Paternal Leakage and Heteroplasmy of Mitochondrial Genomes in *Silene vulgaris*: Evidence From Experimental Crosses

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Manuscript received February 8, 2010

Accepted for publication April 20, 2010

ABSTRACT

The inheritance of mitochondrial genetic (mtDNA) markers in the gynodioecious plant *Silene vulgaris* was studied using a series of controlled crosses between parents of known mtDNA genotype followed by quantitative PCR assays of offspring genotype. Overall, ~2.5% of offspring derived from crosses between individuals that were homoplasmic for different mtDNA marker genotypes showed evidence of paternal leakage. When the source population of the pollen donor was considered, however, population-specific rates of leakage varied significantly around this value, ranging from 10.3% to zero. When leakage did occur, the paternal contribution ranged from 0.5% in some offspring (i.e., biparental inheritance resulting in a low level of heteroplasmy) to 100% in others. Crosses between mothers known to be heteroplasmic for one of the markers and homoplasmic fathers showed that once heteroplasmy enters a maternal lineage it is retained by ~17% of offspring in the next generation, but lost from the others. The results are discussed with regard to previous studies of heteroplasmy in open-pollinated natural populations of *S. vulgaris* and with regard to the potential impact of mitochondrial paternal leakage and heteroplasmy on both the evolution of the mitochondrial genome and the evolution of gynodioecy.

MATERNAL inheritance of the mitochondrial genome seems to be the usual case in angiosperms, with only occasional reports of paternal leakage (BIRKY 2001). The mode of inheritance has several interesting consequences for the evolution of the plant mitochondrial genome and plant mating systems. One is that maternal inheritance contributes to homoplasmy, or within-individual genetic homogeneity, in that it precludes the mixing of mitochondrial genomes of differing origin at the time of fertilization. Homoplasmy is further enforced by repeated sampling events associated with the transmission of a finite number of mitochondria from mother to daughter cells during mitotic or meiotic events (BIRKY 2001). This within-individual genetic drift is sometimes known as vegetative sorting (McCAULEY and OLSON 2008). Paternal leakage would allow the possibility of mitochondrial heteroplasmy (within-individual cytoplasmic genetic diversity) when it leads to some degree of biparental inheritance. With homoplasmy the mitochondrial genome evolves as an effectively asexual

lineage. While intra- or intermolecular recombination associated with repeat sequences often found in non-coding regions of plant mitochondrial genomes can result in structural rearrangements (MACKENZIE and MCINTOSH 1999), there is limited opportunity for such events to generate novel genotypic combinations. Heteroplasmy enhances the possibility that recombination can occur between divergent genomes and generate novel genotypes.

A second consequence of the mode of inheritance concerns the evolution of gynodioecy or the co-occurrence of female and hermaphrodite individuals. This phenomenon is often ascribed to the interaction between mitochondrial genes that confer cytoplasmic male sterility (CMS) and nuclear genes, known as restorers, that counteract the effects of CMS and restore male function (FRANK 1989), a topic that continues to be the object of much study by plant evolutionary biologists (McCAULEY and BAILEY 2009). The evolutionary dynamics of these interactions are usually evaluated on the basis of the assumption of pure maternal inheritance of mitochondrial genes. This maximizes the potential for genetic conflict between a CMS gene and its restorers, since a difference in the mode of inheritance between the mitochondrial and nuclear genomes results in a difference in their respective currency of fitness. With paternal leakage, pollen production is no longer unimportant for the fitness of the mitochondrial genes carried by a hermaphrodite (WADE and McCAULEY 2005).

Supporting information is available online at <http://www.genetics.org/cgi/content/full/genetics.110.115360/DC1>.

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Recently there has been increased appreciation of the potential role of paternal leakage and heteroplasmy in the evolution of the mitochondrial genomes of a broad array of eukaryotes (KMIEC *et al.* 2006; WHITE *et al.* 2008). This includes studies of the plant genus *Silene*, which have provided evidence of at least occasional paternal transmission of mitochondria in several species, as well as mitochondrial heteroplasmy. Observations supporting the possibility of mitochondrial paternal leakage and heteroplasmy in the genus *Silene* are especially intriguing given the occurrence of gynodioecy in this genus. Evidence of paternal leakage comes primarily from two types of observation. First are observations of mitochondrial genotypes that most likely result from intra- or intergenic recombination (see studies by STÄDLER and DELPH 2002 for *S. acaulis* and McCAULEY *et al.* 2005; HOULISTON and OLSON 2006; and McCAULEY and ELLIS 2008 for *S. vulgaris*). Second, direct evidence of heteroplasmy in *S. vulgaris* comes from studies that utilize real time quantitative PCR (q-PCR) to quantify the within-individual diversity of mitochondrial marker genes (WELCH *et al.* 2006; PEARL *et al.* 2009). The likelihood that heteroplasmy is due to paternal leakage in *S. vulgaris* was inferred from observations by PEARL *et al.* (2009) of heteroplasmic offspring of open-pollinated homoplasmic mothers. A second observation by PEARL *et al.* (2009) bears on the inheritance of heteroplasmy. Heteroplasmic mothers were more likely than homoplasmic mothers to produce heteroplasmic offspring, but this heteroplasmy was also lost between generations in many cases, in keeping with the theory of vegetative sorting.

