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Genetic and phenotypic association of the carnauba palm tree evaluated by inter-simple sequence repeat and biometric traits

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ABSTRACT. The carnauba palm *Copernicia prunifera* is the third most important non-timber forest species in Brazil; it is mainly known for the production of carnauba wax. We examined intra-population genetic diversity and correlated genotypic and phenotypic traits of 28 trees from a coastal location in Rio Grande do Norte state. Phenotypic variables tree height, diameter at breast height, curvature of the stem, fresh mass of fruits and seeds, and length and diameter of fruits and seeds were evaluated. Genotypic parameters were examined based on inter-simple sequence repeat (ISSR) polymorphisms. The eight ISSR primers detected 79 loci, of which 76% were polymorphic. The polymorphic information content ranged between 0.408 and 0.500, the Nei's diversity index *h* was 0.327, and Shannon index was 0.470. Although there was no significant association between the matrices of genotypic and phenotypic traits, stepwise multiple regression analysis identified ISSR markers that can be used in a marker-assisted breeding program. The ISSR loci $881_{(2)}$, $880_{(1)}$, and $M1_{(4)}$ were significantly and positively associated with plant height. The sMRA also identified a positive association between DBH and $842_{(5)}$ and a negative association between the curvature of the stem and $857_{(1)}$. The fresh mass of fruits had a significant negative correlation with ISSR locus $840_{(1)}$. The other

traits showed positive and negative associations when the marker(s) of the previous step(s) were included in the succeeding step in sMRA analyses. These results are relevant for breeding programs, with the perspective of obtaining intra specific hybrids that are superior for desirable characters.

Key words: *Copernicia prunifera*; ISSR; genetic diversity; stepwise multiple regression analysis

INTRODUCTION

Studies on genetic diversity are useful for the evaluation, description and classification of genetic resources for preservation (Govindaraj et al., 2015). The results obtained can subsidize strategies for the selection of genotypes that can be used in genetic improvement. In addition, information about the genetic diversity of populations is necessary for the creation of genetic conservation methods (Kagimbo et al., 2018).

Differentiation of genotypes can be quantified from morphological, biochemical or DNA markers (Adhikari et al., 2017). Characterization of genotypes through DNA molecular markers has become popular because DNA markers are stable and will not be confused with environmental, pleiotropic and epistatic effects (Nybom, 2004; Adhikari et al., 2017). Also, the diversity level in DNA markers is greater. In general, the choice among different types of genetic markers depends on the objectives and their applicability in scientific research (Fajardo et al., 2014). DNA markers are unique because of the simplicity of use, cost, type of technology applied, reproducibility, dominance or codominance, and the ability to distinguish between individuals (Adhikari et al., 2017).

Among molecular markers, the ISSRs (inter-simple sequence repeats) are frequently used and have been shown to be efficient. ISSR markers have a high level of polymorphism, with high reproducibility, and they do not require prior knowledge of the genome, making it a relatively low-cost DNA marker (Desai et al., 2015). The use of ISSRs has been shown to be useful in the analysis of the genetic diversity of palm trees, as in *Phoenix dactylifera* L. (Mirbahar et al. 2016), *Elaies guineensis* (Chagas et al., 2015), *Attalea vitrivir* (Santos et al., 2015), *Orbignya phalerata* Mart. (Viana et al., 2015), and *Mauritia flexuosa* (Rossi et al., 2014).

Among species with important economic value in Brazil, we highlight *Copernicia prunifera* (Arecaceae), known in Brazil as carnauba. This species is native to the Brazilian semi-arid region, with predominance in the states of Ceará, Piauí and Rio Grande do Norte (Leitman et al., 2015). Exploitation of *C. prunifera* is mainly based on the extraction of awaxy powder from the leaves, which is then processed into carnauba wax, with a production value of US$140 million in the years 2015-2016 (IBGE, 2016). Fibers of *C. prunifera* are also used in artisanal activities, with enormous economic impact (Sousa et al., 2015).

Despite the importance of *C. prunifera* in the economy of the northeastern region of Brazil, research involving characterization of genotypes is non-existent. Stepwise multiple regression analysis (sMRA) is used to look for associations between phenotypic traits and molecular markers (Khadivi-Khub, 2014; Choudhary et al., 2017). ISSR markers have

frequently been utilized in the sMRA approach to evaluate associations different agronomic and commercial traits (Ganopoulos et al., 2011; Ding et al., 2016). Such studies contribute to the adoption of genetic improvement programs and domestication. To this end, we estimated the intra-population genetic diversity of *C. prunifera* by ISSR markers and examined correlations between phenotypic and genotypic traits among individuals of a local population.

