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Historic pollen and seed dispersal in fragmented populations of the two largest trees of the atlantic forest

Abstract

Cariniana estrellensis and *C. legalis* are two of the largest trees in the Amazon and Atlantic Forest biomes that are currently vulnerable to extinction due to the intense deforestation of these biomes. Strategies for *in* and *ex situ* conservation are urgent and studies of genetic diversity and gene flow are necessary to inform such strategies. Here we investigate the historic pollen and seed flow, dispersal distance and patterns in fragmented populations of both species, using microsatellite markers. All trees found in the populations were mapped, measured for diameter at breast height (DBH), and bark cambium sampled. For both species, high levels of seed (38.5–61.5%) and pollen (80.1–100%) immigration were observed, showing that populations are not genetically isolated. No self–fertilization was detected, but we did find evidence of mating among related trees (8.9–12.5%), suggesting stronger selection against selfed individuals than those originating from mating among relatives. The realized pollen and seed dispersal for both species reached long distances (*C. estrellensis* > 3 km, *C. legalis* up to 385 m), but in general followed a pattern of isolation by distance. The effective size (N_e) in three populations (10–33) was lower than suggested for short term *in situ* genetic conservation ($N_e < 70$). For *in situ* conservation, the results show that the surrounding forest fragments must be preserved to maintain connectivity between the studied populations and other trees of the species and where the N_e was insufficient for short term *in situ* genetic conservation, the N_e must be increased through the introduction of at least 150 individuals.

Keywords: effective population size, gene flow, microsatellite loci, parentage analysis, tropical forests

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Introduction

Cariniana estrellensis (Raddi) Kuntze (jequitibá–branco) and *C. legalis* (Mart.) Kuntze (jequitibá–rosa) belong to the Lecythidaceae family and are the largest South American Neotropical trees in the Amazon, Atlantic, and Savanna forests. *Cariniana estrellensis* can reach up to 45 m in height and 120 cm in diameter at breast height (DBH), and *C. legalis* can reach 60 m in height and 4 m in DBH.[1] Both species are hermaphrodite, mainly bee pollinated, and wind seed dispersed. In general, populations present low density (1<tree/ha), depending on the region of occurrence.[1] Both species are classified as vulnerable to extinction due to extensive exploitation of their wood and the intense deforestation of their biomes over the last several decades.[2,3] Due to their size and long life span, the largest trees in forest biomes provide the environmental conditions required for the survival of many other plant and animal species. Thus, it is necessary to develop *in* and *ex situ* conservation strategies for the remaining natural populations of such species that are currently spatially isolated in small forest fragments across the landscape.

The conservation of trees depends directly on the knowledge obtained through ecological and genetic studies of populations, which requires an understanding of the genetic structure and gene flow of remaining populations.[4–6] Deforestation and forest fragmentation of natural forests causes an environmental imbalance that results in irreversible consequences, such as the extinction of animal and plant species.[7] Without adequate intervention, deforestation and a lack of forest conservation can result in the disappearance of fragments over time.[7] The spatial isolation of tree species populations in small forest fragments reduces the number of reproductive individuals and may cause reproductive isolation by cutting off gene flow through pollen and seeds, which can affect genetic processes, such as genetic drift, mating systems, and natural selection.[8] Such processes can decrease the genetic diversity and effective population size and increase the spatial genetic structure and inbreeding within populations,[9] eventually resulting in local extinction of tree species populations. Loss of alleles, changes in gene frequencies, and a decrease in effective population size are inevitable in tree populations submitted to forest fragmentation and exploitation through selective logging.[4,8,10,11] With fragmentation there is an increase in the fixed genetic load within populations and small and isolated populations often experience reduced fitness.[12] Studies on tropical arboreal species in populations located in fragmented areas show a reduction in the rate of pollen and seed immigration.[13] Genetic problems, coupled with disruptions to the interactions between plant, pollinator, and seed dispersal vectors, and in association with demographic and environmental stochasticity, can further increase the risk of extinction of small isolated populations.[13,14] Furthermore, plant populations in fragmented environments tend to exhibit reduced heterozygosity and adaptability, again increasing the likelihood of extinction.[14]

Understanding gene flow by pollen and seeds is a key aspect in determining the evolutionary potential of tree species, providing support for conservation and management. Gene flow can be defined as the movement of genes among and within populations and it is responsible for the spatial homogenization of genetic diversity within

and between populations. While the effects of natural selection, mutation, and genetic drift lead to differentiation within and among populations, gene flow acts in the opposite direction.[13,15] Estimates of pollen and seed flow are necessary to predict the effects of forest fragmentation on the genetic structure of a tree species. These estimates are also used to define the minimum distance between populations to maintain genetic connectivity[13] for *in situ* conservation, the minimum distance and number of seed trees needed for seed collection for *ex situ* conservation and reforestation for environmental restoration.[16]