One interesting result from WELCH *et al.* (2006) and PEARL *et al.* (2009) is that incidents of heteroplasmy and apparent leakage do not seem to be evenly distributed among the natural populations from which samples were taken. Most of the heteroplasmic individuals documented by WELCH *et al.* (2006) were from just one of the three populations studied. Similarly, while the apparent leakage rate observed by PEARL *et al.* (2009) was ~8% when all 14 study populations are considered together, if the rate is calculated on a population-by-population basis, it exceeds 10% in 3 of them and is zero in 3 others (see their Supplementary Table 2). Population-to-population variation in the rate of leakage might suggest that variable environmental conditions influence leakage or that any genetic variation that influences the traits that determine mode of inheritance is geographically structured.

Much of the current evidence for mitochondrial paternal leakage in *Silene* is indirect in that it is derived from observations of apparent recombinant genotypes or of heteroplasmy. While this evidence is compelling, alternate explanations, such as mutational hotspots within the genes under study, are at least possible. Even the evidence of leakage presented by PEARL *et al.* (2009) was based on mother-offspring comparisons

of individuals collected from natural populations, in which the pollen donor was unknown. Though some evidence for paternal leakage and heteroplasmy reported in McCAULEY *et al.* (2005) comes from controlled crosses of *S. vulgaris*, those crosses were few in number and any incidents of heteroplasmy were based on qualitative observations rather than the q-PCR method used more recently. Thus, it would be valuable to conduct a large number of controlled crosses between *S. vulgaris* individuals of known mitochondrial genotype to assay directly the rate and magnitude of paternal leakage and any resulting heteroplasmy and also to assay the degree to which heteroplasmy is transmitted between generations. Taken together, this information would allow one to begin to ask, not only about the origins of mitochondrial heteroplasmy in *Silene*, but also about the degree to which the frequency of mitochondrial heteroplasmy in natural populations results from gains through paternal leakage *vs.* loss from vegetative sorting. Furthermore, since the among-population heterogeneity in levels of heteroplasmy and leakage summarized above could be due to either real differences between populations in factors promoting these phenomena or simply ascertainment bias associated with differences between populations in the level of polymorphism of the q-PCR markers, it would be valuable to test for a population effect in an experimental setting.

Here we present comparisons of parent and offspring mitochondrial genotypes obtained by q-PCR following three types of controlled crosses in which either (1) the two parents are homoplasmic for different alleles of a marker gene, (2) both parents are homoplasmic for the same allele, or (3) the maternal parent is heteroplasmic. In the first cross type any contribution of the pollen donor to the offspring mitochondrial genotype would be detectable. This quantifies the likelihood of leakage. Knowing the natural population from which the pollen donor and pollen recipient trace their respective ancestry allows investigation of the possibility of a population effect without the confounding effects of varying levels of marker polymorphism present in field studies. In the second cross type, any observed mother-offspring difference would most likely be due to error of some sort (or the unlikely possibility of mutation at the SNP that defines the marker). Thus, these crosses act as a control by estimating the experimental error rate. The third type of cross measures the frequency with which heteroplasmy is transmitted maternally to offspring or is lost. Taken together this study represents what is, to our knowledge, the first attempt to combine experimental crosses and q-PCR methodology to examine mitochondrial genome inheritance and heteroplasmy in a plant species; important information given that it is not yet clear how widespread mitochondrial leakage and heteroplasmy are in the genus *Silene*, in other gynodioecious species, or in other species of plants in general.

MATERIALS AND METHODS

The gynodioecious plant species *S. vulgaris* is an insect-pollinated short-lived perennial native to Europe and widespread in North America. Both females and hermaphrodites produce multiple flowers per flowering season and each flower can produce ≥ 30 seeds. To initiate the controlled crosses reported here, open-pollinated seed capsules were collected from up to 20 individuals from each of 14 populations across the eastern United States. This included eight localities in New York, one in Tennessee, and five in Virginia. Seeds were planted individually in the greenhouse at Vanderbilt University, grown to maturity, and used as either seed parents or pollen donors in the crosses reported in this study. Pairs of individuals consisting of either the same or different haplotypes of the mitochondrial gene *atp1* were crossed (see below for haplotype details). The two individuals participating in a cross could come from the same or different source populations and were chosen according to the availability of fertile flowers at a given time, as well as *atp1* haplotype. Hermaphrodite individuals were used as seed parents in most cases, though all individuals sampled from one of the source populations were female plants and these served as seed parents in a few crosses. Hermaphrodite flowers that received hand pollinations were emasculated prior to anthesis to prevent selfing. After hand pollinations, seed capsules were allowed to mature on the plant and up to 12 offspring per cross were grown in the greenhouse.

