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Pollinator type strongly impacts gene flow within and among plant populations for six Neotropical species

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Abstract

Animal pollinators directly affect plant gene flow by transferring pollen grains between individuals. Pollinators with restricted mobility are predicted to limit gene flow within and among populations, whereas pollinators that fly longer distances are likely to promote genetic cohesion. These predictions, however, remain poorly tested. We examined population genetic structure and fine-scale spatial genetic structure (FSGS) in six perennial understory angiosperms in Andean cloud forests of northwestern Ecuador. Species belong to three families (Gesneriaceae, Melastomataceae, and Rubiaceae), and within each family we paired one insect-pollinated with one hummingbird-pollinated species, predicting that insect-pollinated species have greater population differentiation (as quantified with the F_{ST} statistic) and stronger FSGS (as quantified with the S_P statistic) than hummingbird-pollinated species. We confirmed putative pollinators through a literature review and fieldwork, and inferred population genetic parameters with a genome-wide genotyping approach. In two of the three species pairs, insect-pollinated species had much greater (>2-fold) population-level genetic differentiation and correspondingly steeper declines in fine-scale genetic relatedness. In the Gesneriaceae pair, however, F_{ST} and S_P values were similar between species and to those of the other hummingbird-pollinated plants. In this pair, the insect pollinators are euglossine bees (as opposed to small bees and flies in the other pairs), which are thought to forage over large areas, and therefore may provide similar levels of gene flow as hummingbirds. Overall, our results shed light on how different animal pollination modes influence the spatial scale of plant gene flow, suggesting that small insects strongly decrease genetic cohesion.

KEYWORDS
2b-RAD sequencing, Andean cloud forest understory, fine-scale spatial genetic structure, hummingbird pollination, insect pollination, neotropical plants, population genetic structure

INTRODUCTION

Understanding how plant mutualists influence spatial patterns of genetic diversity is central to plant biology, especially in the present scenario of biodiversity decline due to human-accelerated environmental change (Aguilar et al., 2008, 2019; Dick et al., 2008; Hardy et al., 2006). Animal pollinators directly affect gene flow within and

among flowering plant populations via the transfer of pollen grains (Hamrick et al., 1992; Loveless & Hamrick, 1984). Early reviews on patterns of genetic structure in plants found that wind tends to homogenize plant gene pools, whereas animal pollination is associated with higher population genetic differentiation (Duminil et al., 2007; Hamrick & Godt, 1996) as well as stronger fine-scale spatial genetic structure (FSGS; i.e., the nonrandom spatial distribution of closely related individuals) (Dick et al., 2008; Gelmi-Candusso et al., 2017). However, those studies lumped together all animals, overlooking the effect of different pollinators on gene flow dynamics within and among plant populations. More recently, global reviews on the effects of various factors on population genetic differentiation for 337 plant species (Gamba & Muchhala, 2020) and on the strength of FSGS for 147 plant species (Gamba and Muchhala, unpublished manuscript) found that plants pollinated by small insects have a greater genetic structure than plants pollinated by large insects and vertebrates. These findings are consistent with differences in pollen dispersal among different types of animal pollinators.

Pollen dispersal ultimately depends on the foraging behavior and pollen carryover capacity of pollinators (Levin, 1979). Pollinators with large foraging areas can carry pollen long distances, potentially enhancing gene flow within and among plant populations, while pollinators with local foraging behavior may reduce gene flow. This trend has been predicted in seminal reviews (Levin, 1981; Loveless & Hamrick, 1984), and supported in empirical studies of temperate and subtropical plants (Breed et al., 2015; Kramer et al., 2011; Linhart et al., 1987; Linhart & Grant, 1996), and in a recent study in a set of neotropical Merianieae (Dellinger et al., 2022).

Studies of pollinator movement show that small insects, such as flies, solitary bees, and small beetles, generally forage in relatively small areas, visiting most flowers in a single plant and then moving to nearby plants (Campbell, 1985; Escaravage & Wagner, 2004; Hasegawa et al., 2015). Conversely, large insects such as moths, butterflies, and large bees have larger foraging areas, frequently associated with traplining behavior (i.e., repeatedly visiting a sequence of flowers over several locations) (Levin, 1979; Murawski & Gilbert, 1986; Rhodes et al., 2017; Schmitt, 1980). Similarly, volant vertebrates such as nonterritorial hummingbirds and bats also follow a traplining foraging behavior (Fleming, 1982; Lemke, 1984, 1985; Tello-Ramos et al., 2015), potentially covering even greater distances than large insects (Campbell & Dooley, 1992; Castellanos et al., 2003; Linhart, 1973; Melampy, 1987; Sahley, 2001; Serrano-Serrano et al., 2017; Webb & Bawa, 1983), and flying across fragmented habitats (Breed et al., 2015; Byrne et al., 2007; Hadley & Betts, 2009; Krauss et al., 2017; Levin, 1979; Machado et al., 1998; Sahley, 2001; Solís-Hernández & Fuchs, 2019; Southerton et al., 2004). Therefore, pollination by volant vertebrates should increase the spatial scale of intraspecific gene flow, resulting in larger genetic plant neighborhoods (sensu Webb, 1984, Wright, 1946), relative to pollination by insects (Bezemer et al., 2016; Karron et al., 1995; Krauss, 2000; Krauss et al., 2009).

