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Elevated inbreeding in *Heliconia tortuosa* is determined by tropical forest stand age, isolation and loss of hummingbird functional diversity

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Abstract

Forest conversion and habitat loss are major threats to biological diversity. Forest regeneration can mitigate the negative effects of old-growth forest loss on species diversity, but less is known about the extent to which forest loss reduces genetic diversity in remnant populations and whether secondary forests play a role in the maintenance of genetic diversity. We quantified genetic diversity in a tropical hummingbird-pollinated understorey herb, *Heliconia tortuosa*, across a landscape mosaic of primary and secondary forest regrowth. Using microsatellite genotypes from >850 adult and juvenile plants within 33 forest patches and extensive bird surveys, we examined the effect of contemporary and historical landscape features including forest age (primary vs. secondary forest), stand isolation and pollinator assemblages on genetic diversity and levels of inbreeding in *H. tortuosa*. We found that inbreeding was up to three times higher in secondary forest, and this effect was amplified with reductions in primary forest in the surrounding landscape through reduced observed heterozygosity in isolated fragments. Inbreeding in forest patches was negatively correlated with the local frequency of specialist long-distance foraging traplining hummingbirds. Traplining hummingbirds therefore appear to facilitate mating among unrelated plants—an inference we tested using empirically parameterized simulations. Higher levels of inbreeding in *H. tortuosa* are therefore associated with reduced functional diversity of hummingbirds in secondary forests and forest patches isolated from primary forests. Our findings suggest a cryptic consequence of primary forest loss and secondary forest regeneration through the disruption of mutualistic interactions resulting in the erosion of genetic diversity in a common understorey plant.

KEYWORDS
allelic richness, forest fragmentation, forest regeneration, landscape genetics, mutualistic networks, pollen dispersal, pollinator decline

1 | INTRODUCTION

Contemporary tropical landscapes are often a mosaic of agricultural land, remnant primary forests, degraded forests and regenerating secondary forests (Asner et al., 2009). The small size and isolation of populations in remnant primary forest patches may act to reduce plant population sizes, pollinator abundances and pollinator movement between patches (Brudvig et al., 2015; Volpe et al., 2016), which could increase the likelihood of mating among relatives resulting in inbred individuals and reductions in genetic diversity (Ellstrand & Elam, 1993; Leimu et al., 2006). Secondary forest regeneration could mitigate the negative effects of primary forest loss in fragmented landscapes by increasing the total amount of forest habitat, which could allow for larger populations and increased connectivity between once-isolated old-growth patches (Melo et al., 2013).

Plant and animal species that are characteristic of old-growth forests may occur in lower abundance, differ in their functional attributes or be missing altogether from secondary forests (Betts et al., 2018; Chazdon et al., 2009; Poorter, Craven, et al., 2021). From a functional perspective, habitat generalists are often overrepresented in secondary forests compared to specialists in primary forests (Carrara et al., 2015; Gibson et al., 2011; Kormann et al., 2018). As a result, both the species and functional composition of plant (Santos et al., 2008) and animal (Kormann et al., 2018; Tscharntke et al., 2008) assemblages often differ between primary and secondary forests, with implications for plant–animal mutualistic interactions (Llorens et al., 2012), gene flow (Breed et al., 2015) and the maintenance of genetic diversity across fragmented landscapes.

Genetic diversity in flowering plants is, in part, maintained by outcrossed mating systems and long-distance gene movement facilitated by animal pollinators and seed dispersers within and among plant populations (Gamba & Muchhala, 2020; Hamrick & Godt, 1996). Pollinator species vary in a number of functional traits related to pollination services which can determine the responses of plant populations to forest loss and fragmentation (Llorens et al., 2012). Functional traits of pollinators that might determine population genetic responses in plants include movement capacity, home range size, habitat preferences, degree of morphological specialization and trait matching, and pollination efficacy (Breed et al., 2015). Plant species with less specialized pollinators may be less prone to the effects of habitat fragmentation than species specialized pollinators (Aldrich & Hamrick, 1998; Castilla et al., 2017; Hadley & Betts, 2012). Although knowledge is accumulating on how habitat loss, isolation and forest regeneration interact to determine pollinator functional diversity (Hadley et al., 2014), we lack an understanding of how pollinator species and functional diversity and its loss in disturbed landscapes influence the genetic diversity of plant populations. We also have a limited understanding of how the loss or reduced relative abundance of specialist pollinator species and those with greater movement capacity can influence patterns of mating, genetic diversity and inbreeding in the plants they pollinate.

We studied the role of pollinator functional composition, forest stand age and landscape isolation in determining levels of population genetic diversity of a hummingbird-pollinated forest understorey herb, *Heliconia tortuosa* Griggs, in Costa Rica. Hummingbird pollinators of *H. tortuosa* include morphologically generalist, short-billed species that move short distances within defended territories including rufous-tailed hummingbirds (*Amazilia tzacatl*), and morphologically specialized, curve-billed traplining hummingbirds (e.g., birds that forage over longer distances on repeated routes) that include green hermits (*Phaethornis guy*) and violet sabrewings (*Campylopterus hemileucurus*; Betts et al., 2015). Across our study system, hummingbird species and functional groups have been shown to differ in their responses to forest loss (Hadley et al., 2018), occupancy and movement relative to landscape connectivity (Kormann et al., 2016; Volpe et al., 2016). Territorial bird species in this system, and others, tend to be habitat generalist species that are associated with open areas, forest edges, and both primary and secondary forest interiors, while traplining species are mostly associated with primary forest interiors (Blake & Loiselle, 2001; Kormann et al., 2016; Stouffer & Bierregaard Jr, 1995).

Several lines of evidence indicate that the habitat preferences of these two different functional groups of pollinators may have implications for mating dynamics of *H. tortuosa* in those forest patches of different age. For example, the degree of morphological specialization among resident hummingbird species in our system has been empirically shown to result in different pollination efficiencies in *H. tortuosa*. In controlled aviary experiments, pollination of *H. tortuosa* by traplining birds resulted in a 5-fold greater number of pollen tubes observed in the style compared to territorial birds when controlling for pollen quantity (Betts et al., 2015). Furthermore, a recent study has shown that the relative abundance of traplining birds can affect both the mating system and levels of genetic diversity of *H. tortuosa* seeds. Higher abundances of traplining birds within stands are correlated with increased pollen pool genetic diversity and lower levels of biparental inbreeding in predispersed ungerminated embryos inferred from mother–offspring genotype arrays (Torres-Vanegas et al., 2021). However, additional factors affecting genetic diversity should be studied, such as the persistence of genetic diversity through later juvenile and adult life stages, differences between populations in primary and secondary forest patches, and the impact of landscape variables and bird community species composition.

