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Mating system and intrapopulational genetic diversity of *Copernicia prunifera* (Arecaceae): a native palm from Brazilian semiarid

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ABSTRACT. The aim of this study was to investigate the mating system and genetic diversity of the palm *Copernicia prunifera* using inter-simple sequence repeat markers. We found that the *C. prunifera* has multiple inflorescences with hermaphroditic flowers and pollen viability of 62%. Outcrossing rates at the population level (N = 267) produced a multilocus outcrossing rate (t_m) of 0.878 and a single-locus outcrossing rate (t_s) of 0.738, indicating that *C. prunifera* has a mixed mating system that is preferentially allogamous. The rate of mating among relatives (t_m - t_s) was low, indicating limited outcrossing between closely related individuals. The fixation index between seed trees (F) was negative (-0.200), suggesting an absence of inbreeding, while the correlation of selfing (r_s) was high (0.914). The values of the diversity indices among adults and progenies did not differ statistically (H_E = 0.319 and I = 0.470; H_E = 0.337 and I = 0.505, respectively). In testing for the presence of genetic bottlenecks using the infinite allele model and the stepwise mutation model, we observed a reduction in the effective population, as well as a deficit in heterozygosity (P <

0.0001). The results of this study inform management strategies for the conservation and genetic improvement of the *C. prunifera* palm.

Key words: Caatinga; ISSR; Carnauba palm; Outcrossing rate

INTRODUCTION

The palm *Copernicia prunifera* (Miller) H.E. Moore (Arecaceae), commonly known as carnaúba, occurs across the States of Tocantins, Maranhão, Piauí, Ceará, Rio Grande do Norte, Paraíba, Pernambuco, Alagoas, Sergipe, Bahia, and Mato Grosso (Leitman et al., 2015). The species can be used for a variety of purposes, from urban forestry to wax extraction from its leaves, the main product of the species, which is used in cosmetics, varnishes, and even for polishing fruit (Mota et al., 2006).

The knowledge of the mating systems, as well as preliminary studies, such as reproductive biology, floral structures, and pollinators of a species, are essential to support taxonomy investigations, management strategies, breeding, and domestication of native species (Li et al., 2012). Silberbauer-Gottsberger (1990) argued that it is unlikely the hypothesis of anemophily be the only type of pollination occurring in the family Arecaceae, given the importance of insect pollination reproduction of family representatives. In fact, the most common pollen dispersal agents in palm trees are beetles, followed by bees and flies (Barfod et al., 2003). The protandry where the anthers mature before the stigma is receptive and it is quite common in Arecaceae, which favors the cross-fertilization (Mantovani and Morellato, 2000).

Due to the economic and social importance of *C. prunifera*, determining its mating system is vital, because it is an aspect that must be considered in the management, conservation, and genetic improvement of the species. Mating systems can alter the genetic dynamics of populations by influencing the genetic composition of subsequent generations (Oostermeijer et al., 2003). Besides, the mating system determines the magnitude of inbreeding in the descendant population, and it is important to consider how the recombination of genes in each reproductive event is expressed (Ward et al., 2005).

The mating system of hermaphroditic species can combine selfing with outcrossing, through which both random or correlated mating occurs (Ritland, 2002). Furthermore, most palm species present mixed mating systems, being preferentially allogamous (Ramos et al., 2011; Abreu et al., 2012; Picanço-Rodrigues et al., 2015). Due to the effects of anthropization in the natural populations, and because the species occurs in monodominant groups at high densities, it is expected that *C. prunifera* presents a mixed mating system, with a high rate of outcrossing between related individuals.

Analyses of the mating system of forest species can be performed using dominant (Ferreira et al., 2010) or codominant markers (Wadt et al., 2015). To overcome limitations in the analysis of individual genotypes, Ritland (2002) developed the multilocus model, which includes dominant markers in the evaluation of the mating system of plant species. Among the dominant markers, inter-simple sequence repeat (ISSR) markers are useful (Han et al., 2009) because they are effective in detecting polymorphism, are easily reproduced, and have lower costs than SSR markers, for example. ISSR markers amplify genomic fragments that are abundant and widely distributed throughout the genome of eukaryote individuals and do not require sequencing (Ge et al., 2005). Thus, the aim of this study was to investigate the

genetic diversity and mating system of the palm *C. prunifera* using ISSR markers, generating information that can help us understand the reproduction mechanism of the species.

