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Increased relatedness among the neighboring plants from seedling to adult stages in carnaúba wax palm

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ABSTRACT. The objective of this study was to assess the spatial genetic structure (SGS) at different life stages (cohorts) in a remnant population (N = 101) of *Copernicia prunifera* in the semiarid region of northeastern Brazil. Using seven inter-simple sequence repeat molecular markers, we were able to analyze 93 loci with 100% polymorphism. Seedlings had the highest level of genetic diversity (H_E = 0.411, H_O = 0.599), followed by juveniles (H_E = 0.394, H_O = 0.579) and adults (H_E = 0.267, H_O = 0.427). Based on analysis of molecular variance, the majority of genetic variations were observed to occur within the life stages (93.42%) rather than between the life stages (6.58%). We found a recent reduction in the population size (bottleneck) based on the number of loci with heterozygosity excess for the two models used (infinite allele = 92 and stepwise = 91). All the life stages showed significant SGS, with positive and significant kinship values. *Sp* values were 0.040 for seedlings, 0.093 for juveniles, 0.156 for adults, and

0.053 for the total population. We found an increase in SGS from the seedling to adult stages, indicating that the plants were from related adult progenitors. Data from this study can be used in designing effective management and conservation strategies for the species.

Key words: Northeastern Brazil; ISSR; Molecular markers; *Copernicia prunifera*; Spatial genetic structure

INTRODUCTION

Populations of species exist in various environments that are different in time and space and are affected by many factors, such as weather, natural disasters, pathogens, and herbivore attacks (Condit et al., 2000; Ng et al., 2004), which directly impact the geographical distribution, structure, and genetic diversity of the species (White and Walker, 1997). The distribution patterns of genetic variability in species can reveal ecological and biological aspects, including pollination and seed dispersal mechanisms of the species (Gonçalves et al., 2010; Silva et al., 2011; Brandão et al., 2015). In addition, genetically less diverse populations are particularly vulnerable to extinction, whereas populations with large numbers of genotypes are more likely to be resistant to deleterious influences (Van Treuren et al., 1991). Therefore, genetic diversity is a key factor for development of species over time; it enables the species to adapt to the environmental changes. The genetic composition of a population influences the way its members will adapt to future environmental changes (Hoffmann and Willi, 2008).

The diversity and genetic structure of a species is fundamental for management, conservation, or domestication of the species (Cota et al., 2011). The population genetic structure can be analyzed using molecular markers, such as inter-simple sequence repeat (ISSR; Zietkiewicz et al., 1994). The polymorphism detected by this technique is binary; the technique normally detects only one allele per locus (Reddy et al., 2002). It is possible to assess the diversity of a population, as well as its spatial genetic structure using the ISSR markers. From a conservationist point of view, these observations in the study may contribute directly to strategies aimed at maintaining genetic diversity in reforestation, management, and regeneration programs in degraded areas (Gonçalves et al., 2010; Souza and Lovato, 2010).

To develop an *in situ* conservation plan, it is essential to understand the levels of genetic variability and the spatial distribution of genotypes within the populations. Previous studies on the genetic structure show that the individuals located close to each other tend to be related and usually there is greater genetic diversity within than there is among the life stages (Lacerda and Kageyama, 2003; Rossato et al., 2007). This variation might be the result of different ecological and genetic factors operating in the natural populations, as well as the varying spatial-temporal sampling and statistical methods used in the study (Vekemans and Hardy, 2004). In addition, the scale and magnitude of genetic structure may also differ significantly among the life stages, with seedlings often exhibiting greater structure within populations compared with the adults (Ng et al., 2004).

Copernicia prunifera (Arecaceae), commonly known as carnaúba, naturally occurs in northeastern Brazil. The species has an erect trunk with unique sheaths or residual leaf bases that remain on the trunk (Rodrigues et al., 2013). In rural areas, the trunk is used in construction of houses and animal corrals. The perennial leaves are numerous (from 45 to 60 leaves per individual) and the canopy is round. The species produces a branched inflorescence

that is longer than the leaves; the flowers are pollinated by bees and the flowering is subannual (Rocha et al., 2015). *C. prunifera* has diploid number of chromosome (2n = 36) (Môro et al., 1999). The fruits are spherical with a smooth, dark epicarp when mature and are dispersed by bats (Sousa et al., 2015). The fasciculate roots have medicinal properties (Rodrigues et al., 2013). The present study aimed to determine the variability of spatial genetic structure (SGS) among the different life stages (seedlings, juveniles, and adults) in a carnaúba population using ISSR molecular markers.

