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INVITED REVIEW

Recent advances in the study of gynodioecy: the interface of theory and empiricism

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• *Background* In this review we report on recent literature concerned with studies of gynodioecy, or the co-occurrence of female and hermaphrodite individuals in natural plant populations. Rather than review this literature in its entirety, our focus is on the interplay between theoretical and empirical approaches to the study of gynodioecy.
• *Scope* Five areas of active inquiry are considered. These are the cost of restoration, the influence of population structure on spatial sex-ratio variation, the influence of inbreeding on sex expression, the signature of cyto-nuclear coevolution on the mitochondrial genome, and the consequences of mitochondrial paternal leakage.
• *Conclusions* Recent advances in the study of gynodioecy have been made by considering both the ecology of female:hermaphrodite fitness differences and the genetics of sex expression. Indeed theory has guided empiricism and empiricism has guided theory. Future advances will require that some of the methods currently available only for model organisms be applied to a wider range of species.

Key words: Breeding system, gynodioecy, cytoplasmic male sterility, restoration, sex ratio, inbreeding, population structure, genetic conflict.

INTRODUCTION

Gynodioecy is the circumstance in which female and hermaphrodite individuals co-occur in the same population. In gynodioecious species individuals classified as females typically produce much-reduced anthers and little or no viable pollen, whereas in hermaphroditic flowers both sexes are functional (for an example, see Fig. 1). Gynodioecy is a fairly common phenomenon within the angiosperms, estimated as being present in about 7 % of species (Richards, 1997). Gynodioecy has long been of considerable interest to plant biologists for several reasons. First, it is thought by some to be an intermediate step in the transition from a strictly hermaphroditic to a dioecious breeding system (Darwin, 1877; Lloyd, 1974; Charlesworth and Charlesworth, 1978). Secondly, it can represent a prime example of genetic conflict (e.g. Frank, 1989; Burt and Trivers, 2006) because sex determination in gynodioecious systems is often cyto-nuclear. In that case a cytoplasmic male sterility (CMS) element, usually associated with the mitochondrial genome, can render an individual functionally female unless its phenotypic consequences are countered by one or more nuclear genes that act to restore male function. An individual would be considered female if it carried a CMS element without the proper nuclear gene known as a restorer. It would be a hermaphrodite if it either (*a*) carried a fertile cytoplasm, or (*b*) carried a CMS element paired with the proper nuclear restorer. It appears that gynodioecious systems can contain multiple forms of CMS, each requiring its own mode of restoration (de Haan *et al.*, 1997*b*; Frank, 1997; Charlesworth and Laporte, 1998; Dudle *et al.*, 2001; van Damme *et al.*, 2004).

Genetic conflict arises in such systems owing to differences in the mode of inheritance of mitochondrial and nuclear genes. Mitochondrial genes (mtDNA) are typically maternally inherited (Birky, 2001). If so, their currency of evolutionary fitness is seed production. It is to the evolutionary advantage of a CMS element, then, to cause a phenotype that produces more or better seeds. In fact, it is a common observation in gynodioecious plant species that females produce more and/or higher quality seeds than hermaphrodites (a phenomenon sometimes called female compensation; Shykoff *et al.*, 2003). The greater number of seeds is most likely due to the diversion of energy, otherwise devoted to pollen production, to seed. Additionally, since the majority of gynodioecious species are self-compatible the hermaphrodite morph can often self-fertilize (Meagher, 2007; Ehlers and Schierup, 2008). As a consequence higher quality seed production by females can result from the inability of that morph to self, thereby limiting inbreeding depression in their offspring (e.g. Thompson and Tarayre, 2000; Delph, 2004; Chang, 2007). Other observed female–hermaphrodite differences that could relate to fitness through seed include differences in flower number and fruit set (Shykoff *et al.*, 2003; Meagher, 2007).

Because nuclear genes are bi-parentally inherited their fitness derives from transmission through seed and pollen. Thus, there are circumstances in which an unrestored CMS element increases its own fitness through enhanced seed production but diminishes the fitness of the nuclear genes with which it is associated owing to the loss of pollen production. Similarly a restorer gene can diminish the fitness of a CMS element with which it is associated through diminished female function, while enhancing its own fitness through increased pollen production.

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Hermaphrodite   Female

Fɪɢ. 1. Female and hermaphroditic flowers of *Silene vulgaris*, a gynodioe-cious species. Note the reduced anthers within the female flower. (Photograph courtesy of Steven J. Baskauf)

Of course there are also well-known cases of gynodioecy in which sex determination is under nuclear control, such as *Schiedea adamantis* (Sakai *et al.*, 1997) and *Fragaria viginiana* (Ashman, 2003). With pure nuclear sex determination, the theoretical conditions allowing for the persistence of gynodioecy differ from the cytonuclear case, since the loss of fitness through pollen must be compensated proportionally by increased fitness through seed (Charlesworth and Charlesworth, 1978; Bailey and Delph, 2007*a*). Empirical data supports model predictions of very high seed fitness among females in nuclear gynodioecious species (Bailey and Delph, 2007*a*).

The study of gynodioecy has followed many paths. One has been the development of mathematical models whose goal is to understand the circumstances under which gynodioecy can evolve and persist (reviewed in Bailey and Delph, 2007*a*). An important problem in this regard is to define the conditions that permit the cyto-nuclear polymorphism needed for the cyto-nuclear form of gynodioecy to persist, i.e. under what circumstances can two or more forms of cytoplasm co-exist in the same population (either a fertile form and two or more CMS forms or two or more different CMS types) simultaneous to the coexistence of multiple nuclear sex-determining genotypes (restorer and non-restorer types or multiple restorer types matched to different CMS types). Central to these models is the form of balanced polymorphism derived from rare advantage frequency-dependent selection. At its simplest, gynodioecy could not persist if both females and hermaphrodites (and the sex-determining genes) did not become less fit when most common. Discussed here will be two factors affecting polymorphism stability that have received considerable attention: cost of restoration and population structure.

