

# Male sterility, fitness gain curves and the evolution of gender specialization from distyly in *Erythroxyllum havanense*

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## Abstract

The evolution of dioecy from a monomorphic hermaphroditic condition requires two mutations, one producing females and one producing males. Conversely, a single mutation sterilizing one sexual function in one morph of distylous species would result in functional dioecy because such a mutation also affects the complementary function in the other morph. In this study, we tested these ideas with *Erythroxyllum havanense*, a distylous species with morph-biased male sterility. Based on sex allocation theory we evaluated whether the invasion of thrum females is favoured over the maintenance of this morph cosexuals. Completely male sterile thrum plants obtained higher fitness returns than hermaphrodites or partial male sterile individuals of the same morph, thus favouring the invasion of female thrum plants. We concluded that because fruit production of pin individuals depends on the pollen produced by thrum plants, the invasion of thrum females would result on the evolution of functional dioecy.

## Introduction

Dioecy is a rare reproductive system occurring in only 6% of the angiosperm species (14 620/240 000 species) and spread through half of the families of flowering plants (157 of 365, Renner & Ricklefs, 1995). Given that flowering plants are predominantly hermaphroditic, the occurrence of dioecy on several relatively independent families suggests that dioecy has repeatedly evolved from cosexual forms (Lloyd, 1980; Ross, 1982; Webb, 1999). Although five evolutionary pathways to dioecy are generally recognized (Webb, 1999), two of them have been widely documented. First, in the gynodioecy and androdioecy pathways a monomorphic cosexual population is invaded by a sterile mutant (usually male sterility), so establishing a gender dimorphism. A subsequent mutation sterilizing the complementary sex (usually female sterility) in the remnant hermaphroditic

individuals is necessary to establish a dioecious population. Male sterility (the gynodioecious path) is by far the most common sterility change among the angiosperms (543 species, 178 genera and 50 families, Dem'yanova 1985, cited in Jacobs & Wade, 2003) and has been conceived as a key factor in the evolutionary pathway to sex specialization (Lewis, 1941; Lloyd, 1975; Charlesworth & Charlesworth, 1978). Second, dioecy has also evolved from cosexual, already dimorphic ancestors, as is the case of distyly and heterodichogamy (Darwin, 1877; Lloyd, 1979; Casper & Charnov, 1982; Ross, 1982; Webb, 1999). The classical explanation for the evolution of dioecy from distyly proposes that pollinator-driven asymmetrical pollen transfer between the floral morphs would favour maleness in the morph with the higher pollen donation efficiency (usually thrum), and femaleness in the morph receiving more pollen (usually pin) (Darwin, 1877; Ornduff, 1966; Lloyd, 1979; Beach & Bawa, 1980; but see Ornelas *et al.*, 2004; García-Robledo, 2007; for the opposite pattern). According to this hypothesis, the evolutionary force driving gender specialization depends on ecological changes, i.e. loss of the most efficient pollinators. This selective regime is

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expected to favour a gradual and simultaneous process of sexual divergence between the floral morphs and eventually the evolution of separated sexes. Although dioecy and distyly are correlated at the family level supporting a distylous ancestor for dioecy (Baker, 1958; Pailler *et al.*, 1998) and several studies have shown the existence of asymmetrical pollen flow between the floral morphs (Stone, 1995; Lau & Bosque, 2003; García-Robledo, 2007), no study has proven a causal relationship between pollinator driven asymmetrical pollen flow and the evolution of dioecy (Beach & Bawa, 1980; Muenchow & Grebus, 1989).

Distylous populations may also be invaded by sterility mutations as those characterizing the gynodioecious pathway (Domínguez *et al.*, 1997). Sterility mutations are differentially expressed between the floral morphs and the presence of male sterile individuals in already dimorphic populations may have important consequences for the evolution of gender specialization (Domínguez *et al.*, 1997; Pailler *et al.*, 1998; Del-Carlo & Buzato, 2006). Moreover, a mutational driven explanation does not require any directionality in the pattern of morph specialization and could explain the opposite pattern of gender specialization observed in some distylous species (thrusms as females and pins as males, see Wyatt, 1983; Avila-Sakar & Domínguez, 2000).

Most distylous species are characterized by two attributes enforcing disassortative mating between the floral morphs, reciprocal herkogamy and heteromorphic incompatibility. Because seed production of one floral morph depends on the availability of pollen of the opposite morph, the sterilization of one sexual function in one morph would render the complementary function in the other morph useless. Consequently, unlike the gynodioecy and androdioecy pathways, the establishment of functional dioecy from distylous ancestors requires a single sterilization step. Nonetheless, despite the invasion of sterile individuals having the potential to drive gender specialization in dimorphic reproductive systems, this evolutionary pathway has been poorly explored (Baker, 1958; Avila-Sakar & Domínguez, 2000; Del-Carlo & Buzato, 2006).

Theoretically, in order to invade a population of monomorphic hermaphrodites, females must compensate the reproductive disadvantage that results from male sterility (Fisher, 1958; Charnov, 1982). Depending on the mode in which male sterility is inherited (nuclear, cytoplasmic or nuclear-cytoplasmic) the reproductive compensation of females may range from only a small advantage to twice the fitness of hermaphroditic plants (Lewis, 1941; Schultz, 1994; Charlesworth, 1999, 2002). In contrast, if male-sterility is linked to one sexual morph of dimorphic species, invasion is possible when the fitness of females surmounts that of hermaphrodites of the same morph (Pannell & Verdú, 2006). In this context, classical sex allocation theory provides the analytical tools allowing determining whether hermaphroditism is

evolutionarily stable against the invasion of unisexual individuals (Charnov, 1982, 1984; Campbell, 1998, 2000). Sex allocation analyses are based on the functional relationship between resource allocation to male function and male and female fitness (fitness gain curves, hereafter). With diminishing returns fitness curves, hermaphroditism is generally evolutionarily stable (Charnov, 1982; Lloyd, 1984). Accelerating fitness gains per unit of investment, in contrast, favour the invasion of unisexuals (i.e. the evolution of dioecy). When one fitness function accelerates and the other decelerates, the condition for hermaphroditism to be an evolutionarily stable strategy (ESS) depends on the exponents determining the shape of the fitness functions (Charnov, 1982; Lloyd, 1984).

