University of São Paulo
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Contemporary gene flow, mating system, and spatial genetic structure in a Jequitibá-rosa (*Cariniana legalis* Mart. Kuntze) fragmented population by microsatellite markers

Evandro Vagner Tambarussi

Thesis presented to obtain the degree of Doctor in Science. Program: Plant Genetics and Breeding

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Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA - DIBD/ESALQ/USP

Tambarussi, Evandro Vagner
Contemporary gene flow, mating system, and spatial genetic structure in a Jequitibá-rosa (*Cariniana legalis* Mart. Kuntze) fragmented population by microsatellite markers / Evandro Vagner Tambarussi.- - Piracicaba, 2013.
120 p: il.

Tese (Doutorado) - - Escola Superior de Agricultura "Luiz de Queiroz", 2013.

1. Mata atlântica brasileira 2. Conservação genética 3. Marcadores microssatélites 4. Genética de populações 5. Espécies de árvores tropicais I. Título

CDD 634.97342
T154f

This thesis is dedicated to my parents Adelaide and Altair. I dedicate to you not only this work, but also my life…

After much effort and patience, we are getting where we aimed. I need to say thank you. Because of my absence, I missed many important moments; I gave up many great times with you to complete this step. But you were always in my thoughts.

I love you! Thank you Mom and Dad. I am very proud to be the son of such honest and good people.

To my sisters Rosana and Elaine, nephews, Welton, Leandro, Aislan and nieces, Amanda and Júlia, I offer this thesis

ACKNOWLEDGEMENTS

I must first thank God, provider of supreme energy and source of wisdom;

I thank the Escola Superior de Agricultura "Luiz de Queiroz" (Esalq/USP), especially the Departamento de Genética and Ciências Florestais for providing both the intellectual and physical support necessary to complete this work;

I would also like to thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for granting me a PhD scholarship (grant 2010/12354-3);

The Instituto Florestal de São Paulo (IF) in name of Dr. Miguel Luiz Menezes Freitas for support in collecting the plant material and for enabling Wladimir Correa and Dirceu de Souza to help us during fieldwork;

I am especially grateful to Prof. Dr. Roland Vencovsky for supervising this Doctoral project, for giving me personal and intellectual support, and for creating a welcoming environment for research and constant discussions. I would like to thank him for all the excellent classes in population genetics, for believing in my work from the beginning, and providing me with some good advice when I needed it. Prof. Vencovsky is an exemplary professor and an important force behind population genetics, breeding and biometric research in Brazil. I am very proud to have learned about these topics from him;

I am very grateful to Prof. Dr. Alexandre Magno Sebbenn who introduced me to population genetics analyses. He has provided me Doctoral co-supervision, friendship, guided me to study in Oxford and helped extensively in this research. I have learned so much from his vast experience. Dr. Alexandre is one of the best researchers in this area and I am so happy to have had the opportunity to learn from him;

I thank Dr. David Boshier for supervising me during my time at Oxford;

Kate, Anna and Angous (the Boshier Family) for having me in their home, making me feel welcome, and sharing many good times together in Oxford. I am very grateful to Angous (my little friend) for teaching me the British accent and rugby;

I would like to acknowledge the Department of Plant Sciences, University of Oxford, England for hosting me as an Academic visitor. I especially thank Dr. Roni McGowan, Dr. Caroline Pannell, Dr. Denis Filer, Mrs. Caroline Benfield, Mrs. Pauline Cull, Ms. Serena Marner, Mr. John Baker, Miss Beth Williams. Thank you all very much!

Elza M. Ferraz and Maria Andréia Moreno for helping with the lab work and project coordination; Tiago Gabassi for helping me in the nursery trial;

I thank my best friends Evelyn K. Abe, Kelly Boschetti and Déia Moreno for always being there for me. My life during the PhD was easier because of you. "Our angel uses the others lips to talk to us. Pay attention." (Paulo Coelho);

Alexandre de Vicente Ferraz and Frederico Almeida de Jesus for 12 years of being roommates. Sorry for some things and thank you so much for everything!

Thank you to Tereza Venilha de Andrade Bruno for everything!!!

To my good friends: Bruna Ibañes, Luana Rodrigues, Giullia Forti, Rebeca F. Correr, Lia Maris, João R. B. F. Rosa, Bruno M. de Lima, F.H. Toledo, Bruna Mendes de Oliveira, Valentina de Fátima de Martin, Antonio Sinigaglia (from Italy), Pa Moreno and Zé Rubens, Alex Rodrigues, Luis Ricardo Sesso, Rafael and Léo, Ricardo Manoel, Maria Cecília Perantoni Fuchs, Gustavo Mori, Frederico Domene, Fausto Correr, Flávio Gandara and Magali R. da Silva;

I thank Prof. Edson S. Mori, for introducing me to a life in research. Much of this is because of you;

Prof. Dr. Paulo Y. Kageyama for providing the lab for microsatellites analyzes;

Lilian Farias (Imp. e Exp. de Medidores POLIMATE LTDA) for providing the microsatellite analyzes;

Valentina de Fátima de Martin for sequencing the *C. legalis* DNA;

Léia, the secretary of PPG in Genética e Melhoramento de Plantas (Esalq/USP), for her attention throughout the PhD;

The staff of the Departamento de Genética: Berdan, Fernando, Valdir, Antônio, Maídia, Carlinhos, Salete, Fernanda, Rogério e Vilma;

Aline Cristina Miranda and Paulo H. Miller da Silva for all opportunities in Ipef (Instituto de Pesquisas e Estudos Florestais). I have enjoyed working with you and contributing to the Forest Genetics program;

Eliana Maria Garcia for her help in finalization of the text (Library);

Dr. Evelyn Nimmo for editing the English of the Thesis;

A special thanks to all ESALQ employees who always made our day easier and more enjoyable ... To you my honest thanks!

Thank you all!

"Enquanto estiver vivo, sinta-se vivo.
Se sentir saudades do que fazia, volte a fazê-lo.
Não viva de fotografias amareladas...
Continue, quando todos esperam que desistas.
Não deixe que enferruje o ferro que existe em você.
Faça com que em vez de pena, tenham respeito por você.
Quando não conseguir correr através dos anos, trote.
Quando não conseguir trotar, caminhe.
Quando não conseguir caminhar, use uma bengala.
Mas nunca se detenha."

Madre Teresa de Calcuta

SUMMARY

RESUMO

Fluxo gênico contemporâneo, sistema de reprodução e estrutura espacial de genótipos em população fragmentada de jequitibá-rosa (*Cariniana legalis* Mart. O. Kuntze) utilizando marcadores microssatélites

Cariniana legalis Mart. O. Kuntze (Lecidiaceae) é a maior árvore da Mata Atlântica. Para contribuir com a conservação *in* e *ex situ* nós investigamos a diversidade genética, endogamia, estrutura genética espacial intrapopulacional (EGE), sistema de reprodução e fluxo contemporâneo de pólen em três populações fragmentadas da espécie. Encontrámos 65 árvores adultas na população Ibicatu, 22 em MGI, e quatro em MGII. As sementes foram colhidas e amostradas hierarquicamente entre e dentro de frutos diretamente da copa de 15 árvores matrizes em Ibicatu (n = 40), cinco em MGI (n = 50), e duas em MGII (n = 100). Treze locos microssatélites foram desenvolvidos e validados em 51 indivíduos de *C. legalis*. Onze deles foram polimórficos, revelando um máximo de dois a 15 alelos por loco. Usando os genótipos das progênies e matrizes, foi investigada a herança mendeliana, ligação genética e desequilíbrio genotípico de sete locos isolados de *C. legalis* e dois heterólogos. Não foram detectados desvios notáveis da segregação mendeliana, de ligação, ou desequilíbrio genotípico. A riqueza alélica média de adultos de Ibicatu foi 11,65 e 14,29 para MGI-II e para as sementes foi de 14,18 em Ibicatu e 10,85 na MGI-II, a heterozigosidade média observada para adultos em Ibicatu foi 0,811 e 0,838 para MGI-II, para as sementes foi de 0,793 em Ibicatu e 0,786 em MGI-II, a heterozigosidade média esperada para adultos de Ibicatu foi 0,860 e 0,900 para MGI-II, para as sementes foi de 0,856 em Ibicatu e 0,853 em MGI-II. O índice médio de fixação foi significativamente maior do que zero para adultos e sementes de ambas as populações. A taxa de cruzamento Multilocus (t_m) nas três populações foi significativamente menor do que a unidade (1,0), especialmente para MGII (t_m = 0,830). A taxa de acasalamento entre parentes foi significativa apenas para Ibicatu ($t_m - t_s = 0.266$). A correleção de paternidade foi substancialmente maior dentro do que entre os frutos. O coeficiente médio de coancestria (Θ) foi maior e variação de tamanho efetivo (N_e) foi menor do que o esperado para progênies de meio-irmãos em todas as populações. O número estimado de árvores matrizes necessárias para a coleta de sementes para se obter um tamanho efetivo de 150 foi de 54-58 árvores. A taxa de imigração de pólen foi baixa, especialmente para os fragmentos menores (máximo de 0,4% para MGI), indicando isolamento genético significativo. O raio efetivo de polinização foi baixo em MGI (68 m) e MGII (191 m). Para MGII também encontramos níveis mais elevados de autofecundação (18%) do que para Ibicatu (6%) e MGI (6,4%). O isolamento genético substancial desses estandes sugerem que podemos esperar um aumento na EGE e que estratégias para aumentar o fluxo gênico e tamanho efetivo da população, como o transplante de indivíduos nas populações, são desejáveis para o longo prazo. Em conclusão, este estudo gerou informações valiosas para a gestão de populações fragmentadas de *C. legalis*, contribuindo para programas de melhoramento e fornecendo orientações para a coleta de sementes destinadas a programas de conservação e reflorestamento.

Palavras-chave: Mata atlântica brasileira; Conservação genética; Marcadores microssátelites; Genética de populações; Espécies de árvores tropicais

ABSTRACT

Contemporary gene flow, mating system, and spatial genetic structure in a jequitibá-rosa (*Cariniana legalis* Mart. Kuntze) fragmented population by microsatellite markers

Cariniana legalis Mart. O. Kuntze (Lecidiaceae) is the largest tree of the Atlantic Forest. To contribute to *in situ* and *ex situ* genetic conservation programs for the species, herein we investigate the genetic diversity, inbreeding, intrapopulation spatial genetic structure (SGS), mating system and contemporary pollen flow in three fragmented populations of this species. We found 65 adult trees in the Ibicatu population, 22 in MGI, and four in MGII. Seeds were hierarchically sampled among and within fruits directly from the canopy of 15 seed-trees in Ibicatu (n= 40), five seed-trees in MGI (n= 50), and two seed-trees in MGII (n= 100). Thirteen specific microsatellite loci were developed and validated for 51 *C. legalis* trees. Eleven loci were polymorphic, revealing a maximum of two to 15 alleles per locus. Using the progeny arrays and seed-tree genotypes, we investigated the Mendelian inheritance, genetic linkage and genotypic disequilibrium of seven microsatellite loci specifically isolated for *C. legalis* and two previously developed heterologous microsatellite loci. No notable deviations from the expected Mendelian segregation, linkage, or genotypic disequilibrium were detected. The average allelic richness in the adult cohort of Ibicatu was 11.65 and 14.29 for MGI-II and for seeds it was 14.18 in Ibicatu and 10.85 in MGI-II; the average observed heterozygosity for adults of Ibicatu was 0.811 and 0.838 for MGI-II and for seeds it was 0.793 in Ibicatu and 0.786 in MGI-II; the average expected heterozygosity for adults of Ibicatu was 0.860 and 0.900 for MGI-II and for seeds it was 0.856 in Ibicatu and 0.853 in MGI-II. The average fixation index was significantly greater than zero for adults and seeds from both populations. Multilocus outcrossing rate (t_m) in the three populations was significantly lower than unity (1.0), especially in MGII (t_m = 0.830). The rate of mating among relatives was significant when compared to zero only for Ibicatu ($t_m - t_s = 0.266$). Paternity correlation is substantially higher within than among fruits. The average coancestry coefficient (Θ) was higher and variance effective size (N_e) was lower than expected for half-sib progenies in all three populations. The number of seed-trees necessary for seed collection to obtain progeny arrays with an effective size of 150 was estimated between 54 to 58 seed-trees. The pollen immigration rate was low, especially for the small stands (maximum of 0.4% for MGI), indicating significant genetic isolation of MGI and MGII. The effective pollination radius was also low in MGI (68 m) and MGII (191 m). For MGII, we also found higher levels of selfing (18%) than for Ibicatu (6%) and MGI (6.4%). The substantial genetic isolation of these stands suggest that we can expect an increase in SGS in the future and strategies to increase gene flow and effective population size, such as transplanting individuals among the populations, are desirable for long term *in situ* conservation. In conclusion, this study produced valuable information for the management of fragmented populations of *C. legalis*, contributing to breeding programs and providing guidelines for seed collection aimed at conservation and reforestation programs.

Keywords: Brazilian Atlantic Forest; Conservation genetics; Microsatellite markers; Population genetics; Tropical tree species

1 INTRODUCTION

Forest fragmentation is a global problem that has affected the survival of many populations of tree species around the world. Globally, forest loss is estimated at 9.4 million hectares per year, of which 2.3 million occurred in the humid forests of Brazil. In the past, the Atlantic Forest covered a vast amount of the Brazilian territory but today only small forest fragments remain (<83%, RIBEIRO et al., 2011).

Anthropogenic influences have significant evolutionary implications for tree species populations (ECKERT et al., 2009). Forest fragmentation has negative impacts on genetic diversity, as well as spatial genetic structure, mating system and gene flow of populations (AGUIAR et al., 2009; ECKERT et al., 2009; KAMM et al., 2010; SEBBENN et al., 2011; QUESADA et al., 2013). Eckert et al. (2009) argued that human activities modify the landscape more than the combined impacts of biotic and abiotic variables. It may modify the mating system and the movement of pollen and seeds between populations disrupting ecological processes, evolutionary changes, genetic makeup, and neighborhood history (HAMILTON, 1999; HAMRICK, 2004).

Pollen flow is one of the most important factors influencing the genetic structure of tree species (BURCZYK et al., 2004). Extensive gene flow via pollen promotes high levels of genetic diversity and effective population size within populations and low levels of differentiation among populations (PETIT; HAMPE, 2006). It is often considered the most important process in maintaining the genetic cohesion of a species (FUCHS; HAMRICK, 2010; SEBBENN et al., 2011). When populations become genetically isolated, there is a risk of a loss of genetic diversity, which is critical to the long-term survival of populations. Studies in tropical tree species have found signs of decreased genetic diversity and increased levels of inbreeding and spatial genetic structure in fragmented and exploited populations (MILLAR et al., 2013).

Big trees are among the oldest and most important species of all living organisms in forest biomes. In general, these organisms comprise less than 2% of a forest's tree population but due to their vast energy-absorbing canopies, they can constitute up to 25% of a forest's total biomass. As a result, large trees are keystone species for forest biomes, producing abundant crops of fruits, flowers, leaves and other food that many animals rely on for survival (RICHARDS, 1998; LAURANCE, 2000, 2012). These trees grow to such a large size

because of specific genes and genetic combinations (LAURANCE, 2012). However, large, old trees are being lost in record numbers around the world. These organisms are disproportionately vulnerable in many ecosystems as a result of accelerated rates of forest fragmentation (LINDENMAYER et al., 2013). Therefore, these species depend on their ability to respond to environmental changes, which is directly related to the amount of genetic variation present in each population (AGUILAR et al., 2008).

Cariniana legalis Mart. O. Kuntze (Lecidiaceae) (Figure 1) is endemic to the Atlantic Forest; it is one of the largest trees in the biome and it generally occurs in low population densities (< 1 tree/ha). Individuals can reach up to 60 m in height and 4 m in diameter at breast height (CARVALHO, 1994). Its flowers are hermaphroditic and pollinated by bees and its seeds are dispersed by wind. It has a light wood that is used in internal civil construction and for furniture, as the wood is not very resistant to decay (CARVALHO, 1994). The species is considered endangered (INTERNATIONAL UNION FOR THE CONSERVATION OF NATURE - IUCN, 2002) and strategies for *in* and *ex situ* conservation of the remaining populations are urgent.

As mentioned above, large tropical tree species are especially sensitive to changes in the landscape, such as forest fragmentation, because these species generally occur at low population densities, have complex self-incompatible mating mechanisms, high rates of outcrossing (MURAWSKI et al., 1994; LOWE et al., 2005; LANDER et al., 2010; QUESADA et al., 2013), and specialized interactions with pollinators and seed dispersers (WARD et al., 2005). In order to genetically conserve the remaining populations of *C. legalis*, a better understanding of the genetic diversity, inbreeding, spatial genetic structure, mating system, and gene flow are needed. Previous studies have investigated the mating system and genetic diversity of *C. legalis* (SEBBENN et al., 2000); however, no research published to date has examined pollen flow in fragmented populations of this endangered species.

Figure 1 - View of the Ibicatu forest fragment: (A) a *C. legalis* individual which dominates the forest canopy; (B) seed-tree J04 with 3.0 m in dbh and a height of more than 40 m

In our study we developed a set of microsatellite loci for *C. legalis* to investigate genetic diversity, inbreeding, spatial genetic structure, mating system and gene flow in three fragmented populations of the species. This molecular information is necessary to understand how the genetics of *C. legalis* have been affected by the process of intense fragmentation. As mentioned by Breed et al. (2013), forest density is an important factor in determining mating systems for many tropical trees. As such, microsatellite markers are suitable for these studies due to their high polymorphism, in terms of number of alleles, and their discriminatory power; they have become a popular tool in population and conservation genetics (CHASE et al., 1996; FERREIRA; GRATTAPAGLIA, 1998). Moreover, our scenario provides unique insight into understanding the effects of the process of extreme forest fragmentation because it includes populations of varying sizes (72 ha in Ibicatu and 7.2 ha in Mogi-Guaçu). Within this context, we investigated the genetic diversity and structure, inbreeding, intrapopulation genetic structure, mating system, contemporary pollen flow and inbreeding depression of three fragmented populations of *C. legalis*, located in the Ecological Stations of Ibicatu and Mogi-Guaçu, São Paulo State, Brazil. This study produced valuable information for the management of fragmented populations of the species, contributing to breeding, conservation and reforestation programs.

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2 MICROSATELLITE MARKERS FOR *Cariniana legalis* (Lecythidaceae) AND THEIR TRANSFERABILITY TO *C. estrellensis*

Abstract

Microsatellite primers were developed in the neotropical tree species *Cariniana legalis* (Lecythidaceae) to investigate its genetic diversity, mating system, and gene flow. We identified 96 clones containing 82 repeat motifs from a genomic library enriched for $(CT)_8$ and $(GT)_8$ motifs. Primer pairs were developed for 13 microsatellite loci and validated in 51 *C. legalis* specimens and 26 *C. estrellensis* specimens. Eleven loci were polymorphic, revealing a maximum of two to 15 alleles per locus in *C. legalis* and three to 12 in *C. estrellensis*. For *C. legalis*, the observed (H_o) and expected (*He*) heterozygosities ranged from 0 to 0.99 and from 0.07 to 0.90, respectively. For C. estrellensis, H_o and H_e ranged from 0 to 0.96 and from 0.14 to 0.91, respectively. The primers identified polymorphic loci that are suitable to study genetic diversity and structure, mating system, and gene flow in *C. legalis* and the related species *C. estrellensis*.

Keywords: Brazilian Atlantic forest; *Cariniana legalis*; Conservation genetics; Microsatellite markers; Population genetics; Tropical tree species

2.1 Introduction

Cariniana Casar. (Lecythidaceae) is a genus of trees native to South America, and many of these tree species are harvested for timber. *Cariniana legalis* (Mart.) Kuntze and *C. estrellensis* (Raddi) Kuntze are two endangered tropical trees with winddispersed seeds that are pollinated by bees (CARVALHO, 1994). These species are endemic to the Atlantic Forest in Brazil and have a low population density (less than 1 tree/ha). However, the Atlantic Forest has become increasingly fragmented in recent centuries, and today just 12–16% of the original forest remains (RIBEIRO et al., 2009). Forest fragmentation isolates and decreases the natural populations of these trees. These changes affect gene flow among populations and can increase the selfing rate, correlated matings, genetic structure, and the relatedness in subsequent generations (JUMP; PENUELAS, 2006; O'CONNELL et al., 2006). Recent advances in molecular techniques, such as microsatellite markers, have created new opportunities for conservation research that can be used to minimize the negative implications of population fragmentation. Here, we describe the development of 12 nuclear microsatellite markers for *C. legalis* and the transferability of these markers for studying *C. estrellensis*.

2.2 Development

2.2.1 Material and methods

Total genomic DNA was extracted from fresh leaves collected from a single *C. legalis* tree using the protocol proposed by Doyle and Doyle (1987). A microsatellite-enriched genomic library was constructed following the protocol of Billotte et al. (1999). The *Rsa* I enzyme (Invitrogen, Carlsbad, California, USA) was used to digest genomic DNA from one genotype of *C. legalis*, enriched in microsatellite fragments using $(CT)_8$ and $(GT)_8$ motifs. These enriched fragments were cloned into pGEM-T Easy Vector (Promega Corporation, Madison, Wisconsin, USA), and ligation products were used to transform Epicurian Coli XL1-Blue *Escherichia coli* –competent cells (Stratagene, Agilent Technologies, Santa Clara, California, USA). Transformed cells were cultivated on agar plates containing 100 μ g/mL ampicillin, 50 μ g/mL X-galactosidase, and isopropyl β- D-1-thiogalactopyranoside (IPTG). Single white colonies were selected and stored at −80 ° C. A total of 96 recombinant colonies were obtained and sequenced using the adapters *Rsa* 21 (5′-CTCTTGCTTACGCGTGGACTA-3′) and *Rsa* 25 (5′-TAGTCCACGCGTAAGCAAGAGCACA-3′) in a 3730xl DNA Analyzer sequencer (Applied Biosystems, Foster City, California, USA) using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems).

Ninety-six positive clones from the library were sequenced, and the microsatellites were found in 82 of them. Dinucleotide motifs were the most abundant, followed by mono-, tetra-, tri-, and hexanucleotide motifs (approximately 75.6%, 14.6%, 5%, 2.4%, and 2.4%, respectively); pentanucleotide motifs were not found. Only 13 simple sequence repeat (SSR) markers were selected for primer design because their sequences presented more than five tandem repeats. Vector segments were removed from each of the sequences by VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). Thirteen pairs of primers were designed for SSR fl anking regions using Primer3Plus (UNTERGASSER et al., 2007) according to the following criteria: annealing temperature ranging from 52 - 60 ° C and GC content between 40% and 60%. Each primer pair was designed to amplify a fragment ranging between 150 and 300 bp. Microsatellite loci were amplified by PCR in a final volume of 15 μ L using GoTaq Colorless Master Mix (Promega Corporation) containing 7.5 μ L GoTaq Colorless Master Mix (2×), 10 μ M of each primer (F and R), 3.0 μ L Nuclease-Free Water, and 7.5 ng template DNA. The amplification program for all primers consisted of an initial denaturing step at 94°C for 1 min, followed by 35 cycles of amplification (94°C for 1 min,

followed by 1 min at the specific annealing temperature of each primer pair [Table 1], 72 ° C for 1 min), and a final elongation step at 72 ° C for 10 min. Amplifications were performed with a Mastercycler (Eppendorf, Hamburg, Germany). Thirteen pairs of primers were designed, but only 12 yielded successfully amplified fragments. The Cle13 locus did not amplify despite using a considerable range of temperatures (40- 65°C). The amplification products (2μL of the total reaction volume) were separated on a Fragment Analyzer Automated CE System (Advanced Analytical Technologies [AATI], Ames, Iowa, USA) using the Quant-iT PicoGreen dsDNA Reagent Kit, 35–500 bp (Invitrogen). Raw data were analyzed using PROSize version 2.0 software (AATI).

2.2.2 Results and Discussion

We sampled a total of 51 adult *C. legalis* trees from two populations (25 from Floresta Estadual de Ibicatu and 26 from Floresta Estadual de Mogi-Guaçu) and 26 adult *C. estrellensis* trees from Floresta Estadual de Ibicatu in São Paulo State, Brazil (Appendix). Genetic diversity, fixation index (*F*), and linkage disequilibrium were estimated for each species using the FSTAT program (GOUDET, 2002). To test if *F* was significantly different from zero and to test linkage disequilibrium, we used 1,000 Monte Carlo permutations (alleles among individuals) and a Bonferroni correction (95%, $\alpha = 0.05$). As noted above, of the 13 tested primers in both species, one (Cle13) showed no amplification (Table 2.1). The other 12 loci worked well for both species (Table 2.1). For *C. legalis*, 100 alleles were found (ranging among loci from two to 14, with an average of 8.3) from Ibicatu, and 112 alleles were found from Mogi-Guaçu (ranging among loci from two to 15, with an average of 9.3). For *C. estrellensis*, we identified 103 alleles (ranging from three to 12, with an average of 8.6) (Table 2).

