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The Neotropical tree *Ilex paraguariensis* A. St. Hil. (Aquifoliaceae): pollen and seed dispersal in a fragmented landscape

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Abstract. *Ilex paraguariensis* is a dioecious, Neotropical tree endemic to South America with wide cultural, economic and ecological significance. However, due to extensive fragmentation and deforestation throughout its natural area of occurrence, studies on gene flow and genetic diversity are needed to drive genetic conservation and improvement strategies. Based on the sampling of all adult and juvenile trees in two *I. paraguariensis* populations, we investigate the realized pollen and seed flow and dispersal distance, spatial genetic structure (SGS) and genetic diversity using microsatellite markers. We found limited genetic differentiation between populations ($G'_{st} = 0.033$), but significant differences in terms of genetic diversity (R: 4.7 vs 3.9, H_0: 0.495 vs 0.371, H_e: 0.445 vs 0.375), pollen (10 vs. 23.3%) and seed immigration (0 vs. 3.3%), mating among relatives (t_r: 16 vs 30%) and biparental inbreeding (F_r: 0.253 vs. 0.345). Within populations, the genetic diversity was similar between adults and juveniles, but adults presented significantly lower fixation index than juveniles, suggesting selection against inbred individuals between juvenile and adult life stages. We also found similar mean pollen (255 vs. 293 m) and seed (385 vs. 323 m) dispersal distances within populations. Our results show that the studied populations are not genetically isolated and some mating occurs among related trees due to SGS; however, the frequency of biparental inbred individuals decrease over the life course due to inbreeding depression. These results contribute directly to species management and seed collection and inform *in situ* and *ex situ* conservation programs.

Keywords: conservation genetics, gene flow, mixed ombrophilous forest, microsatellite markers, population genetics

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Introduction

Ilex paraguariensis A. St. Hil. (Aquifoliaceae) is an economically important tree used to prepare the traditional beverage called 'mate', which is consumed throughout subtropical South America (Carvalho 1994). Mate has extensive cultural commercial value, as well as commercial value in the cosmetic and pharmaceutical industries in South America and is one of the most important non-timber forest products in Brazil (Almeida et al. 2009, Burris et al. 2012, Debat et al. 2017). The species is endemic to South America (21º and 30º S, 48º38' and 56º10' W), and in natural conditions can reach 1 m in diameter at breast height (dbh) and 35 m in height (Diaz et al. 2013). The species is a long-living, understory tree found in the *mountainous subtropical* forests in the southern regions of Brazil, Paraguay and Northeastern Argentina, where it can reach densities of hundreds of individuals per hectare (Carpanezzi 1995). It is functionally dioecious, exhibiting diclinous flowers in which one of the sexes is sterile or abortive. Fructification starts in individuals at about two years of age, with dbh > 1 cm (Carvalho 1994). Its flowers are insect pollinated, mainly by the orders of Diptera, Hymenoptera, Coleoptera and Hemiptera, and seed dispersal is mainly by zoochory, particularly birds (Ferreira et al. 1983).

Because of the cultural and economic importance of *I. paraguariensis*, as well as consistent exploitation of the species and the extensive fragmentation and deforestation of its natural area of occurrence, scientific analyses of its ecology and genetics are necessary to guide genetic conservation and improvement strategies (Sturion et al. 2017). Fragmentation of natural forests and human modifications of the landscape have converted once continuous ecosystems into small and isolated forest fragments. In Brazil, the conversion of the Atlantic Forest biome into rural and urban landscapes has reduced the forest into fragments unevenly distributed across its original landcover, and recent assessments suggest that the forest now covers less than 7% of its original area (Ribeiro et al. 2009). Forest fragmentation may have negative effects on species abundance, decrease species population sizes and genetic diversity, while also reproductively isolating populations. It can affect mating systems and pollen and seed immigration and dispersal patterns, not only due to isolation, but also the changing quality of the forest structure in the fragment, modifying pollen and seed dispersal and foraging behavior (Martins et al. 2012, Bezemer et al. 2016, Ismail et al. 2017, Manoel et al. 2017, Spoladore et al. 2017, Moraes et al. 2018, Hardy et al. 2019). These negative impacts may lead to the local extinction of species' populations in fragmented landscapes.

Spatially isolated populations are expected to suffer significant effects of reproductive isolation. This is due to the fact that new generations will originate from mating among the remaining reproductive individuals inside the forest fragment, and if populations are small, new generations will consist of a greater incidence of related individuals (Sebbenn et al. 2011, Duminil et al. 2016, Manoel et al. 2017, Ismail et al. 2017, Monthe et al. 2017, Spoladore et al. 2017, Moraes et al. 2018, Garcia et al. 2019, Potascheff et al. 2019). In the future, we would expect that relatedness would strongly increase, resulting in spatial genetic structure (SGS), mating among related trees and, consequently, inbreeding. At the population level, such effects have specific outcomes due to differences in sexual system, mating system patterns, pollen and seed dispersal vectors, genetic diversity, genetic load, effective population size and level of spatial isolation of remaining populations in fragments. Many studies on tropical trees that are insect and animal pollinated with seeds dispersed by animals, have detected several negative effects of fragmentation, including decreases in genetic diversity and effective population size, changes in mating and pollen dispersal patterns and increases in SGS and inbreeding (Sebbenn et al. 2011, Martins et al. 2012, Tarazi et al. 2013, Lower at al. 2015, Bezemer et al. 2016, Manoel et al. 2017, Tambarussi et al. 2017, Spoladore et al. 2017, Moraes et al. 2018). Gene flow is essential for the maintenance of genetic diversity of natural forest populations. Studies on genetic diversity, mating system and pollen and seed immigration and dispersal distance are important for tropical tree conservation as they help to define strategies for *in situ* and *ex situ* genetic conservation, as well as seed collection for environmental reforestation and tree breeding (Sebbenn 2006). Such studies can be conducted using genetic markers, such as microsatellite loci, which are highly polymorphic in terms of number of alleles per locus (Ashley 2010, Leonarduzzi et al. 2012, Ellstrand 2014). As such, it is an appropriate tool to predict the status of genetic conservation in the long term.

