

INVITED PAPER

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Postpollination discrimination between self and outcross pollen covaries with the mating system of a self-compatible flowering plant¹

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PREMISE OF THE STUDY: Variation in the mating system of hermaphroditic plant populations is determined by interactions between genetic and environmental factors operating via both pre- and postmating processes. Models predicting the maintenance of intermediate outcrossing rates in animal-pollinated plants often assume that the mating system is primarily controlled by floral morphology and pollinator availability, but rarely has the influence of postpollination processes on variation in outcrossing been examined.

METHODS: We investigated the influence of stylar discrimination between illegitimate and legitimate pollen-tube growth and the pollen-load capacity of stigmas on mating-system variation in the annual, tristylous species *Eichhornia paniculata* using controlled crosses and genetic markers. This species exhibits an exceptionally broad range of outcrossing rates in natural populations.

KEY RESULTS: There was significant variation among populations in the pollen-load capacity of stigmas and the ability of styles to discriminate between illegitimate vs. legitimate pollen. There was strong correspondence between stylar-discrimination ability and variation in outcrossing rate among populations and style morphs. The combination of stigmatic pollen-load capacity and stylar discrimination explained more than 80% of the variation in outcrossing rates among populations.

CONCLUSIONS: The finding that stigmatic pollen-load capacity and stylar-discrimination ability contributed significantly to explaining the wide range of outcrossing rates in *E. paniculata* suggests that postpollination mechanisms play an important role in governing mating patterns in this species. The difference in levels of stylar discrimination between outcrossing and selfing populations may reflect a trade-off between selection for increased outcrossing and greater reproductive assurance.

KEY WORDS cryptic self-incompatibility; tristily; heterostyly; pollen competition; pollen-tube attrition; mixed mating; sexual interference; reproductive assurance; *Eichhornia paniculata*; Pontederiaceae

The frequency of selfing and outcrossing in hermaphroditic plants plays an important role in influencing the distribution of genetic variation within and among populations and consequently governing the tempo and mode of evolution (Hamrick and Godt, 1990, 1997; Charlesworth, 1992; Charlesworth, 2003). With the advent of genetic markers, considerable effort has been spent on measuring variation in mating systems including investigation of the ecological and genetic correlates of outcrossing rate and the complex

forces governing its evolution (Schemske and Lande, 1985; Aide, 1986; Barrett and Eckert, 1990; Husband and Schemske, 1996; Goodwillie et al., 2005; Eckert et al., 2010). Models for the evolution of mating systems under inbreeding load (i.e., the decline in fitness due to inbreeding within populations; Charlesworth and Willis, 2009) make clear predictions that the development of strong barriers to selfing are favored when the fitness of selfed offspring is less than half that of outcrossed offspring (inbreeding depression, $\delta = 1 - w_s/w_o$; where w_s and w_o are the fitnesses of selfed and outcrossed offspring, respectively; Lande and Schemske, 1985). This threshold is generated because of the 2-fold advantage to self-fertilization, as two copies of the maternal genome are transmitted to self-fertilized seeds compared with only one in outcrossed seeds. Consequently, when the fitness cost of selfing is less than one-half, there should be

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“automatic selection” favoring selfed offspring. Consideration of the effects of inbreeding depression leads to the prediction that plant populations should be predominantly selfing or outcrossing with mixed mating less common.

The distribution of outcrossing rates was initially thought to be consistent with the simple predictions based on the coevolution of selfing rate and inbreeding depression (Lande and Schemske, 1985; Aide, 1986); however, more extensive sampling of outcrossing rates over the past few decades indicates that at least ~40% of all estimates involve mixed mating (Goodwillie et al., 2005). Moreover, several plant species clearly have mixed mating strategies (e.g., *Impatiens*, *Viola*, *Lamium*) producing both closed (cleistogamous) flowers that automatically self and open (chasmogamous) flowers that may produce outcrossed seed (Oakley et al., 2007). These observations have prompted consideration of the ecological factors that may affect the evolution of outcrossing rate. For example, Holsinger (1991) predicted that across a range of values of inbreeding depression, the outcrossing rate could depend on the density of plants in the population and the level of pollen discounting (the loss of outcrossed siring success due to selfing). There is also evidence that the production of mixtures of selfed and outcrossed offspring in animal-pollinated species is often due to geitonogamy, and it is unlikely that such environmentally determined mixed mating is adaptive (Lloyd, 1992; Barrett, 2003). Many species possess floral mechanisms that promote delayed selfing (Lloyd and Schoen, 1992), and it is now recognized that reproductive assurance (the ability to produce selfed offspring when pollinators or mates are unavailable) is a major determinant of mating system variation in angiosperms (Goodwillie et al., 2005).

It is often assumed that in self-compatible (SC) plants the number of selfed progeny produced is primarily a function of the relative proportions of self and outcross pollen deposited on stigmas (e.g., the mass-action model of Holsinger, 1991). For example, large floral displays are often associated with an increase in the amount of self pollen on stigmas as a result of geitonogamous self-pollination resulting in increased selfing (Harder and Barrett, 1995; Karron and Mitchell, 2012). Although ecological factors affecting stigma-load composition undoubtedly play a role in influencing mating patterns in self-compatible species, postpollination mechanisms that influence the siring success of self and outcross pollen can also promote outcrossing through discrimination against self pollen (Bowman, 1987; Cruzan and Barrett, 1996; Pannell and Labouche, 2013). In the extreme case with classical physiological self-incompatibility (SI), most self pollen fails during germination or pollen-tube growth (Hiscock and McInnis, 2003); however, there is evidence that some SC species possess mechanisms that result in more subtle discrimination between self and outcross pollen, which are only evident after pollination with mixtures of the two pollen types (cryptic self-incompatibility, CSI; Bateman, 1956; Weller and Ornduff, 1977; Bowman, 1987; Casper, 1988; Cruzan and Barrett, 1993; Eckert and Allen, 1997). Cryptic self-incompatibility can be thought of as functionally analogous to delayed selfing as it allows plants to preferentially outcross, but also reproduce through selfing when pollinator service is low—such mating strategies that are context dependent have been viewed as “the best of both worlds” (Bowman, 1987; Becerra and Lloyd, 1992; Cruzan and Barrett, 1996).

