BIOSCIENCE JOURNAL

GENETIC DIVERSITY AND STRUCTURE IN NATURAL POPULATIONS OF CAJUI FROM BRAZILIAN CERRADO

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How to cite: GOMES, M.F.C., et al. Genetic diversity and structure in natural populations of Cajui from Brazilian cerrado. *Bioscience Journal*. 2021, **37**, e37080. https://doi.org/10.14393/BJ-v37n0a2021-53974

Abstract

Cajui (*Anacardium* spp.) is a native fruit tree (small cashew) of the Brazilian Cerrado and possesses the potential for commercialization. However, cajui exploitation occurs exclusively through extractivism in the absence of conservation strategies. The lack of conservation strategies may lead to a decrease in genetic diversity of *Anacardium*. In this work, the genetic diversity and population structure of three natural populations in Sete Cidades National Park (PNSC; PI, Brazil) were assessed using ISSR analysis of 56 cajui accessions and two *A. occidentale* accessions (outgroup) from Pacajus (CE, Brazil). A total of 112 markers were obtained, 93 (83.04%) of which were polymorphic. The diversity indices of these populations indicated moderate levels of genetic diversity. According to AMOVA, 96.17% of the genetic variability lay within populations, with low genetic differentiation among populations (Φ_{ST} = 0.03828). Furthermore, STRUCTURE analysis indicated the existence of four connected genetic groups. The findings show that the individuals from the three collection sites did not represent different subpopulations, likely due to the high gene flow (Nm = 6.7) favored by the floral biology of *Anacardium*, pollinators and small-animal seed dispersers. This research identifies genetically divergent individuals (C-03, C-05, C-22, C-26, C-34 and C-39), which should be considered priority individuals for conservation and can inform conservation programs for *Anacardium* spp.

Keywords: *Anacardium.* Conservation. Genetic resources. Population structure.

1. Introduction

The Brazilian Cerrado is considered a hotspot of biodiversity and presents a richness of fruit species with great potential for economic utilization (Myers et al. 2000). The native fruit species of this biome are traditionally used by the local population and provide an alternative source of income (Campos et al. 2015).

Among the commonly used fruit species in the Brazilian Cerrado, cajui (*Anacardium* spp.) has particular significance. The fruit and pseudofruit are rich in nutrients, being consumed in different ways by the local population in areas of natural occurrence of these species (Maia et al. 2019). The term 'cajui' (small cashew fruit) is used to describe species of the genus *Anacardium* L. that produce small nuts (cashews) and peduncles (Borges et al. 2018). The genus *Anacardium*, family Anacardiaceae, is native to Neotropical regions and presents two centers of diversity: one in the Central Amazon and the other in the Brazilian Cerrado (Mitchell and Mori 1987). Both the nut (fruit) and the peduncle (pseudofruit) can be consumed *in natura* or in the form of juices and sweets (Campos et al. 2015; Porto et al. 2016). In addition, the peduncles can be used to produce granola, which is highly nutritious, and liqueurs (Oliveira et al. 2011; Souza and Silva 2015). The peduncle presents high contents of vitamin C and antioxidant compounds indicated for the prevention or therapy of pathologies in cells under oxidative stress (Barbosa-Filho et al. 2014).

However, although to its economic potential, the lack of conservation strategies may lead to this genus is susceptible to genetic erosion (Cota et al. 2017). The loss of genetic variability may compromise the long-term viability of populations and the potential of species to adapt to environmental changes (Fajardo et al. 2017). Thus, it is necessary to increase our knowledge of this genus and develop strategies for its conservation.

Native populations of cajui are found in Sete Cidades National Park (PNSC) in northeastern Brazil. Several studies regarding PNSC vegetation have been conducted, but knowledge of the diversity and genetic structure of the park's native fruit species is scarce. Nonetheless, information regarding genetic diversity is key for developing strategies for conserving genetic resources. Genetic variation can be evaluated by using molecular markers, such as inter-simple sequence repeats (ISSRs). ISSR analysis is a simple and efficient technique that does not require previous knowledge of the genome of the taxon under study (Duarte et al. 2018; Tian et al. 2018; Mariano et al. 2019).

