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Gender specialization in *Palicourea demissa* (Rubiaceae), a distylous, hummingbird-pollinated treelet

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Received: 24 October 2011 / Accepted: 7 February 2012 / Published online: 6 March 2012
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Abstract Distyly has been interpreted as a mechanism that promotes cross-pollination among conspecific plants and as one of the routes leading to the evolution of dioecy. In one of the possible evolutionary pathways, pollinators may disrupt intermorph pollen flow, and, as a consequence, floral morphs may gradually specialize as either male or female (functional dioecy). Natural patterns of pollen deposition and fruit and seed production were estimated in *Palicourea demissa* (Rubiaceae) and used as parameters to assess functional gender differences between floral morphs. Pollen flow was asymmetrical in *P. demissa*. Long-styled flowers were more effective than short-styled flowers in pollen deposition towards compatible stigmas, whereas short-styled flowers were more effective in legitimate pollen receipt. Accordingly, short-styled plants produced more fruits and viable seeds than long-styled plants. The contributions of male and female function to the potential functional gender were equivalent in both morphs. However, the realized functional gender deviated significantly from the potential functional gender in both morphs, in which short-styled plants were more successful through their female function, but long-styled plants through their

male function. If pollinators disrupt the complementarities of pollen transfer between the two morphs (asymmetric pollen flow), the expression of a more profitable gender is expected in each morph. Thus, our results support the hypothesis that dioecy may evolve in distylous populations through the gradual specialization of each morph as either male or female.

Keywords Asymmetric pollen flow · Distyly · Functional dioecy · *Palicourea demissa* · Reciprocal herkogamy

Introduction

Distyly is a genetically controlled polymorphism where two floral morphs coexist within a population (Barrett 1992, 2002). Populations are typically composed of individuals that display flowers with long styles and short stamens (hereafter LS) and individuals that exhibit flowers with short styles and long stamens (hereafter SS) (Barrett 1992, 2002; Dulberger 1992). Besides the reciprocal position of anthers and stigmas (reciprocal herkogamy), floral morphs of distylous species can be heteromorphic self-incompatible and exhibit other polymorphisms that include anthers and stigmas size, size and amount of pollen grains, and exine/papillae dimorphism (Ganders 1979; Barrett 1990, 1992, 2002; Barrett and Richards 1990; Barrett et al. 2000; Dulberger 1992; Webb and Lloyd 1986; Richards and Barrett 1992).

Reciprocal herkogamy has been interpreted as a mechanism that promotes cross-pollination between floral morphs by means of animals (Darwin 1877). According to this hypothesis, pollen in anthers positioned at different height levels could adhere on different body parts of the pollinators, which would correspond to those areas where

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compatible stigmas contact the pollinators (Barrett and Glover 1985; Lloyd and Webb 1992; Barrett 2002; De Jong and Klinkhamer 2005). Reciprocal herkogamy reduces pollen wastage by promoting an accurate placement of pollen of the morphs where the stigma of the opposite morphs touches the body of pollinators (Barrett 1992, 2002; De Jong and Klinkhamer 2005). Although wastage of pollen may occur through self-pollination, distyly solves the spatial conflict between male and female functions within the flower. The effectiveness of reciprocal herkogamy on cross-pollination between distylous morphs has mainly been evaluated through the examination of stigmatic pollen loads in floral morphs under both natural (Olesen 1979; Ganders 1979; Barrett and Glover 1985; Ornelas et al. 2004a; Hernández and Ornelas 2007; García-Robledo 2008) and experimental conditions (Stone 1995; Lau and Bosque 2003; Ornelas et al. 2004a). This method is useful for determining pollen flow between floral morphs when there is some pollen dimorphism (Ree 1997; Ornelas et al. 2004a; García-Robledo 2008).

Although distyly is suggested to promote disassortative pollination, i.e. pollen flow between opposite floral morphs (Darwin 1877; Lloyd and Webb 1992), pollen flow may differ between morphs, with individuals of one morph playing a major role as males through pollen donation or as females through pollen receipt (reviewed in Freeman et al. 1997). A discrepancy between the potential (ovule and pollen production) and the realized (pollen donation and fruits and seed production) functional genders may promote local gender specialization (González et al. 2005; García-Robledo 2008), and a transitional stage to the evolution of dioecy from distyly (Lloyd and Webb 1992; Naiki and Kato 1999; Pannell and Verdú 2006; Li et al. 2010; Machado et al. 2010). Floral morphs of distylous species can exhibit differences in reproductive success through their male and female functions (Lloyd 1979; Casper 1992). Most evidence for gender specialization comes from differences in fruit and seed production in distylous populations with 1:1 morph ratios (e.g., Ornelas et al. 2004a; González et al. 2005; García-Robledo 2008). Morph differences in stigmatic pollen loads have been reported in several distylous species (Feinsinger and Busby 1987; Stone 1995; Lau and Bosque 2003; Ornelas et al. 2004a; García-Robledo 2008). If pollen flow between floral morphs is symmetric, and morphs exhibit equivalent potential functional gender, it is expected that the floral morphs within a population will display equivalent male and female reproductive success. Conversely, if pollen flow is asymmetric, morph differences in male and female reproductive success are expected within the population. Given that reciprocal herkogamy promotes intermorph cross-pollination, morph differences in reproductive success may occur in

populations where reciprocity of anthers and stigmas differs between directions of pollen flow.