Gene flow can be effectively assessed using genetic markers, particularly microsatellite loci (SSRs), because they are abundant and uniformly distributed throughout the genome, have high polymorphism in terms of number of alleles within the loci, are reproducible, require small amounts of DNA and have codominant inheritance, which allows the distinction between homozygous and heterozygous genotypes, and are multi–allelic.[17] These characteristics enable the quantification of genetic diversity and structure, inbreeding, relatedness, intrapopulation spatial genetic structure, mating system, pollen and seed dispersal patterns, among other functions in population genetics.[17–19] Studies of gene flow based on genetic markers can be classified as historical or realized, that occurred in the past, or contemporary or effective, that occur in the present.[20] Such estimates of gene flow in trees can be obtained using a direct method based on parentage analysis of samples of established individuals, as saplings, seedlings and juveniles and all reproductive trees in the populations.[20] Direct estimates of gene flow based on parentage analysis requires samples of regenerants (realized) and/or open–pollinated seeds of known mothers (effective) and all reproductive plants in the population, i.e., the possible parents of the regenerants and seeds. However, between the mating stage and the establishment of seedlings or juveniles (historical or realized pollen dispersal) many stochastic and deterministic processes may occur,[20] such as random mortality, predation, dispersal of seeds at a distance, and selection. The dispersal distances of pollen and seeds can be measured by sampling established regenerants.[8]

The aims of this study were to use microsatellite loci to investigate the inbreeding, effective population size, and historic pollen and seed flow and distance in small fragmented populations of *C. estrellensis* and *C. legalis*. We specifically address the following two questions:

1) What is the rate of historic pollen and seed immigration and the distance and patterns within populations?

Is the effective population size (N_e) sufficient for *in situ* conservation? As the studied species are mainly bee pollinated and wind seed dispersed, both of which have the potential for long distance gene dispersal, we expect to detect both pollen and seed immigration. Further, because the size of the studied populations in general is lower than 100 trees, we expect that N_e is insufficient for *in situ* conservation.

Material and methods

Studied species

Cariniana estrellensis occurs in the Amazon, Atlantic, and Savanna forests of Brazil (from southern Bahia to Rio Grande do Sul and Acre States), Bolivia, Paraguay and Peru, preferentially in moist and deep soils.[1] The species is monoecious with hermaphrodite flowers, which are pollinated by small insects, mainly bees of the genus *Melipona* and *Trigona*.[1] The seeds are winged and dispersed by anemochory, although seed dispersal can be facilitated by monkeys (*Alouatta caraya*) that consume the seeds during the dry season.[21] Reproduction occurs by outcrossing[22,23] and fruiting begins at about ten years of age.[1]

Cariniana legalis is a tree endemic to Brazil, being one of the largest trees found in the Atlantic Forest.[1] The species occurs in the states of Alagoas, Bahia, Espírito Santo, Mato Grosso, Minas Gerais, Paraíba, Paraná, Pernambuco, Rio de Janeiro, and São Paulo.[1] The trees are monoecious with hermaphrodite flowers that are also pollinated by bees of the genus *Melipona* and *Trigona*.[21] Reproduction occurs mainly by outcrossing.[4,24,25] Fruiting starts in trees at approximately 20 years of age and each fruit can contain more than ten seeds that are dispersed by gravity and anemochory.[1]

Study sites and sampling

Samples were collected from two *C. estrellensis* populations located in Ibicatu (IB), São Paulo State, and Bataguassu (BA), Mato Grosso do Sul State, and two *C. legalis* populations in Ibicatu (IB) and Mogi–Guaçu (MG), São Paulo State, Brazil (Figure 1). The Ibicatu State Forest (22°46' S, 47°43' W, altitude 448 to 576 m asl) is a 72 ha, spatially isolated forest fragment located near Piracicaba.[4,24] The forest fragment is a remnant of a semi–deciduous forest that is currently bounded by agriculture, including sugarcane, eucalyptus, and pasture for livestock and about 30 years ago the stand was subjected to several episodes of selective logging.[4] Ibicatu is isolated from other populations or individuals of the species by at least 4 km.[4] The climate is characterized as humid and mesothermal, with a mean annual temperature of 23.9°C and a mean annual precipitation of approximately 1,320 mm.[26] The Bataguassu site covers 448.2 ha, consists of forest fragments and isolated trees (21°38'00" S, 52°14'02" W, 273.3 m asl), and is located in the city of Bataguassu, Mato Grosso do Sul State, near the Pardo River.[23] The region is characterized as a transition zone between the Savanna and Atlantic Forest biomes[27] and is isolated, surrounded by monoculture (sugarcane and eucalyptus) and pasture.[23] The climate is tropical humid with rainy summers and dry winters, a mean temperature of 23.1°C, and mean annual rainfall ranging from 1,200 to 1,500 mm.[27] The Mata da Figueira (MGI) is a small, 7.2 ha, riparian forest fragment in the semi–deciduous plateau and part of the Mogi–Guaçu Ecological Station (22°16' S 47°11' W, mean altitude of 600 m asl).[4] Located approximately 2.9 km from MGI is a cluster of four reproductively mature *C. legalis* trees, denominated MGII.[4] MGI and MGII (MG) are isolated from other populations by at least 4 km and are located approximately 75 km from Ibicatu.[4] The climate is the same as that described for IB. All found *C. estrellensis* and *C. legalis* trees in the IB site, *C. estrellensis* in the BA site and *C. legalis* trees in MG were sampled (foliar or bark cambium), mapped (GPS III–Garmin, USA), genotyped, measured for diameter at breast height (DBH) and estimated for age based on the annual mean DBH increment.[1] The mean annual DBH increment for *C. estrellensis* is 0.61 cm/year and for *C. legalis* is 0.79 cm/year.[1]

