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Genetic diversity and structure of populations in *Pilosocereus gounellei* (F.A.C.Weber ex K.Schum.) (Cactaceae) in the Caatinga biome as revealed by heterologous microsatellite primers

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ABSTRACT

Microsatellite transferability was used as a method to examine the genetic diversity and structure of populations in *Pilosocereus gounellei* seedling samples that have potential to implement effective restoration strategies for degraded and disturbed areas of the Caatinga biome. Genomic DNA was extracted from 85 seedlings obtained from fruit collected from plants growing in native areas in the Brazilian states of Piaui (PI), Rio Grande do Norte (RN), and Bahia (BA). Six microsatellite primers were polymorphic. AMOVA showed higher genetic variation within (72%) than among (28%) the samples from the three states. The high level of similarity between the seedlings from PI, BA, and RN indicated that samples collected at any of the three sites can be used to represent the genetic diversity of the species. Seeds of plants from the three States are recommended as samples for germplasm banks and/or the production of plantlets to *i*) plant in areas of strategic reserves for forage, *ii*) deploy new cultivation areas, *iii*) restore degraded areas in the semi-arid Northeast, and *iv*) maintain ecological reserve banks and fodder with genetically divergent plants.
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1. Introduction

Pilosocereus gounellei (F.A.C.Weber ex K.Schum.) (Cactaceae), popularly known as xique–xique, is a common cactus species found in northeast Brazil, exclusively located in areas throughout the Caatinga biome (Taylor and Zappi, 2002). The Caatinga, the only exclusively Brazilian biome, occupies 11% of Brazil and is home to unique flora and fauna, with many species that are found nowhere else on the planet. This biome is species-rich but floristically poor compared with tropical rainforests. The

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Caatinga has 11 strictly protected areas, representing less than 1% of the region, which is the smallest protected area of any major Brazilian biome (Leal et al., 2005). Brazil is the third largest center of cactus diversity in the Americas, with the Caatinga biome having the largest number of endemic species of cacti (61), of which the Cactaceae family is the dominant vegetation. Despite this diversity, Brazil has the highest proportion of threatened species (18%) compared with others countries due to high rates of deforestation and habitat fragmentation resulting from mechanized agriculture (Klink and Machado, 2005; Leal et al., 2005; Espírito-Santo et al., 2009). The Caatinga climate is an anomaly and serves as an important laboratory for studying how plants adapt to highly variable rainfall and environmental stress. Ecologically, the Cactaceae species has an important role in the equilibrium of ecosystems in the Caatinga and is also an important food resource.

P. gounellei grows from Maranhão to Bahia, preferring sandy soil and rocky outcrops (Rocha and Agra, 2002). It is primarily found in the states of Ceará, Rio Grande do Norte and Bahia (Silva et al., 2005; Menezes et al., 2011). *P. gounellei* is one of the cactus species chiefly used for feeding herds of cattle and flocks of sheep and goats reared extensively in the Caatinga (Araujo et al., 2010). The added nutritional value and benefits of xique–xique plants for weight gain and/or milk production in feed for cattle, sheep, and goats has been strongly emphasized by Silva et al. (2010a, 2011, 2012). Additionally, this species is used as a "famine food" by people in times of food scarcity. Flour for preparing couscous can be produced from the cladode, and this food can also be eaten boiled or roasted (Nascimento et al., 2012). The consumption of *P. gounellei* involves artificial selection, which favors the survival and propagation of those plants with attributes preferred by people, such as a thinner peel with fewer spines.

The high rates of deforestation and habitat fragmentation resulting from mechanized agriculture and also the continuous consumption and management of the xiquexique plants for food are factors that can cause habitat degradation and fragmentation as well as the loss of genetic diversity. Knowledge of the genetic diversity of *P. gounellei* growing in the Caatinga biome is particularly important for restoration and recovery of degraded/disturbed areas. In restoration projects, the seedlings should be composed of genetic material with high genetic variability since the use of seedlings with low genetic variability may make unfeasible the recovery in the short, medium or long term. The genetic diversity of *P. gounellei* remains unknown, and it is difficult to implement effective restoration strategies for degraded/disturbed areas. The xiquexique plants can represent a first step for restoring degraded areas since that survive in extreme conditions and is a species of the Caatinga biome that produces attractive fruits for birds, bats and other animals.