Our conclusions about the mode of mitochondrial inheritance and the occurrence of heteroplasmy were based on comparisons of the *atp1* genotypes of parents and offspring as determined by q-PCR. Two separate assays of the same individuals were used to distinguish different SNPs within *atp1*. These previously identified SNPs were part of the sequence differences that define *atp1* haplotypes A, B, D, and E as described by PEARL *et al.* (2009) and McCAULEY and ELLIS (2008). Haplotype C is rare and was not incorporated into these crosses. Haplotypes D and E cannot be distinguished by the methods used here and are considered jointly as the same marker (D/E). The two assays are referred to as the A/B and A/D assays.

A/B assays: The A/B assay quantifies the relative representation within a DNA sample of alleles of the SNP that distinguishes the *atp1* haplotype A from the B and D/E haplotypes (PEARL *et al.* 2009) and was performed as follows. A leaf closest to the flower that was used during the cross was collected from each parent and a leaf was collected from the first set of true leaves from up to twelve offspring produced by that cross. Total genomic DNA was isolated from ~ 200 mg of fresh leaf tissue using the Applied Biosystems 6100 Nucleic Acid PrepStation DNA and the associated protocols. In preparation for the q-PCR assay, aliquots of each DNA sample were digested by restriction enzymes to create reciprocal “knockbacks,” as described in WELCH *et al.* (2006). This knockback digestion prevents or reduces interference in amplification of one allele in a heteroplasmic sample, increasing the chances of detecting any rare competing haplotype, or “cryptic heteroplasmy,” during q-PCR analysis (WELCH *et al.* 2006; WOLFF and GEMMELL 2008). For each digestion, 5 μ l of genomic DNA template was used in a 10- μ l reaction mixture following the manufacturer’s suggested protocol (New England Biolabs). The restriction enzyme *BsmI* was used to cut the *atp1* haplotype A sequence whereas *AhaI* cuts the *atp1* haplotype B and D/E sequences. Aliquots of template DNA digested with each enzyme were then used as the templates for the q-PCR assays.

A q-PCR assay of a given individual consisted of two 25- μ l reactions containing ~ 10 ng whole genomic DNA treated with

one or the other restriction enzymes, as above, 12.5 μ l TaqMan Universal PCR master mix (Applied Biosystems), 2.25 μ l of both forward and reverse PCR primers (10 μ M), and 2 μ l of each of the two 2.5- μ M fluorescent probes (Applied Biosystems) used in the A/B assay. Reactions were run for 40 cycles of 15 sec at 95° followed by 1 min at 60° [see WELCH *et al.* (2006) and PEARL *et al.* (2009) for primer and probe sequences and additional details]. ABI Prism 7300 SDS v. 1.3.1 software (Applied Biosystems) was used to calculate the Ct value, or the number of thermal cycles required to generate a significant increase in fluorescent signal from a given probe, relative to that of a background passive reference dye.

Thus, for the A/B assay each individual received a Ct value associated with the A haplotype (in the B/D/E knockback reaction) and a Ct value associated with the B type (in the A knockback reaction). The difference in Ct values reflects the relative representation of each haplotype in the original sample, since the number of cycles required to reach the threshold depends inversely on the number of copies of the target molecule at the start of the assay. When a sample is homoplasmic for one variant there is no Ct value in the assay of the other type and an arbitrary Ct value of 50 was assigned. Equations presented in PEARL *et al.* (2009) that incorporate the difference in Ct values were then used to quantify the relative proportions of each variant in a given sample.

In PEARL *et al.* (2009), heteroplasmy was defined arbitrarily as the case in which a minority mitochondrial marker haplotype was present in an individual at a level ≥ 0.005 , relative to a majority haplotype. Any greater representation of the majority haplotype defined homoplasmy. In the crosses described below we retain the 0.005 cut-off to define heteroplasmy in offspring, but define homoplasmic mothers more strictly as those whose majority haplotype is present ≥ 0.998 (in most cases it is 1). The more rigorous definition of homoplasmy in mothers is used to reduce the probability that heteroplasmy in an offspring of an apparently homoplasmic mother is due to vegetative sorting of preexisting heteroplasmy, rather than paternal leakage.

Crosses can be grouped into three types. For the A/B assay, the first type consists of crosses between homoplasmic individuals that differ with regard to the SNP that distinguishes *atp1* haplotype A from the other known *atp1* haplotypes. This includes A $\times$ B and A $\times$ D/E crosses (and their reciprocals). In such crosses any contribution by the pollen donor to the mitochondrial genome of the offspring should be evident from the A/B assay, and the offspring haplotype score quantifies the representation of a marker haplotype not present in a homoplasmic mother. For example, in an $A_{\text{mother}} \times B_{\text{father}}$ cross, an offspring could range from being homoplasmic for A (resulting in a haplotype score of 0), as with strict maternal inheritance, to being A/B heteroplasmic with biparental inheritance (yielding a haplotype score between 0.005 and 0.995), to being homoplasmic for B if paternal leakage is so extensive as to result in pure paternal inheritance (yielding a haplotype score of 1). These crosses are referred to as experimental.