Accordingly, in this study we used field observations and experiments to estimate whether or not male sterile thrum individuals have higher fitness gains than hermaphrodites of the same morph and evaluated if this ecological scenario favours the evolution of gender specialization in a distylous species. To this end, we used *Erythroxylum havanense* Jacq. (Erythroxylaceae), a tropical shrub with morph-biased male sterility. Analyses of pollen viability revealed that the frequency of females (completely male-sterile individuals) in *E. havanense* is 23% for thrums and 5% for pins and levels of male sterility (proportion of sterile pollen) in partially male sterile individuals are also greater for thrum plants ( $0.81 \pm 0.02$  and  $0.64 \pm 0.02$  (mean  $\pm$  1 SD hereafter), for thrum and pin plants, respectively; Rosas *et al.*, 2005). A previous study showed that gender specialization in this species is not related to classical explanations, as no evidence of asymmetrical pollen flow or self-compatibility was found (Domínguez *et al.*, 1997). Given that *E. havanense* has hermaphrodite (pin) and both partial and totally male sterile (thrum) individuals, it represents an excellent opportunity to assess the influence of male sterility on the initial steps of the evolution of sexual specialization in heterostylous systems. Specifically, in this paper we estimated both male and female fitness gain curves of thrum individuals varying in the level of male sterility and tested if the invasion of females is favoured over the maintenance of cosexuals.

## Materials and methods

### Study site

This study was carried out at the Chamela Biological Station located on the Pacific coast of the state of Jalisco, Mexico ( $19^{\circ}30'N$ ,  $105^{\circ}03'W$ ). The site is characterized by a marked dry season from November to May, with 80% of the precipitation falling from July to October (707 mm year). The vegetation comprises a dense tropical deciduous forest. A detailed description of the flora and the climate of the region can be found in Lott *et al.* (1987) and Bullock (1986) respectively.

### Study plant

*Erythroxylum havanense* (Erythroxylaceae) is a perennial distylous shrub with a sporophytic self-incompatibility system impeding fruit production after same-morph crosses and selfing (Domínguez & Dirzo, 1995). *Erythroxylum havanense* is characterized by morph-biased male sterility and a compensatory female advantage of thrums (Domínguez *et al.*, 1997; Avila-Sakar & Domínguez, 2000). Thrum individuals produce 46% more seeds than pins and its progeny outperforms that of pins (Domínguez *et al.*, 1997; Avila-Sakar & Domínguez, 2000). Because there is no modification in the incompatibility system, pin ovules are fertilized by thrum pollen and vice versa. Repeatability analysis showed that 47% of the variance in male sterility is explained by permanent differences among individuals ( $F_{(1-22)} = 15.8$ ,  $P = 0.0006$ , Rosas *et al.*, 2005). Although there is not information for *E. havanense*, environmental or age differences among individuals might account for the remaining variance in male sterility (Sheoran & Saini, 1996). Plants grow in groups of 20–100 individuals with 1 : 1 morph ratio. Flowering initiates 7–10 days after the first intense rains that mark the beginning of the wet season (Domínguez & Dirzo, 1995). Once flowering is triggered, individual plants flower for a very short period ( $2.8 \pm 0.1$  day). Flowers are small ( $\sim 1$  cm diameter), mainly pollinated by bees of the genus *Trigona* (Apidae) and *Xylocopa* (Anthophoridae), and stigma receptivity is limited to the first half of the day. Fruits are single seeded ovoid red drupes (1 cm long). All observations and experiments performed in this study were done in the Ardilla population, a permanent plot that we have studied intensively since 1995 and representing the general situation of *E. havanense* in the Chamela region (Cuevas *et al.*, 2005).

### Data collection

#### Male sterility

Data on the amount of variation in male sterility (MS) were obtained in a previous study performed in the same population and the same plants we worked with in this paper (Rosas *et al.*, 2005). For each plant we estimated the level of male sterility by means of *in vitro* pollen cultures during the reproductive seasons of 1998 and 1999 (see Cuevas *et al.*, 2005 for a detailed description). We calculated the fraction of germinated and total pollen grains and this measure was used as an estimation of the level of male sterility per plant. Previous analyses have shown that pollen production does not differ among thrum plants from the Ardilla population ( $F_{24, 334} = 1.29$ ,  $P = 0.17$ ; F. Rosas, unpublished data), and that MS is positively related with female fertility, thus suggesting that MS reflects the allocation of a limiting resource between competing functions (see below for further justification of the usage of MS).

#### Female reproductive output

During the reproductive season of 2000, we followed the flowering and fruiting phenology of all the reproductive plants from the Ardilla population ( $N = 76$ ). For each plant, we made daily census to record the number of flowers, immature fruits, and ripe fruits. Census were initiated the first day a plant produced flowers (7 June 2000) and finished when all fruits in the population had ripened (12 July 2000).

#### Male reproductive output

Because we were interested in the estimation of the relationship between male sterility and male fitness, we took advantage of the ability of *E. havanense* to flower in response to watering. We designed a field experiment in which we induced four thrum plants (sires) and two pin plants (dams) to flower simultaneously. As we already knew MS levels of most plants from the Ardilla population (Rosas *et al.*, 2005), thrum plants were selected to embrace all the range of variation in MS. We chose one plant with high ( $\geq 1$  SD above the mean MS value,  $0.81 \pm 0.09$ ), two with medium ( $< 1$  SD around the mean MS) and one with low ( $\geq 1$  SD below the mean MS value) level of MS. This experiment was replicated 10 times 1 month before the beginning of the rainy season of 1999. Watering was scheduled to induce synchronous flowering within a replicate and flowering in sequence among replicates. Each plant was initially watered with 20 L of water and subsequently with 10 L every 2 days. Watering of the first replicate started on May 12 and a period of 2 days was included between the irrigation of subsequent trials. The last trial was watered until June 8. Because experimental plants flowered before the natural flowering period, and given that each pin dam had only four possible pollen donors, this procedure increased the probability to assign the genetic parentage in the paternity analysis. Once plants within each replicate flowered, they were actively visited by the same species of social bees that pollinate this species in natural conditions (Domínguez & Dirzo, 1995). Leaf cutter-ants (*Atta* sp.) attacked some experimental plants, thus reducing the initial sample size to five replicates with a single pin dam and four pollen donors and five replicates with a single pin dam and three pollen donors. Because the number of flowers and the distance between mates is likely to influence the probability of pollen donation-reception, these variables were measured and included in the statistical analysis as covariates.

