



An association between chloroplast DNA haplotype and gender in a plant metapopulation

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Abstract. Theoretical models have shown that metapopulation structure can influence the evolution of the sex ratio in gynodioecious plants when the fitness of females and/or hermaphrodites varies among populations with the local sex ratio. Such frequency-dependent fitness has been demonstrated previously in natural populations of *Silene vulgaris*, a gynodioecious plant in which sex determination is cytonuclear. We investigated whether there was an association between chloroplast DNA (cpDNA) haplotype and sex expression, with the assumption that cpDNA haplotypes could be associated with specific male sterility variants likely found in the mitochondrial DNA, owing to co-transmission of the two cytoplasmic genomes. Two cpDNA haplotypes were studied in a collection of 111 individuals of known sex taken from 20 natural populations found in Virginia, USA. The set of haplotype 1 individuals consisted of 71.4% hermaphrodites, where as only 7.7% of the haplotype 2 individuals were hermaphroditic; a highly statistically significant difference. By extension of a previous model it was also shown that the differential clustering of these two haplotypes into local populations contributes to among-population sex ratio variation and has the potential to lower the fitness of haplotype 2 by greater than 50%, relative to that expected with no population structure.

Key words: chloroplast DNA, CMS, gynodioecy, metapopulation, *Silene vulgaris*

Introduction

A metapopulation can be defined as a system in which individuals are distributed into spatially distinct populations, and in which the populations display a high rate of turnover owing to recurrent extinction and recolonization (e.g. Hanski, 1997). In such a system one must consider evolution as a process that occurs both within ephemeral populations and at a more regional scale according to how those populations contribute to a pool of potential colonists. Evolution in metapopulations has been addressed by several types of models, including those that consider the adaptive evolution of life histories, such as

dispersal strategies, within the context of metapopulation dynamics (e.g. Olivieri *et al.*, 1995; Gandon and Michalakis, 1999; Ronce *et al.*, 2000) and others that focus on the impact of extinction/recolonization on the spatial structure of neutral genetic variation (Slatkin, 1977; Wade and McCauley, 1988; and others). The link between the impact of metapopulation dynamics on adaptive evolution and on neutral genetic structure is not always obvious (McCauley, 1993; Barton and Whitlock, 1997). This paper will illustrate one way in which metapopulation dynamics can influence fitness and the response to selection by focusing on the evolution of the sex ratio in a gynodioecious plant metapopulation, and in particular on how genetic structure could influence the fitness of a gene associated with sex expression.

In the traditional view of evolution in temporally stable structured populations recurrent selection within populations can result in local adaptation. Spatially varying selection pressures can then lead to the evolutionary divergence of populations, the magnitude of which is limited by gene flow. When populations are much less temporally stable, selection within a given population recurs for, at most, a few generations. Since populations are ephemeral, local adaptation is limited. However, selection can influence the number and genetic composition of the individuals that those populations contribute to the pool of colonists available to found new populations. When the fitness of phenotypes depends on their local frequency, and when that frequency varies among populations, the metapopulation-wide evolution of the trait can depend explicitly on the population structure. The effect occurs because, with substructuring, most individuals bearing a trait reside within demes that have a relatively high frequency of that trait (Wilson, 1980). Population structure therefore increases the absolute fitness of traits that are positively affected by their own frequency, and decreases the absolute fitness of traits that are negatively affected by their own frequency. This effect of frequency-dependent selection in structured populations is the basis for the theory of the evolution of many social behaviors (Wade, 1978; Wilson, 1979; Uyenoyama and Feldman, 1980; Goodnight *et al.*, 1992).