MATERIAL AND METHODS

Study site and sampling

The study population is a natural *C. prunifera* stand located in Macaíba (5°53'57"S, 35°22'59"W), Rio Grande do Norte State, Brazil. The region has an average annual precipitation of 1243.5 mm/year. According to the Köppen classification, the climate is a transition between As (tropical) and BSh (dry) climates, with dry summers and rainy winters. The local vegetation is predominantly Caatinga (arboreal and shrubby), with transitional vegetation of the Atlantic Forest (Cestaro and Soares, 2004); it has suffered a high degree of human interference, such as deforestation and road construction. This remnant *C. prunifera* population was chosen because it clearly presents the three main stages of ecological succession: seedlings (height ranging between 0.20 to 1.00 meters), juveniles (greater than 1.00 meters tall, non-reproductive), and adults (reproductive). The study area is also being subjected to desertification, and is, thus, a priority for conservation.

Within the population, all the 101 individuals were sampled and X and Y coordinates were recorded (Figure 1). Plant height was estimated using a steel measuring tape. We assessed the presence or absence of reproductive structures, such as flower buds, flowers, and fruit of all the individuals present in the area, to identify adult individuals. The leaf samples were collected and stored in a freezer at -20°C until DNA extraction.

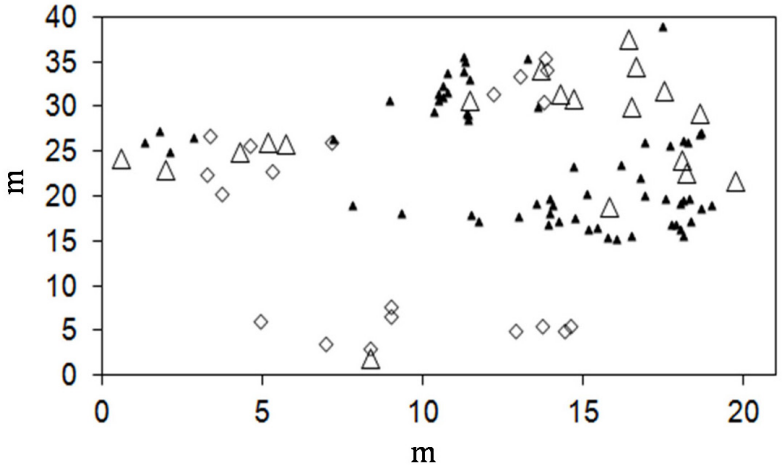

Figure 1. Position and spatial distribution of *Copernicia prunifera* individuals. Large triangles indicate adults; diamonds indicate juveniles; filled triangles indicate seedlings.

DNA extraction, amplification of ISSR, and electrophoresis

DNA was extracted using the CTAB (cetyltrimethylammonium bromide) method, proposed by Doyle and Doyle (1990). Polymerase chain reaction (PCR) was subsequently carried out using 17 ISSR universal primers designed at the University of British Columbia (UBC). The PCR mix consisted of 10X buffer, 1.0 mg/mL BSA, 50 mM $MgCl_2$, 2.5 mM dNTPs, 2 μM primer, 0.5 U Taq polymerase, a 1:50 dilution of DNA, and ultrapure water. The PCRs were conducted using a Veriti automatic thermocycler in which the samples were initially denatured at 94°C for 5 min, followed by 37 amplification cycles of 94°C for 15 s, 47°C for 30 s, and 72°C for 1 min. The reaction was concluded with a final extension step of 72°C for 7 min, followed by cooling at 4°C. The PCR products were analyzed by horizontal electrophoresis on 1.5% agarose gel, in 0.5X Tris-Borate EDTA buffer, at 120 V. The gels were stained with GelRed™ and photographed under ultraviolet light using E-Box VX2 equipment to observe the DNA fragments.

