Genetic structure of *Pilosocereus gounellei* (Cactaceae) as revealed by AFLP marker to guide proposals for improvement and restoration of degraded areas in Caatinga biome

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ABSTRACT. Amplified fragment length polymorphism (AFLP) analysis was used to evaluate DNA polymorphism in *Pilosocereus gounellei* with the aim of differentiating samples grown in different Brazilian semiarid regions. Seven primer pairs were used to amplify 703 AFLP markers, of which 700 (99.21%) markers were polymorphic. The percentage of polymorphic markers ranged from 95.3% for the primer combination E-AAG/M-CTT to 100% for E-ACC/M-CAT, E-ACC/M-CAA, E-AGC/M-CAG, E-ACT/M-CTA, and E-AGG/M-CTG. The largest number of informative markers (126) was detected using the primer combination E-AAC/M-CTA. Polymorphism of the amplified DNA fragments ranged from 72.55% (in sample from Piauí State) to 82.79% (in samples from Rio Grande Norte State), with an

average of 75.39%. Despite the high genetic diversity of AFLP markers in xiquexique, analysis using the STRUCTURE software identified relatively homogeneous clusters of xiquexique from the same location, indicating a differentiation at the molecular level, among the plant samples from different regions of the Caatinga biome. The AFLP methodology identified genetically homogeneous and contrasting plants, as well as plants from different regions with common DNA markers. Seeds from such plants can be used for further propagation of plants for establishment of biodiversity conservation units and restoration of degraded areas of the Caatinga biome.

Key words: Xiquexique; Cactus; Semiarid region; Genetic diversity; AFLP

INTRODUCTION

In the semiarid region of Northeastern Brazil, several cacti are important for the regional fauna and flora (Rocha and Agra, 2002). Fruits of the cacti also constitute a food resource for humans (Albuquerque and Andrade, 2002; Almeida et al., 2007). Cactus, popularly known as xiquexique, belongs to the species *Pilosocereus gounellei* (Web.) Byl. & Rowl. (Byles and Rowley, 1957) and is found in sandy-stony soils and rock outcrops (Rocha and Agra, 2002) as well as in degraded soil areas and regions with irregular rainfall distribution. Xiquexique can be considered an option to repopulate areas where it is impossible to grow traditional crops (Cavalcanti and Resende, 2007). Its distribution mainly covers the states of Ceará, Rio Grande do Norte, and Bahia (Silva et al., 2005), which are part of the Caatinga biome.

P. gounellei has been used as forage for ruminants such as dairy cattle (Silva et al., 2005), ovines (Silva et al., 2010a), caprines (Cavalcanti and Resende, 2007; Silva et al., 2011), and calves (Silva et al., 2010b). Xiquexique fruits are consumed fresh by humans (Barbosa et al., 2007; Lucena et al., 2012) and their pulp is used to prepare cookies, pies, and pastries (Almeida et al., 2007). Xiquexique plants constitute a wild natural resource with an important contribution to the livelihood of local people in semiarid region of the Brazilian Northeast.

Owing to several uses of the plants endemic to the semiarid regions, utilization of Caatinga resources in a sustainable way is a challenge. In addition to biodiversity conservation, restoration of degraded areas has been a growing concern and has encouraged studies for the genetic characterization of these resources. Such characterization is essential for the successful conservation of plant resources, ensuring their sustainable use (Arif et al., 2010). For this purpose, molecular tools developed in recent years, such as amplified fragment length polymorphism (AFLP) markers provide an efficient means to evaluate known and unknown plant taxa. According to Vos et al. (1995), AFLP technique is based on the detection of genomic restriction fragments via Polymerase Chain Reaction (PCR) and has proven to be an extremely effective tool to distinguish between closely related genotypes. AFLP analysis has great potential as a technique for examining plant genetic diversity because a large number of polymorphisms can be detected with no prior sequence information and many markers can be analyzed in short time. Compared to other molecular methods, this technique requires relatively less DNA and has great discriminatory power and reproducibility (Georgnaras et al., 1999). This technique has been used for the study of some cacti, such as the species of genus *Opuntia* (Labra et al., 2003; Nilsen et al., 2005; García-Zambrano et al., 2009), *Gymnocalycium* (Repka and Mrácek, 2012), and *Cereus* (Faria-Tavares et al., 2013).