Consideration of CSI has generally assumed that the degree of stylar discrimination against self pollen is genetically determined and has the potential to respond to selection. However, this has

never been investigated empirically, and surprisingly little is known about variation in CSI among populations, particularly in species with wide variation in outcrossing rate. In this context, the evolution of outcrossing rate can be viewed as an outcome of the balance between selection to avoid the deleterious consequences of inbreeding and selection favoring reproductive assurance when mates or pollinators are in short supply. In circumstances in which postpollination mechanisms play an important role in maintaining high outcrossing, we might predict that selection to avoid selfing will be associated with high inbreeding depression and strong stylar discrimination. In contrast, in populations in which the fitness cost of self-fertilization is not as severe, we might predict that stylar discrimination should be weaker.

When stylar discrimination between self and outcross pollen is based on pollen-tube growth rate, this bias toward outcross pollen has the potential to produce a flexible mating system (i.e., CSI) that may be adaptive when the supply of outcross pollen is unpredictable (Cruzan and Barrett, 1996). Alternatively, stylar discrimination based on pollen-tube attrition (i.e., failure of pollen tubes due to the cessation of growth before fertilization; Cruzan, 1989) results in partial or complete SI and does not provide a flexible mating system that favors outcross pollen when it is more abundant. Cryptic self-incompatibility based on differences in pollen-tube growth rate would be advantageous in plants that may be subject to variable pollination environments, such as occurs in species that occupy ephemeral habitats and that rely on colonization of new sites for persistence (Pannell and Barrett, 1998; Cheptou, 2012). Under such circumstances, mating patterns are likely to be influenced by plant density, pollinator service, and delivery of outcross pollen.

The potential for stylar discrimination to result in selfing avoidance will depend to a large degree on the amount of pollen delivered to stigmas. Our previous work has shown that the promotion of outcrossing through stylar discrimination due to differential pollen-tube growth rate is enhanced with increasing pollen-load size (Cruzan and Barrett, 1996). Large stigmas capture more pollen and may therefore be advantageous in environments where pollinator visits are infrequent since they allow plants to maintain higher outcrossing rates. On the other hand, large stigmas may result in conflict between maternal and paternal sex functions, including the capture of more self pollen and reducing male reproductive success by interfering with efficient pollen dispersal (Barrett, 2002). From these considerations, we can predict that the pollen-load capacity of stigmas may respond to prevailing pollination conditions; in environments where pollinator service is reliable, stigmas may be smaller to reduce sexual interference, whereas larger stigmas might be favored when pollinator visitation is chronically low.

Here, we investigated two components of CSI—stylar-discrimination ability and stigma-load capacity—in tristylous *Eichhornia paniculata* (Pontederiaceae). This species has a wide range of mating patterns ($t = 0.014\text{--}0.961$) associated with evolutionary transitions from outcrossing to selfing as a result of the breakdown of tristylous (Barrett et al., 1989, 1993; Barrett and Husband, 1990). The populations we chose for our study represent a wide range of outcrossing rates and morphological stages in the breakdown of tristylous (Fig. 1, Table 1). This wide range of variation in outcrossing rates and morphology allowed us to examine the extent to which stylar-discrimination ability and stigma-load capacity covary with measured outcrossing rates. Trimorphic populations, containing the long-, mid-, and short-styled morphs (hereafter L-, M-, S-morphs), occur in regions of northeastern Brazil with reliable pollinator

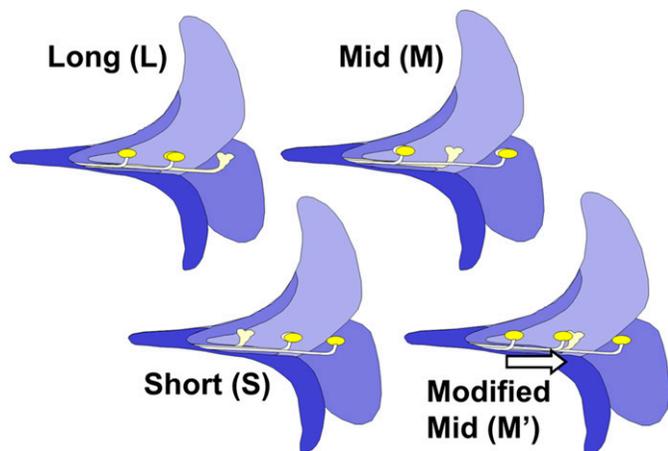


FIGURE 1 The positions of stigmas and anthers in the long-, mid-, and short-styled morph and the modified selfing variant of the mid-styled morph of *Eichhornia paniculata*. Flowers are depicted with one petal and a portion of the floral tube removed. Stigmas and styles are white; anthers are yellow. The arrow indicates the displacement of one or more short-level anthers to the mid-position in the M' morph.

service and have mating patterns ranging from mixed mating to predominant outcrossing. Breakdown of tristylous typically commences with the loss of the S-morph, resulting in dimorphic populations that consist of the L- and M-morph. Subsequent stages in the breakdown of floral polymorphism occur when selfing variants of the M-morph (hereafter M' -morph) invade dimorphic populations or populations fixed for the M-morph, and this transition to the M' morph ultimately gives rise to monomorphic populations with high frequencies of selfing. Selfing variants benefit from reproductive assurance in small populations or at low density because they have the capacity for autogamous self-pollination due to elongation of “short”-level stamens so that they become adjacent to mid-level stigmas (Fig. 1). Selfing variants dominate in Jamaican and Cuban populations of *E. paniculata* where pollinator service is limited, but they also occur in dimorphic and monomorphic populations in northeastern Brazil. Thus, the wide range of mating patterns and demographic conditions that occur in *E. paniculata*, and

TABLE 1. Information on the location, style morph composition, and outcrossing frequency for the nine populations of *Eichhornia paniculata* investigated in this study. Outcrossing data are from Barrett and Husband (1990). The average stigma-load capacities (G_m) and stylar discrimination for legitimate and illegitimate pollen (D) are estimated in the current study. The anther levels l, m, and s refer to long-, mid-, and short-level anthers, respectively. Pollen from B81 was substituted for crosses for recipients from population J27.