Table 2.1 - Microsatellite primers developed in *C. legalis*. The forward (F) and reverse (R) sequence, repeat type, size of the original fragment (bp), annealing temperature when run individually (Ta^{o}C), and the GenBank accession number are shown for each primer pair. All values are based on a variable number of samples representing two populations located in Ibicatu and Mogi-Guaçu, São Paulo, Brazil

Locus	Primer sequence	Repeat motif	Size[a]	Size[b]	Ta(^{o}C)	GenBank accession no.
Cle01	F: TTCTCTTCCCCCTCTTCCTC R: TCCTTTCCAAACCAAACCAC	$(AC)_{18}$	154-184	160-181	60	JX466851
Cle02	F: TCTCAAAACTCCCCCTCAAG R: CCGAAGAAATCATCACCTCA	$(AC)_{11}$	150-202	140-202	62	JX466852
Cle03	F: GCCTGTCTACTGATGCCAGA R: GTATTCCTTGGTTTCTTTGCTG	$(AC)_{8}$	222-241	202-230	62	JX466853
Cle04	F: CAAAGGTTGAGGGTATAAATGC R: GGGAGAACTATCCACATCAAGA	$(TG)_{10}T(TGTA)_{5}$	266-300	256-296	60	JX466854
Cle05	F: CAAGCCGCACCTTTATCTAT R: GCAGCCAAACAGGATAGCA	$(CA)_{8}$	188-226	186-232	62	JX466855
Cle06	F: CTCTCCTGCCATTGATTTTG R: ATGACTGACTCTAAATCTTG	$(TG)_{8}A(GA)_{11}$	198-230	176-202	60	JX466856
Cle07	F: GGGTAGTGACCAACAATCTCG R: ATGATGCTGCCAAGGTAATG	$(CA)_{8}$	150-160	148-208	56	JX466857
Cle08	F: GCAATCCTCCAAACAGCATT R: CCCTCTCTCCATGACCGTTA	(AG)19	152-180	150-180	62	JX466858
Cle09	F: TGGGACAACACATCACAACC R: GAATGAATTGGGAGAAAGTG	$(GT)_{14}$	156-186	158-180	58	JX466859
Cle10	F: AAGTAGAAACCACCTGGCAGA R: CCCTATTTCATCCTCAGCAG	$(TG)_{16}$	156-170	160-182	60	JX466860
Cle11	F: ATGACGCTGATGATGCTGAA R: TGCTCCCTTCTGGCTACTTG	$(GT)_{7}$	226-230	224-230	58	JX466861
Cle12	F: GCCTTGTTAGATGTTGCCTGT R: TTGGTTAGTCTCCCTGTTAGC	$(AG)_{16}$	202-224	202-244	56	JX466862
Cle13	F: TGCCCAACTCAATTCTGAAAC R: TGACTTCTCCACCTTCAACG	$(TC)_{19}(AC)_{8}$	NA	NA	40 to 65	JX466863

[a]Allele size found for *C. legalis*; [b]Allele size found for *C. estrellensis*; NA - not amplified; Ta(^{o}C) = annealing temperature

Table 2.2 - Results of initial primer screening in two populations of *C. legalis* (Ibicatu and Mogi-Guaçu) and one population of *C. estrellensis* (Ibicatu) from São Paulo, Brazil

Locus	*C. legalis*[a] (N = 25)				*C. legalis*[b] (N = 26)				*C. estrellensis*[a] (N = 26)			
	A	H_o	H_e	*F*	*A*	H_o	H_e	*F*	*A*	H_o	H_e	*F*
Cle01	10	0.60	0.81	0.262	15	0.58	0.88	0.347*	11	0.65	0.87	0.247*
Cle02	8	0.96	0.81	-0.18	11	0.92	0.87	-0.056	12	0.88	0.86	-0.029
Cle03	6	0.75	0.76	0.014	5	0.69	0.74	0.066	10	0.30	0.86	0.642*
Cle04	13	0.80	0.90	0.114	14	0.88	0.88	0.001	8	0.54	0.69	0.209
Cle05	7	0.99	0.69	-0.465*	10	0.96	0.86	-0.114	8	0.96	0.73	-0.316
Cle06	6	0.40	0.72	0.448*	7	0.65	0.81	0.196	9	0.27	0.84	0.678*
Cle07	4	0.76	0.56	-0.357	6	0.69	0.73	0.054	6	0.16	0.65	0.754*
Cle08	12	0.76	0.88	0.140	15	0.81	0.90	0.109	11	0.33	0.91	0.635*
Cle09	14	0.68	0.82	0.18	7	0.60	0.76	0.021*	8	0.38	0.72	0.462*
Cle10	8	0.76	0.78	0.024	7	0.96	0.80	-0.203	8	0.70	0.78	0.109
Cle11	2	0.00	0.22	1.000*	2	0.00	0.07	1.000*	3	0.00	0.14	1.00*
Cle12	10	0.46	0.82	0.448*	13	0.96	0.87	-0.104	9	0.51	0.76	0.322*
Mean	8.3	0.66	0.73	0.099*	9.3	0.72	0.77	0.029	8.5	0.47	0.73	0.352*

Note : H_e = expected heterozygosity; H_o = observed heterozygosity; *N* = sample size for each population; *A* = number of alleles per loci; *F* = fixation index; *Significant departures from Hardy–Weinberg equilibrium at $P < 0.05$

[a]Ibicatu, 22°46’32”S/ 47°49’03” W

[b]Mogi-Guaçu, 22°17’25”S/47°10’55” W

For *C. legalis*, the observed (H_o) and expected (*He*) heterozygosities ranged from 0 to 0.99 and from 0.22 to 0.90, respectively, for Ibicatu and from 0 to 0.96 and from 0.07 to 0.90, respectively, for Mogi-Guaçu. For *C. estrellensis* , H_o and H_e ranged from 0 to 0.96 and from 0.14 to 0.91, respectively. Following Bonferroni correction, *F* was signifycantly different from zero for three loci of *C. legalis* from Mogi-Guaçu and four loci and means over loci from Ibicatu. In *C. estrellensis*, significant *F* values were observed in eight loci and means over loci (Table 2.2). After Bonferroni correction, no linkage disequilibrium was detected in the studied populations. Of the 13 loci developed, only Cle11 and Cle13 were not useful due to low polymorphism and lack of amplification, respectively. The remaining 11 loci are suitable for studying genetic diversity and structure, mating system, gene flow, and parentage analysis in both species.

2.3 Conclusions

The microsatellite loci showed high levels of polymorphism in both *C. legalis* and *C. estrellensis*. Our data suggest that the microsatellite markers developed in this study are suitable for population genetic studies in both *Cariniana* species. We are currently using these markers for inferring genetic diversity, spatial genetic structure, and gene flow in *C. legalis*. These studies will produce valuable information for managing fragmented populations, including information for breeding, conservation, and reforestation plans.

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Appendix

List of vouchers of *C. legalis* and *C. estrellensis* used in this paper. Vouchers are deposited in the Herbarium of the Universidade de São Paulo, São Paulo, Brazil (ESA).

Code	Species	Country	Locality (State)	Voucher number
IBI	*C. estrellensis*	Brazil	Ibicatu (SP)	120064
IBI	*C. legalis*	Brazil	Mogi-Guaçu (SP)	120065
MoG	*C. legalis*	Brazil	Mogi-Guaçu (SP)	120489

3 MENDELIAN INHERITANCE, GENETIC LINKAGE, AND GENOTYPIC DISEQUILIBRIUM AT NINE MICROSATELLITE LOCI OF *Cariniana legalis* (Mart.) O. Kuntze

Abstract

Cariniana legalis is one of the largest tropical trees with a wide distribution in the Brazilian Atlantic rainforest. We investigated the Mendelian inheritance, genetic linkage, and genotypic disequilibrium at seven microsatellite loci specifically isolated for *C. legalis*, and at two previously developed heterologous microsatellite loci. Forty to 100 open-pollinated seeds were collected from 22 seed trees in two populations. Using the Bonferroni correction, no remarkable deviations from the expected Mendelian segregation, linkage, or genotypic disequilibrium were detected in the nine studied loci. Only 3.7% of the tests were significant for investigations of the Mendelian proportions. On the other hand, only 2.8% of tests for linkage detection showed significance. In addition, among all pairwise tests used for investigating linkage disequilibrium, significance was found in 9.7% of the loci pairs. Our results show clear evidence that the nine simple sequence repeat loci can be used without restriction in genetic diversity, mating system, and parentage analyses.

Keywords: Brazilian Atlantic forest; Conservation genetics; Microsatellite; Population genetics; Tropical tree species

3.1 Introduction

Cariniana legalis (Mart.) O. Kuntze grows naturally throughout southeastern and northeastern Brazil, and is one of the most important historically harvested timber species. Its wood is light and is used in civil construction only for internal rooms, as well as in furniture manufacturing, because it is not very resistant to wood decay attacks. *C. legalis* is an endangered tropical tree species (FAO, 1996) that is pollinated by bees with wind-dispersed seeds (CARVALHO, 1994). This species is endemic to the Atlantic Forest in Brazil and has a low population density (< 1 tree/ha). Effective genetic conservation of a species requires knowledge of its mating system, genetic diversity, spatial genetic structure, and gene flow. Microsatellite markers or simple sequence repeats (SSRs) are suitable for such studies due to their very high polymorphism in terms of number of alleles (ASHLEY, 2010). However, for SSR markers to be used as genetic markers, it is necessary to know if their inheritance follows Mendelian rules (BRONDANI et al., 1998; TARAZI et al., 2010), as well as if loci are linked. Such information is particularly necessary for studies of genetic diversity, intra-population spatial genetic structure, mating systems, and gene flow because multilocus estimates are used and population genetic models are based on assumptions of Mendelian inheritance, absence of

genetic linkage, and linkage equilibrium. Thus, studies related to Mendelian inheritance, absence of genetic linkage, and linkage equilibrium should be evaluated and reported when novel SSRs are developed. These were the aims of the present study. We investigated these genetic properties in seven microsatellite loci isolated from *C. legalis* by Tambarussi et al. (2013) and two heterologous microsatellite markers previously developed by Guidugli et al. (2010).

3.2 Development

3.2.1 Material and Methods

Sampling

Open-pollinated seeds were collected from 15 seed trees at the Floresta Estadual de Ibicatu (22° 46' S, 47° 43' W, 540 m) and from seven seed trees in Mogi-Guaçu (22° 16' S, 47° 11' W, 568m), both located in Sao Paulo state, Brazil. In Ibicatu, 40 seeds were collected per seed tree and in Mogi-Guaçu, 50 seeds from five seed trees, and 100 seeds from two seed trees were collected. All fruits were directly collected from the canopy of the trees to ensure that all seeds were siblings. Cambium tissue was also collected from the trunk of the seed trees for DNA analysis. We also collected cambium tissue from another 40 adult trees in Ibicatu and a further 19 trees in Mogi-Guaçu.

Microsatellite analysis

From all adult trees, deoxyribonucleic acid (DNA) was extracted from 100 mg adult stem bark material per tree using AnalytikJena DNA isolation kits. Seeds were germinated in vermiculite until the cotyledons emerged, and then DNA was extracted from 15 to 20 day-old seedlings using the method of Doyle and Doyle (1990).

Nine primers were used in this study. Seven of the primers were developed by Tambarussi et al. (2013) and two (Ce07 and Ce18) were developed by Guidugli et al. (2010). Microsatellite loci were amplified with polymerase chain reaction (PCR) in a 15 μL final volume using GoTaq® Colorless Master Mix containing 7.5 μL 2X GoTaq® Colorless Master Mix, 10 μM each primer, forward and reverse (F and R, respectively), 3.0 μL nuclease-free water, and 7.5 ng template DNA. The amplification program for all primers consisted of an initial denaturing step at 94°C for 1 minute; followed by 35 cycles each of amplification at 94°C for 1 minute, 1 minute at the specific annealing temperature of each primer pair (TAMBARUSSI et al., 2013), and 72°C for 1 minute; and a final elongation step at 72°C for

10 minutes. Amplifications were performed using a Mastercycler (Eppendorf, Hamburg, Germany). The amplification products (2 µL total reaction volume) were separated on a Fragment Analyzer™ Automated CE System (Advanced Analytical Technologies, Inc. [AATI], Ames, IA, USA) using the dsDNA Reagent Kit, 35–500 bp. Raw data were analyzed using the PROSize version 2.0 software (AATI).

Analysis of inheritance

We used the Gillet and Hattemer (1989) method to investigate the Mendelian inheritance of the *C. legalis* SSR loci. This method compares the genotype of a heterozygous maternal tree with the segregation of its open-pollinated progeny. This method assumes that the loci have regular segregation and its alleles follow classic Mendelian inheritance patterns, which is based on three main requirements: i) regular meiotic segregation during production of ovules; ii) random fertilization of ovules by each type of pollen; iii) no selection occurred between the moment of fertilization and genotyping of the seeds. The model also assumes that there is a co-dominant relationship among all alleles. The method further requires that the following conditions are met: 1) all progeny of a tree must possess a maternal allele, and 2) in cases of heterozygous parent trees (e.g., A_iA_j, i ≠ j): a) among offspring, each individual must possess an allele of the maternal tree, A_i or A_j; b) the number of heterozygous progeny A_iA_j (n_{ij}) must equal the sum of homozygous progeny A_iA_i (n_{ii}) and A_jA_j (n_{jj}), or $n_{ij} = n_{ii} + n_{jj}$; and c) the number of heterozygous progeny A_iA_k (n_{ik}) must equal the number of heterozygous progeny A_jA_k (n_{jk}), or $n_{ik} = n_{jk}$, where $k \neq$ i, j. The phenotypes observed in each heterozygous seed tree were compared with the expected 1:1 segregation pattern by means of a maximum likelihood *G*-test (SOKAL and ROHLF, 1981) based on the following formula (Equation 1):

$$G = 2\left[n_i \ln\left(\frac{n_i}{E(n)} \right) + n_j \ln\left(\frac{n_j}{E(n)} \right) \right] \qquad \text{Equation (1)}$$

where n_i and n_j are the observed number of genotypes containing alleles A_i and A_j, respectively, ln is the natural logarithm, and $E(n)$ is the expected number of genotypes for the alleles A_i and A_j based on Equation (2):

$$E(n) = 0.5\ (n_i + n_j)$$ Equation (2)

The *G*-test determines if the deviation between the observed and expected segregation is statistically significant or if deviations may be explained by chance. We also applied the Bonferroni correction for multiple comparisons (95%, α= 0.05) to avoid false positives.

Analysis of genetic linkage between pairwise loci

To confirm the independence of allele segregation among different loci, we carried out a test of linkage between pairwise loci using genetic information from parent trees that were doubly heterozygous for two loci, and observed segregation in their progeny. In this case, the null hypothesis (H_0) is regular Mendelian segregation of 1:1:1:1. The hypothesis of regular segregation between pairwise loci was accepted or discarded based on a maximum likelihood *G*-test (SOKAL; ROHLF, 1981), shown in Equation 3, performed for each progeny. For each cell the expected frequency under the null hypothesis of segregation 1:1:1:1 was calculated as:

$$G = 2\left[n_{ik} \ln\left(\frac{n_{ik}}{E(n)}\right) + n_{il} \ln\left(\frac{n_{il}}{E(n)}\right) + n_{jk} \ln\left(\frac{n_{jk}}{E(n)}\right) + n_{jl} \ln\left(\frac{n_{jl}}{E(n)}\right)\right]$$ Equation (3)

where, n_{ik}, n_{il}, n_{jk}, and n_{jl} are the observed number of phenotypes A_iB_k, A_iB_l, A_jB_k, and A_jB_l, respectively, $E(n)$ is the expected number of genotypes A_iB_k, A_iB_l, A_jB_k, and A_jB_l, respectively, ln is the natural logarithm, and $E(n)$ is calculated as in Equation (4):

$$E(n) = 0.25\ (n_{ik} + n_{il} + n_{jk} + n_{jl})$$ Equation (4)

We again applied the Bonferroni correction for multiple comparisons (95%, α= 0.05) to avoid false positives.

Analysis of linkage disequilibrium

The genotypic disequilibrium test was performed only for adult trees, since genotypic disequilibrium is obviously expected in progeny arrays because all descendants always receive a maternal allele, which generates an "apparent genotypic imbalance." The genotypic disequilibrium test was carried out using the FSTAT program (GOUDET, 1995). The H_0 was tested and the probability of the test was used to determine the imbalance between all pairwise

loci. For the avoidance of false positives, we used a Bonferroni correction at 95% probability (P= 0.05).

3.2.2 Results and discussion

The results showed a significant deviation from the expected 1:1 Mendelian segregation pattern in only 22 cases of 589 tests (3.7%) (Table 3.1). For the Ce07, Cle12, and Cle04 loci, no deviation was observed. In the other loci, some deviations were detected in different progenies.

Table 3.1 - Mendelian inheritance tests for nine microsatellite loci in *C. legalis*

(continue)

Loci	**Seed-Trees**	**Genotypes**	**n1**	$n_{ij} : n_{ii} + n_{jj}$	G_1	**n2**	$n_{ik} : n_{jk}$	G_2
Ce07	J04	170/186	40	20:20	0.00	0	NE	0.00
	J16	180/186	24	6:18	6.27	15	6:9	0.60
	J22	170/186	34	20:18	1.06	3	1:2	0.34
	J23	176/184	24	5:19	8.70	16	8:8	0.00
	J27	168/186	26	6:20	7.95	9	3:6	1.09
	J41	172/182	25	7:18	5.01	12	3:9	3.13
	J49	180/186	25	7:18	2.01	14	13:1	12.2
	J61	178/202	31	25:6	12.51	8	5:3	0.50
	J67	184/202	27	15:12	0.33	13	7:6	0.07
	J70	168/184	18	10:8	0.22	21	8:13	1.02
	1M	176/184	23	18:5	7.79	20	16:4	7.70
	2M	164/184	17	11:6	1.49	22	13:9	0.73
	3M	166/174	22	13:9	0.73	22	18:4	9.63
	4M	160/180	21	10:11	0.04	23	11:12	0.04
	5M	170/176	29	15:14	0.03	12	3:9	3.13
	6M	162/182	60	37:23	3.30	40	20:20	0.00
Cle09	J16	160/174	20	19:1	19.79*	20	9:11	0.20
	J23	160/176	30	19:11	2.15	10	5:5	0.00
	J27	178/186	12	8:4	1.36	28	21:7	7.32
	J28	160/176	26	16:10	1.39	14	5:9	1.15

Table 3.1 - Mendelian inheritance tests for nine microsatellite loci in *C. legalis*

(continuation)

	Loci	Seed-Trees	Genotypes	n1	$n_{ij} : n_{ii} + n_{jj}$ G_1	n2	$n_{ik} : n_{jk}$	G_2
	J30	176/182	6	0:6	NE	31	22:9	5.62
	J41	154/168	26	18:8	3.94	14	14:0	NE
	J49	152/158	22	17:5	6.91	18	15:3	8.73
	J61	170/180	16	13:3	6.70	24	23:1	24.91*
	J67	154/170	38	17:21	0.42	1	0:1	NE
	J70	152/168	26	11:15	0.61	14	8:6	0.28
	1M	168/198	34	17:17	0.00	13	12:1	10.97
	4M	150/166	39	14:25	3.14	6	0:6	NE
	5M	152/166	40	22:18	0.40	4	1:3	1.04
	7M	150/158	78	43:35	0.82	21	12:9	0.43
Cle10	J04	160/166	35	30:5	19.81*	5	5:0	NE
	J06	160/166	30	17:13	0.53	9	9:0	NE
	J16	156/162	19	16:3	9.75	21	5:16	6.05
	J22	160/168	20	12:8	0.80	20	20:0	NE
	J23	162/168	26	22:4	13.72	13	6:7	0.07
	J27	162/168	24	20:4	11.64	16	10:6	1.01
	J28	160/168	31	25:6	12.51	9	9:0	NE
	J29	162/170	23	18:5	7.80	16	11:5	2.30
	J30	160/166	27	21:6	8.82	13	8:5	0.69
	J36	160/166	25	12:13	0.04	15	11:4	3.39
	J41	152/166	28	21:7	7.32	11	6:5	0.09
	J49	150/156	19	19:0	NE	20	15:5	5.23
	J61	150/164	26	9:17	2.50	14	7:7	0.00
	J67	152/166	22	11:11	0.00	16	16:0	NE
	J70	150/166	13	3:10	3.97	27	23:4	14.78
	1M	150/154	39	24:15	2.09	11	8:3	2.35
	2M	146/156	29	15:14	0.03	17	9:8	0.05
	3M	150/164	39	30:9	11.92	10	7:3	1.64

Table 3.1 - Mendelian inheritance tests for nine microsatellite loci in *C. legalis*

(continuation)

	Loci	**Seed-Trees**	**Genotypes**	**n1**	$n_{ij} : n_{ii} + n_{jj}$ G_1	**n2**	$n_{ik} : n_{jk}$	G_2
	4M	150/164	40	32:8	15.41*	10	6:4	0.40
	5M	146/162	27	11:16	0.93	22	8:14	1.65
	6M	150/166	49	27:22	0.51	48	36:12	12.55
	7M	150/156	77	52:25	9.67	22	11:11	0.00
Cle12	J16	202/216	16	9:7	0.25	24	11:13	0.17
	J22	202/216	18	8:10	0.22	21	6:15	3.90
	J28	204/210	15	2:13	9.01	26	18:8	3.94
	J30	206/220	13	9:4	1.97	27	14:13	0.03
	J36	202/216	21	4:17	8.66	19	13:6	2.64
	J41	198/204	9	1:8	6.19	30	15:15	0.00
	J49	198/216	8	5:3	0.50	31	20:11	2.65
	J61	202/218	25	9:16	1.98	14	12:2	7.92
	J67	202/220	33	18:15	0.27	7	1:6	3.90
	J70	200/230	24	16:8	2.71	16	3:13	6.73
	1M	214/220	38	26:12	5.28	10	4:6	0.40
	2M	220/230	26	19:7	5.75	23	20:3	14.07
	3M	202/216	27	12:15	0.33	23	4:19	10.63
	4M	200/230	20	9:11	0.20	30	16:14	0.13
	5M	196/222	16	10:6	1.01	34	14:20	1.06
	6M	202/230	62	33:29	0.25	38	17:21	0.42
	7M	200/214	68	31:37	0.53	31	15:16	0.03

Table 3.1 - Mendelian inheritance tests for nine microsatellite loci in *C. legalis*

(continuation)

Loci	**Seed-Trees**	**Genotypes**	**n1**	$n_{ij} : n_{ii} + n_{jj}$	G_1	**n2**	$n_{ik} : n_{jk}$	G_2
Cle04	J04	268/272	27	14:13	0.03	10	6:4	0.40
	J06	268/272	22	8:14	1.65	17	8:9	0.05
	J16	270/288	13	4:9	1.97	26	15:11	0.61
	J22	266/274	9	1:8	6.19	29	13:16	0.31
	J23	270/284	17	3:14	7.72	21	18:3	11.89
	J27	270/284	22	3:19	12.97	17	13:4	5.01
	J28	268/276	11	3:8	2.35	29	14:15	0.03
	J29	270/284	15	5:11	1.69	24	23:1	24.96*
	J30	270/274	28	12:16	0.57	12	8:4	1.35
	J36	268/274	22	8:14	1.65	18	11:7	0.89
	J41	286/290	20	2:18	14.72	19	8:11	0.47
	J49	272/288	2	0:2	NE	38	20:18	0.10
	J61	276/294	15	14:1	13.45	25	10:15	1.00
	J67	286/300	24	14:10	0.67	16	12:4	4.18
	J70	286/290	14	5:9	1.15	25	9:16	1.98
	1M	280/300	34	24:10	5.93	15	11:4	3.30
	2M	278/290	27	24:3	18.59*	23	9:14	1.09
	3M	272/286	35	9:26	8.61	11	1:10	8.54
	4M	278/288	25	12:13	0.04	25	5:20	6.63
	5M	284/294	29	26:3	20.91*	18	11:7	0.89
	6M	278/300	17	12:5	2.96	72	35:37	0.05
	7M	270/284	50	19:31	2.90	46	5:41	32.1*
Cle08	J06	156/174	22	4:18	9.64	18	12:6	2.03
	J22	176/180	7	2:5	1.33	33	27:6	14.40
	J23	152/176	25	3:22	16.31*	12	1:11	9.75
	J27	156/162	12	6:6	0.00	26	21:5	10.59
	J28	156/162	11	3:8	2.36	29	15:14	0.03
	J30	156/166	22	4:18	9.64	19	5:14	4.43

Table 3.1 - Mendelian inheritance tests for nine microsatellite loci in *C. legalis*

(continuation)

