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Effects of fragmentation on density and population genetics of a threatened tree species in a biodiversity hotspot

S. M. Ganzhorn[1,2,*], B. Perez-Sweeney[1], W. W. Thomas[2], F. A. Gaiotto[3], J. D. Lewis[1]

[1]Louis Calder Center – Biological Field Station, and Department of Biological Sciences, Fordham University, Armonk, NY 10504, USA

[2]The New York Botanical Garden, Bronx, NY 10458, USA

[3]Departamento de Ciências Biológicas — Universidade Estadual de Santa Cruz (UESC), Ilhéus, BA 45662-900, Brazil

ABSTRACT: Fragmentation threatens biodiversity globally. *Manilkara maxima* (Sapotaceae) is listed by the IUCN as threatened and is an economically and ecologically important tree species endemic to the Atlantic forest of southern Bahia, Brazil, a biodiversity hotspot. The objectives of this study were to examine the effect of fragment size on density and genetic diversity of this threatened species. We surveyed and sampled 222 individuals across 2 large forest sites and 1 site comprising 8 small fragments. We focused on 5 microsatellite loci that provided comparable genetic information (average 21 alleles locus^{-1}) as 2 congeners in other studies. Fragment size accounted for 71 and 56% of density variation in adult and sapling trees, respectively, but did not account for genetic variation. Rather, density accounted for 80% of allelic diversity and 70% of allelic richness in both life stages. The lack of relationship between genetic diversity and fragment size was driven in part by a co-variation in density and genetic diversity in 25 ha fragments. These small forests can have high densities of *M. maxima*, and in turn, can have as much genetic diversity and conservation value as larger fragments. However, the larger fragments are of unique conservation value because they hold the greatest number of reproductively mature individuals, the ones necessary for the recruitment of new individuals. Our results suggest that relatively high levels of gene flow are contributing to the high genetic diversity of saplings found in some of the small fragments.

KEY WORDS: Bahia · Brazil · *Manilkara maxima* · Population genetics · Fragmentation · Neotropics · Sapotaceae

INTRODUCTION

Fragmentation of continuous habitats into smaller, isolated fragments is recognized as one of the greatest threats to biodiversity (Fahrig 2003). The acute effects of fragmentation have caused many species to exist only as small, isolated populations and be listed on The International Union for Conservation of Nature (IUCN) Red List of Threatened Species (IUCN 2013). The predicted genetic consequences of fragmenting tree species populations include reduced population size and gene flow resulting in a loss of alleles, which over successive generations will lead to increased genetic drift, reduced heterozygosity, increased inbreeding, increased genetic differentiation among populations, and potentially lower fitness (Young et al. 1996, Nason & Hamrick 1997, Aguilar et al. 2008). However, such effects are not always predictable, in part because some species have naturally scattered distributions and low densities, are long-lived and ex-

*Corresponding author: ganzhorn@fordham.edu

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perience long-distance dispersal and may thus be less affected by fragmentation, particularly relatively recent fragmentation (Kramer et al. 2008, Bacles & Jump 2011). Our study species, *Manilkara maxima* T.D. Penn. (Sapotaceae), shares the aforementioned characteristics and is listed by the IUCN as 'Vulnerable' (Pires-O'Brien 1998).

It is important to examine the effects of fragmentation on *M. maxima* and estimate its baseline of genetic diversity because it is an economically and ecologically important species. According to reports (Pennington 1990, Jardim 2003) and herbarium collections, *M. maxima* is endemic to ca. 350 km of forest in southern Bahia (see Fig. 1) in the highly fragmented Brazilian Atlantic forest biodiversity hotspot (Myers et al. 2000). Only 18% of the original forest remains in this region, and 95% of the remaining fragments are <100 ha (Landau et al. 2008, Ribeiro et al. 2009). *M. maxima* is economically important as a timber source and is selectively logged (S. M. Ganzhorn pers. obs.) due to its insect- and fungus-resistant wood (Pennington 2004). Additionally, *M. maxima* is ecologically important to the endangered golden-headed lion tamarin (GHLT) *Leontopithecus chrysomelas* as a source of dietary nectar (Raboy & Dietz 2004), and was identified as an 'extremely valuable' tree species for GHLT conservation (Oliveira et al. 2010).

We performed population genetic analysis on *M. maxima* to better understand the effects of fragmentation on density and genetic diversity of a low-density tree species with a scattered distribution and long distance dispersal. Greater understanding of the effects of fragmentation on low-density species, particularly those at risk of extinction, is critical for developing science-based conservation and restoration projects. We used microsatellite analysis of 5 loci to determine (1) if fragment size affects the density and genetic diversity of adults and saplings, and (2) if density affects the genetic diversity of adults and saplings. Our genotype data contained a large number of alleles that were similar to those found in 2 other congeners (Azevedo et al. 2005, Moraes et al. 2013), underscoring the informative value of our 5 loci. We defined adult trees as plants >1 cm diameter at breast height (dbh), and saplings as plants ≤1 cm dbh and ≥20 cm total height (ht). Tropical trees ≤1 cm dbh are estimated to be <20 yr old (Welden et al. 1991, Hubbell 2004). Most of the fragmentation of the study area occurred in the early 1970s (Mori & Silva 1979, Mendonça et al. 1994), about 35 yr before the present study. Accordingly, these life stages enabled us to conservatively estimate indices of population genetic diversity for adults (>20 yr) and saplings (<20 yr) that were established pre- and post-fragmentation, respectively.