In *Palicourea demissa* Standl. we have observed spatial reciprocity between anthers of LS flowers and stigmas of SS flowers, but reciprocity was reduced in the opposite direction (Valois-Cuesta et al. 2011a). If legitimate pollen deposition on stigmas is predominantly towards SS flowers, SS plants should produce more fruits and seeds than LS plants. Here, we report data on spatial clustering of floral morphs, pollen and ovule production, pollen deposition and donation, and fruit and seed production to evaluate the functional gender expression of SS and LS plants. If asymmetrical pollen flow between floral morphs is the process producing a discrepancy between the potential and the realized functional genders, this would indicate that interactions with pollinators could promote gender specialization in distylous species.

Materials and methods

Study site

Fieldwork was carried out from September 2007 to May 2011 at La Mucuy Bird Observatory (8°38'N, 71°02'W; at 2,300–2,400 m a.s.l) in the Sierra Nevada National Park, Mérida, Venezuela. In the study area, mean annual temperature is 14°C, and mean annual precipitation varies between 2,800 and 3,400 mm, with peaks in April–May and October–November. Horizontal precipitation (fog) represents about 300 mm (Ataroff and Rada 2000).

Study species

Palicourea demissa is an abundant treelet (3–8 m in height) commonly found in disturbed areas of Andean cloud forests of Colombia, Venezuela and Perú. In La Mucuy cloud forest, the studied population is morphologically distylous and exhibits morph differences in most ancillary floral polymorphisms and spatial reciprocity of the sexual organs (Valois-Cuesta et al. 2011a). SS flowers display larger corollas, stigmatic lobes, anthers and pollen grains than those of LS flowers (see also Fig. 1). Similarly, the anther-stigma separation (intramorphic herkogamy) is larger in SS than in LS flowers. However, the position of anthers of LS flowers and stigmas of those SS flowers is highly reciprocal (SS stigma height: 13.4 ± 0.14 mm, $n = 100$; LS anthers height: 13.3 ± 0.15 mm, $n = 100$; $t = 0.37$, $P = 0.70$), whereas in the opposite direction the reciprocity is reduced (LS anthers height: 19.6 ± 0.23 mm, $n = 100$; LS stigma height: 17.1 ± 0.20 mm, $n = 100$; $t = 8.31$, $P < 0.0001$; Valois-Cuesta et al. 2011a). Flowers are visited by bumblebees, butterflies and hummingbirds. Of the six

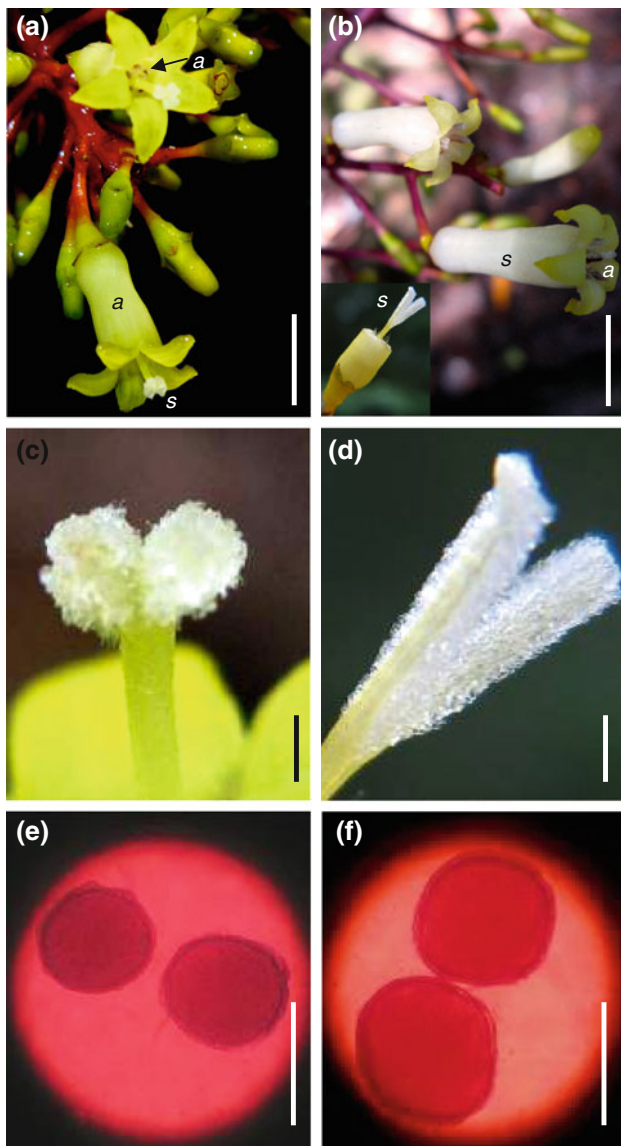


Fig. 1 Morph differences in corolla (position of anthers *a* and stigma *s*), stigma and pollen size of LS (**a**, **c**, **e**) and SS (**b**, **d**, **f**) flowers of *Palicourea demissa*. Bar = 1 cm in **a** and **b**; 1 mm in **c** and **d**; 100 μ m in **e** and **f**

hummingbird species that visit the flowers (95% of floral visits per plant), *Coeligena torquata* (34%), *Heliangelus spencei* (22%) and *Adelomyia melanogenys* (19%) individuals are the most frequent floral visitors (Valois-Cuesta and Novoa-Sheppard 2006; Valois-Cuesta et al. 2011b). These hummingbird species differ in the size of their bills as well as in their foraging behaviors; *C. torquata* is a long-billed (33 mm) and non-territorial forager, whereas *H. spencei* and *A. melanogenys* are short-billed (18 and 13 mm), territorial and non-territorial, respectively. However, they are legitimate floral visitors of both floral morphs (Valois-Cuesta et al. 2011b). Floral morphs in La Mucuy cloud forest display a 1:1 morph ratio (Valois-Cuesta and