Loci	Seed-Trees	Genotypes	n1	$n_{ij} : n_{ii} + n_{jj}$	G_1	n2	$n_{ik} : n_{jk}$	G_2
	J36	156/176	23	11:12	0.04	16	2:14	10.12
	J41	156/160	33	17:16	0.03	7	7:0	NE
	J49	172/178	14	2:12	7.93	25	16:9	1.98
	J61	154/172	24	11:13	0.17	13	11:2	6.85
	J67	152/168	24	9:15	1.52	14	5:9	1.15
	J70	158/172	18	13:5	3.69	20	14:6	3.29
	1M	148/164	24	16:8	2.71	10	4:6	0.40
	2M	148/154	18	15:3	8.73	24	1:23	24.95*
	3M	154/162	13	7:6	0.07	37	21:16	0.67
	4M	156/160	16	4:12	4.18	33	17:16	0.03
	5M	156/158	16	11:5	2.30	35	15:20	0.71
	6M	150/156	31	3:28	23.26*	58	10:48	27.08*
	7M	154/170	54	39:15	11.04	44	14:30	5.95
Cle01	J22	160/166	22	22:0	NE	15	4:11	3.39
	J23	170/182	20	11:9	0.20	20	17:3	10.82
	J28	162/170	18	8:10	0.23	22	12:10	0.18
	J30	162/170	23	19:4	10.63	16	9:7	0.25
	J36	168/180	23	10:13	0.39	16	5:11	2.31
	J41	160/166	23	8:15	2.17	18	3:15	8.73
	J49	166/168	15	3:12	5.78	24	16:8	2.72
	J61	178/184	20	13:7	1.83	20	16:4	7.71
	J67	172/180	25	16:9	1.99	15	8:7	0.07
	J70	170/178	16	9:7	0.25	21	14:7	2.38
	1M	176/184	26	18:8	3.94	21	18:3	11.88
	2M	170/232	21	16:5	6.05	29	11:18	1.70
	3M	170/176	31	23:8	7.57	18	5:13	3.68
	4M	158/166	18	17:1	17.22*	30	10:20	3.39

Table 3.1 - Mendelian inheritance tests for nine microsatellite loci in *C. legalis*

(continuation)

	Loci	**Seed-Trees**	**Genotypes**	**n1**	$n_{ij} : n_{ii} + n_{jj}$ G_1		**n2**	$n_{ik} : n_{jk}$	G_2
	5M	176/180	26	21:5	10.58	24	20:4	11.64	
	6M	162/176	51	38:13	12.80	44	22:22	0.00	
	7M	156/162	44	28:16	3.31	53	17:36	3.20	
Cle05	J04	190/202	22	16:6	4.72	18	18:0	NE	
	J06	190/202	33	21:12	2.49	5	4:1	1.92	
	J16	190/196	39	38:1	44.76*	1	1:0	NE	
	J22	190/196	38	33:5	23.09*	2	2:0	NE	
	J23	190/196	39	30:9	11.93	1	0:1	NE	
	J27	190/196	39	30:9	11.93	1	0:1	NE	
	J28	190/200	24	22:2	19.50*	16	7:9	0.25	
	J29	190/226	11	9:2	4.82	29	18:11	1.70	
	J30	188/194	29	21:8	6.04	11	8:3	2.35	
	J36	188/194	17	13:4	5.02	22	14:8	1.65	
	J41	188/196	11	3:8	2.36	27	14:13	0.03	
	J49	186/190	16	13:3	6.74	19	4:15	6.78	
	J61	188/202	23	19:4	10.63	17	12:5	2.96	
	J67	188/202	29	19:10	2.84	11	8:3	2.35	
	J70	188/202	19	14:5	4.44	20	14:6	3.29	
	1M	186/200	32	30:2	29.39*	7	4:3	0.14	
	2M	186/200	28	24:4	15.84*	14	10:4	2.65	
	3M	184/186	17	0:17	NE	29	9:20	4.27	
	4M	182/196	29	21:8	6.04	16	11:5	2.30	
	5M	186/190	38	21:17	0.42	9	6:3	1.01	
	6M	180/226	40	29:11	8.39	58	37:21	4.47	
	7M	186/196	56	51:5	43.93*	41	23:18	0.61	

Table 3.1 - Mendelian inheritance tests for nine microsatellite loci in *C. legalis*

(Conclusion)

Loci	Seed-Trees	Genotypes	n1	$n_{ij} : n_{ii} + n_{jj}$	G_1	n2	$n_{ik} : n_{jk}$	G_2
Ce18	J04	166/170	15	11:4	3.39	24	12:12	0.00
	J06	170/182	35	21:14	1.41	5	5:0	NE
	J16	164/172	21	6:15	3.99	19	13:6	2.64
	J22	166/218	21	10:11	0.05	19	16:3	9.77
	J27	166/226	15	6:9	0.60	25	23:2	20.72*
	J28	162/172	17	7:10	0.53	23	13:10	0.39
	J29	162/172	25	16:9	1.99	14	8:6	0.29
	J30	162/214	18	7:11	0.89	23	21:2	18.29
	J36	162/172	17	11:6	1.49	22	16:6	4.72
	J49	164/172	25	17:8	3.31	14	10:4	2.66
	J61	162/170	24	19:5	8.70	18	11:7	0.89
	J67	166/176	17	11:6	1.49	23	19:4	10.63
	J70	162/218	25	11:14	0.36	15	9:6	0.60
	1M	158/212	23	18:5	7.79	22	17:5	6.91
	2M	166/218	22	21:1	22.36*	24	16:8	2.71
	3M	166/180	30	21:9	4.93	16	11:5	2.30
	4M	164/184	21	17:4	8.66	23	10:13	0.39
	5M	166/218	28	23:5	12.53	19	10:9	0.05
	6M	162/218	30	23:7	8.99	65	42:23	5.63
	7M	160/228	58	53:5	46.33*	40	20:20	0.00

n1 and 2 = sample size; G_1 and G_2 = maximum likelihood G statistics for the hypothesis of $n_{ij} = n_{ii} + n_{jj}$ and $n_{ik} = n_{jk}$, respectively with one degree of freedom. *Significance after Bonferroni's correction for $\alpha = 0.05$ (χ^2 Table = 15.14). NE=Not estimated.

The *G*-test was used to compare the observed values with those expected under the null hypothesis for the 1:1:1:1 segregation of genotypes between two heterozygous SSR loci. After the Bonferroni correction, only 2.8% of the 594 linkage tests performed (Table 3.2) were significant. However, in all cases in which significant linkage was observed, it occurred in different pairs of loci of different sampled progenies. Moreover, in the largest group of sampled progenies (N = 100), all pairwise loci adjusted to the expected 1:1:1:1 Mendelian

segregation. On the other hand, the majority of progeny adhered to the expected 1:1:1:1 Mendelian inheritance for the same pairs of loci analyzed.

Table 3.2 - Maximum likelihood G-test for testing the hypothesis of independent segregation between pairs of microsatellite loci (1:1:1:1) in *C. legalis*. *Significance after Bonferroni's correction for α= 0.05, 0.00047 (χ^2 Table=21.45). G= G-test for three degree of freedom. NE= not estimated (continue)

Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G
Ce07xCle09	J16	12.95	Ce07xCle12	J16	14.47	Ce07xCle04	6M	1.52	Ce07xCle01	6M	0.56	Ce07xCe18	3M	5.62
Ce07xCle09	J23	7.66	Ce07xCle12	J22	2.83	Ce07xCle04	7M	5.00	Ce07xCle01	7M	3.47	Ce07xCe18	4M	3.12
Ce07xCle09	J27	12.38	Ce07xCle12	J41	10.08	Ce07xCle08	J22	11.88	Ce07xCle05	J04	4.63	Ce07xCe18	5M	4.60
Ce07xCle09	J41	8.37	Ce07xCle12	J49	11.91	Ce07xCle08	J23	17.86	Ce07xCle05	J16	13.17	Ce07xCe18	6M	3.04
Ce07xCle09	J49	9.67	Ce07xCle12	J61	11.22	Ce07xCle08	J27	15.04	Ce07xCle05	J22	5.48	Ce07xCe18	7M	2.65
Ce07xCle09	J61	9.37	Ce07xCle12	J67	4.91	Ce07xCle08	J41	18.71	Ce07xCle05	J23	11.43	Cle09xCle10	J16	5.15
Ce07xCle09	J67	3.36	Ce07xCle12	J70	2.73	Ce07xCle08	J49	6.40	Ce07xCle05	J27	5.94	Cle09xCle10	J23	0.28
Ce07xCle09	J70	13.11	Ce07xCle12	1M	1.74	Ce07xCle08	J61	5.67	Ce07xCle05	J41	8.34	Cle09xCle10	J27	8.60
Ce07xCle09	1M	2.73	Ce07xCle12	2M	13.60	Ce07xCle08	J67	7.77	Ce07xCle05	J49	9.15	Cle09xCle10	J28	2.41
Ce07xCle09	4M	12.80	Ce07xCle12	3M	2.87	Ce07xCle08	J70	2.67	Ce07xCle05	J61	4.88	Cle09xCle10	J30	8.50
Ce07xCle09	5M	11.57	Ce07xCle12	4M	6.79	Ce07xCle08	1M	1.42	Ce07xCle05	J67	1.98	Cle09xCle10	J41	12.59
Ce07xCle09	7M	1.29	Ce07xCle12	5M	5.22	Ce07xCle08	2M	4.76	Ce07xCle05	J70	14.05	Cle09xCle10	J49	11.31
Ce07xCle10	J04	4.26	Ce07xCle12	6M	0.29	Ce07xCle08	3M	6.26	Ce07xCle05	1M	0.49	Cle09xCle10	J61	28.07*
Ce07xCle10	J16	17.65	Ce07xCle12	7M	0.31	Ce07xCle08	4M	0.37	Ce07xCle05	2M	2.89	Cle09xCle10	J67	6.62
Ce07xCle10	J22	23.76*	Ce07xCle04	J04	7.09	Ce07xCle08	5M	7.31	Ce07xCle05	3M	1.70	Cle09xCle10	J70	11.24
Ce07xCle10	J23	6.29	Ce07xCle04	J16	8.72	Ce07xCle08	6M	8.71	Ce07xCle05	4M	2.85	Cle09xCle10	1M	2.72
Ce07xCle10	J27	2.62	Ce07xCle04	J22	2.89	Ce07xCle08	7M	3.79	Ce07xCle05	5M	4.08	Cle09xCle10	4M	13.43

Table 3.2 - Maximum likelihood G-test for testing the hypothesis of independent segregation between pairs of microsatellite loci (1:1:1:1) in *C. legalis*. *Significance after Bonferroni's correction for α= 0.05, 0.00047 (χ^2 Table=21.45). G= G-test for three degree of freedom. NE= not estimated

(continuation)

Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G
Ce07xCle10	J41	10.90	Ce07xCle04	J23	18.23	Ce07xCle01	J22	2.92	Ce07xCle05	6M	2.09	Cle09xCle10	5M	2.75
Ce07xCle10	J49	14.92	Ce07xCle04	J27	11.01	Ce07xCle01	J23	15.70	Ce07xCle05	7M	1.15	Cle09xCle10	7M	0.45
Ce07xCle10	J61	6.58	Ce07xCle04	J41	11.19	Ce07xCle01	J41	16.05	Ce07xCe18	J04	8.09	Cle09xCle12	J16	8.06
Ce07xCle10	J67	5.26	Ce07xCle04	J49	2.47	Ce07xCle01	J49	9.81	Ce07xCe18	J16	17.04	Cle09xCle12	J28	29.58*
Ce07xCle10	J70	1.17	Ce07xCle04	J61	2.02	Ce07xCle01	J61	4.66	Ce07xCe18	J22	2.60	Cle09xCle12	J30	5.11
Ce07xCle10	1M	2.20	Ce07xCle04	J67	6.12	Ce07xCle01	J67	5.08	Ce07xCe18	J27	15.81	Cle09xCle12	J41	0.76
Ce07xCle10	2M	3.71	Ce07xCle04	J70	3.00	Ce07xCle01	J70	3.71	Ce07xCe18	J49	9.97	Cle09xCle12	J49	4.47
Ce07xCle10	3M	6.5	Ce07xCle04	1M	5.84	Ce07xCle01	1M	12.70	Ce07xCe18	J61	7.17	Cle09xCle12	J61	33.60*
Ce07xCle10	4M	3.26	Ce07xCle04	2M	5.53	Ce07xCle01	2M	5.87	Ce07xCe18	J67	9.70	Cle09xCle12	J67	4.96
Ce07xCle10	5M	3.42	Ce07xCle04	3M	9.57	Ce07xCle01	3M	7.59	Ce07xCe18	J70	2.08	Cle09xCle12	J70	4.13
Ce07xCle10	6M	2.55	Ce07xCle04	4M	1.54	Ce07xCle01	4M	2.10	Ce07xCe18	1M	8.91	Cle09xCle12	1M	1.53
Ce07xCle10	7M	0.85	Ce07xCle04	5M	9.59	Ce07xCle01	5M	13.19	Ce07xCe18	2M	4.30	Cle09xCle12	4M	6.36

Table 3.2 - Maximum likelihood G-test for testing the hypothesis of independent segregation between pairs of microsatellite loci (1:1:1:1) in *C. legalis*. *Significance after Bonferroni's correction for α= 0.05, 0.00047 (χ^2 Table=21.45). G= G-test for three degree of freedom. NE= not estimated

(continuation)

Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G
Cle09xCle12	5M	1.62	Cle09xCle01	J23	3.18	Cle09xCe18	J30	4.11	Cle10xCle04	J22	9.46	Cle10xCle08	J67	5.76
Cle09xCle12	7M	2.44	Cle09xCle01	J28	0.66	Cle09xCe18	J49	5.79	Cle10xCle04	J23	13.48	Cle10xCle08	J70	18.33
Cle09xCle04	J16	1.64	Cle09xCle01	J30	0.85	Cle09xCe18	J61	12.97	Cle10xCle04	J27	13.38	Cle10xCle08	1M	2.80
Cle09xCle04	J23	26.50*	Cle09xCle01	J41	5.98	Cle09xCe18	J67	8.75	Cle10xCle04	J28	5.18	Cle10xCle08	2M	9.64
Cle09xCle04	J27	13.50	Cle09xCle01	J49	8.22	Cle09xCe18	J70	3.22	Cle10xCle04	J29	2.30	Cle10xCle08	3M	2.81
Cle09xCle04	J28	1.31	Cle09xCle01	J61	11.35	Cle09xCe18	1M	9.11	Cle10xCle04	J30	1.46	Cle10xCle08	4M	0.36
Cle09xCle04	J30	14.49	Cle09xCle01	J67	3.41	Cle09xCe18	4M	6.69	Cle10xCle04	J36	9.32	Cle10xCle08	5M	0.55
Cle09xCle04	J41	1.73	Cle09xCle01	J70	6.63	Cle09xCe18	5M	4.45	Cle10xCle04	J41	3.53	Cle10xCle08	6M	5.86
Cle09xCle04	J49	3.26	Cle09xCle01	1M	7.36	Cle09xCe18	6M	1.65	Cle10xCle04	J49	4.14	Cle10xCle08	7M	2.76
Cle09xCle04	J61	10.23	Cle09xCle01	4M	15.80	Cle10xCle12	J16	4.54	Cle10xCle04	J61	2.93	Cle10xCle01	J22	16.78
Cle09xCle04	J67	6.15	Cle09xCle01	5M	6.06	Cle10xCle12	J22	22.94*	Cle10xCle04	J67	4.55	Cle10xCle01	J23	2.41
Cle09xCle04	J70	2.50	Cle09xCle01	7M	9.11	Cle10xCle12	J28	10.14	Cle10xCle04	J70	12.76	Cle10xCle01	J28	6.20
Cle09xCle04	1M	5.06	Cle09xCle05	J16	0.03	Cle10xCle12	J30	1.74	Cle10xCle04	1M	5.06	Cle10xCle01	J30	0.60
Cle09xCle04	4M	7.14	Cle09xCle05	J23	1.41	Cle10xCle12	J36	7.59	Cle10xCle04	2M	1.26	Cle10xCle01	J36	10.96
Cle09xCle04	5M	6.03	Cle09xCle05	J27	15.48	Cle10xCle12	J41	3.30	Cle10xCle04	3M	21.11	Cle10xCle01	J41	10.34

Table 3.2 - Maximum likelihood G-test for testing the hypothesis of independent segregation between pairs of microsatellite loci (1:1:1:1) in *C. legalis*.
*Significance after Bonferroni's correction for α= 0.05, 0.00047 (χ^2 Table=21.45). G= G-test for three degree of freedom. NE= not estimated

(continuation)

Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G
Cle09xCle04	7M	7.60	Cle09xCle05	J28	0.42	Cle10xCle12	J49	4.41	Cle10xCle04	4M	4.26	Cle10xCle01	J49	5.73
Cle09xCle08	J23	25.94*	Cle09xCle05	J30	3.20	Cle10xCle12	J61	17.76	Cle10xCle04	5M	4.30	Cle10xCle01	J61	8.12
Cle09xCle08	J27	18.44	Cle09xCle05	J41	2.26	Cle10xCle12	J67	11.15	Cle10xCle04	6M	3.48	Cle10xCle01	J67	5.06
Cle09xCle08	J28	0.32	Cle09xCle05	J49	7.53	Cle10xCle12	J70	19.02	Cle10xCle04	7M	4.85	Cle10xCle01	J70	11.89
Cle09xCle08	J30	11.77	Cle09xCle05	J61	18.18	Cle10xCle12	1M	0.86	Cle10xCle08	J06	4.26	Cle10xCle01	1M	4.04
Cle09xCle08	J41	7.21	Cle09xCle05	J67	3.40	Cle10xCle12	2M	8.23	Cle10xCle08	J22	30.61*	Cle10xCle01	2M	5.16
Cle09xCle08	J49	17.37	Cle09xCle05	J70	0.59	Cle10xCle12	3M	14.26	Cle10xCle08	J23	18.78	Cle10xCle01	3M	1.22
Cle09xCle08	J61	12.72	Cle09xCle05	1M	1.81	Cle10xCle12	4M	7.50	Cle10xCle08	J27	11.67	Cle10xCle01	4M	4.86
Cle09xCle08	J67	13.84	Cle09xCle05	4M	10.57	Cle10xCle12	5M	0.38	Cle10xCle08	J28	1.26	Cle10xCle01	5M	8.33
Cle09xCle08	J70	4.48	Cle09xCle05	5M	2.34	Cle10xCle12	6M	5.85	Cle10xCle08	J30	1.34	Cle10xCle01	6M	3.82
Cle09xCle08	1M	3.15	Cle09xCle05	7M	1.52	Cle10xCle12	7M	1.40	Cle10xCle08	J36	9.26	Cle10xCle01	7M	5.67
Cle09xCle08	4M	7.17	Cle09xCe18	J16	5.35	Cle10xCle04	J04	0.42	Cle10xCle08	J41	2.28	Cle10xCle05	J04	12.95
Cle09xCle08	5M	4.51	Cle09xCe18	J27	23.92*	Cle10xCle04	J06	1.25	Cle10xCle08	J49	9.34	Cle10xCle05	J06	4.31
Cle09xCle08	7M	2.41	Cle09xCe18	J28	2.01	Cle10xCle04	J16	1.22	Cle10xCle08	J61	10.34	Cle10xCle05	J16	7.10

Table 3.2 - Maximum likelihood G-test for testing the hypothesis of independent segregation between pairs of microsatellite loci (1:1:1:1) in *C. legalis*. *Significance after Bonferroni's correction for α= 0.05, 0.00047 (χ^2 Table=21.45). G= G-test for three degree of freedom. NE= not estimated

(continuation)

Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G
Cle10xCle05	J22	21.19	Cle10xCe18	J61	7.34	Cle12xCle08	J30	1.24	Cle12xCle01	7M	5.84	Cle12xCe18	3M	7.20
Cle10xCle05	J23	3.88	Cle10xCe18	J67	9.97	Cle12xCle08	J36	10.79	Cle12xCle05	J16	0.60	Cle12xCe18	4M	5.09
Cle10xCle05	J27	5.27	Cle10xCe18	J70	16.53	Cle12xCle08	J41	3.84	Cle12xCle05	J22	2.08	Cle12xCe18	5M	0.90
Cle10xCle05	J28	3.33	Cle10xCe18	1M	6.88	Cle12xCle08	J49	5.58	Cle12xCle05	J28	5.68	Cle12xCe18	6M	3.27
Cle10xCle05	J29	2.40	Cle10xCe18	2M	0.89	Cle12xCle08	J61	16.32	Cle12xCle05	J30	6.13	Cle12xCe18	7M	2.21
Cle10xCle05	J30	3.72	Cle10xCe18	3M	5.80	Cle12xCle08	J67	13.65	Cle12xCle05	J36	7.17	Cle04xCle08	J06	3.25
Cle10xCle05	J36	5.82	Cle10xCe18	4M	3.11	Cle12xCle08	J70	2.31	Cle12xCle05	J41	2.55	Cle04xCle08	J22	13.90
Cle10xCle05	J41	0.62	Cle10xCe18	5M	1.63	Cle12xCle08	1M	1.42	Cle12xCle05	J49	12.83	Cle04xCle08	J23	26.05*
Cle10xCle05	J49	11.72	Cle10xCe18	6M	9.72	Cle12xCle08	2M	18.68	Cle12xCle05	J61	14.64	Cle04xCle08	J27	12.67
Cle10xCle05	J61	12.00	Cle10xCe18	7M	1.99	Cle12xCle08	3M	3.03	Cle12xCle05	J67	4.20	Cle04xCle08	J28	4.04
Cle10xCle05	J67	4.29	Cle12xCle04	J16	1.51	Cle12xCle08	4M	4.34	Cle12xCle05	J70	1.05	Cle04xCle08	J30	0.91
Cle10xCle05	J70	12.85	Cle12xCle04	J22	2.55	Cle12xCle08	5M	2.00	Cle12xCle05	1M	0.57	Cle04xCle08	J36	7.18
Cle10xCle05	1M	1.57	Cle12xCle04	J28	2.99	Cle12xCle08	6M	7.67	Cle12xCle05	2M	13.35	Cle04xCle08	J41	12.95
Cle10xCle05	2M	2.89	Cle12xCle04	J30	2.89	Cle12xCle08	7M	4.50	Cle12xCle05	3M	3.71	Cle04xCle08	J49	2.52
Cle10xCle05	3M	3.39	Cle12xCle04	J36	6.71	Cle12xCle01	J22	3.76	Cle12xCle05	4M	7.03	Cle04xCle08	J61	9.70

Table 3.2 - Maximum likelihood G-test for testing the hypothesis of independent segregation between pairs of microsatellite loci (1:1:1:1) in *C. legalis*. *Significance after Bonferroni's correction for α= 0.05, 0.00047 (χ^2 Table=21.45). G= G-test for three degree of freedom. NE= not estimated

(continuation)

Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G
Cle10xCle05	4M	2.48	Cle12xCle04	J41	6.26	Cle12xCle01	J28	4.82	Cle12xCle05	5M	0.97	Cle04xCle08	J67	5.82
Cle10xCle05	5M	2.52	Cle12xCle04	J49	2.86	Cle12xCle01	J30	0.96	Cle12xCle05	6M	3.01	Cle04xCle08	J70	3.20
Cle10xCle05	6M	1.08	Cle12xCle04	J61	16.98	Cle12xCle01	J36	4.77	Cle12xCle05	7M	1.16	Cle04xCle08	1M	2.67
Cle10xCle05	7M	0.20	Cle12xCle04	J67	8.43	Cle12xCle01	J41	14.18	Cle12xCe18	J16	4.40	Cle04xCle08	2M	14.92
Cle10xCe18	J04	2.01	Cle12xCle04	J70	3.96	Cle12xCle01	J49	6.93	Cle12xCe18	J22	13.38	Cle04xCle08	3M	8.88
Cle10xCe18	J06	5.29	Cle12xCle04	1M	7.11	Cle12xCle01	J61	18.01	Cle12xCe18	J28	4.58	Cle04xCle08	4M	6.04
Cle10xCe18	J16	4.55	Cle12xCle04	2M	12.19	Cle12xCle01	J67	3.27	Cle12xCe18	J30	9.43	Cle04xCle08	5M	3.34
Cle10xCe18	J22	27.58*	Cle12xCle04	3M	13.40	Cle12xCle01	J70	4.18	Cle12xCe18	J36	12.00	Cle04xCle08	6M	2.30
Cle10xCe18	J27	33.14*	Cle12xCle04	4M	13.35	Cle12xCle01	1M	10.37	Cle12xCe18	J49	5.38	Cle04xCle08	7M	6.45
Cle10xCe18	J28	6.04	Cle12xCle04	5M	1.41	Cle12xCle01	2M	9.83	Cle12xCe18	J61	16.70	Cle04xCle01	J22	0.38
Cle10xCe18	J29	1.27	Cle12xCle04	6M	0.59	Cle12xCle01	3M	7.18	Cle12xCe18	J67	12.40	Cle04xCle01	J23	21.41*
Cle10xCe18	J30	12.11	Cle12xCle04	7M	7.78	Cle12xCle01	4M	5.26	Cle12xCe18	J70	4.23	Cle04xCle01	J28	5.98
Cle10xCe18	J36	11.77	Cle12xCle08	J22	14.36	Cle12xCle01	5M	5.39	Cle12xCe18	1M	4.01	Cle04xCle01	J30	3.54
Cle10xCe18	J49	4.46	Cle12xCle08	J28	1.75	Cle12xCle01	6M	0.49	Cle12xCe18	2M	10.02	Cle04xCle01	J36	2.33

