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FLORAL VARIATION, NECTAR PRODUCTION, AND REPRODUCTIVE SUCCESS OF TWO *DRYMONIA* (GESNERIACEAE) SPECIES WITH MIXED POLLINATION SYNDROMES

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Premise of research.   Pollination syndromes are suites of correlated characters that reflect selection by most frequent and effective pollinators. However, the floral phenotypes of some angiosperm species combine traits from different pollination syndromes. The aim of this study was to assess the correspondence between floral traits and floral visitors, considering the breeding systems of two co-occurring *Drymonia* species with mixed floral phenotypes.

Methodology.   We assessed floral morphology, nectar production patterns, frequency of floral visitation, breeding systems, and natural fruit set in *Drymonia strigosa* and *Drymonia oinochrophylla* species at Los Tuxtlas, Veracruz, Mexico, during 2011–2014. We also conducted a field test of reproductive assurance in *D. strigosa*, the only species with a high potential for autonomous self-pollination.

Pivotal results.   In *D. strigosa*, morphology and nectar production patterns correspond to the ornithophilous syndrome; however, anthers have poricidal dehiscence, a trait associated with bee pollination. Hummingbirds were the most frequent floral visitors, and stingless bees were less frequent visitors acting mostly as nectar robbers. The species has a high autofertility potential, but autonomous self-pollination contributed little to fruit set during the study years. In *D. oinochrophylla*, most floral traits correspond to the melittophilous syndrome; however, high nectar production and large flower size resemble traits of chiropterophilous flowers. Primary pollinators were bees with less frequent visits by butterflies and moths; bats were not observed during the study period. *Drymonia oinochrophylla* is self-compatible, but it has little potential for autonomous self-pollination; therefore, seed production is highly dependent on pollinators.

Conclusions.   Our results suggest that mixed floral phenotypes in the two *Drymonia* species are not likely a consequence of visitation by multiple pollinator functional groups. Instead, incomplete correspondence of floral traits with classic pollination syndromes may represent undetected floral visitors in disturbed environments or plesiomorphies retained throughout the evolutionary history of the genus *Drymonia*.

Keywords: autonomous self-pollination, *Drymonia*, Mexico, nectar, pollination syndromes, reproductive success.

Introduction

Pollination syndromes are convergent suites of floral traits exhibited by species from many different Angiosperm lineages (Faegri and van der Pijl 1978; Rosas-Guerrero et al. 2014). Floral traits commonly used to describe pollination syndromes include corolla color and shape, nectar volume, sugar content and sugar type in nectar, presence and type of scent, and timing of anthesis (Faegri and van der Pijl 1978). The concept of syndromes implies that flowers evolve under pollinator-mediated selection to enhance visitation and pollen transfer efficiency by particular floral visitors (Fenster et al. 2004). However, the

idea that correlated suites of floral traits have evolved in response to pollinator-mediated selection has been questioned because syndromes do not always correspond to expected floral visitors and because plants are often visited by multiple animal taxa (Waser et al. 1996; Ollerton et al. 2009). Earlier on, Stebbins (1970) proposed that the most frequent and effective pollinators are likely responsible for the evolution of floral traits. In support of this hypothesis, recent reviews on the subject demonstrate that syndromes generally predict the most effective pollinators of plants, despite the common occurrence of secondary pollinators (Fenster et al. 2004; Rosas-Guerrero et al. 2014; Ashworth et al. 2015).

Pollination syndromes have also been detected as patterns of convergent evolution associated with pollinator shifts in comparative studies across different plant lineages (van der Niet and Johnson 2012), including the family Gesneriaceae (Martén-Rodríguez et al. 2010). It has been suggested that evo-

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lutionary transitions between different pollination systems may occur through an intermediate stage where floral traits attract more than one functional group of pollinators (Stebbins 1970; Wilson et al. 2006). In this scenario, plants may be subject to selection exerted by multiple pollinators, resulting in floral phenotypes that do not clearly correspond to traditional pollination syndromes. For example, in the predominantly bat-pollinated genus *Burmeistera*, the species *Burmeistera tenuiflora* has crepuscular anthesis, variation in corolla color from light green to red, and nocturnal nectar production. Both bats and hummingbirds were described as effective pollinators of *B. tenuiflora* (Muchhala 2003), suggesting that selection by the two pollinator groups has influenced the evolution of mixed floral traits in this species. However, it is not clear whether this kind of mixed floral phenotype represents a transitional stage or an evolutionarily stable state.

Another possible explanation for floral mixed phenotypes is the presence of historical or developmental constraints that restrict change of particular floral traits. For example, *Penstemon roseus* (Plantaginaceae)—a hummingbird-pollinated species—has nectar production according to the bird syndrome, but it retains morphological traits associated with bee pollination (Lara and Ornelas 2008). Since bee pollination is the ancestral condition in the genus *Penstemon* (Wilson et al. 2007), floral traits associated with melittophily in *P. roseus* possibly reflect common ancestry. Plant species with floral traits that do not clearly fit a single pollination syndrome allow exploration of the possible causes behind the apparent lack of predictability of pollination syndromes that is currently discussed in the literature (Ollerton et al. 2007, 2009; Ashworth et al. 2015).

The predictability of pollination syndromes may also be influenced by plant breeding systems, since species strictly dependent on pollinators for reproduction (e.g., self-incompatible, dioecious, and fully dichogamous plants) may experience stronger selection on floral traits to ensure pollinator visitation and reproductive success (Ashworth et al. 2015; e.g., Anderson and Busch 2006). In contrast, selection may be more relaxed in species that do not depend on pollinators to set seed (e.g., species capable of autonomous self-pollination). For instance, a worldwide literature survey of angiosperms demonstrated that the most effective pollinator group corresponded better with that predicted by the syndrome in pollinator-dependent than pollinator-independent plant species (Ashworth et al. 2015). Thus, in order to achieve a full understanding of the importance of pollinators in floral trait evolution, it is critical to have knowledge of plant breeding systems.

Equally important is determining the contribution of self-pollination to reproduction under natural conditions, since having a self-compatible breeding system does not imply that self-pollination significantly contributes to fruit and seed set (Martén-Rodríguez and Fenster 2010). A number of studies have demonstrated that self-pollination does provide reproductive assurance under certain environmental conditions (Eckert et al. 2006; Kennedy and Elle 2008; Martén-Rodríguez and Fenster 2010). However, the success of this reproductive assurance mechanism depends on various factors, such as levels of inbreeding depression, pollen and ovule discounting, pollinator visitation and effectiveness, and the environment (Busch and Delph 2012). In particular, reproductive assurance through self-pollination has been associated with ecological conditions

such as low densities of conspecifics and pollinators, early successional stages, and harsh weather (Busch and Delph 2012). Furthermore, it has been proposed that epiphytes (i.e., plants growing on trees) should have self-compatible breeding systems because of the ephemeral nature of their habitat and low population densities (Bush and Beach 1995; Cascante-Marín et al. 2006). In this study, we tested for self-compatibility and autonomous self-pollination in two epiphytic *Drymonia* species and evaluated the idea that self-pollination provides reproductive assurance in *Drymonia strigosa*.

