

## MITOCHONDRIAL DNA DIVERSITY, POPULATION STRUCTURE, AND GENDER ASSOCIATION IN THE GYNODIOECIOUS PLANT *SILENE VULGARIS*

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**Abstract.**—A highly variable mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) locus is used to assess the population structure of mitochondrial genomes in the gynodioecious plant *Silene vulgaris* at two spatial scales. Thirteen mtDNA haplotypes were identified within 250 individuals from 18 populations in a 20-km diameter region of western Virginia. The population structure of these mtDNA haplotypes was estimated as  $\theta_{ST} = 0.574$  ( $\pm 0.066$  SE) and, surprisingly, genetic differentiation among populations was negatively correlated with geographic distance (Mantel  $r = -0.246$ ,  $P < 0.002$ ). Additionally, mtDNA haplotypes were spatially clumped at the scale of meters within one population. Gender in *S. vulgaris* is determined by an interaction between autosomal male fertility restorers and cytoplasmic male sterility (CMS) factors, and seed fitness is affected by an interaction between gender and population sex ratio; thus, selection acting on gender could influence the distribution of mtDNA RFLP haplotypes. The sex ratio (females:hermaphrodites) varied among mtDNA haplotypes across the entire metapopulation, possibly because the haplotypes were in linkage disequilibrium with different CMS factors. The gender associated with some of the most common haplotypes varied among populations, suggesting that there is also population structure in male fertility restorer genes. In comparison with reports of mtDNA variation from other published studies, we found that *S. vulgaris* exhibits a large number of mtDNA haplotypes relative to that observed in other species.

**Key words.**—Caryophyllaceae, gene flow, genetic structure, mitochondrial DNA, seed dispersal, sex ratio, spatial structure.

Received March 23, 2001. Accepted September 20, 2001.

Genetic markers are commonly used to assess the effects of genetic drift and gene flow on structuring genetic diversity within and among populations (Slatkin 1985; Neigel 1997). When it is known that the frequency of genetic markers is influenced by selection, however, that influence on spatial structure must also be considered (Whitlock and McCauley 1999). One such case can be found in gynodioecious plant species in which gender is determined in part by cytoplasmic male sterility (CMS) factors that are located in the mitochondrial genome (Saumitou-Laprade et al. 1994). CMS factors are maternally inherited and block the production of viable pollen. It has been suggested that the population structure of highly polymorphic mitochondrial DNA (mtDNA) restriction fragment length polymorphisms (RFLP) might reflect both the effects of selection and random processes on the dispersion of CMS genes in natural populations, if these mtDNA variants mark different CMS lineages (Olson and McCauley 2000).

Approximately 7% of Angiosperm species exhibit gynodioecious breeding systems in which individuals are either female or hermaphroditic (Richards 1997). It has been known for nearly a century that the genetics underlying sex expression in gynodioecious species can be quite complex, involving an interaction between CMS factors and autosomal male fertility restorers (Correns 1906; Grun 1976; Kheyr-Pour 1981). In well studied model systems, for example maize and *Brassica napus* (Schnable and Wise 1998), multiple CMS factors have been identified that trace to the mitochondrial genome and differ in the mechanisms by which they suppress pollen production. Associated with each CMS factor, autosomal restorer genes can be found that counteract the ster-

ilizing mechanisms in a gene-for-gene fashion, reinstating viable pollen production (Frank 1989; Schnable and Wise 1998).

Population sex ratios in gynodioecious species can be highly variable. For example, female frequencies range from 0% to 76% in different populations of *Beta vulgaris* (Cuguen et al. 1994), from 0% to 75% female in *Silene vulgaris* (McCauley et al. 2000a), and from 5% to 95% in *Thymus vulgaris* (Dommée et al. 1983). High variability in sex ratio can be detected between populations separated by 1 km or less (Cuguen et al. 1994; Laporte et al. 2001; this study). Compared to hermaphrodites, females usually benefit from a fitness advantage through seed production (Delph and Lloyd 1991), unless female frequencies are high (McCauley et al. 2000a). Theoretical investigations suggest that high frequencies of females can occur when their restorer frequencies are low (Frank 1989; Gouyon et al. 1991). High frequencies of females can result from random processes such as those during founder events when a CMS type colonizes a new site and restorers are initially absent or in low frequency (Manicacci et al. 1996). Population variation in sex ratio might therefore reflect the population genetic structure of genes controlling sex expression.

A first step toward understanding the relationship between CMS factors and variation in population sex ratio is to assess the population structure of molecular markers that cosegregate with CMS genes. In model systems CMS genes have been traced to the mitochondrial genome (Hanson 1991). The entire mitochondrion is inherited as a single linkage unit. Thus, a polymorphic marker anywhere in the genome could be in linkage disequilibrium with a CMS factor, creating nonrandom associations between mtDNA haplotypes and sex expression in natural populations. In fact, such associations have been identified in French coastal populations of *B. vulgaris* (Cuguen et al. 1994) and in populations of *T. vulgaris*

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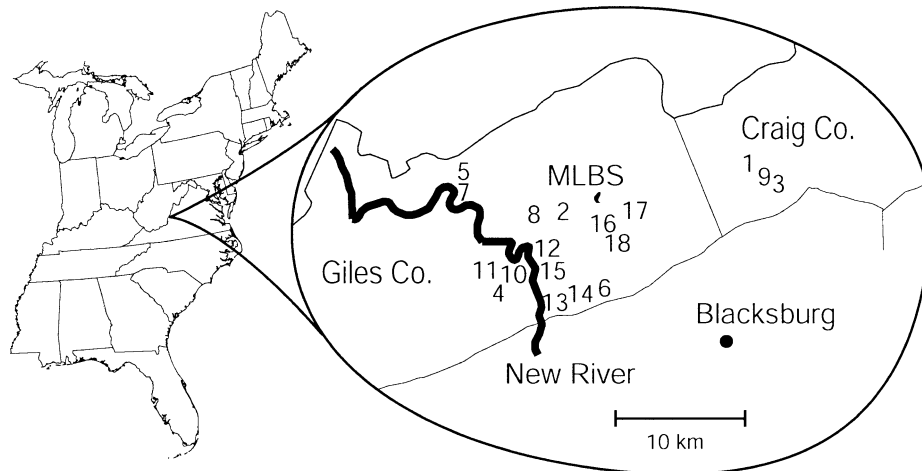


FIG. 1. Geographic distribution of the 18 *Silene vulgaris* populations sampled in this study. MLBS, Mountain Lake Biological Station.

in southern France (Belhassen et al. 1993). However, in four populations of *Plantago lanceolata*, for which the association between CMS types and mtDNA haplotypes is known (De Haan et al. 1997a), one CMS type was generally associated with females but no statistically significant correlation between sex expression and CMS type was apparent (De Haan et al. 1997b), possibly because the frequencies of male fertility restorers varied among populations.

