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Study of flow and contemporary pollen dispersal, mating system, spatial distribution of genotypes and inbreeding depression in fragmented population *Cariniana estrellensis* (Raddi) Kuntze, using microsatellite loci

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Tese apresentada à Faculdade de Engenharia do Campus de Ilha Solteira – UNESP como parte dos requisitos para obtenção do título de Doutor em Agronomia. Especialidade: Sistema de Produção.

Prof. Dr. ALEXANDRE MAGNO SEBBENN
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TÍTULO DA TESE: Estudo do fluxo e dispersão contemporânea de pólen, sistema de cruzamento, distribuição espacial de genótipos e depressão por endogamia em população fragmentada de Cariniana estrellensis (Raddi) Kuntze, utilizando locos microssatélites

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RESUMO

Cariniana estrellensis (Raddi) Kuntze (Lecythidaceae), popularmente conhecida como jequitibá-branco, é uma espécie arbórea tropical típica de estágios sucessionais avançados, característica de florestas clímax. Apesar da sua importância ecológica, a espécie encontra-se ameaçada de extinção, principalmente devido à intensa exploração e degradação de seu ambiente natural. O objetivo deste trabalho foi investigar a diversidade genética, a estrutura genética espacial intrapopulacional (EGE), o sistema de cruzamento e o fluxo gênico contemporâneo de uma população de *C. estrellensis*, localizada em um fragmento florestal (448,2 ha) na cidade de Bataguassu (Estado do Mato Grosso do Sul, Brasil), utilizando marcadores microssatélites. Foram mapeadas, medidas (altura e diâmetro a altura do peito) e genotipadas todas as 285 árvores adultas encontradas na área e coletadas sementes de 20 árvores matrizes, 32 sementes por árvore para as análises de forma hierárquica dentro e entre frutos. Utilizando os genótipos de adultos e progênies foram investigadas a herança Mendeliana, ligação genética e o desequilíbrio genotípico de nove locos de *C. estrellensis*, os quais exibiram herança Mendeliana, não estão ligados e segregam de forma independente. Embora a riqueza alélica (R), heterozigozidade observada (H_o) e esperada (H_e) foram similares entre adultos (R = 8,3, H_o = 0,648, H_e = 0,686) e sementes (R = 7,8, H_o = 0,640, H_e = 0,682), estes índices foram significativamente menores nas sementes. O índice fixação médio (F) não foi significativamente maior do que zero, sugerindo ausência de endogamia nos adultos e nas sementes. A taxa de cruzamento multilocos (t_m) foi significativamente menor que a unidade (1,0), sugerindo autofecundações. A taxa de cruzamento entre indivíduos parentes ($t_m - t_s$) foi significativamente maior do que zero (0,062) e a correlação de paternidade foi maior dentro ($r_{p(w)}$ = 0,835) do que entre frutos ($r_{p(a)}$ = 0,062). O coeficiente médio de coancestria (Θ) foi maior e o tamanho efetivo (N_e) foi menor do que o esperado para progênies de populações panmíticas. O número estimado de árvores matrizes para a coleta de sementes para obter um tamanho efetivo de 150 foi de 52. A taxa de imigração de pólen foi de 9,4%. O raio efetivo de dispersão de pólen (r_{ep}) foi de 974 m. A análise de modelagem de dispersão de pólen de Kernel indicou o modelo de dispersão exponencial como o que melhor explica a dispersão de pólen, com média de dispersão de pólen de 610,9 m. Portanto, a população de *C. estrellensis* não está reprodutivamente isolada devido à dispersão de pólen a longas distâncias e apresenta grande potencial para fins de conservação genética *in situ* e *ex situ*.

Palavras-chave - Conservação genética. Espécie arbórea tropical. Fluxo gênico. Marcadores microssatélites.

ABSTRACT

Cariniana estrellensis (Raddi) Kuntze (Lecythidaceae), popularly known as jequitibá-branco, is a tropical tree species typical of advanced successional stages, characteristic of climax forests. Although it ecological importance, the species is threatened with extinction, mainly due to the intense exploitation and degradation of its natural environment. The objective of this study was to investigate the genetic diversity, intrapopulation spatial genetic structure (SGS), the mating system and contemporary gene flow of a population of *C. estrellensis*, located in a forest fragment (448.2 ha) in the city of Bataguassu (State of Mato Grosso do Sul, Brazil), using microsatellite markers. Were mapped, measured (height and diameter at breast height) and genotyped all 285 adult trees found in the area and collected seeds from 20 matrices trees, 32 seeds per tree for the hierarchical analyses within and among fruits. Using the genotypes of adults and progenies were investigated Mendelian inheritance, genetic linkage and genotypic disequilibrium of nine loci of *C. estrellensis*, which exhibited Mendelian inheritance, are not linkaged and segregate independently. Although the allelic richness (R), observed heterozygosity (H_o) and expected (H_e) were similar among adults (R = 8.3, H_o = 0.648, H_e = 0.686) and seeds (R = 7.8, H_o = 0.640, H_e = 0.682), these indexes were significantly lower in the seeds. The average fixation index (F) was not significantly greater than zero, suggesting absence of inbreeding in adults and seeds. The rate of multilocus outcrossing (t_m) was significantly less than unit (1.0), suggesting selfing. The outcrossing rate between related individuals ($t_m - t_s$) was significantly greater than zero (0.062) and the paternity correlation was higher within ($r_{p(w)}$ = 0.835) than that among fruits ($r_{p(a)}$ = 0,062). The average coefficient of coancestry (Θ) was higher and the effective size (N_e) lower than expected for progenies of panmitic populations. The estimated number of matrices trees to collect seeds to obtain the effective size of 150 was of 52. The immigration rate of pollen was 9.4%. The effective radius of pollen dispersal (r_{ep}) was of 974 m. The analysis of Kernel pollen dispersion modeling indicated the exponential dispersion model as the best explanation for pollen dispersion, with a pollen dispersion average of 610.9 m. Therefore, the population of *C. estrellensis* is not reproductively isolated due to the dispersion of pollen over long distances and presents great potential for *in situ* and *ex situ* genetic conservation purposes.

Keywords - Genetic conservation. Tropical tree species. Gene flow. Microsatellite markers.

SUMÁRIO

1 INTRODUCTION

The trees because of their large size and long life are the key organisms of forest ecosystems, creating habitat for the survival of many other animal and plant species. However, forest species that live in natural conditions are under heavy threat due to the intense fragmentation, which isolates spatially and genetically populations and individuals (AGUILAR et al., 2008; KRAMER et al., 2008; GAINO et al., 2010; SEBBENN et al., 2011; BREED et al., 2012). The fragmentation of forest biomes is a problem that has affected the survival of many tree species populations around the world, especially in the tropics, since the high species diversity occur on a simple hectare. The tree species conservation is necessary in ecosystems so that future generations can enjoy the same benefits they provide us today, such as the extraction of natural resources, shelter, food for fauna and flora, besides the maintenance and improvement of climatic and environmental conditions.

The survival of natural populations depends on their genetic diversity and their effective size, once these are essential conditions for adaptation, evolution and survival of populations, mainly in situations of environmental changes such as forest fragmentation and diseases. The genetic diversity of populations is also the raw material for genetic improvement, since the genetic diversity absence implies the impossibility of progress with the selection (RAJORA; MOSSELER, 2001). The gene flow is one of the most important factors that influence the genetic structure in tree species (DICK et al., 2003). So pollen flow and the seed dispersal promote the distribution of genetic diversity in plant populations and allow determining the ideal sampling within the population for genetic conservation purposes (SEBBENN; KAGEYAMA; VENCOVSKY, 2003).

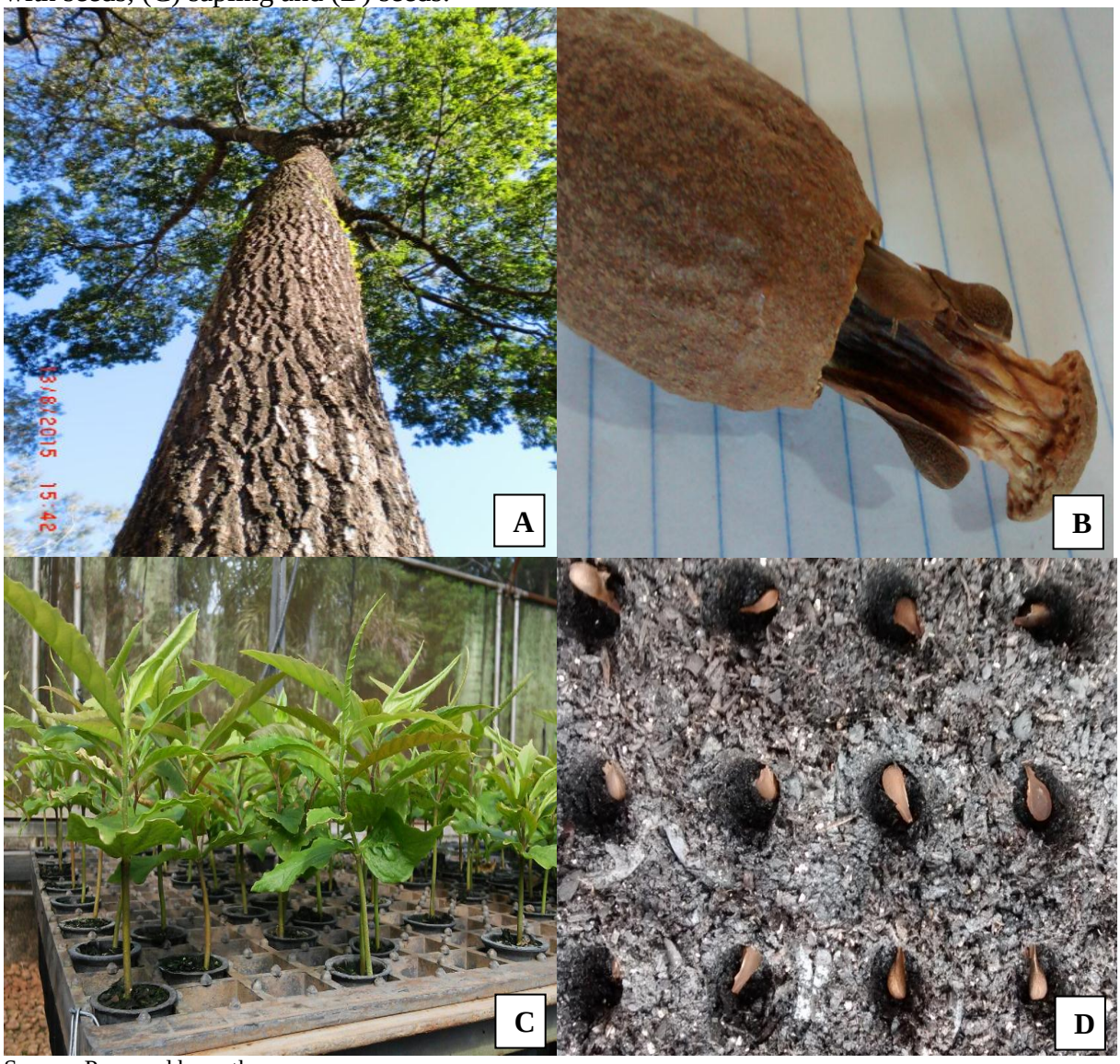
The effects of fragmentation over time can modify the genetic makeup of individual fragments as well as the landscape as a whole (HAMRICK, 2004). A fundamental change in populations that begins immediately after fragmentation is the increase in internal inbreeding with a consequent reduction in effective population size due to the increase in the frequency of identical alleles by offspring that may impact the survival of populations and lead to long-term extinction by genetic effects. The short-term effects can be detected in samples of juveniles and open-pollinated seeds, immediately after the fragmentation (AGUILAR et al., 2008; KRAMER et al., 2008). These effects have been detected in studies on the impacts of fragmentation on diversity and genetic isolations of tree populations as Collevatti et al. (2001) - *Caryocar brasiliense*; Lemes et al. (2003) - *Swietenia macrophylla*; Bittencourt and

Sebbenn (2007) - *Araucaria angustifolia*; Gaino et al. (2010) - *Myracrodruon urundeuva*; Sebbenn et al. (2011) - *Copaifera langsdorffii*; Quesada et al. (2013) - C*eiba aesculifolia*; Baldauf et al. (2014) - *Himatanthus drasticus*; Arruda et al. (2015) - *Bagassa guianensis*; Manoel et al. (2015) - *Genipa americana*; Tambarussi et al. (2015) - *Cariniana legalis*; Guidugli et al. (2016) - *Cariniana estrellensis* and others.

Cariniana estrellensis (Raddi) Kuntze (Figure 1), popularly known as jequitibá-branco, jequitibá-mestiço, binga-de-macaco and caixão, belonging to the family Lecythidaceae is a tree species of great ecological importance in reforestations (DURIGAN; NOGUEIRA, 1990). Under natural conditions, may be found individuals up to 35-45 m in height and 90-120 cm in diameter at breast height (LORENZI, 2002). The species is hermaphrodite and the pollination is carried out by small insects, mainly by bees of the genera *Melipona* and *Trigona*. The period of flowering and fruiting varies greatly according to region and the reproductive process begins at ten years old, in plantations. The species has a wide geographical distribution, its area of occurrence covers countries as Bolivia, Paraguay, Peru and in Brazil in the States of Acre, Bahia (Chapada Diamantina and South), Espírito Santo (North), Goiás (South), Minas Gerais (Wood Zone), Mato Grosso (South), Paraná (East and North), Rio de Janeiro (East) and São Paulo (CARVALHO, 2003). The wide geographical distribution suggests the species presents high genetic diversity. However, the species is included in the list of endangered species in the vulnerable category according to Figliolia et al. (2000), thus it raises the interest in the studies to elaborate conservation and genetic improvement strategies of the specie.