The family Gesneriaceae provides the opportunity to investigate the correspondence between pollination syndromes, effective pollinators, and breeding systems because it exhibits a great diversity of floral phenotypes. Most Neotropical Gesneriaceae species studied to date exhibit functional specialization in pollination systems (SanMartín-Gajardo and Sazima 2005*a*, 2005*b*; Martén-Rodríguez et al. 2009, 2015). However, species in various genera have floral traits that correspond to more than one pollination syndrome (Perret et al. 2001; SanMartín-Gajardo and Sazima 2005*a*; Martén-Rodríguez et al. 2009). In particular, the genus *Drymonia* is characterized by a wide variety of corolla forms, but most species have poricidal anthers (Wiehler 1983). The breeding systems of Neotropical Gesneriaceae studied to date are mostly self-compatible but vary in the form and degree of dichogamy, the temporal separation of sexual functions within and among flowers (Wiehler 1983); therefore, the potential for self-pollination is highly variable among species (Martén-Rodríguez et al. 2015).

In this study, we characterized the floral phenotypes, pollination biology and breeding systems of *D. strigosa* and *Drymonia oinochrophylla* (Gesneriaceae), two species with mixed floral phenotypes in the region of Los Tuxtlas, Mexico. Here, we define a mixed pollination syndrome as a floral phenotype conformed by traits that correspond with more than one previously defined syndrome (Faegri and van der Pijl 1978). *Drymonia strigosa* has scentless flowers with contrasting bright pink to red calyces and yellow tubular corollas (traits associated with ornithophily, i.e., bird pollination syndrome), but it has poricidal anthers (traits associated with melitophily, i.e., bee pollination syndrome). *Drymonia oinochrophylla* has white campanulate corollas with purple lobes and an extended petal lobe (resembling a landing platform), traits usually associated with large bee pollination; however, it has large stout corollas, generally associated with chiropterophyly (i.e., bat pollination syndrome). The three main goals of this study were to (1) obtain a quantitative characterization of floral traits relevant to pollination syndromes, including morphology and nectar production patterns; (2) evaluate the correspondence between floral traits and pollinators predicted by syndromes; and (3) describe breeding systems to determine the potential importance of pollinators as agents of selection on floral traits.

We characterized floral phenotypes (objective 1) through measurements of morphological traits, nectar production, sugar content in nectar, and nectar replenishment experiments. We also estimated the degree of floral integration, since the pollination syndrome concept implies that suites of floral traits should evolve in a correlated way to promote effective pollination (Stebbins 1970; Fenster et al. 2004). Additionally, it has been proposed that species with self-compatible breeding systems should have greater floral integration than self-incompatible

species (Armbruster et al. 1999; Fornoni et al. 2015). Thus, we estimated correlation coefficients among floral traits and the floral integration index to pollination syndromes and breeding systems in the two *Drymonia* species.

In order to assess the correspondence between pollinators and floral traits (objective 2), we determined the frequency of floral visitation for both *Drymonia* species in the field. To describe the breeding systems of each species (objective 3), we conducted pollination experiments in the wild. We tested for self-compatibility (hand-self vs. hand-outcross treatments) and potential for autonomous self-pollination (bagged vs. hand-outcrossed treatments). Although apomixis has not been reported in Gesneriaceae, we also conducted emasculation bagging treatments to confirm lack of apomixis. In order to determine the contribution of autonomous self-pollination to female reproductive success, we compared the fruit set of open-pollinated and emasculated flowers. This experiment was conducted only for *D. strigosa* because breeding system results indicated that only this species had a high potential of autonomous self-pollination.

Material and Methods

Study Sites

We evaluated the pollination biology of *Drymonia strigosa* and *Drymonia oinochrophylla* at the Los Tuxtlas Biosphere Reserve in Veracruz, Mexico (300–1680 m asl), from 2012 to 2014. Specifically within the reserve, fieldwork was conducted at the Ejido Ruiz Cortínez (18°31′38.7″, 95°08′15.8″) for *D. strigosa* and at the private reserve La Otra Opción

A.C. (18°22′37.4″, 94°56′19.8″), for *D. oinochrophylla*. Both *Drymonia* species are found in the two study sites; however, for safety reasons, we could not conduct all fieldwork at Ejido Ruiz Cortínez, the site where we initially started the study of *D. strigosa*. The selected forests at these sites are characterized by trees that can reach 30 m in height and abundant epiphytes (Miranda and Hernández 1963). Mean annual temperature is 27°C; the warmest month is May, with a mean temperature of 35°C, and the coolest month is February, with a mean temperature of 13ºC. Mean annual precipitation is 4900 mm (Soto and Gama 1997).

Study Species

The two study species are epiphytic shrubs that grow on tree trunks and branches of different tree species. They were observed growing 1–15 m aboveground. Individual plants may reach large sizes growing several meters up and around tree trunks. Both species produce solitary flowers.

Drymonia strigosa is an epiphytic species endemic to Mexico from southern Veracruz to Chiapas. It flowers from mid-February to mid-April at Los Tuxtlas (E. Ramírez-Aguirre, personal observation). Corollas are bright yellow with tubular pink calyces (fig. 1). The plants grow on tree trunks and branches. The species is protandrous (i.e., male phase develops first), and flowers last 3 d. Fruits are fleshy capsules with around 600 small seeds (E. Ramírez-Aguirre, unpublished data), and fruiting occurs during mid-April to June.