Given what is known about differences in movement capacity and pollination efficiency among different functional groups of pollinators in this system, we hypothesized that if landscape structure and forest age influence species and functional variation among hummingbird pollinator communities, there is potential for a cascading effect on realized pollen movement within and among forest patches. This would have implications for plant genetic diversity across different life stages in different forest types with variation in levels of isolation. We examined these interactions and their consequences for variation in genetic diversity and inbreeding of *H. tortuosa* across a gradient in forest patch sizes, adjacent primary and secondary forest cover (a proxy for isolation), and forest disturbance history. First, we hypothesized that the functional composition of pollinator communities in secondary forest would differ

from primary forest with a greater prevalence of generalist territorial hummingbirds in secondary forests (Blake & Loiselle, 2001; Stouffer & Bierregaard Jr, 1995) and a loss or reduction of specialist traplining birds (Kormann et al., 2016). Second, to the extent that generalist territorial hummingbird pollinators are dominant in secondary forest, are less efficient pollinators (or effectively nonpollinators) and potentially move pollen shorter distances, we expected a greater frequency of short-distance mating among *H. tortuosa*, resulting in higher inbreeding levels in secondary forest populations. Third, we hypothesized that primary forest in the surrounding landscape of each patch could maintain genetic diversity in secondary forests via the provisioning of traplining hummingbirds and subsequent increased pollen movement between less related individual plants from adjacent populations. More primary forest in the landscape surrounding *H. tortuosa* populations should therefore reduce inbreeding. Alternatively, if hummingbird pollinators are reluctant to cross gaps in isolated patches, they will tend to remain within a patch (Hadley & Betts, 2009; Kormann et al., 2016; Volpe et al., 2016), in which case patch area rather than the total amount of forest surrounding the patch, regardless of age, should be the most important driver of genetic diversity and inbreeding.

We tested these hypotheses using contemporary and historical forest landscape data, microsatellite genetic data from more than 850 adult and juvenile plants from 33 populations of *H. tortuosa*, and measures of hummingbird occupancy in a subset of those focal patches. We validated our inferences with an empirically parameterized simulation model to examine how shifts in the functional composition of hummingbird communities in different forest types would be expected to affect realized pollen movement and determine levels of mating among related individuals in *H. tortuosa* to further explore mating system implications of the second hypothesis considering known differences in movement capacity of different hummingbird pollinator species.

2 | MATERIAL AND METHODS

2.1 | Study species and populations

The study was conducted in an ~31,000-ha area of Coto Brus county in southern Costa Rica (8.78437N, −82.95870W) surrounding the Organization for Tropical Studies' Las Cruces Biological Station. The majority of land clearing in Coto Brus occurred between 1947 and 1980 and remaining forest patches span a range of sizes from 1 to >1000 ha across a gradient in forest amount from 1% to 99% forest within 1 km of each patch (Zahawi et al., 2015). We used remote imagery to dating back to 1947 to classify forest age and successional status; 1947 is the first year for which aerial photography of the region was available (Zahawi et al., 2015). These images suggest that only 1.8% of the region had been deforested in 1947, followed by rapid deforestation by migrants into the area. We considered stands that in 2014 had continuous forest cover in images from 1947, before widespread deforestation, to be primary forest. Forest patches were classified as secondary forest if a stand that had lost forest cover in any period since 1947 had regained forest cover in 2014 images. We calculated the size of each focal forest patch and the percentage cover of primary and secondary forest within 1 km of each sampling point using ARCGIS (Zahawi et al., 2015). A 1-km radius was chosen because previous work demonstrated that the forest amount within 1 km is an adequate proxy for isolation. First, it is the distance that best explains the reproductive response of *Heliconia tortuosa* (e.g., pollen tube abundances and seed set) to forest isolation and fragmentation (Hadley et al., 2014). Second, 1 km is also the estimated maximum daily foraging distance for traplining birds (Hadley & Betts, 2009; Volpe et al., 2016). This proxy for forest isolation was used due to the difficulty of calculating true measures of isolation of a given forest patch given the complex fragmented heterogeneous landscape in Coto Brus (Figure S1).

We selected *H. tortuosa* as a focal species for our study because it is a keystone nectar resource visited by both generalist and specialist hummingbird pollinators (Borgella Jr et al., 2001) and, unlike many plant species characteristic of old-growth forests, *H. tortuosa* is also common and relatively abundant in young secondary forests. *H. tortuosa* has a mixed-mating system; it has been shown to be successfully pollinated by both self- and outcrossed pollen (Kress, 1983). However, significant variation exists in the pollination success of *H. tortuosa* by different hummingbird species (Betts et al., 2015). Therefore, given its mixed mating system (unlike strictly self-incompatible species), we might expect to see variation in the genetic diversity of *H. tortuosa* populations in part due to variation in local hummingbird pollinator community composition. *H. tortuosa* seeds are dispersed by many bird species, including generalist clay-coloured thrushes (*Turdus grayii*). *H. tortuosa* adult ramets can live up to 12 years or longer, although the maximum age of genets is unknown. Juvenile individuals are typically <1 year old before flowering and reproducing. Because of the clonal nature of *H. tortuosa*, it is likely that the age of many genets in old-growth forests in our study could potentially pre-date the widespread deforestation and fragmentation in this system.

2.2 | Sampling and genotyping

We chose 37 patches according to a stratified random sample spanning a range of patch sizes and forest amount (forest cover within a 1000-m radius; sensu Hadley et al., 2014; Kormann et al., 2018; Figure S1). Within each patch, we collected fresh leaf material from a maximum of 16 nonreproductive seedling juveniles and 16 adult reproductive plants. In smaller forest patches where we collected fewer than 16, we sampled all individuals within a patch. Collected individuals were spaced at least 2 m apart to avoid sampling ramets of the same genet. Leaf material was immediately placed on silica gel, dried and transported to Oregon State University, USA, for genetic analyses. Genomic DNA from leaf material was extracted using Qiagen DNAeasy plant kits (Qiagen). We used a set of microsatellite primers described for related members in the genus and modified for

