Genetics of sex determination in the gynodioecious species *Lobelia siphilitica*: evidence from two populations

DANA A. DUDLE*, PIA MUTIKAINEN† & LYNDIA F. DELPH

Department of Biology, Indiana University, Bloomington, IN 47405, U.S.A.

In order to determine whether interactions between multiple sex-determining genes might be partly responsible for the wide variation in female frequency among populations of *Lobelia siphilitica*, we used progeny sex ratios from field-collected plants and from controlled crosses within and between two populations. We demonstrate that multiple cytoplasmic male-sterility types are present in a gynodioecious population where female frequency exceeds 50%. These male-sterility types each have corresponding nuclear alleles that can restore pollen fertility. Restoration of one male-sterility type appears to be controlled by a single, dominant allele, but restoration of a second cytoplasmic type is not easily explained with simple genetic models — perhaps multiple nuclear loci and/or epistatic interactions are involved. In addition, the crosses show that pollen from some hermaphrodite plants in a population containing no females restores male fertility to plants from a geographically distant gynodioecious population that have male-sterile cytoplasm. Furthermore, some plants in that hermaphrodite population carry a male-sterile cytoplasm. Taken together, these results fit theoretical predictions that female frequency might be highly variable among populations when sex is determined by interactions between several nuclear and cytoplasmic genetic factors, some of which may not be present in all populations. The data also illustrate the need for more theoretical and empirical work investigating the evolutionary impact of nuclear restorer genes with complex action, and explaining the existence of nuclear restorers and cytoplasmic male-sterility genes in a population where females are very rare.

Keywords: cytoplasmic male sterility, gender dimorphism, gynodioecy, *Lobelia siphilitica*, plant breeding systems, sex determination.

Introduction

Gynodioecious populations, in which female and hermaphrodite individuals coexist, often show wide variation in female frequency (Webb, 1999). For example, in populations of *Lobelia siphilitica*, the subject of this study, female frequency ranges from 0 to 69% (Dudle, 1999). Resource compensation (i.e. the reallocation of resources not used in male function to enhanced female fecundity or viability in male-sterile individuals) and avoidance of inbreeding depression form the basis for several theoretical models of the maintenance of females in populations where gender is determined by nuclear

genes (Lloyd, 1975; Charlesworth & Charlesworth, 1978; Charlesworth, 1989). These models may explain the sex ratio dynamics of some gynodioecious species, e.g. *Phacelia linearis* (Eckhart, 1992). However, Ross (1978) and Couvet *et al.* (1986) have suggested that much of the empirical data on female frequency, self-fertilization rate, inbreeding depression, and resource compensation in gynodioecious species do not fit these nuclear-inheritance models of gynodioecy. Some populations of gynodioecious species have higher frequencies of females than predicted by these models (Dommée *et al.*, 1978), and in other species, plants do not show enough inbreeding depression or resource compensation for females to exist at all under the models' assumptions (Krohne *et al.*, 1980). In addition, these models of nuclear gynodioecy do not explain the existence of self-incompatible gynodioecious species, such as *Plantago lanceolata* (Van Damme, 1983).

*Correspondence and present address: Department of Biology, DePauw University, Greencastle, IN 46135 U.S.A.

E-mail: ddudle@depauw.edu

†Present address: Section of Ecology, Department of Biology, University of Turku, FIN-20014 Turku, Finland.

Theory (e.g. Ross, 1978; Charlesworth, 1981; Couvet *et al.*, 1986; Frank, 1989) and experimental evidence from the agricultural and molecular literature (e.g. Hanson, 1991; Williams & Levings, 1992; Crouzillat *et al.*, 1994) suggest that persistence of females in gynodioecious populations may be explained in part by the mode of gender inheritance in these plants. In a majority of gynodioecious species, nuclear genes alone do not determine the sex of individuals (reviewed in Charlesworth & Laporte, 1998). Instead, gender is determined by the interaction of cytoplasmic male-sterility genes, which prevent successful pollen production, with nuclear genes, which can restore male function to individuals that have male-sterile cytoplasm. In this case, females have cytoplasmic male sterility (CMS), but not the correct complement of nuclear 'restorer' allele(s). Hermaphrodites may have a male-fertile cytoplasm, or a male-sterile cytoplasm interacting with a nuclear restorer allele. In addition, several male-sterile cytoplasmic genotypes, or cytotypes, could arise in a single species. Genetic studies of several species have shown that, if cytoplasmic male-sterility is coupled with a slight seed-fertility advantage of females relative to hermaphrodites, multiple male-sterile cytotypes can exist in one population, each with its specific set of nuclear restorer alleles (Van Damme, 1983; Belhassen *et al.*, 1991; Koelewijn & Van Damme, 1995a; Charlesworth & Laporte, 1998).

Several models of nuclear–cytoplasmic interactions in gynodioecious species have demonstrated that the high female frequencies observed in some natural populations may be explained by intragenomic interactions, in combination with a seed fertility advantage in females, without invoking self-fertilization and inbreeding depression as factors (Couvet *et al.*, 1986; Frank, 1989). Additional theoretical models (Gouyon *et al.*, 1991; Hurst, 1992; Frank, 1994), as well as empirical work on *Thymus vulgaris* populations with high female frequency (Belhassen *et al.*, 1991), have provided some evidence that the interactions between the cytoplasmic and nuclear genomes in individuals may drive the dynamics of sex ratio in some species, causing the frequency of females to cycle in a predictable way without regard to external environmental factors. However, other field studies indicate that environmental conditions such as water availability, level of disturbance, and pollinator abundance may also have a strong impact on the maintenance and frequency of females, as in *Hebe strictissima* (Delph, 1990), *Thymus vulgaris* (Couvet *et al.*, 1986), *Bidens* (Sun & Ganders, 1986), and *Lobelia siphilitica* (Dudle, 1999).

