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Gene dispersal via seeds and pollen and their effects on genetic structure in the facultative-apomictic Neotropical tree *Aspidosperma polyneuron*

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Abstract

Facultative apomictic trees can produce offspring with a genotype identical to the mother due to asexual propagation through the embryo derived from cells in the maternal ovule tissues. These trees can also produce offspring with a genotype different from the mother due to genetic recombination. For many trees, these reproductive processes remain largely unexplored. Herein, we use microsatellite markers to identify apomictic and sexual reproduction in samples of adult and juvenile trees of the tropical, insect pollinated and wind seed dispersed *Aspidosperma polyneuron*, within a conservation area in Brazil. We also investigate seed and pollen flow and dispersal patterns and compare the genetic diversity, inbreeding, and intrapopulation spatial genetic structure (SGS) between adults and juveniles in two plots. Our results show that the species present both apomictic and sexual reproduction. Sexual reproduction occurred mainly by outcrossing, but we did detect instances of self-fertilization and mating among relatives, which explains the inbreeding observed in juveniles. Seed dispersal distance was shorter than pollen dispersal distance in one of the plots, suggesting that insect vectors are more efficient in gene dispersal than wind for seed dispersal in a high density tropical forest. The patterns of pollen and seed dispersal showed isolation by distance, explaining the SGS detected for adults and juveniles. Our results show that both seed and pollen flow increase the allelic diversity in the population. The regeneration of apomictic individuals may guarantee the continuation of genotypes adapted specifically to the study site, while sexual reproduction results in new genotypes.

Keywords: *Apomictic, conservation genetics, gene flow; propagule dispersal, tropical rainforest*

Introduction

The Neotropical tree *Aspidosperma polyneuron* Müll. Arg (Apocynaceae) is a facultative apomictic tree, presenting sexual reproduction and asexual reproduction by apomixis (Ramos, 2012). In Brazil, the species occurs in Atlantic and Amazon Forest, from Rondônia to Paraná State (Carvalho, 2003). *Aspidosperma polyneuron* is hermaphroditic, pollinated by moths and butterflies, shade tolerant, reaching up to 50 m in height (H), 390 cm in diameter at breast height (dbh), and it can live for up to 1,200 years (Carvalho, 2003). Reproduction starts between 20 to 30 years of age, when dbh ≥ 10 cm and H > 8.6 m, but individuals do not flower annually and only produce seeds every 2 to 4 years (Carvalho, 2003). The fruits are dehiscent with 2 to 5 seeds per fruit; seeds are winged, wind dispersed and has no period of dormancy (Carvalho, 2003). The wood of the species is used to make furniture, and in construction, shipbuilding, and energy industries (Carvalho, 2003). The species has been logged intensively due to the high economic value of its wood; currently, it is classified as endangered on the IUCN Red List of Threatened Species (Version 2016-3) and requires urgent biological and genetic studies to support *ex* and *in situ* conservation programs.

Apomixis is asexual propagation through seeds where the embryo is derived solely from cells in the maternal ovule tissues (Koltunow, 1993). Apomixis and facultative apomixis are strategies to maintain the genetic diversity of plant populations (Murawski, 1995). The combination of apomixis and vegetative division allows for the development of a large clonal population and the persistence of the population over long periods of time. This can lead to the formation of numerous morphological distinctions and these species can occupy disturbed areas, environments where the growing season is short, such as arctic and alpine sites, or areas where other barriers inhibit the successful crossing of compatible individuals, such

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as among widely dispersed individuals within a tropical rainforest (Asker and Jeling, 1992). Species with facultative apomixis could also be advantageous in cases where populations have been significantly reduced; it enables reproduction in the absence of pollination and allows for increases in population size, conserves heterozygosis originating from sexual reproduction, and counteracts the negative effects of inbreeding depression and genetic drift (Murawski, 1995). On the other hand, apomictic species are unable to avoid unfavorable mutations because recombination and segregation may be absent and populations may have a restricted niche (Koltunow, 1993). Apomixis could also be advantageous when there is individual asynchronous flowering leading to variations in effective neighborhood pollination area and effective population size (Koltunow, 1993).

Apomictic reproduction can also affect the intrapopulacional spatial genetic structure (SGS). Intrapopulacional spatial genetic structure is the nonrandom distribution of genotypes within populations, resulting in near-neighbor individuals more related then individuals located at long distance (Vekemans and Hardy, 2004). This pattern of SGS is common phenomenon of many tropical tree populations, being mainly determined by the dispersal of seeds and pollen at short distance (Hardy et al., 2006; Degen and Sebbenn, 2014), as well as by vegetative propagation (Silva et al., 2011; Dering et al., 2015; Spoladore et al., 2017). Studies comparing clonal and non-clonal trees have been showed that vegetative propagation increased the SGS (van Loo et al., 2007; Vaughan et al., 2007; Silva et al., 2011; Dering et al., 2015; Spoladore et al., 2017). If seeds (including apomictic and non apomictic) are dispersed close to the mothers, we can expect an increase in SGS due increase in the relatedness in the neighbor individuals, due to not random distribution of genotypes inside populations. Furthermore, if pollen is also dispersed over short distances, mating among apomictic individuals will probably occur, resulting in production of inbred seeds. In contrast, if seeds are disperse over long distance, apomictic reproduction will probably not produce SGS, due the random distribution of genotypes in the populations and mating among related individuals will occur only if the pollen is disperse over long distance.

Despite the numerous studies of mating system, pollen and seed flow and dispersal distance and patterns, SGS, genetic diversity and inbreeding in a wide variety of tree species, such analysis of apomictic or facultative apomictic tropical tree species, such as *A. polyneuron*, remain largely unexplored. These studies are keys to developing strategies for conservation genetics of the remaining populations of endangered species. Genetics studies based on genetic markers, such as microsatellite markers, can help to illuminate such ecological and genetic processes in plant populations (Burczyk et al., 2004; Ashley, 2010). To assess these ecological and genetic processes using genetic markers, samples from adults, open-pollinated seeds, and/or regenerants (seedlings and juveniles) of the focus species must be used along with techniques such as parentage analysis (Burczyk et al., 2004). Paternity analysis based on open-pollinated seeds enables the investigation of effective pollen flow and dispersal patterns, while maternity and paternity analysis based on regenerants enables the investigation of realized pollen and seed flow and dispersal patterns (Burczyk et al., 2004; Ashley, 2010). Furthermore, genetics studies in forest remnants can help provide insight into the mechanisms that maintain apomictic populations, such as *A. polyneuron*.