Drymonia oinochrophylla is also an epiphytic species distributed from Mexico to Honduras. In Mexico, it is distributed in the states of Veracruz, Oaxaca, and Chiapas. This species
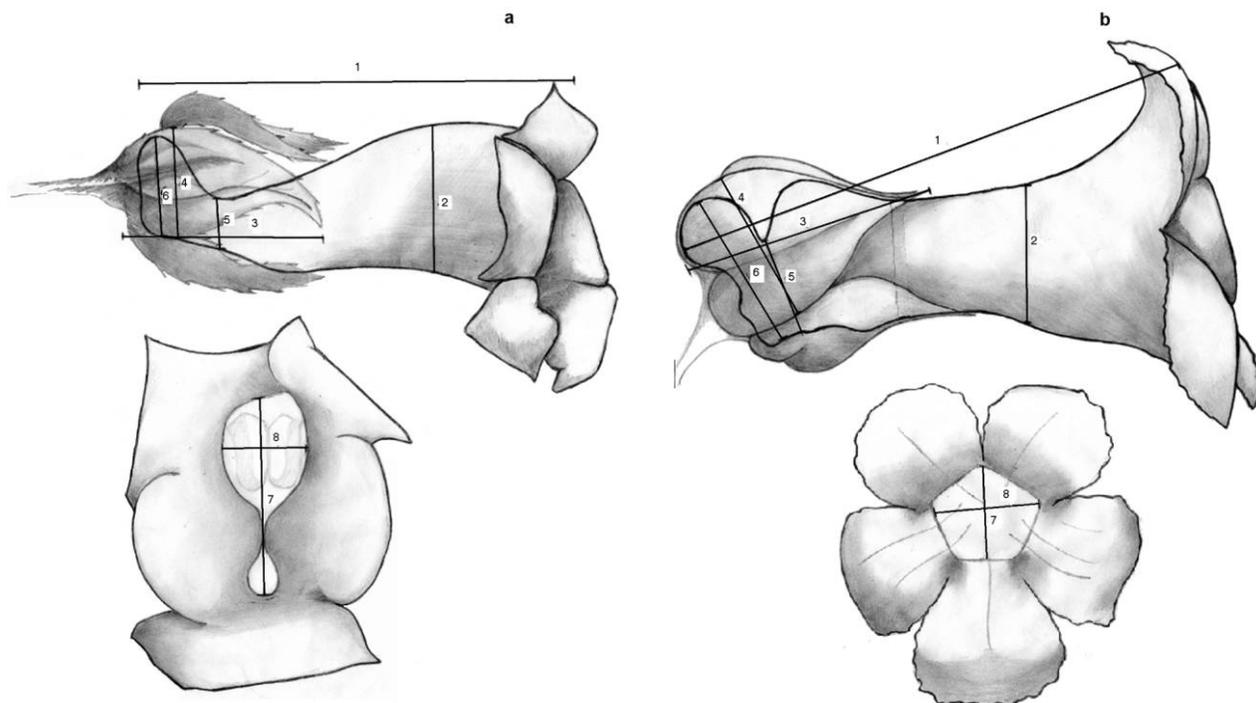

Fig. 1 Floral characters measured in *Drymonia strigosa* (*a*) and *Drymonia oinochrophylla* (*b*). *1*, corolla length; *2*, corolla width; *3*, calyx length; *4*, calyx width; *5*, nectary chamber constriction; *6*, nectary chamber; *7*, corolla opening length; *8*, corolla opening width.

flowers from June to August at Los Tuxtlas. Corollas are campanulate, with white tubes and purple lobes (fig. 1). Flowers are protandrous, and both sexual phases last 1 d. Fruits are fleshy capsules with many small seeds, and fruiting occurs during mid-August to October.

Variation of Morphological Characters

To assess variation in morphological characters, we selected 26 plants of *D. strigosa* and 18 plants of *D. oinochrophylla* and measured two flowers on each plant. We measured the following floral traits (fig. 1): (1) corolla length, (2) corolla width, (3) calyx length, (4) calyx width, (5) nectary chamber constriction, (6) nectar chamber length, (7) corolla opening length, (8) corolla opening width, (9) stamen length, and (10) pistil length. We calculated means and coefficients of variation for each morphological trait.

We calculated the floral integration index to describe patterns of morphological correlation and covariation among floral traits for each species. Stamen and pistil length were excluded from the analysis because they change with time as a result of protandry. The floral integration index was calculated as the variance of eigenvalues (calculated from a correlation matrix) minus a correction factor, divided by k (the number of characters measured minus 1, divided by n measurements; Wagner 1984). Statistical analyses were conducted in R (ver. 3.1.3; R Development Core Team 2014).

Nectar Production and Replenishment

Since nectar is a floral trait that affects pollen deposition, we measured nectar production in 10 plants of *D. strigosa* and 13 plants of *D. oinochrophylla* in the following two experiments. In the first experiment, we measured total nectar volume and sugar concentration in male and female flowers. Flowers were bagged with bridal's veil the evening before anthesis to measure male nectar production and the evening before female phase started, and nectar volume and concentration were measured at 1400 hours. Nectar concentration was measured with a hand refractometer (0°–50° Brix units; American Optical, Buffalo, NY) and volume was measured with a Hamilton microsyringe of 50 μL. With nectar concentration and volume, we calculated the total amount of sugar in milligrams produced per flower, as described by Bolten et al. (1979). To test for differences in nectar volume and sugar content, we ran ANOVAs with sexual phase as a fixed factor and plant as a random factor, using the nlme package in R (R Development Core Team 2014). To compare nectar concentration between sexual phases, we used a generalized linear mixed model (GLMM) with treatment set as a fixed factor and plant as a random factor in the lme4 package in R; since nectar concentration was measured as a percentage, a binomial family and logit link function were specified for this test (R Development Core Team 2014).

Nectar replenishment has been associated with hummingbird pollination in some species (Ordano and Ornelas 2004). Thus, we conducted a second experiment to evaluate nectar replenishment in response to removal. In a different set of covered flowers, in both staminate and pistillate phase, we removed nectar every 3 h in the same focal flower from 0800 to 1700 hours and measured nectar volume and sugar concentration. Nectar production was always absent after the third removal, so we report production from 0800 to 1400 hours. To test for differences in nectar production and sugar content between a single nectar removal and the sum of repeated removals, we conducted an ANOVA for each sexual phase, with treatment as a fixed factor and plant as a random factor. The statistical analysis was conducted with the nlme package in R program (R Development Core Team 2014).

Floral Visitors and Visitation Rates

To assess whether observed floral visitors corresponded with visitors predicted by pollination syndromes, we conducted direct observations and video recordings for both species. We observed 12 individuals of *D. strigosa* and 15 individuals of *D. oinochrophylla* for 30-min periods from 0800 to 1700 hours during 2012–2014. Videos were taken with a Panasonic SDR-H101 camera. Observed individuals of both *Drymonia* species were located 2–6 m aboveground. We also conducted nocturnal observations during 2013–2014 to assess the possibility of bat pollination to the large flowers of *D. oinocrophylla*, using a Samsung SCD86 video camera. Nocturnal observations were conducted from 2100 to 0100 hours for a total of 15 h of video on six plants. For both *Drymonia* species, visits were considered legitimate when contact with the reproductive organs of the flower could be observed or inferred. Nectar robbery was recorded when observed. Visitors were identified to the lowest possible taxonomic level in videos and from direct observations. We also collected insect visitors whenever possible for further identification. To describe floral display, we counted the number of flowers per plant observed. Since both species produce solitary flowers, pollinator observations are reported as the number of visits per flower per 30-min period.

