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Fragmentation and spatial genetic structure in *Tabebuia ochracea* (Bignoniaceae) a seasonally dry Neotropical tree

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ABSTRACT

In this study we investigate the effect of fragmentation and disturbance on the spatial genetic structure, heterozygosity and inbreeding in *Tabebuia ochracea* (Bignoniaceae) in a seasonally Neotropical dry forest in the medium São Francisco River basin, Centre-East Brazil, based on the polymorphism at seven microsatellite loci. Four populations with different histories of disturbance and fragmentation were sampled: two continuous population (CP1 and CP2), with no history of recent disturbance and two fragmented and isolated population (FP1 and FP2), with recent history of disturbance due to logging for pasture establishment. Fragmented and continuous populations did not differ in any estimated parameter. However, all populations showed low levels of polymorphism and genetic diversity and high levels of inbreeding. Also, no spatial genetic structure was detected for populations using SPAGeDI software and no differentiation between these four populations was detected by Bayesian analyses performed with STRUCTURE software ($K = 1$). Differentiation measure by Wright's θ (0.032) and Hedrick G_{ST} (0.032) were significant but low. Our results strongly suggest that continuous populations are seed sources for the fragmented populations and that fragmentation and disturbance have been affecting these populations of *T. ochracea* in the Centre-East Brazil, leading to low levels of polymorphism and genetic diversity, and high inbreeding. Therefore, conservation efforts should increase in this region, with a reduction of agriculture expansion and the remove of cultivated areas and cattle from the Mata Seca and Lagoa do Cajueiro State Parks.

1. Introduction

Habitat fragmentation is one of the main causes of biodiversity loss (Sala et al., 2000). Forest fragmentation reduces continuous habitats into small and isolated remnants, leading to a decrease in the effective population size and a disruption on ecological and genetic processes (see Aguilar et al., 2008; Ewers and Didham, 2006 for reviews). In small and isolated populations, the number of alleles may decrease due to genetic drift and inbreeding is unavoidable due to small effective size and low gene flow among isolated populations (Frankham, 2003; Gilpin and Soulé, 1986). The consequent loss of genetic variability may lead to a reduction in individual fitness and in population evolutionary potential (Lande, 1988; Reed and Frankham, 2003).

Plant reproduction is generally negatively affected by fragmentation (see Aguilar et al., 2006; Lowe et al., 2005 for reviews). 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 (e.g. Cascante et al., 2002; Dick, 2001; Goverde et al., 2002; Quesada et al., 2004). Fragmentation can also affect seed dispersal by changing abundance, richness or behavior of animal seed dispersers (Ghazoul, 2005). In the same manner, habitat fragmentation can affect recruitment due to disruptions in ecological process such as competition with invasive plant species (Ewers and Didham, 2006), and also change the spatial genetic structure that is ultimately affected by ecological and genetic processes (e.g. Born et al., 2008; Nason et al., 1997).

The spatial genetic structure (SGS) is the non-random distribution of genotypes within a population (Vekemans and Hardy, 2004) as the outcome of gene flow, genetic drift and selection (Wright, 1940). Besides, recent colonization events and density of maternal seed sources may foster SGS within populations (Jones et al., 2005). Although some studies have shown the effects of fragmentation on population genetic structure of Neotropical tree species (e.g. Aldrich and Hamrick, 1998; Nason and Hamrick, 1997), few studies were performed with seasonally dry tropical forest tree species (but see Cascante et al., 2002).

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Seasonally dry tropical forests are one of the most endangered ecosystems in the world (Miles et al., 2006) but conservation efforts have been neglected (Sánchez-Azofeifa et al., 2005). The largest remnants of seasonally dry tropical forest are found in Southeast and Central Brazil (Miles et al., 2006). However, these areas are threatened because of agricultural and pasture expansion, harvesting for wood products and the increase of fire frequency due to agricultural practices (Espírito-Santo et al., 2009).