In the present study, we propose microsatellite transferability as an alternative method to examine the genetic diversity and structure of populations in *P. gounellei* seedling samples that have potential for use in effective restoration strategies of Caatinga biome. Reduced genetic diversity and a narrow genetic basis for the species is expected due to the consumption and indiscriminate exploitation of this natural resource.

2. Materials and methods

2.1. Samples of *Pilosocereus grounelli*

Genomic DNA was extracted from 85 seedlings obtained from fruit collected from different *P. grounelli* plants growing in areas of natural occurrence in three Brazilian states: Piaui (PI), Rio Grande do Norte (RN), and Bahia (BA). The seeds collected from the xique–xique plants were germinated in aseptic conditions to form plantlets representative of part of the Caatinga region in Brazil.

Seeds were removed from the mature fruit (one fruit per xique–xique plant) and then washed in tap and distilled water to remove any remaining mucilage. The seeds were then soaked in commercial detergent for 25 min, washed 3–4 times in distilled water, surface sterilized with 10% sodium hypochlorite for 5 min, washed in distilled water and soaked in sterilized water in a sterile chamber (Petri dishes sealed) for 24 h, as described by Carvalho et al. (2008). After pre-soaking in water, the seeds were placed in flasks containing culture medium. Seeds were germinated in aseptic conditions in KC medium (Knudson, 1946) and maintained in an acclimatized chamber at 30 °C with a photoperiod of 12 h. Eight- to twelve-month-old plantlets were used for DNA extraction.

2.2. DNA extraction and primers transferability

DNA was extracted from 200 mg of plantlet tissue as described by Resende et al. (2007). After DNA extraction, DNA quantity and quality were determined by 0.8% agarose gel electrophoresis buffered with 1× TAE (0.04 M Tris–acetate and 0.001 M EDTA). A standard DNA ladder (λ phage, 50, 100 and 150 ng) was used as a marker. The gel was stained with 0.5 μg/mL ethidium bromide, and the image was visualized with a Molecular Image Loccus L-PIX – HE (Loccus do Brasil Ltda., São Paulo, SP., Brazil) using the Picasa 3 software.

The microsatellite loci transferability was analyzed by testing a total of 33 primer pairs from *Polaskia chichipe* (seven primer pairs), *Astrophytum asterias* (six primer pairs), *Ariocarpus bravoanus* (eight primer pairs), and *Echinocactus grusonii* (twelve primer pairs). The polymerase chain reaction (PCR) was performed using 0.2 mL microtubes in a Techne TC-512 thermocycler. A 20 μL reaction was carried out using 20 ng of genomic DNA, 2.0 μL of 1× buffer containing Tris–HCl (10 mM Tris–HCl pH 8.3 and 50 mM KCl), 2.5 mM of $MgCl_2$, 0.1 μM dNTPs, 1 U of Taq-DNA Polymerase (Invitrogen), 0.5 μM each of the forward and reverse primers, and Milli-Q water to bring the reaction to the final volume. Microsatellite amplification was initially performed by Touchdown PCR (Don et al., 1991). The products that were not clearly amplified by

Touchdown PCR were amplified again as described by Albert and Schmitz (2002), and the annealing temperature was optimized to between 50 and 59 °C to achieve clear products. Electrophoresis was performed in a 4% Metaphor agarose gel using 0.5× TBE buffer (0.45 M Tris–borate and 0.001 M EDTA) at 60 V for 4 h. Then, the gels were stained with ethidium bromide at 0.5 μg mL^{-1}, and images were captured with a Molecular Image Loccus L-PIX – HE using the Picasa 3 software. The sizes of the PCR fragments were determined using a 100 bp DNA Ladder (Invitrogen) (Table 1).