Reproductive System and Female Reproductive Success

During 2012, we selected 35 plants of *D. strigosa* and 20 of *D. oinochrophylla* and marked five flowers on each plant. Each flower received one of the following pollination treatments: (1) apomixis, flowers were emasculated; (2) autonomous pollination, flowers were left intact; (3) hand-self-pollination, flowers were pollinated with pollen from flowers of the same plant (i.e., geitonogamous pollination); (4) hand-cross pollination, flowers were pollinated with pollen obtained from two donors at least 10 m away from the focal plant; and (5) natural pollination, flowers were left intact and exposed to natural pollinators. Flowers from treatments 1–4 were bagged before anthesis and after manipulations to exclude pollinators. Emasculation was conducted on floral buds for treatments 1, 3, and 4 the day before anthesis. Sample sizes were according to availability of plants with at least five accessible floral buds. Some flowers/fruits were lost before harvest; therefore, sample sizes for some treatments were lower than the number of marked plants. In order to obtain a better estimate of natural pollination in the study populations, we quantified the fruit set of additional plants that had fewer than five accessible flowers in both species.

Fruit production was recorded for each treatment 2 mo after the experiment was conducted. We compared fruit set among

treatments using a GLMM with treatment set as a fixed factor and plant as a random factor. Because of the nature of the data (i.e., proportions), binomial family and logit link function were used. To minimize intertreatment variance, apomixis treatment was excluded from the analysis, because values in both species were mostly zeros. We conducted analyses with the lme4 package in R (R Development Core Team 2014). To compare breeding systems between species, we calculated the self-incompatibility index and autofertility index (Lloyd and Schoen 1992). The self-incompatibility index was calculated by dividing hand-self fruit set by hand-cross fruit set. The autofertility index was calculated dividing autonomous fruit set by hand-cross fruit set.

Reproductive Assurance in Drymonia strigosa

Since the potential for autonomous fruit production was high in *D. strigosa*, we conducted a reproductive assurance experiment to test for the contribution of autonomous self-pollination to fruit production under natural conditions. We marked two flowers from 20 plants during 2013; one flower was emasculated before anthesis, and the other flower was left intact. Both flowers were left open to natural pollination. Anther removal in *Drymonia* is not expected to interfere with pollinator attraction for two reasons: (1) anthers in these species are inserted within the corolla tube and therefore not visible as attractive structures to floral visitors; (2) hummingbirds visit flowers for nectar, not pollen; therefore, emasculations do not interfere with reward availability. We collected fruits 2 mo later and compared the fruit set between the two groups of flowers using a mixed model with the lme4 package in R (R Development Core Team 2014). This model had treatment as the fixed factor and plant as a random factor, with binomial family and logit link function.

Results

Variation in Floral Morphology

Flowers of *Drymonia strigosa* were smaller and narrower than flowers of *Drymonia oinochrophylla*. Stamens and pis-

til exsertion (protrusion from corolla opening) was higher in *D. strigosa* than in *D. oinochrophylla* (table 1). The coefficients of variation of floral traits were similar for both *Drymonia* species, varying between 2% and 13% for *D. strigosa* and between 6% and 13% for *D. oinochrophylla* (table 1).

Drymonia strigosa had stronger correlations among most morphological traits than *D. oinochrophylla* (fig. 2). Accordingly, the floral integration index of *D. strigosa* was higher (13.1%) than the integration index of *D. oinochrophylla* (9.7%). For *D. oinochrophylla*, significant correlations primarily included traits that describe corolla size, such as corolla width, corolla length, and corolla opening length (fig. 2).

Nectar Production and Replenishment

There were no significant differences in nectar volume, total sugar content sugar, and concentration between sexual phases in *D. strigosa* (staminate phase: $F_{1,6} = 0.45$, $P = 0.52$; pistillate phase: $F_{1,6} = 0.22$, $P = 0.61$; GLMM: Wald's $\chi^2 = 0.02$, $P = 0.87$; table 2). In *D. oinochrophylla*, nectar volume and amount of sugar were higher in the male phase than in the female phase (staminate phase: $F_{1,6} = 59.50$, $P < 0.05$; pistillate phase: $F_{1,7} = 57.95$, $P < 0.05$; table 2), but sugar concentration did not vary among sexual phases (GLMM: Wald's $\chi^2 = 0.07$, $P = 0.70$; table 2).

Nectar replenishment—indicated by a higher production of nectar after removal—was positive in both sexual phases of flowers of *D. strigosa* (staminate phase: $F_{1,10} = 6.86$, $P > 0.05$; pistillate phase: $F_{1,4} = 12.74$, $P > 0.05$; fig. 3). In contrast, sugar content remained constant in the staminate phase ($F_{1,10} = 2.85$, $P > 0.05$) but increased in the pistillate phase ($F_{1,4} = 32.03$, $P < 0.05$; fig. 3).

Nectar was not replenished after repeated removal in *D. oinochrophylla* in the staminate phase ($F_{1,10} = 5.97$, $P < 0.05$) or in the pistillate phase ($F_{1,8} = 3.26$, $P > 0.05$; fig. 3). There were significant differences in nectar volume between treatments during the staminate phase, but these reflected an inhibition in nectar production. Sugar content did not respond to removal in the staminate phase ($F_{1,10} = 4.96$, $P > 0.05$) or in the pistillate phase ($F_{1,7} = 4.03$, $P > 0.05$) in *D. oinochrophylla* (fig. 3).

Table 1

Morphological Measures of Floral Characters of Two *Drymonia* Species

Floral character	D. strigosa			D. oinochrophylla		
	Mean ± SE (mm)	N	CV (%)	Mean ± SE (mm)	N	CV (%)
Corolla length	37 ± 5.0	37	8	70 ± 10.4	19	6
Corolla width	10 ± 1.1	37	6	16 ± 3.2	19	8
Calyx length	23 ± 5.0	37	13	30 ± 8.2	19	12
Calyx width	16 ± 2.7	37	10	23 ± 5.5	19	11
Nectary chamber length	10 ± 1.2	37	8	18 ± 2.5	19	6
Nectary chamber constriction	5 ± .6	37	8	12 ± 3.6	19	13
Corolla opening length	10 ± 1.4	37	9	15 ± 3.8	19	11
Corolla opening width	4 ± 1.10	37	2	16 ± 4.6	19	12
Stamens length	29 ± 4.0	26	8	28 ± 5.2	9	8
Pistil length	34 ± 6.3	20	11	31 ± 6.8	10	9