The second type of cross was between homoplasmic individuals whose haplotypes should not appear different in an A/B assay. These included A $\times$ A, B $\times$ B, D/E $\times$ D/E, and B $\times$ D/E crosses (and the reciprocals). These comprise the control cross, which estimates the rate at which mitochondrial paternal leakage might be indicated in error, rather than due to actual paternal transmission. Possible experimental errors include inadvertent mistakes in labeling of samples during field collection, greenhouse cultivation and DNA extraction, and labeling errors and contamination during q-PCR. Even isolated errors are critical when studying presumably rare events such as paternal leakage, and they are

TABLE 1

Crosses performed between various *Silene vulgaris atp1* haplotype combinations and assignment of each to one of three cross types based on the A/B and A/D assays as described in the text

Haplotypes	A/B assay cross type	A/D assay cross type
A × A	Control (8)	Control (8)
B × B	Control (7)	Control (10)
D/E × D/E	Control (4)	Control (4)
B × D/E, D/E × B	Control (41)	Experimental (44)
A × B, B × A	Experimental (45)	Control (44)
A × D/E, D/E × A	Experimental (45)	Experimental (43)
B(h) × B	Heteroplasmic (1)	Heteroplasmic (1)
D/E(h) × D/E	Heteroplasmic (1)	Heteroplasmic (1)
B(h) × D/E	Heteroplasmic (2)	—
B(h) × A	Heteroplasmic (1)	Control (1)
D(h) × A	Heteroplasmic (1)	Heteroplasmic (2)
D(h) × B	—	Heteroplasmic (1)

The number of crosses (*N*) assigned to each category in the two assays is also given. Haplotype descriptions can be found in McCauley and Ellis (2008) and Pearl *et al.* (2009). (h), heteroplasmic mother.

impossible to eliminate entirely. Thus, it is important to demonstrate statistically that cases in which mother–offspring genotype differences point to paternal leakage in the experimental crosses do, in fact, occur more often than such differences in the controls. In control crosses, heteroplasmy in offspring was quantified relative to the apparent homoplasmic genotype of the mother, as above. Note that the control crosses do not detect error in which actual heteroplasmy or paternal leakage is missed by our protocol. Thus, our estimation of error is conservative with respect to the null hypothesis of strict maternal inheritance.

The final class of crosses is known as the heteroplasmic cross type. Here, the mother is known to be heteroplasmic using the 0.005 criteria mentioned above and the father homoplasmic, or nearly homoplasmic, for the majority haplotype found in the mother. In these crosses any detectable heteroplasmy in the offspring must be inherited from the mother. With strict maternal inheritance from a heteroplasmic mother, her offspring would be predicted to display an equivalent heteroplasmic mixture of haplotypes unless the mixture is altered by vegetative sorting. Note that if any paternal leakage were to occur in these crosses it would decrease the level of heteroplasmy in the offspring. In this type of cross we report the level of heteroplasmy in both mother and offspring as the frequency of the minority haplotype in the heteroplasmic mother. Thus, a mother whose haplotype representation is 95% haplotype A would have a heteroplasmy score of 0.05. One of her offspring with 98% A would have a score of 0.02, whereas an offspring with only A would be considered homoplasmic.

The crosses and their cross types are listed in Table 1. Between 2 and 12 offspring were genotyped per cross (mean/cross of 8.2). The number of each type of cross largely reflects the availability of flowering parents of given genotypes.

A/D assays: These same individuals were also genotyped by the A/D assay described by Pearl *et al.* (2009). Here, different Taqman probes were used to distinguish *atp1* haplotypes A and B from haplotype D/E. In this assay most of the B × D and D × B crosses listed in Table 1 are experimental, rather than being considered controls. Similarly, most of the A × B and B × A crosses join the control group. Most other crosses remain as the same cross type, with the exception of four crosses that were considered heteroplasmic under the A/B assay and either control or experimental in the A/D assay, and three

crosses that were considered to be control or experimental under the A/B assay and heteroplasmic under the A/D assay.

The protocol for the A/D assay is identical to that outlined above for the A/B assay, except that different enzymes were used for the reciprocal knockbacks and different probes were used to distinguish SNPs. In knockbacks used in this assay the restriction enzyme *Taqα1* cut haplotypes A and B and the enzyme *Msp1* cut haplotype D/E. PCR primers were identical to those used in the A/B assay. Information about the probes in the A/D assay can also be found in Pearl *et al.* (2009).