Recently, there has been considerable interest in the effects of population subdivision and associated metapopulation dynamics on the evolution of the sex ratio in gynodioecious plants (Frank, 1997; McCauley and Taylor, 1997; Olivieri and Gouyon, 1997; Pannell, 1997; Couvet *et al.*, 1998). Gynodioecy refers to the mating system in which females and hermaphrodites co-occur. Sex expression in gynodioecious systems is often, but not always, the result of an epistatic interaction between cytoplasmic and nuclear genes. Typically, females arise owing to the action of cytoplasmic male sterility (CMS) factors which, if they are maternally inherited, can spread through a population when they confer an advantage in transmission through seed (Frank, 1989). CMS factors are thought of as selfish elements, both because their fitness interests are in

conflict with the nuclear genome (Cosmides and Tooby, 1981; Hurst *et al.*, 1996; Werren and Beukeboom, 1998), and because females do not contribute to pollen production, which is needed to fertilize the seeds produced by all members of the population. The expression of these CMS factors can be modified by alleles at one or more nuclear loci that restore male function and whose fitness is determined through both seeds and pollen. When sex expression is cytonuclear the local sex ratio is then determined by allele frequencies at CMS loci and nuclear restorer loci, as well as by how those alleles are combined into multi-locus genotypes within individuals.

Attempts to model the evolution of the sex ratio in gynodioecious systems have considered this complex inheritance of sex expression, as well as the potential conflict between maternally and bi-parentally transmitted genes (Lewis, 1941; Lloyd, 1974; Charlesworth and Ganders, 1979; Frank, 1989; and others). One common observation of natural gynodioecious systems is that the sex ratio (i.e. the relative frequencies of females and hermaphrodites) varies considerably among local populations (Frank, 1989). Presumably this sex ratio variation arises owing to the spatial structure of genes controlling sex expression.

A recent model by McCauley and Taylor (1997) demonstrates how the average fitness of females and hermaphrodites can be altered by a population structure that leads to spatial variation in sex ratio, and how that can influence the evolution of the average sex ratio when there is frequent extinction and recolonization. Since hermaphrodites are the sole source of pollen, spatial variation in the sex ratio favors hermaphrodites relative to females and can act as one factor limiting the spread of CMS elements. With sex ratio variation, those females clustered together in some populations might suffer pollen limitation, whereas the clustering of hermaphrodites into other populations enhances the opportunity for that sex to outcross.

Over the past several years we have been investigating whether the McCauley–Taylor model of phenotypic selection might apply to *Silene vulgaris*, a weedy gynodioecious plant. We have studied frequency-dependent fitness in arrays of both artificial and natural populations, focusing on fitness through seed because that is the appropriate currency for cytoplasmic genes. In a study in which the local frequency of hermaphrodites was varied experimentally among artificial populations, components of fitness through seed were shown to be a positive linear function of the frequency of hermaphrodites for both sexes (McCauley and Brock, 1998). A similar result was found more recently in a study that took advantage of the natural sex ratio variation found among local populations of *S. vulgaris* located in Giles County, Virginia (McCauley *et al.*, 2000). Depending on the sex and study, the measures of fitness through seed that increased linearly with the local frequency of hermaphrodites were fruit set, number of seeds per fruit, and seed germination. It

was assumed that females suffer pollen limitation when common, whereas the fitness effects of sex ratio on hermaphrodites were attributed to inbreeding depression following selfing, which was assumed to occur more frequently when hermaphrodites are rare.

To date we have focused on phenotypic fitness. In this paper we now begin to consider how population structure might influence the fitness of genes controlling, or at least linked to, sex expression. Charlesworth and Laporte (1998) and Taylor *et al.* (2001) provide evidence from crosses that genetic control of sex expression in *S. vulgaris* is cytonuclear, and that numerous maternally inherited CMS factors must exist. In an unstructured population, fitness differences between these CMS factors would depend on the strength of female fitness advantage through seed, and on the probability that carrying a given CMS factor results in the female phenotype. In structured populations the influence of the spatial distribution of CMS factors on among-population sex ratio variation must also be considered, especially if fitness is frequency-dependent as modeled by McCauley and Taylor (1997).