In this study, we used microsatellite markers to identify apomictic and sexual reproduction as well as investigate the mating system, pollen and seed flow and dispersal patterns, quantify genetic diversity, and assess inbreeding and SGS in *A. polyneuron* adult and juvenile samples from two plots established in a subtropical forest conservation area in Brazil. To our knowledge, this is the first study that assesses this information in natural populations of tropical trees with facultative apomictic reproduction. We specifically sought to answer the following questions: i) Is reproduction predominantly apomictic or sexual? ii) What is the rate of selfing and mating among relatives? iii) What is the rate of pollen and seed immigration into the study plots? iv) What is the distance of pollen and seed dispersal in the study plots? v) Is SGS present at the adult and juvenile stages? vi) Are there differences in genetic diversity and inbreeding between adult and juvenile stages?

Material and Methods

Study area and sampling

The study was carried out in the "Mata dos Godoy" conservation area (23°27' S and 51°15'W, 700 m altitude), located in Northern Paraná State, Southern Brazil (Figure 1). This conservation area is one of the last remnants of Seasonal Semideciduous Atlantic Forest in Paraná State. It includes a legally protected area of about 681 ha and it is one of the best-preserved forest fragment that has not been subjected to selective logging and other anthropogenic influences (Torezan et al., 2005). The climate is humid subtropical with hot summers and no defined dry season. The mean temperature during the hottest months (December to February) is 28° C, during the coldest months (June to August) the temperature ranges from 16 to 17° C, and annual rainfall ranges from 1,400 to 1,600 mm (Köppen, 1948). The study was carried out in two plots: plot-1 has 9 ha (300 x 300 m) and plot-2 has 5.06 ha (225 x 225 m). Close to the middle of the plots we established one subplot of 0.25 ha (50 x 50 m) to sample juveniles (Figure 1). Adults are defined as individuals who reach the canopy (dbh > 10 cm and H > 8.6 m, Carvalho, 2003). All adult individuals within the plots and juveniles within subplots were mapped using GPS (Garmin Colorado 300, Olathe, KS, USA). Total height (H) was measured for adults and juveniles, as well as dbh for adults, and root collar diameter (rcd) for juveniles using calipers. We collected two cambium tissue samples from each adult and four leaf tissue samples from each juvenile. The cambium tissue was stored in Eppendorf tubes containing CTAB 2 % and the collected leaf tissue from juveniles was stored in silica gel until dry. Plot-1 is a mature forest with old trees, a closed canopy, and a well-defined sub-stratum. In this plot, we sampled 88 adults (9.8 trees/ha) and 425 juveniles. Plot-2 was visibly different from plot-1, mainly because of the large number of lianas and clearings. From this plot we sampled 131 adults (25.9 trees/ha) and 200 juveniles. The dbh and H of adults were lower in plot-2 (mean

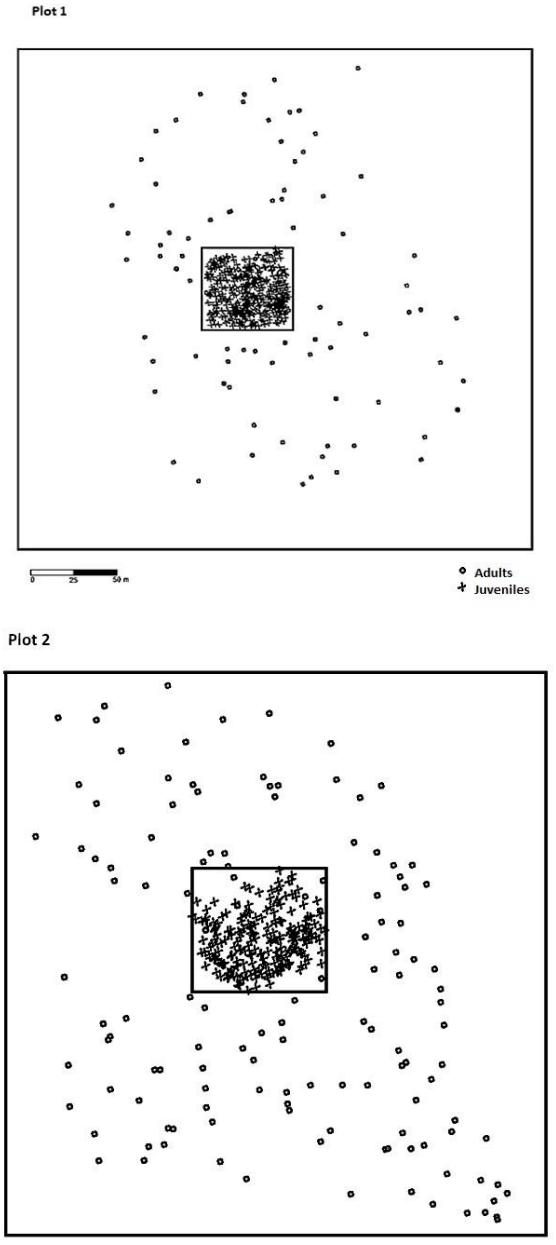


Figure 1
Spatial distribution of adult and juvenile Aspidosperma polyneuron individuals in plot-1 and plot-2

dbh of 16.7 cm, maximum of 66.2 cm; mean H of 9.9 m, maximum of 26.7 m) than plot-1 (mean dbh of 55.6 cm, maximum of 189.7 cm; mean H of 19.2 m, maximum of 40.3 m).

DNA extraction and microsatellite analysis

DNA extraction from adult and juvenile samples was carried out using the protocol described by Doyle and Doyle (1990). A total of nine microsatellite loci were amplified, but due the genotyping errors, the DNA analyses were based on seven microsatellite loci (Apn20, Apn21, Apn22, Apn23, Apn24, Apn26, and Apn27) developed by Ramos et al. (2011).

Analysis of genotypic diversity and disequilibrium

Individuals genotyped for less than seven loci were excluded from statistical analyses (24 and 36 juveniles of plot-1 and plot-2, respectively) to increase the accuracy of the estimates. The presence of apomictic adult and juvenile individuals was investigated in each plot, using the program CERVUS 3.0 (Kalinowski et al., 2007). Genotypic diversity was estimated as $R = (G\text{-}1)/(n\text{-}1)$, where G is the number of distinct genotypes and n is the number of individuals analyzed (Dorken and Eckert, 2001). The genotypic disequilibrium between pairwise loci was estimated for unique multilocus genotypes of adults and juveniles (non-apomictic) in each plot, using the FSTAT program (Goudet, 1995).