In this study we examined if, relative to hummingbird pollination, insect pollination is in fact associated with: (1) greater genetic differentiation between populations, and (2) stronger FSGS structure within populations. We focused on six frequent and locally abundant perennial understory angiosperms in the Andean cloud forest of northwestern Ecuador, a highly diverse but threatened ecosystem. Species belong to three families, and within each family we paired one insect-pollinated species (euglossine bees, or small buzzing bees, or hoverflies and wasps) with one species predominantly pollinated by traplining hummingbirds (Table 1). All six focal species share a similar geographic range, are putatively outcrossing, and mostly similar in their seed dispersal (see Methods: Study species and pollinators). Therefore, we expect that any trend of variation in population

TABLE 1 Characteristics of studied species and sites where they were sampled.

Species	Growth form	Pollinators (source)	Fruit type	Sites
Drymonia brochidodroma (Gesneriaceae)	Herbaceous	Euglossine bees (this study)	Fleshy capsule	SL, T
Drymonia tenuis (Gesneriaceae)	Subshrub	Traplining hummingbirds[a]	Fleshy capsule	SL, P, B
Miconia rubescens (Melastomataceae)	Shrub	Small buzzing bees[b]	Berry	SL, P, B
Meriania tomentosa (Melastomataceae)	Shrub	Traplining hummingbirds/bats[a,c]	Dry capsule	SL, P, B
Notopleura longipedunculoides (Rubiaceae)	Subshrub	Wasps/flies/bees (this study)	Berry	SL, P, B
Palicourea demissa (Rubiaceae)	Shrub	Traplining hummingbirds[a]	Berry	SL, B

Abbreviations: B, Bellavista; P, El Pahuma; SL, Santa Lucía; T: Las Tángaras.
[a]Weinstein & Graham, 2017.
[b]Renner, 1989; Gamba & Almeda, 2014.
[c]Muchhala & Jarrin-V, 2002; Dellinger et al., 2019.

genetic differentiation and FSGS across species will be due primarily to pollination mode. We confirmed putative pollinators through fieldwork, and we used a genome-wide genotyping approach to obtain data for estimates of genetic structure.

METHODS

Study sites

We performed this study in Santa Lucía (0.12 N, 78.6 W), El Pahuma (0.02 N, 78.6 W), Bellavista (0.01 S, 78.7 W), and Las Tángaras (0.08 S, 78.8 W), four private reserves located on the northwestern slope of the Andean cordillera of Ecuador, in the province of Pichincha ~40 km northwest of Quito. Sites are 5–23 km apart, historically connected by continuous forest that is now selectively logged, composed of secondary and primary cloud forest ranging from 1800 to 2500 m in elevation, and part of the southern end of the "biogeographic Choco" (Mordecai et al., 2009). Because they are nearby and similar in elevation, they share many species, yet the distance between them potentially imposes a physical barrier for the movement of pollinators, making them ideal for studying the effect of different animal pollinators on plant gene flow.

Study species and pollinators

To select our focal species, we compiled a list of species occurring at all sites using the Tropicos.org database of the Missouri Botanical Garden and visited the sites to observe abundances. We selected six perennial understory angiosperms from three families, based on their high abundance and frequency along transects of the reserves. We chose one insect-pollinated and one hummingbird-pollinated species per family, including *Drymonia brochidodroma* Wiehler and *Drymonia tenuis* (Benth.) J.L.Clark (Gesneriaceae), *Miconia rubescens* (Triana) Gamba & Almeda and *Meriania tomentosa* (Cogn.) Wurdack (Melastomataceae), and *Notopleura longipedunculoides* (C.M.Taylor) C.M.Taylor and *Palicourea demissa* Standl. (Rubiaceae; with the hummingbird-pollinated species listed second in each case). Pairing by family allowed us to account for phylogenetic autocorrelation when comparing F_{ST} and S_P values between insect-pollinated vs hummingbird-pollinated plants, although we note that pairs differed in how closely related they were to each other: the Gesneriaceae species pair was the most closely related (same genus: *Drymonia*), followed by the Rubiaceae species pair (same tribe: Palicoureeae), and last by the Melastomataceae species pair (same subfamily: Melastomatoideae).