MATERIALS AND METHODS

Study species

Manilkara maxima is listed as 'Vulnerable' by the IUCN due to its restricted range, habitat loss, and fragmentation (Pires-O'Brien 1998). *M. maxima* grows to 30 m in height with a diameter of 100 cm. This species can be identified by the presence of latex, broad cuneiform leaves with abaxial appressed ferruginous indumentum, and solitary white flower with 6 staminodes (Pennington 1990). The genus *Manilkara* is slow-growing (O'Farrill et al. 2006) and has a mating system which is predominately outcrossed by pollen vectors that include flies, bees, bats, and primates, and has seed vectors that include birds, bats, and primates (Pennington 2004, Azevedo et al. 2007). Bat and bird species travel between forest fragments and contribute to long-distance dispersal (Howe & Smallwood 1982, Cosson et al. 1999, Shilton et al. 1999).

Study area

The study was conducted during 2009 in southern Bahia, Brazil, in the Nova Esperança Forest, the Una Ecopark, and the Lemos Maia Experimental Station (Fig. 1). These 3 forests are protected from logging and appear to be well preserved, with a mature forest structure (a 25 m high canopy and numerous large epiphytes and lianas), and a mean canopy cover of 94%. This region has a monthly average temperature of 24°C, annual average relative humidity of 80 to 90%, and evenly distributed annual average precipitation of 1400 to 2000 mm (Rocha 1976, Mori et al. 1983, Landau et al. 2003). The natural vegetation of the study area is classified as lowland tropical moist forest or, locally, tabuleiro forest (Thomas & Barbosa 2008).

Study sites

The Nova Esperança (NE) study site (Fig. 1) is 200 ha of privately owned forest 50 km north of Ilhéus, Bahia, Brazil (14° 20′ 31″ S, 39° 02′ 33″ W). The Una Ecopark (UE) study site (Fig. 1) is a 400 ha Private Natural Heritage Reserve owned and managed by the Institute for Social and Environmental Studies

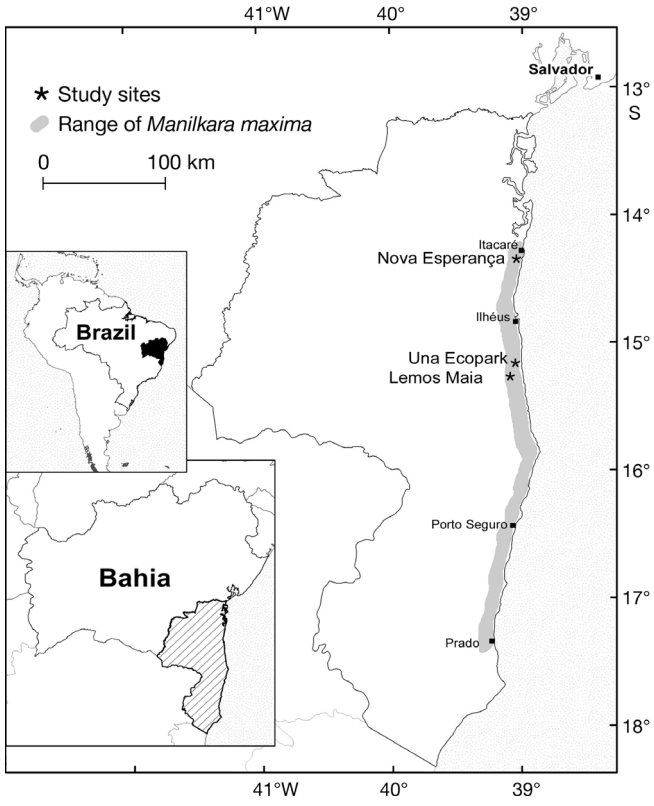

Fig. 1. Locations of study sites (★; Nova Esperança, Una Ecopark, and Lemos Maia) and cities (■). The range of *Manilkara maxima* is shaded in solid gray in the main map. Insets show the location of Bahia (black) and southern Bahia region (stippled)

in Southern Bahia (IESB), located 45 km south of Ilhéus, Bahia, Brazil (15° 10′ 4″ S, 39° 03′ 07″ W). We classified both of these sites as large forest fragments for this study.

The Lemos Maia Experimental Station (LM) study site (Fig. 1 & Fig. S1 in the Supplement at www.int-res.com/articles/suppl/n026p189_supp.pdf) is a 400 ha agricultural field station owned and managed by the Executive Cocoa Planting Commission (CEPLAC) located 52 km south of Ilhéus, Bahia, Brazil (15° 15′ 5″ S, 39° 05′ 34″ W). Much of the fragmentation in southern Bahia occurred in the early 1970s (Mendonça et al. 1994), and many forests are remnants of this recent fragmentation. The station was established in 1975 (J. I. Lacerda pers. comm.), and is a mosaic of recently fragmented forests, surrounded by dirt roads, agricultural fields, regenerating forests, and agroforests. We selected 8 forest fragments (5, 10, and 25 ha) to survey (hereafter LM1, LM2, LM3…) from the small forest fragment site (LM): three 25 ha forests, two 10 ha forests, and three 5 ha forests (Fig. S1). Forest fragment distance to closest forest (large fragment >100 ha) ranged from 1.5 to 3.5 km and had a mean distance of 2.6 km. The distance among fragments ranged from 0.2 to 1.6 km and had a mean distance of 0.5 km.