A second area of active research involves contrasting hermaphrodite:female fitness through seeds and pollen, including differences generated by inbreeding, especially in the context of structured populations. These studies have been conducted in both the field and greenhouse and particularly focus on how hermaphrodite:female fitness differences vary according to locality and/or circumstance. A third area of current interest involves studies of the mitochondrial genome of gynodioecious

species – both its transmission and evolution. Of particular interest here is whether mitochondrial gene sequences bear the signature of the various types of selection that could be associated with cyto-nuclear coevolution in gynodioecious systems.

The gynodioecy literature is large and growing. In a recent review, McCauley and Olson (2008) report that the Web of Science search engine retrieved 220 references published since 1997, when the term gynodioecy was used as the key term in the search. As of April 2009 that number stands at 260. Thus a comprehensive review of the gynodioecy literature is not feasible in this venue and is not the goal of this review. Rather, the goal is to illustrate how some recent advances in the theory of gynodioecy have influenced its empirical study in natural populations, and how the results of such studies have influenced the development of theory. The topic of gynodioecy has been the subject of several other recent reviews (e.g. Budar *et al.*, 2003; Jacobs and Wade, 2003; Shykoff *et al.*, 2003; Delph and Wolf, 2005; Delph *et al.*, 2007; Bailey and Delph, 2007*a*; McCauley and Olson, 2008) and the reader is directed to those sources for consideration of topics not covered here. Further, because CMS–restorer systems are utilized as breeding tools in a number of economically important crops, the underlying molecular and cell biology of CMS–restorer systems has been described for a number of model or economically important plant species not naturally gynodioecious, and this has also been reviewed elsewhere (e.g. Hanson and Bentolila, 2004; Chase, 2007).

Two re-occurring and inter-related themes run through this review: (1) it is a common observation that females and hermaphrodites are not randomly distributed within and among populations of the same species (see Literature Cited); (2) several components of female and/or hermaphrodite fitness are dependent on the relative local proportion of females and hermaphrodites, i.e. they are frequency dependent in that they depend on the local sex ratio. As will be seen below, these observations have two consequences for the study of gynodioecy: (1) in efforts to understand how external environmental factors influence the relative fitness of females and hermaphrodites, this spatial sex ratio variation has been studied in the context of environmental variation that might affect the two sexes differently; (2) this sex ratio variation allows the frequency-dependent processes integral to the maintenance of gynodioecy to be studied to the degree that fitness differences between the sexes are sex-ratio dependent. Of course, distinguishing between instances in which sex ratio variation is driven by spatial variation in external factors influencing the relative fitness of the two sexes and cases in which the sex ratio variation is the cause of spatial variation in the relative fitness of the two sexes is problematic.

MODELS OF CYTONUCLEAR GYNODIOECY AND A COST OF RESTORATION

The interplay of experimentation and theory has been exceptionally fruitful for the study of cytonuclear gynodioecy. As crossing data have progressively eliminated simple models of sex determination in cytonuclear gynodioecy, new theoretical models have been developed to incorporate new levels of genetic complexity. Currently, empirical evidence indicates that ancestral male-fertile and derived male-sterile cytotypes

can co-occur in populations (Fenart *et al.*, 2006). Also, it is becoming clear that restoration of male fertility involves several nuclear loci that may act in a manner that is either additive (incremental restoration with increasing numbers of restorer alleles; Koelewijn and van Damme, 1996; Dufay *et al.*, 2008) or threshold (all or none restoration depending on restorer allele number; Ehlers *et al.*, 2005). If restoration is additive, continuous levels of restoration should be seen such that selection in the corresponding nuclear background acts to increase the number of restorer alleles. This may explain the common observation of individuals with intermediate anther phenotypes. Theoreticians have responded with models that incorporate empirical observations into their models such as including male-fertile or non-segregating cytotypes (Dufay *et al.*, 2007) or threshold restoration (Bailey and Delph, 2007*b*).

Theory has also influenced empirical work, especially in the search for a cost of restoration. Theoretical models have repeatedly shown that females can only be maintained at equilibrium in cytonuclear gynodioecy if some force opposes the fixation of nuclear restorers (e.g. Bailey *et al.*, 2003; Dufay *et al.*, 2007). Specifically, a cost of restoration must be a difference in fitness among hermaphrodites such that individuals with fewer restorer alleles have higher fitness than those with many restorer alleles. Many early efforts to find a cost of restoration were confounded by the level of genetic knowledge necessary to demonstrate a cost of restoration. Currently, only two studies have directly demonstrated a cost of restoration (de Haan *et al.*, 1997*a*; Bailey, 2002); however, two more-recent studies have produced encouraging evidence consistent with a cost of restoration acting in nature (Dufay *et al.*, 2008; del Castillo and Trujillo, 2009).

Dufay *et al.* (2008) examined the frequency of male fertile (MF) and CMS cytotypes, rates of restoration of CMS individuals, pollen production, and pollen viability in two populations of *Beta vulgaris* ssp. *maritima*. Using these data they show an inverse correlation at the population level between pollen fitness in MF hermaphrodites and restoration levels, suggesting that hermaphrodites that carry restorers that are not needed (or 'alien') in the MF cytotype background have low fitness, i.e. that there is a cost of restoration. Specifically, Dufay *et al.* (2008) found that restoration appeared additive with many CMS individuals with intermediate anther phenotypes and pollen production levels, but that cost of restoration seemed discrete, producing roughly two types of MF hermaphrodites with either high or low pollen viability. While not conclusive, these are intriguing data that would be interesting to incorporate in new models.

