UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
FACULDADE DE ENGENHARIA
CAMPUS DE ILHA SOLTEIRA

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ESTUDO HIERÁRQUICO DO SISTEMA DE REPRODUÇÃO ENTRE E DENTRO DE FRUTOS, FLUXO DE PÓLEN E ESTRUTURA GENÉTICA ESPACIAL EM UM FRAGMENTO E EM ÁRVORES ISOLADAS NA PASTAGEM DE *Hymenaea stigonocarpa* Mart. ex Hayne NA REGIÃO DE CERRADO

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

Prof. Dr. Alexandre Magno Sebbenn
Orientador

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TÍTULO DA TESE: Estudo hierárquico do sistema de reprodução entre e dentro de frutos, fluxo de pólen e estrutura genética espacial em um fragmento e em árvores isoladas na pastagem de *Hymenaea stigonacarpa* Mart. ex Hayne na região de cerrado.

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pelo exemplo.

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por estar sempre ao meu lado

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Seja humilde, para que ninguém te ofenda.
E continue sendo o que tu és,
para que ninguém te esqueça."

Autor desconhecido.

RESUMO

Os objetivos deste trabalho foram verificar o sistema de reprodução entre e dentro de frutos, a distância e padrões de fluxo de pólen, os níveis de endogamia e diversidade genética, a depressão por endogamia e a distribuição espacial de genótipos em duas populações de *Hymenaea stigonocarpa:* a primeira está presente em um fragmento e a segunda em árvores isoladas na pastagem. Para tanto, foram mapeadas e medidas todas as árvores adultas reprodutivas existentes nos dois locais. Foram coletadas sementes de 15 árvores matrizes no fragmento florestal e em 20 árvores matrizes isoladas na pastagem, sendo 30 sementes por árvore. Esta coleta propiciou a instalação de um teste de progênies na Fazenda de Ensino, Pesquisa e Extensão da FEIS/UNESP. Foram feitas a genotipagem de todas as árvores adultas de ambas as populações e de todas as progênies. Adicionalmente, dentro do fragmento foram também amostrados, mapeados e medida a altura de juvenis. As análises genéticas permitiram avaliar os efeitos da depressão por endogamia para altura e sobrevivência, e as análises dos genótipos foram feitas para seis locos microssatélites, já transferidos para a espécie. O estudo do sistema de reprodução foi baseado no modelo misto de reprodução e modelo de cruzamentos correlacionados. A análise de paternidade das sementes permitiu determinar a distância e o padrão de fluxo efetivo de pólen dentro das populações, bem como o fluxo gênico externo das áreas amostradas. A análise da distribuição espacial dos genótipos foi realizada para árvores adultas localizadas dentro dos fragmentos, utilizando-se estimativas do coeficiente de coancestria entre pares de indivíduos dentro de diferentes classes de distância, tornando-se possível estimar o tamanho efetivo de variância e, assim, estabelecer estratégias para a coleta de sementes. Os resultados permitiram entender o processo de reprodução que indica a presença marcante de progênies de irmãos-completos, formação da estrutura genética espacial, vizinhança genética reprodutiva e depressão por endogamia na geração descendente proveniente de ambas as populações de *H. stigonocarpa*. A coleta de sementes deve ser feita em árvores espaçadas de pelo menos 350 m de distância em 78 árvores com 30 sementes cada árvore para reter o tamanho efetivo de referência de 150 nas amostras para garantir o estabelecimento destas gerações futuras em longo prazo na conservação “ex situ” e/ou reflorestamento em áreas degradadas.

Palavras-chave: Análise de paternidade. Fluxo gênico. Jatobá. Marcadores microssatélites.

ABSTRACT

The aim of this work were to study the mating system within and among fruits, distance and pollen flow patterns, inbreeding levels and genetic diversity, inbreeding depression and the spatial distribution of genotypes in two populations of *Hymenaea stigonocarpa*: first is present in a fragment and the second in isolated trees in the pasture. Therefore, were mapped and measures all existing reproductive adult trees in both sites. It was collected seed of 15 seed trees in the forest fragment and in 20 isolated trees in the pasture, with 30 seeds per tree. This collection allowed the installation of a progeny test in Farm of Teaching, Research and Extension from FEIS/UNESP. It was made genotyping of all adult trees of both populations and all progenies. Additionally, within the fragment were also sampled, mapped and measured the height of juveniles. Genetic analysis allowed to evaluate the effects of inbreeding depression for height and survival, and analysis of genotypes were made for six microsatellite loci, already transferred for the species. The mating system study was based on the mixed mating model and correlated mating model. The paternity analysis of the seeds allowed to determine the distance and the pattern of effective flow of pollen within the fragments, as well as the outside gene flow of the sampled areas. The spatial distribution analysis of genotypes was done for adult trees located within of the fragments, using estimates coancestry coefficient between pairs of individuals within different distances classes, making it possible to estimate the variance effective size and thereby establish strategies for seed collection. The results allowed understanding the process of mating that indicates the strong presence of progeny full-sibs, formation of spatial genetic structure, reproductive genetic neighborhood and inbreeding depression in the descending generation from both populations of *H. stigonocarpa*. Seed collection should be done in trees spaced of at least 350 m away in 78 trees with 30 seeds each tree to retain the effective size of the reference 150 in the samples to ensure the establishment of these future generations in the long-term conservation "ex situ "and / or reforestation in degraded areas.

Keywords: Effective size. Gene flow. Jatoba. Paternity analysis. Population genetics.

SUMÁRIO

1 INTRODUCTION

Landscape genetics is an area of research involving methods of population genetics and ecological genetics. In the context of forest fragmentation, the focus of the genetic landscape is to access the degree to which the landscape facilitates the movement of organisms (landscape connectivity) by gene flow patterns (HOLDEREGGER; WAGNER, 2008), which in plants can occur both the seeds and by pollen. This new area of research uses genetic markers to determine the current rate of immigration and emigration of genes among populations and individuals.

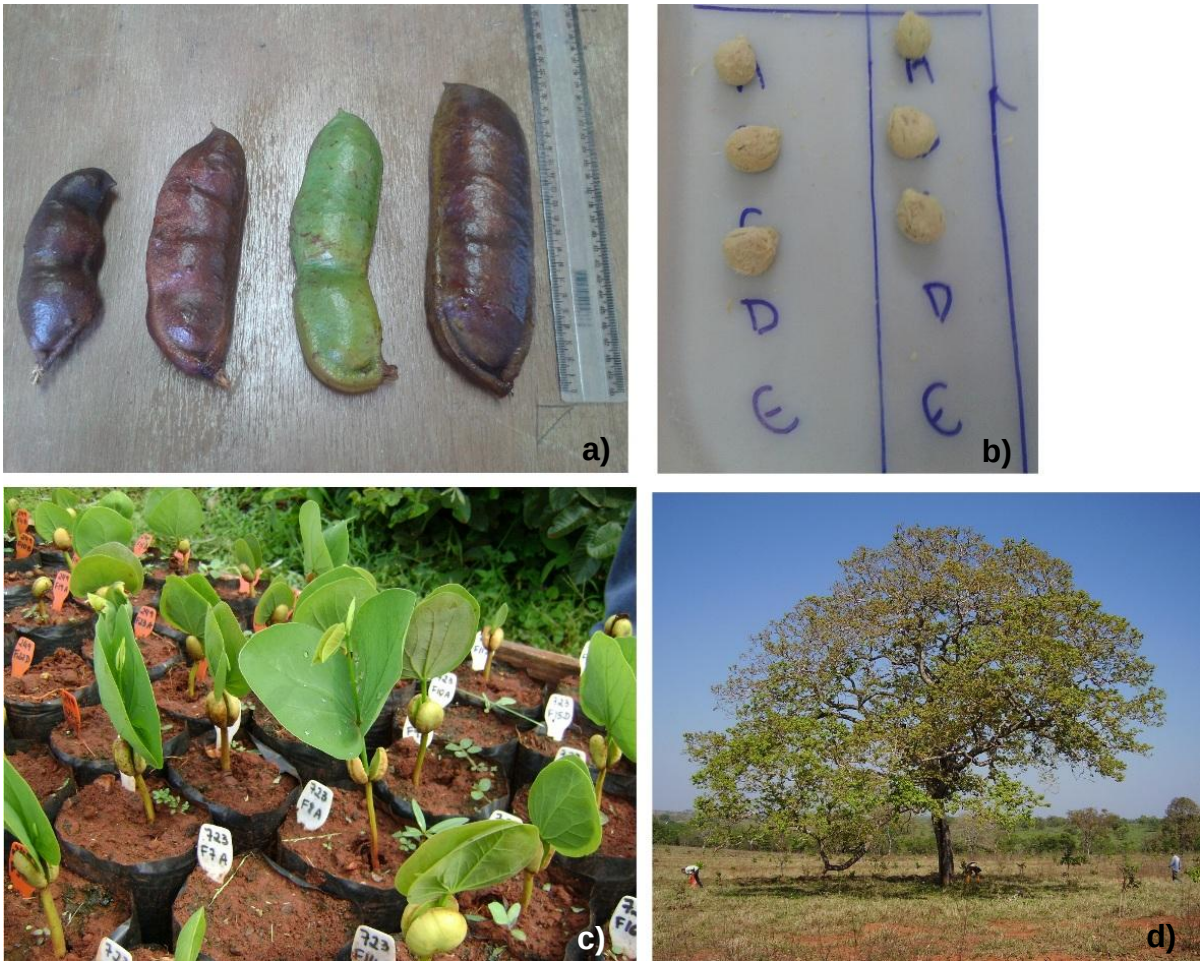
Understand the patterns and the distance of pollen flow in neotropical tree is fundamental to the development of conservation strategies *in situ* and *ex situ*. Rainforests around the world have been extensively fragmented, resulting in mosaics of small fragments and isolated individuals, interspersed with pastures, agriculture, highways and cities. This is especially true for the savannah from Brazil. Studies indicate that over 50% of the Savannah habitat has been extinguished (MACHADO et al., 2004) and the loss continues to give after day, especially now, with the need for large areas for sugarcane cultivation, soybean and livestock. It is obvious that the effects of cutting off are very clear; many populations of tree species are lost (SEBBENN et al., 2008) and some individuals and small forest fragments are isolated in the landscape. Thus, urgent strategies to conserve *in situ* and *ex situ* the remnant populations of tree species are needed, especially for endemic species. Both *in situ* conservation and *ex situ*, information about the movement and pollen dispersal patterns are important to know where populations and individuals spatially isolated in the landscape are genetically isolated.

The absence of pollen flow implies the need to link these forest remnants and individuals for runners of gene flow. In contrast, the intense movement of pollen between these individuals and the remaining populations are highly favorable for *in situ* conservation and seed collection in *ex situ* conservation and environmental reforestation due to the likely high evolutionary potential produced by intense recombination of genes between different individuals.

Hymenaea stigonocarpa, known as jatobá (Figure 1) is a Neotropical species monoecious, deciduous, pollinated by bats. Adult trees can reach 29 m high and 50

cm in diameter at breast height (CARVALHO, 2006). Its flowers are pollinated by at least four different species of bats, including *Glossophaga soricina* and others such as *Platyrhinus lineatus* and *Carolilia perspicillatta* (GIBBS et al., 1999). Whereas bats have the potential to disperse pollen long distances (DUNPHY et al., 2004, DICK et al., 2008, LACERDA et al., 2008), the species is likely to have pollen dispersal over long distances. *H. stigonocarpa* studies involving controlled outcrossings indicate that the species is predominantly outcrossing. Gibbs et al. (1999) observed self-compatibility, however, the species appears to have post-zygotic selection in self-fertilized flowers. Estimates of outcrossing rate based on genetic markers has confirmed the results of controlled outcrosses, showing that the species has a mixed mating system (selfing and outcrossing) (MORAES et al., 2007). Fruits and seeds are dispersed by zoochory (probably dispersed by agrotis) and birds (WEISER and GODOY, 2001; RAMOS et al., 2009). The density of the species among populations is highly variable, ranging from 2 to 43 individuals per 12 hectare (MARIMON et al, 1998; SILVA et al., 2002).

Figure 1 - Details of the species *Hymenaea stigonocarpa*: a) fruits; b) seeds classified of hierarchical mode; c) progeny*;* d) seed-tree isolated on pasture, region from Inocência, MS.



Source: Prepared by author.

While the mating system of *H. stigonocarpa* is relatively well understood (MORAES et al., 2007), information about distance and pollen dispersal patterns are needed to better understand and conserve the species. Estimates of distance and pollen dispersal of abundance are important factors in predicting the dynamics of plant populations and are the basis for designating conservation strategies in highly fragmented environments (AUSTERLITZ; SMOUSE, 2001). In the distance and plenty of pollen dispersal is crucial and significant factors in reproductive neighborhood of pollination in plant populations (AUSTERLITZ; SMOUSE, 2002) which is especially important to calculate the number of seed trees for seed collection in *ex situ* conservation and environmental reforestation. The spatial patterns of tree populations and the connection of individuals via pollen are important aspects of the

mating system (DYER; SORK, 2001) and consequently are determinants of kinship and the effective size of variance of open-pollinated progenies. Genetic processes such as forest fragmentation can affect the mating system and pollen flow among and within populations (ALDRICH; HAMRICK, 1998; DICK et al. 2003; BITTENCOURT; SEBBENN, 2007; DICK et al., 2008.). The spatial isolation pollen donors can result in significant restructuring of the set pollen by increasing the contribution of pollen donor sites (DYER; SORK, 2001).

When if considering populations of low population density, which is typical of many tropical tree species, the isolation and distance between individuals can lead to heterogeneity in the set pollen (MURAWSKI; HAMRICK, 1991). As a result, open-pollinated progeny of such populations suffer an increase in kinship and decrease in the effective size. In contrast, studies have found the opposite, that forest fragmentation can increase the pollen movement and reduce heterogeneity in set pollen received by different seed trees (ALDRICH; HAMRICK, 1998; WHITE et al, 2002; DICK et al., 2003).

Parentage analysis is a direct way to quantify the flow and dispersion distance of pollen and seeds in plant populations (BURCZYK et al., 2004; SMOUSE and SORK, 2004). One advantage of this method in the gene flow study is that it assumes dispersion models and so the observed patterns may represent the true standards, logically if the polymorphism loci are high enough to discriminate between candidates for father.

The parentage analysis has been used intensively in pollen flow study in tree species (DOW and ASHLEY, 1996, WHITE et al, 2002; BITTENCOURT and SEBBENN, 2007). In Brazil, *H. stigonocarpa* occurs between 3°30` S and 22°40´S and is restricted to the savannah habitat in central and south-east (CARVALHO, 2006). Currently, *H. stigonocarpa* is only found in small forest fragments and as isolated trees in the landscape, as a result of intensive fragmentation of the savannah from São Paulo and Mato Grosso do Sul states. Thus, plans for their conservation should involve *ex situ* strategies. Seeds have been collected from the remaining trees in small forest fragments and isolated trees in pastures to establish germplasm banks. However, the mating system of *H. stigonocarpa* has been examined only recently and there is no information about distance and pollen dispersal patterns among individual trees and groups of trees occurring in pastures.

This study will to look for information on the genetics, demography and ecology of the species to better delineate strategies for their conservation.

Another point that no detailed information on *H. stigonocarpa* as well as in almost all other native tree species in Brazil is the inbreeding depression. The inbreeding depression refers to the decline in the values of a quantitative or qualitative character as a direct consequence of inbreeding (WRIGHT, 1977). The inbreeding is increased homozygosity in individuals originating from selfing and outcrossing between related. In natural populations of tree species, inbreeding can be generated by the behavior of pollinators, visiting mainly flowers on the same tree (selfing) due to the internal structure of populations into groups of related individuals, located spatially close, or by reducing the size populations. When there are outcrossing between related and selfing the progeny tend to be less vigorous and less fertile than cross progenies (ALLARD, 1971). Outcrossing populations historically large, who suddenly decline for a few individuals, also reduce variability and fertility (FALCONER; MACKAY 1997). This inbreeding depression is completely removed when inbred individuals are outcrossed and in some circumstances the performance is increased by the hybrid vigor or heterosis (MATHESON et al., 1995).

