seed production is the only currency of mitochondrial fitness, and in most models, of gynodioecy females are considered to be at an advantage due to their increased seed quantity or quality. However, Wade and McCauley (2005) point out that, with paternal leakage, transmission through pollen becomes a component of mitochondrial fitness available to hermaphrodites. They provide a model in which complete paternal inheritance of mitochondria at a low frequency results in CMS–non-CMS polymorphism at the population level, thus promoting the maintenance of gynodioecy. However, paternal leakage can result in biparental inheritance as well as complete paternal inheritance of the mitochondrial genome (including CMS) and the impact of biparental inheritance of CMS on the maintenance of gynodioecy has not been modeled (McCauley and Olson 2008). These authors also speculate that biparental inheritance of CMS could be one mechanism underlying the view that sex ratio inheritance in gynodioecious systems might be best understood using a quantitative genetics rather than a particulate modeling approach (Ehlers et al. 2005; Bailey and Delph 2007).

The paternal leakage and heteroplasmy described here in \( S. vulgaris \) might also explain evidence for recombination provided by analysis of mitochondrial gene sequences in the related gynodioecious species \( S. acaulis \) (Städler and Delph 2002). However, it is not at all clear how widely applicable our results might be to angiosperm species outside the genus \( Silene \), including other gynodioecious species. There have been relatively few studies of mitochondrial inheritance and even fewer investigations of mitochondrial heteroplasm in natural populations of angiosperms. This is perhaps due in part to the rather recent advent of the q-PCR methods that facilitate detection of heteroplasm. Until the possibility of mitochondrial heteroplasm is investigated in a wider array of species its impact on the evolution of the mitochondrial genome of angiosperms remains an open question.

Supplementary Material

Supplementary files one and two are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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Literature Cited


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