Similar to Dufay *et al.* (2008), del Castillo and Trujillo (2009) compare groups that are likely to vary for average rate of restoration, but instead of comparing two populations they compare four sets of crosses from a single population. As part of an experiment looking at inbreeding depression in a natural population of *Phacelia dubia*, open-pollinated fruits were collected and controlled crosses were performed among the progenies. In the course of this experiment, several lineages were identified that did not segregate females in any of the performed crosses (non-segregating or N lineages) and other lineages that did occasionally produce females (segregating or S lineages). The authors reasoned that N lineages either carried a male-fertile mitochondrial type or a CMS allele for which the restorer had fixed in the population. In contrast, S lineages carried a CMS allele for which the restorer and non-restoring alleles were both present in the population. By performing all four possible types of crosses among segregating and non-segregating hermaphrodites (N × N, N × S, S × N, S × S), the authors were able to discern maternal effects such as cytoplasmic type, bi-parental effects such as restorer alleles, and effects specific to interactions between maternal and bi-parental effects such as a cost of restoration. They found that seedlings from crosses with non-segregating hermaphrodites as seed parents and segregating hermaphrodites as pollen donors had significantly lower fitness. Since all these offspring (probably) inherit the maternal cytotype and some of them the alien restorer allele from their father, low fitness on average among progeny from N × S crosses suggests a cost of restoration.

In considering this new evidence of cost of restoration, it can already be seen that some predictions regarding characteristics of cytonuclear gynodioecy may need to be reconsidered. First, both of the most recent examples are from systems that contain hermaphrodite lineages that do not segregate females. Whether these individuals represent ancestral male fertile lineages as in *Beta vulgaris* ssp. *maritima* or if they are CMS cytotypes whose restorers have fixed, these phenomena need to be incorporated into models. Dufay *et al.* (2007) is a helpful first step in this direction. Secondly, seed costs may be more common than theoretically expected (Delph *et al.*, 2007), as both de Haan *et al.* (1997*a*) and del Castillo and Trujillo (2009) found evidence of restorer effects on seed-fitness traits. Thirdly, Dufay *et al.* (2008) suggest that partially restored hermaphrodites may be a result of an additive effect of multiple restorers. These recent papers have benefited from theoretical work that has highlighted the most likely comparisons to reveal costs of restoration in nature (Bailey, 2002; Bailey and Delph, 2007*a*), while the data they produced will in turn suggest new scenarios for modelling. Possibly, recent models could be modified to account for partially restored hermaphrodites, especially in systems that retain MF cytotypes.

CONSEQUENCES OF POPULATION STRUCTURE

Most plant species are not distributed evenly, but rather display patchiness hierarchical in spatial scale. This population structure can result in non-random patterns of mating and, hence, limited movement of genes. As a consequence, gene frequencies can differ from population to population. The genetic consequence of population structure is often measured by the statistic F_{st}, which ranges from 0 (no gene frequency differences between populations) to 1 (fixed allele differences between populations). Gynodioecious systems are often characterized by considerable variation from locality to locality in the relative proportion of female and hermaphrodite individuals (Frank, 1997; Bailey and Delph, 2007*a*). This sex-ratio variation can result from spatial variation in allele frequency at one or more of the loci that determine sex and has been the focus of considerable research effort. There are a variety of possible reasons why the sex ratio can vary from locality to locality. These include chance events associated with genetic drift and spatial variation in the relative fitness

of females and hermaphrodites. This spatial variation in relative fitness could be due to spatial variation in environmental circumstances that affect the sexes differently or due to spatial variation in frequency-dependent processes, such as pollen limitation or the tendency to self-fertilize, that arise once the sex ratio varies from place to place. The impact and origins of this interesting consequence of population structure are of considerable importance for the study of gynodioecy.

Extinction/recolonization dynamics

Sex-ratio variation could be generated by random events, especially if the species in question is subject to local extinction/recolonization dynamics (Manicacci *et al.*, 1996; Frank, 1997). In that case, random events associated with population founding could determine local allele frequencies. In species with a patchy distribution, and a frequent turnover of patches, local populations may be founded by just a few seeds and not persist long enough to permit much modification of the founding allele frequencies by subsequent selection, or by gene flow via seeds or pollen. Mitochondrial CMS elements may be particularly subject to founder effect because plant organellar genomes are often effectively haploid and not capable of gene flow by pollen, owing to maternal inheritance. In general, plant organellar genes show much greater population structure (as measured by F_{st} or its derivatives) than do nuclear genes (Petit *et al.*, 2005). With frequent population turnover, founding events could, by chance, limit associations between particular CMS elements and their restorers in some populations.

This kind of population structure can have interesting consequences for sex-ratio evolution, assuming local pollen limitation. For example, a new population cannot persist if founded only by seeds destined to be females. Further, with local pollen limitation any sex-ratio variation has a greater average effect on female fertility than on hermaphrodite fertility, even when hermaphrodites cannot self-fertilize. This is because, by definition, an increase in sex-ratio variation increases the tendency of females to co-occur in some populations and hermaphrodites to co-occur in other populations, relative to pooling the same set of individuals in a common population (for a numerical example, see Fig. 2). Thus, an increase in sex-ratio variation increases the tendency of hermaphrodites to donate pollen to other hermaphrodites and reduces the availability of pollen to females. Models by Pannell (1997) and McCauley and Taylor (1997) have shown how this kind of population structure favours hermaphrodites over females and reduces the global frequency of females, relative to panmixia. Indeed, McCauley *et al.* (2000) found that some components of female fitness were an inverse function of their local frequency in patches of *Silene vulgaris*, though such frequency-dependent effects on female fitness were not evident in a study of *Kallstroemia gradiflora* (Cuevas *et al.*, 2008), a species which did not appear to be pollen limited.