DNA extraction and microsatellite amplification

The DNA extraction of *C. estrellensis* and *C. legalis* samples were carried out using the method described by Doyle et al.[28] *Carianiana estrellensis* was genotyped for nine microsatellite loci (Cle01, Cle04, Cle05, Ce07, Cle08, Cle09, Cle10, Cle12, and Ce18)[29,30] and *C. legalis* was genotyped for seven SSR loci (Cle01, Cle04, Cle05, Cle08, Cle09, Cle10, and Cle12).[30] Details on amplification and genotyping

of the selected loci for *C. estrellensis* are given in Guidugli et al.[29] & Tambarussi et al.[30] For *C. legalis*, details are described in Tambarussi et al.[30] All loci used for both species present Mendelian inheritance, an absence of genetic linkage, and genotypic linkage equilibrium.[31,32]

Figure 1 Spatial distribution of *Cariniana estrellensis* and *Cariniana legalis* trees sampled in the three study areas: Ibicatu State Forest and Mogi-Guaçu State Forest, Sao Paulo State; and Bataguassu, Mato Grosso do Sul State, Brazil. Source: Google Earth, 2018.

Genetic diversity analysis

Genetic diversity was quantified at the population level. In order to determine if there are differences based on tree age, the population was divided into two groups, 50% of trees with the lowest DBH and the other 50% with the largest DBH. Genetic diversity indices estimated were: allelic richness (R), and observed (H_e) and expected (H_e) heterozygosity according to Hardy–Weinberg equilibrium. Inbreeding was quantified using the fixation index (F) and the statistical significance of F values were tested using Monte Carlo permutation of alleles between individuals. All estimates were carried out using the FSTAT software.[33] In order to test for differences in the indices between samples, an unpaired t–test was used.

Parentage analysis

Combined non–exclusion probability of the first parent (P_1), combined non–exclusion probability of identity (Q_i), and realized pollen and seed dispersal in both species and all populations were carried out by parentage analysis (maternity and paternity), using the software CERVUS 3.0.[34] Cryptic gene flow (C_{gf}) was estimated as described in Dow et al.[35] Parentage analyses were based on the single exclusion method. In order to identify parents for all sampled trees, all sampled individuals within populations were used as candidate parents. However, to accept a candidate parent as the true parent, we adopted the following approach:

i. Self–assignments were excluded;

ii. Positive assignment for descendants was accepted if there was a maximum of two mismatches between the descendant and the assigned putative parent or among the trio: descendant, first, and second assigned parent;

As *C. estrellensis* starts fruiting at ten years of age, which corresponds to a DBH> 7 cm and *C. legalis* starts fruiting at approximately 20 years of age and a DBH> 20 cm,[1] putative *C. estrellensis* parents with less than ten years difference in relation to the descendant tree, and putative *C. legalis* parents with less than 20 years difference in relation to the descendant tree were discarded. To confirm positive assignments, the θ_{xy} was estimated between descendant and assigned putative parents, as well as between assigned parents and trees assigned to the same putative parents, using the SPAGEDI 1.3 software.[36] The expected S value for parent–sib pairs is 0.25, between half–sibs is 0.125, and full–sibs is 0.25. Realized pollen dispersal distance was estimated as the distance between two putative parents when two parents (probable mother and father) were assigned. In cases where at least one putative parent was assigned, realized seed dispersal distance was estimated assuming that the sole assigned parent is the mother. When two putative parents were assigned, realized seed dispersal distance was based on the mean distance between the offspring and first and second parent. The minimum, maximum, and median pollen and seed dispersal distances were also calculated using the Euclidean distance between two points. Self–fertilization (s) was accepted only in cases where there was no mismatching between tested descendant and the assigned putative parent as ovule and pollen donor. Following,[37] to estimate the rate of mating among related trees (t_r), assigned parents with $\theta_{xy} \geq 0.1$ were assumed to be genetically related.

Estimates of variance effective size

The variance effective population size (N_e) was calculated following Manoel et al.[38] as, $N_e = 0.5 / \Theta$,where is the group coancestry (Θ) for the total sample of trees in each population. Group coancestry (Θ) for the total sample of trees in each population was estimated as:

$$\Theta = \frac{\sum_{i=}^{n} 0.5(1 + F_i) + \sum_{i=1}^{n} \sum_{j \neq i}^{n} \theta_{ij}}{n^2},$$

Where F_i is the sample size, F_i is the inbreeding coefficient of the i–th individual, and θ_{ij} is the pairwise coancestry coefficient between the i–th and j–th individuals.[8] The F_i and θ_{ij} values were estimated using the SPAGEDI 1.3 software.[36] As the F_i index was derived from the inbreeding coefficient (ranging from 0 to 1) and true pairwise coancestry coefficient (ranging from 0 to 1), and we estimated F_i and

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θ_{ij} using coefficients of correlation (ranging from –1 to 1), all values lower than zero were assumed as zero. Furthermore, estimates of inbreeding and coancestry from gene markers may be biased due to the occurrence of null alleles, allele dropout, and genotyping errors.[8] Thus, F_i values lower than 0.125, the minimum inbreeding expected for mating between two half–sib individuals, were also assumed as zero, and values greater than 0.25, the minimum inbreeding expected for mating between two full–sib individuals, were assumed as 0.25. The θ_{ij} values lower than 0.125, the minimum coancestry coefficient expected between two half–sib individuals, were also assumed as zero, and N_e values higher than 0.25, the minimum coancestry coefficient expected between two full–sibs, were assumed as 0.25. The relationship between N_e and the sample size (n) was calculated as N_e / n.