Analysis of genetic diversity and inbreeding

We estimated the genetic diversity and fixation index for adults and juveniles in each plot, using distinct genotypes (*G*) and FSTAT (Goudet, 1995). The following indexes were calculated: total number of alleles over all loci (*k*), number of private alleles between adults and juveniles within plots (K_p), mean allelic richness for 45 individual genotypes for the seven microsatellite loci (A_r), mean observed heterozygosity (H_o), and expected heterozygosity at Hardy-Weinberg equilibrium (H_e). Inbreeding was estimated using the fixation index (*F*) and statistical significance was tested using Monte Carlo permutations of alleles among individuals and a sequential Bonferroni correction for multiple comparisons (95 %, α= 0.05). The frequency of null alleles (*Null*) and the fixation index corrected for null alleles (F_{null}) was estimated for adults and juveniles in each plot under a population inbreeding model (PIM) using the INEST 2.0 program (Chybicki and Burczyk, 2009). To test if all estimated indices were significantly different between samples (adults and juveniles) from the plots, we used an unpaired t-test.

Analysis of spatial genetic structure

We assessed the SGS for adults and juveniles based on the coancestry coefficient (θ_{xy}) as described in Loiselle et al. (1995), using SPAGEDI 1.3 (Hardy and Vekemans, 2002). For the analysis of adult trees, we assumed that the parental population had the same allele frequencies found in the present adult generation and for juveniles, we used the allele frequencies of the adult generation of each plot as reference. As many private alleles (K_p) were detected in juveniles of both plots (Table 1), the gene frequency of adults from each plot were re-estimated, subtracting for each locus the sum of the gene frequencies of private alleles from the alleles in adults with highest gene frequency. The θ_{xy} was calculated for all pairs of individuals, averaged over a set of nine distance classes, and then plotted against the distance. To compare adults of the two plots, we used the same distance classes of 40, 60, 80, 100, 120, 140, 160, 180, and 200 m; to compare juveniles we used distance classes of 9, 14, 18, 22, 26, 29, 33, 38, and 43 m. To test if there was significant deviation from a random SGS, the 95 % confidence interval was calculated from 1000 Monte Carlo permutations of individuals among different distance classes. The 95 % confidence interval error was calculated using Jackknife resampling

among loci. We used the *Sp* statistic (Vekemans and Hardy, 2004) to compare the intensity of SGS to the spatial distribution of genotypes between all adults and juveniles and between adult and juvenile genets with adult and juvenile ramets, calculated as $Sp = -b_k/(1-\theta_1)$, where θ_1 is the mean coancestry coefficient within the first distance class (40 m for adults and 9 m for juveniles) and b_k is the slope of regression of θ_{xy} on the logarithm of spatial distance separating individuals $\ln(d_{xy})$. To test the statistical significance of SGS, the spatial position of individuals was permuted 1000 times. The standard error of *Sp* was estimated by $-b_{k(SE)}/1-\theta_1$, where $b_{k(SE)}$ is the standard error of b_k, estimated by jackknifing among loci (Finger et al., 2012).

Parentage analysis

The combined exclusion probability of the first parent (P_p) and combined non-exclusion probability of genetic identity (Q_i) were calculated using CERVUS 3.0 (Kalinowski et al., 2007). Parentage for juveniles from each plot was assigned by comparing genotypes of adults and juveniles based our parentage analysis on a single-exclusion parentage method, accepting a maximum of one mismatch between the loci trio (mother-father-juvenile). If a single parent was identified within the plots for a juvenile, it was assumed to be the maternal parent. If two parents were found inside the plots, the near-neighbor parent was assumed to be the mother and the other parent the father. We based these assumptions on two factors: seeds of *A. polyneuron* are dispersed by wind and pollen is dispersed by insects; and plots were established inside a forest with very high tree density. Thus, seeds are likely dispersed in close proximity to the mother, because the high tree density may restrict seed dispersal distance. This may favor longer pollen than seed dispersal distance for the species in the study area. When more than two putative pollen parents were found inside the plots, the putative fathers were not used to estimate the pollen dispersal distance. For this calculation, we only used juveniles that were assigned one parent pair to reduce ambiguity. The seed flow rate (m_s) was calculated as the proportion of juveniles that had no assigned parents (n_s) inside the plots, relative to the total number of sampled juveniles (n): $m_s = N_s/n$. As there are many apomictic adults and juveniles, pollen flow and dispersal distance were estimated excluding apomictic juveniles (Table 1) as they do not represent pollen immigration, only seed immigration. The pollen flow rate (m_p) was calculated as the proportion of juveniles that had at least one assigned parent inside the plots (n_{sp}), subtracted from the total sample size of juveniles ($n_p = n - n_{sp}$), relative to the total number of sampled juveniles *(n): mp = np/n*. If a juvenile had the same individual as both maternal and paternal parent, but a different multilocus genotype as the parents, it was assumed to be selfed. The selfing rate (*s*) was calculated, excluding apomictic individuals and seed immigration, as: $s = n_{sel}/(n - n_s)$, where n_{sel} is the number of selfed juveniles. To determine the rate of mating among relatives (t_r), we used only juveniles assigned with one parent pair (n_{pair}) and we estimated the coancestry coefficient between the assigned mother and father, using SPAGEDI 1.3 (Hardy and Vekemans, 2002). Based on Ismail et al. (2014), if the coancestry coefficient between assigned mother and father parents of a juvenile is $\theta_{xy} \geq 0.1$, these parents are assumed to be related (θ_r). Thus, t_r was calculated as: $t_r = n_r/n_{pair}$, where *n* is the number of juveniles originating from mating among relatives. We also used the SPAGEDI 1.3 (Hardy and Vekemans, 2002) to estimate the mean individual fixation index (*F*) for juveniles assigned from selfing and mating among relatives. The value of *F* for such juveniles was estimated using the allele frequencies calculated for adults. The expected *F* value for a selfed individual is 0.5. In the case of mating among relatives, the expected value is the same as the coancestry coefficient estimated for the assigned mother and father. As all sampled adults and juveniles of the two plots have known spatial positions, the realized seed dispersal distance was calculated based on the position of the juveniles relative to their putative mothers. To avoid bias in the seed dispersal distance, only juveniles assigned to a single parent were used. The distance of pollen dispersal was estimated using only juveniles assigned with one parent pair, and based on the position of the putative mothers in relation to the putative fathers. The mean, 95 % confidence standard error (2SE), median, and minimum and maximum pollen and seed dispersal distances (*D*) were calculated by the Euclidean distance between two points.