In a theoretical treatment, Frank (1989) emphasized the difficulty of determining the diversity of male-sterility types and nuclear restorer alleles in a single

population: the extent of the polymorphism in these traits may not be evident from conventional crossing designs. He demonstrated that if the frequency of male-sterility types follows frequency-dependent cycles, as suggested by several models (Charlesworth, 1981; Delannay *et al.*, 1981; Gouyon *et al.*, 1991), the study of one population with a particular frequency distribution of various cytoplasmic types will yield little useful information because some of the restorer alleles and/or cytoplasmic types may be rare at any one point in time. Studying several populations with varying frequencies of male-sterile individuals should allow more precise detection of polymorphism in the gender-determination system of gynodioecious species. This has been demonstrated in *Plantago lanceolata* (Van Damme, 1986), *Thymus vulgaris* (Belhassen *et al.*, 1991), *Plantago coronopus* (Koelewijn & Van Damme, 1995a,b) and *Silene vulgaris* (Charlesworth & Laporte, 1998). Inter-population crosses were used to discover several male-sterility cytotypes in each of these species. In addition to contributing important information about the number and distribution of sex determining genes, crosses between populations with different breeding systems might provide insight into the recent evolutionary history of gynodioecious species. Several recent studies have demonstrated the importance of metapopulation dynamics, and of rates of cytoplasmic and nuclear gene flow in gynodioecious species, for explaining the wide range of breeding systems among populations (Frank, 1989; McCauley & Taylor, 1997; Taylor *et al.*, 1999). Crosses between populations should yield evidence regarding the rates at which different cytoplasmic genotypes and nuclear restorer alleles spread and become established.

Here, we present results from a study of sex determination in the gynodioecious plant, *Lobelia siphilitica*, in two populations with different breeding systems. Rather than attempting to describe the precise genetic system of sex determination in this species in full detail, we present the results from several sets of controlled crosses within and between two populations with the goal of addressing three specific questions. (1) Do multiple cytoplasmic types and restorer genes exist in a gynodioecious population of *L. siphilitica* where female frequency is above 50% (Yellowwood State Forest in Brown County, IN)? (2) Do plants in a population containing no females (Hall Lake, Barry Co., MI) carry restorer genes that are effective in restoring male fertility to plants with male-sterile cytotypes in Yellowwood State Forest? (3) Do some plants in a population containing no females (Hall Lake) carry cytoplasmic male sterility that is masked by a high frequency of nuclear restorer genes in the population?

Materials and methods

Study species and populations

Lobelia siphilitica (Lobeliaceae) is an herbaceous, short-lived perennial that inhabits wet sites such as river banks, low woods, and lake edges in the eastern United States (Gleason & Cronquist, 1963; Johnston, 1991a). In natural populations in Indiana and Michigan, plants flower from late July to mid-October, and fruits mature between August and November. *Lobelia siphilitica* produces deep blue/purple flowers in terminal racemose inflorescences (Johnston, 1991b). Polliniferous flowers on hermaphrodites are highly protandrous. When the flower opens, the style is not receptive, but is enclosed by a deep purple anther-cylinder formed by the fusion of five filaments and anthers. The flower's stigmatic surfaces only become receptive after all pollen has been dispensed from the anther-cylinder. Self-pollination is therefore nearly impossible within hermaphrodite flowers, although geitonogamy can occur because several flowers may be open simultaneously on the same plant. *Lobelia siphilitica* is self-compatible, and inbreeding depression is common (Johnston, 1992; Mutikainen & Delph, 1998). Female plants may be distinguished from hermaphrodites by the white colour of the anther-cylinders in pistillate flowers, which do not contain pollen; floral morphology does not vary among male-sterile plants in the populations examined in this study (Dudle, 1999). Some hermaphrodites produce a small number of pistillate flowers under greenhouse conditions, usually at the onset of flowering; however, pistillate flowers have not been observed on hermaphrodites in natural populations (Dudle, 1999). Observations of female individuals over three years indicate that female plants never produce polliniferous flowers (Dudle, 1999).

The two populations used in this study were: Yellowwood State Forest in Brown County, Indiana (39°13'N, 86°21'W) and Hall Lake in Barry County, Michigan (42°38'N, 85°35'W). Plants in the Yellowwood population grow along the forest roads and along a creek bed in the forest interior. The female frequency along the creek bed in the Yellowwood population is 53% (Dudle, 1999) although some sites along the road near the forest contain up to 90% female individuals (Mutikainen & Delph, 1998). The plants at Hall Lake grow on the lake edge. In three surveys during the last three years, only a single female individual has been found in this population. Johnston (1991a) found female plants in one population in the same county in Michigan. These female individuals grew in a few small clumps, and female frequency in

the population was low overall (M. Johnston, personal communication).

Plant culture methods and pollination methods

Seeds from plants of known maternal sex were collected from the two populations during 1993 and 1995. In 1993, seeds were collected from 12 hermaphrodites and 10 females in six sites along the roadside in Yellowwood State Forest (see Mutikainen & Delph, 1998). Each site is separated from the next by 460–500 metres along the road. In 1995, seeds were collected from an additional 20 female and 20 hermaphrodite seed-parents along the creek bed in Yellowwood Forest, and from 30 hermaphrodite parents at Hall Lake. The seed-parents were separated by at least 5 metres. These field-collected seeds were cold-stratified at 5°C for 8–10 weeks on wet soil before being moved to the greenhouse for germination. Seeds produced from subsequent crosses were not cold-stratified. Instead, they were washed with a solution of distilled water, chlorine bleach, and ethanol (16:1:1) and four drops of Triton detergent, and rinsed twice. The seeds were then placed on sterile, moistened filter paper in Petri dishes and allowed to germinate in the greenhouse. Germination after both seed-preparation methods occurred within 12 days. At the rosette stage (6–8 leaves), seedlings were either transplanted into an equal mixture of soil and MetroMix (Scotts-Sierra Horticultural Products, Marysville, OH) in 3- or 4-inch plastic or clay pots and grown in the greenhouse under natural light, or transplanted outside into one of two garden plots at the Tenth Street Botanical Experimental Field at Indiana University. Upon flowering, the sex of each plant was determined by examining three or more flowers per plant. A plant was designated as a hermaphrodite if an anther-cylinder in at least one flower produced pollen. Female plants produced no pollen.

All crosses were completed by hand in a controlled greenhouse environment in July 1994, June 1995, and June 1997. Flowers on hermaphrodite seed-parents were emasculated by removing all pollen from the anther-cylinder, or by completely removing the anther-cylinder before the stigmatic lobes became receptive. Fresh pollen from the designated pollen-parents was dispensed from newly opened flowers onto a clean glass slide. The stigmas of two flowers on the designated seed-parents were saturated with the pollen. The glass slides were cleaned thoroughly with ethanol between pollinations. Pollinators were excluded from the plants with bridal veiling, and the stigmas of pollinated flowers lost receptivity within one day of pollination. Mature seeds were collected in paper envelopes and stored for several months before planting.

