Evolution, 51(4), 1997, pp. 1090–1099

EVOLUTIONARY HISTORY OF THE MATING SYSTEM IN *AMSINCKIA* (BORAGINACEAE)

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Abstract.—A survey of restriction site variation in the chloroplast genome of the annual plant genus *Amsinckia*, together with estimation of outcrossing rates, was conducted to analyze the evolutionary history of the mating system. Species, and in some cases populations within species, differ markedly in their mating system. Five taxa are distylous and predominantly outcrossing, or show mixed mating systems, while the remaining taxa are homostylous and predominantly self-fertilizing. Reconstruction of the molecular phylogeny of the group places different distylous and homostylous taxa at four separate branch tips. When distyly is treated as ancestral in the group, or when the loss of distyly is assumed to be more common than its gain, the results of the phylogenetic analysis support the hypothesis that the self-fertilizing taxa are of recent origin from outcrossing relatives. These findings are discussed with respect to theory for the evolution and breakdown of distyly and the probability of extinction of selfing lineages.

Key words.—cpDNA, distyly, homostyly, phylogeny, self-fertilization.

Received August 15, 1996. Accepted April 14, 1997.

The majority of outcrossing angiosperms have bisexual flowers, a condition from which self-pollination can evolve directly through the modification of self-incompatibility or other floral traits that prevent self-pollination. Indeed, theoretical models for the selection of mating system modifiers indicate that traits promoting self-pollination are selectively advantageous under a wide variety of circumstances (Fisher 1941; Lloyd 1979; Lande and Schemske 1985). Moreover, patterns of floral variation among the angiosperms suggest that characters that prevent self-pollination have been lost independently in many widely separated evolutionary lineages (Stebbins 1974). The apparent ease by which floral modifications can bring about increases in the rate of self-fertilization in angiosperms, together with the often lenient conditions for their selection (Schoen et al. 1996), raises questions about how frequently predominant selfing has arisen in the history of the flowering plants, and why more plant taxa do not reproduce via this mechanism. One way to address such questions is with the tools of phylogenetic analysis, for example, by examining the number of times that selfing has arisen in a clade, the relative evolutionary longevity of selfing lineages, and whether selfing lineages have speciated as often as outcrossing lineages. But despite the potential utility of the phylogenetic approach for examining mating system evolution, population-level analysis has dominated this topic, and only recently have researchers begun to use phylogenetic methods to help interpret patterns of mating system diversity in light of evolutionary theory (e.g., Wyatt 1988; Donoghue 1989; Armbruster 1993; Graham and Barrett 1995; Weller et al. 1995a,b; Barrett et al. 1996; Kohn et al. 1996; Soltis et al. 1996).

Here we report results from a phylogenetic analysis of the genus *Amsinckia*, a group of western North American annual plants that has apparently undergone frequent evolutionary shifts in the mating system (Ray and Chisaki 1957a,b). The genus *Amsinckia* comprises approximately 20 species. Five of these species are distylous. Distyly is a complex morphological and physiological polymorphism—the two floral morphs of a distylous species produce flowers that differ reciprocally in style and stamen lengths, and both self- and intramorph fertilizations are partially or completely prevented by self-incompatibility (Ganders 1979; Barrett 1990). The remaining species of *Amsinckia* lack distyly and show various degrees of floral size reduction (Fig. 1).

Distyly in *Amsinckia* conforms to the classic one-locus genetic model (Ganders 1979) in which the short style–long stamen floral morph (thrum) is dominant to the long style–short stamen morph (pin). Unlike the case in many distylous species, distyly in *Amsinckia* is not coupled with a marked sporophytic incompatibility reaction, though there is evidence of cryptic self-incompatibility (i.e., preferential fertilization by pin pollen when on thrum stigmas, and vice versa) in two taxa (Weller and Ornduff 1977, 1989; Casper et al. 1988). Because they are only cryptically self-incompatible, however, these distylous species need not be complete outcrossers. Indeed, genetic estimates of the mating system in a few distylous species of *Amsinckia* indicate that they are predominantly, but not exclusively, outcrossing (Ganders 1975b, 1976; Ganders et al. 1985; Johnston and Schoen 1996). Less is known about the mating system of the homostylous species, though these taxa often have flowers of reduced size, and they generally self-pollinate spontaneously. Genetic estimates of the mating system in a few homostylous populations indicate that they are predominant to extreme selfers (Ganders et al. 1985; Johnston and Schoen 1995, 1996).

On the basis of morphological and chromosomal evidence, Ray and Chisaki (1957a) suggested that distyly has been lost independently in at least four lineages of *Amsinckia*. The distylous species typically occur in natural habitats such as chaparral borders, serpentine soils, and Pleistocene sand dunes, whereas many homostylous species of *Amsinckia* are

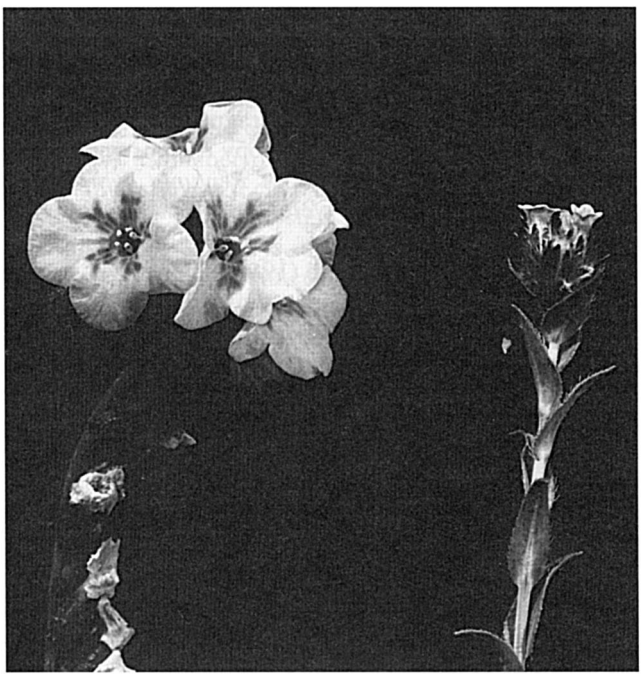

FIG. 1. Flower from the thrum form of distylous *Amsinckia furcata* (left) and its homostylous relative *A. vernicosa* (right) showing the reduction of flower size characteristic of many of the homostylous taxa of the genus.

most abundant as colonizing plants of roadsides, grazed pastures, and agricultural fields. All distylous species are diploid, while many homostylous species are polyploid (Table 1; Ray and Chisaki 1957a). Some populations contain both homostylous and distylous plants (e.g., populations of *A. spectabilis* and *A. lunaris*), suggesting that loss of distyly may occur frequently.