2.3. Polymorphism analysis

The polymorphic SSR loci in the 85 plantlet progeny from the six xique–xique plants of the PI State, seven plants of the RN State, and four plants of the BA State were used to estimate the average number of alleles per locus, the average observed heterozygosity (H_o), and the expected heterozygosity (H_e). The deviation from the Hardy–Weinberg equilibrium was verified for every locus. The *F* statistic of Wright (1965), the deficit of heterozygotes (F_{IS} and F_{IT}), and the genetic diversity between the samples from the three states (Piaui, Rio Grande do Norte, and Bahia) (F_{ST}) were estimated using the POPGENE 1.32 software (Yeh et al., 1999). To explore the hierarchical partitioning of genetic variation within and between the samples from the three states, we performed an Analysis of Molecular Variance (AMOVA, GenAlEx 6.2; Peakall and Smouse, 2006). Polymorphisms from SSR loci were also analyzed using STRUCTURE software 2.0 (Pritchard and Wen, 2003), which evaluates the level of genetic admixture between the 85 *P. gounellei* plantlets. The genotypes were clustered, with the number of clusters (K) ranging from 2 to 12, and were tested using the admixture model with a burn-in period of 5000 repeats followed by 50,000 Markov Chain Monte Carlo (MCMC) repeats, considering the presence and absence of alleles across the sample. 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 using the STRUCTURE HARVESTER, a website and software that are used to visualize STRUCTURE output and to implement the Evanno method (Earl and vonHoldt, 2012) to display a graphical representation.

3. Results

The quantification of genomic DNA indicated that the amount of DNA ranged from 50 to 200 ng μL^{-1}. Five microsatellite primers from the amplified DNA fragments of *Echinocactus grusonii* (mEgR17, mEgR63 and mEgR98), *Polaskia chichipe* (Pchi44), and *Astrophytum asterias* (mAaB6) were obtained, and these primers were polymorphic in *P. gounellei* (Table 2). The eight primers from *Ariocarpus bravoanus* showed no clear SSR-amplifiable products. Microsatellite transferability was 15.5% (5 primers showed amplified products/total of 33 primers tested).

Six alleles were observed at the *AaB6* locus, four alleles were observed at the *mEgR17* and *Pchi44* loci, and two alleles were detected at the *mEgR63* and *mEgR98* loci (Table 2). A total of 18 alleles, with an average of 3.6 alleles per locus, were detected in the *P. gounellei* plantlets. The *AaB6* locus showed the largest number of alleles, but the $AaB6^2$ allele was found only in the plantlets from PI and RN and the $AaB6^6$ allele was found only in the plantlets from PI and BA. The $mEgR63^1$ allele was found only in the plantlets from RN; the $mEgR63^2$ allele was found fixed in the samples from the states of PI and BA (Table 2). The number of polymorphisms in the *AaB6*, *mEgR17*, *mEgR63*, *mEgR98*, and *Pchi44* loci was higher in the plantlet samples from the RN (100%) than in plantlet samples from the PI and BA states (80%). The plantlets from BA showed fewer alleles (Na = 2.8) and a lower effective number of alleles (Ne = 2.1836) than the plantlets from PI and RN (Table 3). The highest values for the expected and observed mean heterozygosity were observed in the plantlets from RN ($H_e = 0.5097$) and PI ($H_o = 0.3632$; Table 3). The Shannon's index was also higher in plantlet samples from PI ($I = 0.9037$) than in the plantlets from the RN ($I = 0.8938$) and BA ($I = 0.7650$) states (Table 3). The highest value of Ho was observed in the *mEgR98* locus (0.5765), and the highest value of He (0.7206) was observed in the *mEgR17* locus (Table 4).

Table 1
Nucleotide sequences of the SSR primers, simple sequence repeats of each primer, number of alleles (Na) detected by each primer in *Pilosocereus gounellei*, and variation in allele size (bp) detected in the original species and in *P. gounellei*.