Novoa-Sheppard 2006), and they do not differ in the number of inflorescences or infructescences per plant, or in the number of pollen grains per flower. The floral morphs are self- and intramorph incompatible; morphs only produce fruits when intermorph crosses are performed (Valois-Cuesta et al. 2011a). Fleshy fruits usually contain two seeds and in both morphs take about 8 months to develop, and they ripen asynchronously during 2 months. At the site of study ripe fruits are consumed by frugivorous birds, *Penelope montagnii*, *Aulacorhynchus prasinus* and *Chlorospingus ophthalmicus* (H. Valois-Cuesta, personal observation). Under controlled cross-pollination conditions, floral morphs do not differ in fruit sets. However, SS plants produce 20% more fruits than LS plants under natural conditions, suggesting that SS plants have higher reproductive success through female function (Valois-Cuesta et al. 2011a), a discrepancy between the potential and the realized functional gender.

Spatial segregation of morphs

Clustering of floral morphs will influence patterns of mating within populations if pollen dispersal is locally limited to short distances (Hodgins and Barrett 2008). To determine whether the style morphs were spatially segregated, we randomly chose focal plants of each morph (32 LS and 34 SS) throughout the study population between May and June 2008 (blooming peak), and measured the distance (m) between each focal plant and its nearest neighbor. The spatial structure of *P. demissa* morphs was tested using Pielou's (1961) coefficient of segregation: $S' = 1 - (O/E)$, where *O* is the observed number of focal and near-neighbor pairs comprised of different morphs and *E* is the expected number. The expected number of each pair type was calculated assuming random pair formation with respect to style morphs. Positive values of the S' coefficient of segregation indicate spatial clumping of the morphs, whereas negative values indicate an affinity between opposite morphs (Pielou 1961).

Pollen deposition patterns

Assuming that natural stigmatic pollen loads result solely from the activity of floral visitors can be misleading because the anthers can spontaneously deposit pollen on stigmas in hermaphroditic flowers (Cariveau et al. 2004). To evaluate the contribution of autonomous self-pollen deposition in explaining natural patterns of stigmatic pollen loads of each morph in *P. demissa*, between May and June 2008 we examined pollen deposition on intact (not emasculated) flowers exposed 12 h to their natural floral visitors ($n = 30$ flowers per morph, one flower per plant) and from bagged not

emasculated flowers ($n = 37$ LS and 30 SS flowers per morph, one flower per plant). In both treatments, stigmas were carefully removed at 1800 hours, individually placed on a microscope slide and examined under a microscope (Leitz Dialux 20 EB) for pollen counting. Autonomous, self-pollen deposition among bagged flowers was minimal (mean ± 2 SE; LS = 0.83 ± 0.8 pollen grains per stigma, $n = 37$; SS = 6.8 ± 4.36 pollen grains per stigma, $n = 30$) compared to those exposed to pollinators (LS = 79 ± 15.0 pollen grains per stigma, $n = 30$; SS = 225 ± 43.4 pollen grains per stigma, $n = 30$; two-way ANOVA; treatment effect, $F_{1,123} = 573.03$, $P < 0.0001$). Morph differences ($F_{1,123} = 69.8$, $P < 0.0001$) were due to variation among flowers exposed to natural visitation (floral morph \times treatment interaction, $F_{1,123} = 26.99$, $P < 0.0001$). The results of this control treatment demonstrate that relatively insignificant numbers of pollen grains are transferred in the absence of pollinators.

Then, to evaluate natural patterns of pollen deposition, we collected additional flowers exposed to their natural pollinators from plants of both morphs every 2 h between 0600 and 1800 hours (210 flowers per morph, 30 flowers per interval, one flower per plant). Collection of stigmas was done at different time intervals because accumulation of legitimate and illegitimate pollen deposition may differ between morphs through time (Ornelas et al. 2004a). Stigmas were removed and placed on microscope slides. Under the microscope, we directly counted the number of legitimate (pollen from the opposite morph) and illegitimate (pollen from same morph) pollen grains on stigmas of each floral morph based on pollen size differences (SS = $119.5 \pm 1.49 \mu\text{m}$, $n = 100$; LS = $92.7 \pm 0.79 \mu\text{m}$, $n = 100$; Valois-Cuesta et al. 2011a). Based on these data we assumed that pollen grains with diameter $>105 \mu\text{m}$ were deposited from SS flowers and those grains with diameter $<100 \mu\text{m}$ were deposited from LS flowers. To compare the capacity of each morph as donor (male function) and recipient (female function) of legitimate pollen we followed Lloyd and Webb (1992):

$$T_{ij} = \frac{(\text{Average stigmatic pollen load})_{ij} \times (\text{no flowers})_j}{(\text{no pollen/flower})_i \times (\text{no flowers})_i} \quad (1)$$

where T_{ij} represents the probability of transfer of pollen type i to a stigma j , i is the donor morph, and j is the recipient morph.