With the lack of information in the literature judged opportune to study the hierarchical mating system within and among fruits, flow, patterns and distance of pollen dispersal, the spatial distribution of genotypes and inbreeding depression in seeds of isolated tree in the pasture and occurring in fragments for *Hymenaea stigonocarpa* species from Inocência region, Mato Grosso do Sul, using six microsatellite loci. Thus the specific aim of the study are: *i*) to determine the outcrossing rates and outcrossing correlated among and within fruit *H. stigonocarpa* occurring in isolated tree on pasture and within the fragment; *ii*) to determine the pollen flow rate in fragment using paternity analysis; *iii*) to estimate parameters of genetic diversity, inbreeding, effective size and pollination neighbor area size; *iv*) to determine the distance and pollen dispersal patterns; *v*) to determine by paternity analysis, progeny generated by selfing and outcrossings and compare the coancestry coefficient and the growth rate in height between progeny of selfing and outcrossing (inbreeding depression); *vi*) to determine sample sizes in terms of number of seed trees for seed collection, in the fragment and isolated trees in pasture for *ex situ* conservation purposes and collecting seeds for environmental reforestation.

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Mendelian inheritance, genetic linkage, and genotypic disequilibrium at six microsatellite loci in *Hymenaea stigonocarpa* Mart. ex Hayne (Fabaceae-Caesalpinioideae)

ABSTRACT

Hymenaea stigonocarpa, locally known as Jatobá, is a deciduous and monoecious Neotropical tree species pollinated by bats and widely used in medicine. Mendelian inheritance, genetic linkage and genotypic disequilibrium were studied in six microsatellite loci in open-pollinated families of the species. Adult and juveniles were sampled and genotyped in two populations. Additionally, open-pollinated seeds from 35 seed trees were collected and genotyped, 30 seeds per tree. Significant deviations from the expected 1:1 Mendelian segregation were detected in only 12 cases (10.6%) out of 113 tests. Genetic linkage between pairwise loci was detected in 15.3% of the tests, but no genotypic disequilibrium was detected between pairwise loci for adult trees and juveniles. Therefore, these set of loci can be used for genetic diversity and structure, mating system and gene flow in *H. stigonocarpa* populations.

Keywords*:* Forest fragment. Jatobá. Microsatellite. Neotropical. Open-pollinated seeds.

2 INTRODUCTION

Hymenaea stigonocarpa Mart ex Hayne belong the Fabaceae-Caesalpinioideae family and has a wide dispersion in the Brazilian savannah. The species is deciduous, monoecious and pollinated by bats. Studies involving controlled mating of *H. stigonocarpa* indicate that the species is self-compatible (GIBBS et al., 1999). Estimates of the outcrossing rate based on genetic markers have confirmed the controlled mating results, showing that the species has a mixed mating system (MORAES; SEBBENN, 2011). The economic importance of the species is associated with the use of the wood for naval and civil construction (LORENZI 1992; BOTELHO et al., 2000). However, the specie has been intensively explored in the past by the naval industry due to the excellent quality of its wood in terms of durability and resistance to rotting. Due to that and also to the widespread destruction of its habitat in the savannah, the species is now found only in small remnant populations or as isolated trees in fields and pastures.

Studies about the genetic consequences of the reduction of natural populations of this species are possible due to the development and characterization of a set of polymorphic microsatellite loci for the species (CIAMPI et al., 2008). However, no study on Mendelian inheritance, genetic linkage and genotypic disequilibrium has been conducted for the developed microsatellite loci. That is important to confirm whether the marker loci are genetic loci. Thus, it was considered appropriate to estimate the Mendelian inheritance, linkage and genotypic disequilibrium in six microsatellite loci of *H. stigonocarpa*, for the robust application in population genetic studies such as genetic diversity and structure, mating system, gene flow and parentage analyses in this species.

2.1 MATERIAL AND METHODS

The study was conducted in an area of 23 x 32 km (736 km^2), using isolated trees and trees occurring in a forest fragment near the city of Inocência (20°07' S, 51°44' W, and 373 m above sea level), in the state of Mato Grosso do Sul, Brazil. The climate condition is tropical with dry winter and wet summer. The average rainfall

is 1,232.2 mm and average annual temperature is 24.5° C. The regional biome is characterized by savannah with high levels of anthropogenic disturbance.

The collection of open-pollinated seeds was made in two populations of *H. stigonocarpa.* The first is located in a pasture area where trees are isolated or in small clusters (POP pasture) where 359 adult trees were sampled and genotyped. The second is located in a forest fragment (POP forest) where 111 adult trees and 219 juveniles were sampled and genotyped. Additionally, open-pollinated seeds from 20 seed-trees (POP pasture) and 15 seed-trees (POP forest) were collected and genotyped, 30 seeds per tree in different fruits, totaling 35 seeds-trees sampled and 1,050 seeds.

The DNA extraction from leaves of seeds-trees and germinated seeds was carried out using the method of Doyle and Doyle (1987). DNA quantification of the samples was made using an electrophotometer. The amplification reactions were performed according to the method presented in Ciampi et al. (2008). The amplification DNA fragments (2-µL total reaction volume) were separated on a Fragment Analyzer™ Automated CE System (Advanced Analytical Technologies Inc. [AATI], Ames, IA, USA) using a dsDNA Reagent Kit, 35-500 bp (DNF-900, Advanced Analytical Technologies Inc.). Raw data were analyzed using PROSize™ (version 2.0) software (AATI). Initially, nine di-nucleotide microsatellite loci, developed by Ciampi et al. (2008) for *Hymenaea courbaril* L., were tested. Of these nine loci, six (HC14, HC17, HC33, HC35, HC40 and HC49) were successfully transferred to *H. stigonocarpa* and used here.

Mendelian inheritance of the microsatellite loci was determined according to Gillet and Hattermer (1989), which is based on comparisons of a heterozygous maternal genotype tree with the segregation of its alleles in an open-pollinated progeny. This method assumes that the loci have regular segregation and that their alleles follow classic Mendelian inheritance patterns, which are based on three main requirements: i) regular meiotic segregation during ovule production; ii) random ovule fertilization by a type of pollen; iii) no selection between the moment of fertilization and the genotyping of the seeds. The model also assumes that there is a co-dominant relationship among all the alleles. The method further requires that the following conditions be met: 1) all the progeny of a tree must possess a maternal allele; 2) in cases of heterozygous parent trees (e.g., A_iA_j, $i \neq j$): a) each individual

offspring 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 be equal to the sum of the homozygous progeny A_iA_i (n_{ii}) and A_jA_j (n_{jj}), $n_{ij} = n_{ii} + n_{jj}$; and c) the number of heterozygous progeny A_iA_k (n_{ik}) must equal the number of heterozygous progeny A_jA_k (n_{ik}), or $n_{ik} = n_{jk}$, where k ≠ i, j. Using this model and the open-pollinated progenies sampled from 35 seed-trees and 1,050 seeds, we proceeded to compare the segregation observed in each progeny of the heterozygous maternal tree for a given loci, with the expectation of a classic Mendelian 1:1 segregation, using a G-test (SOKAL and ROHLF ,1981):

$$G = 2\left[n_{ij} \ln\left(\frac{n_{ij}}{E(n)} \right) + (n_{ii} + n_{ij}) \ln\left(\frac{(n_{ii} + n_{jj})}{E(n)} \right) \right] \quad (1)$$

where ln is the natural logarithm and *E(n)* is the expected number of genotypes for the alleles A_iA_j (n_{ij}) and $A_iA_i + A_jA_j$ ($n_{ii} + n_{jj}$), based on $E(n) = 0.5(n_{ij} + n_{ii} + n_{jj})$. Additionally, Bonferroni´s correction for multiple comparisons (95%, α = 0.05) was used to avoid false positives.

In order to confirm the independence of allele segregation among different loci, a test of linkage was carried out between pairwise loci using genetic information from seed-trees 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 and ROHLF, 1981):

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

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, ln is the natural logarithm, and *E(n)* was calculated as:

$$E(n) = 0.25(n_{ik} + n_{il} + n_{jk} + n_{jl}) \quad (3)$$

Bonferroni´s correction for multiple comparisons (95%, α = 0.05) was also applied.

A genotypic disequilibrium test was conducted for the adult trees and juveniles, since genotypic disequilibrium is expected in juvenile arrays because all the descendants always receive a maternal allele. 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 in order to determine the disequilibrium between all pairwise loci. For the avoidance of false positives, we used Bonferroni´s correction at 95% probability ($\alpha = 0.05$).

2.2 RESULTS

After Bonferroni's correction, the results showed a significant deviation from the expected 1:1 Mendelian segregation pattern in only 12 cases (10.6%) of 113 tests (Table 1). After Bonferroni's correction, only 24 (15.3%) of 156 linkage tests performed (Table 2) were significant, suggesting linkage between some pairs of loci. However, in all cases in which significant linkage was observed, it occurred in different pairs of loci of different sampled progenies. Soon, these loci are not genetic linked. Genotypic disequilibrium was tested in samples of adult trees of the forest and pasture and juveniles of the forest. After Bonferroni's correction, the results showed no significant evidence of genotypic disequilibrium between pairwise loci. Thus, the loci are in linkage equilibrium (Table 3).

Table 1 - Mendelian inheritance tests for six microsatellite loci in *Hymenaea stigonocarpa*.

Seed-tree	Genotype	n	n_1	$n_{ij} : n_{ii} + n_{jj}$	G	Seed-tree	Genotype	n	n_1	$n_{ij} : n_{ii} + n_{jj}$	G
HC14						HC17					
24	126130	19	16	3:13	0.43	37	128132	30	19	7:12	1.33
37	130136	30	26	1:25	27.57*	240	110114	24	21	1:20	21.07*
240	128138	24	18	5:13	3.68	256	120130	24	16	3:13	6.74
358	126136	30	26	11:15	0.62	292	120130	20	15	2:13	9.01
610	128136	18	15	3:12	5.78	313	116120	28	20	3:17	10.82
702	126130	29	28	1:27	30.19*	339	128134	19	16	8:8	0.0
703	128132	29	22	4:18	9.64	342	120128	28	18	8:10	0.22
707	126130	30	29	4:25	16.93	358	126130	30	19	9:10	0.05
712	128132	30	23	1:22	23.66*	368	136140	24	16	7:9	0.25
714	126130	25	21	1:20	21.07*	700	116120	30	22	6:16	4.72
715	122130	30	27	2:25	23.17*	702	130136	29	22	5:17	6.92
724	128132	24	19	3:16	9.77	703	116120	29	20	4:16	7.71
						707	116120	30	22	7:15	2.98
						710	126136	29	19	7:12	1.33
						715	114124	30	23	10:13	0.39
						716	116124	30	23	12:11	0.04
						721	116122	30	28	8:20	5.31
						723	116122	25	20	10:10	0.0
						724	120128	24	24	3:21	15.19

Continuation

Seed-tree	Genotype	n	n_1	$n_{ij} : n_{ii} + n_{jj}$	G	Seed-tree	Genotype	n	n_1	$n_{ij} : n_{ii} + n_{jj}$	G
HC33						HC35					
15	102112	30	27	6:21	8.83	11	280290	27	27	3:24	18.59*
37	110116	30	27	11:16	0.93	15	270280	30	30	2:28	26.89*
368	98110	22	17	8:9	0.06	24	290296	19	18	3:15	8.73
703	112116	29	29	3:26	20.91*	34	280290	18	15	4:11	3.40
715	112116	30	30	15:15	0.0	37	284296	40	23	5:18	7.80
716	112116	30	30	6:24	11.57	240	286292	24	21	5:16	6.06
721	112116	30	29	8:21	6.04	249	290296	29	20	16:4	7.71
722	108112	30	30	5:25	14.56	256	290296	24	17	11:6	1.49
723	108112	25	25	7:18	5.01	272	280290	23	15	5:10	1.70
						313	274286	28	25	9:16	1.99
						334	280290	21	19	3:16	9.77
						339	270280	19	18	6:12	2.04
						342	280290	28	27	6:21	8.83
						358	280290	30	30	2:28	26.89*
						368	280290	22	21	3:18	11.89
						610	286290	18	15	3:12	5.78
						700	270280	30	23	6:17	5.48
						702	280290	29	23	7:16	3.62
						704	280290	30	26	6:20	7.95
						707	280290	30	22	7:15	2.98
						712	276290	30	22	10:12	0.18
						715	280290	30	27	16:11	0.93
						721	280288	30	29	4:25	16.93
						722	278288	30	22	2:20	17.09
						723	286296	25	15	2:13	9.01
						724	276286	26	23	2:21	18.30

Conclusion

Seed-tree	Genotype	n	n_1	$n_{ij} : n_{ii} + n_{jj}$	G	Seed-tree	Genotype	n	n_1	$n_{ij} : n_{ii} + n_{jj}$	G
HC40						HC49					
11	172180	27	24	14:10	0.67	15	106110	30	30	9:21	4.94
15	172178	30	29	19:10	2.84	24	104108	19	16	5:11	2.31
37	174180	30	29	8:21	6.04	37	104108	30	25	15:10	1.01
240	174180	24	18	6:12	2.04	240	104108	24	18	8:10	0.22
249	172178	29	26	15:11	0.62	272	106112	23	22	5:17	6.92
321	176180	19	15	1:14	13.45	292	102106	20	19	6:13	2.64
334	170180	21	21	8:13	1.20	299	100106	16	15	6:9	0.60
339	170178	19	19	8:11	0.48	313	100106	28	28	11:17	1.30
342	174180	28	21	13:8	1.20	321	98102	19	19	8:11	0.48
358	170176	30	23	6:17	5.48	334	98102	21	20	11:9	0.20
610	162172	18	15	9:6	0.60	339	98104	19	18	6:12	2.04
702	174180	29	20	5:15	5.23	342	98102	28	15	8:7	0.07
703	174180	29	20	7:13	1.83	358	102108	30	26	19:7	5.75
704	174180	30	27	2:25	23.17*	368	104110	22	20	15:5	5.23
707	172180	30	25	10:15	1.01	610	106112	18	17	11:6	1.49
710	172180	29	29	6:23	10.63	700	100106	30	20	13:7	1.83
712	174180	30	28	10:18	2.32	702	100106	27	22	11:11	0.00
714	176182	25	22	6:16	4.72	704	106112	30	29	27:2	25.65*
715	174182	30	30	18:12	1.21	707	106112	30	27	20:7	6.53
716	174180	30	30	13:17	0.53	710	106112	29	28	20:8	5.31
721	174180	30	29	19:10	2.84	712	104110	30	23	12:11	0.04
722	172180	30	23	10:13	0.39	715	102108	30	16	7:9	0.25
723	168178	25	23	9:14	1.10	716	104108	30	24	7:17	4.30
						721	106112	30	28	16:12	0.57

n and n_1 = sample size and sample size used for G test; G = maximum likelihood G statistics for the hypothesis of $n_{ij} = n_{ii} + n_{jj}$. * Significance after Bonferroni's correction for α= 0.05 (χ2= 20.13).

Source: Prepared by author.

Table 2 - Maximum likelihood G-test for the hypothesis of independent segregation between pairwise loci (1:1:1:1) of *Hymenaea stigonocarpa*.

