RECONSTRUCTION OF THE EVOLUTION OF REPRODUCTIVE CHARACTERS IN PONTEDERIACEAE USING PHYLOGENETIC EVIDENCE FROM CHLOROPLAST DNA RESTRICTION-SITE VARIATION

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Abstract.—We reconstructed the phylogenetic history of Pontederiaceae using chloroplast DNA restriction-site variation from approximately two-thirds of the species in this family of aquatic monocotyledons. The molecular phylogeny was used to evaluate hypotheses concerning the evolution of reproductive characters associated with the breeding system. The family has four main genera, two of which (*Eichhornia* and *Pontederia*) have tristylous, predominantly outcrossing species, while two (*Monochoria* and *Heteranthera*) have enantiostylous taxa. Self-incompatibility is restricted to some but not all tristylous species. In *Eichhornia* and *Pontederia*, predominantly selfing species with small monomorphic flowers (homostyly) have been hypothesized to result from the multiple breakdown of tristyly. Restriction-site variation provided a well supported phylogeny of ingroup taxa, enabling the mapping of reproductive characters onto trees. Two contrasting optimization schemes were assessed, differing in the relative weights assigned to shifts in character states. The reconstructed sequence of floral character-state change was used to assess competing hypotheses concerning the origin and breakdown of tristyly, and the relationships between tristylous and enantiostylous syndromes. Our results indicate that the class of optimization scheme used was the most critical factor in reconstructing character evolution. Despite some topological uncertainties and difficulty in reconstructing the primitive floral form in the family, several broad conclusions were possible when an unordered, unequally-weighted optimization scheme was used: (1) tristyly originated either once or twice, while the occurrence of enantiostyly in *Monochoria* and *Heteranthera* was always found to have independent origins; (2) tristyly has repeatedly broken down leading to selfing, homostylous taxa; and (3) self-incompatibility probably arose after the origin of floral trimorphism, a sequence of events that conflicts with some evolutionary models.

Key words.—Breeding system, *Eichhornia*, enantiostyly, optimization scheme, phylogeny, Pontederiaceae, self-fertilization, tristyly.

The integration of phylogenetic analyses with studies of character evolution has blossomed over the past decade following the acceptance of cladistic techniques and the introduction of molecular data to systematics. Recognition of the utility of historical reconstruction as a tool for testing evolutionary hypotheses has awakened an interest in phylogenetics among evolutionary ecologists. Several authors have recently pointed out that when competing hypotheses differ in their proposed order of character-state change, phylogenetic analysis can provide a tool for hypothesis evaluation (e.g., Donoghue 1989; Brooks and McLennan 1991; Harvey and Pagel 1991). For at least three reasons, plant breeding-system evolution is well suited for analysis by historical reconstruction: (1) closely related groups often show abundant quantitative and qualitative variation in floral traits influencing mating patterns (Jain 1976; Wyatt 1988; Barrett 1989); (2) theories of breeding-system evolution provide detailed hypotheses that often conflict with respect to the proposed order of character state change as well as the selective forces involved (e.g. Charlesworth and Charlesworth 1978; Barrett 1990; Thomson and Brunet 1990; Lloyd and Webb 1992a, b; Uyenoyama et al. 1993); (3) there exists, at least in some plant groups, a wealth of microevolutionary studies of mating-system variation providing a useful context for macroevolutionary inquiry (Schoen 1982; McNeill and Jain 1983; Weller et al. 1990; Barrett et al. 1992; Fenster and Ritland 1992). A major goal of evolutionary studies should be to find

ways to integrate findings from micro- and macroevolutionary inquiry. Studies of plant breeding-system evolution are likely to provide a useful model in this regard.

In this study we perform phylogenetic analysis on chloroplast DNA (cpDNA) restriction-site variation in the small monocotyledonous family Pontederiaceae to evaluate hypotheses concerning the evolution of reproductive characters associated with the breeding system. Included within this family of some 35 species are four major genera: *Eichhornia* (8–9 spp.), *Pontederia* (6 spp.), *Heteranthera* (10–12 spp.) and *Monochoria* (7–8 spp.) and several smaller, segregate genera containing 1–3 species each: *Eurystemon, Hydrothrix Scholleropsis, Reussia* and *Zosterella*. Three of the smaller genera are included in this study: *Reussia*, allied to or combined with *Pontederia* (Lowden 1973); *Zosterella*, allied to or combined with *Heteranthera* (Horn 1985; Rosatti 1987) and *Hydrothrix*, a monotypic genus placed within the family by Hooker (1887), which may also be associated with *Heteranthera* (Rutishauser 1983; Eckenwalder and Barrett 1986) All species are freshwater aquatics occurring primarily i tropical regions, with the center of diversity for the famil located in lowland South America. Three distinct floral con ditions (Fig. 1) involving the relative positions of male an female sex organs are present in the family: tristyly, a geneti polymorphism found in *Eichhornia* and *Pontederia*; enan tiostyly, a floral polymorphism that predominates among spe

Long   Mid   Short

A. Tristyly

B. Monomorphism (Homostyly)
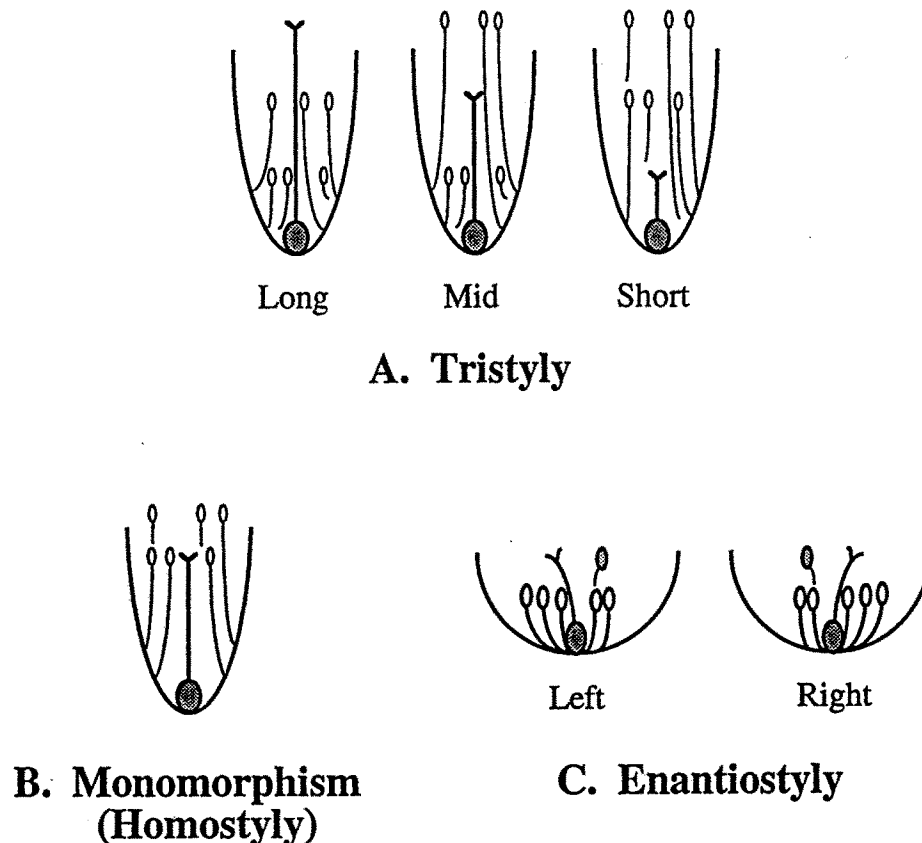
Left   Right

C. Enantiostyly

Fig. 1. Schematic representation of stamen and style configurations in tristylous, enantiostylous and monomorphic floral forms: (A) Tristyly: a genetic polymorphism controls whether individuals produce long-, mid- or short-styled flowers. Within each flower, stamens are positioned at the two levels not occupied by the stigma. (B) Monomorphism: populations are composed of a single floral form with either one or two sets of anthers at the same level as the stigma. Homostylous taxa of Pontederiaceae typically have one set of anthers at the stigma, and this most commonly involves the mid-styled morph (see Barrett 1988a). A homostylous mid-styled flower is shown, with short-level anthers adjacent to the stigma. (C) Enantiostyly: flowers have either left- or right-bending styles. A cryptically colored stamen (one of six stamens in *Monochoria*, one of three in *Heteranthera*) bends in the opposite direction. In contrast with tristyly, individuals can produce both flower types simultaneously.

cies of *Heteranthera* and *Monochoria*; and floral monomorphism, found in all four major genera.

Tristyly is a genetic polymorphism in which populations contain three floral morphs that differ from one another in style length and stamen height (Fig. 1a). The reciprocal arrangement of anther and stigma heights (reciprocal herkogamy) in the three morphs is a mechanism that promotes animal-mediated cross-pollination between morphs (Darwin 1877; Barrett and Glover 1985; Kohn and Barrett 1992; Lloyd and Webb 1992b). In most tristylous species a physiological self-incompatibility system (heteromorphic incompatibility) prohibits both self and intramorph matings by preventing fertilization unless pollen comes from the same level as the stigma. Tristyly is usually associated with a suite of ancillary polymorphisms in which pollen size, exine sculpturing, and stigma papillae length vary among stamen and style levels (Dulberger 1992). In Pontederiaceae, tristyly occurs in three species of *Eichhornia* (*E. azurea*, *E. crassipes*, *E. paniculata*) and in all but one species of *Pontederia*. All tristylous taxa are insect-pollinated and primarily outcrossing. Among tristylous species of Pontederiaceae there is considerable variation both in the strength of the incompatibility system and the expression of ancillary polymorphisms (Barrett 1988a,

1993). This variation ranges from species such as *E. paniculata*, which are highly self-compatible, setting equivalent numbers of seed following self and intermorph pollinations (Barrett 1985), to species of *Pontederia* with strongly developed self-incompatibility (Barrett and Anderson 1985). The strength of self-incompatibility is correlated with the expression of pollen and stigma heteromorphisms. Self-compatible tristylous species have weak pollen and stigma polymorphisms (Barrett 1988a), whereas self-incompatible taxa exhibit pronounced differences in pollen size among stamen levels and well developed stigma polymorphisms (Scribailo and Barrett 1991).