The objectives of the study were to determine the diversity genetic, the intrapopulation spatial genetic structure, the mating system and the contemporary gene flow in the natural population of *C. estrellensis* located in the city of Bataguassu (State of Mato Grosso do Sul, Brazil), using microsatellite markers. The anthropogenic effects knowledge on forest fragmentation is of fundamental importance to understand and apply measures of conservation and environmental recovery of natural populations at genetic decline risk, as is the of species *C. estrellensis*.

Figure 1- General appearance of the species *Cariniana estrellensis*: (A) adult tree, (B) fruit with seeds, (C) sapling and (D) seeds.



Source: Prepared by author.

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2 STUDYING MENDELIAN INHERITANCE, GENETIC LINKAGE, AND GENOTYPIC DISEQUILIBRIUM FOR NINE MICROSATELLITE LOCI IN *Cariniana estrellensis* (Raddi) Kuntze (LECYTHIDACEAE)

ABSTRACT

Cariniana estrellensis, known as jequitibá-branco, is one of the largest trees found in Brazilian tropical forests. The species is typical of advanced stages of succession, characteristic of climax forests, and essential in genetic conservation and environmental restoration plans. In this study, we assessed Mendelian inheritance, genetic linkage, and genotypic disequilibrium in nine microsatellite loci for a *C. estrellensis* population. We sampled and genotyped 285 adult trees and collected seeds from 20 trees in a fragmented forest landscape in Brazil. Based on maternal genotypes and their seeds, we found no deviation from the expected 1:1 Mendelian segregation and no genetic linkage between pairwise loci. However, for adults, genotypic disequilibrium was detected for four pairs of loci, suggesting that this result was not caused by genetic linkage. Based on our results, the analyzed microsatellite loci are suitable for use in population genetics studies assessing genetic diversity, mating system, and gene flow in *C. estrellensis* populations.

Keywords - Fragmented forest . Genetic conservation. Jequitibá-branco. Population genetics. Tropical forests.

2.1 INTRODUCTION

Cariniana estrellensis (Raddi) Kuntze (Lecythidaceae), or jequitibá-branco, has a wide geographic range distributed across Brazil, Bolivia, Paraguay, and Peru. The species is a priority for genetic conservation due to its ecological (reforestation) and commercial (wood and pulp) importance. It is currently threatened with extinction due to intense exploitation of the species as a timber resource (FOOD AND AGRICULTURE ORGANIZATION- FAO, 2002). The species is hermaphroditic and pollinated mainly by bees of the genus *Melipona* and *Trigona*. Its winged seeds are dispersed by wind and periods of flowering and fruiting vary greatly across its range (PRANCE; MORI, 1979; CARVALHO, 2003; LEITE, 2007).

Microsatellite markers (Simple Sequence Repeats, SSR) are a useful tool to analyze the genetics of forest species due to their high degree of polymorphism in terms of numbers of alleles. However, in order to use molecular markers as genetic markers, it is important to determine whether their inheritance follows the rules of Mendelian segregation, and whether the loci are genetically linked (TAMBARUSSI et al., 2013; MANOEL et al., 2015; MORAES et al., 2016). Studies assessing linkage among loci are also necessary because the detected loci are used to calculate averages among loci in population genetic studies; therefore, linked loci can create bias in the estimates (GUIDUGLI et al., 2010). To enable the analysis of genetic diversity and structure, mating system, and gene flow for *C. estrellensis*, herein we assess the Mendelian inheritance, genetic linkage, and genotypic disequilibrium for nine microsatellite loci developed for the species.

2.2 MATERIAL AND METHODS

Study site and sampling

The study was based on samples collected from a highly fragmented forest landscape situated in a transition zone between the Savanna and Atlantic Forest biomes. The study area covers 448.2 ha and is located in the city of Bataguassu (Mato Grosso do Sul State, Brazil), alongside the Pardo River (21°38'00"S, 52°14'02"W), with average altitude of 273.3 m. The climate of the region is tropical humid in summer and dry in winter. The average temperature is 23.1° C, being in the colder months it varies from 15 to 20° C and the dry period can extend from four to five months. Annual precipitation varies from 1200 to 1500 mm (IBGE, 2016).

All 285 adult trees found in the area were sampled and 32 seeds were collected from each of 20 selected seed trees. For the molecular analyses, we used foliar or cambial tissue from adult trees and foliar samples from seeds germinated in a nursery (Appendix 5.1, 5.2, 5.3, 5.4, 5.5 and 5.6).

Microsatellite analysis

Multilocus genotyping of the *C. estrellensis* samples was performed at the HERÉDITAS/GENOMAX laboratory. We used nine microsatellite loci chosen from the 15 loci previously developed for the species (GUIDUGLI et al., 2009). The loci were analyzed in an ABI 3100XL automatic sequencer. Based on these genotypes, a multilocus profile was defined that allows for the identification of each sample individually and enables the analyses of population genetics parameters.

Mendelian inheritance, genetic linkage and genotypic disequilibrium

The study of microsatellite loci inheritance was based on the method described by Gillet and Hattemer (1989), which compares the genotype of a heterozygous maternal tree with the segregation of its open-pollinated progenies. This method assumes that all loci have regular segregation and their alleles follow Mendelian inheritance patterns based on the following conditions: *i*) regular meiotic segregation during ovule production; *ii*) random ovule fertilization by a type of pollen; and *iii*) no selection between the moment of fertilization and the genotyping of seeds. The model also assumes a co-dominant relationship between all alleles. The method further requires that all progeny of a tree must possess a maternal allele, and in cases of a heterozygous mother tree (e.g., A_iA_j, $i \neq j$), the following are required: a) each individual within progeny must have one allele of the maternal tree, A_i or A_j; b) the number of heterozygous progeny A_iA_j (n_{ij}) must be equal to the sum of the number of homozygous progeny A_iA_i (n_{ii}) and A_jA_j (n_{jj}): $n_{ij} = n_{ii} + n_{jj}$; and c) the number of heterozygous progeny A_iA_k (n_{ik}) must be equal to the number of heterozygous progeny A_jA_k (n_{jk}), or $n_{ik} = n_{jk}$, in other words $k \neq i$, j. The observed segregation of each progeny of the heterozygous maternal tree for a given locus was statistically compared to that expected for the segregation hypothesis of 1:1, using the *G*-test (SOKAL; ROHLF, 1981):

$$G = 2\left[n_{ij} \ln\left(\frac{n_{ij}}{E(n1)} \right) + (n_{ii} + n_{ij}) \ln\left(\frac{(n_{ii} + n_{jj})}{E(n1)} \right) \right] \qquad \text{Equation 1}$$

where, ln is the natural logarithm, $E(n1)$ is the expected number of offspring genotypes A_iA_j (n_{ij}) and $A_iA_i + A_jA_j$ ($n_{ii} + n_{jj}$): $E(n1) = 0.5(n_{ij} + n_{ii} + n_{jj})$, or:

$$G = 2\left[n_{ik} \ln\left(\frac{n_{ik}}{E(n2)} \right) + n_{jk} \ln\left(\frac{n_{jk}}{E(n2)} \right) \right] \qquad \text{Equation 2}$$

where, $E(n2)$ is the expected number of genotypes for alleles A_iA_k (n_{ik}) and A_jA_k (n_{jk}): $E(n2) = 0.5(n_{ik} + n_{jk})$. To avoid false positives, the *G*-test was determined only when $n1$ and $n2$ was ≥ 10, and deviation from the *G*-test between the observed and expected segregation

was determined as statistically significant using a Bonferroni correction for multiple comparisons (95%, α = 0.05).

To determine if the loci were genetically linked, a test was carried out between pairs of loci using genetic information from mother trees that were doubly heterozygous for two loci (A_iA_j, B_lB_m). The segregation was recorded in their progeny. In this case, the null hypothesis (H_o) was regular Mendelian segregation of 1:1:1:1. The regular segregation hypothesis between pairs of loci was accepted or rejected based on a maximum likelihood *G*-test (SOKAL; ROHLF, 1981), performed for each progeny:

$$G = 2\left[n_{il} \ln\left(\frac{n_{il}}{E(n)} \right) + n_{im} \ln\left(\frac{n_{im}}{E(n)} \right) + n_{jl} \ln\left(\frac{n_{jl}}{E(n)} \right) + n_{jm} \ln\left(\frac{n_{jm}}{E(n)} \right) \right] \qquad \text{Equation 3}$$

where, n_{il}, n_{im}, n_{jl}, and n_{jm} are the observed numbers of the phenotypes A_iB_l, A_iB_m, A_jB_l, and A_jB_m, respectively, and $E(n)$ is the expected number of each genotype A_iB_l, A_iB_m, A_jB_l, and A_jB_m, calculated by $E(n) = 0.25(n_{il} + n_{im} + n_{jl} + n_{jm})$. We again applied the Bonferroni correction for multiple comparisons (95%, α = 0.05) to avoid false positives.

The genotypic disequilibrium test between pairwise loci was only performed with adult samples. Estimates of gene frequencies based on open-pollinated progeny arrays are biased because each progeny has at least one maternal allele, resulting in a genotypic disequilibrium. This analysis was carried out using the FSTAT software (GOUDET, 2002). The probabilities of the significance test were obtained by permutation of alleles among individuals, associated with a Bonferroni correction for multiple comparisons (95%, α= 0.05).

2.3 RESULTS

After Bonferroni correction, we found no deviation from 1:1 Mendelian segregation for the nine loci analyzed for *C. estrellensis* heterozygous trees (Table 1). Furthermore, after Bonferroni correction, we detected no deviation from 1:1:1:1 Mendelian segregation between pairwise loci, indicating that the nine loci analyzed in this study are not linked (Table 2). However, significant genotypic disequilibrium was detected between four pairs of loci among adult trees after Bonferroni correction: Ces01xCes02, Ces01xCes04, Ces01xCes11, and Ces04xCes11 (Table 3).

Table 1- Mendelian inheritance tests for nine microsatellite loci in *Cariniana estrellensis*.

Locus/ mother	Mother genotype	n_1	$n_{ij} : n_{ii} + n_{jj}$	G_1	n_2	$n_{ik} : n_{jk}$	G_2
Ces01							
1	158/162	6	0:6	NE	26	11:15	0.62
5	148/152	23	11:12	0.04	10	8:2	3.85
15	148/152	23	9:14	1.09	9	1:8	NE
22	152/156	23	4:19	10.63	9	9:0	NE
31	152/156	27	8:19	4.61	5	1:4	NE
66	148/152	31	16:15	0.03	1	0:1	NE
91	152/156	23	5:18	7.80	9	9:0	NE
101	146/156	22	12:10	0.18	10	1:9	7.36
123	146/152	29	17:12	0.86	3	0:3	NE
125	146/150	30	11:19	2.15	2	1:1	NE
136	148/152	23	15:8	2.16	9	0:9	NE
Ces02							
15	170/186	24	12:12	0.00	8	4:4	NE
19	182/186	22	17:5	0.18	10	8:2	3.85
21	170/182	28	9:19	3.65	4	4:0	NE
22	170/182	22	14:8	1.65	10	6:4	0.40
31	170/182	17	10:7	0.53	15	9:6	0.60
35	170/186	25	14:11	0.36	7	6:1	NE
45	170/182	25	15:10	1.00	7	4:3	NE
66	170/186	32	10:22	4.61	0	0:0	NE
96	170/186	22	11:11	0.00	10	7:3	1.64
101	182/186	12	6:6	0.00	20	13:7	1.82
119	170/186	26	12:14	0.15	6	3:3	NE
123	170/186	32	17:15	0.12	0	0:0	NE
125	170/186	32	11:21	3.17	0	0:0	NE
136	170/186	32	16:16	0.00	0	0:0	NE
225	182/186	21	9:12	0.43	11	6:5	0.09

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Table 1- Mendelian inheritance tests for nine microsatellite loci in *Cariniana estrellensis*.

Locus/ mother	Mother genotype	n_1	$n_{ij} : n_{ii} + n_{jj}$	G_1	n_2	$n_{ik} : n_{jk}$	G_2
Ces04							
1	184/204	17	8:9	0.05	15	7:8	0.06
5	204/212	13	7:6	0.07	19	10:9	0.05
15	182/216	10	8:2	3.85	22	11:11	0.00
19	182/204	19	12:7	1.33	13	2:11	6.85
21	206/216	5	1:4	NE	27	16:11	0.93
22	186/212	1	0:1	NE	31	17:14	0.29
31	186/214	15	3:12	5.78	17	12:5	2.97
35	212/216	11	6:5	0.09	21	9:12	0.43
45	182/214	11	6:5	0.09	21	14:7	2.37
64	204/216	5	5:0	NE	27	13:14	0.03
66	204/212	4	1:3	NE	28	16:12	0.57
88	178/216	15	8:7	0.06	17	12:5	2.97
91	182/186	5	3:2	NE	27	15:12	0.33
96	188/214	4	2:2	NE	28	13:15	0.14
101	214/216	15	8:7	0.06	17	7:10	0.53
119	182/216	17	8:9	0.05	15	8:7	0.06
123	204/216	3	3:0	NE	29	13:16	0.31
125	182/216	0	0:0	NE	32	14:18	0.50
136	182/200	6	3:3	NE	26	14:12	0.15
225	208/214	0	0:0	NE	32	20:12	2.02
Ces09							
1	179/183	32	16:16	0.00	0	0:0	NE
15	177/179	26	11:15	0.61	6	1:5	NE
19	177/179	21	8:13	1.20	0	0:0	NE
22	179/183	25	17:8	3.31	7	4:3	NE
35	179/183	32	14:18	0.50	0	0:0	NE
45	179/183	23	13:10	0.39	9	8:1	NE
64	177/179	26	11:15	0.61	6	5:1	NE
66	179/183	27	11:16	0.93	5	3:2	NE
88	177/183	11	4:7	0.82	21	9:12	0.43
96	177/183	5	3:2	NE	27	13:14	0.03
101	179/183	26	10:16	1.39	6	4:2	NE
123	179/183	28	14:14	0.00	4	2:2	NE
225	179/183	32	16:16	0.00	0	0:0	NE
Ces10							
21	197/199	29	14:15	0.03	3	0:3	NE
35	197/199	32	9:23	6.33	0	0:0	NE
91	197/199	32	14:18	0.50	0	0:0	NE
96	197/199	29	9:20	4.27	3	0:3	NE
101	197/199	32	14:18	0.50	0	0:0	NE
119	197/199	32	20:12	2.02	0	0:0	NE
123	197/199	27	14:13	0.03	5	4:1	NE
125	199/203	11	5:6	0.09	21	11:10	0.04
136	197/199	24	12:12	0.00	8	5:3	NE

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Table 1- Mendelian inheritance tests for nine microsatellite loci in *Cariniana estrellensis*.