The loss of observed heterozygosity per generation based on N_e and due to genetic drift was estimated using a model that assumes one locus with two alleles in random mating populations with discrete generations (not overlapping), $H_{o(t)} / H_{o(t)} = \left[1 - \left(1 / 2N_e\right)\right]^t$, and the decrease in the observed heterozygosity in generation t as $H_{o(t)} = H_{o(0)}\left[1 - \left(1 / 2N_e\right)\right]^t$, where $H_{o(0)}$ and $H_{o(t)}$ are actual observed heterozygosity and heterozygosity in generation t, respectively.[39]

Results

Population density, DBH and tree age

In Ibicatu, we sampled all 26 *C. estrellensis* trees (0.36 trees/ha) and 65 *C. legalis* trees (0.93 trees/ha). The DBH of *C. estrellensis* ranged from 13–248 cm (mean of 68.5 cm) and the estimated age ranged from 21–400 years, with a mean of 110 years. The DBH of *C. legalis* ranged from 25–325 cm (mean of 121 cm) and the estimated age ranged from 24–570 years, with a mean of 160 years. In BT, 284 *C. estrellensis* trees (0.63 trees/ha) were found, with the DBH ranging from 21–147 cm (mean of 65.6 cm) and the estimated age ranging from 36–241 years (mean of 108 years). MGI contains 22 *C. legalis* trees and MGII contains four trees, for a total of 26 trees (3.6 trees/ha), with DBH ranging from 21–144 cm (mean of 68 cm), and estimated age ranging from 27–144 years, with a mean of 83 years.

Genetic diversity and inbreeding

The observed (H_e) and expected (H_e) heterozygosity were similar between *C. estrellensis* (IB: $H_o = 0.71$; $H_e = 0.66$; BA: $H_o = 0.65$; $H_o = 0.81$) and *C. legalis* (IB: $H_o = 0.81$; $H_e = 0.86$; MG: $H_o = 0.82$; $H_e = 0.89$) populations (Table 1). The results for fixation index (F) were significantly (P< 0.05) higher than zero for *C. estrellensis* of BA ($F = 0.06$) and *C. legalis* of IB ($F = 0.06$), indicating inbreeding. According to the unpaired t–test, comparing 50% of trees with <DBH and >DBH, F values of *C. estrellensis* in BA (0.06) and *C. legalis* in IB (0.09) were significantly greater than zero in trees with < DBH, suggesting the occurrence of inbreeding.

Parentage analysis

For all trees sampled, the combined non–exclusion probability of the first parent (C_{gf}) was higher in *C. estrellensis* (IB= 0.045008; BA= 0.025563) than in *C. legalis* (IB= 0.002699; MG= 0.000383). This resulted in a cryptic gene flow (C_{gf}) greater in *C. estrellensis* (IB= 0.698; BA= 0.999) than *C. legalis* (IB= 0.161; MG= 0.010) populations, indicating a high probability that pollen and seed flow in *C. estrellensis* may be biased. However, the combined non–exclusion probability of identity (Q_i) was lower for both *C. estrellensis* populations (IB= 0.00000002; BA= 0.0000054) than for *C. legalis* (IB= 0.00044367; MG= 0.00030133), indicating that all adults present different genotypes, which is favorable for the assignment of parents in parentage analysis.

Table 1 Genetic diversity and inbreeding in samples of *Cariniana estrellensis* and *Cariniana legalis*

Sample	n	R (SE)	H_o (SE)	H_e (SE)	F (SE)
Cariniana estrellensis	310				
Ibicatu	26	–	0.71 (0.06)	0.66 (0.03)	–0.07 (0.08)
50%< DBH	13	3.9 (0.3)	0.71 (0.07)	0.64 (0.03)	–0.11 (0.10)
50%> DBH	13	4.7 (0.2)	0.70 (0.06)	0.69 (0.02)	–0.01 (0.08)
Bataguassu[1]	284	–	0.65 (0.03)	0.69 (0.02)	0.06 (0.08)*
50%< DBH	142	5.0 (0.7)	0.64 (0.05)	0.68 (0.05)	0.06 (0.04)*
50%> DBH	142	5.0 (0.6)	0.66 (0.06)	0.69 (0.05)	0.05 (0.05)
Cariniana legalis	91				
Ibicatu[2]	65	–	0.81 (0.02)	0.86 (0.01)	0.06 (0.02)*
50%< DBH	32	7.8 (0.5)	0.75 (0.04)	0.83 (0.03)	0.09 (0.07)*
50%> DBH	33	8.6 (0.5)	0.87 (0.03)	0.88 (0.02)	0.01 (0.05)
Mogi–Guaçu[2]	26	–	0.82 (0.07)	0.89 (0.01)	0.08 (0.07)
50%< DBH	12	9.8 (0.7)	0.82 (0.08)	0.92 (0.01)	0.10 (0.08)
50%> DBH	14	9.6 (0.7)	0.85 (0.06)	0.89 (0.02)	0.04 (0.06)

n is the sample size; R is the allelic richness for ten genotypes in *C. estrellensis* and nine in *C. legalis*; H_e is the observed heterozygosity; H_e is the expected heterozygosity; F is the fixation index; SE is the standard error; [1]Kubota et al.[23]; [2]Tambarussi et al.[40]; *P< 0.05.