Fruit and seed production

Plants ($n = 24$ SS and 26 LS) from different zones of the population were chosen at the beginning of the fruiting season (September 2007) to explore possible differences

in female reproductive success (number of fruits per inflorescence) between floral morphs. In each plant we selected infructescences with initiated fruits from different branches and quantified the number of developing fruits present in each of them. Floral pedicels persist on the infructescence; therefore, we also quantified the number of floral pedicels as an estimate of total flower production per inflorescence throughout the flowering season (see Ree 1997). It is important to note that we observed no damage in the floral buds, open flowers or developing fruits during the study period. Lastly, we explored morph differences in the amount of viable and aborted seeds. In this case, we randomly selected plants in May 2008 and May 2011 ($n = 30$ per morph per year; plants were randomly selected from the population each year) and randomly collected fully ripe fruits ($n = 115$ from SS and 114 from LS plants in 2008, and 116 from SS and 110 from LS in 2011) from different infructescences in each plant (3–4 inflorescences per plant, one fruit per inflorescence). Seed viability was evaluated indirectly by categorizing seeds with very reduced size and amorphous as nonviable or aborted seeds.

Gender expression of floral morphs

To evaluate the sexual expression in *P. demissa*, we first quantified the number of ovules and pollen grains per flower in each morph ($n = 100$ flowers per morph, from 30 plants of each morph) (Kearns and Inouye 1993) and then followed García-Robledo (2008) to calculate the potential functional gender (male and female) of each morph as follows:

$$Gpot_i = \frac{OV_i}{OV_i + (PO_i \times Epot_i)} \quad (2)$$

$$Apot_i = \frac{PO_i \times Epot_i}{OV_i + (PO_i \times Epot_i)} \quad (3)$$

where $Gpot_i$ and $Apot_i$ represent the potential contribution of each morph i to the following generation through female (ovules) and male (pollen donation) functions, respectively. PO_i indicates the number of pollen grains for morph i . $Epot_i$ is an equivalence factor by which pollen units (PO_i) must be multiplied to adjust $Gpot_i$ and $Apot_i$ to the number of ovules available for legitimate pollinations (OV_j), where j is the opposite morph to i as follows:

$$Epot_i = \frac{OV_j}{PO_i} \quad (4)$$

Besides the potential functional gender, we also calculated the realized functional gender in each morph according to García-Robledo (2008) but using the number of viable seeds as predictor (VS_i).

$$\text{Greal}_i = \frac{\text{VS}_i}{\text{VS}_i + (\text{PO}_{ij} \times \text{Ereal}_i)} \quad (5)$$

$$\text{Areal}_i = \frac{\text{PO}_{ij} \times \text{Ereal}_i}{\text{VS}_i + (\text{PO}_{ij} \times \text{Ereal}_i)} \quad (6)$$

In these equations, Greal_i and Areal_i are estimators of the real contribution of each morph to the following generation through its female and male functions, respectively. PO_{ij} indicates the number of legitimate pollen grains of the morph i on stigmas j . Ereal_i is an equivalence factor by which pollen units (PO_{ij}) must be multiplied to adjust Greal_i and Areal_i based on the actual seeds sired by the opposite morph (VS_j), as follows:

$$\text{Ereal}_i = \frac{\text{VS}_j}{\text{PO}_{ij}} \quad (7)$$

Because morphs may differ in the amount of flowers per inflorescence and the number of viable seeds per infructescence, the parameters for all equations were normalized to the inflorescence and infructescence level as follows: OV = number of ovules per flower \times number of flowers per inflorescence, PO = number of pollen grains per flower \times number of flowers per inflorescence, VS = number of viable seeds per fruit \times number of fruits per infructescence, and PO_{ij} = number of pollen grains $_i$ on stigma $_j$ \times number of flowers per inflorescence $_j$.

Finally, to explore whether possible asymmetries in legitimate pollen flow between morphs modify the potential functional gender (male and female) in each morph, the deviation between the potential functional gender and the realized functional gender (G female and A male) was calculated following García-Robledo (2008):

$$\Delta G = \text{Greal}_i - \text{Gpot}_i \quad (8)$$

$$\Delta A = \text{Areal}_i - \text{Apot}_i \quad (9)$$

Statistical analyses

Morph differences in the distance between a focal plant and its closest neighbor were assessed using a Kruskal-Wallis test. The effects of floral morph on variables related to potential and real functional genders (Table 2; Fig. 4), and on pollen donation and pollen receipt were analyzed using Generalized Linear Models (GLMs) with Poisson distribution. For pollen donation and pollen receipt, the GLMs included floral morph (SS and LS) and time of day (2-h intervals) as the main factors, and the resulting two-way interactions. Within-morph differences in the proportion of viable and aborted seeds were assessed using a binomial test. The realized functional gender and its deviation from the potential functional gender within-morph were tested using one-way ANOVAs. Data were \log_{10} and square root ($x + 0.5$) transformed to achieve normality and

homocedasticity. However, untransformed data (mean \pm 2 SE) are reported in text and figures. All analyses were performed using the computer program R (R Development Core Team 2008).

Results

The distance between a focal plant and its closest neighbor was statistically similar among all possible morph combinations (focal plant \rightarrow neighbor; SS \rightarrow SS = 2.25 ± 0.86 m, $n = 15$; SS \rightarrow LS = 2.20 ± 0.66 , $n = 19$; LS \rightarrow LS = 2.57 ± 0.72 , $n = 14$; LS \rightarrow SS = 1.97 ± 0.64 , $n = 18$; $\chi^2 = 1.82$, d.f = 3, $P = 0.60$). The morph of a plant was independent of the morph of its nearest neighbor. The coefficient of segregation was $S' = -0.12$, indicating spatial affinity between morphs.