Sample collection and density estimate

One 10 ha sampling area (250 × 400 m) was surveyed at each of the 2 large fragment sites (NE and UE) and at each of the 25 ha forests for the study species. The one 10 ha sampling area was used at both large fragment sites (NE and UE) instead of smaller replicated plots to sample enough individuals at the large fragment sites (NE and UE), due to the low density and patchy distribution of this species. The entire forest area was surveyed for the study species at each of the two 10 ha forests and three 5 ha forests. In total, we mapped and sampled 222 trees (adults >1.0 cm dbh and saplings ≤1 cm dbh and ≥20 cm ht) from the 3 study sites (NE, N = 60; UE, N = 54; and LM, N = 108), a sampling regime that represented >5% (and in some cases 100%) of the area from each forest, with one exception, where it represented 2.5% of the total area (Table S1 in the Supplement at www.int-res.com/articles/suppl/n026p189_supp.pdf). Density (*D*) was calculated as N (no. of individuals per sample area)/sample area. Cambium and leaf material were collected and placed in re-sealable plastic bags with 50 to 60 g of self-indicating silica gel (Chase & Hills 1991, Colpaert et al. 2005).

Genetic analysis

Genomic DNA was extracted from cambium and leaves of *M. maxima* using the DNeasy™ Plant Mini Kit (Qiagen). Plant material was crushed in liquid nitrogen for tissue homogenization prior to DNA extraction. The DNeasy™ extraction protocol was performed with the addition of 2% PVP-40 (polyvinyl-pyrrolidone) to the lysis buffer.

The 5 microsatellite loci used in this study (Table 1) were selected from those developed for the congener *Manilkara huberi* Ducke (Azevedo et al. 2005). A multiple-tailed M13 primer method was used for genotyping microsatellite loci by synthesizing an additional modified 16 to 17 bp tail (Table 1) to the 5′-end of the forward primer for each primer pair (Oetting et al. 1995). The polymerase chain reaction (PCR) had a final volume of 10 µl, and contained 1.0 µl of 10× buffer (200 mM Tris-HCl pH 8.8, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $(MgSO_4)_7H_2O$, 1% (v/v) Triton X-

Table 1. Five microsatellite loci characteristics for 222 *Manilkara maxima* individuals: locus identifications; repeat motif; oligonucleotide primer sequences; fragment sizes (bp); annealing temperatures (T_a); number of alleles (A); observed heterozygosity (H_o); expected heterozygosity (H_e); inbreeding coefficient (F_{IS}); and null allele frequency. Mean: null alleles corrected by Brookfield method; Mean[a]: with uncorrected null alleles

Locus	Repeat motif	Primer sequence (5′–3′)	Size (bp)	T_a (°C)	A	H_o	H_e	F_{IS}	Null alleles
Mh02	$(CT)_9$	Fwd: CCT TTT CCC GCA AAT CCT Rev: GGG CTG AGA CCA ATG TCA AT	165–187	48	11	0.37	0.51	0.27	0.14
Mh08	$(CT)_{11}$	Fwd: GTA ATG GGA GCC GTT TGA GA Rev: CTG GGT AGC ATT TGT TGC AT	181–229	48	24	0.69	0.88	0.21	0.15
Mh12	$(CT)_9$ $(AC)_6$	Fwd: TGC GGA ACT GTG GAA AGA GT Rev: ATC CAC AGC AAT GAC TGA CG	175–235	50	27	0.68	0.86	0.22	0.16
Mh17	$(CT)_{13}$	Fwd: CAC GAT GAC CTC TCA GTG GA Rev: CCT GTG TAT GCG TTC GAT TG	192–265	50	21	0.45	0.54	0.17	0.14
Mh22	$(CT)_{15}$	Fwd: CCC ATT ATA GCC CTC CAC CT Rev: AGA GAG CAC ATG CAA GCT CA	169–207	50	27	0.76	0.86	0.11	0.07
Mean					22	0.59	0.73	0.19	0.13
Mean[a]					21	0.44	0.66	0.37	

100, 50% (w/v) sucrose, and 20 µg ml^{-1} bovine serum albumin (BSA)), 0.8 µl of 0.2 mM dNTP (New England Biolabs), 1.0 µl of 0.25 µg µl^{-1} BSA (New England Biolabs), 2.0 µl of 5 M betaine, 0.25 µl of 10 µM forward primer (Invitrogen), 0.5 µl of 10 µM reverse primer (Invitrogen), 0.5 µl of 10 µM dye primer, and 0.2 µl of *Taq* DNA polymerase, 2 µl of genomic DNA, and 1.75 µl of nanopure water. The PCR amplifications were carried out on an Eppendorf Mastercycler Pro S thermocycler (Eppendorf North America) under the following conditions: 95°C for 2.5 min, 10 cycles at 95°C for 30 s, locus-specific annealing temperatures (Table 1), and 64°C extension for 1 min, and then 30 cycles at 88°C for 30 s, locus-specific annealing temperatures (Table 1), and 64°C extension for 1 min. After 40 cycles, a final extension at 64°C for 10 min was used. The PCR products were sized with a Beckman Coulter CEQ 8800 sequencer using the Beckman Coulter DNA Size Standard Kit-400 and running the software package CEQ 8800 Genetic Analysis System v.9.0 (Beckman Coulter). The size analysis had a final volume of 40 µl, containing 0.5 µl of size standard, PCR products according to tail type: –R tail 0.5 µl; –40 tail 2.0 µl; and –20 tail 4.0 µl, and formamide up to the final volume of 40 µl. Allele sizes were estimated using the GeneMarker Software v.1.90 (SoftGenetics).