Since there is no information available in literature on the genetic parameters of *P. gounellei*, the present study was aimed to evaluate the genetic diversity among samples obtained from the states of Bahia, Piauí, and Rio Grande do Norte, using the AFLP technique. The application of this technique revealed the genetic structure of these samples that could be useful in guiding proposals for i) the improvement of this species, ii) the use of *P. gounellei* for restoring degraded areas, and iii) the establishment of biodiversity conservation units. The indiscriminate use of plants as forage for animals and for human consumption, and the predominantly vegetative form of propagation for producing seedlings, can reduce the local genetic diversity leading to genetic divergence among samples of *P. gounellei* collected from different states of the Caatinga biome.

MATERIAL AND METHODS

Xiquexique samples

Seeds collected from 21 xiquexique samples from the Northeast Brazilian states of Piauí (PI), Rio Grande do Norte (RN), and Bahia (BA) were germinated under aseptic conditions to form plantlets. Xiquexique plants used as the source of the seeds were grown in home gardens in the cities of Teresina, Campo Maior, and Picos in PI, Oliveira dos brejinhos and Queimada Nova in BA, and in an experimental field in Cruzeta, RN (Figure 1; Table 1). The distance separating the seed-donor plants grown in home gardens was 1000-7000 m (within each city), while the distance between the plants grown in the experimental field of Cruzeta was 5-30 m. Seeds were germinated on KC medium (Knudson, 1946) according to the protocol described by Oliveira et al. (1995) and maintained in a chamber acclimatized at 30°C with a photoperiod of 12 h. Five-to-twelve-month-old plantlets were used for DNA extraction.

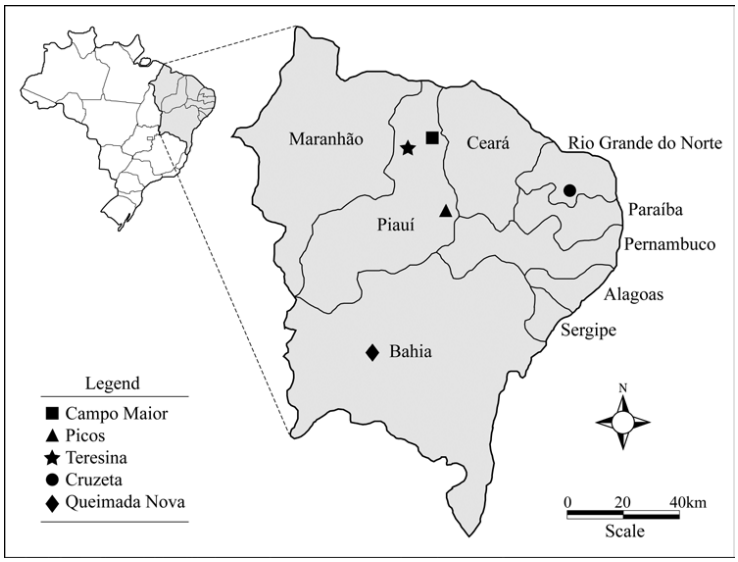

Figure 1. Origin of the *Pilosocereus gounellei* plants whose seeds were germinated to form plantlets representative of the Caatinga biome. Fruit was collected from the plants growing in areas of natural occurrence, gardens, and experimental fields in the cities of the Brazilian states Piauí (PI), Rio Grande do Norte (RN), and Bahia (BA).

Table 1. Collection sites and number of samples of *Pilosocereus gounellei*.

City (State)	Sample	Geographic coordinate
Teresina (PI)	Te-1	5°5'20"S/42°48'07"W/72 m
	Te-2	
	Te-3	
Picos (PI)	Pi-4	7°4'37"S/41°28'1"W/206 m
Campo Maior (PI)	CM-5	4°49'40"S/42°10'7"W/125 m
	CM-6	
Oliveira dos brejinhos, in Queimada Nova (BA)	QN-7	10°56'9"S/41°56'51"W/550 m
	QN-8	
	QN-9	
	QN-10	
	QN-11	
Cruzeta (RN)	Cz-12	6°26'S/36°35'W/230 m
	Cz-13	
	Cz-14	
	Cz-15	
	Cz-16	
	Cz-17	
	Cz-18	
	Cz-19	
	Cz-20	
	Cz-21	

DNA isolation and AFLP assay

DNA was isolated according to the protocol originally described by Aljanabi et al. (1999), incorporating minor modifications proposed by Resende et al. (2010). These modifications included the use of 400 µL 7.5 M ammonium acetate after centrifugation with chloroform/isoamyl alcohol (24:1), and the use of 3 M NaCl and 3% (w/v) CTAB. DNA concentration was estimated using a UV-visible spectrophotometer Picodrop®.