Population	Location	Style morph composition	Anther levels used in crosses	Outcrossing frequency	G_m	D
J27	Jamaica	M'	l, m, s (B81)	0.20	155.3	0.28
B31	Brazil	L, M	l, m, s	0.45	262.9	0.09
B46	Brazil	L, M, S	l, m, s	0.87	171.4	0.61
B63	Brazil	M'	l, s	0.41	292.0	0.12
B69	Brazil	M'	l, s	0.46	184.6	0.29
B72	Brazil	L, M'	l, m, s	0.26	209.7	0.07
B81	Brazil	L, M, S	l, m, s	0.96	450.2	0.38
B85	Brazil	L, M, S	l, m, s	0.81	291.2	0.48
B117	Brazil	L, M, M'	l, m, s	0.91	261.1	0.49

its unusual self-compatible status for a heterostylous species, provide a suitable experimental system to investigate the covariation of mating systems and postpollination mechanisms of discrimination among various pollen types.

We examined stylar discrimination in *E. paniculata* using controlled crosses with mixtures of two types of outcross pollen: illegitimate pollen (analogous to self pollen; see below)—from an anther level that differs from the stigma height and legitimate pollen (analogous to outcross pollen)—from an anther level equivalent in height to the stigma (Fig. 1). In our experiments, these two pollen types could be distinguished based on genetic markers. The siring ability of the two pollen types in mixed pollinations was quantified by assaying seeds produced from crosses (see Cruzan and Barrett, 1993, 1996). By obtaining this information and estimating the pollen-load capacity of stigmas, we were able to use these parameters in multiple regression models to assess their ability to predict the among-population variation in outcrossing rate of the populations we chose for study. We addressed three specific questions: (1) Does stylar discrimination differ among populations and floral morphs? (2) Is there evidence for variation in stigma-load capacity among populations and floral morphs? (3) We used information gathered on the pollen-load capacities of stigmas and differential pollen-tube growth rates for legitimate and illegitimate pollen to predict previously reported outcrossing rates for nine populations and to specifically ask to what extent can variation in stylar discrimination and stigma-load capacity be used to predict the mating system of populations from which our experimental plants were derived. We predicted that the combination of these variables would explain a significant proportion of the variation in outcrossing rate among populations. Our analyses provide insights into the mechanisms influencing mating patterns in plant populations. They also illustrate that postpollination mechanisms that discriminate among pollen types are not restricted to self-incompatible plants but can also be an important influence on mating in self-compatible species.

MATERIALS AND METHODS

Study system—Plants used in this experiment were derived from nine populations from northeastern Brazil and Jamaica (Table 1) for which information on floral biology, outcrossing rates, and population genetic parameters is available (Barrett and Husband, 1990). Previous studies of *E. paniculata* indicate that although the species is highly self-compatible, it possesses a cryptic trimorphic incompatibility system that functions to maintain high outcrossing rates in trimorphic populations (Cruzan and Barrett, 1996). Legitimate pollen sires more offspring than illegitimate and self pollen, which are generally equivalent in their siring ability (Cruzan and Barrett, 1993). In this study, with a few exceptions discussed below, we focused primarily on stylar discrimination in mixtures of legitimate and illegitimate pollen. We chose to primarily use illegitimate as opposed to self pollen in crosses to avoid the potential for post-fertilization embryo abortion due to inbreeding depression to confound our estimates of stylar discrimination. However, it is important to point out that when two pollen types originate from the same anther level in a tristylous species, they generally have equivalent siring ability (e.g., self-pollination of an L-morph with its own mid-level pollen is equivalent to a cross using midlevel pollen of an S-morph; see Cruzan and Barrett, 1993).

Controlled crosses and allozyme assays—We sowed open-pollinated seed families on moist soil in the glasshouse at the University of Toronto and allowed them to germinate before transplanting seedlings to larger pots. We then grew plants to flowering and screened them for their genotype at up to four allozyme loci (*Aat-3*, *Pgi-2*, *Pgi-3*, and *Tpi-1*). We chose individuals as pollen donors and recipients based on their allozyme genotype; with the exception of two populations (B31 and B85), we used donors and recipients that were homozygous for all of the loci assayed, which allowed the unequivocal determination of seed paternity. For the exceptional two populations, we used only homozygous recipients, but one of the two pollen donors was heterozygous at one locus so the fertilization success of each pollen type (see below) was inferred based on the assumption of equal segregation of alleles. Pollen from long-level anthers (l) was substituted for “legitimate” pollen (i.e., mixtures of l and s pollen were used) in two of the monomorphic populations (B63 and B69) because plants with mid-level (m) pollen were not present in these populations. Because the sample of plants from population J27 was small and consistent sources of cross pollen were not available, we evaluated legitimate pollen fertilization ability with pollen mixtures from population B81 (Table 1).