Data analysis

Diversity and genetic structure

We used the POPGENE program version 1.3 to analyze the genetic diversity within the studied population (Yeh et al., 1997), including the number of observed alleles (N_A), effective number of alleles (N_E), Nei's genetic diversity (H_E), and Shannon Index (H_O) for each life stage. The ALERQUIM 3.0 program (Excoffier et al., 2007) was used for analysis of molecular variance (AMOVA) among the different life stages. This analysis uses molecular data to estimate the population differentiation and allows for hypothesis testing related to such differentiation.

SGS

For analyzing the SGS, we estimated the kinship coefficient between pairs of individuals (Hardy, 2003) in the total sample and for each of the three life stages using the SPAGeDi program, v.1.2g (Hardy and Vekemans, 2002). To analyze the intensity of SGS in each cohort, the *Sp* was calculated using the formula $Sp = -b_{log}/(1 - F_{IJ(1)})$, where $-b_{log}$ is the regression slope of the kinship coefficient curve and $F_{IJ(1)}$ is the kinship coefficient in the first distance class.

Detecting genetic bottlenecks

To test our hypothesis about the reduction in the effective population size and consequent reduction in genetic diversity, we used the allele frequencies in the Bottleneck program, version 1.2 (Cornuet and Luikart, 1996), using the models infinite allele model of mutation (IAM) and stepwise mutation model (SMM). These two models were used to calculate the expected heterozygosity of the population at mutation-drift equilibrium, which were then compared to the level of heterozygosity found in the studied population. The mutation model for the ISSR loci is intermediate between the IAM and SMM; therefore, both of these were used (Luikart et al., 1998). The sign test ($\alpha = 0.05$) was used to identify significant recent genetic bottleneck based on the allele frequency (Cornuet and Luikart, 1996).

RESULTS

Genetic diversity and structure

Of the 17 tested primers, 12 amplified the DNA of *C. prunifera*. Seven of these were selected (Table 1), which detected 93 loci with 100% polymorphism. The values of the observed alleles (2.00), polymorphic loci (93), and polymorphic loci percentage (100%) were equal for the total population and for the three cohorts. The seedlings showed the greatest levels of genetic diversity (H_E = 0.411 and H_O = 0.599), followed by juveniles and adults (Table 2).

AMOVA showed that there was more genetic variation within cohorts (93.42%) than between cohorts (6.58%) (Table 3).

Table 1. Nucleotide sequence of ISSR primers, number of loci detected by it, and the PIC value of each primer.

ISSR primer	Sequence (5'-3')	Loci	PIC
UBC 813 (CT)8-T	CTCTCTCTCTCTCTCTT	11	0.280
UBC 827 (AC)8G	ACACACACACACACACG	9	0.264
UBC 840 (GA)8-YT	GAGAGAGAGAGAGAGAYT	12	0.393
UBC 841 (GA)8-YC	GAGAGAGAGAGAGAGAYC	18	0.444
UBC 842 (GA)8-YG	GAGAGAGAGAGAGAGAYG	17	0.419
UBC 857 (AC)8-YG	ACACACACACACACACYG	14	0.079
UBC 859 (TG)8-RC	TGTGTGTGTGTGTGTGRC	12	0.057
Mean		13.28	0.277

R = purine (A or G); Y = pyrimidine (C or T).

Table 2. Genetic diversity and spatial genetic structure for the life stages of *Copernicia prunifera*.

	N	N_E	H_E	H_O	F_{IJ}	b_{log}	*Sp*
Seedlings	62	1.728 ± 0.219	0.411 ± 0.079	0.599 ± 0.086	0.069	-0.036*	0.040
Juveniles	20	1.689 ± 0.242	0.394 ± 0.095	0.579 ± 0.108	0.172	-0.077*	0.093
Adults	19	1.397 ± 0.215	0.267 ± 0.114	0.427 ± 0.146	0.211	-0.123*	0.156
Total	101	1.667 ± 0.209	0.390 ± 0.079	0.576 ± 0.088	0.083	-0.048*	0.053

Bottleneck

Using IAM, it was expected that the number of loci with heterozygosity excess was 37.09, whereas, the results showed 92 loci with heterozygosity excess. Therefore, the observed value of loci was more than twice of the expected value and was significant according to the sign test ($P < 0.0001$). For SMM, the expected value of loci with heterozygosity excess was 42.59; however, the observed value was much higher (91 loci) and was also statistically significant ($P < 0.0001$).