Enantiostyly is a floral polymorphism in which flowers possess either left- or right-bending styles (Fig. 1c). It is most commonly associated with a stamen dimorphism in which one anther is positioned opposite the stigma and the remaining anthers are located together elsewhere in the flower. Such stamen dimorphism is referred to as heteranthery where, as in Pontederiaceae and several other enantiostylous taxa, it reflects a functional division of labor into predominantly attractive, contrastingly colored "feeding" anthers and a cryptically-colored "pollinating" anther (Müller 1883; Iyengar 1923; Vogel 1978; Buchmann 1983; Lloyd 1992; Graham

and Barrett 1995). There have been few attempts to investigate the ecological significance of enantiostyly (Bowers 1975; Fenster 1995) and while its function as a mechanism promoting interflower and, perhaps, interplant pollen dispersal seems likely, experimental evidence in support of this hypothesis is lacking. While the enantiostylous floral syndrome can apparently exist as a true genetic polymorphism (Ornduff and Dulberger 1978), it usually occurs as a somatic polymorphism with right- and left-handed flowers occurring within the same individual, as is the case in enantiostylous species of *Monochoria* and *Heteranthera*.

Among all four major genera of Pontederiaceae are species that lack either the tristylous or enantiostylous floral syndromes. Flowers are uniform with respect to style and stamen characters and we therefore refer to them as monomorphic. In *Eichhornia*, monomorphic species are small-flowered, self-compatible, and largely self-pollinating, with one set of anthers positioned close to the stigma (Fig. 1b). This floral condition is referred to as homostyly following Darwin (1877) and other workers on heterostylous groups (e.g., Ornduff 1972; Ganders 1979), who have assumed that homostylous species are derived from heterostylous ancestors through loss of floral morphs and selection for self-fertilization. Evidence in support of this hypothesis in *Eichhornia* has come from microevolutionary studies in which selfing, homostylous populations have been documented at the margins of the range in each of the three tristylous species (Barrett 1978, 1979, 1985). In *E. paniculata*, the evolutionary pathway from trimorphism and outcrossing to monomorphism and predominant self-fertilization is associated with major changes in floral architecture and has been the focus of intensive microevolutionary investigation over the past decade (Glover and Barrett 1986; Barrett et al. 1989; Barrett and Husband 1990; Barrett et al. 1992; Fenster and Barrett 1994; Kohn and Barrett 1994).

Variation in floral syndromes and mating-systems, a wealth of microevolutionary information, and the relatively small size of the family make Pontederiaceae particularly appropriate for phylogenetic analysis of the evolution of reproductive characters. A major objective in this study was to use molecular phylogenetic evidence to reconstruct the history of evolutionary transitions in floral characters. We posed three questions about the evolution and breakdown of floral syndromes, paying particular attention to character-state changes associated with the buildup and breakdown of tristyly. (1) What is the evolutionary relationship between tristyly and enantiostyly and were there single or multiple origins of each polymorphism? (2) What is the order of character-state changes leading to the buildup of the tristylous floral syndrome? In particular, is self-compatibility the ancestral or derived condition among tristylous species? (3) Is homostyly among selfing taxa of *Eichhornia* homologous or were there multiple transitions from tristylous, outcrossing taxa to homostylous, selfing ones? We next develop the rationale for each of these questions.

Tristyly has been well documented in only four unrelated angiosperm families (Lythraceae, Oxalidaceae, Pontederiaceae, and Amaryllidaceae; reviewed in Barrett 1993) and therefore its origin is likely to have been an infrequent event. The rarity of tristyly is undoubtedly associated with its de-

velopmental and genetic complexity (Charlesworth 1979; Lewis and Jones 1992). Little is known of the floral characteristics of the immediate ancestors of tristylous species. Based on their developmental studies of various tristylous groups, Richards and Barrett (1992) proposed that a within-flower stamen dimorphism was probably a prerequisite for the evolution of tristyly, raising the possibility of an evolutionary linkage to enantiostyly. Several monocotyledonous families (e.g., Commelinaceae, Tecophilaeaceae, Haemodoraceae, Philydraceae), some of which are closely allied to Pontederiaceae (see Graham and Barrett 1995), also contain enantiostylous taxa and it is therefore possible that enantiostyly in Pontederiaceae is homologous to that found in related families.

An important issue concerned with the evolution of heterostyly is the order of establishment of the morphological and physiological components of the syndrome (Barrett 1992). For example, models of the evolution of distyly conflict as to whether heteromorphic incompatibility evolves before reciprocal herkogamy (Charlesworth and Charlesworth 1979) or after it (Lloyd and Webb 1992a,b). Charlesworth (1979) proposed that a distylous condition (with two levels of anthers in each flower) serves as an intermediate stage in the transition from monomorphism to tristyly. In her model, modifiers that increase the strength of heteromorphic incompatibility spread, though perhaps not to fixation, before floral trimorphism becomes established. Where self-compatibility occurs in tristylous taxa, it has usually been assumed to represent a secondary loss from a self-incompatible ancestor (Ornduff 1972; Weller 1992). Where heterostylous taxa within a clade differ with regard to the presence or absence of incompatibility, phylogenetic reconstruction of character-state evolution may allow us to determine the sequence of evolutionary events.

Microevolutionary studies of the dissolution of tristyly in *Eichhornia* suggest that the polymorphism has broken down repeatedly, giving rise to selfing, homostylous species (e.g. Barrett 1988a; Husband and Barrett 1993). However a phylogenetic study of Pontederiaceae involving 34 taxa and 42 morphological characters, 27 of which were reproductive traits, suggested instead that the shift from tristyly to homostyly occurred once, resulting in a monophyletic group of selfing species (Eckenwalder and Barrett 1986). This result was not in accord with previous population-level studies and Eckenwalder and Barrett (1986) proposed that the apparent monophyly of homostylous *Eichhornia* species may have resulted from the repeated evolution of traits associated with a selfing syndrome (see also Wyatt 1988). In the present study, we use restriction-site variation of the chloroplast genome because this source of data is likely to be relatively free of multiple convergences caused by similar selective forces acting on reproductive characters in different lineages.

Reconstruction of character evolution commonly involves mapping traits onto a phylogenetic tree. This is usually achieved by treating all shifts in characters as equally likely events (e.g. Brooks and McLennan 1991). Although details of the optimization method are not always explicitly stated, this has generally been the approach employed in phylogenetic studies concerned with the evolution of reproductive traits in flowering plants (Hart 1985; Eckenwalder and Barrett

1986; Donoghue 1989; Olmstead 1989; Cox 1990; Sytsma et al. 1991; Rieseberg et al. 1992; Armbruster 1993; Bruneau 1993; Weller et al. 1995). Equal weighting is methodologically simpler and is used to avoid subjectivity in weighting various classes of evolutionary change. However, there is abundant evidence that certain evolutionary transitions are more likely than others, such as shifts from diploidy to polyploidy in plants (Stebbins 1950) or the evolution of flightlessness in island-inhabiting birds (Carlquist 1974), compared to the reverse changes. Where external evidence bearing on the likelihood of particular character-state changes is available, it would seem wise to take account of this information in reconstructing the course of evolution (Maddison and Maddison 1992). Hence, an additional goal of our study was to explore the importance of different optimization schemes on the interpretation of the evolution of reproductive characters in Pontederiaceae.

MATERIALS AND METHODS

Taxon Sampling.—Localities for the 24 accessions from Pontederiaceae and three outgroup taxa used in this study are given in the Appendix. The 24 taxa from Pontederiaceae included eight species of *Eichhornia*, four taxa of *Pontederia*, five species of *Heteranthera*, four species of *Monochoria*, the monotypic *Hydrothrix*, and one species each from *Reussia* and *Zosterella*. Outgroup taxa representing Commelinaceae, Philydraceae and Liliaceae were used. Eckenwalder and Barrett (1986) used Liliaceae to root their morphological phylogeny of Pontederiaceae. Based on morphological evidence, Commelinaceae and especially Philydraceae have been closely allied with Pontederiaceae by several workers (Hamman 1966; Takhtajan 1969; Dahlgren et al. 1985; Thorne 1992); evidence from the chloroplast gene *rbcL* provides only limited support for these alliances (Chase et al. 1993; Clark et al. 1993; Graham and Barrett 1995). An undescribed species of *Eichhornia* (referred to here as *Eichhornia* sp.) was originally identified in Eckenwalder and Barrett (1986) as *E. paradoxa* (Mart.) Solms-Laub. Differences in a range of vegetative and reproductive traits between this accession and *E. paradoxa* (S. C. H. Barrett, unpubl. data) led to a study of differentiation between these two taxa. F_1 hybrids proved vigorous but sterile and a study of isozyme differentiation between the taxa showed levels of divergence normally found among congeneric species (Cole and Barrett 1989).