Analysis of seed and pollen flow from the dispersal kernel

We estimated the combined exclusion probability of the first parent (P_p), seed (m_s) and pollen (m_p) flow, selfing rate (*s*), and mean seed (δ_s) and pollen (δ_p) dispersal distance, using the neighborhood model, based on a maximum likelihood method and implemented in the program NM+ 1.1 (Chybicki and Burczyk, 2010). In the neighborhood model, the seed and pollen dispersal distance and patterns are indirectly derived from a spatially explicit mating model (in this case, exponential power), whereas in CERVUS, these parameters are derived from individual paternity assignments. As NM+ accounts for the occurrence of null alleles, we used the null allele frequencies estimated with the INEST program, as described above. The neighborhood size parameter was set to 'infinite' (inf) to include all sampled adults in our study plots (Chybicki and Burczyk, 2010). We modeled pollen and seed dispersal assuming an exponential power dispersal kernel (Austerlitz et al., 2004). We also estimated the scale parameters of the seed (a_s) and pollen (a_p) dispersal kernel and the shape of seed (b_s) and pollen (b_p) dispersal.

Results

Genotypic and genetic diversity

The analysis of genotypic diversity (*R*) indicated that of the 88 and 131 adults and 401 and 166 juveniles sampled in plot-1 and plot-2, respectively, 45 ($R = 0.506$) and 63 ($R = 0.477$) adults and 129 ($R = 0.320$) and 120 ($R = 0.721$) juveniles present distinct multilocus genotypes (Table 1). Considering only adults and juveniles with distinct multilocus genotypes, significant

Table 1
Results of genotypic diversity and genetic diversity (excluding apomictic individuals) in adults and juveniles of the two study plots.

Sample	*n*	*G*	*R*	*k*	k_p	*Ar*	H_o	H_e	*F*	*Null*	F_{Null}
Plot-1: adults	88	45	0.506	42	3	6.00	0.467	0.614	0.239*	0.100	0
Plot-1: juveniles	401	129	0.320	57	18	6.92	0.483	0.622	0.223*	0.091	0
Plot-2: adults	131	63	0.477	36	4	4.89	0.522	0.644	0.199*	0.114	0
Plot-2: juveniles	166	120	0.721	45	13	5.72	0.384	0.644	0.403*	0.004	0.399*

n is the sample size; *G* is the number of distinct multilocus genotypes (including one ramet of each apomictic individual); *R* is the genotypic diversity;*k* is the total number of alleles over all loci; k_p is the number of private alleles between adults and juveniles within plots; A_r is the mean allelic richness for 45 genotypes; H_o and H_e are the observed and expected heterozygosity, respectively; *F* and F_{null} are the fixation index uncorrected and corrected for null alleles, respectively; *Null* is the expected frequency of null alleles; *P< 0.05.

genotypic disequilibrium was detected for 15 (17.9%) of the 84 realized tests between pairwise loci. Thus, the seven loci used in this study are suitable for use in population genetics studies of *A. polyneuron*.

For the entire sample of adults and juveniles from both plots, we found a total of 83 alleles and a mean of 11.7 alleles per locus. Adults of both plots have a lower total number of alleles over loci (*k*) and lower number of private alleles (k_p) than juveniles, suggesting pollen and seed immigration into the plots (Table 1). Based on an unpaired t-test, the indices A_r, H_o, and H_e were not significantly different (P> 0.05) between adults and juveniles of plot-1. The uncorrected mean fixation index (*F*) was significantly greater (P< 0.05) than zero for adults and juveniles of the plots, suggesting inbreeding, but not significant between adults and juveniles of the plots. The mean null gene frequencies ranged among samples from 0.004 to 0.114. The fixation index corrected for null alleles (F_{null}) decreased for the samples, being significantly higher (P< 0.05) than zero only for juveniles of plot-2, suggesting inbreeding.

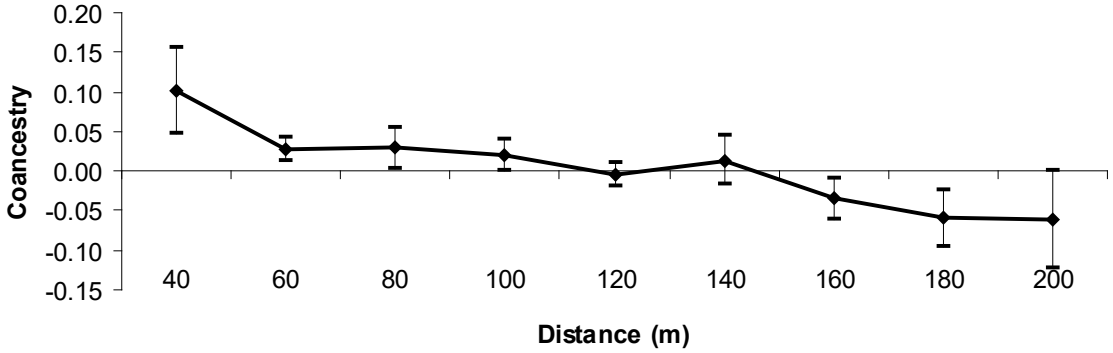
Spatial genetic structure

Significant spatial genetic structure (SGS) was detected for adults and juveniles of both plots, with mean pairwise coancestry coefficient decreasing with increased distance between pairwise individuals (Figure 2). The SGS was significantly higher (P< 0.05) than expected for the hypothesis of absence of SGS for adults (up to 90 m in plot-1 and 60 m in plot-2) and for juveniles (up to 21 m in plot-1 and 13 m in plot-2). The regression slope (b_k) of pairwise coancestry coefficient on the logarithm of spatial distance was significantly negative (P< 0.05) for all adults, genets and ramets of both plots, all juveniles and ramets of plot-1, and all juveniles and genets of plot-2 confirming the presence of SGS (Table 2). The b_k was lower for juveniles ramets than genets of plot-1 and juveniles genets than ramets of plot-2. The *Sp*-statistic was significantly (P< 0.05) greater than zero for all adults, genets and ramets of both plots, all juveniles and ramets of plot-1, and all juveniles and genets of plot-2. The *Sp*-statistic was significantly higher for all adults, genets and ramets than all juveniles, genets and ramets of plot-1, and for all adults and ramets than all juveniles and ramets of plot-2.