Notation

Plants in the 22 maternal lineages collected from Yellowwood State Forest in 1993 were identified by site within the population, the sex of the plant from which the seeds were collected (M refers to hermaphrodites, F refers to females), and that seed-parent's identifying letter. For example, plant 1MA2 is the second individual from the open-pollinated progeny of hermaphrodite A from site 1. Families from Yellowwood and Hall Lake in 1995 are identified by the population (YW or HL), the sex of the plant from which the original seeds were collected (M or F), and the seed-parent's number. For example, HLM34/365 is an open-pollinated progeny from hermaphrodite 34 from Hall Lake. Individual progeny from all families were assigned identifying numbers that follow the population and family designations.

Crossing designs and predictions

Preliminary crosses In 1994, Mutikainen & Delph (1998) used controlled crosses for their investigation of inbreeding depression in female and hermaphrodite lineages of *L. siphilitica*. These maternal lineages were derived from the 10 female and 12 hermaphrodite seed parents in the Yellowwood State Forest population in 1993. The crosses included self-fertilizations of hermaphrodites from female and hermaphrodite lineages, and outcrossed fertilizations of females and hermaphrodites in which the pollen donor and seed-parent originated from lineages in the same subpopulation in Yellowwood Forest. The sex ratios of the progeny from this set of crosses were used to form hypotheses about the inheritance of gender in the Yellowwood population, and to plan subsequent crosses. For example, if a hermaphrodite gave rise to any female progeny after selfing, one hypothesis might be that the hermaphrodite seed-parent carried cytoplasmic male sterility (CMS) and was heterozygous for a dominant nuclear restorer gene. Similarly, if a female seed-parent gave rise to all-female progeny when crossed with three different pollen-parents, one might suspect that the female carried a type of CMS for which restorer genes were relatively rare or absent.

In 1995, five females from different lineages from Mutikainen & Delph's (1998) study were pollinated by one potentially restored hermaphrodite (a hermaphrodite with female siblings). If two females produce different proportions of male-sterile offspring when pollinated by the same pollen-parent, this is evidence that those two females have different genotypes for sex-determining factors. The final set of preliminary crosses included a subset of six hermaphrodite progeny, pro-

duced by selfing, that were from lineages containing females (i.e. the hermaphrodites either had female seed-parents or female siblings). These crosses were intended to shed some light on the inheritance of the restorer alleles. For example, if restoration of pollen production depends on the action of one dominant allele, then hermaphrodite progeny of females, when selfed, should produce about 25% female offspring because they are heterozygous for the dominant restorer.

Question 1: Multiple cytoplasmic types and restorer alleles in the Yellowwood population To determine whether there is more than one cytoplasmic type in Yellowwood State Forest population, reciprocal crosses were made between two hermaphrodites (1MA2 and 6FA16) with female siblings from the Yellowwood population. In addition, several seed-parents were crossed with one set of pollen donors. Seven hermaphrodite plants were pollinated by the same six hermaphrodites from Yellowwood and two hermaphrodites from Hall Lake.

The six pollen donors from Yellowwood Forest were chosen because the preliminary crosses showed that these individuals had female siblings and/or female seed-parents. These donors were suspected to carry at least one effective restorer gene. The remaining two pollen donors, from Hall Lake, were chosen without knowledge of their ability to restore pollen production to plants with male-sterile cytoplasm. However, it seemed likely that Hall Lake hermaphrodites might differ from Yellowwood hermaphrodites in their complement of nuclear restorer alleles, as has been suggested by theory on gynodioecy in separate populations (Frank, 1989). Several of the crosses in this design failed to produce progeny that could be scored for sex because of a high rate of seedling death (see Table 5 for parents' identities and successful crosses).

Monte Carlo $2 \times N$ contingency table simulations (Engels, 1988) were used to test whether, given one parent in the set, the proportions of female progeny are independent of the other parent. The Monte Carlo procedure estimates the probability of a set of sex ratios as extreme as the set being examined, if the data were independent of the second parent. For example, consider a single pollen-parent for which the proportion of female offspring produced is independent of seed-parent; i.e. the probability of randomly getting more extreme differences among the sex ratios in crosses for the pollen-parent and several seed-parents is high. This would imply that all of the seed-parents in this crossing design had the same ability to produce females, given the nuclear genes of the pollen-parent. In other words, all seed-parents are equally well-restored by a given pollen donor. Conversely, if the sex ratio of a single

seed-parent's progeny is contingent on the identity of the pollen-parent in a set, then the set of pollen-parents must be heterogeneous in terms of the nuclear restorers they carry. The results from using this procedure indicate whether there is heterogeneity of cytoplasmic types and restorer genotypes among the small set of individuals used in this design. For each test, the Monte Carlo procedure was run 10 000 times so that the estimated *P*-value had a standard error lower than 0.002.

Question 2: Restorer genes in the Hall Lake population

To determine whether plants at Hall Lake carry nuclear restorer genes that can restore pollen production to plants with male-sterile cytoplasm in the Yellowwood population, four females from Yellowwood were chosen as seed-parents. Each seed-parent was crossed with the same eight pollen-parents from the Hall Lake population. The pollen donors were offspring of plants widely separated on the perimeter of Hall Lake. If any of the female seed-parents from Yellowwood produce hermaphrodite offspring from crosses with Hall Lake pollen donors, it will be strong evidence for the existence of nuclear restorer alleles in the Hall Lake population that match at least one CMS type in Yellowwood Forest.

Question 3: Cytoplasmic male-sterility in the Hall Lake population

Some Hall Lake individuals may carry CMS, but it might be masked if the correct nuclear restorer alleles are fixed or nearly fixed in the population. Most theory does not predict that the same restorers should be fixed in Yellowwood Forest (e.g. Frank, 1989). Therefore, pollen from Yellowwood might expose latent male sterility in the Hall Lake population. To begin the investigation of whether some Hall Lake plants carry cytoplasmic male-sterility factors that are masked by a high frequency of nuclear restorer genes in the population, 10 hermaphrodites from Hall Lake were crossed with pollen from the same five hermaphrodites from Yellowwood.