In summary, the A/B and A/D assays were used to investigate the inheritance of two different SNPs within the mitochondrial gene *atp1*. Because the two assays were applied to the same set of parents and offspring, some individuals could fall into different experimental treatments, depending on which assay is being considered. Very rarely the results of the two assays are contradictory, in that a very low level of heteroplasmy was occasionally found in one assay but not the other, owing to the differential sensitivity of the probes.

Data analysis: Two approaches were taken to analyze the data. A first analysis of the results considers all crosses, regardless of the population of origin of the parents. Of interest are (1) the proportion of offspring showing leakage in the experimental group, as opposed to the proportion indicating errors in the control group; (2) the magnitude of that leakage; and (3) the magnitude of offspring heteroplasmy in the heteroplasmic group, as compared to their respective heteroplasmic mothers. The A/B and A/D assays were initially analyzed separately and then in a combined analysis. In the combined analysis the overall leakage rate in the experimental group was calculated by dividing the total number of cases of leakage observed in the combined results of the A/B and A/D assays by the total number of experimental offspring genotyped. Offspring created by A × D crosses were genotyped as experimental in both the A/B and A/D assays (see Table 1). In the above calculation each of these A × D offspring was counted once, as a case of leakage if leakage was indicated by either assay, or as a case of maternal inheritance if neither assay indicated leakage.

A second analysis of the experimental crosses examines population-specific leakage rates. First, offspring whose male parents were derived from each of the 13 natural populations that were the source of the pollen donors were used to calculate population-specific leakage rates. The statistical

significance of heterogeneity among populations in those rates was evaluated with a Kruskal–Wallis test (SOKAL and ROHLF 1995) using the software package JMP (version 7). In that analysis each offspring individual was assigned a score of 1 (leakage) or 0 (no leakage). In a similar analysis of the same data, offspring were grouped according to the 14 populations of origin of the pollen recipients. The effect of the origins of pollen donors and pollen recipients were not considered simultaneously, nor was their interaction, because only 56 of the 196 possible pairwise combinations of pollen donor and pollen recipient population of origin were represented (see supporting information, Table S1).

RESULTS

A/B assays: Parents from 156 crosses and 1280 offspring resulting from these crosses were genotyped using the A/B assay. Sixty of the 156 crosses, yielding a total of 510 genotyped offspring, constitute the A/B controls. Five offspring individuals (from four different crosses) displayed mitochondrial genotypes not predicted by the recorded genotypes of the parents, resulting in an estimated error rate of ~1%. The apparent cases of error included four heteroplasmic offspring in whom the maternal genotype was numerically dominant in copy number over a genotype that should not be present in the mother or the putative father, and one offspring homoplasmic for a haplotype not carried by either putative parent.

Ninety A/B experimental crosses were performed and 719 offspring from these crosses were genotyped. Of these, 19 (2.6%) displayed a genotype not identical to the mother, distributed over 14 families. At least one instance of leakage was detected by each type of cross in which leakage could be detected by the A/B assay (*i.e.*, in $A \times B$, $B \times A$, $A \times D/E$, and $D/E \times A$ crosses). The offspring included 14 heteroplasmic individuals and five homoplasmic for the paternal genotype, yielding an average paternal contribution of 28.3% in those 19 individuals that do show leakage.

Six A/B heteroplasmic crosses were performed. The average haplotype score for the six mothers involved in these crosses was 0.059. Fifty-one of their offspring were genotyped. The level of heteroplasmy declined in 96% of these offspring relative to that of the respective heteroplasmic mothers. Ten of the 51 offspring were heteroplasmic (19.6%). The average haplotype score of these heteroplasmic offspring was 0.009, with only 2 of the 10 having haplotype scores higher than their respective mothers.

To test whether the observed incidents of paternal leakage and inherited heteroplasmy occur at a frequency greater than the estimate of the error rate, the proportion of nonmaternal genotypes found among the offspring from the control crosses (5 of 510) was first compared to the proportion in the experimental crosses (19 of 719) by a G -test of independence (SOKAL and ROHLF 1995). The difference in proportion was statisti-

cally significant ($G = 4.69$, d.f. = 1, $P = 0.030$). Similarly, the proportion of heteroplasmy in the offspring generated by the heteroplasmic crosses (10 of 51) was significantly greater than the proportion of heteroplasmic offspring (4 of 510) found in the controls ($G = 33.75$, d.f. = 1, $P < 0.001$).