In the few cases that are well studied, CMS has been mapped onto the mitochondrial genome (Saumitou-Laprade *et al.*, 1994). A recent observation by Olson and McCauley (2000) of strong associations between chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) haplotypes in *S. vulgaris* suggests that cpDNA variants might serve as markers for CMS diversity, even if the actual CMS factor is contained within the mitochondrial genome. If so, this would be relevant to the study of gynodioecy in metapopulations of *S. vulgaris* because McCauley (1998) has shown that, in *S. vulgaris*, cpDNA diversity is highly structured at a local spatial scale. Thus, CMS factors might be similarly structured; one possible explanation for the observed sex ratio variation. In this report we investigate associations between cpDNA haplotype and sex expression, and then show how the population genetic structure of such haplotypes could influence their average fitness through seed. This will serve to illustrate our general point that metapopulation dynamics can have interesting and profound effects on traits displaying frequency-dependent fitness.

Materials and methods

The natural populations of *S. vulgaris* that have been used in our studies of fitness through seed (McCauley *et al.*, 2000), and of genetic structure (McCauley, 1998; Taylor *et al.*, 2001), are located in Giles County, Virginia near the Mountain Lake Biological Station. This weedy species is distributed into local populations of 5–100+ individuals along roadsides. Nearest neighbor distances range from 0.5 to 5 km, with approximately 20 km separating the most widely separated of our 20 study populations. Local sex ratios range from

100% hermaphrodite to 75% female (McCauley *et al.*, 2000). The previous study of the population genetics of cpDNA (McCauley, 1998) made use of PCR–RFLP variation and demonstrated a large degree of spatial structure for that genome. However, the leaf material needed for DNA extraction was collected at a time that the sex of individuals could not be determined.

In order to ask whether cpDNA haplotypes differ with regard to the sex ratio of the individuals that carry them, leaf material was collected from 111 individuals distributed among 20 populations in June 1998. Because June is the time of peak flowering, the sex of each study individual was also determined. In addition, a population sex ratio was obtained by sexing the remaining flowering individuals in each population.

DNA was extracted from leaf material using QUIAGEN DNeasy DNA extraction kits for plants (QUIAGEN Inc., Valencia, CA). This DNA was then used as a template for polymerase chain reaction (PCR) using the c & d primers of Taberlet *et al.* (1991) and an annealing temperature of 50 °C. Primers c & d amplify an approximately 600 bp portion of the trnL (UAA) gene, including a group I intron. Noncoding regions are considered good candidates for detecting intraspecific variation in cpDNA (McCauley, 1994). After amplification, each PCR product was digested with the restriction enzyme *MseI*. The resulting fragments were electrophoresed on 3.5% metaphor gels for 4 h at 75 V. Electrophoretic patterns were visualized under UV light after ethidium bromide staining. This method revealed the two most common of the three cpDNA haplotypes studied by McCauley (1998), which differ electrophoretically because of size variation in the restriction fragments (see Fig. 1).

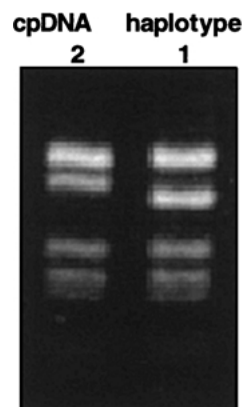


Figure 1. *MseI* restriction digest of PCR product obtained using primers that target the trnL (UAA) region of cpDNA. Shown are two lanes of an agarose gel in which *S. vulgaris* individuals carrying chloroplast haplotypes 1 and 2 are compared. See the text for details concerning PCR and gel protocols. Sequence data has shown that the second fragment of haplotype 2 differs from that of haplotype 1 by a 8 bp deletion.

In order to evaluate the effect of population structure on the fitness of these two haplotypes we also modify the McCauley–Taylor model so that it can be applied to genotypes that differ in sex expression, rather than to phenotypic selection on the sexes themselves. We then use our modified model, along with the results of our previous study of phenotypic selection in natural populations (McCauley *et al.*, 2000), to evaluate the effect of population structure on haplotype fitness.