Data analysis

Null alleles, stuttering, and allelic dropout were tested with Micro-Checker v.2.2.3 (Van Oosterhout et al. 2004). Errors due to stuttering or allelic dropout were not detected, but null alleles were found across all loci, ranging in frequencies from 7 to 16% (Table 1). Null alleles were corrected using the Brookfield method in Micro-Checker v.2.2.3 (Brookfield 1996, Van Oosterhout et al. 2006) and used for the genetic analyses. Data with corrected null alleles and uncorrected null alleles provided similar results. Deviation from Hardy-Weinberg Equilibrium (HWE) was examined with an exact test using the Markov chain (Levene 1949, Guo & Thompson 1992), and linkage disequilibrium was assessed with a likelihood ratio test (Excoffier & Slatkin 1998) in Arlequin v.3.5 (Excoffier et al. 2005). The genetic indices for the 5 polymorphic microsatellite loci of all 222 individuals were characterized for number of alleles locus^{-1} (A), % polymorphic loci (P), observed heterozygosity (H_o), expected heterozygosity (H_e), and the inbreeding coefficient (F_{IS}) using GenAlEx v.6.4 (Peakall & Smouse 2006). Study-site specific A, P, H_e, H_o, and number of private alleles (P_A) were estimated using GenAlEx v.6.4 (Peakall & Smouse 2006) and F_{IS} was estimated using 10000 permutations in Arlequin v.3.5 (Excoffier et al. 2005). The private allele method (Barton & Slatkin 1986) was used to estimate the effective number of migrants generation^{-1} (N_m) using GENEPOP v.4.2 (Raymond & Rousset 1995, Rousset 2008). Additionally, allelic richness (R_S) was estimated by extrapolation to the largest sample size using the ARES package (van Loon et al. 2007) in R v.2.13 (R Development Core Team 2013). This method improves the accuracy of allelic richness estimates for small sample sizes and comparison of uneven sample sizes (Colwell et al. 2004), while rarefaction can result in relatively inaccurate estimates of richness for uneven sample sizes (Gotelli & Colwell 2001).

We tested for recent genetic bottlenecks of populations (Cornuet & Luikart 1996) at the 3 study sites for both life stages by examining differences across loci between Hardy-Weinberg H_e and heterozygosity predicted at mutation-drift equilibrium from the observed number of alleles using the program BOTTLENECK v.1.2.02 (Piry et al. 1999). These differences were statistically tested under the infinite alleles model (IAM), the stepwise-mutation model (SMM), and the 2-phased model of mutation (TPM) using the Wilcoxon sign-rank test, which is traditionally the most powerful and commonly used test (Luikart et al. 1998). Since heterozygosity expected under mutation-drift equilibrium is more sensitive to the loss of low frequency alleles during a bottleneck, a significant excess of Hardy-Weinberg heterozygosity suggests a recent bottleneck (Cornuet & Luikart 1996). The 5 populations that had sample sizes ≤2 were excluded from this analysis.

An analysis of molecular variance (AMOVA) was performed using Arlequin v.3.5 (Excoffier et al. 2005). The AMOVA estimated genetic structure from *F* statistics (Vogel et al. 1999) from a matrix of Euclidian squared distances of allelic content using a nonparametric procedure with 10 000 permutations (Excoffier et al. 1992) based on a hierarchical model (Weir 1996) for both adult and saplings.

Population and genetic data variances were tested for homogeneity of variances using Levene's test (Levene 1960). All data were $\log_{10}$(variable + 1) transformed to meet statistical assumptions of homoscedasticity. The statistical relationships among forest area, density and genetic indices were examined using linear regression analysis. We examined the effects of forest area and density on genetic indices using ANCOVA, where forest area and density were covariates and genetic indices was the dependent variable. To examine the effects of life stage and density on genetic indices, we used ANCOVA generalized linear model (GLM) analysis, with life stage and density as covariates and genetic indices as the dependent variable. All statistical tests were calculated using SYSTAT v.13 software (Systat Software).

RESULTS

Density

There were no *Manilkara maxima* individuals in the LM5 5 ha forest and no *M. maxima* adults in the LM1 5 ha forest (Table S1 in the Supplement). Mean adult densities were 1.0 stems ha^{-1} for small forest fragments and 4.2 stems ha^{-1} for large fragments. Densities of adult trees increased with increasing forest size (r^2 = 0.714, p = 0.029; Fig. 2A).

As with the adults, mean sapling densities in the small fragments (0.7 stems ha^{-1}) were lower than those in large fragments (1.6 stems ha^{-1}). Sapling tree density also increased with increasing forest size (r^2 = 0.565, p = 0.020; Fig. 2B). For these small fragments, the density sample is a good estimate of the actual density since the entire area was surveyed.

Loci

Null alleles were found across all loci, ranging in frequency from 7 to 16% (Table 1). Mean genetic indices with uncorrected and corrected null alleles were similar (Table 1). The results from the analyses were not different between data sets with uncorrected and corrected null alleles. Only analyses using corrected alleles are presented. The 5 microsatellite loci had a total of 110 alleles for the 222 individuals over all populations with a mean of 22 alleles locus^{-1}