Table 3.2 - Maximum likelihood G-test for testing the hypothesis of independent segregation between pairs of microsatellite loci (1:1:1:1) in *C. legalis*. *Significance after Bonferroni's correction for α= 0.05, 0.00047 (χ^2 Table=21.45). G= G-test for three degree of freedom. NE= not estimated

(continuation)

Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G
Cle04xCle01	J41	10.18	Cle04xCle05	3M	13.95	Cle08xCle01	J36	6.13	Cle08xCle05	5M	1.01	Cle01xCle05	J70	2.71
Cle04xCle01	J49	1.70	Cle04xCle05	4M	7.20	Cle08xCle01	J41	7.33	Cle08xCle05	6M	5.69	Cle01xCle05	1M	6.59
Cle04xCle01	J61	2.62	Cle04xCle05	5M	2.56	Cle08xCle01	J49	5.47	Cle08xCle05	7M	1.43	Cle01xCle05	2M	3.40
Cle04xCle01	J67	3.62	Cle04xCle05	6M	4.37	Cle08xCle01	J61	14.35	Cle08xCe18	J06	4.03	Cle01xCle05	3M	4.93
Cle04xCle01	J70	3.53	Cle04xCle05	7M	9.68	Cle08xCle01	J67	3.77	Cle08xCe18	J22	20.45	Cle01xCle05	4M	8.76
Cle04xCle01	1M	10.92	Cle04xCe18	J04	6.46	Cle08xCle01	J70	5.91	Cle08xCe18	J27	NE	Cle01xCle05	5M	6.74
Cle04xCle01	2M	1.88	Cle04xCe18	J06	3.13	Cle08xCle01	1M	7.16	Cle08xCe18	J28	4.99	Cle01xCle05	6M	3.15
Cle04xCle01	3M	15.31	Cle04xCe18	J16	6.79	Cle08xCle01	2M	14.01	Cle08xCe18	J30	12.59	Cle01xCle05	7M	1.75
Cle04xCle01	4M	6.83	Cle04xCe18	J22	9.70	Cle08xCle01	3M	2.79	Cle08xCe18	J36	5.59	Cle01xCe18	J22	18.08
Cle04xCle01	5M	7.49	Cle04xCe18	J27	22.58*	Cle08xCle01	4M	0.90	Cle08xCe18	J49	10.21	Cle01xCe18	J28	4.60
Cle04xCle01	6M	0.84	Cle04xCe18	J28	4.96	Cle08xCle01	5M	6.25	Cle08xCe18	J61	2.87	Cle01xCe18	J30	8.89
Cle04xCle01	7M	8.24	Cle04xCe18	J29	9.58	Cle08xCle01	6M	13.33	Cle08xCe18	J67	8.06	Cle01xCe18	J36	4.66
Cle04xCle05	J04	6.37	Cle04xCe18	J30	8.46	Cle08xCle01	7M	6.00	Cle08xCe18	J70	2.87	Cle01xCe18	J49	8.20
Cle04xCle05	J06	1.47	Cle04xCe18	J36	1.33	Cle08xCle05	J06	6.37	Cle08xCe18	1M	7.60	Cle01xCe18	J61	4.56
Cle04xCle05	J16	1.29	Cle04xCe18	J49	0.51	Cle08xCle05	J22	23.00*	Cle08xCe18	2M	12.14	Cle01xCe18	J67	7.13

Table 3.2 - Maximum likelihood G-test for testing the hypothesis of independent segregation between pairs of microsatellite loci (1:1:1:1) in *C. legalis*. *Significance after Bonferroni's correction for α= 0.05, 0.00047 (χ^2 Table=21.45). G= G-test for three degree of freedom. NE= not estimated

(continuation)

Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G
Cle04xCle05	J22	1.46	Cle04xCe18	J61	4.02	Cle08xCle05	J23	24.01*	Cle08xCe18	3M	4.27	Cle01xCe18	J70	4.77
Cle04xCle05	J23	16.27	Cle04xCe18	J67	12.13	Cle08xCle05	J27	13.44	Cle08xCe18	4M	5.28	Cle01xCe18	1M	6.94
Cle04xCle05	J27	17.35	Cle04xCe18	J70	4.15	Cle08xCle05	J28	1.11	Cle08xCe18	5M	1.52	Cle01xCe18	2M	1.58
Cle04xCle05	J28	0.94	Cle04xCe18	1M	8.41	Cle08xCle05	J30	3.26	Cle08xCe18	6M	12.08	Cle01xCe18	3M	1.32
Cle04xCle05	J29	5.32	Cle04xCe18	2M	3.73	Cle08xCle05	J36	9.68	Cle08xCe18	7M	2.38	Cle01xCe18	4M	4.61
Cle04xCle05	J30	2.51	Cle04xCe18	3M	21.08	Cle08xCle05	J41	0.86	Cle01xCle05	J22	3.51	Cle01xCe18	5M	7.39
Cle04xCle05	J36	6.30	Cle04xCe18	4M	5.51	Cle08xCle05	J49	7.62	Cle01xCle05	J23	5.14	Cle01xCe18	6M	5.57
Cle04xCle05	J41	9.19	Cle04xCe18	5M	2.10	Cle08xCle05	J61	6.58	Cle01xCle05	J28	0.11	Cle01xCe18	7M	7.80
Cle04xCle05	J49	3.20	Cle04xCe18	6M	1.93	Cle08xCle05	J67	4.24	Cle01xCle05	J30	4.45	Cle05xCe18	J04	6.46
Cle04xCle05	J61	2.72	Cle04xCe18	7M	9.43	Cle08xCle05	J70	8.01	Cle01xCle05	J36	9.18	Cle05xCe18	J06	5.02
Cle04xCle05	J67	10.10	Cle08xCle01	J22	14.79	Cle08xCle05	1M	1.38	Cle01xCle05	J41	6.97	Cle05xCe18	J16	10.92
Cle04xCle05	J70	0.52	Cle08xCle01	J23	20.25	Cle08xCle05	2M	13.94	Cle01xCle05	J49	10.42	Cle05xCe18	J22	17.30
Cle04xCle05	1M	3.56	Cle08xCle01	J28	2.29	Cle08xCle05	3M	4.11	Cle01xCle05	J61	5.76	Cle05xCe18	J27	25.77*
Cle04xCle05	2M	2.85	Cle08xCle01	J30	2.43	Cle08xCle05	4M	3.88	Cle01xCle05	J67	1.25	Cle05xCe18	J28	0.29
Cle05xCe18	J49	5.91	Cle05xCe18	J70	1.52	Cle05xCe18	3M	6.56	Cle05xCe18	6M	5.83	Cle05xCe18	J29	2.47

Table 3.2 - Maximum likelihood G-test for testing the hypothesis of independent segregation between pairs of microsatellite loci (1:1:1:1) in *C. legalis*. *Significance after Bonferroni's correction for α= 0.05, 0.00047 (χ^2 Table=21.45). G= G-test for three degree of freedom. NE= not estimated

(conclusion)

Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G	Loci	Seed-trees	G
Cle05xCe18	J61	3.08	Cle05xCe18	1M	8.03	Cle05xCe18	4M	2.63	Cle05xCe18	7M	1.35	Cle05xCe18	J30	13.74
Cle05xCe18	J67	8.30	Cle05xCe18	2M	5.20	Cle05xCe18	5M	2.11				Cle05xCe18	J36	8.55

Genotypic disequilibrium in molecular markers is one of the basic assumptions for use in genetic diversity and structure, mating system, and paternity analyses. After Bonferroni correction, the results showed no significant evidence of genotypic disequilibrium between pairwise loci. Only 9.7% of pairwise loci showed linkage (Table 3.3). This was most likely a consequence of the small sample size within families. Combining this result with that observed in the test of linkage between loci, it appears that this imbalance was not associated with physical linkage between the loci.

These few cases of deviation of Mendelian segregation can be influenced by sex-linkage, physical association with genes under strong selection, centers of recombination, transposable elements, or processes during meiosis such as non-disjunction or meiotic drive (SELKOE and TOONEN, 2006). Some observed deviations were also likely caused by the reduced number of seedlings per progeny (ranging from 40 to100).

Again, it is happened in null hypothesis for the 1:1:1:1 segregation. With the reduced number of seedlings per progenies, deviations were expected by chance. This was apparent by the smaller deviations obtained for Mogi-Guaçu (N = 50 to 100 per seed-tree) when compared with the Ibicatu results (N = 40) samples. This suggests that the deviations found in groups of smaller sample size were sampling artifacts. Tarazi et al. (2010), using 20 seeds per progeny collected from 28 seed trees, found two loci with 20% significant genetic linkage in *Copaifera langsdorffii*. Carneiro et al. (2012) observed similar results in *Hymenaea courbaril* with sample sizes ranging from 13 to 20 seeds per seed tree. Both authors suggested that the small number of progenies were the likely cause of these observed deviations.

These results are expected in studies involving species with relatively large numbers of chromosomes and a small number of markers. In such situations, the probability of finding markers localized in a given chromosome is small. In general, *Cariniana* species have X = 17 chromosomes, and nine microsatellite markers were used in the present study. It is also important to mention that the majority of investigations involving wild species have been carried out using six loci markers. In only 10% of such studies were more than 10 loci used (KOSKINEN et al., 2004). Therefore, our study was based on more loci than are commonly used.

Also, genotypic disequilibrium can be generated by self-pollination, correlated mating, mating among relatives, genetic bottleneck effects, founder effects, and natural and artificial selection. The bottleneck effect is a possible explanation for the observed deviations in the

Ibicatu population, which recently underwent strong forest fragmentation. Belaj et al. (2007) observed that among a total of 308 tests for linkage disequilibrium between pairs of loci, 6% were significant. It is known that population structure increases linkage disequilibrium in the genome. Guidugli et al. (2009) found significant genotypic disequilibrium in only one pair of SSR loci for *C. estrellensis*. However, Tarazi et al. (2010) observed genotypic disequilibrium between all 28 combinations in pairs of SSR loci in *C. langsdorffii*.

3.3 Conclusions

The seven microsatellite loci and the two heterologous microsatellite markers developed for *C. legalis* presented a Mendelian inheritance pattern, no genetic linkage, and negligible genotypic disequilibrium. Therefore, this analysis indicated that this set of SSR loci could be used without restriction in studies on the genetic diversity and structure, mating system, and parentage analysis of *C. legalis*.

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4 THE EFFECTS OF FOREST FRAGMENTATION ON THE MATING SYSTEM OF THE ATLANTIC FOREST'S LARGEST TREE: *Cariniana legalis* MART. KUNTZE (LECITIDACEAE)

Abstract

Forest fragmentation affects mating system of insect pollinated tropical tree species due the changes in reproductive population density as well as the behavior of the pollination population vector. In this study we investigate the impact of population size on genetic diversity, inbreeding, mating system and inbreeding depression in three fragmented populations of the Neotropical tree, *Cariniana legalis*, using seven microsatellite loci. All adult trees found within the three study populations were sampled: Ibicatu (65), MGI (22) and MGII (4). Open-pollinated seeds were hierarchically sampled among and within fruits. No significant association was detected between population size and genetic diversity parameters. However, the multilocus outcrossing rate was significantly lower in the smallest population, MGII, suggesting that low population densities can increase the rate of self-pollination for *C. legalis*. Significant positive associations between individual outcrossing rate, growth and survival of offspring were detected, indicating that selfing produces inbreeding depression in the species. The multilocus paternity correlation within fruits was significantly higher than among fruits in all populations. Our results demonstrate the importance of the size of the reproductive population in seed collection strategies for genetic breeding, conservation and environmental restoration programs. Our analysis shows that *C. legalis* is not self-incompatible and open-pollinated seeds may present inbreeding from both selfing and mating among relatives, especially in small populations. A large pollination neighbor area increases the effective number of pollen donors contributing to progeny arrays and trees show low rates of selfing; therefore, our results suggest that seeds collected from large populations will present higher genetic diversity, increased growth rate and survival than seeds collected from small patches.

Keywords: Brazilian Atlantic Forest; Conservation genetics; Microsatellite markers; Population genetics; Tropical tree species

4.1 Introduction

Fragmentation of forest biomes is a global problem that has affected the survival of many tree species populations around the world. Forest fragmentation is especially problematic in the tropics where there are generally high levels of tree species diversity within a single hectare. The Brazilian Atlantic Forest is a striking example of this problem, where today only 16% of the original forest remains in the state of São Paulo (RIBEIRO et al., 2009). Considering that trees, due to their large size and longevity, are the key organisms in forest ecosystems; conserving these species is critical to preserving many other animal and plant species.

For many tree species, mating systems vary with reproductive biology and spatial genetic structure; in combination, these two factors affect the level and dynamics of genetic diversity (BOSHIER, 2000). In general, tree species have high levels of genetic diversity within populations and low genetic differentiation among populations due to their predominantly outcrossed mating system, extensive pollen and seed flow, long-life, diversifying selection, as well as their large population sizes (PETIT and HAMPER, 2006). However, the mating system of a tree species is environmentally and genetically controlled and, consequently, it may be affected by natural changes in the environment or by anthropogenic processes, such as logging and forest fragmentation (LOWE et al., 2005; WARD et al., 2005; AGUIAR et al., 2009; CARNEIRO et al., 2011).

Understanding the effects of the changes caused by forest fragmentation and logging on mating system patterns of tree species are important for conservation, reforestation, management and breeding programs (LOWE et al., 2005; WARD et al., 2005; CARNEIRO et al., 2011; KAMM et al., 2011; BREED et al., 2012). Studies have shown that forest fragmentation and logging create genetic bottlenecks caused by reductions in pollination neighbor area, decreases in the reproductive population density, as well as the isolation of trees in the landscape; as such, fragmentation may lead to increases in selfing and correlated mating (MURAWSKI and HAMRICK, 1991; DICK et al., 2003; LOWE et al., 2005; WARD et al., 2005; LOURMAS et al., 2007; AGUILAR et al., 2008; CARNEIRO et al., 2011; MOREAS; SEBBENN, 2011; KAMM et al., 2011; BREED et al., 2012).

Tree species are predominantly outcrossing as the result of processes that restrict non-outcrossed mating, such as inbreeding depression, self-incompatibility, and dioecy (PETIT; HAMPER, 2006). Strong early inbreeding depression is common in trees species (SORENSEN 1999; STACY, 2001; HUFFORD; HAMRICK, 2003; BOWER; AITKEN, 2007) ensuring that only outcrossed individuals survive to reach the adult stage. Inbreeding depression occurs over many years in trees species due their typically greater longevity (PETIT and HAMPER, 2006). Understanding the outcrossing rate is very important because the mating system has major evolutionary consequences, especially since it is one of the best predictors of the genetic structure of a population (SCHOEN; BROWN 1991). Tree species also present generally low genetic differentiation among populations due to extensive gene flow (PETIT; HAMPER, 2006). Although many tree species are bisexual (hermaphroditic and monoecious), the self-

incompatibility mechanism has been identified in many of these species, which forces seed production through outcrossing. For example, Bawa et al. (1985), studying the sexual system and incompatibility mechanisms of 333 tropical tree species, found that of the 28 species studied using controlled pollination, 24 were self-incompatible (86%).

Our study aims to investigate the effects of fragment population size on the genetic diversity, inbreeding, mating system and inbreeding depression, of the largest tree in the Atlantic Forest, *Cariniana legalis* Mart. Kuntze (Lecitidaceae), using seven microsatellite loci. This species was chosen because it is representative of the largest trees within the Atlantic Forest, its flowers are hermaphroditic and insect pollinated (bees), its seeds are wind dispersed, and its fruit produce many seeds (average of 7.7 seeds per fruit, Tambarussi personal observations). The tree height ranges from 10 to 60 m, diameter at breast height (dbh) ranges from 0.60 to 4 m, and its wood has many uses (CARVALHO, 1994). *C. legalis* occurs naturally between latitudes 7° S (Paraiba State) and 23° S (São Paulo State), at altitudes ranging from 30 to 1,000 m. It occurs at low population densities (generally < 1 tree per hectare) and is on the list of endangered tree species of the Atlantic Forest (IUCN, 2002).

Due to significant deforestation and forest fragmentation of the Atlantic Forest, many populations of *C. legalis* have been lost and many others have been greatly reduced and are now restricted to forest fragments. These factors may result in a negative impact on the reproductive success of the species (LOWE et al., 2005; WARD et al., 2005; AGUIAR et al., 2009). Forest fragmentation modifies the movement of pollen and seeds in tree populations, disrupting ecological and evolutionary processes (CUERTAS-HERNANDES et al., 2010). Forest fragmentation also affects other organisms in forest ecosystems, such as insects, birds and bats, which are responsible for the pollination of most tropical tree species (CHESE et al., 1996). The current interest in studying *C. legalis* is to support genetic conservation, breeding and reforestations programs (SEBBENN et al., 2000). This study was carried out using a large forest fragment (72 ha), called Ibicatu, and a small fragment (7.2 ha), called Mogi-Guaçu (MGI), both located in São Paulo State, Brazil. Specifically we explored the following issues: i) does the size of the fragmented population affect the levels of genetic diversity and inbreeding in the generations after fragmentation? ii) Does the size of the reproductive population affect the rate of self-pollination and correlated mating? iii) Is there a positive association between individual outcrossing rate and growth and survival of open-pollinated seeds (inbreeding depression)?

4.2 Development

4.2.1 Material and methods

Study sites

Unfortunately, no undisturbed forest remains in which *C. legalis* occurs; therefore, we used forest fragments of varying sizes for our study. The study was carried out at the Ibicatu State Forest (22°46' S, 47°43' W, altitude ranging from 448 to 576 m) and Mata da Figueira in the Mogi-Guaçu Ecological Station (22° 16 'S, 47° 11' W, average altitude of 600 m), both in São Paulo State, Brazil. The Ibicatu forest has an area of approximately 72 ha, while Mogi-Guaçu has only 7.2 ha. Both forests are currently surrounded by agricultural crops (sugarcane, *Eucalyptus* spp.) and pastures. These forest fragments are remnants of the semi-deciduous Atlantic Forest and in the past these regions were subjected to several fires and selective logging. According to Köeppen (1948), the Ibicatu climate is Cwa with an annual average temperature of 23.9° C, average minimum of 16.1° C, average maximum of 25° C, and pluvial annual precipitation of 1,328.1 mm. The soil types are yellow regosol "intergrade" and red podzolic "intergrade". In this study, this forest fragment is referred to as Ibicatu. The Mata da Figueira is a small forest fragment of riparian forest on the Semi-deciduous plateau. According to the Köeppen (1948) classification, the climate is Cwa, characterized as humid and mesothermal, with variation in the average temperature between 14.30° C and 24.65° C. The dry season extends from May to August, with 86.2% of the precipitation (annual average of 1,314 mm) concentrated in the rainy season (September to April). Herein, this small forest fragment is denominated MGI. About 2.9 km from Mata da Figueira, also a riparian forest, we found four reproductive *C. legalis* individuals. This small, spatially isolated group was considered as an isolated population of *C. legalis* and was denominated MGII.

Sampling

To carry out our genetic study, all individuals found in the forest fragments were sampled (bark samples), mapped (using a GPS III-Garmin, USA) and the diameter at breast height (dbh) measured. In total, we found 65 adult trees in Ibicatu (density of 0.9 trees/ha), 22 in MGI (density of 3.0 trees/ha), and four in MGII.

In August of 2011, fruits were harvested directly from the canopy of 15 seed-trees randomly selected in Ibicatu, five trees in MGI, and from two trees in MGII. Because of the large size of the trees (> 40 m), the fruits were collected by climbing the trees. After harvesting the fruits, they were left in the shade for about 15 days to facilitate seed extraction. Fruits were packaged in plastic bags separately by seed-tree. The seeds were germinated separately by fruit and source seed-tree. After seed germination, only fruits with five seedlings were genotyped.

From the Ibicatu population, 40 seeds from eight fruits of each seed-tree were genotyped, totaling 600 seeds. From the MGI population, 50 seeds originated from ten fruits of each seed-tree were genotyped, totaling 250 seeds, and from the MGII population, 100 seeds originated from twenty fruits of each seed-tree were genotyped, totaling 200 seeds. The number of seed-trees sampled in MGI and MGII was lower than Ibicatu because the populations were much smaller.

DNA extraction and SSR amplification

For all adult trees, DNA was extracted from 100 mg per tree of adult stem bark tissue using AnalytikJena DNA isolation kits. Seeds were germinated in vermiculite until the cotyledons emerged; subsequently, DNA was extracted from 15 to 20-day old seedlings from the first leaf pair using the method of Doyle and Doyle (1990). We used seven microsatellite markers specific to *C. legalis*: Cle01, Cle04, Cle05, Cle08, Cle09, Cle10, and Cle12. These microsatellites were selected because they showed Mendelian inheritance, an absence of genetic linkage, and a high level of polymorphism (TAMBARUSSI et al., 2013a, 2013b). Microsatellite loci were amplified by PCR for a final volume of 15 μL using GoTaq® Colorless Master Mix containing 7.5 μL GoTaq® Colorless Master Mix (2×), 10μM of each primer (F and R), 3.0 μL Nuclease-Free Water, and 7.5 ng template DNA. The amplification program for all primers consisted of an initial denaturing step at 94° C for 1 min, followed by 35 cycles of amplification (94° C for 1 min, followed by 1 min at the specific annealing temperature of each primer pair (TAMBARUSSI et al., 2013a), 72° C for 1 min), and a final elongation step at 72° C for 10 min. Amplifications were performed with a Mastercycler (Eppendorf, Hamburg, Germany). The amplification products (2 μL of the total reaction volume) were separated on a Fragment Analyzer™ Automated CE System (Advanced Analytical Technologies, Inc. [AATI], Ames, Iowa, USA) using dsDNA Reagent Kit, 35-500 bp. The raw data were analyzed using the

software PROSize version 2.0 (AATI). Samples with questionable or missing alleles (peaks) or for which mismatches occurred between mothers and progeny were genotyped a second time.

Analysis of genetic diversity, genetic structure, and inbreeding

To assess genetic diversity within populations, we grouped the MGI and MGII populations due to the short distance between them (about 3 km) and the minimal number of adult trees in MGII (4 trees). For adults and progenies, genetic diversity was characterized per locus and as an average across all loci using the following indices: average number of alleles per locus (k), allelic richness (R) estimated using rarefaction (EL MOUSSADIK; PETIT, 1996), observed heterozygosity (H_o), and expected heterozygosity in Hardy-Weinberg equilibrium (H_e). To check whether there is inbreeding in different sampled generations (adults and progeny), we used the within population fixation index (F). The statistical significance of F values was tested by permuting alleles among individuals. All analyses were performed using the FSTAT program (GOUDET, 1995).

Mating system analysis

Analysis of the mating system was based on the mixed-mating model (RITLAND; JAIN, 1981) and the correlated mating model (Ritland, 1989), using Multilocus MLTR program (RITLAND, 2002). The analyses were estimated at the population level and for individual seed-trees, and were calculated separately for Ibicatu, MGI, and MGII. The numerical method used was the maximum likelihood EM (Expectation Maximization) for seed-trees and Newton-Raphson at the population level. The estimated parameters were: multilocus outcrossing rate (t_m), single-locus outcrossing rate (t_s), outcrossing rate among relatives ($t_m - t_s$), multilocus correlation of selfing (r_s), and multilocus paternity correlation ($r_{p(m)}$). As our sample was hierarchical within and among fruits, multilocus paternity correlation was also estimated within ($r_{p(m)w}$) and among ($r_{p(m)a}$) fruits. The estimates of the 95% confidence interval (95% CI) at the population level were obtained using 1,000 re-sampling bootstraps. In Ibicatu, where 15 progenies were sampled, the unit of re-sampling was progeny. In MGI and MGII, where only five and two progenies were sampled, respectively, the unit of re-sampling was individuals within progenies. To estimate the standard error at the progeny level, the unit of re-sampling was also

individuals within progenies. These parameters were used to estimate other genetic and demographic parameters, such as effective number of pollen donors ($\hat{N}_{ep} = 1/\hat{r}_{p(m)}$) and the average coancestry coefficient within progenies, calculated by: $\bar{\theta} = 0.125(1+\hat{F}_p)[4\hat{s} + (\hat{t}_m + \hat{s}\hat{t}_m\hat{r}_s)(1+\hat{r}_{p(m)})]$, where F_p is the parental inbreeding coefficient (RITLAND, 1989). The frequency of pairwise self-sibs (P_{SS}), half-sibs (P_{HS}), full-sibs (P_{FS}) and self-half-sibs (P_{SHS}) within progenies were estimated as: $P_{SS} = s^2$, $P_{HS} = t_{HS}^2(1-r_{p(m)})$, $P_{FS} = t_m^2 r_{p(m)}$ and $P_{shs} = 2st_m$, respectively (SEBBENN, 2006). The effective size within progeny was estimated as:

$$N_{e(v)} = \frac{0.5}{\bar{\theta}\left(\frac{n-1}{n}\right) + \frac{1+\hat{F}_o}{2n}}$$ (COCKERHAM, 1969),

where, *n* is the sample size and F_o is the inbreeding coefficient within progenies, estimated using the fixation index and $s = 1 - t_m$ is the natural selfing rate . The number of seed-trees necessary for seed collection was calculated based on the retention of a reference effective size of 150 in the total progeny array: $\hat{m} = N_{e(referência)} / \hat{N}_{e(v)}$ (SEBBENN, 2006). This estimate is based on three assumptions: i) the seed-trees are not related; ii) the seed-trees do not receive an overlapping pollen pool (each seed-tree mated with a different set of pollen donors); and iii) the selected seed-trees did not mate with each other. Thus, related individuals in the progeny array will occur only within progeny but not among different sampled progeny.

