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The genetic consequences of evolving two sexes: the genetic structure of distylous and dioecious species of *Erythroxylum*

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ABSTRACT

Question: Is the evolution of dioecy associated with increasing levels of genetic variation?

Background and hypothesis: Dioecy and self-incompatibility are seen as alternative mechanisms promoting outcrossing and avoiding the deleterious effects of inbreeding depression. Under this formulation, we would expect similar genetic variation between dioecious and self-incompatible species. In contrast, differences in genetic variation between these two breeding systems would indicate that sexual specialization has genetic effects that go beyond the avoidance of inbreeding.

Organisms and methods: We studied the genetic structure of one distylous and one dioecious species of *Erythroxylum* by means of ISSR markers. We measured the level and distribution of genetic diversity in five populations of the dioecious *E. rotundifolium* and in six populations of the self-incompatible distylous *E. havanense*.

Results: Expected heterozygosity and Shannon genetic diversity in populations of the distylous *E. havanense* were significantly greater than in populations of dioecious *E. rotundifolium*. Differences between species were small but differences among populations accounted for a large fraction of the genetic variation. Levels of differentiation were slightly greater among populations of the dioecious species. Overall, our results indicate that the evolution of dioecy in *Erythroxylum* is associated with a significant reduction in genetic diversity and increasing population differentiation.

Keywords: Baker's hypothesis, dioecy, distyly, *Erythroxylum*, genetic differentiation, genetic diversity, sexual dimorphism.

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INTRODUCTION

Following Darwin's (1877) lead, many authors have proposed that the avoidance of inbreeding has been a major factor in the evolution of plant breeding systems (Lloyd, 1975, 1976; Charlesworth and Charlesworth, 1978; Thomson and Barrett, 1981; Sakai and Weller, 1999). This argument has also been used to explain the evolution of dioecy because the presence of male and female plants completely avoids uniparental inbreeding (Baker, 1959; Charlesworth and Charlesworth, 1978; Thomson and Barrett, 1981). Empirical studies with gynodioecious species have shown that both selfing and inbreeding depression are involved in the maintenance of females within populations, and eventually, in the evolution of sexual specialization (Ashman, 1992; Molina-Freaner and Jain, 1993; Sakai *et al.*, 1997; Thompson and Tarayre, 2000). Based on the observation that self-incompatibility and dioecy were negatively correlated at the family level, Baker (1959) hypothesized that dioecy and self-incompatibility were alternative selfing-avoidance mechanisms (see Sakai and Weller, 1999, for a review). This hypothesis implicitly assumes that dioecious (or self-incompatible) species should have higher levels of heterozygosity and lower population structure than selfing species. It also predicts a similar genetic structure when self-incompatible and dioecious species are compared. Nonetheless, although the avoidance of inbreeding hypothesis has permeated the scientific literature for decades (Bawa, 1982), few direct attempts have been made to evaluate whether the increased outcrossing rates produced by the evolution of dioecy have a concomitant effect on the heterozygosity levels of the population (Waycott *et al.*, 1996; for gynodioecious species, see Gouyon and Couvet, 1987; Cuevas *et al.*, 2006).

The few empirical studies examining the genetic variation of dioecious species have found no general support for the inbreeding avoidance hypothesis (Waycott *et al.*, 1996; Terauchi *et al.*, 1997; Bartish *et al.*, 1999; Rottenberg *et al.*, 1999; Crawford *et al.*, 2001; Dorken *et al.*, 2002; Dorken and Barrett, 2004). For example, allozyme genetic diversity did not differ between self-compatible monoecious and dioecious populations of *Sagittaria latifolia* (Dorken *et al.*, 2002). In contrast, a chloroplast haplotype-based estimation of genetic diversity was more than six times greater among monoecious than dioecious populations (Dorken and Barrett, 2004). The genetic variation of dioecious species from the Juan Fernandez Islands was slightly greater than that of non-dioecious species, but this difference was non-significant (Crawford *et al.*, 2001). Moreover, a study examining the patterns of inbreeding in dioecious and self-compatible mosses showed that both groups have a deficiency of heterozygosity, although this deficiency was higher in self-compatible species (Eppley *et al.*, 2007). Studies on other dioecious species found relatively low levels of genetic diversity (Waycott *et al.*, 1996; Terauchi *et al.*, 1997; Bartish *et al.*, 1999; Rottenberg *et al.*, 1999) and/or heterozygote deficiency [i.e. a significant F_{is} (Lokker *et al.*, 1994; Rottenberg *et al.*, 1999; Luna *et al.*, 2007)]. Overall, the above examples suggest that dioecy does not increase the amount of genetic variation, and could even reduce it.

