

Research review

Evolutionary pathways to self-fertilization in a tristylous plant species

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Summary

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Evolutionary transitions from outcrossing to selfing occur commonly in heterostylous genera. The morphological polymorphisms that characterize heterostyly provide opportunities for different pathways for selfing to evolve. Here, we investigate the origins and pathways by which selfing has evolved in tristylous *Eichhornia paniculata* by providing new evidence based on morphology, DNA sequences and genetic analysis. The primary pathway from outcrossing to selfing involves the stochastic loss of the short-styled morph (S-morph) from trimorphic populations, followed by the spread of selfing variants of the mid-styled morph (M-morph). However, the discovery of selfing variants of the long-styled morph (L-morph) in Central America indicates a secondary pathway and distinct origin for selfing. Comparisons of multi-locus nucleotide sequences from 27 populations sampled from throughout the geographical range suggest multiple transitions to selfing. Genetic analysis of selfing variants of the L- and M-morphs demonstrates recessive control of the loss of herkogamy, although the number of factors appears to differ between the forms. Early stages in the establishment of selfing involve developmental instability in the formation of flowers capable of autonomous self-pollination. The relatively simple genetic control of herkogamy reduction and frequent colonizing episodes may often create demographic conditions favouring transitions to selfing in *E. paniculata*.

Introduction

In many herbaceous plants, morphological and physiological traits that function to reduce the incidence of inbreeding have been lost, leading to high rates of self-fertilization and evolution of the 'selfing syndrome' (Lloyd, 1965; Ornduff, 1969; Ritland & Ritland, 1989; Armbruster *et al.*, 2002). The origin of high levels of self-fertilization (autogamy) from obligate cross-fertilization has been considered the most frequent evolutionary transition in flowering plants (Stebbins,

1974). There are no estimates of the number of origins of selfing, but it is likely that this transition has occurred many hundreds of times. Most transitions probably go undetected because selfing lineages are often short lived and usually occur at the tips of phylogenetic trees (Schoen *et al.*, 1997; Takebayashi & Morrell, 2001; Igic *et al.*, 2008).

The paradox of why selfing should evolve, given its limited evolutionary future and the generally poorer performance of selfed offspring relative to outcrossed offspring, has been an enduring source of curiosity for evolutionary biologists.

Charles Darwin (1878) drew attention to the reproductive advantage possessed by selfing individuals when pollinators are scarce owing to 'reproductive assurance'. Later, Fisher (1941) pointed out that genes that increase the rate of selfing have a transmission bias when they arise in an outcrossing population (Jain, 1976). Since then, this transition has been the focus of considerable theoretical and empirical research aimed at understanding how and why selfing evolves from outcrossing (reviewed in Lloyd, 1980; Uyenoyama *et al.*, 1993; Holsinger, 1996).

Selfers are represented in many floras, especially those in which there is a marked dry season and an abundance of ephemeral habitats. Selfing species are commonly annual and many have prolific colonizing ability and extensive geographical ranges (Jain, 1976; Lloyd, 1980; Barrett *et al.*, 1996). This is associated with the ability of individuals to establish at low density or following long-distance dispersal (Baker, 1955; Pannell & Barrett, 1998). The evolution of selfing from outcrossing can therefore have important ecological, demographic and biogeographical consequences.

Heterostylous groups provide a rich source of reproductive diversity for investigating the evolution of selfing. In many heterostylous taxa, obligate outcrossing, enforced by heteromorphic self-incompatibility, has been replaced by predominant selfing as a result of the origin and spread of self-compatible homostyles (distyly) or semi-homostyles (tristyly). These floral forms have the capacity for autonomous self-pollination because they either have all anthers (homostyles), or one set of anthers (semi-homostyles), closely adjacent to stigmas. The transition to predominant selfing in heterostylous groups is evident from phylogenetic analysis (for example, Pontederiaceae, Kohn *et al.*, 1996; *Amsinckia*, Schoen *et al.*, 1997; *Primula*, Mast *et al.*, 2006), and at the intraspecific level through population-level studies (Crosby, 1949; Ornduff, 1972; Ganders, 1975). Homostyles often occur at range margins, or on islands, a finding consistent with their ability to produce seed in the absence of pollinators or mates (Baker, 1966; Barrett, 1985; Barrett & Shore, 1987). This pattern implicates reproductive assurance as playing an important role in the selection of homostyly.

Heterostylous populations are reproductively subdivided into two (distyly) or three (tristyly) morphologically distinct mating groups. The pathways from outcrossing to selfing therefore have the potential to be more diverse than in non-heterostylous species because of sexual polymorphism. For example, the floral morphs could participate equally in the breakdown process, or, as occurs in some distyly taxa (for example, *Primula*, Charlesworth & Charlesworth, 1979; *Turnera*, Barrett & Shore, 1987), a particular floral morph may be favoured because of distinctive features of its morphology, genetics or mating ability. The morphological and physiological polymorphisms that characterize the heterostylous syndrome therefore provide opportunities for different mechanisms and pathways for selfing to evolve.

Here, we review what is known about the evolution of selfing from outcrossing in *Eichhornia paniculata* (Pontederiaceae), an

annual neotropical tristylous species. Populations of *E. paniculata* occupy ephemeral aquatic habitats, including ponds, drainage ditches, wet pastures and rice fields. The main concentration of populations occurs in north-east Brazil, with smaller foci in Jamaica and Cuba and isolated disjunct populations in Nicaragua and Mexico. In tristylous species, the three floral forms are known as the long-, mid- and short-styled morphs (hereafter L-, M- and S-morphs). *Eichhornia paniculata* has been the subject of extensive research on the ecological and demographic factors responsible for mating system variation (reviewed in Barrett *et al.*, 1992). To provide a context for our current investigations, we briefly summarize earlier studies on the evolutionary processes responsible for the breakdown of tristily. We then present new biogeographical and molecular genetic evidence on the evolutionary pathways by which selfing has evolved based on a wide geographical sampling of populations. In addition, using controlled crosses, we examine the inheritance of mating system modification and describe the expression of selfing modifiers when they first appear in populations.