Statistical analysis

To investigate if the parameters k, R, H_o, H_e and F were significantly different between adult and progeny samples, we used an unpaired *t*-test (SOKAL and ROHLF, 1981). To investigate if the sample size (n) within progeny affects the estimates of the parameters N_{ep}, Θ, N_e, K, R H_o and to determine if N_{ep} increased the Θ and decreased the K, R H_o and N_e within progenies, we estimated the correlation between all these parameters using linear

regression (R^2), calculated with the SAS program (SAS, 1999). We also calculated the correlation of these estimates with height (cm), root-collar diameter (RCD, in cm) and survival, measured in the germinated seeds at the age of 8 months. Additionally, we estimated the correlation between t_m and $t_m - t_s$ with K, R H_o, height (cm), root-collar diameter (RCD, in cm), and survival.

4.3 Results

Genetic diversity, structure and inbreeding of the total sample from all populations, we found 131 different alleles, with 49 private alleles within populations (Table 4.1).

Table 4.1- Genetic diversity and fixation index (F) in seven microsatellite loci of adults and progenies from three *Cariniana legalis* populations. N is the census number; n is the sample size; *k* is the number of alleles; R_{25} and R_{429} are the allelic richness for 21 adults and 192 seeds, respectively; H_o is the observed heterozygosity; and H_e is the expected heterozygosity

(continue)

Locus	Adults: Ibicatu (n= 65)					Adults: MGI-II (N= 26)				
	k	R_{25}	H_o	H_e	F	k	R_{25}	H_o	H_e	F
F11	18	12.50	0.810	0.864	0.062	9	9.00	0.560	0.858	0.347 *
G07	11	9.71	0.908	0.858	-0.058	14	14.00	0.923	0.906	-0.019
H06	17	13.62	0.846	0.891	0.050	17	15.00	1.000	0.904	-0.106
B05	13	11.77	0.810	0.820	0.012	17	17.00	0.924	0.931	0.008
E02	16	12.42	0.794	0.884	0.102	17	17.00	0.846	0.921	0.081
A04	16	13.46	0.817	0.912	0.104	15	14.00	0.653	0.889	0.265 *
B06	9	8.11	0.692	0.788	0.122	14	14.00	0.962	0.894	-0.076
Mean	14.3	11.65	0.811	0.860	0.057 *	14.7	14.29	0.838	0.900	0.069 *
SD	3.4	2.03	0.065	0.043	0.063	2.9	2.69	0.167	0.024	0.173
Total	100		--	--	--	103	--	--	--	--

Table 4.1- Genetic diversity and fixation index (F) in seven microsatellite loci of adults and progenies from three *Cariniana legalis* populations. N is the census number; n is the sample size; *k* is the number of alleles; R_{25} and R_{429} are the allelic richness for 21 adults and 192 seeds, respectively; H_o is the observed heterozygosity; and H_e is the expected heterozygosity

(conclusion)

	Adults: Ibicatu (n= 65)					Adults: MGI-II (N= 26)				
	Progenies: Ibicatu (n= 600)					Progenies: MGI-II (n= 450)				
Locus	k	R_{429}	H_o	H_e	F	k	R_{429}	H_o	H_e	F
F11	17	16.92	0.812	0.893	0.091 *	9	8.01	0.742	0.812	0.086 *
G07	11	11.00	0.826	0.854	0.033	14	10.84	0.797	0.825	0.034
H06	17	16.88	0.795	0.849	0.064 *	15	11.54	0.812	0.875	0.072 *
B05	13	13.00	0.730	0.868	0.159 *	17	12.73	0.872	0.891	0.021
E02	17	16.73	0.781	0.853	0.084 *	17	11.57	0.773	0.869	0.110 *
A04	16	15.73	0.812	0.883	0.080 *	14	10.50	0.700	0.867	0.193 *
B06	9	9.00	0.794	0.794	0.000	14	10.74	0.804	0.834	0.036
Mean	14.3	14.18	0.793	0.856	0.074 *	14.3	10.85	0.786	0.853	0.079 *
SD	3.3	3.22	0.031	0.032	0.050	2.7	1.46	0.055	0.029	0.060
Total	100	--	--	--	--	100	--	--	--	--
General total	101	--	--	--	--	106	--	--	--	--
Total of all pops	131	--	--	--	--	--	--	--	--	--

* P< 0.05 after a Bonferroni correction for multiple tests. SD is the standard deviation.

In Ibicatu, MGI and MGII populations we found 22, 23 and 4 private alleles, respectively. In the total sample from each population (adults + seeds), 101 alleles were detected in Ibicatu and 106 alleles were found in the MGI-II populations (Table 4.1). Comparing adults and seeds of each population, in the Ibicatu population one allele was private to the adults and one to seeds; for MGI, 14 alleles were private to the adults and 8 to seeds; and in MGII, one allele was private to the adults. The total number of alleles was similar among adults and progenies of each population (Table 4.1). The average allelic richness (R) for adults was 11.65 in

Ibicatu and 14.29 in MGI-II and for seeds was 14.18 in Ibicatu and 10.85 in MGI-II (Table 4.1). The average observed heterozygosity (H_o) was similar for adults (Ibicatu = 0.811, MGI-II= 0.838) and for seeds (Ibicatu= 0.793, MGI-II= 0.786); the average expected heterozygosity (H_e) was similar for adults (Ibicatu = 0.860, MGI-II= 0.900) and seeds (Ibicatu= 0.856, MGI-II= 0.853); and the average fixation index was significantly higher than zero for adults and seeds from both populations, suggesting inbreeding (Table 4.1). Using an unpaired t-test, the mean of H_e was significantly lower in adults of Ibicatu than in MGI-II (H_e: t= -2.18, P= 0.05) and H_e was significantly higher in adults than progenies from MGI-II (H_e: t= 3.30, P= 0.01).

Mating system

The multilocus outcrossing rate (t_m) was significantly lower than unity (1.0) in the three populations, indicating that some self-pollination does occur (Table 4.2). According to a 95% CI, the multilocus outcrossing rate was significantly lower in MGII, the smallest population, than in Ibicatu and MGI (Table 4.2), suggesting that a low population density increases the rate of selfing.

Table 4.2 - Mating system parameters in three *Cariniana legalis* populations (95% CI is the 95% confidence interval)

(continue)

Parameter	Ibicatu, mean (95% CI)	MG I, mean (95% CI)	MG II, mean (95% CI)
Number of seed-trees (number of seeds)	15 (600)	5 (250)	2 (200)
Multilocus outcrossing rate: t_m	0.957 (0.914 to 0.988)	0.932 (0.904 to 0.960)	0.830 (0.776 to 0.880)
Single-locus outcrossing rate: t_s	0.691 (0.649 to 0.786)	0.921 (0.882 to 0.958)	0.951 (0.877 to 1.000)
Mating among relatives: $t_m - t_s$	0.266 (0.265 to 0.202)	0.011 (0.002 to 0.022)	-0.121 (-0.120 to -0.101)
Selfing correlation: r_s	0.160 (0 to 0.239)	0.116 (0.040 to 0.178)	0.044 (0.000 to 0.107)
Multilocus paternity correlation (among and within fruits): $r_{p(m)}$	0.391 (0.246 to 0.472)	0.266 (0.230 to 0.301)	0.207 (0.178 to 0.222)
Multilocus paternity correlation (within fruits): $r_{p(m)w}$	0.433 (0.397 to 0.469)	0.856 (0.765 to 0.934)	0.660 (0.498 to 0.797)
Multilocus paternity correlation (among fruits): $r_{p(m)a}$	0.265 (0.251 to 0.279)	0.203 (0.166 to 0.239)	0.187 (0.157 to 0.203)
Effective number of pollen donors (among and within fruits): N_{ep}	2.6 (2.1 to 4.1)	3.8 (3.3 to 4.3)	4.8 (4.5 to 5.6)
Effective number of pollen donors (within fruits): $N_{ep(w)}$	2.3 (2.1 to 2.5)	1.2 (1.1 to 1.3)	1.5 (1.3 to 2.0)
Effective number of pollen donors (among fruits): $N_{ep(a)}$	3.8 (3.6 to 4.0)	4.9 (4.2 to 6.0)	5.3 (4.9 to 6.4)
Percent of pairwise self-sibs: P_{SS} (%)	0.2 (0.0 to 0.7)	0.5 (0.2 to 0.9)	2.9 (1.4 to 5.0)

Table 4.2 - Mating system parameters in three *Cariniana legalis* populations (95% CI is the 95% confidence interval)

(conclusion)

Parameter	Ibicatu, mean (95% CI)	MG I, mean (95% CI)	MG II, mean (95% CI)
Percent of pairwise half-sibs: P_{HS} (%)	55.8 (51.5 to 63.0)	63.8 (62.9 to 64.4)	54.6 (49.5 to 60.2)
Percent of pairwise full-sibs: P_{FS} (%)	35.8 (20.6 to 46.1)	23.1 (18.8 to 27.7)	14.3 (10.7 to 17.2)
Percent of pairwise self-half-sibs: P_{SHS} (%)	8.2 (2.4 to 15.7)	12.7 (7.7 to 17.4)	28.2 (21.1 to 34.8)
Coancestry (among and within fruits): Θ	0.182 (0.158 to 0.200)	0.173 (0.162 to 0.183)	0.190 (0.174 to 0.207)
Variance effective size (among and within fruits): N_e	2.62 (2.39 to 2.99)	2.78 (2.63 to 2.95)	2.59 (2.38 to 2.82)
Number of seed-trees (among and within fruits): m	57 (50 to 63)	54 (51 to 57)	58 (53 to 63)

The single-locus outcrossing rate (t_s) was significantly lower than unity (1.0) in the Ibicatu and MGI populations and significantly lower than the multilocus outcrossing rate in the Ibicatu population, resulting in a substantial and significant difference in $t_m - t_s$. This result suggests the occurrence of mating among relatives. The selfing correlation (r_s) was significantly different from zero only in the MGI population, demonstrating some individual variation in the outcrossing rate (see also Table 4.3).

Table 4.3- Mating system parameters in open-pollinated progenies from three *Cariniana legalis* populations (± SD is the standard deviation)

(continue)

Population/progeny	F_m	F_o	k	R	H_o	t_m ±SD	$t_m - t_s$ ±SD	$r_{p(m)}$ ±SD	$r_{p(m)w}$ ±SD	$r_{p(m)a}$ ±SD	N_{ep}	$N_{ep(w)}$	$N_{ep(a)}$	Θ	N_e
Ibicatu (n= 40/progeny)															
J04	-0.080	-0.389	20	2.86	0.70	0.85±0.06	0.35±0.04	1.00±0.03	1.00±0.00	0.99±0.04	1.0	1.0	1.0	0.26	1.91
J06	0.054	-0.138	33	4.64	0.64	0.71±0.07	0.12±0.05	0.53±0.10	0.70±0.18	0.51±0.10	1.9	1.4	2.0	0.24	2.01
J16	-0.148	-0.300	46	6.49	0.87	1.00±0.01	0.18±0.03	0.23±0.05	0.32±0.17	0.22±0.05	4.4	3.1	4.6	0.15	3.07
J22	-0.043	-0.316	49	6.88	0.88	0.97±0.02	0.19±0.03	0.24±0.05	0.55±0.16	0.19±0.06	4.3	1.8	5.2	0.16	2.97
J23	0.113	-0.276	54	7.56	0.85	1.00±0.01	0.22±0.03	0.22±0.07	0.30±0.20	0.21±0.08	4.5	3.3	4.7	0.15	3.08
J27	0.226	-0.246	46	6.47	0.76	1.00±0.01	0.18±0.02	0.23±0.05	0.52±0.16	0.20±0.05	4.3	1.9	5.1	0.15	3.07
J28	-0.005	-0.184	59	8.25	0.84	1.00±0.01	0.17±0.03	0.16±0.04	0.25±0.30	0.14±0.04	6.5	4.0	7.0	0.15	3.25
J29	0.315	-0.222	70	9.78	0.82	1.00±0.01	0.06±0.01	0.07±0.06	0.18±0.29	0.06±0.10	13.5	5.6	15.9	0.13	3.47
J30	-0.070	-0.179	55	7.73	0.83	0.95±0.03	0.13±0.02	0.21±0.06	0.30±0.24	0.20±0.06	4.8	3.4	5.0	0.16	2.94
J36	0.052	-0.157	61	8.56	0.81	1.00±0.01	0.14±0.02	0.11±0.10	0.28±0.22	0.10±0.16	8.8	3.5	10.5	0.14	3.36
J41	-0.169	-0.108	49	6.91	0.75	0.97±0.02	0.26±0.03	0.21±0.05	0.38±0.16	0.18±0.05	4.9	2.6	5.5	0.16	3.04
J49	-0.117	-0.161	54	7.61	0.82	1.00±0.01	0.18±0.02	0.20±0.05	0.28±0.20	0.19±0.05	5.1	3.6	5.3	0.15	3.14
J61	-0.073	-0.146	51	7.18	0.75	0.97±0.02	0.34±0.02	0.37±0.07	0.70±0.16	0.34±0.07	2.7	1.4	3.0	0.18	2.73
J67	-0.135	-0.154	46	6.48	0.79	0.95±0.03	0.26±0.04	0.31±0.05	0.41±0.13	0.30±0.04	3.2	2.5	3.3	0.17	2.76
J70	-0.210	-0.175	33	4.71	0.78	0.97±0.02	0.30±0.03	0.55±0.06	0.67±0.13	0.54±0.06	1.8	1.5	1.9	0.20	2.44
MGI (n= 50/progeny)															

Table 4.3- Mating system parameters in open-pollinated progenies from three *Cariniana legalis* populations (± SD is the standard deviation)

(conclusion)

Population/progeny	F_m	F_o	k	R	H_o	t_m ±SD	$t_m - t_s$ ±SD	$r_{p(m)}$ ±SD	$r_{p(m)w}$ ±SD	$r_{p(m)a}$ ±SD	N_{ep}	$N_{ep(w)}$	$N_{ep(a)}$	Θ	N_e
1M	-0.053	-0.113	43	6.13	0.82	1.00±0.00	0.08±0.02	0.30±0.03	1.00±0.04	0.24±0.03	3.3	1.0	4.2	0.16	2.95
2M	0.077	-0.049	64	8.96	0.75	0.86±0.05	-0.04±0.03	0.09±0.02	0.81±0.11	0.03±0.01	10.8	1.2	33.3	0.16	2.99
3M	0.084	-0.279	37	5.21	0.86	0.98±0.02	0.03±0.01	0.50±0.07	0.95±0.05	0.44±0.08	2.0	1.1	2.3	0.19	2.55
4M	-0.003	-0.046	53	7.49	0.79	0.82±0.05	-0.03±0.02	0.17±0.02	0.72±0.12	0.11±0.02	5.8	1.4	8.9	0.19	2.57
5M	-0.102	0.009	67	9.04	0.72	1.00±0.00	0.07±0.02	0.12±0.02	0.53±0.10	0.07±0.03	8.7	1.9	13.7	0.14	3.41
MGII (n= 100/progeny)															
6M	0.156	-0.148	44	6.19	0.81	0.91±0.03	-0.14±0.04	0.21±0.01	0.62±0.10	0.19±0.01	4.9	1.6	5.3	0.17	2.89
7M	-0.035	-0.070	44	6.23	0.76	0.75±0.04	-0.12±0.03	0.19±0.02	0.64±0.11	0.17±0.02	5.2	1.6	6.0	0.21	2.36

F_m is the seed-tree fixation index; F_o is the progeny fixation index; k is the total number of alleles; R is the allelic richness; H_o is the observed heterozygosity; t_m is the multilocus outcrossing rate; $t_m - t_s$ is the rate of mating among relatives; $r_{p(m)}$, $r_{p(m)w}$ and $r_{p(m)a}$ are the multilocus paternity correlation among and within fruits, within fruits, and among fruits, respectively; N_{ep}, $N_{ep(w)}$ and $N_{ep(a)}$ are the effective number of pollen donors among and within fruits, within fruits, and among fruits, respectively; Θ is the coancestry coefficient within progenies; N_e is the variance effective size within progenies.

The multilocus paternity correlation within and among fruits ($r_{p(m)}$), within fruits ($r_{p(m)w}$), and among fruits ($r_{p(m)a}$) were significantly greater than zero in all three populations, indicating correlated mating (Table 4.2). However, the multilocus paternity correlation was significantly higher within fruits than among fruits, especially in the MGI population. Consequently, the effective number of pollen donor was significantly lower within fruits ($N_{ep(w)}$) than among fruits ($N_{ep(a)}$), which indicates that between one and three pollen donors fertilized each fruit. Open-pollinated progenies of the three populations are composed mainly of half-sibs (ranging from 54.6% to 63.8%), followed by full-sibs in Ibicatu (35.8%) and MGI (23.1%), and self-half-sibs in MGII (28.2%). The average coancestry coefficient within progenies (Θ, ranging from 0.173 to 0.190), the variance effective size (N_e, ranging from 2.62 to 2.78) and the number of seed trees required for seed collection ($\hat{m}$, ranging from 54 to 58) were not significantly different among population (Table 4.2).

Inbreeding, genetic diversity and mating system within progenies

The fixation index ranged among seed-trees (F_m) from -0.210 to 0.315 and within progenies (F_o) from -0.389 to 0.009 (Table 4.3). The total number of alleles (k) ranged among progenies in Ibicatu from 20 to 70, in MGI from 37 to 67, and in MGII the total number of alleles was 44. The allelic richness (R) ranged from 2.86 to 9.78 in Ibicatu, from 5.21 to 9.04 in MGI, and in MGII the results were 6.19 and 6.23. The observed heterozygosity (H_o) ranged in Ibicatu from 0.64 to 0.88, in MGI from 0.72 to 0.86, and in MGII the results were 0.76 and 0.81.

Substantial variation in the mating system parameters was observed among seed-trees (Table 4.3). The multilocus outcrossing rate ranged among seed-trees in Ibicatu from 0.71 to 1.0, in the MGI population from 0.82 to 1.0, and in MGII we found a rate of 0.75 for one tree and 0.91 for the other. According to the standard error, these estimates were significantly lower than zero for eight seed-trees from Ibicatu, two from MGI, and both from MGII. The difference of $t_m - t_s$ was significantly higher than zero for all seed-trees from Ibicatu (ranging from 0.06 to 0.35) and for three seed-trees from MGI (ranging from -0.04 to 0.08). The paternity correlation was higher within ($r_{p(m)w}$) than among ($r_{p(m)a}$) fruits in all populations, ranging from 0.18 to 1.0 in Ibicatu, from 0.53 to 1.0 in MGI, and in MGII the results were 0.62 and 0.64. Among fruits,

the paternity correlation ($r_{p(m)a}$) ranged from 0.06 to 0.99 in Ibicatu, from -0.07 to 0.44 in MGI, and in MGII the results were 0.17 and 0.19. Due to the higher paternity correlation within fruits, the effective number of pollen donors was equal or lower within fruits ($N_{ep(w)}$) than among fruits ($N_{ep(a)}$). In Ibicatu the number of pollen donors ranged within and among fruits (N_{ep}) from 1.0 to 13.5, within fruits ($N_{ep(w)}$) from 1.0 to 5.6, and among fruits ($N_{ep(a)}$) from 1.0 to 15.9. For the MGI and MGII populations, this number ranged within and among fruits (N_{ep}) from 2.0 to 10.8, within fruits ($N_{ep(w)}$) from 1.0 to 1.9, and among fruits ($N_{ep(a)}$) from 2.3 to 33.3. In the three populations, the average coancestry coefficient within progenies (Θ) ranged among seed-trees from 0.13 to 0.26 and the variance effective size (N_e) from 1.91 to 3.47.

Correlation among the parameters

Using Spearman ranking correlation, no association was detected between the sample size of progenies (n) and all estimated parameters, nor was there an association found with average height and RCD (Table 4.4). However, the height was strongly positively associated with RCD (r = 0.874). The individual outcrossing rate (t_m) was significantly and positively correlated with H_o, height, RCD, and survival. The number of pollen donors (N_{ep}) was significantly and negatively associated with the coancestry within progenies (Θ) and significantly and positively associated with the variance effective size (N_e), total number of alleles (k), and allelic richness (R) of the progenies, indicating that an increase in N_{ep} decreases the Θ and increases N_e, k and R. The coancestry within progenies (Θ) was significantly and negatively associated with the total number of alleles (k), allelic richness (R), and observed heterozygosity (H_o), and Θ was positively associated with survival. These results indicate that an increase in the relatedness decreases the N_e, k, R and H_o, while it increases survival within progenies. As expected, the variance effective size (N_e) within progenies was significantly positively associated with k, R, H_o, and survival within progenies. The parameters k, R and H_o were significantly and positively associated with survival, H_o, as well as with RCD.

Table 4.4- Coefficient of determination (R^2, df= 20) among the sample size (n), multilocus outcrossing rate (t_m), effective number of pollen donors (N_{ep}), average coancestry within progenies (Θ), variance effective size within progeny (N_e), total number of alleles (k), allelic richness (R), observed heterozygosity (H_o), total height (Height, cm), root-collar diameter (RCD, cm), and survival for *Cariniana legalis* open-pollinated progenies measured at eight months

	N_{ep}	Θ	N_e	k	R	H_o	Height	RCD	Survival
n	0.001	0.03	-0.031	-0.009	-0.009	0.001	--	-	--
t_m	--	--	--	0.152	0.152	0.384 **	0.301 **	0.394 **	0.259 *
N_{ep}	--	-0.386 **	0.468 **	0.731 **	0.732 **	0.015	-0.011	-0.003	0.095
Θ	--	--	--	-0.688 **	-0.694 **	-0.339 **	-0.152	-0.164	0.374 **
N_e	--	--	--	0.733 **	0.732 **	0.258 *	0.121	0.143	0.320 **
K	--	--	--	--	--	--	0.025	0.024	0.198 *
R	--	--	--	--	--	--	0.030	0.030	0.207 *
H_o	--	--	--	--	--	--	0.097	0.184 *	0.264 *
Height	--	--	--	--	--	--	--	0.874 **	0.090
RCD	--	--	--	--	--	--	--	--	0.090

* P< 0.05. ** P< 0.01.

4.4 Discussion

The studied *C. legalis* populations have high levels of genetic diversity for the loci analyzed. In the total sample, considering all adults and progenies of the three populations, 131 alleles were detected across the seven loci. In all three populations, the adults showed equal or higher number of alleles than that observed within progenies, suggesting low rates of immigration of alleles through pollen flow (Table 4.1).

Does the size of the fragmented population affect the levels of genetic diversity and inbreeding in post-fragmentation generations?

Our results do not support the hypothesis that there is lower genetic diversity (k, R, H_o and H_e) and higher rates of inbreeding in the smaller MGI-II population than in the larger Ibicatu

population. In general, no differences were observed between adults and progenies from Ibicatu and MGI-II (Table 4.1). In fact, Ibicatu presents significantly lower H_e than the results found for MGI-II.

However, comparing adults and progenies within populations, we observed in the smaller MGI-II population lower expected heterozygosity in progenies (H_e = 0.853) than adults (H_e = 0.900). This result suggests a loss of genetic diversity, which is likely due to selfing and genetic drift.

Does the size of the reproductive population affect the rate of selfing and correlated mating?

One of the most important results found in our study was the low multilocus outcrossing rate (t_m = 0.830) observed in the smallest population MGII in comparison to Ibicatu (t_m = 0.957) and MGI (t_m = 0.932). This indicates that small populations of *C. legalis* may present higher rates of selfing. The effect of the size of the reproductive population or population density has been observed in other studies on tropical tree species. These studies have found higher selfing rates in small or low density populations than in higher density populations (MURAWSKI; HAMRICK, 1991; DICK et al., 2003; CARNEIRO et al., 2011; TARAZI et al., 2013), as well as higher selfing rates for geographically isolated groups of trees or single isolated trees (DICK et al., 2003; LANDER et al., 2010; KAMM et al., 2011; MORAES; SEBBENN, 2011; MANOEL et al., 2012).

For example, Dick et al. (2003) found a selfing rate of 0.144 for *Dinizia excelsa* trees occurring in pastures and in a forest fragment compared to 0.103 for trees occurring in a continuous forest; for *Gomortega keule,* Lander et al. (2010) found a selfing rate of 56% for single isolated trees, 30% in small fragments and 22% in large fragments. Moraes and Sebbenn (2011) found a selfing rate of 26% in isolated trees and 12% in a population of *Hymenaea stigonocarpa*. While for *Copaifera langdorffii,* Manoel et al. (2012) found a selfing rate of 20% for a single, isolated tree, in contrast with 8% in a small isolated forest fragment with 112 adult trees. All these cited studies, including the results discussed herein, support the idea that the density of a reproductive population influences the outcrossing rate. Small, isolated groups of trees present lower outcrossing rates than trees occurring in large and more dense populations. On

the other hand, some studies have not found such differences in populations of different densities (CASCANTE et al., 2002; QUESADA et al., 2004; LOURMAS et al., 2007).

