Diversity and spatial genetic structure of natural populations of *Ziziphus joazeiro* Mart.

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ABSTRACT: This study aimed to characterize the genetic diversity and spatial genetic of natural populations of *Ziziphus joazeiro* using molecular markers. Twelve ISSR markers were used in 77 individuals, distributed between two populations located in the state of Ceará, Brazil. The set of markers generated 220 polymorphic loci for the "Não me Deixes" population (Pop 1) and 74 loci for the "Triunfo" population (Pop 2). The results indicated a higher genetic diversity among individuals of the Pop 2. According to the analysis of molecular variance, most genetic diversity occurred within the populations (59.49%). There was no spatial genetic structure among individuals the Pop 1 population. In contrast, the Pop 2 population presented spatial genetic structure up to the first distance class (132 m). Due to the observed genetic variation, both populations present potential for seed collection and seedling formation to be used in restoration area and genetic conservation programs.

Key words: caatinga biome; genetic resources; seed collection

Diversidade e estrutura genética espacial em populações naturais de Ziziphus joazeiro Mart.

RESUMO: Este estudo objetivou a caracterização da diversidade e estrutura genética espacial existente em populações naturais de *Ziziphus joazeiro* a partir de marcadores moleculares. Doze marcadores ISSR foram utilizados em 77 indivíduos, distribuídos em duas populações, localizadas no estado do Ceará, Brasil. O conjunto de marcadores gerou 220 locos polimórficos para a população Não me Deixes (Pop 1), e 74 locos para a população Triunfo (Pop 2). Os resultados indicaram maior diversidade genética entre os indivíduos da Pop 2 e a maior parte da diversidade genética ocorreu dentro das populações (59,49%). Não houve estruturação genética espacial entre os indivíduos da Pop 1, em contrapartida, a Pop 2 apresentou estruturação genética espacial até a primeira classe de distância (132 m). Devido à variação genética observada, as duas populações apresentam potencial para a coleta de sementes e formação de mudas a serem utilizadas em programas de restauração de áreas e de conservação genética.

Palavras-chave: bioma caatinga; recursos genéticos; coleta de sementes

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Introduction

The drastic modifications in the natural environment caused by anthropic action have resulted in degradation and fragmentation of native areas. The pure exploratory pressure exerted on these remnants has contributed unfavorably to the maintenance of forest ecosystems, mainly in what refers to reduction of genetic diversity and size of natural populations. This has serious consequences, such as the increase in inbreeding rate, risk of loss of alleles, genetic drift and lack of gene flow (Kageyama et al., 1998).

The Caatinga covers approximately 10% of the national territory and stands out as the only ecosystem exclusively Brazilian, presenting a very characteristic phytofisiognomy: dry forests and shrubland vegetation (savanna-steppe), with enclaves of moist montane forests and Cerrado (Tabarelli & Silva, 2003). It is a holder of high biodiversity, with a large number of endemic species. Despite the importance of this biome, the Brazilian Institute of Environment and Renewable Natural Resources (Ibama, 2016) states that degradation in the Caatinga has reached 46% of its total area. Ceará is considered the state where the vegetation was most devastated, since 10% of its territory is at an advanced stage of desertification (Pereira, 2011). This results in the fragmentation and significant reduction in natural habitats and populations present in this ecosystem.

Ziziphus joazeiro Martius, part of the Rhamnaceae, is one of the most typical species of the Caatinga. It is considered a priority for the development of research related to its conservation, motivated by its economic and ecological importance for the northeast region of Brazil (Pareyn, 2010). Despite the justifiable importance of the species, currently in the literature there is only one genetic study related to *Z. joazeiro*, developed by Gois et al. (2014). In light of this, it becomes necessary to search for studies that aim to understand the current situation of natural populations, as well as the dynamics, ecological and genetic patterns in the species.

The characterization of the population allows for the recognition of the real genetic loss, making it possible to carry out inferences about the establishment and adoption of the best forms of conservation, management and sustainable use of genetic resources (Lima et al., 2015). In this context, characterization of genetic diversity in plants has been studied with the aid of molecular marker techniques, highlighting the use of ISSR (*Inter Simple Sequence Repeat*) markers, developed by Zietkiewicz et al. (1994). The use of ISSR has generated satisfactory results in studies regarding genetic structure and diversity on species for which there are no developed molecular markers of codominant nature (Brandão et al., 2015; Alves et al., 2016; Pádua et al., 2016).