Thus, the present study aimed to assess the amount of the genetic differentiation among populations of cajui in the PNSC by ISSR. The genetic information from our study will support conservation programs of *Anacardium* spp.

2. Material and Methods

Study sites and sampling

A total of 56 individuals of *Anacardium* spp. were collected from Cerrado areas in PNSC, which is located between the cities of Brasileira and Piracuruca (PI-Brazil). Although we could not identify the plant material to a species-level, all samples belong to the genus *Anacardium* and correspond to cajui trees due to the small sizes of the chestnut and peduncle. The sampling area represented three natural populations: Descoberta (12 individuals), Lagoa Seca (24 individuals) and 5ª Cidade (20 individuals) (Table 1, Figure 1). Our sampling strategy aimed to achieve samples representative of the various phytophysiognomies in the Brazilian Cerrado from various areas within PNSC (Table 1).

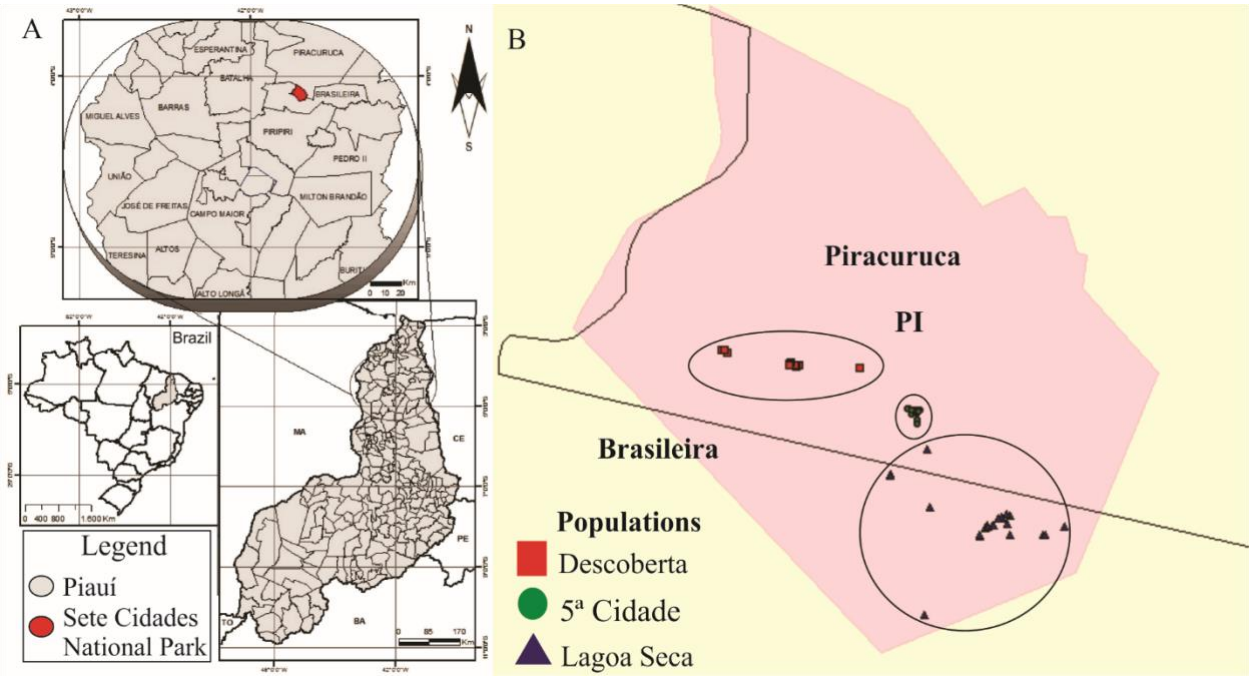

Figure 1. Area of study, Sete Cidades National Park, Piracuruca and Brasileira, Piauí, northeastern Brazil. A – map of Brazil with Sete Cidades National Park highlighted in red (adapted from Costa et al. 2016); B – sampled populations of cajui (*Anacardium* spp.): Descoberta, 5ª Cidade and Lagoa Seca **(**map was created using the speciesMapper tool available from http://splink.cria.org.br/mapper?criaLANG=pt).

Table 1. Cajui populations collected in Sete Cidades National Park, Piracuruca and Brasileira (PI), Brazil.