Molecular Methods.—Total genomic DNA was isolated by the method of Doyle and Doyle (1987) using approximately 1 g of leaf tissue from single individuals ground initially under liquid nitrogen. For *M. cyanea*, DNA was extracted from pooled seedlings instead of an individual plant because we were unable to maintain plants beyond the seedling stage. DNA (approximately 1–3μg) was cut with one of 10 restriction enzymes (*Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Kpn*I, *Pst*I, *Sal*I, *Xho*I) and then separated on 0.8% agarose gels and transferred onto nylon filters (Sambrook et al. 1989). Filters were then sequentially hybridized to 42 clones from the *Nicotiana* chloroplast genome (Sugiura et al. 1986; Olmstead and Palmer 1992). Clones were radiolabeled with ^{32}P-labeled dATP using the random hexanucleotide priming method (Feinberg and Vogelstein 1983) and bands were vi-

sualized using autoradiography (for hybridization methods, see Doyle et al. 1990). Restriction sites were inferred by examination of autoradiogram banding patterns from adjacent cpDNA clones.

Tree Reconstruction.—Taxa were coded 0 for absence and 1 for presence of inferred restriction sites and analyzed using PAUP version 3.1.1 (Swofford 1993). The data matrix is available from the senior author. Sites were scored wherever unambiguous, but some sections of the chloroplast genome for some enzyme-taxa combinations were not mapped due to poor hybridization or difficulty in site reconstruction among divergent taxa. Heuristic searches used tree bisection-reconnection (TBR) branch swapping with the "MULPARS" and "steepest descent" options in effect. To evaluate the effectiveness of the heuristic search, 100 random addition replicates were performed (Maddison 1991). These found no additional trees of equal or shorter length. The ingroup was monophyletic in all trees. Separate analyses were performed in which outgroup taxa were excluded or included. To assess tree robustness, two bootstrap analyses were performed, one with the three outgroup taxa and one without, using the same heuristic search settings as before. The addition sequence was random for each of the 200 bootstrap replicates performed.

Character Optimization.—Reproductive characters were optimized onto trees in two independent sets of analyses, one involving floral form (tristyly, enantiostyly, and monomorphism) and the other the self-incompatibility status of taxa in Pontederiaceae. For both floral conditions and self-incompatibility status, two different types of optimization schemes were assessed. In one, all character state transitions were treated as unordered and equally likely (equally weighted optimization scheme, Table 1a, Fig. 4b). The second type of scheme (hereafter "2:1" weighting; Table 1b, Fig. 4a,c,d) was also unordered but applied weights in the following manner: for floral form, the loss of tristyly or enantiostyly was favored by a two-fold margin over their gain or interconversion; for self-incompatibility, the loss of self-incompatibility was favored by a two-fold margin over its gain. Below, we discuss more fully our rationale for favoring reconstructions that use these unequally weighted schemes. Reconstructions were performed using the "state changes and stasis" option of MacClade version 3.0 (Maddison and Maddison 1992).

Two a posteriori methods were used to root trees resulting from the phylogenetic analysis of ingroup taxa: (1) the most parsimonious rooting, the branch to which the outgroups join to the family when these taxa are included in the searches; and (2) midpoint rooting, the midpoint of the longest path connecting any pair of taxa. The instability of the root position found using outgroup analysis (see results) motivated the examination of the effect of alternative root position on character reconstruction and midpoint rooting was considered as one example of a slightly suboptimal root position. Although Pontederiaceae is a monophyletic group within the monocotyledons (Graham and Barrett 1995), the identity of the sister-group to Pontederiaceae is unclear with respect to current molecular and morphological evidence (Clark et al. 1993; Graham and Barrett 1995). A hypothetical outgroup was therefore placed at the base of trees for the root positions discussed above, and was coded either as monomorphic, en-

TABLE 1. Reconstructed number of shifts of floral form in Pontederiaceae. The range of frequencies of shifts is summarized for all most-parsimonious reconstructions of floral form on the ten shortest chloroplast-based trees. (A) Equally weighted optimization scheme; (B) "2:1" optimization scheme. Outgroup and midpoint rootings were assessed in conjunction with the three possible codings of the outgroup floral form. The primitive floral form in the family is listed as equivocal if ambiguous for at least some of the trees.

(A)

Floral shift	Outgroup coding		
	Monomorphic	Enantiostylous	Tristylous
Equally weighted optimization scheme, outgroup rooting:			
Tristyly gained	4	0–4	0–3
Tristyly lost	0	0–6	1–5
Enantiostyly gained	2	1–3	2
Enantiostyly lost	1	1–5	1–2
Total events and steps	7	7–8	7
Primitive floral form	Monomorphic	Equivocal	Tristylous
Equally-weighted optimization scheme, midpoint rooting:			
Tristyly gained	1–4	0–4	0–1
Tristyly lost	0–4	0–6	4–5
Enantiostyly gained	2	1–3	2
Enantiostyly lost	1–2	1–4	1
Total events and steps	7	8	7
Primitive floral form	Monomorphic	Equivocal	Tristylous

(B)

Floral shift	Outgroup coding		
	Monomorphic	Enantiostylous	Tristylous
"2:1" optimization scheme, outgroup rooting:			
Tristyly gained	0	0–2	0
Tristyly lost	6	4–6	5
Enantiostyly gained	2	1–3	2
Enantiostyly lost	1	1–3	1
Tristyly to homostyly	5	4	4
Tristyly-enantiostyly interconversion	1	2	1
Total events (steps)	8 (10)	8 (11)	7 (9)
Primitive floral form	Monomorphic	Equivocal	Tristyly
"2:1" optimization scheme, midpoint rooting:			
Tristyly gained	0–1	1	0
Tristyly lost	4–6	4	5
Enantiostyly gained	2	1	2
Enantiostyly lost	1	4	1
Tristyly to homostyly	3–5	3	4
Tristyly-enantiostyly interconversion	1	2	1
Total events (steps)	7–8 (10)	8 (10)	7 (9)
Primitive floral form	Equivocal	Enantiostyly	Tristyly

antiostylous, or tristylous to examine the effect of different outgroup codings on the reconstruction of shifts in floral condition within the family. Heteromorphic sporophytic self-incompatibility systems are unknown in the monocotyledons outside Pontederiaceae (Charlesworth 1985; Weller et al. 1995). The homomorphic gametophytic self-incompatibility systems found in some monocotyledonous groups (e.g. in some taxa of Commelinaceae) are unlikely to be homologous with heteromorphic sporophytic incompatibility in Pontederiaceae. The hypothetical outgroup was therefore coded as self-compatible.