Evolutionary pathways to selfing

Large-scale surveys of the frequency of style morphs in populations of *E. paniculata* in north-east Brazil and Jamaica have provided evidence for a primary evolutionary pathway from outcrossing to selfing that has been followed repeatedly (Barrett *et al.*, 1989, Fig. 1; Husband & Barrett, 1993). In north-east Brazil, stilar trimorphism is the most common condition, followed by dimorphism and monomorphism. Most dimorphic populations are missing the S-morph and contain the L-morph and selfing variants of the M-morph (Fig. 2a). Monomorphic populations are dominated by selfing variants of the M-morph. Surveys indicate that approximately 30% of populations are missing at least one style morph, and genetic drift and founder events are largely responsible for the loss of morphs from populations (Husband & Barrett, 1992a,b). Populations missing morphs are significantly smaller than those that are tristylous, and the observed pattern of S-morph loss is that predicted by stochastic models of the influence of finite population size on the two-diallelic locus (*S*, *M*) genetic system controlling tristily (reviewed in Barrett, 1993).

Style morph frequencies on the island of Jamaica are distinct from those in north-east Brazil (Barrett, 1985; Barrett *et al.*, 1989; Husband & Barrett, 1991). No tristylous populations are known in Jamaica and most populations are composed exclusively of selfing variants of the M-morph. A few populations contain low frequencies of the L-morph. Recent surveys of style morph frequencies in Jamaica (2007) and Cuba (2008) confirm these overall patterns; most populations were fixed for selfing variants of the M-morph, and a low frequency of the L-morph occurred in a single population on both islands (Table 1). The absence of the S-morph from the Caribbean is probably the result of a founder event associated with long-distance dispersal from Brazil.

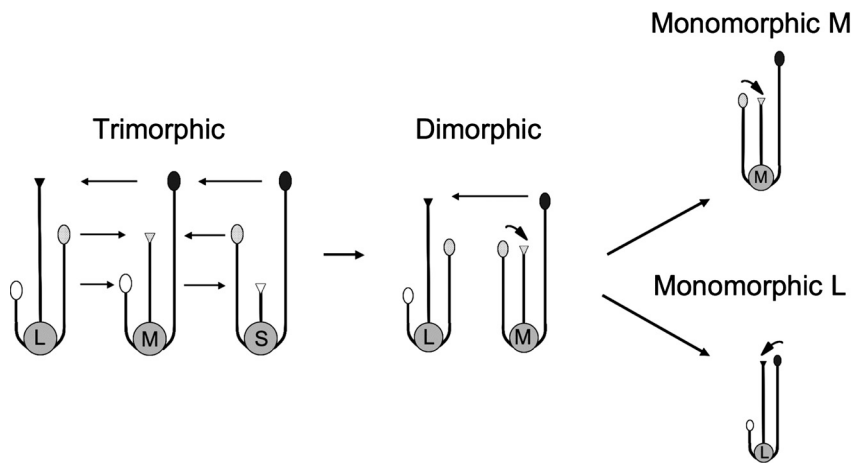


Fig. 1 Evolutionary pathways from cross-fertilization to self-fertilization in tristylous *Eichhornia paniculata* via the origins of semi-homostyly. The primary pathway from trimorphism to dimorphism culminates in the fixation of selfing semi-homostylous forms of the M-morph. A less common pathway leads to populations monomorphic for semi-homostylous forms of the L-morph. Arrows linking anthers and stigmas indicate mating combinations; those linking floral phenotypes indicate evolutionary transitions. The trend to smaller reproductive organs with increased selfing reflects reductions in flower size.

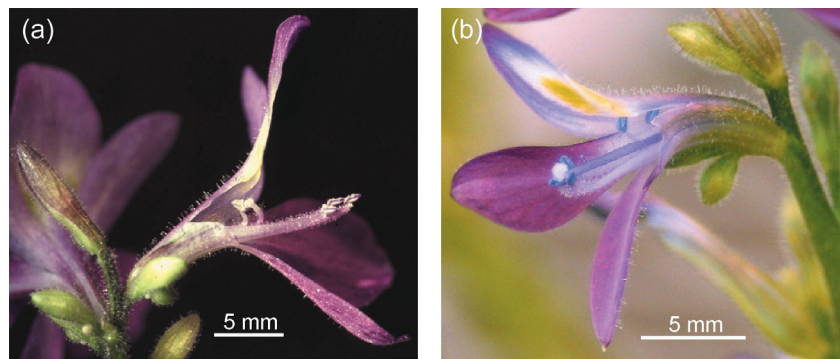


Fig. 2 Two selfing variants of *Eichhornia paniculata* illustrating their contrasting positions of sexual organs. (a) In the M-morph, the first stage in the transition to selfing involves elongation of a single stamen from short-level stamens, so that it takes up a position next to mid-level stigmas, resulting in autonomous self-pollination. Plants with this phenotype are very common in dimorphic populations from north-east Brazil where the individual illustrated originated. (b) Semi-homostylous flower of the L-morph from Mexico. All three stamens are adjacent to the stigma, resulting in autonomous self-pollination. Plants from Nicaragua possess sexual organs in similar positions.

Table 1 Style morph frequencies and the size of *Eichhornia paniculata* populations on the islands of Jamaica and Cuba surveyed in 2007 and 2008, respectively

Population	Locality	Habitat	Population size	Floral morph frequency	
				L-morph	M-morph ¹
Jamaica					
J28	Treasure Beach, St Elizabeth	Wet pasture	200	0.03	0.97
J29	Fullerswood, St Elizabeth	Marsh	28	0	1.0
J30	Catabo, St. Elizabeth	Seasonal pond	20	0	1.0
J31	Slipe, St. Elizabeth	Wet pasture	4	0	1.0
J32	Little London, Westmoreland	Sugar cane field	25	0	1.0
Cuba					
C1	Yara, Granma	Rice field	500	0	1.0
C2	Manzanillo, Granma	Rice field	800	0.15	0.85
C3	Chorrera, Granma	Rice field	120	0	1.0
C4	Baracoa, Guantánamo	Drainage ditch	10	0	1.0
C5	Camelote, Camagüey	Rice field	600	0	1.0

Surveys were conducted following the methods described in Barrett *et al.* (1989).

¹ All plants of the M-morph are semi-homostylous.