Population	Number of individuals	Individual labels	Phytophysiognomy of the Cerrado	Mean distance between populations (km)
Descoberta (A)	12	C1-C12	Typical and Rupestre Cerrado	4.6 (A-B)
Lagoa Seca (B)	24	C13-C30, C32, C34, C36, C38-C40	Typical Cerrado	2.5 (B-C)
5ª Cidade (C)	20	C41-C60	Cerradão	2.2 (A-C)

The characteristics of each phytophysiognomy are as follows: typical Cerrado, a herbaceous vegetation layer and a shrub-tree layer with a height of approximately 5 m, and plants with irregular and twisted branches; Cerrado Rupestre, plants up to 2 m high in rocky environments; and Cerradão, forest vegetation with tall, straight trees with an average height of 7 m (Castro et al. 2010). The mean distances between populations are shown in Table 1. The greatest geographic distance between individuals within PNSC was 6.8 km, between individuals 03 (population Descoberta) and 25 (population Lagoa Seca) (Figure 1). Individuals were georeferenced by GPS. In addition, two *A. occidentale* accessions (Caju I and Caju II) from Pacajus (Ceará - Brasil) were used as an outgroup.

Samples of young, healthy leaves were collected, placed in a saturated solution of cetyltrimethylammonium bromide and sodium chloride (CTAB-NaCl) and stored at -20°C until DNA extraction.

DNA extraction and amplification of ISSRs

Total DNA from 100 mg of each plant sample was extracted following Doyle and Doyle (1987) with the following modifications: The volume of extraction buffer for lysis was increased to 1000L, and the chloroform:isoamyl alcohol (24:1) step was performed twice to increase DNA quality. The DNA was subjected to electrophoresis on 0.8% agarose gels prepared in 0.5X tris-borate-EDTA (TBE) buffer, stained with GelRed™ dye and photographed under ultraviolet light. DNA quantification was performed by comparison with λ DNA standards (100 ng). The purity of the samples was examined on a NanoDrop 2000

spectrophotometer (Thermo Scientific). The DNA from each sample was diluted to 7 ng/µL and stored at -20°C for subsequent PCR.

The DNA amplification reactions were performed in a Veriti 96-well Thermal Cycler (Applied Biosystems®, USA) with an initial denaturation for 1.5 min at 94°C; 40 cycles of denaturation for 40 sec at 94°C, annealing for 45 sec at the optimized temperatures for the individual primers, and extension for 2 min at 72°C; and a final extension of 7 min at 72°C. Each reaction mixture contained 1 µL DNA (7 ng/µL), 0.8 mM dNTPs (dATP, dCTP, dGTP, and dTTP), 1 U Taq DNA polymerase (Invitrogen), 0.8 µM primer, 2.0 mM $MgCl_2$, 1X Taq buffer [20 mM Tris-HCl, pH 8.0; 0.1 mM EDTA; 1 mM DTT; 50% (v/v) glycerol] and ultrapure H_2O in a 10 µL final volume. The amplification products were subjected to electrophoresis on 1.5% agarose gel stained with GelRed™ dye in 0.5X TBE buffer for four hours at 100 V. The gel was visualized under a UV transilluminator and photographed. The sizes of the amplicons were estimated by comparison with 1 kb DNA ladder (Invitrogen).

Twenty primers developed by the University of British Columbia (UBC), Vancouver, Canada, were tested to screen for the best amplification profiles. Among them, eleven primers yielding greater resolution of amplicons and higher levels of polymorphism were selected for further study (Table 2).

Table 2. ISSR primers (UBC) used in the molecular characterization of 58 individuals of *Anacardium* spp.

