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RESEARCH ARTICLE

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Genetic threats to the Forest Giants of the Amazon: Habitat degradation effects on the socio-economically important Brazil nut tree (*Bertholletia excelsa*)

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Societal Impact Statement

The Brazil nut is a highly valuable non-timber forest product from a wild, hyperdominant, emergent tree species that is increasingly vulnerable and exposed to habitat degradation. We provide evidence for how Brazil nut genetic resources are negatively affected by forest degradation and discuss the consequences of this for reproductive success. To avoid negative effects of genetic erosion and inbreeding, we discuss the need to cease large-scale forest conversion and to promote landscape connectivity. This could support gene flow, maintain genetic diversity across individuals reproducing in clustered patterns and contribute to securing the long-termed reproductive viability and resilience of this high socio-economically and ecologically valuable species.

Summary

- Ecosystem degradation in the Amazon drives this biodiverse rainforest toward an ecological tipping point. Sustainable management and restoration of degraded rainforest therein are central to counteract this crisis. One hyperdominant, keystone species of high ecological and socio-economic value, the Brazil nut tree, offers additional benefits as a major carbon sink and a nutritional source of the most prominent globally traded non-timber forest product.
- Despite Brazil nut trees being protected by conservation regulation, forest degradation threatens sufficient gene-flow among Brazil nut tree populations. This has impacts on the reproductive success, genetic diversity, and consequently on the resilience of this species to environmental change.
- We used 13 microsatellite loci to explore the consequences of forest degradation on the reduction in genetic diversity of Brazil nut populations. We examined the clustering of genetically related individuals as fine-scale genetic structure (FSGS) and the variation in genetic diversity and inbreeding across adult trees and seedlings along a categorized forest-degradation gradient ranging from conserved to

degraded areas. In addition, we applied direct and indirect approaches to estimate contemporary pollen-mediated gene flow.

- We found significant levels of FSGS, comparable to other similar tropical tree species. Brazil nut seedlings had consistently lower genetic diversity and higher inbreeding than adults, significantly associated with the degree of forest degradation of their origin. We observed limited pollen dispersal, differential patterns in pollen heterogeneity, and disproportionate paternal-assignment rates from few individuals shaping the effective population size in our dataset. We discuss how this evidence for reproduction vulnerability may affect the genetic resources and undermine the resilience of this ecological and socio-economic system in Peru.

KEYWORDS

Brazil nut, fine-scale genetic structure, genetic diversity, Habitat degradation, inbreeding, non-timber forest product, pollen gene flow, sustainable forest management

1 | INTRODUCTION

The Amazon basin accounts for the world's largest rainforest, is home to globally important biodiversity regions and provisions multiple planetary services, from climate change mitigation to supporting millions of rural livelihoods. However, there is growing concern regarding an ecological tipping point, as this forest becomes destabilized due to rapid deforestation, followed by increasingly frequent droughts and fire associated with land conversion to unsustainable agriculture (Lenton et al., 2019). These environmental changes have already started (Lovejoy & Nobre, 2018) and catalyze the anthropogenic change of a large proportion of this region from rainforest toward savannah (Lovejoy & Nobre, 2018; Sampaio et al., 2007). Forest trees are a foundational component of these systems and policies promoting sustainable management strategies to maintain forest cover are critical (Lenton et al., 2019). So-called hyperdominant tropical tree species, which have very high abundance and can disproportionally contribute to ecosystem services and to the livelihood of people in specific tropical forest types (Fauset et al., 2015; Ter Steege et al., 2013; Thomas et al., 2018), are a special priority.

The Brazil nut tree (*Bertholletia excelsa*) is an exceptional example of an Amazonian hyperdominant tree species producing non-timber forest products (NTFPs) while providing socio-ecological benefits. This species can synergistically contribute to nature conservation as a key-stone species, and to sustainable development providing revenues for thousands of families involved in its seed market (Guariguata et al., 2017; Thomas et al., 2018) while supporting biodiversity and acting as major contributor to climate-change mitigation through carbon sequestration (Fauset et al., 2015). Similarly to other NTFPs, it represents an opportunity to help break the link between poverty and environmental degradation, and thus posits a win-win solution for long-term forest stewardship (Escobal & Aldana, 2003; Guariguata et al., 2011; Kusters et al., 2006; Sunderland et al., 2011). Nevertheless, the rainforest landscapes where it occurs are undergoing increasing processes of forest degradation and land conversion, which puts in risk its population viability, fruit productivity, and value-chain sustainability. This is the case of our study highly biodiverse study region, Madre de Dios, in the Peruvian Amazon, where habitat degradation has been intensified during the latest decades in relation to growth in human settlements, the construction of the Interoceanic Highway that crosses eastern part of the region,, and associated increased access to previously remote areas making them prone to degradation (Finer et al., 2017; Caballero Espejo, 2018; Pinasco, 2018; Nicolau et al., 2019 and Figure S1).

Understanding the effects of anthropogenic habitat disturbance as on the reproductive patterns and genetic resources of Brazil nut populations is essential to be able to maintain sustainable production levels. However, critical aspects of the species' reproductive ecology, including mating system, gene flow, and the effects of forest degradation on these processes, remain understudied. For outcrossing species like the Brazil nut, fragmentation of populations could lead to genetic erosion (Zhang et al., 2012) as a consequence of restricted gene flow and elevated inbreeding (Finger et al., 2011; Ismail et al., 2017).

We hypothesized that the Brazil nut tree, due to limited seed dispersal, will exhibit significant fine-scale genetic structure (FSGS), which was documented in this species by Baldoni et al. (2017) and in other long-lived tropical trees with restricted seed dispersal-mediated gene flow (Ismail & Kokko, 2020; Kettle et al., 2011; Morgan et al., 2017; Shao et al., 2018; Smith et al., 2018; Tito de Morais et al., 2015). Consequently, the reproduction of populations in degraded sites may become increasingly limited, leading to reductions in effective population sizes. This in turn can undermine the genetic resource base of populations, leading to erosion of genetic diversity, with negative effects on seedling growth (Nakanishi et al., 2015; Shao et al., 2018) and mortality (Costa E Silva et al., 2011; Nutt et al., 2016; Tito de Morais et al., 2019). For adult trees, this can reduce productivity by compromising flowering (Engelhardt

et al., 2014), lowering fruit set, and increasing fruit abortion (Jones & Comita, 2008). Tree genetic erosion can also negatively affect population recruitment (Ismail et al., 2014a), reduce resistance of individuals and populations against environmental stressors and risk long-term viability and future adaptive potential of tree populations (Rellstab et al., 2016). All together, these processes can have further negative consequences on the Brazil nut market and on the local communities that depend on it.