In this paper, we focus on estimates of the mating system and on the results of a phylogenetic analysis of mating system evolution aimed at addressing: (1) the number of times that selfing has evolved in the genus; and (2) the relative ages of the selfing taxa.

MATERIALS AND METHODS

Study Species and Populations

The species and populations studied include members of all four sections of the genus in California, the apparent center of diversity of the group (Ray and Chisaki 1957a; Table 1). One population of each distylous species was studied. Many of the distylous species are rare due to habitat destruction, and in some cases only a single population could be located (e.g., *A. grandiflora*). One or more of the putative homostylous relatives of each distylous species, as hypothesized by Ray and Chisaki (1957a), were also included in the analysis. We concentrated our efforts in the three sections of the genus where there are the fewest difficulties associated with species identification (sections Tessellatae, Microcarpae, and Disjunctae). Species in the fourth section, Muricatae, often have similar and overlapping morphology, possibly the result of hybridization in the past (Ray and Chisaki 1957c). This last section does not contain any distylous species. Only one member of it (*A. intermedia*) was included in the present analysis (Table 1).

Mating System Estimation

The mating systems of several species included in the phylogenetic analysis have not been estimated before. Starch gel electrophoresis of seed extracts (Johnston and Schoen 1995, 1996) was used to assay progeny at marker loci coding for the enzymes phosphoglucoisomerase (*A. lunaris*), phospho-

TABLE 1. Description of species and populations studied.

Species (pop. no.)	Floral condition	Ploidy level (chromosome no.)	Mating system (outcrossing rate, *t*)	Geographic location
A. furcata	distylous	2x (14)	$t = 0.99$,[a] disassortative mating[b]	Silver Creek, Fresno Co.
A. vernicosa (1)	homostylous	2x (14)	fully autofertile[c]	Catway, Santa Barbara Co.
A. vernicosa (2)	homostylous	2x (14)	$t = 0.12$,[a] fully autofertile[c]	Potrero, Santa Barbara Co.
A. grandiflora	distylous	2x (12)	cryptic self-incompatibility[d]	Corral Hollow, Contra Costa Co.
A. douglasiana	distylous	4x (24)	$t = 0.75$,[e] cryptic self-incompatibility[f]	Paloma Crk Canyon, Monterrey Co.
A. gloriosa (1)	homostylous	4x (24)	$t = 0.001$,[e] fully autofertile[c]	Paloma Crk Canyon, Monterrey Co.
A. gloriosa (2)	homostylous	4x (24)	fully autofertile[c]	Geneso Rd., San Luis Obispo Co.
A. gloriosa (3)	homostylous	4x (24)	fully autofertile[c]	Truesdale Rd., San Luis Obispo Co.
A. spect. microcarpa	distylous	2x (10)	$t = 0.45$,[e] $t = 0.53$[f]	Nipoma, San Luis Obispo Co.
A. spect. spectabilis (1)	mixed	2x (10)	$t = 0.27$[e]	La Purisima, Santa Barbara Co.
A. spect. spectabilis (2)	homostylous	2x (10)	$t = 0.002$,[e] fully autofertile[c]	Alisal Slough, Monterrey Co.
A. spect. spectabilis (3)	homostylous	2x (10)	fully autofertile[c]	Pescadero St. Beach, San Mateo Co.
A. lunaris	distylous	2x (8)	$t = 0.99$[a]	Hampton Road, Alameda Co.
A. intermedia	homostylous	4x (15)	fully autofertile[c]	Bear Valley Rd., Colusa Co.
Cryptantha flava	distylous	2x	lacks cryptic self-incompatibility[f]	Vernal, Utah

[a] See results section and Table 2.
[b] Progeny tests combined with observations of pin and thrum pollen on stigmas (Ganders 1975).
[c] All fruits with three to four of the ovules developing into seeds, as judged by measuring seed set per flower in five flowers from 10 plants protected from pollinators (see materials and methods).
[d] Weller and Ornduff (1977, 1989).
[e] Johnston and Schoen (1996).
[f] Casper (1985).

glucomutase (*A. douglasiana*), and peptidase (*A. vernicosa*). Many of the homostylous populations were monomorphic for all surveyed enzymes (Schoen, unpubl. data), and mating system estimates could not be obtained for them.

For each polymorphic marker locus, we assayed four to six progeny genotypes from up to 70 maternal parent families per population. Statistical estimation based on the mixed mating model and implemented in a program written by Ritland (1990) was used for estimating mating system parameters from the progeny array data. Standard errors were obtained via bootstrapping. In addition, flowers and fruits on 10 to 15 plants from each population listed in Table 1 were monitored for autonomous seed set in a pollinator-free greenhouse.

Molecular Phylogenetic Analysis

Data on restriction site variation in the chloroplast DNA (cpDNA) were obtained for 14 populations of *Amsinckia* spanning all four sections of the genus in California. As an outgroup species we used *Cryptantha flava*. On the basis of nutlet characteristics, floral venation and pigmentation, and the presence of heterostyly, *C. flava* is similar to *Amsinckia* (Ray and Chisaki 1957b), but its perennial habit, lack of cryptic self-incompatibility (Casper 1985), and geographic location in the Rocky Mountain states set it apart from *Amsinckia*. Because only one outgroup species was employed in this study, the data from the outgroup were used only to help establish the rooting position of the molecular phylogeny, rather than the ancestral character state of the reproductive system (see below). When the choice of outgroup species involves groups that are polymorphic for the character of interest (e.g., as is true of the presence or absence of distyly in the Boraginaceae, see below), it is possible that the use of several outgroups may help to resolve the ancestral character state in the ingroup (Maddison et al. 1984). But given uncertainties about taxonomic relationships in the Boraginaceae, there is no objective means of selecting a set of outgroup species with respect to the evolution of distyly in *Amsinckia*. Even if several outgroups were used, it is likely that a decision about the ancestral state of the reproductive system would be highly influenced by sampling error. That is, with the use of two or three outgroups (as is common in many studies), and application of the procedure suggested by Maddison et al. (1984), one might by chance alone select a set of taxa that lead to an erroneous conclusion about an ancestral state. Thus, we felt it preferable to examine different phylogenetic interpretations by considering different hypotheses about the ancestral state of distyly, and by treating it as a weighted or unweighted character (see below).