Selection in spatially varying environments

The sex ratio can also vary from locality to locality as a local response to natural selection. Females might be expected to be most common where most fit (especially in the absence of pollen limitation). For example, Nilsson and Agren (2006)

No population structure   Population structure : sex ratio variation

70 F
30 H

5 F 20 H   20 F 5 H

20 F 5 H   25 F

$p_H = 0.30$
$p_{HH} = 0.30$
$p_{HF} = 0.30$

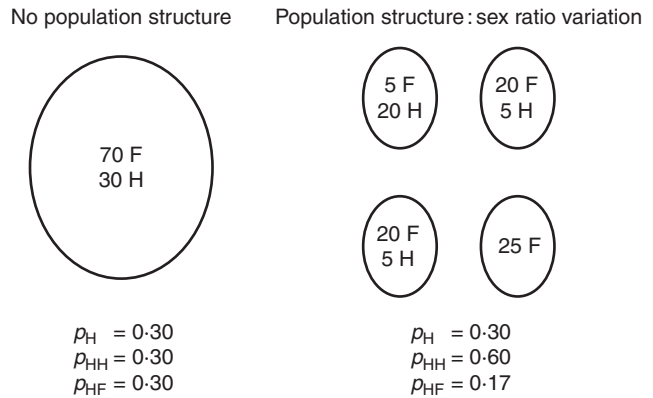$p_H = 0.30$
$p_{HH} = 0.60$
$p_{HF} = 0.17$

FIG. 2. The influence of population structure and the resulting sex ratio variation on pollen availability. It is assumed in the diagram that pollen availability for both females (F) and hermaphrodites (H) is a function of the local frequency of hermaphrodites. Note that an equivalent group of 100 individuals is combined to create a panmictic population (left) or subdivided to create local sex ratio variation (right). In the panmictic population the actual frequency of hermaphrodites (p_H) is the same as the frequency of hermaphrodites experienced by females (p_{HF}) or hermaphrodites (p_{HH}). With population subdivision, the average frequency of hermaphrodites experienced by females (p_{HF}) is less than p_H, whereas the average frequency of hermaphrodites experienced by hermaphrodites (p_{HH}) is greater than p_H. For example, 25 of the 70 females distributed among the population subdivisions live at a locality where no pollen is available. See McCauley and Taylor (1997) for details on the calculation of p_{HH} and p_{HF}.

found a positive correlation among populations of *Plantago maritima* between the frequency of females and the fecundity of females relative to hermaphrodites (though they found evidence that stochastic processes influence the local sex ratio as well). The relative fitness of females and hermaphrodites could vary from locality to locality due to differences in the environment, ranging from variation in soil properties to differences in the biotic community with which the plant interacts. In a study of spatial variation in the sex ratio in *Thymus praecox*, Landergott *et al.* (2009) found that the proportion of hermaphrodites decreases with increasing altitude, presumably as a result of spatial variation in selection pressures. In an experimental study, Barr (2004) found that soil moisture influenced sex differences in seed production in *Nemophila menziesii*, although spatial variation in seed moisture did not correlate with variation in the sex ratio of natural populations. In a study of *Wurmbea biglandulosa*, Vaughton and Ramsey (2004) showed that drier environments promoted the establishment of females by differentially lowering the seed fitness of hermaphrodites. Caruso and Case (2007) found in *Lobelia siphilitica* that female frequency co-varied negatively with soil moisture and fruit predation, and positively with annual temperature. Finally, Alonso and Herrera (2008) found in a study of *Daphne laureola* that the magnitude of sex-specific differences in pollinator visitation varied across light environments. Another intriguing possibility is that the cost of restoration could vary from locality to locality as a consequence of environmental variation and that differences in this cost influence the outcome of sex-ratio evolution (Bailey and Delph, 2007*a*).

One additional factor that must be considered is that hermaphrodites could exhibit phenotypic plasticity with regard to their relative allocation of resources to male and female

function. In particular, the relative allocation to female function (seed set) could be less in harsher environments (Delph, 2003; Delph and Wolf, 2005). This, in turn, could alter female : hermaphrodite fitness relationships and the evolution of the local sex ratio. Indeed, it has been noted in several gynodioecious species that females are more common in poorer sites (Delph, 2003).

Sex-ratio variation and asynchronous limit cycles

Theoretically, sex ratio could also differ from locality to locality owing to natural selection, not as a consequence of differences in the environment, but rather because there need not be a single stable equilibrium point even when identical CMS elements and restorers interact in identical environmental settings. In some mathematical models of cytonuclear gynodioecy CMS and restorer allele frequencies exhibit stable limit cycles through time, with a consequential cycle in the sex ratio (Gouyon *et al.*, 1991). Should different populations be out of synch regarding these cycles those populations could differ in their sex ratio at any given time point. Demonstration of such a phenomenon would require a careful examination of female and hermaphrodite fitness at several locations, probably over long periods of time.