MATERIAL AND METHODS

Plant sampling

The sampled population of *C. prunifera* for the study of the reproductive biology is located in the municipalities of Lagoa de Pedras (6°12'33.51"S and 35°27'38.24"W) and Parnamirim (5°57'59.14"S and 35°08'34"W), Rio Grande do Norte (RN), Brazil. The sampled population of *C. prunifera* for the study of the mating system and genetic diversity is located in the municipality of São Miguel do Gostoso, RN, Brazil (5°07'18"S and 35°41'02"W). The region is characterized by a tropical climate with the dry season (As), according to the Köppen climate classification. The vegetation of the study areas is Caatinga, made up of shrubs and thorny trees. Also, the site presents high levels of anthropization, mainly due to the expansion of wind power plants.

Three individuals were selected to provide the data on the structure of the inflorescence and two for pollen viability. The reproductive parts (flower buds and flower) were collected and subsequently packaged in Falcon tubes containing the FAA 50 solution (10% formaldehyde, 85% ethyl alcohol and 5% acetic acid). To study the mating system, leaf samples and fruits were collected from 16 adult reproductive individuals, in a 0.55-ha area. Due to the limited number of fruits available for some individuals, the number of progenies ranged from 4 to 20. The population was georeferenced using GPS and individuals were mapped with a tape measure for greater accuracy.

Leaf tissue samples of adult individuals were placed in 2-mL plastic tubes containing 2X CTAB (cationic hexadecyltrimethylammonium bromide) and then labeled and transported to the laboratory. For progenies, we collected the first leaves to develop and then stored in a freezer at -20°C until DNA extraction.

DNA extraction, amplification of ISSR, and electrophoresis

DNA was extracted using the CTAB method, proposed by Doyle and Doyle (1990). The PCRs were carried out in a Veriti Thermal Cycler, in a volume of 12 µL, containing diluted genomic DNA, 10X PCR buffer, 1.0 mg/mL BSA, 2.5 mM dNTP, 50 mM $MgCl_2$, 5 U/µL Taq DNA polymerase, 2 µM primer, and ultrapure water. The PCR protocol consisted of an initial denaturation for 2 min at 94°C, followed by 37 amplification cycles of 15 s at 94°C, 30 s at 47°C, 1 min at 72°C, a final extension for 7 min at 72°C, and cooling to 4°C. The PCR products were stained with GelRed™ and analyzed using horizontal electrophoresis, separated on 1.5% agarose gel, in a solution of 1X TAE (Tris-acetate-EDTA), at 100 V for 2.5 h. We used a molecular weight marker (Ladder) of 10,000 bp, and the gels were photographed with ultraviolet light in an E-Box VX2.

Reproductive biology

The structure of the inflorescence was characterized by the length of the rachis (cm); the number of rachillae up in inflorescence; the position of the rachilla, which is subdivided

into three distinct areas (basal, intermediate and apical region); the number of multiple inflorescences and blooms at rachilla (Figure 1A). The sexual type and flower morphological characterization were determined with the aid of a stereoscopic microscope Medilux®.

For pollen viability analysis, there were eight repetitions in blades. The pollen grains were stained with 1% acetic orcein solution (Kearns and Inouye, 1993). Then, the pollen grains once stained were covered with coverslip and mountant for observation in an optical microscope, using the magnifying lens of 40X. To obtain a random sampling of stained pollen grains, we counted 100 pollen grains per blade. The pollen grains were analyzed and classified as normal/viable with cytoplasm stained recorded as normal and as abnormal/inviable recorded for those with little or no cytoplasm evidenced. The percentage of viable pollen was calculated by the equation: pollen viability (%) = number of colored grains / grain number counted x 100.