H. tortuosa (Côrtes et al., 2009; Gowda et al., 2012). Ten polymorphic primer pairs were used to genotype *H. tortuosa* individuals, and fragment analyses were carried out using an ABI 3730 XL (Thermo Fisher Scientific). Electropherograms were scored using the microsatellite add-on package in GENEIOUS (version 6.1.7, Kearse et al., 2012). When markers were designed and modified from the above studies (Table S1), primer combinations showing evidence of null alleles were eliminated from the genotyping panel. Furthermore, mother–offspring arrays examined in a separate study using the same marker system and individuals from many of the same forest patches did not reveal the presence of null alleles (Torres-Venegas et al. 2019, Torres-Vanegas et al., 2021). Finally, to confirm the absence of null alleles, analyses using the program INEST, which jointly estimates null allele frequency and inbreeding levels, were conducted (Chybicki & Burczyk, 2009) for all individual populations in our study. INEST estimates the parameters f (inbreeding coefficient), n (null allele frequencies), b (genotyping failure rate) and models that incorporate a combination of these parameters jointly (fn, fb, nb, the full model nfb and a null model). All possible models were run using 500,000 model updates with a 100,000-update warmup/burn in. Model selection can be performed by choosing the model with the lowest Deviation Information Criterion. We found no evidence for models that included null alleles as being the best fit models so null alleles are not considered in our analyses. Moreover, many population-level models failed to converge in INEST, perhaps due to small samples sizes, complete data (i.e., no genotyping failure) or the lack of null alleles in our data set. Finally, for those models that did give inbreeding estimates (f), these values were not correlated with our estimates of inbreeding described below, perhaps due to bias in estimates for small sample sizes (Campagne et al., 2012).

2.3 | Genetic analyses

Because *H. tortuosa* can reproduce clonally, we identified those plants that had identical multilocus genotypes using the R library POPPR (R version 3.6.1, Kamvar et al., 2014). We found only four pairs of individuals with identical genotypes. Because removal of these individuals did not alter the results, they were retained in the data set. Population genetic calculations were done in the software package GENODIVE (version 2.0178 b23; Meirmans & Van Tienderen, 2004). We calculated the effective number of alleles, average allelic richness, observed heterozygosity (H_O), expected heterozygosity (H_E), and heterozygote deficit using the inbreeding coefficient G_{IS} (a multilocus *F* analogue; Nei, 1987) for juvenile and adults separately in each forest patch.

2.4 | Landscape analyses

We modelled the effect of landscape features on population generic parameters using generalized linear mixed effects models (GLMMs) as implemented in the lme package (Pinheiro et al., 2015) in R statistical software (version 3.6.1). In the final analyses, we included population genetic estimates from patches with five or more adults and juveniles, each. Including patches with as few as 10 total number of sampled individuals was necessary for us to be able to include *H. tortuosa* populations found in the smallest and most isolated patches, a key hypothesis of our study. Our final genotype data set consisted of 449 adults and 408 juvenile *H. tortuosa* from 33 patches (26 primary and seven secondary forests). Patch area ranged from 0.63 to 1358 ha (mean area = 158.2 ha), percentage cover of primary forest surrounding each patch within a 1-km radius ranged from 2.31 to 75.04 (mean = 29.80), and the percentage cover of secondary forest surrounding each patch within a 1-km radius ranged from 8.15 to 42.08 (mean = 24.47).

We modelled allelic richness, effective number of alleles, expected heterozygosity (H_E), observed heterozygosity (H_O) and the inbreeding coefficient (G_{IS}) of each patch as a function of individual-, stand- and landscape-level variables. Specifically, models included plant stage (adult or juvenile), the log of forest patch area in 2014, forest stand age (primary or secondary forest), the percentage cover of primary forest within a 1-km radius of the stand, the percentage cover of secondary forest within 1 km of the forest, and interactions between stand and forest cover, with population identity as a random intercept. Initial tests with univariate and multivariate linear models showed that all population genetic statistics were consistently independent of the log of forest patch area, so we excluded this from subsequent models. The list of final models tested is given below, with the dependent variable indicated by Y:

$$\text{Model 1: } Y \sim \text{stage} + \text{Forest Age} + \%\,\text{primary} + \%\,\text{secondary}$$

$$\text{Model 2: } Y \sim \text{stage} + \text{Forest Age} + \%\,\text{primary}$$

$$\text{Model 3: } Y \sim \text{stage} + \text{Forest Age} + \%\,\text{secondary}$$

$$\text{Model 4: } Y \sim \text{stage} + \text{Forest Age}$$

$$\text{Model 5: } Y \sim \text{stage} + \text{Forest Age}^{*}\,\%\,\text{primary} + \%\,\text{secondary}$$

$$\text{Model 6: } Y \sim \text{stage} + \text{Forest Age}^{*}\,\%\,\text{secondary} + \%\,\text{primary}$$

Variance inflation factors (VIFs) were all below 1.25, both before and after model selection (function "vif," package "car 3.0-2": Fox & Weisberg, 2011), indicating that multicollinearity of predictors was not critical. We found little evidence for spatial autocorrelation in residual variation in our genetic diversity estimates.

2.5 | Fine scale spatial genetic structure

To understand how patterns of relatedness among neighbouring plants within a forest patch may vary as a function of stage and forest age and how this may therefore influence mating patterns within a stand, we analysed fine-scale genetic structure (FSGS) in

adults and juveniles in primary and secondary forests using SPAGEDI software (Hardy & Vekemans, 2002). We estimated pairwise kinship relationships among adults and juvenile stages and how these changed as a function of pairwise spatial distance classes (Loiselle et al., 1995). We used distance classes of 10, 25, 50, 100, 250, 500, 750, 1000, 2500 and 5000 m (sensu Torres-Vanegas et al. (2021)) and the significance of FSGS was determined using 1000 permutations of kinship and distance class estimates.

2.6 | Pollinator communities

To test whether local and landscape variables were associated with differences in the proportions of traplining and territorial hummingbirds observed in the patch, we surveyed hummingbird communities in 49 forest patches (Kormann et al., 2018), including 22 of the forest patches where we collected *H. tortuosa* genetic samples. The same experienced observer visited each forest patch in either 2012 ($n = 40$) or 2013 ($n = 9$) between 5 and 10 a.m. and conducted 12-min fixed-radius point counts ($r = 25$ m) to generate a relative index of hummingbird activity. For this, we sampled three plots in small forest patches (<5 ha) and six plots in large patches (>25 ha) that were at least 50 m apart. Plots were selected to represent the structural heterogeneity of the forest patch. We then used GLMMs with a binomial error distribution to determine the proportion of all hummingbirds that belonged to traplining species. The response variable was a two-column object containing the sum of large trapliner individuals (*Phaethornis guy* and *Campylopterus hemileucurus*) vs. all other observed potential *H. tortuosa* pollinators with short foraging distances (*Amazilia tzacatl*, *A. decora*, *Heliodoxa jacula*, *Phaethornis striigularis*, *P. cuvierii*) detected per point count. These pollinators were placed into functional groups based upon their movement capacities and pollination efficiencies. While some variation surely exists in the degree of territoriality, movement capacity and pollination efficiencies of these different species, the foraging distances of traplining *P. guy* and *C. hemileucurus* are consistently greater than territorial flower visitors and they have been experimentally shown to be more efficient pollinators (Betts et al., 2015). The full model contained point-count-level forest type (secondary vs. primary), $\log_{10}$-transformed forest patch size (ha), and proportions of primary and of secondary forest within 1 km of the site as fixed effects. We standardized and centred on zero all covariates and used forest patch ID as a random intercept to account for spatial nonindependence of point counts within the same patch. The scale parameter (0.85, dispersion glmer in blmeco) and VIFs <1.1 indicated no model overdispersion or collinearity of predictors. Diagnostic plots showed that assumptions for residual normality and homoscedasticity were met, and residual maps indicated no spatial autocorrelation of residuals.