RESULTS

Phylogenetic Structure of the Family.—We scored 356 restriction sites of which 292 were variable and 104 were potentially informative within Pontederiaceae. Twenty most-parsimonious trees of length 492 steps and with a consistency index (CI) of 0.593 (0.464 excluding uninformative characters), retention index (RI) of 0.692, and rescaled consistency index (RC) of 0.411 were found in the analysis of the entire data set. When included, the outgroup taxa joined Pontederiaceae along the terminal branch subtending *E. crassipes* in all most-parsimonious trees. Ten shortest unrooted trees of length 299 steps, CI = 0.582 (0.454 excluding uninformative characters), RI = 0.718, and RC = 0.418 were found when outgroup taxa were excluded. Strict consensus trees of the trees from analyses in which outgroup taxa were either included or excluded are presented in Figure 2. Bootstrap values are indicated on branches seen in 50% or more of replicates. When outgroup taxa were excluded, the phylogenetic structure of the family is well resolved; most branches have bootstrap support of 70% or higher. Simulation studies by Hillis and Bull (1993) suggest that bootstrap values of about 70% or more represent well supported branches. The
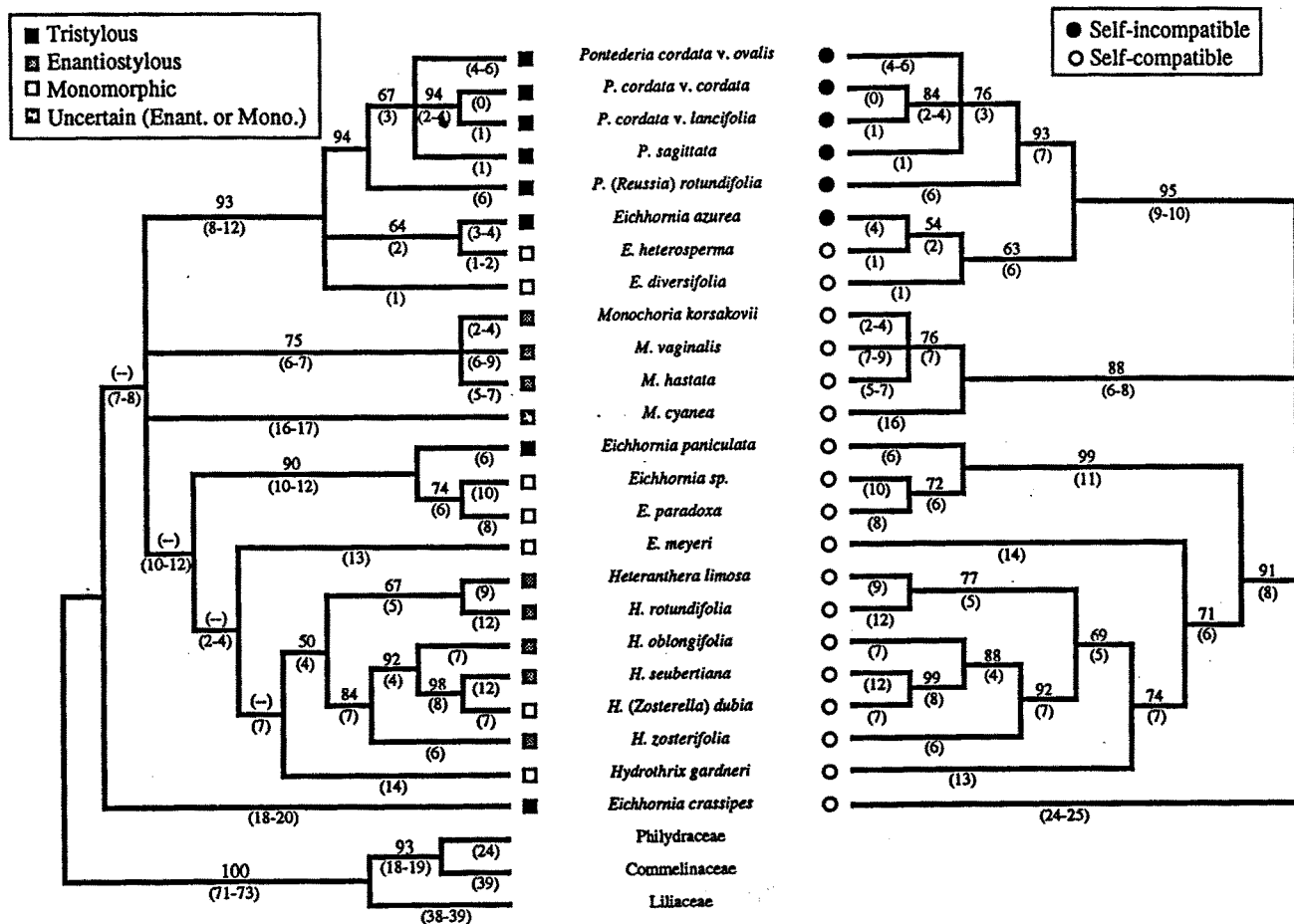
FIG. 2. Strict consensus of the most-parsimonious phylogenetic trees found in heuristic searches based on chloroplast DNA restriction site variation found in Pontederiaceae (see text). Outgroup taxa were either included (left side) or excluded (right side) from the analysis. Bootstrap support for phylogenetic structure in the consensus trees is indicated above each branch. Branches with less than 50% bootstrap support are indicated by "(–)." Indicated below each branch is the range of lengths across the shortest trees (computed using ACCTRAN optimization). Tree lengths and summary statistics are provided in the text. The symbols beside each taxon indicate its floral form and self-incompatibility status.

inclusion of outgroup taxa in the analysis led to a collapse of support for several of the branches at the base of the rooted tree, indicating uncertainty in the position of the root of the family. In particular, the rooting seen in all most parsimonious rooted trees, with *E. crassipes* sister to the rest of Pontederiaceae (Fig. 3), was supported by only 27% of bootstrap replicates.

In all unrooted trees, three of the four main taxonomic groups, *Pontederia s. l.*, *Monochoria*, and *Heteranthera s. l.*, of Pontederiaceae are monophyletic. Two separate clades of *Eichhornia* occur that are each composed of a tristylous species together with two monomorphic species of *Eichhornia*. Tristylous *E. paniculata* is sister to the clade consisting of monomorphic *Eichhornia* sp. and *E. paradoxa*, while tristylous *E. azurea* forms a clade with monomorphic *E. heterosperma* and *E. diversifolia*. *Eichhornia crassipes* and *E. meyeri* were not part of either clade. The two enantiostylous genera (*Monochoria* and *Heteranthera s. l.*) did not form a monophyletic group, but instead were well separated on the trees (Figs. 2, 3).

Relatively minor differences in topology were observed

among the 10 shortest unrooted trees. These varied in three ways (Fig. 3). Within *Pontederia*, *P. sagittata* was seen in the two different positions shown. Within *Monochoria*, a clade consisting of *M. korsakovii*, *M. vaginalis*, and *M. hastata* varied in their relative branching order such that all three possible branching orders of these taxa were found (two of the three are shown). Two major topological classes are defined by the relative positions of *E. crassipes* and the genus *Monochoria* within the family. With respect to the a posteriori rooting indicated in Figure 3, *Monochoria* was either the sister group of the clade consisting of *Pontederia s. l.* and the clade of *Eichhornia* species that includes *E. azurea* (hereafter referred to as "class A topology"), or was the sister group of all taxa in the family excluding *E. crassipes*, ("class B topology"). The 10 most-parsimonious unrooted trees all have topological equivalents among the twenty most-parsimonious rooted trees.

Reconstruction of Reproductive Character Evolution.—Reproductive characters were optimized onto these 10 shortest trees in the separate analyses for floral form and self-incompatibility status. Apart from the two possible positions of *E.*

Class A Topology
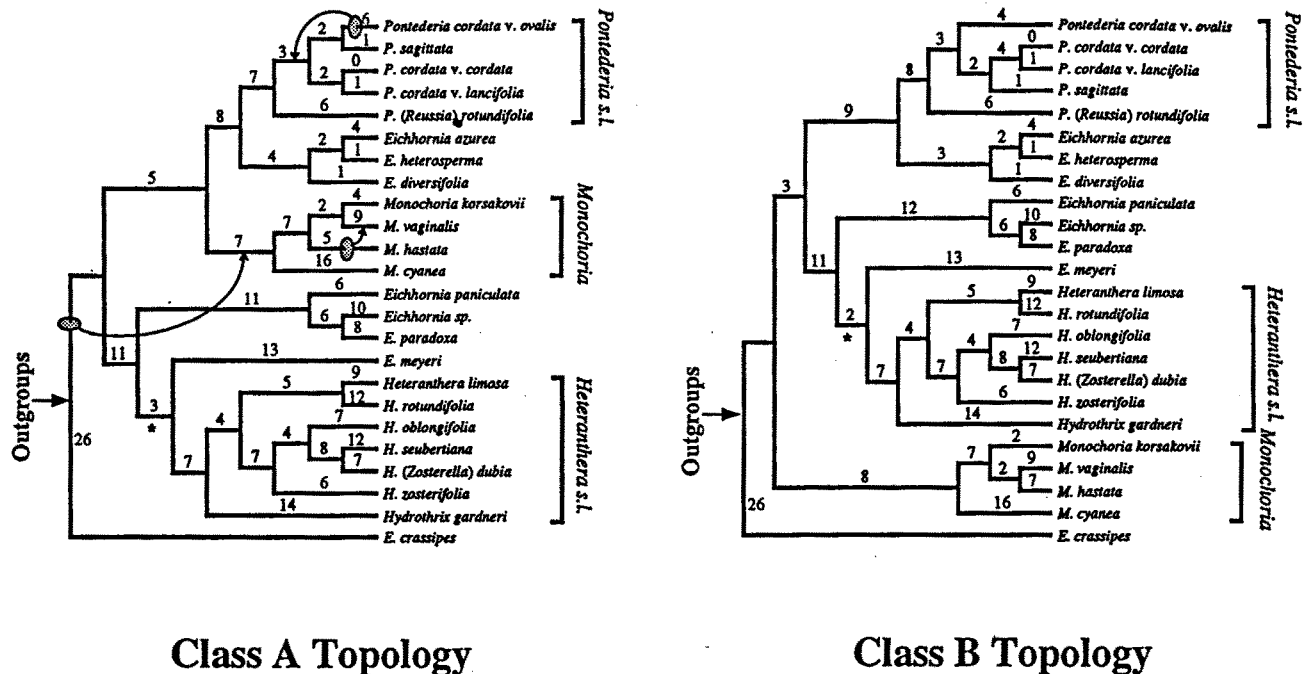
Class B Topology

FIG. 3. Two of the 10 shortest maximum parsimony trees from a heuristic search involving only taxa of Pontederiaceae. The two trees summarize almost all of the topological variation seen among the 10 shortest trees and illustrate the two classes (A and B) of topological variation important for reconstructing the evolution of reproductive characters (see text and Fig. 4). Each class is defined with respect to two possible positions of *Eichhornia crassipes* relative to *Monochoria*. The three arrows indicate topological shifts that would convert one tree into the other. The branch containing the tree midpoint is indicated with an asterisk. ACCTRAN optimization was used to compute all branch lengths.

crassipes relative to *Monochoria* shown in Figure 3, other topological variations among the shortest trees had no effect on character reconstructions since all taxa in the relevant clades possess the same character state.

Table 1a,b summarizes shifts in floral condition reconstructed across the shortest trees for the different outgroup codings and rootings using the two different optimization schemes. Under the first scheme all floral shifts were treated as equally weighted, unordered character-state transitions, and up to four reconstructed gains of tristyly are possible (Table 1a, Fig. 4b). However, several lines of evidence indicate that this optimization scheme is unlikely to reflect the relative likelihood of the gain versus loss of a complex polymorphism such as tristyly (see below). The second optimization scheme was also unordered, but the weights used favored the loss of tristylous or enantiostylous floral syndromes over their gain by a two-fold margin (Table 1b, Fig. 4a,c,d). For trees with class B topology, it is equivocal whether tristyly arose once or twice, if outgroup rooting is employed and the outgroup is coded as enantiostylous (see Fig. 4d). Under such conditions, tristyly arises either a single time prior to the origin of the extant lineages of Pontederiaceae, or it arises twice, once within the *E. crassipes* lineage, and once prior to the origin of the other tristylous taxa. Under all other combinations of root placement, ingroup topology, and outgroup coding using the 2:1 weighting scheme, all instances of tristyly are inferred to be homologous within the family.