Primer	Tm (C°)	Ta (°C)	Sequence (5'-3')	GC (%)
UBC808	48.8	54.0	AGA GAG AGA GAG AGA GC	52.94
UBC818	51.0	49.0	CAC ACA CAC ACA CAC AG	52.94
UBC826	57.2	56.0	ACA CAC ACA CAC ACA CC	52.94
UBC834	49.2	54.0	AGA GAG AGA GAG AGA GYT	50.00
UBC836	48.9	52.0	AGA GAG AGA GAG AGA GYA	50.00
UBC840	47.4	52.0	GAG AGA GAG AGA GAG AYT	50.00
UBC841	48.5	51.0	GAG AGA GAG AGA GAG AYC	55.55
UBC842	48.8	49.0	GAG AGA GAG AGA GAG AYG	55.55
UBC845	48.1	50.0	CTC TCT CTC TCT CTC TRG	55.55
UBC857	54.3	55.0	ACA CAC ACA CAC ACA CYG	55.55
UBC864	43.6	50.0	ATG ATG ATG ATG ATG	33.33

Tm, melting temperature; Ta, annealing temperature; Y = C or T; R = A or G.

Statistical analysis: population genetic diversity

The amplification profiles were scored manually for the presence (as 1) or absence (as 0) of bands, and a binary matrix was created. The Sorensen-Dice coefficient was calculated using PAST v. 3.08 software (Hammer et al. 2001) to evaluate genetic similarity between populations. POPGENE v. 1.32 software (Yeh et al. 1999) was employed to determine genetic diversity within and among the populations, measured as the percentage of polymorphic loci (P), Nei's genetic diversity (h) (Nei 1987), the Shannon index (I) (Lewontin 1972), the coefficient of genetic differentiation (GST) and indirect gene flow (Nm).

Statistical analysis: genetic structure

Analysis of molecular variance (AMOVA), performed with 10,000 permutations to test for significance, and genetic differentiation (ΦST) analysis were conducted using ARLEQUIN v. 3.5.1.2 software (Excoffier and Lischer 2010) to determine the amount of genetic variability within and among the populations. Bayesian analysis using Structure v. 2.3.4 software (Pritchard et al. 2000) was conducted to infer the number of genetic groups (K) that best represented each sampled population. The K values evaluated ranged from K = 1 to K = 7, and an admixture ancestry model using the correlated allele frequency option and the default parameters was used (Evanno et al. 2005). Ten independent runs were performed for each K, using 1,000,000 Monte Carlo Markov Chain (MCMC) simulations and a burn-in of 500,000. The number of genetic groups, K, was identified according to the ΔK method (Evanno et al. 2005) as implemented in the Structure Harvester program (Earl and Vonholdt 2012).

3. Results

ISSR markers

A total of 11 primers were selected for ISSR analysis, which generated 112 fragments, of which 93 were polymorphic (83.04%). The highest number of bands was obtained with primer UBC 834 (14 fragments), and the lowest number was obtained with primers UBC 840 and UBC 845 (7 fragments each), with a mean of ~10 fragments per primer. Amplicon size ranged from 150 to 2200 bp (Table 3). The amplification profile of primer UBC 834 is shown in Figure 2.

Table 3. Primers used, band sizes, and number of amplified and polymorphic loci from 11 ISSR primers.

Primer	Band size (bp)	Loci		Polymorphism (%)
		Total	Polymorphic	
UBC808	300-1500	11	10	90.9
UBC818	250-1550	8	8	100.0
UBC826	400-2000	12	11	91.7
UBC834	150-2000	14	11	78.6
UBC836	200-1800	12	9	75.0
UBC840	300-1500	7	7	100.0
UBC841	500-2200	10	8	80.0
UBC842	200-1650	13	10	76.9
UBC845	500-1850	7	6	85.7
UBC857	400-1300	8	8	100.0
UBC864	250-1100	10	5	50.0
Total	150-2200	112	93	83.04