Aiming to inform conservation strategies, we used eight microsatellite loci to characterize the genetic diversity, SGS and pollen and seed flow of juveniles and adults in two natural *I. paraguariensis* populations in the region surrounding the Iguaçu National Park. One population is located in a forest fragment isolated from the park, and the other is located in a fragment that was recently reconnected to the park through the implementation of a biological corridor. The hypotheses of this study were: (i) adult trees will have higher levels of genetic diversity than juveniles in both populations because they retain genetic diversity from the pre-fragmentation stage, (ii) spatial genetic structure (SGS) is found among adults and juveniles of both populations, (iii) pollen-mediated gene flow will be more restricted than seed-mediated gene flow, both from outside and within the forest fragments, (iv) the recently established ecological corridor, although only four years old at the time of analysis, already helps to enhance both pollen-mediated and seed-mediated gene flow, both from outside and within the forest fragment. Other objectives were to compare the genetic diversity among adults and seedlings, population structure and regeneration, SGS, pollen and seed migration rates and the average distances of gene flow via pollen and seeds across the two study populations.

Material and methods

Study area and sampling

The study was carried out in the region surrounding the Iguaçu National Park (PARNA Iguaçu), located in Southwestern Paraná State, Brazil. With an area of 185,262.2 ha (ICMBio 2012), the park contains one of the largest and most well-preserved Sub-tropical Mixed Ombrophilous Forest remnants in Brazil that is also connected to extensive forested areas in Argentina. The climate of the region is subtropical humid with hot summers and average temperatures ranging from 15 to 25 ºC, with 1500–2000 mm of annual rainfall. The present study was carried out in two forest fragments of similar size (~320 ha), separated by a distance of 18.1 km, that have been isolated from PARNA Iguaçu for decades due to forest clearing for agriculture development (Figure 1). The first forest fragment is located at Fazenda dos Paulistas (25º24’19.5”–25º26’04.1” S, 54º10’33.6”–54º11’33.2” W, mean elevation of 259 m). As this *I. paraguariensis* population remains isolated, it is referred to herein as the non-corridor population (NC). The second area is located at Fazenda Santa Maria (25º29’08”–25º30’15” S, 54º21’26”–54º22’26” W, mean elevation of 296 m). Four years prior to sam-

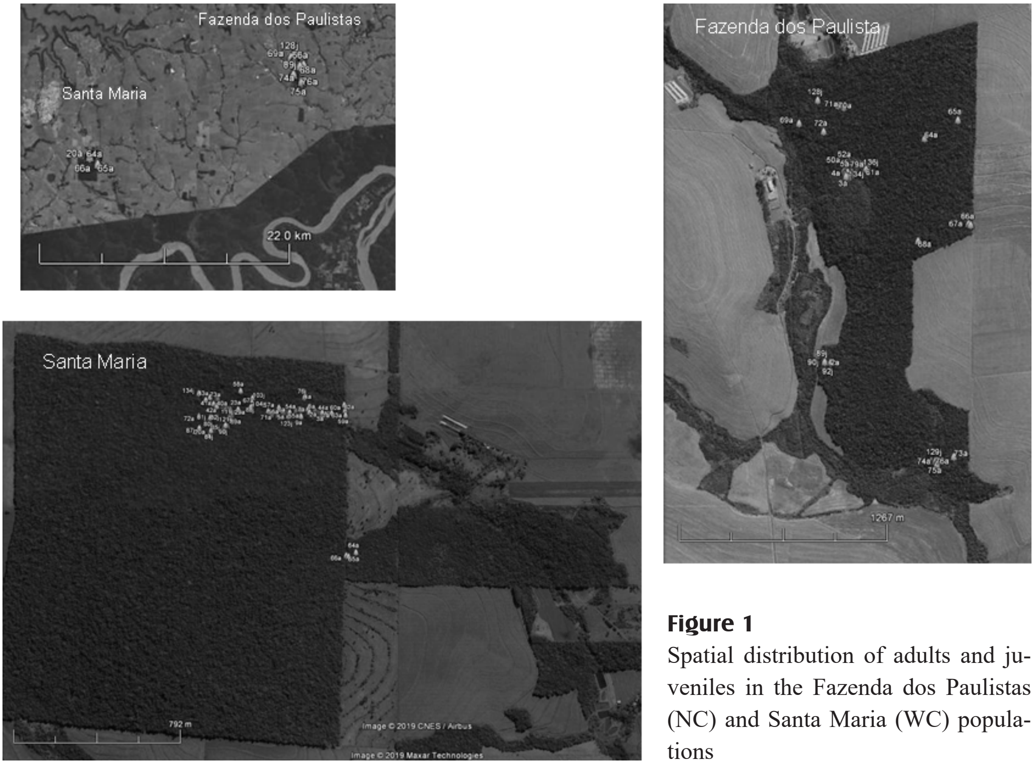

Figure 1
Spatial distribution of adults and juveniles in the Fazenda dos Paulistas (NC) and Santa Maria (WC) populations