Seed-tree	G	Seed-tree	G	Seed-tree	G	Seed-tree	G
HC14xHC17		HC14xHC35		HC14xHC49		HC17xH35	
37	37.45*	342	2.72	334	9.62	610	11.77
358	8.11	358	11.67	339	4.35	700	3.36
368	10.45	610	9.96	342	1.95	702	6.38
700	24.72*	702	5.26	358	9.38	707	0.92
702	7.00	707	0.07	610	13.07	715	13.94
703	4.19	712	9.78	702	4.03	721	25.57*
707	0.07	715	29.01*	707	0.90	723	13.70
714	6.87	724	3.72	715	22.75*	724	4.06
715	25.22*	HC14xHC40		HC17xHC33		HC17xHC40	
724	1.43	240	18.11	37	10.35	37	4.57
715	5.33	334	10.27	368	15.84	240	18.04
716	11.29	339	6.85	703	0.98	339	5.12
721	3.77	342	2.75	715	8.78	342	3.17
722	15.40	358	9.48	716	9.42	358	6.72
723	14.46	610	7.54	721	4.71	610	11.71
HC14xHC33		702	19.67*	723	8.71	702	22.13*
37	46.61*	703	4.45	HC17xH35		703	3.29
703	5.18	707	7.52	37	6.34	707	12.21
715	27.35*	712	20.01*	240	12.87	710	7.37
HC14xHC35		715	29.24*	256	22.31*	714	20.43*
37	33.79*	HC14xHC49		313	5.12	715	8.97
240	13.04	24	9.68	339	3.56	716	0.68
272	24.56*	37	44.00*	342	1.92	721	1.98
334	6.59	240	9.37	358	12.34	723	2.17
339	7.19	272	27.19*	368	14.71		

Seed-tree	G	Seed-tree	G	Seed-tree	G
HC17xHC49		HC33xHC40		HC35xHC40	
37	2.03	15	22.21*	342	4.19
240	26.52*	37	14.36	358	11.15
292	3.82	703	3.13	610	5.49
313	3.71	715	5.33	702	18.59
321	4.57	716	11.29	704	32.29*
339	1.13	721	3.77	707	9.33
342	2.54	722	15.40	712	9.23
358	6.37	723	14.46	715	11.46
368	9.80	HC33xHC49		721	25.08*
610	13.55	15	14.63	722	7.19
700	2.99	37	14.27	723	2.08
702	9.02	368	7.28	HC35xHC49	
707	1.40	715	5.76	15	10.82
710	9.04	716	9.09	24	14.73
715	8.89	721	10.16	37	7.16
716	2.88	723	11.98	HC40xHC49	
721	4.31	HC35xHC40		710	2.02
HC33xHC35		11	13.62	715	2.89
15	23.31*	15	12.08	716	3.31
37	13.51	37	5.09	721	4.16
715	5.95	240	4.92	723	0.80
721	15.41	249	0.55		
722	14.48	334	6.26		
723	20.22*	339	6.02		

*Significance after Bonferroni's correction for α = 0.05, 0.00037 (χ^2= 20.66). G = G-test for three degrees of freedom.

Source: Prepared by author.

Tabela 3 - Genotypic disequilibrium between pairwise microsatellite loci in juveniles and adult trees of *Hymenaea stigonocarpa*.

Pairwise loci	Forest: adults	Forest: juveniles	Pasture: adults
HC33xHC35	0.07533	0.32200	0.47600
HC33xHC40	0.11133	0.05533	0.31333
HC33xHC49	0.71867	0.05733	0.38800
HC33xHC14	0.19200	0.01600	0.43067
HC33xHC17	0.03200	0.05533	0.05200
HC35xHC40	0.02000	0.98533	0.68333
HC35xHC49	0.75133	0.04867	0.04133
HC35xHC14	0.33600	0.03800	0.42467
HC35xHC17	0.58000	0.06467	0.24667
HC40xHC49	0.83600	0.55533	0.31533
HC40xHC14	0.45333	0.70000	0.01533
HC40xHC17	0.45267	0.11200	0.05000
HC49xHC14	0.54000	0.80200	0.43867
HC49xHC17	0.75067	0.06667	0.06200
HC14xHC17	0.87400	0.48133	0.44667

The values represent the probability of genotypic linkage after 1500 permutations of alleles among individuals. Probability of Bonferroni's corrections: $P = 0.00067$ ($\alpha = 0.05$).

Source: Prepared by author.

2.3 DISCUSSION

Overall, the results show that all six loci segregated according to the Mendelian rules of 1:1. Few cases of deviations were detected in some progenies, but these will never occur with all progenies at the same locus, then these six loci segregate according to expected ratios. Studies with microsatellite loci in tree species have detected low segregation deviations. Carneiro et al. (2012) observed deviations from expected 1:1 segregation only in locus HC33 (same locus of this study) in *Hymenaea courbaril*, Tambarussi et al. (2013) observed deviations from expected segregation of 1:1 in 3.7% of tests in *Cariniana legalis.* Manoel et al. (2015) observed deviations from expected segregation of 1:1 in 29% of tests in *Genipa americana*.

The results show also that the loci are not linked and are in genotypic disequilibrium. The genetic linkage is caused by the absence of low recombination rate of loci that are close on the same chromosome, and that keep alleles together when inherited (HARTL; CLARK, 2010). However, in this study the majority of SSR loci pairs revealed absence of linkage. The loci HC14 stands out for being involved in all cases with 25.9% of significant tests. These linkages observed between pairs of loci for several progenies may be a true genetic linkage, or may originate from individual locus deviations from a 1:1 Mendelian segregation (MANOEL et al., 2015). We believe in the second hypothesis, because 11 of 14 significant linkage test evolving loci HC14 were detected in families with significant deviation of segregation. Another reason for some linkage is the sample sizes. Tarazi et al. (2010) studied *Copaifera langsdorffii* and concluded that with the reduced number of seedlings per progenies used and specifically with the high number of alleles per loci, deviation was expected. Those latter authors recommend if possible, a future analysis to adjust the seedlings sample size to the species mean number of alleles in order to get good frequency estimates. Similar results were reported by Carneiro et al. (2012), who analyzed 13 to 20 seeds per family collected from *Hymenaea courbaril* and Tambarussi et al. (2013), who studied two populations of *Cariniana legalis* with samples of 40 to 100 seeds.

Moraes et al. (2007) observed in *Hymenaea stigonocarpa* genotypic disequilibrium in adult between the HC33 and HC49 loci. This population is located

next to the one studied in this work, showing that the linkage disequilibrium can occur in different places. Now, in adults, no linkage disequilibrium that could have broken family structure may be explained by a natural thinning and indirect selection of the loci analyzed during plant development.

2.4 CONCLUSION

The present result of this study provides subsidy for future research of the genetic behavior of natural populations of *H. stigonocarpa.* The six microsatellite loci tested adhered to Mendelian inheritance assumptions and showed no linkage or genotypic disequilibrium, indicating that these loci can precisely estimate important population genetic parameters applied to genetic diversity and structure, mating system, and gene flow studies in *H.stigonocarpa.*

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Absence of differences in genetic diversity and mating system patterns between fragmented and spatial isolated trees in pasture of *Hymenaea stigonocarpa* Mart. ex Hayne in the Brazilian savannah

ABSTRACT

This study investigated the mating system within and among fruits, spatial genetic structure (SGS), and inbreeding in isolated trees in a pasture (PPA) and in an extensive forest fragment (PFF) of *Hymenaea stigonocarpa*, located in the Brazilian Savannah. Sampling was carried out on all existing adult trees and juveniles in the populations.We also sampled seeds from 20 seed trees in the PPA and from 15 seed trees in the PFF. From each seed tree, seeds were hierarchical sample among and within fruits. Both population present SGS for adults (PPA= 250 m; PFF= 350 m) and PFF for juveniles (250 m). The outcrossing rate was similar between populations, but significant lower than the unity (mean of 0.82). The mating among relative rate was high in the populations (> 40%). These results suggest that seeds present inbreeding from selfing and mating among relatives which was confirmed by the estimate of the fixation index (minimum of 42%). Correlated mating were high within and among fruits in both populations (> 70%), indicating that progeny are compound by high frequency of pairwise full-sibs. Due the selfing, inbreeding and correlated mating the variance effective size within progeny was very low (maximum of 1.93), indicating that collection of seeds for *ex situ* conservation, breeding and environmental reforestation must be realized in a great number of seed trees.

Keywords: Genetic conservation. Genetic diversity. Mixed mating system. Savannah.

3 INTRODUCTION

Habitat degradation and deforestation continues across the tropics. This is worrisome because these regions harbouring the vast majority of global biodiversity (KETTEL, 2014). This results in forest fragmentation of tree species and can results in many negative impacts on genetic diversity of trees species, as the loss of genetic diversity, restricted gene flow, increase intrapopulation spatial genetic structure (SGS) and inbreeding of the remaining populations (AGUILAR et al., 2008; ISMAIL et al., 2012; DEGEN; SEBBENN, 2014; FINGER et al., 2014). However, if the populations present enough adaptive genetic variation, the species can respond to these challenges. Thus, understanding how forest fragmentation undermines adaptive genetic variation is vital for tropical tree conservation (KETTEL, 2014).

In a more explicitly biological conservation context, habitat fragmentation and disturbance may change the SGS due to disruptions in ecological process that affect recruitment, such as competition with invasive plant species (EWERS; DIDHAM, 2006), and plant reproduction (AGUILAR et al., 2006). Reproductive success may decrease in fragmented populations because plants may receive fewer flower visitors due to the decline in the richness and abundance of pollinators, modifications in species composition and limitation in movement among patches (GOVERDE et al., 2002).

In this context, several studies on tropical tree species in forest fragments detected an adaptive mechanism for the survival of the species, which is the mixed mating system (FUCHS et al., 2003; RIBEIRO and LOVATO, 2004; LOBO et al., 2005; MORAES and SEBBENN, 2011; FERES et al., 2012; CAMPOS et al., 2013). This mating system consists in which the population outcrossing rate depart significantly from both zero and one (CRUDEN; LYON, 1989) : *i*) a genetically based selfing rate polymorphism can exist, as for instance in the relatively rare case where populations contain both self-compatible and self-incompatible individuals (STONE, 2002); *ii*) species can exhibit heteromorphic flower systems (SCHOEN; LLOYD ,1984; MASUDA et al., 2004), such as cleistogamous (purely selfing) and chasmogamous flowers (both outcrossing and selfing possible); *iii*) by far most common system, individual plants produce a single flower type, and fruits may contain selfed, outcrossed, or a mixture of progeny types (SCHOEN; BROWN,1991).

In this case, the proportions of selfed versus outcrossed progeny are determined by the timing and relative amount of self- and outcross-pollination and post-pollination processes (LLOYD; SCHOEN, 1992; KALISZ et al., 2004). Most strikingly, the mechanisms contributing to the estimated outcrossing rates are unknown for most species. Moreover, for only a small fraction of species surveyed has any quantitative information on the mode of self-fertilization. Although detailed studies of reproductive biology have been undertaken for many plant species, these are often not linked to outcrossing rate estimates, limiting their contribution to our understanding of mating system evolution. Progress will come from the compilation of outcrossing estimates with at least basic functional information on floral biology across a broader range of plant species. Moreover, these mechanisms suggest that, when known, must be taken into greater consideration in the valid application of mating system theory (GOODWILLIE et al., 2005).

Mating system studies have indicated a potential for detecting variation in the rates of selfing (or outcrossing) on a micro-scale level, where variation occurs among fruits within individuals, individuals within populations, among populations and from one flowering event to another (WARD et al., 2005). Thus, the information allow more detailed understanding of adaptive and evolutionary biology of the species, providing support for a more accurate planning strategies for the *in situ* and *ex situ* conservation of tropical species, most of which are threatened endangered.

In favor of the current situation, we consider useful to study the Neotropical tree species, *Hymenaea stigonocarpa*. The species is a deciduous and monoecious tree species, belonging to Fabaceae-Caesalpinioideae family, native to the savannah. It can reach up to 29 m height and 50 cm of diameter at breast height (dbh). It occurs naturally in low chemical fertility of soils and well drained land, is considered heliophytic and no tolerate low temperatures (CARVALHO, 2006). The flowers are pollinated by at least four different species of bats, including *Glossophaga soricina* and others such as *Platyrhinus lineatus* and *Carolilia perspicillatta* (GIBBS et al., 1999). The economic importance of the species is associated with the use of wood for naval and civil construction; its bark that provides a yellow dye used in various segments; and their fruits are edible. It is also important for fauna, serving as food for parakeets, parrots, howler monkeys, rodents, small wolves and insects (LORENZI, 1992; BOTELHO et al., 2000), their dispersal likely.

Knowledge of the mating system of the species was reported by Gibbs et al. (1999), based on hand mating classified the species as prevalent outcrossed and self-compatible; However, the species appears to have post-zygotic selection in self-pollinated flowers. Estimates of outcrossing rate based on genetic markers have confirmed the controlled mating results, showing that the species has a mixed mating system (MORAES; SEBBENN, 2011).

Due the importance of the species, not only in economic question but also the vast production of fruits that provide food for fauna (MORAES; SEBBENN, 2011), its play an important role in the ecological aspects and is indicated for recovery of degraded areas of the Savannah (DURIGAN, 2003). The conservation of the species is of great importance to the Savannah biome. Thus, the aim of this study was to investigate the genetic diversity, the hierarchical mating system within and between fruits, the spatial distribution of genotypes and to determine sample sizes in terms of number of seed trees for seed collection in fragment and in isolated trees in pastures and forest fragments for the purpose of *ex situ* conservation and environmental reforestation, using microsatellite loci. We explore also the outcrossing rate variation among and within fruits. We compared mating system in two populations with different life histories: a large forest fragment and sparse trees located in a pasture. We tested the following hypotheses: (i) the selfing rate and the paternity correlation are higher in a sparse trees in pasture than trees occurring in a forest fragment; (ii) mating among relatives is lower in sparse trees in pasture than in the forest fragment due to SGS in the forest fragment; (iii) the paternity correlation is lower among than within fruits and; (iv) the levels of variance effective size are lower and inbreeding is higher in open-pollinated seeds collected in sparse trees than seed trees occurring in the forest fragment.

3.1 MATERIAL AND METHODS

Study site

The landscape of the study site consists of large extended pastures, sugarcane and eucalyptus, plantation interspersed with small forest fragments and a few isolated trees of *H. stigonocarpa* between fragments. Its location is close to highway MS 444, in the municipality of Inocência-Mato Grosso do Sul state, Brazil

(20°07´S, 51°44´W, and 373 m above sea level). The climate in this region is tropical with a dry winter and humid summer. The average rainfall is 1,232.2 mm and the annual average temperature is 24.5° C. The regional biome is characterized by savannah with high levels of anthropogenic disturbance. Much of the savannah was cutting down in this area between 1970 and 1980 for livestock. Currently, this area is also used for planting sugarcane (after 2002) and eucalyptus.The study was carried out in two populations of *H. stigonocarpa.* The first population (PPA) are grouped or isolated trees in pasture (*Brachiaria* sp) that corresponds to an area of 2.82 km^2 (2.66 x 1.06 km), with estimated density of 0.101 trees/ha. The distance among the trees ranged from 63 to 2,480 m (mean: 1,005 m). The second population (PFF) is located within a large forest fragment with an area of 736 km^2 (23 x 32 km), the estimated density is 0.0094 trees/ha. The distance between the trees, ranged from 63 to 10,289 m (mean: 2,124 m). The distance between populations is approximately 5 km and the vegetation in this area consists of pasture, eucalyptus plantations and some isolated trees *H. stigonocarpa.* All trees in the PPA and PFF are remaining from pre-fragmentation of the area, which occurred about 30 years ago.

Sampling

All adult trees were mapped, sampled and genotyped in the two populations. In PPA were sampled 359 adult trees and PFF were sampled 111 adult trees and 219 juveniles. The juveniles were classified as non-reproductive plants with about 1 m in height. Additionally, we collected and genotyped open-pollinated seeds from 20 seed trees in the PPA and 15 seed trees in PFF, with 30 seeds per tree in different fruits. Each seed was kept record of the seed trees and fruit origin for hierarchical analysis of mating system within and among fruits.