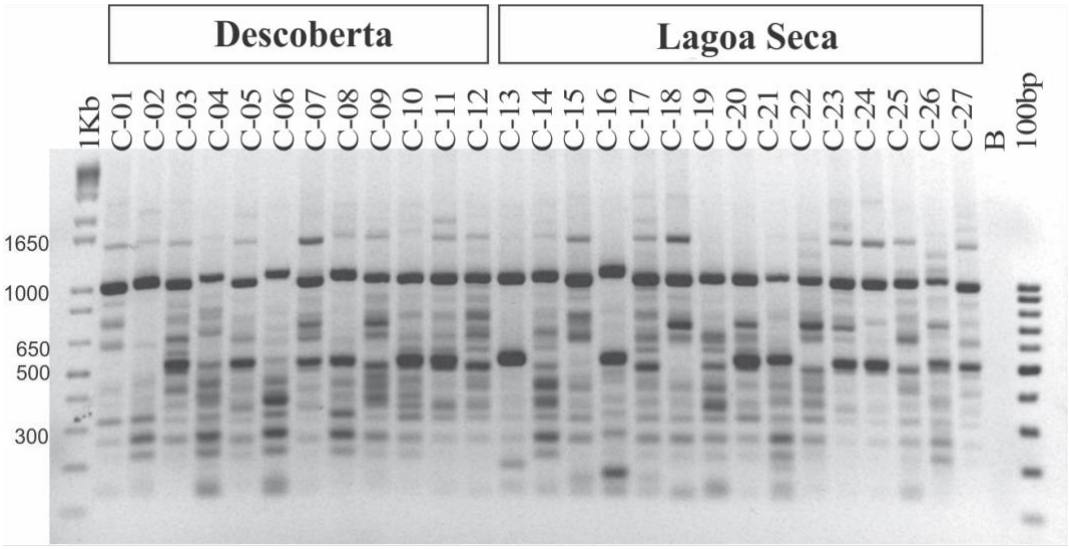

Figure 2. Gel profiles obtained from the DNA amplification of 27 cajui individuals from Sete Cidades National Park, located in Descoberta (12) and Lagoa Seca (15), with primer 834 (UBC). B, blank (negative control).

Genetic similarity

Genetic similarity between individuals of *Anacardium* spp. ranged from 0.60, observed between individuals C-23 (population Lagoa Seca) and Caju-II (BAG), to 0.94, observed between individuals C-26 and C-27; the latter were both collected in PNSC (population Lagoa Seca). The mean similarity was 0.81. In general, the lowest similarity values were found between PNSC and outgroup individuals. The most divergent individuals compared to the outgroup were C-13, C-16, C-22, C-23 and C-44, with average similarity values of 0.63, 0.64, 0.65, 0.61 and 0.63, respectively. The lowest similarity value, 0.67, was found between C-22 and C-26, both belonging to population Lagoa Seca from PNSC.

Population genetic diversity

Among the populations, population Lagoa Seca showed the highest percentage of polymorphism (78.57%), whereas population Descoberta presented the lowest (61.61%). Nei's genetic diversity (h) ranged from 0.22 to 0.27, with a total of 0.26. The Shannon index (I) ranged from 0.33 to 0.40, with a total of 0.39. According to the differentiation index (G_{ST}), only 6.94% of the genetic diversity occurred among the populations. The estimate of average gene flow (Nm) was 6.70, indicating a high level of gene flow among the populations (Table 4).

Table 4. Estimates of genetic diversity, number of migrants and genetic differentiation of the three populations of *Anacardium* spp.

Population	PL	PPL (%)	h	I	Nm	G_{ST}
Descoberta	69	61.61	0.22	0.33		
Lagoa Seca	88	78.57	0.27	0.40		
5ª Cidade	81	72.32	0.24	0.36		
Total	93	83.04	0.26	0.40		
Subpopulations					6.70	0.07

PL, polymorphic loci; PPL, percentage of polymorphic loci; h, Nei's diversity index, I, the Shannon diversity index; Nm, number of migrants; G_{ST}, index of genetic differentiation between populations.

Genetic structure and differentiation

The AMOVA results showed that a higher proportion of genetic variation was explained by differences within populations (96.17%). In addition, low genetic differentiation was observed between the populations (Φ_{ST} = 0.04) (Table 5).

Table 5. Molecular analysis of variance (AMOVA) of three populations of *Anacardium* spp.

Source of variation	DF	Sum of squares	Component of variance	Percentage of variance	Fixing index (ΦST)
Between populations	2	50.45	0.58	3.83	0.04
Within populations	53	778.99	14.70	96.17	Fixing index (ΦST)

DF, degrees of freedom; P-value, probability of significance.

The Bayesian analysis performed using the Structure software indicated that the clusters were not strongly correlated with geographical locations, with individuals from the same collection point being very likely to belong to more than one genetic group. The ΔK statistic indicated K = 4 as the probable number of genetic groups of *Anacardium* spp. (Figure 3).