Using MacClade, the number of gains of particular character states does not necessarily give an accurate account of the total number of homoplastic (i.e., nonhomologous) oc-

currences of each state. This is because a character state can have *x* separate homoplastic instances across a tree, but have only $x - 1$ gains reported. The "missing" gain is a symplesiomorphic (shared primitive) instance of the character retained from the base of the tree, hence cases with zero gains of tristyly in Table 1a,b. In some cases the number of gains of enantiostyly indicated in Table 1b underreports the number of homoplastic incidences. We examined all cases where a single gain of tristyly occurred (Table 1b) for the 2:1 weighting scheme and confirmed that these corresponded to a single origin of tristyly on the tree. In all cases with the 2:1 weighting scheme, there were at least two homoplastic cases of enantiostyly on the tree. In some trees with the outgroup coded as enantiostylous, there was an additional homoplastic reconstruction of its occurrence on the branch leading to the outgroup, hence cases with three gains of enantiostyly (Table 1b). Finally, when 2:1 weighting is used, from three to five shifts from tristyly to homostyly are inferred under all combinations of outgroup coding, root placement, and ingroup topology. A total of seven to eight events (each event representing a single shift in floral form) occurred across all classes of rooting, outgroup coding, and optimization scheme.

Using the "enforce topological constraints" option of PAUP we examined the hypothesis that monomorphic species form a monophyletic clade, as was found in several of the morphological cladograms of Eckenwalder and Barrett (1986, Figs. 1, 2). Shortest trees in our search were 26 steps (5.28%) longer than the most parsimonious unconstrained trees. Constraining only *E. diversifolia, E. heterosperma, E. paradoxa*

and *Eichhornia* sp., three of the four selfing taxa used by Eckenwalder and Barrett (1986), to be monophyletic resulted in shortest trees 23 steps (4.67%) longer than the most-parsimonious trees. Both scenarios therefore require a substantial decrease in parsimony.

Reconstructions of the evolution of heteromorphic incompatibility also differ according to the weighting scheme used to model its gain and loss. With an equal weighting scheme, incompatibility is seen to arise on two separate occasions in the family, once along the terminal branch leading to *E. azurea*, and once in the lineage leading to *Pontederia s. l.* (shown in Fig. 4b; shifts in incompatibility are represented by bars). With a 2:1 weighting scheme for the gain versus loss of incompatibility, however, it is equivocal whether it arises once or twice. When delayed transformation (DELTRAN) is employed to resolve this ambiguity, two origins of incompatibility are inferred on these two branches. With accelerated transformation (ACCTRAN), a single gain of heteromorphic incompatibility is inferred along the branch supporting the clade consisting of *E. azurea, E. heterosperma, E. diversifolia,* and *Pontederia s. l.* (as shown in Fig. 4a,c,d). This single gain is followed by reversions to self-compatibility along the terminal branches leading to the two monomorphic species in this clade. In fact, all weighting schemes favoring the gain over the loss of SI by a greater than twofold margin result in a single origin and two losses of incompatibility in the family. Regardless of which scenario is inferred, incompatibility always arises several branch segments after the origin of tristyly, when the 2:1 weighting scheme is used to reconstruct shifts in floral form, for all topologies and rootings examined here.

DISCUSSION

Analysis of chloroplast DNA restriction-site variation produced a highly resolved phylogenetic hypothesis for Pontederiaceae with many groupings within the family strongly supported by the bootstrap analysis. However, topological uncertainties involving the root placement and the relative positions of *E. crassipes* and *Monochoria* in the family posed complications for reconstruction of the evolution of reproductive characters. In this discussion we first take up the systematic implications of the molecular data and present the implications of infrafamilial relationships for understanding shifts in floral characters. Next we discuss the reconstruction of floral character state transitions using different optimization schemes and alternative topologies. We show that the class of optimization scheme employed is a critical issue in interpreting character evolution, particularly where biological evidence dictates that equal weighting of evolutionary transitions is of dubious validity.

Systematics of Pontederiaceae

Many of the relationships among taxa within Pontederiaceae were well resolved, allowing several conclusions to be drawn from the phylogenetic analysis of chloroplast characters. The degree of divergence of the chloroplast genomes of *Eichhornia* sp. and *E. paradoxa* (Fig. 3) is consistent with the proposed species-level status of each taxon. Based on the root placement indicated by outgroup analysis, *Monochoria, Pontederia s. l.,* and *Heteranthera s. l.* are all monophyletic. However, bootstrap support for this root placement is well below 50%. Bootstrap support for several other branches, including the one supporting the monophyly of *Monochoria,* falls below 50% when outgroups are included in the analysis (Fig. 2). We interpret this collapse in support as being a function of the uncertainty in root placement, since these branches are well supported when outgroups are excluded from the analysis. Finally, the genus *Eichhornia* is an "unnatural" or polyphyletic group, regardless of the placement of *E. crassipes.*

The apparent polyphyly of *Eichhornia* could have a number of causes. It is always possible that the chloroplast "gene" tree is not an accurate reflection of the "species" tree of the organisms in which the chloroplasts reside, due to phenomena such as ancient introgression of chloroplast genomes (reviewed in Doyle 1992). While there is little evidence for hybridization among extant species in Pontederiaceae (S. C. H. Barrett, unpubl. data), historical introgression of chloroplasts could have led, for example, to the capture by the ancestor of *Monochoria* of the chloroplast genome of an early species of *Eichhornia.* Phylogenetic evidence from several unlinked nuclear genes may help clarify whether such events have occurred at this or any other point on the tree. However, it is worth noting that phylogenetic evidence based on morphology also indicates that *Eichhornia* is at least paraphyletic, if not polyphyletic (Eckenwalder and Barrett 1986). Rather than appealing to phenomena such as ancient hybridization to explain the dispersed placement of *Eichhornia* species across Pontederiaceae, it may be safer to assume in the absence of evidence to the contrary, that the unnaturalness of the genus *Eichhornia* is real.

Implications of Infrafamilial Relationships for Reproductive Character Evolution.—Despite the uncertainty of root placement, at least two conclusions are warranted on the basis of

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FIG. 4. Example reconstructions of breeding-system evolution for selected maximum parsimony trees. Reconstruction of character evolution was performed using MacClade version 3 (Maddison and Maddison, 1992). *Monochoria cyanea* was coded as uncertain for floral form (i.e., as monomorphic or enantiostylous). Floral form and self-incompatibility were each assessed independently. Step matrices employed for reconstructions are depicted in each figure. All optimization schemes were unordered. The outgroup was coded as self-compatible for all reconstructions of shifts in compatibility status. (A) Character state reconstruction using unequal weighting schemes, class A tree topology (see Fig. 3), the outgroup coded as monomorphic for floral form, and the root location found by outgroup analysis (see Fig. 2). The reconstruction of self-incompatibility was equivocal under 2:1 weighting and was resolved here using ACCTRAN optimization (see text). (B) Same conditions as Figure 4a, except that all character transformations were weighted equally. (C) Same conditions as Figure 4a, except that midpoint rooting was employed. (D) Unequal weighting schemes, Class B topology (see Fig. 3), outgroup rooting, and the outgroup coded as enantiostylous. This reconstruction illustrates a scenario where the unequal weighting scheme for floral form can lead to two origins of tristyly.
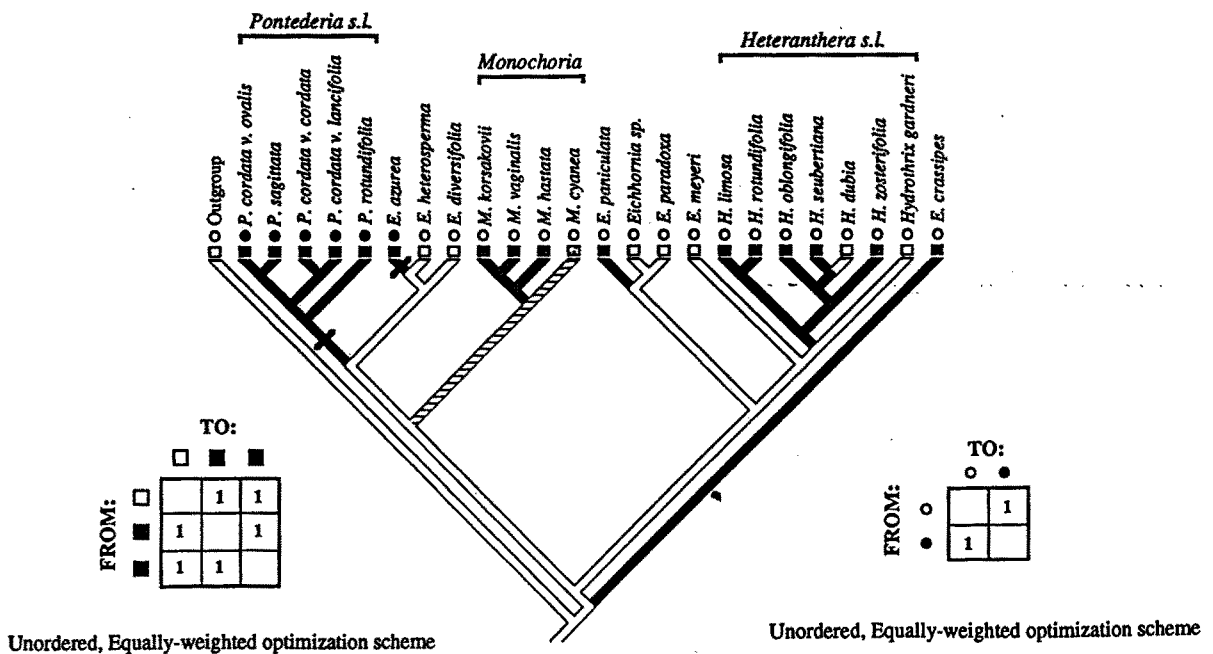
A