We obtained information on pollination mode from peer-reviewed literature (Clark et al., 2015; Dellinger et al., 2019; Gamba & Almeda, 2014; Muchhala & Jarrin-V, 2002; Renner, 1989; Weinstein & Graham, 2017), and by videotaping plants in the field (Table 1). Specifically, for species with little information on pollination mode (*D. brochidodroma* and *N. longipedunculoides*), we confirmed putative pollinators by videotaping flowers with four high-definition Sony digital camcorders for 4 days at each site. Cameras simultaneously videotaped four individuals per day (one species per day, eight individuals per species per site). Flowers were videotaped in the morning (6:30 AM to 11:30 AM) and in the afternoon (01:30 PM to 06:30 PM). Among hummingbird-pollinated species, *M. tomentosa* is also pollinated by nectar bats (Muchhala & Jarrin-V, 2002) and, along with *D. tenuis* and *P. demissa*, can also be visited by territorial hummingbirds (Dellinger et al., 2019, Weinstein & Graham, 2017).

Based on our field observations, the spatial distribution of all six species appeared widespread and consistent within sites, with occasional clusters of conspecifics. Geographic distributions are also similar; all species occur along western Ecuador, with our study sites near the center of their ranges (Global Biodiversity Information Facility). Little information is known about their seed dispersal and floral biology, although fruit and floral morphology and previous field studies in closely related species gave some clues. Most study species have fleshy fruits (Table 1) that are presumably consumed by understory birds, as reported for *M. rubescens* (Kessler-Rios & Kattan, 2012) and closely related species (Loiselle & Blake, 1999), as well as for *Psychotria*, which is related to *Palicourea* and similar in habit and habitats (Loiselle et al., 1995; Loiselle & Blake, 1993; Theim et al., 2014). *Drymonia* have fleshy capsules, often termed "display-capsules" in understory Gesneriaceae, because of their presumed role in animal attraction (Clark et al., 2012). The limited reports suggest these are also consumed by understory birds, as well as frugivorous bats and monkeys (Wiehler, 1983). The capsular fruits of *M. tomentosa* probably have wind- or gravity-dispersed seeds, as in many understory Melastomataceae with the same type of fruit (Renner, 1989), potentially making this species the most limited in seed dispersal. All six species have mechanisms to reduce selfing, either via marked herkogamy (i.e., spatial separation of stigma and anthers via style elongation) in the Melastomataceae (Renner, 1989), protandry (i.e., temporal separation of male and female phases, with anthers releasing pollen and dying-off before stigma is receptive) in the Gesneriaceae (Clark et al., 2012; Wiehler, 1983), or distyly (i.e., polymorphism in style length within a population in which flowers in one individual are monomorphic) in the Rubiaceae (Bawa & Beach, 1983). Self-incompatibility (via intramorph incompatibility) is common in the

Rubiaceae (Bawa & Beach, 1983), but less clear in the Gesneriaceae and Melastomataceae, where both self-incompatibility and self-compatibility have been documented (Ramírez-Aguirre et al., 2016; Renner, 1989).

Molecular work and genotyping

We collected leaf tissue and extracted DNA from 20 individuals per species from each of the three study sites (please refer to Table 1 for sampled sites per species). We largely followed available trails in the reserves, making sure sampled individuals were at least 20 m apart from each other, and taking GPS coordinates for each. We used a genome-wide restriction site-associated DNA sequencing technique termed 2b-RAD (Wang et al., 2012) to build loci de novo (Catchen et al., 2013) and obtain 100–1000 of unlinked SNPs (please refer to Appendix S1: Sections S1 and S2). For estimating population genetic parameters, we removed ~20% of total individuals genotyped due to >50% missing data (2–17 individuals per species; Appendix S1: Table S1). Please refer to Gamba and Muchhala (2022) for all geographic and genetic data.

Inference of population genetic parameters

We used the program GenoDive v3.0 (Meirmans & Van Tienderen, 2004) to calculate genetic diversity statistics for each species. We assessed population genetic structure using the *F*-statistics derived from an Analysis of Molecular Variance (AMOVA; Excoffier et al., 1992). AMOVA determines the proportion of genetic variance partitioned within individuals, among individuals within subpopulations, and among subpopulations. Related *F*-statistics were obtained with an infinite allele model; therefore, they are equivalent to *G*-statistics (Nei, 1973; Nei & Chesser, 1983). These include F_{IT} (the mean reduction in heterozygosity of an individual relative to the total population), F_{IS} (the inbreeding coefficient among individuals within sites), and F_{ST} (the global genetic differentiation among sampled sites). The statistical significance of these diversity statistics was assessed using 1000 random permutations of the data, while their standard deviations were obtained by jackknifing over loci.