Results

Open-pollinated progeny and preliminary crosses

Progeny resulting from natural pollinations tended to be of the same sex as their seed-parent in both populations (see Table 1). Among 34 hermaphrodites from Yellowwood Forest, only seven produced any female offspring. Thirteen out of 30 females from Yellowwood Forest produced some hermaphrodite offspring, but among these, the progeny sex ratios were predominantly female. These data strongly suggest a cytoplasmic role in the inheritance of male sterility that is complicated by nuclear factors. Among Hall Lake hermaphrodites, two of 30 plants produced female offspring after natural pollination. No families from Hall Lake seed-parents produced more than three female offspring. These data suggest that male sterility does exist at Hall Lake, although it is rare. It is not clear from these data that male sterility is cytoplasmically inherited in the Hall Lake population.

Sex ratio data from the crosses used by Mutikainen & Delph (1998) are presented in Table 2. Among hermaphrodite plants, selfing produced some female offspring in 11 of 20 families, including six out of the seven plants with female seed-parents. Few hermaphrodites produced females after pollination by a plant from the same subpopulation (five of 20) or from a different subpopulation (five of 20, Table 2). In general, female frequencies were low in these preliminary crosses; in fact, several hermaphrodites (2FA, 2MB, 4MB, 4 MD, and 5MB) produced no females in any of the four types of crosses.

Female seed-parents crossed with pollen from siblings or unrelated plants produced female progeny at relatively higher frequencies than hermaphrodite seed-parents (Table 2). With one exception, all crosses involving female seed-parents resulted in at least one female offspring, including seven crosses that produced 100% female offspring. Although the number of

Table 1 Sex phenotypes in the progeny of open-pollinated *Lobelia siphilitica* plants from two natural populations

Population and year	Seed parent's sex	Number of maternal plants			Mean proportion of females in segregating families
		Segregating	Nonsegregating females	Nonsegregating hermaphrodites	
Yellowwood 1993	Hermaphrodite	4	0	10	0.172
	Female	7	3	0	0.756
Yellowwood 1995	Hermaphrodite	3	0	17	0.255
	Female	6	14	0	0.707
Hall Lake 1995	Hermaphrodite	2	0	28	0.107

Table 2 Results from controlled self-pollinations and outcrossed matings between *Lobelia siphilitica* plants from female and hermaphrodite lineages in Yellowwood State Forest. Dashes indicate unsuccessful crosses or insufficient data for analysis

Lineage of seed parent	Sex of grandmother	Cross type				
		Selfed		Outcrossed		
		<i>N</i>	Prop. female	Pollen lineage	<i>N</i>	Prop. female
Hermaphrodite seed parents						
1FB	Female	7	0.71	1MA	13	0.08
2FA	Female	9	0.00	2MB	20	0.00
2FB	Female	6	0.50	2MB	18	0.06
3FB	Female	10	0.10	3MB	9	0.00
5FA	Female	8	0.75	5FB	19	0.68
6FA	Female	8	0.13	6MB	10	0.00
6FB	Female	9	0.11	6MB	18	0.00
1MA	Hermaphrodite	11	0.00	1MB	19	0.00
1MB	Hermaphrodite	6	0.17	—	—	—
2MA	Hermaphrodite	3	0.00	2MB	15	0.00
2MB	Hermaphrodite	13	0.00	2MA	10	0.00
3MA	Hermaphrodite	11	0.09	3MB	15	0.00
3MB	Hermaphrodite	8	0.13	3MA	9	0.00
4MA	Hermaphrodite	6	0.00	4MB	13	0.18
4MB	Hermaphrodite	8	0.00	4MA	11	0.00
4MC	Hermaphrodite	8	0.25	4MB	21	0.00
4MD	Hermaphrodite	8	0.00	4MB	17	0.00
5MA	Hermaphrodite	7	0.00	5MB	16	0.06
5MB	Hermaphrodite	15	0.00	5MA	17	0.00
6MB	Hermaphrodite	13	0.63	5MA	17	0.00
Female seed parents						
1FA	Female		N/A	1MB	9	0.89
1FB	Female		N/A	1MA	14	0.29
2FA	Female		N/A	2MB	16	0.56
2FB	Female		N/A	2MB	11	0.64
3FB	Female		N/A	3MA	10	0.80
5FA	Female		N/A	5MB	13	0.46
5FB	Female		N/A	5MA	10	1.00
6FA	Female		N/A	6MA	12	0.00
6FB	Female		N/A	6MB	8	1.00

offspring from each cross is small, these data suggest some potential differences among cytoplasmic genotypes of the lineages. If a cross yields 100% female progeny, a simple explanation might be that the pollen-parent does not carry nuclear alleles that restore pollen function to the seed-parent's cytoplasm. Because the pollen-parent is clearly capable of producing pollen, it follows from this result that the pollen-parent must not carry the same male-sterility factor as that of the seed-parent in its own cytoplasm. As one example of this logic, the crosses in Table 2 suggest a difference between the cytoplasm of the 5FB lineage and the cytoplasm of both the 5MA and 3MA lineages. However, this method of discerning different cytoplasmic types is not infallible, especially when sample sizes are small. This is illustrated by the 100% female progeny produced by a female from the

6FB lineage with pollen from a hermaphrodite maternal sibling. No restoration is visible among the six progeny that were scored for this cross, although the pollen-parent should carry the same cytoplasm as the seed-parent, and presumably carries nuclear genes that can restore that male-sterile cytoplasm.

More conclusive evidence of different cytoplasmic types is provided by the results from the crosses between one pollen-parent and several female seed-parents (Table 3). The sex ratio of the progeny produced through pollen from 1MA2 crossed with different seed-parents was contingent on the identity of the seed-parent, as demonstrated by a significant Monte Carlo contingency test. For three seed-parents (1FB12, 2FA14, and 3FB19), pollination by 1MA2 produced hermaphrodite progeny at frequencies between 48.2%

Table 3 Progeny sex ratios (females/total) resulting from fertilization of five females of *Lobelia siphilitica* with pollen from one hermaphrodite (1MA2). The pollen donor had female siblings. Monte Carlo simulations were run to test for the independence of the sex ratio regarding the seed parent; the test statistic and estimated *P*-value are reported here

Seed parent	<i>N</i>	Prop. female
1FB12	52	0.404
2FA14	12	0.333
3FB19	36	0.528
5FA18	70	1.000
6FA19	32	0.938
Observed <i>T</i>		44.10
Estimated <i>P</i>		<0.001

and 66.7%. For two other seed-parents (5FA18, and 6FA19), pollination by 1MA2 rarely or never produced hermaphrodite progeny. One explanation for this result is that 1MA2 bears a restorer or restorers for the CMS factor(s) carried by the first three females, but not for the CMS factor carried by the last two females.