The overarching aim of this study is to advance our understanding of the role of genetic processes in shaping the resilience of the Brazil nut system in the Peruvian Amazon region of Madre de Dios. Specifically, we quantify the scale of FSGS and the levels of genetic diversity across a forest-degradation gradient. To better understand how landscape context may influence state of Brazil nut genetic resources, we evaluated pollen-mediated gene flow within different sites across this gradient and examined the evidence that fragmentation genetics could undermine the reproductive patterns and genetic diversity of Brazil nut populations. To this end, we asked the following: What is the historical scale of FSGS prior to forest degradation of Brazil nut adult populations in comparison to other long-lived tropical tree species? Are there signs of loss of genetic diversity and of inbreeding in Brazil nut populations undergoing processes of forest degradation? What is the distance and heterogeneity of pollen-mediated gene flow of Brazil nut populations and are these disrupted by forest degradation? For this, we used genotypic data from 13 microsatellite loci from adults as mother trees and potential pollen donors, and from nursery-germinated seedlings in common garden experiments. We discuss the evidence from the results of these questions and the implications of these findings for sustainable management of Brazil nut ecological and socio-economic systems in Madre de Dios and the Amazon more broadly.

2 | METHODS

2.1 | Study species

The Brazil nut is an emergent and one of the tallest Amazonian tree species that can grow up to 60 meters, live for hundreds of years (Ortiz, 2002; Ribeiro et al., 2014; Schöngart et al., 2015; Zuidema, 2003), and it is source to one of the most important globally traded NTFP collected from the wild (Guariguata et al., 2017). Bolivia, Brazil, and Peru are the main producers, together annually generating tens of millions of US dollars (Coslovsky, 2014), particularly and traditionally to North-American and European (Collinson et al., 2000), and recently increasingly to emergent Asian markets (Tridge, 2019). The collection of Brazil nuts does not cause any damage to individual trees as the fallen fruits are collected from the grounds (Zuidema, 2003), and for the combination of its ecological and socio-economic attributes, the forests where it occurs are promoted to be preserved in order to enhance its production (Guariguata et al., 2017).

As an outcrossing species (Cavalcante et al., 2012; O'Malley et al., 1988), the Brazil nut is dependent on wild, solitary carpenter and other large-bodied bee species for pollination, particularly within the genera *Xylocopa*, *Eulaema*, and *Centris* (Cavalcante et al., 2012; Motta Maués, 2002; Wiederkehr-Guerra, 2018), some of whose distribution and activity might depend on the quality of the habitat in their ecosystem (Hadley & Betts, 2012). Seeds are dispersed by agouties at very short distances (*Dasyprocta spp*; Haugaasen et al., 2010; Scoles & Gribel, 2015). These interactions are crucial to the extent of gene flow of this species: Bumble bees can fly over several dozens of kilometers, although pollinating mating distances can range from neighboring to distantly located adult trees. Based on molecular markers, Martins et al. (2018) assigned pollination distances in between 33 and 372m, although parentage was assigned to only less than 20% of their studied progeny, suggesting that most realized gene flow occurred beyond their 25-ha plots. Seed dispersal is restricted to several dozens to few hundreds of meters. In the same study and parentage analyses, Martins et al. (2018) estimated seed dispersal from assigned mother trees to range from 58 to 655 m for significant assignments obtained within their 25ha plots. These results were based on a relaxed 80% threshold for parentage analyses, and contrast with previous results using traditional methods, with much more restricted dispersal rates of rarely more than 25m away from adult trees (Haugaasen et al., 2010).

2.2 | Study sites

This study was established in the highly biodiverse region of Madre de Dios in the Peruvian Amazon. In this region, several human activities, mainly mining, logging, and land conversion to agriculture (Finer et al., 2017; Caballero Espejo, 2018; Pinasco, 2018; Nicolau et al., 2019 and Figure S1), have since contemporary times degraded the landscapes region at wide range. In this region and covering a total of 15.18 km^2, we studied Brazil nut populations by identifying adult trees for collection of DNA samples for genetic analysis from scattered study sites following a categorized gradient of forest degradation. The concept of this gradient is based on the process of complete conversion from pristine, old-growth forests to deforested landscapes converted for agriculture, and includes logged-over and secondary forests as intermediate steps. This concept was illustrated by Van Noordwijk and Sunderland (2014), as shown in Figure S2 to exemplify how our forest-degradation categories fit into that transition.

Thus, the scheme for sample collection consisted of four categories, each with study sites as replicates using a concentric approach at varying distances from regionally intensely populated areas (Figure 1) and based on their forest concession management. The categories are as follows: (a) Conservation (C) for areas with concessions designated to conservation with no logging taking place; (b) logging (L) in non-protected state-owned areas with concessions designated to selective logging (Chavez, 2009; Duchelle, 2009), both of these in remote zones with large distances from main urban areas; (c) partially degraded forest (F), where forest is protected but has previously been logged; and (d) degraded areas (D), where activities have led to complete forest

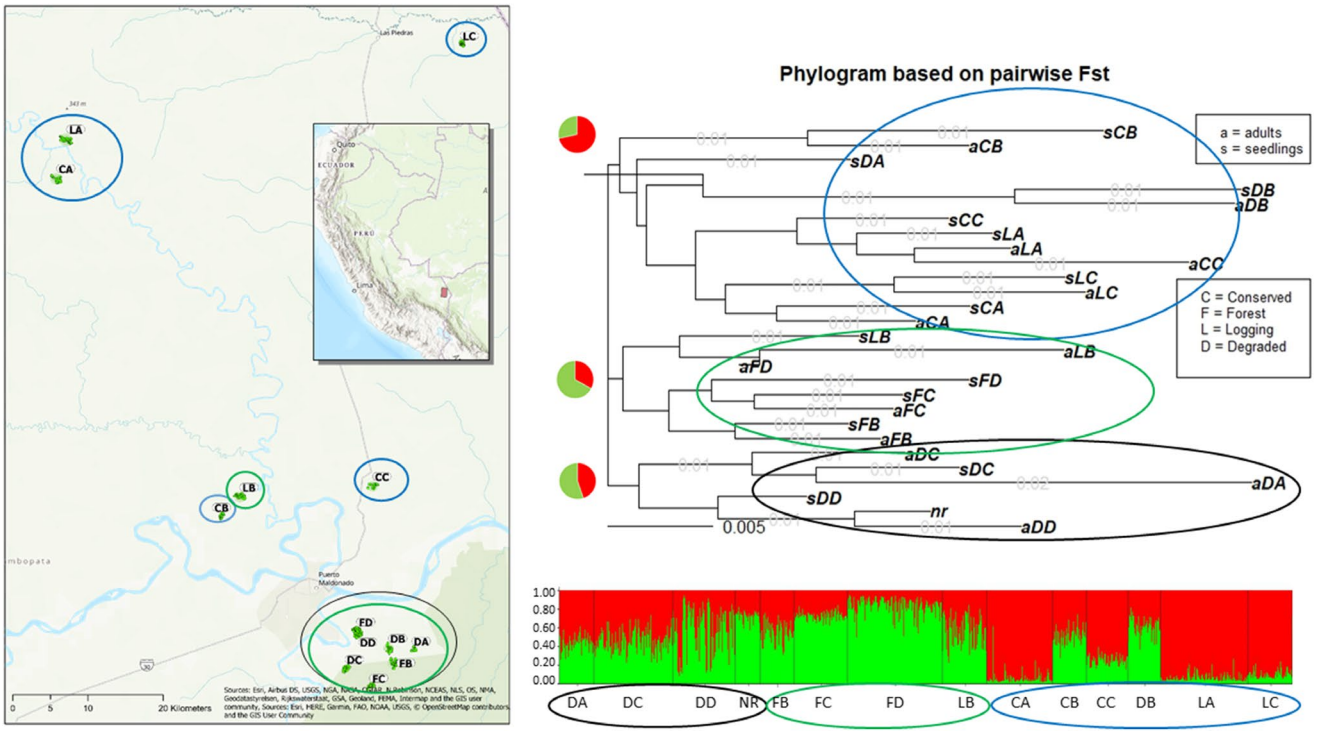

FIGURE 1 Left: Study region with labels of each study site from which seeds and genetic material were collected. Right: Fst-based phylogram of samples and study sites together with corresponding overall allele frequencies and population structure. The labels for phylogram groups denote in order: sample type (adult or seedling), forest-degradation category (C, F, L, or D), and replicate (A, B, or C)

removal and land conversion. The first three categories had three study-site replicates each, whereas the 4th category had 4 (Table 1). F and D were, respectively, located inside and at the buffering zone of the East part of the Tambopata Nature Reserve.