Microsatellite genotyping

The DNA extraction from leaves of adult trees, juveniles and germinated seeds (progeny) was carried out according to the cetyl trimethylammonium bromide method (DOYLE and DOYLE, 1987). DNA quantification of the samples was conducted using an electrophotometer. The amplification reactions and procedures

of microsatellite analysis followed the methods proposed by Ciampi et al. (2008) and Moraes and Sebbenn (2011). Amplification of the DNA fragment (2µL 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 (DNF-900, Advanced Analytical Technologies Inc.). Raw data were analyzed using the PROSize™ (version 2.0) software (AATI). Initially, nine di-nucleotide microsatellite loci, developed by Ciampi et al. (2008) for *Hymenaea courbaril* L., were tested. Of these nine loci, six were successfully transferred to *H. stigonocarpa* and used here.

Genetic diversity

The intrapopulation genetic diversity has been characterized by allelic richness (R), calculated by rarefaction, observed heterozygosity (H_o) and expected heterozygosity under Hardy–Weinberg equilibrium (H_e). The presence of inbreeding was investigated by the fixation index (F) and their statistical significance was estimated using permutation of alleles among individuals. The genetic diversity indexes, the fixation index and permutation were calculated using the FSTAT program (GOUDET, 1995).

Spatial genetic structure (SGS)

This analysis of SGS was carried out for adults and juveniles of PFF and adults of PPA based on the estimated coancestry coefficient (θ_{xy}) between pairs of individuals into previously determined distance classes, using coancestry estimator described in Loiselle et al. (1995) and the Spagedi 1.3 program (HARDY and VEKEMANS, 2002). The statistical significance of the coefficient θ_{xy} was obtained by comparing the limits of the confidence interval at 95% probability of the average estimate θ_{xy} for each distance class, calculated by Monte Carlo permutation of individuals between distance classes. To compare the extension of SGS between the two adults of PPA and PFF and between adults and juveniles of PFF, the S_p-statistic (VEKEMANS; HARDY, 2004) was calculated by $-b_k/(1-\theta_1)$, where θ_1 is the mean

coancestry coefficient calculated between all pairwise individuals within the first distance class (100 m) and b_k is the slope of the regression of coancestry coefficient on the logarithm of spatial distance (0-900 m). To test for SGS statistic significance, spatial position of the individuals were permuted (1,000 times) to obtain the frequency distribution of b_k under null hypothesis that θ_1 and ln(d_{xy}) were uncorrelated.

Mating system analysis

The mating system was characterized in level of individuals and populations by the mixed mating model (RITLAND and JAIN, 1981) and correlated mating model (RITLAND, 1989), using the MLTR 3.1 program (RITLAND, 2002). Estimates were based on the Expectation maximization numerical method (EM). The indexes estimated were: a) the multilocus outcrossing rate (t_m); b) the single-locus outcrossing rate (t_s); c) the mating among relatives rate ($t_m - t_s$); d) the correlation of selfing (r_s); e) the correlation of paternity within and among fruits (r_p). As our sample was hierarchical within and among fruits, the correlation of paternity also was estimated within ($r_{p(w)}$) and among fruits ($r_{p(a)}$). The standard deviation of the parameters was estimated by 1,000 *bootstrap* resampling. The effective number of pollen donors ($N_{ep} = 1/r_p$) was estimated based on Ritland (1989). In order to know the genetic structure of the progenies was estimated average coancestry coefficient (Θ) among plants within progenies, calculated as derivations Sebenn (2006). The genetic representation within progenies was measured by the variance effective size (N_e) based on sample variance of allelic frequencies (COCKERHAM, 1969). The number of seed-trees for seed collection was calculated assuming that the objective was to retain in the total sample, the effective 150 reference size, $m = N_{e(reference)} / N_e$ (SEBBENN, 2006).

3.2 RESULTS

Genetic diversity and fixation index

The average allelic richness (R) ranged among samples from 12.3 to 17.8, the average observed heterozygosity (H_o) ranged from 0.428 to 0.540 and the expected heterozygosity (H_e) ranged from 0.763 to 0.878 (Table 4). The fixation index (F) ranged from 0.371 to 0.511 and was significantly higher from zero for all samples, suggesting inbreeding.

Table 4 - Genetic diversity and fixation index (F) for adult trees, juveniles and progenies of *Hymenaea stigonocarpa* in a forest fragment (PFF) and a pasture (PPA). n is the sample size; R is allelic richness for all individuals genotyped in the six loci; H_o is the observed heterozygosity; H_e is the expected heterozygosity.

Sample	n	R ±SD	H_o ±SD	H_e ±SD	F ±SD
PFF: adults	111	16.2±3.1	0.54±0.14	0.86±0.04	0.37±0.15*
PPA: adults	359	17.8±1.6	0.52±0.18	0.87±0.05	0.39±0.21*
PFF: juveniles	219	16.6±4.1	0.43±0.21	0.88±0.04	0.51±0.24*
PFF: progeny	419	12.3±2.1	0.45±0.17	0.76±0.07	0.42±0.19*
PPA: progeny	457	12.9±1.8	0.45±0.07	0.82±0.05	0.45±0.07*

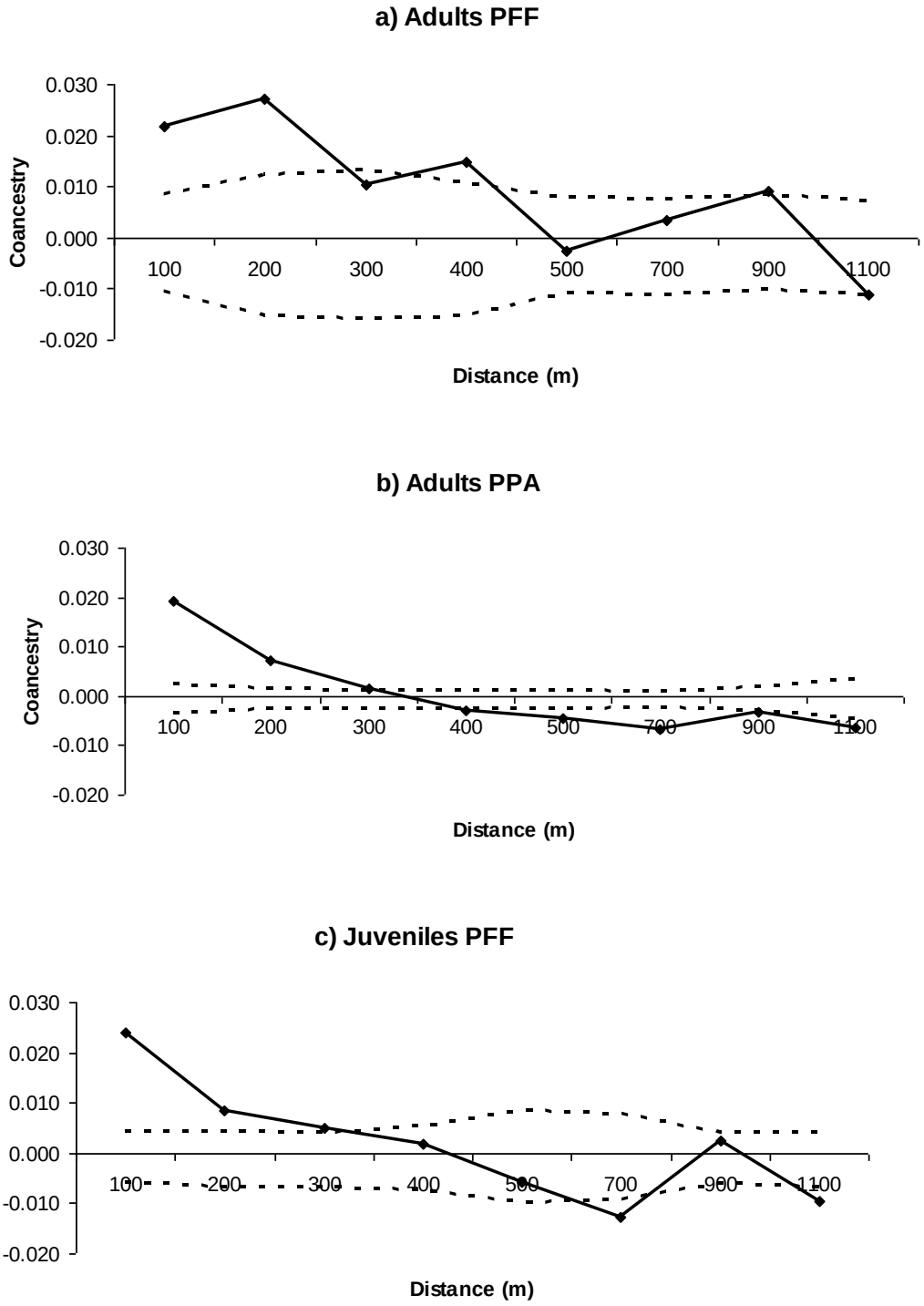
* P< 0.05. SD is the standard deviation.

Source: Prepared by author.

Intra-population spatial genetic structure

The spatial distribution of genotypes from the estimate of coancestry coefficient between pairs of trees by distance class present spatial genetic structure (SGS) significant for adults and juveniles of PFF and adults in PPA (Figure 2). PFF's adults the SGS was significant up to distance of 350 m and juveniles up to 250 m. For adults of PPA the SGS was significant up to distance of 250 m. The average pairwise θ_{xy} values for the first distance class (0-100 m) was similar between adults of PFF (θ_{xy} = 0.022) and PPA (θ_{xy} = 0.019) and juveniles of PFF (θ_{xy} = 0.024). The regression slope b_k of pairwise coancestry coefficient on the logarithm of spatial distance (0-900 m) was significant negative for both populations for adults (PFF= -0.00727; PPA= -0.0094) and juveniles in PFF (-0.0095), confirming the presence of SGS. The intensity of SGS, measured by S_p -statistic was also similar between adults of PFF (S_p = 0.0074) and PPA (S_p = 0.0095) and juveniles of PFF (S_p = 0.0097).

Figure 2 – Intrapopulation spatial genetic structure in adult trees of PFF (a), adult trees of PPA (b) and juveniles of PFF (c) of *Hymenaea stigonocarpa*. The continuous line represents the average coefficient of coancestry estimated and the dashed lines represent the confidence interval at 95% probability of the hypothesis of no spatial genetic structure (H0: $\theta_{xy} = 0$).



Source: Prepared by author.

Mating system at the population level

The estimates of multilocus outcrossing rate (t_m) in progenies of PFF (0.813) and PPA (0.821) were significantly lower than unity (1.0), which indicates the presence of selfing (Table 5). However, these values were not statistically different each other. The single-locus outcrossing rate (t_s) was also significantly lower than unity (1.0) at the PFF and PPA and significantly smaller than the multilocus outcrossing rate. Consequently, the differences between the multilocus and single-locus outcrossing rates ($t_m - t_s$) was high and significantly different from zero in the PFF (0.413) and PPA (0.465), suggesting mating among relatives individuals. The correlation of selfing (r_s) was significantly higher from zero in both populations, but although it was higher in PFF (0.124) than in PPA (0.031), the values were low, which indicates individual variation in outcrossing rate. The correlation of paternity within and among fruits (r_p) was significantly higher from zero in PFF (0.742) and PPA (0.756) which indicates that most of the seed trees were effectively fertilized effectively by a few number of pollen donor (about 2). The progenies were compound in highest proportion by full-sibs pairs (P_{fs}). Thus, the coancestry coefficient (Θ) and effective size (N_e) within progenies were close to expected in full-sibs progenies (Θ = 0.25, N_e = 2) in both populations. Thus, the number of seed trees (m) for seed collection retain an effective size of 150 was high (minimum of 78 seed trees).

The hierarchical analysis of correlation of paternity was similar and not significantly different among ($r_{p(a)}$) and within ($r_{p(w)}$) fruit in both the populations, which indicates similar probability of to find full-sibs individuals among and within fruits. As a result, the effective number of pollen donors was similar among ($N_{ep(a)}$) and within ($N_{ep(w)}$) fruits.

Table 5 – Mating system parameters for *Hymenaea stigonocarpa* in two populations (95% CI is the 95% confidence interval).

Index	PFF mean (95% CI)	PPA mean (95% CI)
Multilocus outcrossing rate: t_m	0.813 (0.746-0.887)	0.821 (0.780-0.863)
Single-locus outcrossing rate: t_s	0.400 (0.351-0.496)	0.356 (0.324-0.431)
Mating among relatives: $t_m - t_s$	0.413 (0.391-0.395)	0.465 (0.432-0.456)
Correlation of selfing: r_s	0.124 (0.048-0.197)	0.031 (0.0-0.063)
Correlation of paternity within and among fruits: r_p	0.742 (0.573-0.868)	0.756 (0.568-0.843)
Effective number of pollen donors (among and within): N_{ep}	1.3 (1.2-1.7)	1.3 (1.2-1.8)
Proportion of self-sibs pairs: P_{ss}	0.04 (0.01-0.06)	0.04 (0.02-0.05)
Proportion of self-half-sibs pairs: P_{shs}	0.30 (0.20-0.38)	0.29 (0.24-0.34)
Proportion of half-sibs pairs: P_{hs}	0.17 (0.10-0.24)	0.16 (0.12-0.26)
Proportion of full-sibs pairs: P_{fs}	0.49 (0.32-0.68)	0.51 (0.35-0.63)
Coancestry within progeny: Θ	0.24 (0.24-0.25)	0.26 (0.23-0.28)
Variance effective size (among and within): N_e	1.93 (1.88-1.98)	1.81 (1.71-2.04)
Number of seed-trees for seed collection: m	78 (76-80)	83 (73-88)
Correlation of paternity within fruits: $r_{p(w)}$	0.728 (0.544-0.865)	0.745 (0.565-0.836)
Correlation of paternity among fruits: $r_{p(a)}$	0.755 (0.593-0.877)	0.769 (0.570-0.860)
Effective number of pollen donors (within): $N_{ep(w)}$	1.4 (1.2-1.8)	1.3 (1.2-1.8)
Effective number of pollen donors (among): $N_{ep(a)}$	1.3 (1.1-1.7)	1.3 (1.2-1.8)

Source: Prepared by author.

Genetic diversity and mating system at the level of progenies

In both populations, the fixation index of seed trees (F_m) was lower (ranged from -0.08 to 0.24) than the fixation index within progenies (F_o, ranged from 0.34 to 0.64), suggesting selection against homozygous individuals between seed to adult stage (Tables 6 and 7). In PFF, the total number of alleles ranged from 18 to 32 and observed heterozygosity ranged from 0.33 to 0.53. The PPA showed the same behavior with values of the total number of alleles also showed the same interval of variation (18 to 32 alleles) and the observed heterozygosity ranged from 0.31 to 0.55.

The estimates of the mating system index were significantly higher from zero in both populations. The outcrossing rate (t_m) in progenies of PFF ranged from 0.60 to 1.00 and in PPA ranged from 0.58 to 0.94 (Tables 6 and 7). The correlation of paternity among and within fruits (r_p) ranged from 0.32 to 0.99 for both populations. The correlation of paternity within fruits ($r_{p(w)}$) ranged from 0.27 to 0.99 for PFF and 0.34 to 0.99 for PPA and the correlation of paternity among fruits $r_{p(a)}$ ranged from 0.36 to 0.99 for PFF and 0.00 to 0.99 for PPA. The effective numbers of pollen donors among and within fruits (N_{ep}), within fruits ($N_{ep(w)}$) and among fruits ($N_{ep(a)}$) showed similar values in the two populations (Tables 6 and 7). The coancestry coefficient (Θ) and the variance effective within progeny was also no different among populations.

Table 6 - Estimates of mating system and genetic structure for each family in PFF.