In this study, we compared the genetic structure of a dioecious species with that of a closely related self-incompatible species. If both dioecy and self-incompatibility are inbreeding-avoidance mechanisms (Baker, 1959), dioecious species should have greater genetic variation than self-compatible but not self-incompatible species. Dioecy has repeatedly evolved from self-incompatible distylous ancestors (see Webb, 1999), and therefore no differences in genetic diversity should be apparent between these two breeding systems. Accordingly, we measured the amount of genetic diversity in five populations of the dioecious species *Erythroxylum rotundifolium*, and in six of its distylous relative *E. havanense*. Similar levels of genetic variation between the two species would be consistent with Baker's statement. Higher or lower levels of genetic variation in *E. rotundifolium* would indicate

that sexual specialization has population genetic effects that go beyond the avoidance of inbreeding.

METHODS

Study sites

We studied six populations of *E. havanense* distributed along the tropical deciduous forests of the Pacific coast of Mexico. We sampled three populations from the state of Jalisco (Ranchitos, Limón, and Ardilla), one from Guerrero (Ixtapa), and two from Oaxaca (Huatulco and Nizanda). The distribution of *E. rotundifolium* along the Pacific coast of Mexico closely resembles that of *E. havanense*, although the former is also found in the tropical dry forests of southeast Mexico. Thus, we sampled three populations from the Pacific coast in the state of Oaxaca (Concepción Papalo, Otaltepec, and Huatulco), one from the Atlantic coast in the state of Yucatán (Celestún), and one from the Caribbean sea (Cobá, Quintana Roo; Fig. 1).

Study organisms

Erythroxylum (Erythroxylaceae) is a Pantropical genus comprising 250 species of distylous shrubs and trees, although some derived dioecious species also occur (Ganders, 1979; Barrett, 1992;

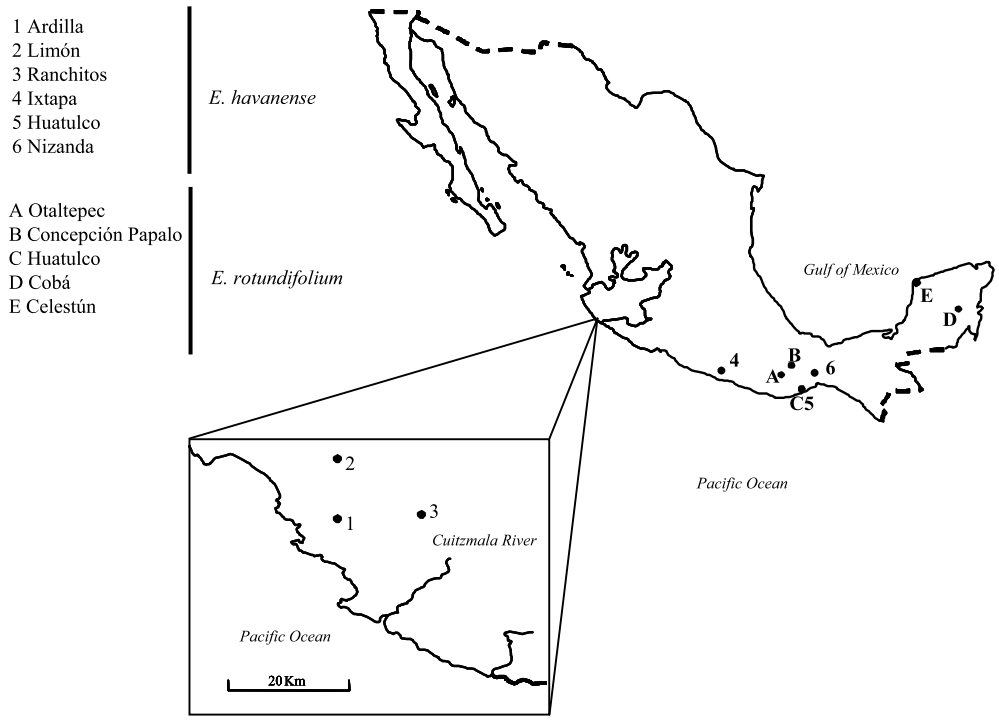

Fig. 1. Map showing the location of the six populations of *Erythroxylum havansne* (in numbers) and the five populations of *Erythroxylum rotundifolium* (in letters) sampled in this study.