Locus/ mother	Mother genotype	n_1	$n_{ij} : n_{ii} + n_{jj}$	G_1	n_2	$n_{ik} : n_{jk}$	G_2
Ces11							
1	206/212	15	3:12	5.78	17	9:8	0.05
5	206/210	20	13:7	1.82	12	9:3	3.13
19	208/232	14	6:8	0.28	18	12:6	2.03
21	206/232	14	6:8	0.28	18	12:6	2.03
22	230/232	15	3:12	5.78	17	8:9	0.05
31	206/230	12	6:6	0.00	20	8:12	0.80
35	210/234	6	3:3	NE	26	8:18	3.94
45	206/208	16	8:8	0.00	16	7:9	0.25
64	206/232	8	4:4	NE	24	9:15	1.51
66	206/208	18	10:8	0.22	14	7:7	0.00
88	206/218	8	2:6	NE	24	10:14	0.66
91	208/212	9	4:5	NE	23	11:12	0.04
123	206/208	25	11:14	0.36	7	4:3	NE
125	206/208	21	8:13	1.20	11	10:1	8.54
136	206/208	32	18:14	0.50	0	0:0	NE
Ces13							
1	130/142	22	14:8	1.65	10	3:7	1.64
5	140/148	13	3:10	3.97	19	12:7	1.33
15	130/142	19	13:6	2.64	13	4:9	1.97
19	130/142	22	11:11	0.00	10	7:3	1.64
21	138/142	24	14:10	0.66	8	0:8	NE
22	142/148	6	3:3	NE	26	15:11	0.61
31	130/148	4	3:1	NE	28	14:14	0.00
45	140/142	20	7:13	1.82	12	5:7	0.33
64	130/142	12	4:8	1.35	20	12:8	0.80
66	130/148	4	2:2	NE	28	14:14	0.00
88	142/148	12	3:9	3.13	20	11:9	0.20
91	138/142	18	14:4	5.88	14	5:9	1.15
101	140/148	7	3:4	NE	25	12:13	0.04
119	142/148	18	11:7	0.89	14	11:3	4.85
123	138/142	15	6:9	0.60	17	6:11	1.49
125	138/148	21	13:8	1.20	11	5:6	0.09
136	138/140	23	8:15	2.16	9	5:4	NE
225	140/142	21	5:16	6.05	11	10:1	8.54
Ces14							
15	182/184	19	8:11	0.47	13	4:9	1.97
31	184/188	24	5:19	8.70	8	2:6	NE
45	182/184	24	8:16	2.71	8	2:6	NE
88	182/184	25	12:13	0.04	7	3:4	NE
91	184/188	32	14:18	0.50	0	0:0	NE
96	182/184	30	13:17	0.53	2	1:1	NE
125	182/184	22	9:13	0.73	10	4:6	0.40
136	184/188	26	13:13	0.00	6	4:2	NE
225	182/184	32	11:21	3.17	0	0:0	NE

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Table 1- Mendelian inheritance tests for nine microsatellite loci in *Cariniana estrellensis*.

Locus/ mother	Mother genotype	n_1	$n_{ij} : n_{ii} + n_{jj}$	G_1	n_2	$n_{ik} : n_{jk}$	G_2
Ces18							
1	168/180	11	6:5	0.09	21	4:17	8.66
15	174/180	13	2:11	6.85	19	14:5	4.43
19	168/170	25	9:16	1.98	7	0:7	NE
35	168/170	28	12:16	0.57	4	2:2	NE
45	168/180	11	6:5	0.09	21	9:12	0.43
64	168/180	9	2:7	NE	23	9:14	1.09
66	170/180	15	3:12	5.78	17	5:12	2.97
88	170/180	30	14:16	0.13	2	1:1	NE
91	168/170	27	11:16	0.93	5	1:4	NE
96	166/174	9	4:5	NE	23	10:13	0.39
119	174/178	8	3:5	NE	24	21:3	15.18*
123	166/168	17	5:12	2.97	14	9:5	1.15
125	166/170	22	13:9	0.73	10	5:5	0.00
136	168/180	13	5:8	0.70	19	14:5	4.43
225	170/176	16	13:3	6.73	16	7:9	0.25

n_1 and n_2 are the sample size; G_1 and G_2 are the maximum likelihood G statistics for the hypothesis of $n_{ij} : n_{ii} + n_{jj}$ and $n_{ik} : n_{jk}$, respectively, for one degree of freedom; * Significance after Bonferroni correction for α = 0.05 (χ^2 = 12.18). NE is not estimated due to a sample size of less than ten progeny.

Table 2- Values of maximum likelihood *G* -test for the hypothesis of independent segregation between pairwise loci (1:1:1:1) of *Cariniana estrellensis*.

	Ces1xCes2	Ces1xCes4	Ces1xCes9	Ces1xCes10	Ces1xCes11	Ces1xCes13	Ces1xCes14	Ces1xCes18
1	0.35 (15)	5.12 (1)	6.45 (1)	0.35 (91)	8.32 (1)	1.14 (1)	4.76 (15)	6.64 (1)
2	6.92 (22)	1.32 (5)	10.08 (15)	3.16 (101)	5.22 (5)	1.68 (5)	1.59 (31)	5.38 (15)
3	1.60 (31)	0.40 (15)	10.20(22)	0.26 (123)	10.30 (22)	2.37 (15)	1.60 (91)	1.74 (66)
4	5.74 (66)	5.42(22)	1.84 (66)	3.23 (125)	5.38 (31)	7.91 (22)	8.02 (125)	2.16 (91)
5	3.50 (101)	2.08 (31)	5.83 (101)	8.37 (136)	2.46(66)	0.45 (31)	11.51 (136)	2.16 (101)
6	4.28 (123)	0.23 (66)	6.36 (123)		2.46 (91)	1.27 (66)		2.35 (123)
7	1.74 (125)	0.40 (91)			7.32 (123)	3.87 (91)		8.02 (125)
8	3.31 (136)	1.60 (101)			6.64 (125)	0.84 (101)		11.34 (136)
9		0.35 (123)			8.03 (136)	0.90 (123)		
10		1.73 (125)				3.00 (125)		
11		6.49 (136)				10.12 (136)		
	Ces2xCes4	Ces2xCes9	Ces2xCes10	Ces2xCes11	Ces2xCes13	Ces2xCes14	C2xC18	Ces4xCes09
1	0.29 (15)	10.08 (15)	0.33 (21)	1.12 (19)	1.92 (5)	6.51 (15)	3.06 (15)	5.17 (1)
2	3.38 (19)	12.51 (19)	7.63 (35)	8.22 (21)	1.12 (19)	2.17 (31)	4.04 (19)	9.70 (15)
3	2.81 (21)	0.92 (22)	4.00 (6)	7.10 (22)	8.76 (21)	13.12 (45)	6.68 (35)	14.04 (19)
4	1.40 (22)	3.26 (35)	7.61 (101)	1.19 (31)	1.19 (22)	5.15 (96)	5.67 (45)	1.27 (35)
5	3.89 (31)	3.92 (45)	1.71 (119)	7.14 (35)	1.79 (31)	11.51 (125)	3.69 (66)	7.17 (45)
6	3.50 (35)	3.25 (66)	3.75 (123)	3.71 (45)	5.94 (45)	2.49 (136)	2.02 (96)	2.56 (64)
7	4.16 (45)	0.23 (96)	2.21 (125)	5.08 (66)	2.19 (66)	2.67 (225)	5.66 (119)	3.66 (66)
8	3.13 (66)	7.71 (101)	0.55 (136)	6.14 (123)	3.75 (101)		7.33 (123)	0.44 (66)
9	0.94 (96)	1.94 (123)		0.35 (125)	4.14 (119)		0.97 (125)	1.11 (96)
10	6.17 (101)	1.64 (125)		0.76 (136)	2.49 (123)		15.97 (136)	13.09 (101)
11	2.67 (119)				0.67 (125)		0.05 (225)	6.03 (123)
12	3.98 (123)				1.34 (136)			7.51 (225)
13	0.80 (125)				0.52 (225)			
14	1.51 (136)				0.52 (225)			
15	0.44 (225)							
	Ces4xCes10	Ces4xCes11	Ces4xCes13	Ces4xCes14	Ces4xCes18	Ces9xCes10	Ces9xCes11	Ces9xCes13
1	3.69 (21)	6.34 (1)	3.00 (1)	7.06 (15)	3.71 (1)	7.79 (35)	6.85 (1)	4.21 (1)
2	9.54 (35)	1.94 (5)	2.43 (5)	1.01 (31)	1.63 (15)	3.89 (96)	16.80 (19)	9.80 (15)
3	0.98 (91)	5.05 (15)	1.50 (19)	2.89 (45)	5.61 (19)	2.74 (101)	4.19 (22)	7.67 (19)
4	3.82 (96)	5.42 (21)	10.13 (21)	0.72 (88)	3.23 (35)	6.22 (123)	2.17 (35)	2.04 (22)
5	2.96 (101)	5.20 (22)	2.13 (22)	0.58 (91)	2.69 (45)		8.28 (45)	0.17 (35)
6	0.30 (119)	1.53 (131)	4.59 (31)	1.97 (6)	2.70 (64)		2.64 (64)	3.54 (45)
7	1.35 (123)	1.90 (35)	0.76 (45)	6.27 (119)	2.81 66)		0.44 (66)	4.36 (64)
8	1.94 (125)	0.33 (45)	0.65 (64)	5.67 (125)	1.52 (88)		0.11 (88)	1.74 (66)
9	0.17 (136)	1.25 (64)	0.98 (66)	2.32 (136)	1.38 (91)		0.06 (96)	1.56 (88)
10		0.18 (66)	0.18 (88)	5.27 (225)	1.78 (96)		8.51 (123)	9.34 (101)
11		0.00 (88)	0.40 (91)		6.84 (119)			4.47 (123)
12		0.50 (91)	6.20 (101)		6.54 (123)			4.15 (225)
13		0.24 (96)	1.84 (119)		1.12 (125)			
14		5.23 (123)	4.75 (123)		9.26 (136)			
15		0.58 (125)	0.48 (125)		4.99 (225)			
16		1.65 (136)	4.07 (136)					
17			3.54 (225)					

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Table 2- Values of maximum likelihood *G* -test for the hypothesis of independent segregation

	Ces9x Ces14	Ces9x Ces8	Ces10x Ces11	Ces10x Ces13	Ces10x Ces14	Ces10x Ces18	Ces11x Ces13	Ces11x Ces14
1	14.29 (15)	6.31 (1)	2.53 (21)	9.19 (21)	0.90 (91)	12.5 (35)	3.53 (1)	1.76 (31)
2	4.57 (45)	8.02 (15)	7.68 (35)	1.68 (91)	7.38 (96)	2.47 (91)	1.90 (5)	2.54 (45)
3	0.38 (88)	10.04 (19)	0.06 (91)	1.37 (101)	2.49 (125)	4.30 (96)	2.56 (19)	3.23 (88)
4	3.70 (96)	2.85 (35)	7.20 (123)	5.43 (119)	4.82 (136)	4.77 (119)	8.73 (21)	1.83 (91)
5	10.42 (225)	5.99 (45)	3.04 (125)	1.47 (123)		3.88 (123)	8.57 (22)	7.89 (125)
6		1.79 (64)	1.00 (136)	2.15 (125)		3.93 (125)	0.90 (31)	3.78 (136)
7		3.81 (66)		4.15 (136)		7.02 (136)	1.90 (45)	
8		1.46 (88)					2.79 (64)	
9		2.36 (96)					1.30 (66)	
10		5.84 (123)					0.30 (88)	
11		7.48 (225)					5.42 (91)	
12							4.75 (123)	
13							2.00 (125)	
14							1.73 (136)	

	Ces11x Ces18	Ces13x Ces14	Ces13x Ces18	Ces14x Ces18
1	4.99 (1)	7.69 (15)	3.40 (1)	7.52 (15)
2	2.82 (19)	1.71 (31)	0.06 (5)	3.74 (45)
3	2.84 (35)	7.29 (45)	3.08 (15)	0.63 (88)
4	2.72 (45)	1.48 (88)	4.14 (19)	5.94 (91)
5	2.14 (64)	0.79 (91)	143 (22)	3.23 (96)
6	1.91 (66)	8.56 (125)	3.21 (45)	11.50 (125)
7	1.68 (88)	4.49 (136)	0.50 (64)	11.96 (136)
8	2.25 (91)	2.17 (225)	2.12 (66)	
9	4.49 (123)		1.82 (88)	
10	0.59 (125)		1.85 (91)	
11	5.79 (136)		7.13 (119)	
12			5.60 (123)	
13			1.63 (125)	
14			5.70 (136)	
15			1.52 (225)	

* Significance after Bonferroni correction for α = 0.05, (χ^2 = 20.73). G = G-test for three degrees of freedom.