Tabebuia ochracea (Cham.) Standl. (Bignoniaceae) is a widely distributed Brazilian tree species, commonly found in seasonal savannas (cerrado *sensu stricto*) and also in seasonally dry tropical forests of Central-East Brazil, and Costa Rica in Central America. The species is pollinated by large bees, such as bumblebees and carpenter bees (Barros, 2001) and seeds are small and wind-dispersed.

In this paper we report on the genetic diversity and spatial genetic structure of *T. ochracea* over both continuous and fragmented/disturbed forests. Our working hypothesis was that populations from fragmented and disturbed forests have lower levels of genetic diversity and higher levels of inbreeding than populations from continuous forest. We also tested the hypothesis that spatial genetic structure is reinforced by forest fragmentation and disturbance due to restriction in gene flow and disruption in ecological process related to population dynamics.

2. Materials and methods

2.1. Study site, sampling and DNA extraction

The study was conducted in the Mata Seca State Park (14°56′59″S 44°04′12″W) and Lagoa do Cajueiro State Park (14°92′90″S 43°92′15″W), in the medium São Francisco River basin, Centre-East Brazil (Fig. 1). The region is inside the Ecological Unit 3A *sensu* Silva and co-workers (Silva et al., 2006) characterized

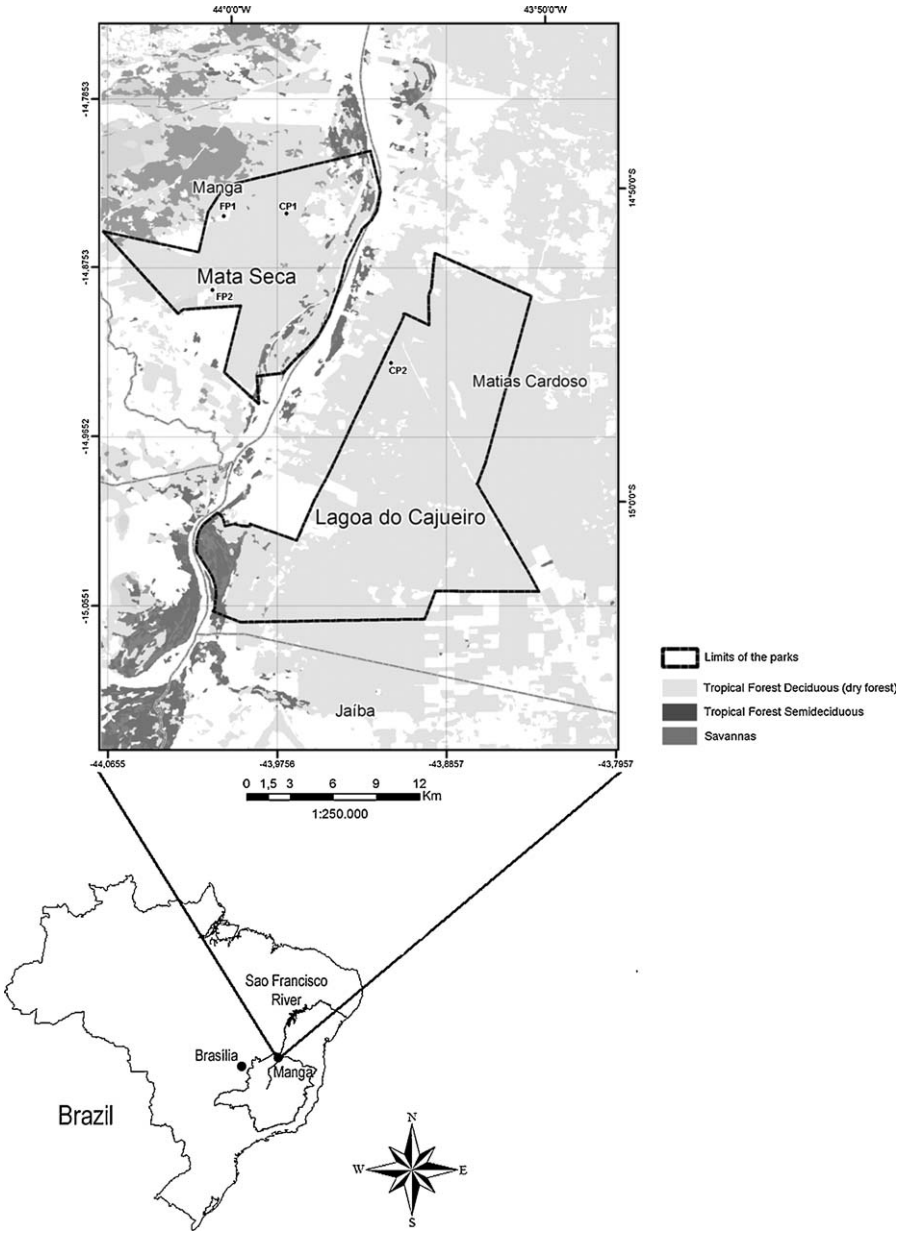

Fig. 1. Localization of *Tabebuia ochracea* populations sampled in the Mata Seca State Park (CP1, FP1 and FP2) and Lagoa do Cajueiro State Park (CP2), in the medium São Francisco River basin, Centre-East Brazil. The distance between each pair of populations is: CP1 and CP2 10 km; CP1 and FP1, 4 km; CP1 and FP2, 5 km; FP1 and FP2, 6 km; FP1 and CP2, 13 km; FP2 and CP2, 12 km.

by plain land with some rolling terrain dominated by a mosaic of deciduous and semi-deciduous forest and with some dense savanna. It has a very old history of human disturbance, since the 17th Century (Rodrigues, 2000) and the landscape is now dominated by a mosaic of small remnants of seasonally dry forests in different successional stages and crops and pasture. Both parks are conservation areas of seasonally dry forests with great extensions of abandoned pastures and crop areas and also by illegal settlement of farmers (IEF, 2000). In addition, there are large pasture areas around both parks that may lead to an increase of fire frequency and also cattle incursion into the parks (IEF, 2000). Both banks of São Francisco River inside the parks are occupied by fishery activities. As the region has an old history of human occupation it is difficult to disentangle the effects of fragmentation (reduction in area) and disturbance causing secondary succession.