Of particular significance is the observation that, in dimorphic populations in both Jamaica and Cuba, the L-morph exhibits conspicuous herkogamy. This limits opportunities for autonomous self-pollination in contrast with selfing variants of the M-morph. The morph-specific difference in herkogamy has a profound influence on mating patterns and fertility of the two morphs. Plants of the L-morph are largely cross-pollinated by the M-morph, but have low seed set, whereas those of the M-morph produce large quantities of self-fertilized seed (Fig. 1; Barrett *et al.*, 1989). Theoretical models of selection in dimorphic populations indicate that this asymmetrical mating system is difficult to maintain and that fixation of selfing variants occurs under most conditions (Barrett *et al.*, 1989; Pannell & Barrett, 2001).

Collectively, these style morph surveys, in combination with field studies of fitness components, support a pathway involving two main stages: (1) stochastic loss of the S-morph from trimorphic populations through drift and founder events; (2) selective loss of the L-morph by spread and fixation of self-pollinating variants of the M-morph as a result of automatic selection and reproductive assurance. The pathway to selfing represents an example of how a transition from outcrossing to selfing can be triggered by genetic drift. It has been identified as one of the few cases that meet several of the key conditions in Sewall Wright's shifting balance theory of evolution (Coyne *et al.*, 1997).

Additional exploration in Central America has revealed a second pathway for the evolution of selfing in *E. paniculata* (Fig. 1). Single isolated populations in Nicaragua and Mexico were both composed of a single floral phenotype not observed elsewhere in the range. At the present time, only a single population is known from Mexico and three from Nicaragua (A. Novelo R, pers. comm., Instituto de Biología, Universidad Nacional Autónoma de México, Mexico). Plants in both populations that were sampled are semi-homostylous forms of the L-morph, rather than the M-morph (Fig. 2b). They possess purple pigmentation of styles, a feature typical of the L-morph, and, when crossed to homozygous plants of the M-morph (*ssMM*), the progeny are uniformly mid-styled. This demonstrates that they are derived from the L-morph rather than from the other two style morphs (S. C. H. Barrett, unpublished). The Nicaraguan and Mexican populations exhibit the smallest flowers observed in *E. paniculata*, and 'mid-level anthers' in these populations are in contact with stigmas of the long style. Plants from these populations grown under pollinator-free glasshouse conditions in Toronto produce 100% fruit set as a result of autonomous self-pollination. Although we have not measured mating patterns in these populations, they are likely to be highly autogamous, given their strong facility for autonomous self-pollination. The origin of these semi-homostylous L-morph populations is unclear. They could be descended from a single long-distance dispersal event from north-east Brazil or, more likely, from a dimorphic population from the nearby Caribbean islands of Jamaica

or Cuba. Molecular evidence presented below is equivocal on this question.

Multiple origins of selfing

Evidence from genetic relations among populations

Patterns of allozyme variation at 24 loci from 44 populations of *E. paniculata* from north-east Brazil provided evidence for multiple origins of selfing in the M-morph (Husband & Barrett, 1993). Selfing populations were dispersed across a dendrogram of genetic relationships and, for the most part, were more genetically similar to nearby trimorphic populations than to more geographically distant selfing populations. Here, we present additional evidence for multiple transitions to selfing using DNA sequence data from 10 expressed sequence tag (EST)-derived nuclear loci sampled from 4–12 individuals from each of 27 populations, for a total of 229 individuals [for detailed localities of the populations sampled and methods, see Table S1 and Methods S1 (Supporting Information), respectively]. The populations investigated here represent a broader geographical sample and involve different populations from those investigated previously. We have not estimated outcrossing rates in these populations, but assume, based on our previous work, that the morph structure of populations (trimorphic, dimorphic, monomorphic), especially the occurrence of self-pollinating variants in all monomorphic populations sampled, has a major influence on their mating patterns (see Barrett & Husband, 1990; Barrett *et al.*, 1992).

Using the program SplitsTree (Huson & Bryant, 2006), we estimated the network of genetic relationships among populations by randomly sampling one individual per population and concatenating all 10 loci (Fig. 3). In a neighbour network, single nucleotide polymorphisms (SNPs) that are not consistent with a simple bifurcation process, either as a result of incomplete lineage sorting or recombination, are represented by reticulation in the network. This method is more appropriate for depicting the genetic relationships among sequences for a species with recombination than are methods of historical reconstruction that assume a bifurcating process of divergence. To ensure that our results were robust, we also generated the neighbour network for all 229 individuals in our sample. The resulting network was qualitatively similar to that displayed in Fig. 3. We also ran the SplitsTree program for each of the 10 loci individually, and in seven of the 10 cases the same overall topology was evident. The three instances in which this was not the case involved sequences with fewer informative segregating sites. In addition, we tested other phylogenetic methods [for example, Bayesian analysis (Mr. Bayes), neighbour joining and parsimony (PAUP*)] by concatenating all sequences for each individual. The trees obtained from each of these methods were concordant with the overall patterns obtained in Fig. 3, with four deep clusters separating the major geographical regions occupied by *E. paniculata*.