Each species in this study was represented by one to four populations. Because of the low level of restriction site variation within the group and the small plant size, DNA was extracted from five to seven plants per population and pooled for analysis. DNA was extracted from *Amsinckia* using about three to five grams of leaf tissue frozen and ground in liquid nitrogen following the methods of Doyle and Doyle (1987). Chloroplast DNA from *C. flava* was isolated (by Dr. Richard Olmstead) using cesium chloride centrifugation. DNAs were digested with the following restriction enzymes: *AvaI*, *BamHI*, *BclI*, *BglII*, *CfoI*, *ClaI*, *DraI*, *EcoRV*, *HaeIII*, *HindIII*, *HpaI*, *KpnI*, *NcoI*, *NruI*, *NsiI*, *PstI*, *PvuII*, *SacI*, *SalI*, *SmaI*, *XbaI*, *XhoI*, *XmnI*. Digestion products were separated in 1.0% agarose gels, followed by transfer of DNA from the gels to nylon filters (Nytran™). Cloned fragments encompassing most of the small and large single-copy regions and inverted repeats of the cpDNA molecule of *Pentunia hybrida* (provided by Dr. Jeffrey Palmer) were used as probes in hybridization experiments. The probes and their positions are illustrated in Sytsma and Gottleib (1986). Clones were labeled with digoxigenin-11-dUTP (Genius Kit™, Boehringer-Mannheim) and used to probe the digestion products. DNA fragment lengths were determined in relation to size standards. Restriction site gains were inferred by the presence of new bands whose estimated lengths totaled to that of the missing fragment in taxa lacking the new bands. Presence or absence of restriction sites was used as data to implement the phylogenetic analyses.

Use of a clonally inherited molecule such as cpDNA to reconstruct a species phylogeny is not without some problems (Doyle 1992). Of particular concern is the possibility that gene trees may not accurately reflect species trees, especially when the study group in question contains polyploid taxa that may have arisen by past hybridization (such as may be true of several homostylous taxa included in this analysis; see Table 1). For example, studies of other groups where hybridization is thought to occur suggest that the reticulate nature of evolutionary history would not be revealed clearly by phylogenetic analysis of a clonally inherited molecule (Soltis and Kuzoff 1995). For this reason, we also carried out separate phylogenetic analyses for the diploid subset of the species in Table 1, and compared the results and interpretations with regard to mating system evolution to those obtained using the full complement (diploid plus polyploid) of taxa.

Two approaches were used for reconstructing the cpDNA phylogeny of taxa in Table 1. Phylogenetic trees were first constructed by parsimony methods, under the assumption that restriction site mutations represent unordered characters, and that site gains and losses are equally weighted. The program package PAUP (vers. 3.1.1; Swofford, 1993) was employed to find the most parsimonious tree for the restriction site data, using the branch and bound algorithm (Hendy and Penny 1982) with the collapse zero-length branches option in effect. Bootstrapping of the site data (Felsenstein 1985) was performed (as implemented in PAUP) to explore the degree of statistical support for the tree.

Maximum-likelihood methods, assuming the Jukes and Cantor (1969) model of base change, as implemented in the program RESTML in PHYLIP, vers. 3.5 (developed by Joseph Felsenstein), were used as a second method to reconstruct trees from the restriction site data (Smouse and Li 1987; Felsenstein 1992) This algorithm assumes equality of transitions and transversions, and that base frequencies are equal, but relaxation of these assumptions renders tree estimation computationally intractable (Felsenstein 1992).

Differences in evolutionary rates between all pairs of populations were evaluated using a two-tailed Wilcoxon matched-pair sign test, as described by Templeton (1983).

Mapping Mating System Evolution onto the Molecular Phylogeny

To explore the evolution of distyly in *Amsinckia*, the mating system character states were mapped onto the cpDNA phylogeny under a number of different assumptions about the evolution of distyly as a character and of the primitive character state of the mating system in the genus. The simplest assumption about distyly is to treat it as an unordered character with transitions in either direction between distyly and homostyly being equally likely (equal weighting). But it has also been argued that the origin of a complex trait such as distyly is likely to be less common than its loss (Kohn et al. 1996). Thus, as an alternative to equal weighting we also explored the consequences to the reconstructed evolution of distyly of a character weighting scheme in which the loss of distyly is favored over the gain by a 2:1 margin (2:1 weighting) (Kohn et al. 1996).

The primitive character state of the mating system in *Amsinckia* is not well established. Apart from *Amsinckia*, many genera in the Boraginaceae contain distylous members, for example, *Anchusa*, *Arnebia*, *Cordia*, *Cryptantha*, *Echioides*, *Lithodora*, and *Lithospermum* (Ganders 1979), but distyly is unknown in other Boraginaceous genera. To address this uncertainty, we conducted separate historical reconstructions of mating system evolution, assuming distyly or homostyly as ancestral in *Amsinckia*. Data from the outgroup, *Cryptantha flava*, (i.e., restriction site presence and absence) was used only to root the ingroup (*Amsinckia*) phylogeny, so that the consequences of different assumptions about distyly as a primitive or as advanced (or unordered or weighted) could be examined.

Resolution of Polytomies

The cpDNA phylogenies obtained by the methods outlined above contained either one polytomy (when diploid taxa were analyzed alone) or two polytomies (diploid and polyploid taxa analyzed together). These were interpreted as "soft polytomies" (i.e., uncertainties about the evolutionary relationships of the taxa involved; Maddison and Maddison 1992). Because polytomies interfere with the reconstruction of character evolution (Maddison 1989), it was necessary to explore the different possible resolutions of the polytomies with respect to the evolutionary history of distyly. This was done by randomly resolving the polytomies 1000 times using the algorithm described by Maddison (1989), implemented in MacClade, vers. 3.01 (Maddison and Maddison 1992). The different resolutions were examined in light of the minimum number of transitions required to account for the evolution of the mating system. Trees requiring the minimum number of evolutionary steps to account for the evolution of the distyly were retained and compared with other reconstructions.

RESULTS

Mating System Estimation

Mating system estimates were obtained for three species of *Amsinckia* for which no prior estimates were available (Table 2). The distylous species *A. furcata* was found to be predominantly outcrossing, while distylous *A. lunaris* exhibited a mixed mating system. The homostylous *A. vernicosa* is predominantly selfing (Table 2). In pollinator-free growth conditions, all homostylous species set three to four seed per fruit (out of a possible maximum of four seeds), while flowers of the distylous species set no seed.

TABLE 2. Mating system estimates in three species of *Amsinckia*.

Species	Number of families	Family size	Outcrossing rate estimate (95% C.I.)[1]
A. furcata	33	6	0.98 (0.89–1.30)
A. lunaris	20	6	0.44 (0.28–0.62)
A. vernicosa	71	6	0.12 (0.05–0.22)

[1] From bootstrapping of progeny within families.