Kelly, 2001). Phylogenetic relationships among species of *Erythroxylum* are poorly understood (Johnson *et al.*, 2005). Nonetheless, our results indicate (see below) much lower genetic differentiation between *E. havanense* and *E. rotundifolium* than among other *Erythroxylum* species (Johnson *et al.*, 2005), suggesting that they are closely related.

Erythroxylum havanense is a tropical distylous shrub with a heteromorphic incompatibility system preventing seed production from self- and same-morph crosses (Domínguez *et al.*, 1997). Flowering initiates soon after the first heavy rains of the wet season and results in a marked reproductive synchrony within and among individuals [3 ± 0.1 days and 6 ± 0.9 days for individual and population flowering periods, respectively (Domínguez and Dirzo, 1995)]. Fruit maturation is also highly synchronized among plants within a patch (Gryj and Domínguez, 1996). Mass flowering and synchronous ripening produce a high and ephemeral abundance of resources for both pollinators and frugivores (Domínguez *et al.*, 2005), which in turn concentrates their foraging bouts within a flowering (fruiting) patch. *Erythroxylum rotundifolium* is a dioecious tree that also flowers in response to the first heavy rains, but unlike *E. havanense* it has an extended flowering period and a marked reproductive asynchrony among individuals. Plant flowering periods may vary from 18 to 40 days, while that of the population lasts up to 3 months (Martínez-Bauer, 2007). Flowers of female plants have reminiscences of stamens, while those of males present a sterile pistiloid, thus indicating its derived condition (Kelly, 2001; Martínez-Bauer, 2007).

Accordingly, differences in flowering phenology between these two species could also influence the patterns of genetic structure. While the high levels of genetic variation and population structure found in *E. havanense* may be explained by the effect of synchronous reproduction on both pollinators and frugivores (Domínguez *et al.*, 2005), the marked flowering asynchrony observed in *E. rotundifolium* suggests that effective mating occurs only between those individuals that flower in synchrony with each other. Thus, temporal reproductive isolation has the potential to subdivide the population into smaller mating groups.

Material collection

Fresh tissue was collected from the leaves of 20–30 individuals of each population during the summers of 2001 and 2002. In total, 300 plants were sampled in this study. Tissue samples were frozen in liquid nitrogen for transport to the laboratory where they were stored at −80°C.

DNA extraction

DNA was extracted from 1 g of fresh tissue (leaves) with a modified mini-prep technique (Doyle and Doyle, 1987). Once DNA was extracted, it was re-suspended in TE (10 $mmol \cdot l^{-1}$ tris-HCl, pH 8.0; 1 $mmol \cdot l^{-1}$ EDTA) and stored at −20°C.

ISSR amplification

Four simple sequence repeats (ISSR) were used as primers to generate a total of 119 polymorphic bands in single-primer reactions. The ingredients per reaction (20 µl in total in each) were the same for each of the four primers: 2 $mmol \cdot l^{-1}$ $MgCl_2$, 0.2 U Taq polymerase, 2.5 µl DNA, 0.2 $mmol \cdot l^{-1}$ dNTPs, 1 $\mu mol \cdot l^{-1}$ primer, and 1 × Taq DNA polymerase buffer.

The polymerase chain reaction (PCR) was conducted in a Techne thermocycler, Touchgene gradient, and the program was 5 min at 94°C; 27 × 30 s at 94°C, 45 s at 52°C, 2 min at 72°C, 7 min at 72°C; 6°C soak.

Following PCR, 1.5 µl bromophenol blue marker dye was added to each reaction and the samples were loaded onto a 6% polyacrylamide gel in 1 × TBE buffer. We used acrylamide gels because more bands were solved for each primer than in agarose gels. Additionally, 1 Kb Ladder (Gigco/BRL) and positive and negative controls were loaded onto each gel. Gels were ran at constant voltage (500 V) for 2.5 h. Each gel was stained with silver nitrate and digitized using a Sony DSC-P50 cyber shot camera (2.1 megapixels). Images were analysed using the Labworks software pakage (4.5 version for Windows 98/Nt/2000 pro/xp pro), which assigns a fragment size to each band using an algorithm based on the 1 Kb ladder. Bands for each assigned locus were scored as diallelic (1 for band present and 0 for band absent).