Variation in mtDNA allele frequency among populations could develop from either stochastic processes or selective factors influencing the spread of alleles. The relative influence of these two mechanisms may be elusive. For instance, if seed dispersal among populations is an event that occurs only rarely, each population may have only a few mitochondrial haplotypes tracing back to the original colonists. However, if there are differences among populations in the frequencies of male fertility restorers, specific CMS factors may be favored in some populations, through female advantage, but not in others. In either event, two individuals within the same population have a higher probability of being the same mtDNA haplotype than two individuals selected at random from the metapopulation. A study of mtDNA diversity in a gynodioecious plant must consider both the spatial distribution of haplotypes and their association with gender.

*Silene vulgaris* is a gynodioecious plant species in which sex expression is determined through an interaction between maternally inherited CMS genes and biparentally inherited nuclear male fertility restorers (Charlesworth and Laporte 1998; Taylor et al. 2001). Fitness through seed is affected by both the gender of the individual and the population sex ratio (McCauley et al. 2000a). Females produce more seeds per fruit than hermaphrodites when females are rare within populations (presumably because of the absence of pollen limitation), whereas females produce fewer seeds per capsule than hermaphrodites when females are in high frequency (McCauley and Brock 1998; McCauley et al. 2000a). A previous study of maternally inherited cpDNA alleles in *S. vulgaris* populations in Virginia indicated that the population structure of maternally inherited haplotypes may be three times as great as that of biparentally inherited autosomal allozyme loci (McCauley 1998). In a subset of these popu-

lations, variation in progeny sex ratio was largely attributed to differences in the mitochondrial genetic contribution from mothers drawn from different populations (Taylor et al. 2001), a result consistent with a hypothesis that much of the natural variation in population sex ratio results from population differences in resident CMS factors. However, the extent and spatial structure of mtDNA variation in natural populations is not well studied, nor is the association between mtDNA haplotype and gender. It is also not known whether haplotype diversity is structured on an even smaller scale within populations, as was found in a congener growing in the same region of Virginia (McCauley et al. 1996).

The present investigation describes patterns of diversity of mitochondrial haplotypes and their relation to gender in the gynodioecious plant *S. vulgaris* within a localized geographic region in southwestern Virginia (Fig. 1). We used Southern blotting techniques to identify mtDNA haplotypes associated with restriction site variation flanking the *cytochrome oxidase I (COI)* gene. These mtDNA haplotypes were used to assess mitochondrial variation at two spatial scales. In 18 populations the total diversity and between-population variability in mtDNA haplotype was estimated using  $\theta_{ST}$ . The among-population spatial patterning of these haplotypes was also interpreted using Mantel's  $r$  correlation index. Within one focal population the spatial distribution of all individuals was mapped and analyzed by spatial autocorrelation to assess small scale within-population clumping of mtDNA haplotypes. In addition, associations between haplotype and gender were evaluated across and within populations. As a point of discussion, we present a comparison of this study to previously published studies of mitochondrial population structure in seed plants.

## MATERIALS AND METHODS

### *Study Species*

*Silene vulgaris* (bladder campion) is a short-lived perennial common to roadsides in the mid to northeastern United States. Individuals begin flowering in Virginia in mid-May and flowering continues throughout the growing season. Hermaphroditic and female individuals can be easily discriminated in

TABLE 1. Sampling and population data from the *Silene vulgaris* sites used in this study. The gene diversity index ( $h$ ) was calculated according to Nei (1973).

Population	Sample size (females/hermaphrodites)	Population size	Sex ratio (% female)	No. mtDNA haplotypes	Gene diversity ( $h$ )
1	8 (5/3)	8	0.78	2	0.500
2	9 (5/4)	9	0.56	1	0.000
3	20 (11/9)	47	0.54	3	0.265
4	19 (10/9)	>100	0.52	3	0.385
5	13 (6/7)	74	0.46	2	0.497
6	16 (7/9)	27	0.44	3	0.477
7	10 (4/6)	18	0.33	2	0.420
8	14 (6/8)	33	0.29	1	0.000
9	12 (4/8)	21	0.27	2	0.375
10	13 (5/8)	20	0.25	2	0.355
11	16 (6/10)	>250	0.24	5	0.624
12	16 (6/10)	>250	0.23	3	0.570
13	16 (6/10)	>250	0.23	1	0.000
14	11 (4/7)	>250	0.12	1	0.000
15	16 (1/15)	30	0.07	3	0.555
16	21 (3/18)	31	0.06	2	0.907
17	15 (1/14)	22	0.05	2	0.391
18	5 (0/5)	5	0.00	2	0.320
Total	250 (90/160)		0.36	13	0.862

the field by observing the presence or absence of mature anthers. Hermaphroditic flowers are capable of either self or outcross fertilization, and insects are the primary pollination vector.

Populations in the Allegheny Mountains of southwestern Virginia, where this study was conducted (Giles and Craig Counties, VA), are patchily distributed (Fig. 1). The study populations ranged in size from fewer than 10 to more than 1000 individuals, and populations were separated from one another by a minimum of 500 m (Table 1). Spatial proximity among populations was determined by plotting the populations on a 1:150,000 scale map, and measuring the shortest distance between populations with a ruler.

During the summers of 1999 and 2000, all populations were visited and the population-specific sex ratio was estimated. In small populations, sex ratio was calculated based on the sexes of all flowering plants. In larger populations

TABLE 2. Restriction fragment sizes (in kilobases) for mtDNA haplotypes associated with *COI* probes in this *Silene vulgaris* study. Haplotypes *a*, *b*, *c*, *d*, *e*, *f*, *g*, *j*, and *l* are labeled according to Olson and McCauley (2000); because a direct association between the haplotypes *m-p* and those in Olson and McCauley (2000) could not be confirmed, new labels were assigned to these haplotypes.