Spatial genetic structure

The developmental stages (seedlings and juveniles) of the population showed positive relatedness within the first distance class, from 0 to 4 m (Figures 2A and B, respectively), whereas the adults (Figure 2C) and total population (Figure 2D) showed significant relatedness up to the second distance class, i.e., from 0 to 8 and 0 to 9 m, respectively. The seedlings showed negative relatedness in the fourth distance class (about 13 meters). In the subsequent

distance classes, the distribution was random. The genetic divergence between the juveniles, adults, and all the individuals was significant in the third distance class, whereas the juveniles showed dissimilarity at 17 and 33 m.

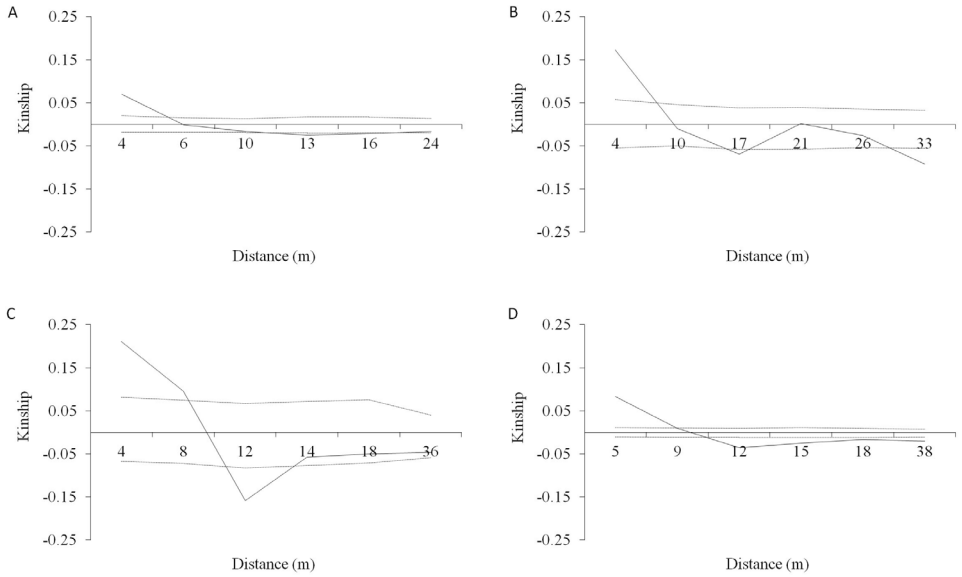

Figure 2. Correlogram of kinship coefficients of seedlings (**A**), juveniles (**B**), adults (**C**) and for all the *Copernicia prunifera* individuals (**D**). Dotted line indicates confidence interval.

The results of *Sp* statistics showed the same genetic structure in the total population as well as in the developmental stages ($P < 0.05$) (Table 2). In the first distance class, the *Sp* value was highest for adults (0.156), followed by juveniles (0.093), and seedlings (0.040).

DISCUSSION

Our study showed 100% polymorphism (93 loci), using seven selected ISSR primers. Srivashtav et al. (2013) used two ISSR primers to assess the genetic diversity of the species *Phoenix dactylifera* L. (Arecaceae) from a region in India. Total thirteen loci were found, among which 10 were polymorphic and three were monomorphic. Roncal et al. (2007) assessed the taxonomic distinction of two varieties of *Geonoma macrostachys* Mart. (Arecaeae) in the Amazon region using ISSR markers and observed 99 loci, among which 51.52% were polymorphic.

We found the lowest levels of diversity among the *C. prunifera* adults ($H_E = 0.267$ and $H_O = 0.427$), possibly because they are remnants of a population that has suffered a genetic bottleneck. This conclusion is supported by the test for genetic bottleneck, discussed below. The significant differences between the levels of genetic diversity across generations within the population might be the result of natural selection or influx of genotypes from other populations (Epperson and Alvarez-Buylla, 1997; Vieira et al., 2010). Vieira et al. (2012) noted that environmental disturbances, limited seed dispersal near the mother plant, and low density of reproductive adults can determine the varying levels of relatedness between individuals and between life stages, as well as the degree of genetic diversity of the population.