Family	F_m	n	k	H_o	F_o	$t_m \pm SD$	$t_m - t_s \pm SD$	$r_p \pm SD$	$r_{p(w)} \pm SD$	$r_{p(a)} \pm SD$	N_{ep}	$N_{ep(w)}$	$N_{ep(a)}$	Θ	N_e
610	0.07	18	26	0.46	0.42	0.78±0.11	0.52±0.08	0.93±0.09	0.99±0.04	0.84±0.21	1.08	1.01	1.19	0.273	1.68
700	0.04	30	32	0.47	0.41	0.87±0.07	0.30±0.06	0.32±0.10	0.27±0.12	0.36±0.10	3.13	3.70	2.78	0.197	2.34
702	0.17	29	26	0.38	0.52	0.73±0.08	0.38±0.05	0.65±0.08	0.64±0.10	0.66±0.10	1.54	1.56	1.52	0.285	1.66
703	0.16	29	25	0.48	0.40	1.00±0.00	0.53±0.06	0.47±0.10	0.45±0.12	0.49±0.11	2.13	2.22	2.04	0.213	2.17
704	-0.09	30	23	0.41	0.48	0.83±0.07	0.49±0.05	0.93±0.07	0.90±0.09	0.97±0.07	1.08	1.11	1.03	0.228	2.04
707	0.10	30	26	0.41	0.48	0.60±0.09	0.35±0.06	0.69±0.15	0.63±0.20	0.75±0.17	1.45	1.59	1.33	0.303	1.57
710	-0.02	29	21	0.40	0.50	0.66±0.09	0.33±0.05	0.67±0.13	0.65±0.17	0.69±0.14	1.49	1.54	1.45	0.254	1.84
712	0.04	30	32	0.53	0.34	0.97±0.12	0.43±0.11	0.45±0.11	0.42±0.12	0.47±0.12	2.22	2.38	2.13	0.188	2.45
714	0.00	25	30	0.51	0.36	1.00±0.00	0.64±0.04	0.86±0.09	0.82±0.13	0.90±0.08	1.16	1.22	1.11	0.233	2.00
715	-0.05	30	21	0.52	0.34	0.63±0.09	0.40±0.06	0.99±0.15	0.99±0.14	0.99±0.16	1.01	1.01	1.01	0.269	1.77
716	0.21	30	18	0.42	0.48	0.81±0.07	0.43±0.05	0.83±0.10	0.84±0.11	0.82±0.11	1.20	1.19	1.22	0.297	1.61
721	0.06	30	20	0.43	0.46	0.64±0.09	0.38±0.06	0.98±0.09	0.95±0.14	0.99±0.07	1.02	1.05	1.01	0.300	1.59
722	0.19	30	21	0.46	0.42	1.00±0.00	0.67±0.67	0.88±0.08	0.87±0.10	0.89±0.09	1.14	1.15	1.12	0.280	1.70
723	0.02	25	23	0.49	0.38	0.88±0.09	0.50±0.07	0.90±0.09	0.88±0.11	0.92±0.10	1.11	1.14	1.09	0.248	1.88
724	0.23	24	18	0.33	0.58	0.79±0.09	0.50±0.06	0.97±0.05	0.99±0.04	0.93±0.09	1.03	1.01	1.08	0.316	1.49

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

Table 7 - Estimates of mating system and genetic structure for each family in PPA.

Family	F_m	n	k	H_o	F_o	$t_m \pm SD$	$t_m - t_s \pm SD$	$r_p \pm SD$	$r_{p(w)} \pm SD$	$r_{p(a)} \pm SD$	N_{ep}	$N_{ep(w)}$	$N_{ep(a)}$	Θ	N_e
11	0.04	27	28	0.51	0.40	0.89±0.09	0.32±0.07	0.32±0.08	0.34±0.12	0.31±0.11	3.13	2.94	3.23	0.193	2.37
15	0.09	30	18	0.34	0.60	0.63±0,09	0.44±0.06	0.99±0.01	0.99±0.05	0.99±0.01	1.01	1.01	1.01	0.308	1.54
24	0.07	19	27	0.40	0.52	0.90±0.12	0.52±0.10	0.99±0.02	0.99±0.04	0.99±0.05	1.01	1.01	1.01	0.253	1.79
34	-0.01	18	31	0.47	0.44	0.94±0.14	0.41±0.13	0.56±0.14	0.57±0.15	0.52±0.25	1.79	1.75	1.92	0.190	2.28
37	0.11	30	30	0.43	0.49	0.73±0.08	0.49±0.06	0.95±0.08	0.98±0.06	0.89±0.13	1.05	1.02	1.12	0.293	1.62
237	-0.08	16	27	0.54	0.36	0.88±0.13	0.47±0.11	0.67±0.21	0.72±0.21	0.34±0.32	1.49	1.39	2.94	0.187	2.29
240	0.24	24	29	0.42	0.50	0.83±0.09	0.48±0.06	0.83±0.11	0.74±0.15	0.93±0.11	1.20	1.35	1.08	0.299	1.57
249	-0.08	29	28	0.47	0.45	0.79±0.07	0.52±0.06	0.99±0.05	0.97±0.07	0.99±0.05	1.01	1.03	1.01	0.239	1.96
256	-0.05	24	30	0.54	0.36	0.92±0.11	0.47±0.10	0.81±0.14	0.82±0.15	0.80±0.14	1.23	1.22	1.25	0.206	2.21
262	0.02	14	21	0.45	0.46	0.86±0.13	0.50±0.07	0.83±0.15	0.83±0.15	0.00±0.00	1.20	1.20	0.00	0.221	1.94
272	0.06	23	27	0.41	0.51	0.92±0.11	0,43±0.09	0.76±0.13	0.69±0.15	0.84±0.15	1.32	1.45	1.19	0.226	2.01
292	0.00	20	27	0.40	0.53	0.90±0.12	0.46±0.10	0.65±0.17	0.72±0.16	0.55±0.35	1.54	1.39	1.82	0.201	2.18
299	0.19	16	23	0.39	0.54	0.75±0.11	0.46±0.08	0.99±0.02	0.99±0.10	0.99±0.33	1.01	1.01	1.01	0.312	1.47
313	0.09	28	25	0.35	0.59	0.77±0.08	0.52±0.06	0.99±0.05	0.99±0.06	0.99±0.06	1.01	1.01	1.01	0.285	1.65
321	0.12	19	21	0.31	0.64	0.58±0.11	0.31±0.06	0.72±0.17	0.84±0.21	0.57±0.42	1.39	1.19	1.75	0.313	1.47
334	0.09	21	28	0.48	0.44	0.91±0.12	0.51±0.10	0.85±0.09	0.90±0.12	0.78±0.15	1.18	1.11	1.28	0.240	1.90
339	0.11	19	23	0.45	0.47	0.84±0.10	0.56±0.08	0.99±0.06	0.97±0.11	0.99±0.01	1.01	1.03	1.01	0.282	1.63
342	0.01	28	32	0.55	0.34	0.86±0.07	0.43±0.05	0.53±0.12	0.50±0.15	0.56±0.15	1.89	2.00	1.79	0.213	2.18
358	0.06	30	22	0.49	0.41	0.77±0.07	0.44±0.05	0.86±0.10	0.77±0.14	0.91±0.09	1.16	1.30	1.10	0.267	1.78
368	0.06	22	28	0.50	0.41	0.86±0.10	0.53±0.08	0.92±0.10	0.95±0.10	0.89±0.16	1.09	1.05	1.12	0.262	1.77

± SD is the standard deviation; * P< 0.05; F_m and F_o are the fixation index of seed-trees and within families. respectively; n is sample size within of families; k is the total number of alleles; H_o is the observed heterozygosity; t_m is the multilocus outcrossing rate; r_p , $r_{p(w)}$ and $r_{p(a)}$ are the correlations of paternity within and among fruits within fruits and between fruits, respectively; N_{ep} , $N_{ep(w)}$ and $N_{ep(a)}$ are the effective numbers of pollen donors between and within of fruits, within of fruits and among fruits, respectively; Θ and N_e are the coefficients of coancestry and the variance effective size within of families, respectively.

3.3 DISCUSSION

3.3.1 Genetic diversity and inbreeding

Our study comparing a population located in an extensive forest fragment and one in a pasture of the bat pollinated and animal seed disperse tree *H. stigonocarpa* showed no differences in levels of genetic diversity, inbreeding and mating system patterns. Similar levels of genetic diversity among adult trees were expected, because probably it consists of only a population and has been fragmented in the past by pre-fragmentation process. Although the levels of genetic diversity were decrease in open-pollinated seeds, the levels were also similar in both sites. The levels of genetic diversity in descent populations are the product of mating patterns in parental population, as selfing rate, mating among relatives and correlated mating. As these mating patterns were similar in both PPA and PFF, the levels of genetic diversity were also similar in the seeds. The decrease of genetic diversity in seeds occurred due genetic drift caused by selfing, mating among relatives and correlated mating.

In view of maintaining genetic diversity in the long-term, allelic richness it plays an important role because it is more sensitive to bottlenecks than expected heterozygosity, it reflects better past fluctuations in population size (NEI et al., 1975; CORNUET and LUIKART, 1996; LUIKART et al., 1998; LEBERG, 2002). In this study, the loss of observed heterozygosity of adults to progeny was about 13.5% in PPA and 16.7% in PFF and the loss of the allelic richness was more drastic, with 27.5% in PPA and 24.1% in PFF, showing that allelic richness is much more susceptible to environmental disturbances than the observed heterozygosity.

3.3.2 Spatial genetic structure

Both adult populations and juveniles of PFF presented significant SGS, similar average pairwise θ_{xy} values for the first distance class (ranging from 0.019 to 0.024) and intensity of SGS, as measured by S_p-statistic (ranging from 0.0074 to 0.0097). The results showed that near-neighbor adult trees up to 350 m in PFF and 250 m in PPA are probably related individuals. Juveniles of PFF also present significant SGS

(250 m). As all adult trees in the PPA and PFF are remaining from pre-fragmentation times of the area, the differences in SGS distance is probably a not anthropogenic effect, but can be explained the different population density. In PFF, the adult population density is lower (0.0094 trees/ha) than in PPA (0.101 trees/ha), thus the trees are more aggregated in PPA (mean of 1,005 m) than PFF (mean of 2,124 m), resulting in a lowest SGS distance in PPA.

Ecological processes (BORN et al., 2008), breeding system, life form (VEKEMANS; HARDY, 2004) and pollen and seed dispersal mode (HARDY et al., 2006) are factors that can directly interfere with the SGS. However, Collevatti et al. (2010) studied three species of the Savannah and observed that seed dispersal is the decisive factor to explain the degree of SGS, being more important than pollination. It is noteworthy that in these study the species that had dispersal by animal (*Caryocar brasiliense* and *Dipteryx alata*) were self-incompatible, and this may have contributed to the dispersal of seeds is most important that the pollen dispersal. The other species (*Tibouchina papyrus*) is self-compatible and has wind seed dispersal. In the present case of *H. stigonocarpa*, it is not possible affirm if seed or pollen if the most important factor producing the SGS, because both pollen and seeds are dispersed by animals with potential for long gene dispersal. For to do that, we need a parentage analysis to determined the mother and father of juveniles.

The distance of SGS observed for adults and juveniles of *H. stigonocarpa* (minimum of 250 m) is in agreement with the review of Degen and Sebbenn (2014), which observed than wind seed disperse tropical tree species with low population density generally present higher SGS distance than animal and gravity seed disperser tropical trees species with high population density.

3.3.3 Mating system and its variation

Hymenaea stignocarpa is monoecious and self-compatible species, therefore produces seeds by outcrossing and selfing. Estimates of multilocus outcrossing rate of the two populations confirm this expectation (GIBBS et al.,1999; CARVALHO, 2006; MORAES and SEBBENN, 2011). Our results confirm that seeds are produced by both outcrossing and selfing, although outcrossing is predominantly (minimum t_m

of 0.813). However, we expected that the PPA had higher outcrossing rate and correlated mating and smaller mating among relative rate than PFF, due to environmental conditions and spatial distribution of trees. The results refuted these expectations. PPA and PFF showed similar levels of outcrossing rate, mating among relative rate and correlated mating. As the adult trees of both populations are remaining from pre-fragmentation time and SGS was similar between PPA and PFF, the results suggest that environmental conditions did not affect in the behavior of pollinators. Thus, the remaining isolated trees in pasture could serve as ecological corridors between PPA and PFF.

In contrast, mating system studies of Neotropical tree species based on genetic markers between isolated trees occurring in pastures with those in forest fragments or continues forest has been in general show contrasting differences for the outcrossing rate, indicating that isolated trees have higher selfing rate than trees in fragments or continues forest, as observed for *Samanea saman* (CASCANTE et al., 2002), *Dinizia excelsa* (DICK et al., 2003), *Paquira quinata* (FUCHS et al., 2003; RYMER et al., 2013), *Dipteryx panamensis* (HANSON et al., 2008), *Hymenaea stignocarpa* (MORAES; SEBBENN, 2011) and *Copaifera langsdorffii* (MANOEL et al., 2012).

Plants with mixed mating system have higher evolutionary processes than the obligatorily selfing or outcrossed species, with the presence of several strategies to perpetuate the species. Goodwille et al. (2005) studied several species of plants and found that the mixed mating system is more frequently in species that are pollinated by animals (46.4% of 267 species) than those pollinated by wind or water (26. 9% of 78 species). According with our estimate of individuals outcrossing rate, *H. stigonocarpa* present a mixed mating system with predominance of outcrossing; ranging from 0.58 to 1.0 (Tables 3.3 and 3.4).

3.3.4 Inbreeding

The estimate of the fixation index (F) indicates inbreeding in all samples. The level of families seed-trees are well below the fixation index compared to the progeny showing the strong presence of natural selection in the study sites. As the mating among relative rate was higher ($t_m - t_s$) than the selfing rate ($s = 1 - t_m$), the

inbreeding in seeds of both populations was probably special originated from $t_m - t_s$. However, for adult and juveniles trees, the observed inbreeding was not expected because *H. stigonocarpa* present post-zygotic selection in self-pollinated flowers, suggesting inbreeding depression (GIBBS et al., 1999). Seeds from selfing and from mating among relatives may present inbreeding depression, due to the homozygous combinations of identical by descent alleles. As the populations present SGS, the Wahlund effect maybe is the explanation for inbreeding in adults and juveniles (HEDRICK, 2000; BITTENCOURT; SEBBENN, 2007). An alternative explanation, it the presence of null alleles, due the fact that the microsatellite loci used here were transferred from *H. courbaril* to our study species. Null alleles result in homozygous genotypes of heterozygous true genotypes, due the absence of amplification of some alleles, resulting in bias in the estimates of fixation index.

3.3.5 Correlated paternity among and within fruits and effective number of pollen donors

One hypothesis was that when individuals *H. stignocarpa* occurs in high population density, pollinators have the habit of to visit several flowers of different tree, minimizing selfing and correlated mating. Our results not support such hypothesis. The PFF with lower population density showed similar levels of correlated mating among and within fruits (r_p), within fruits ($r_{p(w)}$) and among fruits ($r_{p(a)}$) than PPA. All r_p, $r_{p(w)}$ and $r_{p(a)}$ indexes showed that the number of pollen donors fertilizing the seed trees, fruits within tree and among fruits of each trees was low (< 2) and the families are compound mainly by full-sibs. The correlation mating suggesting that outcrossing occurs between nearest neighboring trees or due the tendency pollinator carrying pollen derived from same individual plants (RITLAND, 1989).

Interesting, the hierarchical analysis of correlation of paternity was similar between within ($r_{p(w)}$) and among ($r_{p(a)}$) fruits in both PPA and PFF populations, which indicates similar probability to find full-sibs individuals among and within fruits. On the other hand, analyzing these indexes at family level, we observed greater variation between families (Tables 3.3 and 3.4), but there was no difference between the indexes of each family and between PPA and PFF. This result is a surprise,

because other studies show in many species with multi-seed fruit, full-sibs occur at a higher frequency within fruits than among fruits, indicating a hierarchical pattern of correlated mating (RITLAND, 1989; MUONA et al., 1991; QUESADA et al., 2001; CASCANTE et al., 2002; HARDY et al., 2004; LOBO et al., 2005; TAMAKI et al., 2009; SILVA et al., 2011; FERES et al., 2012).