Genetic diversity

To determine the genetic diversity parameters, we built a binary array based on the presence (1) and absence (0) of loci on gels. The polymorphic information content (PIC) was used to test the efficiency of the ISSR markers to detect polymorphism between two individuals, through the presence or absence of loci. According to Botstein et al. (1980), molecular markers are classified as satisfactory in informational content when the PIC value is greater than 0.5. Values from 0.25 to 0.5 are moderately informative, and values below 0.25 have little information. For this, we used the formula proposed by Anderson et al. (1993):

$$PIC_i = 1 - \sum_{j=1}^{n} P_{ij}^2$$

where Pij is the frequency of allele "j" at marker "i".

The genetic data were used to calculate the percentage of polymorphic loci, the number of effective alleles, the number of observed alleles, Nei's genetic diversity (H_E), and Shannon diversity index (I). The adult individuals and progenies were evaluated, and analyses were carried out using the POPGENE v. 1.32 program (Yeh et al., 1997). Genetic identity was obtained using the NTSYS program (Rohlf, 1993), with the goal of constructing a dendrogram of unweighted pair group method with arithmetic mean (UPGMA) for the 16 seed trees, based on Nei (1978)'s genetic identity. Analysis of Nei's genetic distance was conducted with the POPGENE program v. 1.32 (Yeh et al., 1997).

Mating system

The mating system was assessed using the mixed mating model and the correlated mating model using the MLTR program (Ritland, 2004). The standard deviations of the estimates were obtained by 1000 bootstraps. The estimated parameters were: a) multilocus outcrossing rate (t_m); b) single-locus outcrossing rate (t_s); c) rate of mating among relatives (t_m - t_s); d) selfing rate (s = 1 - t_m); e) fixation index between seed trees (F); f) expected fixation index F = [(1 - t_m) / (1 + t_m)]; g) correlation of selfing (r_s); h) multilocus paternity correlation ($r_{p(m)}$); i) single-locus paternity correlation ($r_{p(s)}$); j) correlation of the estimate of t_m (r_t); and k) the relatedness between pollen donor trees ($r_{p(s)}$ - $r_{p(m)}$).

Genetic bottleneck detection

To verify the presence of a genetic bottleneck that resulted in a reduction in the effective size of the population over generations, we used the infinite allele model (IAM) and the stepwise mutation model (SMM). The analyses were performed using the Bottleneck 1.2.02 program (Cornuet and Luikart, 1996).

RESULTS

Reproductive biology

The species presented multiple inflorescences, being made up of hermaphroditic flowers, with a yellowish coloration (Figure 1B). Besides, the flowers were composed of 3 sepals, 3 petals, 3 stamens, and 6 carpels. The average percentage of viable pollen was 62% (Figure 1C).

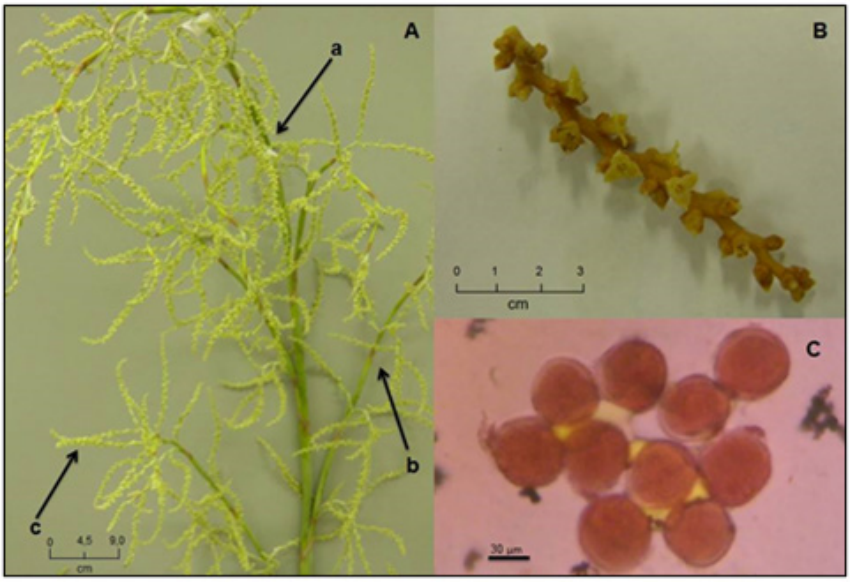

Figure 1. Reproductive structures of *Copernicia prunifera*. **A.** Rachis (a), rachilla (b), and portion of rachilla (c). **B.** Multiple inflorescences of *C. prunifera*. **C.** Pollen grains of palm *C. prunifera*, viewed in the objective lens of 40X.