All mature fruits from pin plants were collected and seeds were germinated in 5-cm jiffy pots. Once seeds germinated, 511 seedlings from 10 maternal plants ( $51 \pm 3.7$  seeds per plant) were transplanted in a fully randomized design within an exclusion constructed in the forest. In November 1999, we collected sample of leaves from each of the 460 surviving seedlings and from all parental plants. Although this sampling procedure may include early selection, we preferred it instead of

sampling germinated seeds because we were interested in the fate of seedlings originated from parents varying in their level of male sterility. Moreover, early mortality was independent of sire identity, suggesting that our final sample is not biased with regard to male sterility ( $F_{34,460} = 1.3$ ,  $P = 0.12$ ; F. Rosas, unpublished data). Leaves were bagged, marked and transported in liquid nitrogen to the laboratory where they were kept in an ultra freezer ( $-80\text{ }^{\circ}\text{C}$ ). Paternity analysis was performed with a set of polymorphic ISSRs (inter simple sequence repeats) genetic markers by employing laboratory standard techniques. The procedures and details used to determine the multilocus genotypes of the analysed progeny are described in the Supporting Information.

#### *Paternity assignment*

We followed the standard procedure for paternity exclusion (Ellstrand, 1984). First, we compared the multilocus genotype of each seedling with that of its mother, and all shared bands were excluded from the analyses. Informative bands were then used to identify the actual sire of each seedling by excluding all the two or three potential sires. This information was used to calculate the relative contribution of each sire to the paternity of the progeny produced in its experimental replicate.

#### **Data analyses**

In order to estimate the functional relationship between MS and male and female fitness (seed production), we fitted power functions with the nonlinear platform implemented in JMP 5.0.1. (SAS, 2003). Power functions were used instead of simple regression procedures because sex allocation analyses are based on the comparison of the parameters defining the shape of the fitness gain functions (power functions rendered lower values of the Akaike's information criterion (Akaike, 1974) than linear ones, thus indicating that the former better fit the data). Male fitness ( $m$ ) is assumed to increase with the amount of resources ( $r$ ) allocated to this function as defined by the power equation  $m = k_m r^b$ .  $b$  determines the shape of the male fitness gain curve, while  $k_m$  is the male fitness of a plant assigning all its resources to the male function. As resources not used in the male function are assumed to be invested in the female function ( $1 - r$ ), female fitness ( $f$ ) is determined by the power function  $f = k_f (1 - r)^c$ . Equivalently,  $k_f$  represents the fitness of a plant allocating all its resources to the female function. Although we have good estimations of male and female fitness for this species, we were unable to measure the slight differences in androecium biomass that may exist between the flowers of both hermaphrodite and male sterile thrum individuals. Both types of plants produce full-developed anthers that only differ in the amount of fertile pollen (Domínguez *et al.*, 1997), thus we preferred to use  $1 - \text{MS}$  (MS is inversely proportional to male fertility) as a surrogate of  $r$  in these

analyses. Pollen is high in nitrogen-based constituents, amino acids, and proteins (Stanley & Linskens, 1974), compounds that plants might need in many other functions. If the resources male-sterile plants save by not provisioning pollen are allocated to seeds, then male-sterile plants could produce more or better seeds. Resource compensation has proved to be a common mechanism maintaining male sterility in several species (Shykoff, 1988; Atlan *et al.*, 1992; Eckhart, 1992; Poot, 1997; Ashman, 1999; Koelewijn, 2003) and previous studies demonstrate that it also occurs in *E. havanense* (Avila-Sakar & Domínguez, 2000). The higher seed set of thrum dams, as well as the superior performance of their progeny indicate that thrum plants of *E. havanense* (the more male-sterile morph) devote more resources to female function and lesser to the male one.

Finally, sex allocation theory establishes that for hermaphroditism to be an ESS the following condition must be satisfied  $b + c > 2bc$  (Lloyd, 1984). So, we used this criterion to evaluate whether natural selection favours the evolution of female thrum plants.

#### *Male sterility and female fecundity*

Female fitness gain curves were estimated by using MS measures performed in 1998 and 1999 and fecundity data obtained in 2000. Although we combined data from different years, our repeatability estimations showed that plants maintain their MS level through time (Rosas *et al.*, 2005). So, we are confident that we have a stable estimate of MS. Preliminary analyses showed that flower number has a strong effect on the number of fruits produced by a given plant (results not shown). Thus, in order to avoid this effect we used fruit set instead of fruit production as a measure of female fitness ( $f$ ) and estimated the parameters of the power function between MS and fruit set [ $f = k_f (\text{MS})^c$ ] by means of the nonlinear platform implemented in JMP 5.0.1. (SAS, 2003).

#### *Male sterility and siring success*

We first evaluated the effect of MS, the number of flowers per plant, and the distance between pollen donors and maternal plants on the siring success of thrum plants by means of a covariance analysis. To control for the effect of variation among replicates, the effect of Block was also included in the analysis. Siring success (the proportion of seeds sired for a given thrum plant within a replicate) was arcsine transformed (arcsine  $\sqrt{\text{siring success}}$ ) and used as the response variable in this analysis. We verified that all the ANCOVA assumptions were met by our data before analysis.

The male fitness curve was obtained by fitting a power function [ $m = k_m (1 - \text{MS})^b$ ] with the nonlinear platform implemented in JMP 5.0.1. (SAS, 2003). Siring success was used as the response variable ( $m$ ) and  $1 - \text{MS}$  was used as a surrogate of  $r$ . Because preliminary analyses showed that besides MS, flower number had also a significant effect on siring success, we calculated the

residuals of a regression analysis between siring success and this variable. The goal of this analysis was to obtain an estimation of siring success once the influence of flower number was removed. Given that both estimations produced qualitatively similar power functions, and because the effect of flower number was meagre, we only present the results based on siring success of MS plants.