Mitotype	<i>EcoRI</i>	<i>HindIII</i>	Overall frequency
<i>a</i>	2.5	6.5	0.064
<i>b</i>	2.1	5.7	0.136
<i>c</i>	1.9	3.7	0.064
<i>d</i>	2.7	6.3	0.080
<i>e</i>	2.4	4.7	0.104
<i>f</i>	2.5	7.3	0.340
<i>g</i>	2.4	4.2	0.108
<i>j</i>	2.2	5.8	0.044
<i>l</i>	3.1	7.3	0.008
<i>m</i>	2.5	6.1	0.012
<i>n</i>	1.9, 4.0, 5.5, 7.9	3.9, 4.6, 8.7, 9.2	0.004
<i>o</i>	3.0	2.7	0.020
<i>p</i>	2.0	4.1, 5.0	0.016

(> 250 individuals), the sexes of 50+ randomly selected individuals from throughout the population were used to estimate the overall population sex ratio. Concurrently, several leaves from up to 21 randomly selected plants in each population were collected and stored on ice until DNA could be extracted using a Qiagen DNeasy DNA extraction kit for plants (Qiagen, Inc., Valencia, CA). Plants were selected to include individuals from throughout the spatial extent of the population.

RFLP variation was assessed for the *HindIII* and *EcoRI* restriction sites flanking the *COI* mitochondrial gene in two separate assays, using techniques outlined in Olson and McCauley (2000). Briefly, total genomic DNA was digested with either *HindIII* or *EcoRI*, electrophoresed on an agarose gel, and transferred to a Hybond N<sup>+</sup> membrane (Amersham Pharmacia, Buckinghamshire, U.K.) via a capillary blot. A 1.5-kb segment of the *COI* gene was amplified using polymerase chain reactions (PCRs) and radioactively labeled. This labeled probe was hybridized to the total genomic blots overnight, washed at moderate stringency (50°C), and placed on film. We chose to screen mtDNA RFLPs associated with the *COI* probe because this region reveals a high number of haplotypes relative to other mtDNA regions (Olson and McCauley 2000). Nonetheless, other mtDNA regions are known to reveal additional variation within some of these mtDNA haplotypes (Olson and McCauley 2000).

Most digests revealed a single band that hybridized with the *COI* probe, although there were two haplotypes that did not follow this pattern (*n* and *p*, Table 2). It is currently unknown whether these multiple bands represent heteroplasmy or duplications of all or part of the *COI* gene, but PCR-RFLP analyses indicate that they do not result from a restriction site within the *COI* gene. Individuals were assigned the same allele (haplotype) only if all restriction fragments matched for both restriction digests (Table 2). When possible, haplotypes were labeled according to Olson and McCauley (2000); otherwise, when a direct association between the hap-

lotypes could not be confirmed, new labels were assigned to the haplotype.

One natural population, population 5 (also known as the Couch population), was selected for further investigation of the within-population spatial distribution of mtDNA haplotypes and individual sex expression. This population was selected because it was known to be polymorphic for mtDNA type, and has a sex ratio of approximately 1:1 (female:hermaphrodite), which is more female biased than the global sex ratio across all populations (Table 1). In the summer of 2000 this population consisted of 74 individuals (> 90% of the total were flowering) that were distributed over a 20 × 20-m area. In early June 2000 the locations of all individuals in population 5 were mapped to the nearest centimeter using a triangulation method. For all flowering individuals gender was noted and the mtDNA genotypes assayed by the method outlined above.

#### Statistical Analyses

The population genetic structure of mtDNA haplotypes was assessed by calculating  $\theta_{ST}$ , a statistic analogous to Wright's  $F_{ST}$  (Weir 1996). The estimate of  $\theta_{ST}$  included a sample size correction and was calculated using the GDA computer program (Lewis and Zaykin 2001).  $\theta_{ST}$  was chosen because it describes the contribution of among-population genetic variation to the total genetic variation. Additionally, within each population gene diversity ( $h$ ) was calculated according to Nei (1973) as  $h = 1 - \sum_{i=1}^m x_i^2$ , where  $x_i$  is the frequency of allele  $i$  and  $m$  is the number of mtDNA haplotypes in the population.

Pairwise genetic distance between populations was computed by calculating  $\theta_{ST}$  for each pair of populations using GDA (Lewis and Zaykin 2001). The correlation of the between population pairwise  $\theta_{ST}$  matrix and the pairwise geographic distance matrix was estimated as Mantel's  $r$  using the R computer program, version 4.0 (Legendre and Vaudor 1991). The significance of the correlation was computed by calculating the Mantel  $r$  statistic for 10,000 random permutations of the matrices and was equal to the frequency of permutations with a correlation as high as or higher than the two original matrices (Legendre and Vaudor 1991).

Associations between mtDNA haplotype and gender were tested for the seven most common haplotypes pooled across all of the populations in which each occurred using likelihood chi-square test statistics (SAS Institute 1995). Four of these haplotypes were each found in at least four populations and in more than 10% of individuals overall (Table 2). For these four haplotypes, Fisher's exact tests were used to assess whether the association of each with gender was consistent across populations. Because multiple independent hypothesis tests were conducted, the critical value for rejecting the null hypothesis was divided by the number of tests in accordance with a Bonferroni adjustment. For most populations, sample sizes were too small to test for within-population associations between mtDNA haplotype and sex expression. However, in population 5 we sampled all 69 flowering individuals for gender and mtDNA haplotype and tested their association using a contingency test.

The null hypothesis that the spatial distribution of individuals within population 5 is random with regard to mtDNA

haplotype was assessed using spatial autocorrelation analysis with Moran's  $I$  as the test statistic (Sokal and Oden 1978). In this analysis, distances between pairs of plants were based on a nearest neighbor criteria, rather than absolute metric distance. That is, Moran's  $I$  was first calculated by comparing the haplotype of each individual with its nearest neighbor, using a (0, 1) coding of the two haplotypes. Moran's  $I$  was then recalculated by pairing each individual with its second nearest neighbor, third nearest neighbor, etcetera up to the tenth nearest neighbor.