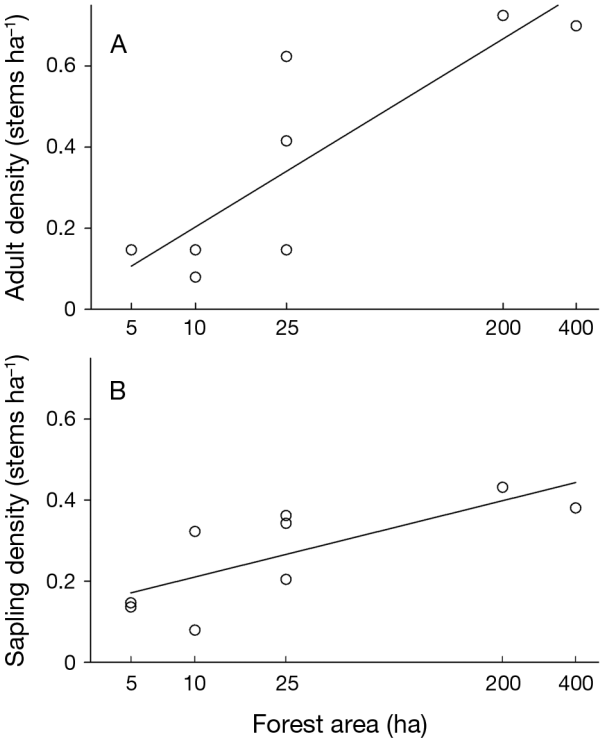

Fig. 2. (A) Linear regression of adult *Manilkara maxima* population mean density (stems ha^{-1}) and forest area (ha) (N = 8, r^2 = 0.714, p = 0.029). (B) Linear regression of sapling population mean density (stems ha^{-1}) and forest area (ha) (N = 9, r^2 = 0.565, p = 0.020). Note the change of scale on the *x*-axis. α = 0.05

(Table 1). A total of 28 private alleles (Table 2) were detected for both life stages, 86% of which had a frequency <5%. The mean % of polymorphic loci was 92% across all populations (Table 2). The 5 loci exhibited significant departure from HWE, but no pairs of loci had significant linkage disequilibrium. The values for H_o ranged from 0.37 to 0.69 and were lower than H_e, which ranged from 0.51 to 0.88 indicating an excess of homozygotes across all loci (Table 1).

Population genetics

For the adults, a total of 98 alleles were found, ranging from 1.8 to 11.6 alleles locus^{-1} (Table 2), with large fragments averaging 8.0 alleles locus^{-1} and small fragments averaging 5.7 alleles locus^{-1}. Indeed, one of the 25 ha small fragments had the highest allelic diversity (LM8 A = 11.6) and another 25 ha fragment (LM2 A = 10.2) was similar to the large NE fragment (A = 10.2) (Table 2). Adults in these same 3 sites had 53% of the total private alleles (NE = 8, LM2 = 2, LM8 = 5; Table 2). Similarly, high adult allelic richness was found in the same two 25 ha small fragments (LM2 R_S = 80 and LM8 R_S = 67.0; Table 2). Adult trees did not exhibit a significant genetic bottleneck for any population except for one small forest fragment site, LM7, that exhibited a significant bottleneck using the IAM, SMM, or TPM ($p < 0.05$). Adult H_o ranged from 0.40 to 0.69 (Table 2), with moderate values for both large (H_o = 0.56) and small fragments (H_o = 0.53). Gene diversity of adults (measured as H_e) ranged from 0.30 to 0.84, with moderately high values for the adults of both large (H_e = 0.57) and small fragments (H_e = 0.61) (Table 2). Adult F_{IS} ranged from −0.08 to 0.52 (Table 2), and was lower in the large (F_{IS} = 0.02) compared to the small fragments (F_{IS} = 0.23). Significant inbreeding was found for adults at the NE, LM2, LM3, LM7, and LM8 populations (Table 2).

For the saplings, a total of 86 alleles were detected ranging from 1.8 to 9.4 alleles locus^{-1} (Table 2), with large fragments averaging 5.6 alleles locus^{-1} and

Table 2. Mean population and genetic diversity indices (±SE) of the 3 study sites (NE = Nova Esperança, UE = Una Ecopark, and LM = Lemos Maia Experimental Station), showing number of *Manilkara maxima* individuals in the sample area (N); density of stems ha^{-1} (D); percent polymorphic loci (P); number of alleles per locus (A); number of private alleles (P_A); allelic richness (R_S); observed heterozygosity (H_o); expected heterozygosity (H_e); inbreeding coefficient (F_{IS}). $^{\star}p < 0.05$, $^{\star\star}p < 0.01$, $^{\star\star\star}p < 0.001$. Mean: null alleles corrected by Brookfield method; Mean[a]: with uncorrected null alleles