MATERIAL AND METHODS

Study site and sampling

The study was carried out in a planted population at coordinates 5°57'59.14"S and 35°08'34"W in the municipality of Parnamirim, Rio Grande do Norte state, Brazil. The planting was established approximately 25 years ago from seeds of an open pollinated population. The climate of the region is type As, tropical with dry summer and rainy seasons, according to the Köppen classification (Alvares et al., 2013).

Twenty-eight individuals were sampled. Measurements included the height of the plants, DBH (diameter at breast height – approximately 1.3 meters) and curvature of the stem by a qualitative scale (1 - rectilinear, 2 - rectilinear with curvature and 3 - tortuous). Plant height was estimated considering the distance between the soil surface and the last living branch of the plant. The DBH value was obtained through the formula DBH = CBH/π, where CBH is the circumference at breast height. Leaf samples were collected and conditioned in 2 mL plastic tubes containing 2X CTAB (cetyltrimethylammonium bromide).

Ripe fruits were collected from all 11 adult plants that presented fruits during the study period. We collected 15 fruits from each plant, totaling 165 fruits. For each plant, the length (mm), diameter (mm) and fresh mass (g) of the fruits and seeds were recorded with a digital caliper and an electronic scale.

DNA extraction, PCR, and electrophoresis

The DNA was extracted following a standardized protocol for *C. prunifera* (Vieira et al., 2015). The DNA samples were quantified using an Epoch™ spectrophotometer. The PCRs were performed in a Veriti 96-well thermocycler. Twenty-seven ISSR primers were tested, with a length between 13 and 18 nitrogenous bases and with %CG between 44 and 80%. The PCR solution was run in a total volume of 12 μL with Buffer (10 X, IC PHT™), BSA (1.0 $mg.mL^{-1}$), $MgCl_2$ (50 mM), dNTP (2.5 mM), primer (2 μM), Taq polymerase ($U.\mu L^{-1}$), DNA (50 ng), and ultrapure water. The PCR cycle consisted of denaturation at 94°C for 5 min, followed by 37 cycles at 94°C for 15 s, 47°C for 30 s, and 72°C for 1 min. The process was finalized at 72°C for 7 min and cooled to 4°C.

PCR products were subjected to electrophoresis on a 1.5% agarose gel (v/v) in TAE buffer (1.0 X, EDTA Tris-acetate). We ran the electrophoresis at 110 V and utilized a molecular weight marker (ladder) of 1 kb. The gels were stained with GelRed™ and photographed with ultraviolet light in an E-Box™ VX2.

Data analysis

Descriptive statistics and genetic diversity

The phenotypic characteristics were analyzed through descriptive statistics, including position and dispersion measures using SPSS 23 software (IBM, 2015). Deviations from the normal distribution were evaluated by skewness (*S*) and kurtosis (*K*) values.

The genotypes were evaluated by a binary matrix, according to the presence (1) or absence (0) of the ISSR marker. The polymorphism information content (PIC) was calculated using the formula $PIC = 1 - \Sigma^{n}_{j=1} P^{2}_{ij}$, where P_{ij} is the frequency of the allele "j" in the marker "i" (Anderson et al., 1993). The percentage of polymorphic loci (%*P*), number of alleles observed (n_a), number of effective alleles (n_e), genetic diversity of Nei (*h*, also called H_E) and Shannon Index (*I*) were calculated using POPGENE 1.32 software (Yeh et al., 1997).

The pairwise genetic similarities between genotypes were evaluated using a Dice coefficient. The clustering procedure was the method UPGMA (Unweighted Pair-Group Method with Arithmetic Mean). The cophenetic correlation (r_C) and bootstrap support values were calculated considering 1,000 replicates. This analysis was performed using the software PAST version 3.18 (Hammer et al. 2001). The resulting dendrogram was drawn using TreeView 1.6.6 software (Page, 1996).

Association among traits and stepwise multiple regression analysis

The phenotypic traits were standardized to *Z* scores prior to obtaining the Euclidean distance matrix in the SPSS software. The genetic distance matrix was obtained based on Nei's standardized genetic distance (*Ds*) using FreeTree 0.9.1.50 software (Hampl et al., 2001). The correlation between the phenotypic matrices (Euclidean distance) and the genotypic matrix (*Ds*) was evaluated by the Mantel (r_M) test using GenAlex 6.503 software (Peakall and Smouse, 2012). The significance value of the r_M was obtained through the Monte Carlo test with 999 permutations.