The forest-structure difference in these categories was controlled using data collected from three of the categories (C, F, and D), while due to logistical limitations L was not possible to be controlled in the same way. For this approach, concentric transects were placed for 45 randomly selected adult trees across the analyzed study sites. A transect for each cardinal direction with 5 m × 50 m was placed for each adult tree, giving a total of 1,000 m^2 per analyzed tree, and thus 45,000 m^2 of analyzed forest. In each transect, trees bigger than 100 cm in DBH were monitored and recorded for stem density (number of tree stems per total transect area), stand basal area SBA (cumulative stem area), and above-ground biomass AGB estimated in weight (kg) based on Selaya et al. (2017) and volumes (m^3). This data set was collected collaboratively with Jansen et al., under review and Capurso (2018), which were developed in parallel to the present study and altogether shared same study sites.

2.3 | Sampling design and collection of genetic material

Seeds were collected between February and April 2016, which is the usual Brazil nut harvesting season in Madre de Dios. In each of the described study sites from the first four forest-degradation categories (C, L, F, D), we randomly chose 10 fruit-producing adult trees (called "mother trees" in the analyses) with a circumference around breast height greater than 2 m (DBH > 0.64 cm) and which had no obvious signs of injuries or lianas infestations. From each of these trees, we collected all seeds from five random fruits under each tree's crown. Permission for this was granted by all of the owners of the concessions. Fruit collection was possible for the great majority of trees except for very few exceptions when less than five fruits were found due to trees already harvested and remaining fruits already dispersed away by mammals. It is worth noting that these fruits can weigh up to 2 kg, and for the height they fall from, they create a depression in the ground, which allowed excluding secondary dispersed fruits. Sampled mother trees were at least 50m apart from the closest conspecific neighbor and the fruits were collected under each tree crown. Seeds from each fruit were stored in independent mesh bags coded with each mother-tree unique identifier. In total, 755 fruits containing 12,593 seeds were collected for germination.

We were not given permission to take cambium DNA samples directly from the tree trunks because local communities are very sensitive to physical damage to trees and fear injury or mortality associated with this sampling approach. As a feasible alternative to climbing each mother tree to collect fresh leaf material, we collected freshly fallen leaves at the tree's base or under its crown. Because Brazil nut trees leaves are rather large (20–30 cm long, roughly) and heavy, it can be expected that they fell vertically from their crowns and hence there is a very high likelihood that they belonged to the

TABLE 1 Number of censed and genotyped individuals across forest degradation and study site categories and their geographical attributes

Forest degradation and use category	Study site	Mother trees	Potential pollen donors mapped (genotyped)	Total adults (genotyped)	Proportion genotyped adults	Circular study-site area (km2)	Circular tree density (adult trees/km2)	Seedlings genotyped	Natural regeneration (genotyped)	Total genotyped samples
Conservation (C)	CA	10	32 (32)	42 (42)	1	1.8	23.32	53	1 (0)	95
	CB	10	26 (0)	36 (10)	0.28	0.54	67.21	39	0 (0)	49
	CC	10	23 (0)	33 (10)	0.3	1.46	22.58	48	2 (0)	58
Forest (F)	FB	9	41 (0)	50 (9)	0.18	1.51	33.17	40	1 (0)	49
	FC	11	23 (22)	34 (33)	0.97	0.88	38.65	39	4 (0)	74
	FD	13	68 (47)	81 (60)	0.74	1.18	68.66	51	0 (0)	111
Logging (L)	LA	10	52 (52)	62 (62)	1	2.22	27.93	59	4 (0)	121
	LB	10	41 (0)	51 (10)	0.2	1.61	31.65	54	8 (0)	64
	LC	10	34 (0)	44 (10)	0.23	0.5	88.77	54	1 (0)	64
Degraded (D)	DA	10	16 (0)	26 (10)	0.38	0.45	58.12	38	1 (0)	48
	DB	7	36 (1)	43 (8)	0.19	1.44	29.93	39	11 (5)	52
	DC	12	41 (31)	53 (43)	0.81	0.9	58.81	68	23 (0)	111
	DD	11	20 (8)	31 (19)	0.61	0.71	43.85	64	44 (26)	109
	Total	**133**	**453 (193)**	**586 (326)**				**646**	**101 (34)**	**1,005**

sampled individual. In the sporadic cases that we found naturally regenerated seedlings or saplings, leaf cuts were taken directly from the sampled individual, to incorporate this data into exploratory analyses at the population level. Finally, to include all potential pollen donors within a 0.5km radius of each sampled mother tree, we carefully identified and sampled all adult trees within that distance from sampled mother trees within all study sites. All sampled individuals were georeferenced with GPS coordinates.

All meshed seed bags containing all seeds from each sampled fruit were randomized, placed in bigger meshed sacks, and submerged in running water for an average period of 2–3 weeks, which is normally done by local technicians for softening the shell and, thus, allow opening it without harming the seeds. Shelling the seeds was carefully done using bench vices and garden scissors, and only visually intact seeds were considered as viable and taken for the next steps. Shelled seeds were treated with a broad-spectrum fungicide solution (Vitavax® 300) for 2 hr, followed by overnight drying and thereafter planted in four germination beds containing river sand sterilized with boiling water. Tracking the codes for fruit origin and following a common-garden scheme, seeds were randomized at the fruit level across the germination beds, and leaf cuts from each of the emerging seedlings were collected and stored after germination. The genetic material (leaf cuts) from all sampled individuals (mother trees, conspecific neighbors, natural regeneration, and germinated seedlings) was stored in silica for drying directly after collection.