AFLP amplification was performed using AFLP® Analysis System I and AFLP® Starter Primer Kit (Invitrogen). The sequence of the adaptors and pre-selective amplification primers are given in Table 2. Genomic DNA (300 ng) was digested with *Eco*RI and *Mse*I (3 U each) in a 25 µL standard reaction mix for 3 h at 37°C in a Techne TC-512 thermocycler. Thereafter, the restriction endonucleases were heat inactivated at 70°C for 15 min followed by ligation of the DNA fragments to *Eco*RI and *Mse*I adapters in a ligation reaction containing 24 µL adaptor solution [*Eco*RI/*Mse*I adapters, 0.4 mM ATP, 10 mM Tris-HCl (pH 7.5), 10 mM Mg-acetate, 50 mM K-acetate] and 1 µL T4 DNA ligase (1 U/mL). The ligation was carried out at 20°C for 2 h 30 min in a Techne TC-512 thermocycler generating the template DNA for amplification. The fragments were pre-amplified according to the method of Labra et al. (2003). The pre-amplification reactions were performed with 2.5 µL DNA containing the adaptors (obtained after the ligation), 20 µL primer mixture for the pre-amplification, 2.5 µL 10X PCR buffer with Mg^{2+} and 0.5 µL Platinum Taq DNA polymerase (5 U/µL) in a total volume of 25.5 µL.

The selective amplification reactions were performed in 10 µL solution containing the following: 2.5 µL pre-amplified DNA, 0.05 µL Platinum Taq DNA polymerase (5 U/µL), 1.0 µL 10X PCR buffer with Mg^{2+}, 3.95 µL distilled water, 2.25 µL *Mse*I primer, and 0.25 µL *Eco*RI primer. PCR was performed in a Techne TC-512 thermocycler for 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 60 s. The sequences of selective primers used to amplify each of the 21 DNA samples are provided in Table 2.

Table 2. Sequences for the adapters and selective primers used for the AFLP analysis in DNA samples of *Pilosocereus gounellei*.

Adapters/primers	Sequence (5'-3')
Adapters	
*Eco*RI	5'-CTCGTAGACTGCGTACC-3'
	3'-CATCTGACGCATGGTTA-5'
*Mse*I	5'-GACGATGAGTCCTGAG-3'
	3'-TACTCAGGACTCAT-5'
Primers	
E-AAG	5'-GACTGCGTACCAATTCAAG-3'
M-CTT	5'-GATGAGTCCTGAGTAACTT-3'
E-ACC	5'-GACTGCGTACCAATCACC-3'
M-CAT	5'-GATGAGTCCTGAGTAACAT-3'
E-ACC	5'-GACTGCGTACCAATCACC-3'
M-CAA	5'-GATGAGTCCTGAGTAACAA-3'
E-AGC	5'-GACTGCGTACCAATTCAGC-3'
M-CAG	5'-GATGAGTCCTGAGTAACAG-3'
E-AAC	5'-GACTGCGTACCAATTCAAC-3'
M-CTA	5'-GATGAGTCCTGAGTAACTA-3'
E-ACT	5'-GACTGCGTACCAATTCACT-3'
M-CTA	5'-GATGAGTCCTGAGTAACTA-3'
E-AGG	5'-GACTGCGTACCAATTCAGG-3'
M-CTG	5'-GATGAGTCCTGAGTAACTG-3'

E = selective primer for *Eco*RI; M = selective primer for *Mse*I.

Four µL PCR product was added to 3 µL denaturation buffer (99% formamide, 10 mM EDTA, pH 8.0, 1 mg/mL xylene cyanol, and 1 mg/mL bromophenol blue), and the samples were heated at 94°C for 3 min. After denaturation, the samples were loaded on an 8% denaturing polyacrylamide gel (Creste et al., 2001). Electrophoresis was performed in a vertical chamber (FB-SEQ-3445, Fisher Scientific; Life Technologies do Brasil Ltda, São Paulo) for 3 h 15 min at 65 W in 1X TBE buffer. After electrophoresis, the gel was fixed with 0.5% acetic acid and 5% ethanol for 20 min and then stained with silver nitrate, according to the protocol of Beidler et al. (1982). After staining, the gel was again fixed using 10% acetic acid for 20 min and then stored in water.