Under natural conditions SS stigmas received more conspecific pollen grains (mean \pm 2 SE; 132.9 ± 14.4 pollen grains per stigma, $n = 210$) than LS stigmas (52.8 ± 6.2 pollen grains per stigma, $n = 210$; $F_{1,406} = 158.74$, $P < 0.0001$). After classifying pollen grains by size differences, we found that stigmas of SS flowers received more legitimate (78.6 ± 9.0 , $n = 210$) and illegitimate (54.4 ± 8.9 , $n = 210$) pollen grains than stigmas of LS flowers (legitimate = 13.1 ± 2.2 , $n = 210$; $F_{1,406} = 419.75$, $P < 0.0001$; illegitimate = 39.7 ± 5.2 , $n = 210$; $F_{1,406} = 5.15$, $P = 0.02$). SS stigmas received proportionally more legitimate pollen than LS stigmas ($F_{1,406} = 204.44$, $P < 0.0001$; Fig. 2). Heterospecific pollen grains (7.3%) were excluded from further analyses.

Stigmas of both floral morphs accumulated pollen grains (legitimate and illegitimate) throughout the day (time-of-day effect; $F_{6,406} = 49.29$, $P < 0.0001$). However, stigmas of SS flowers accumulated significantly larger amounts and received proportionally more legitimate pollen over time

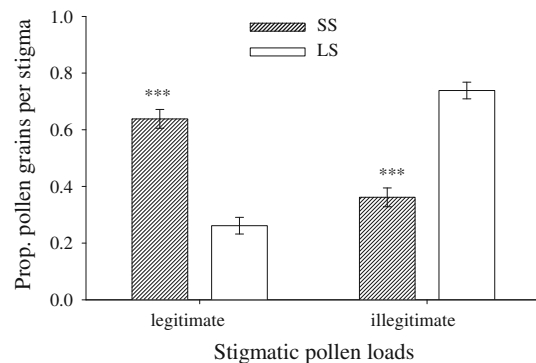


Fig. 2 Proportion of legitimate (compatible) and illegitimate (incompatible) pollen classified by size on stigmas of SS and LS flowers of *Palicourea demissa*. Data indicate mean \pm 2 SE. Differences between morphs: *** $P < 0.0001$

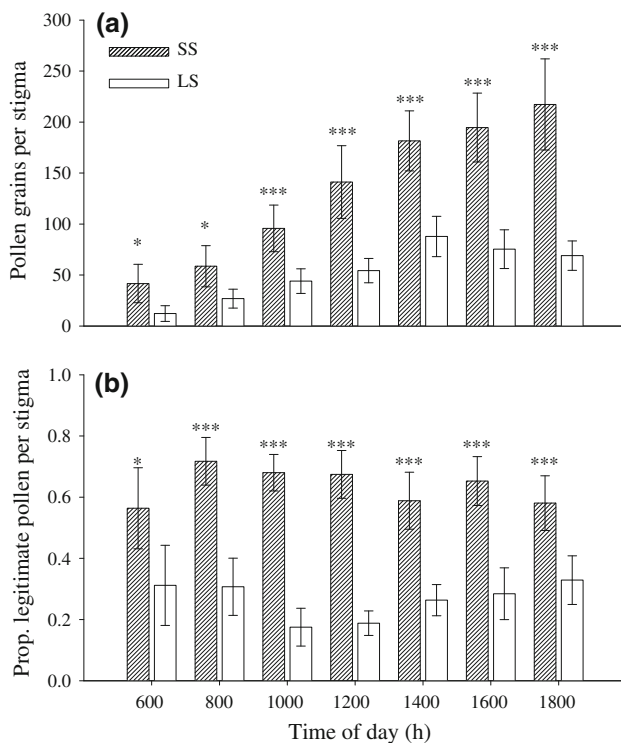


Fig. 3 Rate of pollen deposition on stigmas of SS and LS flowers of *Palicourea demissa*. **a** total stigmatic pollen load, **b** proportion of legitimate pollen within the stigmatic pollen load. Data indicate mean \pm 2 SE. Differences between morphs: * $P < 0.05$; *** $P < 0.0001$

than those of LS flowers (floral morph \times time-of-day interaction, $F_{6,406} = 2.42$, $P = 0.03$; Fig. 3). From the female function perspective (pollen receipt), the probability analysis indicated that SS stigmas had 1.4 times more chance of receiving legitimate (LS) than illegitimate (SS) pollen grains, whereas the probability of receiving pollen grains from the opposite floral morph was lower in LS stigmas (Table 1). When data were analyzed from the male function perspective (pollen donation), LS pollen grains had two times more chances of being deposited on stigmas of the opposite morph, whereas for SS pollen grains the chances were four times lower (Table 1).

Floral morphs did not differ in the number of flowers per inflorescence, but their infructescences differed significantly in the number of initiated fruits, developing 20% more successfully in SS than LS plants (Table 2). In both

Table 1 Probability of pollen transfer on a stigma (T_{ij}) under conditions of natural pollination in *Palicourea demissa*

Donor (pollen)	Recipient (stigma)	
	Long-styled	Short-styled
Long-styled	0.92×10^{-3}	1.83×10^{-3}
Short-styled	0.31×10^{-3}	1.27×10^{-3}

floral morphs the number of viable seeds was significantly larger than the number of aborted seeds [2008 (SS: viable = 215, aborted = 15, $n = 115$ fruits; binomial test, $P < 0.0001$; LS: viable = 194, aborted = 34, $n = 114$ fruits, $P < 0.0001$); 2011 (SS: viable = 206, aborted = 26, $n = 116$ fruits; $P < 0.0001$; LS: viable = 164, aborted = 56, $n = 110$ fruits, $P < 0.0001$)]. However, infructescences of SS plants produced more viable seeds per fruit than LS plants over time (Table 2).