We assessed the strength of cryptic incompatibility for each style morph in each population by making crosses with mixtures of genetically marked pollen over a 14-d period when temperatures in the glasshouse ranged from 25° to 30°C. Equal mixtures of within-population legitimate and illegitimate pollen were created for each cross. For dimorphic and trimorphic populations these mixtures contained pollen from three anther levels (l, m, and s), and two anther levels (l and s) were used for monomorphic populations (Table 1). Each day, 4–6 plants of each style morph from two populations were chosen as recipients in an incomplete block design. We collected pollen from each anther level from two outcross donors of the same allozyme genotype within each population, such that each anther level was represented by a unique allozyme banding profile. Equal numbers of anthers were collected for all pollen types and mixed in clean glass vials before application to stigmas, a treatment that has previously been shown to produce nearly equal and homogeneous mixtures (Cruzan and Barrett, 1996). The proportion of each pollen type in the mixture (P_i for pollen type i) is assumed to be roughly equal for all pollen types present ($P_i = 0.5$ and 0.33 for mixtures of two and three pollen types, respectively; Table 1). Note that the actual frequencies of each pollen type may differ from these expectations due to small differences in pollen production among anther levels (Barrett, 1985), but this bias did not appear to have affected our results (see Discussion). Stigmas of each recipient were saturated with pollen by evenly coating the entire surface. By using this approach, we were able to estimate the stigma-load capacity (G_m) and maximum legitimate pollen fertilization advantage (F_1), as discussed fully below. We excised stigmas 6 h after pollination, and these were stored in 70% ethanol before estimating stigma-load capacity. Our previous work demonstrated that excision of stigmas has no apparent effects on fruit or seed set in the short-lived flowers (6–8 h) that characterize this species (Cruzan and Barrett, 1993).

We quantified pollen loads using a video image analysis system after staining with 0.1% w/v malachite green and acetocarmine (a saturated solution of carmine in acetic acid) added to each stigma in 70% ethanol. The number of pollen grains on stigmas was estimated from images of the entire stigma with attached pollen grains using methods detailed in Wang and Cruzan (1998). The total number of pollen grains on the stigma (i.e., stigma-load capacity: G_m)

was calculated by dividing the total area of grains by the average area of an individual pollen grain estimated from a subsample of slides representing all populations used in crosses.

We estimated the siring success of each pollen type by assaying 10–20 seeds from each of 6–12 fruits (only 2 fruits for M' in B117) of each morph in each population (3530 seeds total). Each seed was assayed for all allozyme loci listed above on 10% hydrolyzed starch gels using the methods of Cruzan and Barrett (1993). The number of seeds sired by each pollen type in each cross was determined from allozyme profiles and was used to calculate the proportion of seeds sired ($F_i = S_i/S$ and $F'_i = F_i - P_i$ where S is the number of seeds assayed, S_i is the number of seeds sired by pollen type i , and P_i is based on the pollen mixtures described above; Cruzan and Barrett, 1996). We used pollen from the same anther level as the stigma (F_1 : legitimate pollen) as a measure of the siring advantage (or occasionally disadvantage) of outcross pollen. Treating legitimate as equivalent to outcross pollen is reasonable because illegitimate pollen types in a tristylous population can be either self or outcross, whereas legitimate pollen can only be from outcross sources.

Estimating stigma-load capacity and stylar-discrimination ability—

The legitimate pollen fertilization advantage (F_1) described above estimates the degree to which legitimate pollen has a greater siring success than illegitimate pollen, but it confounds the effects of pollen-load size (G_m) and the discrimination ability of the style (D). The ability of styles to discriminate among different pollen types can be estimated from the ratios of observed to the expected siring frequencies of legitimate (f_l) and illegitimate (f_e) fertilization. Each frequency can be expressed as a ratio of the number of ovules fertilized and the expected number of fertilizations, based on the number of pollen grains of each type present on the stigma (i.e., $f_l = F_1 O / P_l G_m$ and $f_e = F_e O / P_e G_m$ for the legitimate and illegitimate pollen success frequency, respectively; where O is the number of ovules in a flower). In *E. paniculata*, estimates of ovule number per flower range from 103.7 to 118.4 and do not differ significantly among style morphs; mean = 108.9 across the three style morphs in two populations (Manicacci and Barrett, 1996). For the purposes of our calculations, we assume that $O = 109$.

Stylar-discrimination ability (D) was estimated as the difference between the ratios of legitimate and illegitimate fertilization ($D = f_l - f_e$). Values of D generally range from positive 1.0 (all seeds fertilized by legitimate pollen) to negative 1.0 (all seeds fertilized by illegitimate pollen), with zero indicating no difference in the siring abilities of different pollen types. In a few cases where seed set was limited by pollen-tube attrition, i.e., failure of pollen tubes to enter the ovary and fertilize ovules for unknown reasons (Cruzan and Barrett, 1996), estimates of D for individual crosses were greater than 1.0 and were assumed to be equal to one for the purposes of data analysis. Unlike the legitimate pollen fertilization advantage (F_1), the stylar-discrimination ability is insensitive to differences in the stigma-load size and represents a standardized estimate of the relative siring advantage of legitimate pollen. This measure of stylar discrimination reflects the overall siring advantage of legitimate pollen and integrates the individual effects of differential pollen tube attrition and competitive ability (Cruzan and Barrett, 1996).

We used the combination of mean stigma-load capacity (G_m) and stylar discrimination (D) for each population to predict expected outcrossing rates (\hat{t}) under conditions of equal frequencies of pollen from each anther level. We assumed that if the number of pollen grains present on the stigma were equal to the ovule number,

then the proportion of outcrossed seeds produced would be equal to the frequency of outcross pollen on the stigma, and that when G_m exceeds the ovule number, the proportional outcross pollen advantage would be determined by D (Cruzan and Barrett, 1996). The predicted outcrossing rate is then the sum of the frequency of legitimate pollen on the stigma (P_1) and D scaled by G_m relative to the ovule number (assuming $O = 109$ as described above):

$$\hat{t} = P_1 + D \frac{G_m}{O}$$

We calculated \hat{t} for each population to assess the effect of pollination environments on the observed outcrossing rates.