To visualize population genetic structure for each species, we used principal component analyses (PCA). After SNP calling and filtering, we obtained a VCF for each species with the final set of SNPs from the program "populations" on our pipeline (Appendix S1: Section S2). We then used the R program (Core Team, 2018) SNPRelate (Zheng et al., 2012) to convert the VCF to a GDS file with *snpgdsVCF2GDS* and to compute PCA on each species' SNP set with *snpgdsPCA*.

Inference of fine-scale spatial genetic structure

We evaluated FSGS for each species via spatial autocorrelation analyses at the individual level (Vekemans & Hardy, 2004) using the program SPAGeDi v.1.3a (Hardy & Vekemans, 2002). We first transformed individuals' decimal degree coordinates into the Universal Transverse Mercator coordinate system, which is compatible with the SPAGeDi version we used. We then assessed genetic relatedness between all pairs of individuals i and j with Nason's kinship coefficient, F_{ij} (Loiselle et al., 1995). We specified five distance intervals for each species and allowed the program to define their maximal distance such that the number of pairwise comparisons within each interval was kept approximately constant. F_{ij} values were regressed on the natural logarithm of the spatial distance separating pairs of individuals, $\ln(d_{ij})$, to quantify regression slopes, b. To test for significant fine-scale spatial structure, spatial positions of individuals were permuted 1000 times to obtain a frequency distribution of b under the null hypothesis that F_{ij} and $\ln(d_{ij})$ are not correlated. We quantified the strength of FSGS with the S_P statistic (Vekemans & Hardy, 2004), which is calculated as $-b/(1 - F_1)$, where F_1 is the mean F_{ij} between all pairs of individuals in the first distance interval containing nearest neighbors (< ~1 km for all species). The S_P statistic mainly depends on the slope of the kinship–distance curve, allowing direct comparisons of FSGS among species (Vekemans & Hardy, 2004). Standard errors of all FSGS statistics were obtained by jackknifing over loci. To visualize FSGS, we plotted the mean F_{ij} at each distance interval over the five distance intervals for each species.

RESULTS

Pollinators

We recorded, in total, 10 individuals and 30 h (i.e., ~3 h/individual) for *D. brochidodroma*, and 12 individuals and 35 h (i.e., ~2.9 h/individual) for *N. longipedunculoides*. From these videos, we observed that *D. brochidodroma* was exclusively visited by euglossine bees (also please refer to Clark et al., 2015), with five bee visits lasting ~10 s each, whereas *N. longipeduncoloides* was visited by wasps, hoverflies, and small bees. We recorded 18 wasp visits lasting ~60 s each, 10 hoverfly visits ~30 s each, and five bees visits ~15 s each. Although we did not record pollinators for

M. rubescens, their anther morphology and small flowers conformed to the buzz-pollination syndrome common to this genus, adapting them to small buzzing bees that shake anthers to release pollen from tiny apical pores (Brito et al., 2016). *Drymonia tenuis*, *M. tomentosa*, and *P. demissa* are primarily pollinated by traplining hummingbirds; for a complete list please refer to Weinstein and Graham (2017).

Filtered genetic datasets

After SNP calling and quality control using different filtering procedures, we obtained a mean of 2,797,308 SNP loci per species (± 1,091,949 SD; range: 879,138–4,151,836), with average coverage ranging from 14.0–95.1 read depth per locus across species (Appendix S1: Table S1). After removing individuals with >50% missing data, final sample sizes of individuals per species per study site ranged from 8–18 (mean = 13 ± 3 SD), and the number of variant loci ranged from 1044–4907 (mean = 2699 ± 1427 SD) across species, with missing data across species ranging from 24%–38% (mean = 33% ± 5 SD) (Appendix S1: Tables S2 and S3).

Gene diversity was similar across species; total expected heterozygosity (H_T) ranged from 0.21–0.25 (mean = 0.23 ± 0.02; Appendix S1: Table S2) and mean expected heterozygosity within sites (H_S) ranged from 0.17–0.26 (mean = 0.22 ± 0.02). Additionally, all species showed statistically significant levels of inbreeding, as indicated by significant G_{IS} values depending on whether these were pooled across sites (mean = 0.30 ± 0.14 SD; Appendix S1: Table S2) or analyzed separately by site (mean = 0.32 ± 0.16 SD; Appendix S1: Table S3).

Population-level genetic structure

AMOVA results revealed that, in all species, most of the genetic diversity resides within populations/sites, whereas less genetic diversity resides among sites (Appendix S1: Table S4). AMOVA F_{IT} showed that for most species a large proportion of individuals across study sites were out of Hardy–Weinberg equilibrium, and this was likely to be due to inbreeding. In fact, AMOVA F_{IS} was significant for all species, congruent with our G_{IS} estimates above, and confirming that there was substantial inbreeding within sites across studied species. Furthermore, AMOVA F_{ST} was variable (range = 0.03–0.21, average = 0.10 ± 0.06) but significant for all species, demonstrating considerable genetic differentiation among study sites (Table 2). Regarding pollination systems, for two of the three pairs of species (Melastomataceae and Rubiaceae), F_{ST} values were more than twice as high for the insect-pollinated species, while in the final pair (Gesneriaceae), F_{ST} values were comparable for the insect- and hummingbird-pollinated species (Table 2, Figure 1). A PCA of filtered SNPs showed some separation of individuals following their site of origin (Appendix S1: Figure S1), but the percentage variation explained by PC1 and PC2 was generally <15%, suggesting admixture between sites. In *M. rubescens* and *N. longipedunculoides*, however, PC1 and PC2 explained ~20 and 35%, respectively, of the SNP variation, in accordance with their higher F_{ST} values.