Molecular Variation and Phylogeny

The chloroplast DNA of *Amsinckia* is approximately 155 kb in length. We detected 536 restriction sites (representing approximately 1.7% of the cpDNA molecule), of which 44 were polymorphic (Table 3). An additional 12 restriction-site polymorphisms were found between *C. flava* and all members of *Amsinckia* in the study (Table 3). With the exception of a single site mutation in *A. spectabilis*, no intraspecific variation was detected. Sequence divergence values among the different populations (Nei and Tajima 1981) are shown in Table 4.

When both diploid and polyploid species were included in the analysis, the consensus cpDNA tree found using PAUP contained polytomies at two positions (Fig. 2). This tree has a length of 56 steps, with a consistency index of 0.95 (excluding uninformative characters), and a retention index of 0.99. Bootstrap support for the internal nodes of the tree was high. The four terminal nodes of the tree are each occupied by different combinations of distylous and homostylous taxa. This topology coincides with current taxonomic descriptions of the genus (Ray and Chisaki 1957a,b; Ganders 1993). The branch connecting the outgroup connects roughly in the middle of the longest branch of the *Amsinckia* phylogeny (Fig. 2). The maximum likelihood estimate of the phylogeny is congruent with the most parsimonious tree, and the relative variation in branch lengths is similar to that observed in the parsimony analysis (tree not shown). A molecular clock could not be rejected for any pairwise comparison of taxa.

For the analysis of the diploid species alone, the consensus cpDNA tree found using PAUP contained a single polytomy (Fig. 3). The tree has a length of 54 character steps, a consistency index of 0.96, and a retention index of 0.98. Bootstrap support for the internal nodes was $\geq$ 60%.

Notable in the cpDNA trees for both the entire group of species as well as the diploid subset (Figs. 2, 3) are the short or zero-length branches connecting each distylous species to one or more homostylous taxa. This is in comparison with the longer branches separating distylous species from one another. On average, the branches separating distylous species from their nearest distylous relative are nine to 10 times longer than those separating the homostylous species from their nearest distylous relatives. The only exception to this pattern is the polytomy involving the two distylous species (*A. grandiflora* and *A. douglasiana*), along with homostylous

TABLE 3. Chloroplast DNA restriction site changes in *Amsinckia* and *Cryptantha*.

Character	Probe	Enzyme	Change[a]	Mutated DNAs[b]
1	Pst3	*AvaI*	5.1 = 4.2 + 0.9	FUR, VERN, GRAND, DOUG, GLOR
2	Pst1	*BamHI*	8.1 = 6.6 + 1.5	GRAND, DOUG, GLOR
3	Pst10	*BamHI*	10.0 + 5.1 = 15.1	*Amsinckia*
4	Pst3	*BamHI*	3.8 = 2.1 + 1.7	LUN, INT
5	Sal8	*BamHI*	20.2 + 3.7 = 23.9	FUR, VERN, GRAND, DOUG, GLOR, LUN, INT
6	Pst12	*BamHI*	1.3 + 1.3 = 2.6	FUR, VERN, GRAND, DOUG, GLOR
7	Pst1	*BclI*	6.6 + 2.6 = 9.2	*Amsinckia*
8	Pst20	*BclI*	3.1 = 1.6 + 1.5	SPECT, MICRO
9	Sal8	*BclI*	2.4 + 0.6 = 3.0	GRAND, DOUG, GLOR, SPECT, MICRO, LUN, INT
10	Sal11	*BclI*	6.2 = 0.8 + 5.4	*Amsinckia*
11	Pst6	*BglII*	3.8 + 1.2 = 5.0	LUN, INT
12	Pst3	*BglII*	6.2 = 4.2 + 2.0	FUR, VERN, GRAND, DOUG, GLOR
13	Pst1	*CfoI*	3.0 + 2.8 = 5.8	FUR, VERN, GRAND, DOUG, GLOR
14	Pst14	*CfoI*	2.5 + 0.3 = 2.8	FUR, VERN
15	Pst14	*CfoI*	2.2 + 0.6 = 2.8	INT
16	Pst10	*CfoI*	8.1 + 2.2 = 10.3	LUN, INT
17	Pst8	*CfoI*	6.1 = 3.9 + 2.2	LUN, INT
18	Pst6	*CfoI*	4.1 + 3.6 = 7.7	FUR, VERN, GRAND, DOUG, GLOR
19	Pst6	*CfoI*	7.7 + 1.9 = 9.6	FUR, VERN, GRAND, DOUG, GLOR
20	Pst3	*CfoI*	4.7 + 3.7 = 8.4	*Amsinckia*
21	Pst8	*ClaI*	2.4 + 2.4 = 4.8	FUR, VERN
22	Pst3	*ClaI*	4.4 + 1.9 = 6.3	*Amsinckia*
23	Pst16	*ClaI*	5.7 + 0.3 = 6.0	INT
24	Pst8	*DraI*	1.5 + 1.5 = 3.0	FUR, VERN, GRAND, DOUG, GLOR
25	Pst3	*DraI*	2.1 + 3.5 = 5.6	SPECT (population 3)
26	Pst3	*DraI*	7.6 = 6.2 + 1.4	FUR, VERN
27	Pst3	*DraI*	3.3 + 2.5 = 5.8	GRAND, DOUG, GLOR
28	Sal8	*DraI*	1.8 = 0.8 + 1.0	*Amsinckia*
29	Sal11	*DraI*	3.2 = 1.7 + 1.5	*Amsinckia*
30	Pst1	*HaeIII*	4.9 = 3.3 + 1.6	FUR, VERN, GRAND, DOUG, GLOR
31	Sal11	*HaeIII*	1.1 + 0.8 = 1.9	SPECT, MICRO, LUN, INT
32	Sal8	*HindIII*	8.6 + 6.0 = 14.6	SPECT, MICRO, LUN, INT
33	Pst4	*HindIII*	5.6 + 1.1 = 6.7	VERN
34	Pst12	*HpaI*	1.4 + 0.5 = 1.9	*Amsinckia*
35	Pst10	*HpaI*	3.9 = 2.0 + 1.9	GRAND, DOUG, GLOR
36	Pst10	*HpaI*	0.6 + 0.2 = 0.8	SPECT, MICRO
37	Pst6	*HpaI*	1.9 + 0.4 = 2.3	GRAND, DOUG, GLOR
38	Sal8	*HpaI*	1.6 = 0.8 + 0.8	*Amsinckia*
39	Pst4	*HpaI*	2.1 + 1.8 = 3.9	FUR, VERN
40	Pst1/4	*NsiI*	10.0 + 0.8 = 10.8	FUR, VERN, GRAND, DOUG, GLOR
41	Pst1/4	*NsiI*	10.8 = 5.6 + 5.2	FUR, VERN, GRAND, DOUG, GLOR
42	Pst6	*NsiI*	6.2 = 4.8 + 1.4	LUN
43	Pst3	*NsiI*	2.1 = 1.6 + 0.5	SPECT, MICRO
44	Pst10	*PstI*	32.3 = 26.4 + 5.9	FUR, VERN
45	Pst8	*PvuII*	12.2 = 11.7 + 0.9	SPECT, MICRO, LUN, INT
46	Pst3	*SacI*	23.1 = 19.5 + 3.6	SPECT, MICRO, LUN, INT
47	Pst12	*XbaI*	4.8 + 2.3 = 7.1	*Amsinckia*
48	Pst3	*XbaI*	11.4 = 6.5 + 5.9	GRAND, DOUG, GLOR
49	Pst3	*XbaI*	12.9 + 1.6 = 14.5	SPECT, MICRO
50	Pst1/4	*XhoI*	23.0 + 2.0 = 25.0	FUR, VERN, GRAND, DOUG, GLOR
51	Pst3	*XhoI*	27.6 = 18.4 + 9.2	FUR, VERN
52	Pst14	*XmnI*	4.1 = 3.6 + 0.5	GRAND, DOUG, GLOR
53	Pst6	*XmnI*	4.0 = 2.5 + 1.5	SPECT, MICRO, LUN, INT
54	Sal8	*XmnI*	1.5 + 0.6 = 2.1	SPECT, MICRO