## RESULTS

### Patterns of Mitochondrial DNA Variation

Thirteen mitochondrial haplotypes were identified in the 250 individuals sampled from 18 populations. The most widely dispersed haplotype (haplotype *f*) was found in only half of the populations and six haplotypes were found in only one population (mean = 3 populations/haplotype; Fig. 2). The most common haplotype (*f*) was found in 34% of all individuals sampled, whereas the least common (*n*) was found in less than 1%. Seven common mtDNA haplotypes (*a*, *b*, *c*, *d*, *e*, *f*, and *g*) each comprised more than 5% of the total number of individuals sampled; each of these were found in at least three populations (Fig. 2). Three or fewer mtDNA haplotypes were identified in all but one of the populations (mtDNA haplotypes/population: range = 1–5, mean = 2.3). We did not detect an association between haplotype number and population size; populations with more than 50 individuals did not have a significantly different number of haplotypes than those with fewer than 50 individuals (Kruskal-Wallis  $\chi^2 = 1.36$ ,  $df = 1$ ,  $P > 0.20$ ). There also was no significant correlation between population sex ratio and within-population genetic diversity (Nei's  $h$ , Table 1;  $r = -0.249$ ,  $P > 0.35$ ). The distribution of mtDNA haplotypes among populations is illustrated in Figure 2 and yielded an estimate of  $\theta_{ST}$  of 0.574 ( $\pm 0.066$  SE).

Pairwise genetic distance (as measured by pairwise  $\theta_{ST}$ ) between populations ranged from 0.0 to 1.0. We found no evidence that increasing spatial distance between populations was positively associated with increased genetic distance, as might be expected with isolation by distance (Slatkin 1993). On the contrary, there was a statistically significant negative correlation between increasing geographic distance and genetic distance (Mantel  $r = -0.246$ ,  $P < 0.002$ ).

Sex expression was associated with mtDNA haplotype at two scales: across all populations and within populations. Across all populations, the common haplotypes (those which each accounted for >5% of the total number of individuals sampled) were nonrandomly associated with sex expression (likelihood  $\chi^2 = 20.4$ ,  $df = 7$ ,  $P < 0.005$ ; Fig. 3). Haplotype-specific sex ratio ranged from 63% female in haplotype *g* to only 10% females in haplotype *d*.

Four haplotypes were sufficiently common in more than one population to test the consistency of the association between haplotype and sex expression across different populations (Fig. 4). For two of these haplotypes (*b* and *g*), association with gender varied significantly among populations even when the critical values for statistical significance were adjusted according to Bonferroni criteria ( $0.05/4 = 0.0125$ ;

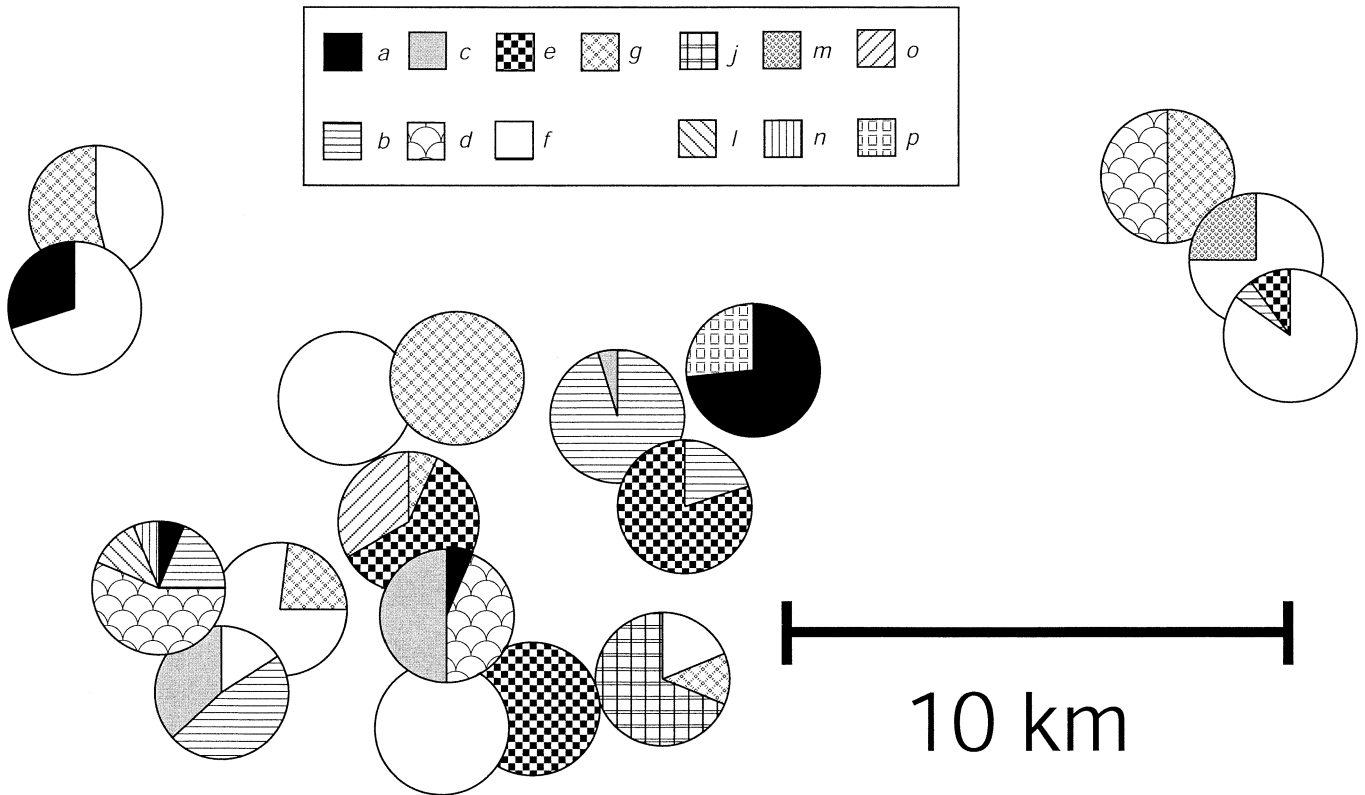


FIG. 2. Geographical distribution of the 13 mitochondrial DNA haplotypes among the 18 studied populations. Each pie chart represents the frequency of different mitochondrial DNA haplotypes found in each population. Pie charts are placed in the geographic positions of each population (see Fig. 1). Haplotypes *a, b, c, d, e, f, g, j, l* are labeled according to Olson and McCauley (2000); because a direct association between the haplotypes *m-p* and those in Olson and McCauley (2000) could not be confirmed, new labels were assigned to these haplotypes. Common haplotypes (*a-g*) are defined as those that comprise more than 5% of all individuals and are found in at least three populations. Each population is separated from its nearest neighboring population by a minimum of 0.5 km.