Association between ISSR markers (as independent variables) and the phenotypic traits (as dependent variables) was estimated through stepwise multiple regression analysis (sMRA) according to Virk et al. (1996), using the software SPSS. The stepping criteria employed to select independent variables for entry and removal was *F* values with 0.045 and 0.099 probabilities, respectively.

RESULTS

Phenotypic traits

The height and DBH ranged, respectively, from 3.20 to 6.25 m, and 31.21 to 45.22 cm. The mean fruit average length, diameter, and fresh mass were, respectively, 26.12 mm, 16.25 mm and 2.64 g (Table 1). The standard error values (s.e. < 1.0) indicated good accuracy in the sampling of phenotypic traits.

The *CV* values indicated that the fresh mass (FM) of fruits and seeds varied more than other traits, with a high *CV* of 67.44 and 66.76%, respectively. The kurtosis values indicated that all the traits had a platykurtic distribution ($K < 0$), indicating wide dispersion of the data around the mean.

Table 1. Descriptive statistics of the phenotypic traits of *Copernicia prunifera*.

Traits	*n*	Minimum	Mean (s.e.)	Maximum	*CV* (%)	*S*	*K*
Plants' height (m)	28	3.20	4.69 (0.16)	6.25	17.94	-0.09	-0.66
DBH (cm)	28	31.21	38.85 (0.77)	45.22	10.61	-0.31	-1.06
Fruits (FM) (g)	165	0.61	2.64 (0.13)	7.15	67.44	1.07	-0.18
Fruits (L) (mm)	165	20.20	26.12 (0.23)	33.80	11.52	0.48	-0.25
Fruits (D) (mm)	165	6.60	16.25 (0.21)	22.00	16.65	-0.0003	-0.04
Seeds (FM) (g)	165	0.17	1.25 (0.07)	3.41	66.76	0.80	-0.55
Seeds (L) (mm)	165	11.10	16.22 (0.20)	22.90	15.62	0.33	-0.62
Seeds (D) (mm)	165	4.20	10.55 (0.23)	17.00	27.89	0.15	-1.00

DBH, diameter at breast height; FM, fresh mass; L, length; D, diameter; *n*, sample size; s.e., standard error; *CV*, coefficient of variation; *S*, skewness; *K*, kurtosis.

ISSR markers and genetic diversity

The eight selected ISSR primers detected 79 loci (Table 2), of which 76% were polymorphic. The number of loci ranged from 6 to 13 per primer. The PIC ranged between 0.408 and 0.500.

Table 2. Nucleotide sequence of the ISSR primers, number of loci (NL), and the polymorphic information content (PIC) for each primer.

Primer ISSR	Sequence (5' – 3')	NL	PIC
825 (AC)8-T	ACACACACACACACACT	11	0.495
840 (GA)8-YT	GAGAGAGAGAGAGAGAYT	10	0.488
842 (GA)8-YG	GAGAGAGAGAGAGAGAYG	6	0.496
857 (AC)8-YG	ACACACACACACACACYG	9	0.449
873 (GACA)4	GACAGACAGACAGACA	13	0.409
880 (GGAGA)3	GGAGAGGAGAGGAGA	8	0.408
881 (GGGTG)3	GGGTGGGGTGGGGTG	11	0.500
M1 CAA (GA)5	CAAGAGAGAGAGA	11	0.444
Mean		9.88	0.461

Y = pyrimidine (C or T)

The sampled population had an observed number of alleles n_a = 1.76 (s.e. 0.05), number of effective alleles n_e = 1.59 (s.e. 0.04), Nei's genetic diversity h = 0.327 (s.e. 0.02), and Shannon index I = 0.470 (s.e. 0.03). Figure 1 presents the clustering analysis generated for all genotypes.