3.3.6 Implications of the results for conservation genetics

Most populations of tropical tree species at risk of extinction are spatially fragmented, causing a structuring of populations in reduced and sometimes isolated groups of trees. This has can result in an erosion of the genetic variation and the increase in inbreeding of remaining populations. In this context, the presence of increased inbreeding and high relatedness in the seeds was confirmed by the high proportions of full-sibs pairs (50%). The average coefficient of coancestry within progenies (Θ) showed a value expected for full-sibs progeny (Θ = 0.25) and the variance effective size among and within fruits (N_e) was lower than expected in panmitic populations (N_e = 4). For *ex situ* conservation of the species, the seeds collection should be done at distances greater than 250 m in PPA and 350 m in PFF, to avoid seed collection in trees relatives, which reduces the effective size of total sample seeds. It is recommended, in this way, seeds collection in at least seed trees 83 in PPA and 78 in PFF to retain the effective size of the 150.

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Gene flow and inbreeding depression in the bat-pollinated *Hymenaea stigonocarpa* Mart. ex Hayne. (Fabaceae-Caesalpinioideae) in the Brazilian Savannah

ABSTRACT

The Neotropical species *Hymenaea stigonocarpa* presents desirable characteristics in wood, commonly used in ship and civil building, which is why demand and cut off. It also presents an interesting feature, due its fruits are edible, being recommended for reforestation in degraded and remaining areas to attract local wildlife. Due the importance of the species, deemed opportune to study pollen and seed flow and dispersal patterns and inbreeding depression in progeny and juveniles in two populations of *H stigonocarpa*. We sampled all existing reproductive tree and juveniles in pasture area (PPA) and forest fragment (PFF) located in the savannah region in the Inocência city, Mato Grosso do Sul Sate, Brazil. Also were collected seeds from 20 seed tree at PPA and in 15 seed trees in the PFF. In each seed tree, the seeds were sampled hierarchically within and among fruit. The results show high pollen immigration (~30%) and selfing rate (39-45%) for both populations. For the juveniles of PPA, the seed immigration (53%) was higher than pollen (33%). Pollen was dispersed over long distance (average ranged from 1,596 to 4,658 m). The effective pollen neighborhood area was extensive in both PPA (4,378 ha) and PFF (6,170 ha). The average seed dispersal distance was similar to pollen dispersal at PFF (4.988 m). The progeny showed a strong inbreeding depression for survival and height of plants. The results showed that PPA and PFF are linked by intensive gene flow and, thus both populations must be conserved.

Key words: *In situ* and *ex situ* conservation. Neotropical species. Pollen and seed dispersal.

4 INTRODUCTION

Habitat fragmentation is a globally pervasive problem that continues to drive changes to woody plant ecosystems (FAO, 2012). As most species of woody plants are animal-pollinated (OLLERTON et al., 2011), studying the impacts of forest fragmentation on woody plant–pollinator interactions seems particularly important, especially because significant amounts of biodiversity rely on these interactions (BREED et al., 2013). Forest fragmentation and the resulting spatial isolation of tree species can modify the activity of pollinators by reducing the density of potential food resources and increasing the distance between those resources (SIH; BALTUS, 1987). The reduction of floral resources results in longer travel distances between menaining and feeding areas and often results in pollinators crossing disturbed areas that are dominated by agriculture (KEARNS et al., 1998). When the distance between plants is greater than the home range of the pollinators, their density will decrease in the disturbed areas and this will result in fewer pollinator visits (KEARNS et al., 1998). Specialist pollinators that are less flexible in exploiting food resources are the most susceptible to local extinction (ESTRADA et al., 1993, COSSON et al., 1999; STONER, 2002).

For populations are less susceptible to deforestation they need to present some robustness levels: (1) woody plants tend to have many overlapping generations within populations; (2) trees are long-lived and many adults may be from pre-fragmentation stage and, (3) undergo regular far-reaching gene flow, mediated by their pollinators, even in fragmented landscapes (PETIT; HAMPE, 2006; KREMER et al, 2012). These traits result in substantial genetic inertia within populations of animal-pollinated woody plants, which generally maintains genetic diversity within fragmented populations (VRANCKX et al., 2011). Additionally, species with less mobile pollinators should theoretically be more sensitive to the drivers of these fitness effects (HEINRICH; RAVEN, 1972; CHARNOV, 1976; HADLEY; BETTS, 2012). As plant densities decline, animal pollinators are less likely to shift from one plant to another because of the increased costs of doing so the theory of optimal foraging (HEINRICH; RAVEN, 1972; CHARNOV, 1976; OTTEWELL et al., 2009). A pollinator foraging on a plant that is a self-compatible hermaphrodite for longer periods of time increases the probability of selfing (increases either via autogamy or geitonogamy; that is, pollination from the same or a different flower on the same

plant; KARRON et al., 2009). As a consequence of increased self-pollen being received, an increase in pollen discounting is expected (BARRETT, 1998). A recent review of outcrossing rates in undisturbed versus disturbed plant populations across 27 species confirmed the expectation of decreased outcrossing rate in disturbed plant populations (ECKERT et al., 2010).

Pollen dispersal is a component of the mating system and is influenced by population density, flower phenology, vectors of dispersal, mechanism that reduces the selfing rate, and also by factors such as the climate, forest fragmentation, and logging. The main vector of pollen dispersal in most temperate tree species is the wind, and most tropical tree species are pollinated by animals. Seeds can be dispersed in both temperate and tropical trees by wind and animals, and in some cases water serves as vector of seed dispersal. Species pollinated by wind generally have lower genetic differences among populations compared to species pollinated by animals. For animal-pollinated tree species, the type of pollinator partly determines the distances of pollen dispersal (DICK et al., 2008), and pollen dispersal is also affected by the population density, because at low densities, individuals are more widely dispersed and the pollinator needs to invest more energy to fly among co-flowering trees. Pollen dispersal distances are generally longer in low density compared to high-density populations (WARD et al., 2005; DICK et al., 2008).

Studies on pollen dispersal in Neotropical tree species have increased considerably in the last decades (NASON et al.,1998; WHITE et al., 2002; DICK et al., 2003; LATOUCHE-HALLÉ et al., 2004; DUNPHY and HAMRICK, 2007; LACERDA et al., 2008; BITTENCOURT; SEBBENN, 2007, SEBBENN et al., 2011; SEBBENN et al., 2012; MANOEL et al., 2012; TAMBARUSSI et al., 2015). These studies indicate long-distance pollen flow and reproduction mainly by outcrossing in Neotropical tree species (WARD et al., 2005). For instance, trees pollinated by large bees may present pollen dispersal distance greater than 500 m (*Platypodium elegans*, HAMRICK and MURAWSKI, 1990; *Dicorynia guianensis*, LATOUCHE-HALLÉ et al., 2004; *Myracrodruon urundeuva*, GAINO et al., 2010), while trees pollinated by small-size insects may present more variable pollen dispersal distance, ranging from ~100 to more than 600 m (WARD et al., 2005; DICK et al., 2008; ODDU-MURATORIO; KLEIN, 2008). For self-compatible species, outcrossing rate may be highly variable due to differences in pollinator availability and flowering

phenology (BARRET, 2003). Studies of bat-pollinated species show variable patterns of pollen dispersion distances with minimum distance of 115 m found in *Hymenaea courbaril* (LACERDA et al., 2008) at the distances up to 18 km found in *Ceiba pentandra* (GRIBEL; LEMOS, 1999).

The capacity for long-distance pollen movement may be crucial for plants in a fragmented landscape, where inbreeding and genetic drift threaten the continued survival of small relictual populations. Plants pollinated by bats in particular may be expected to experience relatively high levels of gene flow because bats are strong fliers capable of traveling long distances (DUNPHY et al., 2004). For example, two species of bats fitted with radio transmitters in southeastern Australia moved up to 6.9 km nightly from roosting to foraging sites (LUMSDEN et al., 2002). Even in the face of habitat fragmentation, many species of bats continue to visit plants in forest remnants, readily flying over open areas (LAW et al., 1999). In fragmented Australian tropical rain forests, bats flew up to 5.8 km (mean, 1 km) across cleared land (LAW and LEAN, 1999). Home ranges encompassed 12–1,796 ha and often contained multiple Forest fragments. Bats fed only 1.2 minutes per tree (yet carried more pollen than birds) and frequently moved more than 200 m between trees, indicating the possibility of long-distance pollen movement (DUNPHY et al., 2004). For the Costa Rican tree *Bauhinia ungulata*, Heithaus et al. (1982) found that the distance that bats flew from a roosting site to a flowering tree (range, 270–1420 m) did not affect pollination success, indicating that trees could obtain adequate pollen over all distances examined. In Mexico, the Jamaican fruit bat (*Artibeus jamaicensis*) has been found to fly nightly 8 km from roosting to foraging areas (MORRISON, 1978). In Costa Rica, 22 bats of five species carrying pollen of *Ceiba aesculifolia* were captured an average of 0.56 km from the lone tree of that species in the study site (HEITHAUS et al., 1975). Two larger bat species (*A. jamaicensis* and *Phyllostomus discolor*) were often caught over 1 km away.

Understanding current gene flow effects across environmental gradients will improve predictions on the adaptive capacity of species under climate change scenarios (SEXTON et al., 2013). While the mating system of *Hymenaea stigonocarpa* is relatively well understood (MORAES et al., 2007), information about distance and pollen dispersal are needed to better understand and conserve the species. Estimates of distance and abundance of pollen are important factors in

predicting the dynamics of plant populations and are the basis for designating conservation strategies in highly fragmented environments (AUSTERLITZ; SMOUSE, 2001). The distance and abundance of pollen is a significant determinant of effective pollination neighborhood are in plant populations (AUSTERLITZ; SMOUSE, 2002) which is especially important to calculate the number of mother trees to collect seeds for *ex situ* conservation and environmental reforestation.

Parentage analysis is a direct way to measure the flow and dispersion distance of pollen and seeds in plant populations (BURCZYK et al., 2004; SMOUSE; SORK, 2004). One advantage of this method in the gene flow study is that it assumes dispersion models and so the observed patterns may represent the true pattern. The paternity analysis has been extensively used to study pollen flow in tree species (DOW; ASHLEY, 1996; WHITE et al., 2002; BITTENCOURT and SEBBENN, 2007; KAMM et al., 2009; NIELSEN; KJAER, 2010).

In Brazil, *H. stigonocarpa* occurs between 3°30` S and 22°40´S and is restricted to the savannah habitat in central and south-east country (CARVALHO, 2006). Currently, *H. stigonocarpa* is only found in small forest fragments and as isolated trees in the landscape, as a result of intensive fragmentation of the savannah in the states of São Paulo and Mato Grosso do Sul. Thus, plans for their conservation strategies should involve *ex situ* strategies. Seeds have been collected from the remaining trees in small forest fragments and isolated trees in pastures to establish germplasm banks. However, the mating system of *H. stigonocarpa* has been examined only recently and there is no information about distance and pollen dispersal patterns among individual trees and groups of trees occurring in pastures. In this context, this study aims to provide additional information about the pollen and flow and dispersal pather for the species in order to better inform efforts and conservation practices. Thus, we consider the following questions: (1) What is the pollen flow rate and immigration in a fragmented population and in population occurring in a pasture of *H. stigonocarpa*? (2) What is the distance and patterns of pollen dispersal within fragmented populations and in the isolated trees in a pasture? (3) There are differences in selfing rate between seed trees occurring in a forest fragment and in isolated trees in a pasture? (4) Selfing and mating among relative trees produce inbreeding depression in the species?

4.1 MATERIAL AND METHODS

Species studied

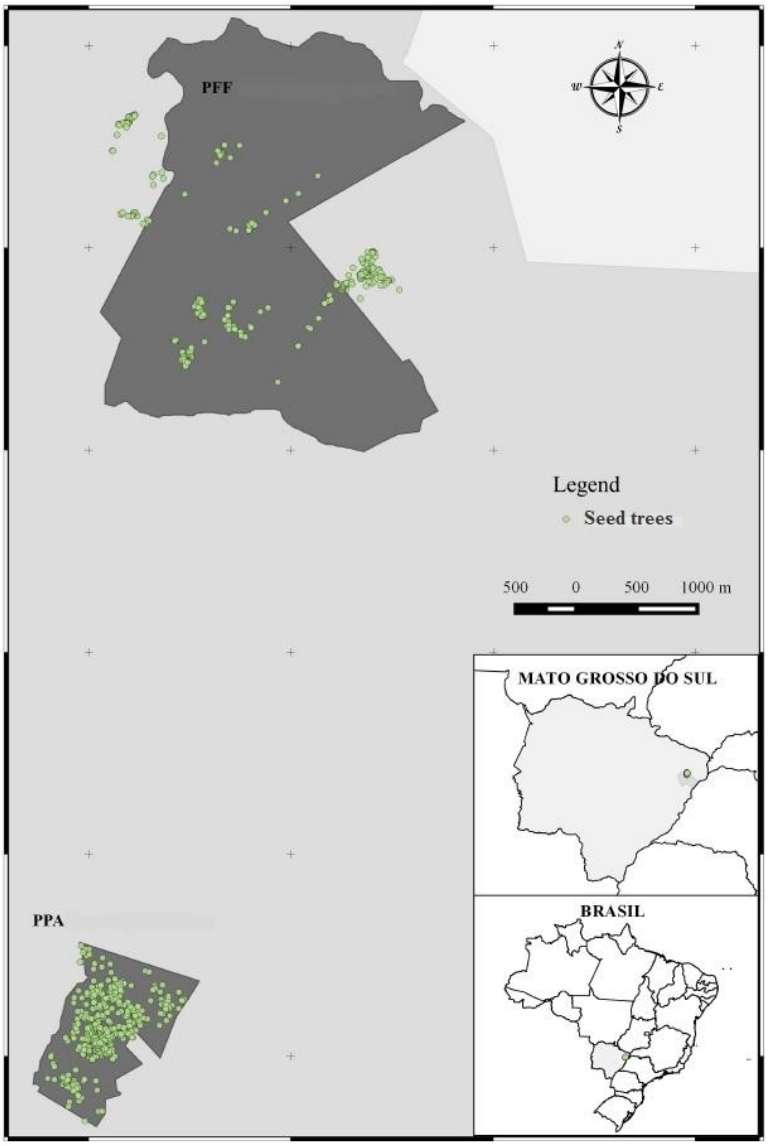
Hymenaea stigonocarpa Mart. ex Hayne. (Fabaceae-Caesalpinioideae) has a wide dispersion in the Savannah of Brazil and adult trees can reach 29 m in height and 50 cm in diameter at breast height (DBH). The species has hermaphrodite flowers (CARVALHO, 2006) and is pollinated by at least four species of bats, including the specialized *Glossophaga soricina* and less frugívorous specialized *Platyrhinus lineatus* and *Carolilia perspicillatta* (GIBBS et al., 1999). Studies based on hand pollination indicate the species as outcrossing, self-compatible and there is evidence of post-zygotic selection in self-pollinated flowers (GIBBS et al., 1999). The species is a key ecological role by producing annually lot of fruits that serve as food for terrestrial fauna (MORAES et al., 2007). These features are important for forest restoration programs, since *H. stigonocarpa* is among the species recommended for planting and restoration in Savannah regions, in well-drained soil conditions (DURIGAN, 2003).