Fig. 4). For the other two haplotypes, *e* and *f*, sex expression did not vary significantly across populations (Fig. 4).

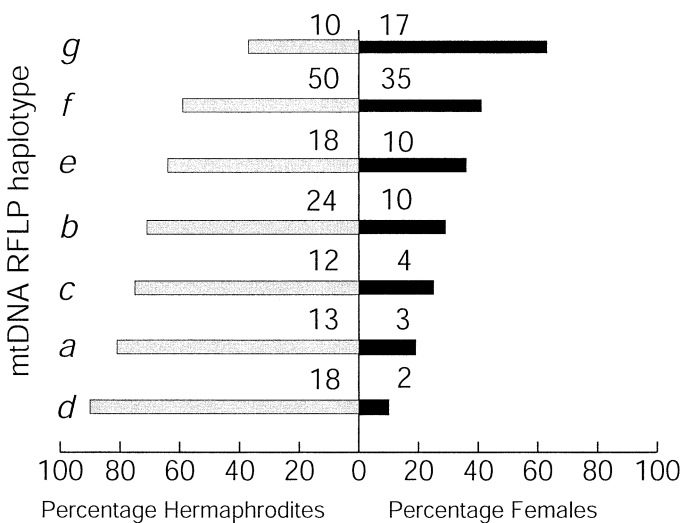


FIG. 3. Percentage hermaphrodites and percentage females associated with each of the eight common mitochondrial DNA haplotypes. Common haplotypes were defined as those found in at least 5% of all individuals and in at least three populations. Individuals were pooled across populations. Sample sizes are indicated above each histogram.

*Spatial Associations within Population 5*

In population 5 a total of 74 plants were mapped (Fig. 5), of which 32 were classified as female, 37 as hermaphrodite, and five did not flower. There was a strong association between mtDNA and sex expression within the 69 flowering individuals ( $\chi^2 = 12.3, df = 1, P < 0.0004$ ); 63.4% (26 of 41) of individuals with haplotype *g* were female, whereas only 21% (six of 28) of individuals with haplotype *f* were female. Examination of Figure 5 suggests that the individuals bearing a given haplotype are distributed into distinct clusters. This is supported by the results of the spatial autocorrelation analysis, in which statistically significant positive autocorrelation is found out to the fourth nearest neighbor (Fig. 6). That is, two individuals living near one another have a higher probability of carrying the same mtDNA haplotype than two individuals selected at random from the entire sample. Although we were tempted to assess the spatial clumping of gender within mtDNA haplotype, there were too few individuals within in one gender x haplotype class (hermaphrodites with haplotype *f*) to permit a robust test of this association.

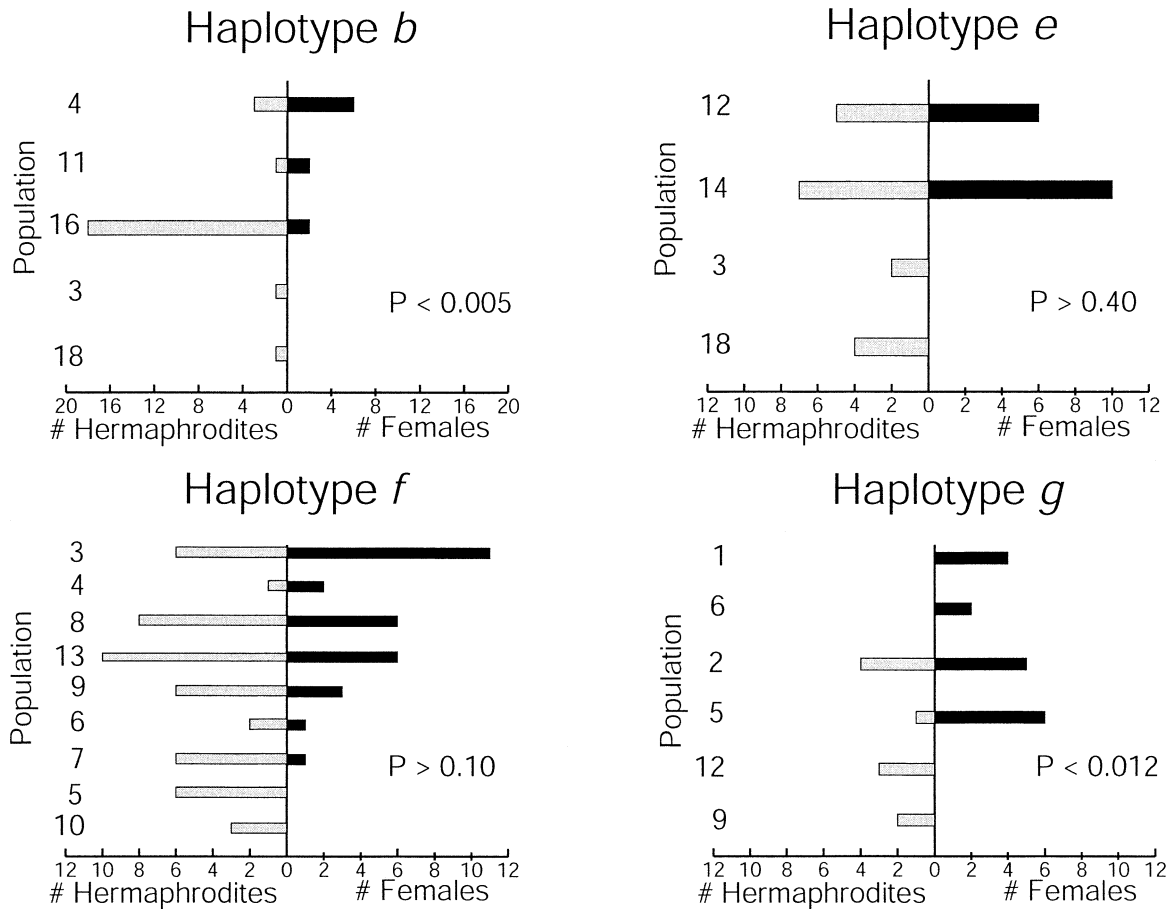


FIG. 4. The numbers of hermaphrodites and females associated with each of the four most common mitochondrial DNA haplotypes across the populations in which they were found.  $P$ -values refer to the results of Fisher's exact tests.