Data analyses

Because of the dominant nature of ISSR markers, it is not possible to distinguish between dominant homozygotes and heterozygotes. Consequently, Hardy-Weinberg equilibrium must be assumed for genetic analyses. We followed this approach and used Lynch and Milligan's (1994) criteria to calculate unbiased allelic frequencies. We then estimated the average percentage of polymorphic loci (P) and the average expected heterozygosity (H_e) per population (Hedrick, 2000). Because our sampling procedure included a hierarchical arrangement (species, populations within species, and individuals within populations and species), population genetic structure was analysed following the procedures outlined by Weir and Cockerham (1984) and implemented in the TFPGA program (Miller, 1997). Finally, to gain a thorough understanding of the genetic structure of the studied species, we performed independent estimations of the F-statistics.

Because the Hardy-Weinberg assumption could be easily violated (see Culley and Wolfe, 2001), a complementary estimation of the genetic variation based on the Shannon information measure was performed (Lewontin, 1972; King and Schaal, 1989; Bussell, 1999; Domínguez *et al.*, 2005). We calculated the amount of genetic diversity for each species (H'_{sp}), the average genetic variation among populations from each species (H'_{pop}), and the total diversity (H'_{tot}). Population genetic structure was estimated following the same hierarchical structure we used in the allelic frequencies analysis. We estimated the proportion of genetic diversity explained by differences between species [$(H'_{tot} - H'_{spp})/H'_{tot}$, where H'_{spp} equals the average genetic diversity among species], and among populations within species [$(H'_{tot} - H'_{pops})/H'_{tot}$, where H'_{pops} equals the genetic variation among populations averaged across species]. Genetic structure was then independently estimated for each species. We calculated two measures of genetic differentiation, one for each species, as $(H'_{sp} - H'_{pop})/H'_{sp}$. Finally, we used Neighbour-Joining and Euclidean distances to construct a phenogram of the populations with the Phyllip program [version 3.6 (Felseinstein, 2004)].

RESULTS

Genetic analyses revealed a large amount of genetic variation in both species (Table 1). *Erythroxylum havanense*, the distylous species, showed significantly greater polymorphism (P) than the dioecious *E. rotundifolium* (89.63% and 73.6% for *E. havanense* and

Table 1. Estimates of genetic diversity for six populations of distylous *Erythroxylum havanense* and five populations of dioecious *Erythroxylum rotundifolium* from Mexico

Species	Population	n	P	H_e	H'
Erythroxylum havanense	Ranchitos	23	88.2	0.29	0.6795
	Ardilla	18	81.5	0.26	0.6369
	Limón	21	91.6	0.31	0.7322
	Huatulco	28	92.4	0.32	0.7622
	Ixtapa	27	95.8	0.34	0.7627
	Nizanda	21	88.2	0.31	0.7622
Erythroxylum rotundifolium	Cobá	20	73.31	0.22	0.5313
	Otaltepec	18	69.7	0.21	0.4791
	Celestún	20	69.7	0.2	0.5302
	Concepción Papalo	20	69.7	0.21	0.5132
	Huatulco	21	85.7	0.24	0.624

Note: Expected heterozygosity (H_e) and percentage of polymorphic loci (P) were calculated assuming that populations are at Hardy-Weinberg equilibrium. Shannon's index (H') provides a measure of genetic diversity that does not rely on the Hardy-Weinberg equilibrium. n = number of individuals scored.

E. rotundifolium, respectively; $U = 1.0$, $P = 0.01$). Expected heterozygosity in *E. havanense* ranged from 0.26 to 0.34 (average $H_e = 0.30$), while that of *E. rotundifolium* varied between 0.20 and 0.24 (average $H_e = 0.21$). The amount of genetic variation in populations from the heterostylous species was significantly higher than in dioecious populations (Mann-Whitney $U = 2.73$; $P = 0.0001$).

Hierarchical analysis of *F*-coefficients showed that differences between species accounted for only a small proportion of the total genetic variation (Table 2). Differences among populations within species were also significant and accounted for almost 30% of the genetic variation (Table 2). Independent estimations of population differentiation, one for each species, were in agreement with our previous analysis and showed that populations differ significantly from each other in both species (Table 3). Population differentiation in *E. rotundifolium*, the dioecious species, was 17% higher than that of the distylous *E. havanense*.