2.3.1 | DNA extraction and genotyping

We genotyped a total of 1,005 individuals using 13 loci nSSR based on 19 microsatellite loci with already developed markers by Sujii et al. (2013) and Reis et al., (2009) (12 and 7 loci, respectively) sampled across the four forest-degradation categories and age classes (Table 1). The genotyped individuals presented in this study consisted in 646 experimental seedlings (through this study denominated as "seedlings" to specify nursery-germinated individuals and distinguish them from naturally regenerated individuals), which represented a subsample of the total of around 4,000 germinated seedlings, and 326 adult trees, which included 133 mother trees and 193 potential pollen donors (Table 1). Genotyping efforts from the total collected bank of genetic material from potential pollen donors (conspecific adult neighbors to mother trees) were larger for the Southern part of our study region (Categories F and D, Figure 1), to be able to have a closer examination of gene flow there, as study sites were closer to each other. Genotype data were produced as following:

For DNA amplification of each sample, ~20 mg of silica-dried leaf was inserted in 96-wells plates, with three metal beads in each of them and homogenized by at 30 Hz for 3 min in average. The homogenized samples were then taken directly to DNA extraction using the reagents, workflow, and machinery of the KingFisher™ Flex system (ThermoFisher Scientific). Extractions were stored in 50-uL elution buffer, from which we took aliquots and made 1:5 dilutions to be used for subsequent PCRs. Dilutions were made to avoid reaction inhibitors, which during the first PCR series showed to prevent successful reactions. PCR trials showed that only 15 of the 19 available loci were polymorphic and kept for continued laboratory work. These loci were divided into three pseudo-multiplex groups based on the literature information of their expected fragment sizes, and used the four standard reporter fluorescent dyes 6-FAM, VIC, NED, and PET (Schuelke, 2000), integrated into the PCR reactions. This was made to differentiate overlapping of genotypes across loci within each multiplex group and designed based on grouping suggestions acquired with the Multiplex Manager 1.0 software (Holleley & Geerts, 2009). For this pseudo-multiplex strategy, DNA amplification was done separately for each individual loci across all samples, with a touch-down PCR program with 5-min denaturation at 95°C; followed by 15 cycles of denaturation at 95°C for 0.5 min, annealing starting at 63°C and reduced by 1°C per each of cycle, and extension for 1 min at 72°C. The program continued with 20 similar cycles with a fix annealing temperature at 53°C and terminated for final extensions during 10 min at 72° before cooling down. After this, 1 uL of each PCR product per sample template was pooled together according to the designed multiplex before fragment analysis with a capillary sequencer ABI3730 (Applied Biosystems). All laboratory work was conducted in collaboration with the Genetic Diversity Centre (GDC), ETH Zurich, and genotyping was performed with Geneious 9.1.8 by scoring the amplified fragments using the reference of LIZ 500 size standard. For consistency in the genotyping, binning of peak-called alleles was made using the results from adult trees, to thereafter add the fragments from seedlings to the program and genotype them according to the bins established from adults genotypes. The genotype output revealed a locus that could still be regarded as lacking polymorphism and one with more than 50% missing data. Further loci exclusion due to missing data was not possible due to large variation of missing data depending on sample type and study site for each approach, and, thus, data from all remaining 13 loci were used for analyses.

2.4 | Data analysis

2.4.1 | Forest structure

Differences in stem density, stem basal area SBA, and above ground biomass AGB shown in Figure S2 were tested with mixed-effect linear models using the lmer function in the lme4 R package (Bates et al., 2015). These differences were highly significant for all metrics of forest structure across forest-degradation categories, as seen in Table S1.

2.4.2 | Fine-scale genetic structure

The patterns of historical FSGS within adult samples were explored using matrices of pairwise geographic distances obtained with

GenAlEx 6.5 (Peakall & Smouse, 2012), grouped in discreet distance classes, and autocorrelated with their pairwise relatedness coefficients acquired with SPAGeDi (Hardy & Vekemans, 2002) following Tito de Morais et al., (2015) and Smith et al., (2018). The intensity of FSGS was quantified by using the Sp statistics (Vekemans & Hardy, 2004). We compared our observed patterns of FSGS with published intensity of FSGS using SP from a range of other tropical tree species with similar reproductive traits found in Kettle et al., (2011); Harata et al., (2012); Ismail et al., (2012); Tito de Morais et al., (2015); Smith et al., (2018), and Ismail and Kokko (2020). Microsatellites as the used genetic marker were used as a criterion for selecting these datasets, to ensure compatibility with our comparisons.

2.4.3 | Genetic diversity and inbreeding

Genetic diversity was calculated at the study-site level by observed (Ho) and expected (He) heterozygosity using GenAlEx 6.5 (Peakall & Smouse, 2012) and allelic richness with FSTAT 2.9.4 (Goudet, 2003). At the individual level, genetic diversity was estimated as standardized multilocus heterozygosity (sMLH), which is a metric that controls for potential bias due to missing data at particular loci and is calculated by the ratio between individual and mean population heterozygosity (Coltman et al., 1999; Nutt et al., 2016); Slate et al., 2004; Spiering et al., 2011, and was estimated using the inbreedR package (Stoffel et al., 2019) in R. Inbreeding coefficients F were calculated with GenAlEx 6.5 (Peakall & Smouse, 2012). All results derived from these programs were exported to R for comparative analyses. Genotypic information as well as a general overview and averages of metrics of genetic diversity per microsatellite locus are shown in Table S2.

The effect of age stage (adults and seedlings) and forest-degradation category on He, Ho, F, allelic richness, and sMLH were tested separately using linear mixed-effects models with the lmer function in the lme4 (Bates et al., 2015) R packages, using study sites as the random factor for the mixed effect models and following the formula: *Genetic metric ~ Type +Category + Type:Category + (1 | Study site)*, in which Genetic metric was replaced with each metric for genetic diversity, respectively; Type related to either adults or seedlings; and Category was the forest-degradation category. The models were tested at the population (study-site) level for all genetic-diversity metrics except for sMLH, which is a metric estimated and analyzed at the individual level.

2.5 | Estimates of contemporary pollen-mediated gene flow

To estimate pollen-mediated contemporary gene flow, microsatellite genotypes of adult trees (Mother trees and potential pollen donors) and sampled progeny from known mothers were used to perform a range of different direct and indirect approaches to estimate pollen dispersal and heterogeneity in different landscape contexts. First, separate paternity analyses using Cervus 3 (Kalinowski et al., 2007) using the whole dataset, the data from study sites in the Southern cluster of study sites (categories F and D within region A in Figure 1) within our study region, and for each study site separately. Parentage-assignment models were performed using all 13 loci and allowed a minimum of 7 loci genotyped per individual. Furthermore, to be able to control for pollination distances elucidated by different confidence levels of parentage analyses, we used the results from both the relaxed and strict assignment levels, which were set up at 80% and 95%, respectively. Pollination distances as proxies for pollen-borne gene flow from significant trio assignments (seedling, mother, and candidate father) were retrieved from distance matrices previously obtained for the FSGS analyses. The differences of pollination distances were statistically associated with parentage analysis confidence level (relaxed 80% vs. strict 95%) and dataset approach (whole data set vs. Southern study-site cluster) as explaining variables using linear models. Furthermore, pollen contribution at the study-site level was analyzed as the differences in observed proportion of assignments. This was done using linear models for each confidence level, the effect of forest-degradation category, and proportion of genotyped individuals. As a complementary approach to estimate pollen-mediated gene flow, we also used TwoGener within the POLDISP software package (Robledo-Arnuncio et al., 2007) separately for each study site to indirectly estimate differentiation or heterogeneity of pollen dispersal kernels comparatively across sites and forest-degradation categories using linear models. Normal pollen dispersal, individual tree coordinates, and the density of adult trees from each study site (Table 1) were included as the required parameters for this approach. All graphic visualizations of datasets were performed using the ggplot2 3.2.1 package (Wickham, 2016). This together with all statistical analyses in R were performed with Rstudio (Rstudio Team, 2016).