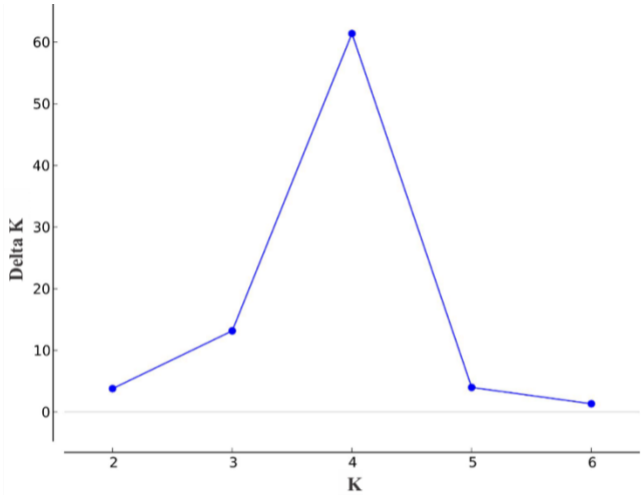

Figure 2. Values of ΔK estimated for populations of *Anacardium* spp., as proposed by Evanno et al. (2005).

All individuals were differentiated into four different genetic groups to varying degrees (Figure 4). The genetic group that appeared in the greatest proportion is indicated in Figure 4 in blue (42%) for the Descoberta population, yellow (31%) for the Lagoa Seca population and green (38%) for the 5ª Cidade population. However, no predominance was observed, with no group having a proportion greater than 50%. This result indicated a considerable mixture of genotypes among these genetic groups. Only the outgroup sample presented a genetic group with a predominance of greater than 80%, represented by red in the figure, highlighting the divergence of the outgroup individuals from the others (Figure 4). The results agreed with the genetic similarity analysis of the populations.

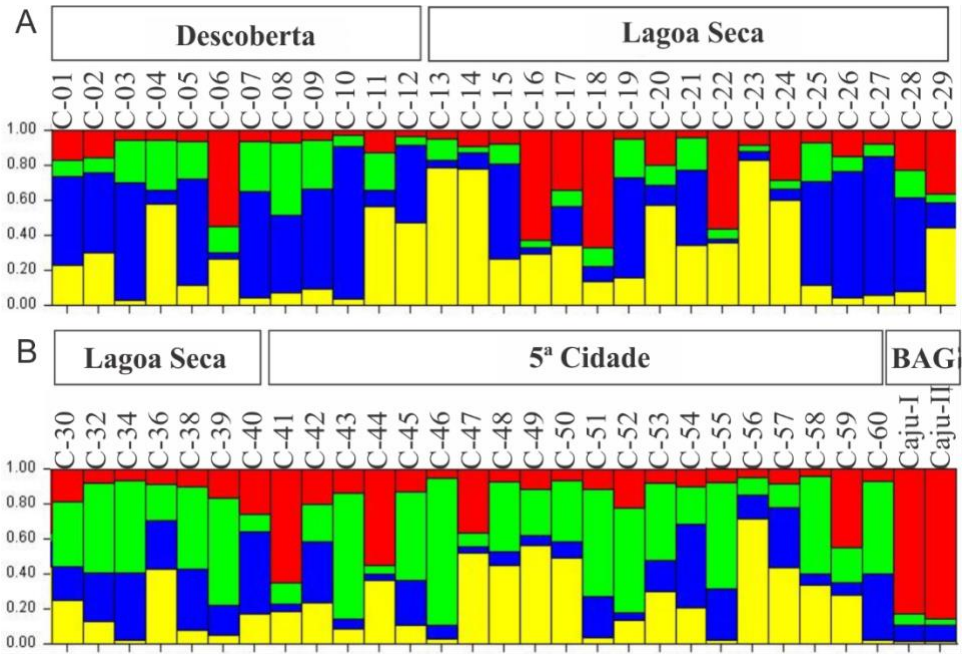

Figure 3. Graphical representation of the Structure analysis results, showing the individuals of *Anacardium* spp. distributed into four genetic groups. A – strucuture genetic of Descoberta and Lagoa Seca populations; B – structure genetic of Lagoa Seca and 5ª Cidade populations and two accessions from Pacajus (Caju-I and Caju-II).