Class A Topology, Outgroup Rooting
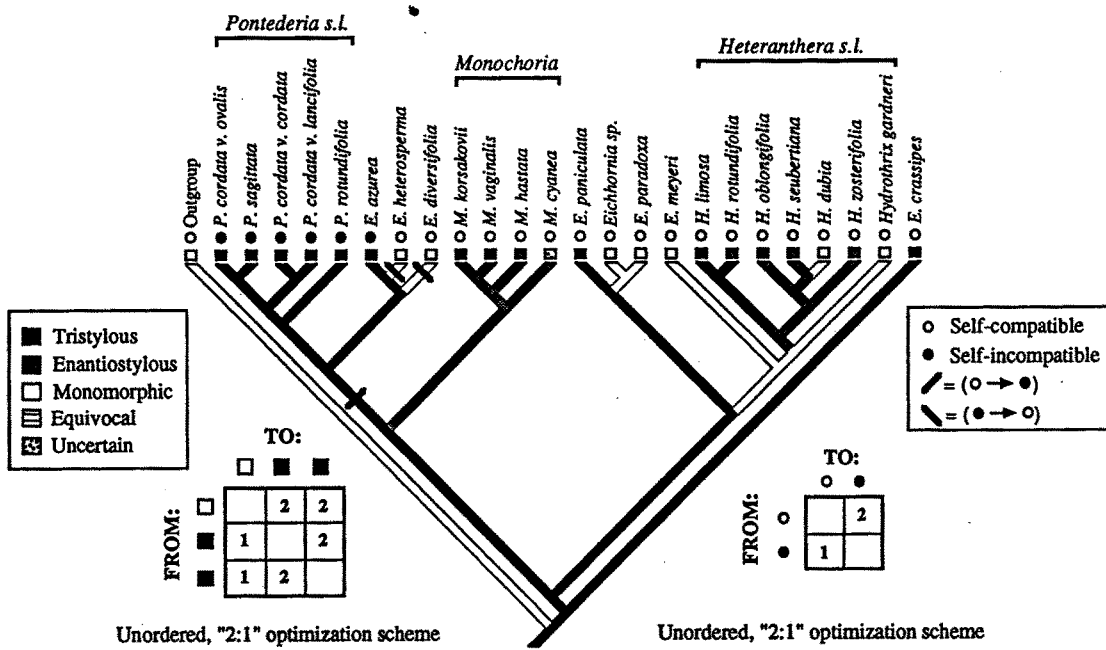
Floral Form
(8 events)

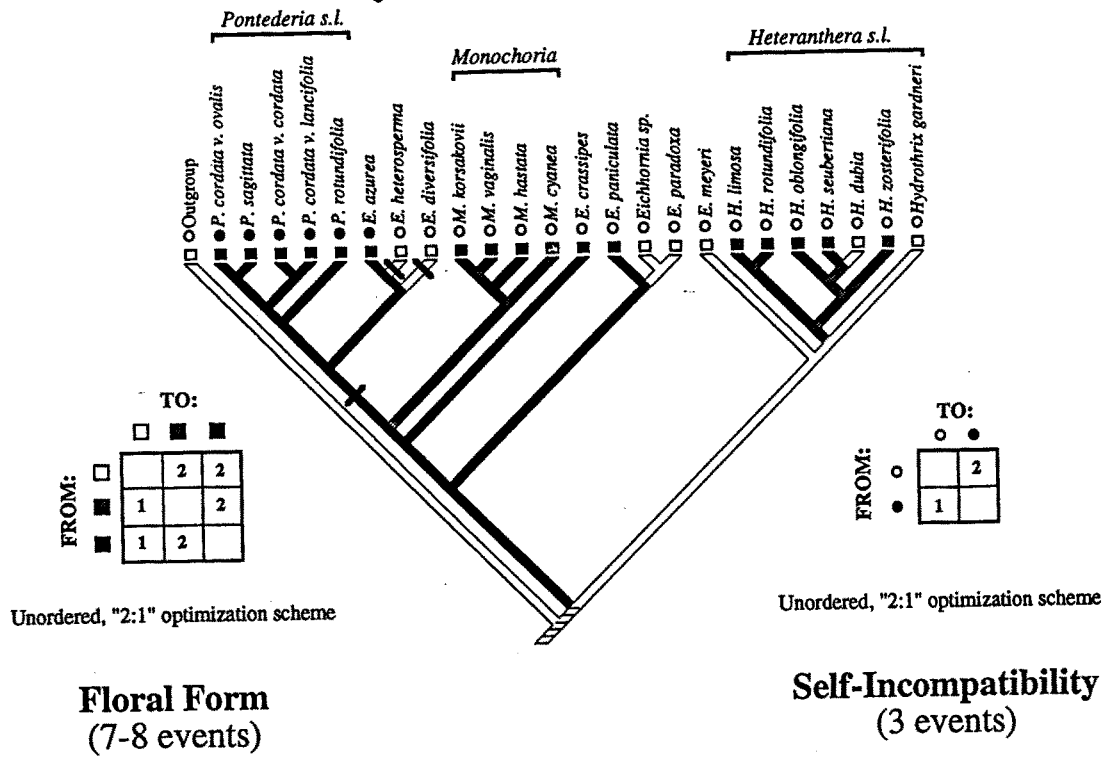**Self-Incompatibility**
(3 events)

B

Class A Topology, Outgroup Rooting
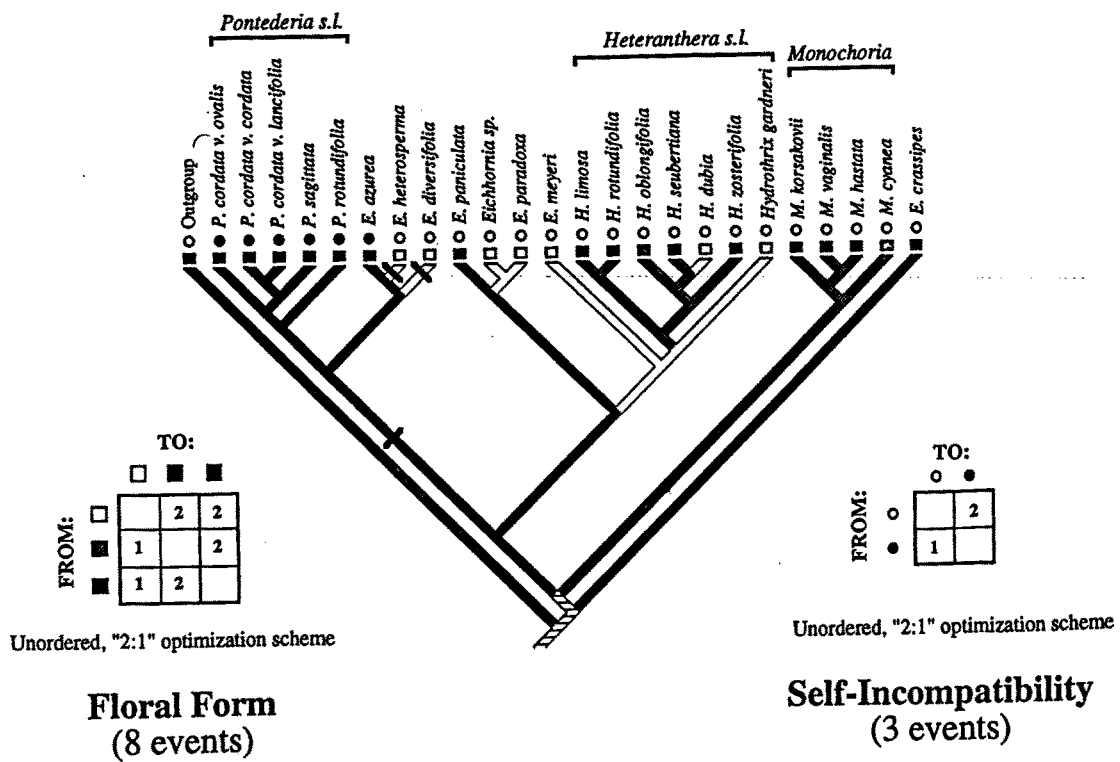
Floral Form
(7 events)

Self-Incompatibility
(2 events)

C

Class A Topology, Midpoint Rooting

Floral Form
(7-8 events)

Unordered, "2:1" optimization scheme

Self-Incompatibility
(3 events)

Unordered, "2:1" optimization scheme

D

Class B Topology, Outgroup Rooting

Floral Form
(8 events)

Unordered, "2:1" optimization scheme

Self-Incompatibility
(3 events)

Unordered, "2:1" optimization scheme

ingroup topology alone. The naive expectation that species sharing the same complex floral syndrome would be grouped together is not supported by molecular data. In fact, because of the manner in which tristylous and enantiostylous groups are interspersed on the tree, at least one of these complex polymorphisms must have multiple origins in the family regardless of root placement or ancestral floral form. Second, monomorphic species of *Eichhornia* are not monophyletic. Both the bootstrap analysis and use of the "constraints" option in PAUP confirm that trees which unite the monomorphic species of *Eichhornia* are highly unlikely.