Note. Data were recorded at Los Tuxtlas Biosphere Reserve, Mexico, during the 2012 flowering season. The floral integration index (*D. strigosa*: 13.1%; *Drymonia oinochrophylla*: 9.7%) was calculated excluding stamen and pistil measures because these organs do not mature at the same time. CV, coefficient of variation.
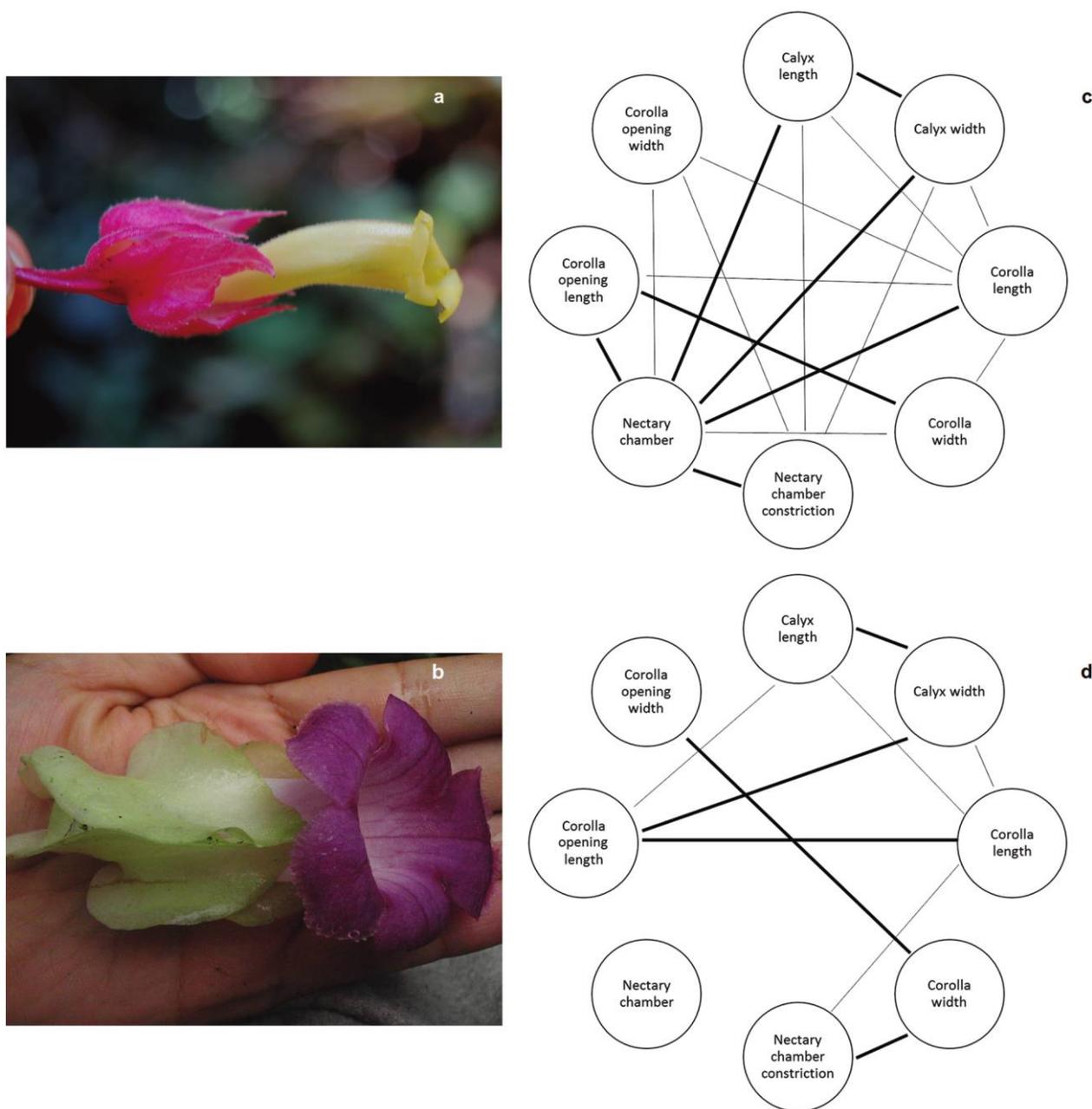
Fig. 2 Flowers of *Drymonia strigosa* (*a*) and *Drymonia oinochrophylla* (*b*) from Los Tuxtlas, Mexico. Patterns of correlation among floral traits of *D. strigosa* at $P < 0.05$ (*c*) and *D. oinochrophylla* (*d*). Thin lines represent significant correlations with $r < 0.5$, and thicker lines represent correlations with $r > 0.5$.

Floral Visitors and Visitation Rates

We recorded 63 diurnal hours of observation for *D. strigosa* and 48 h for *D. oinochrophylla* and calculated visitation rates for all visitors and pollinator functional groups (fig. 4). The most frequent floral visitors in *D. strigosa* were hummingbirds (Trochilidae), which accounted for 85% of 126 legitimate floral visits observed. The most frequent hummingbird species was *Campylopterus curvipennis excellens* (78% of all hummingbird visits), a subspecies endemic to the region of Los Tuxtlas; there were also visits by three other hummingbird spe-

cies (table 3). The bees *Trigona fulviventris* (Meliponini) were also frequent visitors, but most times they were observed chewing a hole at the base of the corolla to steal nectar (78% of their visits); less frequently, these bees landed on the corolla lobes and entered the floral tube. It was not possible to determine whether bees that entered the flower were able to deposit or remove pollen, but given their small size, it is unlikely that they would trigger pollen release from the poricidal anthers of *D. strigosa*.

The most frequent diurnal visitors of *D. oinochrophylla* were bees, which accounted for 90% of all legitimate floral visits.

Table 2

Mean Total Nectar Volume, Sugar Concentration, and Sugar Amount of Two *Drymonia* Species

	D. strigosa	N	D. oinochrophylla	N
Nectar volume (μL):				
Staminate phase	14 ± 3.8^A	10	261 ± 14.7^A	11
Pistillate phase	17 ± 5.1^A	5	114 ± 15.6^B	10
Sugar concentration (°Brix):				
Staminate phase	24.1 ± 2.21^A	10	36.1 ± 6.87^A	11
Pistillate phase	23.5 ± 3.01^A	5	35.2 ± 4.51^A	10
Sugar content (mg):				
Staminate phase	4 ± 1.1^A	10	110 ± 5.9^A	11
Pistillate phase	5 ± 1.5^A	5	42 ± 6.5^B	10

Note.   Mean total nectar volume, sugar concentration, and sugar amount ($\pm$ SEM) measured in first- and second-day flowers of *D. strigosa* and *D. oinochrophylla*. Data were recorded at Los Tuxtlas Biosphere Reserve, Mexico, during the 2012 flowering season. Means with different letters are significantly different ($P < 0.05$).

Anthophorini and Eucerini bees were the most common bee groups (60% of all bee visits). Other visitors included *Trigona fulviventris* bees and one butterfly species (table 3). During nocturnal observations, we observed two visits by moths crawling in the flowers of *D. oinochrophylla*, but bats were not recorded. Four hummingbird species and *T. fulviventris* were observed robbing nectar from the base of the corolla. *Trigona fulviventris* was also observed chewing holes at the base of the flower. Butterflies visited *D. oinochrophylla* flowers less frequently, and their proboscides rarely made contact with the flower´s reproductive organs; therefore, these visitors may be considered occasional pollinators and nectar thieves.