pling for this study, the area was reconnected to other natural forests through the implementation of an ecological corridor. One section of the corridor connects the forest fragment to the north with a 60 m wide and 4 km long linear plantation of pioneer native tree species. This section aimed to reconnect the forest fragment to the green belt surrounding the Itaipu reservoir and hydroelectric plant which was established in the 1980s. The other section includes a recovered riparian forest of about 60 m wide and 4 km long, along the rivers that pass through the study forest fragment and flow into the park. This *I. paraguariensis* population is referred to herein as with corridor (WC). The NC and WC populations are located at a distance of 3.9 and 3.7 km, respectively, from PARNA Iguaçu.

We sampled leaves, mapped (GPS Garmin GPWCAP 76S ®) and measured the dbh of all *I. paraguariensis* adults and total height of all juveniles in both forest fragments. In NC, we found 88 adults (0.27 trees/ha) and sampled 50 juveniles. The dbh of adults ranged from 1.1 to 33.3 cm, with a mean of 4.7 ± 4.5 cm (± 1.96SE, where SE is the standard error) and median of 2.8 cm. The height of juveniles ranged from 15–131 cm, with a mean of 97 ± 32 cm and median of 106 cm. The distance among adults ranged from 2–2357 m (mean of 360 m), among juveniles from 1–2414 m (mean of 551 m) and among adults and juveniles from 1–2436 m (mean of 433 m). In WC, we identified and sampled 75 adults (0.23 trees/ha) and 60 juveniles. The dbh of adults ranged from 0.9–41.2 cm, with a mean of 5.8 ± 6.5 cm (± 1.96SE) and median of 3.3 cm, and the height of juveniles ranged from 7–140 cm, with a mean of 83 ± 35 cm and median of 85 cm. The spatial distribution of *I. paraguariensis* in WC was aggregated, with individuals occurring predominantly in soils saturated by the riverbanks and springs of the fragment. The distance among adults ranged from 2–1105 m (mean of 332 m), juveniles from 2–672 m (mean of 229 m) and adults and juveniles from 1–1154 m (mean of 290 m). In dioecious species such as *I. paraguariensis,* it is possible to distinguish the sex based on flower morphology or the presence of seeds in female plants. However, no flowering or fruiting plants were identified in the two populations during the sample collection period from March to September 2011, despite the fact that this period overlaps with the flowering and fruiting range of *I. paraguariensis* reported in the literature for Paraná State (Carvalho 1994).

Analysis of microsatellite markers

Genomic DNA was extracted from the sampled leaves using an adaptation of the protocol described by Doyle and Doyle (1987), including DNA purification with ammonium acetate (Oliveira et al. 2007). Details of microsatellite analyses for the eight loci used (Ppg07, Ipg19, Ipg37, Ipg43, Ipg49a, Ipg49b, Ipg50, and Ipg52) are reported in Diaz et al. (2013).

Analysis of genetic diversity

To verify if there are associations among alleles from different loci, we estimated the genotypic linkage disequilibrium for the total sample (adults + juveniles) of each population and tested the significance of the values using a sequential Bonferroni correction for multiple comparisons. For each population, the genetic diversity for the total sample, adults and juveniles was quantified by the total number of alleles across all loci (*K*), total number of private alleles (P_a), mean allelic richness (*R*), observed heterozygosity (H_0) and expected heterozygosity (H_e). Levels of inbreeding were assessed by calculating the fixation index (*F*); to verify if the *F* values were significantly different from zero, we used Monte Carlo permutations of alleles among individuals as well as a sequential Bonferroni correction for multiple comparisons. The genetic differentiation between populations was estimated for the total sample of each population using the standardized G'_{st} statistic (Hedrick 2005). All analyses were run using the software FSTAT 2.9.3.2 (Goudet 1995). To verify if there are statistical differences between the samples for the indices, Jackknife resampling among loci was used.

Spatial genetic structure

The analysis of spatial genetic structure (SGS) was carried out in each population for the total sample, adults and juveniles using the coancestry coefficient (θ_{xy}) as described in Loiselle et al. (1995) and the SPAGEDi 1.3 software (Hardy & Vekemans 2002). To visualize SGS, the mean θ_{xy} was calculated for pairwise adult and juvenile individuals in eight distance classes for NC (0–15, 15–30, 30–40, 40–60, 60–100, 100–750, 750–1500, and 1500–2357 m), and WC (0–15, 15–40, 40–75, 75–100, 100–300, 300–500, 500–750, and 750–1105 m) and in ten classe for entire sample for each population (0–10, 10–35, 35–75, 75–100, 100–300, 300–500, 500–750, 750–1154, 1154–1750, and 1750–2436 m). To verify if the θ_{xy} values within each distance class were significantly different from zero, the 95% confidence interval was calculated by 1000 Monte Carlo permutations of individuals among different distance classes. To compare the extent of SGS between populations, we estimated the *Sp* statistic, $Sp = -b_k/(1 - \theta_1)$, where θ_1 is the average coancestry coefficient calculated in the first distance class, and b_k is the slope of the logarithm linear regression between coancestry in relation to the spatial distance between individuals (Vekemans & Hardy 2004).