2.5.1 | Population genetic structure

To evaluate levels of genetic differentiation over the scale of the entire study region, neighbor-joining models of the study sites were constructed in R with the nj (neighbor-joining) function in the ape 5.3 package (Paradis & Schliep, 2019), which is based on the neighbor-joining construction method by Saitou and Nei (1987) and is visualized as phylogram in Figure 1. The scale of population genetic structure in terms of shared ancestry across the whole study region was analyzed separately for adult, seedling, and the complete genotype dataset with STRUCTURE 2.3.4 (Porras-Hurtado et al., 2013), using 10 runs of 250,000 Burn-in steps and 500,000 MCMC replications and comparing across combinations of all models of admixture and prior-origin information. From the results of all these models, the most likely number of populations *K* was estimated with the Evanno method (Evanno et al., 2005) using Structure harvester (Earl & VonHoldt, 2012). Maps and densities of adult trees were constructed with ArcGIS Pro 2.4.0 (ESRI, 2019), using minimum geometry circular areas for each study site and using the geographical information from all mapped adult trees.

3 | RESULTS

3.1 | Fine-scale genetic structure

We observed strong, significant patterns of FSGS as indicated by autocorrellograms of significant pairwise kinship coefficients, particularly within the first 500 m and up to 1000 m pairwise distance in between individuals (Figure 2). This was noticeable for the total range of up to 100 km of pairwise distances of genotyped individuals (Figure S3). The FSGS was estimated to have a Sp value of 0.0055 (standard deviation 0.0015) which is comparable with other tropical tree species (Figure 2).

3.2 | Genetic diversity and inbreeding

Adult trees showed similar levels of He and Ho across all sample sites, ranging from 0.482 to 0.670 for He and 0.446 to 0.658 for Ho, while seedlings and natural regeneration consistently showed lower levels of Ho compared to He, ranging within 0.523 and 0.643 for He and 0.481 to 0.590 for Ho (Figure 3, Table 2 and Figure S4). Additional differentiation of genetic diversity between life stage and forest-degradation category is shown in Figure S5 for allelic richness, which ranged in between 2.190 and 2.560 for adults and 2.180 and 2.430 for seedlings, and sMLH, calculated at the individual level and ranging from 0.284 to 1.847 in adults and 0.142 to 1.705 in seedlings. For these metrics, allelic richness was similar for adults and seedlings regardless of degradation state except degraded forest, while for sMLH adults scored higher than seedlings in degraded forest and conservation forest. Furthermore, seedlings showed also consistently to have higher levels of inbreeding (F) ranging from 0.018 to 0.207 for seedlings and −0.045 to 0.125 for adults, which was also associated with forest-degradation categories, particularly for seedlings in conserved and degraded areas (Figure 4). The statistical results from all these associations are shown in Table 3 and the estimates from the linear models are shown in Table S3.

3.3 | Contemporary pollen-mediated gene flow

We found large variation in pollen-mediated gene flow distances from Cervus paternal analyses, which for the approach including the whole data set had median of 0.33 km (StDev 2.66 km) at the strict and 3.89 km (StDev = 2.82) at the relaxed confidence levels (Figure 5). For the second approach entailing the more extensively genotyped Southern cluster (sites in categories D and F, Figure 1), the median for gene-flow distances was 0.47 km (StDev 3.20 km) at the strict and 4.41 km (StDev 2.80) at the relaxed confidence levels. This was based on distances calculated only for significant trio (seedling, mother, and pollen donor) parental assignments, and accounted for 90 seedlings assigned at the relaxed confidence level in the whole data set, for which 24 were assigned above the strict level. For the approach focused on the Southern cluster, counts of assigned seedlings were 81 and 21 for the relaxed and strict levels, respectively.

Thus, pollen-mediated gene-flow distances varied in between neighboring trees up to several kilometers. The distances were, however, significantly lower for the strict (95%) confidence level compared to the relaxed (80%) one (linear model, $p = .003$). We observed a higher proportion of parentage assignments in logging concessions, with almost 100% assignment rate there, compared to the other categories where assignments were as low as around 25% depending on the forest-degradation category and confidence level (Figure S6). This

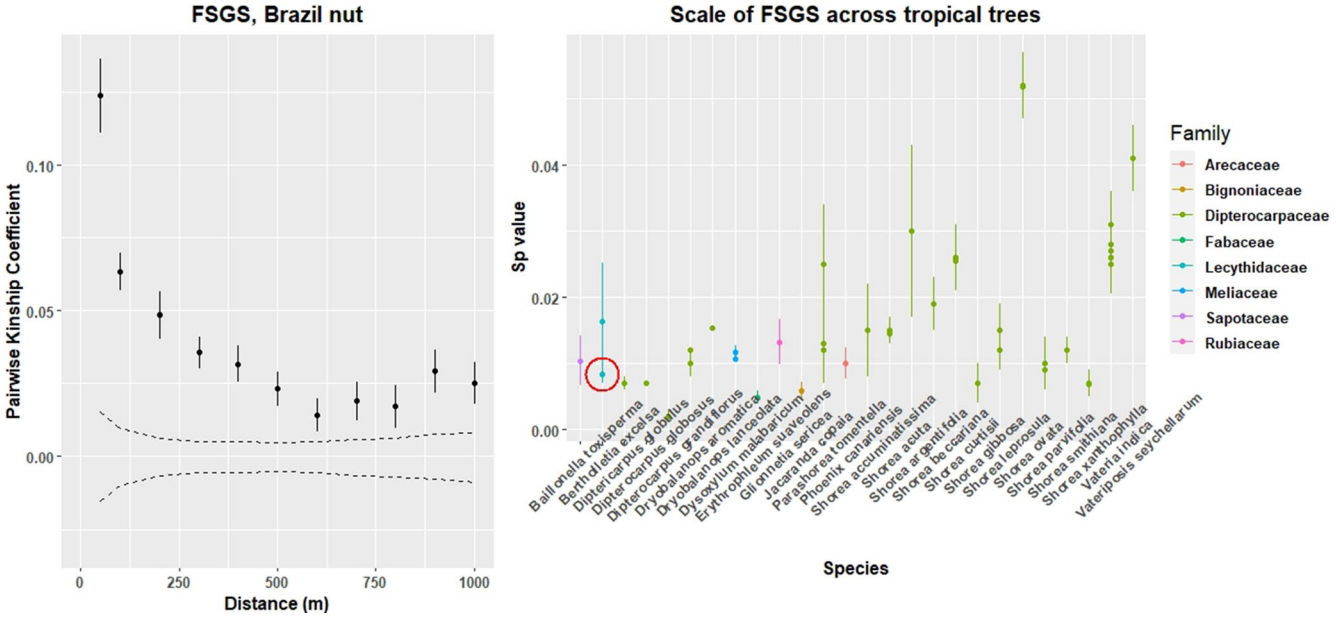

FIGURE 2 Fine-scale genetic structure (FSGS) in Brazil nut trees. Left: Spatial autocorrelations of pairwise distance between individuals and their relatedness as Kinship Coefficients. Right: Sp value marked in red compared to Baldony et al 2017 mmmmand other tropical tree species