Results

Population-specific haplotype frequencies and census sex ratios are presented in Figure 2. Sample sizes range from 3 to 11 individuals sampled per population. Haplotype 2 was found in the same five populations in which it was seen in a previous study (McCauley, 1998), and at about the same frequency. It can be seen that while haplotype 2 is rare overall, it is locally quite common in three of the 20 populations. The cpDNA haplotype and sex of the 111 study indi-

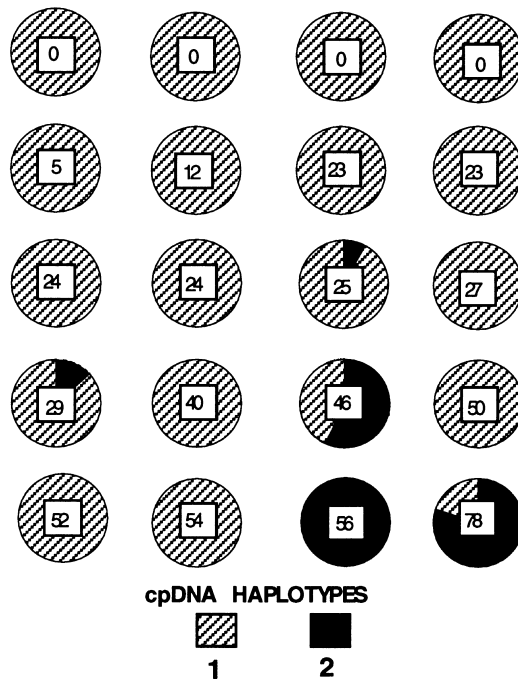


Figure 2. Pie diagrams representing haplotype frequencies in 20 populations of *S. vulgaris* located in Virginia, USA. Populations are arranged in order of increasing percentage of females, which is indicated by the number within each rectangular insert. Note: haplotype frequencies are based on sample sizes ranging from 3 to 11 per population; sex ratios are based on field censuses.

Table 1. The sex and cpDNA haplotype of 111 *S. vulgaris* individuals collected from 20 local populations in Southwest Virginia. The number in parenthesis indicates the number of populations found to contain each haplotype

Sex	Haplotype	
	1 (19)	2 (5)
Female	28	12
Hermaphrodite	70	1

viduals, pooled across populations, is given in Table 1. Overall, 71.4% of the carriers of haplotype 1 were hermaphroditic, whereas only 7.7% of the carriers of haplotype 2 were hermaphrodites. The large sex ratio difference seen between the carriers of the two haplotypes is highly statistically significant ($p < 0.001$, Fisher's Exact Test).

Our previous studies have shown that, on average, females enjoy higher fitness than hermaphrodites for all of the components of fitness through seed that we have measured (McCauley and Brock, 1998; McCauley *et al.*, 2000). Averaged across populations, females comprise about 28% of the individuals in the region of Virginia in which we carry out our studies. Thus, without population structure we would expect cpDNA haplotype 2 to enjoy a fitness advantage relative to haplotype 1 owing to the fact that it is almost always carried by females and because, globally, females are fairly rare. However, given the sex ratio variation and genetic structure known to exist in our system, we must now consider the impact that population subdivision could have on the average fitness of the two genotypes.

Accommodating the model to genotypic fitness

In the McCauley–Taylor model the net effect of population structure is to shift the equilibrium sex ratio in favor of hermaphrodites. The metapopulation-wide fitness of each sex is effectively a weighted average of the population-specific averages, since the contribution of each population to the pool of colonists must be weighted by the number of individuals of a given sex in that population, relative to other populations. This effect is captured in the model by the use of subjective frequencies (Wilson, 1980), or the frequency with which individuals of either sex ‘experience’ hermaphrodites within their local population. That is

$$p_{HH} = p_H + V_H/p_H \quad (1)$$

and

$$p_{HF} = p_H - V_H/(1 - p_H) \quad (2)$$

where p_{HH} and p_{HF} are the subjective frequencies of hermaphrodites from the perspective of hermaphrodites and females, respectively. The p_H is the meta-population-wide frequency of hermaphrodites and V_H is the variance among populations in the local frequency of hermaphrodites. It is assumed that all demes are of equal size. Notice that V_H is effectively the measure of population structure and that as V_H increases the fitness of hermaphrodites increases relative to that of females.