Four populations with different histories of disturbance and fragmentation were sampled: two continuous population with no history of recent disturbance (<50 years), CP1 and CP2, and two fragmented and isolated population, with recent history of disturbance due to logging for pasture establishment (FP1 and FP2). Populations CP1, FP1 and FP2 are localized in the Mata Seca State Park, in the left bank of the São Francisco River, and population CP2 is localized in the Lagoa do Cajueiro State Park, in the right bank (Fig. 1). In each population, all individuals in a permanent plot of 1000 m^2 (20 m × 50 m) were sampled. Individuals were classified into two stages: juveniles (non-reproductive) and adults (reproductive individuals). Expanded leaves were collected from all individual plants and stored at −80 °C. Genomic DNA extraction followed the standard CTAB procedure (Doyle and Doyle, 1987).

2.2. Microsatellite analysis

Seven microsatellite loci (Tau 07, Tau 12, Tau 15, Tau 17, Tau 28, Tau 30, Tau 31) previously developed for *Tabebuia aurea* and transferred for other *Tabebuia* species (Braga et al., 2007) were used to genotype all sampled individuals. Microsatellite amplifications were performed in a 10 μL volume containing 10.0 μM of each primer, 1 unit of Taq DNA polymerase (Phoneutria, BR), 250 μM of each dNTP, 1× reaction buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 0.25 μg of BSA and 10.0 ng of template DNA. Amplifications were performed using PE9700 thermal controller (Applied Biosystems, CA) under the following conditions: 94 °C for 5 min (one cycle); 94 °C for 1 min, 48 to 62 °C for 1 min (according to each primer), 72 °C for 1 min (35 cycles); and 72 °C for 30 min (one cycle). The PCR products were electrophoresed on an ABI Prism 3130*xl* automated DNA sequencer (Applied Biosystems, CA) and were sized by comparison to a 500 internal lane standard ROX (Applied Biosystems, CA). Fluorescent PCR products were automatically sized using Genescan and Genotyper softwares (Applied Biosystems, CA).