[a] Change shown with respect to the outgroup species *Cryptantha flava*.
[b] Taxon abbreviations: FUR = *A. furcata*, VERN = *A. vernicosa*, GRAND = *A. grandiflora*, DOUG = *A. douglasiana*, GLOR = *A. gloriosa*, SPECT = *A. spectabilis* var. *spectabilis*, MICRO = *A. spectabilis* var. *microcarpa*, LUN = *A. lunaris*, INT = *A. intermedia*; *Amsinckia* refers to all *Amsinckia* species in study.

A. gloriosa. The distylous species *A. grandiflora* is highly restricted geographically, being known from only one or two nearby localities in northern California, and it may represent the product of a recent speciation event.

The Evolution and Breakdown of Distyly in Amsinckia

Figure 4 shows phylogenies for the combined phylogenetic analysis of the diploid and polyploid species, representing the most parsimonious reconstructions of the evolution of the mating system in *Amsinckia* given the cpDNA phylogeny, resolution of the polytomous branches, and four sets of assumptions about distyly as a character. When distyly is assumed to be primitive in *Amsinckia* and the mating system is treated either as an unordered or weighted character, the single most parsimonious reconstruction of mating system evolution involves four separate losses of distyly at the tips

TABLE 4. Chloroplast DNA divergence (as 100 × *P*) values among populations of *Amsinckia*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. *A. furcata*	—														
2. *A. vernicosa* 1	0.02	—													
3. *A. vernicosa* 2	0.02	0.00	—												
4. *A. grandiflora*	0.23	0.25	0.25	—											
5. *A. douglasiana*	0.23	0.25	0.25	0.00	—										
6. *A. gloriosa* 1	0.23	0.25	0.25	0.00	0.00	—									
7. *A. gloriosa* 2	0.23	0.25	0.25	0.00	0.00	0.00	—								
8. *A. gloriosa* 3	0.23	0.25	0.25	0.00	0.00	0.00	0.00	—							
9. *A. spect. microcarpa*	0.53	0.55	0.55	0.51	0.51	0.51	0.51	0.51	—						
10. *A. spect. spectabilis* 1	0.53	0.55	0.55	0.51	0.51	0.51	0.51	0.51	0.00	—					
11. *A. spect. spectabilis* 2	0.53	0.55	0.55	0.51	0.51	0.51	0.51	0.51	0.00	0.00	—				
12. *A. spect. spectabilis* 3	0.55	0.56	0.56	0.53	0.53	0.53	0.53	0.53	0.02	0.02	0.02	—			
13. *A. lunaris*	0.51	0.53	0.53	0.49	0.49	0.49	0.49	0.49	0.20	0.20	0.20	0.22	—		
14. *A. intermedia*	0.53	0.54	0.54	0.51	0.51	0.51	0.51	0.51	0.22	0.22	0.22	0.24	0.05	—	
15. *C. flava*	0.51	0.53	0.53	0.53	0.53	0.53	0.53	0.53	0.38	0.38	0.38	0.40	0.40	0.42	—

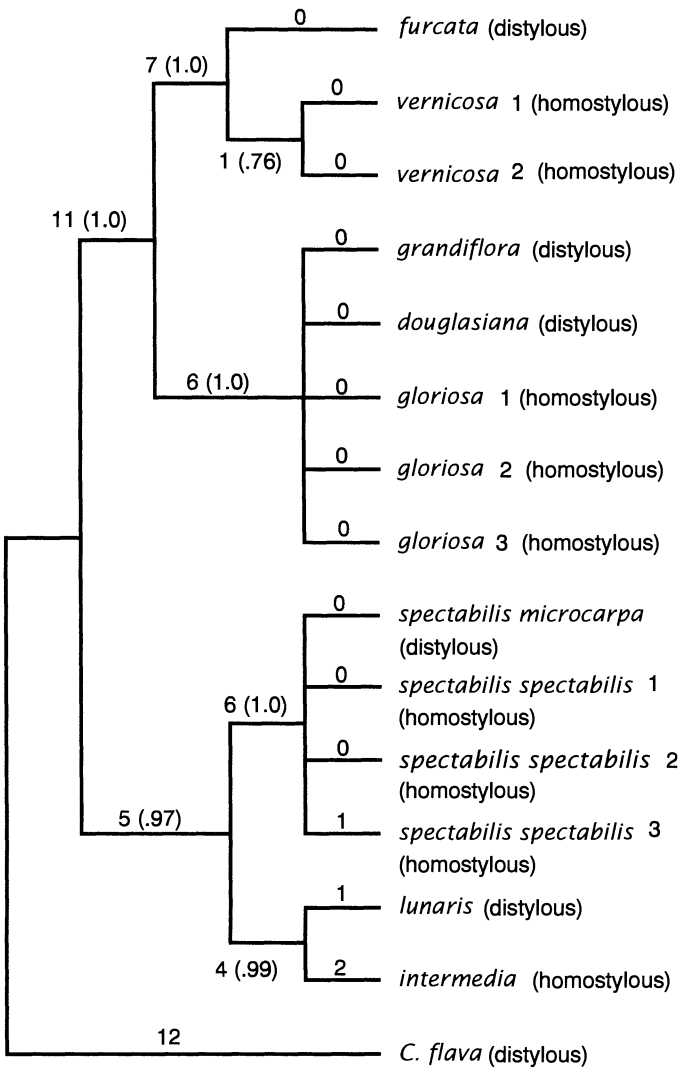

FIG. 2. Phylogeny of diploid and polyploid taxa of *Amsinckia* based on variation in the presence and absence of restriction sites in cpDNA. Numbers along the branches are the number of restriction site mutations and the proportion (in parentheses) of trees in the bootstrap analysis containing the clade.