The length of the rachis averaged 1.29 m; the ratio of the number of rachilla by inflorescence ranged from 4.00 to 15.00. The rachilla exhibited greater length in the basal portion, averaging 62.50 cm. The number of subrachilla and flowers by rachilla were concentrated with higher proportions in the basal portion, with average values of 6.17 and 1735.50, respectively. In the studied population, the "sanhaçú do coqueiro" (*Tangara palmarum*) was found visiting the tops of some individuals (Figure 2A). Floral visits were also recorded by the "maribondo-caboclo" (*Polistes canadensis* Linnaeus) and by "irapuá" (*Trigona spinipes* Fabricius) (Figure 2B and C).

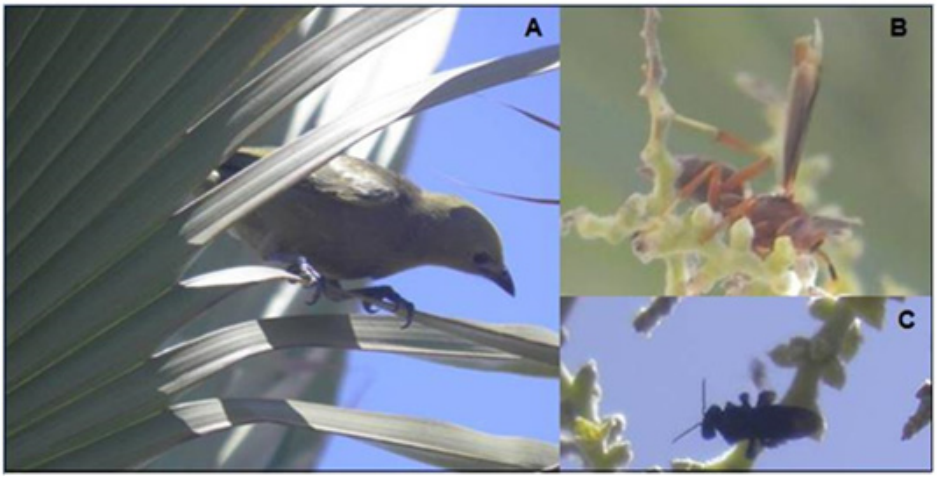

Figure 2. *Tangara palmarum* (**A**), *Polistes canadensis* (**B**), and *Trigona spinipes* (**C**) in individual *Copernicia prunifera*.

Polymorphism and PIC

The markers showed large numbers of loci, as well as a good resolution for the analyzed fragments. Eight ISSR markers were used, producing 104 loci with 100% polymorphism (Table 1). The number of loci ranged from 10 to 17, with an average of 13 per marker. The PIC of markers ranged from 0.416 to 0.500, with an average of 0.477.

Table 1. Nucleotide sequence of the ISSR markers, number of loci, and the PIC value for each primer.

ISSR primer	Sequence (5'-3')	Loci	PIC
UBC 825 $(AC)_8$-T	ACA CAC ACA CAC ACA CT	17	0.498
UBC 827 $(AC)_8$-G	ACA CAC ACA CAC ACA CG	11	0.484
UBC 840 $(GA)_8$-YT	GAC AGA GAG AGA GAG AYT	14	0.500
UBC 851 $(GT)_8$-YG	GTG TGT GTG TGT GTG TYG	12	0.416
UBC 857 $(AC)_8$-YG	ACA CAC ACA CAC ACA CYG	13	0.492
UBC 859 $(TG)_8$-RC	TGT GTG TGT GTG TGT GRC	13	0.439
UBC 860 $(TG)_8$-RA	TGT GTG TGT GTG TGT GRA	10	0.494
UBC 873 $(GACA)_4$	GAC AGA CAG ACA GAC A	14	0.495
Average		13	0.477

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

Diversity and genetic identity

For the parameters of genetic diversity (Table 2), the adults showed 84.62% polymorphic loci, while the progenies presented 100% polymorphism. The number of alleles (N_A) and the number of effective alleles (N_E) observed were higher among progenies than among adults [2.000 (± 0.000) and 1.575 (± 0.020), respectively]. No statistical differences in the results for Nei's genetic diversity (H_E), assuming Hardy-Weinberg equilibrium, and the Shannon index (I) between adults and progenies were found.