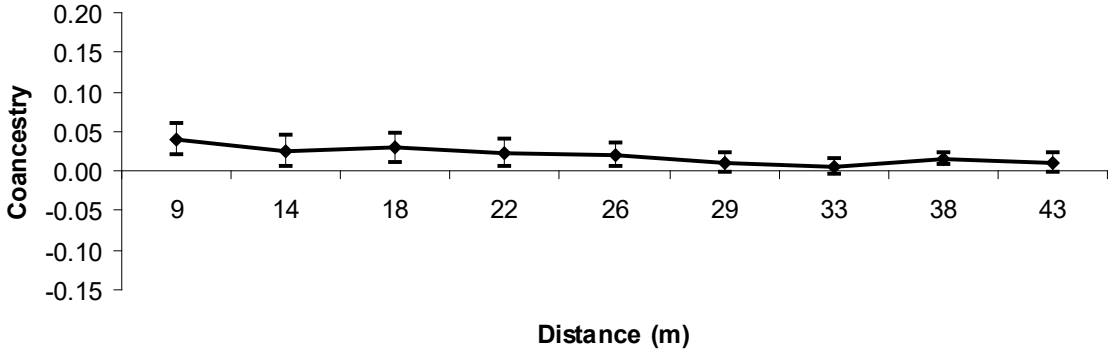
Parentage analysis

The hypothetical combined exclusion probability of the first parent (P_p) estimated using CERVUS (plot-1= 0.832098; plot-2= 0.883024), was similar to that estimated using NM+ (plot-1= 0.816577; plot-2= 0.900259). From CERVUS, the combined non-exclusion probability of identity (Q_i) for adults in plot-1 (0.000054) and plot-2 (0.000006) was low. The high values of P_p and low values of Q_i indicate that the loci used are suitable for distinguishing parents in parentage analysis. Of the 401 juveniles from plot-1 and 166 juveniles from plot-2, we assigned at least one parent for 390 (97.3 %) and 130 (78.3 %) individuals, respectively, using CERVUS (Table 3). Assuming that one of these identified parents is the mother tree, the seed flow was lower in plot-1 (2.7 %) than plot-2 (21.7 %). We detected more than one parent for 59 and 52 juveniles with unique multilocus genotypes in plot-1 and plot-2, respectively, indicating lower levels of pollen flow into plot-1 (43.3 %) than plot-2 (49.5 %). The mean selfing rate, the rate of mating among relatives, and seed and pollen dispersal distances were calculated excluding ambiguous juveniles, those with more than one putative apomictic parent, or those assigned more than one parent pair or pollen donor. The selfing rate (*s*) was lower in plot-1 (5.9 %) than plot-2 (14.3 %). The mean fixation index estimated for juveniles originating from selfing (F_s) in plot-1 was near to that expected (0.5) and in plot-2 was lower than expected. We also detected 6 juveniles in plot-1 and 13 in plot-2, whose assigned pairwise parents showed a coancestry coefficient (0_r) ≥ 0.1, indicating a rate of mating among relatives (t_r) of 27.3 % in plot-1 and 39.4 % in plot-2. The mean pairwise coancestry coefficient between mother and father trees that are assumed to be genetically related ($\theta_r \geq 0.1$), according to the standard error (SE) was not significantly different (P> 0.05) between plot-1 (0.393) and plot-2 (0.213). The mean fixation index estimated for juveniles originating from mating among relatives (F_r) in plot-1 (0.255) and plot-2 (0.284) were not significantly different (P< 0.05) from the mean pairwise coancestry between the related parents. The mean pollen dispersal distance for juveniles assigned for t_r was not significant (P> 0.05) different in plot-1 (65 m) and plot-2 (82 m), with a maximum of 213 and 121 m, respectively. The mean seed dispersal distance was higher in plot-1 (102 m) than in plot-2 (62 m), with a maximum of 190 and 107 m, respectively. The mean pollen

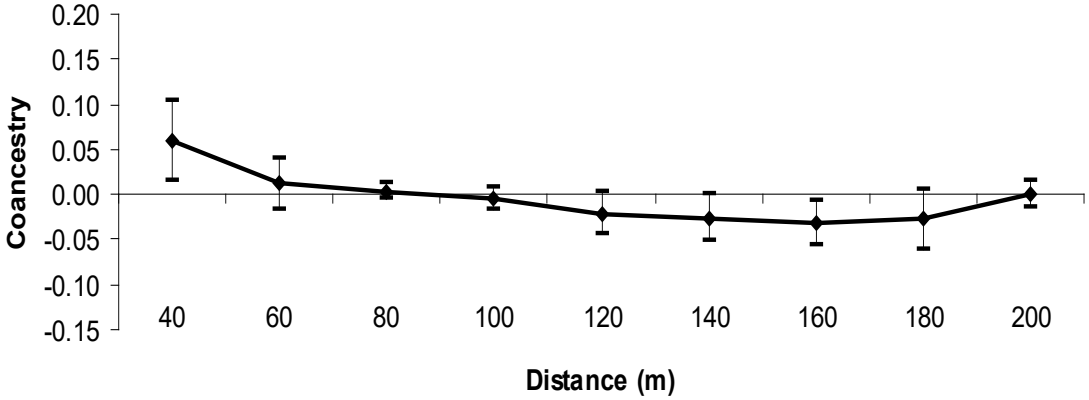
a) Plot-1: Adults



b) Plot-1: Juveniles



c) Plot-2: Adults



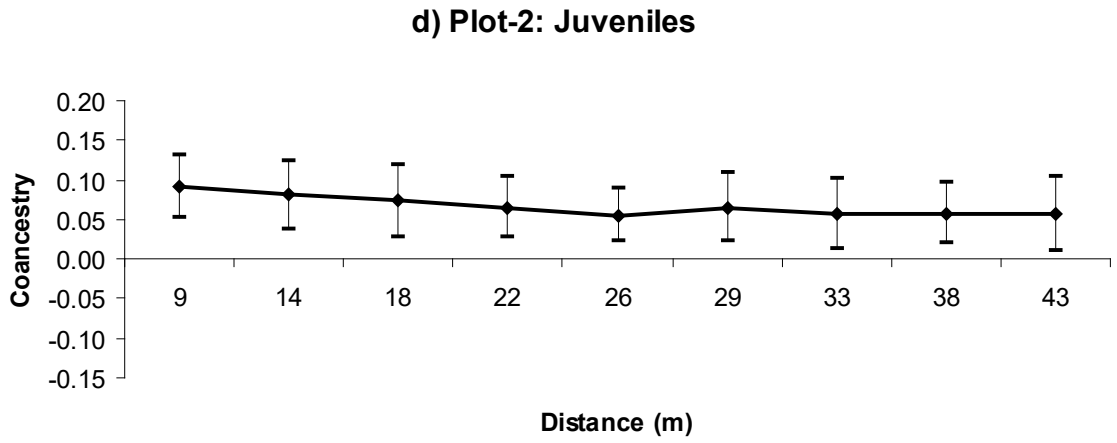


Figure 2
Correlogram with the average pairwise coancestry coefficient (continuous line) for adult trees (a, c) within 20 m distance classes and juveniles (b, d) from 3 to 5 m distance classes in plot-1 and plot-2. The vertical lines represent the 95% confidence interval of the average pairwise coancestry coefficient

Table 2
Estimate of spatial genetic structure indices for adults and juveniles of both plots.