2.3. Genetic diversity and spatial genetic structure

Microsatellite loci were characterized for the number of alleles per locus and observed (H_o) and expected heterozygosity under Hardy–Weinberg equilibrium (H_e) (Nei, 1978), based on adult individuals. Fixation index (f), for each locus and over all loci, were also estimated (Nei, 1978). Departure from linkage equilibrium was verified for all pairs of loci. Analyses and randomization based tests with Bonferroni correction were performed with the software FSTAT 2.9.3.2 (Goudet et al., 1996; Goudet, 2002).

To verify if fragmentation leads to a reduction in genetic diversity and increase in inbreeding, observed and expected heterozygosities, fixation index and allelic richness were estimated and significance tests were performed for each population and for juveniles and adults separately, also using FSTAT software. Permutation tests implemented in the software FSTAT were also used to test for differences in the above mentioned population parameters between each pair of populations and between juveniles and adults within populations.

The effect of fragmentation on SGS was assessed by an autocorrelation analysis. Pairwise kinship coefficients (F_{ij}) were estimated based on Nason equation (see Loiselle et al., 1995), among individuals from a set of distance class using the software SPAGeDi (Hardy and Vekemans, 2002). Permutation tests (1000 permutations) were performed to verify significance of kinship for each distance class and the regression and the kinship values were plotted against the distance classes to visualize SGS. Standard errors (SE) were estimated by jackknife over loci.

To verify if all sampled individuals comprise a single gene pool or if they belong to different demes or populations, a Bayesian analysis of population structure was carried out with the software STRUCTURE 2.2 (Pritchard et al., 2000, 2007). The analysis is based on a clustering method for inferring population structure using genotype data consisting of unlinked markers to identify the presence of population structure and distinct genetic populations (Pritchard et al., 2000). A burn-in period of 100,000 generations and 100,000 steps of Markov Chain Monte Carlo simulations were used to estimate $\ln \Pr(X/K)$, F_{ST} and Q (individual ancestry) for different values of K (number of populations). The analyses were run for $K = 1$ to $K = 8$. Admixture model (individuals may have mixed ancestry), a reasonably model for dealing with many of the complexities of real populations, and correlated allele frequencies model (frequencies in the different populations are likely to be similar probably due to migration or shared ancestry), which performs better with inbreeding were considered for all analyses (see Pritchard et al., 2007). For each K value, 10 runs were carried out to verify the consistency of the results. We also assessed genetic differentiation among populations using Wright's F-statistics, F, θ, and f (Wright, 1951), obtained from an analysis of variance of allele frequencies (Cockerham, 1969; Weir and Cockerham, 1984) and by G'_{ST} (Hedrick, 2005), a standardized measure of genetic differentiation which better deals with the high mutation rate of microsatellite loci. The analyses were performed using FSTAT, version 2.9.3.2 (Goudet, 2002). A significance test of differentiation with Bonferroni correction was performed by randomizing genotypes among samples to obtain the log-likelihood G statistics (Goudet et al., 1996).

3. Results

All pairs of microsatellite loci were in linkage equilibrium (all $p > 0.002381$, adjusted nominal 5% level with Bonferroni correction). For most loci, the observed heterozygosity was lower than the expected under Hardy–Weinberg equilibrium, with fixation indexes significantly different from zero (Table 1). Nevertheless, the combined probability of paternity exclusion ($QC = 0.9073$) was high and the probability of identity (IC) was very low, $\sim 10^{-22}$ (Table 1), showing that the battery of loci is suitable for population genetic analyses.

Although fragmented population FP1 presented the highest density of juveniles, it presented no adults (Table 2). Continuous populations presented higher density of adults than fragmented populations (Table 2).

Allelic richness was very similar between continuous populations, and also between stages in continuous population (Table 2). Fragmented population FP2 presented a slightly lower allelic richness but differences were not significant ($p > 0.57$ for all comparisons). Also, genetic diversity was not statistically different between populations or stages within populations ($p > 0.22$ for all comparisons). Fixation index was high and significant for all

Table 1
Characterization of seven microsatellite loci, based on a sample of 138 adult individuals of *Tabebuia ochracea* from Mata Seca State Park and Lagoa do Cajueiro State Park, in the medium São Francisco River basin, Centre-East Brazil.