Parentage analysis

Pollen and seed flow and dispersal distance patterns for juveniles of each population were estimated using a simple exclusion method of parentage analysis and the CERVUS 3.0 software (Marshall et al. 1998). CERVUS was also used to estimate the combined non-exclusion probability for the first parent (P_1) and the combined non-exclusion probability of identity (Q_i) for adult samples. Cryptic gene flow (C_{gf}), or the probability of finding a compatible parent candidate within the plot when the true parent is outside the plot, was estimated following Dow & Ashley (1996). The most probable female and male parents of each juvenile were determined using the gene frequency estimates for adult trees of each population. Paternity and maternity assignments were only accepted in cases where there was no mismatch between the paired juvenile and mother or father, and the trio juvenile-mother-father. As identifying the sex of adults was not possible during sampling, all adults were used as putative female and male candidate parents. Pollen immigration was determined by the proportion of juveniles not assigned to a pair of parents within the population and seed immigration was determined by the proportion of juveniles not assigned any parent within the population. The distance between the parent pair within the population was used to estimate the mean, median, minimum and maximum pollen dispersal distances. The distance between juveniles and assigned parents within the populations was used to estimate the minimum and maximum mean, median, and minimum and maximum seed dispersal distances. To assess the association between the frequency and distance of pollen dispersal and between the frequency of assigned juveniles and the distance to the assigned parents, the Spearman's rank correlation coefficient (ρ) was used. To confirm parentage assignments, the pairwise coancestry coefficient between juveniles and first (θ_{JP1}) and second assigned (θ_{JP2}) parents was estimated. The expected coancestry between parents and offspring is 0.25. The rate of mating among related trees (t_r) and the pairwise coancestry coefficient between first and second assigned parents (θ_{P1P2}) was estimated, as described above and using the SPAGEDi 1.3 software (Hardy & Vekemans 2002). Parents were accepted as related if $\theta_{P1P2} > 0.125$, in which case the individual fixation index assigned juveniles as inbred due to mating among relatives (F_r).

Results

Genetic diversity

Significant genotypic disequilibrium was

Table 1 Results of genetic diversity for the total sample (All), adults and juveniles of Fazenda dos Paulistas (NC) and Santa Maria (WC) populations

	n	K	P_a	$R \pm 1.96SE$	$H_0 \pm 1.96SE$	$H_e \pm 1.96SE$	$F \pm 1.96SE$
NC: All	138	33	9	4.7 ± 0.3^{a}	0.495 ± 0.032^{A}	0.445 ± 0.026^{A}	$-0.112 \pm 0.028^{A*}$
WC: All	135	27	3	3.9 ± 0.3^{B}	0.371 ± 0.029^{B}	0.375 ± 0.031^{B}	0.012 ± 0.038^{B}
NC: Adults	88	31	4	4.1 ± 0.2^{a}	0.510 ± 0.034^{a}	0.448 ± 0.026^{a}	$-0.137 \pm 0.033^{a*}$
NC: Juveniles	50	27	0	3.9 ± 0.2^{a}	0.463 ± 0.029^{a}	0.433 ± 0.027^{a}	-0.068 ± 0.022^{b}
WC: Adults	75	25	3	3.5 ± 0.1^{a}	0.394 ± 0.034^{a}	0.371 ± 0.032^{a}	-0.061 ± 0.035^{a}
WC: Juveniles	60	24	2	3.4 ± 0.1^{a}	0.342 ± 0.025^{a}	0.379 ± 0.030^{a}	0.098 ± 0.041^{b}
Total	273	36					

Note. Abbreviations: n - sample size, K - total number of alleles, P_a - number of private alleles, R - allelic richness for 134 individuals for the total sample and 50 individuals for the adult and juvenile samples, H_0 - observed heterozygosity, H_e - expected heterozygosity, F - fixation index, SE - standard error, $*P < 0.05$ after Bonferroni correction. Different capital letters means significant differences across all samples and lowercase letters means significant differences between adults and juveniles of each population at 95% confidence interval, estimated by Jackknife resampling among loci.

detected in adults and juveniles of both populations for pairwise loci Ipg49a and Ipg49b (data not shown). As locus Ipg49a showed a low number of alleles, it was excluded from the analysis to avoid bias in the estimates. For the total sample of adults and juveniles of both populations (*n* = 273), we detected 36 alleles (*K*), ranging between samples from 24 to 33 (Table 1). The allelic richness (*R*), observed heterozygosity (H_0), and expected heterozygosity (H_e) were significantly higher and the fixation index (*F*) significantly lower in the total sample of NC than WC. The *R*, H_0 and H_e values were not significantly different between adults and juveniles of both populations. The value of *F* was significantly lower in adults than juveniles of both populations. Adults of NC presented a significantly lower than zero *F* value, suggesting selection for heterozygous trees. The genetic differentiation (G'_{st}) between populations was low (0.033, 0.027–0.039; mean, 95% confidence interval).