Thus, the present work aims to characterize the variability and spacial genetic structure of *Z. joazeiro* populations using ISSR markers, generating information for the adoption of strategies, as well as conservation of the species' natural populations.

Material and Methods

Location of study area, sampling and collection of material

Two *Ziziphus joazeiro* populations located on private property, in the state of Ceará (Brazil), were studied. The first population (Pop 1) is located on the "Não me Deixes" farm, which is a Private Natural Heritage Reserve (RPPN) in the city of Quixadá, under the coordinates 4°48'45' S and 38°58'21'' W. The second population (Pop 2) is located in the "Triunfo" farm, in the city of Ibaretama, under the coordinates 4°44'20'' S and 38°44'51'' W. The distance between the two populations is 26 miles.

In the "Não me Deixes" population (Pop 1), 30 *Z. joazeiro* trees were sampled, and in the "Triunfo" population (Pop 2), 47 trees were sampled, with a minimum distance of twice the height of the tree between each individual being considered, as recommended by Sebbenn (2006). All trees were georeferenced with the aid of GPS and demarcated with aluminum platelets. Young leaves were collected from each selected individual, wrapped in aluminum foil and plastic bags, then placed in thermal boxes containing ice and sent to the Forest Seed Laboratory of Embrapa Florestas in Colombo city, state of Paraná (Brazil). The samples were kept in a refrigerator until the extraction of genetic material.

DNA extraction and ISSR reactions

For the extraction of genomic DNA, the Wizard® Genomic DNA Purification kit from the Promega company was used, following the protocol established by the manufacturer. 40 mg of leaf tissues from each individual, manually macerated in liquid nitrogen, were used. The PCR reaction was prepared for a final volume of 25 μL, containing 25 ng of DNA; 10 mM Tris pH 8.0; 1.5 mM $MgCl_2$; 0.2 mM dNTP; 0.8 μM of each ISSR marker, and two units of Taq polymerase. The amplification conditions were: initial denaturation for five minutes at 94ºC, 30 one minute cycles at 94ºC for denaturation, one minute at 52°C for girdling, and two minutes at 72°C for DNA strands extension. After 30 cycles, a final 72ºC extension was performed for seven minutes. The amplified products, stained with GelRed, were separated by horizontal 1.2% agarose gel electrophoresis at 200 V for two hours. Subsequently, the fragments were visualized in a photodocumentation system (Duarte, 2015).

Data analysis

The ISSR markers were genotyped regarding the presence (1) and absence (0) of locus, generating a binary matrix. For analysis of genetic diversity in the populations, POPGENE software version 1.32 was used (Yeh et al., 1997), through which the genetic diversity of Nei (H_e) (Nei, 1973), the Shannon index (I), and the percentage of polymorphic locus ($P\%$) were estimated.

The analysis of molecular variance (AMOVA) (Excoffier et al., 1992) was performed through the GENES software, version 2014.6.1 (Cruz, 2008), for genetic structuring and division of the variance components within and between

the populations, assuming that all individuals within these populations had the same pattern of crossing. The significance of variance components was tested with 1,000 permutations. Moreover, based on AMOVA, the Φ_{ST} statistic (Excoffier et al., 1992) was calculated. This parameter is analog to Fst and explain genetic differentiation between the populations.