Data analysis

All 21 plantlets were scored for the presence or absence of AFLP bands (a score of 1 was assigned for presence and 0 for absence of the homologous band), and the data were fed into a binary data matrix as discrete variables. The NTSYS-pc package (Rohlf, 1989) was used to compare the data from individual plantlets. Polymorphisms among the AFLP markers were analyzed using STRUCTURE software 2.0 (Pritchard and Wen, 2003), which evaluated the level of genetic admixture between the samples from the three Northeastern States. The genotypes were clustered, with the number of clusters (K) ranging from 2 to 8, and tested using the admixture model with a burn-in period of 5,000 interations followed by 50,000 Markov Chain Monte Carlo (MCMC) interations, considering the presence or absence of AFLP markers (bands) across the samples. The true number of populations (K) is often identified using the maximal value of Δ (K) returned by the software. The most probable number (K) of subpopulations was identified as described by Evanno et al. (2005). The graphical output display of the STRUCTURE results was taken as input data for STRUCTURE HARVESTER, a web-based software for visualizing the STRUCTURE output and implementing the Evanno method (Earl and von Holdt, 2012) to obtain a graphical representation.

RESULTS

The fingerprint pattern of the xiquexique samples showed a high genetic diversity among the samples. The seven primer pairs amplified 703 AFLP markers, of which 700 (99.57%) were polymorphic (Table 3). The percentage of polymorphic fragments ranged from 95.3% for the primer pair E-AAG x M-CTT to 100% for E-ACC x M-CAT, E-ACC x M-CAA, E-AGC x M-CAG, E-ACT x M-CTA, E-ACT x M-CTA, and E-AGG x M-CTG pairs. The largest number of informative markers (126) was detected using the primer combination E-AAC x M-CTA, while the E-AAG x M-CTT combination revealed the lowest number of polymorphic fragments (48), in the xiquexique samples.

Table 3. Number of fragments obtained with the seven primers combinations and the polymorphisms (PAF) detected in the 21 samples of *Pilosocereus gounellei*.

Primer combination	Amplified fragments	PAF	% P
E-AAG × M-CTT	48	46	95.3%
E-ACC × M-CAT	82	82	100.0%
E-ACC × M-CAA	96	96	100.0%
E-AGC × M-CAG	122	122	100.0%
E-AAC × M-CTA	126	125	99.2%
E-ACT × M-CTA	118	118	100.0%
E-AGG × M-CTG	111	111	100.0%
Total	703	700	99.21%

Percentage of polymorphic fragments ranged from 72.55% in the PI sample to 82.79% in the RN sample. In BA samples, the percentage of polymorphic fragments was 70.84%. The values of Nei's identity (I; Nei, 1978) varied from 0.1393 (between the Pi-4 and Cz-18 samples) to 0.7020 (between the Te-1 and Te-2 samples).

The clustering of the 21 xiquexique samples according to a model-based Bayesian algorithm is shown in Figure 2. Each bar in the graph represents a plant and its inferred proportion of AFLP marker admixture. The optimal K value determined by the Bayesian analysis indicated that the plants were grouped into 4 clusters ($\Delta K2 = 0.00$; $\Delta K3 = 0.1779$; $\Delta K4 = 2.1683$; $\Delta K5 = 1.0511$; $\Delta K6 = 1.0816$; $\Delta K7 = 0.2739$; $\Delta K8 = 0.3873$; $\Delta K9 = 0.0701$; and $\Delta K10 = 0.00$). The bar plot obtained for the K value ($K = 4$; $\Delta K = 2.1686$) and the results were consistent with the evidence of plants with a mixture of DNA fragments of 2, 3, and 4 groups, and plants with fragments predominantly of one of the groups.

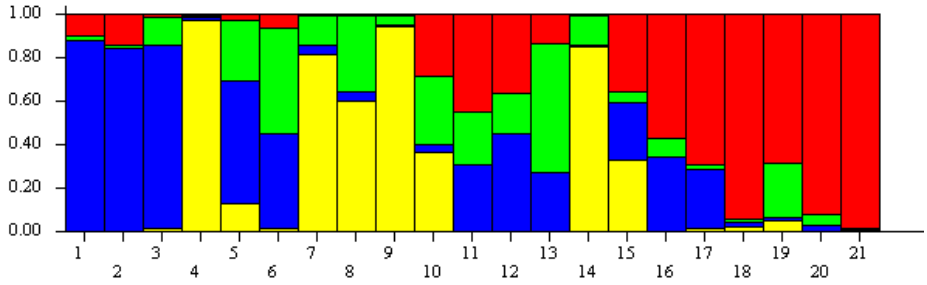

Figure 2. Bar plot-like population structure, based on AFLP markers for plants of *Pilosocereus gounellei* from Teresina (1-3), Picos (4), Campo Maior (5-6), Queimada Nova (7-11), and Cruzeta (12-21) within the K clusters. Each plant is represented by a single vertical bar broken in K colored segments (K = 4), with lengths proportional to each of the K inferred clusters. Each color represents the proportion of fragments for each individual, represented by a vertical bar.