Spatial variation in rates of self-fertilization

It has been hypothesized that population structure and sex-ratio variation can also influence another important attribute of fitness in some gynodioecious species, i.e. the frequency with which hermaphrodites self-fertilize. As populations become increasingly female-biased the tendency for self-compatible hermaphrodites to self-fertilize should increase, owing to the concomitant decline in the availability of outcross pollen. Indirect evidence for this comes from studies of the relationship between the sex ratio and the germination rate of seeds produced by hermaphrodites. If hermaphrodites self-fertilize more frequently when rare, one might expect an inverse relationship between the frequency of hermaphrodites and the level of inbreeding depression in their offspring. This relationship has been found in studies of *Silene vulgaris* in which the rate of seed germination declined with a decrease in the frequency of hermaphrodites among artificial populations in which the sex ratio had been manipulated experimentally (McCauley and Brock, 1998) or among populations in which the sex ratio varied naturally (McCauley *et al.*, 2000). Miyake and Olson (2009) found direct evidence for this in another experimental array study of *S. vulgaris* that also manipulated the sex ratio of artificial populations, but in which the out-crossing rate of hermaphrodites was assayed using genetic markers to compare mothers and offspring. Indeed, the frequency of self-fertilization was an inverse function of the frequency of hermaphrodites in that study.

A related phenomenon would be an increase in self-fertilization as the absolute number of hermaphrodites decreases, for much the same reasons. While the number and frequency of hermaphrodites might often co-vary positively, this need not always be the case. Consider an extreme example in which a population consists of a single hermaphrodite. The frequency of hermaphrodites in that population would be 100 %, but that lone hermaphrodite could only reproduce via self-fertilization. Given the ability to self-fertilize, single hermaphrodites might make better colonists than single females, but their ability to found new populations would be tempered by any inbreeding depression in their offspring. Taylor *et al.* (1999) illustrate this in a third study of artificial populations of *S. vulgaris*. Here the reproductive success of individual plants was monitored as a function of distance from a pollen source. Individual females situated >80 m from a pollen source displayed very little reproductive success, whereas similarly situated hermaphrodites were able to produce viable seed, albeit in numbers below the seed production of hermaphrodites closer to a source of outcross pollen. Genetic studies showed that offspring produced by those more isolated hermaphrodites were, on average, more likely to be produced by self-fertilization. As argued by Miyake and Olson (2009), the influence of context-dependent self-fertilization should be incorporated more fully into future models of the evolution of gynodioecy, especially those that consider meta-population dynamics.

Fine-scale population structure

Finally, in gynodioecious species the sexes can be spatially aggregated within populations whose member individuals otherwise show little evidence of fine-scale patchiness (e.g. Laporte *et al.*, 2001; McCauley and Olson, 2003; Olson *et al.*, 2006). It has been suggested that this is due to limited movement of CMS via seed, an argument whose logic follows from observations that mtDNA markers can exhibit structure at a similar fine scale. This fine-scale clumping of the sexes could further limit the availability of pollen to females relative to panmixia (Graff, 1999; Olson *et al.*, 2006). Graff (1999) found in a study of *Sidalcea malviflora malviflora* that, in fact, the neighbourhood sex ratio (the frequency of hermaphrodites) did influence seed set in both sexes, resulting in a net gain in fitness for hermaphrodites relative to that expected with a random spatial distribution of the sexes. Similarly Olson *et al.* (2006) found in one population of *S. vulgaris* that fruit set in females increased with the local neighbourhood frequency of hermaphrodites.

In summary it appears that spatial variation in the sex ratio can be a result of chance events or a response to selection. The random/selective dichotomy is a bit simplistic, however, since some of the reasons for female:hermaphrodite fitness differences appear to be frequency-dependent. As such, sex-ratio differences initially due to chance can have subsequent consequences for fitness and the response to selection. This interaction has not been modelled fully.

INBREEDING AND THE PRODUCTION OF FEMALES

The fact that the hermaphroditic morph of many gynodioecious species is capable of self-fertilization has obvious consequences. As emphasized above it would limit the tendency of hermaphrodites to be pollen limited even when rare or isolated but could also result in inbreeding depression in any seeds produced by such events. Indeed, as stated above, lowered quality of seeds produced by hermaphrodites as a consequence of

self-fertilization is one factor thought to drive the evolution of gynodioecy. Other phenotypic consequences for the offspring produced by self-fertilization are less well studied. Emery and McCauley (2002) found that in *Silene vulgaris* hermaphrodites that were self-fertilized during experimental crosses produced a greater proportion of female offspring than did outcrossed hermaphrodites. In fact, that proportion was essentially equivalent to the proportion of female offspring produced by females. Bailey and McCauley (2005) also used experimental crosses to extend this result to a much larger number of *S. vulgaris* populations, including some in which no females were produced in the experiment by out-crossing hermaphrodites, and in which naturally occurring females were virtually non-existent.

At first glance increased production of female offspring with selfing might be expected. Since restorer alleles are often dominant to non-restorers (Frank, 1989), it must be that many hermaphrodites are, in fact, heterozygous at loci that restore male function. It is well known that self-fertilization increases the probability of homozygosity in offspring, relative to outcrossing. Thus, self-fertilization of restorer heterozygote mothers who carry CMS would increase the probability of production of non-restorer homozygote (i.e. female) offspring. However, Bailey and McCauley (2005) show that the magnitude of the increase in production of female offspring with selfing exceeds that possible with a single locus dominant restorer. This suggests the possibility of multi-locus restoration with some kind of epistasis among restorer loci. Glaettli and Goudet (2006) argue that differential loss of male function could be a general consequence of inbreeding depression in *S. vulgaris* and not necessarily a consequence of allelic state at specific restorer loci. It should be noted that Gomez and Shaw (2006) also saw an increase in the production of male-sterile flowers with experimental inbreeding in a study of *Nemophila menzeii*. One wonders what proportion of the females seen in natural populations are the offspring of self-fertilized hermaphrodite mothers, as opposed to outcrossed female and hermaphrodite mothers, and whether they differ with regard to other aspects of the phenotype. As far as is known this phenotypic effect of self-fertilization has not been incorporated explicitly into models of gynodioecy (see also Gomez and Shaw, 2006).