Estimations of genetic variation based on the Shannon index were in general agreement with those derived from allelic frequencies (Table 1). Populations of *E. havanense* were significantly more diverse than those of the dioecious *E. rotundifolium* ($H'_{pop} = 0.71$ and 0.53 for *E. havanense* and *E. rotundifolium*, respectively; $U = 2.73$; $P = 0.0001$). Both approaches (allelic frequencies and Shannon index) showed that genetic variation in the more variable dioecious population (Huatulco) was lower than that of the less variable distylous population (Ardilla). These results indicate that the dioecious species contains less genetic variation than the distylous species.

Analyses of genetic structure showed that differences between species accounted for a small proportion of the total genetic variation. In contrast, differences among populations within species explained a substantial amount of the genetic variation (Table 2). Analyses of the genetic structure performed independently for each species also showed high values of population differentiation in both species (Table 3). Genetic structure was higher among dioecious than among distylous populations (Table 3).

Table 2. Hierarchical analysis of the genetic structure in both *Erythroxylum havanense* and *Erythroxylum rotundifolium*

$F_{(ct)}$	0.052
$(H'_{tot} - H'_{sp})/H'_{tot}$	0.073
$F_{(sc)}$	0.26
$(H'_{tot} - H'_{pop})/H'_{tot}$	0.33

Note: Hierarchical analysis was calculated from allele frequencies and the Shannon information index. $F_{(ct)}$ and $(H'_{tot} - H'_{sp})/H'_{tot}$ represent genetic differences between species; and $F_{(sc)}$ and $(H'_{tot} - H'_{pop})/H'_{tot}$ represent genetic differences among populations between species. For details, see data analyses.

Table 3. Independent estimations of the genetic differences among populations of each species

Erythroxylum havanense	$F_{(st)}$	0.24
	$(H'_{sp} - H'_{pop})/H'_{sp}$	0.24
Erythroxylum rotundifolium	$F_{(st)}$	0.29
	$(H'_{sp} - H'_{pop})/H'_{sp}$	0.31

Note: Each estimation was derived from allele frequencies $F_{(st)}$ and Shannon's index $[(H'_{sp} - H'_{pop})/H'_{sp}]$. For details, see data analyses.

Neighbour Joining produced two main groups, one for each species (Fig. 2). Populations of *E. rotundifolium* were separated into two groups, each corresponding to geographic affinity (Otaltepec and Concepción Papalo, from Oaxaca; and Celestún and Cobá, from the Yucatán peninsula). Although the Huatulco population is also located in the state of Oaxaca, it showed the greatest genetic distance within the *E. rotundifolium* group. Populations of *E. havanense* showed no pattern regarding geographic distances, since populations from distant localities were grouped together. Independent analyses of isolation by distance showed no relationship between genetic and geographic distances for either species ($r = 0.19$, $P = 0.60$ and $r = 0.25$, $P = 0.66$ for *E. rotundifolium* and *E. havanense*, respectively).

DISCUSSION

The most important single result of this study is that genetic diversity of the dioecious species, *E. rotundifolium*, was significantly less than that of the distylous *E. havanense*. Thus, although both dioecy and self-incompatibility are efficient inbreeding-avoidance mechanisms (Baker, 1959), the genetic consequences of following one of these evolutionary paths are not equivalent. Our results suggest that even though both species maintain relatively high levels of genetic variance, the evolution of dioecy in this genus was associated