Our analysis on sexual expression in *P. demissa* indicated that SS plants were not statistically different from LS plants in their potential functional gender (Table 2; Fig. 4a). However, in the realized or observed functional gender, floral morphs showed significant differences (Table 2), and SS plants showed higher female reproductive success than LS plants, whereas LS plants were more successful through their male function (Fig. 4b). The realized functional gender deviated from the potential functional gender in both floral morphs of *P. demissa* (Fig. 4c). The female contribution was higher (one-way ANOVA; $F_{1,94} = 5.89$; $P = 0.017$), and the male contribution was lower ($F_{1,94} = 7.48$; $P = 0.007$) in SS morphs than that expected from their potential functional gender. In contrast, the female contribution was lower ($F_{1,94} = 7.48$; $P = 0.007$), and the male contribution was higher ($F_{1,94} = 5.89$; $P = 0.017$) in LS morphs than that expected from their potential functional gender (Fig. 4a–c).

Discussion

Spatial segregation of morphs

Morph-specific clumping was not observed in the studied population of *P. demissa*. The lack of spatial aggregation suggests that the intermixed distribution of mating types within the population should favor disassortative pollen transfer through the foraging activity of hummingbirds, which typically move from one plant to its nearest neighboring plants (De Jong and Klinkhamer 2005; Valois-Cuesta et al. 2011b). In addition to floral morphology and spatial distribution of morph types, other factors including vegetative reproduction, floral display size, local pollinator foraging, limited seed dispersal and the extent of heteromorphic incompatibility can also influence mating patterns in distylous populations (e.g., Bawa and Beach 1983; Boege and Domínguez 2008; Hodgins and Barrett 2008; Brys and Jacquemyn 2010). In *P. demissa* in which vegetative reproduction is uncommon (H. Valois-Cuesta, personal observation), and seeds dispersed by birds and heteromorphic incompatibility govern mating patterns, the style morphs should exhibit weak or no local spatial structure (see also Ree 1997; Ornelas et al. 2004b).

Table 2 Differences in gamete and fruit and seed production between long- (LS) and short-styled (SS) morphs of *Palicourea demissa*

	<i>n</i>	SS	<i>n</i>	LS	MS factor/error	$F_{\text{ndf,ddf}}$
Flower, fruit and seed production						
No. flowers per inflorescence	48	122.4 ± 7.3	55	109.5 ± 5.9	7.08/4.9	1.45 _{1,101} ns
No. developing fruits per infructescence (2007)	48	68.4 ± 4.9	55	52.5 ± 3.2	39.8/3.44	11.54 _{1,101} **
No. viable seeds per fruit per infructescence (2008)	115	1.9 ± 0.003	114	1.7 ± 0.004	0.15/0.01	9.92 _{1,227} **
No. viable seeds per fruit per infructescence (2011)	116	1.78 ± 0.06	110	1.49 ± 0.08	0.41/0.02	21.55 _{1,224} ***
Potential functional gender						
No. ovules per inflorescence	48	244.9 ± 14.6	48	219.2 ± 11.8	14.2/9.8	1.45 _{1,101} ns
No. pollen grains per inflorescence	48	230,956 ± 13,802	48	207,767 ± 11,169	12,008.8/ 9,263.3	1.30 _{1,101} ns
Realized functional gender						
No. pollen grains _i deposited per inflorescence _j	55	1,435 ± 77.2	48	9,625 ± 5,722	87,941.9/243.6	360.97 _{1,101} ***
No. viable seeds per infructescence	48	139.7 ± 9.4	55	89.3 ± 5.5	129.1/6.27	20.58 _{1,101} ***

ns non-significant

** $P < 0.01$, *** $P < 0.0001$

Asymmetric pollen flow

In *P. demissa* we observed the opposite pattern of most heterostylous species: stigmas of SS flowers received significantly more compatible pollen than those of LS flowers. This pollen deposition pattern has been documented in several species of Rubiaceae, in which stigmas of SS flowers are larger and shaped differently than those of LS flowers (see Sobrevila et al. 1983; Feinsinger and Busby 1987; Stone and Thompson 1994; Stone 1995; Ree 1997; Lau and Bosque 2003; Ornelas et al. 2004a; Hernández and Ornelas 2007; García-Robledo 2008; Wu et al. 2010; Machado et al. 2010; Maruyama et al. 2010). In *P. demissa* we observed that stigmas of SS flowers are reciprocally positioned in height to LS anthers (Valois-Cuesta et al. 2011a). Although stigma size and its relative position in relation to the anthers of the opposite morph may in part explain morph differences in pollen receipt (Barrett 2002; Ornelas et al. 2004a; Hernández and Ornelas 2007), the observed asymmetric pollen flow pattern in *P. demissa* might have been triggered by a pollinator shift. Long-billed hummingbirds are more efficient in the transfer of pollen grains between reproductive organs at low levels (from anthers of LS flowers toward stigmas of SS flowers), whereas short-billed hummingbirds would be more efficient in the transfer of pollen grains from anthers of SS flowers toward stigmas of LS flowers (Valois-Cuesta et al. 2011b). Preliminary data on pollinator effectiveness in *P. demissa* showed that long- and short-billed hummingbirds differ in their deposition capacities of legitimate pollen towards either direction of the pollen flow (SS→LS or LS→SS); long-billed *C. torquata* hummingbirds deposited twice as many legitimate pollen grains after a visit on emasculated SS flowers than emasculated LS flowers, whereas short-billed hummingbirds (*A. melanogenys* and