In order to evaluate the effect of population structure on the fitness of cytoplasmic genes McCauley and Taylor (1997) make the simplifying assumption that there are only two classes of cytoplasm, a form causing CMS unless restored and a form that does not cause CMS in any nuclear background. A more realistic situation might be one in which two or more CMS factors coexist, but in which those factors differ in probability of restoration across nuclear backgrounds. That is the likely case with our two cpDNA haplotypes. Understanding how metapopulation structure influences the average fitness of those haplotypes requires a mechanism that translates their population genetic structure into among-population sex ratio variation and a corresponding effect on phenotypic selection. This is easily accomplished using the concept of subjective frequencies, given the simplifying assumption that CMS factors are randomly associated with nuclear backgrounds, both within and among populations.

Consider CMS factors or cpDNA haplotypes 1 and 2, which produce hermaphrodites with probabilities X_1 and X_2 , respectively. Let the metapopulation-wide frequency of factor 1 be p_1 and the frequency of factor 2 be p_2 , and let the among-population variance in the relative frequencies of 1 and 2 be V_{12} . Following Equations (1) and (2), the subjective frequencies of the two factors from the perspective of CMS 1 are

$$p_{11} = p_1 + V_{12}/p_1 \quad (3)$$

and

$$p_{21} = p_2 - V_{12}/p_1 \quad (4)$$

CMS factor 1 then 'experiences' hermaphrodites with a frequency

$$p_{H1} = (p_1 + V_{12}/p_1)X_1 + (p_2 - V_{12}/p_1)X_2 \quad (5)$$

This can be rearranged to

$$p_{H1} = p_1X_1 + p_2X_2 + (V_{12}/p_1)(X_1 - X_2) \quad (6)$$

Similarly

$$p_{H2} = p_1X_1 + p_2X_2 + (V_{12}/p_2)(X_2 - X_1) \quad (7)$$

Thus, the McCauley–Taylor effect comes into play again as genetic structure (V_{12}) increases, and as the absolute magnitude of $(X_1 - X_2)$ increases. Population structure increases the fitness of the CMS factor or cpDNA haplotype with the higher probability of being carried by a hermaphrodite, and decreases the fitness of the other. When $|X_1 - X_2| = 1$ the pure cytoplasmic inheritance case of McCauley and Taylor (1997) is recaptured.

Application of the model to the cpDNA data

This logic can be applied to the empirical results presented here by designating $X_1 = 0.714$ and $X_2 = 0.077$; yielding $(X_1 - X_2) = 0.637$. Given the high degree of genetic structure documented by McCauley (1998) and the strong frequency-dependent selection acting on the two sexes (McCauley and Brock, 1998; McCauley *et al.*, 2000), the among-population sex ratio variation known to exist in this system should have quite different effects on the two haplotypes.

In the population survey haplotype 2 was found in 5 of 20 populations (and was fixed in one small population), yet comprised only 11.7% of the 111 individuals sampled. With no population structure the frequency of hermaphrodites (from the perspective of either sex) is then calculated from our data and Equations (6) or (7) to be $[(0.883)(0.714) + (0.117)(0.077)] = 0.639$. Evaluating the effect of population structure requires an estimate of V_{12} . Wright's F_{st} calculated for haploid cpDNA data equaled 0.624 in the McCauley (1998) study. Recognizing that a general equation for F_{st} for a gene with two alleles is

$$F_{st} = V_{12}/p_1p_2 \quad (8)$$

where V_{12} , p_1 , and p_2 are defined as above, the subjective frequency of hermaphrodites from the perspective of haplotype 1 can be rewritten as

$$\begin{aligned} p_{H1} &= p_1X_1 + p_2X_2 + p_2F_{st}(X_1 - X_2) \\ &= 0.69. \end{aligned} \quad (9)$$