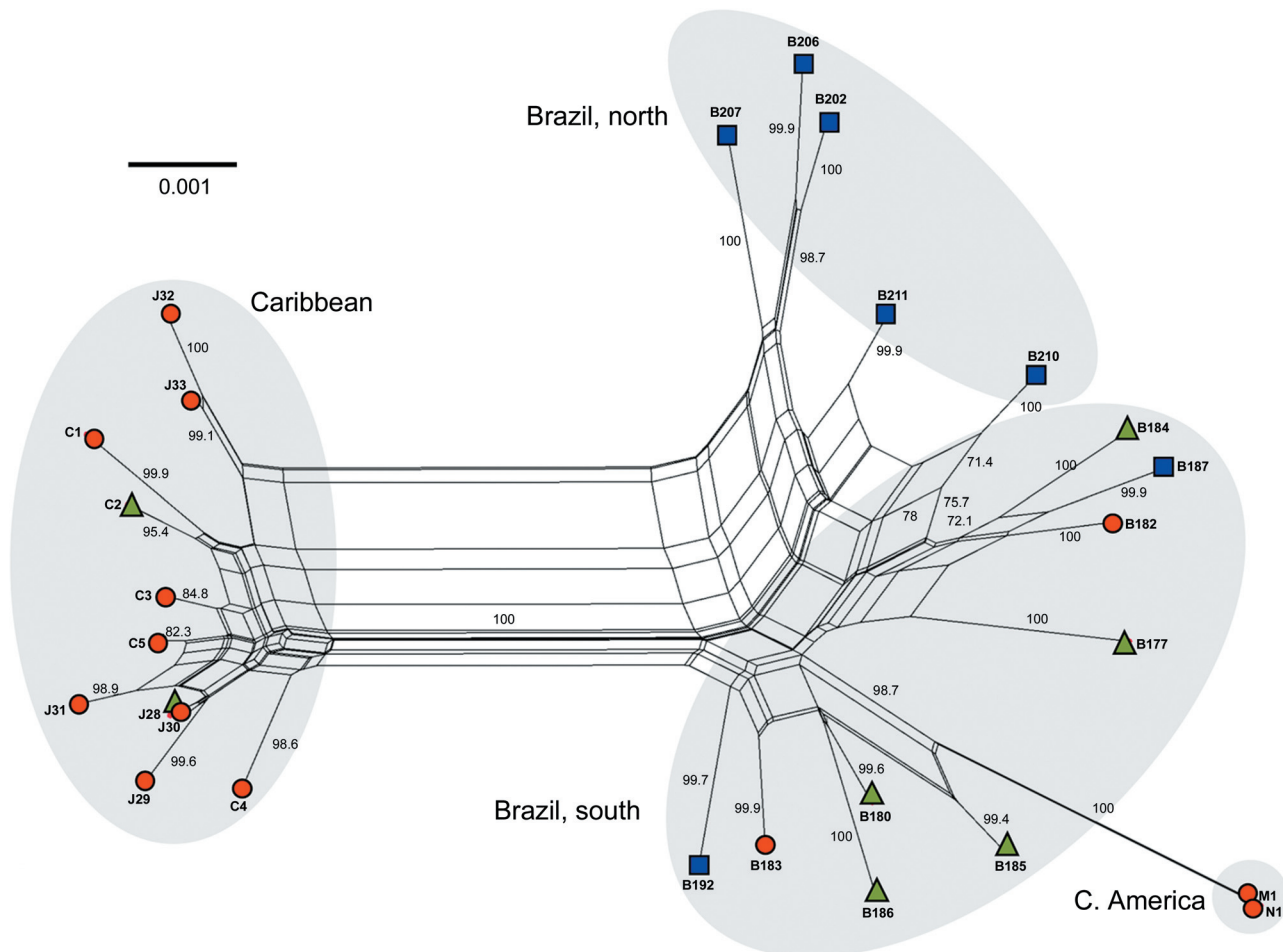


Fig. 3 Neighbour network of 10 expressed sequence tag (EST)-derived nuclear loci displaying relationships among 27 populations of *Eichhornia paniculata* sampled throughout the geographical range. Population codes are indicated at the branch tips. Brazilian, Cuban, Jamaican, Mexican and Nicaraguan populations are represented by codes that begin with B, C, J, M and N, respectively. The morph structure of populations is indicated with squares, triangles or circles, representing trimorphic, dimorphic or monomorphic populations, respectively. We constructed the network with the program SplitsTree (Huson & Bryant, 2006) by randomly sampling one individual per population and concatenating the sequences from all 10 loci. Bootstrap values from 10 000 replicates are displayed for nodes with more than 70% support.

Within north-east Brazil, populations are geographically subdivided into northern and southern ranges separated by an extensive arid zone (see Fig. 2 in Barrett *et al.*, 1989). This subdivision was evident in our data with a split in the network (78.1% bootstrap support) that probably reflects a barrier to gene flow imposed by arid conditions inimical for aquatic plant growth. Within the southern cluster, selfing monomorphic populations (B182, B183) were separated on the tree and each clustered with nearby dimorphic and trimorphic populations. However, bootstrap support for relationships among the Brazilian populations in the southern range was weak, perhaps reflecting limited divergence and/or gene flow.

Populations from Jamaica and Cuba formed a distinct cluster (100% bootstrap support), indicating that they are likely to be descended from a single dispersal event to the Caribbean from mainland South America. Lastly, population samples from Mexico and Nicaragua formed a fourth cluster separated from the Brazilian and Caribbean populations by a long branch,

indicating a long period of isolation. The dispersed distribution of monomorphic populations across the network is consistent with the occurrence of multiple transitions from outcrossing to selfing.

The distribution of SNPs among populations of *E. paniculata* provides additional support that selfing has probably arisen on multiple occasions. If populations of selfers are derived from a particular source region, they are likely to be less differentiated from that source than from other selfing populations that are independently derived from a different region. To test this proposition, we used alignments of all 10 loci for all individuals, excluding the Central American plants, for which we did not have adequate population samples. Using the software package SITES (Hey & Wakeley, 1997) we estimated F_{st} for each locus among all pairs of populations (Fig. 4a). Average pairwise differentiation (F_{st}) among selfing populations from the Caribbean (CRB) ($F_{st,CRB} = 0.402$, SE = 0.053) is lower than the differentiation of these populations from