We assessed whether patches with a greater frequency of pollinator community shifts towards territorial hummingbirds were associated with greater *H. tortuosa* inbreeding by combining patch-specific data on *H. tortuosa* inbreeding coefficients and hummingbird communities for the 22 fragments for which we had overlapping bird and plant genetic data. Using a linear mixed effect model, we related the inbreeding coefficient (G_{IS}) of each fragment to the relative proportion of trapliners and the plant stage (juvenile or adult). We used forest patch ID as a random intercept and implemented an exponential variance structure (varExp) to improve homoscedasticity of the residuals.

2.7 | Modelling genetic consequences of pollinator functional shifts

Given the underlying spatial genetic structure of our study system that we determined by genotyping *H. tortuosa* individuals and further explored in analyses of FSGS, we explored the implications of hypothesized pollinator shifts within different forest patches and examined our second hypothesis using simple distance-dependent mating simulations. We simulated pollen movement by hummingbirds among adult plants with known genotypes across our study system under three pollination scenarios ("territorial hummingbird only" community, a "trapliner only" community, a "mixture" community with half trapliners and half territorial birds). Another scenario was parameterized based on empirically measured pollen dispersal ("empirical dispersal") using data from a small study on pollen transfer among neighbouring plants in an old-growth fragment that was dominated by trapliners. To parametrize the empirical scenario, we applied a fluorescent pollen analogue (RadgloR, Radiant Colour) before dawn to a total of six freshly opened *H. tortuosa* flowers on two single plants in a primary forest patch in the study region near the field station. Our previous mist-netting efforts showed that the hummingbird community in the patch is dominated by trapliners (75% trapliners, $n = 36$; Hadley et al., 2018). The next day, we collected 69 1-day-old flowers up to 213 m away from the treated flowers, stored them in individual bags to avoid cross-contamination, and recorded the GPS coordinates of the flowers. We counted the number of fluorescent particles on the stigmas, using a 40× magnification with an epifluorescence microscope. We fit an exponential distribution to the dye dispersal distances using the maximum-likelihood estimator implemented in the "fistdist" package (Delignette-Muller & Dutang, 2015), and used the resulting λ to parametrize the pollen dispersal distances for the empirical scenario (estimated $\lambda \pm SE = 0.0126 \pm 0.0003$, corresponding to a 78.2-m mean dispersal distance). Further dye experiments in different forest types with different pollinator communities were prevented by the COVID-19 pandemic during 2020 and 2021.

To simulate pollinator movement, we first generated distributions of pollinator movement distances for each scenario. We drew 1000 random numbers from a negative exponential distribution of the form $x \sim \text{Exp}(\lambda)$, where x is the realized pollen dispersal distance and $1/\lambda$ = mean pollen dispersal distance. We parameterized the functions with $\lambda = 1/100$ for the trapliners and $\lambda = 1/20$ for the territorial pollinators, corresponding to mean pollen dispersal distances of 100 and 20 m, respectively (Linhart, 1973). For the mixture scenario, half of the dispersal events were drawn from the territorial

scenario, and half from the trapliner distance distribution. These estimates correspond to pollen dispersal distances found for *Heliconia* pollinated by territorial hummingbirds (Linhart, 1973) and trapliners (Côrtes et al., 2009), respectively.

Based on these four dispersal distance distributions, we then determined the relatedness of the genotyped plant pair whose spatial distance matched most closely to the random draw. The absolute difference between the simulated pollen dispersal distances and the spatial distance of the corresponding empirical parents was small. The mean absolute difference between empirical distances between *H. tortuosa* in our populations and the random draws from each of the four scenarios was <0.1 m and the 95% quantiles for all four scenarios showed a difference between the random draws and empirical distances was <7.3 m, indicating that the simulated matings had closely matching empirical counterparts. We used DyadML as a pairwise relatedness estimator (Wang, 2011). Alternative relatedness estimators (TrioML, Ritland, LynchRD) did not qualitatively change our results. Finally, we compared the distributions of the resulting median relatedness, and the dispersal distances between the four scenarios using Wilcoxon ranked sign tests (wilcox. test, package "stats," Bonferroni-adjusted p-value corresponding to $\alpha = 0.05$).

3 | RESULTS

3.1 | Population-level genetic diversity and inbreeding

Our data set consisted of genotypic data for 449 adults and 408 juvenile *Heliconia tortuosa* from 33 patches (26 primary and seven secondary forests). Adult and juvenile genetic diversity (H_E) averaged 0.58 (range 0.48–0.73) and 0.57 (range 0.49–0.62; Table S2). Secondary forest populations had higher allelic richness compared to primary forests ($F = 4.4$, $p = .044$, Figure 1a). Expected heterozygosity (H_E) was lower in primary vs. secondary forests (Figure 1b, $F = 8.47$, $p = .007$) Observed heterozygosities (H_O) did not differ between forest types (Figure 1c, $F = 1.629$, $p = .21$). We found support for the hypothesis that populations within secondary forests show higher inbreeding coefficients than populations from primary forests (Figure 1d, $F = 11.11$, $p < .001$).

Across all patches, adults and juveniles showed, on average, positive inbreeding coefficients (Table S2). We found that populations of juvenile *H. tortuosa* showed higher inbreeding coefficients than adults. Adults had an inbreeding coefficient of $G_{IS} = 0.044$ [0.012, 0.076] (mean, [lower CI, upper CI]) and juveniles had an inbreeding coefficient approximately twice that, $G_{IS} = 0.095$ [0.063, 0.127] (Table S2). Overall, 24% of adult populations sampled showed significantly positive G_{IS} values compared to 42% of juvenile populations (Table S2).