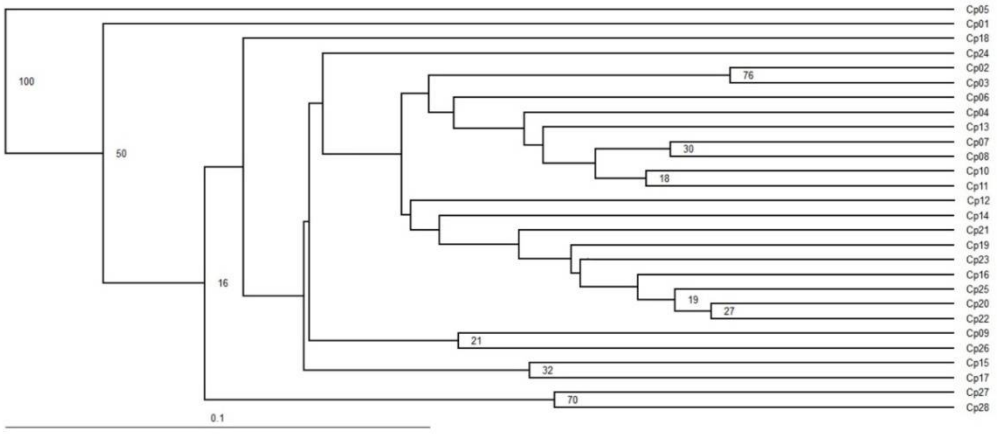

Figure 1. Cluster analysis of genotypes of *Copernicia prunifera*, based on Dice coefficient similarity. Bootstrap values greater than 15% are shown.The cophenetic correlation was r_C = 0.8.

Association among traits and stepwise multiple regression analysis

There was not significant correlation between the Euclidean distance matrix (for plant height, DBH, and stem curvature) and the genetic distance matrix (Mantel test, r_M = 0.096; P = 0.151) and between the Euclidean distance matrix (for fruits and seeds) and the genetic distance matrix (r_M = 0.006; P = 0.366).

However, that the ISSR loci $881_{(2)}$, $880_{(1)}$, and $M1_{(4)}$ were significantly and positively correlated with plant height (sMRA analysis, Table 3). The sMRA also identified a positive association between DBH and $842_{(5)}$ and a negative association between stem curvature and $857_{(1)}$. The fresh mass of fruits significantly and negatively correlated with the ISSR locus $840_{(1)}$. The other traits showed positive and negative associations when the marker(s) of the previous step(s) were included in the succeeding step in the sMRA analyses.

Table 3. ISSR markers associated with phenotypic traits in *Copernicia prunifera* as revealed by multiple regression analysis.

Traits	ISSR Markers$_{(Locus)}$	r	Adjusted R^2	$F_{ANOVA(P< 0.05)}$	β	t value	P value
Plant height (m)	$881_{(2)}$	0.547	0.272	10.7	0.481	3.528	0.002
	$+880_{(1)}$	0.472	0.451	11.7	0.370	2.659	0.014
	$+M1_{(4)}$	0.465	0.527	10.6	0.308	2.194	0.039
DBH (cm)	$842_{(5)}$	0.454	0.174	6.5	0.454	2.545	0.017
Stem	$857_{(1)}$	-0.466	0.186	6.9	-0.466	-2.635	0.014
Fruits (FM) (g)	$840_{(1)}$	-0.790	0.577	13.3	-1.097	-15.806	0.000
	$+840_{(5)}$	0.187	0.917	50.9	0.612	9.102	0.000
	$+881_{(10)}$	0.018	0.966	86.5	-0.212	-3.330	0.016
Fruits (L) (mm)	$873_{(10)}$	0.846	0.680	20.1	0.936	17.452	0.000
	$+825_{(5)}$	-0.023	0.843	25.2	-0.551	-10.067	0.000
	$+840_{(4)}$	-0.478	0.931	41.4	-0.272	-5.311	0.003
	$+825_{(8)}$	0.269	0.980	110.5	0.202	3.950	0.011
Fruits (D) (mm)	$880_{(7)}$	-0.745	0.499	10.0	-1.060	-15.446	0.000
	$+880_{(8)}$	0.276	0.825	22.3	0.709	10.327	0.000
	$+873_{(7)}$	-0.023	0.966	86.4	0.363	5.477	0.002
Seeds (FM) (g)	$840_{(1)}$	-0.755	0.517	10.6	-1.092	-14.695	0.000
	$+840_{(5)}$	0.187	0.827	22.5	0.595	8.267	0.000
	$+881_{(10)}$	-0.112	0.961	75.3	-0.343	-5.024	0.002
Seeds (L) (mm)	$840_{(1)}$	-0.736	0.484	9.5	-0.827	-7.585	0.000
	$+881_{(11)}$	-0.550	0.800	19.0	-0.661	-5.945	0.001
	$+857_{(3)}$	0.113	0.902	28.6	-0.333	-2.878	0.028
Seeds (D) (mm)	$840_{(1)}$	-0.720	0.458	8.6	-1.072	-14.433	0.000
	$+840_{(5)}$	0.167	0.708	11.9	0.553	7.683	0.000
	$+881_{(10)}$	-0.236	0.961	75.3	-0.466	-6.833	0.000

r, Pearson's correlation coefficient; Adjusted R^2, the square of R multiple adjusted for the number of predictors in the model, the multiple correlation coefficient; β, standardized beta coefficients; +, denotes that the marker(s) of the previous step(s) are included in the succeeding step.