	Adults			Juveniles		
	θ_1 (40m)	$b_k \pm 2SE$	$Sp \pm 2SE$	θ_1 (9m)	$b_k \pm 2SE$	$Sp \pm 2SE$
Plot-1						
All	0.101	-0.050 ± 0.024*	0.056 ± 0.013*	0.040	-0.015 ± 0.008*	0.016 ± 0.004*
Genets	0.084	-0.039 ± 0.016*	0.042 ± 0.017*	0.069	0.002 ± 0.006	-0.002 ± 0.003
Ramets	0.133	-0.061 ± 0.032*	0.071 ± 0.018*	0.067	-0.026 ± 0.008*	0.028 ± 0.004*
Plot-2						
All	0.060	-0.045 ± 0.018*	0.048 ± 0.019*	0.092	-0.018 ± 0.014*	0.020 ± 0.008*
Genets	0.040	-0.033 ± 0.030*	0.035 ± 0.016*	0.136	-0.038 ± 0.014*	0.044 ± 0.016*
Ramets	0.089	-0.063 ± 0.042*	0.069 ± 0.023*	0.108	0.005 ± 0.018	-0.006 ± 0.010

θ_1 is the mean pairwise coancestry coefficient in first distance class; and b_k is the regression slope of θ_1 on log of spatial distance; *SP* is the intensity of spatial genetic structure; ± 2*SE* is the standard error. P< 0.05.

dispersal distance was similar in plot-1 (95 m) and plot-2 (76 m), with a maximum of 213 and 121 m, respectively (Table 3). However, the median seed and pollen dispersal distance was shorter than the mean, indicating a tendency toward short distance seed and pollen dispersal.

Seed and pollen flow and dispersal kernel

Although seed (m_s) and pollen (m_p) flow were higher in plot-2 (m_s = 47.7 %; m_p = 52.5 %) than plot-1 (m_s = 29.6 %; m_p = 45.8 %), using NM+ (Table 4) we found no significant differences between plots. The selfing rate (*s*) was similar and not significantly different between plot-1 (12.3 %) and plot-2 (13.5 %). The seed and pollen dispersal kernel in the plots were fat-tailed (b_s and b_p < 1), indicating a high probability of long-distance seed and pollen dispersal and immigration. The mean seed (δ_s) and pollen (δ_p) dispersal distance were not significantly different between the plots, but δ_s was significantly lower than δ_p in plot-1.

Table 3
Results of seed and pollen flow and dispersal distance for the two study plots.

	Plot-1: seed	Plot-2: seed	Plot-1: pollen	Plot-2: pollen
Total sample size: *n*	401	166	401	166
Number of apomictic individuals	297	63	297	63
Number of unique genotypes	104	103	104	103
Gene flow	11 (2.7%)	36 (21.7%)	45 (43.3%)	51 (49.5%)
Assigned juveniles within plot	390	130	59	52
Non-ambiguous assignments: n_1	155	66	22	33
Selfing: *S*	-	-	6 (5.9%)	11 (14.3%)
Fixation index for selfed: $F_S \pm 2SE$	-	-	0.508 ± 0.188	0.438 ± 0.161
Mating among relatives: t_r	-	-	6 (27.3%)	13 (39.4%)
Coancestry among relatives: $\theta_r \pm 2SE$	-	-	0.393 ± 0.190	0.213 ± 0.056
Fixation index for juveniles from t_r: $F_r \pm 2SE$	-	-	0.255 ± 0.356	0.284 ± 0.102
Mean pollen distance for t_r (m) ± 2SE	-	-	65 ± 20	82 ± 58
Minimum/maximum distance for t_r (m) ± 2SE	-	-	17/82	27/121
Mean distance (m) ± 2SE	102 ± 6	62 ± 56	95 ± 8	76 ± 12
Median distance (m)	96	59	82	57
Minimum/maximum distance (m) ± 2SE	2/190	5/107	23/213	27/121

n_1 is the number of juveniles assigned to only one pollen parent (non-ambiguous); ± 2SE is the standard error.

Table 4
Pollen and seed dispersal kernel for the two study plots.

Sample	m_s (%)	$m_p \pm 2SE$ (%)	$s \pm 2SE$ (%)	$\delta_s \pm 2SE$ (m)	$\delta_p \pm 2SE$ (m)	a_s	a_p	b_s	b_p
Plot-1	29.6 ± 12.0	45.8 ± 16.2	12.3 ± 12.2	62 ± 6	119 ± 36	31	177	0.999	0.950
Plot-2	47.7 ± 9.6	52.5 ± 14.6	13.5 ± 9.8	72 ± 68	142 ± 132	32	12	0.948	0.561

m_s and m_p are the seed and pollen flow; *s* is the selfing rate; δ_s and δ_p are the mean seed and pollen dispersal distance, respectively; a_s and a_p are the scale parameters of seed and pollen dispersal kernel; b_s and b_p are the shape of seed and pollen dispersal; ± 2SE is the standard error.

Discussion

Apomixis

Our results confirm that *A. polyneuron* is a facultative apomictic species, producing seeds by apomixis and sexual reproduction, as reported previously by Ramos (2012), who found an apomixis rate of 22.8% in open-pollinated seeds from a small population of the species. The analyses of genotypic diversity (*R*) indicates that established juveniles from plot-1 (1- *R* = 0.680) and adults from plot-2 (1- *R* = 0.523) originated mainly from apomixis (1- > 0.5). In adults of plot-1 (1- *R* = 0.494) and juveniles of plot-2 (1- *R* = 0.279) the rate of apomixis was also substantial (minimum 1- *R* of 28 %). Propagation by facultative apomixis in *A. polyneuron* might favor individual fitness through the establishment of individuals adapted specifically to local conditions. It can also lead to increases in species population density. In this study, we only consider individuals to be apomictic if they present a multilocus genotype identical to other adult or juvenile individuals within the plots.