of the tree (Fig. 4a). Constraining the phylogeny to allow only one, two, or three separate losses of distyly results in increases of 60%, 52%, and 15% in the number of steps in the tree. When homostyly is assumed to be primitive, and the mating system is treated as a weighted character, the most parsimonious reconstruction of mating system evolution in-

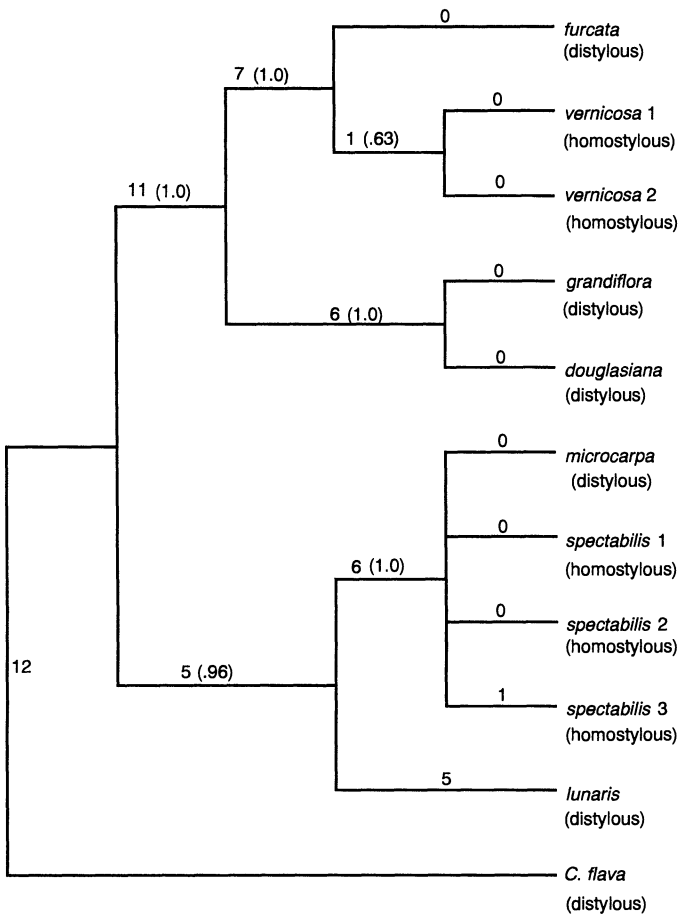

FIG. 3. Phylogeny of the diploid taxa of *Amsinckia* alone, based on variation in the presence and absence of restriction sites in cpDNA (see Fig. 2 for explanation of measures).

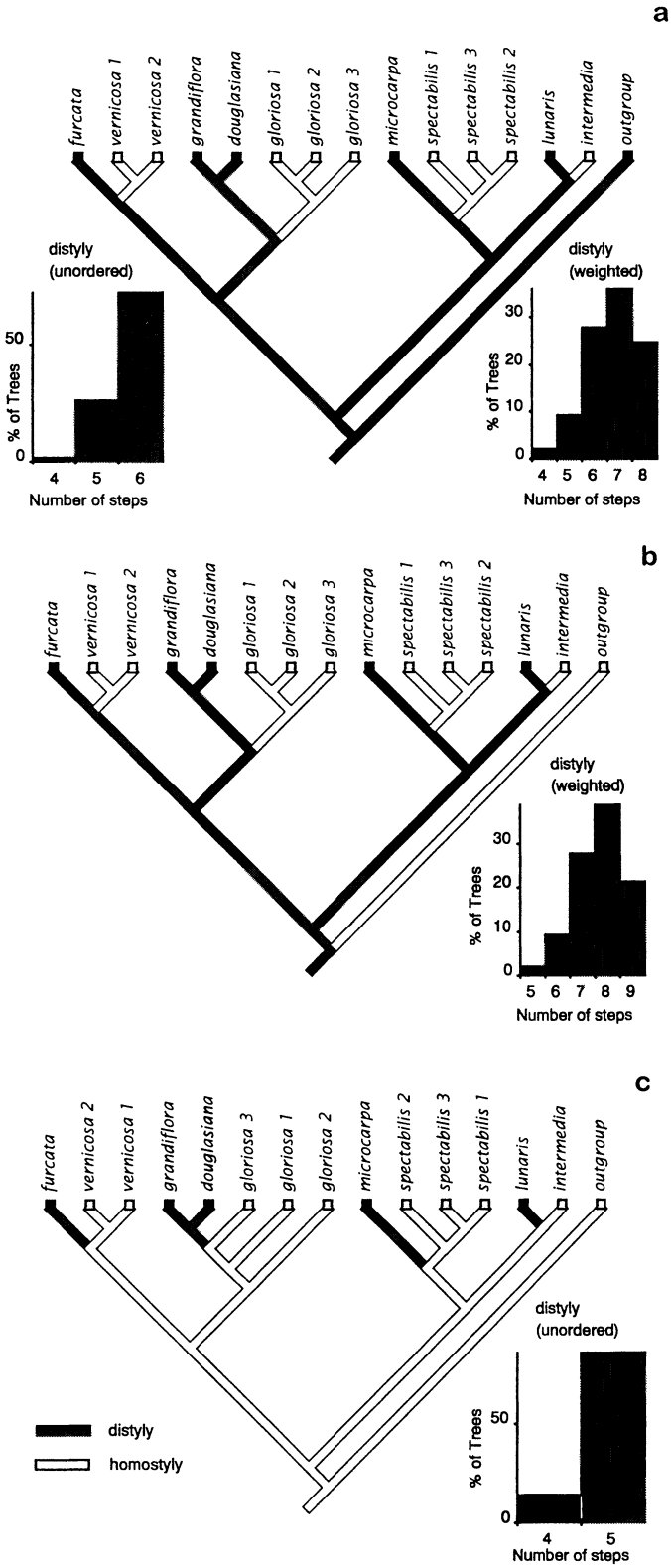

FIG. 4. Parsimonious reconstructions of the evolution of distyly in diploid and polyploid taxa under different assumptions about the character state of the hypothesized outgroup and about likelihood of loss versus gain of distyly (as indicated by the character weightings for distyly). The phylogenies illustrated are representative of the most parsimonious resolutions of polytomies with respect to the evolution of distyly. Solid lines are lineages with distyly; open lines are lineages with homostyly. The histograms show distributions of numbers of steps for the most (as illustrated) and less parsimonious resolutions of polytomies. (a) Outgroup distylous and distyly treated as unordered (i.e., loss of distyly weighted equally to gain of distyly) or as weighted (i.e., loss of distyly weighted 2:1 over gain of distyly); (b) Outgroup homostylous and loss of distyly weighted 2:1 over gain; (c) Outgroup homostylous and distyly treated as unordered.

volves one gain at the base of the tree and four losses of distyly at the tips (Fig. 4b). When homostyly is assumed to be primitive and the mating system is treated as an unordered character, the most parsimonious reconstruction of mating system evolution involves four gains of distyly (Fig. 4c).