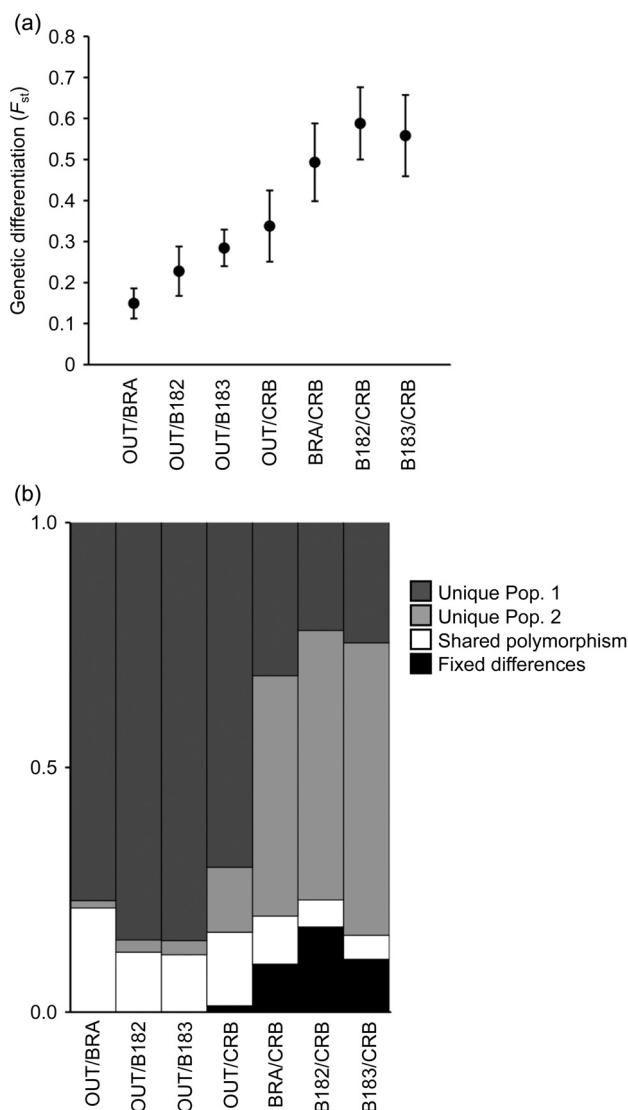


Fig. 4 Pairwise distribution of single nucleotide polymorphisms (SNPs) from 10 expressed sequence tag (EST)-derived nuclear loci from outcrossing (OUT, $n = 111$ individuals) and selfing populations of *Eichhornia paniculata*. Selfing populations from Jamaica and Cuba are combined (CRB, $n = 64$ individuals) and Brazilian populations (B182 and B183, $n = 10$ and 12 individuals, respectively) are combined in pairs as BRA. The first population group listed in the axis labels is Group 1 and the second is Group 2. (a) Mean pairwise differentiation (F_{st}) between population groups based on alignments of 10 sequences. Each point represents the mean across all loci and population pairs, with error bars indicating $\pm 1SE$. (b) Proportion of unique and shared SNPs and fixed differences between population groups. We calculated all statistics using the program SITES (Hey & Wakeley, 1997). Nucleotide sequences were collected from 4–12 individuals per population for a total of 6678 bp per individual.

selfing populations from Brazil (BRA) ($F_{st,CRBvsBRA} = 0.493$, $SE = 0.095$), but higher than the differentiation of Caribbean populations from outcrossing populations from Brazil (OUT) ($F_{st,CRBvsOUT} = 0.338$, $SE = 0.087$). Furthermore, the two monomorphic selfing populations from Brazil (B182 and

B183) are more differentiated from one another than each is from trimorphic outcrossing populations ($F_{st,B182vsB183} = 0.562$, $SE = 0.088$; $F_{st,B182vsOUT} = 0.284$, $SE = 0.060$; $F_{st,B183vsOUT} = 0.227$, $SE = 0.044$). Lastly, selfing populations from the Caribbean are more differentiated from Brazilian selfing populations (BRA) than from outcrossing populations from Brazil ($F_{st,BRAvsCRB} = 0.493$, $SE = 0.095$). These patterns would be predicted if different portions of the allelic variation segregating in outcrossing Brazilian populations were sampled during the origin of selfing populations in the Caribbean and north-east Brazil.

The proportions of fixed, shared and unique SNPs in these populations provide further insight into the patterns of differentiation (Fig. 4b); 93.4% of nucleotide polymorphisms in selfing populations from Brazil were shared with outcrossing populations, and there were no fixed differences between the groups. This pattern is consistent with the hypothesis that these populations are of relatively recent origin and represent early stages in the breakdown of tristylly. Morphological studies of these populations support this hypothesis (Vallejo-Marín & Barrett, 2009, and see below). Comparisons of nucleotide variation between selfing populations from the Caribbean and outcrossing populations from Brazil indicated that these populations share only 15% of nucleotide variation, and there were three fixed differences between the two regions (Fig. 4b). Further, comparisons of selfing populations from the Caribbean vs Brazil indicate that populations from the two regions have more fixed differences (11) and share few polymorphisms (9.1%). These patterns indicate that most of the variation in selfing populations represents different subsamples of variation maintained among outcrossing populations, a pattern consistent with multiple origins.

Evidence from genetic control of mating system modification

Feenster & Barrett (1994) used controlled crosses of selfing and outcrossing phenotypes of the M-morph from Brazil and Jamaica to investigate the inheritance of variation in herkogamy, the principal floral trait governing selfing rates in *E. paniculata*. Their results indicated that the loss of herkogamy causing selfing was fully recessive to the ancestral outcrossing condition. They also provided evidence that different recessive modifiers were responsible for the loss of herkogamy, based on crosses among selfing phenotypes from different regions, a finding consistent with multiple origins of selfing.

To investigate further the genetic basis for the loss of herkogamy, we conducted controlled crosses of outcrossing and selfing phenotypes of the M- and L-morphs. Here, we present selected crosses to illustrate general patterns. A more detailed treatment will be presented elsewhere (M. Vallejo-Marín and S. C. H. Barrett, unpublished). For the M-morph, we crossed an individual with a single stamen adjacent to the stigma (Fig. 2a) from a monomorphic selfing population to an unmodified M-morph from a trimorphic outcrossing

population. Both plants were from north-east Brazil. In the case of the L-morph, we used a semi-homostylous individual of the L-morph from Mexico (Fig. 2b) and crossed it to an unmodified (outcrossing) L-morph from a trimorphic population in Brazil. Crosses were performed in both directions, using plants in each cross as maternal and paternal parents. To generate an F₂ population, we self-fertilized a single F₁ plant per cross type, including separate F₁ parents for crosses performed in both directions. Because we found no evidence that the direction of crosses had a significant influence on the distribution of herkogamy variation, we pooled the data presented in Fig. 5.

Our results support previous findings that the loss of herkogamy in the M-morph is controlled by recessive factors. F₁ offspring strongly resembled the outcrossing parent with respect to their herkogamy values (Fig. 5). Under a simple model of monogenic or oligogenic inheritance of herkogamy modification, we would expect that a fraction of the F₂ population would recover the parental selfing phenotype. The distribution of herkogamy values in the F₂ population indicates a discontinuous distribution showing two main classes of plants: one with unmodified flowers with a mode near the outcrossing parent (> 2 mm), and a second class of individuals with herkogamy values (< 2 mm) closer to the selfing parent (Fig. 5a). The segregation ratio for these two classes of plant was 142 : 17, which is statistically different from Mendelian expectations for either one ($\chi^2 = 17.36$, 1 df, $P < 0.001$) or two ($\chi^2 = 5.35$, 1 df, $P = 0.02$) recessive gene(s) controlling the loss of herkogamy.