Intrapopulation spatial genetic structure

The spatial genetic structure (SGS) for the combined sample of adults and juveniles was significantly greater than zero up to about 55 m in NC and 22 m in WC. For adults and juveniles of NC, SGS was significantly greater than zero up to 22 m and 35 m, respectively, and in WC up to 57 and 28 m for adults and juveniles, respectively (Figure 2). The regression slope for the coancestry coefficient between pairs of individuals coancestry on the logarithm of spatial distance between individuals (b_k) was significantly lower than zero, and the intensity of SGS (S_p) was significantly higher than zero for the total sample of

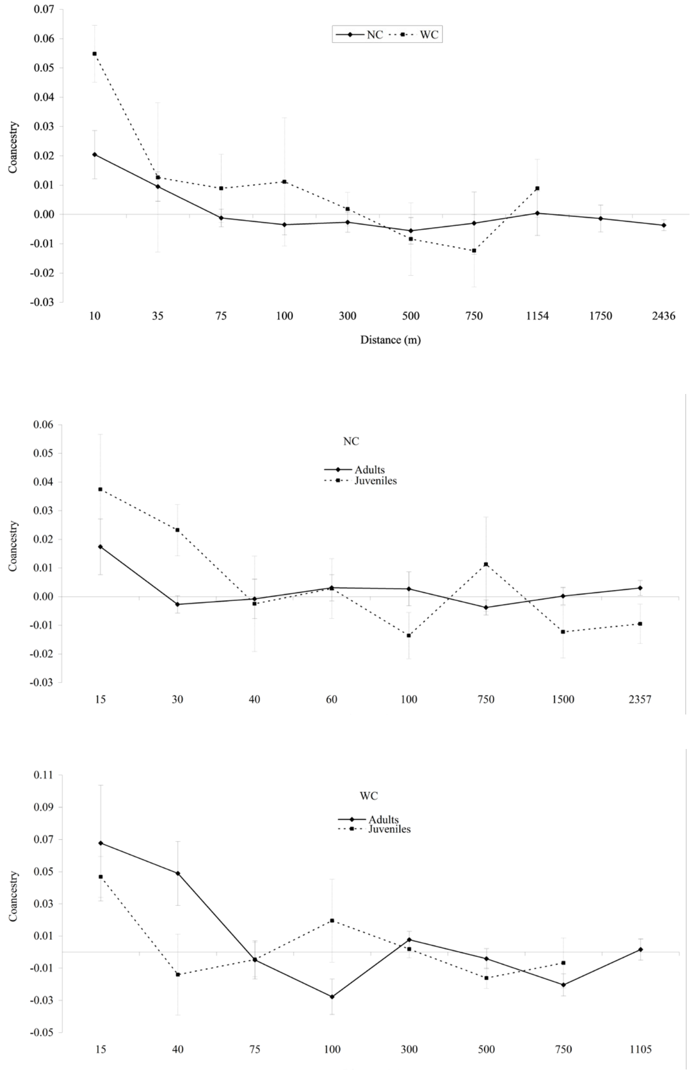

Figure 2 Correlogram of the spatial genetic structure in the Fazenda dos Paulistas (NC) and Santa Maria (WC) populations of the entire sample, adults and juveniles, with the mean and 95% confidence interval of the coancestry coefficien θ_{xy}

Table 2 Spatial genetic structure indices for the total sample (All), adults and juveniles of Fazenda dos Paulistas (NC) and Santa Maria (WC) populations

	n	First distance class (m)	θ_1	$d_{[m]}$	b_k	S_p (95% CI)
NC-All	138	0-10	0.021*	0-2436	-0.0025*	0.003 (0.001-0.004)*
WC-All	135	0-10	0.055**	0-1154	-0.0155*	0.016 (0.008-0.025)*
NC-Adults	88	0-15	0.017*	0-2357	-0.0003	0.001 (-0.001-0.001)
NC-Juveniles	50	0-15	0.038*	0-2414	-0.0060*	0.006 (0.001-0.011)*
WC-Adults	75	0-15	0.067*	0-1154	-0.0255*	0.027 (-0.009-0.064)
WC-Juveniles	60	0-15	0.046*	0-672	-0.0141*	0.015 (0.010-0.020)*

Note. Abbreviations: n - sample size, θ_1 - coancestry in the first distance class, b_k - regression slope of the coancestry on the logarithm of spatial distance between individuals, d - distance where b_k was calculated, Sp - statistic that measures the extent of the spatial genetic structure in the first distance class, 95% CI is the 95% confidence interval, $^{**}P < 0.01$; $^{*}P < 0.05$.

both NC (0.003) and WC (0.016), juveniles of NC (0.006), and juveniles (0.015) of WC (Table 2). However, the S_p statistic was not significantly different between populations nor between adults and juveniles of the same population.