Figure 5 shows the most parsimonious reconstructions of the evolution of the mating system when the diploid species of *Amsinckia* are analyzed separately, given the cpDNA phylogeny obtained for them, resolution of the single polytomous branch, and the four sets of assumptions about distyly discussed above. Again, when distyly is assumed to be primitive in *Amsinckia* and the mating system is treated either as an unordered or weighted character, the single most parsimonious reconstruction of mating system evolution involves separate losses of distyly at the tips of the tree (Fig. 5a). If homostyly is assumed to be primitive and the mating system is treated as a weighted character, the most parsimonious reconstruction of mating system evolution involves one gain at the base of the tree and losses of distyly at the tree tips (Fig. 5b). When homostyly is assumed to be primitive and the mating system is treated as an unordered character, the most parsimonious reconstruction of mating system evolution also involves one gain at the base of the tree and two losses of distyly at the tips (Fig. 5c).

DISCUSSION

Mating System Variation in the Distylous and Homostylous Taxa

Estimates of the mating system and observations of seed set in the absence of pollinators in *A. furcata* indicate that it is predominantly outcrossing (Table 2). These results accord well with progeny tests and stigmatic pollen load observations by Ganders (1975b, 1976). The homostylous relative of *A. furcata*, *A. vernicosa*, has a significantly lower rate of outcrossing, a result that is in accord with observations from other comparisons of related distylous and homostylous taxa in this genus (Ganders et al. 1985; Johnston and Schoen 1996). The third species whose mating system was examined here, *A. lunaris*, has an intermediate mating system (mixed selfing and outcrossing). While distylous, *A. lunaris* shows relatively reduced stigma-anther separation when compared with other distylous species in the genus such as *A. furcata*, *A. douglasiana*, and *A. grandiflora* (Table 1; Ray and Chisaki 1957a,b; Ganders 1993). A relationship between outcrossing rate and stigma-anther separation has also been reported among populations of *A. spectabilis* (Ganders et al. 1985). It would be useful to determine whether reduced stigma-anther separation, lack of cryptic self-incompatibility, or both are responsible for the lower outcrossing rates measured in these

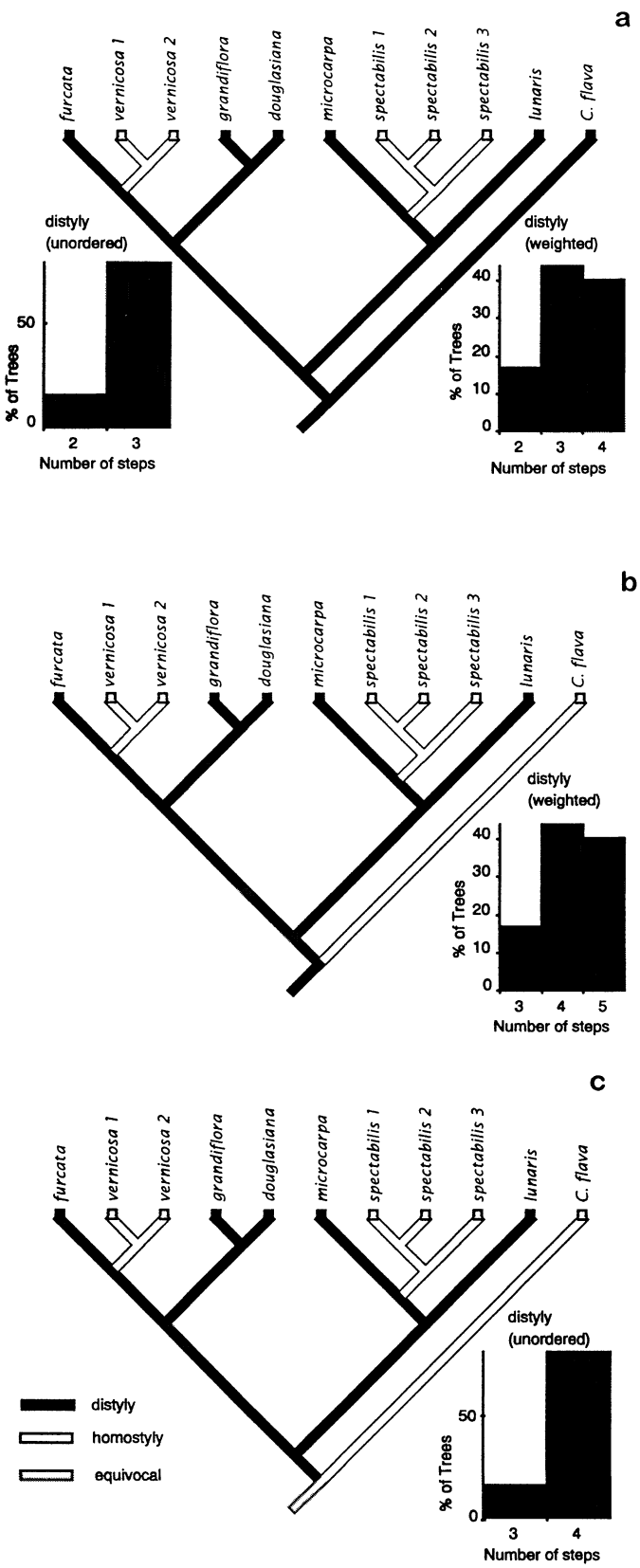

FIG. 5. Parsimonious reconstructions of the evolution and loss of distyly for diploid taxa alone under different assumptions pertaining to the ancestral state of and the likelihood of loss versus gain of distyly. See legend for Figure 4.

latter two species, especially because competing theories for the evolution of heterostyly hinge on the order of acquisition of incompatibility (Charlesworth and Charlesworth 1978; Lloyd and Webb 1992). Viewed in the context of what is known about the mating system of other *Amsinckia* species (Table 1), the overall results of the mating system studies reported here fit the pattern of a close association of outcrossing with distyly, and of selfing with homostyly.