DISCUSSION

Phenotypic traits

Copernicia prunifera typically reaches 7-10 m in height and can reach up to 15 m (Lorenzi, 2010). In the study population, the plants were relatively small, with an average height of 4.69 m. This difference is probably due to environmental factors. The plant height and DBH variables presented $S < 0$, indicating that tall individuals and with higher DAP predominate in this population.

There was significant variability in the fresh mass of fruit and seed. This is probably due to environmental effect (e.g. availability of water), which is relevant for the production

of fleshy fruits (Chenet al., 2017). The mass and the length of the fruits showed positive skewness ($S > 0$), indicating that fruits with smaller length and mass predominate in this sample. The diameter of the fruit presented $S < 0$, evidencing asymmetric distribution to the left, meaning a tendency of fruits with a larger diameter in the sample. The seeds presented $S > 0$, indicating a predominance of seeds with less mass, length and diameter in the sample.

Phenotypic studies including biometric observations of fruits and seeds are useful for understanding the intra-population variation (Vieira and Carvalho, 2009), and can support conservation and genetic improvement programs. Freitas et al. (2015) studied *Spondias* sp. genotypes and generated valuable information for selection based on biometric parameters of the fruits. Júnior et al. (2011) studied *Prunus persica* and provided relevant data for selection of superior genotypes regarding fruit quality. Yadav et al. (2017) biometrically characterized fruits and seeds of *Annona squamosa* and indicated that this approach is useful for tree selection in genetic improvement programs.

Genetic diversity

The PIC demonstrates the efficiency of the ISSR primers used to detect the polymorphism. All the markers were moderately informative, according to the Botstein et al. (1980) classification. Vieira et al. (2015) studied *C. prunifera* genotypes and found PIC values ranging from 0.057 to 0.444. The PIC observed here (0.46) was higher than in other studies of palm trees using ISSR, including *Borassus flabellifer* (0.23) (Vinayagam et al., 2009), *Phoenix dactylifera* (0.32) (Sabir et al., 2014), and *Attalea vitrivir* (0.45) (Santos et al., 2015).

The h value in this study (0.327) was higher than expected for long-lived perennial and out crossing species (0.25 and 0.27, respectively) (Nybom, 2004). Our results were similar to Pinheiro et al. (2017) that found $h = 0.341$ and $I = 0.505$ for adults individuals of *C. prunifera*, but superior to those of Vieira et al. (2016), who found $h = 0.267$ and $I = 0.427$ in a natural population of *C. prunifera*.

Association among traits and stepwise multiple regression analysis

Silva et al. (2007) also did not identify an association between the biometric characteristics of the fruits and genetic distance of the palm tree *Geonoma schottiana*. Given that the phenotype is the result of the action of the genotype under the influence of the environment, the phenotypic characteristics of the fruits are probably affected mainly by the availability of water, which is the primary determinant of fruit mass (Chenet al., 2017).

Although we did not find a significant correlation between genotypic and phenotypic traits based on the Mantel test, the sMRA analysis identified ISSR markers that can be used in a marker-assisted breeding program. These results are relevant for breeding programs, with the perspective of obtaining intraspecific hybrids for desirable characters. Integrating phenotypic traits with molecular markers has recently become a useful tool for estimating genetic divergence and consequently select plants for breeding (Choudhary et al., 2017). Additional studies using assemblages of DNA-based markers, biological and agronomic traits, are necessary to create a database for *ex situ* conservation and domestication of *C. prunifera* genotypes.

In conclusion, the population of *C. prunifera* showed considerable genetic diversity among the plants, based on the ISSR markers. The phenotypic traits evaluated were also diverse, especially the fresh mass of fruits and seeds. There was no correlation between the matrices of phenotypic and genotypic traits, indicating significant environmental influence in the set of observations. However, a positive and significant correlation was observed between some ISSR markers and phenotypic traits, indicating that such characteristics can be determined by genetic factors and thus should be considered in a breeding program.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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