Table 3- Genotypic disequilibrium between pairwise microsatellite loci for sampled *Cariniana estrellensis* adult trees.

Pairwise loci	P-value	Pairwise loci	P value
Ces01xCes02	0.00069	Ces04xCes13	0.00278
Ces01xCes04	0.00069	Ces04xCes14	0.10833
Ces01xCes09	0.20833	Ces04xCes18	0.12847
Ces01xCes10	0.00139	Ces09xCes10	0.60556
Ces01xCes11	0.00069	Ces09xCes11	0.84514
Ces01xCes13	0.03611	Ces09xCes13	0.09653
Ces01xCes14	0.26458	Ces09xCes14	0.32431
Ces01xCes18	0.06042	Ces09xCes18	0.00556
Ces02xCes04	0.08681	Ces10xCes11	0.43750
Ces02xCes09	0.61111	Ces10xCes13	0.01111
Ces02xCes10	0.19931	Ces10xCes14	0.08264
Ces02xCes11	0.00139	Ces10xCes18	0.85000
Ces02xCes13	0.09028	Ces11xCes13	0.01181
Ces02xCes14	0.03403	Ces11xCes14	0.17361
Ces02xCes18	0.06597	Ces11xCes18	0.41667
Ces04xCes09	0.16319	Ces13xCes14	0.02222
Ces04xCes10	0.20486	Ces13xCes18	0.00417
Ces04xCes11	0.00069	Ces14xCes18	0.52431

The P-values represent the probability of genotypic disequilibrium after 1440 permutations of alleles among individuals. Value at which results are deemed significant after Bonferroni correction: P = 0.00069 ($\alpha = 0.05$).

2.4 DISCUSSION

Confirmation of Mendelian segregation for individual loci was confirmed based on the expected 1:1 Mendelian segregation test. Independent segregation of alleles between different loci was performed through the linkage test based on 1:1:1:1 segregation, using genetic information of doubly heterozygous mother trees and observed segregation in progenies. We found that the nine loci assessed herein present Mendelian segregation and are not linked. Thus, these molecular markers developed by Guidugli et al. (2010) can be considered as genetic markers and our results support the hypothesis that they are not located in the same chromosome linkage group. However, genotypic disequilibrium was detected between four pairs of loci for adults. Genotypic disequilibrium is largely caused by genetic linkage, natural and artificial selection, genetic bottleneck, founder effect, and genetic drift (HARTL; CLARK, 2010). Among these, genetic bottleneck and genetic drift can be the result of forest fragmentation due to decreases in effective population size, resulting in a limited number of pollen donors participating in reproduction. In studying a different population of the same species, Guidugli et al. (2010) found no significant genotypic disequilibrium between the same pairwise loci assessed herein. Thus, the genotypic disequilibrium we detected may be attributed to genetic drift caused by forest fragmentation in the study region. Studies on other tree species have also found an absence of genetic linkage with a presence of genotypic disequilibrium, including *Araucaria angustifolia* (MEDINA-MACEDO et al., 2014), *Copaifera langsdorffii* (TARAZI et al., 2010), *Cariniana legalis* (TAMBARUSSI et al., 2013), and *Genipa americana* (MANOEL et al., 2015).

2.5 CONCLUSIONS

The nine microsatellite loci evaluated in this study exhibit Mendelian inheritance, are not linked, and segregate independently. These loci are therefore suitable for population genetics analyses, which can generate precise estimates of genetic diversity, spatial genetic structure, mating system, and contemporary gene flow for *C. estrellensis*.

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3 GENETIC DIVERSITY, MATING SYSTEM, EFFECTIVE SIZE, AND SPATIAL STRUCTURE IN A LARGE POPULATION OF *Cariniana estrellensis* (Raddi) Kuntze (LECYTHIDACEAE)

ABSTRACT

Large continuous tropical forests are rapidly being reduced to fragments due to intense deforestation. Tropical tree species, such as *Cariniana estrellensis,* are in danger of extinction and require strategies for conservation. This study uses nine microsatellite loci to investigate genetic diversity, mating system, effective population size, and spatial genetic structure (SGS) in a large population of *C. estrellensis*. We sampled all 285 adult trees found in the population and collected seeds from 20 maternal trees, at 32 seeds per tree. The open pollinated seeds were sampled hierarchically within and among fruit. Our results demonstrate that adults present a higher total number of alleles (75) than seeds (73). The allelic richness for 280 genotypes (R) was significantly higher and the fixation index (F) significantly lower in adults (R = 8.3; F = 0.058) than seeds (R = 7.8; F = 0.064). Significant SGS was detected at a distance up to 150 m for adults. The difference $t_m - t_s$ was significantly different from zero, indicating mating among related individuals. The multilocus paternity correlation (within and among) fruit (r_p = 0.246) was significantly greater than zero, suggesting that a limited number of pollen donors fertilized the seed trees (N_{ep} = 4.1). The multilocus paternity correlation was higher within ($r_{p(w)}$ = 0.835) than among ($r_{p(a)}$ = 0.062) fruit, suggesting that the number of pollen donors that fertilized each fruit ($N_{ep(w)}$ = 1.2) was lower than the number of pollen donors fertilizing fruit within trees ($N_{ep(a)}$ = 16.1). The coancestry within progeny (Θ = 0.162) was higher and the effective size (N_e = 2.89) lower than expected in panmictic populations (Θ = 0.125, N_e = 4). In terms of species conservation, it is necessary to collect seeds from a minimum of 52 seed trees located at a distance of at least 150 m to decrease the likelihood of collecting seeds from related trees.

Keywords: Conservation. Effective population size. Microsatellite loci. Tropical tree species.

3.1 INTRODUCTION

A plant's mating system, which can include outcrossing, correlated mating, selfing and apomixis, determines how an individual or a population transfers genetic information from one generation to the next. Thus, the mode of reproduction and mating system for each species has a marked effect on the genetic composition of populations. Understanding the mating system makes it possible to comprehend the crossing patterns that influence genetic diversity. As such, we can assess the distribution of genotypic frequencies that control the potential recombination within populations, which can lead to the moderation or acceleration of new genetic combinations, influencing evolutionary processes in natural populations (WRIGHT, 1921; RITLAND; JAIN, 1981; HEDRICK, 1990; ZANETTINI; CAVALLI, 2003). Yet, several ecological variables have been proposed as factors that can affect the patterns of mating in tree species, including spatial isolation (CASCANTE et al., 2002; FUCHS et al., 2003), flowering phenology (ODDOU-MURATORIO et al., 2006), plant density (MURAWSKI; HAMRICK, 1991), and pollinator activity (HIRAO et al., 2006).

Cariniana estrellensis (Raddi) Kuntze (Lecythidaceae) is a hermaphroditic species, with slow growth and wide geographic distribution. It occurs mainly in Atlantic and subtropical forests on the coast of Brazil, from southern Bahia to Rio Grande do Sul State (CARVALHO, 2003a; LEITE, 2007). Adult trees can reach 35-45 m in height and 90-120 cm in diameter at breast height (LORENZI, 2002). Pollination occurs by small insects, mainly bees of the genera *Melipona* and *Trigona*, and the flowering and ripening of fruits is highly variable depending on where individuals occur across the species' range. The dispersal of the fruits and seeds is by wind. However, monkeys play an important role in dispersal by removing the operculum (lid) of the fruit, and facilitating seed dispersion by wind (CARVALHO, 2003b).

The process of forest fragmentation and consequent population size reduction can have an immediate impact on the mating system of a species by increasing correlated crosses, resulting in greater levels of kinship among offspring. Therefore, in this study, we used microsatellite loci to assess the genetic diversity, mating system, effective size, and spatial genetic structure of a large population of *C. estrellensis*, with the goal of supporting genetic conservation plans for the species.

3.2 MATERIAL AND METHODS

Study site and sampling

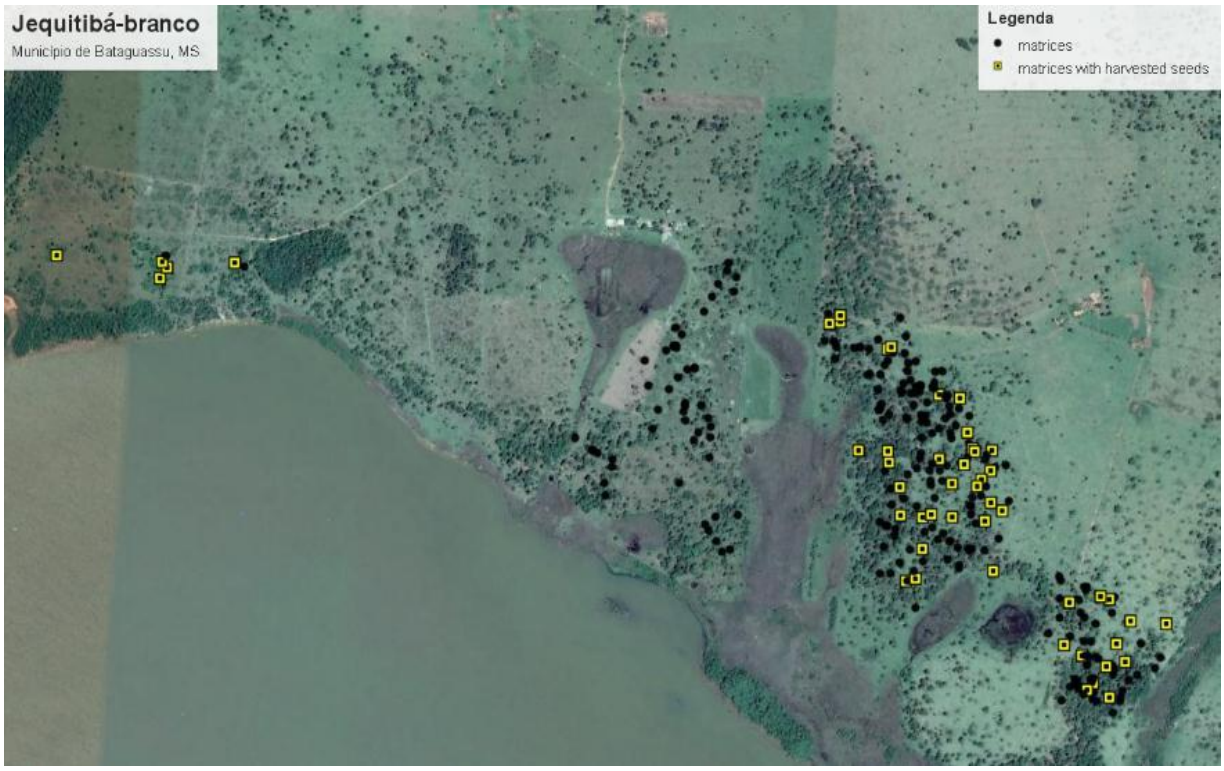
Within a Savanna landscape composed of forest fragments and isolated trees (Figure 2), we sampled all 285 *C. estrellensis* trees found in the study area of 448.2 ha. The study site has an average altitude of 273.3 m and it is located in the city of Bataguassu, Mato Grosso do Sul State, Brazil, near the Pardo River. Adult trees are distributed across two privately owned farms "Olhos d' Água" and "Vó Ida" (21° 38'00" S, 52° 14'02" W). The population is isolated, surrounded by monoculture crops (sugar cane and *Eucalyptus*), pasture, and urban development. The predominant soil in the region is dark red Oxisols of medium texture and low natural fertility. The climate is tropical humid with rainy summers and dry winters. The average temperature is 23.1°C, with annual rainfall ranging from 1200 to 1500 mm, and the region is characterized as a transition zone between the Savanna and Atlantic Forest biomes (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA- IBGE, 2016).

All adult trees were georeferenced (GPS-Garmin), measured for total height and diameter at breast height (DBH), sampled (foliar or cambial tissue), and genotyped. We also collected 32 seeds from each of 20 maternal trees. The seeds were planted in a nursery and foliar tissue samples were later taken from seedlings for genotyping (Appendix 5.1, 5.2, 5.3, 5.4, 5.5 and 5.6).

Microsatellite analysis

All genetic analyses (adult trees and progeny) were done in the HERÉDITAS/GENOMAX laboratory using nine microsatellite loci based on the 15 loci previously developed for the species by (GUIDUGLI et al., 2009).

Figure 2- Location of the sampled *Cariniana estrellensis* individuals in the study site.



Source: Prepared by author (2015).

Genetic diversity and inbreeding

Genetic diversity for adults and seeds was assessed for each locus and as an average across loci for the following indices: total number of alleles per locus (k); allelic richness for 280 genotypes (R) using a rarefaction method (EL MOUSADIK; PETIT, 1996); and observed (H_o) and expected (H_e) heterozygosity according to Hardy-Weinberg equilibrium. The presence of inbreeding was evaluated based on the fixation index (F). The statistical significance of F was tested using Monte Carlo permutation of alleles between individuals (1000 replicates). All analyses were performed using the FSTAT program (GOUDET, 1995). For adults and seeds, we also estimated the frequency of null alleles ($Null$) under a population inbreeding model (PIM) using the INEST 2.0 software (CHYBICKI; BURCZYK, 2009). However, because each plant within a family receives at least one maternal allele, the within family fixation index ($F_o = 1-(H_o / H_e)$) can be biased due to an overestimation of gene frequencies of maternal alleles. This results in an overestimation of the expected heterozygosity under Hardy-Weinberg equilibrium ($H_e = 1-\sum_{i=1}^{k} p_i^2$, where p_i is the frequency of the $i-th$ allele), because high frequency alleles contribute more to the estimate of H_e than low frequency alleles. Thus, H_e was estimated based on the gene frequencies of

the pollen donor parents, calculated using the MLTR 3.4 software (RITLAND, 2002), and H_o was estimated for seeds using the FSTAT program (GOUDET, 1995).