Pollen and seed flow

The combined non-exclusion probability of the first parent (P_1) calculated for adults was low in NC (P_1 = 0.376175) and WC (P_1 = 0.435484). This resulted in a high probability of cryptic pollen and seed gene flow in both populations (1.0), suggesting that the cryptic gene flow may bias our estimates. However, the combined non-exclusion probability of identity (Q_i) was low in both populations (NC = 0.000524, WC = 0.001916, respectively), indicating that adult genotypes are unique, which is highly favorable for parentage analysis. Male and female parents were assigned to 45 and 46 juveniles in NC and WC, respectively, indicating a pollen immigration of 10 and 23.3%, respectively (Table 3). At least one parent was assigned in NC to all 50 juveniles (100%) and to 58 juveniles in WC (96.7%), indicating no seed immigration in NC and 3.3% seed immigration in WC. Pollen dispersal distance in NC ranged from 2–1918 m, with a mean of 255 m, and in WC ranged from 7-938 m, with a mean of 293 m. The seed dispersal distance in NC ranged from 3-1967 m, with a minimum and maximum mean of 222 and 385 m, respectively; in WC it ranged from 9-914 m, with a minimum and maximum mean of 165 and 323 m (Table 3, Figure 3). According to a 95% standard error, the mean pollen and seed dispersal distances were not significantly different within and among populations. However, the mean distances were shorter than the median distances in both populations, indicating a dispersal pattern of isolation by distance (IBD). About 86.7% of pollen in NC and 82.6% in WC were dispersed up to 500 m, while 84.2% of seeds in NC and 86.7% in WC were dispersed up to 500 m. The Spearman's rank correlation coefficient was significantly negative between the frequency and distance of pollen dispersal in NC (ρ = -0.693, P = 0.009) and WC (ρ = -0.601, P = 0.023) and between the frequency of assigned juveniles and the distance to their assigned parents in WC (ρ = -0.845, P > 0.001). In NC, it was marginally (<0.1) significantly negative between the frequency of assigned juveniles and the distance to their assigned parents (ρ = -0.518, P = 0.070), also indicating the IBD pattern of seed and pollen dispersal. The mean pairwise coancestry coefficient between juveniles and the assigned first and second parent (NC: θ_{JP1} = 0.25, θ_{JP2} = 0.26; WC: 0.36 and 0.37, respec-

Table 3 Pollen and seed flow and dispersal distance in juveniles from Fazenda dos Paulistas (NC) and Santa Maria (WC) populations

	NC	WC
Sample size: *n*	50	60
Assigned within for at least one parent (%)	50 (100)	58 (96.7)
Assigned within for two one parent (%)	45 (90)	46 (76.7)
Pollen immigration (%)	5 (10)	14 (23.3)
Seed immigration (%)	0	2 (3.3)
Mean pollen dispersal distance: ±SE (m)	255 ± 122	293 ± 59
Median pollen dispersal distance (m)	81	233
Minimum/maximum pollen dispersal distance (m)	2/1918	7/938
Minimum/maximum mean seed dispersal distance: ±SE (m)	222 ± 133/385 ± 165	165 ± 38/323 ± 52
Minimum/maximum median seed dispersal distance (m)	46/86	105/271
Minimum/maximum seed dispersal distance (m)	3/1967	9/914
Coancestry juvenile-first parent: θ_{JP1} ± SE	0.249 ± 0.030	0.363 ± 0.039
Coancestry juvenile- second parents: θ_{JP2} ± SE	0.254 ± 0.026	0.369 ± 0.035
Coancestry mother-father: θ_{P1P2} ± SE	0.015 ± 0.041	0.120 ± 0.050
Coancestry for related parents: θ_r ± SE	0.231 ± 0.042	0.315 ± 0.040
Mating among related trees: t_r(%)	8 (16)	18 (30)
Fixation index of inbred individuals: F_r ± SE	0.253 ± 0.115	0.345 ± 0.077
Mean distance between relatives: ±SE (m)	168 ± 122	268 ± 90
Minimum/maximum distance (m)	6/478	7/618

Note. Abbreviation: SE is the 95% standard error (1.96SE).

tively) was similar to that expected between offspring and parents (0.25). The coancestry between assigned parents (θ_{P1P2}) was low in NC (0.015) and WC (0.120) and between assigned related parents (θ_r) in NC (0.231) and WC (0.315). Mating among related individuals (t_r) was lower in NC (0.16) than WC (0.315), as was the mean fixation index of inbred juveniles