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At least one putative parent was found for 16 and 123 *C. estrellensis* trees of IB and BA, respectively, and for 25 and 10 *C. legalis* trees of IB and MG, respectively (Table 2). These results suggest a respective seed immigration rate of 38.5 and 56.7% for *C. estrellensis*, and for *C. legalis* of 61.5% for both populations. Two putative parents within the sample areas were detected for five and 28 *C. estrellensis* trees of IB and BA, respectively, and for five *C. legalis* trees of IB, suggesting a rate of pollen flow of 80.8 and 90.1% for *C. estrellensis*, and for *C. legalis* of 92.3 and 100%. No trees were themselves assigned as the parent tree, indicating an absence of realized self–fertilization. In general, mean coancestry coefficient between trees (θ_{xy}) and assigned putative parents was lower than expected for *C. estrellensis* in IB (0.18) and for *C. legalis* in IB (0.22) and MG (0.10). Assuming a minimum coancestry coefficient of 0.1 ($\theta_r \geq 0.1$) to determine parents as related individuals, we found two pairs in IB ($\theta_r = 0.17$) and 11 pairs in BA ($\theta_r = 0.17$) for *C. estrellensis*, and three pairs ($\theta_r = 0.20$) in IB for *C. legalis* to be related parents. Thus, the realized mating among related trees (t_r) for *C. estrellensis* in IB was 0.125 and in BA was 0.089 and in *C. legalis* in IB it was 0.120. The pairwise coancestry coefficient between assigned trees with at least one common parent ranged from –0.01 to 0.15. The fixation index (t_r) for assigned trees with related parents (t_r) ranged from –0.12 to 0.14. The mean distance between related parents for *C. estrellensis* ranged from 398 to 615 m and for *C. legalis* the distance was 312 m.

Table 2 Seed and pollen flow, mean pairwise coancestry, inbreeding and effective population size for *Cariniana estrellensis* and *C. legalis*

	Cariniana estrellensis		***Cariniana legalis***	
	Ibicatu	**Bataguassu**	**Ibicatu**	**Mogi–Guaçu**
Sample size: n	26	284	65	26
Pollen	–	–	–	–
Number of not assigned trees (%)	21 (80.8)	256 (90.1)	60 (92.3)	26 (100)
Number of assigned trees	5	28	5	0
Mean (SD) dispersal distance (m)	338 (88)	850 (888)	312 (80)	–
Maximum distance (m)	465	3309	385	–
Median (m)	339	516	322	–
Seeds				
Number of not assigned trees (%)	10 (38.5)	161 (56.7)	40 (61.5)	16 (61.5)
Number of assigned trees	16	123	25	10
Maximum mean (SD) distance (m)	308 (227)	615(697)	393 (236)	72 (28)
Maximum distance (m)	878	3483	851	131
Maximum median distance (m)	325	340	397	67
Coancestry and effective population size				
Assigned trees and parents: θ_{xy} (SD)	0.18 (0.04)	0.25 (0.00)	0.22 (0.01)	0.10 (0.04)
Mean among related parents: $\theta_r \geq 0.1$ (SD)	0.17 (0.06)	0.29 (0.04)	0.20 (0.03)	–
Rate of mating among related: t_r	0.125	0.089	0.120	–
Fixation index for assigned trees for Θ: Θ (SD)	–0.12 (0.06)	0.08 (0.03)	0.14 (0.07)	–
Group coancestry: Θ	0.03473	0.00236	0.00747	0.01375
Variance effective size: N_e	10	119	33	15
N_e / n	0.37	0.42	0.50	0.57
Decrease rate in the heterozygosity: $H_{o(t=50)}$ (%)	7.7	81.0	46.6	18.4

SD is the standard deviation. $H_{o(t=50)}$ is the decrease rate in the heterozygosity after 50 (t) generations by genetic drift.

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Pollen and seed dispersal distance

The pollen dispersal distance for *C. estrellensis* reached 3309 m, with a mean of 338 and 850 m in IB and BA, respectively (Table 2) (Figure 2). For *C. legalis*, the pollen dispersal distance reached 385 m, with a mean of 312 m in IB. For *C. estrellensis*, the median pollen dispersal distance was lower (IB= 330 m, BA= 516 m) than the mean, indicating a pattern of isolation by distance. However, for *C. legalis* the median (322 m) was larger than the mean, indicating a random pollen dispersal pattern. Seed dispersal distance for *C. estrellensis* reached 3483 m, with a mean maximum of 308 m in IB and 615 m in BA. For *C. legalis* seed dispersal distance reached 851 m, with a mean maximum of 393 m in IB, and in MG it was 72 m. The median seed dispersal distance was lower than the mean for the BA population of *C. estrellensis* and for both *C. legalis* populations, indicating a pattern of isolation by distance.