Self-fertilizations of hermaphrodites whose seed-parents or siblings were female yielded somewhat unexpected results. The six selfed hermaphrodites fall into two distinct groups. Three of the plants (1FB22, 2FA26, 3FB29) produced females at frequencies between 19% and 25% (Table 4). These sex ratios are statistically indistinguishable from each other (Monte Carlo contingency test, estimated $P = 0.485$) and from a sex ratio of 25% females (Fisher's exact test, $P = 0.215$). These data are consistent with a genetic model including a single-locus, dominant restorer gene: because these three hermaphrodites had female seed-parents, they should be heterozygous for a dominant restorer, and thus selfing should produce 25% female offspring that do not carry the dominant restorer allele. However, data from self-crosses of hermaphrodites from the same lineages performed by Mutikainen & Delph (1998) do not match

Table 4 Progeny sex ratios (females/total) of *Lobelia siphilitica* resulting from self-fertilization of six hermaphrodites with female seed parents or female siblings

Selfed hermaphrodite	<i>N</i>	Prop. female
1FB22	63	0.190
2FA26	18	0.222
3FB29	20	0.250
1MA2	47	0.021
6FA16	45	0.067
6MB14	13	0.000

this interpretation; comparisons show that the two sets of self-fertilizations yielded significantly different sex ratios (Monte Carlo contingency test, estimated $P = 0.030$, compare 'Self-crosses' column in Table 2 with Table 4). Although there is some evidence supporting the hypothesis that restoration of the pollen fertility for the CMS type carried by the 1FB, 2FA, and 3FB lineages is controlled by a simple dominant gene, some uncertainty persists, indicating the need to investigate other hypotheses for the control of gender inheritance in these plants.

The other three selfed hermaphrodites from the 1995 crosses (1MA2, 6FA16, and 6MB14) produced much lower frequencies of females than the hermaphrodites discussed above (Table 4). The sex ratios from these three plants are statistically indistinguishable from each other (Monte Carlo contingency test, estimated $P = 0.363$), and from a sex ratio of 0% females (Fisher's exact test, $P = 0.061$). Superficially, this result would be consistent with the hypothesis that these pollen-parents are homozygous for a single dominant restorer. However, the results from these crosses do not closely correspond to the results from self-crosses within plants from the same lineages (Monte Carlo contingency test, estimated $P < 0.001$ compare 'Self-crosses' column of Table 2 with Table 4). Furthermore, hermaphrodites in each of these three lineages produced some females as a result of selfing, contrary to predictions for selfed progeny if the hermaphrodites were indeed homozygous for a single dominant restorer gene. Together, these inconsistencies indicate that a more complex explanation is required for the progeny sex ratios of selfed hermaphrodites.

Cytoplasmic male-sterility types and nuclear restorers in Yellowwood Forest

Diversity of cytoplasmic male-sterility types The reciprocal crosses between 1MA2 and 6FA16 yielded significantly different proportions of females ($\chi^2_1 = 5.86$, $P = 0.02$). When 1MA2 was used as the seed parent, the proportion of female individuals in the progeny was 0.04 (1/24), and when 6FA16 was used as the seed parent, the proportion of females in the progeny was 0.39 (11/28). The reciprocal difference in progeny sex ratios between these crosses is evidence that these two plants have different cytoplasmic sex-determining genotypes. In addition, these two plants have different mitochondrial genotypes, as determined by Southern blot hybridizations using the cob gene cut with multiple restriction enzymes (M. Bailey and L. Delph, unpublished data).

The evidence from the crosses between female seed-parents and pollen from 1MA2, and from the reciprocal

crosses between 1MA2 and 6FA16 that there are multiple cytoplasmic types in Yellowwood Forest is further supported by data from crosses between seven female seed-parents and eight pollen-parents (Table 5). Highly significant Monte Carlo contingency tests for each of the pollen-parents show that sex ratios of the progeny were highly dependent on the seed-parent (see last column of Table 5). More specifically, four of the seven seed-parents (5FB27, 6FB30, YWM16, and YWF21) did not produce more than 13% hermaphrodites in any crosses (Table 5). When pooled across pollen-parents, the progeny sex ratios of 5FB27, 6FB30, and YWM16 are statistically indistinguishable (Monte Carlo contingency test, estimated $P=0.081$). YWF21 was not included in this analysis because of the comparatively small number of successful crosses involving this seed-parent. Two of the seed-parents (1FB2 and 3FB9) produced between 32.3% and 71.4% hermaphrodites in every cross (Table 5). When pooled across pollen-parents, the sex ratios of these two seed-parents are statistically indistinguishable from each other (Fisher's Exact test, $P=0.342$). This is strong evidence that the Yellowwood Forest population contains plants with at least two different male-sterile cytoplasmic types, one of which can be restored by pollen from each of the pollen-parents included in this design, and one which can not be restored by pollen from the same pollen-parents.

The progeny sex ratios of the six seed-parents listed above (1FB2, 3FB9, 5FB27, 6FB30, YWM16, YWF21) were not contingent on the identity of the pollen-parent; that is, crosses between one of these seed-parents and any of the set of eight pollen-parents yielded progeny sex ratios that are statistically indistinguishable by the Monte Carlo procedure (see last row of Table 5). In contrast, the progeny sex ratio was dependent on the identity of the pollen-parent for the seventh female in this design (YWF8). The sex ratios of the seed progeny of plant YWF8 ranged from 21.6% female to 100% female (Table 5). These results suggest that YWF8 differs in its sex-determining genotype from the other six seed-parents. A possible explanation that would fit the data is that YWF8 has a third male-sterile cytotype, one for which a subset of the pollen-parents in this design carries the corresponding restorer(s). Further crosses between YWF8, her progeny, and an expanded set of pollen-parents might help distinguish the proposed explanation from more complex explanations.

Action and diversity of restorer alleles The data from seed-parents 1FB2 and 3FB9 are consistent with a genetic model including a CMS type that corresponds to single dominant restorer allele, for which all eight pollen-parents used in the crosses are heterozygous.