Intrapopulation spatial genetic structure

We assessed intrapopulation spatial genetic structure (SGS) based on the coancestry coefficient (Θ_{xy}) between pairs of trees in previously determined distance classes, as described in Loiselle et al. (1995). The statistical significance of the coancestry coefficient (Θ_{xy}) was obtained by comparing the limits of the confidence interval at 95% probability for the average coancestry for each distance class, calculated by Monte Carlo permutations of individuals between distance classes. The coancestry coefficient and the standard error were estimated using the SPAGEDI program (HARDY; VEKEMANS, 2002). To compare SGS between adult trees, the Sp statistic (VEKEMANS; HARDY, 2004) was calculated as $Sp = -b_k /(1-\Theta_l)$, where Θ_l is the coancestry coefficient calculated between all pairs of individuals in the first distance class (0-25 m) and b_k is the slope of regression of the coancestry coefficient on the logarithm of spatial distance between plants (0-1000 m). To test for significance of SGS, the spatial position of each individual was permuted 1000 times to obtain the frequency distribution of b_k under the null hypothesis that Θ_{xy} and $\ln(d_{xy})$ are uncorrelated.

Mating system

The mating system analysis was based on the mixed mating model (RITLAND; JAIN, 1981) and correlated mating model (RITLAND, 1989) and was carried out using the MLTR program (RITLAND, 2002). The analyses were estimated at the maternal tree level and at the population level. The Maximization Expectation (ME) numerical method was used at the maternal tree level and the Newton-Raphson method at the population level. The estimated indices were multilocus outcrossing rate (t_m), single-locus outcrossing rate (t_s), outcrossing rate between related individuals ($t_m - t_s$), correlation of selfing (r_s), correlation of t among loci (r_l), and multilocus paternity correlation (r_p). As our sample was hierarchical, the multilocus paternity correlation also was also estimated within ($r_{p(w)}$) and among ($r_{p(a)}$) fruit. The confidence interval of the estimates was obtained by 1000 bootstrap resampling. For the population analysis, the resampling units were the progenies and for individual analysis, resampling was within progeny. These indices were also used to estimate the effective

number of pollen donors, $N_{ep} = 1/r_p$ (RITLAND, 1989). In order to determine the genetic structure of progenies, we calculated the average coancestry coefficient (Θ) among plants within progenies, as described by Sebbenn (2006). The genetic representation within progenies was measured by the variance effective size (N_e) based on sample variance of allelic frequencies (COCKERHAM, 1969). The number of trees necessary for seed collection was calculated assuming that the objective was to retain an effective reference size of 150 in the total sample, $m = N_{e(r)} / N_e$ (SEBBENN, 2006).

Statistical analyses

To investigate whether the indices R, H_o, H_e, and F were significantly different between adults and seeds, we used the t-test based on loci information (SOKAL; ROHLF, 1981). To investigate whether the selfing rate ($s = 1 - t_s$) plus mating among relatives ($t_m - t_s$) decreased F, and if k and H_o increased F within families, we used the Spearman ranking correlation with the SAS program (SAS, 1999).

3.3 RESULTS

Genetic diversity and inbreeding

For the entire sample of 925 *C. estrellensis* genotypes (285 adults trees + 640 seeds), we found a total of 148 alleles. The total number of alleles (k) for adults was 75 and 73 in seeds (Table 4). The allelic richness (R), observed heterozygosity (H_o), and expected heterozygosity (H_e) were similar between adults (R = 8.3; H_o = 0.648; H_e = 0.686) and seeds (R = 7.8; H_o = 0.640; H_e = 0.682). The fixation index (F) was significantly (P< 0.05) higher than zero and similar between adults (0.058) and seeds (0.064), suggesting the occurrence of inbreeding. Based on the t-test, the indices R, H_o, and H_e were significantly (P< 0.05) higher and F was significantly lower in adults than in seeds. The expected frequency of null alleles ranged in adults from 0.031 to 0.150 and in seeds we detected an absence of null alleles.

Table 4- Genetic diversity in adult trees (A) and seeds (S); n is the sample size; k is the number of alleles per locus; R is the allelic richness for 280 genotypes; H_o is the observed heterozygosity; H_e is the expected heterozygosity; F is the fixation index; *Null* is the expected frequency of null alleles.

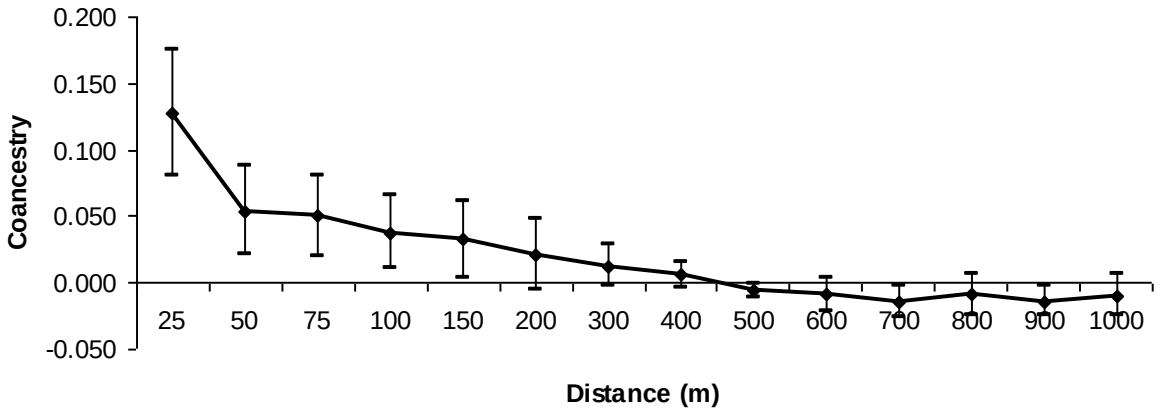
	n		k		R		H_o		H_e		F		*Null*	
Locus	A	S	A	S	A	S	A	S	A	S	A	S	A	S
Ces01	281	638	8	8	8.0	8.0	0.530	0.575	0.766	0.737	0.308*	0.220*	0.15	0
Ces02	284	640	6	5	6.0	5.0	0.673	0.602	0.670	0.636	-0.003	0.055	0	0
Ces04	280	640	20	17	20.0	15.6	0.839	0.870	0.883	0.876	0.049	0.007	0	0
Ces09	282	640	4	5	4.0	4.9	0.461	0.517	0.447	0.496	-0.032	-0.042	0	0
Ces10	284	639	7	6	7.0	6.0	0.613	0.548	0.587	0.544	-0.043	-0.006	0	0
Ces11	284	640	12	13	12.0	12.7	0.789	0.762	0.774	0.761	-0.019	-0.002	0	0
Ces13	284	640	5	6	5.0	5.4	0.771	0.766	0.776	0.78	0.006	0.019	0	0
Ces14	281	640	5	5	5.0	4.9	0.345	0.405	0.480	0.523	0.281*	0.227*	0.13	0
Ces18	282	640	8	8	8.0	8.0	0.812	0.713	0.793	0.789	-0.025	0.097	0	0
Mean	282	640	8.3	8.3	8.3	7.8	0.648	0.640	0.686	0.682	0.058*	0.064*	0.03	0
Se	-	-	24.7	17.6	1.6	1.2	0.030	0.022	0.022	0.019	0.018	0.010	-	-
Total	-	-	75	73	-	-	-	-	-	-	-	-	-	-

*P< 0.05. SE is the standard error.
Source: Prepared by author.

Intrapopulation spatial genetic structure

We found significant spatial genetic structure (SGS) up to approximately 150 m, with the mean pairwise coancestry coefficient decreasing to non-significant or significantly lower than zero in the other distance classes, a typical pattern of isolation by distance (Figure 3). The SGS intensity measured by the S_p statistic was 0.0107.

Figure 3- Correlogram of the average coancestry coefficient (Θ_{xy}) for 14 distance classes of *Cariniana estrellensis* adults. The continuous line represents the average coancestry coefficient with their respective confidence intervals at 95% of the distribution of the mean.



Source: Prepared by author.

Mating system at the population level

The fixation index for maternal trees (F_m = -0.041) was not significantly different from zero (Table 5). The estimate of the population multilocus outcrossing rate (t_m = 0.970) was not significantly different from unity (1.0), suggesting an absence of selfing. The single-locus outcrossing rate (t_s = 0.908) was significantly lower than unity (1.0) and the difference $t_m - t_s$ (0.062) was significantly greater than zero, indicating mating among relatives. The selfing correlation (r_s = 0.031) was not significantly different from zero. The fraction of apparent selfing due to uniparental inbreeding (r_l = 0.373) was significantly higher than zero and indicates that the inbreeding detected in seeds was mainly the result of mating among relatives (1-r_l = 0.627). The paternity correlation among and within fruit ($r_{p(m)}$ = 0.246) was significantly greater than zero, suggesting a limited number of effective pollen donors that fertilized the seed trees (N_{ep} = 4.1). The coancestry coefficient (Θ) was 0.162 and the variance effective size (N_e) within families was 2.89. This result indicates that it is necessary

to collect seeds from 52 seed trees (m) to retain an effective size of 150 in progeny arrays. For hierarchical analysis of paternity correlation, we assessed the multilocus paternity correlation within ($r_{p(w)}$) and among fruit ($r_{p(a)}$). The results were significantly greater than zero ($r_{p(w)}$ = 0.835; $r_{p(a)}$ = 0.062), indicating that the effective number of pollen donors was lower within ($N_{ep(w)}$ = 1.2) than among fruit ($N_{ep(a)}$ = 16.1).

Table 5- Mating system indices at the population level (95% CI is the confidence interval at 95% probability).

Index	Mean (95% CI)
Fixation index of mother trees: F_m	-0.041 (-0.163–0.010)
Multilocus outcrossing rate: t_m	0.970 (0.952–1.000)
Single-locus outcrossing rate: t_s	0.908 (0.875–0.954)
Mating among relatives: $t_m - t_s$	0.062 (0.033–0.077)
Selfing correlation: r_s	0.031 (-0.999–0.064)
Correlation of s (or t) among loci: r_l	0.373 (0.113–0.847)
Multilocus paternity correlation (within and among fruit): r_p	0.246 (0.193–0.291)
Effective number of pollen donors: N_{ep}	4.1 (3.4–5.2)
Coancestry coefficient: Θ	0.162 (0.154–0.169)
Variance effective size: N_e	2.89 (2.78–2.99)
Number of seed trees: m	52 (50–54)
Multilocus paternity correlation (within fruit): $r_{p(w)}$	0.835 (0.758–0.899)
Multilocus paternity correlation (among fruit): $r_{p(a)}$	0.062 (0.018–0.093)
Effective number of pollen donors (within fruit): $N_{ep(w)}$	1.2 (1.1–2.3)
Effective number of pollen donors (among fruit): $N_{ep(a)}$	16.1 (10.8–55.6)

Source: Prepared by author.

Genetic diversity, inbreeding, and mating system at the progeny level

For mother trees from which seeds were collected, the fixation index (F_m) ranged from -0.39 to 0.34, total number of alleles (k) ranged from 30 to 42, observed heterozygosity (H_o) from 0.53 to 0.74, and the fixation index within progenies (F_o) ranged from -0.10 to 0.25 (Table 6). The multilocus outcrossing rate (t_m) was significantly different from unity (1.0) in 9 of the 20 progeny. The rate of mating among relatives ($t_m - t_s$) ranged among progenies from 0.02 to 0.14. The multilocus paternity correlation within and among fruit (r_p) ranged from 0.12 to 0.38. The within fruit paternity correlation ($r_{p(w)}$) ranged from 0.48 to 1.00 and among fruit ($r_{p(a)}$) from 0.03 to 0.23. Therefore, the effective number of pollen donors within and among fruit (N_{ep}) ranged from 2.6 to 8.8. Within fruit, the $N_{ep(w)}$ ranged from 1.0 to 2.1, which is lower than the $N_{ep(a)}$ among fruit, which ranged from 4.4 to 37.0. Consequently, the coancestry coefficient within progenies (Θ) ranged from 0.142 to 0.202 and the variance effective size (N_e) ranged from 2.38 to 3.25.

Spearman correlation

We found no association between the combined selfing rate and mating among related individuals [$s + (t_m - t_s)$] and k (r= -0.309; P= 0.184). The spearman ranking correlation was significantly negative for $s + (t_m - t_s)$ compared to H_o (r= -0.842; P= 0.000) and significantly positive for $s + (t_m - t_s)$ and F_o (r= 0.808; P< 0.000).

Table 6- Genetic diversity, inbreeding and mating system indices for open-pollinated progeny arrays (n= 32).