DISCUSSION

*Patterns of Mitochondrial DNA Variation*

This survey of the mitochondrial genome in *S. vulgaris* uncovered a strikingly high level of haplotype diversity and a profound level of population structure, given the limited geographic scale over which the study was conducted. To place our study in context, we present a compilation of intraspecific mtDNA diversity and population structure measures in both gymnosperm and angiosperm taxa (Appendix). Compared with mitochondrial diversity in other plant species, *S. vulgaris* exhibits a large number of haplotypes. An equal or greater number of haplotypes were found in only five other species. Although the Appendix does not present a random sample of gymnosperms and angiosperms (in particular six of the 14 angiosperms were gynodioecious) and sampling effort varied among studies, it is noteworthy that in this sample angiosperms displayed higher numbers of haplotypes than did gymnosperms (Wilcoxon rank sum test,  $P < 0.04$ ; see the Appendix for sampling details), despite the fact that there were not significantly different numbers of populations sampled within the two divisions (Wilcoxon rank sum test,  $P > 0.29$ ). This relationship was still significant when only studies that used Southern blots were analyzed ( $P < 0.02$ ). This pattern underscores the potential utility of mtDNA RFLP markers for population genetic studies in angiosperms.

The level of population structure of mtDNA RFLP haplotypes in this study was high, especially considering the study populations represented a small portion of the total range of *S. vulgaris*. Our estimate of  $\theta_{ST} = 0.54$  is comparable

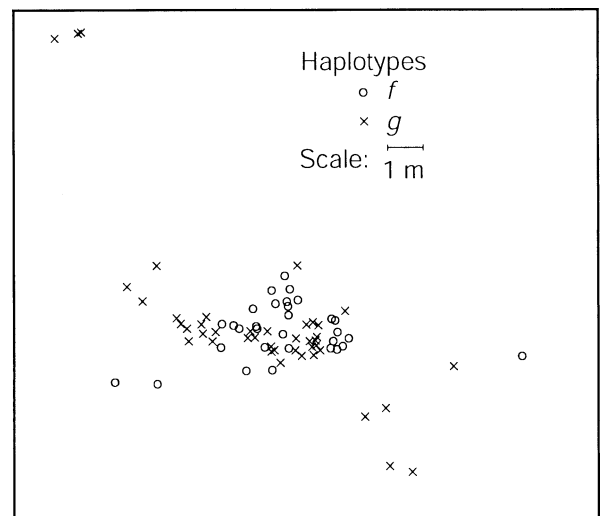


FIG. 5. Map of the 74 individuals in population 5 and their mitochondrial DNA haplotypes.

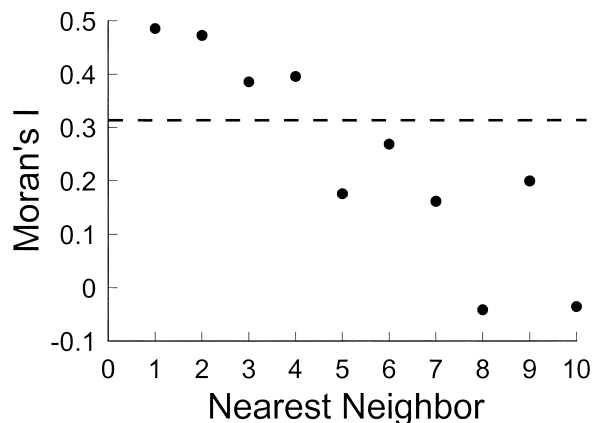


FIG. 6. The spatial autocorrelation of mitochondrial DNA haplotypes from the 10 nearest neighbors in population 5. Because each individual has only one nearest, second nearest, third nearest, etcetera neighbor, the sample size ( $n = 74$ ) for each test is constant. The critical value ( $\alpha$ ) for  $P = 0.05$  is  $I = 0.31$ . Because  $\alpha$  is a function of the sample size, it is the same for all tests and is represented by the horizontal dashed line.

to that found across the entire range of many seed plants (Appendix). In *S. vulgaris*, high  $\theta_{ST}$  might result either from limited among-population gene flow via seeds, which is typical of the ruderal herbaceous gynodioecious species (McCauley 1994) or from localized selection causing different CMS types to be favored in different populations resulting in hitchhiking effects on the mtDNA RFLP haplotypes. It is difficult to disentangle these effects because both may result in the same expectation of high  $\theta_{ST}$ .

In another study of 16 of these same *S. vulgaris* populations, population structure of the chloroplast genome was estimated at  $F_{ST} = 0.62$  (CI = 0.37–1.05) using three chloroplast DNA (cpDNA) haplotypes (McCauley 1998). It is not surprising that the estimates of population structure of haplotypes based on cpDNA and mtDNA are similar because both genomes are maternally inherited in *S. vulgaris* (Olson and McCauley 2000). However,  $F_{ST}$  estimated with each of the genomes might be quite different if the mtDNA haplotypes are prone to convergent evolution (Palmer 1992; Wu et al. 1998). This does not appear to be the case with *S. vulgaris* (Olson and McCauley 2000). The large number of mtDNA haplotypes provide new insight into population structure. For instance, only a few populations are fixed for mtDNA haplotype, whereas in the cpDNA study most populations were fixed. The biological implication is that multiple maternal lineages are commonplace within populations. Either populations are founded by seeds from more than one maternal line, or gene flow via seed into preexisting populations is common.

Spatial structuring of cytoplasmic haplotypes occurs on a scale of meters within a population, as has been found in other gynodioecious and weedy plant species (McCauley et al. 1996; Tarayre et al. 1997; Laporte et al. 2001). Based on long-term census records, population 5 has persisted for at least 15 years (J. Antonovics, pers. comm.), an amount of time sufficient for several generations to pass, but not so long as to exclude the possibility that some original colonists may still be alive. Whatever the colonization history, limited seed

dispersal must contribute to the within-population spatial structure of haplotypes, either because the haplotypes of the original colonists were spatially structured and limited seed dispersal has allowed this structure to persist, or because the spatial structure was created after colonization by drift and/or selection combined with limited seed movement.

Limited seed movement within populations did not predict patterns in genetic similarity between populations. Rather, genetic distance (as measured by pairwise  $\theta_{ST}$ ) between populations was negatively associated with increasing geographic distance, suggesting that factors structuring haplotype diversity within and among populations differ. Such differences could result when most seeds disperse close to parents within populations but the few that disperse long distances do so at random with respect to distance from the source. In addition, the spread of a novel CMS type may be affected by selection through restorers present in nearby populations. For example, a novel CMS type may only spread through female advantage if its restorers are at low frequencies in its own and neighboring populations.