Crosses between selfing and outcrossing phenotypes of the L-morph also indicate that the loss of herkogamy involves the action of recessive factors. Herkogamy values in the F₁ population resembled the outcrossing parent (Fig. 5b). The distribution of herkogamy values in the F₂ generation was continuous, but included two modes: one roughly intermediate between the two parental values and a second close to values for the selfing parent. This distribution suggests quantitative genetic control, but involving the segregation of both minor and major genes.

Developmental instability in the formation of selfing flowers

An unusual feature of the early stages of the evolution of selfing in *E. paniculata* is the occurrence of plants exhibiting developmental instability in the production of flowers capable of autonomous self-pollination (Barrett, 1985; Seburn *et al.*, 1990; Barrett & Harder, 1992; Vallejo-Marín & Barrett, 2009). This conspicuous within-plant variation is a feature of the M-morph only. In some flowers, usually one, sometimes two, short-level stamens elongate to a position close to mid-level stigmas, resulting in self-pollination. In the remaining flowers, there is no modification to the positions of short-level stamens. This phenomenon results in a bimodal distribution of

herkogamy values within a plant and has the potential to promote mixed mating. To our knowledge, this type of discontinuous variation in stigma–anther separation is unique among angiosperm species and raises the question of the proximate mechanisms responsible and their ecological and evolutionary consequences.

The genetic basis of developmental instability in *E. paniculata* is not well understood, but it seems probable that it is associated with inbreeding. To initiate investigations on the inheritance of developmental instability, we self-pollinated an individual of the M-morph that displayed a bimodal distribution of herkogamy values for two generations. In the S₂ generation ($n = 75$ plants), we measured the stigma–anther separation on an average of 9.7 (SE = 0.25) flowers per plant and classified them as either modified (herkogamy < 2 mm) or unmodified (herkogamy ≥ 2 mm). The frequency distribution of herkogamy values among all flowers produced by S₂ plants was strongly bimodal (Fig. 6a), resembling the distribution observed in the parental plant (B189-7-1 from Fig. 5 in Vallejo-Marín & Barrett, 2009). However, despite all S₂ plants sharing the same level of inbreeding, there was considerable variation among individuals in the proportion of modified flowers. The majority of plants (67%) produced only one class of flower, either modified (31%) or unmodified (36%), whereas the remaining plants (33%) displayed instability in herkogamy (Fig. 6b).

The expression of herkogamy in *E. paniculata* is also influenced by environmental factors. Using cloned genotypes grown under different resource levels, Barrett & Harder (1992) and Vallejo-Marín & Barrett (2009) were able to modify the frequency of self-pollinating flowers. Under more stressful conditions, plants produced more flowers with near-zero herkogamy values. Vallejo-Marín & Barrett (2009) further demonstrated genetic variation in the level of plasticity in stigma–anther separation using genotypes sampled from trimorphic, dimorphic and monomorphic populations from north-east Brazil. Reductions in water availability, nutrients and pot size resulted in significantly smaller herkogamy values in some, but not all, genotypes. Plants from trimorphic and monomorphic populations exhibited much less developmental instability in herkogamy. By contrast, plants from dimorphic populations produced a mixture of flowers with ‘high vs low’ herkogamy values. These results obtained under uniform glasshouse conditions confirm field observations, indicating that developmental instability is most common in dimorphic populations where selfing modifiers first spread.

Discussion

‘Some species exist under two forms, the one bearing conspicuous flowers adapted for cross-fertilisation, the other bearing inconspicuous flowers adapted for self-fertilisation’

Charles Darwin (1878, p. 445)

As recognized by Darwin (1878), some plants exhibit intraspecific variation in mating system, including predominant

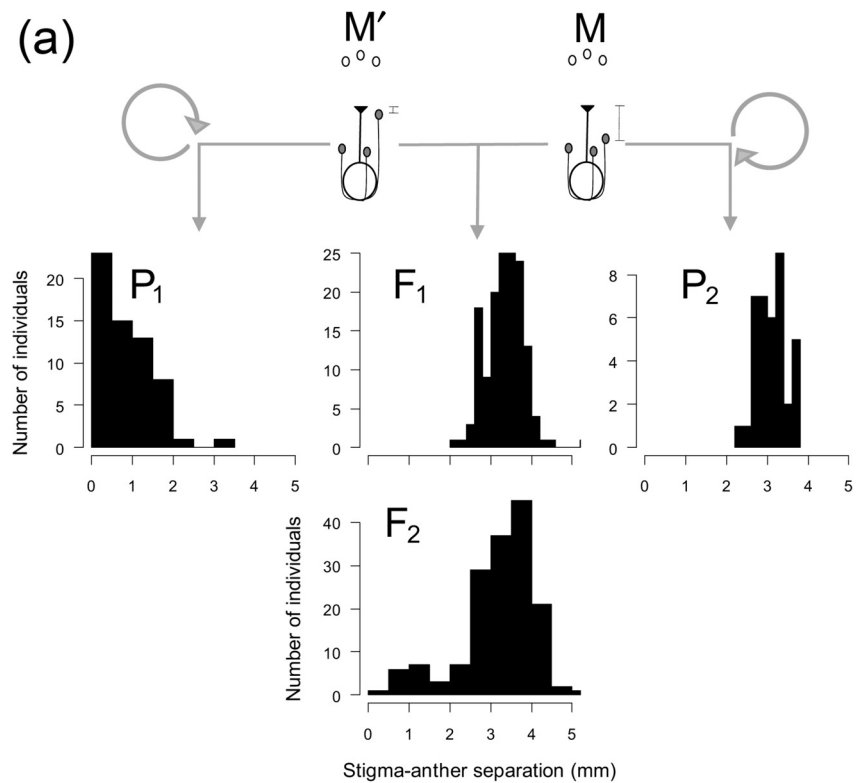
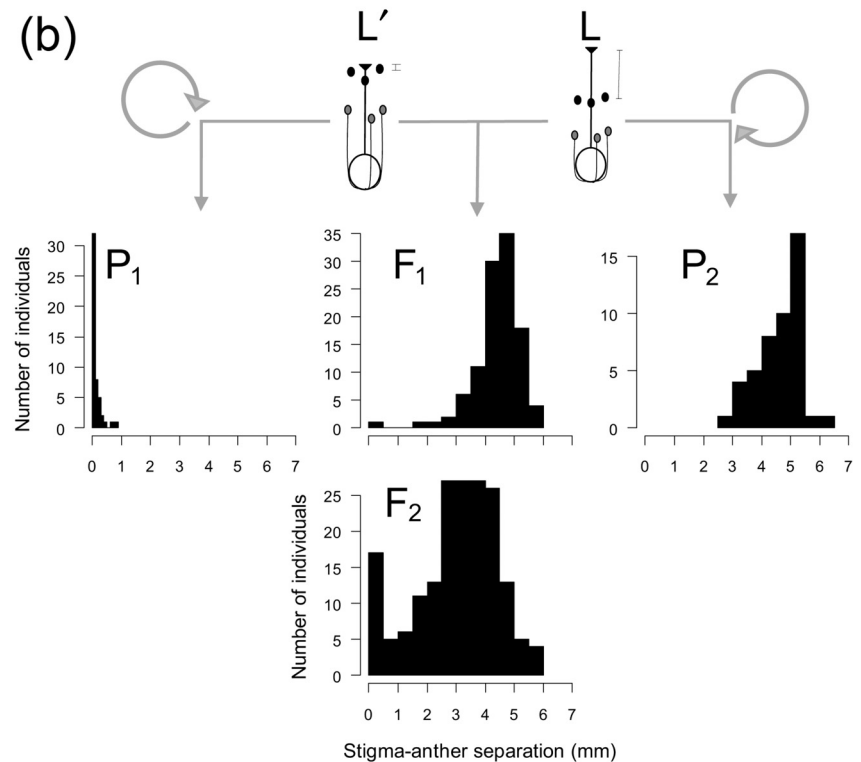