Locus	*A*	H_e	H_o	*f*	*Q*	*I*
Tau 07	10	0.406	0.410	-0.247^{ns}	0.317	0.0005
Tau 12	8	0.581	0.145	0.711	0.383	0.0000
Tau 15	8	0.187	0.190	-0.061^{ns}	0.282	0.0012
Tau 17	3	0.482	0.314	0.623	0.260	0.0013
Tau 28	3	0.054	0.025	0.664	0.128	0.0426
Tau 30	6	0.630	0.721	-0.254^{ns}	0.354	0.0002
Tau 31	7	0.500	0.269	0.360	0.261	0.0018
Over all loci	6.42	0.406	0.296	0.220	$QC = 0.9073$	$IC = 7.655 \times 10^{-22}$

A, number of alleles; H_e, expected heterozygosity; H_o, observed heterozygosity; *f*, fixation index, *Q*, probability of paternity exclusion; *QC*, combined probability of paternity exclusion; *I*, probability of genetic identity; *IC*, combined probability of genetic identity. Values followed by ns did not statistically differ from zero, for $p = 0.005$, Bonferroni adjusted *p*-value for a nominal level of 5%.

Table 2
Characterization of *Tabebuia ochracea* populations sampled in the Mata Seca State Park (CP1, FP1 and FP2) and Lagoa do Cajueiro State Park (CP2), in the medium São Francisco River basin, Centre-East Brazil, based on seven microsatellite loci. Sample size was 1000 m^2 for all populations.

Population	Stage	*N*	Density (Ind/m^2)	*A*	H_e	H_o	*f*
CP1	Juveniles	56	0.056	4.29	0.380	0.251	0.340
Continuous	Adults	56	0.056	4.43	0.398	0.285	0.285
	Overall	112	0.112	4.36	0.388	0.268	0.310
CP2	Juveniles	12	0.012	3.57	0.465	0.400	0.145
Continuous	Adults	58	0.058	4.43	0.453	0.368	0.189
	Overall	70	0.070	4.00	0.459	0.384	0.167
FP1	Juveniles	89	0.089	5.00	0.425	0.331	0.222
Fragmented/isolated/disturbed	Adults	0	–	–	–	–	–
	Overall	89	0.089	5.00	0.425	0.331	0.222
FP2	Juveniles	06	0.006	2.57	0.508	0.409	0.251
Fragmented/isolated/disturbed	Adults	24	0.024	3.71	0.464	0.377	0.192
	Overall	30	0.030	3.14	0.486	0.393	0.221
Overall		301		4.13	0.439	0.344	0.230

N, number of individuals sampled in each area; *A*, allelic richness; H_e, expected heterozygosity; H_o, observed heterozygosity; *f*, fixation index overall loci. All values of *f* are significant ($p < 0.002$, adjusted nominal 5% level with Bonferroni correction).

populations (Table 2). These patterns were maintained even comparing only juveniles from fragmented and continuous populations (Table 2).

Kinship was not significant ($p > 0.05$) for all distance classes and no spatial genetic structure was detected in all analyzed populations (Fig. 2; for CP1 $b = 0.8 \times 10^{-8}$, $p = 0.800$; for CP2 $b = 0.1 \times 10^{-3}$, $p < 0.001$; for FP1 $b = -0.2 \times 10^{-2}$, $p = 0.051$). When juveniles and adults from CP1 were analyzed separately, the same pattern was found (Fig. 3, for juveniles $b = -0.2 \times 10^{-3}$, $p = 0.517$; for adults $b = -0.1 \times 10^{-7}$, $p = 0.570$). The SGS analysis was not performed for population FP2 because of the low number of individuals. Also, analyses of juveniles and adults separately could not be performed for populations CP2 and FP2 because of the low number of individuals in each life stage.