Fine-scale spatial genetic structure

FSGS was significant for all studied species, in that regression slopes *b* of pairwise kinship coefficients on the natural logarithm of spatial distance were always significantly negative (Table 3). The extent of FSGS as quantified with the S_P statistic varied by an order of magnitude across species, ranging from 0.009 to 0.089 (mean = 0.04 ± 0.03 SD). This variation is evident in our FSGS visualizations (Figure 2; Appendix S1: Tables S5–S10), which showed that species pollinated by insects tended to have steeper average kinship–distance slopes (Figure 2 a,c,e) than species pollinated by hummingbirds (Figure 2 b,d,f). Given that standard errors associated with each average F_{ij} are vanishingly small (Appendix S1: Tables S5–S10), they are not observable in Figure 2. S_P values were higher for all insect-pollinated species relative to hummingbird-pollinated ones (average = 0.054 ± 0.03 SD vs. 0.017 ± 0.01 SD). Among species pairs, this pattern was most pronounced in the Melastomataceae and Rubiaceae pairs, with little difference in the Gesneriaceae pair (Table 3, Figure 1).

DISCUSSION

The contrasting effect of different pollinators on plant gene flow has remained largely unexplored, especially in the Neotropics (Wessinger, 2021, but please refer to Dellinger et al., 2022). Our study highlights that pollinator type can have a strong impact on genetic structure: among our species pairs, two of the three species pollinated by insects had greater levels of population genetic differentiation and stronger FSGS than their hummingbird-pollinated counterparts (Tables 2 and 3, Figure 1). Our findings support the idea that pollinator movement during foraging affects the spatial scale of intraspecific plant gene flow, with the limited movement of small insects restricting gene flow within and among populations, and the traplining behavior of hummingbirds promoting genetic cohesion.

TABLE 2 Estimates of population genetic structure for each studied species.

Species	*N* total	*N* loci	AMOVA F_{IT} (SE)	AMOVA F_{IS} (SE)	AMOVA F_{ST} (SE)
Drymonia brochidodroma	35	4907	0.42 (0.007)	**0.37** (0.007)	**0.08** (0.004)
Drymonia tenuis	29	1044	0.56 (0.014)	**0.51** (0.015)	**0.10** (0.010)
Miconia rubescens	34	2171	0.50 (0.009)	**0.43** (0.010)	**0.13** (0.005)
Meriania tomentosa	32	3883	0.29 (0.008)	**0.24** (0.008)	**0.06** (0.003)
Notopleura longipedunculoides	41	1815	0.35 (0.013)	**0.17** (0.014)	**0.21** (0.008)
Palicourea demissa	30	2376	0.22 (0.012)	**0.19** (0.012)	**0.03** (0.003)

Note: *N* total, number of genotyped individuals in the final genetic dataset; *N* loci, number of variant loci in the final genetic dataset; AMOVA F_{IT} represents the deviation from Hardy–Weinberg equilibrium within individuals relative to the expected heterozygosity in the total population; AMOVA F_{IS} represents the inbreeding coefficient among individuals within sites; AMOVA F_{ST} represents the global genetic differentiation among sampled sites. Significance of statistics (F_{IS} and F_{ST}) is denoted in bold ($p = 0.001$) and is based on 1000 permutations of the data. The insect-pollinated species is listed first for each pair.