A/D assays: Parents of 159 crosses and 1370 offspring resulting from these crosses were genotyped using the A/D assay. Sixty-seven crosses (and 545 offspring) were A/D controls. Of the offspring, one displayed a genotype that could not be contributed by either parent, resulting in an error rate estimate of 0.2%. Eighty-seven experimental crosses yielded 775 offspring. Ten offspring (1.3%) displayed a genotype not identical to the mother, including one individual homoplasmic for the pollen donor genotype. The observations of leakage were distributed among nine families. At least one case of leakage was detected in each type of experimental cross (*i.e.*, the $A \times D/E$, $D/E \times A$, $B \times D/E$, and $D/E \times B$ crosses). Of the 10 offspring showing evidence of paternal leakage the average paternal contribution was 27.0%. Finally, under the A/D assay, five families and 50 offspring represent the heteroplasmic group. The average haplotype score for the five mothers involved in these crosses was 0.029. The level of heteroplasmy declined in 96% of the offspring, though seven (14%) were heteroplasmic with an average score of 0.043. The level of heteroplasmy was greater than the mother in one of these individuals and equivalent to the mother in another.

When the proportion of error obtained with the A/D assay controls (1 in 545) is compared to the proportion of leakage in the experimental crosses (10 of 775) the difference in proportion is again statistically significant ($G = 5.76$, d.f. = 1, $P = 0.016$). Similarly, the difference in proportion of heteroplasmy in the controls and in the heteroplasmic group (7 of 50) is statistically significant ($G = 29.74$, d.f. = 1, $P < 0.001$).

Combined analysis: The frequency of leakage detected by the experimental crosses was significantly higher than the frequency of errors detected in the control crosses for both the A/B and A/D assays, when considered separately ($P = 0.030$ and $P = 0.016$, respectively, see above). One way to evaluate the overall significance of this difference across both assays would be to conduct a combined probabilities test (SOKAL and ROHLF 1995, pp. 194–195) that computes an overall probability that the null hypothesis of no real difference is true, based on P -values obtained from individual, independent, tests of the same null hypothesis. On the basis of this test, the difference between the frequency of leakage in all experimental crosses and the frequency of errors in all control crosses is highly significant ($P > 0.005$).

Pooling the results of the A/B and A/D experimental cross assays yields 29 cases of paternal leakage out of 1152 experimental offspring genotyped (see Table S2),

FIGURE 1.—The distribution of the relative magnitudes of the paternal contribution to the *atp1* genotypes of the 29 offspring from the experimental crosses that show paternal leakage. The number of offspring showing paternal leakage in the A/D assay (shaded bars) is stacked above the number obtained from the A/B assay (solid bars).

or an overall leakage rate of 2.5% [95% confidence limits $L_1 = 1.7\%$ and $L_2 = 3.8\%$ by the method of ZAR (1999)]. The markedly bimodal distribution of the magnitude of the paternal contributions observed in these 29 cases is illustrated in Figure 1. The 29 cases of leakage are not distributed randomly among the natural populations to which the parents trace their ancestry. When the source of pollen donors is considered, ~10.3% of offspring derived from one population in Virginia showed leakage, whereas none of the offspring of fathers derived from three of the remaining populations showed leakage (Figure 2). This heterogeneity is statistically significant ($\chi^2 = 25.0$, d.f. = 12, $P = 0.015$). When the population of origin of pollen recipients is considered, the leakage rates range from ~8% for one population to none in four others (data not shown), and the effect is not statistically significant ($\chi^2 = 16.4$, d.f. = 13, $P = 0.230$).

Pooling the results of both the A/B and A/D heteroplasmic crosses yields 17 cases of offspring heteroplasmy, including 3 in which the magnitude of heteroplasmy in the offspring was greater than the mother. No heteroplasmy was transmitted to the remaining 84 offspring (Figure 3).

DISCUSSION

The experimental crosses show that in *S. vulgaris*, maternal inheritance of the mitochondrial genome is the rule in the vast majority of cases. However, paternal

FIGURE 2.—The distribution of 29 paternal leakage events observed in the experimental crosses among the 13 natural populations of *Silene vulgaris* from which pollen donors were derived. For each source population the proportion of offspring displaying leakage is presented along with the total number of offspring fathered by all pollen donors derived from that population.

leakage does occur in a small proportion of crosses, and when it does occur the paternal contribution ranges from <1 to 100%. Offspring heteroplasmy observed in the heteroplasmic crosses are a consequence of transmission of the mitochondrial genome from the heteroplasmic mother, as expected with vegetative sorting, though maternal heteroplasmy is more often lost between generations than retained.

The results reported here support the qualitative conclusions PEARL *et al.* (2009) made from studies of natural populations of *S. vulgaris* in which the pollen donor was unknown. In that report offspring grown from field-collected seeds produced by homoplasmic mothers differed in genotype from the mother in ~8% of cases, either in that the offspring were heteroplasmic or that they were homoplasmic for an *atp1* or *cox1* genotype other than that of the mother. As in the current study, the distribution of the apparent paternal contribution was bimodal. Seeds taken from heteroplasmic mothers had a higher probability of being heteroplasmic (~16%), though heteroplasmy was often lost between generations.