Table 2. Genetic diversity parameters for the population of *Copernicia prunifera*.

Population	N	Lp (%)	N_A	N_E	H_E	I
Adults	16	84.62	1.846 ± 0.090	1.558 ± 0.087	0.319 ± 0.044	0.470 ± 0.061
Progenies	251	100	2.000 ± 0.000	1.575 ± 0.020	0.337 ± 0.009	0.505 ± 0.011
Total	267	100	2.000 ± 0.000	1.607 ± 0.018	0.353 ± 0.008	0.526 ± 0.010

Sample size (N), percentage of polymorphic loci (Lp%), number of alleles observed (N_A), number of effective alleles (N_E), Nei's genetic diversity index (H_E), Shannon index (I). Values represent the average ± standard error.

Based on Nei's genetic identity (1978), the UPGMA dendrogram grouped the population into two groups: one formed by individuals 1, 3, 5, and 9; and the other made up of the remaining individuals (Figure 3). Individuals 9, 10, and 12 showed less genetic similarity regarding the others.

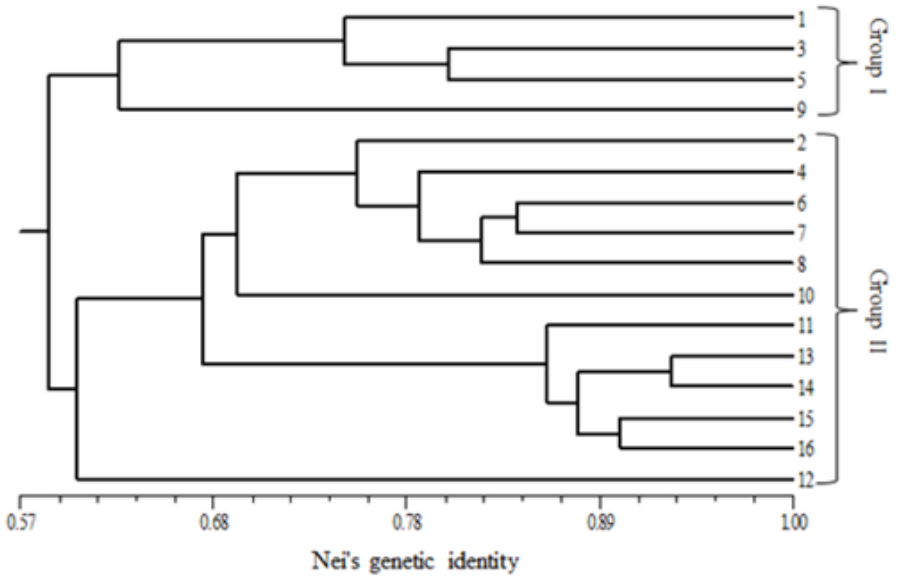

Figure 3. Dendrogram of Nei's genetic identity between *Copernicia prunifera* individuals.

Estimates of Nei's genetic distance between individuals were observed in those that have less genetic similarity based on the identity analysis and greater genetic distance: primarily between individuals 9 and 2 (0.838), 10 and 3 (0.693), 10 and 5 (0.693), 10 and 11 (0.501), 12 and 9 (0.732), 12 and 15 (0.637).

In the analysis to determine a reduction in the effective population, both the IAM and the SMM detected genetic bottleneck in the population. In the IAM, it was expected that the number of loci with heterozygosity excess was 38.56, whereas, the results showed 93 loci with heterozygosity excess. For SMM, the expected value of loci with heterozygosity excess was 44.65; however, the observed value was much higher, 88 loci. Additionally, the signal test showed a significant deficit of heterozygosity based on the evaluated models ($P < 0.0001$).