4. Discussion

In this study, genetic polymorphism in the populations was evaluated through the use of molecular markers that have the advantage of not being influenced by environmental factors (Laakili et al. 2018). Dominant markers, such as ISSRs, allow rapid, simple, and reproducible analysis and exhibit sufficient polymorphism to assess genetic diversity or identify closely related cultivars in many species, including fruit trees (Santos et al. 2015; Paschoa et al. 2018). Therefore, we used an ISSR approach to evaluate the diversity and genetic structure of cajui from three natural populations in PNSC and two accessions from Pacajus (Caju I and Caju-II).

The percentage of polymorphic loci indicates the effectiveness of molecular markers for estimating genetic variability in populations (Grativol et al. 2011). From the 11 ISSR primers employed, 112 loci were obtained, of which 83.04% were polymorphic. Hence, these ISSR markers were found to be effective for characterizing and discriminating genetic diversity in the individuals of *Anacardium* spp.

The most similar individuals, C-26 and C-27 (94.0%), and the most divergent ones within PNSC, C-22 and C-26 (32.5%), belong to the same collection population (population Lagoa Seca). Additionally, the most geographically distant individuals (C-03 and C-25) were very similar (87.3%). Thus, the distribution of genetic diversity was not significantly influenced by geographic distance. The Sorensen-Dice coefficient among the individuals ranged from a maximum of 0.94 (between individuals C-26 and C-27) to a minimum of 0.60 (between C-23 and Caju-II). Considering only the individuals from PNSC, the range of diversity was low (6-32.5%), which indicated low to moderate genetic variability between individuals. This low variability may be related to the small number of founding genotypes of these populations and/or genetic drift due to the occurrence of fires in the park region (Souza and Pereira 2019).

The finding of moderate estimated genetic variability was supported by the diversity indexes. In general, Nei's diversity index (h) ranges from 0 to 0.5 for dominant markers. In this study, Nei's diversity index was estimated at 0.26, and the Shannon index (I) was 0.40. The largest population examined, Lagoa Seca (24 individuals), presented the highest values of both Nei's diversity index (h = 0.27) and the Shannon index (I = 0.40). In contrast, the Descoberta population, with 12 individuals, presented the lowest genetic diversity (h = 0.22, I = 0.33), which was possibly influenced by the small sample size. Therefore, the three populations presented moderate genetic variability, possibly due to the small sample sizes in this study, the genetic constitution of the founding genotypes, and/or genetic drift.

Analysis of the genetic diversity of natural cashew populations (*Anacardium humile*) in GO-Brazil, by RAPD, revealed higher Nei (0.3842) and Shannon (0.5662) diversity rates than our work, indicating greater genetic diversity of cajui in the cerrado of the Brazil (Santos et al. 2019). This reinforces the need for conservation strategies in the PNSC's cashew species.

AMOVA indicated that most genetic variability lies within the populations (96.17%) and that there is low differentiation among them. This result is consistent with the reproductive biology of members of the genus *Anacardium*, which are predominantly allogamic (Aliyu 2008; Chhajer et al. 2018). In addition, although the inflorescences of these species comprise male and hermaphrodite flowers, self-fertilization is mechanically limited, which limits genetic differentiation among populations (Eradasappa and Mohana 2016).

The GST (0.07) and index of fixation (0.04) obtained by AMOVA were similar and indicated low genetic differentiation among the populations. This finding suggested a high level of gene flow, which was supported by the high number of migrants (Nm = 6.70) per generation. Gene flow between populations may directly affect the distribution of genetic variation, since it represents the introduction of new allelic variants that can reduce genetic differentiation among populations (Hamrick 2012). One or more migrants per generation are considered sufficient to avoid population substructure (Sheidai et al. 2016). Thus, the high value of Nm (6.7028) explains the low differentiation found among the cajui populations of PNSC.

The Bayesian analysis obtained with Structure software and the similarity values together indicate high admixture of genotypes in the PNSC population, demonstrating that the individuals sampled at the different sites within PNSC do not represent structured subpopulations. The observed population admixture may be attributed to factors such as high gene flow, favored by the morphological and reproductive characteristics of the flowers of *Anacardium* spp., pollinators and small-animal seed dispersers. However, there is differentiation between the cajui species and the two individuals comprising the outgroup (*A. occidentale*).