While the results of the experimental crosses presented here are in qualitative agreement with the inferences made from studies of natural populations by PEARL *et al.* (2009), there are quantitative differences. Most notable is the difference between the leakage rate of ~8% inferred by PEARL *et al.* (2009) [$L_1 = 0.06$, $L_2 = 0.10$, calculated from data in their supplemental table

FIGURE 3.—Offspring from the heteroplasmic crosses standardized as a proportion of the heteroplasmy found in their respective heteroplasmic mothers. Owing to the standardization, all mothers have a score of one and an offspring with a score of one would indicate no change between generations. Results from the A/B assay are presented on the right and the A/D assay on the left side. Note that in four cases the thickness of the line is indicative of multiple individuals with approximately equivalent trajectories, with the number of individuals contributing to those lines indicated. The scale of the upper end of the A/D assay offspring axis has been altered to accommodate a single observation of offspring heteroplasmy that is nearly nine times that of its mother.

2 by the method of ZAR (1999)] and the 2.5% rate measured directly here. PEARL *et al.* (2009) felt that the real rate of leakage in their populations was higher than their estimate since the pollen donor is unknown in natural populations, resulting in a considerable number of fertilization events in which there is undetected leakage from a pollen donor who carries the same marker genotype as the pollen recipient.

Several explanations for the difference in the leakage rate estimated by the two studies are possible. There might be differences in the mechanics of artificial pollination *vs.* natural insect pollination that somehow influence the probability of leakage. Moreover, the occasional multiple paternity within fruits, possible with open pollination, might somehow increase the probability of paternal leakage. It is also possible that the difference results from attributes of the populations used in the studies. Evidence that the propensity for leakage and heteroplasmy differs from population to population of *S. vulgaris* was found in both WELCH *et al.* (2006) and PEARL *et al.* (2009), though variation among populations in the magnitude of marker polymorphism confounds differences in the power to detect leakage with real differences in the rate of leakage in these open-pollinated systems. In the experimental crosses reported here considerable differences in leakage rates were found when the source population of the pollen donor was considered. Perhaps, by chance, populations more prone to leakage made a proportionally greater contribution to the data used to evaluate leakage in the field than to our experimental crosses. Because there was only partial overlap in the specific populations used

in the two studies this possibility cannot be evaluated directly.

The population effect detected here warrants additional discussion. Because it was found in controlled crosses it cannot be due to either detection bias or the direct influence of unknown environmental differences among field sites. There may be genetic differences among populations that influence inheritance of mtDNA. A population effect was detectable when the pollen donor was considered, but not the pollen recipient, suggesting that the effect might be due to variable characteristics of pollen. Whatever the cause, such population effects could have interesting evolutionary consequences. For example heteroplasmy increases the likelihood that recombination within the mitochondrial genome will create novel genotypes. McCAULEY and ELLIS (2008) found evidence that recombination in the mitochondrial genome of *S. vulgaris* generated novel multilocus genotypes, but when populations were examined individually, only two populations contained haplotype diversity suggestive of recombination. While this could be due to sampling error, populations with a higher rate of leakage should harbor higher levels of the heteroplasmy needed for recombination to generate diversity.

Variation among populations in the rate of leakage could also influence the evolutionary dynamics of gynodioecy. One motivation for studying mitochondrial paternal leakage and heteroplasmy in *S. vulgaris* is that this species is gynodioecious, with likely cytonuclear sex determination. McCAULEY and OLSON (2008) discuss some of the possible consequences of leakage and heteroplasmy for gynodioecious species with cytonuclear sex determination. Most obvious is that, when carried by hermaphrodites, the fitness of a mitochondrial genome capable of leakage depends partly on transmission through pollen. As shown by WADE and McCAULEY (2005) paternal leakage of the male-fertile mitochondrial genome can stabilize gynodioecious systems by enhancing the cytoplasmic fitness of hermaphrodites, especially when they are rare. The simplest model presented by WADE and McCAULEY (2005) considers competition between a male-sterile and a male-fertile cytotype (nuclear restoration is ignored in this model). Depending on the magnitude of any ovular advantage to females (s) and the rate of leakage (L), paternal leakage can prevent the male-sterile cytotype from sweeping to fixation (see their equation 6). Leakage comparable to the overall leakage rate of $L = \sim 0.025$ estimated here would have a modest impact on stability, given values of s in the range of 1–2. In that model a value of $L = 0.025$ and $s = 1$ results in an equilibrium frequency of females of $\sim 90\%$ (see WADE and McCAULEY 2005; their figure 1). Since leakage in the Wade–McCauley model was assumed to result in pure paternal inheritance, an outcome seen in only 0.4% of offspring in this study, the impact of the average

leakage rate documented here on the maintenance of cytoplasmic and gender polymorphism could be even less. However, our experimental crosses suggest that some populations may have leakage rates as high as 0.10, which would have a substantial impact on the maintenance of gynodioecy. In the Wade–McCauley model with $L = 0.10$ and $s = 1$ the equilibrium frequency of females is 50%. In contrast, there would obviously be no impact of leakage in those populations where $L = 0$, and females would sweep to fixation. Thus, the impact of leakage on the evolution of gynodioecy may have to be considered on a population-by-population basis, rather than as an average effect.