Study site and sampling

The landscape of the study site consists of large extended pastures, sugarcane and eucalyptus, plantation interspersed with small forest fragments and a few isolated trees of *H. stigonocarpa* between fragments. Its location is close to highway MS 444, in the municipality of Inocência, Mato Grosso do Sul state, Brazil (20°07´S, 51°44´W, and 373 m above sea level). The climate in this region is tropical with a dry winter and humid summer. The average rainfall is 1,232.2 mm and the annual average temperature is 24.5° C. The regional biome is characterized by savannah with high levels of anthropogenic disturbance. Much of the savannah was cutting down in this area between 1970 and 1980 for livestock. Currently, this area is also used for planting sugarcane (after 2002) and eucalyptus. The study was carried out in two populations of *H. stigonocarpa* (Figure 3). The first population (PPA) are grouped or isolated trees in pasture (*Brachiaria* sp) that corresponds to an area of 2.82 km^2 (2.66 x 1.06 km), with estimated density of 0.101 trees/ha. The distance among the trees ranged from 0.063 to 2,48 km (mean: 1,0 km). The second

population (PFF) is located within a large forest fragment with an area of 736 km^2 (23 x 32 km), the estimated density is 0.0094 trees/ha. The distance between the trees, ranged from 0.063 to 10,3 km (mean: 2,1 km). The distance between populations is approximately 5 km and the vegetation in this area consists of pasture, eucalyptus plantations and some isolated trees *H. stigonocarpa.* All trees in the PPA and PFF are remaining from pre-fragmentation of the area, which occurred about 30 years ago.

Figure 3. Spatial distribution of *Hymenaea stigonocarpa* trees in the pasture population (PPA) and forest fragment (PFF) occurring in the savannah region of Inocência city, MS, Brazil.



Source: Prepared by author.

The sampling was done so mapped, sampled and genotyped all existing adult trees in the two populations. In PPA were sampled 359 adult trees and PFF were sampled 111 adult trees and 219 juveniles. The juveniles were classified as non-reproductive plants with about 1 m in height. Additionally, we collected and genotyped open-pollinated seeds from 20 seed trees in the PPA and 15 seed trees in PFF, with 30 seeds per tree in different fruits. Each seed was kept record of the seed trees and fruit origin for hierarchical analysis of mating system within and among fruits.

DNA extraction and genotyping

The DNA extraction from leaves of adult trees, juveniles and germinated seeds (progeny) was carried out according to the cetyl trimethylammonium bromide method Doyle and Doyle (1987). DNA quantification of the samples was conducted using an electrophotometer. The amplification reactions and procedures of microsatellite analysis followed the methods proposed by Ciampi et al. (2008) and Moraes and Sebbenn (2011). Amplification of the DNA fragment (2µL 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 (DNF-900, Advanced Analytical Technologies Inc.). Raw data were analyzed using the PROSize™ (version 2.0) software (AATI). Initially, nine di-nucleotide microsatellite loci, developed by Ciampi et al. (2008) for *Hymenaea courbaril* L, were tested. Of these nine loci, six were successfully transferred to *H. stigonocarpa* and used here.

Parentage analysis

The estimate of the contemporary gene flow via pollen and seed analysis and fine-scale mating system were obtained using paternity analysis and CERVUS 3.0 program (MARSHALL et al., 1998). Analyses were conducted from maternal trees genotypes and their progeny, juveniles other reproductive adult trees of the Forest (PFF) and pasture (PPA) as candidates for paternal and maternal parents (in the case of juveniles). Seeds and juveniles who have not been determined father into the

forest and pasture were considered as pollen migrants and juveniles who were not determined mother into the forest and pasture were determined to seed immigrants. Seeds and juveniles who had the same given individual as maternal and paternal parenting were considered to originate from selfing. Since all seed trees, adult and juvenile had a certain spatial position (x and y coordinates), it determined the distance of pollen and seed dispersion, effective pollination neighbour area and effective ratios of pollen dispersal distance. To investigate if the reproductive success was a function of the distance between trees, we compared the frequency distribution of pollen dispersal to the frequency distribution of the distance between all the trees of both populations, using the Kolmogorov-Smirnov test (SOKAL and ROHLF, 1995). The effective pollination area (A_{ep}) was calculated for the population assuming a circular area around this ($A_{ep} = 2\pi\sigma^2$, LEVIN, 1998), where σ^2 is the axial variance of pollen dispersal. The effective ratios of pollen dispersal was estimated by $r_{ep} = \sqrt{A_{ep} / 3.1415}$ (AUSTERLIZ; SMOUSE, 2001).

Estimate of Inbreeding depression

Eighteen months after planting the progeny test, it was measured the height of plants and the survival rate. From this data it was estimated inbreeding depression due to high mortality rate. We estimated the realized the selfing rate (s), outcrossing rate (t), outcrossing rate from non-related individuals (t_u) and from related parents (t_r) with base in the genotyped germinated progeny (n_1). The selfing rate (s) was estimated as the proportion of seeds determined to have the same seed tree as the pollen donor (n_s) in relation to the n_1 by $s = n_s / n_1$ and the outcrossing rate was calculated by $t = 1 - s$. The progeny originated from outcrossing were split in progeny originated from among not related individuals (t_u) and from related parents (t_r). To determined progeny originated from mating among relatives parents we estimated the relatedness among the seed trees and the assigned fathers by paternity analyses by the coancestry coefficient (θ), which was calculated using the method described in Loiselle et al. (1995), and implemented in the Spagedi 1.3 program (HARDY and VEKEMANS, 2002). Following Ismail et al. (2014), if the coancestry between parents

(θ) was ≥ 0.1, then we assumed the seed was inbred due to mating among relative parents and the outcrossing from non-related individuals was calculated by $t_u = n_u / n_1$ and from related parents by $t_r = n_r / n_1$, where n_u and n_r are the number of progeny that originated from non-related parents and from related parents, respectively.

Inbreeding depression was estimated in terms of survival and height at eighteen months of age for the trees in the progeny test. Inbreeding depressions were estimated in terms of selfing (δ_s), mating among relatives (δ_r) and the total (δ_T) according to the following expression:

$$\delta_s = 1 - \left(\frac{\bar{x}_s}{\bar{x}_t} \right),\ \delta_r = 1 - \left(\frac{\bar{x}_r}{\bar{x}_t} \right) \text{ and } \delta_T = 1 - \left(\frac{\bar{x}_i}{\bar{x}_t} \right),$$

where $\bar{x}_t$, $\bar{x}_r$, $\bar{x}_s$ and $\bar{x}_i$ are the means of the traits for the individuals that were outcrossed between not relatives, outcrossing between relatives, those that were selfed, and all inbred individuals (i.e., selfed + mating among relatives), as determined by the paternity analyses and coancestry coefficient between parents.

4.2 RESULTS

Paternity analysis of the open-pollinated seeds

To 420 seeds sampled in PFF (Table 8), a father parent was found for 294 seeds (70%), being 218 seed (52%) pollinated by trees within PFF, 76 (18%) pollinated by trees in PPA and 126 (30%) by trees out of both PFF and PPA, suggesting a pollen immigration rate in PFF (m_p) of 48% (30% + 18%). To 458 seeds sampled in PPA, a father parent was found for 297 seeds (65%), being 278 seeds (61%) pollinated by trees within PPA, 19 seeds (4%) pollinated by trees in PFF and 161 (35%) by trees out of both PFF and PPA, suggesting a pollen immigration rate in PPA (m_p) of 39% (35% + 4%). From 294 seeds of PFF and 297 of PPA assigned for a pollen donor, the same seed tree was determined as the pollen donor for 164 and 206, suggesting a selfing rate of 39% and 45%, respectively (Table 8).

Table 8 - Results of paternity analysis from pollen and seed dispersal in both populations of *Hymenaea stigonocarpa*.

Parâmetros	PFF Pollen	PPA Pollen	PFF Seeds	PFF Pollen
Sample (*n*)	420	458	227	227
Assigned (%)	294 (70%)	297 (65%)	106 (47%)	151 (67%)
Within (%)	218 (52%)	278 (61%)	76 (33%)	73 (32%)
Pollen immigration rate PFF or PPA (%)	76 (18%)	19 (4%)	30 (13%)	78 (34%)
Pollen immigration rate outside (%)	126 (30%)	161 (35%)	121 (53%)	76 (33%)
Selfing (%)	164 (39%)	206 (45%)	34 (15%)	-
Mean SD (km)	4.7 ± 3.1	1.6 ± 2.6	4.9 ±2.8	2.4 ± 2.9
Median (km)	6.7	0.31	6.5	0.65
Min-max (km)	0.04-8.4	0.04-7.8	0.03-8.6	0.02-8.2
Effective pollination neighbor area (A_{ep}) (ha)	6.17	4.38	-	5.42
Ratios of pollen dispersal distance (r_{ep}) (km)	4.4	3.7	-	1.7

SD: standard deviation.

Source: Prepared by author.

Parentage analysis of the juveniles

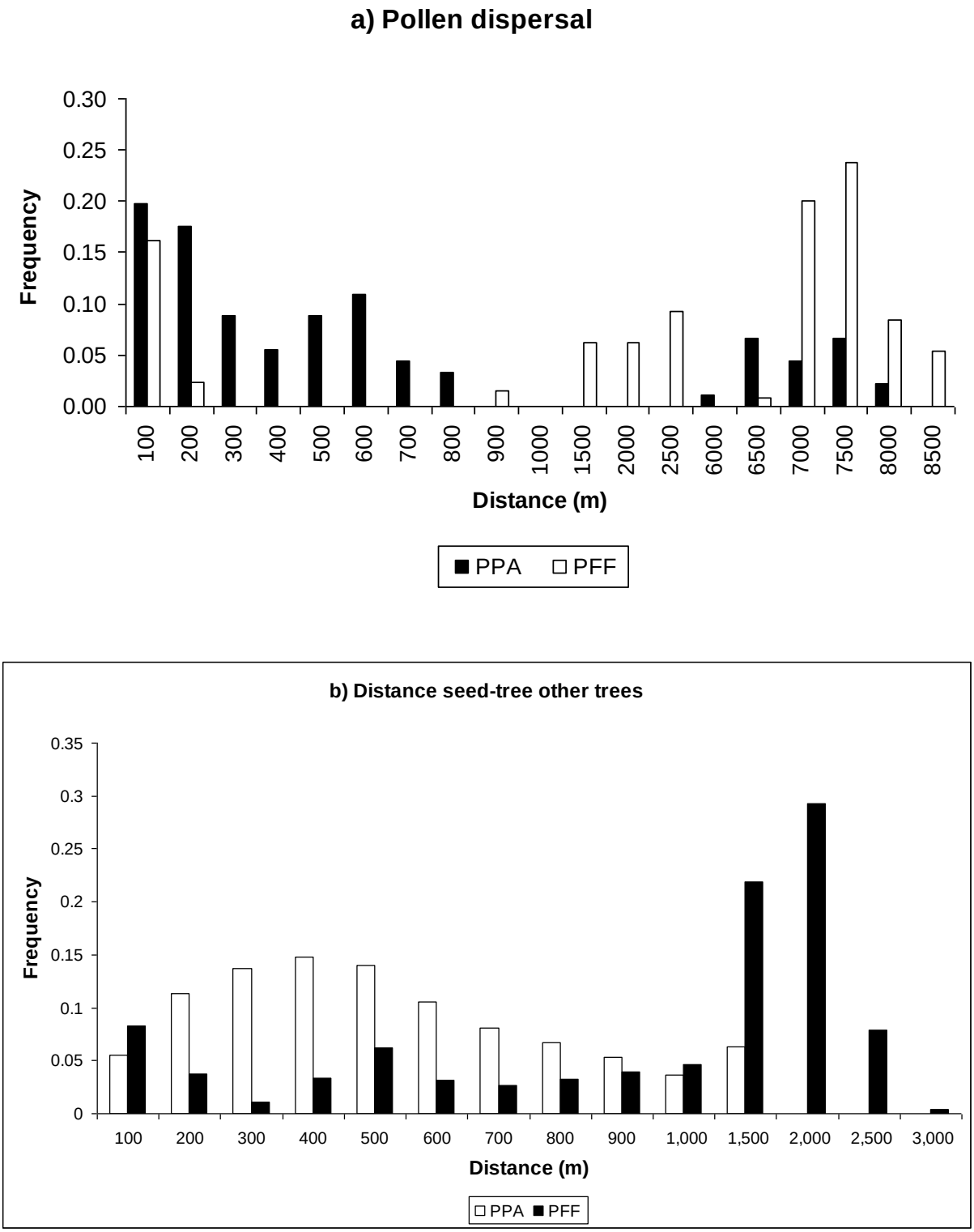
For 227 juveniles sampled in the PFF, a putative maternal parent was found for 106 (47%), being 76 juveniles (33%) originated from maternal trees in PFF, 30 (13%) were originated from trees in PPA and 121 (53%) by trees outside the two populations, suggesting a seed immigration rate of 66% (13%+ 53%). For 227 juveniles sampled in the PFF, a putative pollen parent was found for 151 (67%), being 73 juveniles (32%) originated from pollen donors in PFF, 78 (34%) were originated from trees in PPA and 76 (34%) by trees outside the two populations,

suggesting a pollen immigration rate in PFF of 67% (34%+ 33%). For the juveniles, the same seed tree has been determined as the pollen donor for 34 individuals, suggesting a selfing rate of 15% (Table 8).

Distance and patterns of effective dispersion of pollen for progeny

For the seeds of the PFF and PPA which had the father found within the both populations, the average distance of pollen dispersal was 4.7 km to 1.6 km and the median of 6.7 km and 0.31 km, respectively, which indicates long-distance dispersal (Table 8). The effective pollination neighbor area (A_{ep}) was very high (minimum of 4.38 ha in PPA), indicating an effective pollination neighbor radius (r_{ep}) of 4.4 km and 3.7 km in PFF and PPA, respectively. In PFF, the comparison between the frequency distribution of pollen dispersal distance with the frequency distribution of the distance among all trees in PFF and PPA, using the Kolmogorov-Smirnov test (Figure 4) indicated that these are statistically different in both PFF ($D = 0.326$, $P < 0.0001$), and PPA ($D = 0.607$, $P < 0.0001$). Therefore the spatial distance between the trees not explain pollen dispersal patterns in the populations.

Figure 4. Effective distance of pollen dispersal in the PFF and PPA (a), distance seed-trees other trees in both populations (b), determined by analysis of relatedness in progeny of *Hymenaea stigonocarpa*.