## Results

### ISSRs variation

The screening of ISSR markers provided six polymorphic primers that amplified 111 different fragments of DNA. Overall, we obtained 72 (64.8%) polymorphic bands (see Table S1). The number of amplified fragments and the fraction of polymorphic bands by primer varied from 10 to 28 and from 0.45 to 0.71 respectively. The frequency of the recessive allele  $q$  (from  $q^2$ ) for each polymorphic loci varied from 0.42 to 0.75. The average frequency of the recessive allele for the 72 polymorphic loci was 0.586.

### Paternal assignment

The high level of variation expressed by the ISSR markers coupled with the experimental design resulted in a high exclusion power. We were able to assign a single sire for 89.3% of the analysed offspring. The paternity of 4–6 seedlings (8–13%) per experimental trial remained undetermined and they were not considered for further analyses. Mean siring success was  $0.28 (\pm 0.23)$  and ranged from 0 to 0.88. Thrum plants with MS higher than 0.9 (23%) not sired any offspring. Hence, these individuals can be considered completely male sterile (functional females).

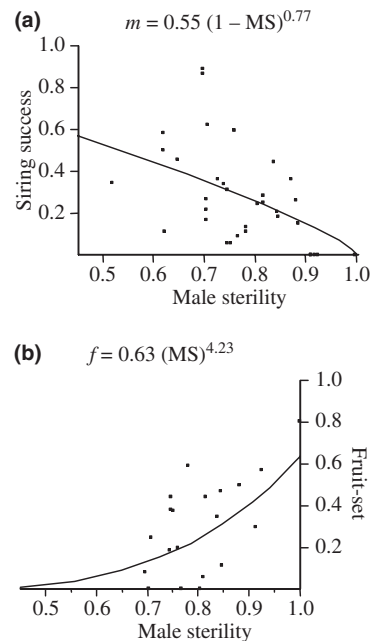
### Factors influencing siring success

Analysis of covariance explained 82% of the total variance in siring success ( $F_{21,34} = 8.53$ ,  $P = 0.0001$ ) and revealed significant effects of MS, Block, the number of flowers per plant and the interaction MS\*Block. Conversely, the variance in siring success was independent of distance between mates ( $F_{1,34} = 3.6$ ,  $P = 0.08$ ). As expected, there was a strong negative relationship between siring success and MS ( $F_{1,34} = 46.5$ ,  $P < 0.001$ ), while this relation was positive for flower number ( $F_{1,34} = 12$ ,  $P = 0.004$ ). In order to compare the slopes associated to each of the explanatory variables, the analysis was redone with reduced variables ( $\frac{x_i - \bar{x}}{\sigma}$ ). The slope relating MS and siring success ( $-0.27 \pm 0.03$ ) was 6.7–1.6 times higher than those of distance between mates ( $-0.04 \pm 0.029$ ) and flower number ( $0.17 \pm 0.033$ ), respectively. Finally, there was a significant effect of the interaction MS \* Block ( $F_{9,34} = 6.35$ ,  $P = 0.0016$ ), indicating that the relationship between siring success and MS changes depending on the replicate.

### Fitness gain functions and male sterility

As expected from sex allocation theory, there was a significant relationship between the extent of male sterility and fitness gained by a given sexual function. There was a positive and significant relationship between MS and fruit set ( $F_{1,21} = 4.21$ ,  $P = 0.05$ ), although the regression model only explained 13.2% of the variance. Although male sterile plants have higher female reproductive efficiencies, they paid a strong reproductive cost that was expressed as a lower efficiency as paternal parents. As indicated by the covariance analysis, a 10% increase in male sterility reduces siring success by 27.6%.

Nonlinear fitting produced two contrasting fitness gain curves (Fig. 1). While the fitness function for females showed a marked accelerating behaviour (Fig. 1b), the male curve was slightly decelerating (Fig. 1a). The exponent defining the shape of the female fitness function was  $c = 4.23$  (asymptotic 95% CI, 0.6–8.42), whereas that for the male function was  $b = 0.77$  (asymptotic 95% CI, 0.32–1.7). Because the covariance analysis showed a significant effect of the interaction MS \* Block, the parameter  $b$  was recalculated 10 times excluding one block at each run.  $b$ -Values ranged from



**Fig. 1** Estimated fitness gain curves showing the relationships between male (siring success,  $m$ ) and female fitness (fruit set,  $f$ ) and the extent of male sterility of thrum individuals of *Erythroxylum havanense* (male sterility is used as a surrogate of  $r$ ). The gain curve for the male function (a) showed decelerating fitness returns, whereas the female gain curve (b) accelerates at increasing values of male sterility. Plotted values include only the range values of male sterility observed in the field.

0.71 to 0.96 with a mean of 0.78 ( $\pm 0.071$ ), thus suggesting that our estimation of the  $b$  parameter is robust. Overall, our results indicate that *E. havanense* do not satisfy the condition for hermaphroditism to be an ESS ( $b + c > 2bc$ , Charnov, 1982; Campbell, 2000). Accordingly, these findings predict the fixation of male sterile thrum plants and consequently the establishment of a functional dioecious reproductive system.