Reproductive System and Female Reproductive Success

Both *Drymonia* species are self-compatible. Flowers exposed to hand self-pollination produced fruits in the two species (table 4). However, fruit set in autonomous, hand-cross, and natural pollination treatments were higher in *D. strigosa* than in *D. oinochrophylla* (table 4). Apomixis was absent in both *Drymonia* species. The production of two fruits in the apomixis treatment in *D. strigosa* is attributed to a possible error in emasculation. Fruit set did not differ among autonomous pollination, hand pollination, and natural pollination in *D. strigosa* (GLMM: Wald's $\chi^2 = 4.07$, $P > 0.05$) or in *D. oinochrophylla* (GLMM: Wald's $\chi^2 = 6.07$, $P > 0.05$).

Reproductive Assurance in Drymonia strigosa

In the experiment to test for the contribution of self-pollination to female reproductive success, the fruit set of emasculated flowers of *D. strigosa* was 0.3 ± 0.09, and the fruit set of intact flowers was 0.5 ± 0.09; however, no significant differences in fruit set were found between treatments (GLMM: Wald's $\chi^2 = 1.26$, $P > 0.05$).

Discussion

Correspondence of Floral Traits with Pollination Syndromes

The results of this study show that despite having mixed pollination syndromes, most floral characters in *Drymonia*

strigosa and *Drymonia oinochrophylla* corresponded to ornitophilous and melitophilous syndromes, and the main pollinators of each species were the ones predicted by the syndrome (hummingbirds and bees, respectively). However, floral traits that did not fit pollination syndromes did not always correspond with less frequent pollinators. In *D. strigosa*, which has poricidal anthers, secondary pollinators were bees, while in *D. oinochrophylla*, with nectar volumes typically associated with the bat syndrome, secondary pollinators were butterflies. The presence of traits that do not correspond with classical pollination syndromes could be the result of selective pressures exerted by different groups of pollinators or by developmental or historical constraints.

A recent comparative analysis of the genus *Drymonia* indicates that melitophilous flowers are most likely the ancestral condition from which ornithophilous floral phenotypes are derived (Clark et al. 2015). In the same study, poricidal anther dehiscence is proposed to be the ancestral condition to longitudinal dehiscence, which appears to be associated with transitions to ornithophily. *Drymonia strigosa* is primarily a hummingbird-pollinated species, which indicates that poricidal anthers represent a plesiomorphy that is currently functional under hummingbird pollination. This may also be the case of other *Drymonia* species pollinated by hummingbirds (e.g., *Drymonia rubra*; Feinsinger et al. 1986); however, the pollination mechanism needs to be further studied.

Poricidal anthers are uncommon in hummingbird-pollinated plants, but they occur in some members of the Ericaceae and Melastomataceae families. For example, in *Gaylussacia* (Ericaceae), pollen release from poricidal anthers occurs when the beak of the bird presses the base of the anthers (Freitas et al. 2006). The presence of poricidal dehiscence is a distinctive character of the families Ericaceae and Melastomataceae generally linked to buzz pollination by bees (Buchmann 1983; De Luca and Vallejo-Marín 2013). In contrast, pollen release in *Drymonia* does not require buzzing; rather, the anthers are tipped over by the pollinator, changing the orientation of anther pores downward and delivering pollen onto the pollinator's beak or head (Wiehler 1983; Clark et al. 2015; E. Ramírez-Aguirre and S. Martén-Rodríguez, personal observation). The genus *Drymonia* is within a larger clade of gesneriads characterized by longitudinal anther dehiscence not associated with buzz pollination. Interestingly, poricidal anthers
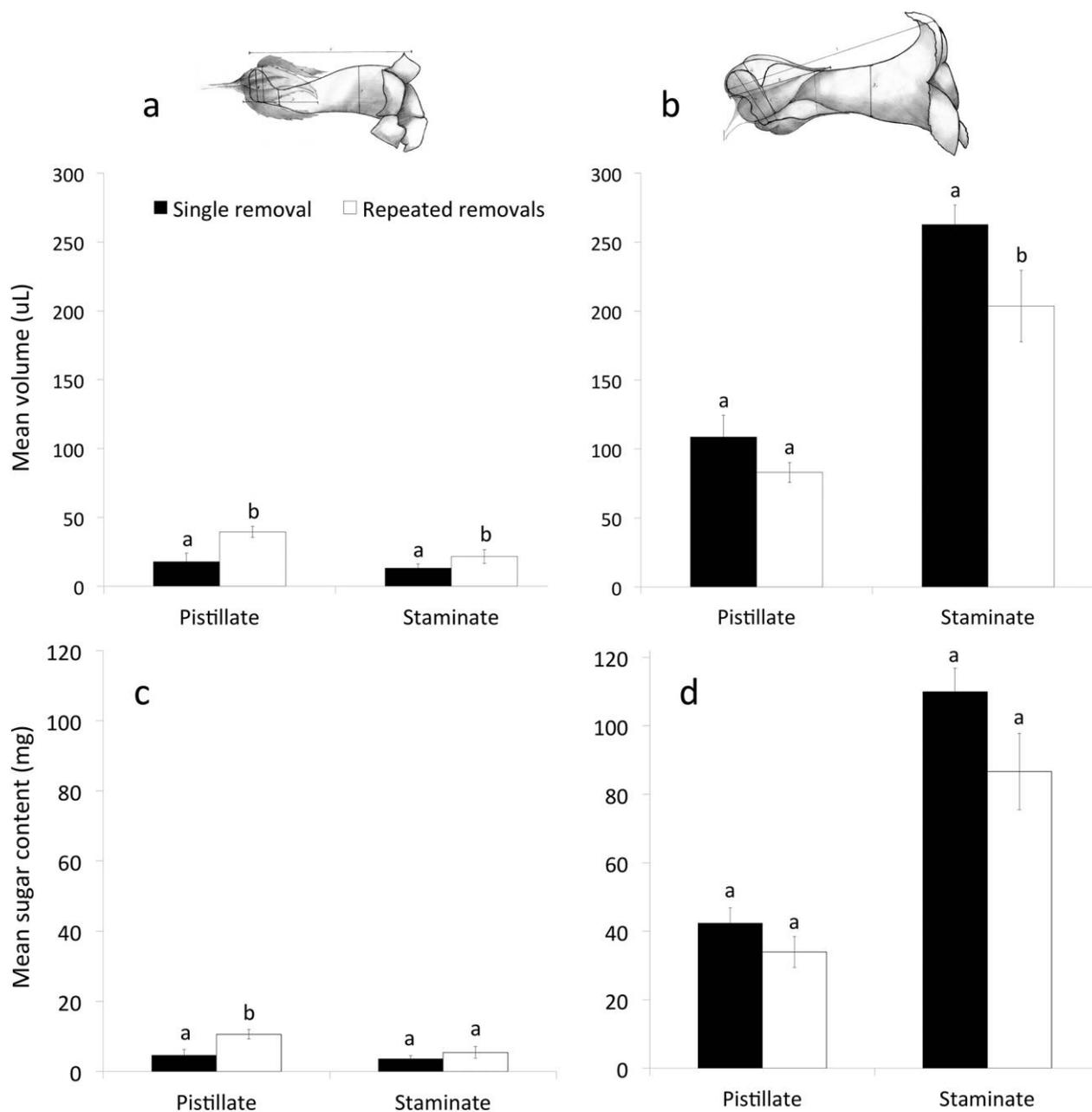
Fig. 3 Mean nectar volume and sugar production for *Drymonia strigosa* (*a*, *c*) and *Drymonia oinochrophylla* (*b*, *d*). Error bars represent ±1 SE. One removal indicates volume or sugar measured at 1400 hours in pistillate or staminate flowers. Repeated removals equal the sum of three removals measured every 3 h on the same flower per sexual phase. Different letters represent significant differences among treatments at $P < 0.05$.

in this genus allow effective pollination by bees as well as by other floral visitors able to trip the pollen-dispensing mechanism, as is the case for *D. strigosa*.