Data analysis—We determined whether there were differences in stigma-load capacity and stylar discrimination among populations and style morphs, and we also examined the relation between these variables and the previously determined mean multilocus outcrossing rate (t_m) of each population. We estimated stylar discrimination (D) for each cross from the data on fertilization advantage and stigma-load capacity using the equations above. Stigma-load capacities were \log_{10} -transformed, and the F'_1 and D values were arcsine-square-root-transformed before analysis. After transformation, the resulting distributions were approximately normal. Differences

among populations and style morphs were analyzed using a fixed-effects nested analysis of variance with pollination date nested within population (Proc GLM and MIXED; SAS, 2002). We examined the contribution of stylar discrimination and stigma-load capacity to variation in outcrossing rate among populations using multiple regression analysis with each reproductive attribute treated as an independent variable (Proc REG; SAS, 2002).

RESULTS

Variation in stigma-load capacity and stylar-discrimination ability—

There were significant differences among populations for average stigma-load capacity and stylar-discrimination ability (Fig. 2). Stigma-load capacity (G_m) varied substantially among populations ($F_{8,121} = 20.0$, $P < 0.0001$), but there was no strong correspondence between average stigma-load capacity and the morph structure (trimorphic, dimorphic, monomorphic) of populations (Fig. 2A). Although a priori contrasts revealed that trimorphic populations tended to have larger stigmas than in monomorphic populations ($F_{1,121} = 11.06$, $P = 0.0011$), there was no difference between trimorphic and dimorphic populations ($F_{1,121} = 2.76$, $P = 0.099$). In contrast, population morph structure was a better predictor of stylar-discrimination ability (D), with the three trimorphic and one dimorphic population having D values significantly greater

than zero (Fig. 2B; $F_{8,121} = 5.09$, $P < 0.0001$, for the overall difference in D among populations). This pattern is reflected in the contrasts, where trimorphic populations had significantly higher levels of discrimination ability than all other population morph structures combined ($F_{1,121} = 18.41$, $P < 0.0001$), and there was no significant difference between monomorphic and dimorphic populations ($F_{1,121} = 0.72$, $P = 0.399$).

Differences among style morphs were apparent for both stigma pollen-load capacity and stylar-discrimination ability (Fig. 3). Stigma-load capacity (G_m) decreased as style lengths became shorter for the outcrossing tristylous morphs (i.e., from L to M to S styles), but were smallest for the selfing M' morph (Fig. 3A; $F_{3,121} = 8.92$, $P < 0.0001$). Stylar-discrimination ability (D) was significantly different from zero for the L-, M- and S-morphs, but not for the M' morph (Fig. 3B). Plants possessing short-styled flowers had the highest values for discrimination ability, followed by the L- and M-morphs, and the lowest values of D were evident for the M' morph (Fig. 3B); however, these differences were not statistically significant ($F_{3,121} = 1.80$, $P = 0.151$).

Stigma-load capacity, stylar-discrimination ability and outcrossing rate—

The majority of the variation in outcrossing rate among populations of *E. paniculata* was explained by multiple regression models that combined population averages for pollen-load capacities of stigmas and stylar-discrimination ability

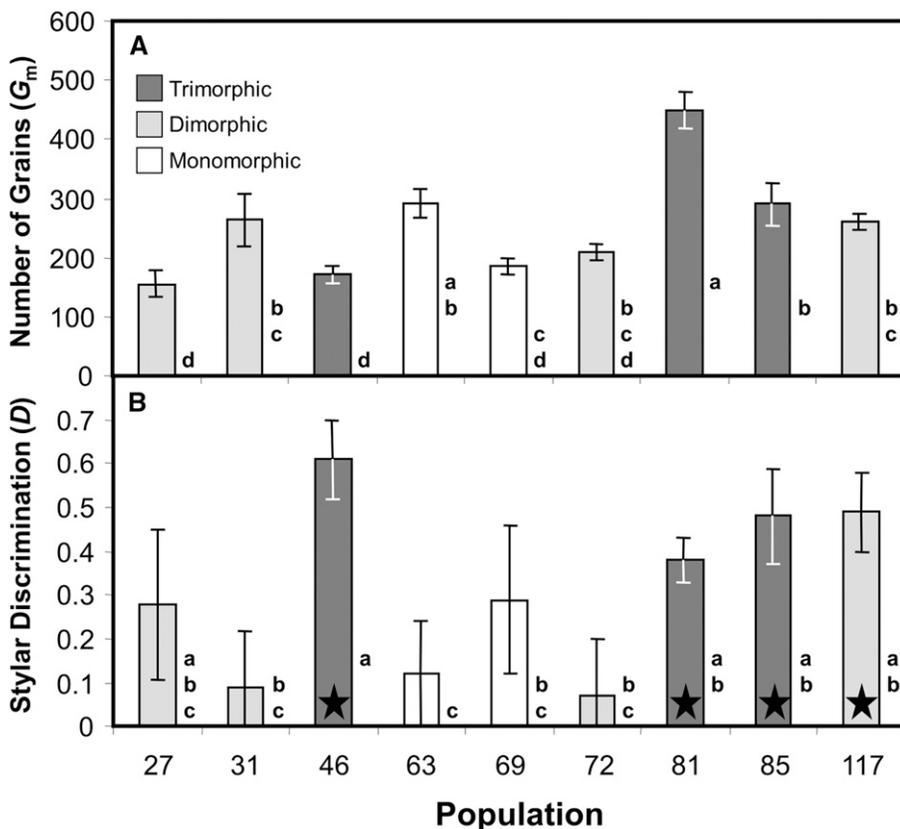


FIGURE 2 Variation among populations in (A) stigma-load capacity (G_m) and (B) stylar-discrimination ability (D) in *Eichhornia paniculata*. Vertical bars represent standard errors of the mean, and populations having the same lowercase letter are not significantly different from one another (based on Tukey's test for differences among means). The stars indicate discrimination abilities that are significantly different from zero (panel B only).