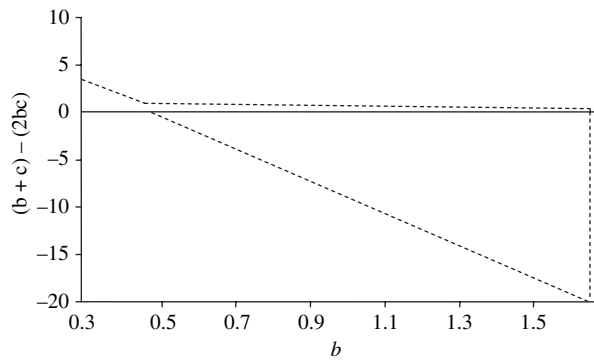
## Discussion

In this study, we have shown that the expression of sexuality among thrum individuals of *E. havanense* ranges from hermaphroditism (plants able to produce both seeds and fertile pollen), to full male sterility (functional females). Hence, we took advantage of the continuous distribution of male sterility and explored if a dimorphic cosexual reproductive system (distyly) is the ESS (Charnov, 1982; Lloyd, 1984) for this species. We used the rationale of sex allocation theory and estimated both male and female fitness gain curves for thrum individuals varying in the extent of male sterility. Our results revealed that completely male-sterile thrum individuals obtain higher fitness returns than hermaphrodites or partially male-sterile plants of the same morph, thus indicating that hermaphroditism is not an ESS for thrum plants. Accordingly, we predict that completely male-sterile thrum individuals will invade the population, which in turn would result in the evolution of functional dioecy. Because *E. havanense* possesses a heteromorphic incompatibility system preventing seed production after same-morph crosses, the invasion of completely male-sterile thrum individuals would render the female function of pin plants useless. In short, our results suggest that *E. havanense* is experiencing a process of gender specialization driven by sterility mutations associated with the thrum morph. Moreover, this is one of the few studies that have evaluated the fitness gain curves for both sexual functions (Campbell, 2000) and, to our knowledge, the first in applying this approach to dimorphic cosexual reproductive systems.

The evolutionary scenario depicted above for *E. havanense* could appear as a particular case, as it involves a continuous distribution of male-sterility, occurs in a cosexual dimorphic species and is morph-biased. In what follows, we argue that these phenomena are neither rare, nor restricted to *E. havanense*. First, theoretical and empirical approaches evaluating the role of male sterility on the evolution of sexual specialization have focused on the comparison of hermaphroditic and male-sterile individuals of gynodioecious species (Gouyon & Couvet, 1987; Shykoff, 1988; Frank, 1989; Maurice *et al.*, 1994; Delph *et al.*, 1999; Nilsson & Ågren, 2006). This approximation has almost ignored the presence of partial male-sterile plants in well-known gynodioecious species (Atlan *et al.*, 1992; Ashman, 1999; Koelewijn & Hundscheid, 2000; Rodriguez-Riaño &

Dafni, 2007). An examination of the available evidence suggests that more than an exception, continuous distributions of male-sterility in both gynodioecious and other sexual systems (Hanson, 1991; Schnable & Wise, 1998; van der Hulst *et al.*, 2004; Elkonin, 2005; Gomez & Shaw, 2006) could be the rule. Secondly, in spite of its apparent rareness, male-sterility in dimorphic cosexual species has been reported in the literature (Domínguez *et al.*, 1997; Paillet *et al.*, 1998; Naiki & Kato, 1999; Del-Carlo & Buzato, 2006). Some of these studies have also shown differences in male-sterility between the reproductive morphs (Naiki & Kato, 1999; Rosas *et al.*, 2005; Del-Carlo & Buzato, 2006). For example, at least five *Chassalia* species from Mauritius and La Reunion islands are morphological distylous but functionally dioecious (i.e. male-sterility is associated with only one sexual morph; Paillet *et al.*, 1998). Thus, the evolution of dioecy from distylous ancestors (Baker, 1958; Ornduff, 1966; Vuilleumier, 1967; Bawa & Opler, 1975; Opler *et al.*, 1975; Ganders, 1979; Beach & Bawa, 1980) indicates that morph-biased male-sterility has repeatedly evolved in heterostylous species. Nonetheless, although our data clearly indicate that morph-biased male sterility has played an important role in the process of gender specialization in *E. havanense*, the observation that some morphologically distylous but functionally dioecious species have MS linked to pins suggests that ecological hypothesis cannot be ruled out.

Despite the widespread occurrence of partial male sterility, there have been very few attempts to evaluate its consequences on the evolution of plant reproductive systems. In this study we have shown that natural selection may act on this variation, and depending on the conditions, favour the evolution of a gynodioecious like system. Such a system, however, differs from those derived from monomorphic species because of the presence of heteromorphic incompatibility and morph-specific sex specialization. Our analyses of fitness gain curves predict the invasion of completely male-sterile thrum plants. According to Lloyd (1984) the condition for hermaphroditism to be an ESS is  $b + c > 2bc$ . Given that the exponent of the female fitness function was  $c = 4.23$  and that of the male gain curve was  $b = 0.77$  (see Results), the condition for the maintenance of hermaphrodite thrum plants is not satisfied in *E. havanense*. Nonetheless, because the 95% CI for the  $b$  and  $c$  parameters were relatively wide (see Results) and could cast doubts about the validity of our interpretation, we explored the parameter space defined by all possible combinations of these two parameter values. This analysis revealed that only 13% of the possible combinations satisfied the condition  $b + c > 2bc$ , suggesting that hermaphroditism has a relatively low probability of being an ESS for thrum plants (Fig. 2). These findings support the proposal that natural selection is favouring the invasion of completely male-sterile thrum plants, which in turn, would



**Fig. 2** Parameter space defined by all the possible combinations between the parameter values defining the shape of male ( $b$ ) and female ( $c$ ) fitness functions included within the asymptotic 95% confidence intervals (CI) obtained from nonlinear fitting. For each value of  $b$  in the  $x$ -axis, we calculated the difference  $[(b + c) - (2bc)]$  for all  $c$  values included within the 95% CI. Accordingly, the area above zero in the  $y$ -axis corresponds to combinations of  $b$  and  $c$  where the condition for hermaphroditism to be an evolutionarily stable strategy is satisfied. The area below zero in the  $y$ -axis represents combinations where gender specialization is favoured by natural selection.

result in the replacement of distyly by a morphological gynodioecious (hermaphroditic pins and female thrums) but functional dioecious reproductive system. This interpretation was further reinforced by the examination of  $k_m$  and  $k_f$  values (the fitness gained by a thrum plant reproducing only as males or females, respectively), indicating that a thrum plant functioning exclusively as female would obtain a higher fitness gain ( $k_f = 0.63$ ) than a plant specialized as a male ( $k_m = 0.55$ ). Overall, our results suggest that we are witnessing an unfinished process of gender specialization in this species. Whether other populations are already dioecious remains as an unanswered question because detailed studies would be necessary to determine if morphological distylous populations are functioning as dioecious. Nonetheless, the presence of dioecious species within this distylous genus (Ganders, 1979), indicates that *E. havanense* is not the only species following this evolutionary path within the *Erythroxylum* group.