Mother tree	F_m	t_m ±SE	$t_m - t_s$ ±SE	r_p ±SE	N_{ep}	k	H_o	F_o	Θ	N_e	$r_{p(w)}$ ±SE	$r_{p(a)}$ ±SE	$N_{ep(w)}$	$N_{ep(a)}$
1	0.07	0.93±0.02	0.05±0.02	0.34±0.03	2.9	33	0.61	0.11	0.191	2.47	0.81±0.39	0.23±0.02	1.2	4.4
5	0.30	0.99±0.01	0.09±0.01	0.12±0.01	8.3	41	0.65	0.08	0.184	2.56	0.56±0.27	0.05±0.01	1.8	21.7
15	0.04	0.96±0.00	0.03±0.01	0.15±0.01	6.8	40	0.73	-0.10	0.157	2.99	0.76±0.36	0.03±0.01	1.3	30.3
19	0.26	0.94±0.00	0.05±0.01	0.15±0.02	6.8	35	0.63	0.03	0.198	2.40	0.48±0.22	0.09±0.02	2.1	10.6
21	-0.00	0.99±0.01	0.10±0.01	0.14±0.01	6.9	38	0.53	0.25	0.145	3.13	0.68±0.32	0.05±0.01	1.5	22.2
22	0.02	0.99±0.01	0.06±0.01	0.15±0.01	6.7	42	0.68	-0.03	0.149	3.13	0.76±0.36	0.03±0.01	1.3	33.3
31	-0.00	0.99±0.01	0.09±0.01	0.12±0.01	8.4	41	0.59	0.15	0.142	3.22	0.49±0.23	0.04±0.01	2.0	26.3
35	-0.05	0.99±0.01	0.14±0.03	0.16±0.01	6.1	34	0.53	0.23	0.147	3.09	0.89±0.42	0.05±0.01	1.1	18.5
45	-0.04	0.96±0.00	0.05±0.01	0.15±0.01	6.9	34	0.69	-0.04	0.151	3.09	0.83±0.40	0.03±0.01	1.2	31.2
64	0.34	0.99±0.00	0.04±0.01	0.19±0.01	5.4	39	0.74	-0.10	0.202	2.37	0.98±0.47	0.03±0.01	1.0	37.0
66	-0.01	0.91±0.00	0.09±0.04	0.38±0.07	2.6	30	0.53	0.25	0.188	2.48	1.00±0.48	0.22±0.05	1.0	4.50
88	0.19	0.93±0.00	0.09±0.03	0.23±0.03	4.3	35	0.61	0.05	0.199	2.38	0.94±0.44	0.08±0.02	1.1	12.3
91	-0.20	0.94±0.00	0.02±0.01	0.23±0.02	4.4	32	0.66	0.03	0.166	2.83	0.82±0.39	0.13±0.01	1.2	7.75
96	0.22	0.93±0.00	0.01±0.01	0.14±0.01	7.0	40	0.71	-0.09	0.192	2.48	0.86±0.41	0.03±0.01	1.2	37.0
101	0.01	0.99±0.01	0.07±0.01	0.11±0.01	8.8	38	0.68	0.01	0.142	3.25	0.75±0.36	0.03±0.01	1.3	32.3
119	0.25	0.99±0.01	0.07±0.01	0.24±0.03	4.1	32	0.69	-0.04	0.196	2.44	1.00±0.48	0.10±0.01	1.0	10.0
123	-0.39	0.99±0.01	0.09±0.01	0.19±0.01	5.2	36	0.65	0.04	0.151	3.08	0.77±0.37	0.08±0.01	1.3	12.8
125	-0.26	0.99±0.01	0.13±0.02	0.22±0.02	4.5	37	0.59	0.16	0.155	2.98	0.72±0.35	0.13±0.02	1.4	7.7
136	-0.34	0.99±0.01	0.08±0.01	0.16±0.01	6.4	34	0.65	0.04	0.146	3.17	0.87±0.42	0.04±0.01	1.1	22.7
225	0.13	0.85±0.01	0.02±0.00	0.13±0.01	7.6	41	0.65	0.05	0.200	2.38	0.59±0.28	0.07±0.01	1.7	13.5

± SD is the standard deviation; * P< 0.05; F_m and F_o are the fixation index for maternal trees and progenies, respectively; k is the total number of alleles; H_o is the observed heterozygosity; t_m is the multilocus outcrossing rate; $t_m - t_s$ is rate of mating among relatives; r_p, $r_{p(w)}$, and $r_{p(a)}$ are the correlations of paternity within and among fruit, within fruit, and among fruit, respectively; N_{ep}, $N_{ep(w)}$, and $N_{ep(a)}$ are the effective numbers of pollen donors among and within fruit, within fruit, and among fruit, respectively; Θ and N_e are the coancestry coefficient and the variance effective size within progenies, respectively. Source: Prepared by author.

3.4 DISCUSSION

Genetic diversity and inbreeding

Our results show that the studied *C. estrellensis* population presents higher levels of genetic diversity among adults than seeds due to selfing and mating among relatives [$s + (t_m - t_s)$]. However, this pattern may vary over time as a result of inbreeding depression, or mortality of inbred seeds between seed and adult stages, as has been observed in the congener *Cariniana legalis* (TAMBARUSSI et al., 2016). Genetic diversity within populations has important implications for species survival because maintaining this diversity is the basis for species conservation (YEEH et al., 1996). High levels of genetic diversity are desirable because they enable populations to better adapt to local conditions and colonize new environments, due to the substantial potential for genotypic recombination in subsequent generations (SEOANE et al., 2000).

Intrapopulation spatial genetic structure

The *C. estrellensis* population analyzed herein presents spatial genetic structure (SGS); seeds are dispersed and established near to maternal trees and in general trees are genetically related up to a distance of 150 m (Figure 3). Within the population, the coancestry coefficient (Θ_{xy}) of adult trees diminishes with an increase in the distance between trees, as expected in a classic model of isolation by distance. SGS is determined by various factors, such as ecological processes, sexual and mating system, and the dispersion of pollen and seeds. However, the absence of SGS beyond 150 m does not mean that at these distances individuals are unrelated; trees may be related but they are not grouped (LOISELLE et al., 1995; HARDY et al., 2006; SATO et al., 2006; BORN et al., 2008). Another factor that must be considered is that the *C. estrellensis* population is located in an extensively fragmented forest landscape and deforestation in the area may have modified the original SGS. The pattern of SGS found in this study is consistent with that expected for natural tree populations (DEGEN; SEBBENN, 2014) and similar to the results reported for many tropical trees species, such as *Genipa americana* (SEBBENN; KAGEYAMA; VENCOVSKY, 1998), *Symphonia globulifera* (DEGEN et al., 2004), *Araucaria angustifolia* (BITTENCOURT; SEBBENN, 2007), *Myracrodruon urundeuva* (GAINO et al., 2010), *Copaifera langsdorffii* (SEBBENN et al., 2011; TARAZI et al., 2013), and *C. estrellensis* (GUIDUGLI et al., 2016).

Mating system at the population level

Our estimates of the multilocus outcrossing rate (t_m) at the population and family levels indicate that seeds originate mainly from outcrossing (minimum of 0.85). This is similar to results previously reported for another population of the species (t_m = 0.999, GUIDUGLI et al., 2016) and indicates that the species is self-compatible. Outcrossing favors the continuation of genes and genetic recombination among adults and maintains the genetic diversity in populations (MANOEL et al., 2012). The same mating pattern of a predominance of outcrossing has also been found in the congener *C. legalis* (SEBBENN et al., 2000; TAMBARUSSI et al., 2016).

The rate of mating among relatives ($t_m - t_s$ = 0.062) can be explained by the detected SGS and suggests some mating between related individuals located at distances shorter than 150 m. The paternity correlation among and within fruit (r_p) showed that, on average, a low number of pollen donors fertilized the seed trees (N_{ep} = 4.1). Furthermore, the hierarchical analysis of the paternity correlation among ($r_{p(a)}$) and within ($r_{p(w)}$) fruit shows that there is a greater probability of finding full-sib individuals within fruit ($r_{p(w)}$ = 0.835) than among fruit ($r_{p(a)}$ = 0.062). Thus, the effective number of pollen donors is higher among ($N_{ep(a)}$ = 16.1) than within fruit ($N_{ep(w)}$ = 1.2). Hierarchical studies of paternity correlation for tree species have shown that individual fruits are pollinated by a lower number of pollen donors than the fruit within a tree, as has been found for *Centaurea corymbosa* (HARDY et al., 2004), *Ceiba pentandra* (LOBO et al., 2005), *Theobroma cacao* (SILVA et al., 2011), *Tabebuia roseo-alba* (FERES et al., 2012), *Genipa americana* (MANOEL et al., 2015), *C. estrellensis* (GUIDUGLI et al., 2016), and *C. legalis* (TAMBARUSSI et al., 2016).

Genetic diversity, inbreeding and mating system at the progeny level

We found greater levels of inbreeding in adults than families (F_o), with a higher level of inbreeding in seeds (Table 7). The presence of endogamy may compromise the long-term survival of individuals (GAINO et al., 2010). The observed heterozygosity (H_o) was high with the largest value being 0.73, suggesting that the population adheres to the proportions of the Hardy-Weinberg equilibrium. The seeds collected within the fragment show significant values of $t_m - t_s$, r_p, $r_{p(w)}$ and $r_{p(a)}$.

Due to selfing, correlated mating, and inbreeding in some seed trees and families, the coancestry coefficient (Θ) was higher and the variance effective size (N_e) within progeny was lower than expected in half-sibling progenies (Θ = 0.125; N_e = 4). Consequently, for seed collection for *ex situ* conservation, environmental reforestation, and tree breeding, seeds must be collected from at least 52 seed trees.

3.5 CONCLUSIONS

The genetic diversity is higher and inbreeding is lower in adults than seeds due to selfing and mating among relatives. Mating among related individuals is the result of the SGS found in the population. In terms of seed collection for *ex situ* conservation and environmental restoration, due to higher levels of coancestry within than among fruit, seeds should be collected from several fruit of each maternal tree to reduce the levels of coancestry and increase the variance effective size in the progeny arrays. The minimum number of seed trees indicated for seed collection is 52. Furthermore, due to the presence of SGS, seed trees chosen for seed collection must be located at least 150 m apart within the population to avoid the collection of seeds from related individuals.

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4 POLLEN FLOW AND DISPERSION IN *Cariniana estrellensis* (Raddi) Kuntze (LECYTHIDACEAE)

ABSTRACT

The drastic reduction in the size of tropical forests through deforestation and forest fragmentation has spatially isolated many populations and individuals. This, in turn, can result in smaller effective population sizes and may lead to reproductive isolation due to decreases in pollen and seed flow. Gene flow can increase genetic diversity and maintain the evolutionary potential of tree species populations. In this study, we use nine microsatellite loci to investigate the effects of spatial isolation caused by forest fragmentation on pollen flow and dispersal patterns in a large *Cariniana estrellensis* population. All 285 adult trees found in the study area of 448.2 ha, located in a Savanna biome in Mato Grosso do Sul State, Brazil, were mapped, sampled, measured for height and diameter at breast height (DBH), and genotyped. We also collected seeds from 20 trees, at 32 seeds per tree. The parentage analysis detected 9.4% pollen immigration into the population. The average pollen dispersal distance was 597 m, effective pollination neighbor area (A_{ep}) was 298 ha, and effective pollination radius (r_{ep}) was 974 m. The pollen dispersion Kernel can be explained by an exponential power dispersal curve, indicating a pattern of isolation by distance, but with a long tail, suggesting some instances of long distance dispersal. Our results show that the ideal seed collection strategy is to collect seeds from trees at distances greater than 600 m to avoid collecting paternally related seeds from different trees.

Keywords - Gene Flow. Microsatellite loci. Parentage analysis. Tropical forests.

4.1 INTRODUCTION

In the last few decades, extensive deforestation and fragmentation of tropical forests has resulted in the spatial isolation of forest populations and individuals. This isolation can result in the reproductive isolation of populations and individuals because of decreased gene flow through pollen and seeds (TOWSEND et al., 2006). One of the most dramatic consequences of fragmentation is the extinction of populations and species. Excessive exploitation of an environment can trigger a decline in biodiversity and such a loss of species may be irreversible (PRIMACK; RODRIGUES, 2006).

The movement of genes between populations directly influences a population's genetic structure and gene flow has an important evolutionary role in introducing new alleles into populations and maintaining the genetic potential of tree species to the respond to environmental disturbances (NASON; HAMRICK, 1997; AGUILAR et al., 2008; KRAMER et al., 2008). Thus, studies involving estimates of the spatial and temporal dynamics of pollen and seed flow are fundamental for understanding the genetic changes resulting from fragmentation in tropical tree species populations. Furthermore, this information can help ensure the survival of species and support the effective conservation of forest remnants (HAMILTON, 1999).

Having a solid understanding of the effective pollen dispersal distances and mating system as a function of tree density are fundamental in the development of strategies for the management and conservation of tree populations. A reduction in population size associated with low population density tends to limit the number of reproductive individuals. Further, if the movement of pollination and seed dispersal vectors is also affected by fragmentation, populations can become genetically and demographically isolated, with small effective sizes and species vulnerable to local extinction (NASON; HAMRICK, 1997; SORK et al., 2002).

Gene flow can be quantified by direct and indirect analysis through genetic studies based on molecular marker data. Indirect methods use genetic differentiation among populations (F_{ST}), presence of private alleles, and spatial genetic autocorrelation based on historical gene flow or gene flow that occurred in the past. Direct methods are more robust and use contemporary, or present day, gene flow inferred through parentage analyses (maternity and paternity) based on highly polymorphic neutral and co-dominant genetic marker data, such as microsatellite loci (ASHLEY, 2010; ELLSTRAND, 2014).

Cariniana estrellensis (Raddi) Kuntze (Lecythidaceae), known as jequitibá-branco, is a slow growing tree with a wide geographic distribution. The species occurs mainly in Atlantic and subtropical forests along the coast of Brazil, from southern Bahia to Rio Grande do Sul State, as well as in Bolivia, Paraguay, and Peru (CARVALHO, 2003a; LEITE, 2007). Adult trees can reach 35 to 45 m in height and 90 to 120 cm in diameter at breast height (LORENZI, 2002). Their hermaphroditic flowers are pollinated mainly by bees of the genera *Melipona* and *Trigona*. The flowering and ripening of fruit varies according to geographic location across its range. Monkeys remove the operculum (lid) from the fruit, thus facilitating seed dispersal by wind (CARVALHO, 2003b). The species is currently included on the list of endangered species as vulnerable (FIGLIOLIA et al., 2000).