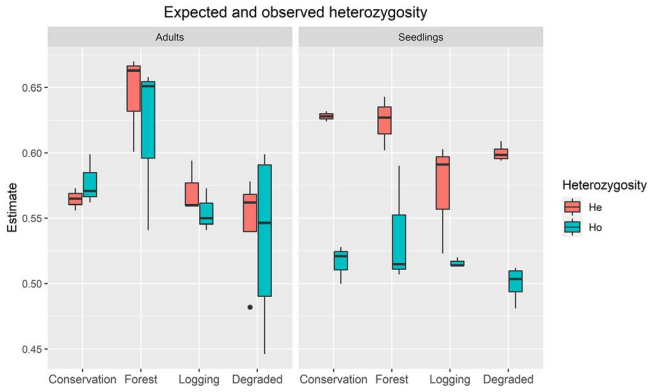

FIGURE 3 Expected and observed heterozygosity for adults and nursery-reared seedlings originated from each forest-degradation category

difference in proportions of parentage assignments across categories was, however, not statistically significant, but it was significantly attributed to the confidence level of parentage analyses (linear model, $p < .001$) and the interaction between confidence level and proportion of genotyped individuals (linear model, $p < .001$). Additionally, we observed that few pollen donors were accounted for the parental assignments and at different frequencies, with few individuals assigned to 2 up to 8 seedlings depending on the applied confidence level (Figure S7). These trio parental assignments were sometimes in between the same adult individuals, sometimes with diverse ones, and in ranges from 83m (neighboring) to several kilometers apart.

Pollen heterogeneity from the TWOGENER approach showed no significant relation between pairwise distance in between mother trees and the Phift estimator of pollen-cloud differentiation, but showed significant differences across forest-degradation categories, with the partially degraded forest as the most heterogeneous category, followed by logging (Figure S8). Finally, generalized linear models showed that effective density of adult trees was significantly associated with pollen heterogeneity, while the density of all mapped adults was not, which potentially indicates a disproportionate low ratio of trees effectively contributing to fertilizing seedlings. The relationship among TWOGENER output parameters are showed in Figure S9.

3.4 | Population structure

All tests performed using all combinations of STRUCTURE's models of ancestry (admixture and no admixture) and allele frequency (correlated and independent) yielded consistently a $k = 2$ as the most likely number of populations after inspecting results with Structure Harvester. These was consistent for runs using and not using sampling locations as prior, and with dataset from all individuals, as well as for adults and seedlings analyzed separately. Fst values varied in between 0.005 to 0.106 for adults and 0.008 to 0.059 in seedlings, with clear differences across study sites and forest-degradation categories (Figure S10). A bar graph showing population assignments of each study site, together with overall allele frequency linked to the phylogenetic clusters in our study region and how they relate to the K = 2 populations from structure is shown in Figure 1.

4 | DISCUSSION

Brazil nut populations in this study demonstrated significant and strong patterns of fine-scale genetic structure (FSGS), particularly for shorter distances than 700–1000 meters. This is in line with our initial hypothesis given the life history and dispersal mechanisms of this species and as hypothesized by Thomas et al. (2018). We observed significantly lower genetic diversity and higher inbreeding for seedlings compared to adult trees, and these differences were associated with the degree of forest degradation, of which differences in forest structure across categories were confirmed to be highly significant. We observed most pronounced differences in genetic diversity in degraded areas and, contrary to our expectation, we observed this also in conserved areas, whereas no significant differences were seen in logging concessions and in previously degraded, protected study sites. Pollen-mediated gene-flow patterns varied across forest-degradation categories, although this was highly dependent on the trio-assignment confidence level used and the proportion of adults genotyped. Direct and indirect gene flow analysis indicated significant divergence between the census and effective population sizes, with relatively few individuals contributing to the majority of fertilization events in our sampled populations. These results are consistent with the idea that landscape connectivity is important for the long-term viability and productivity of the species. Below we discuss the evidence for how vulnerable Brazil nut reproduction is to habitat degradation and the implications of this for future management and sustainable production.

What is the evidence for historical FSGS of Brazil nut populations?

As an obligate outcrossing species, the Brazil nut is dependent on the extent of gene flow in the landscape and how this shapes its reproductive patterns. As we hypothesized, our studied adult populations showed a significant historical FSGS, which was strongest within the 500 to 1000 m of pairwise distance between individuals. Its scale, parameterized by means of Sp statistics, was comparable to other tropical tree species (Figure 2) and overlapped with the results of FSGS in Brazil nuts published by Baldoni et al. (2017). Tropical tree species included in the comparisons included species with similar reproductive traits but also with noticeably higher Sp values. For example, both the highest and almost lowest Sp values were attributed to the Dark Red Meranti *Shorea ovata* and for *Dipterocarpus grandiflorus*, which are both monoecious Dipterocarps from lowland tropical forests (Tito de Morais, 2016) pollinated by bees, similarly to the hawkmoth-pollinated *Glionnetia sericea* from the Seychelles (Finger et al., 2014). In our study, we observed consistent patterns of FSGS despite of the large variation in pollination distances. This has also been documented in other tree species, for example, for *Glionnetia sericea*, of which pollination distances range from some hundreds of meters to more than 12 km (Finger et al., 2014). Hence, FSGS is likely to be mostly due to short distance seed dispersal.

TABLE 2 Means of the different parameters used for genetic diversity and inbreeding, with standard error in parenthesis