Species	Primer	Nucleotide sequence	Simple sequence repeated	Na	Base pair in original specie	Base pair in *Pilosocereus gounellei*
Polaskia chichipe Otero-Arnaiz et al. (2004)	Pchi44 *AY147834*	ATTCAAACAGGCCCACACAG [(F)] GGGTGTTAGAAGGAATAATAGCTTG[(R)]	$(CA)_{17}$	4	137 bp	250–313 bp
Astrophytum asterias Terry et al. (2006)	AaB6	ATGCGAACAGATTGAAAAGAGGG[(F)] CTCAGGAAAGACTTACACCATGG[(R)]	$(GA)_{13}$	6	83–99	80–140 bp
Echinocactus grusonii Hardesty et al. (2008)	mEgR17	ATCGTTGAAAGAGGCGAAA [(F)] TCCCTCTTCTTCGTCAGAGC [(R)]	$(AG)_{11}$	4	330–347 bp	350–400 bp
	mEgR63	TTGAATTGGGTGCTTCACTG [(F)] CCCTAAATATGCTGCCGATG [(R)]	$(AG)_3T(GA)_{12}$	2	238–253 bp	23–90 bp
	mEgR98	ACCCTAGTGGGGTCGAGAAT [(F)] GTCGCCCAGAACCCTAGTCT [(R)]	$(AG)_{12}AA(AG)_4$	2	172–187 bp	190–367 bp
Total				18		

Table 2
Frequency of alleles for the five SSR loci evaluated in plantlets of *Pilosocereus gounellei* from Piaui (PI), Rio Grande do Norte (RN) and Bahia (BA).

Locus	Alleles	PI	RN	BA
Pchi44	*1*	0.0926	0.1795	–
	2	0.4444	0.5256	0.3333
	3	0.3148	0.2692	0.6667
	4	0.1416	0.0256	–
AaB6	*1*	0.0179	0.0385	0.0556
	2	0.1607	0.0769	–
	3	0.5179	0.4872	0.3889
	4	0.0536	0.2821	0.4167
	5	0.1429	0.1154	0.1111
	6	0.1071	–	0.0278
mEgR17	*1*	0.2963	0.3421	0.2222
	2	0.3889	0.3421	0.2222
	3	0.0926	0.2895	0.4444
	4	0.2222	0.0263	0.1111
mEgR63	*1*	–	0.0769	–
	2	1.0000	0.9231	1.0000
mEgR98	*1*	0.6786	0.3333	0.5833
	2	0.3214	0.6667	0.4167

The largest effective number of alleles per locus and the highest genetic diversity (He > 0.50) were detected in the *mEgR17*, *AaB6*, *Pchi44*, and *mEgR98* loci, suggesting that these four loci are adequate to allow polymorphism among the *P. gounellei* plantlets from the three states (PI, BA, RN). The allele frequencies were analyzed for the five SSR loci. A departure from the Hardy–Weinberg equilibrium was observed for 67% of the *P. gounellei* plantlets. The *Pchi44*, *AaB6* and *mEgR17* loci showed a departure from the Hardy–Weinberg equilibrium in plantlet samples from PI, RN, and BA while the *mEgR98* loci showed a departure from the Hardy–Weinberg equilibrium only in plantlet samples from RN.

The use of *F* statistics as parameters for genetic diversity (Table 4) indicated a global deficit of heterozygotes (F_{IS}) in the *P. gounellei* plantlets. The F_{IS} was 0.3183, which seemed either high or low depending on the individual SSR locus analyzed. The analysis of the loci *mEgR17* ($F_{IS} = 0.5596$) indicated the highest value for homozygote excess, while at the *mEgR98* and *mEgR63* loci, the F_{IS} values were negative, indicating heterozygote excess. The positive global value of F_{IS} (0.3183) indicated a 31.83% deficit in heterozygous plants. Analyses of genetic divergence among plantlets from PI, RN, and BA (F_{ST}) indicated a global value of 0.0614, with three SSR loci (*mEgR98*, *mEgR63*, and *Pchi44*) having values higher than 0.05 (Table 4) and indicating a moderate difference among these samples (Wright, 1978). The *mEgR98* locus had the highest proportion of genetic diversity ($F_{ST} = 0.0851$); therefore, the *mEgR98* primer was most appropriate to characterize the different *P. gounellei* plantlets from PI, BA, and RN by allelic frequency. The gene flow estimated from F_{ST} was high (Nm = 3.8238), indicating the migration of alleles among the three states.

The Nei identity (Nei, 1972) values calculated between the *P. gounellei* plantlets from PI, BA, and RN varied from 0.9075 (between PI and BA) to 0.9209 (between RN and BA), indicating less than 10% genetic divergence between the plantlets from PI and BA. AMOVA showed higher genetic variation within (72%) than among (28%) the samples from the three states.