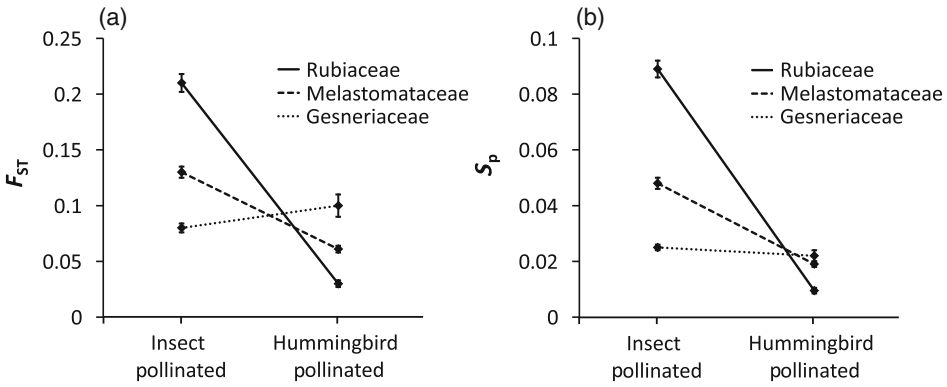

FIGURE 1 Genetic parameters evaluated in three insect-pollinated vs three hummingbird-pollinated plants paired by taxonomic family, corresponding to (a) AMOVA F_{ST} values and (b) S_P values. Error bars surrounding dots correspond to standard errors obtained through jackknifing over loci. Lines connect species pairs by family: solid line for the Rubiaceae, dashed line for the Melastomataceae, and dotted line for the Gesneriaceae.

TABLE 3 Estimates of FSGS parameters for each studied species.

Species	*N* pairs	F_1 (SE)	*b* ln(distance)	S_P (SE)
Drymonia brochidodroma	595	**0.053** (0.003)	−**0.024** (0.001)	0.025 (0.001)
Drymonia tenuis	406	**0.044** (0.007)	−**0.021** (0.002)	0.022 (0.002)
Miconia rubescens	561	**0.105** (0.005)	−**0.043** (0.002)	0.048 (0.002)
Meriania tomentosa	496	**0.051** (0.002)	−**0.018** (0.001)	0.019 (0.001)
Notopleura longipedunculoides	820	**0.180** (0.006)	−**0.073** (0.003)	0.089 (0.003)
Palicourea demissa	435	**0.018** (0.002)	−**0.009** (0.001)	0.009 (0.001)

Note: *N* pairs, number of comparisons between all pairs of conspecific individuals; F_1, kinship coefficient between individuals in the first distance interval (separated by <1 km); *b* ln(distance), slope of the regression of kinship coefficients on the natural logarithm of spatial distance; S_P, intensity of FSGS for each species. Standard errors (SE) were obtained through jackknifing over loci. Significance of parameters (F_1 and *b*) is denoted in bold ($p < 0.01$) and is based on 1000 permutations of individual locations. The insect-pollinated species is listed first for each pair.

Our chosen study species allowed us to control for other factors that might impact plant population genetic structure and FSGS, increasing the probability that differences in F_{ST} and S_P values are in fact due directly to animal pollination mode rather than a confounding variable. For example, pairing species within families allowed us to control for phylogenetic autocorrelation in our dataset. Furthermore, all species belong to cloud forest understory sites inside the southern end of the Choco Andean corridor