		Expected Heterozygosity – He		Observed Heterozygosity – Ho		Allelic Richness		standard Multilocus Heterozygosity – sMLH		Inbreeding coefficient – F	
Forest degradation and use category	Study site	Adults	Seedlings	Adults	Seedlings	Adults	Seedlings	Adults	Seedlings	Adults	Seedlings
Conservation	CA	0.573 (0.045)	0.628 (0.052)	0.562 (0.057)	0.528 (0.067)	2.28 (0.11)	2.31 (0.16)	1.037 (0.04)	0.948 (0.035)	0.005 (0.066)	0.207 (0.1)
	CB	0.556 (0.054)	0.624 (0.041)	0.599 (0.079)	0.521 (0.046)	2.34 (0.169)	2.29 (0.113)	1.09 (0.083)	0.809 (0.052)	−0.012 (0.112)	0.131 (0.076)
	CC	0.565 (0.074)	0.632 (0.052)	0.571 (0.076)	0.50 (0.06)	2.36 (0.194)	2.39 (0.135)	1.042 (0.049)	0.899 (0.051)	−0.022 (0.032)	0.189 (0.069)
Degraded	DA	0.482 (0.081)	0.594 (0.045)	0.446 (0.105)	0.481 (0.051)	2.19 (0.21)	2.39 (0.113)	0.849 (0.069)	1.037 (0.053)	0.102 (0.119)	0.181 (0.059)
	DB	0.559 (0.045)	0.609 (0.042)	0.588 (0.074)	0.512 (0.045)	2.36 (0.124)	2.34 (0.083)	1.006 (0.092)	1.128 (0.049)	−0.045 (0.092)	0.136 (0.059)
	DC	0.565 (0.048)	0.596 (0.048)	0.505 (0.049)	0.509 (0.052)	2.28 (0.13)	2.18 (0.11)	0.928 (0.049)	0.838 (0.035)	0.073 (0.068)	0.129 (0.063)
	DD	0.578 (0.054)	0.601 (0.047)	0.599 (0.067)	0.498 (0.047)	2.37 (0.138)	2.41 (0.133)	0.899 (0.099)	0.963 (0.044)	−0.034 (0.064)	0.162 (0.041)
Forest	FB	0.601 (0.048)	0.627 (0.035)	0.541 (0.07)	0.507 (0.054)	2.46 (0.144)	2.43 (0.119)	0.922 (0.147)	0.963 (0.049)	0.125 (0.083)	0.178 (0.075)
	FC	0.663 (0.035)	0.643 (0.046)	0.658 (0.04)	0.59 (0.048)	2.55 (0.102)	2.42 (0.102)	1.211 (0.038)	1.052 (0.048)	0 (0.04)	0.06 (0.059)
	FD	0.67 (0.038)	0.602 (0.052)	0.651 (0.05)	0.515 (0.056)	2.56 (0.11)	2.40 (0.13)	1.164 (0.031)	0.983 (0.039)	0.017 (0.061)	0.113 (0.074)
Logging	LA	0.594 (0.045)	0.523 (0.064)	0.573 (0.057)	0.52 (0.085)	2.35 (0.113)	2.32 (0.116)	1.051 (0.035)	0.98 (0.037)	0.025 (0.06)	0.018 (0.125)
	LB	0.56 (0.066)	0.603 (0.045)	0.541 (0.077)	0.514 (0.054)	2.36 (0.188)	2.39 (0.127)	0.969 (0.088)	0.942 (0.033)	0.028 (0.078)	0.151 (0.071)
	LC	0.56 (0.062)	0.591 (0.055)	0.55 (0.071)	0.514 (0.06)	2.39 (0.188)	2.34 (0.163)	0.947 (0.091)	0.926 (0.036)	0.044 (0.097)	0.092 (0.096)
Natural regeneration		—	0.57 (0.049)	—	0.523 (0.046)	—	—	—	—	—	0.12 (0.045)

The significant FSGS results found in this study suggest that this species could be vulnerable to elevated inbreeding under reproductive isolation due to the agglomeration of related individuals in close clusters. The degree of FSGS is essential to be taken into account for resilient forest management (Bessega et al., 2016; Tito de Morais et al., 2015), in which landscape connectivity is essential for maintaining genetic admixture in between clusters and maintaining sufficient levels of diversity of the populations' genetic resources. However, to discuss the whole picture it is also essential to monitor changes in genetic diversity, and to understand how the extents of gene flow are affected by the same processes of forest degradation.

Is there evidence for loss of genetic diversity and increased inbreeding in Brazil nut populations undergoing processes of forest degradation?

Seedlings consistently showed substantially lower genetic diversity and higher inbreeding levels than adults. As we hypothesized, these differences appear to be associated with the most severe levels of forest degradation (Figures 3 and 4). Nevertheless, these patterns were also apparent in the conserved areas, which is counter to our initial hypothesis that seedlings would less inbreed in more pristine locations. Partially degraded forest and selective-logging categories showed intermediate levels of inbreeding.

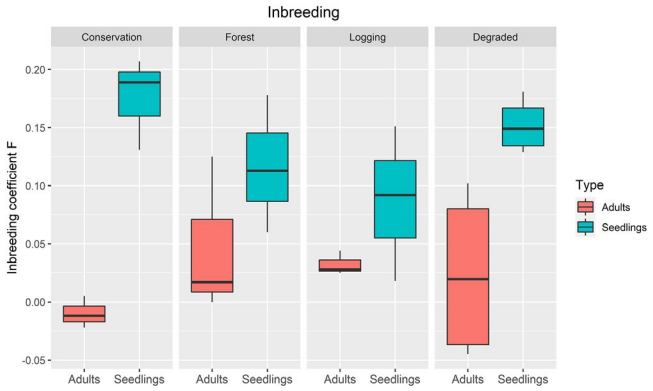

FIGURE 4 Inbreeding coefficients for adults and nursery-reared seedlings originated from each forest-degradation category

The consistent signals of low genetic diversity and high inbreeding were also observed in exploratory observations comparing genotypic data produced by this project with samples taken from 33 naturally regenerated individuals sporadically found in our study areas. As these data points could not follow the same forest-degradation categories of the rest of the study, they were not included in the presented statistical models, but overview purposes their average estimations are also included in Table 2 and Figure S5. This overall consistent low genetic diversity and high inbreeding in progeny compared to adults is likely to be a natural process in our study species, similar to other tree species with strong FSGS (Nakanishi et al., 2015). These patterns further indicate the potential of purging (Wu et al., 2016; Yang et al., 2018) or strong selective forces against inbred seedlings for germination, establishment (Finger et al., 2011), growth, and survival (Nutt et al., 2016; Tito de Morais et al., 2019), and in favor for outbred individuals (Ismail et al., 2014b). In our study, since seedlings were nursery germinated, they were not subject to potential the selective forces that would likely shape their patterns in germination success in natural conditions, favoring less inbred progeny. However, it is noteworthy that a strong bottleneck in the transition of Brazil nut seedlings to saplings was reported by Porcher et al. (2018), which is consistent with high selection in early-age cohorts.

We further acknowledge that our germination-dependent methodology for sampling genetic material can only elucidate patterns of genetic diversity of seeds that successfully germinated, and cannot say how this relates to the complete bank of seeds produced by sampled trees. As inbreeding processes are strong in early life stages (Costa E Silva et al., 2011; Ishida, 2006), among related individuals (Ferriol et al., 2011) and can compromise seed germination (Del Castillo & Trujillo, 2008), we would hypothesize that inbreeding in non-germinated seeds was even more pronounced, as only about half of the about 8,000 seeds we sowed germinated successfully. Further studies, which genotype naturally established Brazil nut individuals would elucidate if less inbred individuals accrue with increasing age classes. We acknowledge further that our approach was restricted to the use of genetic material of only one harvesting season and, thus, neglects the potential temporal variation in

TABLE 3 Significance levels of the linear -model and random effects of life stage (adults and seedlings), forest-degradation category, and the interaction between them on each metric of genetic diversity

		Significance level of the model			
Metric of genetic diversity or inbreeding	**Linear-model analysis level**	**Life stage**	**Forest-degradation category**	**Life stage Category**	**Random-effect variation**
Expected Heterozygosity – He	Population	*	**	.	0.000
Observed Heterozygosity – Ho	Population	**	.		0.025
Allelic richness	Population		**		0.020
Standard Multilocus Heterozygosity – sMLH	Individual	***	***	***	0.056
Inbreeding – F	Population	***		.	0.032

Signif. codes: <0.001 = '***'; 0.001–0.01= '**'; 0.01–0.05= '*'; 0.05–0.1 = '.'.