In the clustering of the 85 plantlet according to a model-based Bayesian algorithm, the bar plot obtained for the *K* value ($K = 8$; $\Delta K = 5.7152$), and the results were consistent with the evidence that many alleles are migrants or are admixed among the samples from the three states. In all samples from the three states, the plantlets shared alleles from the eight groups.

4. Discussion

The use of the primers Pchi44, AaB6, mEgR63, mEgR17, and mEgR98 was an important alternative strategy to characterize the genetic diversity in *P. gounellei*. The expected heterozygosity in the SSR loci of *P. gounellei* ranged between 0.4562 and 0.5097 (plantlets from BA and RN). The mean He value (0.5236) of the plantlet progeny from xique–xique plants distributed across the three selected states of northwestern Brazilian was high; however, it was lower than the He values reported from

Table 3
Number of alleles (Na) and number of effective alleles (Ne) per polymorphic SSR locus, mean observed heterozygosity (H_o) and expected heterozygosity (H_e), percentage of polymorphic locus (%*P*), and Shannon indices (*I*) in the samples of *Pilosocereus gounellei* plants from Piaui (PI), Rio Grande do Norte (RN), and Bahia (BA).

State	Number of plantlets	Na	Ne	Ho	He	%*P*	*I*
PI	28	3.4	2.4473	0.3632	0.4966	80	0.9037
RN	39	3.4	2.3374	0.3293	0.5097	100	0.8938
BA	18	2.8	2.1836	0.3000	0.4562	80	0.7650
Total	85	3.6	2.5307	0.3339	0.5236	100	0.9439

Table 4
Number of alleles (Na) and number of effective alleles (Ne) per polymorphic SSR locus, mean observed heterozygosity (H_o) and expected heterozygosity (H_e), Shannon indices (I) in *Pilosocereus gounellei* plantlets, as well as fixation coefficients F ($F_{(IS)}$, $F_{(IT)}$, $F_{(ST)}$; Wright 1965) and gene flux (Nm), in the three samples of plants from Piaui (PI), Rio Grande do Norte (RN), and Bahia (BA).

Locus	Na	Ne	Ho	He	F_{IS}	F_{IT}	F_{ST}	Nm
Pchi44	4	2.7579	0.2976	0.6374	0.4961	0.5342	0.0756	3.0578
AaB6	6	3.2428	0.4235	0.6916	0.3508	0.3809	0.0464	5.1412
mEgR17	4	3.5627	0.3012	0.7206	0.5596	0.5805	0.0475	5.0094
mEgR63	2	1.0731	0.0706	0.0681	−0.0833	−0.0263	0.0526	4.5000
mEgR98	2	2.000	0.5765	0.5000	−0.2632	−0.1557	0.0851	2.6868
Mean	3.6	2.5307	0.3339	0.5236	0.3183	0.3793	0.0614	3.8238

other cactus species. In seven SSR loci of *Polaskia chichipe*, the He values ranged between 0.188 and 0.797 (Otero-Arnaiz et al., 2004), and in twelve SSR loci of *Echinocactus grusonii*, the He values ranged between 0.235 and 0.785 (Hardesty et al., 2008). Hughes et al. (2008) observed He values ranging between 0.201 and 0.688 in eight SSR loci from *Ariocarpus bravoanus.*

The high rates of deforestation and habitat degradation and the continuous cultivation and consumption of *P. gounellei* are factors that may explain the lowest heterozygosity values that were found in samples of *P. gounellei* in the Caatinga biome. The occurrence of allele fixation (the *mEgR63*^{2} fixed in the plantlets from PI and BA) may also explain the lowest heterozygosity values that were found in samples of *P. gounellei*. This allele fixation may result from the casual cultivation of these plants or, alternatively, it may be associated with selectable characteristics. The consumption of *P. gounellei* involves artificial selection, which favors the survival and propagation of those plants with attributes preferred by people, such as thicker peel with fewer spines (Almeida et al., 2007).

The consumption of *P. gounellei* occurs occasionally in nature, but frequently, the plants are burned off to remove the thorns and to produce flour used in human and animal food (Nascimento et al., 2012). The burning of plants can indiscriminately eliminate many genotypes and contribute to a deficit of heterozygotes. Clonal multiplication through cuttings is another common form of propagation of *P. gounellei* (Silva et al., 2010a), which leads to the aleatory selection of homozygous or heterozygous genotypes.