In bee-pollinated *Drymonia* species, such as *D. oinochrophylla*, poricidal anthers may represent a pollen-dispensing mechanism (Clark et al. 2015). As proposed by the pollen presentation theory, feeding behaviors and patterns of floral visitation by different types of pollinators possibly select for different pollen-dispensing strategies that regulate pollen removal by pollinators when pollen is consumed and wasted (Harder and

Thomson 1989). Poricidal anthers in bee-pollinated *Drymonia* may represent a strategy to limit pollen removal by bees that also collect pollen to feed their brood.

Nectar is another character that has been related to pollination syndromes (Baker and Baker 1983; Cruden et al 1983); however, in some Gesneriaceae species, nectar traits appear to be conserved. For example, in the genus *Sinningia*, changes in pollination syndromes were not related to changes in nectar sugar composition (Perret et al. 2001). In *D. oinochrophylla*, most morphological traits and nectar concentration (36%) cor-
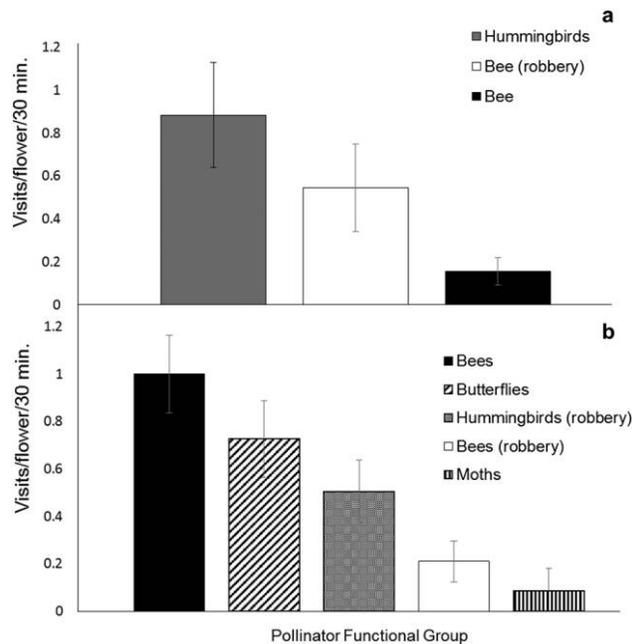
Fig. 4 Pollinator visitation frequency to flowers of *Drymonia strigosa* (*a*) and *Drymonia oinochrophylla* (*b*) at Los Tuxtlas, Mexico, during the 2012–2014 flowering seasons. Error bars represent ± 1 SE.

respond to the bee pollination syndrome (Baker 1975); however, mean nectar volume produced per day (114–261 μL; table 2) is higher than the volumes reported for bee-pollinated species (0.14–7.4 μL; Baker 1975; Cruden et al. 1983). Instead, mean nectar volume and sugar content in nectar (table 2) are more similar to the traits of bat-pollinated species (volume: 265 μL; sugar content: 45 mg/d; Ornelas et al. 2007). Bats were not recorded during the study period; thus, the available evidence does not support that nectar traits evolved under bat-mediated selection. Interestingly, *Drymonia serrulata*, a bee-pollinated species closely related to *D. oinochrophylla* (Clark et al. 2006), has large flowers and high nectar production (Steiner 1985). It is possible that allometric relationships between flower size and nectar production underlie the occurrence of high nectar production in species pollinated by large bees (Galetto and Bernardello 2004). Allometric relationships between corolla length and nectar volume have been documented in hummingbird-pollinated species (Ornelas et al. 2007), but they have not yet been tested in the Gesneriaceae. Alternatively, flowers of *D. oinochrophylla* are pollinated by both bees and bats, but bats were not detected in our sampling. If bat visitation is low or variable because of habitat disturbance, it could have been unrecorded during the study period.

A different pattern of nectar production was recorded for *D. strigosa*: nectar was dilute, and moderate volumes were recorded (table 2), similar to nectar production in other hummingbird-pollinated species (mean volume per day: 22.5 μL; Ornelas et al. 2007). Also, nectar sugar production in *D. strigosa* (table 2) is according to nectar sugar concentration (12%–34%) and sugar content (5.9 mg) of other hummingbird-pollinated species (Baker 1975; Ornelas et al. 2007). In *D. strigosa*, nectar was replenished in response to removal in both sexes, and sugar

was replenished in the female phase. This pattern of nectar production ensures a reliable reward available to the hummingbird community throughout the flowering period of the species. *Drymonia strigosa* may be a particularly important food reward for the subspecies of hummingbird *Campylopterus curvipennis excellens*, endemic to Los Tuxtlas region.

Nectar replenishment may promote frequent pollinator visitation and pollen deposition on stigmas (Aizen and Basilio 1998). Consistent with this idea, we recorded frequent visitation by hummingbirds in *D. strigosa*. Other hummingbird-pollinated plant species also replenish nectar (Cruden et al. 1983; Ordano and Ornelas 2004), and this trait may describe nectar production in ornithophilous flowers (Wilson et al. 2006; Pérez-Crespo et al. 2016). In *D. oinochrophylla*, a bee-pollinated species, nectar was not replenished, possibly because the high amount of nectar produced is enough to ensure high visitation by bees. However, other bee-pollinated species replenish nectar fast and in small quantities, depending on the frequency of bee visitation, to avoid pollen consumption (Castellanos et al. 2002). Furthermore, nectar production may be costly, and it can reduce fruit production in some plant species, so absence of replenishment may save resources (Ordano and Ornelas 2005).