In this context, we used microsatellite loci to assess the effects of spatial isolation due to forest fragmentation on patterns of pollen flow in a population of *C. estrellensis*. We specifically address the following questions: *i*) What is the rate of pollen immigration and the distance and patterns of pollen dispersal within the large fragmented landscape? *ii*) Does a reduction in population size due to fragmentation affect the population mating system, for example, by increasing the rate of selfing and correlated mating within the population?

4.2 MATERIAL AND METHODS

Study site and sampling

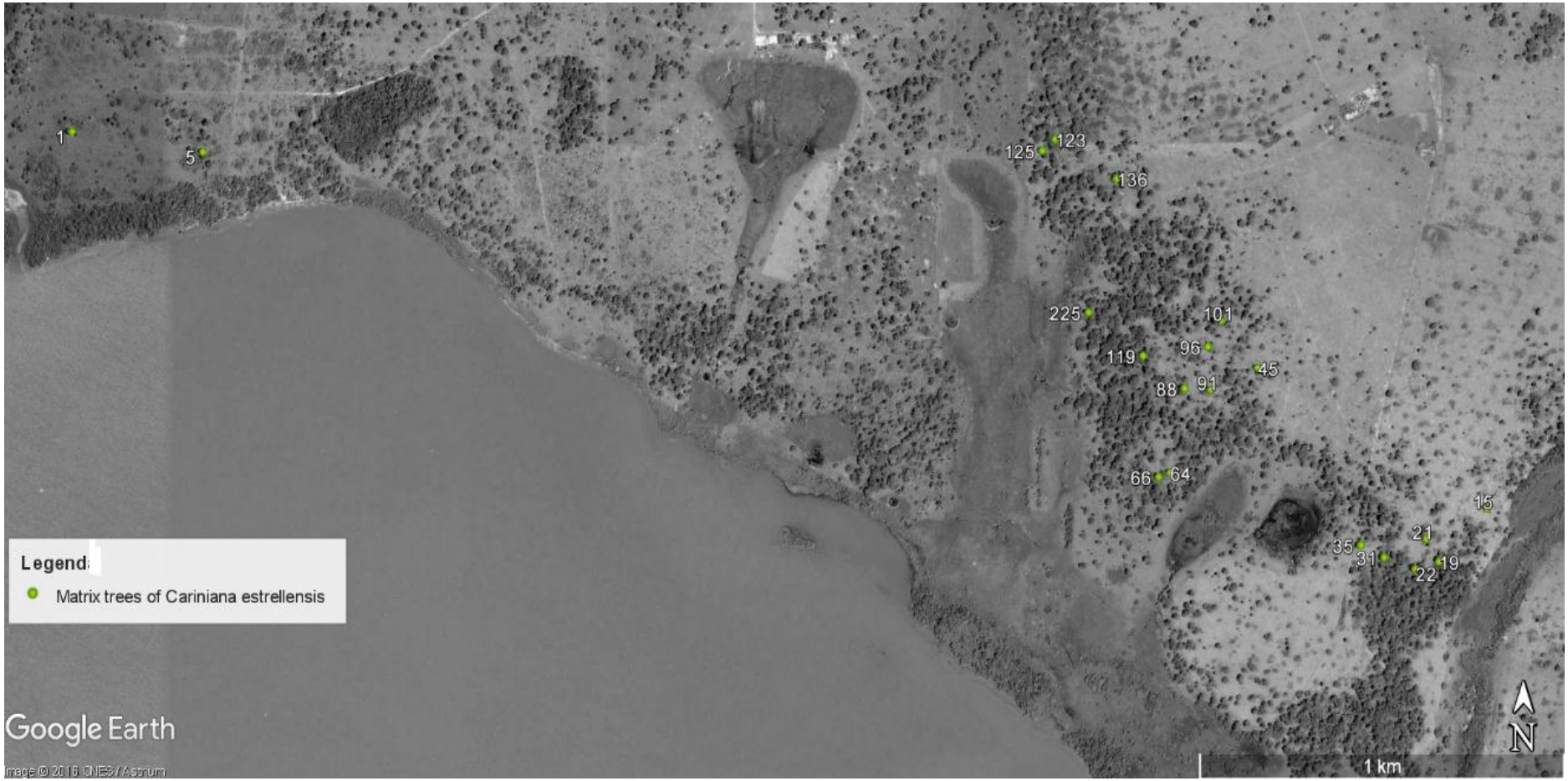
The study was conducted in a forest fragment (21° 38’00” S, 52° 14’02” W, average altitude of 273.3 m) located in the city of Bataguassu, Mato Grosso do Sul State, Brazil. The study site is situated alongside the Pardo River and is surrounded by monoculture crops and pastures. The 448.2 hectare fragment occurs in a transition zone between the Savanna and Atlantic Forest biomes (Figure 4). The predominant soil in the area is dark red Oxisols, with medium texture and low natural fertility. The climate is tropical humid with rainy summers and dry winters. The average temperature is 23.1°C and during the coldest months, temperatures range from 15 to 20ºC. Annual rainfall varies from 1200 to 1500 mm (IBGE, 2016).

All individuals of *C. estrellensis* found in the fragment were mapped and measured for full height and diameter at breast height (DBH). We then sampled foliar or cambial tissue of adult trees and progenies after germination for molecular analyses. The total sample included 285 adult trees and 640 seeds (32 seeds collected from each of 20 selected maternal trees) (Appendix 5.1, 5.2, 5.3, 5.4, 5.5 and 5.6).

Microsatellite analysis

The genotyping of microsatellite markers was performed at the laboratory HERÉDITAS/GENOMAX, Technology in DNA. Of the 15 microsatellite loci developed for the species (GUIDUGLI et al., 2009), we selected nine loci for use in this study. The genomic analysis technique was based on Polymerase Chain Reaction (PCR) amplification for primers of a defined sequence (microsatellites) and detection of alleles by laser fluorescence in an ABI 3100XL automatic sequencer. Based on these genotypes, a multilocus profile was defined that allowed us to identify each sample individually and carry out population genetic analyses.

Figure 4- Spatial distribution of *Cariniana estrellensis* trees sampled in the forest fragment.



Source: Prepared by author (2015).

Parentage analysis

We estimated contemporary pollen flow using the paternity analysis implemented in the CERVUS 3.0 software (MARSHALL et al., 1998). The paternity analysis was based on the genotypes of all adult trees and seeds in the population. To determine the putative pollen donors for seeds, we used all adult trees as potential pollen candidate parents. Paternity of each seed was determined based on the Δ statistic (MARSHALL et al., 1998), defined as the difference between the "LOD score" of the first most likely father candidate and the "LOD score" of the second most likely candidate. The significance was determined by paternity test simulations in CERVUS. The cryptic Δ was estimated based on a confidence level of 95%, as suggested by Marshall et al. (1998), using 10,000 repetitions, 0.01 as the ratio of genotyping errors, and 50% as the proportion of pollen donors sampled within the population. If a seed had no potential pollen donor within the population, the seed was considered as having received pollen from outside the population (pollen immigration). In the analysis, we also considered the possibility of self-fertilization (s) which was estimated as the proportion of seeds identified as having the same mother tree as pollen donor (n_s) in relation to the total number of sampled seeds (n). The standard error of the mean rate of selfing was estimated assuming a binomial distribution, as SE (s) = $\sqrt{s(1-s)/m_{st}}$, where m_{st} is the number of sampled maternal trees in the fragment (SLAVOV et al., 2005). The pollen immigration rate (m_p) was calculated as the proportion of seeds for which a pollen donor was not found within the population (n_i) relative to total number of sampled seeds (n), as $m_p = n_i/n$ (BURCZYK et al., 2004). As all sampled trees were genotyped and their spatial position known (x and y coordinates), the seeds assigned to a pollen donor were used to determine the minimum, maximum, mean, and median pollen dispersal distance, as well as the standard deviation of pollen dispersal. Pollen dispersal distance (D) was calculated as Euclidean distance between two points: $D = \sqrt{(x_i - x_j)^2 + (y_i - y_j)^2}$, where x_i and y_i are the spatial coordinates of the mother tree, and x_j and y_j are the spatial coordinates of the assigned pollen donor. To determine whether reproductive success was a function of the distance between trees, we compared the frequency distribution of pollen dispersal with the frequency distribution of the distance between all trees using the Kolmogorov-Smirnov test (SOKAL; ROHLF, 1995). The effective pollination area (A_{ep}) was calculated for the population assuming a circular area around the seed tree ($A_{ep} = 2\pi\sigma^2$, LEVIN, 1998), where σ^2 is the axial variance of pollen

dispersal. The effective ratio of pollen dispersal was estimated as $A_{ep} = \sqrt{A_{ep}/3.1415}$ (AUSTERLIZ; SMOUSE, 2001). It is important to note that the parameter A_{ep} corresponds to the circular area in which 63% of pollen donors that fertilized a mother tree are expected to be located (LEVIN, 1998).

Pollen dispersal Kernel

A maximum likelihood procedure was used to determine the pollen dispersal distance probability density function based on seedling (n = 640) and adult genotypes (n = 285) (Table 9), with the NEIGHBOURHOOD model (BURCZYK et al., 2002) implemented in the NM+ 1.1 software (CHYBICKI; BURCZYK, 2010). The NEIGHBOURHOOD model uses a maximum-likelihood fractional paternity assignment approach where, for each sampled seed, paternity may result from: 1) a paternal tree located outside the study plot due to pollen immigration, with probability m_p; or 2) a paternal tree located within the study plot, with probability 1-m_p. In the latter case, seeds may be the result of selfing, with probability s. The NEIGHBOURHOOD parameter was set to 'infinite' to include all sampled adults in our study as the NEIGHBOURHOOD size (CHYBICKI; BURCZYK, 2010). Pollen dispersal was modeled using Exponential, Weibull, Geometric, and Bivariate Student t (2Dt) distribution (AUSTERLITZ et al., 2004; CHYBICKI; BURCZYK, 2010; OTTEWELL et al., 2012; CÔRTES et al., 2013), which provides the a and b parameters (scale and shape, respectively) from which the average pollen dispersal distance (δ_p) is estimated. The shape parameter b describes the shape of the dispersal kernel tail. Results of b< 1 indicate a fat-tailed dispersal, meaning that long range decline of probability is low; b> 1 indicates that the dispersal is thin-tailed, with a rapid decrease of the dispersal function, implying few long distance dispersal events (AUSTERLITZ et al., 2004).

4.3 RESULTS

Parentage analysis

Of the 640 sampled seeds, a putative pollen donor was found for 580 seeds, indicating a pollen immigration rate (m_p) of 9.4% (n = 60) from outside the sample population (Table 7). Of the seeds assigned a pollen donor within the sample area, 27 showed the same pollen donor as mother tree, indicating a mean selfing rate (s) of 4%, 84 seeds were assigned a pollen donor who is a related individual (t_p =13%), and 529 showed mating among unrelated individuals (t_u = 83%) (Table 8). The mean pairwise coancestry for related individuals (Θ_r) was 0.18 and for unrelated individuals Θ_u was -0.01. The mean distance between mother trees and related pollen donors for inbred seeds was 583 m. The mean fixation index for selfed seeds (F_s) was 0.51. The fixation index for outcrossed seeds resulting from mating among related individuals (F_p) was 0.25, and for unrelated individuals F_u was 0.03.

Pollen dispersal distance

The mean pollen dispersal distance ranged from 20 to 3519 m. The effective pollination area (A_{ep}) was 298 ha, with an effective pollination radius (r_{ep}) of 974 m (Table 7). Based on the Kolmogorov-Smirnov test, the comparison between the distribution curve of pollen dispersal frequencies and the distance frequency curve between all trees (D=0.254, P = 0.001, Figure 5) indicates that the curves are statistically different. Therefore, the distance between trees does not explain the pollen dispersal pattern.

Table 7- Results of pollen flow, distance, and dispersal patterns for *Cariniana estrellensis*.

Family	n	m_p (proportion)	Within	Within outcross	Pollen distance				
					Mean ± SE (m)	Median (m)	Min/Max (m)	A_{ep} (ha)	r_{ep} (m)
287	32	4 (0.125)	28	26	1792 ± 213	2161	378/3320	740	1535
320	32	4 (0.125)	28	28	1480 ± 267	1682	55/3464	1253	1997
353	32	4 (0.125)	28	27	519 ± 115	233	20/2126	224	845
386	32	10 (0.313)	22	19	851 ± 140	932	48/1964	233	861
419	32	6 (0.188)	26	25	633 ± 166	128	74/3519	433	1175
452	32	3 (0.094)	29	29	607 ± 101	567	30/1837	186	770
485	32	7 (0.219)	25	25	573 ± 62	555	82/1271	60	438
518	32	2 (0.063)	30	28	625 ± 120	524	70/3352	252	895
551	32	1 (0.031)	31	30	254 ± 33	219	105/1032	20	253
584	32	8 (0.250)	24	24	387 ± 70	177	29/1116	74	486
617	32	1 (0.031)	31	27	641 ± 103	621	95/2771	179	755
650	32	1 (0.031)	31	29	251 ± 50	145	44/1068	46	383
683	32	1 (0.031)	31	29	291 ± 41	239	126/1123	31	313
716	32	1 (0.125)	28	26	713 ± 121	851	75/2813	241	876
749	32	0 (0)	32	32	238 ± 50	121	22/1065	51	403
782	32	0 (0)	32	32	407 ± 51	312	90/1065	53	411
815	32	0 (0)	32	32	298 ± 61	197	20/1565	74	486
848	32	1 (0.031)	31	31	581 ± 84	418	60/1502	137	660
881	32	3 (0.094)	29	29	539 ± 81	304	74/1367	121	619
914	32	0 (0)	32	25	483 ± 46	431	233/1023	33	323
Overall	640	60 (0.094)	580	553	597 ± 59	319	20/3519	298	974

n is the sample size; m_p is the pollen flow; ± SE is the standard error; A_{ep} is the effective pollination neighbor area; r_{ep} is the effective pollination radius.