In a previous study of the mating system in three *C. legalis* populations, including the Ibicatu population, the multilocus outcrossing rate was also significantly lower than unity (Jequitibas: t_m = 0.990 ± 0.009; Ibicatu: t_m = 0.976 ± 0.011; Vassununga: t_m = 0.901 ± 0.025), again suggesting that some seeds were produced by selfing (SEBBENN et al., 2000). These results, along with the results of the current study, supports the idea that this species is self-compatible.

The correlation of selfing was low and significant only in the MGI population (r_s = 0.116), indicating low variation in the individual outcrossing rate. Furthermore, the multilocus outcrossing rate ranged among seed-trees from 0.71 to 1.0 in Ibicatu, from 0.82 to 1.0 in MGI, and in MGII the results were 0.75 in one of the trees and 0.91 in the other. However, it is possible that these values are overestimated due to the potential occurrence of inbreeding depression between the fertilization stage and the analysis of the microsatellite genotypes, as has been observed for *Platypodiun elegans* (HUFFORD; HAMRICK, 2003). Our results clearly show the effect of inbreeding depression; the correlation among the individual multilocus outcrossing rate was significantly and positively associated with height (R^2 = 0.301, P< 0.01), RCD (R^2 = 0.394, P< 0.01), and survival (R^2 = 0.259, P< 0.05), which indicates that seed-trees with a high outcrossing rate (or a low selfing rate) produce sibs which are taller, have a larger RCD and are more likely to survive.

Mating among relatives

Substantial mating among relatives was detected in Ibicatu ($t_m - t_s$ = 0.266). At the individual seed-tree level, we also detected mating among relatives for all seed-trees of the Ibicatu population and for three seed-trees in MGI. This result can be attributed to the presence of intra-population spatial genetic structure in these populations. In Ibicatu, the average pairwise coefficient of coancestry was significantly different from zero up to 150 m, indicating that individuals located within this range are likely related (TAMBARUSSI et al., unpublished). Mating among related individuals was also detected for the same species in the same study populations ($t_m - t_s$ = 0.059), as well as in two other populations (0.070 and 0.091) discussed in a

previous study (SEBBENN et al., 2000). Thus, open-pollinated seeds may present inbreeding not only from selfing, but also from mating among relatives.

Paternity correlation among and within fruits

The multilocus paternity correlation within fruits ($r_{p(m)w}$) was higher than among fruits ($r_{p(m)a}$) in the three populations, especially in MGI (Table 4.2). Consequently, the effective number of pollen donors was significant lower within ($N_{ep(w)}$) than among fruits ($N_{ep(a)}$). One to three pollen donors fertilized each fruit and there are higher proportions of full-sibs within fruits than among fruits. Bees are the main pollinators of *C. legalis* and they probably visit flowers carrying a set of pollen with low variation that is collected from previously visited conspecifics. This pattern leads to seeds from the same fruit that may have the same father, while seeds from different fruits have different fathers. This may explain the rate of correlated mating within fruits that is higher than among fruits. Similar results have been reported for other plant species (SCHOEN; CLEGG, 1984; SCHOEN, 1985; RITLAND, 1989; MUONA et al., 1991; SILVA et al., 2011; FERES et al., 2012). Correlated mating may also occur when the number of potential parents is low or when mating occurs between near neighbors (SURLES et al., 1990). This can explain why our estimates of correlated mating were significantly higher in the two smallest populations, MGI and MGII, as compared to Ibicatu. The low number of potential male parents, due to the small size of the MGI and MGII populations, may result in increased mating between a limited numbers of individuals.

Inbreeding from selfing and mating among relatives

In the individual analysis of the mating system, for 12 of the 22 seed-trees we detected selfing and for 18 seed-trees we found mating among relatives; however, no inbreeding was detected using the estimate of the fixation index (F_o) at the level of progenies (Table 4.3). Some studies of mating systems based on genetic markers and progeny arrays have found evidence of selfing, and in some cases mating among relatives, but no inbreeding was detected in the progenies (KAMM et al., 2011; DANNER et al., 2013). Kamm et al. (2011) explain this phenomenon by the existence of natural selection or inbreeding depression working against inbred offspring between the stages of fertilization and adulthood. This apparent incongruence is

associated with the way that inbreeding is estimated in the progeny array samples. We do not directly estimate the inbreeding coefficient; rather, inbreeding is inferred from the fixation index. These two parameters are different as the inbreeding coefficient ranges from zero to one and can only be estimated using pedigree control. On the other hand, the fixation index, which ranges from -1 to 1, can be estimated using genetic markers. Significantly negative fixation index values means selection toward heterozygosity and significantly positive values means endogamy.

However, when we estimate the fixation index using progeny arrays, the calculated value may not be significantly different from zero or the value may be lower than zero and will, therefore, not demonstrate the inbreeding produced by selfing and/or mating among relatives. This can happen because both inbred and non-inbred offspring are included in the analysis; it is particularly problematic when the rate of selfing and the rate of mating among relatives are low and only a small proportion of trees are inbred. In such a case, the majority of the offspring will not be inbred and thus the fixation index not will reflect inbreeding in some of the offspring. As such, there will be an absence of significant positive F_o values.

Inbreeding depression

Our results showed that selfing and mating among relatives limited growth in height and RCD of the seedlings, indicating the occurrence of inbreeding depression. In another exploratory study of the effects of mating system parameters on growth in *Swietenia magrophylla*, Breed et al. (2012) found that both selfing and paternity correlation limited growth in relation to tree volume at 5 years of age. However, our results showed minimal association between paternity correlation and height and RCD. This could be the result of the age of the seedlings used in our study (8 months). Inbreeding depression may occur throughout the life-cycle of the trees. Thus, the effects of selfing, mating among relatives, and paternity correlation may change with seedling development. Our sampled seedlings are now established in a provenance and progeny test in the Selvíria Experimental Station, at UNESP Ilha Solteira, and we will continue to monitor the impacts of mating system parameters on the growth of these trees.

4.5 Conclusions and conservation recommendations

The results of our analysis of genetic diversity parameters (k, R, H_o and H_e) and fixation index do not support the hypothesis that the smallest MGI-MGII populations present

lower genetic diversity than the larger Ibicatu population. Adult trees in both populations are remnants of pre-fragmentation and may retain the genetic signature of this earlier era in which anthropogenic activities were not a factor. This last hypothesis is based on the comparison between adults and progenies, for which we detected a lower expected heterozygosity (H_e) in progenies than in adults of the smallest MGI-II population. This indicates a loss of genetic diversity which is likely due to selfing and genetic drift. Furthermore, our results clearly show a decrease in outcrossing rate with a decrease in the size of the populations. This indicates that small *C. legalis* populations present lower outcrossing rate and, as expected, present higher inbreeding depression, as demonstrated by the positive association between individual outcrossing rate and growth traits and survival. As selfing was higher in the two smaller populations, MGI and MGII, a practical interpretation of this result is that seeds collected from seed-trees located in small and isolated populations may produce seeds with lower growth and survival rates than seeds collected from larger forest fragments.

The Ibicatu population showed substantial mating among relatives due to the presence of intrapopulation spatial genetic structure, as individuals within a range of 150 m are likely related. Consequently, this indicates that the species is self-compatible and open-pollinated seeds may present inbreeding not only from selfing, but also from mating among relatives. Moreover, the paternity correlation is substantially higher within than among fruits, indicating the importance of collecting several fruits from seed-trees and mixing the seeds from these fruits to increase genetic diversity, paternity contribution and effective size for breeding, conservation and reforestation.

The results show the importance of the size of the reproductive population from which seeds are collected for genetic breeding, conservation and environmental restoration plans. Large pollination neighbor areas may increase the effective number of pollen donors (N_{ep}) contributing to progeny arrays. Thus, as we detected significant association between N_{ep} and Θ, N_e, k and R, with a larger pollination neighbor area, and the consequent increase in N_{ep}, the coancestry coefficient within progenies will be reduced and the number of alleles and effective size will increase. These factors are highly favorable to genetic breeding, conservation and environmental restoration programs. Furthermore, as Θ is negatively associated with H_o, a decrease in coancestry due to an increase in N_{ep} will increase the heterozygosity within progenies.

Finally, due to the detected selfing and correlated mating in all populations, the average coancestry coefficient within progenies (Θ) was significantly higher and variance effective size (N_e) in all populations was also lower than expected for panmictic populations ($\Theta = 0.125$, $N_e \approx 4$). Due to the absence of panmixis in the populations, the number of seed-trees from which to collect seeds, aiming at retaining the reference effective size of 150 for genetic breeding, conservation and environmental reforestation, our results suggest that seeds must be collected from at least 54 seed-trees. This minimum value is within the range estimated for 30 tropical tree species (ranging from 45 to 114) although it is lower than the estimated average of 67 seed-trees (SEBBENN, 2006). We note that the seed-trees must be unrelated and they must be located at a sufficient distance to avoid overlapping pollen pools to ensure that each seed-tree mated with a different set of pollen donors.

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5 PATERNITY ANALYSIS REVEALING SIGNIFICANT ISOLATION OF POLLEN FLOW AND A NEAR NEIGHBOUR POLLEN DISPERSAL PATTERN IN SMALL *Cariniana legalis* MART. KUNTZE POPULATIONS IN THE BRAZILIAN ATLANTIC FOREST

Abstract

Around the world, large trees are becoming increasingly rare. *Cariniana legalis* is the tallest tree species found in the Atlantic Forest, reaching up to 60 m in height. However, due to the significant fragmentation of the Atlantic Forest, the remaining *C. legalis* populations have become spatially isolated and, as such, strategies for conserving the remaining populations are necessary. For *in situ* and *ex situ* genetic conservation to be effective, it is important to understand the levels of genetic diversity, spatial genetic structure (SGS), and gene flow. In this study we investigate genetic diversity, pollen flow and SGS in three small, isolated *C. legalis* populations using microsatellite loci. We measured, mapped, and sampled all *C. legalis* trees occurring in these three populations: 65 trees from the Ibicatu population, 22 trees from the MGI population, and four trees from the MGII population. We also collected and genotyped 600 seeds from Ibicatu, 250 seeds from MGI, and 200 seeds from MGII. High levels of genetic diversity were detected in all populations. Significant SGS was detected in Ibicatu up to 150 m and substantial levels of pollen flow were only detected in Ibicatu (8%), and not in MGI (0.4%) or MGII (0%). Selfing was highest in MGII, the smallest group of trees, at 18%, as compared to Ibicatu (6%) and MGI (6.4%). In MGI and MGII, we also detected a strong pattern of mating among near-neighbours. Our results can be used to guide seed collection strategies for genetic breeding, conservation and environmental reforestation. Our conclusion is that seeds must be collected from seed trees located at distances greater than 350 m and from several forest fragments.

Keywords: Conservation genetics; Microsatellites; Population genetics; Tropical tree species; Inbreeding; Fragmentation

5.1 Introduction

Throughout the last two centuries human activities have caused extensive forest fragmentation altering the structure, distribution, and functioning of natural ecosystems, particularly in tropical rainforests (AGUILAR et al., 2008; RIBEIRO et al., 2009). Forest fragmentation has serious impacts on tree genetic diversity, as well as on other organisms associated with forest ecosystems (LAURANCE, 2012). Although forest fragmentation is a global problem, it is believed to be particularly problematic in the tropics, where there is a high

diversity of tree species, many of which have low population densities (NASON; HAMRICK, 1997; LAURANCE 2012).

In tropical forests, tall trees with large canopies are crucial to forest survival (LAURANCE et al., 2000) as they provide fruits, flowers and shelter for animal populations and countless others species (LAURANCE et al., 2000; LAURANCE, 2012). Considering the size and longevity of these trees, they are a keystone species of forest ecosystems, creating optimal environmental conditions for many other plants and animals, such as insects, birds, and bats, which are the main pollinations of most tropical tree species (CHASE et al., 1996). The viability of keystone tree populations is fundamental for biodiversity conservation in fragmented tropical landscapes and requires knowledge of their responses to habitat fragmentation (HANSON et al., 2008). Today, the Brazilian Atlantic semi-deciduous rainforest is significantly fragmented and only 11 to 17% of the original forest cover remains (RIBEIRO et al., 2009, 2011). It is expected that reductions in forest cover, and the subsequent spatial isolation of tree species populations, have a negative impact on the trees reproductive success and gene flow (NASON; HAMRICK, 1997; LOWE et al., 2005; AGUILAR et al., 2008; MANOEL et al., 2012).

Forest fragmentation modifies the movement of pollen and seeds within tree populations, disrupting ecological and evolutionary processes (CUERTAS-HERNANDES et al., 2010). These disruptions may lead to lower rates of outcrossing for isolated trees and small forest fragments, as well as lower genetic diversity, increased correlated mating, inbreeding, coancestry, and decreased progeny vigor (WHITE et al., 2002; BURCZYK et al. 2004; SEBBENN et al., 2011; BREED et al., 2012). As trees are predominantly outcrossing (WARD et al., 2005; PETIT; HAMPE, 2006), they can accumulate deleterious recessive alleles, creating a high genetic load, thus creating a greater potential for inbreeding depression (LOWE et al., 2005; PETIT; HAMPE, 2006; BREED et al., 2012). High rates of selfing have been found in extremely low density tree populations (DICK et al., 2003; LANDER et al., 2010; MORAES; SEBBENN, 2011; MANOEL et al., 2012) and small forest fragments (VRANCKX et al., 2012; TARAZI et al., 2013) resulting from a change in the foraging behavior of pollinators as a result of forest fragmentation (HARDER; BARRETT, 1995; GHAZOUL 2005). Studies of tropical trees have also found signs of decreased genetic diversity in fragmented populations (MANOEL et al., 2012). When the population size declines, genetic diversity may be lost. Identifying drastic population decreases remains an important issue because it can increase the spatial genetic structure (SGS) in

fragmented populations. Increases in SGS result in an increase in local genetic drift as a result of the reduced number of reproductive individuals (DICK et al., 2008). However, long-distance pollen dispersal may guard against the negative genetic consequences of forest fragmentation (WHITE et al., 2002; DICK et al., 2003; LANDER et al., 2010). Extensive gene flow via pollen promotes high levels of genetic diversity, larger effective population size within populations, and low genetic differentiation among populations (FORTUNA et al., 2008; CUERTAS-HERNADEZ et al., 2010).

Determining the influence of the movement of pollen on the effective population size or reproductive neighbourhood area requires detailed analyses of the genetic and reproductive structure of populations. These analyses include identifying the pollen donor and its distance from the mother tree (NASON; HAMRICK, 1997; BURCZYK et al., 2004), defining reproductive patterns within populations (variation in flowering phenology), as well as estimating the rate of immigration into the population (APSIT et al., 2001). Furthermore, the effective population size is a critical parameter in population genetics because it measures the rate of genetic drift and inbreeding (VENCOVSKY; CROSSA, 2003).

In this study we assess the effects of spatial isolation due to forest fragmentation on pollen flow, mating patterns and intrapopulation spatial genetic structure (SGS) in three populations of the tallest tree in the Atlantic Forest, *Cariniana legalis* Mart. O. Kuntze (Lecidiaceae). This species is endemic to the Atlantic Forest, it occurs at low densities (< 1 tree/ha), and can reach 60 m in height and 4 m in diameter at breast height (dbh). Its flowers are hermaphroditic and pollinated by bees of the genus *Melipona* and *Trigona* (PRANCE; MORI, 1979). Fruits can contain more than 10 seeds and its seeds are dispersed by gravity and anemochory (CARVALHO, 1994). Its wood is light and used in indoor civil construction and for furniture as it is not resistant to decay. The species is considered endangered (IUCN, 2002) and strategies for *in situ* and *ex situ* conservation of the remaining populations are necessary. The genetic conservation of the remaining populations of this species requires an understanding of its genetic diversity, inbreeding, spatial genetic structure, mating system and gene flow. As such, herein we address the following questions: i) Is there intra-population spatial genetic structure? ii) Are populations that have been isolated spatially due to forest fragmentation reproductively isolated? iii) What are the patterns and distance of pollen dispersal within and between forest fragments?

iv) Do larger trees have more descendants or is their size not a factor in the number of offspring generated?

5.2 Development

5.2.1 Material and Methods

Study sites

Our genetic study of pollen flow was carried out in three *C. legalis* fragments (Figure 5.1). One fragment is located within the Ibicatu State Forest (22°46' S and 47°43' W, average altitude of 542 m), Piracicaba, São Paulo State, Brazil. The Ibicatu State Forest belongs to the Forestry Institute of São Paulo and it has a total area of 72 ha. According to Köppen classification, the climate is Cwa, with a mean temperature in January of 23.9° C (the hottest month) and in July of 16.1° C (the coldest month). The Cwa climate is characterized as humid and mesothermal, with variation in the monthly mean temperature from 14.3 to 24.7° C (KÖEPPEN, 1948). The State Forest is currently surrounded by agriculture (sugarcane, *Eucalyptus*) and pastures. The fragment is a remnant of a semi-deciduous forest and in the past the fragment was subjected to several fires and selective logging. The Mata da Figueira (MGI) is a small forest fragment of 7.2 ha of riparian forest on a semi-deciduous plateau. The fragment is part of the Mogi-Guaçu Ecological Station run by the Forestry Institute of São Paulo (22°16'S 47°11' W, average altitude of 600 m). In this region, the dry season extends from May to August, with 86.2% of the precipitation (1,314 mm annually) concentrated during the rainy season. Located approximately 2.9 km from MGI is a cluster of four *C. legalis* individuals. We considered this small group as population MGII (Figure 5.1, yellow arrow). The Ibicatu population contains 65 *C. legalis* adult trees (0.93 trees/ha) with dbh ranging from 0.25 to 3.25 m (average of 1.21 m). MGI contains 22 adults trees (3.6 trees/ha), with dbh ranging from 0.25 to 1.14 m (average of 0.68 m), and MGII consists of four adults trees, with dbh ranging from 0.46 to 0.73 m (average of 0.52 m).

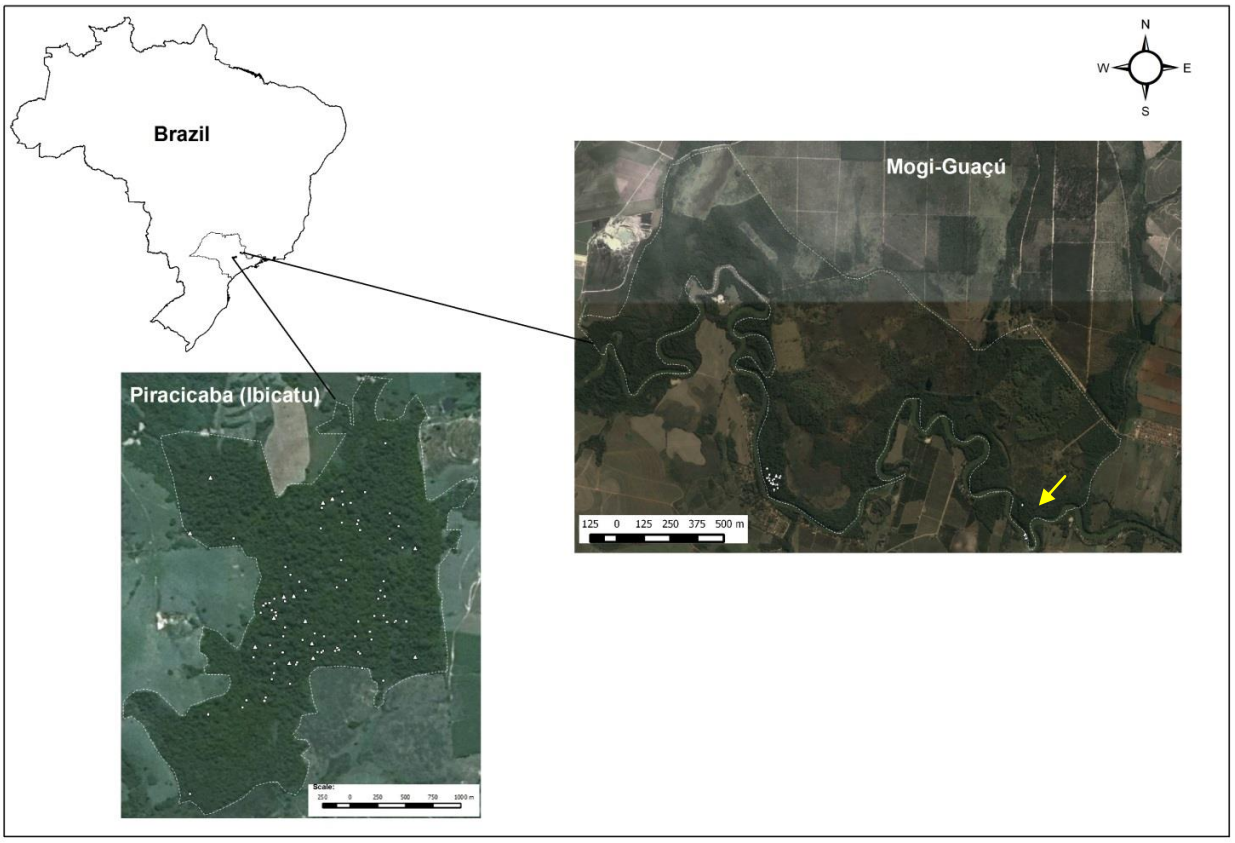

Figure 5.1- Spatial distribution of *Cariniana legalis* trees sampled in the Ibicatu State Forest and Mogi-Guaçu. Yellow arrow = Four isolated trees (MGII). Triangle=seed-trees; circle = adults trees

Sample size

Stem bark tissue and fruits were collected from reproductive adult trees in the Ibicatu, MGI and MGII populations. In Ibicatu all 65 identified adult individuals were mapped (*x* and *y* coordinates obtained using a GPS III-Garmin, USA) and dbh measured. In MGI and MGII, all identified trees (22 and four, respectively) were mapped, dbh measured, sampled (cambium tissue), and genotyped. Open-pollinated seeds were collected directly from the canopy of 15 seed trees chosen randomly in the Ibicatu population. Seeds were randomly collected from five trees in MGI and two trees in MGII by climbing the trees and collecting the seeds from the canopy. The fruits contained an average of 7.7 seeds (Tambarussi, personal observation) and seeds were germinated in vermiculite until the cotyledons emerged and DNA was subsequently extracted. From Ibicatu, 40 seeds (five seeds per fruit) from each seed-tree were genotyped, totaling 600

seeds. For MGII, 50 seeds (five seeds per fruit) from each seed-tree were genotyped, totaling 250 seeds and from MGII, 100 seeds (five seeds per fruit) were genotyped, totaling 200 seeds.

DNA extraction and SSR amplification

For all adult trees, DNA was extracted from 100 mg per tree of adult stem bark tissue using AnalytikJena DNA isolation kits. Seeds were germinated in vermiculite until the cotyledons emerged and then DNA was extracted from the first leaf pair of 15 to 20-day old seedlings using the method of Doyle and Doyle (1990). We used seven specific microsatellite markers for *C. legalis*: Cle01, Cle04, Cle05, Cle08, Cle09, Cle10 and Cle12. These microsatellite loci were selected because they show Mendelian inheritance, a high level of polymorphism (TAMBARUSSI et al., 2013a) and because they are unlinked (TAMBARUSSI et al., 2013b). Microsatellite loci were amplified by PCR for a final volume of 15 μL using GoTaq® Colorless Master Mix containing: 7.5 μL GoTaq® Colorless Master Mix (2×), 10μM of each primer (F and R), 3.0 μL Nuclease-Free Water, and 7.5 ng template DNA. The amplification program for all primers consisted of an initial denaturing step at 94° C for 1 min, followed by 35 cycles of amplification (94° C for 1 min, followed by 1 min at the specific annealing temperature of each primer pair (see TAMBARUSSI et al., 2013a), 72° C for 1 min), and a final elongation step at 72° C for 10 min. Amplifications were performed with a Mastercycler (Eppendorf, Hamburg, Germany). The amplification products (2 μL of the total reaction volume) were separated on a Fragment Analyzer™ Automated CE System (Advanced Analytical Technologies, Inc. [AATI], Ames, Iowa, USA) using the dsDNA Reagent Kit, 35-500 bp. Raw data were analyzed using the PROSize version 2.0 software (AATI). Samples with suspicious or missing alleles (peaks), or samples for which mismatches occurred between mothers and progeny, were genotyped a second time.

Analysis of genetic diversity

By grouping adults and seeds from each population, genetic diversity was estimated by total number of alleles (k), average number of alleles per locus (A), observed (H_o) and expected heterozygosity (H_e) at Hardy-Weinberg equilibrium over all loci. All these analyses were performed using the FSTAT program (GOUDET, 1995).