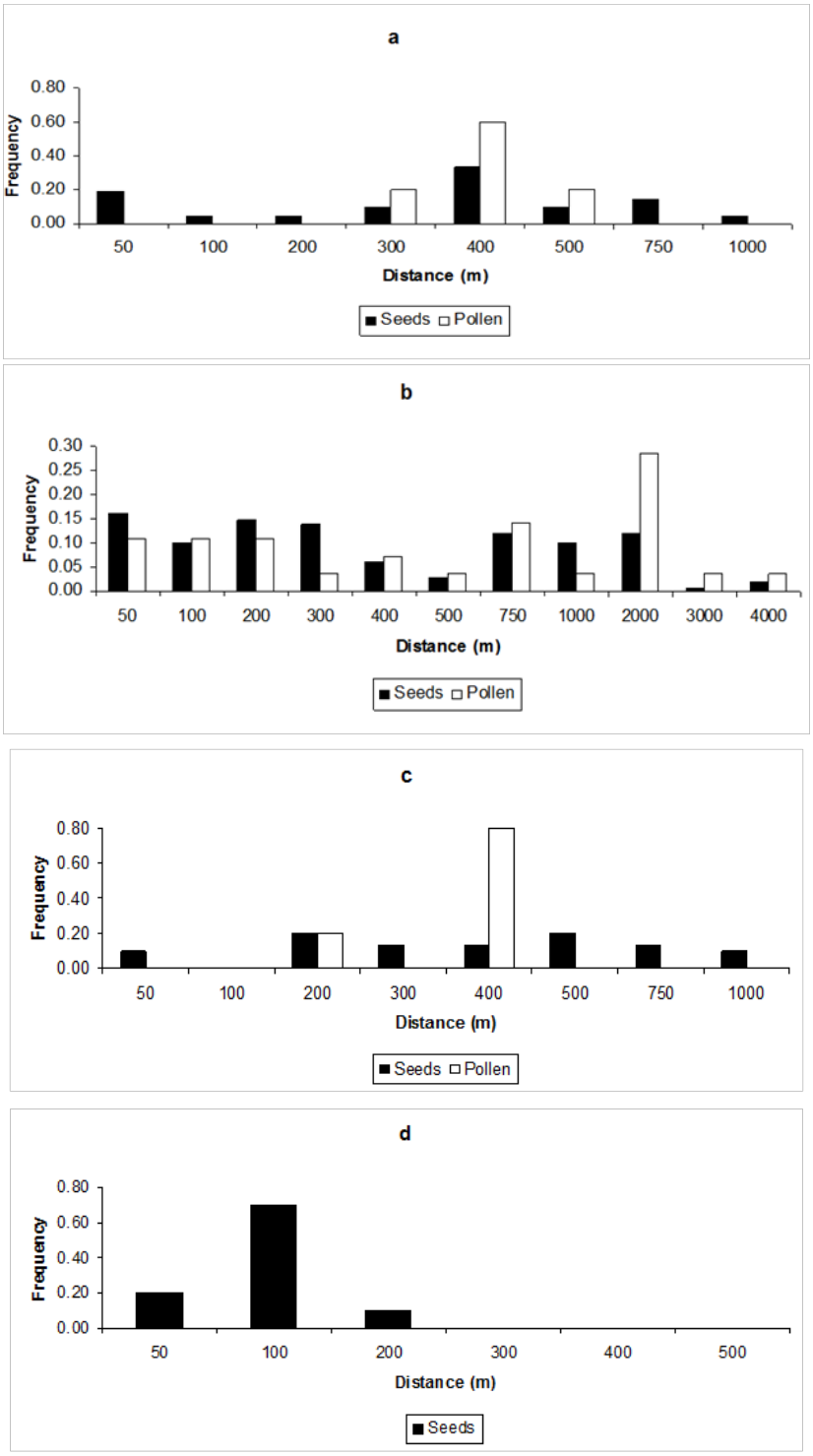

Figure 2 Frequency distribution of seed and pollen dispersal distance in *Cariniana estrellensis* of Ibicatu (a), Bataguassu (b), *Cariniana legalis* of Ibicatu (c) and Mogi-Guaçu (d).

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Effective population size

The group coancestry coefficient (Θ) was low among all pairwise *C. estrellensis* (0.03473–0.00236) and *C. legalis* (0.00747–0.01375) trees, suggesting that in random mating, the expected inbreeding from mating among related trees is very low, <5% (Table 2). The variance effective population size (N_e) shows that the 26 and 284 *C. estrellensis* trees of IB and BA correspond to about 10 ($N_e / n = 0.37$) and 119 ($N_e / n = 0.42$) unrelated and non–inbred individuals. For *C. legalis*, the estimated $N_e / n = 0$ suggests that of the 65 and 26 trees of IB and MG, represent to about 33 ($N_e / n = 0.57$) and 15 ($N_e / n = 0.57$) are unrelated and non–inbred individuals. The estimated loss of heterozygosity after 50 generations ($H_{o(t=50)}$), based on the calculated N_e indicates that the observed heterozygosity for *C. estrellensis* in IB and BA is expected to be only 7.7 and 81.0%, respectively, of the actual. For *C. legalis* in IB and MG, the actual heterozygosity is expected to be 46.6 and 18.4%, respectively, of the actual.