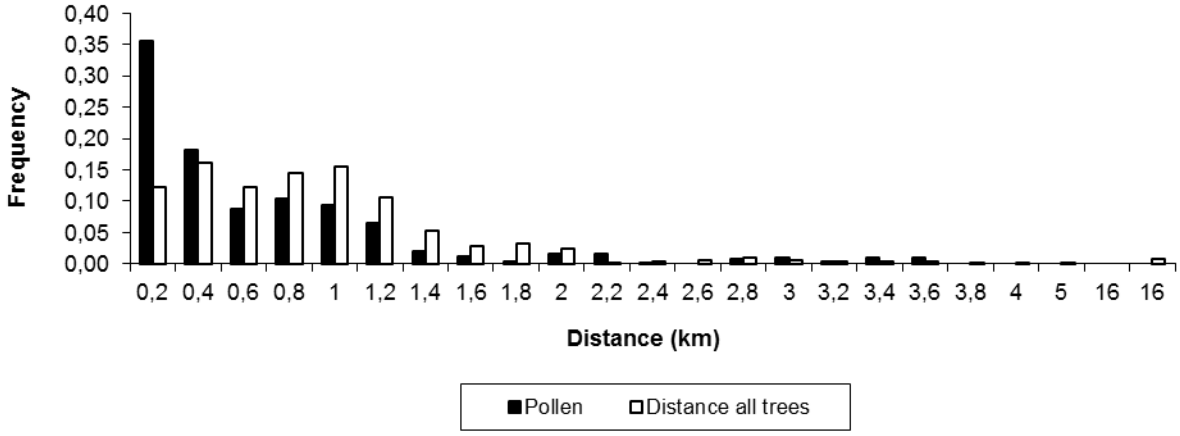
Source: Prepared by author.

Table 8- Mating system, coancestry, and inbreeding at the family level for *Cariniana estrellensis*.

Seed-tree	n_w	s (proportion)	F_s	t_p (proportion)	D (m)	Θ_r	F_p	t_u (proportion)	Θ_u	F_u
287	28	2 (0.06)	0.55	0	0	0	0	30 (0.94)	0.03	0.09
320	28	0	0	1 (0.03)	1811	0.16	0.23	31 (0.97)	0.02	0.02
353	28	1 (0.03)	0.28	1 (0.03)	318	0.15	0.08	30 (0.94)	-0.07	-0.08
386	22	3 (0.09)	0.73	5 (0.16)	654	0.20	0.16	24 (0.75)	0.04	0.11
419	26	1 (0.03)	0.52	9 (0.28)	170	0.22	0.27	22 (0.69)	0.01	0.08
452	29	0	0	4 (0.13)	1038	0.15	0.23	28 (0.88)	0.00	0.01
485	25	0	0	8 (0.25)	511	0.17	0.26	24 (0.75)	-0.03	0.11
518	30	2 (0.06)	0.14	5 (0.16)	655	0.18	0.25	25 (0.78)	0.01	0.16
551	31	1 (0.03)	0.37	6 (0.19)	244	0.19	0.23	25 (0.78)	-0.01	-0.03
584	24	0	0	0	0	0	0	32 (1.00)	-0.03	0.03
617	31	4 (0.12)	0.55	15 (0.47)	544	0.23	0.31	13 (0.41)	0.05	0.27
650	31	2 (0.06)	0.80	18 (0.56)	149	0.19	0.27	12 (0.38)	0.01	0.03
683	31	2 (0.06)	0.57	0	0	0	0	30 (0.94)	-0.03	-0.02
716	28	2 (0.06)	0.54	2 (0.06)	900	0.15	0.24	28 (0.88)	-0.07	-0.01
749	32	0	0	0	0	0	0	32 (1.00)	-0.05	-0.01
782	32	0	0	0	0	0	0	32 (1.00)	-0.04	-0.04
815	32	0	0	1 (0.03)	371	0.13	0.43	31 (0.97)	0.01	-0.01
848	31	0	0	3 (0.09)	1037	0.18	0.29	29 (0.91)	0.01	0.11
881	29	0	0	2 (0.06)	162	0.15	0.12	30 (0.94)	0.01	0.01
914	32	7 (0.22)	0.61	4 (0.13)	181	0.22	0.33	21 (0.66)	-0.06	-0.15
Mean	580	27 (0.04)	0.51	84 (0.13)	583	0.18	0.25	529 (0.83)	-0.01	0.03

n_w is the number of seeds assigned a pollen donor within the sample area; s is the mean selfing rate; t_p and t_u are the rate of mating among related individuals and unrelated individuals, respectively; D is the mean distance between maternal trees and related pollen donors (pollen dispersal distance for inbred seeds); Θ_r and Θ_u are the mean pairwise coancestry coefficient for related and unrelated individuals, respectively; F_s, F_p, and F_u are the mean fixation index for selfing, outcrossing among related and unrelated individuals, respectively. Source: Prepared by author.

Figure 5- Frequency distribution of pollen dispersal distance and distance between trees in the studied *Cariniana estrellensis* population.



Source: Prepared by author.

Pollen dispersal Kernel

The distribution of pollen dispersal was analyzed using the Exponential, Weibull, Geometric, and 2Dt models (Table 9). The Weibull model showed the lowest value for pollen flow (m_p 0.310). The Geometric model and 2Dt showed the same values (m_p = 0.405), while the Exponential model resulted in a pollen flow of 0.352. The selfing rate was higher in the Geometric and 2Dt (s = 0.037) models than in the Exponential (s =0.035) and Weibull (s = 0.036) models. The mean pollen dispersal distance was higher in the 2Dt model (4788.4 m), followed by Geometric (784.9 m), Exponential (610.9 m), and Weibull (570.5 m). The pollen dispersal Kernel scale parameters (α_p) varied from 83.6 (Exponential) to 623.4 (Weibull), and pollen dispersion shape (b_p) was 0.626 (Exponential), 1.365 (Weibull), 1.525 (2Dt), and 4.343 (Geometric) (Table 9). The Exponential pollen dispersal function presented the lowest Log value (-10867.5200) and thus best explains the pollen dispersal pattern in the population.

Table 9- Pollen dispersal Kernel for *Cariniana estrellensis*.

Model	Log	$m_p \pm 2SE$	$s \pm 2SE$	$\delta_p \pm 2SE$ (m)	α_p	b_p
Exponential	-10867.5200	0.352 ± 0.028	0.035 ± 0.008	610.9 ± 113.0	83.6	0.626
Weibull	-10871.5045	0.310 ± 0.029	0.036 ± 0.008	570.5 ± 76.1	623.4	1.365
Geometric	-10868.0084	0.405 ± 0.032	0.037 ± 0.008	784.9 ± 381.7	527.2	4.343
2Dt	-10867.6378	0.405 ± 0.032	0.037 ± 0.008	4788.4 ± 1952.8	230.5	1.525

Log is the log-likelihood; m_p is the pollen flow; s is the selfing rate; δ_p is the mean pollen dispersal distance; α_p is the scale of pollen dispersal kernel; b_p is the shape of pollen dispersal; ± 2SE is the standard error. Source: Prepared by author.

4.4 DISCUSSION

Gene flow

The results show a substantial rate of pollen flow from outside the studied population (9.4%), indicating the population and the individuals within are not reproductively isolated (Table 7). Pollen flow introduces new alleles into a population, enabling increases in genetic diversity and effective population size. Ghazoul (2005) notes that fragmented populations can be sustained if pollinators are able to travel over long distances, as is the case with *C. estrellensis*, whose main pollinating vectors are bees that have the potential to transport nectar and pollen over distances of up to 2 km (CARVALHO, 2003a; PIERROT; SCHLINDWIN, 2003). This is supported by our results, shown in Table 7, with a maximum dispersal distance of 3519 m. Similar pollen flow rates have been detected for other tropical trees occurring in highly fragmented landscapes, such as a rate of 10% for *Araucaria angustifolia* (BITTENCOURT; SEBBENN, 2007), 8% for *Copaifera langsdorffii* (SEBBENN et al., 2011), and 8% for *Cariniana legalis* (TAMBARUSSI et al., 2015). Our results suggest there are currently favorable conditions for maintaining the genetic diversity of the studied population, and indicates its utility as a site for *ex situ* conservation of this vulnerable species.

Nevertheless, the pollen dispersal pattern found in this study cannot be explained by the distance between trees and our results show that mating within the population was not random, as can be seen in the rates of selfing (4%) and mating among relatives (13%). Selfing and mating among relatives can explain the inbreeding detected in the seeds. The minimum expected level of inbreeding from selfing is 0.5 and for eight of the 11 seed trees showing selfing, we found a level of inbreeding higher than 0.5. The other three instances can be explained by the fact we used only nine loci. As such, the probability of a selfed seed presenting a heterozygous genotype is 50%. Inbreeding from mating among relatives is expected to be equal to the coancestry between parents. The mean coancestry coefficient between mother trees and pollen donors assigned for seeds within families ranged from 0.13 to 0.23, suggesting mating among related individuals ranging from half-sibs (0.125) to full-sibs (0.25). In general, inbreeding from mating among relatives was higher than the coancestry coefficient among parents. This difference can also be attributed to the fact that we only used nine loci.

Pollen dispersal distance

Our results show long distance pollen dispersal reaching up to 3.5 km, but following a pattern of isolation by distance. Tree species are sessile organisms that depend on pollinating vectors and seed dispersers to achieve reproductive success (GHAZOUL, 2005). The effective pollination area (A_{ep}) represents a circular area around a mother tree in which 63% of the pollen donors are located (LEVIN, 1998). Thus, the effective pollination area (A_{ep}) for this fragment is 298 ha, with an effective pollination radius (r_{ep}) of 974 m (Table 8). These results can be explained by flowering phenology (hermaphroditic flowering that varies widely according to geographical location) and pollinator behavior (mainly bees), as well as stochastic factors, such as predation, mortality, and natural selection which eliminates inbred individuals. For other tree species pollinated by bees, studies have reported maximum pollen dispersal distances of 887 m for *M. urundeuva* (GAINO et al., 2010) and 170 m for *C. langsdorffii* (SEBBENN et al., 2011).

Our results emphasize that the type of pollinator has a significant impact on the success of plant reproduction, especially if pollinators, such as bees, have the capacity to travel long distances. However, we must also consider the question of population density; lower tree density means that trees are more dispersed, with greater isolation between stands. Therefore, in order for the species to successfully reproduce, pollinators must travel longer distances. The physical isolation of a forest fragment due to anthropogenic interference such as highways, pastures, and monoculture crops, limits the movement of pollinators, reducing pollen immigration rates or even eliminating pollen immigration into the fragment. Guidugli et al. (2016) studied a small *C. estrellensis* fragment and detected a high rate of pollen immigration ranging from 23.5 to 53%; however, gene flow in their study occurred at short distances, with a mean of 69.9 to 146.9 m.

Pollen dispersal Kernel

Kernel dispersal modeling estimates the frequency distribution of dispersal distances in contrast to the pollen delivery pattern detected through paternity analysis (ODDOU-MURATORIO et al., 2005). The Exponential Kernel dispersion model was the most adequate function to explain pollen dispersal in the studied population. Although this model suggested a similar rate of selfing (s = 3.5%) and mean pollen dispersal distance (δ_p = 610.9 m), it presented higher rates of pollen flow (m_p = 35.2%) than the results found using the CERVUS software (s = 4%; δ_p = 597 m; m_p = 9.4%). Pollen dispersal contributes to genetic diversity in populations and pollen dispersal curve estimates are essential for predicting changes to disturbed environments, such as forest fragments.

4.5 FINAL CONSIDERATIONS FOR GENETIC CONSERVATION

Our results show that the studied population and the individuals within are not reproductively isolated due to pollen flow from outside the study area. Pollen flow, dispersal distances, and patterns provide fundamental information to develop strategies for seed collection for *ex situ* conservation, environmental reforestation, and tree breeding, as these parameters define the distance at which mating between related individuals occurs. The use of seeds that are inbred or related, but from different seed trees, must be avoided for such conservation purposes as they can decrease the effective size of sampled progeny arrays. Our results indicate that seeds must be collected from seed trees located at least 600 m apart to avoid seed collection from related seed trees. *Cariniana estrellensis* is a tropical tree species that is vulnerable to extinction and the results reported herein on pollen and seed flow and dispersion will inform strategies aimed at the *in situ* and *ex situ* conservation of the species.

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APPENDIX

5.1- *Cariniana estrellensis* in different environments in the natural fragment.

Source: Prepared by author.

5.2- Collect of fruits of *Cariniana estrellensis*.

Source: Prepared by author.

5.3- Fruits of *Cariniana estrellensis*.

Source: Prepared by author.

5.4- Plantation of seeds of *Cariniana estrellensis*.

Source: Prepared by author.

5.5- Seedlings of *Cariniana estrellensis* with 60 days.

Source: Prepared by author.

5.6- Plantation of seedling of *Cariniana estrellensis* in the field.

Source: Prepared by author.