Bayesian analyses showed no population structuring ($K = 1$, $\ln P(X/K) = -1984.2$). The assignments were roughly symmetric to all populations ($\sim 1/K$) when $K > 1$ and no individuals were strongly assigned indicating that there was no population structure. This result was confirmed by Wright's *F*-statistics and by G'_{ST}. Although significantly different from zero, differentiation measured by θ (0.032 ± 0.016, $p < 0.001$) and by G'_{ST} (0.032, $p < 0.001$) was very low. However, *F* (0.259 ± 0.161, $p < 0.001$) and *f* (0.234 ± 0.158, $p < 0.001$) were high and significant.

4. Discussion

Although some loci presented a significant excess of homozygotes, the low value of combined probability of identity (*IC*) showed that the battery of loci is suitable for kinship analysis. The analysis of raw data in MICRO-CHECKER software (Oosterhout et al., 2004) showed that the results were not affected by genotyping errors (results not shown).

Fragmented and continuous populations did not differ in any estimated parameter. Conversely, fragmented populations tended to present slightly higher genetic diversity and allelic richness than continuous population and all four populations presented high levels of inbreeding. Polymorphism was low, comparing to other Neotropical tree species (e.g. Collevatti et al., 2001; Lemes et al., 2003; Braga et al., 2007). The low polymorphism and high fixation indexes may be caused by restricted pollen dispersal. *T. ochracea* has a highly synchronous flowering in a short time during the dry season favoring a high within plant and patch residence time of bee pollinators, increasing biparental inbreeding. This behavior may be reinforced by the isolation of adults in some remnant forests in this region.

Low polymorphism and genetic diversity may also be caused by fragmentation and disturbance history. Although the continuous population have no history of recent disturb (at least 50 years), the region of São Francisco River basin, Centre-East Brazil, is highly disturbed and was occupied in the 17th Century. Due to the high soil fertility this region has been receiving governmental financial support for agribusiness improvement and irrigation projects, such as Jaíba Irrigation project (see www.projetojaiba.com.br). Thus, the remnant forests have been isolated for many generations (Espírito-Santo et al., 2009).

The long distance seed dispersal may be responsible for the absence of SGS in all populations analyzed. Besides, Bayesian analyses showed that individuals of *T. ochracea* from all populations belong to the same gene pool. Also, θ and G'_{ST} were very low, and *F* and *f* were high, showing that differentiation due to genetic

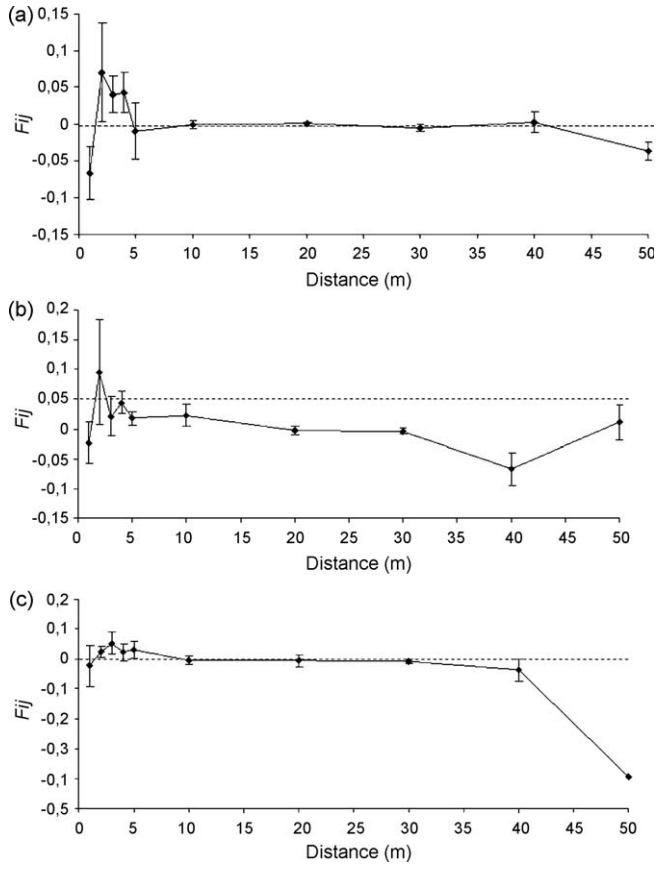

Fig. 2. Relationship between kinship ($F_{ij} \pm$ SE) and distance for populations of *Tabebuia ochracea* sampled in the Mata Seca State Park and Lagoa do Cajueiro State Park, in the medium São Francisco River basin, Centre-East Brazil. (a) All individuals from CP1; (b) all individuals from CP2; (c) all individuals from FP1.