3.2 | Landscape results—Population isolation

The proportion of primary forest cover with 1 km had a positive effect on the effective number of alleles (Table 1a; Table S4a, $p < .05$). The proportion of primary forests within 1 km had a positive interaction with forest age in determining observed heterozygosity (Figure 2a; Table S4c, $p < .05$), with models 2 and 5 showing approximately equivalent AICc scores (Table S4d). The interaction between forest age and proportion of primary forests showed a greater positive effect of observed heterozygosity on secondary forests surrounded by primary forests than primary forest stands (Figure 2a). The best overall model predicting inbreeding in *H. tortuosa* was one that included greater inbreeding in secondary forests, increased greater inbreeding in juveniles vs. adults, and decreased inbreeding in patches surrounded by increased proportion of primary forests (Figure 2b; Table S4e). Therefore, populations of *H. tortuosa* that exist in landscapes that have lost the most primary forest cover around each patch tended to have lower observed heterozygosity and higher rates of inbreeding.

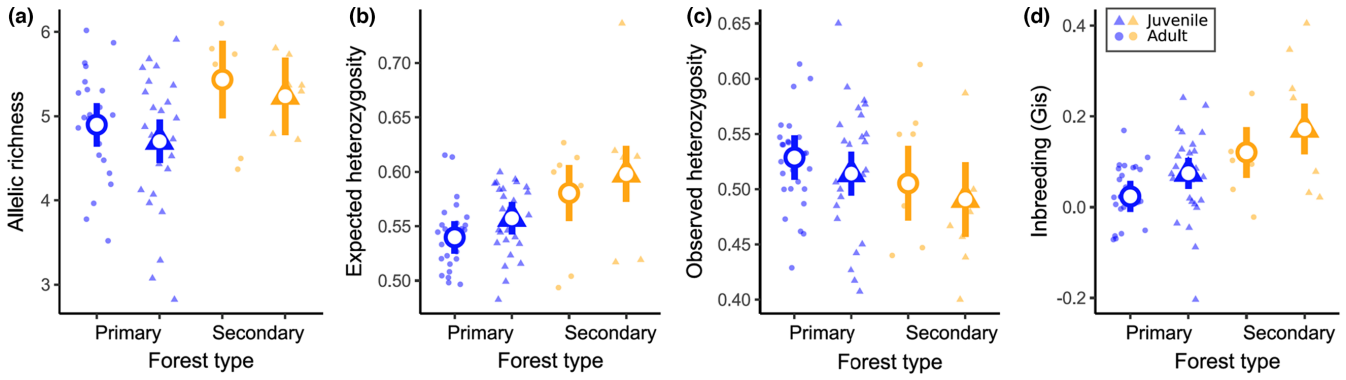

FIGURE 1 Plots of genetic diversity in *Heliconia tortuosa* adult and juvenile plants in Costa Rica. (a) The effective number of alleles is greater in plants in secondary forests (yellow symbols) than primary forests (blue symbols, $p < .05$). (b) Differences in expected heterozygosity in adults (triangles) and juveniles (circles) in secondary forests and primary forests ($p < .05$). (c) Differences in observed heterozygosity across juvenile and adults in primary and secondary forests ($p > .05$). (d) Inbreeding (heterozygote deficit) in juveniles and adults in secondary forests relative to primary forests (both $p < .05$). Shown are model predictions (large circles and triangles), 95% confidence intervals (bars) and empirical patch-level data points (semitransparent dots and triangles) [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 (a) Summary of best fit model parameters for stand- and landscape-level independent variables, generalized linear mixed model predictors and genetic diversity measures for *Heliconia tortuosa* populations in Coto Brus, Costa Rica. (b) For traplining birds, odds ratio estimates are given for models of bird occupancy in different forest ages and landscape contexts

			Primary		Secondary		Landscape context			
	Model	Delta AIC	Adult	Juvenile	Adult	Juvenile	% primary	% secondary	Forest age * % primary	Forest age * % secondary
(a) *H. tortuosa*										
Effective no. of of alleles	2	0.34	4.86	4.67	5.57	5.37	0.21	–	–	–
Allelic richness	4	0.43	2.76	2.78	3.03	3.05	–	–	–	–
H_E	4	0.44	0.54	0.56	0.58	0.59	–	–	–	–
H_O	5	0.32	0.53	0.51	0.54	0.52	0.05	0.004	−0.04	–
G_{IS}	2	0.56	0.03	0.08	0.10	0.15	−0.03	–	–	–
(B) Birds										
Trapliners	3	0.54	−0.66		−1.43		–	−0.74	–	–

Note: Model is the best fit model number; delta AIC is the relative value of AIC for the chosen model against all other models. Parameters are given for effective number of alleles (eff # A), allelic richness (A rich), expected heterozygosity (H_E), observed heterozygosity (H_O), and the inbreeding coefficient (G_{IS}). Parameters are given for adults (A) and juveniles (J) in primary and secondary forests, % primary and % secondary are the percentage of primary and secondary forest within 1 km of the forest patch. Forest Age * % Primary and Forest Age * % Secondary are interactions between forest age and % forest cover in the surrounding 1 km. See Tables S3 and S4 for full model selection criteria and all model parameters, respectively.

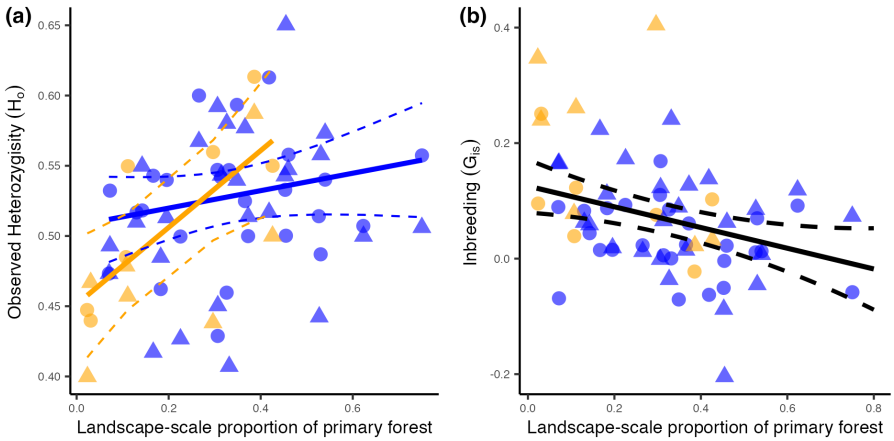

FIGURE 2 (a) The effect of the proportion of primary forest within 1 km surrounding a patch on observed heterozygosity (H_O) in adult (circles) and juvenile (triangles) *Heliconia tortuosa* from primary (blue) and secondary (yellow) populations (best fit model 5). (b) The effect of the proportion of primary forest within 1 km surrounding a patch on the inbreeding statistic (best fit model 2). Shown are model predictions (solid line), 95% confidence intervals (dashed lines) and empirical patch-level data points [Colour figure can be viewed at wileyonlinelibrary.com]

3.3 | Fine scale genetic structure

Our analysis of FSGS revealed that adults and juveniles in primary forest show similar levels and spatial scales of pairwise kinship out to distances of 0–25 m in primary forests (Figure S2a). Adults in secondary forests show similar levels of nearest neighbour kinship compared to adults and juveniles in primary forest to scales of 25 m, but juveniles show close to twice the level of nearest neighbour kinship as adults in those forests at distances out to 25 m (Figure S2b).