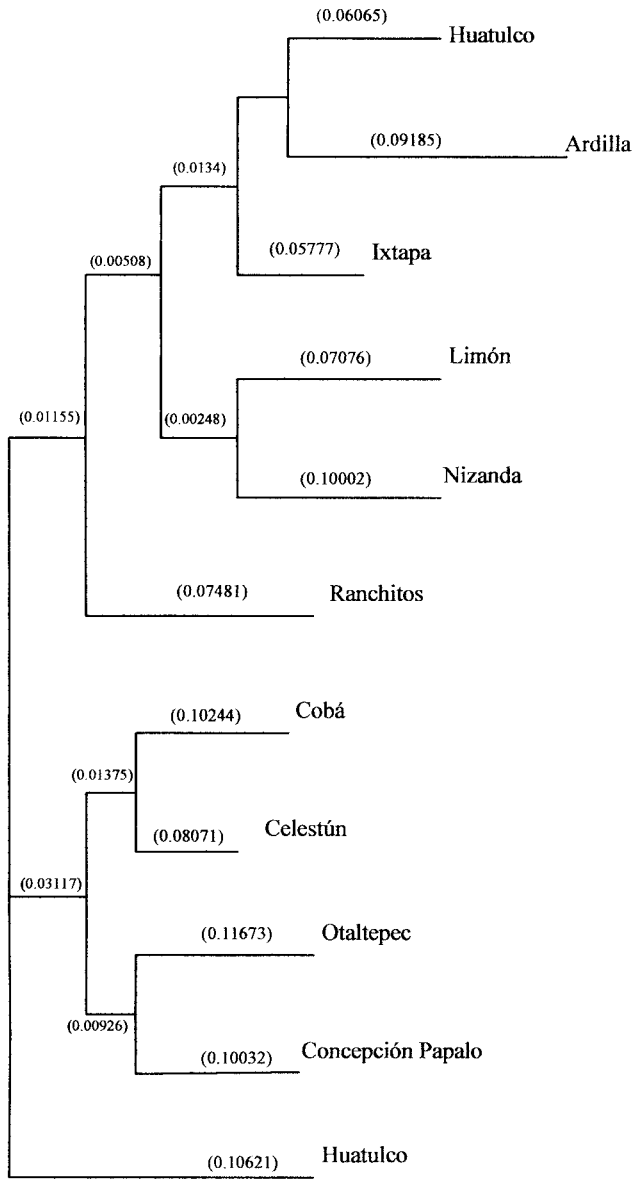

Fig. 2. Phenogram built with the Neighbour-Joining method and Euclidian distances (Excofier 1992) in the Phyllip program [version 3.6 (Felseinstein, 2004)]. Numbers indicate branch length.

with a significant decline in the amount of genetic diversity and an increase in the extent of population differentiation. Nonetheless, the genetic differences observed in this study could also be a consequence of species-specific differences in life-history traits (Hamrick and Godt, 1996), or in the evolutionary history of the species analysed. Our findings, however, do not represent an isolated case, since other dioecious species varying in life and/or evolutionary history also show reduced genetic diversity when compared with non-dioecious species

(Waycott *et al.*, 1996; Terauchi *et al.*, 1997; Bartish *et al.*, 1999; Rottenberg *et al.*, 1999; Crawford *et al.*, 2001; Dorken *et al.*, 2002; Dorken and Barrett, 2004). The approach adopted in this study differs from that in previous ones because, to our knowledge, this is the first study to compare the genetic structure of a dioecious species with that of a closely related self-incompatible species.

There are two possible proximal explanations for the relatively low level of genetic variation observed in the dioecious *E. rotundifolium*. First, it is likely that *E. havanense* possesses an exceptionally high level of genetic diversity producing the false impression of a relatively low amount in the dioecious species. Second, the genetic diversity of *E. havanense* falls within the range of other outcrossing tropical perennial plants and, consequently, *E. rotundifolium* actually has low levels of genetic variance. As revealed by allozyme-based studies (Ganders *et al.*, 1985; Hamrick and Loveless, 1986; Pérez-Nasser *et al.*, 1993; Loiselle *et al.*, 1995), the genetic diversity of distylous species usually falls within the range reported for other long-lived perennial outcrossing plants (Hamrick and Godt, 1996). Nonetheless, the results of this and previous studies (Domínguez *et al.*, 2005) show that *E. havanense* has particularly high levels of genetic variation ($H_e = 0.431 \pm 0.04$ and 0.30 ± 0.03 for RAPD and ISSR-based estimations, respectively) that are almost double the average values of other long-lived perennial outcrossing species [$H_e = 0.214 \pm 0.117$, $H_e = 0.220 \pm 0.08$, and $H_e = 0.230 \pm 0.08$ for RAPD, ISSR, and AFLP-based estimations, respectively (Nybom and Bartish, 2000; Nybom, 2004)]. Thus, even though the genetic variation of *E. rotundifolium* was significantly lower than that of its distylous relative, it falls within the range observed for other long-lived perennial outcrossing species (Nybom and Bartish, 2000; Nybom, 2004). In short, although this result could suggest that both breeding systems represent alternative outbreeding mechanisms, the *magnitude* of the genetic diversity maintained by the distylous species is one-third higher than that of the dioecious species. Under this scenario, it would be pertinent to ask what mechanisms may generate these differences, as well as the consequences.