The bar plot indicates the division of the xiquexique samples, from the three states, according to their genetic relatedness. Table 4 shows that the largest proportion of plants of *P. gounellei* from PI was in the blue group (59.6%), that of the plants from BA was in the yellow group (54.8%), and that of the plants from the RN was in the red group (56.2%). The differential distribution of the highest proportions of plants of each state in different groups (59.6% in the blue group, 54.8% in the yellow group, and 56.2% in red group) indicates a genetic divergence at the molecular level of these plants.

Table 4. Proportion of plants of *Pilosocereus gounellei* distributed in each group and number of plants in each state Piauí (PI), Bahia (BA), and Rio Grande do Norte (RN).

State	Group				Number of plants
	Red	Green	Blue	Yellow	
PI	0.057	0.155	0.596	0.191	6
BA	0.147	0.217	0.088	0.548	5
RN	0.562	0.140	0.167	0.131	10
Mean/total	**0.255**	**0.171**	**0.284**	**0.290**	**21**

DISCUSSION

The polymorphism yielded by AFLP markers in *P. gounellei* was 99.57%, which was significantly higher than that reported previously from research with other cactus species. In a study evaluating the genetic diversity at the DNA level in mandacaru (genus *Cereus*), six primer pairs were used, to amplify 348 AFLP markers, of which 282 (81%) were polymorphic (Faria-Tavares et al., 2013). In the genus *Opuntia*, AFLP analysis was carried out on one sample each of *O. ficus-indica*, *O. megacantha*, *O. amyclaea*, *O. undulate*, and *O. spinulifera* to elucidate genetic relationship amongst species plotted in the first cluster of the cpSSR neighbor joining tree (Nilsen et al., 2005). In this study four primer combinations used to amplify the DNA obtained from the *Opuntia* samples generated 169 bands of which 131 (77.51%) were polymorphic. In addition, 86% of the sites were detected to be polymorphic. The high polymorphism of the amplified fragments of DNA in *P. gounellei* is an important and promising trait to encourage the use of xiquexique in the restoration of degraded areas in Northeastern Brazil and also for proposing genetic improvements in it. The improvement in *P. gounellei* should aim to achieve plants with attributes preferred by humans and animals, such as sweeter fruit, thinner peel, and fewer spines (Almeida et al., 2007).

However, despite the high genetic diversity of AFLP markers in xiquexique, the similarity between plants from the same place was well defined as illustrated in the bar plot (Figure 2). The model-based Bayesian algorithm in the STRUCTURE software is efficient in assigning individuals to their populations of origin (Evanno et al., 2005). Thus, the identification of relatively homogeneous clusters of xiquexique reflecting their origin is evident in the bar plot. The vegetative propagation as the predominant form of multiplication of xiquexique plants may explain the high genetic similarity between plants from the same location and seems to be the cause of the differentiation at molecular level among the plant samples from different regions of the Caatinga biome.

Polymorphism of the amplified fragments of DNA was higher (82.79%) in the Cruzeta samples than in the ones from Piauí and Bahia. The genetic variation in samples from Cruzeta as revealed by microsatellite markers was also higher than that in the plantlets from PI and BA (Monteiro et al., 2015). However, due the highest proportion of plants in the red group (56.2%) should be very important to increase genetic diversity in plants for use in the restoration of degraded

areas, and also to select samples of contrasting plants for crosses in the breeding program of xiquexique, a wild natural resource important to the livelihoods of local people. The introduction of xiquexique plants with different origins, from other neighboring states that are also part of the Caatinga (Paraíba, Ceará, Maranhão, Pernambuco, Alagoas, and Sergipe), into the experimental field of Cruzeta is needed to promote the natural exchange of genetic material. Such exchanges are required for establishment of biodiversity conservation units and also for broadening of the genetic base of the species.

The AFLP methodology is important to identify genetically homogeneous and contrasting plants, as well as plants sharing DNA markers of different origins. In principle, along with local plants, those sharing genetic material from different origins are ideal source of seeds to produce plants that can restore degraded areas of the Caatinga biome. Restoration of degraded areas in the Caatinga biome is a nationwide concern in Brazil, and for this reason, the biome is included in the National Action Plan for the Conservation of Cactaceae, which is coordinated by the Chico Mendes Institute for Biodiversity Conservation in partnership with international institutions.

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