Estimates of group coancestry and variance effective size

Group coancestry for adults within populations was estimated as the average coancestry coefficient between all pairwise individuals (Θ) using the Nason coancestry estimator (Loiselle et al., 1995) and the SPAGEDI 1.3 program (Hardy and Vekemans, 2002):

$$\hat{\Theta} = \left[0.5N(1+\hat{F}) + \sum_{i=1}^{n}\sum_{i\neq j}^{n}\hat{\theta}_{ij}\right] / N^2$$

(LINDGREN et al., 1997), for a sample of N individuals. This includes the coancestry of individuals with themselves. The variance effective population size was calculated as

$$N_e = \frac{0.5}{\bar{\theta}\left(\frac{N-1}{N}\right) + \frac{1+\hat{F}}{2N}} = 0.5/\hat{\Theta}$$

(COCKERHAM, 1969), where θ_{ij} is the pairwise coancestry coefficient between individuals *i* and *j*, $\bar{\theta}$ the average value of the $N(N-1)$ θ_{ij} pairs of different individuals and $\hat{F}$ the corresponding average fixation index. The fixation index was estimated as $\hat{F} = 1-(\hat{H}_o / \hat{H}_e)$, where H_o and H_e are the observed and expected heterozygosity, which is adequate for sufficiently large N, using the FSTAT program (GOUDET, 1995). To test if $\hat{F}$ values were significantly different from zero, we permutated alleles among individuals (Monte Carlo permutations), also using FSTAT and a sequential *Bonferroni* correction (95%, α= 0.05). The relationship between N_e and the census size of the population (*N*), was calculated as N_e/*N*.

Analysis of intrapopulation spatial genetic structure

The intrapopulation spatial genetic structure (SGS) was investigated for adults in each population using the estimate of coancestry coefficient between pairwise individuals (θ_{ij}), as proposed by Nason and described in Loiselle et al. (1995). To visualize the SGS in the Ibicatu population, mean values were calculated for pairs of individuals within seven distance classes (0-100, 100-200, 200-300, 300-400, 400-500, 500-600, 600-700 m). The number of pairs in each of

these seven classes was 132, 257, 273, 353, 361, 298 and 188, respectively. To test whether the mean values were significantly different from zero, a confidence interval of 95% probability was calculated for each value in each distance class using 1,000 permutations per individual between different distance classes. The confidence interval was used to construct the correlogram. All these analyses were carried out using SPAGEDI 1.3 program (HARDY; VEKEMANS, 2002).

Paternity analysis

Contemporary pollen flow and dispersal was carried out using a categorical paternity analysis, implemented in the Cervus 3.0 program (MARSHALL et al., 1998; KALINOWSKI et al., 2007). The paternity analysis for Ibicatu, MGI and MGII was conducted using the genotypes of all adult trees (65, 22 and four individuals, respectively), seed trees (15, five and two seed trees, respectively) and seeds (600, 250 and 200 seeds, respectively) from each population. To determine the likely pollen donors for seeds, all adult trees in each population were used as putative pollen donor candidates. Due to the proximity of the MGI and MGII populations (< 2.9 km), we grouped all 26 adult trees of both populations (22 and four individuals, respectively) as pollen donor candidates. In the paternity analysis, the most likely pollen donor was determined using the reference allele frequencies calculated in the adult populations as suggested by Meagher and Thompson (1987). 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 with the paternity tests simulated by Cervus. The Δ cryptic was determined based on a confidence level of 80%, as suggested by Marshall et al. (1998), using 10,000 repetitions, 0.01 as the ratio of genotyping errors, 70% as the proportion of pollen donors sampled within each population (given the high degree of isolation of the populations). The minimum number of loci necessary to determine the paternity of a seed was fixed at six. If a seed had no potential pollen donor within the population, this seed was considered as having received the pollen from outside the population (pollen immigration). In the analysis we also considered the possibility of one single mismatch in the seed-tree-seed trio and putative pollen donor and the possibility of self-pollination.

The selfing rate (s) was estimated as the proportion of seeds identified as having the same seed tree as pollen donor (n_{selfed}) in relation to the total number of sampled seeds (n_{total})

as: $\hat{s} = \hat{n}_{selfed} / n_{total}$. The standard error of average selfing was estimated assuming binomial distribution, as $SE(\hat{s}) = \sqrt{\hat{s}(1-\hat{s})/m_{st}}$, where m_{st} is the number of sampled seed trees in each forest fragment (SLAVOV et al., 2005). The pollen immigration rate (m) was calculated as the proportion of seeds for which a pollen donor candidate was not found within the populations ($n_{immigrant}$) relative to total number of sampled seeds (n_{total}) within populations, $\hat{m} = \hat{n}_{immigrant} / n_{total}$ (BURCZYK et al., 2004; SMOUSE; SORK, 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. The pollen dispersal distance (D) was calculated by the Euclidean distance between two points ($\hat{D} = \sqrt{(x_i - x_j)^2 + (y_i - y_j)^2}$, where x_i and x_j are the spatial x coordinates of seed tree i and putative pollen donor j, assigned by paternity analysis, and y_i and y_j are the spatial y coordinates of seed tree i and the pollen donor j, respectively).

To investigate whether reproductive success was a function of the distance between trees, we compared the frequency distribution of pollen dispersal with the frequency distribution of distance between all trees using the Kolmogorov-Smirnov test (SOKAL; ROHLF, 1995). The effective pollination neighbour area (A_{ep}) was calculated by assuming a circular area around a seed tree, $\hat{A}_{ep} = 2\pi\hat{\sigma}^2$ (LEVIN, 1998), where σ_p^2 is the axial pollen dispersal variance. It is important to note that the parameter A_{ep} corresponds to the circular area in which 63% of pollen donors that crossed with a seed tree are expected to be located (LEVIN, 1988). The circular pollination radius was estimated as $\hat{r}_{ep} = \sqrt{\hat{A}_{ep} / 3.1415}$ (AUSTERLIZ; SMOUSE, 2001). The cryptic gene flow (C_{GF}), or the probability of finding a compatible paternal candidate of a seed within the population when the real father is located outside of the population, was calculated from the probability of detecting immigrant pollen grains (d) in paternity analysis, given a local population of candidate pollen donors (SMITH; ADAMS, 1983): $\hat{C}_{GF} = 1 - \hat{d}$. The parameter d is estimated as $\hat{d} = 1 - \sum_{i=1}^{t} \hat{h}_i$, ($i = 1,2,...,t$), using the Pollen Flow program (SLAVOV et al., 2004), where h_i is the frequency of local pollen grain i in the background population, or the idealized population in which all reproductive trees could pollinate any of the sampled seed trees

of the studied forest fragments (SLAVOV, 2004), and t is the total number of distinct local pollen grains (SMITH; ADAMS, 1983, SLAVOV et al., 2005). Thus, the pollen immigration rate (m), estimated from Cervus 3.0, was corrected to an unbiased pollen immigration rate, and calculated using the probability of detecting immigrant pollen grains (d) in paternity analysis, given a local population of candidate pollen donors: $\hat{m} = \hat{b} / \hat{d}$ (SMITH; ADAMS, 1983), (b) is the detected pollen immigration. The standard error of pollen immigration [$SE(m_p)$] was estimated as $SE(\hat{m}) = \hat{m}\sqrt{(1-\hat{b})/(\hat{b}n)}$ (SLAVOV et al., 2005), where n is the number of seeds sampled from each seed tree. We also calculated the number of pollen donors that mated with each seed tree (N_{ep}) and from this parameter we estimated the paternity correlation within progenies, $r_p = 2 / N_{ep}$ (RITLAND, 1989).

5.2.2 Results

Genetic diversity

We found high levels of genetic diversity in the three studied populations: in Ibicatu, for the entire sample of 665 individuals (65 adults and 600 seeds), we found a total of 101 alleles, an average of 14.3 alleles per locus, observed heterozygosity of 0.811 and expected heterozygosity of 0.860; in MGI and II, for the total sample of 476 individuals (22 adults in MGI and four in MGII plus 450 seeds), we found 106 alleles, with an average of 14.3 alleles per locus, observed heterozygosity of 0.786, and expected heterozygosity of 0.853. In Ibicatu, 22 private alleles were identified, in MGI 23 were found, and in the MGII population four private alleles were identified. In comparing adult and seed cohorts, one allele was unique to adults of Ibicatu and one to seeds, 14 alleles were exclusive to adults and eight to seeds in MGI, while in MGII, one allele was exclusive to adults.

Intra-population spatial genetic structure

The coefficient of coancestry decreases with increased distance between trees in all three populations (Figure 5.2a). However, significant spatial genetic structure was found only in the

Ibicatu population up to 150 m, suggesting that trees which occur within this distance may be related.

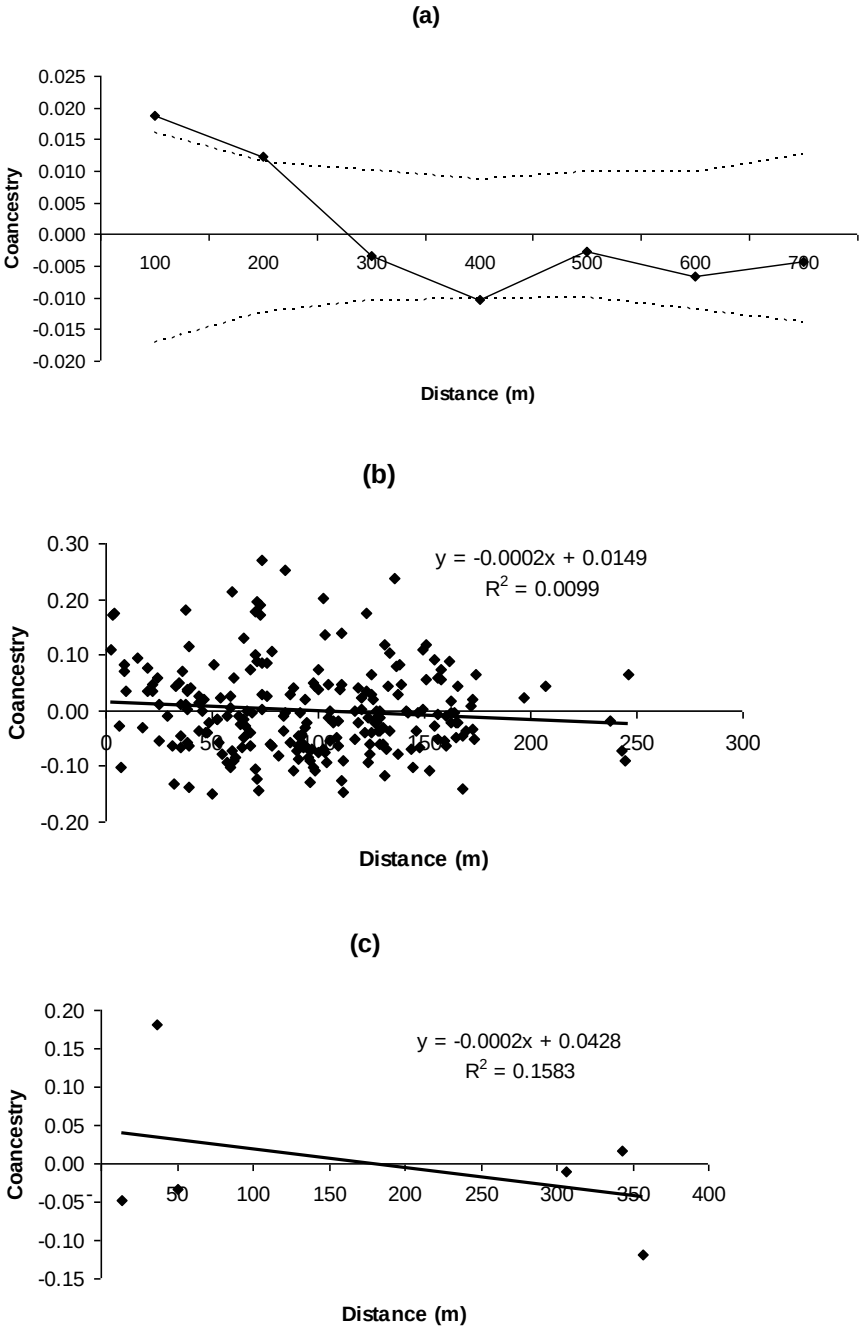

Figure 5.2 - Correlograms of average coancestry coefficients (θ_{ij}) of *Cariniana legalis* adult trees from Ibicatu (a), MGI (b) and MGII (c). In the figure a, the solid line represents the average θ_{ij} value. The dashed lines represent the 95% (two-tailed) confidence interval of the average θ_{ij} distribution calculated by 1,000 permutations of spatial distance among pairwise of adult trees

Effective population size of the adult population

Of the three populations, the average group coancestry of adult trees within populations was higher in the smallest population, MGII, with a result of $\Theta = 0.12441$ (Table 5.1).

Table 5.1 - Census number, group coancestry, effective population size and relationship between the effective size and census number (N_e / N) in three *Cariniana legalis* populations

Parameter	Ibicatu	MGI	MGII
Census number: N	65	22	4
Fixation index in the parental population: F	0.057	0.078 *	0.011
Group coancestry: Θ	0.00747	0.02245	0.12441
Effective population size: N_e	32.5	10.9	2.3
N_e / N	0.50	0.49	0.57

* $P < 0.05$

The effective population size was lower than the number of individuals within populations. Our results suggest that of the 65, 22 and four adult trees of Ibicatu, MGI and MGII, correspond to approximately 33, 11 and threes individuals not related and nor inbred. Consequently, the relationship among the effective population size and the census (N_e/N) was lower than unity for all populations.

Gene flow and pollen dispersal

Of the 600 sampled seeds in the Ibicatu population, 250 in MGI and 200 in MGII population, a putative paternal parent was assigned to 553 (92.2%), 249 (99.6%), and all 200 seeds (100%), respectively (Table 5.2). This suggests a pollen immigration rate of only 7.8% for Ibicatu, 0.4% for MGI, and 0% for MGII. The 47 seeds in Ibicatu and the single seed from MGI that have no detected paternal parent within the populations likely represent pollen immigration

from trees located outside of the forest fragments. In MGI, the single seed without an identified pollen donor within MGI was assigned a pollen donor from MGII. In Ibicatu, the detected pollen immigration rate (b) ranged among seed trees from zero to 15%, with an average of 7.8%. On the other hand, for MGI b ranged from zero to 2% and for MGII the result was zero (Table 5.3). However, the probability that an immigrant pollen grain (d) has a detectable genotype (detected probability) inside the forest fragments ranged among seed trees in Ibicatu from 0.978 to 0.994, with an average of 0.985, in MGI from 0.995 to 0.997, with an average of 0.997, and in MGII d was 0.997 for both seed trees, with an average of 0.997. This resulted in an unbiased average pollen immigration ($\hat{m} = \hat{b}/\hat{d}$) of 8, 0.4 and 0%, for Ibicatu, MGI, and MGII, respectively. The mean cryptic gene flow (C_{GF}) estimated using the *Pollen Flow* program was very low, with results of 0.015, 0.004 and 0.003 in Ibicatu, MGI and MGII, respectively. This result indicates that the levels of cryptic gene flow do not bias our pollen flow estimates.

Table 5.2- Results of the analysis of pollen dispersal for the sampled *Cariniana legalis* populations

Population	*n*	Pollen flow (% relative)	Paternity (% relative)	Selfing (% relative)	Mean ± SD (m)	Median (m)	Min-max (m)	$\hat{A}_{ep}$ (ha)	$\hat{r}_{ep}$ (m)
Ibicatu	600	47 (7.8%)	553 (92.2%)	36 (6%)	352 ± 244	341	19-922	37	345
MGI	250	1 (0.4%)	249 (99.6%)	16 (6.4%)	63 ± 194	35	4-154 [A]	1.44 [A]	68 [A]
MGII	200	0 (0%)	200 (100%)	36 (18%)	130 ± 135	37	14-344	11	191

n is the sample size; SD is the standard deviation; A_{ep} is the effective area of pollination, r_{ep} is the effective radius of pollination

[A] was calculated excluding the single seed pollinated by a tree of MGII population. This unique mating event represents a pollen dispersal distance of 2,929 m

Table 5.3- Results of paternity analysis in level of seed tree for three *Cariniana legalis* forest fragments. n is the sample size; b is the detected pollen immigration; d is the probability that an immigrant pollen grain has a detectable genotype (detected probability) inside the stand; m is the unbiased pollen immigration rate; $SE(m)$ is the standard error of pollen immigration; C_{GF} is the unbiased cryptic pollen flow; s is the selfing rate; $t_m - t_s$ is the rate of mating among relatives; δ is the pollen dispersal distance estimated without selfing; A_{ep} and r_{ep} are the effective pollination and radius of neighbourhood area, respectively; N_{ep} is the effective number of pollen donors; r_p is the paternity correlation; Θ and N_e are the coancestry coefficient and variance effective size within progenies, respectively; SD is the standard deviation

(continue)

Seed tree	N	$\hat{b}$	$\hat{d}$	$\hat{m} = \hat{b}/\hat{d}$	$SE(\hat{m})$	$\hat{C}_{GF}$	$\hat{s}$	$\hat{t}_m - \hat{t}_s$	$\hat{\delta}$ (mean ± SD) (m)	$\hat{A}_{ep}$ (ha)	$\hat{r}_{ep}$ (m)	N_{ep}	r_p	$\hat{\Theta}$	$\hat{N}_e$
Ibicatu															
J04	40	0	0.981	0	0	0.019	0.15	0.80	60 ± 81	4.15	115	2	0.50	0.238	2.04
J06	40	0.2	0.984	0.203	0.064	0.016	0.12	0.40	183 ± 233	39.14	353	7	0.14	0.188	2.53
J16	40	0	0.978	0	0	0.022	0	0.30	436 ± 224	30.30	311	10	0.10	0.144	3.28
J22	40	0.125	0.982	0.127	0.053	0.018	0.02	0.25	546 ± 162	16.78	231	10	0.10	0.143	3.27
J23	40	0.025	0.987	0.025	0.025	0.013	0	0.43	686 ± 179	21.22	260	15	0.07	0.137	3.41
J27	40	0	0.991	0	0	0.009	0	0.40	354 ± 208	23.75	275	15	0.07	0.132	3.54
J28	40	0.05	0.984	0.051	0.035	0.016	0.05	0.33	419 ± 175	21.02	259	12	0.08	0.145	3.23
J29	40	0.05	0.994	0.050	0.035	0.006	0	0.10	309 ± 200	20.67	257	18	0.06	0.129	3.61
J30	40	0.1	0.983	0.102	0.048	0.017	0.05	0.45	235 ± 192	13.04	204	11	0.09	0.156	3.02

Table 5.3- Results of paternity analysis in level of seed tree for three *Cariniana legalis* forest fragments. n is the sample size; b is the detected pollen immigration; d is the probability that an immigrant pollen grain has a detectable genotype (detected probability) inside the stand; m is the unbiased pollen immigration rate; $SE(m)$ is the standard error of pollen immigration; C_{GF} is the unbiased cryptic pollen flow; s is the selfing rate; $t_m - t_s$ is the rate of mating among relatives; δ is the pollen dispersal distance estimated without selfing; A_{ep} and r_{ep} are the effective pollination and radius of neighbourhood area, respectively; N_{ep} is the effective number of pollen donors; r_p is the paternity correlation; Θ and N_e are the coancestry coefficient and variance effective size within progenies, respectively; SD is the standard deviation

(continuation)

Seed tree	N	$\hat{b}$	$\hat{d}$	$\hat{m} = \hat{b}/\hat{d}$	$SE(\hat{m})$	$\hat{C}_{GF}$	$\hat{s}$	$\hat{t}_m - \hat{t}_s$	$\hat{\delta}$ (mean ± SD) (m)	$\hat{A}_{ep}$ (ha)	$\hat{r}_{ep}$ (m)	N_{ep}	r_p	$\hat{\Theta}$	$\hat{N}_e$
J36	40	0.075	0.985	0.076	0.042	0.015	0	0.28	263 ± 152	13.97	211	14	0.07	0.132	3.51
J41	40	0.05	0.983	0.051	0.035	0.017	0.17	0.25	224 ± 89	5.31	130	8	0.13	0.183	2.62
J49	40	0.15	0.985	0.152	0.057	0.015	0	0	297 ± 206	29.53	307	9	0.11	0.145	3.21
J61	40	0.1	0.988	0.101	0.048	0.012	0.07	0.15	324 ± 218	32.50	322	9	0.11	0.157	3.01
J67	40	0.125	0.985	0.127	0.053	0.015	0.15	0	551 ± 264	51.29	404	8	0.13	0.177	2.69
J70	40	0.125	0.986	0.127	0.053	0.014	0.05	0.30	245 ± 174	17.16	234	3	0.33	0.185	2.58
Mean		**0.078**	**0.985**	**0.080**	**0.037**	**0.015**	**0.06**	**0.30**	**342.1**	**22.66**	**258.2**	**10.1**	**0.10**	**0.159**	**3.04**
MGI															
1M	50	0.02	0.995	0.020	0.020	0.005	0	0.28	15 ± 13	0.11	19	3	0.33	0.286	1.72
2M	50	0	0.996	0.	0	0.004	0.10	0.20	141 ± 437	120.10	618	7	0.14	0.163	2.94

Table 5.3- Results of paternity analysis in level of seed tree for three *Cariniana legalis* forest fragments. n is the sample size; b is the detected pollen immigration; d is the probability that an immigrant pollen grain has a detectable genotype (detected probability) inside the stand; m is the unbiased pollen immigration rate; $SE(m)$ is the standard error of pollen immigration; C_{GF} is the unbiased cryptic pollen flow; s is the selfing rate; $t_m - t_s$ is the rate of mating among relatives; δ is the pollen dispersal distance estimated without selfing; A_{ep} and r_{ep} are the effective pollination and radius of neighbourhood area, respectively; N_{ep} is the effective number of pollen donors; r_p is the paternity correlation; Θ and N_e are the coancestry coefficient and variance effective size within progenies, respectively; SD is the standard deviation

(conclusion)

Seed tree	N	$\hat{b}$	$\hat{d}$	$\hat{m} = \hat{b}/\hat{d}$	$SE(\hat{m})$	$\hat{C}_{GF}$	$\hat{s}$	$\hat{t}_m - \hat{t}_s$	$\hat{\delta}$ (mean ± SD) (m)	$\hat{A}_{ep}$ (ha)	$\hat{r}_{ep}$ (m)	N_{ep}	r_p	$\hat{\Theta}$	$\hat{N}_e$
3M	50	0	0.996	0	0	0.004	0	0	31 ± 6	0.02	8	3	0.33	0.309	1.60
4M	50	0	0.997	0	0	0.003	0.14	0	112 ± 44	1.21	62	5	0.20	0.175	2.76
5M	50	0	0.996	0	0	0.004	0	0	58 ± 50	1.56	70	10	0.10	0.138	3.44
Mean		**0.004**	**0.996**	**0.004**	**0.004**	**0.004**	**0.05**	**0.10**	**71.4**	**24.60**	**155.4**	**5.6**	**0.18**	**0.214**	**2.49**
MGII															
6M	100	0	0.997	0	0	0.003	0.10	0.34	145 ± 128	10.22	180	3	0.33	0.172	2.85
7M	100	0	0.997	0	0	0.003	0.27	0.32	104 ± 137	11.80	194	3	0.33	0.209	2.36
Mean		**0**	**0.997**	**0**	**0**	**0.003**	**0.18**	**0.33**	**124.5**	**11.01**	**187**	**3.0**	**0.33**	**0.191**	**2.61**

[A] ± Standard error.

In Ibicatu, of the seeds with an assigned father (Table 5.2), we identified the same individual as both seed tree and pollen donor for 36 seeds, representing a selfing rate of 6%. In the smaller forest fragments, 16 seeds from MGI and 36 seeds from MGII had the same individual as both seed tree and pollen donor, representing a selfing rate of 6.4 and 18%, respectively. Of the 65 sampled reproductive trees in Ibicatu, 56 (86%) fathered at least one seed (ranging from 1 to 45 seeds). Of the 22 and 4 reproductive trees in MGI and MGII, 19 (86%) and 4 (100%) fathered at least one seed (ranging in MGI from 1 to 37 seeds and MGII from 42 to 60 seeds).

Distance and patterns of pollen dispersal

For the seeds that had the putative pollen donor identified within the forest fragments, excluding selfing, the pollen dispersal distance ranged in Ibicatu from 19 to 922 m, with an average of 352 m and a median of 341 m, in MGI from 4 to 154 m, with an average of 63 m and a median of 35 m, and in MGII from 14 to 344 m, with an average of 139 m and a median of 37 m (Table 5.2, Figure 5.3).