Molecular Phylogeny

The cpDNA phylogeny of *Amsinckia* presented here places three of the distylous species, *A. furcata*, *A. grandiflora*, and *A. douglasiana*, in one major clade, and the other two distylous species, *A. spectabilis* and *A. lunaris*, in a second (Fig. 2). Each of these taxa shares separate branch tip positions with one or more different homostylous species. This arrangement is consistent with the classification suggested by Ray and Chisaki (1957b), which is based on chromosome numbers and morphology. While phylogenies based on a single molecule may not always yield accurate representations of the true evolutionary history of the group in question (Doyle 1992), the congruence of separate phylogenetic analyses performed in this study using two different methods (parsimony and maximum likelihood) together with the similarity of the molecular phylogeny to species relationships proposed on the basis of chromosome numbers and morphology (Ray and Chisaki 1957b), suggest that the trees in Figures 2 and 3 reflect the organismal phylogeny.

Evolutionary History of the Mating System

The patterns of mating system evolution shown in Figures 4 and 5 are based on a number of different assumptions about the mating system. They are best viewed as working hypotheses, each of which may be tested as new data are collected. It is, however, possible to cite a number of arguments in support of the notion that heterostyly has broken down recently and repeatedly in the genus, as opposed to a pattern of repeated evolution of distyly from homostylous ancestors. First, from observations in other heterostylous groups, it has been postulated that the breakdown of distyly to homostyly has a simple genetic basis, being brought about by recombination in the distyly locus or by changes at modifier loci (Lewis and Jones 1992). Second, existing theoretical treatments for the evolution of distyly suggest that it is unlikely to have arisen in a simple one-step process (Charlesworth and Charlesworth 1978; Lloyd and Webb 1992), supporting the notion that distyly should be treated as a weighted character (Figs. 4a,b, 5a,b). Third, most homostylous species and populations of *Amsinckia* are found in ecologically marginal habitats such as roadsides and grazed fields, whereas distylous species are found in natural habitats, suggesting that the homostylous species may be of recent origin. Fourth, in two of the lineages containing homostylous taxa, homostyly and polyploidy are coupled. This may be explained by the hypothesis that selfing arose in conjunction with past hybridization of separate taxa and chromosome doubling in the hybrid progeny; that is, if this scenario is correct, only the autogamous breakdown products of the hybrid distylous parents would have been capable of producing offspring.

Clearly, more information (e.g., additional morphological data and/or studies of molecular variation in the nuclear or mitochondrial genome) would be useful in resolving the order of events associated with mating system evolution in the group, particularly the use of additional outgroup species. This would help confirm or reject the hypothesis of distyly as the primitive mating system in *Amsinckia*.

Evolutionary Longevity of Selfing Taxa

If we assume that outcrossing is the primitive state of the mating system in *Amsinckia*, then the selfing taxa have apparently been derived repeatedly from different outcrossing taxa in the genus and are of recent origin compared with the degree of divergence separating the outcrossing taxa from one another. The exact time durations under discussion cannot be estimated accurately, however, due to uncertainties in the rate of cpDNA evolution. Nevertheless, the repeated pattern of short phylogenetic branch lengths separating selfers and their nearest outcrossing relatives in each of the four lineages suggests that there are no ancient self-fertilizing taxa in *Amsinckia*. This finding is consistent with the idea that self-fertilizing taxa have limited evolutionary potential (Stebbins 1957). Stebbins (1957) argued that the loss of variation accompanying the evolution of selfing means that taxa adopting this mode of reproduction would become an evolutionary dead end. In support of this hypothesis he cited evidence that suggested that selfing lineages do not give rise to new groups. The notion that loss of variation accompanies (or follows) the evolution of selfing is well supported on the basis of allozyme surveys of species with contrasting mating systems (Hamrick and Godt 1990; Schoen and Brown 1991).

Another, more recent hypothesis for limited evolutionary longevity of selfing taxa stems from theoretical analyses that examine the demographic consequences of accumulation of deleterious mutations in selfing populations (Lynch et al. 1995). Specifically, when the rate of population growth is assumed to be a function of deleterious mutational load, the probability of extinction of selfing populations is predicted to be greater than that of outcrossing populations. If extinction rates in selfing populations are indeed elevated, one might expect that most extant selfing lineages of *Amsinckia* would be of recent origin, found primarily at the tips of trees connecting the outcrossing taxa to one another. This is consistent with what is seen in each of the two of the hypothetical mating system phylogenies illustrated above (Figs. 4a,b, 5). Independent support for the validity of this hypothesis comes from the estimation of deleterious mutation rates (U) in several homostylous taxa of *Amsinckia* (Johnston and Schoen 1995). The deleterious mutation rate estimates fall near values where models of mutational meltdown predict significant differences in extinction probabilities of selfing and outcrossing taxa ($U \simeq 1$ per genome per generation) (Lynch et al. 1995). Some uncertainty as to how such mutation rates may influence extinction probability in polyploid taxa remains, however, as mutational load may be reduced in polyploids (Lande and Schemske 1985). Short branch lengths in phylogenetic analyses of related sexual and asexual taxa, another instance where mutational load is expected to differentially influence the likelihood of lineage extinction (Lynch et al. 1995), have also been reported (e.g., amphibians; Moritz et al. 1989).

Not all investigations of the evolutionary history of plant mating systems have shown a pattern of selfing evolving at the branch tips of the phylogeny. For example, Kohn et al.'s (1996) recent molecular phylogenetic investigation of mating system evolution in the plant family Pontederiaceae suggests that there may be several long-lived selfing taxa in the group, and Armbruster's (1993) investigation of mating system evolution in the genus *Dalechampia* suggests reversals from selfing to outcrossing in several lineages. Whether the autogamous species in these groups are extreme selfers (as in the case of *Amsinckia*) is not known with certainty, a factor that could be significant if mutation accumulation is the cause of elevated extinction rates in selfers.

Conclusions

The adoption of self-pollination is perhaps the most common evolutionary trend in the angiosperms (Stebbins 1974). The majority of recent studies concerned with the evolution of selfing have focused primarily on population level questions (Uyenoyama et al. 1993). Relatively few studies have exploited the tools and inferences available through phylogenetic analysis. Results from the present investigation suggest that floral barriers preventing selfing have broken down repeatedly in the genus *Amsinckia*, but that the breakdown products are of short evolutionary duration. Whether this type of result is a general trend in mating system evolution must await the results of further study in other plant groups.

Acknowledgments

We thank S. Armbruster, S. Barrett, Y.-B. Fu, J. J. Doyle, J. Kohn, M. Morgan, B. Ornduff, and K. Ritland for discussing this problem with us and for providing comments on the manuscript; and P. Bates, M. Borchert, B. Casper, F. Ganders, K. Marshall, R. Olmstead, B. Ornduff, F. Stermitz for help in locating populations and locating materials from several of the species studied here. This research was supported by an operating grant from the Natural Sciences and Engineering Research Council of Canada to DJS.

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