drift is low but non-random mating may be important in shaping the genetic structure of these populations. This result was unexpected because the continuous population CP2 is 10 km distant from CP1 (the nearest *T. ochracea* population) and in the opposite river bank (Fig. 1). Hence, our results strongly suggest that

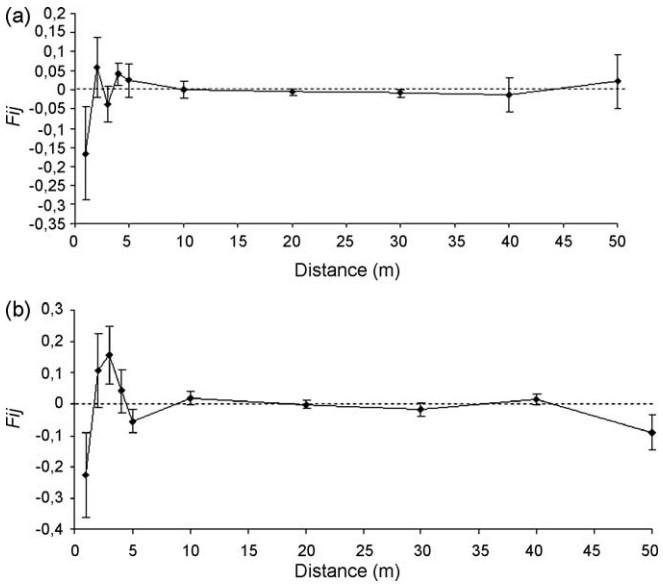

Fig. 3. Relationship between kinship ($F_{ij} \pm$ SE) and distance for the continuous population CP1 of *Tabebuia ochracea* sampled in the Mata Seca State Park, in the medium São Francisco River basin, Centre-East Brazil. (a) Juveniles and (b) adults.

T. ochracea presents long distance seed dispersal. Seed dispersal from continuous populations to the fragmented population FP1, that has no adults, may be responsible for the lack of significant genetic differentiation. Wind may promote long distance seed dispersal (Horn et al., 2001), and wind-dispersed plants are likely to be relatively immune from disturbances of landscapes (Ghazoul, 2005) because wind dispersal may be facilitated by fragmentation and trees with small seeds dispersed by wind are favored over animal-dispersed trees with large fruits (Cordeiro and Howe, 2003; Fore et al., 1992; Tabarelli and Peres, 2002). The fragmented populations FP1 and FP2 were clear-cut for pasture establishment. In the last 9 years the pastures have not been managed and populations of *T. ochracea* is colonizing the area. Our results suggest that the populations nearby FP1 are seed source for the fragmented populations. This may explain the high allelic richness of fragmented population FP1. Nevertheless, fragmented population FP2 presented the lowest allelic richness. This may be due to demographic stochasticity. This population presented the lowest density of juveniles showing that successful recruitment is low.

Inferring the effects of habitat fragmentation can be especially difficult for species with high distance gene flow because they tend to have relatively low levels of differentiation among populations (Waples, 1998). Additionally, the high polymorphism typically showed by microsatellite loci could mask important losses of heterozygosity that could only be detected after a certain threshold of disturbance is attained consequently taking too long or very drastic events for any fragmentation or isolation process to significantly affect heterozygosity and inbreeding at a detectable level (Collevatti et al., 2001). Nevertheless, our results strongly suggest that fragmentation and disturbance have been affecting the populations of *T. ochracea* in the seasonally dry forest of the medium São Francisco River, leading to low levels of polymorphism and genetic diversity, and high inbreeding. Therefore, conservation efforts should increase in this region, with a reduction of agriculture expansion, the remove of cultivated areas and cattle from the Mata Seca and Lagoa do Cajueiro State Park.

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