The above conclusion is based on the comparison of the fitness gains obtained by thrum plants varying in their levels of male-sterility. Such a conclusion is also supported by previous studies showing that thrum and pin plants of *E. havanense* express strong reproductive asymmetries (Domínguez *et al.*, 1997; Avila-Sakar & Domínguez, 2000; Rosas *et al.*, 2005). Although there are no differences in the number of flowers or the amount of pollen produced by each morph, thrum plants produce at least twice as many seeds than pin plants (Domínguez *et al.*, 1997; Cuevas *et al.*, 2005; Rosas *et al.*, 2005). Such differences are not because of either

asymmetrical pollinator visitation or pollen flow (Domínguez *et al.*, 1997), but are intrinsic to each sexual morph. The seeds produced by thrum plants also have greater germination rates, a superior vegetative performance, and a greater probability of reproduction (Avila-Sakar & Domínguez, 2000). Thus, thrum plants efficiently compensate for the loss of the male function by producing more seeds of greater quality than pin plants. Taken together, the results of this and previous studies strongly support our conclusion that natural selection is favouring the evolution of male-sterile thrum individuals of *E. havanense*. Furthermore, the extent of fitness compensation of male-sterile thrum individuals not only was sufficient to overcome the total fitness of hermaphroditic thrum plants, but also surpassed the female fitness of pin individuals. Theoretical modelling by Pannell & Verdú (2006) assessing the conditions under which male sterility (or female-sterility) can invade a heterodichogamous or a distylous population, found that even a slight specialization in the functional gender of one of the sexual morphs might relax the conditions for the invasion of females (or males). Here we have shown that gender specialization in thrum individuals of *E. havanense* is strong enough as to drive the evolution of a functionally dioecious reproductive system in this species.

Finally, given that most of our conclusions are based on the estimation of the fitness gain curves, and the variables involved in these functions are difficult to measure (Campbell, 2000), it is worth to ask about their robustness. First, we used  $1 - MS$  as a surrogate of the amount of resources invested in the male function ( $r$ ) (Charnov, 1984). There is no general consensus on which currency (biomass, carbon, or nutrients) best measures this parameter (Ashman, 1994; Campbell, 1998, 2000) and some authors have even used measures as flower number (Meagher, 1991; Devlin *et al.*, 1992; Emms *et al.*, 1997). In this study, we chose to use  $1 - MS$  as a measure of  $r$  because both pin and thrum plants of *E. havanense* produce full-developed anthers that differ in the amount of fertile pollen (Domínguez *et al.*, 1997), thus precluding a biomass-based measure. Both pin and thrum plants of *E. havanense* produce only one-seed fruits and no further differences among flowers have been detected. As far as we know, the only measurable difference among flowers of thrum individuals is related to pollen fertility (Cuevas *et al.*, 2005 and see above). Moreover, repeatability analysis suggests that some of the phenotypic variation in male sterility is the result of genetic differences among individuals (Rosas *et al.*, 2005). In addition, the opposite relationships observed between male-sterility and male and female fitness, support the assumption of a trade-off in allocation. Consequently, the usage of male-sterility level as a surrogate of  $r$  assumes that variance in female investment is relatively constant and randomly distributed among thrum individuals. The reliability of seed production as a measure of female fitness depends on the

assumption that more seeds results into higher number of surviving progeny (Lloyd, 1984; Campbell, 1998). Although we do not have data regarding seedling survival in the wild, data obtained from previous field experiments showed that crop size and seedling survivorship after 8 years are positively related in *E. havanense* (Number of surviving seedlings =  $-0.164 + 0.70 \times$  number of seeds,  $R^2 = 0.80$ ; and see Avila-Sakar & Domínguez, 2000). Hence, we are confident that a seed production based measure of fitness is robust enough as to produce a clear description of the relationship between male sterility and female fitness. Finally, genetic markers were used to determine the number (proportion) of seeds sired by an individual. This estimation of male fitness may be biased if cryptic gene flow is present in the studied population (i.e. the siring of seeds by plants outside of the reference population; Roeder *et al.*, 1989). As our experiments consisted of only four possible pollen donors for each replicate, this design completely eliminated this bias and increased the probability of paternity assignment.

In conclusion, the results of this and previous studies support the interpretation that natural selection is favouring the evolution of complete male-sterile thrum individuals of *E. havanense*. We propose that this process, in turn, will result in the evolution of a functionally dioecious reproductive system. Hence, as pointed out by this and theoretical studies (Pannell & Verdú, 2006), the evolution of dioecy from cosexual ancestors may represent a shorter pathway to sexual specialization.