Similarly, for haplotype 2

$$\begin{aligned} p_{H2} &= p_1X_1 + p_2X_2 + p_1F_{st}(X_2 - X_1) \\ &= 0.29. \end{aligned} \quad (10)$$

Thus, if fitness is proportional to the local frequency of hermaphrodites, population structure increases the fitness of cpDNA haplotype 1 by increasing the subjective frequency of hermaphrodites. The effect is small because individuals carrying haplotype 1 are usually hermaphroditic and haplotype 1 is globally common. In contrast, population structure reduces the fitness of haplotype 2 by more than 50% because bearers of that haplotype are usually female, and that haplotype is globally rare.

Application of the preceding analysis to *S. vulgaris* requires that the properties of natural populations resemble those assumed in the model. That this is at least approximately the case can be illustrated by calculating the average expected fitness of females carrying haplotype 2 using the population-specific estimates of fitness given in McCauley *et al.* (2000). That study showed that the relative fitness of females, as measured by the number of seeds per fruit, varied among natural populations of *S. vulgaris* as a positive function of the local frequency of hermaphrodites. Eleven of the twelve females found to be carrying haplotype 2 in the present study were collected from populations in which the local frequency of females was much higher than the global average. Figure 3 places these 12 females onto the sex ratio/fitness relationship demonstrated by McCauley *et al.* (2000) by considering them in the context of the sex ratio of the population from which they were drawn, and compares this to what would be expected with no among-population sex ratio variation.

Discussion

In most models of gynodioecy a CMS factor that is rarely restored should spread through a population if females have a general fitness advantage

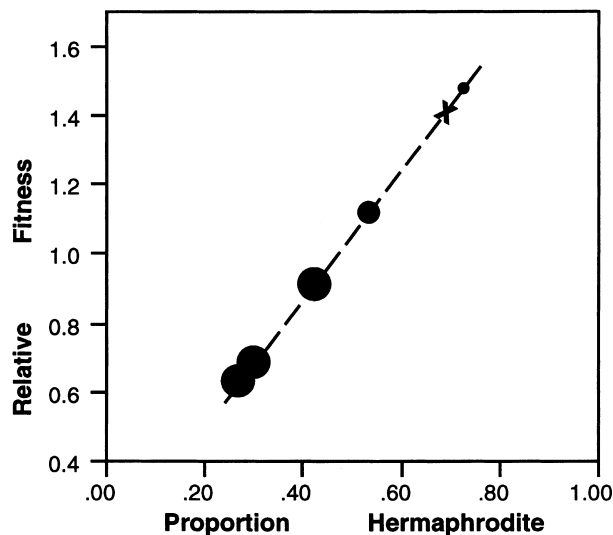


Figure 3. The relative fitness of females carrying cpDNA haplotype 2 (●), based on the proportion of hermaphrodites found in the local population from which they were drawn and the linear relationship (---) between sex ratio and relative fitness as measured by seeds/fruit in McCauley *et al.* (2000). Females were drawn from five populations; circle size is proportional to sample size. The x symbol represents the fitness expected if all local populations displayed the metapopulation-wide average sex ratio; that is, if there were no population structure.

through seed (e.g. Charlesworth and Ganders, 1979). McCauley and Taylor (1997) showed that frequency-dependent fitness, combined with sufficient population structure, could reverse this and prevent the spread of CMS factors that would otherwise be favored. It is interesting that, at least based on the fitness components we have studied, the spread of CMS factors marked by cpDNA haplotype 2 would appear to be limited by the population structure of *S. vulgaris*, even though that haplotype produces mostly females. Our results suggest that once that CMS factor becomes locally common, the fitness of the females that carry it is reduced relative to females (and hermaphrodites) in other populations.