An important factor that promotes gene flow among *Anacardium* populations is the floral biology of the plant. The hermaphrodite flower has short stamens that do not reach the single long pistil, which makes self-fertilization difficult (Eradasappa and Mohana 2016). In addition, the higher production of male flowers than of hermaphrodite flowers, which has been detected for *A. occidentale* commercial species, early dwarf cashew and *Anacardium giganteum*, promotes reproduction by allogamy (Sousa et al. 2007; Takehana et al. 2013). The characteristics of *Anacardium* floral morphology, such as nectar quality, pollen grain traits, aroma, flower color, structure and size of the flowers and quantity of flowers produced, contribute significantly to the attraction of bees as pollinators (Takehana et al. 2013; Eradasappa and Mohana 2016). Therefore, floral biology directly affects the pollination mode of the plant, favoring cross-pollination in *Anacardium*. Gene flow is also facilitated by the small distances between populations. Bees, especially *Apis mellifera*, are the main dispersing agents of *Anacardium* pollen and can travel long distances in search of floral foods (Hagle et al. 2011). Since the greatest distance between the cajui individuals within PNSC did not exceed 6.8 km, gene flow was likely promoted by pollen transport by bees in the region.

Gene flow through seed dispersion is also possible; the fruit is associated with a fleshy and succulent peduncle that attracts small animals, mainly frugivorous bats, which can disperse the fruit over long distances (Galindo-González 1998). The peduncle is also a food source for parrots and parakeets, which are frequent visitors to cajui plants in PNSC and possibly act as seed-dispersing agents. Thus, the high dispersal potential of *Anacardium* species via pollinators and seed dispersers, the high pollen viability in the genus, which exceeds 90% in two cajui species (*Anacardium microcarpum* and *Anacardium pumilum*), and the high germination percentages, culminate in high gene flow (Eradasappa et al. 2014; Moreira et al. 2016).

In addition to having economic, medicinal, and ecological potential, the populations of *Anacardium* evaluated in the present study were under adverse environmental conditions, such as drought and poor soil. Because they can survive in such conditions, these individuals may contain genes responsibleS for plant defense under abiotic stress. Fires of criminal origin are common within PNSC (Souza and Pereira 2019), and alleles with potential for exploitation by breeding programs may be lost; thus, *ex situ* conservation of these materials is necessary. For the *ex situ* conservation of these materials we suggest the most divergent cajui individuals analyzed: C-03, C-05, C-22, C-26, C-34 and C-39.

5. Conclusions

The set of ISSR markers tested in this study was effective for characterizing the *Anacardium* spp. individuals and represents a valuable tool for genetic breeding projects. The genetic diversity and population structure generated can allow the identification of high-priority genotypes for conservation.

Authors' Contributions: GOMES, M.F.C: conception and design, acquisition of data, analysis and interpretation of data, drafting the article, critical review of important intellectual content; BORGES, A.N.C.: acquisition of data, analysis and interpretation of data, critical review of important intellectual content; BATISTA, G.S.S.: acquisition of data; LUZ, G.A.: acquisition of data, analysis and interpretation of data, critical review of important intellectual content; OLIVEIRA, M.E.A.: acquisition of data; LOPES, A.C.A.: critical review of important intellectual content; ARAÚJO, A.S.F.: critical review of important intellectual content; GOMES, R.L.F.: critical review of important intellectual content; BRITTO, F.B.: analysis and interpretation of data, drafting the article, critical review of important intellectual content; LIMA, P.S.C.: conception and design, analysis and interpretation of data, drafting the article, critical review of important intellectual content; VALENTE, S.E.S.: conception and design, analysis and interpretation of data, drafting the article, critical review of important intellectual content. All authors have read and approved the final version of the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

Ethics Approval: Not applicable.

Acknowledgments: The authors would like to thank the funding for the realization of this study provided by the Brazilian agencies FAPEPI (Fundação de Amparo a Pesquisa do Estado do Piauí - Brasil), and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil), Finance Code 001.

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Received: 25 April 2020 | **Accepted:** 14 December 2020 | **Published:** 29 December 2021