Mating system

Estimates of population-level outcrossing (Table 3) showed rates of t_m = 0.878, t_s = 0.738, and s = 0.122. Mating among relatives (t_m - t_s) was positive (0.140). The main coefficient of selfing between seed trees was negative (-0.200), and lower than expected (0.065). For selfing and multilocus and single-locus paternity correlation, we found high rates of direct selfing correlation (0.914) and low rates of single-locus paternity correlation (0.017). The level of relatedness between pollen donors in the population was -0.296.

Table 3. Estimates of mating system parameters for the *Copernicia prunifera* population.

Parameters	Average
Multilocus outcrossing rate: t_m	0.878 (0.037)
Single-locus outcrossing rate: t_s	0.738 (0.037)
Mating among relatives: t_m - t_s	0.140 (0.037)
Selfing rate: s = 1 - t_m	0.122
Fixation index between seed tree: F	-0.200 (0.023)
Fixation index expected: $F = (1 - t_m) / (1 + t_m)$	0.065
Correlation of selfing: r_s	0.914 (0.110)
Multilocus paternity correlation: $r_{p(m)}$	0.313 (0.043)
Single-locus paternity correlation: $r_{p(s)}$	0.017 (0.030)
Correlation of the estimate of t_m: r_t	0.597 (0.095)
Relatedness between pollen donors: $r_{p(s)}$ - $r_{p(m)}$	-0.296 (0.041)

Values in parentheses mean standard deviation.

DISCUSSION

Souza et al. (2002) reported that pollen viability in forest species is only considered high for values above 70%. Information on pollination in palm trees is incipient, and under the existing entomophily (pollination by insects) and the wind (pollination by wind action) have been reported as the main systems of pollination, with highlight to entomophily (Oliveira et al., 2003). Regarding the observation of insects in individuals of *C. prunifera*, the population of Parnamirim has been the most frequent. Nevertheless, Silveira et al. (2010), in a study conducted with individuals from *Vaccinium myrtillus* (Ericaceae), identified that *Trigona spinipes* is harmful to the species, especially at the time of flowering, fruiting, and fruit with reduced size. The presence of insects observed in the population is an indication that they are likely pollinators of the species.

In tropical vegetation, the zoochory is the dominant dispersal syndrome (Bollen et al., 2004). Purificação et al. (2015) verified that in individuals from *Schefflera morototoni* (Araliaceae), the *Tangara palmarum* presents itself as one of the main dispersers of the fruits of this species. Additionally, in remarks carried out in individuals of *Cecropia pachystachya*, it was noted that *Tangara palmarum* is omnivorous, with a habit of visiting and reap the rewards in plants (Gonçalves and Vitorino, 2014).

The number of loci evaluated in this study was high (N = 104) in comparison with other studies on the genetic diversity of palms using dominant markers, with results ranging between 47 and 93 (Chagas et al., 2015; Vieira et al., 2016). Using ISSR markers to study the palm species *Phoenix dactylifera* and *Mauritia flexuosa*, the percentage of polymorphic found by Mirbahar et al. (2014) and Rossi et al. (2014) was similar to that found in the present study. However, Chagas et al. (2015) found low levels of genetic polymorphism in a population of

Elaeis guineenses. Thus, estimates of the level of genetic variability in a population can be influenced by the percentage of polymorphic loci.

The molecular polymorphism of ISSRs used in this study was moderate, according to the classification by Botstein et al. (1980). Vieira et al. (2016), testing seven ISSR markers for *C. prunifera*, found PIC values ranging from 0.079 and 0.444, with an average of 0.277. The PIC may vary depending on the type of molecular marker used. According to Buonaccorsi et al. (1999), among all genetic markers, microsatellite markers offer greater information content.