Breeding Systems, Pollinator Visitation, and Plant Female Reproductive Success

Other floral characters that may be under pollinator-mediated selection and relevant during pollinator shifts are those associated with plant breeding systems. For example, in self-compatible species, pollinator foraging could promote geitonogamous self-pollination and pollen discounting, favoring traits associated with the spatial and temporal separation of sexual functions (i.e., dichogamy and herkogamy, respectively; Lloyd and Webb 1986; Webb and Lloyd 1986). Both *Drymonia* species were protandrous; however, overlap of sexual phases allowed self-pollination to different degrees: *D strigosa* had high fruit set

Table 3

Number of Potential Pollinators and Nectar Robbers Observed in Two *Drymonia* Species

Floral visitors	*D. strigosa*	*D. oinochrophylla*
Campylopterus curvipennis excellens	32	15[a]
Campylopterus hemileucurus	5	11[a]
Phaethornis striigularis	3	3[a]
Phaethornis longirostris	1	3[a]
Trigona fulviventris	19/66[a]	34
Anthophorini	1	23
Eucerini	1	26
Euglossa sp.	…	2
Lepidoptera	1	7/13[a]
Nocturnal Lepidoptera	1	2
No. observation hours	62.5	47.5/15
No. plants observed	12	15

Note. Data were recorded at Los Tuxtlas Biosphere Reserve, Mexico, during the 2012–2014 flowering season. Total number of observation hours for *D. oinochrophylla* was divided into diurnal (left) and nocturnal (right) observations; moths were observed during nocturnal observations.

[a] Illegitimate floral visits associated with nectar robbing.

Treatment	D. strigosa	N	D. oinochrophylla	N
Apomixis	.06 ± .04	33	0	14
Autonomous	.83 ± .063[A]	35	.14 ± .144[A]	14
Hand-self	.83 ± .079[A]	23	.29 ± .093[A]	10
Hand-cross	.71 ± .080[A]	31	.47 ± .114[A]	19
Natural	.87 ± .032[A]	38	.47 ± .054[A]	52
AFI	1.16	31	.30	14
SCI	1.17	23	.64	10

Note.   Mean fruit set ± SEM of pollination treatments and breeding system indices of two *Drymonia* species at Los Tuxtlas, México. Means with different letters are significantly different after Tukey's honest significant difference ($P < 0.05$). N, number of plant replicates. Sample sizes are not the same for all treatments because some flowers/fruits were lost during development. Autofertility (AFI) and self-compatibility (SCI) indices calculated according to Lloyd and Schoen (1992).

under autonomous self-pollination (83%), whereas *D. oinochrophylla* had a lower fruit set value (14%; table 4).

Interestingly, in *D. strigosa*, the high visitation frequency by hummingbirds and the high fruit set under natural pollination suggest that these animals were highly effective pollen vectors during the study years. For instance, *Campylopterus* and *Phaethornis* species are considered trapliners that follow foraging routes promoting cross-pollination with distant conspecific plants (Feinsinger and Colwell 1978; Stiles 1978). Furthermore, autonomous pollination did not significantly increase fruit set during the study period in *D. strigosa*, indicating that pollination by hummingbirds effectively achieved near-maximum values of fruit set (table 4). Nevertheless, the high capacity for self-pollination in *D. strigosa* suggests that autonomous self-pollination may be used as a reproductive assurance mechanism under temporal or spatial variation in pollinator visitation (Darwin 1877; Lloyd 1992; Fenster and Martén-Rodríguez 2007).

For *D. oinochrophylla*, high visitation frequency by bees also appears to ensure maximum fruit set, although fruit set values under natural pollination and hand-outcross pollination were lower than those of *D. strigosa*, suggesting resource limitation. In *D. oinochrophylla*, autonomous self-pollination is minimal, revealing that this species is highly dependent on pollinators for fruit set (table 4). Large bees were frequent visitors to flowers of *D. oinochrophylla*, visiting few flowers on each plant and contacting stigmas and anthers with their thorax. Large bees are known to travel long distances to find food resources, suggesting that these animals may also promote cross-pollination (Zurbuchen et al. 2010).

Patterns of Morphological Variation and Covariation

Stebbins (1970) proposed that floral characters should be integrated to promote reproductive success via the most frequent and effective pollinator. In this view, the concept of pollination syndromes implies that floral traits do not evolve independent of each other, and there should be detectable patterns of phenotypic correlations among floral traits. The pattern of correlations among floral traits was stronger in hummingbird-pollinated *D. strigosa* than in *D. oinochrophylla*, but integration indices for both species fell between the 10%–20% observed in a previous study of 36 angiosperm species (Ordano et al. 2008). The idea that patterns of correlations among sets

of floral traits independent of correlations among vegetative characters was proposed by Berg (1960; i.e., correlation pleiades). Later on, Berg demonstrated that plants with specific pollinators showed these correlation pleiades, while species lacking specific pollinators (e.g., pollination by unspecialized insects, wind, and self-pollination) also lacked correlation pleiades. Derived from these findings, it has been hypothesized that species with specialized pollination should have higher correlations among floral traits than less specialized species (Armbruster 1999). Following this rationale, it has also been proposed that floral integration should be higher in species with outcrossing than selfing breeding systems (Anderson and Bush 2006; Rosas-Guerrero et al. 2010; Fornoni et al. 2015).

Both *Drymonia* species had a major functional group of pollinators that accounted for more than 80% of all legitimate visits and less frequent groups of floral visitors (table 3). According to the criteria defined by Fenster et al. (2004), these species could be considered functionally specialized; therefore, differences in floral integration among the two *Drymonia* species do reflect differences in pollination specialization. However, while both *Drymonia* species are self-compatible, the two species differed in the degree of pollinator dependence (potential autonomous self-pollination: 0.83 in *D. strigosa* vs. 0.14 in *D. oinochrophylla*). *Drymonia strigosa* showed twice as many significant correlations among floral traits as *D. oinochrophylla* (fig. 2), a result that is consistent with recent findings that pollinator-independent species have higher floral integration than pollinator-dependent species (Pérez et al. 2007; Rosas-Guerrero et al. 2010; Fornoni et al. 2015; but see Anderson and Busch 2006). This interpretation has the caveat that pollinators were highly effective during the study years, despite the fact that *D. strigosa* has high potential for self-pollination (table 4). Future studies should include long-term experiments of reproductive assurance across different plant populations to determine the role of animal pollinators as potential agents of selection on floral traits.

Conclusions

Overall, our findings support the pollination syndrome hypothesis, since most floral traits of the two *Drymonia* species corresponded with the main pollinator functional group predicted by the syndrome. However, floral phenotypes did not

fully correspond to a single pollination syndrome, suggesting transitional stages between pollination systems or stable mixed pollination systems. The results of this study did not support the hypothesis of mixed pollination systems in *Drymonia*. Mixed syndromes most likely reflect historical or developmental constraints. Given the recent evolutionary history of Neotropical Gesneriaceae (Perret et al. 2013), it is possible that the lack of correspondence between some floral traits and observed pollinators may be related with limited time for the evolution of floral adaptations during pollination system transitions. A better understanding of the role of pollinators and breeding systems on floral transitions and pollination syndromes requires more work on the floral biology and pollination ecology of the family Gesneriaceae.

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