Null alleles, genetic diversity, and inbreeding

The expected frequency of null alleles (*Null*) varied between samples (Table 1). Null alleles can produce an excess of homozygote individuals, increases estimates of *F* due to the fact that some alleles are not amplified during microsatellite analysis (Chybicki and Burczyk, 2009). This occurred herein, through the significantly positive uncorrected *F* values for adults and juveniles of both plots. The *F* values for all samples decreased after correction for null alleles (F_{null}) and inbreeding was detected only in juveniles of plot-2 (0.399), where mean null alleles frequency was lowest (0.004). Self-fertilization and mating among relatives produce inbreeding in hermaphroditic, self-compatible tree species, such as *A. polyneuron*, which can explain the inbreeding detected in the juveniles of plot-2. Inbreeding in tropical trees may result in inbreeding depression, which is more pronounced in the initial life stages, and adult trees are expected to present lower levels of inbreeding than seeds, seedlings and juveniles (Degen and Sebbenn, 2014). Although we did not find significant differences between adults and juveniles for F_{null}, the absence of inbreeding in adults and the

observed inbreeding in juveniles of plot-2 is consistent with the hypothesis of selection against inbred individuals between juvenile and adult life stages. Similar results have been found in other studies on tree species (Bittencourt and Sebbenn, 2007; Duminil et al., 2016; Degen and Sebbenn, 2014). Null alleles can also affect the parentage analysis due to homozygote-homozygote mismatches between offspring and parents, resulting in no assignment of parents for some individuals and overestimates in seed and pollen flow (Chybicki and Burczyk, 2010).

Spatial genetic structure (SGS)

Consistent with the pattern of seed and pollen dispersal, the population is spatially genetically structured at both adult and juvenile stages (Figure 2) and many near neighbor individuals are related within the plots. SGS in plants is shaped by genetic drift, natural selection, and restricted seed and pollen dispersal, as well as by clonal reproduction (Silva et al., 2011; Dering et al., 2015), such as apomictic reproduction. Apomictic reproduction, seed dispersal and recruitment near to the mother tree and short distance pollen dispersal, result in the spatial structuring of *A. polyneuron* genotypes due to an increase in the mean pairwise coancestry among near neighbor individuals. However, the *Sp* -statistic indicates that adults present greater spatial genetic aggregation than juveniles (Table 2). Adults and juveniles represent different gene dispersal events; therefore, greater SGS in adults than juveniles can be explained by the fact juveniles are the product of recent seed and pollen dispersal events and adult trees reflects historic seed and pollen dispersal. Mortality of juveniles, due to local competition, natural selection, predation and disease probably decrease SGS in juveniles.

The extent of SGS, measured by the *Sp* -statistic, in adults (minimum of 0.048) is higher than the results reported for other tree species whose seeds are dispersed by wind (0.023; Dick et al., 2008). This is likely the result of apomictic reproduction in *A. polyneuron*, as well as short distance seed dispersal. Dering et al. (2015) revised their estimates of *Sp* -statistic in clonal and non-clonal tree and shrub species and observed that clonality tends to increase the SGS. With the exception of juveniles from plot-2, that showed low levels of apomictic reproduction (Table 1), the other samples presented higher *Sp* for ramets than for genets (Table 2), which is consistent with Dering et al. (2015). Thus, apomictic reproduction increases SGS. As apomictic individuals are not the product of outcrossing, this result also indicates that seed dispersal, rather than pollen dispersal is the main factor causing SGS.

CERVUS versus NM+ gene flow estimates

Estimates of gene flow and selfing rate were different using CERVUS and NM+softwares (Tables 3 and 4). CERVUS indicated lower seed and pollen flow, especially in plot-1, lower selfing rate and mean pollen dispersal distance, and higher mean seed dispersal distance in plot-1 than NM+. The differences can be attributed to the fact that, using CERVUS, our estimates were based on a single exclusion method, assuming a maximum of one mismatch among juveniles and assigned parents, and the assumption that the near neighbor or the only assigned parent is the mother, with the more distant parent as the father. In CERVUS, these assumptions can underestimate seed and pollen flow and distances of gene dispersal due to cryptic gene flow, or the false-positive assignment of parents inside plots, when the true parents are located outside of the study areas. In contrast, NM+ estimates are based in the neighborhood model, it requires no particular assumptions, accounts for null alleles, seed and pollen dispersal patterns are derived from parameters estimated from the model, which describes patterns of parentage of juveniles or open-pollinated seeds located spatially within the study areas (Chybicki and Burczyk, 2010). Thus, our gene dispersal and selfing estimates from NM+ are likely more accurate and the following discussion is based solely on the NM+ results. CERVUS results will used only to discuss the rate of mating among relative individuals as pollen dispersal within plots is expected to show less bias because it does not require assumptions about who is the mother or father assigned parent.

Seed and pollen flow

The juveniles of both plots present a greater number of private alleles (minimum k_p of 13), suggesting seed and pollen immigration. Both seed (minimum of 29.9 %) and pollen (minimum of 45.8 %) flow indicate extensive gene immigration into the study area (Table 4). However, our results for gene flow are probably overestimates due to genotyping errors and parent tree mortality. Some private alleles detected in both adults and juveniles may be an artifact of genotyping errors, resulting in no parentage assignment for some juveniles and overestimates of seed and pollen flow. We also note that our study is based on juveniles established in the plots and mortality in the adult population cannot be ignored. Tree mortality could contribute to the observed levels of gene flow, because deceased parent trees cannot be assigned to their juvenile descendants. Thus, gene flow may be lower than that detected herein. However, any level of gene flow can increase the actual levels of genetic diversity and effective population size of the populations due to the immigration of new alleles and genotypes into the plots.

Seed dispersal distance

The detected fat-tailed seed dispersal kernel pattern in the plots ($b_s < 1$) suggests a high probability of long-distance seed dispersal, but with the frequency of juvenile establishment decreasing with increased distance from the mother, in a typical isolation by distance (IBD) seed dispersal pattern (Table 4). The juveniles were frequently established in close proximity (< 100 m) to mother trees. Seed dispersal vectors and tree height have impact on seed dispersal distance (Soons et al., 2004), as well as forest porosity (Gaino et al., 2010). Seed dispersal by wind in trees that occur in the upper stratum of the forest, such as *A. polyneuron*, would favor long distance seed dispersal. However, the high tree density in the Atlantic Forest may act as a barrier for long distance seed dispersal by wind, as

was found for *M. urundeuva* (Gaino et al., 2010), thus decreasing the probability of regeneration at long distances from the mother. Nevertheless, extreme weather events such as storms and high winds probably enable seed dispersal over long distances. The combination of a high frequency of near neighbor seed dispersal with a low frequency of long distance seed dispersal can produce IBD. The IBD seed dispersal pattern seems to be common for many tropical tree species whose seeds are wind dispersed, such as *Jacaranda copaia* (Jones and Hubbell, 2006), *Aucoumea klaineana* (Born et al., 2008), *M. urundeuva* (Gaino et al., 2010), and *Himatanthus drasticus* (Baldauf et al., 2014).