Finally, it is worth pointing out that without fairly large sample sizes, both in terms of number of crosses and total number of offspring assayed, paternal leakage rates on the order of 2% could be easily missed, a statistical point raised earlier by MILLIGAN (1992). Detection of a population effect suggests that any study of the possibility of leakage in a given species should survey multiple populations rather than study just one population intensively. Furthermore, levels of heteroplasmy resulting from biparental inheritance in which the minority genotype often occurs at low frequency within individuals would be difficult to detect without methods of quantification such as q-PCR. Studies of a number of additional plant species, both gynodioecious and nongynodioecious, are needed using these statistical and methodological considerations.

We thank Stephanie Pearl and Mark Welch for their contribution to the development of the q-PCR assays that form the core of this article and Michael Wade for his constructive comments. This work was supported by National Science Foundation Award 0621867 (to D.E.M.).

LITERATURE CITED

- BIRKY, C. W., 2001 The inheritance of genes in mitochondria and chloroplasts: laws, mechanisms, and models. *Annu. Rev. Genet.* **35**: 125–148.
- FRANK, S. A., 1989 The evolutionary dynamics of cytoplasmic male sterility. *Am. Nat.* **133**: 345–376.
- HOULISTON, G. J., and M. S. OLSON, 2006 Nonneutral evolution of organelle genes in *Silene vulgaris*. *Genetics* **174**: 1983–1994.
- KMIEC, B., M. WOLOSZYNSKA and H. JANSKA, 2006 Heteroplasmy as a common state of mitochondrial genetic information in plants and animals. *Curr. Genet.* **50**: 149–159.
- MACKENZIE, S., and I. MCINTOSH, 1999 Higher plant mitochondria. *Plant Cell* **11**: 571–585.
- MCCAULEY, D. E., and M. F. BAILEY, 2009 Recent advances in the study of gynodioecy: the interface of theory and empiricism. *Ann. Bot.* **104**: 611–620.
- MCCAULEY, D. E., and J. R. ELLIS, 2008 Recombination and linkage disequilibrium among mitochondrial genes in structured populations of the gynodioecious plant *Silene vulgaris*. *Evolution* **62**: 823–832.
- MCCAULEY, D. E., and M. S. OLSON, 2008 Do recent findings in plant mitochondrial and population genetics have implications for the study of gynodioecy and cytonuclear conflict? *Evolution* **62**: 1013–1025.
- MCCAULEY, D. E., M. F. BAILEY, N. A. SHERMAN and M. Z. DARNELL, 2005 Evidence for paternal transmission and heteroplasmy in the mitochondrial genome of *Silene vulgaris*, a gynodioecious plant. *Heredity* **95**: 50–58.
- MILLIGAN, B. G., 1992 Is organelle DNA strictly maternally inherited? Power analysis of a binomial distribution. *Am. J. Bot.* **79**: 1325–1328.
- PEARL, S. A., M. E. WELCH and D. E. MCCAULEY, 2009 Mitochondrial heteroplasmy and paternal leakage in natural populations of *Silene vulgaris*, a gynodioecious plant. *Mol. Biol. Evol.* **26**: 537–545.
- SOKAL, R. R., and F. J. ROHLF, 1995 *Biometry*, Ed. 3. W. H. Freeman, New York.
- STÄDLER, T., and L. F. DELPH, 2002 Ancient mitochondrial haplotypes and evidence for intragenic recombination in a gynodioecious plant. *Proc. Natl. Acad. Sci. USA* **99**: 11730–11735.
- WADE, M. J., and D. E. MCCAULEY, 2005 Paternal leakage sustains the cytoplasmic polymorphism underlying gynodioecy but remains invisible by nuclear restorers. *Am. Nat.* **166**: 592–602.
- WELCH, M. E., M. Z. DARNELL and D. E. MCCAULEY, 2006 Variable Populations within variable populations: quantifying mitochondrial heteroplasmy in natural populations of the gynodioecious plant *Silene vulgaris*. *Genetics* **174**: 829–837.
- WHITE, D. J., J. N. WOLFF, M. PIERSON and N. J. GEMMELL, 2008 Revealing the hidden complexities of mtDNA inheritance. *Mol. Ecol.* **17**: 4925–4942.
- WOLFF, J. N., and N. J. GEMMELL, 2008 Combining allele-specific fluorescent probes and restriction assay in real-time PCR to achieve SNP scoring beyond allele ratios of 1:1000. *Biotechniques* **44**: 193–197.
- ZAR, J. H., 1999 *Biostatistical Analysis*, Ed. 4. Prentice Hall, Upper Saddle River, NJ.

Communicating editor: D. CHARLESWORTH