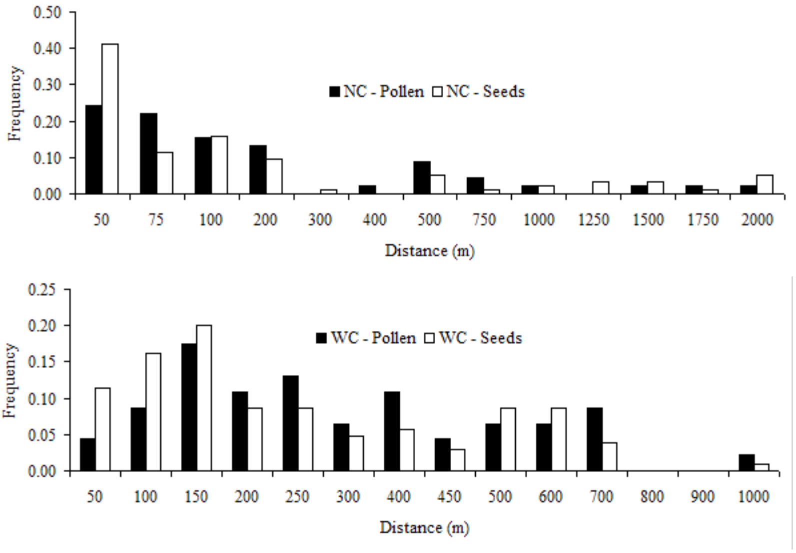

Figure 2 Correlogram of the spatial genetic structure in the Fazenda dos Paulistas (NC) and Santa Maria (WC) populations of the entire sample, adults and juveniles, with the mean and 95% confidence interval of the coancestry coefficient θ_{xy}

(F_r: NC = 0.253, WC = 0.345) and distance between related parents (NC = 168 m, WC = 268 m).

Discussion

Genetic diversity and structure

We assessed genetic diversity, inbreeding, relatedness, pollen and seed immigration and dispersal patterns in two fragmented *I. paraguariensis* populations. Our results show that genetic diversity in terms of number of private alleles, allelic richness (*R*), observed heterozygosity (H_0) and expected heterozygosity (H_e) were higher in the NC population than the WC population. However, in terms of gene frequencies, there is limited genetic differences between the two populations (G'_{st} = 0.033). The low level of differentiation between populations can be explained by the short distance between them (18.1 km), long-distance pollen and seed dispersal of the species and the fact that estimates of genetic differentiation between populations are mainly determined by high frequency alleles. In *I. paraguariensis*, pollen is dispersed by insects and seeds dispersed by birds, both of which have potential for long-distance dispersal, which may homogenize the genetic diversity of proximal populations (Wendt et al. 2009). Due to the short distance between the populations, the frequency of the most frequent alleles is similar in both populations, resulting in limited genetic differentiation between them.

Spatial genetic structure

To understand the spatial pattern of genetic diversity of the two *I. paraguariensis* populations, it is necessary to consider the ecology of the species and local conditions. The species has high plasticity to environmental conditions and their natural range covers various ecological formations, from the tropical rainforest to savanna. However, the most favorable area for the species is the Sub-tropical Mixed Ombrophilous Forest, where it reaches a population density greater than one hundred individuals per hectare (Carpanezzi 1995). Our study was conducted in a region of suboptimal conditions for *I. paraguariensis*, in which populations are small, occur at low densities and are distributed primarily in areas with moisture in the soil. Both populations present aggregated genetic distribution with individuals occurring close together (up to 57 m), suggesting greater probability of neighboring individuals being more closely related than individuals located at greater distances. This pattern of distribution may be related to factors such as fertility or soil humidity and specific microclimate conditions. Thus, the distribution of individuals suggests that there are differential conditions for germination and/or seedling establishment according to soil characteristics. The genetic spatial aggregation of individuals is a common feature for most tropical trees (Hardy et al. 2006; Degen & Sebbenn 2014).

Both populations present SGS at distances ranging from 22 to 57 m, indicating high probability of related individuals occurring within these distances. However, the *Sp* statistic indicates difference between populations (NC: *Sp* = 0.006, WC: *Sp* = 0.016) and the value for WC is similar to those reported based on microsatellite loci (*Sp* = 0.016) for *Virola michellii*, another tropical, dioecious, insect-pollinated tree with seeds dispersed by birds (Hardy et al. 2006). Higher values have been reported for other species that are insect- and animal-pollinated with seeds dispersed by barochory, mammals and wind, or asexual reproduction by root propagation, including: the insect-pollinated and wind seed dispersed *Myracrodruon urundeuva* (*Sp* = 0.024-0.047, Gaino et al. 2010) and *Apidosperma polyneuron* (adults: *Sp* = 0.035-0.071, Chaves et al. 2017); *Swartzia glazioviana* which is insect-pollinated with seeds dispersed by barochory (*Sp* = 0.022-0.071, Spoladore et al. 2017); and *Theobroma cacao* which is insect pollinated, with seeds dispersed by small animals and birds and vegetative propagation (*Sp* = 0.021, Silva et al. 2011). The limited SGS in *I. paraguariensis* can be explained by the significant potential for long-distance pollen and seed dispersal by vector species (bees and birds, respectively). For tropical trees with pollen dispersed by insects and seeds dispersed by birds, such as *I. paraguariensis*, long-distance seed dispersal has been suggested as the main cause of limited SGS (Hardy et al. 2006, Collevatti et al. 2010, Sebbenn et al. 2011, Bauldauf et al. 2014). Regeneration of individuals originated from long-distance seed and pollen dispersal decreases relatedness between near-neighbor trees within populations and consequently decreases the SGS. Through parentage analysis, we found a lower minimum mean seed dispersal distance than pollen, supporting the idea that seed dispersal is the cause of the observed SGS and that both long-distance seed and pollen dispersal can lead to low levels of SGS.