Study site	N	D	P	A	P_A	R_S	H_o	H_e	F_{IS}
Large fragments									
NE Adult	43	4.3	100	10.2 (1.50)	8	77.7 (2.87)	0.58 (0.09)	0.65 (0.11)	0.11**
NE Sapling	17	1.7	100	7.0 (1.30)	1	35.0 (1.91)	0.64 (0.06)	0.67 (0.08)	0.08
UE Adult	40	4.0	100	5.8 (1.11)	2	50.1 (0.93)	0.54 (0.15)	0.49 (0.14)	−0.08
UE Sapling	14	1.4	80	4.2 (0.86)	0	22.8 (1.14)	0.50 (0.14)	0.47 (0.13)	−0.03
Small fragments									
LM1 Adult	0	–	–	–	–	–	–	–	–
LM1 Sapling	2	0.4	100	2.6 (0.40)	0	12.0 (0.59)	1.00 (0.00)	0.58 (0.05)	−0.54
LM2 Adult	16	1.6	100	10.2 (1.20)	2	80.0 (2.79)	0.65 (0.03)	0.84 (0.02)	0.26***
LM2 Sapling	13	1.3	100	9.4 (1.36)	3	53.4 (3.24)	0.71 (0.05)	0.82 (0.01)	0.17**
LM3 Adult	4	0.4	100	4.0 (0.55)	0	29.7 (1.66)	0.45 (0.09)	0.64 (0.08)	0.43*
LM3 Sapling	6	0.6	100	4.6 (0.81)	3	29.5 (1.55)	0.53 (0.14)	0.62 (0.10)	0.22
LM4 Adult	2	0.2	80	2.6 (0.51)	1	19.1 (0.97)	0.60 (0.19)	0.48 (0.13)	0.08
LM4 Sapling	11	1.1	80	4.4 (1.32)	1	24.0 (1.24)	0.49 (0.14)	0.52 (0.15)	0.10
LM6 Adult	2	0.4	60	1.8 (0.37)	0	12.1 (0.26)	0.40 (0.19)	0.30 (0.13)	0.01
LM6 Sapling	2	0.4	60	1.8 (0.37)	0	12.0 (0.59)	0.28 (0.11)	0.30 (0.13)	0.01
LM7 Adult	4	0.4	100	4.2 (0.66)	0	41.0 (1.20)	0.40 (0.19)	0.68 (0.08)	0.52**
LM7 Sapling	2	0.2	100	2.4 (0.25)	0	15.7 (0.75)	0.90 (0.10)	0.53 (0.05)	−0.50
LM8 Adult	32	3.2	100	11.6 (1.10)	5	67.0 (2.50)	0.69 (0.04)	0.75 (0.05)	0.09*
LM8 Sapling	12	1.2	100	8.0 (0.84)	2	45.8 (2.83)	0.55 (0.06)	0.71 (0.09)	0.27***
Mean			92	5.6 (0.86)		37.0 (1.59)	0.59 (0.12)	0.59 (0.09)	0.07
Mean[a]			85	5.1 (0.39)		34.4 (1.01)	0.42 (0.04)	0.50 (0.03)	0.16

small fragments averaging 4.7 alleles locus^{-1}. Similar to the adults, saplings in 2 of the 25 ha forests had the highest allelic diversity (LM2 A = 9.4, LM8 A = 8.0; Table 2) and allelic richness (LM2 R_S = 53.4, LM8 R_S = 45.8; Table 2). Saplings in the three 25 ha fragments harbored 29% of the total private alleles (LM2 = 3, LM3 = 3, LM8 = 2; Table 2). Hardy-Weinberg heterozygosity and expected heterozygosity at mutation-drift equilibrium did not differ significantly using the IAM, SMM, or TPM, suggesting a lack of evidence for recent genetic bottlenecks for the saplings. Sapling H_o ranged from 0.28 to 1.00 (Table 2), with moderately high means for both large (H_o = 0.57) and small fragments (H_o = 0.58). Similar to allelic diversity and richness, saplings in 2 of the 25 ha sites (LM2 H_e = 0.82 and LM8 H_e = 0.71) exhibited the highest gene diversity of all populations (Table 2). Sapling inbreeding also had a wide range (–0.54 to 0.52; Table 2) with low means for both large (F_{IS} = –0.03) and small fragments (F_{IS} = –0.04). Significant inbreeding was found for saplings in the LM2 and LM8 populations (Table 2).

There were no significant forest fragment size effects on the genetic indices for either adults or saplings. There were significant density effects for adult A and R_S (r^2 = 0.640, p = 0.017 and r^2 = 0.573, p = 0.030, respectively) and sapling A, R_S and F_{IS} (r^2 = 0.670, p = 0.007; r^2 = 0.571, p = 0.019; r^2 = 0.463, p = 0.044, respectively). When taking both density and area into account using an ANCOVA, A and R_S did not vary clearly with area for either adults or saplings (p > 0.05), indicating that differences in density accounted for more of the variation among study sites than differences in area. The ANCOVA GLM showed significant density effects for A (N = 18, r^2 = 0.802, p = 0.001), R_S (N = 18, r^2 = 0.702, p = 0.019), and no effect of life stage or an interaction of density and life stage for either adults or saplings (Fig. 3A,B). However, the ANCOVA GLM showed a significant interaction of density and life stage (p = 0.010) for F_{IS} (Fig. 3C).

The AMOVA revealed that adult trees and sapling trees had moderate differentiation (F_{ST} = 0.10 and 0.15, respectively) among the 3 study sites. However, the low adult and sapling genetic differentiation among the 3 study sites was not significantly different (adults: 3.47%, p = 0.194; saplings: 5.12%, p = 0.213). Most of the adult and sapling genetic variation was found within the study sites (adults: 90.16%, p = 0.001; saplings: 84.95%, p = 0.001). However, the low adult and sapling genetic differentiation among the small fragments was significant (adults: 6.37%, p = 0.001; saplings: 9.93%, p = 0.001). The effective N_m across all populations using the private allele method (Barton & Slatkin 1986) was N_m = 6.49 after correction for sample size. Additionally, the effective N_m after correction for sample size for adults was N_m = 6.58 and for saplings was N_m = 5.23. It generally only takes 1 migrant per population to homogenize a population.