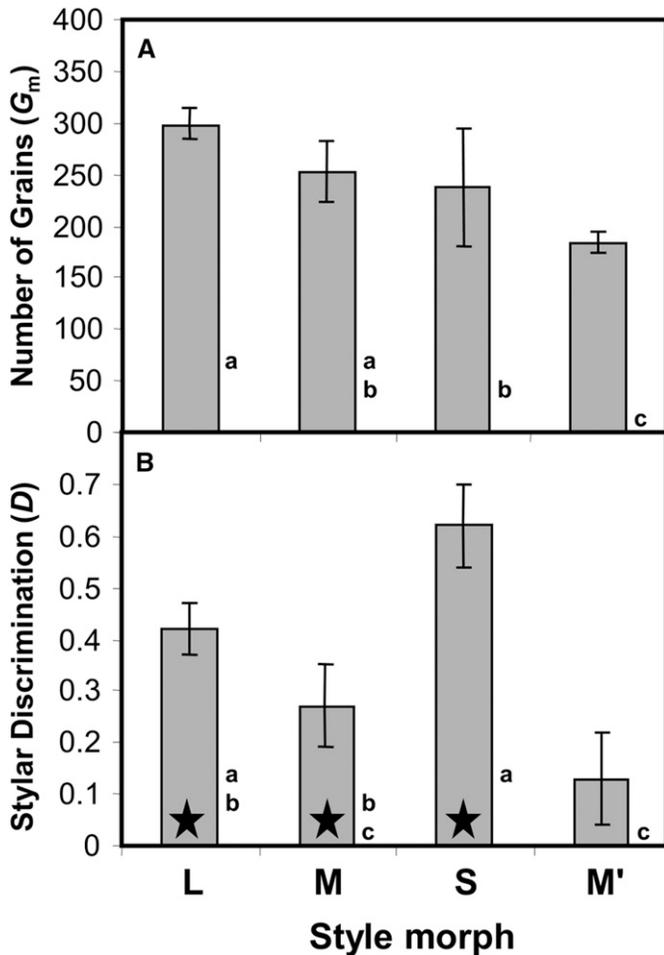


FIGURE 3 Variation among style morphs in (A) stigma-load capacity (G_m) and (B) stylar-discrimination ability (D) in *Eichhornia paniculata*. Vertical bars represent standard errors of the mean, and populations having the same lowercase letter are not significantly different from one another (based on Tukey's test for differences among means). The stars indicate discrimination abilities that are significantly different from zero (panel B only).

(Fig. 4). A regression model that combined these two parameters explained a large proportion of the variation in outcrossing rate among populations ($R^2 = 0.847$, $F = 23.29$, $P = 0.002$). In this model, the average level of stylar-discrimination ability (D : slope = 1.13, $t = 5.66$, $P = 0.002$, $N = 9$; Fig. 4) and stigma-load capacity (G_m : slope = 1.05, $t = 3.26$, $P = 0.017$, $N = 9$) were both positively associated with the average frequency of outcrossed progeny produced in populations. Observed outcrossing rates were generally similar to those predicted based on mean stylar discrimination and stigma-load capacities for each population (Fig. 4). With the exception of B46, all populations displayed outcrossing rates that were lower than predicted.

DISCUSSION

Variation among populations in outcrossing rate in *Eichhornia paniculata* appears to be strongly influenced by a combination of

stigma-load capacity and the ability of styles to discriminate between legitimate and illegitimate pollen. In our experiment we assumed that illegitimate pollen sources are a reliable predictor of the performance of self pollen in competition with legitimate pollen, as we previously demonstrated (Cruzan and Barrett, 1993). The nine populations examined differed substantially in style-morph composition, outcrossing rate, pollen-load capacity and stylar discrimination, as estimated from the siring success of legitimate and illegitimate pollen. The variation in the discrimination ability of styles combined with differences in the pollen-load capacities of stigmas explained a substantial proportion of the variation in outcrossing rate among populations. The wide range of stylar discrimination abilities is significant and suggests that mixed mating in *E. paniculata* may be due to a combination of intrinsic floral characteristics as well as environmental and demographic variation that affects the size and composition of stigma pollen loads.

While the measure of stylar-discrimination ability used in this study was strongly associated with outcrossing rate, these estimates were made under glasshouse conditions and did not examine a wide range of pollen load size and composition. However, our estimates of stylar discrimination are repeatable, as evidenced by the consistency of results from particular crosses (Cruzan and Barrett, 1993, 1996, this study), and CSI based on stylar discrimination has been found in a diverse collection of species (e.g., Bateman, 1956; Weller and Ornduff, 1977; Bowman, 1987; Casper, 1988; Cruzan and Barrett, 1993; Eckert and Allen, 1997). Our measure of stylar discrimination represents the fertilization advantage of legitimate pollen on a per grain basis that is independent of pollen-load size. The large amount of variation we observed in discrimination ability among populations when plants were grown in a common glasshouse environment suggests that a significant component of the variation in this trait is genetically determined and therefore may be subject to selection. It is important to note that the predictive ability of this parameter is strongly influenced by the size and composition of the stigmatic pollen load (Cruzan and Barrett, 1993, 1996). Consequently, the predictive power of stylar discrimination for outcrossing rate may be dependent on levels of pollinator activity and hence the size and composition of the stigma load (i.e., the proportion of self and outcross pollen). As pollinator activity declines, we might expect the power of stylar discrimination for predicting variation in outcrossing rate to erode.