Fig. 5 (a) Inheritance of stigma–anther separation (herkogamy) in the M-morph of *Eichhornia paniculata* from north-east Brazil. Two individuals of the M-morph (parental generation), representing contrasting selfing and outcrossing phenotypes, were used to generate two filial generations (F₁ and F₂). The parental plant on the left represents a ‘modified’ (selfing) phenotype (M’) collected from a monomorphic population with values of herkogamy near zero. The parental plant on the right represents an ‘unmodified’ phenotype (M) with higher herkogamy values (~3 mm) from a tristylous population. (b) Inheritance of stigma–anther separation (herkogamy) in the L-morph of *E. paniculata* from Mexico and north-east Brazil. The Mexican plant was semi-homostylous (L’) with no stigma–anther separation (Fig. 2b); the Brazilian plant was an unmodified L-morph (L) from a tristylous population. The histograms in (a) and (b) illustrate the frequency distribution of herkogamy values for offspring from each self or cross type. F₁ was produced by crossing the two parents and F₂ by selfing a single F₁ individual from each cross-type. P₁ and P₂ illustrate the segregation of herkogamy variation following self-fertilization in the modified and unmodified parents, respectively. Sample sizes for flowers measured per plant were 2.76 ± 0.08 (mean ± SE) for F₁, P₁ and P₂, and 9.59 ± 0.13 for F₂.



outcrossing and high levels of self-fertilization. In species maintaining outcrossing and selfing forms, it is of interest to determine the pathway(s) by which selfing has evolved and how often it has originated. Although questions related to the

origins of selfing have often been addressed using species-level phylogenies (for example, Barrett *et al.*, 1996; Kohn *et al.*, 1996; Schoen *et al.*, 1997; Goodwillie, 1999), there have been fewer efforts to consider how often the shift to selfing may

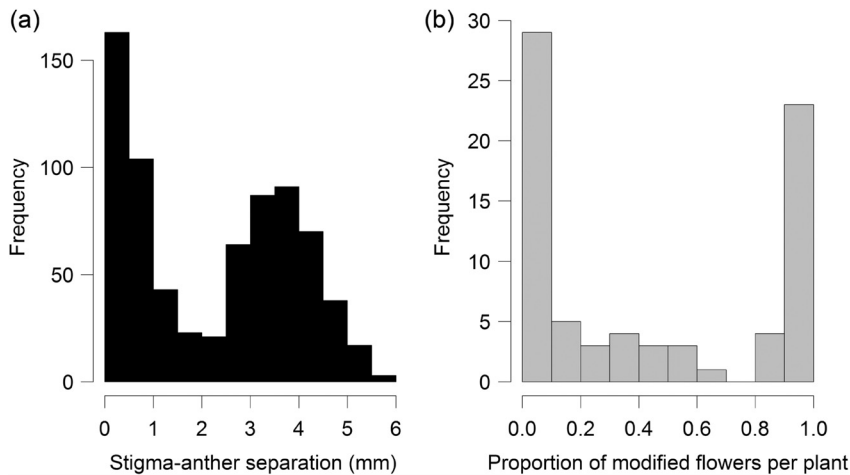


Fig. 6 Developmental instability of stigma–anther separation (herkogamy) in *Eichhornia paniculata* from north-east Brazil.

(a) Bimodal distribution of herkogamy values in 724 flowers from 75 individuals in the S_2 generation. This line is the product of two generations of selfing by single seed descent of an individual (B189-7-1) that displayed a bimodal distribution of herkogamy values (see Fig. 5 in Vallejo-Marín & Barrett, 2009). (b) The average proportion of modified flowers relative to total flowers ($n = 9.66$, $SE = 0.25$) in each plant. Developmental instability occurs in individuals producing both ‘modified’ and ‘unmodified’ flowers. Among the 75 plants, 33.3% displayed developmental instability.

occur within species (Allen *et al.*, 1991; Wyatt *et al.*, 1992), presumably because species displaying wide intraspecific variation in mating patterns are relatively uncommon.

Biogeographical surveys have revealed two distinct selfing forms of *E. paniculata* independently derived from the L- and M-morphs of heterostylous populations (Figs 1, 2). Over most of the geographical range, the principal form involves modified variants of the M-morph with one (Fig. 2a) to three short-level stamens close to mid-level stigmas. Semi-homostylous M-morphs have been reported in several other tristylous species and appear to be the most common type of breakdown product (Stout, 1925; Ornduff, 1972; Barrett, 1988). Nevertheless, the two populations of *E. paniculata* from Central America were composed exclusively of the semi-homostylous L-morph and, to our knowledge, these forms occur nowhere else. Significantly, both of the semi-homostylous forms of *E. paniculata* have also been described from the related *E. heterosperma* and *E. diversifolia* (Barrett, 1988), where they co-exist within populations rather than occurring allopatrically, as in *E. paniculata*. Although polymorphism involving two selfing forms could potentially occur in dimorphic populations of *E. paniculata*, the L-morph in these populations exhibits well-developed herkogamy (Fig. 1). Why are selfing variants of the L-morph absent from Jamaica and Cuba, the closest concentrations of populations to Central America? This question is particularly perplexing as the ecological conditions on these islands clearly favour selfing, as indicated by the predominance of semi-homostylous M-plants.