H. spencei) deposited 2.3 times more legitimate pollen on emasculated LS flowers than emasculated SS flowers (Valois-Cuesta et al. 2011b). A similar pattern was documented for *P. padifolia* in a study using stuffed hummingbirds (Ornelas et al. 2004a), in which long-billed hummingbirds transferred significantly more pollen from LS towards SS flowers than in the opposite direction. However, they found no differences between short- and long-billed hummingbirds in the transfer of legitimate pollen between morphs in both directions and suggested that a non-evaluated behavioral component of hummingbird foraging may explain in part the morph differences in pollen deposition rates and may play a functional role in facilitating legitimate pollination in one direction.

In *P. demissa*, a long-billed, non-territorial hummingbird (*C. torquata*) was the most frequent floral visitor in both floral morphs (Valois-Cuesta et al. 2011b). Then, it is also possible that the asymmetric pollen flow, in which SS flowers received more legitimate pollination than LS flowers, may be explained by the higher visitation rate of *C. torquata* associated to a morphological fit between the size of its bill, length and width of corolla-tube, and reciprocal position of low-height sexual organs. The fact that SS stigmas received more pollen than LS stigmas could be related to the larger stigma size of SS flowers and the smaller but exerted stigma of LS flowers. Thus, the match between the exerted stigma and hummingbird bill entering the corolla tube is not good in LS flowers. Although visitation rate and morphology may explain pollen deposition patterns on stigmas (Barrett 2002; Engel and Irwin 2003), the foraging behavior of pollinators may also play an important role in the pollination success of each morph. In *P. demissa*, short-billed hummingbirds (*H. spencei*, *A. kingi* and *A. melanogenys*) were territorial and commonly observed cleaning their bills after probing several flowers

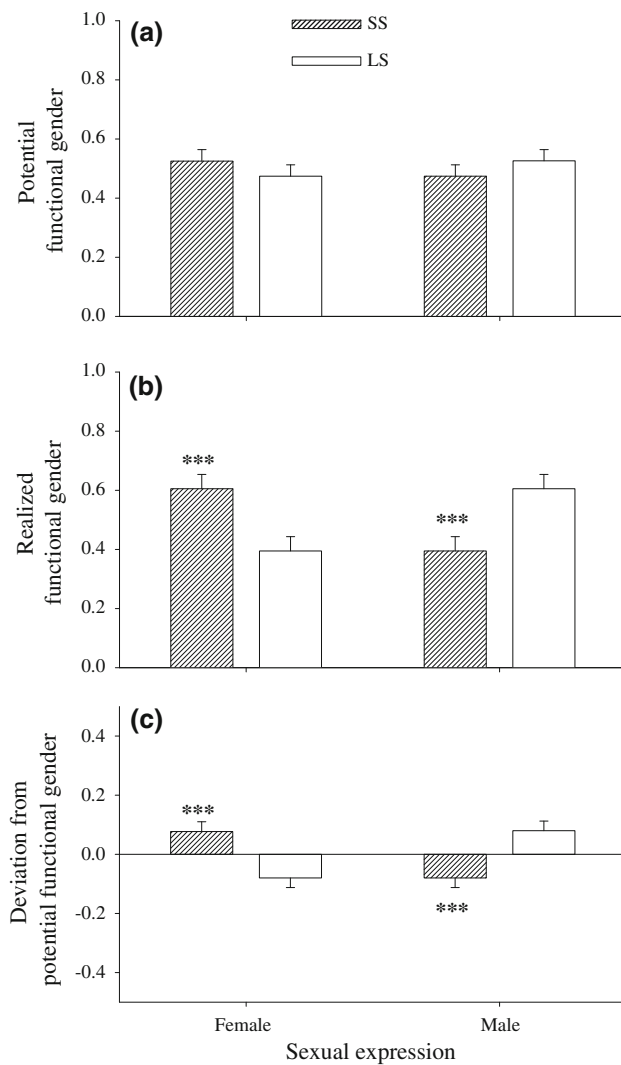


Fig. 4 Functional gender in SS and LS plants of *Palicourea demissa*. **a** Contribution of male and female functions to the potential functional gender (Apot, Gpot). **b** Contribution of male and female reproductive functions to the observed or realized functional gender (Greal, Areal). **c** Deviation of the realized (ΔA) from the potential functional gender (ΔG) in SS and LS plants. Data indicate mean \pm 2 SE. Differences between morphs: *** $P < 0.0001$

(see also Contreras and Ornelas 1999). These behaviors should increase the chances of geitonogamous crosses and seed abortion (De Jong and Klinkhamer 2005). If territoriality enforces self-pollination by territory defending birds (Justino et al. 2012), this may be compensated in populations of *P. demissa* where non-territorial, long-billed hummingbirds are more frequent, leading to the observed asymmetric pollen flow.