Using allozyme markers, Conte et al. (2003) observed no significant differences in the genetic diversity (Nei index) between the seedlings, juveniles, and adults of *Euterpe edulis* Mart. They attributed the similarity in genetic diversity to the large number of adult individuals in the population, which might compensate for the variability that is naturally lost through genetic drift. In our study, the significant differences observed in the levels of genetic diversity between the life stages and the greater molecular variation within the life stages can be associated with an influx of genotypes from other populations or random crossing among adult individuals in the studied population. Thus, further studies are necessary to evaluate the outcrossing rate of *C. prunifera* and its association with genetic structure.

The genetic bottleneck analysis showed that the population suffered a strong reduction in the effective size, possibly as a consequence of human activities in the region. We observed the evidence of cutting-down of *C. prunifera* and other species in the study area. On the other hand, according to Deshpande et al. (2001), the large number of loci with heterozygosity excess might be related to the fact that ISSR markers tend to generate more polymorphism than other markers, such as AFLP and RAPD. In this case, the simultaneous use of different molecular markers might shed light on this hypothesis.

Using microsatellite markers, Seoane et al. (2005) assessed the reproductive system of two *E. edulis* populations, one fragmented and the other continuous, and found a greater kinship value among the progeny of the continuous population (F_{LJ} = 0.222) than that in the progeny of the fragmented population (F_{LJ} = 0.193). Furthermore, Brandão et al. (2011) used 10 ISSR primers to identify the genetic distribution of *Myrcia splendens* (SW.) DC in a fragment-corridor system in southeastern Brazil. They found positive kinship values in two vegetation corridors, at distances of 20 and 70 m. Several authors have suggested that genetic structuring between the life stages might be common among the tropical tree species (Conte et al., 2003; Seoane et al., 2005; Vieira et al., 2012). A possible explanation for SGS being found in the first distance class for *C. prunifera* is its restricted seed dispersal, where large number of fruits fall near the mother plant.

In this study, we observed that the SGS increases from seedling to the adult life stages, suggesting that the adult plants are from related parents. Similar results were found by Latouche-Hallé et al. (2003), whereas Kalisz et al. (2001) found a lower genetic diversity index for adults, supporting the hypothesis that more significant SGS is inversely related to the genetic diversity. In fact, the initial life stages of *C. prunifera* had greater H_E and less SGS, whereas the final life stage (adults) have lower H_E and higher SGS (Tables 1 and 3).

Implications for conservation

Our results show that *C. prunifera* plants have spatial structure up to a distance of 5 to 6 m in all the life stages, with spatial correlation between seedlings and adults, which indicates restricted dispersion around the parental plants. Among the developmental stages of *C. prunifera*, the regeneration stage (seedlings) was most genetically diverse and the majority of genotypic variations were found within the life stages. The life stages with the greatest genetic similarity were the juveniles and seedlings. In addition, the genetic bottleneck analysis suggests that the population was greatly reduced and consequently suffered a reduction in genotypes. Finally, SGS was found up to the second distance class for all the life stages, indicating that the individuals located in close proximity to one another are closely related.

To date, few studies have been conducted to analyze the genetic diversity of *Copernicia* species using molecular markers (Vieira et al., 2015). Therefore, this study presents novel

genetic information not only about the genus *Copernicia*, but also about *C. prunifera*, which is important for further understanding of the Arecaceae family. Although it is not present in the list of endangered species, *C. prunifera* is increasingly being threatened by agricultural practices in northeastern Brazil (Sousa et al., 2015). Therefore, the preservation of this palm is of fundamental importance because, besides the ecological relationships that the species has with the natural environment, it is a source of income for many rural families and is an important factor in economic and social development. The data obtained in this study might contribute to the development of strategies for conservation and proper management of *C. prunifera*. For example, using such information, it is possible to select progeny arrays to produce seedlings with high levels of genetic diversity (Lowe et al., 2005). Based on our results, we recommend that trees selected for seed collection should be located at least 12 m apart, thereby, greatly reducing the probability of progeny arrays being closely related and contribute to the molecular diversity of the population. In addition, conservation plans must ensure that the size and structure of the population (e.g., number of individuals of reproductive age) presents sufficient genetic diversity to enable the species to maintain itself for a long time within the environment in which it occurs (Harris and Johnson, 2004).

Conflicts of interest

The authors declare no conflict of interest.

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