Discussion

Mating system and inbreeding

All trees assigned through parentage analysis of both species were the result of outcrossing, but 8.9 to 12.5% of the mating events occurred between related individuals (t_r). Previous studies based on samples of germinated seedlings (effective mating system) detected some self–fertilization (t_s) and mating among related trees (t_r) in *C. estrellensis* ($s = 4\%, t_r = 13\%$)[23] and *C. legalis* ($s = 5.1\%$, $t_r = 35.7\%$).[37] Considering some levels of self–fertilization previously observed in germinated seeds, the absence of established selfed individuals in our study suggests stronger selection against selfed *C. estrellensis* and *C. legalis* individuals between seedling to adult stages in natural populations. Furthermore, selfing (t_s :2.4–9.9%) and mating among related trees (t_r : 5.9–9.1%) were detected in *C. legalis* juveniles of 17 years of age from established provenance and progeny test,[24] again suggesting stronger selection against selfed *C. legalis* individuals in natural populations than in experimental trials. It is probable that, due to environmental controls in experimental trials, selfed individuals may survive for longer than in natural conditions, where natural selection and stochastic factors, such as random mortality, predation, diseases, among others, may be more predominant.

As no selfing was detected by parentage analysis, the inbreeding found in populations of both species can be attributed to mating between relatives. Both *C. estrellensis* and *C. legalis* are subject to inbreeding depression (ID). Kubota et al.[23] detected stronger ID for survival in *C. estrellensis* at the seedling stage (8 months of age) for individuals produced from self–fertilization (2.6%) than from mating among relatives (0.2%). Tambarussi et al.[37] also found greater ID for survival in *C. legalis* seedlings produced from self–fertilization (3.9–36%) than from mating among relatives (0.2–30.4%). The stronger ID for selfing than mating among related trees is supported by the absence of selfed individuals in our study populations, with inbred individuals originating from mating among relatives surviving up to 91 years of age in *C. estrellensis* and 122 years of age in *C. legalis*. Selfing results in inbreeding of at least 50% ([$100\%(0.5(1 + F_m)$], where F_m is the inbreeding in the mother) and mating among related individuals results in inbreeding equal to the coancestry between related parents ($F_r = \theta_r$); for example, if the parents are full–sibs ($\theta_{fs} = 0.25$), inbreeding (F_r) of 25% is expected in their descendants. Thus, inbreeding originating from mating among related individuals is expected to result in lower homozygosis for identical by descent alleles than from selfing. As deleterious genes that produce ID generally occur at low frequencies, mating among related individuals has a low probability that it will result in homozygosis for such genes and, consequently, some mating among related individuals do not result in ID. The fact that inbreeding at early stages was only detected for *C. estrellensis* in BA ($F = 0.06$) and for *C. legalis* in IB ($F = 0.09$) may be explained by the fact that the genetic load or the occurrence and frequency of deleterious alleles is variable between populations and individuals due to the life histories of mutation in the populations.[37]

Gene flow

The results for both species suggest greater levels of pollen (80.1–100%) than seed immigration into the populations (38.5–61.5%). The coefficient of coancestry expected between parents and sibs is 0.25, or on average 25% of genes of parents and descendants are identical by descent. Mean pairwise coancestry coefficient between trees and assigned putative parents was lower than expected for both *C. estrellensis* (0.18–0.25) and *C. legalis* (0.10–0.22). The differences between the estimated and expected values (0.25) are due to difficulties in estimating kinship using genetic markers.[9] However, when several loci are used simultaneously, the mean value of loci tends to be similar to the expected value. Thus, we can consider that the estimates obtained herein reflect the expected relationship between parents and descendants. This may also explain the lower expected values for *C. legalis* which is likely an artifact of the sampling as the number of loci used (7 loci) was lower than for *C. estrellensis* (9 loci). Nevertheless, the estimates of pairwise coancestry between parents and descendants were greater than zero, supporting the hypothesis that these individuals are indeed related, and the trees assigned to parents are likely correct. The majority of *C. estrellensis* and *C. legalis* trees that were not assigned a mother or father are remnants of the pre–fragmentation era (< 100 years) in the study populations, indicating that parents were logged from the area around or within the forest fragments, died before sampling, or the trees originated from seed immigration from populations located outside of the sample areas.

Pollen dispersal distance

Realized pollen dispersal for *C. estrellensis* reached longer distance (3309 m) than for *C. legalis* (IB= 385 m) and mean pollen dispersal distance for *C. estrellensis* was greater in BA (850 m) than in IB (338 m) and that detected for *C. legalis* in IB (312 m). However, it is important to note that these results are likely underestimates due to pollen immigration or mortality of pollen parents. In both populations of *C. estrellensis*, pollen was dispersed in a pattern of isolation by distance (IBD), with a higher frequency of pollination occurring between trees in closer proximity than more distant trees. However, in IB for *C. legalis* the pattern was random, which may be due to the underestimation of pollen dispersal distance due to pollen immigration, death or logging of the pollen parents. The distances and realized patterns of pollen dispersal for both species can be attributed to many factors, such as population and individual variation in flowering phenology, self–incompatible systems, inbreeding depression, and particularly sample size design. Studies carried out in small areas will result in lower mean and maximum dispersal distances than in larger areas. This can explain the longer pollen dispersal distance detected for *C. estrellensis* in BA, where the study area was larger (448.2 ha)

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than in IB (72 ha) for both species. Furthermore, as the sampling in this study did not include seeds, the results for both species represent the realized pollen dispersal and not the effective pollen dispersal, which occurs at fertilization. Based on samples of open–pollinated seeds of *C. estrellensis* from BA, effective pollen dispersal reached up to 3519 m (mean of 597 m) and also followed an IBD pattern.[23] For samples of open–pollinated seeds of *C. legalis*, pollen reached up to 922 m in IB (mean of 352 m) and followed a pattern of IBD.[4] We can see that the effective and realized maximum, mean, and pattern of pollen dispersal do not differ widely. The minimal differences can be due to many factors that can occur between fertilization and the current life cycle of the sampled trees, such as inbreeding depression, random mortality, disease, predation, and logging, which can change the effective pattern of pollen dispersal in relation to that, detected herein, the realized pollen dispersal pattern.