Our study was conducted at two small spatial scales within a small portion of the introduced range of *S. vulgaris*, and thus the population structure most likely developed within the past 300 years via processes such as limited gene flow or selection. The high level of local population structure found in this study underscores the requirement that studies that focus on larger spatial scales also incorporate smaller scale sampling. Otherwise, the scale at which populations are genetically structured cannot be accurately determined and phylogeographic patterns may be obscured.

#### Associations between Sex Expression and Haplotype

Associations between mtDNA haplotypes and sex expression have been found in natural populations of wild beets (*Beta maritima*, Cuguen et al. 1994), plantains (*P. lanceolata*, De Haan et al. 1997b), thyme (*T. vulgaris*, Belhassen et al. 1993; Manicacci et al. 1996), and now bladder campion (*S. vulgaris*). Sex expression in *S. vulgaris* is cytonuclear (Charlesworth and Laporte 1998; Taylor et al. 2001), and crossing studies using plants from the studied populations have detected the presence of multiple CMS factors that are statistically associated with different mtDNA haplotypes (Taylor et al. 2001). We have reported elsewhere on a simple association between cpDNA (which is coinherited with mtDNA; Olson and McCauley 2000) and gender that involves only two cpDNA haplotypes and a smaller sample (McCauley et al. 2000b). Here we utilize the greater diversity in mtDNA to show a more complex association between mtDNA haplotype and gender in 18 natural populations. These results, combined with our observation that sex expression differs for plants with different mtDNA haplotypes in natural populations of *S. vulgaris*, support the contention that differences in mtDNA haplotype in some way reflect differences in CMS factors. It is still unclear whether more than one RFLP haplotype could be associated with one CMS type or vice versa.

We found that the sex expression of individuals carrying the same mtDNA haplotype varied across populations for two of the four most common haplotypes. This pattern can result from any of three phenomena; either there are between-pop-

ulation differences in the frequencies of male fertility restorers, linkage disequilibrium between CMS factors and restorers differs among populations, or RFLP haplotypes in different populations are associated with different CMS types. The latter could result from convergent evolution of RFLP haplotypes and/or rapid evolution of CMS genes relative to RFLP haplotypes. Although possible, it would seem improbable that this would occur in two different RFLP lineages, given that RFLP variation appeared to evolve more quickly than CMS variation in other species (Groenendijk et al. 1997), and given the low incidence of convergent evolution of mtDNA haplotypes detected in these populations (Olson and McCauley 2000). Similarly, it would seem improbable that linkage disequilibrium between the cytoplasmic and nuclear genes would differ among populations for more than one CMS/restorer system, although the presence of selfing will decrease the rate of decay of any linkage resulting from founder effects (Schnabel and Asmussen 1989). It seems reasonable, however, that the pattern of between-population differences in the associations between gender and mtDNA haplotype reflects the presence of population structure in the male fertility restorer genes, although possibly less structure than in the CMS genes (Taylor et al. 2001). Lower structure in nuclear genes might be expected because maternally inherited CMS genes are dispersed only through seeds, whereas autosomal male fertility restorers are dispersed through seeds and pollen. Accordingly, in a comparative study of cytoplasmic and nuclear population structure in some of these same *S. vulgaris* populations, the maternally inherited cpDNA haplotypes were more highly structured than nuclear allozyme alleles (McCauley 1998).

#### Implications for Sex Ratio Evolution

We suspect that the population distribution of CMS genes is reflected by the distribution of mtDNA RFLP haplotypes in these populations of *S. vulgaris*. Crossing studies that found spatial structure at maternally inherited loci in the same populations support this contention (Taylor et al. 2001). Because most populations harbored individuals with more than one RFLP haplotype, seed dispersal must occur at a sufficiently high rate as to allow multiple CMS factors to colonize the same population over time. The observation that the same mtDNA haplotype was associated with different genders in different populations might also suggest that the distributions of autosomal male fertility restorers are spatially structured. This is consistent with disequilibrium between autosomal restorers and CMS types, a condition for maintenance of a joint polymorphism of CMS and restorer genes in some theoretical models (Frank 1989; Gouyon et al. 1991). However, it is not known whether these patterns result more commonly from coadaptive cycling of the frequencies of CMS and restorer genes in persistent populations (Gouyon et al. 1991), or if they result from instances when CMS genes escape into populations with low frequencies of restorers (Frank 1989). It is now clear, however, that the joint population structure of both CMS and nuclear restorer genes must be determined to fully understand sex ratio selection and the spread of CMS and restorer genes in structured populations of cytonuclear gynodioecious species.

Theoretical and empirical studies of phenotypic selection suggest that among-population sex ratio variation will tend to limit the spread of CMS factors in a metapopulation because individuals expressing female gender will tend to be clustered within some populations and hermaphrodites into others (McCauley and Taylor 1997; McCauley et al. 2000b). The population structure documented here suggests that there is a nonrandom distribution of haplotypes, as well as sexes, among populations. We found that fine-scale structuring within a population results in nearest neighbors sharing mitochondrial types more often than individuals drawn from the population at random. Such fine-scale structuring means that the cytoplasmic genomes of neighbors share an evolutionary history and are likely to express the same gender within a population. Thus, it may be possible to extend our view of phenotypic selection in structured populations to an explicitly genetic case. Accomplishing this goal will require reciprocal crossing studies to determine the numbers of CMS factors that are present in *S. vulgaris* as well as their associations with mtDNA haplotypes.

#### ACKNOWLEDGMENTS

We thank L. Zweibel for help in the lab and Mountain Lake Biological Station for providing logistical support. We thank M. Morgan and two anonymous reviewers for comments that improved this manuscript. Financial support was provided through a National Science Foundation/Alfred P. Sloan Postdoctoral Fellowship in Molecular Evolution (DBI-9750033) as well as a grant from the National Science Foundation (DEB-0078531).

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#### APPENDIX

Intraspecific population surveys of mitochondrial DNA variation in gymnosperms and angiosperms.