Pollen dispersal distance

The mean pollen dispersal distance was 119 and 142 m in plot-1 and 2, respective, and observed pollen dispersal pattern was the IBD (Table 4). Studying a small forest fragment (7.2 ha), Ramos (2012) also found an IBD pattern for pollen dispersal in open-pollinated seeds of *A. polyneuron*. Pollen dispersal distance and patterns in tropical tree species are influenced by dispersal vectors, population density, synchronicity in individual flowering, mechanisms that reduce the selfing rate, as well as factors such as climate, forest fragmentation, and logging (Dick et al., 2008; Degen and Sebbenn, 2014). Flowers of *A. polyneuron* are pollinated by moths and butterflies and these insects have the potential to disperse pollen over long distances (Torezan et al., 2005). In populations of animal pollinated tropical trees with density greater than 5 trees/ha, such as *A. polyneuron*, the mean pollen dispersal distance is generally lower than 300 m and the pattern of pollen dispersal is generally IBD, with mating occurring at high frequencies between near-neighbor synchronous flowering trees (Dick et al., 2008; Degen and Sebbenn, 2014). Our results are consistent with these expectations. However, our results for pollen dispersal distance may be underestimated due to the possibility that some adult pollen donors may have died before sampling for the study occurred.

Seed versus pollen dispersal distance

In general, for insect-pollinated tree species, pollen dispersal distances are greater than seed dispersal distances (Dick et al., 2008; Oddou-Muratorio and Klein, 2008; Degen and Sebbenn, 2014). Our results are consistent with this pattern in plot-1 (Table 4), where the mean pollen dispersal distance (119 m) was significantly greater than the mean seed dispersal distance (62 m). The difference between plots may be attributed to the low population density in plot-1 (9.8 trees/ha). Population density may affect gene dispersal by influencing insect behavior and the restricting the movement of wind (Duminil et al., 2016). Pollen dispersal is affected by the distance between conspecifics and wind seed dispersal is affected by forest density, determined not only by the density of the studied species, but by other trees in the area as well. In low density populations, the distance among reproductive trees is greater than in high density populations and pollinators must fly greater distances to reach other conspecifics, increasing pollen dispersal. In contrast, high density forests may act as a barrier for wind seed dispersal by restricting the distance of seed dispersal.

Greater pollen than seed dispersal distance suggests that insects disperse pollen over longer distances than wind disperses seeds. Similar results were reported for *M. urundeuva*, an insect pollinated tree with wind dispersed seeds, which showed a mean seed dispersal distance of 124 m and minimum mean pollen dispersal distance of 138 m (Gaino et al., 2010). In contrast, *Simarouba amara* (Hardesty et al., 2006) and *C. langsdorffii* (Tarazi et al., 2013), insect pollinated tropical trees with animal dispersed seeds, show greater mean seed dispersal distances than pollen, suggesting that seed dispersal by animals is more effective than wind. However, more studies are needed to gain a better understanding of the effectiveness of these processes.

Mating system

Due to difficulties in estimating relatedness from the genetic markers, caused by genotyping errors, presence of null alleles and homozygous genotypes for alleles non identical by descent, we assumed that a pairwise coancestry coefficient between the mother and father of assigned juveniles greater than 0.1 means that the parents are related. Based on this assumption, the mean pairwise coancestry coefficient between mother and father of assigned juveniles in plot-1 (0.393) fell between that expected for full-sibs (0.25) and selfed or apomictic individuals (0.5). In plot-2 (0.213), the result fell between that expected among half-sibs (0.125) and full-sibs (0.25). The rate of mating among related individuals (Table 3) was lower in plot-1 (t_r = 27.3 %) than plot-2 (t_r = 39.4 %), but the selfing rate (Table 4) was similar between plot-1 (s = 12.3 %) and plot-2 (s = 13.5 %). Selfing and mating among relatives explains the inbreeding detected in juveniles and suggests that the inbreeding was mainly the result of mating among related individuals. The higher t_r in plot-2 is probably associated with SGS, population density, and pollinator behavior as the distance of SGS for adults is shorter (60 m) and the population density (25.9 trees/ha) is greater than plot-1 (90 m and 9.8 trees/ha). High population densities result in an aggregation of related individuals that are reproductive, which may increase the probability of mating among related individuals, as insects may forage more nectar locally than in low density populations (Degen and Sebbenn, 2014). The minimum expected inbreeding from selfing is 0.5 and the expected inbreeding in individuals originating from mating among relatives is equal to the coancestry coefficient between mother and father (Lindgren and Mullin, 1998). The mean fixation index estimated for selfed juveniles in plot-1 (F_s = 0.508) was similar to that expected (0.5), while in plot-2 (F_s = 0.438) it was lower than that expected (0.5). For juveniles originating from mating among relatives, the inbreeding (F_r) in plot-1 (0.255) was lower (but not significant different) than the estimated coancestry coefficient between the mother and father (θ_r = 0.393), while in plot-2 inbreeding (0.284) was higher than the estimated coancestry coefficient between the mother and father (θ_r = 0.213). The difference between the observed and expected F_s and F_r values is a

consequence of the number of loci used in this study (seven loci). For more accurate estimates of relatedness, approximately 20 loci are required (Blouin et al., 1996).

Conclusions

Our results show that the *A. polyneuron* population studied herein presents both asexual and sexual reproduction. Asexual reproduction occurs by apomixis. Sexual reproduction is realized mainly through outcrossing among unrelated individuals. The regeneration of apomictic individuals may guarantee the continuation of genotypes adapted specifically to the study site, while sexual reproduction by outcrossing results in new genotypes. Inbreeding in juveniles of both plots is explained by selfing and mating among relatives. The greater rate of inbreeding in juveniles than adults of plot-2 suggests selection against inbred individuals between juvenile and adult stages. Both seeds and pollen are dispersed in a IBD pattern, producing SGS in the population and resulting in mating among related individuals. These results are likely linked to the effectiveness of insect vectors in pollen dispersal as compared to wind seed dispersal in the studied population.

Finally, both seed and pollen flow contribute to increases in genetic diversity in the study area by incorporating new genotypes and alleles. Our study indicates for collection of seeds, seed trees should preferentially being located distant from each other in at least 110 m in plot-1 and 70 m in plot-2. *Aspidosperma polyneuron* has a dynamic reproductive system, which maintains the genetic diversity and the survival of the populations. Possibly the main role of the apomictic here is to maintain the population size and survival of the species, since apomictic are highly adapted to the specific environmental conditions of the site. Then, the *in situ* survival depends of the maintenance of the dynamic balance in the populations.

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