3.4 | Pollinator functional composition across forest types

Hummingbird pollinators were common throughout the study areas and were detected in 73% of all point counts, with traplining birds detected in 23% of all point counts. The proportion of trapliner observations in secondary forest was almost half of that in primary forest plots ($z = -2.194$, $p = .028$, Table 1b, Figure 3a), and territorial hummingbird species numerically dominated the pollinator community when there was a higher proportion of secondary forest

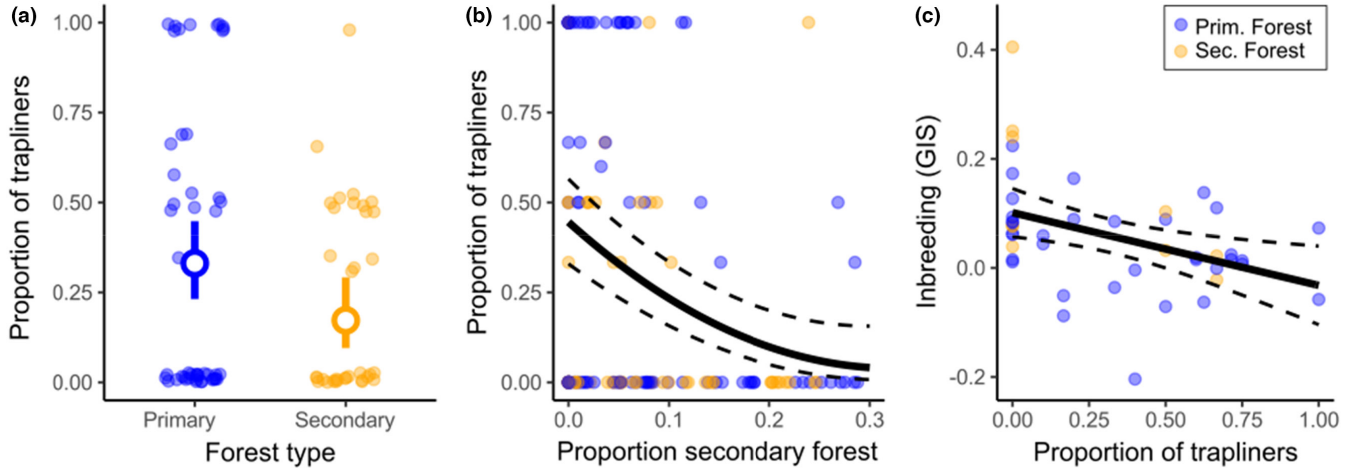

FIGURE 3 The effect of local and landscape-scale forest characteristics on hummingbird pollinator communities and inbreeding observed during point counts in southern Costa Rica. Shown is the proportion of trapliners in the *Heliconia tortuosa* pollinator community as a function of (a) primary and secondary forest type and (b) the amount of secondary forest cover within 1 km of each point count. We also found a relationship (c) between the proportion of trapliners in each forest and levels of inbreeding in each *H. tortuosa* population [Colour figure can be viewed at wileyonlinelibrary.com]

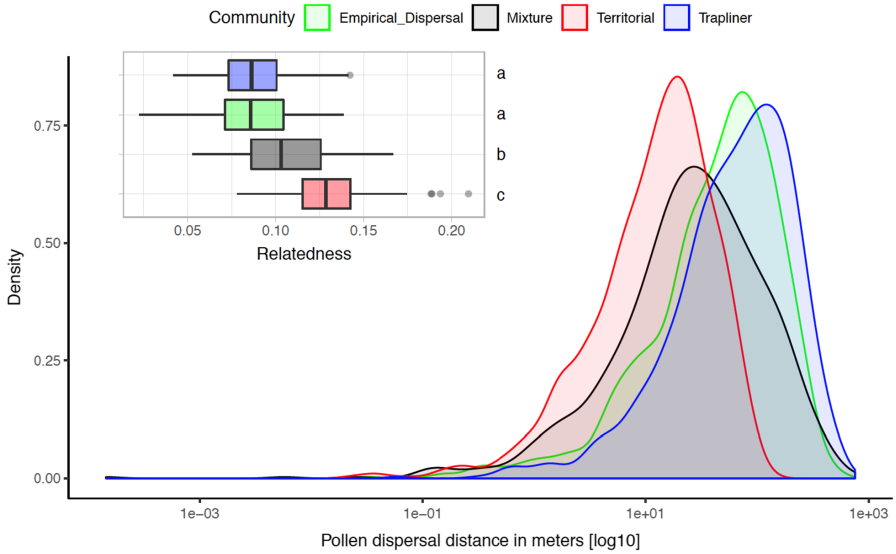

FIGURE 4 Simulated matings between genotyped adult *Heliconia tortuosa* plants under four different pollinator scenarios ("trapliner only," "territorial hummingbirds only," "mixture," "empirical primary forest"). Curves show the frequency distribution of 1000 simulated pollen dispersal distances under four scenarios. The inset shows boxplots of the corresponding pairwise relatedness between individuals drawn from genotyped adult plants according to the simulated pollen dispersal distances. The "territorial hummingbirds only" scenario has a mean pollen dispersal distance of 20 m, and the "trapliner only" scenario a mean pollen dispersal distance of 100 m. The mixture scenario corresponds to a community with 50% trapliners and 50% territorial hummingbirds. The empirical scenario is based on field-based measurement of artificial pollen (i.e., fluorescent dye) in a primary forest dominated by trapliners (mean pollen dispersal distance = 78.9 m). Letters in the inset indicate significant differences at $\alpha = 0.05$ (Wilcoxon ranked sign tests, Bonferroni-adjusted) [Colour figure can be viewed at wileyonlinelibrary.com]

surrounding a patch ($z = -3.429$, $p < .001$, Figure 3b). Specifically, an increase of secondary forest cover from 0% to 30% surrounding a patch increased the proportion of territorial birds up to nine-fold, to the point where large trapliners were virtually absent from patches embedded in landscapes with a large amount of secondary forest (Figure 3b). Finally, we found a negative relationship between the inbreeding coefficient of *H. tortuosa* and the proportion of traplining birds relative to all hummingbirds observed in a forest patch ($F = 7.5$, $p = .012$, Figure 3c). The effect of traplining birds on the propensity of inbreeding did not differ between adult and juvenile *H. tortuosa* ($F = 2.23$, $p = .15$).