Eckenwalder and Barrett (1986) suspected that monomorphic species of *Eichhornia* grouped together in their morphological phylogenetic reconstruction because of convergent evolution of characters associated with selection for a selfing syndrome. When morphological and molecular phylogenetic data conflict, it sometimes may be difficult to determine which form of data is more reliable. However, there is a widespread belief that the evolution of the selfing syndrome involves multiple morphological convergences (e.g. Ray and Chisaki 1957; Lloyd 1965; Ornduff 1969; Wyatt 1984; Ritland and Ritland 1989). The possibility that apparently synapomorphic morphological characters associated with selfing are actually homoplastic suggests that classes of characters not known to be involved in this syndrome, such as the cpDNA characters used here, are particularly useful when testing phylogenetic hypotheses concerning the evolution of autogamy. Based on their intuition and limited cytogenetic evidence, Eckenwalder and Barrett (1986) predicted particular associations between tristylous and homostylous species of *Eichhornia*: *E. paradoxa* with *E. paniculata*, *E. heterosperma* with *E. azurea*, and *E. diversifolia* with *E. crassipes*. Our data lend support to some of these suggestions. *Eichhornia paradoxa* and *Eichhornia* sp. represent a clade sister to *E. paniculata* while *E. heterosperma* is allied with *E. azurea*. However, *E. diversifolia* is sister to *E. azurea* and *E. heterosperma*, rather than having its suspected close relationship to *E. crassipes*.

Reconstruction of Character Evolution

Conclusions concerning the number and direction of character-state transitions require careful reconstruction of reproductive characters on the phylogenetic trees. Progress has been made in reconstructing the evolutionary history of character-state transitions despite uncertainties in outgroup identity and character state, root placement, and ingroup structure. These difficulties make it hard to determine the precise points of particular evolutionary shifts, but the following broad conclusions can be drawn: (1) tristyly broke down to homostyly on multiple occasions within the family; (2) instances of enantiostyly in *Monochoria* and *Heteranthera* are not homologous; (3) there was probably one, but perhaps also a second, origin of tristyly; and (4) heteromorphic incompatibility arose after the origin of floral trimorphism.

Optimization Schemes.—The conclusions stated above are sensitive to whether character-state transformations are uniformly or nonuniformly weighted (Table 1a,b; Fig. 4a–d). They largely depend upon acceptance of the unequally

weighted optimization scheme for floral form. How can we justify choosing between different optimization scenarios?

In the absence of external evidence concerning the relative difficulty of particular evolutionary shifts, it may be operationally simpler to assume an equally weighted optimization scheme. However, equally weighted optimization schemes are themselves strong assumptions (Swofford and Olsen 1990). Several lines of evidence indicate that the origin of tristyly is an improbable occurrence relative to its evolutionary dissolution. Tristyly is perhaps the most complex breeding system in flowering plants. Its relatively elaborate developmental basis (Richards and Barrett 1992) and great rarity within the angiosperms (Barrett 1993) certainly argue that it is difficult to evolve. A range of microevolutionary evidence also demonstrates that, at least at the population level, tristyly readily breaks down to yield predominantly self-fertilizing floral variants both in Pontederiaceae (Barrett et al. 1989; Husband and Barrett 1992, 1993; Fenster and Barrett 1994) and other tristylous families (e.g., Stout 1925; Mayura Devi and Hashim 1966; Ornduff 1972; Eckert and Barrett 1994). Where such "external" evidence exists concerning the relative difficulty of particular evolutionary events, it would be unwise to ignore it (Maddison and Maddison 1992; p. 71). Given that all possible shifts in floral form are not equally probable, it would consequently be inappropriate to give all possible character-state transformations equal weighting during character reconstruction.

Dollo parsimony (Le Quesne 1975; Farris 1977a,b) is an extreme example of unequal weighting which traditionally has been used as a criterion for modeling the evolution of complex characters, or for using those characters in the reconstruction of phylogenies. An infinitely large cost is used to reject multiple gains of a complex character, but multiple losses are possible under the logic that complexity is much easier to lose than originate. Dollo weighting has been criticized on the grounds that the infinite penalty against homoplasy in complex structures is unrealistic (Swofford and Olsen 1990; Albert et al. 1992). Our 2:1 weighting scheme is an example of a "relaxed" Dollo criterion (Swofford and Olsen 1990) whereby single-gain, multiple-loss scenarios are preferred, but not demanded, during character reconstruction.

Linear, quadratic, or logarithmic transformations have been used to translate the probabilities of particular evolutionary events into weighting schemes (Felsenstein 1981; Williams and Fitch 1989, 1990; Wheeler 1990). The two-fold weighting imbalance we employed may correspond to greater than two-fold differences in the relative probabilities of transformation. Weighting imbalances similar to the one we use have been suggested for use when reconstructing phylogenie with molecular data (e.g., Albert et al. 1992). There is empirical evidence that different classes of change at individual nucleotides or restriction sites occur at different rates. The origin of a floral polymorphism such as tristyly, or a physiological mechanism such as self-incompatibility, is assuredly a much more complex evolutionary event than a substitution event in a DNA sequence, but it is less obvious how to rate the evolutionary difficulty of the origin or dissolution of such characters.

With equal weighting, tristyly may arise up to four times in the family (Table 1a; Fig. 4b). Under the 2:1 scheme for

shifts in floral form, a dramatic shift occurs in the pattern of reconstructed events; Tristyly is gained only once or perhaps twice, but lost on multiple occasions (Table 1b; Fig. 4a,c,d). A greater than two-fold imbalance does not change this conclusion (S. W. Graham et al., unpubl. results), and less than two-fold biases can still suffice to reject multiple origins of tristyly in Pontederiaceae (Graham and Barrett 1995). The boundary weighting imbalance needed to yield this shift depends on details of tree structure, root position, and outgroup coding (S. W. Graham et al., unpubl. results), but clearly the bias required to favor the scenario with a single origin of tristyly over multiple origins is not very large.

Enantiostyly is also a relatively elaborate floral form. We treated both floral polymorphisms (tristyly and enantiostyly) equivalently in the 2:1 weighting scheme, i.e., two steps for their gain, and one step for a shift to floral monomorphism. Interconversion between them was also assigned two steps. This approach is conservative in that it does not downwardly weight gains of enantiostyly, compared to gains of tristyly, even though enantiostyly is arguably a less complex floral polymorphism than is tristyly. Although the same pattern of weights was assigned to shifts involving the two polymorphic floral forms, in all reconstructions enantiostyly is homoplastic, whereas in most cases instances of tristyly are homologous across the tree. At least one shift between tristyly and enantiostyly is necessary in all reconstructions examined (Table 1b).

Outgroup Identity and Coding.—We assessed reconstructions with the outgroup coded, in turn, as each of the three floral forms seen in the family. However, tristyly is an improbable candidate character state for the outgroup. Apart from an isolated case in the genus *Narcissus* (Amaryllidaceae) (Fernandes 1935; Lloyd et al. 1990; Barrett et al. 1996), it has not been reported in other monocotyledonous families. Several potential outgroup families are enantiostylous or include enantiostylous taxa (e.g., Commelinaceae, Haemodoraceae, Philydraceae; see Graham and Barrett 1995), and floral monomorphism of one form or another is the predominant condition within the monocotyledons. Employing different outgroup codings had a marginal effect on the broad pattern of reconstructed shifts in the family, compared with changing the optimization scheme (Table 1a,b). One obvious effect is on the reconstructed primitive floral condition in the family. This was strongly influenced by the character state assigned to the outgroup, but was not always the same as the outgroup state (Table 1b). The issue of the primitive floral condition will remain unresolved until we have a clearer idea of the phylogenetic placement of Pontederiaceae within the monocotyledons.

Uncertainty in Tree Topology.—We performed character reconstructions using the two most-parsimonious positions for *Monochoria* relative to *E. crassipes,* and two alternative root positions: the most-parsimonious rooting and the midpoint of ingroup taxa. As with outgroup coding, the topological differences analyzed had little effect on broad patterns of character evolution, in comparison to the effect of the different optimization schemes. Under only one combination of topology and outgroup coding was tristyly found to be potentially homoplastic, while enantiostyly was homoplastic under all optimizations using 2:1 weighting. In addition, mul-

tiple transitions from tristyly to homostyly were always inferred (Table 1b).

Origin of Self-Incompatibility.—Whether its gain is equally or unequally weighted, self-incompatibility is shown to arise after the one or two origins of floral trimorphism inferred when shifts in floral form are unequally weighted. Models that posit the origin of incompatibility before floral heteromorphism (Charlesworth and Charlesworth 1979; Charlesworth 1979) thus fail to find support, since self-compatibility can be a primitive condition among tristylous species. This finding contradicts the common assumption that self-compatibility in heterostylous taxa must necessarily represent a secondary loss from a self-incompatible ancestor, although reversions to self-compatibility are required in scenarios where a single gain of incompatibility is observed (Fig. 4).