Pollen and seed dispersal

Both study populations are not genetically isolated, particularly the WC population which showed greater pollen (23.3%) and seed (3.3%) immigration than NC (pollen= 10%, seeds= 0%), suggesting than the corridor in WC` was effective to promote gene flow. Although the two study sites are isolated by an anthropogenically influenced matrix composed mainly of agriculture and intensive grazing pasture, the proximity of the study sites to the PARNA Iguaçu suggests some external source of pollen and seed migration, specially in WC, were the area was reconnected to other natural forests through the implementation of an ecological corridor. However, our estimates of pollen and seed immigration may be biased due to the high probability of cryptic gene flow, despite the combined non-exclusion probability of identity (Q_i) showing that adult genotypes are unique within populations and thus highly favorable for parentage analysis. Furthermore, the mean pairwise coancestry coefficient between juveniles and the assigned first (θ_{JP1}) and second (θ_{JP2}) parents in both populations were similar to that expected between offspring and parents and offspring (0.25), suggesting that parentage assignments are likely correct. The differences between the observed and expected values is due to difficulties in estimating pairwise relatedness using genetic markers, which depends on correct estimates of the parental gene frequencies and number of loci used (Moraes et al. 2012, Ackerman et al. 2017).

Although the mean pollen and seed dispersal distance was similar between populations, both were dispersed over greater distances in NC than WC, reaching 1918 m for pollen and 1967 m for seeds. The lower spatial aggregation and greater distances among adults and juveniles in NC (1-2436 m) than WC (1-1154 m) can explain this difference. The distance among NC adults likely forced pollen dispersal vectors to fly longer distances between reproductive trees. However, in both populations the mean pollen and seed dispersal distances were lower than the median, indicating a dispersal pattern of isolation by distance (IBD). This means that the realized seed dispersal resulted in juveniles establishing at greater frequencies near to the mother tree and that mating predominantly occurred between proximal female and male trees. In general, IBD pollen and seed dispersal is a commonly observed pattern in tropical trees (Sant'Anna et al. 2013, Degen and Sebbenn 2014, Moraes et al. 2018, Garcia et al. 2019). The distance between reproductive female and male trees must be the main cause of IBD gene dispersal in the studied populations.

Mating system and inbreeding

Some mating occurred among related trees (t_r: NC= 16%; WC= 30%) and some juveniles present biparental inbreeding (F_r: 0.253–0.345). The mean distance between related parent pairs was lower (NC = 168 m, WC = 268 m) than the mean pollen dispersal distance (NC = 255 m, WC = 293 m), but it was higher than the SGS detected for adults (NC = 22 m, WC = 57 m). This result shows that there are related individuals at distances greater than the SGS and fertilization can occur between spatially distant males and females, thus, mating can occur among related individuals. Furthermore, our results indicate that inbred juveniles remain in the populations, even with substantial levels of inbreeding. This suggests low genetic load, which can have implications for the future, particularly in WC, where the biparental inbreeding was high ($F_r = 0.345$). To date, no studies have assessed inbreeding depression in the species, but our results suggest that inbreeding depression occurs between juvenile and adult life stages. The mean fixation index (F) for adults and juveniles suggests an absence of inbreeding within populations. However, adults present lower F values than juveniles in both populations, indicating selection for heterozygous individuals between the juvenile and adult stage. Similarly, lower levels of inbreeding in *I. paraguariensis* adults than offspring were also reported for a seedling seed orchard (Wendt et al. 2009). Thus, we can expect that the number of inbred individuals will decrease as they move to the adult life stages due to inbreeding depression.

Final considerations

Pollen and seed immigration increase the genetic diversity and effective size of populations and reduce the genetic divergence among them (Hamrick & Trapnell 2011). The limited genetic differentiation between populations found herein is due to pollen and seed immigration in each fragment.

The SGS for adults indicates the distance at which there is greater probability of the occurrence of related trees and this information can be used to determine the minimum distance for seed collection for *ex situ* conservation, tree breeding and environmental conservation (Sebbenn 2006). Our results indicate that seeds must be collected from trees located at least 22 m apart in NC and 57 m in WC, to avoid collecting seeds from related trees, and to ensure the retention of an appropriate effective size in the sample.

Despite the cultural and economic importance of the cultivation of *I. paraguariensis* in Brazil, there are reasons for concern regarding the conservation of the species; it is a species typical of the understory of Sub-tropical Mixed Ombrophilous Forest , a severely threatened forest formation of the Atlantic Forest biome of which few protected areas remain. This can bring about a critical situation for the species as it exposes natural populations to significant potential for genetic erosion. The loss of genetic diversity of natural *I. paraguariensis*s populations and the consequent ecological and genetic impacts may compromise breeding programs in the medium and long term, with serious economic implications for *ervateira* (commercial erva-mate exploitation) activities in Brazil. A loss of genetic diversity can contribute to the risk of extinction of *I. paraguariensis* in natural populations. It is, therefore, necessary to create *in situ* conservation programs, especially in unprotected areas where the species naturally occurs, for example in the studied populations, to help maintain the genetic diversity of the species. Finally, we note that further studies are needed on mating patterns and pollen dispersal based on open-pollinated seeds sampled from natural populations, as well as the effects of inbreeding depression (due to biparental inbreeding resulting from mating between related trees) on growth traits (dbh, height, survival, stem form, etc.). Such information is necessary for species conservation, breeding and the use of seeds collected in natural populations for environmental and commercial reforestation.

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