To determine the spatial genetic structure (SGS) between the genotypes of each population, the coancestry value was estimated by the *kinship* coefficient for dominant data, according to Hardy (2003), using the SPAGeDI program version 1.4 (Hardy & Vekemans, 2002). For the analysis, the inbreeding coefficient was considered equal to zero. The *kinship* coefficient is given by $F_{ij} = (Q_{ij} - Q_m)/(1 - Q_m)$, where F_{ij} is the *kinship* coefficient of genetic coancestry between i and j; Q_{ij} is the probability of the i and j random samplings being identical by coancestry; Q_m is the mean probability that the random sampling conducted on the population is identical by coancestry. Ten distance classes were used, and the standard error of the mean of the estimates was obtained by *jackknife* resampling, from which 95% confidence intervals of probability of the mean coancestry coefficient, estimated by distance class, were obtained. In order to test the occurrence of the SGS within each class of distance, 1,000 permutations were made. The magnitude of the SGS was calculated using the statistic S_p (Vekemans & Hardy, 2004): $S_p = -b_{log}/(1 - F_{ij}(1))$, in which b_{log} is the slope of the regression curve of the coancestry coefficient; $F_{ij}(1)$ is the average of the coancestry coefficient of the first class of distance (F_{ij}). The values of S_p were used to compare the extent of the spatial genetic structure among the populations.

Results and Discussion

Of the 21 ISSR markers initially tested, 12 showed to be adequate to access the genetic diversity of *Ziziphus joazeiro*, producing robust fragments. The performance of each marker varied between the two populations studied. For the "Não me Deixes" population (Pop 1), the 12 markers used generated 220 locus, ranging from 11 to 25 locus per marker. For the "Triunfo" population (Pop 2), 74 locus were generated, ranging from 10 to five locus per marker. In both populations, the size of the fragments ranged between 200 and 1,500 base pairs (Table 1).

In order to adopt strategies for conservation or management of any species *in situ*, it is necessary to know the structure and genetic diversity contained in the target populations. The use of the ISSR markers to access the genetic diversity of *Z. joazeiro* has been shown to be satisfactory in the present study, because due to the use of the 12 ISSR markers selected, it was possible to access the level of polymorphism in the two populations studied. Studies developed with different plant species have demonstrated the efficiency of the use of ISSR in initial studies of genetic diversity in natural populations, such as those developed by Brandão et al. (2015), Alves et al. (2016), Pádua et al. (2016) and Yang et al. (2016).

Genetic diversity is a relevant aspect and should primarily be studied in natural populations, because according to Gonçalves et al. (2016), high levels of genetic variability allow for many new genotypical combinations, resulting in an increase in evolution potential of the species due to the greater capacity of adaptation to the environment.

The Pop 1 showed values of Nei's genetic diversity (assuming Hardy-Weinberg equilibrium) and the *Shannon* Index equal to 0.23 and 0.38, respectively. In regards to the Pop 2, higher values were observed, those being 0.39 for Nei's genetic diversity and 0.57 for the *Shannon* Index. Both populations showed a polymorphism rate of 98.7% (Table 2).

Table 2. Genetic parameters for the populations of *Ziziphus joazeiro.* N: number of individuals; H_e: Nei's genetic diversity; I: *Shannon* Index; P: percentage of polymorphic locus.

Population	N	H_e	I	P
Não me Deixes (Pop 1)	30	0.23 (0.11)	0.38 (0.15)	98.7
Triunfo (Pop 2)	47	0.39 (0.11)	0.57 (0.14)	98.7
Pop 1 + Pop 2	77	0.43 (0.08)	0.62 (0.09)	-

( ): Standard deviation.

Table 1. ISSR markers and their sequences, total of fragments amplified (*TFA*) and the range of variation of the locus size in base pairs (*SBP*) produced in *Ziziphus joazeiro* for each population studied.

Primer	Sequence (5' – 3')	Não me Deixes (Pop 1)		Triunfo (Pop 2)	
		TFA	SBP	TFA	SBP
UCB 808	GAG AGA GAG AGA GAG AT	20	900 – 200	10	800 – 200
UBC 809	GAG AGA GAG AGA GAG AC	21	700 – 300	7	700 – 300
UCB 810	GAG AGA GAG AGA GAG AA	23	1000 – 300	6	1500 – 200
UCB 811	CTC TCT CTC TCT CTC TG	11	700 – 300	6	1000 – 200
UBC 812	AGA GAG AGA GAG AGA GG	21	1000 – 300	5	800 – 300
UCB 815	AGA GAG AGA GAG AGA GC	25	1000 – 500	5	900 – 500
UCB 817	TCT CTC TCT CTC TCT CC	13	700 – 300	6	900 – 400
UCB 818	TCT CTC TCT CTC TCT CA	13	900 – 400	8	900 – 300
UCB 819	GTG TGT GTG TGT GTG TA	14	900 – 500	0*	–
UCB 822	CAC ACA CAC ACA CAC AA	19	1500 – 600	6	1500 – 400
UCB 823	ACA CAC ACA CAC ACA CT	21	1000 – 400	8	1500 – 400
UCB 825	CAC ACA CAC ACA CAC AG	19	1000 – 400	7	1000 – 400
Average		18		7	

* There was no amplification of DNA fragments using the *primer* UBC 819 in the population of Triunfo.