3.5 | Consequences of pollinator functional shifts on plant inbreeding

Our simulations predicted increased mating among related individual plants for communities dominated by territorial birds vs.

traplining birds (Figure 4, Wilcoxcon signed rank test: $p < .001$) and in those with a mixture of these pollinators (Wilcoxcon signed rank test: $p < .001$). Importantly, the simulations based on empirically inferred pollen dispersal distances from a trapliner-dominated primary forest patch mirrored the relatedness results based on the trapliner-only scenario (Figure 4, Wilcoxcon signed rank test: $p = .800$).

4 | DISCUSSION

Pollinator decline is a worldwide phenomenon with largely unknown future effects on natural populations across intact and degraded landscapes (Herrera, 2019; Potts et al., 2010). In bee-pollinated systems, the loss of specialist long-tongued pollinators has been associated with local extinction of plant species across England that depend upon these pollinators (Biesmeijer et al., 2006) and the evolution of shorter corolla lengths as generalist short-tongued pollinators dominate the community (Miller-Struttmann et al., 2015). In our study system, generalist and specialist hummingbirds differ in beak and tongue lengths. We show that the local loss or reduction of long-beaked specialist pollinators leads to altered mating patterns and increased inbreeding in *Heliconia tortuosa* juveniles and adults. Unlike the above studies, however, we still do not know if the loss of specialist pollinators has resulted in phenotypic differentiation of *H. tortuosa* floral structures or if increased inbreeding or loss of pollinator services will lead to population decline. We note that both generalist and specialist hummingbird pollinators are present in the metacommunity in our study region, but they differ in their occupancy and movement through primary and secondary forest habitats. Our results suggest that secondary forest regeneration may provide suitable habitat for some species (e.g., *H. tortuosa*) but less so for mutualists of those species (e.g., traplining hummingbirds) with a resultant effect on the genetic diversity of plants in those habitats.

Fragmentation of primary forest and regeneration of secondary forests have complex effects on the species, functional and genetic diversity of interacting animal and plant populations. Many plants rely on pollinators to move their genes across the landscape and these pollinators have the potential to maintain plant population genetic diversity. By combining historical data on forest disturbance history, genotypic data, bird surveys and simulations, our results show that combinations of fragmentation and regeneration can lead to the reduction and loss of species and functional diversity of pollinators, altering mating patterns within and among plant populations, and ultimately eroding within-individual and population genetic diversity in isolated and regenerating forest patches. Our empirical and simulation results indicate populations of *H. tortuosa* that have higher inbreeding are associated with pollinator communities that have lower abundances of traplining birds. While this is true in secondary forest, we also note that the landscape context of a population, in particular the amount of primary forest habitat surrounding a population, has a positive effect on observed heterozygosity, particularly in regenerating secondary forests (Figure 2a). This increase in heterozygosity is probably due increased connectivity across the landscape for traplining birds, which promotes greater pollen-mediated gene flow, increased outcrossing and less within-patch biparental inbreeding.

We demonstrate a negative relationship between inbreeding in *H. tortuosa* and the proportion of traplining birds in the pollinator community sampled at that location (Figure 3c). In a related study that genotyped maternal plants and ungerminated embryos and used these in a TWOGENER analysis, Torres-Vanegas et al. (2021) found that pollen haplotype diversity increased and biparental inbreeding decreased with increases in traplining hummingbirds observed within a patch. Furthermore, traplining birds facilitate significant departures from non-nearest neighbour mating in primary forest *H. tortuosa* populations (Torres-Venegas, unpublished data). Our results are consistent with those of Torres-Vanegas et al. (2021), at least in terms of the effect of hummingbird occupancy, and we show that these hummingbird-mediated effects on the genetic diversity of ungerminated embryos in seeds probably persist in later stages after seed dispersal and recruitment. Furthermore, our analyses of FSGS show that higher levels of kinship in juveniles in secondary forests out to spatial scales of 25 m may reflect more limited pollen dispersal in secondary forests mediated by territorial hummingbirds. Movement capacity and pollinator efficacy are therefore key pollinator functional traits (Breed et al., 2015) that maintain genetic diversity and heterozygosity found in *H. tortuosa* populations across multiple life cycle stages.

We found that the amount of primary forest surrounding a patch was positively correlated with observed heterozygosity and negatively correlated with levels of inbreeding in both primary and secondary forest populations. The amount of primary forest around a focal patch therefore plays a critical role in the maintenance of genetic diversity in *H. tortuosa* particularly in secondary forests. We hypothesize that the importance of primary forest surrounding a patch in increasing observed heterozygosities and reducing inbreeding in all forest populations is due to the habitat preferences of traplining hummingbirds and potential "mass effects" (Leibold et al., 2004) that these forests have in facilitating the movement of traplining birds and unrelated pollen between surrounding populations of *H. tortuosa*. Our data showing higher proportions of traplining hummingbirds in primary forest support this, but to date, we do not have movement data between these habitats and nor do we understand how territorial hummingbirds and traplining birds may competitively interact to influence habitat occupancy. We also do not have direct genetic estimates of pollen movement among patches that enable direct tests of hummingbird functional connectivity in primary vs. secondary forest.

Other ecological and evolutionary processes that differ among different habitats could also play role here. Ultimately, stand-level differences in seedling mortality between different forest habitats or forests surrounded by different amounts of primary and secondary forest must be correlated with a fitness disadvantage (e.g., inbreeding depression) assuming pollinator-mediated genetic differences among populations are only part of the answer. Excess heterozygote deficit in secondary vs. primary forests could be additionally explained if inbreeding depression differed among habitats. For example, if inbred

individuals had lower fitness in primary forest due to different ecological conditions found in these habitats (e.g., lower light, presence of enemies, differences in competitive dynamics) this could contribute to the results presented here. However, while this could explain the effect of forest age on genetic diversity estimates and inbreeding within a forest stand, it would not explain why we found that the amount of primary forest in the surrounding forested landscape within 1 km of a stand would lead to higher observed heterozygosities and lower inbreeding within the stand. Therefore, we suspect that the effect of the amount of primary forest surrounding both primary and secondary forests in increasing observed heterozygosity and decreasing inbreeding is probably due to the effect this has on facilitating traplining pollinator movement across the landscape.

Founding events from a single or few source populations can typically lead to lower genetic diversity in colonizing populations (Sezen et al., 2005; Slatkin, 1977) and the reduced genetic diversity can persist for decades, even in understorey herbs (Vellend, 2004). However, founding events from a limited number of source populations are unlikely to explain the observed genetic differences between primary and secondary forest populations observed in our study. Our results showed that secondary forest populations of *H. tortuosa* exhibit higher allelic richness than those of primary forest, suggesting colonization from multiple populations. Furthermore, this greater allelic richness in secondary forest was consistent across the life stages examined (i.e., juveniles and adults; Figure 1a), potentially leading to greater expected heterozygosity in secondary forest populations than in primary forests.