Self-fertilization (and other close-relative mating) increases offspring homozygosity in specific pedigrees, relative to other pedigrees within the population produced without inbreeding. At the population level, homozygosity can increase by genetic drift in small populations, relative to levels found in populations not subject to genetic drift. Thus, the same effect of inbreeding on offspring sex ratio seen by comparing inbred and outcrossed hermaphrodites within populations might also be seen by comparing the offspring of hermaphrodites sampled from large to those sampled from small populations. Byers *et al.* (2005) compared the offspring sex ratio of hermaphrodite and female *Lobelia spicata* mothers from large and small populations and found that, indeed, hermaphrodites from small populations produced a higher proportion of female offspring than hermaphrodites from large populations, even controlling for local sex-ratio variation. Since this was a study based on naturally occurring fruits, rather than controlled crosses, it is possible that self-fertilization is more common in

small populations. If so, this would confound the effects of genetic drift in small populations with the effects of self-fertilization.

DOES CMS–RESTORER CO-EVOLUTION RESULT IN BALANCED OR TRANSIENT POLYMORPHISM?

It is interesting to consider the long-term dynamics of the evolutionary interaction between mitochondrial CMS elements and their nuclear restorers. The question arises as to whether the cyto-nuclear polymorphism necessary for some forms of gynodioecy represents a continuing interaction between a limited number of mitochondrial lineages and their restorers, maintained by balancing selection, or a series of transient polymorphisms that result as a succession of CMS elements arise, interact with novel restorers, and are replaced. The two scenarios are illustrated in Fig. 3. (Note that the specific players involved in both the balanced and transient
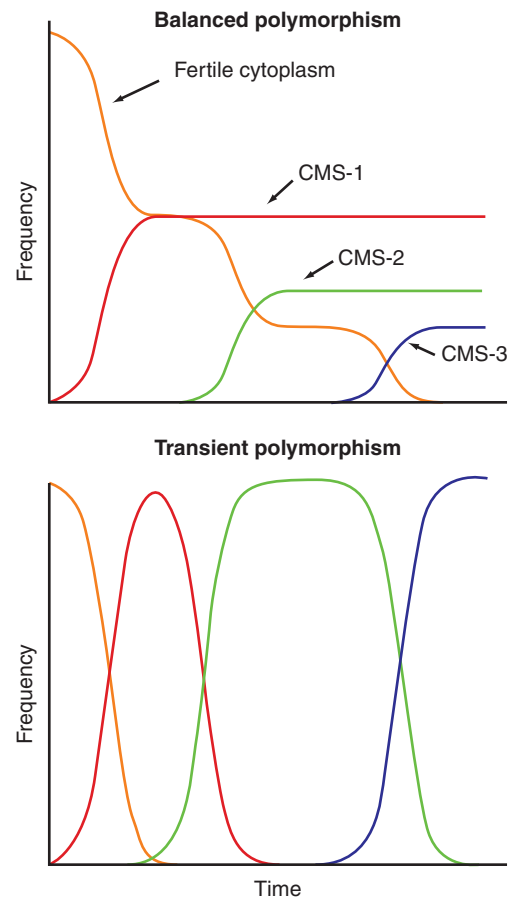

FIG. 3. A schematic in which a series of CMS types (1–3) are sequentially formed by mutation and preserved in the population by selection as the fertile type is replaced (above), contrasted to a case where a series of CMS types (1–3) are formed by mutation and replace a fertile type and then one another (bottom). With balancing selection the mitochondrial genome associated with each CMS type is preserved long enough to diverge from other CMS bearing mitochondrial genomes at loci additional to the CMS locus. With transient polymorphism, mitochondrial genomes do not coexist for a sufficiently long period of time to accumulate polymorphic variation at loci other than the CMS locus.

polymorphism scenarios could differ in different parts of a species range.) Recent studies comparing organellar gene diversity in gynodioecious species and their non-gynodioecious relatives support both arguments.

An interesting property of the mitochondrial genomes of some members of the genus *Silene* is the high level of sequence diversity seen in the coding regions of some species (Städler and Delph, 2002; Houliston and Olson, 2006; Barr *et al.*, 2007). This observation runs counter to the prevailing wisdom that the sequences of the coding regions of plant mitochondrial genes are quite highly conserved (e.g. Wolfe *et al.*, 1987). It has been argued that the high level of intra-specific variation is a consequence of the balancing selection inherent to gynodioecious systems (Städler and Delph, 2002; Houliston and Olson, 2006; Touzet and Delph, 2009). That is, rare-advantage frequency-dependent selection driven by the coevolution of multiple forms of CMS with their respective restorers, and perhaps by pollen limitation, would allow mitochondrial lineages to persist in populations long enough to accumulate sequence variation in portions of the mitochondrial genome not directly involved in CMS. Alternatively, it has also been suggested that recurrent episodes of mutation create novel forms of CMS that are then favoured by selection until the appropriate restorer also evolves and they become vulnerable to replacement by some other form of CMS not widely restored. These recurrent episodes of mutation and selective sweep would cause a high turnover rate of mitochondrial lineages, limiting the opportunity for accumulation of sequence diversity in mitochondrial genes.