However, there are at least two potential problems in accepting this reconstruction of events. First, bootstrap analysis indicates that the position of the root is uncertain, and many root positions are possible with only slight increases in tree length. For the present study we examined only two rootings in detail. Attaching the outgroup taxa at the midpoint requires only three extra steps (495 steps or 0.6% longer) compared to the most-parsimonious root position (492 steps), but other nearly optimal root positions are possible (S. W. Graham et al., unpubl. data). Not all root positions necessarily lead to the conclusion found here concerning the order of origin of floral trimorphism and heteromorphic self-incompatibility. This issue will be explored in detail in a future paper.

Weller et al. (1995) treat self-incompatibility as a qualitative character during the reconstruction of its evolutionary history, as we do here. However, there is a growing body of evidence indicating that the expression of self-incompatibility can vary quantitatively, and in these cases can be difficult to distinguish from inbreeding depression (Charlesworth 1985; Seavey and Bawa 1986; Barrett 1988b). Where incompatibility is quantitative, some judgment is required in classifying species as self-incompatible or self-compatible (reviewed in Rigney et al. 1993). In our study, the self-compatibility status of taxa was based upon the criterion of full or near full ($\geq$ 75%) seed set upon selfing. We coded *E. paniculata* and *E. crassipes* as self-compatible because they satisfy this criterion. However, more detailed studies of pollen-pistil interactions in *E. paniculata* indicate that this species exhibits cryptic self-incompatibility (sensu Bateman 1956). In all three style morphs, outcross pollen from the same level as the stigma has higher siring success than self pollen or outcross pollen from other anther levels. This pattern may represent a weaker, but homologous, form of the strong incompatibility found in species of *Pontederia* (Cruzan and Barrett 1993). These considerations suggest that incompatibility might better be treated as a quantitative rather than a qualitative character in phylogenetic analysis. Quantitative measures of the strength of the incompatibility reaction in all tristylous taxa would be needed to utilize comparative methods developed for quantitative traits (e.g., Harvey and Pagel 1991, and references therein). Such measures, particularly competitive abilities of different pollen types when placed in mixtures on stigmas, are unavailable for most of the taxa under study. However, even if quantitative data were available for each species, it seems unlikely that the weak

· incompatibility found in *E. paniculata* and perhaps *E. crassipes*, would be derived relative to the strong incompatibility expressed in *E. azurea* and *Pontederia s. l.*, given the topologies examined in this study.

The Evolution of Selfing in Eichhornia.—All reconstructions using 2:1 weighting indicate that at least three transitions from tristyly to homostyly have occurred. Stebbins (1957) suggested that selfing species, because of low genetic variability, were often evolutionary dead ends and rarely gave rise to new phyletic lines. If true, phylogenetic reconstructions generally should show relatively short branch lengths between selfing taxa and their outcrossing relatives, and speciation should be rare within selfing lineages. Recent work by Schoen et al. (unpubl.) on the phylogeny of *Amsinckia* is consistent with this view. Their work suggests that there were multiple shifts from distyly to homostyly in the genus, and they found little evidence of either great longevity of selfing lineages or speciation within these lineages. Our data, in contrast, are not entirely consistent with this view.

Homostyly is probably a shared derived condition for *E. paradoxa* and *Eichhornia* sp. (e.g., Fig. 4a,c,d), indicating that speciation occurred within this lineage subsequent to the origin of self-fertilization. Under some reconstructions (e.g., Fig. 4b,c) the floral monomorphism found in *E. meyeri* may be a retained primitive condition, whereas in others, it arises as a consequence of the loss of tristyly (e.g. Fig. 4a,d). Unlike other selfing species of *Eichhornia*, all six stamens are at the level of the stigma in *E. meyeri* and it does not possess "residual" pollen heteromorphism (Barrett 1988a). These distinctive features could either be a consequence of a substantial time depth since the loss of tristyly, or may simply reflect a retained primitive monomorphic condition for this lineage. Regardless of which interpretation is correct, it is clear from the lengths of the branches following the origin of monomorphism (Figs. 2, 4) that monomorphism is of ancient origin in this lineage. Both speciation within one monomorphic lineage and the ancient origin of monomophism in another indicate that not all selfing species are phylogenetically evanescent (see also Armbruster 1993).

Conclusion

Uncertainties in a variety of factors can impede the reconstruction of character evolution. Our study investigated the effect of a number of these factors on the reconstruction of historical shifts in floral syndromes in Pontederiaceae. The choice of optimization scheme is perhaps the most critical issue when mapping evolutionary shifts, more important in our study than the topological uncertainties we encountered. Just as microevolutionary studies should not be performed without reference to phyletic history, phylogenetic investigations of character-state changes need to be integrated with other lines of biological evidence concerning the likelihood of occurrence of particular evolutionary transitions. Considerable genetic, developmental, and ecological information is available for many heterostylous groups, and hence these taxa and their reproductive adaptations provide useful model systems for the analysis of character evolution.

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APPENDIX

Source and locality of specimens used for analysis of chloroplast DNA restriction site variation.

Taxon	[1]Source	Locality (collector)
Eichhornia azurea (Swartz) Kunth	-na-	In cultivation at Van Ness Water Gardens, Upland, California, U.S. (S. C. H. Barrett)
E. crassipes (Mart.) Solms-Laub.	Barrett 814	Boca de Jari, Pará, Lower Amazon Basin, Brazil (S. C. H. Barrett)
E. diversifolia (Vahl) Urb.	Barrett 1122	Boca de Jari, Pará, Lower Amazon Basin, Brazil (S. C. H. Barrett)
E. heterosperma (Alex.)	Barrett 1400	Quixadá, Ceará, Brazil (S. C. H. Barrett)
E. meyeri Schulz	Barrett 1409	Nuevo Asunción, Paraguay (Billiet & Jadin)
E. paniculata (Spreng.) Solms-Laub.	Barrett 1401	Population B46, Quixadá, Ceará, Brazil (S. C. H. Barrett)
E. paradoxa (Mart.) Solms-Laub.	Barrett 1402	Patos, Paraíba, Brazil (S. C. H. Barrett)
[2]*Eichhornia* sp.	Barrett and Shore 1399	Propriá, Sergipe, Brazil (S. C. H. Barrett and J. S. Shore)
Heteranthera limosa (Swartz) Willd.	Barrett 1054	Cañas, Guanacaste Province, Costa Rica (S. C. H. Barrett)
H. oblongifolia Mart.	Barrett and Shore 1402	Pto. Real do Colegio, Alagoas, Brazil (S. C. H. Barrett and J. S. Shore)
H. rotundifolia Griseb.	Barrett 1411	Pernambuco, Brazil (S. C. H. Barrett)
H. seubertiana Solms-Laub.	Barrett 1412	Pernambuco, Brazil (S. C. H. Barrett)
H. zosterifolia Mart.	Barrett 1413	Brazil (J. Bogner)
H. (Zosterella) dubia (Jacq.) MacMill.	Barrett and Graham 1406	Queens University Biology Stn., Chaffeys Lock, Ontario, Canada (S. C. H. Barrett)
Hydrothrix gardneri Hook f.	Barrett 1414	Brazil (J. Bogner)
[3]*Monochoria cyanea* (F. Muell.) F. Muell.	-na-	Tortilla Flats, Northern Territory, Australia (S. MacIntyre)
M. hastata (L.) Solms-Laub.	Barrett 1407	In cultivation at Singapore Botanical Garden, Singapore ex Royal Botanic Gardens, Kew, U.K. (S. C. H. Barrett)
M. korsakovii Reg. and Maack	Barrett 1415	Japan (T. Morita)
M. vaginalis (Burm f.) Presl	Barrett 1111	Rice field, Biggs Rice Research Stn., Butte County, California, U.S. (S. C. H. Barrett)
Pontederia cordata L. var. *cordata*	Barrett 1403	Paugh Lake, near Barrys Bay, Ontario, Canada (S. C. H. Barrett)
P. cordata var. *lancifolia* (Muhl.) Torr.	Barrett 1404	Brunswick, Georgia, U.S. (S. C. H. Barrett)
P. cordata var. *ovalis* (Mart.) Solms-Laub.	Barrett 1124	Rondonópolis, Mato Grosso Brazil (S. W. Barrett)
P. sagittata Presl	Barrett 1416	Vera Cruz, Vera Cruz, Mexico (D. E. Glover)
P. (Reussia) rotundifolia L.	Barrett 1405	Boca de Jari, Pará, Lower Amazon Basin, Brazil (S. C. H. Barrett)
Chlorophytum sp. (Liliaceae)	-na-	In cultivation at Botany Greenhouse, University of Toronto, Toronto, Ontario, Canada (J. R. Kohn)
Philydrum lanuginosum Gaertner (Philydraceae)	Graham and Barrett 1	In cultivation at Botany Greenhouse, University of Toronto, Toronto, Ontario, Canada (S. W. Graham)
Tradescantia sp. (Commelinaceae)	-na-	In cultivation at Botany Greenhouse, University of Toronto, Toronto, Ontario, Canada (J. R. Kohn)

[1] Each voucher (deposited at the Vascular Plant Herbarium, Botany Department, Royal Ontario Museum, Toronto, Ontario, Canada) is a representative individual of material under cultivation at Toronto. Source populations are listed in the locality column. One to several individuals from each population were used for DNA extractions.

[2] An undescribed species of *Eichhornia* (referred to here as *Eichhornia* sp.) was identified in Eckenwalder and Barrett (1986) as *E. paradoxa*.

[3] DNA extracted from pooled seedlings grown from seed from this source.