Our measurements of stigma-load capacity varied substantially among populations, but were relatively consistent among plants within a population (Fig. 2A). It is of interest to ask why all plants do not produce large stigmas because there may be an advantage to being able to capture larger amounts of pollen in terms of increased outcrossing (i.e., due to stylar discrimination), greater gametophytic competition and higher seed set. Given the level of variation in stigma size among populations (>50% based on stigma-load capacity), it is probable that there are fitness trade-offs associated with stigmas that have larger pollen-load capacities. For example, there may be substantial energetic costs associated with providing carbohydrate resources for large populations of pollen tubes growing in styles (Mascarenhas, 1975). Also, a reduction in reproductive success may be incurred as a result of stigmas interfering with pollen dispersal (e.g., Keller et al., 2014; but see Kohn and Barrett, 1992a), or increasing stigma size may promote higher rates of self-pollen deposition (Thomson and Stratton, 1985). Alternatively, stigma size variation may covary to some extent with flower size, although the much wider range in stigma-load capacity (Fig. 2A)

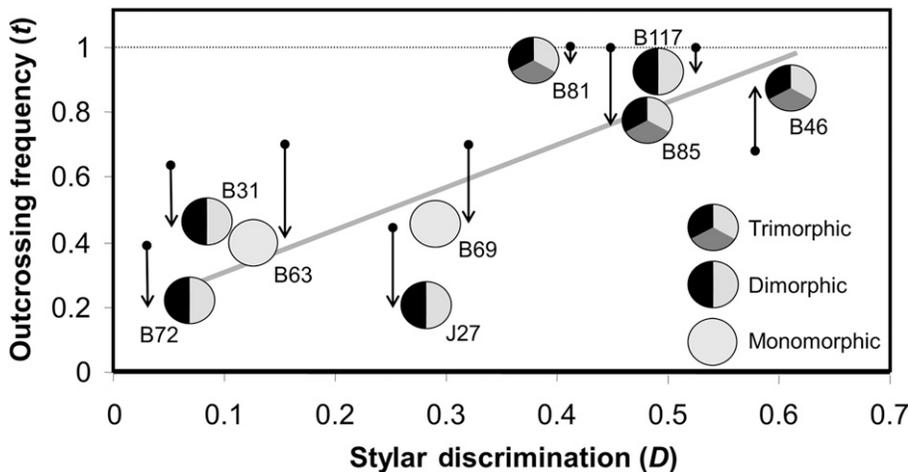


FIGURE 4 The relation between stylar-discrimination ability (D) and the mean multilocus population outcrossing rate (t_m) for nine population of *Eichhornia paniculata* from Brazil and Jamaica. Populations are classified according to their style morph structure (monomorphic, dimorphic, trimorphic). See text for further explanation. The line is based on the best-fit least squares regression for the relationship ($y = 1.21x + 0.21$; $R^2 = 0.63$). Predicted outcrossing rates (\hat{t}) based on mean stylar discrimination and stigma-load capacity for each population are indicated by the location of arrow origins, and the length of the arrows indicates the departure from expected outcrossing for each population.

compared with flower size variation among populations is not consistent with this hypothesis. Variation in stigma size among populations contributed significantly to variation in outcrossing rates, suggesting that the effect of pollen competition on mating system variation was greater than any increase in the capture of self pollen by larger stigmas. The fact that stigma-load capacity varied in a predictable manner among the style morphs within a population suggests that variation in this trait among the outcrossing floral morphs (i.e., excepting the M' morph—see below) may be due to a trade-off between selection for increased outcrossing vs reductions in sexual interference.

We predicted that stylar discrimination would be associated with the mating system of populations, with a lower ability to discriminate between pollen types in highly selfing populations, compared with those that are mostly outcrossing. This prediction was supported, because stylar discrimination was significantly different from zero for all style morphs, except selfing variants (M') of the M -morph (Fig. 3B). Significantly, the stigma size of M' plants was also smaller, compared with those from the L -, M -, and S -morphs from polymorphic populations, suggesting that the stigmas of these plants would be less efficient at capturing outcross pollen compared with stigmas of the other floral morphs. The reduced size of stigmas of M' plants may be a consequence of allometric relations with flower size and perhaps also selection to reduce investment in the reproductive structures of the smaller flowers of this selfing morph (Cruden, 1977; Ritland and Ritland, 1989; Elle and Carney, 2003). Given the close proximity of modified stamens to stigma of the M' morph (Fig. 1), it is not surprising that these plants have the capacity for autonomous self-pollination and display high selfing rates in natural populations (Barrett et al., 1989; Kohn and Barrett, 1992b). Indeed, the frequency of selfing M' variants in *E. paniculata* populations is correlated with the population mean selfing rates (Barrett and Husband, 1990). Flowers and sex organs in selfing populations are significantly smaller in size than those from outcrossing populations

(Barrett, 1985; Morgan and Barrett, 1989), which is consistent with the “selfing syndrome” that has evolved by convergent selection in numerous other plant families (e.g., Runions and Geber, 2000; Kalisz et al., 2011; Sicard and Lenhard, 2011)

We were able to explain more than 80% of the variation in outcrossing rate among populations of *E. paniculata* based on the combination of stylar-discrimination ability and pollen-load capacity of stigmas. As noted above, this result implies that the contribution of environmental variation (i.e., pollination conditions) to outcrossing rates among most of the sampled populations of *E. paniculata* may have been relatively low (i.e., pollinator activity was high) at the time outcrossing rates were estimated (Barrett and Husband, 1990; Barrett et al., 1993). The outcrossing rate for two of the three style morphs (L and M) can be strongly affected by levels of pollen competition (Cruzan and Barrett, 1993, 1996) and should therefore be sensitive to differences in pollen-load size. Small pollen loads or low rates of pollen deposition as a result of limited pollinator activity would

be expected to reduce the outcrossing rate and to decrease the predictive powers of the reproductive characters measured in this study. Hence, it appears that most of the populations we examined received sufficient levels of pollinator activity to maintain outcrossing rates consistent with our predictions from glasshouse-measured levels of stylar discrimination.