Table 5 Results of controlled crosses between seven female seed parents of *Lobelia siphilitica* from Yellowwood State forest and eight different pollen parents. Number of progeny and proportion of progeny that were female are given, as well as the results of Monte Carlo contingency tests of independence for the progeny sex ratios for each seed parent and pollen parent. Dashes indicate unsuccessful crosses or insufficient data for analysis

Pollen parent	Seed parent															
	1FB2		3FB9		5FB27		6FB30		YWM16		YWF21		YWF8			
	N	Prop. ♀	N	Prop. ♀	N	Prop. ♀	N	Prop. ♀	N	Prop. ♀	N	Prop. ♀	N	Prop. ♀		
1MA2	54	0.481	59	0.475	49	0.959	58	1.000	—	—	—	—	73	0.959	59.29	<0.001
1MB22	54	0.574	19	0.421	8	1.000	34	1.000	44	1.000	58	0.879	65	0.492	51.07	<0.001
3MA1	21	0.286	23	0.652	—	—	43	1.000	—	—	—	—	62	0.613	38.61	<0.001
6FA16	62	0.677	46	0.391	42	1.000	—	—	45	1.000	—	—	66	0.970	53.23	<0.001
YWF6/331	64	0.547	44	0.523	—	—	—	—	11	1.000	—	—	46	0.543	41.58	<0.001
YWF10/202	—	—	61	0.344	30	1.000	—	—	26	1.000	—	—	74	0.216	54.41	<0.001
HLM15/317	25	0.480	3	0.667	26	1.000	34	1.000	18	1.000	10	1.000	12	1.000	24.96	<0.001
HLM47/304	52	0.519	60	0.567	2	1.000	31	1.000	62	1.000	3	1.000	33	1.000	59.89	<0.001
T-value	5.934		5.562		2.357		0		0		1.499		94.2			
Estimated P	0.067		0.090		0.353		1		1		0.393		< 0.001			

This model predicts a 50 : 50 sex ratio of progeny for crosses between a heterozygous pollen-parent and a female with the correct CMS type, and the data in Table 5 for 1FB2 and 3FB9 are statistically indistinguishable from a 50:50 sex ratio (Fisher's exact test, $P=0.824$). It is not surprising that the pollen-parents from the Yellowwood lineages in the crossing design include many heterozygotes: six of the eight pollen-parents had female seed-parents or female siblings, so they were likely to have only one copy of the restorer(s), at least for their own cytotypes. Subsequent crosses should determine whether the cytoplasm of the six pollen-parents from Yellowwood carry the same cytoplasm as 1FB2 and 3FB9. It is intriguing that the two pollen donors from Hall Lake appear to fit the model as heterozygous carriers of a nuclear restorer; this result should be confirmed with future crosses.

The results for the restoration of the second CMS type, carried by the hermaphrodite plants designated as 5FB27, 6FB30, and YWM16 (and perhaps YWM21), are more difficult to interpret. The pollen-parents used in this design appear unable to restore pollen fertility to progeny carrying this cytoplasm (progeny sex ratios are indistinguishable from 100% female, Fisher's exact test, $P=0.441$, Table 5). In the preliminary crosses, a female with the 5FB cytoplasm did not produce any hermaphrodites in two crosses with outcrossed pollen (see Table 2), which is consistent with the pattern shown in Table 5. In contrast, the preliminary crosses suggest that plants with 6FB cytoplasm can be restored, because the naturally pollinated plant gave rise to a hermaphrodite that produced many hermaphrodite seed progeny in all four types of crosses performed by Mutikainen & Delph (1998) (see Table 2). It is somewhat perplexing that the female plant from the 6FB lineage (6FB30) did not produce any hermaphrodite progeny in eight crosses (see Table 5), but these data are not blatantly inconsistent with the preliminary crosses. It is possible that more than one gene is responsible for the restoration of pollen fertility for this cytoplasmic type, as has been suggested for some CMS types in *Plantago lanceolata* (Van Damme, 1983), *Thymus vulgaris* (Belhassen *et al.*, 1991 in Charlesworth & Laporte, 1998), and *Silene vulgaris* (Charlesworth & Laporte, 1998). However, it is impossible to understand fully the action of the restorer(s) of this cytoplasmic type, given the limited information provided by the crosses described herein.

The most puzzling result from the seed-parents with the second CMS type is not the large number of females produced in the crosses, but the few hermaphrodites that emerge from some crosses. To our knowledge, no model of sex determination in gynodioecious species predicts 3%–4% hermaphrodite progeny. Despite the statistical assurances that these crosses give sex ratios

indistinguishable from 100% female, the presence of these few hermaphrodites may be biologically important. Perhaps they indicate spontaneous reversion to male-fertility in plants with cytoplasmic male sterility, rare biparental inheritance of mitochondria, environmentally labile sex expression, or epistatic gene action. Both labile sex expression and epistatic interactions between sex-determining genes have been implicated in explanations of progeny sex ratios in families of the gynodioecious species *Plantago coronopus* (Koelewijn & Van Damme, 1995b), *Silene vulgaris* and *Thymus vulgaris* (Charlesworth & Laporte, 1998). Further studies should investigate the nature of these rare hermaphrodites produced in primarily female families of *L. siphilitica*.

Restorer genes in the Hall Lake population

Most crosses between female seed-parents from Yellowwood Forest and pollen-parents from Hall Lake yielded only female progeny (Table 6). However, two of the four seed-parents produced some hermaphrodite progeny when crossed with some Hall Lake pollen-parents. These 'restorable' females did not produce hermaphrodites in combination with the same pollen-parents (Table 6). However, pollen from plant HLM15/317 produced hermaphrodites in combination with two Yellowwood females (1FB2 and 3FB9) in the investigation of cytoplasmic diversity at Yellowwood Forest (Table 5) as well as with YWF2/346 in this crossing design (Table 6). These data indicate that pollen from some Hall Lake lineages can restore male fertility for some females with cytoplasm derived from the Yellowwood Forest population, although the data do not suggest a simple genetic explanation for the widely different sex ratios in the progeny.