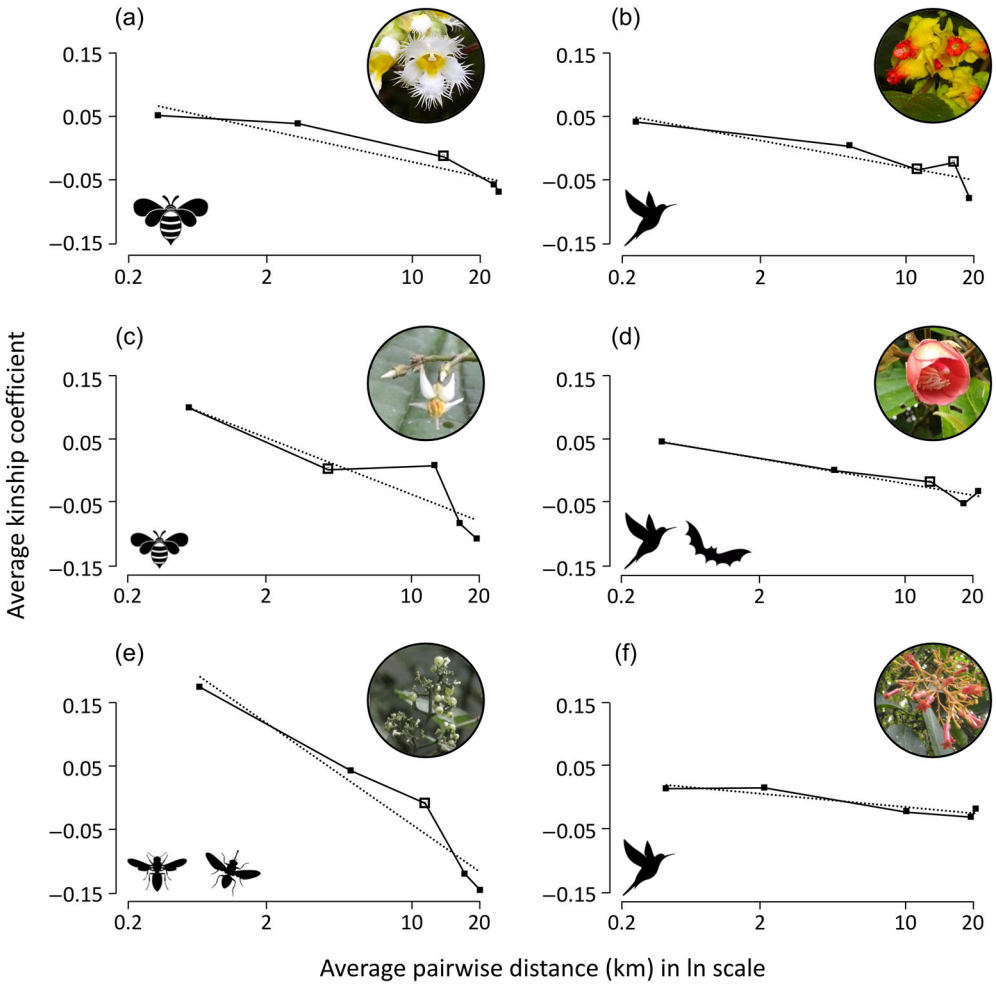

FIGURE 2 Average kinship–ln(distance) curves of each studied species. Filled symbols represent significant ($p < 0.05$) average kinship coefficient values based on 1000 permutations of individual spatial locations among all individuals. Open symbols represent non-significant ($p > 0.05$) average kinship coefficient values. Dotted lines correspond to linear regressions. For associated standard errors of average F_{ij} at each distance interval refer to Appendix S1: Tables S5–S10. (a) *Drymonia brochidodroma*. (b) *Drymonia tenuis*. (c) *Miconia rubescens*. (d) *Meriania tomentosa*. (e) *Notopleura longipedunculoides*. (f) *Palicourea demissa*. Photographs by Diana Gamba.

(Mordecai et al., 2009) that are relatively well connected by a continuous corridor of forests. Therefore, pollinator movement between sites for all species should be constrained by the same geographic barriers. Similarly, the geographic distribution of our study species greatly overlaps, especially in Ecuador and Colombia, and study sites are always well within their distributions (rather than at the edges, which might affect genetic structure; Global Biodiversity Information Facility). *Miconia rubescens* has the widest distribution, *Notopleura longipedunculoides* extends to Panama, *Meriania tomentosa* and *Palicourea demissa* to Peru (the former also to Venezuela), whereas the two *Drymonia* are restricted to Ecuador and Colombia. Seed dispersal across species is likely to be similar; seeds either fall under mother plants (Noé, E. Toapanta, personal observation, March 2016) or are predominantly consumed by understory frugivores (observed by birders and guides in the study sites). Additionally, most species pairs have the same type of fruit: *D. brochidodroma* and *D. tenuis* have fleshy capsules, and *N. longipedunculoides* and *P. demissa* have berries. The exception is the Melastomataceae pair, in which *M. tomentosa* has dry capsules and *M. rubescens* has fleshy berries. We would expect capsular seeds to be dispersal limited and therefore correspond with higher F_{ST} and S_P values than a species with fleshy berries, which is likely to be dispersed by animals. Our data instead found that *M. tomentosa* has lower F_{ST} and S_P values than *M. rubescens*, suggesting that vertebrate pollination (by hummingbirds and bats) in this species overrides any dispersal limitation imposed by the dry capsules. Furthermore, all species exhibited occasional clusters of individuals in our

study sites. In fleshy fruited plants, this clustering is often associated with the foraging behavior of understory birds that aggregate around preferred food sources (Kessler-Rios & Kattan, 2012; Loiselle & Blake, 1993; Smith, 2001) therefore potentially limiting gene flow via seed dispersal.

Our study species have various mechanisms to reduce autogamy (via protandry, herkogamy, or distyly; see Methods: Study species and pollinators), yet they all exhibited significant levels of inbreeding as evidenced by their F_{IS} values (Table 2). This could be due to spatial distributions and mating between close relatives, or to pollinators transferring pollen between flowers on the same plant (geitonogamy), as all species typically have multiple flowers open at any given time. Interestingly, the Rubiaceae species showed the lowest F_{IS} values, which makes sense given that the distyly and intramorph incompatibility exhibited by these species (Bawa & Beach, 1983) is likely to be a more effective mechanism at preventing geitonogamy than protandry or herkogamy, given that individual plants are either short or long styled (i.e., all flowers on a plant have the same morphology). Observed differences in inbreeding levels, however, do not seem to underlie the differences we found in population genetic differentiation or strength of FSGS. Inbreeding can increase genetic structure by increasing genetic drift (Duminil et al., 2007; Vekemans & Hardy, 2004) however, among our focal species, inbreeding coefficients (AMOVA F_{IS} in Table 2) were not correlated with AMOVA F_{ST} ($r = -0.06$, $t = 0.11$, $p = 0.9$) nor with S_P ($r = -0.25$, $t = 0.52$, $p = 0.6$).