While pollinator activity may have been generally high among most populations, there were still substantial departures from predicted outcrossing rates in the majority of populations we investigated. For example, most of the outcrossing rates for trimorphic populations are similar to or above predictions based on stylar discrimination and stigma-load capacity, while estimates for the other populations all fall well below the predicted values in Fig. 4. The higher-than-expected outcrossing in one population (B46) may be due to relatively high frequencies of outcross pollen on stigmas. The elevated rates of selfing in the other populations may be explained by the presence of selfing variants of the midstyled morph (M' ; Barrett and Husband, 1990), which produce relatively few outcrossed seeds owing to autonomous self-pollination (Barrett et al., 1989; Kohn and Barrett, 1992b). However, there did not appear to be a consistent effect of the presence of the selfing variant on deviations from expected outcrossing rates when populations with (B72 and B117) and without (dimorphic population B31, and trimorphic populations B46, B81, and B85) the M' morph are compared (Fig. 4). While autonomous self-pollination due to the presence of the M' morph certainly contributed to variation in outcrossing, the deviations from expected outcrossing rates in most populations may be better explained by variation in pollinator visitation and frequencies of self pollen in stigma pollen loads.

With the observed inbreeding load that occurs in outcrossing populations of *Eichhornia paniculata* (Barrett and Charlesworth, 1991), increased levels of stylar discrimination may be favored as a mechanism to avoid inbreeding depression. This hypothesis is supported by our predicted estimate of complete outcrossing for some

of the populations (B81, B85, and B117); however, the observed outcrossing rates for these populations were lower than our predicted values (Fig. 4). The lack of more stringent discrimination against self pollen would have ensured consistently high outcrossing rates, but complete outcrossing has not been observed as a general feature among populations of *E. paniculata* (Barrett and Husband, 1990). This observation indicates that either an increase in the strength of incompatibility in *E. paniculata* is not possible because of evolutionary constraints or selection favors weaker discrimination ability because of the benefits of reproductive assurance when pollinators and mates are limiting. The well-developed trimorphic self-incompatibility in closely related taxa, e.g., *E. azurea* (Bianchi et al., 2000) and *Pontederia subovata* (Puentes et al., 2013) and variation in stylar discrimination in *E. paniculata* suggest that the evolution of stronger discrimination against self or illegitimate pollen should be possible in *E. paniculata* if ecological conditions permitted the evolution of stronger self-incompatibility. It seems more likely, given the annual life history of *E. paniculata* and its colonization of ephemeral habitats (Barrett and Husband, 1997), that these features of the ecology of the species can explain the persistence of weak stylar discrimination as a mechanism enabling reproductive assurance when pollinator service is unreliable. In *E. paniculata* the advantage of a flexible mating system that allows seed production when pollinators are infrequent probably outweighs the potential deleterious effects of producing inbred offspring.

It is likely that selection for reproductive assurance associated with the annual life history of *E. paniculata* prevents evolution toward obligate outcrossing based on trimorphic incompatibility. However, the lack of transition to complete selfing in populations that have low inbreeding load such as those in Jamaica (Barrett and Charlesworth, 1991) requires an explanation. With a low inbreeding load, we would predict that the transmission advantage of selfing would strongly favor the spread of mutations favoring complete selfing. Genetic marker evidence indicates that selfing variants of *E. paniculata* have originated multiple times resulting in the evolution of monomorphic populations (Husband and Barrett, 1993; Barrett et al., 2009). In the Caribbean and Central America, most populations are fixed for selfing variants and possess low levels of genetic diversity, but significantly, these populations are not completely selfing and always have a low level of outcrossing (Barrett and Husband, 1990). Such low levels of outcrossing in selfing species is commonplace and may arise because of the benefits of recombination thus preventing the build-up of gametic disequilibria, which can lead to selective interference and limit the rate of adaptation (Kamran-Disfani and Agrawal, 2014). In contrast, in Brazil trimorphic populations are most common, and dimorphic and monomorphic populations with varying frequencies of selfing variants are less frequent. The monomorphic and dimorphic populations containing the M' morph that we investigated from Brazil exhibited a wide range of values for stylar discrimination, some of which were significantly greater than zero, indicating that outcrossing should be favored. The discrimination against self pollen by the M' morph indicates that there remains some advantage to postpollination mechanisms that favor the production of outcrossed offspring even in populations with moderate to high frequencies of selfing or that the loss of stylar discrimination is not yet complete in plants that are evolving toward an autogamous syndrome.

In conclusion, our study is the first to examine the covariation of the mating system with postpollination mechanisms affecting

cross- and self-fertilization in a flowering plant. Our finding that the level of stylar discrimination between illegitimate and legitimate pollen (i.e., CSI) is a strong predictor of population outcrossing rate is novel and is not predicted by existing models of mating system evolution in hermaphroditic organisms (Goodwillie et al., 2005). This observation contrasts with existing models of mating system evolution based on inbreeding avoidance and suggests that mixed mating may be a stable feature of these populations and not just a consequence of levels of pollinator activity or the frequency of selfing variants. The importance of reproductive success for fitness in annual *E. paniculata*, and the strong associations found among determinants of the mating system in this species make it unlikely that the variation we observed has arisen by neutral processes and is nonadaptive. Stigma-load capacity and the strength of stylar discrimination in populations we investigated likely reflect the effects of historical selection on the mating system. Future marker gene studies of other self-compatible flowering plants displaying wide variation in mating patterns would be valuable to determine whether similar mechanisms of stylar discrimination to those that we have documented also occur.

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