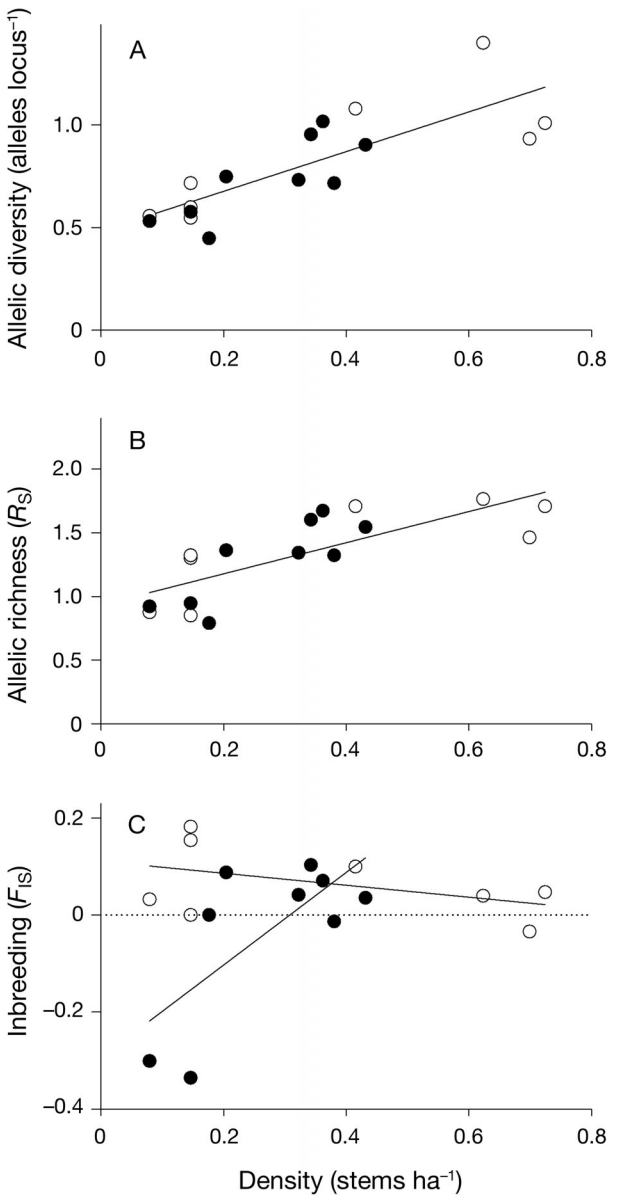

Fig. 3. Effects of *Manilkara maxima* life stage (O = adults; ● = saplings) and density (stems ha^{-1}) analyzed with ANCOVA generalized linear model. (A) Allelic diversity (alleles locus^{-1}) (N = 18, r^2 = 0.802, p = 0.001). (B) Allelic richness (R_S) (N = 18, r^2 = 0.702, p = 0.019). (C) Inbreeding (F_{IS}) significant interaction of life stage and density (N = 18, p = 0.010). All data were $\log_{10}$(variable + 1) transformed. α = 0.05

DISCUSSION

Adults

Our results suggest that while density decreased with decreasing forest fragment size, it varied substantially among 25 ha fragments. In addition, small fragments can harbor high levels of genetic diversity comparable to large fragments. Finally, density was a good predictor of genetic diversity and more reliable than fragment size, even though adult densities decreased with decreasing forest fragment size. This was primarily because one of the small fragments (LM8) had a stem density similar to that found in the large fragments (NE and UE). Additionally, adult trees in some small fragments (LM2 and LM8) had high levels of genetic diversity that were higher than or similar to the genetic diversity of trees found in large fragments (NE and UE).

The wide range in adult density across the 25 ha fragments (0.4 to 3.2 stems ha^{-1}) may reflect the naturally (pre-fragmentation) scattered and patchy distribution of *Manilkara maxima,* as this variable distribution of trees is consistent with density expectations for spatially scattered populations unaffected by fragmentation (Kramer et al. 2008, Bacles & Jump 2011), where more trees may be found in some fragments than others, even if the fragments are the same size and in close proximity. Despite their small size, some 25 ha forests are as important for maintaining *M. maxima* adult densities and genetic diversity as large forests. The adults in the LM2 25 ha fragment had the highest allelic diversity and richness, even though their density was half that of another 25 ha fragment (LMB) and the large fragments (NE and UE).

The heterozygosity, gene diversity and inbreeding observed for adults did not vary with forest size or density as we hypothesized, because the adult generation was established prior to the relatively recent forest fragmentation. One small population, LM7, comprising only 4 adults, had one of the highest gene diversity values (H_e = 0.68), and adds support to the contention that small populations have conservation value for maintaining genetic diversity (Schneller & Holderegger 1996, Finger et al. 2012). The adult mean inbreeding value found in the small fragments was higher than that reported by Azevedo et al. (2007) (F_{IS} = 0.18) for a congener, *Manilkara huberi*, in a continuous forest, but lower than the mean inbreeding we found in the large fragments. However, the mean inbreeding for all the adults (F_{IS} = 0.18) is consistent with that reported by Azevedo et al. (2007) for *M. huberi*. The relatively high inbreeding in some of the adult populations might be explained by sampling error or mating between related individuals that occurred pre-fragmentation.

The low and insignificant differentiation of the adult cohort indicates that only 3.47% of the genetic variation is distributed among the 3 study sites. Most of the distribution of the genetic variation was found within the study sites (90.16%), suggesting extensive pre-fragmentation gene flow or common ancestry. Also, the relatively high gene flow (N_m = 6.58) of the adults represents pre-fragmentation gene flow (Bossart & Prowell 1998). Indeed, outcrossing species generally demonstrate high levels of genetic variation within populations and lower genetic differentiation among populations (Loveless & Hamrick 1984), and similar results have been found in other fragmented Atlantic forest tree species (Seoane et al. 2000, 2005, Auler et al. 2002, Salgueiro et al. 2004, Silva et al. 2008).