Ingvarsson and Taylor (2002) provide evidence for the selective sweep scenario by comparing chloroplast DNA (cpDNA) diversity in gynodioecious *S. vulgaris* and non-gynodioecious *S. latifolia*. (Given that mtDNA and cpDNA are both maternally inherited selection acting on one cytoplasmic genome should influence the evolutionary dynamics of the other.) There was no evidence for higher cpDNA diversity in the gynodioecious species; in fact there was evidence that purifying selection associated with selective sweeps had acted on the cytoplasmic genomes of both species. Recently, however, Touzet and Delph (2009) have compared mitochondrial gene diversity in several gynodioecious and non-gynodioecious *Silene* species. Indeed, the diversity in the gynodioecious species is considerably higher, as predicted by the balancing selection scenario, though the genealogical structure of the haplotypes did not resemble the signature of either purifying or balancing selection. In another study of sequence diversity in the mitochondrial genes of *S. vulgaris*, Houliston and Olson (2006) found statistical evidence for balancing selection acting on at least one gene. Barr *et al.* (2007) point out, however, that differences between mitochondrial genes in standing levels of variation and apparent differences between genes in mutation rates complicate the relationship between mode of selection and genetic diversity. Interestingly, recent evidence for mitochondrial heteroplasmy and inter-genic recombination (see below) could suggest that hitch hiking between the CMS genes targeted by selection in gynodioecious systems and other mitochondrial genes or chloroplast genes need not be absolute. If so, this would temper both arguments (see also Sloan *et al.*, 2008). Further,

more complex forms of selection are possible. Ingvarsson and Taylor (2002) suggest that their results are also consistent with one in which multiple forms of CMS are maintained in populations by selection, but rather than achieving stable frequencies undergo the strong oscillations in frequency mentioned above. Such oscillations could lead to the chance loss of lineages in finite populations that could mimic the signature of recurrent selective sweeps. An added complication is that the spatial scale at which selective sweeps or oscillations occur depends on the population structure and could occur independently from locality to locality.

CONSEQUENCES OF PATERNAL LEAKAGE AND MITOCHONDRIAL HETEROPLASMY

The studies documenting levels of mitochondrial gene diversity suggestive of the effects of balancing selection also revealed mtDNA sequence patterns most likely generated by intragenic or intergenic recombination among mitochondrial lineages (Städler and Delph, 2002; Houliston and Olson, 2006; see also McCauley *et al.*, 2005; McCauley and Ellis, 2008). Recombination most easily generates novel products when the recombining molecules differ (i.e. when the individual is heteroplasmic). Heteroplasmy could, in turn, result from occasional bi-parental inheritance of the mitochondrial genome, rather than the strict maternal inheritance most often assumed (Kmiec *et al.*, 2006). Direct evidence of mitochondrial heteroplasmy in a gynodioecious species has come from two recent studies that have employed quantitative PCR to estimate the relative representation of different *atp1* or *cox1* haplotypes within *Silene vulgaris* individuals. While most individuals carried just one haplotype (i.e. were homoplasmic), a small fraction of them were found to be heteroplasmic (Welch *et al.*, 2006; Pearl *et al.*, 2009). Further, Pearl *et al.* (2009) compared the quantitative PCR profiles of mothers and offspring grown from seeds collected from those mothers in the field. In a small fraction of cases, homoplasmic mothers produced heteroplasmic offspring, providing evidence for mitochondrial leakage during fertilization (the fathers were unknown in these open pollinations).

Evidence for paternal leakage of the mitochondrial genome in gynodioecious species implies that CMS elements (or their fertile counterparts) could occasionally be transmitted through pollen in some species, contradictory to the usual assumption. This possibility motivated Wade and McCauley (2005) to develop a mathematical model of gynodioecy that included paternal leakage of the mitochondrial genome. In these models, even rare transmission of the mitochondrial genome through pollen stabilizes the cyto-nuclear polymorphism responsible for gynodioecy. This is because the fact that an individual produces pollen (i.e. is a hermaphrodite) implies that individual either carries a fertile cytoplasm or a restored form of CMS. As the sex ratio becomes increasingly female biased, the per capita pollination success of the hermaphrodite morph increases (especially assuming no pollen limitation). Ordinarily this success only contributes to nuclear fitness but with paternal leakage it could also contribute to the fitness of fertile cytoplasms or those that carry CMS elements associated with the proper locally common restorer. At some sex ratios the seed compensation enjoyed by females carrying un-restored

CMS is offset by the fitness advantage of transmission of fertile or restored mitochondrial genomes through pollen, providing another mechanism that contributes to balancing selection. The general applicability of these models depends on whether paternal leakage is a fairly general phenomenon among gynodioecious species or is restricted to *S. vulgaris*.

SYNTHESIS

Many interlocking issues have been addressed in this review including fitness effects of restorers, population structure, inbreeding effects, and the evolutionary genetics of the mitochondrial genome. Considered together these topics lead to some interesting questions. Consider three examples. First, what are the consequences for the progeny sex ratio of gene flow between structured populations of gynodioecious plants? Recall that inbreeding by hermaphrodites may enhance the production of female offspring. If so, gene flow events should maximize outbreeding and hence minimize the production of female offspring. On the other hand, if the co-evolution between CMS and restorers occurs on a local spatial scale then the union of gametes from different localities should maximize the probability of CMS/restorer mismatches and hence the probability of producing female offspring. Taken together one could imagine there is an optimal outcrossing distance for producing hermaphrodite offspring – far enough to alleviate inbreeding effects but proximate enough to minimize CMS/restorer mismatch. Several recent studies are relevant to this question. The observation that male sterility is often seen in the F_1 hybrids of two otherwise hermaphroditic species (Frank, 1997) leads to the prediction that local co-evolution occurs when reproductive isolation is complete or nearly complete. For example, Fishman and Willis (2006) revealed a male-sterile phenotype in crosses between two closely related species of *Mimulus*. It was later found that only some local populations of one of the species contained a form of CMS that was typically restored, and that combining this CMS with the naïve nuclear genome of the other species resulted in the male-sterile phenotype (Case and Willis, 2008). Barr (2004) found that an area within the distribution of *Nemophila menziesii* in which the frequency of females was particularly high corresponded to an apparent hybrid zone between two geographically distinct forms of the species that differ in corolla colour. This was supported by her observation that experimental crosses between colour morphs tended to produce more female offspring than did within-morph crosses. While some other studies have shown enhanced production of females following long-distance among-population crosses (e.g. Frank, 1997; Manicacci *et al.*, 1997), Gigord *et al.* (1998) found no effect of distance on progeny sex ratio when individuals originating from *Thymus vulgaris* populations spatially separated by varying degrees were crossed. Bailey and McCauley (2005) found in a study of *Silene vulgaris* that longer-distance among-region crosses actually produced fewer female offspring than did crosses among populations within regions. Thus, the impact of gene flow on the progeny sex ratio remains an open question whose answer might depend on the system under consideration and the spatial scale at which the gene flow occurs.