Several points must be made about the assumptions underlying our analysis. First, we are not proposing that the genetic region responsible for differences average sex expression lies in the cpDNA. Rather, we are suggesting that there are cpDNA differences that mark maternal lineages, or classes of lineages, that also differ in the CMS factor they carry. Such CMS effects are most likely specific to variation at some unknown region in the mitochondrial DNA. Olson and McCauley (2000) have shown a statistical association between cpDNA and mitochondrial haplotypes, which might be expected if they are both inherited maternally. If so, perhaps what we have really described here is a case of hitch-hiking selection acting on cpDNA. This is of potentially broad significance since the genetic structure of cpDNA polymorphism has been used to infer rates of gene flow by seed (e.g. Ennos, 1994; McCauley, 1997, 1998; Provan *et al.*, 2001) under an assumption of selective neutrality.

Second, we are not proposing that there are just two CMS factors coexisting in our populations. We have reported on the cpDNA haplotype designations for which we have extensive population information. Olson and McCauley (2000) have reported greater cpDNA diversity in *S. vulgaris* by including information from two other regions of cpDNA that are amplified by two other sets of primers. Even greater diversity was detected by Olson and McCauley in mtDNA using an RFLP method. Given this extensive diversity in maternal lineages coexisting in our study populations, it is perhaps surprising that the cruder method presented here could detect such clear differences in sex ratio between haplotypes.

Third, we have ignored the effect of nuclear genes on sex expression by assuming that, averaged across individuals, the haplotypes we have studied reside in the same nuclear background. This would not be true, for example, if there were strong CMS-restorer coevolution within populations, which might generate strong cytonuclear disequilibrium. We are encouraged in this regard by the observation that, while globally fairly rare, haplotype 2 was found in five populations at varying local frequency. It seems to be associated with a female phenotype whether locally common or rare, an observation not in keeping that expected with strong local coevolution.

Fourth, we are assuming that CMS type has no effect on fitness other than through sex expression. Our two cpDNA haplotypes could be linked to CMS factors that differ in the mechanism of male sterility. If so, it is possible that females bearing the two haplotypes differ in fitness for reasons other than the local sex ratio that they experience or that the two classes of hermaphrodites could differ in fitness owing to differences in the mechanism of restoration.

This leads to a final point about the assumptions inherent in our analysis. The McCauley–Taylor model assumes populations go extinct each generation, following seed dispersal. That is, genetic structure, and thus local sex ratio, is determined almost entirely by founding events derived from that seed dispersal. Only one episode of selection can act within a given population. Because *S. vulgaris* is a weedy species that inhabits disturbed habitat we feel that it is safe to assume that local populations are ephemeral, and we have in fact documented several local extinctions over a 5 year period. However, most local populations clearly persist for more than one generation. Theoretical studies need to be directed at the question of how selection and population structure interact at the levels of population turnover that probably typify most plant metapopulations (i.e. populations that persist on the order of 2–20 generations). Such theoretical studies could then be specialized to address the specific question of CMS-restorer coevolution under metapopulation dynamics and frequency-dependent fitness.

It should also be noted that the disadvantage that population structure imposes on females, as discussed here, arises from pollen limitation, a condition likely to occur in *S. vulgaris* (McCauley and Brock, 1998; McCauley *et al.*, 2000), but certainly not in all gynodioecious plants. Models that do not assume pollen limitation (e.g. Couvet *et al.*, 1998) suggest that metapopulation dynamics would increase the fitness of cpDNA haplotypes carried predominately by females. In the absence of pollen limitation, females make better colonists because of their tendency to higher seed production and because repeated founding events could limit the potential for CMS-restorer coevolution.

The general point of this paper has been that metapopulation dynamics can influence the trajectory of adaptive evolution, especially for traits with frequency-dependent fitness. The metapopulation dynamics serve two purposes. Extinction/recolonization can create population structures favorable for among-population variation in fitness through the genetic structure resulting from founder effect. Simultaneously, the ephemeral nature of populations requires that long term fitness be related to the contribution of a genotype to a pool of colonists. Sex ratio evolution in structured populations of *S. vulgaris* appears to be one specific example of this general phenomenon. It is our feeling, however, that these conditions are met in many species of plants that occupy disturbed, patchy habitat.

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