Saplings

Similar to the adults, our results for saplings suggest that density decreased with decreasing forest size, but varied substantially among 25 ha fragments, that small fragments can harbor high levels of genetic diversity, and that density was a better indicator of genetic diversity than fragment size. As with the adult populations, 2 of the 25 ha fragments (LM2 and LM8) had sapling densities similar to the large fragments. Saplings in these same two 25 ha small fragments also had the highest allelic diversity and richness (Table 2). Also, as with the adults, the high density and genetic diversity of saplings in these small fragments provides support for the importance of small fragments for *M. maxima*.

The highest sapling genetic diversity detected in 2 of the 25 ha fragments may result from either long-distance dispersal from other forest fragments, or dispersal from adult trees within the LM fragmented area. Indeed, gene flow was relatively high for the saplings in the small fragments (N_m = 5.23), and indicates recent gene flow (Yamamichi & Innan 2012). The LM2 site had no reproductively mature adult trees in the sampled area (S. M. Ganzhorn unpubl. data) and is located between the LM8 site and the closest forest >100 ha at distances of 0.6 km and 1.5 km, respectively. The LM2 site may offer an admixture of saplings between the LM8 and the closest forest >100 ha, and may act as a stepping

stone to promote long-distance seed dispersal from larger to smaller fragments and a site for recruitment of younger trees from adult trees in LM8 and other larger fragments. The LM8 site had a similar adult density compared to the larger fragments (Table 2) with many reproductively mature individuals (S. M. Ganzhorn unpubl. data). The high quality matrix surrounding these sites, composed of advanced regenerating forests and agroforests, may help facilitate long-distance dispersal by organisms (e.g. bats, birds, and primates). The high sapling density and sapling allelic diversity in LM2 and LM8 makes these 25 ha fragments potentially important sources of genetic variation once the saplings mature.

The sapling H_o and H_e did not clearly vary with forest size or density. We expected such results because fragmentation in our study sites has been recent and genetic diversity declines only after successive generations (Young et al. 1996). Similar to the adults, 2 of the 25 ha fragments had the highest gene diversity (Table 2) and 1 small population, LM3, comprised of only 6 saplings, had one of the highest gene diversity values (H_e = 0.62), supporting other studies that show that small populations of saplings have conservation value for genetic diversity (Schneller & Holderegger 1996).

The mean sapling inbreeding in the large (F_{IS} = −0.02) and small (F_{IS} = −0.04) fragments was low. These values were lower than those found for *M. huberi* (F_{IS} = 0.24) progeny in an undisturbed habitat (Azevedo et al. 2007). However, we found significant sapling inbreeding in 2 of the 25 ha forests (Table 2), suggesting either sampling error or that mating between related individuals is occurring in some of the small forests. Similar to our results, Franceschinelli et al. (2007) found similar inbreeding values for the insect-pollinated and bird-dispersed Atlantic forest tree species *Myrciaria floribunda* among various sizes of fragments, suggesting that long-distance pollen and seed dispersal act as potential mechanisms for maintaining gene flow. Primates may also contribute to the low inbreeding given that GHLT, for example, can move between fragments (S. M. Ganzhorn pers. obs.) and have daily ranges (1.4 to 2.2 km) that are within the inter-fragment distances (0.2 to 1.6 km) in the LM study site (Raboy & Dietz 2004). The GHLT lick nectar from the flower and may visit up to 60 *M. maxima* individuals in 1 d (B. E. Raboy pers. comm.). *M. maxima* comprises 5.3% of the GHLT frugivorous diet, but the seeds are spit out near the parent tree (Cardoso et al. 2011). The GHLT may be more important for long-distance pollen dispersal when consuming floral nectar.

Despite the moderate differentiation (F_{ST} = 0.15) of the saplings, only 5.12% of the variation is found among the study sites, whereas 84.95% is found within the study sites. We expected this pattern because fragmentation has been recent relative to the generation time of these trees, and gene flow appears to be relatively high (N_m = 5.23). Similar results of moderate differentiation have been found in other fragmented young Atlantic forest tree species (Seoane et al. 2000, 2005, Auler et al. 2002, Salgueiro et al. 2004, Silva et al. 2008).

CONCLUSIONS

Our baseline data suggest that larger forest fragments maintain higher tree densities of the threatened tree species *Manilkara maxima*; this is alarming considering only 5% of forests in this region are fragments >100 ha (Landau et al. 2008). However, some smaller, 'intermediate' sized forests also exhibited high tree densities and high levels of genetic diversity in both adults and saplings. Conserving reproductively mature individuals of *M. maxima* is necessary for the recruitment of new individuals into the population and for the species' long-term persistence, but we found that forests with few reproductively mature individuals have a promising amount of genetic diversity worth conserving as well. Our results suggest that relatively high levels of gene flow are contributing to the high genetic diversity of saplings found in some of the small fragments. Additionally, reforestation projects should use seeds from forests near project sites to preserve the genetic diversity found within local forests, even if the fragments are small. Finally, our results suggest that fragment size alone cannot be used as a predictor of genetic diversity; rather, tree density can offer a better predictor of diversity. Although density varied with fragment size (suggesting fragment size both directly and indirectly affects diversity), the results of our analyses of covariance suggest that density has a greater effect on allelic diversity and richness. For example, some small fragments exhibited genetic diversity comparable to large fragments, reflecting comparatively high density in these fragments. This study advances our understanding of how fragmentation affects the genetic diversity of this threatened tree species, and will aid in developing science-based biodiversity conservation and restoration projects.

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