The results obtained from the AMOVA show that the largest degree of genetic diversity observed is concentrated within populations (59.69%). In addition, the statistic Φ_{ST} presented a value of 0.41, suggesting a weak structuring of the populations (Table 3).

The *Shannon* Index values found in the present study demonstrated that there is low genetic diversity in the Pop 1 (0.38) and moderate diversity in the Pop 2 (0.57), considering that the smaller the observed value for this index, the lower the diversity of the population (Estopa et al., 2006). The results for the diversity observed in this study are similar to those verified by Gois et al. (2014) when studying three populations of *Z. joazeiro* located at Sergipe state, using RAPD markers. It is believed that the low diversity observed in the Pop 1 is related to the number of individuals sampled, and/or to the history of exploitation of the property, since the area had been used for agriculture and livestock before being made a RPPN, indicating that many individuals must had been cut.

Autecology and the characteristics of each species, such as geographic distribution, reproductive system and seed dispersal are factors responsible for the distribution of the genetic variability within and among populations (Nybom & Bartish, 2000). In general, the natural populations of perennial and allogamous species, such as *Z. joazeiro*, have higher rates of genetic diversity within populations and lower rates among populations (Nybom & Bartish, 2000), confirming the results found in this study. Moreover, the Φ_{ST} statistic suggests a weak genetic structuring in the populations, demonstrating that there is a gene flow between them. The values are similar to those described by Nybom & Bartish (2000) for the Φ_{ST}, which were estimated by using ISSR markers (Φ_{ST}=0.35 ± 0.25).

The plant populations that exhibit effective gene dispersion allow allele flow over long distances, thus maintaining the high levels of genetic variation within populations (Loveless & Hamrick, 1984). Although the results for the genetic structuring are in agreement with the ones mentioned in the literature, that is the largest proportion of the observed diversity is within populations, the variation value among populations (40.51%) can be considered high. In their study, Gois et al. (2014) pointed out that the high variation level observed within the populations of *Z. joazeiro* can be a result of not only from the geographical distance between the studied areas, but also from the ecological characteristics of the species. In addition, Gonçalves et al. (2016) state that the variation in estimates of genetic variability can

also be affected by the particular characteristics of each environment. In natural ecosystems the fragmentation areas, relief or large water courses (like reservoirs) could works as environmental barriers and prevent the gene flows, changed the genetic variability.

Knowing the distribution of genetic diversity within and among populations becomes important for decision-making. Thus, with the results found for the two populations studied when considering the adoption of conservation strategies such as seed collection and seedling production, it is appropriate to have the concern in sampling as many individuals as possible within each population, rather than a large number of populations, as recommended by Yang et al. (2016).

In relation to spatial genetic structure, the Pop 1 presented positive coancestry coefficient (F_{ij} = 0.018), but not significant (P = 0.509) in the first distance class (96 m), indicating a random distribution of individuals within the population (Figure 1). On the other hand, the Pop 2 presented genetic structure up to the first distance class (132 m), with positive and significant coancestry coefficient (F_{ij} = 0.160; P = 0.003), which implies that there is a genetic structuring between individuals that are up to 132 meters distanced from each other (Figure 1).

Spatial genetic structure (SGS) can be defined as the way in which individuals are distributed in space, which may be random or aggregate. Regarding the conservation practices, knowing the SGS in natural populations allows for sample maximization, expanding genetic variability, either for improvement programs, recovery of degraded areas or even collecting seeds (Gonçalves et al., 2016).