Cytoplasmic male sterility in the Hall Lake population

Crosses between 10 hermaphrodite seed-parents from Hall Lake and five pollen-parents from Yellowwood Forest yielded almost entirely hermaphrodite progeny (data not shown). Two of the 50 crosses produced female progeny: HLM36/340 $\times$ YWM7/213 produced eight females (female frequency = 0.347), and HLM36/340 $\times$ YWM20/206 produced eight females (female frequency = 0.174). This suggests that plant HLM36/340 may have a male-sterile cytoplasm for which the restorer(s) are not fixed in the Yellowwood population. The extent of CMS in the Hall Lake population can not be determined without evidence from back-crosses between the progeny produced here and their pollen-parents and/or from selfing of these progeny. If many of

Table 6 Results of controlled crosses between *Lobelia siphilitica* plants from two populations. Seed parents are female plants whose maternal parents are from Yellowwood State Forest in Indiana. Pollen donors are hermaphrodites whose maternal parents are from Hall Lake, in Michigan. Dashes indicate unsuccessful crosses or insufficient data for analysis

Pollen parent	Seed parent							
	YWF2/346		YWM5/115		YWF18/143		YWF22/431	
	<i>N</i>	Prop. female	<i>N</i>	Prop. female	<i>N</i>	Prop. female	<i>N</i>	Prop. female
HLM5/440	—	—	42	1.000	11	1.000	66	1.000
HLM6/232	56	0.982	45	1.000	60	1.000	43	1.000
HLM9/321	41	0.927	43	1.000	47	1.000	50	1.000
HLM11/442	58	0.259	61	1.000	50	1.000	68	1.000
HLM15/317	41	0.220	30	1.000	22	1.000	46	1.000
HLM28/337	55	1.000	53	1.000	52	1.000	45	1.000
HLM32/328	54	1.000	63	0.238	—	—	14	1.000
HLM34/365	55	1.000	10	0.800	48	1.000	56	1.000
Estimated <i>P</i>	< 0.001		< 0.001		1.00		1.00	

these crosses generate male-sterile progeny, it will be evidence that CMS is common at Hall Lake, but masked by a high frequency of restorer alleles. Alternatively, if few or no male-sterile progeny are produced through back-crossing and selfing, it is likely that plants with male-sterile cytoplasm are rare at Hall Lake.

Discussion

Overall, these data can be used to address the three questions posed at the beginning of this investigation. There are several lines of evidence supporting the existence of multiple cytoplasmic types in Yellowwood Forest. At least two CMS types are present; in addition, the crosses described here suggest a potential third male-sterility cytotype and a male-fertile cytotype, as shown by the lineages that did not produce females in any crosses (Table 2). In the Hall Lake population, genes appear to be present at low frequency that can restore male fertility to some plants from Yellowwood Forest with cytoplasmic male sterility. Finally, the naturally pollinated progeny and controlled crosses demonstrate that some hermaphrodites from Hall Lake are capable of producing male-sterile progeny through seeds, although it is not clear that the genetic basis of male sterility at Hall Lake is the same as at Yellowwood Forest.

That multiple CMS types exist within Yellowwood Forest is not surprising. Cytoplasmic male-sterility arises relatively often in plants, usually as a result of a rearrangement of the structurally labile mitochondrial genome that causes a chimeric protein to be transcribed that interferes with pollen production (Hanson, 1991; Williams & Levings, 1992). Because it is maternally inherited, CMS will persist in a population if male-sterile individuals have higher seed fitness than male-fertile individuals, and if nuclear restorer alleles do not

spread to fixation, masking the effects of the CMS (Charlesworth, 1981; Delannay *et al.*, 1981). Theory suggests that multiple CMS types, once they arise, may coexist with their corresponding nuclear restorers under a wide set of parameters, and that sex ratios among populations may vary widely due in large part to interactions between the sex-determining genes (Frank, 1989; Gouyon *et al.*, 1991). Investigations of sex determination in other gynodioecious species indicate that multiple CMS factors often occur within and among populations (Van Damme, 1983; Belhassen *et al.*, 1991; Koelewijn & Van Damme, 1995a,b; de Haan *et al.*, 1997; Charlesworth & Laporte, 1998), as shown here for *L. siphilitica*. It is probable that the complex mode of sex determination in *L. siphilitica* is responsible in large part for the persistence of females at high frequencies in many populations such as Yellowwood Forest. This is especially likely because the action of the nuclear restorer(s) for at least one of the cytoplasmic types is not simply dominant. The difficulties associated with selection on a recessive restorer or on a set of epistatic restorers suggest that female frequencies might be high in populations where restoration is not controlled by simple dominance (Charlesworth & Laporte, 1998). Further crosses that examine the action of the restorer alleles for each of the CMS types, the costs of carrying the restorers, and the relative frequency of the different CMS types should clarify which aspects of the genetic system of sex determination have the greatest role in the persistence of females in gynodioecious populations of *L. siphilitica*.

The interpopulation crossing design employed in this investigation yielded information that will be important in understanding the evolution of male sterility across populations of *L. siphilitica*. Despite the extreme rarity of female individuals in the Hall Lake population over three seasons (less than 1% female in the natural

population), genetic evidence demonstrates that nuclear restorer alleles and male sterility are present at Hall Lake. The results from the crosses also suggest that none of the restorer(s) corresponding to the CMS types found in the Yellowwood population are fixed at Hall Lake. This conflicts with the hypothesis that the same CMS types carried by Yellowwood plants are common among Hall Lake plants but masked by widespread nuclear restorers. One explanation for the relatively rare (but not absent) restorer alleles and male-sterile individuals includes recent migration of male-sterility to the Hall Lake population from southern populations. More interpopulation crosses among the two populations used here as well as geographically intermediate populations will shed more light on this possibility. Alternatively, an as-yet-undetected set of sex-determining factors unique to the Hall Lake population may be responsible for low frequencies of male-sterile individuals. A generation of backcrosses between pollen-parents from Yellowwood Forest and F₁ seed-parents with Hall Lake cytoplasm should reveal any cytoplasmic male sterility that may still be masked among Hall Lake plants, addressing this possibility. Finally, habitat differences may exist between the populations that allow females to persist at high frequencies in Yellowwood but not Hall Lake, for reasons that are not dependent on the genetics of sex determination. Although the complexity of the genetic system of sex determination probably explains much of the variation in sex determination among populations of *L. siphilitica*, it is also possible that ecological factors contribute to the persistence of females at high frequencies in some populations.