We note that differences in F_{ST} and S_P values were more pronounced between the Rubiaceae species pair (7-fold and 10-fold, respectively), followed by the Melastomataceae pair (2.2-fold and 2.5-fold, respectively), and last by the Gesneriaceae pair (almost equivalent values; Tables 2 and 3, Figure 1). *Notopleura longipedunculoides* (Rubiaceae) is largely pollinated by tiny wasps and hoverflies that probe most flowers in the same individual and stay among nearby plants (D. Gamba, personal observation, January 2018), consistent with it having the greatest observed F_{ST} and S_P values. *Miconia rubescens* (Melastomataceae) is pollinated by relatively small pollen-collecting bees (e.g., *Melipona* and *Trigona*; Renner, 1989), consistent with the intermediate F_{ST} and S_P values. Finally, *D. brochidodroma* (Gesneriaceae) is pollinated by euglossine bees, which are larger and have been reported to fly long distances (Janzen, 1971; López-Uribe et al., 2008), in line with *D. brochidodroma* having the smallest F_{ST} and S_P values among our insect-pollinated plants. Therefore, differences between insect pollinators may explain this pattern. Among our vertebrate-pollinated species, *P. demissa* is visited by ~15 hummingbird species, *M. tomentosa* is visited by ~eight hummingbird species and by nectar bats (Muchhala & Jarrin-V, 2002), and *D. tenuis* is visited by ~seven hummingbird species (Weinstein & Graham, 2017). Some of these hummingbirds are territorial, but most are traplining (Weinstein & Graham, 2017), therefore the latter should override the potential isolating effect of the former. The fact that the two *Drymonia* species had such similar F_{ST} and S_P values suggests that euglossine bees and hummingbirds may be similar in their pollen dispersal ability. Overall, our genetic structure results are also consistent with direct measures of pollen dispersal based on paternity analyses, in that bats and hummingbirds can transport pollen for several kilometers, large insects such as euglossine bees for more than 600 m, while insects smaller than a honeybee rarely transfer pollen more than 300 m (Dick et al., 2008; Webb & Bawa, 1983).

We might also expect different vertebrates to vary in pollen dispersal ability in the same way that insects do. For instance, foraging behavior among hummingbirds can strongly impact plant gene flow (Cuevas et al., 2018; Murawski & Gilbert, 1986; Schmidt-Lebuhn et al., 2019), as evidenced by the fact that territorial hummingbirds move pollen much shorter distances than traplining hummingbirds (Betts et al., 2015; Ohashi & Thomson, 2009; Wolowski et al., 2013). Hummingbirds and bats may also differ, as the latter have been found to carry pollen more efficiently (Muchhala & Thomson, 2010) and to longer distances than hummingbirds (Lemke, 1984, 1985; Tello-Ramos et al., 2015). We encourage future work to look more in depth at how plant gene flow is affected by behavioral differences within pollinator guilds. For example, foraging ranges of pollinators might predict the spatial scale of plant gene flow, but this could be complicated by behaviors such as grooming or differences in pollen retention on fur, feathers, and insect hairs. A larger sample with details on pollinator behavior would clarify the effects of these differences on plant population structure.

Our study provides new evidence on the contrasting effect that different animal pollinators can have on the spatial scale of intraspecific plant gene flow. We found that plants pollinated by small insects have considerably higher population genetic differentiation and stronger FSGS than hummingbird-pollinated plants. Our results also suggest that large insects, such as euglossine bees, can connect plant populations as effectively as traplining hummingbirds. Therefore, the effect of different animal pollinators on neotropical plant gene flow can be significantly different at local (within populations) and regional (among populations) scales (e.g., Dellinger et al., 2022). Our results are also relevant to conservation efforts, suggesting that plants pollinated by small insects are likely to be very susceptible to habitat fragmentation

(more so than vertebrate-pollinated plants, e.g., Côrtes et al., 2013), as this can further isolate populations and result in the loss of genetic variability due to increased genetic drift (Aguilar et al., 2008, 2019). Nevertheless, focal studies show that plants pollinated by hummingbirds and bats can also experience detrimental effects due to habitat fragmentation (Hadley et al., 2018; Hadley & Betts, 2009; Nunes et al., 2017; Wanderley et al., 2020). Future studies should seek to compare how animal foraging behavior, animal flying distances, and their related effect on plant gene flow might be altered due to anthropogenic disturbance.

AUTHOR CONTRIBUTIONS

Diana Gamba and Nathan Muchhala planned and designed the research. Diana Gamba collected and analyzed the data. Diana Gamba wrote the initial draft of the manuscript. Diana Gamba and Nathan Muchhala contributed equally to substantial revisions of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data (Gamba & Muchhala, 2022) available from Dryad: https://doi.org/10.5061/dryad.rr4xgxdbf.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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