The rate of H_E in this study was higher than expected for long-lived perennial species and outcrossing (H_E = 0.25 and 0.27, respectively) (Nybom, 2004). Our results were very similar to those seen in other natural population of *C. prunifera* (I = 0.44 and H_E = 0.228) (Vieira et al., 2016), and relatively higher than the Shannon index of a natural population of *Phoenix dactylifera*, with values between 0.290 and 0.097 (Marsafari and Mehrabi, 2013). As such, the differences between the values of genetic diversity indices reflect the effects of life history traits (Nybom, 2004). All these factors may result in the loss of rare alleles, a reduction in heterozygosity, and increased inbreeding (Rossi et al., 2014).

The population analyzed presented genetic bottleneck, based on IAM and SMM ($P < 0.01$). Therefore, there was no balance between mutation and drift in the sampled population. This result was similar to that found for a population of *Elaeis guineenses* where the authors also reported a reduction in the effective population size (Chagas et al., 2015). A reduction in the effective population size can be the result of human intervention in the region, through the installation of wind turbine towers and the introduction of cattle in the study area. These disturbed environments can lead to an increased risk of extinction of local populations, as well as decrease the evolutionary potential of species due to changes in the natural environment (Hamrick, 2004).

The mating system parameters indicate that *C. prunifera* is a mixed mating species ($t < 0.95$), that is preferentially allogamous (t_m = 0.878). Furthermore, the single-locus outcrossing rate was high (t_s = 0.738). These values were consistent with those found for other tropical palms, which are predominantly outcrossing, such as *Acrocomia aculeata* (Abreu et al., 2012) and *Bactris gasipaes* (Picanço-Rodrigues et al., 2015). Ward et al. (2005) in 36 studies surveyed found > 90% outcrossed mating for 45 hermaphroditic or monoecious species. Another parameter that defines allogamy is the rate of selfing (s); the result from the present study (s = 0.122) falls within the range expected for a predominantly allogamous species (s < 20%) (Winn et al., 2011).

The outcrossing rate in hermaphroditic species, such as *C. prunifera*, depends on factors including pollinator behavior, which is influenced by the density of flowering individuals in the population; selective abortion of fruits and seeds from outcrossing; presence and intensity of self-incompatibility mechanisms; and the degree of protogyny and protandry (Murawski and Hamrick, 1991). The rate of mating among relatives (t_m - t_s) showed that, although outcrossing in the population is high, some individuals are the product of mating between relatives (0.140). Picanço-Rodrigues et al. (2015) found a similar result for the *Bactris gasipaes* palm. Additionally, the correlation of selfing was high (r_s = 0.914), indicating that some plants produce more descendants from selfing than outcrossing.

The fixation index between seed trees (F = -0.200) indicates an absence of inbreeding among reproductive individuals. Ramos et al. (2011) and Abreu et al. (2012) also identified an absence of inbreeding in natural populations of the palms *Astrocaryum aculeatum* and *Acrocomia aculeata*. The lack of inbreeding in the study population is consistent with the low

level of mating among relatives. Therefore, despite the occurrence of *C. prunifera* individuals in an anthropogenic area, in monodominant clusters, and at high densities, we can reject the assumption of a high rate of outcrossing between related individuals expected for the study population. In addition, we found low levels of relatedness between pollen donor trees ($r_{p(s)}$ - $r_{p(m)}$ = -0.296).

Implications for conservation and management

The study area suffers constant anthropogenic pressure, primarily through the advancement of wind energy facilities in the region. Without proper planning, these facilities can have a negative impact on the studied *C. prunifera* population, which can lead to habitat fragmentation and a loss of genetic variability. The genetic bottleneck detected through our analysis may be associated with anthropogenic interventions in the study population.

With the aim of supporting species conservation, our study shows that the local population and wind energy companies must be better informed about the importance of maintaining the existing population. Furthermore, the exploitation of the species must be carried out sustainably. For this, government programs should be developed to reduce the anthropogenic impacts on *C. prunifera*. Considering future genetic improvement studies and programs for the species, we propose the formation of a base population of seeds of different individuals and populations. Besides, to conserve the current genetic diversity, we suggest the creation of a genbank, based on different genotypes. Our study shows a clear need for conservation of populations with large numbers of individuals.

Conflicts of interest

The authors declare no conflict of interest.

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