Table 3. Analysis of molecular variance (AMOVA) for populations of *Ziziphus joazeiro.*

Source of variation	DF	SS	Components of variance	Total variance (%)
Within populations	75	818.27	10.91	59.49
Total	76	1101.26	18.34	100.00
Φst	0.41			

DF: degrees of freedom; SS: sum of square of the deviations.
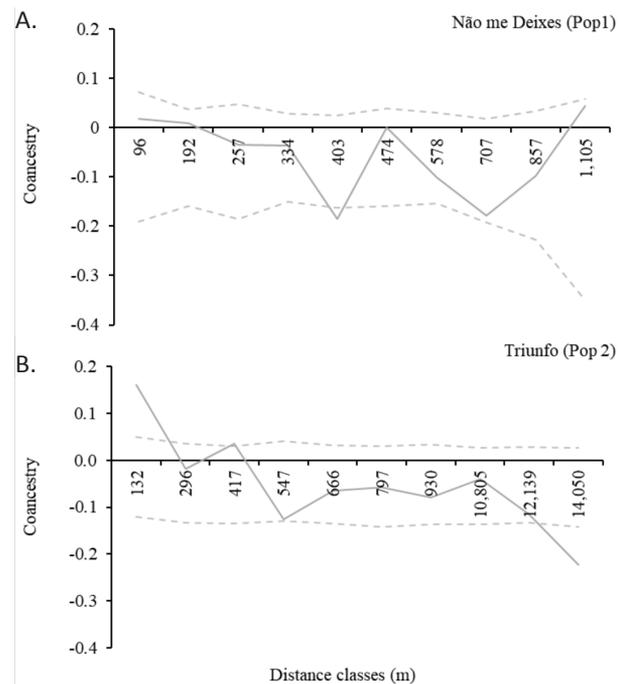
Figure 1. Correlograms of coancestry coefficients (*kinship*) estimated by distance classes in the studied populations of *Ziziphus joazeiro* (– Coancestry Coefficient; --- confidence interval of 95%).

The correlograms (Figure 1) indicated the absence of spatial genetic structure in the Pop 1 up to the first distance class, that is the individuals are distributed in the environment. In this population, for the purposes of conservation or genetic improvement, it is recommended that samplings are performed respecting the minimum distance of 96 m between individuals, since SGS has not been tested for distances lower this one. In contrast, the Pop 2 presented spatial genetic structure up to the first distance class, 132 m. It is essential that this distance between individuals is respected when collecting the seeds so that the adoption of conservation strategies that aims at gathering the greatest genetic diversity is possible.

Also, it was possible to observe a similar behavior in the two populations, where individuals located next to each other had genetic similarities (positive values of coancestry), which decreased with an increase of geographical distances between the stock plants, thus presenting negative values of coancestry (Figure 1). The same patterns were observed in populations of *Eremanthus erythropappus* (DC.) MacLeish (Pádua et al., 2016).

The genetic structure implies a spatial structure of the individuals, a result of the limited mobility of the plants. Thus, Loveless & Hamrick (1984) point out that in natural populations, it is possible to observe structured genetic patterns without a structured distribution of individuals in the environment. On the other hand, a population may exhibit a structured spatial distribution and a random genetic structure, once the genetic and spatial patterns are often the result of environmental differences and selection pressures.

Considering the results observed, several factors need to be taken into account for the adoption of conservation strategies, such as the levels of diversity within and among populations, spatial genetic structure, autecology of the species, and environmental factors. Moreover, these factors will allow for the proper inferences about the population behaviors and decision making concerning species conservation. Thus, based on the results of this study some strategies could be draw to avoid the decline of these populations. As an example of strategies could be cited the selection of mother trees for seed collection, production of seedlings for reforestation, the implantation of ecological corridors and introduction of pollinators could facilitate the gene flow and improve the genetic diversity in these populations.

Conclusions

Through ISSR markers it was possible to detect both low genetic diversity in the Pop 1 (Não me Deixes) and moderate diversity in the Pop 2 (Triunfo).

Most of the genetic diversity occurs within the populations studied.

There was no spatial genetic structure among individuals the Pop 1. In contrast, the Pop 2 presented spatial genetic structure up to the first distance class (132 m).

Acknowledgments

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