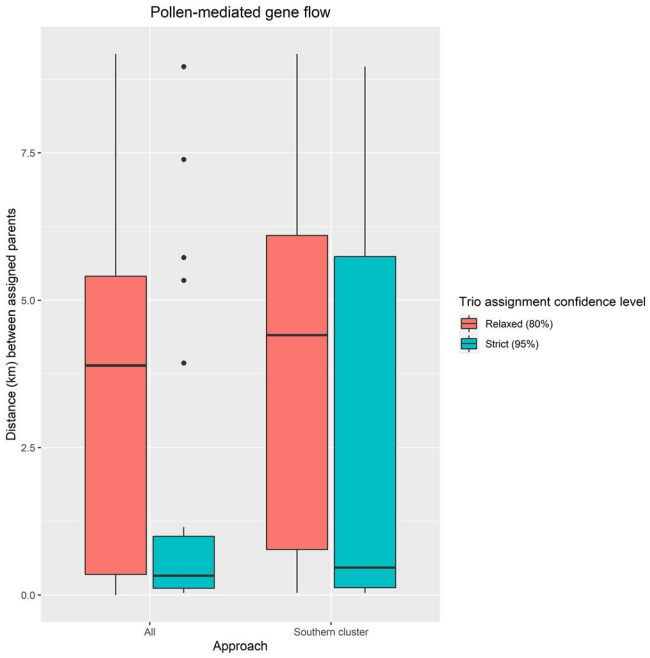

FIGURE 5 Distances of pollen-mediated gene flow between mother trees and significantly assigned pollen donors based on relaxed (80%) and strict (95%) confidence levels of trio parentage analyses. Results from the analyses are shown for all data set (left) and the more intensely genotyped Southern cluster (right)

reproductive patterns (Braga & Collevatti, 2011), which would be interesting to further investigate in this tree species.

4.1 | Is there evidence for changes in pollen-mediated patterns of gene flow?

Pollination distances analyzed in this study as proxies for pollen-mediated gene flow varied for the different analytical approaches and confidence levels acquired with parentage analyses with Cervus (Figure 5). This could mean that there are high chances that pollination occurs at distance scales between trees situated several kilometers apart. However, our approach using the strictest scenario at a confidence level of 95% indicated a median pollination distance below 500m. These results are consistent with the idea that a high proportion of pollen fertilization is likely to happen within relatively short distances, which are lower than the flight distance that carpenter bees have been documented to cover (Tonhasca et al., 2002). These short pollination distances are also consistent with the observed patterns of FSGS over short distances in our populations, and high levels of inbreeding on offspring generally, as well as with lower fruit production of Brazil nut trees that grow at very close distances from their closest neighbors (Thomas et al., 2018). The consequences of short-distance pollen-mediated gene flow under fragmentation lead to the creation of reproductive islands or clusters. However, given the caveat that fragmentation is relatively recent in our study sites compared to the age of the trees, it may be too early to detect significant effects and to detect further population structure and genetic fragmentation in progeny.

Based on the TwoGener approach, the diversity of pollen donors was greater in forest and logging categories (Figure S8), which is consistent with our observations of genetic diversity, in which seedlings in these category had more genetic diversity and were less inbred than those in the other categories. This suggests that these patterns could peak at intermediate disturbances, as they were low in conserved and very degraded, and highest at intermediate categories. High diversity of pollen donors to isolated trees in fragmented landscapes has been previously reported, for example, by Ismail et al., (2012), in which isolated trees received pollen from a larger number of conspecific trees and, thus, from a management perspective, were important as they captured increased proportions of the population's genetic resources. Additionally, we observed a disproportional contribution of few pollen donors to the significant pollen assignments obtained by Cervus (Figure S7), which suggests that a reduced number of individuals were actively reproducing. Additional evidence to this was observed of the multivariate general linear-model analyses conducted with the TwoGener results, in which effective tree density and the interaction between this and forest degradation significantly explained the patterns of pollen heterogeneity in the analyzed populations, independent of forest-degradation category. This once again signals the potential of non-random mating of fewer, effective pollen donors in our study populations and supports further the perspective that landscape connectivity is important for maintaining genetic diversity in this species.

4.2 | Management implications and research suggestions

The significant scale of FSGS and loss of genetic diversity in degraded areas observed in this study are likely to affect the density, productivity and viability of the studied Brazil nut populations (Thomas et al., 2018). We found consistent evidence of inbreeding in seedlings, which signals the potential of strong selective pressures that undermine their survival and development and could be one explanation for previously reported bottlenecks in between early life stages (Porcher et al., 2018). However, areas with selective logging and partially degraded forests, at least at the levels we sampled, can still maintain genetic resources and preserve reproductive viability for Brazil nut populations. This signals the hypothetical potential of intermediate disturbances to be tested for its role in the preservation of genetic diversity and gene flow, which is simultaneously congruent with the idea that sustainable selective logging or protected areas should not be further degraded and completely converted into agricultural lands. We found evidence for short pollination distances leading to effective pollination, which is likely associated with a disproportionately small number of individuals dominating mating, and can lead to a deduction in effective (reproducing) population size. Based on these evidences, we highlight the importance of reproductive landscape connectivity; encourage the continued conservation

of biodiverse areas and support sustainable selective logging as an alternative to land conversion, thus, supporting the viability and productivity of this ecologically and socio-economically important long-lived tree species.

As further studies, we suggest exploring the relationship among genetic diversity, inbreeding, and fitness traits, e.g., seedling establishment and adult-tree productivity, of either natural (Hansson & Westerberg, 2002) or experimental individuals (Rodríguez-Quilón et al., 2015; Vranckx et al., 2014) across a gradient of forest degradation, and thus explore signs of inbreeding depression in studied populations (Grueber et al., 2008). This is crucial to assess the effects of anthropogenic disturbances, conservation status, restoration potential, and future viability of outcrossing tree species (Engelhardt et al., 2014; Graudal et al., 2014) and contributes to the design of management strategies (Armbruster & Reed, 2005; Hedrick & Garcia-Dorado, 2016). Using genome-wide high-throughput genetic markers with high resolution for estimating individual genetic diversity, e.g., SNPs (Fischer et al., 2017; Lemopoulos et al., 2019; Miller et al., 2014; Rellstab et al., 2013), would provide greater power to explore the role of genetic diversity on productivity, resilience, and population viability.

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AUTHOR CONTRIBUTIONS

C-A.F and K.C.J designed the project rationale and directed the project development, decided which methodological scope and analyses to apply, and discussed the interpretation and implications of the results. J. M. established the first sets of study sites and tree selection. C.G.R and B.L.R supplied crucial infrastructure, logistics, and expertise in practice for nursery experiments. G.M. contributed with establishment of the project in the local context and collaboration with the local University. C-A.F performed the sampling and shipping of genetic material, nursery experiments, molecular and laboratory work, genotypic data analysis, and manuscript writing. I.S.A. guided through the performance and interpretation of the different models used for examining reproductive patterns in the data set. J. M., T.E., and I.S.A. contributed to the analysis of the results, manuscript revision, and provided central feedback for suitable statistical analysis and writing adjustments.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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