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## References

- Akaike, H. 1974. Factor analysis and AIC. *Psychometrika* **52**: 317–332.
- Ashman, T.L. 1994. Reproductive allocation in hermaphrodite and female plants of *Sidalcea oregana* ssp. *spicata* (Malvaceae) using four currencies. *Am. J. Bot.* **81**: 433–438.
- Ashman, T.L. 1999. Determinants of sex allocation in a gynodioecious wild strawberry: implications for the evolution of dioecy and sexual dimorphism. *J. Evol. Biol.* **12**: 648–661.
- Atlan, A.P., Gouyon, H., Fournial, T. & Couvet, D. 1992. Sex allocation in an hermaphrodite plant: the case of gynodioecy in *Thymus vulgaris* L. *J. Evol. Biol.* **5**: 189–203.
- Avila-Sakar, G. & Domínguez, C.A. 2000. Parental effects and gender specialization in a tropical heterostylous shrub. *Evolution* **54**: 866–877.
- Baker, H.G. 1958. Studies in the reproductive biology of West African Rubiaceae. *J. West. Afr. Sci. Assoc.* **4**: 9–24.
- Bawa, K.S. & Opler, P.A. 1975. Dioecism in tropical forest trees. *Evolution* **29**: 167–179.
- Beach, J.H. & Bawa, K.S. 1980. Role of pollinators in the evolution of dioecy from distyly. *Evolution* **34**: 1138–1142.
- Bullock, S.H. 1986. Climate of Chamela, Jalisco, and trends in the south coastal region of México. *Arch. Meteorol. Geophys. Bioklimatol.* **36**: 297–316.
- Campbell, D.R. 1998. Variation in lifetime male fitness in *Ipomopsis aggregata*: test of sex allocation theory. *Am. Nat.* **152**: 338–353.
- Campbell, D.R. 2000. Experimental tests of sex-allocation theory in plants. *Trends Ecol. Evol.* **15**: 227–232.
- Casper, B.B. & Charnov, E.L. 1982. Sex allocation in heterostylous plants. *J. Theor. Biol.* **96**: 143–149.
- Charlesworth, D. 1999. Theories of the evolution of dioecy. In: *Gender and Sexual Dimorphism in Flowering Plants* (M.A. Geber, T.E. Dawson & L.F. Delph, eds), pp. 33–60. Springer Verlag, Berlin.
- Charlesworth, D. 2002. What maintains male-sterility factors in plant populations? *Plant Popul. Genetics* **89**: 408–409.
- Charlesworth, B. & Charlesworth, D. 1978. A model for the evolution of dioecy and gynodioecy. *Am. Nat.* **112**: 975–997.
- Charnov, E.L. 1982. *The Theory of Sex Allocation*. Princeton University, Princeton.
- Charnov, E.L. 1984. Behavioral ecology of plants. In: *Behavioral Ecology, An Evolutionary Approach* (J.R. Krebs & N.B. Davies, eds), pp. 362–379. Sinauer, Sunderland MA.
- Cuevas, G.E., Molina-Freaner, F., Eguiarte, L.E. & Domínguez, C.A. 2005. Patterns of male sterility within and among populations of the distylous shrub *Erythroxylum havanense* (Erythroxylaceae). *Plant Ecol.* **176**: 165–172.
- Darwin, C. 1877. *The Different Form of Flowers of the Same Species*. John Murray, London.
- Del-Carlo, S. & Buzato, S. 2006. Male sterility and reproductive output in distylous *Erythroxylum suberosum* (Erythroxylaceae). *Biol. J. Linn. Soc.* **88**: 465–474.
- Delph, L.F., Bailey, M.A. & Marr, D.L. 1999. Seed provisioning in gynodioecious *Silene acaulis* (Caryophyllaceae). *Am. J. Bot.* **86**: 140–144.
- Devlin, B., Clegg, J. & Ellstrand, N.C. 1992. The effect of flower production on male reproductive success in wild radish populations. *Evolution* **46**: 1030–1042.
- Domínguez, C.A. & Dirzo, R. 1995. Rainfall and flowering synchrony in a tropical shrub: variable selection on the flowering time of *Erythroxylum havanense*. *Evol. Ecol.* **9**: 204–216.
- Domínguez, C.A., Avila-Sakar, G., Vázquez-Santana, S. & Márquez-Guzmán, J. 1997. Morph-biased male sterility in the tropical distylous shrub *Erythroxylum havanense* (Erythroxylaceae). *Am. J. Bot.* **84**: 626–632.
- Eckhart, V.M. 1992. Resource compensation and the evolution of gynodioecy in *Phacelia linearis* (Hydrophyllaceae). *Evolution* **46**: 1313–1328.