A second question follows from the issue of whether the cytoplasmic genetic polymorphism needed to maintain gynodioecy in systems with cyto-nuclear sex determination need be one with a fertile cytoplasm co-existing with one or more forms of CMS, or whether it is one in which multiple forms of CMS coexist. In the latter case, it is assumed that different forms of CMS require different restorers. Dufay *et al.* (2007) point out that there are a number of gynodioecious species in which no true fertile lines have been found (at least some females segregate from all maternal lines), but others in which fertile cytoplasms persist (no females segregate from some maternal lines). Modelling efforts suggest that maintaining a fertile/CMS cytoplasmic polymorphism may be more difficult than a CMS1/CMS2 polymorphism, though Dufay *et al.* (2007) have derived reasonable conditions for the maintenance of fertile cytoplasms in gynodioecious systems. This brings up an additional possible scenario within the selective sweep/balancing selection discussion. Perhaps balancing selection could maintain a fertile/CMS polymorphism, but the specific form of CMS could undergo replacement via a series of partial selective sweeps (as illustrated in Fig. 4). This could leave rather different signatures if mitochondrial gene sequences taken from individuals carrying CMS cytoplasms and fertile cytoplasms were to be analyzed separately. As far as is known this 'mixed model' has not been considered when patterns of mitochondrial gene diversity have been used to address the balanced polymorphism versus selective sweep issue.

A final question that might be raised concerns the potential impact of paternal leakage and heteroplasmy on sex determination in cytonuclear gynodioecious systems. Recall that two recent models have treated sex determination as a polygenic threshold trait (Ehlers *et al.*, 2005; Bailey and Delph, 2007b). This is motivated by the empirical observation that the genetics of restoration is often complex, involving multiple nuclear loci and therefore many possible multi-locus restorer genotypes. While two or more types of CMS might coexist in the same population it is assumed in these models that the cytoplasmic component of sex determination is invariant within individuals. This is because uni-parental (maternal) inheritance of the mitochondrial genome enforces homoplasmy. Recent studies of *S. vulgaris* have provided evidence
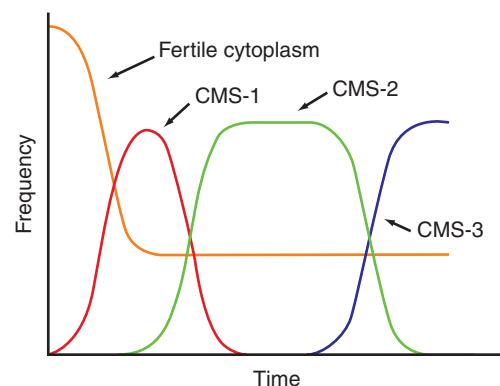

FIG. 4. Schematic of a mixed model in which an ancestral fertile type is maintained by balancing selection when in competition with three successive CMS types (1–3), which replace one another in a series of partial selective sweeps.

of occasional paternal leakage of mitochondrial genes (Welch *et al.*, 2006; Pearl *et al.*, 2009). It is now becoming clear that this paternal leakage is more likely to result in bi-parental inheritance and heteroplasmy than in uni-parental inheritance through pollen. Pearl *et al.* (2009) suggest that that the varying levels of heteroplasmy they documented were consistent with the 'quantitative' model of organelle transmission proposed by Birky (2001). Assuming that CMS genes reside in the mitochondrial genome, it would seem that paternal leakage could result in heteroplasmic mixes of different forms of CMS or CMS/fertile cytotypes. What effect might this have on sex expression? Consider a heteroplasmic mix of fertile and CMS cytotypes against a non-restoring nuclear background. What proportion of CMS is required to generate the female phenotype? Could a lower proportion of CMS generate the partial male phenotypes sometimes seen in gynodioecious systems? The same argument might also apply to a heteroplasmic mix of two CMS types against a nuclear background that only restores one of them.

This review has focused on the evolution and maintenance of gynodioecy in natural populations. As pointed out by Meagher (2007) the evolutionary–ecology approach focuses more on identifying the selective factors maintaining the gender variation than on the specific underlying molecular and developmental mechanisms responsible for floral phenotypes. Recent advances in our understanding of plant mitochondrial genomes (McCauley and Olson, 2008), plant developmental genetics (Meagher, 2007) and mechanisms of restoration (Delph *et al.*, 2007) have come primarily from studies of model organisms, not of those species amenable to the study of the ecology of gynodioecy in nature. Perhaps in this age of expanding genomic resources, made possible by faster and cheaper next generation genome sequencing technology (Rokas and Abbot, 2009), methods once restricted to so-called model organisms can be applied to those gynodioecious species for which there is already a rich history of ecological study.

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