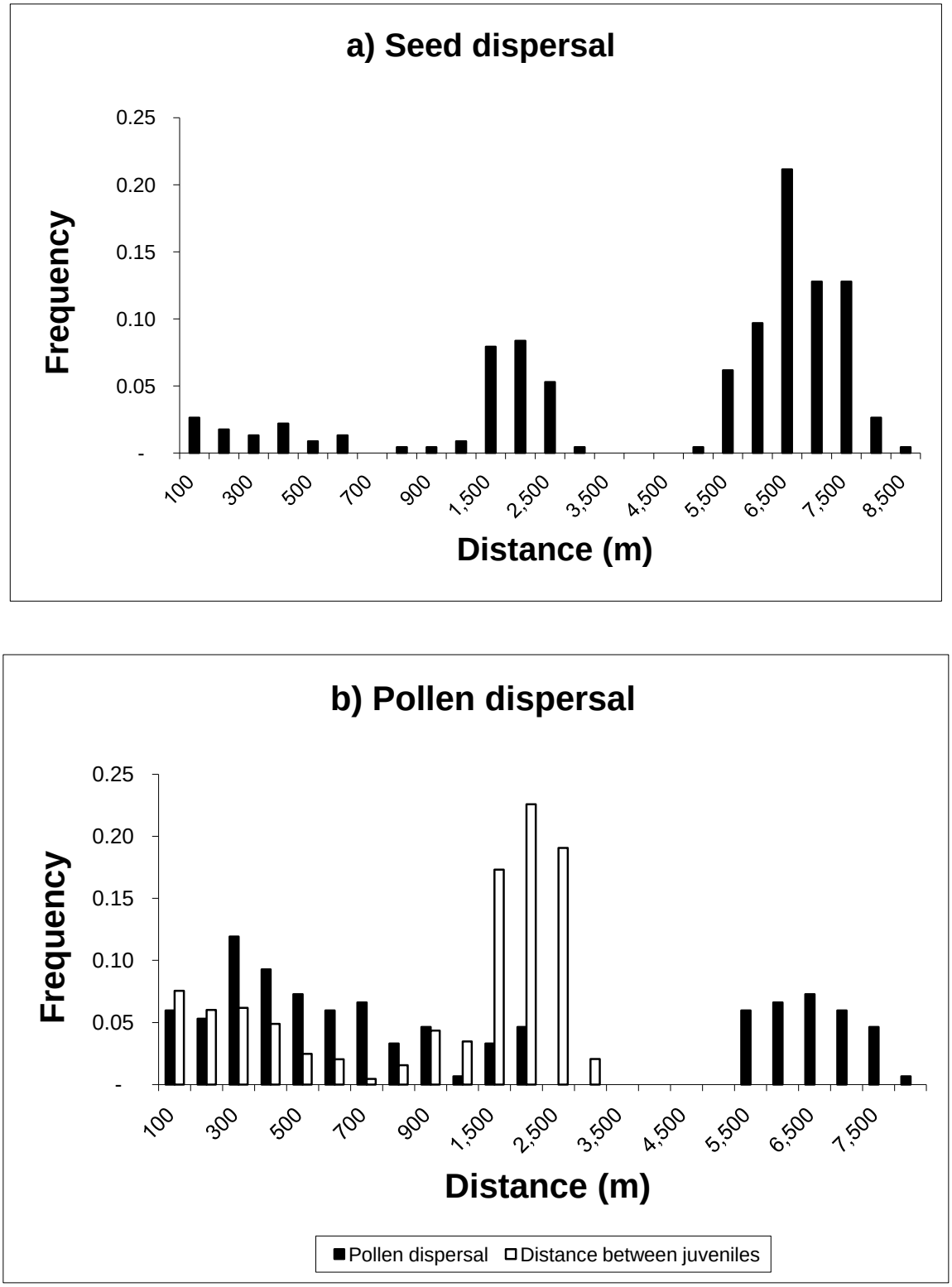


Source: Prepared by author.

Distance and patterns of dispersion realized of pollen and seeds for juveniles

The juveniles in PFF who had the father and mother found within the PFF and PPA, the average distance of seed dispersal distance was high (5.0 km, median of 6.5 km), which also indicates long-distance seed dispersal. For juveniles who had found his father into the PFF, the average distance of pollen dispersal was 2.5 km and the median was 0.65 km, showing a isolation by distance of pollen dispersal. The effective area of pollination (A_{ep}) was 5.42 ha and the pollination radius (r_{ep}) was 1.7 km (Table 8). The comparison between the frequency distribution of pollen dispersal distance with the frequency distribution of the distance among all trees in PFF and PPA (Figure 5b) was also statistically different (D = 0.133, P < 0.0001) and thus the spatial distance between the trees not also explain pollen dispersal patterns.

Figure 5 - Distance realized seed (a) and pollen (b) dispersal in the PFF, determined by analysis of relatedness in juveniles of *Hymenaea stigonocarpa*.



Source: Prepared by author.

Coancestry, survival and growth rate and Inbreeding depression

The estimate of the average pairwise coancestry coefficient between assigned parents assumed as not relatives ($\theta_{xy} < 0.1$) was very low in both populations (maximum of 0.012 in PPA), between the expected for half-sibs to full-sibs in parents assumed as relatives ($\theta_{xy} \geq 0.1$, Table 9).

The total survival in the progeny test was low (58.9%), being PFF (63.6%) greater than the PPA (54.6%). The outcrossed progeny had higher levels of survival in PFF (77.8%) and PPA (77.2%) than the outcrossed progeny from mating among related individuals (67.4% and 57.6%, respectively) and selfed progeny (53.1% and 43.2%, respectively). In PFF, we also detected that the outcrossed progeny had a significantly higher height value (61.4 cm) than the outcrossed progeny from related individuals (58.5 cm) (Table 9).

Table 9. Analysis of relatedness among progeny, survival and height of the both populations of *Hymenaea stigonocarpa*. t_u, t_r and s are the rate of outcrossing among non-related individuals, among related individuals, and selfing, respectively; 95% CI is the 95% confidence interval.

PFF (95% CI)	Coancestry between parents: $\overline{\theta}$	Survival	Height (cm)
t_u	0.008 (-0.117-0.096)	77.8%	61.4 (30.0-85.0)
t_r	0.201 (0.113-0.353)	67.4%	58.5 (27.0-85.6)
s	-	53.1%	47.1 (13.0-82.0)
PPA (95% CI)			
t_u	0.012 (-0.127-0.092)	77.2%	54.9 (24.0-88.5)
t_r	0.231 (0.112-0.467)	57.6%	52.4 (20.0-83.0)
s	-	43.2%	51.6 (16.0-86.5)

Source: Prepared by author.

The present results showed inbreeding depression for inbred seedlings for all two studied traits (Table 10). The greater inbreeding depression was in the selfed progeny in terms of survival in both populations and height in PFF than in the progeny was done by mating among relatives. The total inbreeding depression (δ_T) in terms of survival was greater in PPA than in the PFF, but in terms of height the PFF was greater than the PPA.

Table 10. Inbreeding depression (ID) in percent (%) due to mating between relatives (δ_r), selfing (δ_s) and total (δ_T) in terms of survival (SUR) and height (H) in the PFF and PPA of *Hymenaea stigonocarpa*.

	PFF (%)		PPA (%)	
ID	SUR	H	SUR	H
δ_r	13.31	4.75	25.46	4.74
δ_s	31.80	23.31	44.05	6.13
δ_T	22.25	12.63	35.59	5.40

Source: Prepared by author.

4.3 DISCUSSION

4.3.1 Pollen flow

Our results showed high rate of pollen flow in both PFF and PAA populations (minimum m_p = 38%). Thus, the fragmented populations and spatially isolated trees in the landscape are not reproductively isolated. For understanding of pollen flow of the species, it is imperative to know the pollinator behavior and studied plant. It is

estimated that the bat need to consume 1 mg sugar or about 5 µl nectar per day, whereas on flower nectar has a sugar concentration of 20% (NASSAR et al., 1997). In Brazil, Bobrowiec and Oliveira (2012) studied in the savannah region in relationship of pollination x plant in four species pollinated by bats: *Bauhinia holophylla, H. stigonocarpa*, *Luehea grandiflora* and *Caryocar brasiliense*. These species *Hymenaea stigonocarpa* showed the most producing nectar (430 µl/h), higher concentration of sugar in nectar (701 mg sugar) and fewer flowers visited by tree (5 flowers). Another important point is the morphology of the pollen grain of the species pollinated by bats. Pollen grain is large, dense, oval shape, which adheres easier the animal, making it common to mix pollen when the bat feeds (STROO, 2000). These considerations may explain the fact that both the populations present high rate of pollen immigration and selfing. High rate of pollen immigration has also been detected in other Neotropical tree species, animal pollinated, as for example 30% in *Carapa guianensis* (MARTINS et al., 2012), 74% in *Copaifera langsdorffii* (TARAZI et al., 2013), minimum of 34% in *Dipteryx panamensis* (HANSON et al., 2008), 38-55% in *Hymenaea courbaril* (CARNEIRO et al., 2011, LACERDA et al., 2008), 31-35% in *H. stigonocarpa* (MORAES and SEBBENN, 2011), and 37-50%, in *Swietenia macrophylla* (SEBBENN et al., 2012).

4.3.2 Mating system

The effective selfing rate, measured in open-pollinated seeds was similar between the populations (39 and 45%), but was higher than the realized selfing rate, measured in the juveniles of PFF (15%). This result suggest selection against inbreed individuals between seed to juvenile stage. This phenomenon has been also reported in *Platipodium elegans* (HUFFORD; HAMRICK, 2003).

The estimates of the effective selfing rate in *H. stigonocarpa* were consistent with the observed for other bat pollinated tree species of the savanan region, *Caryocar brasiliense*, 39% (COLLEVATI et al., 2009). Interestingly, the authors point out that in one of the seed trees showed 75% of selfing, confirming the above idea that when the plants are pollinated by bats, because of their mobility habits and food, it is common show high rates of self-fertilization.

4.3.3 Pollen dispersal distance

The present study shows that the extent of pollen dispersal distance reach great distance (8.5 km), but most pollination events occurred at distances less than 0.200 km (37%) for PPA and 7,0 to 7,5 km (43.8%) to PFF. These differences in distance between populations can be explained by the way the trees are arranged in the landscape. In PPA the trees are in a range of distance between 0.30 m to 0.50 km. In contrast, in the PFF, about 51% of the sampled trees are in a range of distance of 1,5 to 2,0 km. As there is gene flow between the two populations, the average pollen dispersal distance (1.6 km in PFF and 4.7 km in PPA) in *H. stigonocarpa* was greater than the distance found in other species pollination by bats. For example, Mexico, Quesada et al. (2013) studied the species *Ceiba aesculifolia* in a forest fragment (disturbed area, 3 trees/ha) and an ecological reserve (undisturbed area, 6 trees/ha), observed an average dispersal of 0.315 km and 0.217 km, respectively. In the Brazilian Amazon forest, Lacerda et al. (2008) observed in *Hymenaea courbaril*, with a density of 0.23 trees/ha, the average pollen dispersal distance was 0.827 km. In Costa Rica, Rymer et al. (2013) studying *Pachira quinata* occurring in a pasture (14 tree/ha) close to a rainforest, observed an average pollen dispersal distance of 0.438 km. In Brazil, the savannah region, close the present study area, Moraes and Sebbenn (2011) observed in *H. stigonocarpa* in the pasture, with a density of approximately 0.0094 tree/ha, an average pollen dispersal distance of 0.719 m, with 72% of pollination occurring in shorter distances than 1,0 km. In contrast, the greatest distance reported pollinated by bats was in species of low density found in the tropical rainforest *Ceiba pentandra* with 18 km (GRIBEL and LEMOS, 1999). To illustrate the bat's ability to travel long distances for pollination than other insect pollinated trees species, pollen dispersal has been detected until 4.5 km in the bee pollinated *Swietenia humilis* (WHITE et al., 2002), moths run 0.28 km in *Cordia alliodora* (BOSHIER et al., 1995), insects run up to 0.35 km in *Pithecellobium elegans* (CHASE et al., 1996), birds run up to 2.8 km in *Symphonia globulifera* (CARNEIRO et al., 2009), bees run 1.5 km in *Dinizia excelsa* (DICK et al., 2003) and wasps run up to 14.2 km in *Ficus dugandii* (NASON et al., 1998). At the time, pollination by bats is efficient because of isolated trees in pasture serve as "perch" for rest of these animals, allowing traverse long distances, but with the rapid deforestation of the savannah, this situation can change quickly. As a preventive

measure, it would be appropriate to take conservation measures of these populations in the study sites to preserve the environment ecosystem dynamics, in terms of relative pollinator x tree.

4.3.4 Pollination neighborhood area

The effective pollination neighboring area (A_{ep}) refers to the area where 63% of the mating events occurring (LEVIN, 1988). Our estimate indicates high A_{ep} for the studied populations (PFF= 4.38 ha, PPA= 6.17 ha). The cause is the low population density in both populations, associated to the fact pollinator vector is capable of flying great distances in foraging trip. Other factor that contribute to the high A_{ep}, is that the populations have large areas of pollination can not refer to a great reproductive area, whereas in the case of *H. stigonocarpa* pollinator vector, visit few flowers by individuals, due to the high production of nectar per flower, and move to the next tree.

To get an idea of the extent of the area in the literature is find area of reproductive neighborhood of 25 ha for species *Cordia alliodora* pollinated by moths (BOSHIER et al., 1995), from 6.36 ha to 209 ha for species pollinated by bees *Swietenia humilis* and *Carapa guianensis,* respectively (CLOUTIER et al., 2007; WHITE et al., 2002), 0.88 ha for species *Symphonia globulifera* pollinated by birds (DEGEN et al., 2004), 10.5 ha to 63.3 ha for species pollinated by wasps *Ficus obtusifolia* and *Ficus dugandii,* respectively (NASON et al., 1998) and 14 ha to 715 ha for species pollinated by animals *Dinizia excelsa* (DICK et al., 2003).

4.3.5 Seed dispersal distance

The *H. stigonocarpa* has an ecological importance very great, because its fruits are attractive to wildlife, which are the possible seed dispersers. Our results showed that the seed were dispersed in long distance reaching and average of 5 km. Considering the area of the forest fragment of 736 km^2 (23 x 32 km), can be said that the animal dispersers of seeds has great mobility on site, then possibly they feed on fallen fruit (or not) nearby trees, and after then go to their dens, and so the seeds are

dispersed over long distances. Remembering that around this fragment has pasture and eucalyptus plantations, so the animals concentrate most foraging part within the fragment.

The average seed dispersal distance *H. stigonocarpa* is higher than has been found for animal and wind seed dispersal species. For example, the seed dispersal by animals ranged from 5 m (*Theobroma cacao,* SILVA et al., 2011) to 300 m (*Bagassa guianensis,* SILVA et al., 2008) and by wind ranged from 30 m (*Machaerium villosum,* GIUGICE-NETO and KAGEYAMA, 2000) to more than 1,000 m (*Aucoumea klaineana,* BORN et al., 2008).

4.3.6 Inbreeding depression and your implication for conservation genetic

Another point that lack information on *H. stigonocarpa* as well as in almost all other native tree species in Brazil is about the inbreeding depression. The inbreeding depression refers to the decline of the values of a quantitative or qualitative as a direct consequence of inbreeding (WRIGHT, 1977). It is increased homozygosis in individuals originating from selfing and mating among related individuals. In natural populations of tree species, inbreeding can be generated by the behavior of pollinators, visiting mainly flowers on the same tree (selfing) due to the internal structure of populations in groups of related individuals, located spatially close, or by reducing the size populations (ALLARD, 1971). This happened in the present study. Initially, it observed high rates of abortive seeds within the fruit were subsequently observed high mortality in progeny test. Thus, it was deemed appropriate to estimate the cause of mortality, as initial hypothesis, the strong presence of inbreeding depression.

The results of this study show evidence of inbreeding depression especially for selfing progeny in both characters studied in two populations. The degree of relationship between the parents has been confirmed on the values of coancestry coefficient that was between to the expected in half-sibs to full-sib parent. This is worrying, because the reduction of individuals in populations of *H. stigonocarpa*, increases the number of relatives individuals as a result of bats pollinate the same trees, making it strong inbreeding depression. Thus, all the ecosystem is affected

both the bats as the disperser animals seeds, because the production of fruits and seeds can decrease with increasing degree of relatedness of trees, causing the animals leave the area in search food. Another point is in relation to seed production is the fact that if the seeds have inbreeding depression, there is a tendency to have an increase of aborted seeds. This may influence the seed bank that would be in the area for the emergence of regenerants the area, or will interfere with the maintenance of the population. Therefore is very important the conservation of the areas studied for the survival of the populations, such as *in situ* conservation and to establish *ex situ* conservation strategies from the collection of seeds. The inbreeding depression suggested that we must collect about 50% more seed than expected (at least 30 seeds per tree), as the high mortality rate of the progeny in the field.

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ATTACHMENT

A6- Fruit and seeds of *Hymenaea stignocarpa.*

Source: Prepared by author

A7- Seeds classified in hierarchical form of *H. stignocarpa.*

Source: Prepared by author.

A8- Seedlings of *H. stignocarpa.*

Source: Prepared by author.

A9- Progeny test of *H. stignocarpa.*

Source: Prepared by author.

A10- Overview of the pasture population of *H. stignocarpa.*

Source: Prepared by author.

A11- Overview of the forest fragment population of *H. stignocarpa.*

Source: Prepared by author.

A12- Juvenile of the forest fragment population of *H. stignocarpa.*

Source: Prepared by author.