Ghazoul et al.[41] notes that fragmented populations can be sustained if pollinators are able to travel long distances. The results of this study for *C. estrellensis* and *C. legalis*, which are mainly pollinated by bees that have the potential for long distance pollen dispersal,[41] confirm this expectation. Our results suggest that, currently, there are favorable conditions for maintaining the genetic diversity of the studied populations, thus indicating their utility as sites for *in situ* conservation of these vulnerable species.

Seed dispersal distance

Seed dispersal in *C. estrellensis* also reached long distances in the BA population (3483 m). The explanation for this result is the same as that discussed for pollen; the ability to measure gene dispersal depends on the sample design and as the sample area of BA was larger than the other studied populations of both species, we were able to detect longer seed dispersal distances. This also explains why mean seed dispersal distance for *C. estrellensis* was longer in BA (452–615 m) than in IB (308 m) and for *C. legalis* it was greater in IB (338–393 m) than MG (72 m). However, the seed dispersal pattern was isolation by distance.

The maximum seed dispersal was greater than pollen dispersal in both populations of *C. estrellensis* and in IB for *C. legalis*. This can be explained by the fact that for both species, seeds are dispersed by wind and pollen dispersed mainly by bees, both of which have the potential for long distance dispersal. Furthermore, aggregation of regenerants near to the parent trees can result in high mortality rates among juveniles due to predation, thus leading to lower levels of aggregation at advanced life stages,[42] which may result in longer average seed dispersal distances.

Final considerations

Cariniana estrellensis and *C. legalis* are Neotropical trees that are vulnerable to extinction and the results reported herein on pollen and seed flow and dispersal are key in developing strategies aimed at their *in* and *ex situ* conservation. For *in situ* conservation, the results show that the studied populations of both species are not reproductively and genetically isolated due to pollen flow from outside the study areas. However, it is important to maintain connectivity of the studied populations with other reproductive populations that were not included in this study to ensure continuation of gene flow. Thus, other forest fragments and trees of the species surrounding the forest fragments must be preserved.

The variance effective population size (N_e) was lower than census size (n) in all populations. This is related to the occurrence of SGS and inbreeding in the populations.[4,23] With the exception of the *C. estrellensis* population in BA ($N_e = 119$), the other populations showed a N_e (*C. estrellensis*: IB= 10; *C. legalis*: IB= 33, MG= 15) that is lower than that suggested ($N_e = 70$) for short term genetic conservation to avoid the effects of genetic drift, such as the occurrence of inbreeding.[43] If the N_e is low, genetic drift in the populations will quickly decrease heterozygosity, resulting in a decrease in population fitness.[43] The loss of heterozygosity after 50 generations ($H_{o(t=50)}$) based on actual N_e was higher in populations with low N_e (7.7–46.6%) than with larger N_e (*C. estrellensis*: BA= 81.0%). However, as both species present overlapping generations, the loss in heterozygosity is expected to be greater than that observed using this model due to the occurrence of mating between related trees. To avoid inbreeding depression due to genetic drift in small populations, for *in situ* conservation the N_e must be increased to at least 70. The N_e can be increased for *C. estrellensis* in IB and *C. legalis* in IB and MG through the introduction of about 60, 40, and 55 non–inbred and unrelated individuals, respectively. The introduced individuals must originate from other populations of the species within the same region to avoid further genetic problems, including out–breeding depression.[16] For practical reasons, following the recommendation,[11] we suggest the introduction at least 150 individuals to mitigate against the possible mortality of the introduced trees due to random mortality, predation, disease, and other factors.

Conclusion

Our results indicate that in natural populations of both species, inbreeding depression is stronger for selfed trees than for those originated from mating among related individuals. Inbred individuals originating from mating among relatives can survive up to 91 years of age in *C. estrellensis* and 122 years of age in *C. legalis*. The inbreeding at early stages detected for *C. estrellensis* in BA and for *C. legalis* in IB indicates that the genetic load or the occurrence and frequency of deleterious alleles are variable between populations and individuals. The spatial isolation of populations of both species did not result in reproductive isolation due to pollen and seed immigration from outside the study area. Estimates of pollen and seed dispersal distance are dependent on sample design, with a greater ability to estimate long distances in large rather than small sample areas. The realized dispersal distance of pollen and seeds for both species reached long distances, but in general followed a pattern of isolation by distance. For *in situ* conservation, the results show that it is important to maintain connectivity of the studied populations with other trees of the species surrounding the forest fragments, especially populations or individuals located at distances lower than 3.5 km, the maximum dispersal distance of pollen and seeds that we detected gene immigration. For *C. estrellensis* in IB and *C. legalis* in IB and MG populations, where the effective population size (N_e) is insufficient for short term genetic conservation, the N_e must be increased for *in situ* conservation, through the introducing of at least 150 individuals originating from other populations of the species within the same region, but located in distances higher than 3.5 km to avoid introduce related individuals, which can decrease the effective size of introduced sample.

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Conflict of interest

Authors declare there is no conflict of interest.

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