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References

- BELHASSEN, E., DOMMÉE, B., ATLAN, A., GOUYON, P. *ET AL.* 1991. Complex determination of male sterility in *Thymus vulgaris* L. Genetic and molecular analysis. *Theor. Appl. Genet.*, **82**, 137–143.
- CHARLESWORTH, D. 1981. A further study of the problem of maintenance of females in gynodioecious species. *Heredity*, **46**, 27–39.
- CHARLESWORTH, D. 1989. Allocation to male and female function in hermaphrodites, in sexually polymorphic populations. *J. Theor. Biol.*, **139**, 327–342.
- CHARLESWORTH, D. AND CHARLESWORTH, B. 1978. A model for the evolution of dioecy and gynodioecy. *Am. Nat.*, **112**, 975–997.
- CHARLESWORTH, D. AND LAPORTE, V. 1998. The male-sterility polymorphism of *Silene vulgaris*: analysis of genetic data from two populations and comparison with *Thymus vulgaris*. *Genetics*, **150**, 1267–1282.
- COUVET, D., BONNEMAISON, F. AND GOUYON, P. 1986. The maintenance of females among hermaphrodites: the importance of nuclear–cytoplasmic interactions. *Heredity*, **57**, 325–330.
- CROUZILLAT, D., DE LA CANAL, L., VEAR, F., SERIEYS, H. *ET AL.* 1994. Mitochondrial DNA RFLP and genetical studies of cytoplasmic male sterility in the sunflower (*Helianthus annuus*). *Curr. Genet.*, **26**, 146–152.
- DE HAAN, A. A., LUYTEN, R. M. J. M., BAKX-SCHOTMAN, T. J. M. T. *ET AL.* 1997. The dynamics of gynodioecy in *Plantago lanceolata* L. I. Frequencies of male-steriles and their cytoplasmic male-sterility types. *Heredity*, **79**, 453–462.
- DELANNAY, X., GOUYON, P. AND VALDEYRON, G. 1981. Mathematical study of the evolution of gynodioecy with cytoplasmic inheritance under the effect of a nuclear restorer gene. *Genetics*, **99**, 169–181.
- DELPH, L. F. 1990. Sex ratio variation in the gynodioecious shrub *Hebe strictissima* (Scrophulariaceae). *Evolution*, **44**, 134–142.
- DOMMÉE, B., ASSOUAD, M. W. AND VALDEYRON, G. 1978. Natural selection and gynodioecy in *Thymus vulgaris*. *Bot. J. Linn. Soc.*, **77**, 17–28.
- DUDLE, D. A. 1999. *Maintenance and Consequences of Females in the Gynodioecious Plant, Lobelia siphilitica*. PhD Thesis, Indiana University.
- ECKHART, V. M. 1992. Resource compensation and the evolution of gynodioecy in *Phacelia linearis* (Hydrophyllaceae). *Evolution*, **45**, 1313–1328.
- ENGELS, B. 1988. Statistical tests for categorical data, version 1.01. University of Wisconsin, Madison, WI.
- FRANK, S. A. 1989. The evolutionary dynamics of cytoplasmic male sterility. *Am. Nat.*, **133**, 345–376.
- FRANK, S. A. 1994. Recognition and polymorphism in host–parasite genetics. *Phil. Trans. Soc. Lond. B.*, **346**, 283–293.
- GLEASON, H. A. AND CRONQUIST, A. 1963. *The New Britton and Brown Illustrated Flora of the Northeastern United States and Adjacent Canada*. Hafner, New York.
- GOUYON, P., VICHOT, F. AND VAN DAMME, J. M. M. 1991. Nuclear–cytoplasmic male sterility: single point equilibria versus limit cycles. *Am. Nat.*, **137**, 498–514.
- HANSON, M. R. 1991. Plant mitochondrial mutations and male sterility. *Ann. Rev. Genet.*, **25**, 461–486.
- HURST, L. D. 1992. Intragenomic conflict as an evolutionary force. *Proc. R. Soc. B.*, **248**, 135–140.
- JOHNSTON, M. O. 1991a. Natural selection on floral traits in two species of *Lobelia* with different pollinators. *Evolution*, **45**, 1468–1479.

- JOHNSTON, M. O. 1991b. Pollen limitation of female reproduction in *Lobelia cardinalis* and *L. siphilitica*. *Ecology*, **72**, 1500–1503.
- JOHNSTON, M. O. 1992. Effects of cross and self-fertilization on progeny fitness in *Lobelia cardinalis* and *L. siphilitica*. *Evolution*, **46**, 688–702.
- KOELEWIJN, H. P. AND VAN DAMME, J. M. M. 1995a. Genetics of male sterility in gynodioecious *Plantago coronopus*. I. Cytoplasmic variation. *Genetics*, **139**, 1749–1758.
- KOELEWIJN, H. P. AND VAN DAMME, J. M. M. 1995b. Genetics of male sterility in gynodioecious *Plantago coronopus*. II. Nuclear genetic variation. *Genetics*, **139**, 1759–1775.
- KROHNE, D. T., BAKER, I. AND BAKER, H. G. 1980. The maintenance of the gynodioecious breeding system in *Plantago lanceolata*. *Am. Midl. Nat.*, **103**, 269–279.
- LLOYD, D. G. 1975. The maintenance of gynodioecy and androdioecy in angiosperms. *Genetica*, **45**, 325–339.
- MCCAULEY, D. E. AND TAYLOR, D. R. 1997. Local population structure and sex ratio: evolution in gynodioecious plants. *Am. Nat.*, **150**, 406–419.
- MUTIKAINEN, P. AND DELPH, L. F. 1998. Inbreeding depression in gynodioecious *Lobelia siphilitica*: among-family differences override between-morph differences. *Evolution*, **52**, 1572–1582.
- ROSS, M. D. 1978. The evolution of gynodioecy and subdioecy. *Evolution*, **32**, 174–188.
- SUN, M. AND GANDERS, F. R. 1986. Female frequencies in gynodioecious populations correlated with selfing rates in hermaphrodites. *Am. J. Bot.*, **73**, 1645–1648.
- TAYLOR, D. R., TRIMBLE, S. AND MCCAULEY, D. E. 1999. Ecological genetics of gynodioecy in *Silene vulgaris*: Relative fitness of females and hermaphrodites. *Evolution*, **53**, 745–751.
- VAN DAMME, J. M. M. 1983. Gynodioecy in *Plantago lanceolata* L. II. Inheritance of three male sterility types. *Heredity*, **50**, 253–273.
- VAN DAMME, J. M. M. 1986. Gynodioecy in *Plantago lanceolata* L. V. Frequencies and spatial distribution of nuclear and cytoplasmic genes. *Heredity*, **56**, 355–364.
- WEBB, C. J. 1999. Empirical studies: Evolution and maintenance of dimorphic breeding systems. In: Geber, M. A., Dawson, T. E. and Delph, L. F. (eds) *Gender and Sexual Dimorphism in Flowering Plants*, pp. 61–95. Springer-Verlag, Berlin.
- WILLIAMS, M. E. AND LEVINGS, III, C. S. 1992. Molecular biology of cytoplasmic male sterility. *Plant Breed. Rev.*, **10**, 23–51.