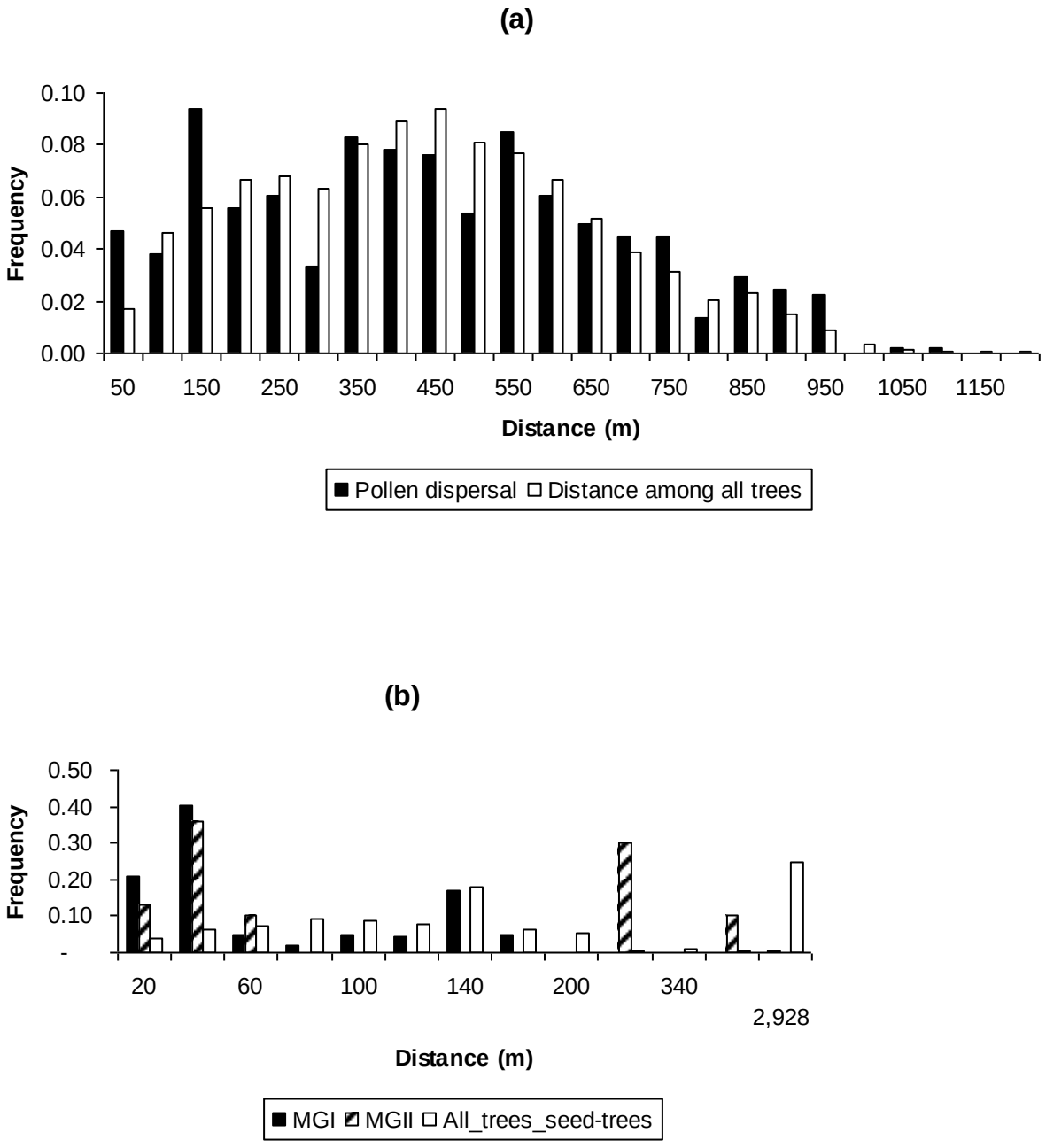

Figure 5.3 - Pollen dispersal distance determined by paternity analysis in progenies and distance among all adult trees of *Cariniana legalis* in Ibicatu (a) and MGI and MGII (b) populations

The single occurrence of pollen immigration found in MGI from MGII represents a pollen dispersal distance of 2,929 m. The average effective pollination neighbour area (A_{ep}) was estimated as 37 ha in Ibicatu, 1.44 ha in MGI (and considering the observed unique pollen immigrant, 23 ha), and 11 ha in MGII. This result in an average effective neighbour pollination radius (r_{ep}) of: 345 m in Ibicatu; 68 m in MGI; and 191 m in MGII (or 274 m considering the single pollen immigration event). Comparing the frequency curve of effective pollen dispersal with the frequency curve measured among all reproductive trees using the Kolmogorov-Smirnov test, we observed significant differences in Ibicatu ($\hat{D} = 0.18$, $P < 0.001$), MGI ($\hat{D} = 0.461$, $P < 0.001$), and MGII ($\hat{D} = 0.511$, $P < 0.001$), suggesting a non-random distribution of pollination distance in the three forest fragments (Figure 5.3). Moreover, there was a significant association between the number of seeds fertilized by pollen donors and the distance between the paternal and maternal trees in Ibicatu (R^2 = 0.40, df= 17, P< 0.01) and MGI (R^2 = 0.35, df= 11, P< 0.05). In MGII, there was no significant association between the number of seeds fertilized by pollen donors and the distance between the paternal and maternal trees (R^2 = 0.001, df= 2, P> 0.05).

Male fertility

A linear regression analysis was used to determine if fertility of pollen donors or capacity to generate more offspring was associated with dbh. We found a significant association in the Ibicatu (R^2 = 0.17, df= 54, P< 0.01) and MGII (R^2 = 0.99, df= 2, P< 0.01) populations, indicating that the number of seeds produced by pollen donors is strongly associated with the size of the dbh. No association was found for MGI (R^2 = 0.01, df= 17, P> 0.05).

Individual seed trees

Pollen immigration corrected for cryptic pollen flow (m) ranged among seed trees from zero to 0.203 (with a selfing rate ranging from zero to 0.15) in Ibicatu, zero to 0.14 in MGI, and 0.1 to 0.27 for the two seed trees in MGII (Table 5.3). The pollen dispersal distance (δ) was

higher for seed trees from Ibicatu than observed for the MGI and MGII populations, ranging from 60 to 686 m in Ibicatu, 15 to 141 m in MGI, and 104 and 145 m in MGII. Effective neighbour area (A_{ep}) ranged from 4.15 to 51.29 ha in Ibicatu, 0.02 to 120.1 m in MGI, and 10.22 to 11.80 m in MGII. The radius of effective neighbour area (r_{ep}) ranged from 4.15 to 51.29 ha in Ibicatu, 0.02 to 120.1 m in MGI, and from 10.22 to 11.80 m in MGII. The number of pollen donors (N_{ep}) per seed tree in Ibicatu ranged from 2 to 18, with an average of 10.1, in MGI from 3 to 10, with an average of 5.6, and in MGII the number of pollen donors was 3, resulting in an average paternity correlation (r_p) of 0.10, 0.18 and 0.33, respectively. Due to selfing and correlated mating, the average coancestry coefficient within progeny (Θ_i) was 0.159, 0.214 and 0.191 in Ibicatu, MGI and MGII, respectively. Thus, the average variance effective size (N_e) within progeny was 3.04, 2.49 and 2.61 in Ibicatu, MGI and MGII, respectively.

5.2.3 Discussion

Our results showed high levels of genetic diversity, in terms of number of alleles and heterozygosity in the three studied adult populations. However, we found some levels of inbreeding in the MGI population and evidence of SGS in Ibicatu; we also detected some level of relatedness between adult individuals in all populations. Thus, mating among relatives may occur in all populations. Due to inbreeding in the MGI population and the presence of a high number of related individuals in all populations, the estimated effective population size was lower than the number of individuals (N) within populations; the 65, 22, and four adult trees of Ibicatu, MGI, and MGII correspond to about 33, 11 and three individuals that are not related, nor inbred, considering an idealized population, resulting in a N_e/N relationship that is lower than unity (with a maximum of 0.57). In general, such results indicate that without pollen flow, the populations have a low evolutionary potential because even with high levels of genetic diversity, the high frequency of related individuals inside the populations may increase inbreeding due to mating among relatives, thus resulting in inbreeding depression.

Intrapopulation spatial genetic structure

Significant spatial genetic structure (SGS) was found only in the Ibicatu population up to 150 m, suggesting the likelihood that trees located at distances up 150 m are related (Figure 5.1a). The coancestry coefficient of adult trees decreased with increased distance between trees. However, in analysing the values of coancestry between pairwise individuals up to a distance of 150 m, there are many high values: 29% of the values ranged between 0.062 to 0.486 (distances ranging from 146 to 179 m), as expected for first cousins (θ_{xy} = 0.0625) and self-sibs (θ_{xy} = 0.5). It is also noteworthy that even at greater distances (180 to 1,184 m), we observed high levels of coancestry, with 23% of values ranging from 0.062 to 0.406 (distances ranging from 180 to 1,123 m). This result suggests that despite the fact that the analysis of SGS was not significant over longer distances, many individuals within populations are related, even at greater distances. SGS in natural populations can be caused by short distance seed and pollen dispersal (BACLES et al., 2006). Our analysis of pollen dispersal distances for the Ibicatu population showed that short-distance dispersal could be contributing to SGS because approximately 30% of mating occurred between trees located within 150 m (Figure 5.3a). Furthermore, many of these trees are related and there is a substantial rate of mating among related trees (ranging among seed trees from zero to 0.8, with an average of 0.30).

Mating pattern and pollen flow

Our results from paternity analysis suggest that selfing may be higher in populations of *C. legalis* with fewer individuals. We detected higher rates of selfing in MGII, the smallest stand, at 18%, as compared to the selfing rates in MGI (6.4%) and Ibicatu (6%). Other studies with tropical tree species have observed a similar pattern (MURAWSKI; HAMRICK, 1991; DICK et al., 2003; LANDER et al., 2010; KAMM et al., 2011; MORAES; SEBBENN, 2011; MANOEL et al., 2012; TARAZI et al., 2013). For example, in a recent study of mating system in the insect pollinated, tropical tree *Gomortega keule*, the authors found a selfing rate of 56% in single isolated trees, 30% in small stands, and 22% in large stands (LANDER et al., 2010).

Our results show that the spatially isolated Ibicatu and MGI populations are not reproductively isolated (Table 5.2 and 5.3); however, the four isolated trees (MGII) were reproductively isolated. Furthermore, the rate of pollen flow for Ibicatu and MGI were low (8% and 0.4%, respectively). In the region surrounding the Ibicatu population, we found some reproductive *C. legalis* individuals in two areas, one located at a distance of about 2.5 km and the other at about 5.4 km. Although there are other forest fragments in the region, these trees are the likely pollen donors providing the 8% pollen immigration, which demonstrates the efficiency of bees in enabling gene flow for tree species. In the MGI population, the closest conspecifics are the four trees of the MGII population, located at a distance of 2.9 km. Our results showed that one of the MGII trees was the pollen donor of the single seed detected as coming from immigrant pollen. There are some *C. legalis* trees located about 4.9 km from MGI and about 2.2 km from MGII; however, based on the reproductive event in 2011, our results suggest that MGI and MGII did not receive pollen from these trees. Obviously, if the rates of pollen immigration detected herein are consistent across all reproductive events within Ibicatu and MGI, the rate of pollen immigration may be enough to increase genetic diversity and effective population size and reduce inbreeding and SGS. Nevertheless, it is very important to note that we investigated effective pollen dispersal based on open-pollinated seeds collected from the canopy of the seed-trees, not the realized pollen dispersal, as measured through regeneration or seedlings. There difference between the seed dispersal phase and the seedling or juvenile phases is significant. In fact, although many trees were fruiting and producing a large number of fruits in both the Ibicatu and MGI populations, we did not find substantial regeneration in these forest fragments, suggesting that the rate of seedling establishment is low. We do not have enough data to investigate the cause of this phenomenon. However, according to our results for selfing (maximum 18%) and mating among relatives (maximum of 30%), inbreeding depression is not the cause because the majority of the seeds in all populations were produced by outcrossing.

Distance and patterns of pollen dispersal

Pollen dispersal distance within Ibicatu reached longer distances (922 m, average of 352 m) than in MGI (154 m, average of 63 m) and MGII (344 m, average of 139 m), although we did detect a singular event of pollen immigration from MGII to MGI of 2,929 m. The type of

pollinator partly determines the distances over which pollen is transported (DICK et al., 2008; MITCHELL et al., 2009); however, pollen dispersal is also affected by population density because at low densities, individuals are more widely dispersed and the pollinator vector must fly longer distances than in populations with a high density. Our results support the latter argument in that the differences in pollen dispersal distance occurred as a result of differences in the population densities in Ibicatu (0.93 trees/ha) and MGI (3.6 trees/ha). It is important to note that the observed maximum pollen dispersal distance for both Ibicatu and MGI are underestimated due to the detected pollen immigration into both populations.

The patterns of pollen dispersal were also different among the studied fragments. In Ibicatu, the mean (352 m) and the median (341 m) dispersal distance were similar, while in MGI and MGII, the median dispersal distance (both at 37 m) was substantially lower than the mean dispersal distance (63 and 130 m, respectively), clearly indicating the tendency of mating among near neighbour individuals in these fragments (Figure 5.3). Furthermore, there was a significant association between the number of seeds fertilized by pollen donors and the distance between the paternal and maternal trees in Ibicatu and MGI. The Kolmogorov-Smirnov test, which compares the frequency curve of effective pollen dispersal with the frequency curve measured among all reproductive trees, suggested non-random mating in the three forest fragments (Figure 5.3). Bees may show fidelity to very small fragments even though the location of the stand may be several kilometers from the hive (CHAPMAN et al., 2003; MONZON et al., 2004). Thus, isolation of forest fragments will be mediated by the foraging range and behaviour of their pollinators (GHAZOUL 2005; MITCHELL et al., 2009; WANG et al., 2010), which tend to forage in small areas or within a few trees of a population. This type of pollination behavior by bumblebees was observed by Goverde et al. (2002) for *Betonica officinalis* which visited a high number of inflorescences but tended to remain longer in smaller stands. Gérard et al. (2006) note that a non-random spatial assortment of mating events among conspecifics is mediated by pollinators. Thus, pollen donors do not distribute their pollen evenly among seed trees, but rather among a non-random subset of available seed trees (FORTUNA et al., 2008).

Ghazoul (2005) conducting a review of the pollen and seed dispersal among widely dispersed plants, found that for 123 species belonging to 59 families, approximately 68% of the studied species were pollinated by some kind of bees. Dick et al. (2008) showed that of 40 tropical tree species for which pollen flow was studied, 25% were pollinated by bees. Thus, in

tropical forests worldwide, bees are the predominant pollinator that transports genetic material in forest fragments (DICK et al., 2008). Analyzing the distance of pollen dispersal in 13 species pollinated by bees, pollen dispersal in continuous forests has reportedly reached 3,500 m, while in 10 species occurring in fragmented populations, pollen dispersal reached 16 km (Table 5.4). In studies of seven species pollinated by insects, pollen dispersal in continuous forests was recorded at a distance of 23.6 km, while for four species occurring in fragmented populations, pollen dispersal ranged from 944 to 3,133 m (Table 5.4). However, the distance of *Ceratosolen arabicus* Mayr pollen dispersed by wind-borne small insects was detected up to 165 km and the mean distance for confirmed successful pollination was 88.6 km (AHMED et al., 2009).

Table 5.4 - A summary of the results of pollen flow (realized and effective), average, median and maximum distance for tree species pollinated by bees and insects

(continue)

Species	Pollinator	Isolation distance	Realized Pollen flow (%)	Effective Pollen flow (%)	Average (m)	Median (m)	Maximum (m)	Authors
Carapa guianensis	Bees	Continuous	29.8	--	195	181	430	Martins et al. 2012
Carapa guianensis	Bees	Continuous	18.3	--	175	169	397	Martins et al. 2012
Copaifera langsdorffi	Bees	Continuous	--	64	74	39	297	Tarazi et al. 2013
Dicorynia guianensis	Bees	Continuous		29	142	--	350	Latouche-Hallé et al. 2004
Dinizia excelsa - Twogener	Bees	Continuous	--	--	1509	--	--	Dick et al. 2003
Dipteryx panamensis	Bees	Continuous	--	--	240	--	--	Hanson et al. 2008
Dipterocarpus tempehes	Bees	Continuous	--	44	222	--	--	Kenta et al. 2004
Dipterocarpus tempehes	Bees	Continuous	--	33	192	--	--	Kenta et al. 2004
Guaiacum santum	Bees	Continuous	--	19.2	1276	--	3500	Fuchs and Hamrick 2011
Kandelia candel	Bees	Continuous	--	--	15.2	--	--	Geng et al. 2008
Myracrodruon urundeuva	Bees	Continuous	2.7	--	138	64	863	Gaino et al. 2010
Myracrodruon urundeuva	Bees	Continuous	--	1.6	252	192	890	Gaino et al. 2010
Neabalanocarpus heimii	Bees	Continuous	--	21	187	--	524	Konuma et al. 2000
Neabalanocarpus heimii	Bees	Continuous	--	34-69	195	--	--	Konuma et al. 2000
Prunus avium	Bees	Continuous	--	39	-	100	694	Cottrell et al. 2009

Table 5.4 - A summary of the results of pollen flow (realized and effective), average, median and maximum distance for tree species pollinated by bees and insects

(continuation)

Species	Pollinator	Isolation distance	Realized Pollen flow (%)	Effective Pollen flow (%)	Average (m)	Median (m)	Maximum (m)	Authors
Swietenia humilis	Bees	Continuous	--	78	347	141	2547	Rosas et al. 2011
Swietenia macrophylla	Bees	Continuous	0-40	--	75-255	78-189	576	Sebbenn et al. 2012
Variation			**2.7-40**	**1.6-78**	**15.2-1509**		**297-3500**	
Albizia lebbeck	Bees	Fragmented	--	44-100	--	--	--	Dunphy and Hamrick 2005
Copaifera langsdorffi	Bees	Fragmented	4.7	--	94	86	229	Sebbenn et al. 2011
Copaifera langsdorffi	Bees	Isolted tree	--	8	63	53	183-1420	Manoel et al. 2012
Copaifera langsdorffi	Bees	Fragmented	--	75	--	--	1420	Manoel et al. 2012
Dinizia excelsa - Twogener	Bees	Disturbed	--	--	212	--	--	Dick et al. 2003
Dipteryx panamensis	Bees	Fragment	--	--	343	--	1000	Hanson et al. 2008
Dipteryx panamensis	Bees	Pasture	--	--	557	--	2300	Hanson et al. 2008
Eucalyptus regnas	Bees	Seed orchard	--	37.5	--	40	-	Burczyk et al. 2002
Eurycorymbus cavaleriei	Bees	Fragmented	--	39.3	85	--	1107	Wang et al. 2010
Eurycorymbus cavaleriei	Bees	Fragmented	--	42.6	164	--	325	Wang et al. 2010
Eucalyptus loxophleba	Bees	Agroforestry	--	67	70.3	--	1940	Sampson and Byrne 2008

Table 5.4 - A summary of the results of pollen flow (realized and effective), average, median and maximum distance for tree species pollinated by bees and insects

(continuation)

Species	Pollinator	Isolation distance	Realized Pollen flow (%)	Effective Pollen flow (%)	Average (m)	Median (m)	Maximum (m)	Authors
Eucalyptus loxophleba	Bees	Agroforestry	--	42.5	91.1	--	1940	Sampson and Byrne 2008
Prunus mahaleb	Bees	Fragmented	--	9.5	--	62.9	548	García et al. 2007
Sorbus domestica	Bees	Fragment	--	9.7	1200		16000	Kamm et al. 2009
Swietenia humilis	Bees	Fragment	--	36-100	1.6- >4500	--	> 4500	White et al. 2002
Swietenia humilis	Bees	Fragment	--	74	841	285	2014	Rosas et al. 2011
Variation				**8-100**	**1.6-4500**		**183-16000**	
Castanopsis sieboldii	Insect	Continuous	--	35.1	35.1	--	--	Nakanishi et al. 2012
Cordia alliodora	Insect	Continuous	--	--	--	--	280	Boshier et al. 1995
Dysoxylum malabaricum	Insect	Continuous HD	--	8	1205	106	23600	Ismail et al. 2012
Dysoxylum malabaricum	Insect	Continuous LD	--	--	600	56	--	Ismail et al. 2012
Kandelia candel	Insect	Continuous	--	--	15.2	--	--	Geng et al. 2008
Tectona grandis	Insect	Continuous	--	0	--	--	414	Prabha et al. 2011
Theobroma cacao	Insect	Continuous	--	61.3	28	28	67	Silva et al. 2011

Table 5.4 - A summary of the results of pollen flow (realized and effective), average, median and maximum distance for tree species pollinated by bees and insects

(conclusion)

Species	Pollinator	Isolation distance	Realized Pollen flow (%)	Effective Pollen flow (%)	Average (m)	Median (m)	Maximum (m)	Authors
Shorea acuminata	Insect	Continuous	--	53.8	100	--	--	Naito et al. 2008
Variation				**0-61.3**	**15.2-1205**		**67-23600**	
Entandrophragma cylindrum	Insect	Unlogged	--	70	338	--	1027	Lourmas et al. 2007
Entandrophragma cylindrum	Insect	Midle logged	--	74	266	--	944	Lourmas et al. 2007
Entandrophragma cylindrum	Insect	Severy logged	--	66	385	--	2095	Lourmas et al. 2007
Dysoxylum malabaricum	Insect	Isolated trees	--	--	6525	5316	--	Ismail et al. 2012
Ficus sycomorus	Insect	1-81.6 km	--	--	26-141	--	165000	Ahmeda et al. 2009
Gomortega keule	Insect	Large sites	--	57	709	--	2510	Lander et al. 2010
Gomortega keule	Insect	Small sites	--	--	709	--	2833	Lander et al. 2010
Gomortega keule	Insect	Single tree	--	--	709	--	3133	Lander et al. 2010
Variation				**57-74**	**26-6525**		**944-165000**	

Male fertility

A very interesting aspect of reproductive biology is understanding male fertility or if the largest trees are more efficient pollinators than smaller trees. Male mating success has been shown to increase with proximity, flower intensity and tree size (BURCZY et al., 1996; KLEIN; ODDOU-MURATORIO, 2008; MORI et al., 2009). In this study, a linear regression analysis was used to verify whether fertility of pollen donors or capacity to generate more offspring was associated with dbh. We found significant association between dbh and the number of fathered seeds in the Ibicatu and MGII populations and between the number of seeds fertilized by pollen donors and the distance between the paternal and maternal trees in Ibicatu and MGI. These results indicate that the number of fathered seeds by pollen donors is strongly associated with both their dbh and the distance between paternal and maternal trees. The effect of dbh size on fertility can be related to the occurrence of large crowns in the forest and easy access for bees and other insects (PLOWRIGHT; GALEN, 1985). The tall crowns of flowering trees are conspicuous and pollination vectors may use them as landmarks to help orient their foraging over long distances (PLOWRIGHT; GALEN, 1985). Positive correlation between dbh size and male fertility has also been found in *Gleditsia triacanthos* (SCHNABEL; HAMRICK, 1995), *Pinus attenuate* (BURCZY et al., 1996), *Quercus macrocarpa* (DOW; ASHLEY, 1998), and *Prunus ssiori* (MORI et al., 2009). However, Oddou-Muratorio et al. (2005) concluded that for the bee pollinated, temperate tree species *Sorbus torminales*, the spatial position of a pollen donor in relation to seed tree is just as important for mating success and it is an ecological determinant of male fecundity.

Effective pollination neighbour area

Effective pollination neighbour area (A_{ep}) was variable among seed trees (ranging from 0.02 to 51.3) and among populations (average of 37, 1.4 and 11 ha in Ibicatu, MGI and MGII, respectively). This estimate assumes that A_{ep} represents a circular area around a seed tree where we can find 63% of the pollen donors that mated with the seed tree (LEVIN, 1988); in our study, this produces an average radius of effective neighbour area (r_{ep}) of 345, 68 and 191 m, in Ibicatu, MGI and MGII, respectively. Thus, the seed trees received pollen from a large area and long

distances. For example, our estimate of A_{ep} in *C. legalis* is much higher than the results detected for another bee-pollinated and Neotropical tree species (*Copaifera langdsdorffi*), in which A_{ep} ranged among seed trees from 0.05 to 4.15 ha, with an average of 0.68 ha (MANOEL et al., 2012).

5.3 Conclusions

Our results show that while the populations studied herein present high levels of genetic diversity only the Ibicatu population has a substantial effective population size, due to the greater number of individuals than the other populations. Although the studied populations were spatially isolated, in the single reproductive event evaluated we found a substantial level of pollen flow in the Ibicatu population, which is essential in promoting genetic diversity, increasing the effective population size, and reducing inbreeding and SGS. However, in the smaller stands, MGI and MGII, the detected gene flow was very low. We also found the tendency that small stands have higher rates of selfing. Thus, seeds in smaller stands may have higher levels of inbreeding than seeds collected from larger populations. Our results can be used to inform seed collection strategies for genetic breeding, conservation and environmental reforestation. For these purposes, it is very important to collect seeds from: i) non-related seed trees; ii) seed trees that do not mate with each other; iii) seed trees that do not receive an overlapping pollen poll. Our results of SGS analysis in the Ibicatu population indicate that to adhere to the first requirement (i), seed trees must be located at a distance of at least 150 m in order to avoid collecting seeds from related seed trees. However, to adhere to the other two requirements (ii and iii), our results of average pollen dispersal distance (352 m) and radius of effective pollinator neighbour area (r_{ep} = 345 m) suggest that seed trees must be located at a distance of at least 352 m. Obliviously, even in the largest fragment, Ibicatu, these requirements will result in the collection of seeds from a small number of seed trees. Thus, we must collect seeds from other populations to reach a minimum number of seed trees for genetic breeding, conservation and environmental reforestation (45 seed trees, SEBBENN, 2006).

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