- Elkonin, L.A. 2005. Dominant male sterility in sorghum: effect of nuclear background on inheritance of tissue-culture-induced mutation. *Theor. Appl. Genet.* **111**: 1377–1384.
- Ellstrand, N.C. 1984. Multiple paternity within the fruits of the wild radish, *Raphanus sativus*. *Am. Nat.* **123**: 819–828.
- Emms, S.K., Stratton, D.A. & Snow, A.A. 1997. The effect of inflorescence size on male fitness: experimental tests in the andromonoecious lily, *Zigadenus paniculatus*. *Evolution* **51**: 1481–1489.
- Fisher, R.A. 1958. *The Genetical Theory of Natural Selection*, 2nd edn. Clarendon Press, Oxford.
- Frank, S.A. 1989. The evolutionary dynamics of cytoplasmic male sterility. *Am. Nat.* **133**: 345–376.
- Ganders, F.R. 1979. Heterostyly in *Erythroxylum coca* (Erythroxylaceae). *Bot. J. Linn. Soc.* **78**: 11–20.
- García-Robledo, C.. 2007. Asymmetry in pollen flow promotes gender specialization in morphs of the distylous neotropical herb *Arcytophyllum lavarum* (Rubiaceae). *Evol. Ecol.* (In press), doi: 10.1007/s10682-007-9198-0.
- Gomez, N.N. & Shaw, R.G. 2006. Inbreeding effect on male and female fertility and inheritance of male sterility in *Nemophila menziesii* (Hydrophyllaceae). *Am. J. Bot.* **93**: 739–746.
- Gouyon, P.H. & Couvet, D. 1987. A conflict between two sexes, females and hermaphrodites. In: *The Evolution of Sex and its Consequences* (S.C. Stearns, ed.), pp. 245–261. Birkhauser Verlag Basel, Boston.
- Hanson, M.R. 1991. Plant mitochondrial mutations and male sterility. *Annu. Rev. Genet.* **25**: 461–486.
- van der Hulst, R.G., Meirmans, M.P., van Tienderen, P.H. & van Damme, J.M.M. 2004. Nuclear–cytoplasmic male-sterility in diploid dandelions. *Heredity* **93**: 43–50.
- Jacobs, M.S. & Wade, M.J. 2003. A synthetic review of the theory of gynodioecy. *Am. Nat.* **161**: 837–851.
- Koelwijn, H.P. 2003. Variation in restorer genes and primary sexual investment in gynodioecious *Plantago coronopus*: the trade-off between male and female function. *Proc. R. Soc. Lond. B Biol. Sci.* **270**: 1939–1945.
- Koelwijn, H.P. & Hunscheid, M.P.H. 2000. Intraspecific variation in sex allocation in hermaphroditic *Plantago coronopus* (L.). *J. Evol. Biol.* **13**: 302–315.
- Lau, P. & Bosque, C. 2003. Pollen flow in the distylous *Palicourea fendleri* (Rubiaceae): an experimental test of the disassortative pollen flow hypothesis. *Oecologia* **135**: 593–600.
- Lewis, D. 1941. Male sterility in natural populations of hermaphrodite plants. *New Phytol.* **40**: 56–63.
- Lloyd, D.G. 1975. The maintenance of gynodioecy and androdioecy in angiosperms. *Genetica* **45**: 325–329.
- Lloyd, D.G. 1979. Evolution toward dioecy in heterostylous populations. *Plant. Syst. Evol.* **131**: 71–80.
- Lloyd, D.G. 1980. The distribution of gender in four angiosperm species illustrating two evolutionary pathways to dioecy. *Evolution* **34**: 123–134.
- Lloyd, D.G. 1984. Gender allocations in outcrossing cosexual plants. In: *Perspectives on Plant Population Ecology* (R. Dirzo & J. Sarukhán, eds), pp. 277–300. Sinauer, Sunderland MA.
- Lott, E.J., Bullock, S.H. & Solís-Magallanes, J.A. 1987. Floristic diversity and structure of upland and arroyo forest of coastal Jalisco. *Biotropica* **19**: 228–235.
- Maurice, S., Belhassen, E., Couvet, D. & Gouyon, P.H. 1994. Evolution of dioecy: can nuclear–cytoplasmic interactions select for maleness? *Heredity* **73**: 346–354.
- Meagher, T.R. 1991. Analysis of paternity within a natural population of *Chamaelirium luteum*. Patterns of male reproductive success. *Am. Nat.* **137**: 738–752.
- Muenchow, G.E. & Grebus, M. 1989. The evolution of dioecy from distyly: reevaluation of the hypothesis of the loss of long long-tongued pollinator. *Am. Nat.* **133**: 149–156.
- Naiki, A. & Kato, M. 1999. Pollination system and evolution of dioecy from distyly in *Mussaenda parviflora* (Rubiaceae). *Plant Species Biol.* **14**: 217–227.
- Nilsson, E. & Ågren, J. 2006. Population size, female fecundity, and sex ratio variation in gynodioecious *Plantago maritima* J. *Evol. Biol.* **19**: 825–833.
- Opler, P.A., Baker, H.G. & Frankie, G.W. 1975. Reproductive biology of some Costa Rican *Cordia* species (Boraginaceae). *Biotropica* **7**: 234–247.
- Ornduff, R. 1966. The origin of dioecism from heterostyly in *Nymphoides* (Menyanthaceae). *Evolution* **20**: 309–314.
- Ornelas, J.F., Gonzalez, C., Jimenez, L., Lara, C. & Martinez, A.J. 2004. Reproductive ecology of distylous *Palicourea padifolia* (Rubiaceae) in a tropical montane cloud forest. II. Attracting and rewarding mutualistic and antagonistic visitors. *Am. J. Bot.* **91**: 1061–1069.
- Pailler, T., Humeau, L., Figier, J. & Thompson, J.D. 1998. Reproductive trait variation in the functionally dioecious and morphologically heterostylous island endemic *Chassalia coral-lioides* (Rubiaceae). *Biol. J. Linn. Soc.* **64**: 297–313.
- Pannell, J.R. & Verdú, M. 2006. The evolution of gender specialization from dimorphic hermaphroditism: paths from heterodichogamy to gynodioecy and androdioecy. *Evolution* **60**: 660–673.
- Poot, P. 1997. Reproductive allocation and resource compensation in male-sterile and hermaphroditic plants of *Plantago lanceolata* (Plantaginaceae). *Am. J. Bot.* **84**: 1256–1265.
- Renner, S.S. & Ricklefs, R.E. 1995. Dioecy and its correlates in the flowering plants. *Am. J. Bot.* **82**: 596–606.
- Rodríguez-Riño, T. & Dafni, A. 2007. Pollen–stigma interference in two gynodioecious species of Lamiaceae with intermediate individuals. *Ann. Bot.* **100**: 423–431.
- Roeder, K.M., Devlin, B. & Lindsay, B.G. 1989. Application of maximum likelihood methods to population genetic data for the estimation of individual fertilities. *Biometrics* **45**: 363–379.
- Rosas, L.F., Pérez-Alquicira, J. & Domínguez, C.A. 2005. Environmentally induced variation in fecundity compensation in the morph-biased male-sterile distylous shrub *Erythroxylum havanense* (Erythroxylaceae). *Am. J. Bot.* **92**: 116–122.
- Ross, M.D. 1982. Five evolutionary pathways to subdioecy. *Am. Nat.* **119**: 297–318.
- SAS. 2003. *JMP User's Guide, Release 5.0.1*. SAS Institute, Cary, NC.
- Schnable, P.S. & Wise, R.P. 1998. The molecular basis of cytoplasmic male sterility and fertility restoration. *Trends Plant Sci. Rev.* **3**: 175–180.
- Schultz, S.T. 1994. Nucleo-cytoplasmic male sterility and alternative routes to dioecy. *Evolution* **48**: 1933–1945.
- Sheoran, I. & Saini, H. 1996. Drought-induced male sterility in rice: changes in carbohydrate levels and enzyme activities associated with the inhibition of starch accumulation in pollen. *Sex. Plant Rep.* **9**: 161–169.
- Shykoff, J.A. 1988. Maintenance of gynodioecy in *Silene acaulis* (Caryophyllaceae): stage-specific fecundity and viability selection. *Am. J. Bot.* **75**: 844–850.

- Stanley, R.G. & Linskens, H.F. 1974. *Pollen, Biology, Biochemistry, Management*. Springer, New York.
- Stone, J.L. 1995. Pollen donation patterns in a tropical distylous shrub (*Psychotria suerrensis* Rubiaceae). *Am. J. Bot.* **82**: 1390–1398.
- Vuilleumier, B.S. 1967. The origin and evolutionary development of heterostyly in the angiosperms. *Evolution* **21**: 210–226.
- Webb, C.J. 1999. Empirical studies: evolution and maintenance of dimorphic breeding systems. In: *Gender and Sexual Dimorphism in Flowering Plants* (M.A. Geber, T.E. Dawson & L.F. Delph, eds), pp. 61–95. Springer Verlag, Berlin.
- Wyatt, R. 1983. Pollinator-plant interactions and the evolution of breeding systems. In: *Pollination Biology* (L. Real, ed.), pp. 51–95. Academic Press, New York, USA.

## Supporting information

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

**Table S1** Number of loci, number of polymorphic loci and percentage of polymorphic loci for six ISSR primers on plants of *Erythroxylum havanense*.

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