The genetic architecture of tristylous and semi-homostylous morphs may help to explain these puzzling geographical patterns. The most probable historical scenario is that Cuba or Jamaica was originally colonized from Brazil by a self-pollinating heterozygous variant of the M-morph (*ssMm*), with the L-morph (*ssmm*) then arising through segregation. The very similar patterns of nucleotide variation among populations from these islands suggest that they are descended from a single long-distance dispersal event (Fig. 3). In dimorphic populations, the semi-homostylous M-morph is predominantly selfing,

whereas the L-morph is largely outcrossed by pollen from long-level anthers of the M-morph (Barrett *et al.*, 1989; Fig. 1). This asymmetrical mating pattern combined with M-morph abundance will cause the recessive alleles governing the L-morph to spend much of their time sheltered from selection in heterozygous M-genotypes. Such an effect should reduce the intensity of selection for selfing on the L-morph, as well as guarantee a long persistence time of this morph on these islands despite its lower fertility in comparison with the M-morph (for example, Haldane, 1924).

Studies of inheritance indicate that selfing modifiers causing the loss of herkogamy in the M-morph are morph limited in expression (Fenster & Barrett, 1994; Vallejo-Marín & Barrett, 2009). Although the alleles are transmitted to the L-morph through segregation in dimorphic populations, they do not alter the stigma–anther separation or mating patterns of this morph. Selfing in the semi-homostylous L-morph involves different genetic modifiers affecting the position of mid-level rather than short-level stamens. These modifiers were only evident in Central American populations. This indicates that selfing has arisen by two distinct genetic pathways in *E. paniculata*, although, in both cases, the modifications are under recessive gene control (Fig. 5). Favourable recessives have a low chance of establishment in large random mating populations because of the rarity of homozygotes for low-frequency alleles (‘Haldane’s sieve’; Haldane, 1924). However, in populations with moderate to high rates of self-fertilization, there is both theoretical and empirical evidence that favourable recessive mutations can play a role in the evolution of adaptations (Charlesworth, 1992). Genetic drift can also result in chance fixation of recessive alleles in small populations because of reductions in effective population size (Pollak, 1987). As discussed earlier, both selfing and drift are characteristic features of *E. paniculata* populations, and the finding that selfing modifiers in both the L- and M-morph are recessive is therefore not entirely unexpected.

Long-distance dispersal of the L-morph to Central America would result in monomorphic populations, because

self-fertilization of this morph can only result in L-morph progeny. In Central America, strong selection for reproductive assurance may have led to the evolution of semi-homostylous forms of the L-morph. It is possible that, in contrast with Jamaica and Cuba, the absence of the M-morph from this region may have fostered the evolution of semi-homostyly in the L-morph. Selfing modifiers may be more rapidly fixed in the M-morph than the L-morph because fewer alleles of larger effect appear to be involved (Fig. 5). According to this hypothesis, the dynamics of selfing evolution in *E. paniculata* may be conditional on which morphs are represented in populations because of the different genetic pathways involved in the origins of selfing. However, the coexistence of both selfing morphs within populations of related *Eichhornia* species (Barrett, 1988) argues against this interpretation, unless their co-occurrence has arisen secondarily after polytypic origins of semi-homostyly.

The patterns of molecular genetic variation among our sample of 27 populations of *E. paniculata* are consistent with multiple independent transitions to self-fertilization. However, our analyses cannot provide a concrete estimate of the number of independent origins. Gene flow among populations could allow recombination between nuclear markers and the loci responsible for the selfing phenotypes, resulting in a decoupling of the evolutionary history of loci within the genome. However, our data on the distribution of SNPs among populations, and the proportion that are fixed, shared and unique, indicate that populations containing selfing variants have probably been derived from outcrossing populations in different parts of the geographical range, a pattern repeated across 10 presumably putatively unlinked nuclear markers. This result, in conjunction with the occurrence of two distinct semi-homostylous phenotypes derived independently from the L- and M-morphs, and genetic evidence indicating different selfing modifiers in geographically distinct populations, argues strongly against a single origin for selfing in *E. paniculata*. However, it is possible to explain the patterns of molecular genetic differentiation observed with alternative scenarios in which all of the selfing populations derived from the M-morph resulted from a single origin. This is because differentiation among selfing populations is likely to have been affected after the evolution of selfing by gene flow, as well as drift and isolation. These factors will complicate inferences on the origins of selfing from patterns of differentiation alone. Although we cannot confidently estimate the number of independent transitions to selfing with our data, especially in north-east Brazil where gene flow is most likely, it seems unlikely that all selfing populations are descendants of a single ancient transition to selfing in *E. paniculata* based on the different lines of genetic evidence presented here.

Developmental instability is determined by a variety of factors, including inbreeding and homozygosity at regulatory loci, mutation, breakdown of adapted gene complexes and various environmental stressors (Polack, 2003). Early stages in the establishment of selfing involve developmental instability

in the production of flowers capable of autonomous self-pollination. Plants of the M-morph in dimorphic populations carry selfing modifiers producing both unmodified and modified flowers. Our studies have demonstrated both genetic and environmental components to this variation. The widespread occurrence of developmental instability in dimorphic populations of *E. paniculata* raises the issue of whether it is maladaptive, as is often assumed, or whether this form of within-plant variation represents a mixed strategy, enabling plants to adjust their mating to match heterogeneous environmental conditions. Although we do not know the answer to this question in *E. paniculata*, the relatively simple genetic basis for selfing modifiers, frequent colonizing episodes and the species capacity for long-distance dispersal seem likely to often create conditions favouring transitions to selfing.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Methods S1 Details of population samples, marker development, sequencing and the analyses of molecular data.

Table S1 Population samples of *Eichhornia paniculata* used for molecular genetic analyses

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