Artificial selection by silvicultural management, with a possible exchange (migration) of plantlets for clonal multiplication, explains why progeny from samples of the three states share alleles in the eight groups shown in the bar plot data, which were obtained using a model-based Bayesian algorithm. These data represent the highest genetic variation within the samples of *P. gounellei* plants from the three states. The high level of similarity between the seedlings of *P. gounellei* from PI, BA, and RN indicated a narrow genetic base (0.9075–0.9209) and that samples collected at any of the three sites can be used to represent the genetic diversity of the species. Seeds of plants from the three States (PI, RN, and BA) are recommended as samples for germplasm banks and/or the production of plantlets to *i*) plant in areas of strategic reserves for forage, *ii*) deploy new cultivation areas, *iii*) restore degraded areas in the semi-arid Northeast, and *iv*) maintain ecological reserve banks and fodder with genetically divergent plants. In particular, maintaining the genetic diversity of *P. gounellei* should be one of the objectives of the Campo Experimental e de Produção de Cruzeta (RN). The Campo Experimental e de Produção de Cruzeta is an area of the EMPARN where studies have examined the nutritional content and nutritional value of xique–xique plants (Almeida et al., 2007); the digestibility of plants and weight gain in cattle (Lima, 1996; Silva et al., 2010a), sheep and goats (Cavalcanti and Resende, 2007; Silva et al., 2010b, 2011) fed with xique–xique plants; and milk production (Silva et al., 2005, 2011).

Despite the global deficit of heterozygotes ($F_{IS} = 0.3183$) estimated for the five SSR loci in seedlings of *P. grounellei*, the level of genetic diversity revealed by our study ($H_e = 0.5236$) as well as the mixture of ancestral alleles of the eight groups observed in the bar plot, shows that these plants are indicated for restoration and recovery of areas degraded/disturbed in the Caatinga biome. The areas of anthropic action in the Caatinga biome have been mapped and analyzed, and are shown on report of monitoring of the deforestations in the Brazilian biomes by satellite (2011) [http://www.mma.gov.br/estruturas/sbf_chm_rbbio/_arquivos/relatrio_tcnico_caatinga_72.pdf], drafted by the Ministry of Environment (MMA) and Brazilian Institute of Environment and Natural Resources Renewable (IBAMA). Although the functional significance of SSR loci (*Pchi44, AaB6, mEgR63, mEgR17,* and *mEgR98*) in the *P. grounellei* species is still unknown, the high level of observed and expected heterozygosities indicate, in theory, the high potential this species to colonize new areas. High heterozygosity has been considered an indicator that the plant population has a substantial amount of adaptive genetic variation (Allendorf and Luikart, 2007). Moreover, the value of expected heterozygosity indicates that there is a greater chance of finding plants that respond differently to changes or pressures exerted by the environment.

The level of heterozygosity in samples of *P. grounellei* growing in areas of natural occurrence in three Brazilian states (PI, RN, and BA) is higher than those reported for the following other species of the *Pilosocereus* genus, distributed in South America, using isozyme markers: *P. lanuginosus* ($H_e = 0.274$), *P. aureispinus* ($H_e = 0.284$), *P. viloboensis* ($H_e = 0.292$), *P. machrissii* ($H_e = 0.380$), and *Pilosocereus tillianus* ($H_e = 0.352$) (Nassar et al., 2003; Moraes et al., 2005; Figueredo et al., 2010). The microsatellite transferability proved to be an interesting approach that was used to *i*) reveal both the highest genetic polymorphism in *P. gounellei* and how the plants are genetically related in three parts of the Caatinga biome; *ii*) select SSR loci candidates (*mEgR98*, *Pichi44*, and *mEgR63*) to investigate genetic diversity in xique–xique plants from others regions of the

Caatinga; *iii*) indicate the need of broader the genetic base of the species to obtain most genetically divergent plant groups with potential for use in breeding programs. Moreover, polymorphisms in SSR loci of *P. gounellei* may prove to be a useful tool in monitoring cultivated plants and the restoration of degraded areas in the Caatinga biome.

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