There are at least three non-exclusive explanations for the observed reduction in the extent of genetic diversity of the dioecious species. First, because the evolution of dioecy is usually accompanied by the presence of sexual dimorphisms [floral display, amount of rewards, etc. (see Geber, 1999)], pollinators may discriminate against the less attractive individuals [usually the females (Delph, 1996; Eckhart, 1999; Vamosi and Otto, 2002)], reducing effective population size [N_e (Hedrick, 2000)]. It is well known that N_e is directly related to the magnitude of the genetic variation within a population (Wright, 1930; Charlesworth, 2003). Second, biases in sex ratio may also reduce the effective population size (Hedrick, 2000). Because $N_e = 4N_f N_m / N_f + N_m$ [where N_f and N_m denote the number of females and males within a population, respectively (Hedrick, 2000)], any deviation from equal representation of males and females results in $N_e < N$ (Hedrick, 2000). Third, a lack of reproductive synchronization between male and female individuals may reduce the effective population size by producing temporal fluctuations in population sex ratio. It should be noted that the three mechanisms would result in increased variance in the reproductive success between the sexes and among individuals within a sex.

Preliminary observations performed in the Chamela *E. rotundifolium* population indicate that pollinator visitation differs between the sexes, with females receiving twice the number of visits observed in male plants (Martínez-Bauer, 2007). Although the sex ratio of the population is 1:1, the flowering onset of individual plants is not synchronous, thus resulting in a marked temporal variation in the number of individuals of each sex that flower simultaneously (Martínez-Bauer, 2007). In contrast, the flowering pattern of *E. havanense* is

highly synchronous both within and among individuals (Domínguez and Dirzo, 1995), and pollinators do not discriminate between Pin and Thrum plants (Domínguez *et al.*, 1997). Moreover, the population density of *E. havanense* is much higher than that of *E. rotundifolium* (1320 and 35 individuals per hectare in *E. havanense* and *E. rotundifolium*, respectively). Thus, the combined effects of female-biased pollinator foraging, the lack of flowering synchronization between most male and female plants, and the relatively low population density of *E. rotundifolium* likely result in high reproductive variances and low effective population sizes. Overall, differences in the reproductive biology and demographic traits of *E. rotundifolium* and *E. havanense* may explain why the dioecious species has less genetic variation and higher population differentiation than its distylous relative.

Finally, we thought that the differences in the foraging behaviour of pollinators observed in this study may have originated as a by-product of the selective forces favouring the evolution of separated sexes. Although both male and female flowers of *E. rotundifolium* produce nectar, the secretory tissue of female flowers shows marked differences from that of males. Female flowers have functional and well-developed nectaries located in the wall of the ovary. The secretory tissue of male flowers is also located in the wall of the vestigial ovary, but in contrast with the female nectary, it consists of groups of secretory cells without a clear differentiation (Martínez-Bauer, 2007). Given that the secretory tissue is associated with the ovary wall, and because the evolution of separated sexes involves the sterilization of the female function in male flowers, it would be possible that the evolution of dioecy in *E. rotundifolium* also reduced the ability of male flowers to produce nectar. This scenario is in accordance with previous proposals that the evolution of sexual dimorphisms may influence the response of pollinators to individuals of each sex (Vamosi and Otto, 2002). Such differences have the potential to increase the variance in reproductive success and, therefore, to reduce the effective population size of dioecious populations (Charlesworth, 2003).

Overall, our results and those of other studies indicate that dioecious species have lower levels of genetic variance than other non-dioecious species (Waycott *et al.*, 1996; Terauchi *et al.*, 1997; Bartish *et al.*, 1999; Rottenberg *et al.*, 1999; Crawford *et al.*, 2001; Dorken *et al.*, 2002; Dorken and Barrett, 2004). Thus, although both dioecy and self-incompatibility may function as effective inbreeding-avoidance mechanisms (Baker, 1959), the evolution of unisexuality seems to reduce the amount of genetic variation. In *E. rotundifolium*, such a reduction is probably a consequence of the evolution of sexually dimorphic attributes influencing the effective population size. Whether this phenomenon is widespread among other dioecious species requires further work involving multiple phylogenetically independent comparisons.

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