Species (breeding system) <sup>1</sup>	No. populations (N)	No. haplotypes (probes/enzymes) <sup>2</sup>	$\theta_{ST}$	Geographic/taxonomic range	Reference <sup>3</sup>
<b>Gymnosperms</b>					
<i>Abies firma</i> (H)	7 (161)	7 (2/2) <sup>a</sup>	0.88	central and southern Japan	1
<i>Abies homolepis</i> (H)	8 (155)	7 (2/2) <sup>a</sup>	0.44	central and southern Japan	1
<i>Abies mariesii</i> (H)	7 (237)	1 (2/2) <sup>a</sup>	—	central Japan	1
<i>Abies sachalinensis</i> (H)	5 (142)	6 (2/2) <sup>a</sup>	0.21	northern Japan (Hokkaido)	1
<i>Abies veitchii</i> (H)	12 (299)	4 (2/2) <sup>a</sup>	0.02	central and southern Japan	1
<i>Pinus attenuata</i> (H)	4 (96)	11 (13/2) <sup>a</sup>	0.78	California	2
	4 (89)	3 (1/4)	0.86	California	3
<i>Pinus banksiana</i> (H)	8 (378)	6 (2/1) <sup>a</sup>	0.04 <sup>d</sup>	New Brunswick to Manitoba, Canada	4
<i>Pinus contorta</i> Dougl. (H)					
among subspecies	8 (363)	5 (2/1) <sup>a</sup>	0.31	California to Colorado to British Columbia	4
within subspecies			0.56–0.82		
<i>Pinus flexilis</i> (H)	7 (277)	3 <sup>c</sup>	0.68	Colorado/Nebraska	5
	40 (704)	8 <sup>ac</sup>	0.80	southwestern U.S.	6
<i>Pinus muricata</i> (H)	6 (171)	11 (13/2) <sup>a</sup>	0.77	California and northern Baja	2
	10 (107)	6 (1/4)	0.88	California and northern Baja	3
<i>Pinus ponderosa</i> (H)	8 (328)	3 <sup>ac</sup>	1.0 <sup>e</sup>	Idaho and Montana	7
<i>Pinus radiata</i> (H)	3 (76)	6 (13/2) <sup>a</sup>	0.79	California and Guadalupe Island	2
	5 (72)	3 (1/4)	0.83	California and Guadalupe Island	3
<i>Pinus sylvestris</i> (H)	20 (466)	2 (1/2)	0.37	Scotland	8
	7 (126)	3 (2/1) <sup>a</sup>	0.82	Spain	9
	23 (747)	2 <sup>c</sup>	0.59	western, central, and northern Europe	10
<b>Angiosperms</b>					
<i>Beta vulgaris</i> (GD)	100 (414)	20 (3/1) <sup>a</sup>	0.48 ± 0.04 <sup>b</sup>	southern France and Spain	11
	38 (190)	11 (2/1)	0.47	coastal France	12
<i>Daucus carota</i> (GD)	7 (80)	13 (3/2) <sup>a</sup>	0.078	100-km diameter area in southern France	13
<i>Fagus crenata</i> (H)	17 (409)	8 (3/2)	0.97	Japan	14
	16 (96)	11 (5/3) <sup>a</sup>	0.80 ± 0.10 <sup>b</sup>	Japan	15
<i>Fagus japonica</i> (H)	3 (18)	3 (5/3) <sup>a</sup>	0.66 ± 0.07 <sup>b</sup>	central Japan	15
<i>Fagus hayatae</i> (H)	1 (15)	1 (5/3) <sup>a</sup>	—	Lalashan Nature Reserve, northern Taiwan	16
<i>Hevea brasiliensis</i> (H)	34 (395)	212 (14/2) <sup>ag</sup>	—	Amazon basin (Brazil, Colombia, Peru)	17
<i>Hordeum vulgare</i> (H)	12 (12)	6 <sup>af</sup>	—	Greece and southwest Asia	18
<i>Hordeum spontaneum</i> (H)	13 (13)	12 <sup>af</sup>	—	Greece and southwest Asia	18
<i>Penstemon haydenii</i> (H)	9 (65)	8 (3/3) <sup>a</sup>	—	200-km diameter area in Nebraska, USA	19
<i>Plantago lanceolata</i> (GD)	8 (518)	>20 (1/1) <sup>a</sup>	0.21 ± 0.08 <sup>b</sup>	Netherlands	20
	24 (88)	9 (4/5)	0.35 ± 0.11 <sup>bh</sup>	Netherlands	21
<i>Quercus</i> spp. (H) <sup>i</sup>	378 (1749)	3 <sup>af</sup>	—	southern France	22
<i>Rosmarinus officinalis</i> (GD)	3 (27)	11 (3/3) <sup>a</sup>	—	1.5-km diameter region in Spain	23
<i>Silene vulgaris</i> (GD)	18 (250)	13 (1/2) <sup>a</sup>	0.54 ± 0.07	20-km diameter area in Virginia	this study
<i>Thymus vulgaris</i> (GD)	3 (52)	11 (1/2) <sup>a</sup>	0.42 ± 0.29 <sup>b</sup>	southern France	24
	13 (86)	53	—	southern France	25

<sup>1</sup> Breeding system acronyms: H, hermaphrodite; GD, gynodioecious.

<sup>2</sup> Symbols: —  $F_{ST}$  could not be estimated; (a) study used in test of angiosperms versus gymnosperms; (b) estimated from data in paper using GDA; (c) PCR-RFLP methods used; (d) estimate does not include hybrid population; (e) estimate does not include populations in a transition zone; (f) RFLP analysis on entire mtDNA genome cleaved with 16 restriction enzymes; (g) 14 random mtDNA clones combined into five sets of probes; (h) sampling within populations not randomized; (i) four species of hybridizing white oaks.

<sup>3</sup> References: (1) Tsumura and Suyama 1998; (2) Wu et al. 1998; (3) Strauss et al. 1993; (4) Dong and Wagner 1993; (5) Latta and Mitton 1997; (6) Mitton et al. 2000; (7) Latta and Mitton 1999; (8) Sinclair et al. 1998; (9) Sinclair et al. 1999; (10) Soranzo et al. 2000; (11) Desplanque et al. 2000; (12) Cuguen et al. 1994; (13) Ronfort et al. 1995; (14) Tomaru et al. 1998; (15) Koike et al. 1998; (16) Kato et al. 2000; (17) Luo et al. 1995; (18) Holwerda et al. 1986; (19) Caha et al. 1998; (20) De Haan et al. 1997b; (21) Groenendijk et al. 1997; (22) Dumolin-Lapègue et al. 1998; (23) Hildago-Fernandez et al. 1999; (24) Belhassen et al. 1993; (25) M. Tarayre, unpubl. study in Tarayre et al. 1997.