Gender expression of SS and LS plants

The main finding of our study is that asymmetric pollen flow promoted a divergence between the potential and the

realized functions in both morphs of the *P. demissa* population. Unequal pollen flow and gender function contradict the hypothesis of disassortative pollen transfer in distylous plants to ensure cross-pollination between opposite floral morphs originally posed by Darwin (1877). The floral morphs of several distylous species specialize in their reproductive effort through male and female function (Lloyd 1979). Some species exhibit such extreme gender specialization that they are functionally dioecious (Opler et al. 1975; Naiki and Kato 1999; Litrico et al. 2005), but less pronounced gender specialization has been commonly reported for naturally pollinated individuals (reviewed by Casper 1992). The evidence that morphs specialize in functional gender mostly comes from differences among populations in seed production and non-equilibrium morph ratios (Casper 1992). In *P. demissa*, our results suggest that pollinators have disrupted the complementarities of pollen transfer between the two morphs, so that SS receives more legitimate pollen than it disperses, and LS disperses more pollen than it receives, eliciting a more profitable gender in each morph. Furthermore, LS flowers received three times more illegitimate than legitimate pollen, a pollen transfer pattern that cannot solely be explained by morph reciprocity.

Distyly is suggested as an ancestral state in the evolution of separate sexes among flowering plants (Beach and Bawa 1980), and there is some phylogenetic evidence that a shift to morphologically true dioecy from functional dioecy has occurred in several groups in Rubiaceae independently (Razafimandimbison et al. 2009). In this evolutionary pathway, pollinators may promote an asymmetry in pollen flow that disrupts the complementarities of pollen transfer between the two floral morphs. As a consequence, the realized functional gender may diverge from the potential functional gender, and the floral morphs may gradually specialize as either male or female (Charlesworth 1999). This evolutionary pathway is likely in Rubiaceae given that dioecy is considerable in this family (10% of genera; Robbrecht 1988). Although some cases of evolution to dioecy from distyly have been reported (Beach and Bawa 1980; Naiki and Kato 1999; Li et al. 2010), they seem to be very isolated and, to our knowledge, not yet reported for any *Palicourea* species. Interestingly, *P. demissa* was pollinated mainly by long-billed, non-territorial hummingbirds, an unusual situation because most of the dioecious plants that may have distylous ancestors are thought to have evolved through a replacement of long-tongued insects by short-tongued ones (Beach and Bawa 1980). Nonetheless, our study was limited to one population, and since pollinator species and other characteristics of the plants can vary among populations, gender specialization can be local, and further study considering several populations at the same time is required to confirm this hypothesis.

In cases where dioecious species have evolved from distylous ancestors, male individuals originated from SS plants, whereas female individuals derived from LS plants (Bawa 1980). The accumulated evidence for distylous species in Rubiaceae, particularly in *Palicourea* species, is not consistent with this interpretation. In *P. demissa*, SS plants showed more female reproductive success than LS plants, whereas LS plants were more efficient than SS plants in legitimate pollen donation. The same pattern has been documented in distylous *Palicourea fendleri* (Lau and Bosque 2003), *P. padifolia* (Ornelas et al. 2004a; Hernández and Ornelas 2007; but Ree 1997) and *Arcytophyllum lavarum* (García-Robledo 2008). Little is known about the genetic basis and/or selective mechanisms that govern the evolutionary transition from distyly to dioecy (Barrett 2002; Barrett and Shore 2008). It is possible that pollinators play an important role as mediators of asymmetric pollen flow between morphs leading to a gradual specialization, towards a certain gender function (Beach and Bawa 1980; Naiki and Kato 1999; Ornelas et al. 2004a). Gender specialization has been reported, notwithstanding in the Rubiaceae (e.g., González et al. 2005; Litrico et al. 2005; García-Robledo 2008), but some force should make distyly stable enough to avoid the transition to at least functional dioecy in most cases.

Although SS plants showed higher female reproductive success than LS plants between years (Table 2), in other distylous species morphs may shift their sexual expression through time. González et al. (2005) found that SS plants of *P. padifolia* showed higher female reproductive success than LS plants in 1998 and 1999, but the functional gender shifted in 2002. It is possible that this variation in functional gender expression is due to changes in the frequency, abundance and composition of pollinators in a population across years (González et al. 2005). On the other hand, among-population data in pollen receipt, as an indirect measure of female fitness, also suggested that the SS morph was more successful in most studied populations of *P. padifolia* (Hernández and Ornelas 2007).

In conclusion, our results showed that asymmetric pollen flow could be promoted by long-billed pollinators, which result in a divergence between the potential and realized functional genders, a fundamental process that may lead to functional dioecy (García-Robledo 2008). The transition to this pathway may gradually promote the evolution of dioecy in distylous species. However, gender specialization needs to be confirmed considering at the same time several populations because pollinators and other floral characteristics of the plants can vary among populations.

Acknowledgments We are grateful to S. Ecce-Homo, Z. Betancourt, C. Rengifo, R. Pacheco, J.H. Castaño, and J. Murillo for

assistance during fieldwork. We thank M.E. Naranjo, J. Estrada, C. García, A. Jaramillo, N. Ramírez, Marlies Sazima and two anonymous reviewers for their constructive comments on an earlier draft of the manuscript. This work was funded by the Red Latinoamericana de Botánica (code: RLB06-M03 and RLB08-SP-1 to H.V.-C.) and the Consejo de Desarrollo Científico, Humanístico y Tecnológico of the Universidad de Los Andes, Mérida-Venezuela (code: C-1557-08-01EM to H.V.-C. and P.S.). Staff from La Mucuy Bird's Observatory, Sierra Nevada National Park and the Laboratorio de Ecología Animal "A" of the Universidad de Los Andes, Mérida, Venezuela provided logistical support.

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