Despite higher allelic richness in secondary forests (Table 1), there is a trend towards lower observed heterozygosity in secondary forest populations, although these are not different from primary forest populations (Figure 1c). However, observed heterozygosity in each forest stand type does increase in populations with higher amounts of primary forests surrounding them (Table 1, Figure 2a). That we found no to weak relationships between landscape variables and expected heterozygosity and allelic richness and effective number of alleles suggests a decoupling of seed dispersal processes from forest area and landscape configurations–perhaps due to the long-distance movement of habitat generalist seed dispersal agents, primarily clay-coloured thrushes (*Turdus grayi*). We presume that all previously deforested landscapes were initially colonized by seeds. Contrary to pollinators, the abundance and diversity of the community of seed dispersers of *H. tortuosa* was little affected by deforestation or landscape configurations. Seed dispersal simulations in other studies have shown that most of the seeds stayed within 30 m of the maternal plant (63.30%) or landed in the same forest patch (16.10%), 5.21% dispersed to a different forest patch, and 15.4% were lost to the non-forested landscape matrix (Arias-Medellín, et al. unpublished data).

Movement of seeds from multiple populations, particularly into regenerating secondary forest populations, would be expected to increase allelic diversity of founding populations under a propagule pool model of colonization, where founding individuals come from multiple populations, in contrast to an island model of colonization where founding seeds come from a single population or parent (Hamrick & Nason, 1996; Whitlock & McCauley, 1990). However, observed heterozygote deficits could also be caused by a Wahlund effect (Wahlund, 1928) due to intrapopulation genetic structure within secondary forest and/or isolated habitats that could be caused by founding events due to seed dispersal from genetically differentiated populations. However, we note that we see no evidence of stronger spatial differentiation in reproductive *H. tortuosa* adults in secondary forests relative to primary forests in our FSGS analyses (Figure S2) as might be expected if strong subpopulation structure were giving rise to heterozygote deficits in each of our secondary forest habitats.

Pollinators can play a role in counteracting the effect of founding events and drift in small populations through pollen-mediated gene flow. For example, in a study of founder effects in secondary forests for the palm *Iriatea deltoidea*, Sezen et al. (2005) found that pollen movement from nearby adults in primary forests increased genetic diversity of the offspring of founding individuals in secondary forest within a generation. However, *Iriatea* is pollinated by a diverse assemblage of native and introduced bees (Sezen et al., 2007). Generalist pollinators have been shown through fractional paternity analyses in the tropical tree *Miconia* to increase pollen movement and connectivity (Castilla et al., 2017), buffering the effects of forest loss and fragmentation on genetic diversity.

Our results suggest that the reduction of specialist pollinators in secondary forests and the forest structure and composition in the surrounding landscape presumably affect hummingbird movement and occupancy, which can lead to increased mating among relatives and decreased pollen-mediated gene flow. Biparental inbreeding leads to reduced heterozygosity and greater levels of fine-scale genetic structure in subsequent generations in secondary forests. Higher mating among related individuals is supported by our finding that neighbouring juveniles within a patch were 2-fold more closely related in secondary forests at small distance classes (0–25 m) compared to adults, while primary forest adults and juveniles show almost identical levels of relatedness in the smallest (0–25 m) distance classes (Figure S2). Assuming seed dispersal processes do not differ between habitats, higher relatedness in the smallest distance classes could be attributed to differences in mating among relatives and/or differences in selective factors acting on juveniles in these different forests. Alternatively, more widespread seed and pollen movement in primary forests could explain the similar levels of genetic structure seen in the smallest distance classes, while increased near-neighbourhood mating and reduced movement of seeds could explain the observed fine-scale genetic structure differences in adults and juveniles in secondary forests.

Secondary tropical forests, as they undergo succession, often have lower plant species richness than primary forest communities for up to 100 years after initial regeneration (Chazdon et al., 2009) and the regeneration of old-growth species in those patches may take centuries to fully recover (Rozendaal et al., 2019). This could also be true for genetic diversity in some secondary forest populations of plants (Vellend, 2004), but few longitudinal studies have been conducted on genetic diversity dynamics. Our results imply

that secondary forests provide habitat for *H. tortuosa* and populations in those forests have higher allelic genetic diversity probably because of widespread seed dispersal during initial colonization of open habitats. However, within-individual genetic diversity, expressed in terms of observed vs. expected heterozygosity, is lower in secondary forest populations than in remnant primary forest. Observed heterozygosity is also lower and inbreeding higher in all forest types that are less well connected to primary forest, regardless of the size of the stand. Over time, inbreeding depression could result in loss of allelic diversity in populations with inbred individuals. Alternatively, as forests mature, traplining birds and the pollination services they provide may eventually return to these forests and rescue these populations through pollen-mediated gene flow. Future work could monitor genotyped individuals across life stages to determine if inbreeding depression exists in *H. tortuosa* and the stages at which it is strongest. Seeds or seedlings originating in different forest habitats could also be reciprocally transplanted to look for fitness differences associated with different forest types and levels of landscape isolation. Furthermore, an opportunity exists to examine the effect of pollinator loss on phenotypic selection on floral traits and mating system evolution of *H. tortuosa*.

Here, we demonstrate that pollination networks can be altered in regenerating forests due to differences in the species and functional diversity of pollinator communities relative to those in old-growth forests (Poorter, Rozendaal, et al., 2021). Our results, and the results of Torres-Vanegas et al. (2021), indicate that the pollination services of traplining pollinators are critical for the maintenance of within-individual genetic diversity in *H. tortuosa* across multiple life stages, from seeds to reproductive adults. Higher inbreeding, if coupled with inbreeding depression or the increased effect of drift in isolated populations, could lead to the loss of genetic diversity in the species and erode the adaptive potential of populations. Primary tropical forests continue to be lost at an alarming rate (Betts et al., 2017; Hansen et al., 2013). Given that future increases in forest cover can only occur through secondary forest regeneration, we show that secondary forests can differ from old forest in their ability to maintain species, functional and genetic diversity, emphasizing the critical role of primary forests in conservation and management of biological diversity.

AUTHOR CONTRIBUTIONS

F.A.J., A.S.H., M.G.B. and U.G.K. designed the study; A.S.H., M.G.B. and U.G.K. carried out field collections; K.B. genotyped the plants; F.A.J. and U.G.K. analysed the data; A.S.H., U.G.K., W.D.R. and R.A.Z. contributed data and support; F.A.J. wrote the manuscript. All authors contributed to the final version of the manuscript and approved it for publication.

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

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DATA AVAILABILITY STATEMENT

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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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