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Genetic evidence of multiple reproductive strategies in a microendemic and threatened cactus (Cactaceae: *Discocactus* Pfeiff) in Bahia, Brazil

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ABSTRACT

Discocactus zehntneri subsp. *petr-halfari,* an endangered taxon, is represented by a single population in an anthropized area of Bahia, Brazil, where it is suffering due to extreme extractivism. Thus, information about this cactus, such as its reproductive patterns, is urgently needed to support conservation strategies. A population genetics approach was used to determine if this subspecies has a preferential pattern of reproduction. We sampled 18 individuals, both with and without connection to parental plants, from five clumps and assessed their diversity and genetic structure using five ISSR markers. The results revealed two clumps that are genetically supported by the presence of genetically equal individuals. The other three groups presented individuals that are genetically different and similar to individuals in other clumps. These findings suggest that this subspecies has sexual and clonal reproduction and that its environmental distribution might be shaped by events of dispersion. In addition, a possible hybrid origin may explain its rates of genetic diversity. Despite all these factors, this taxon is in danger and so the development of conservation strategies to preserve its population are urgently needed, including *in situ* and *ex situ* actions such as the micropropagation *in vitro*, living collections and cryopreservation.

Keywords: *Discocactus*, ISSR, genetic variabilty, microendemism, reproductive strategies

Introduction

Discocactus is a genus of the family Cactaceae that occurs in Brazil, Bolivia and Paraguay (Machado 2004; Machado *et al.* 2005); this genus comprises approximately 10 to 25 species, depending on the classification system adopted (Machado 2004; Machado *et al.* 2005; Braun & Esteves 2008; Santos 2013). This incongruence is basically the result of the high morphological variability within the genus, which hinders the identification of the real number of species (Buining 1980; Anderson 2001; Machado 2004).

Discocactus is one of the most critical genera of Cactaceae; its members have a high habitat specificity and live in small populations with limited geographical distribution (Machado *et al.* 2005). In addition, this genus suffers from extreme extractivism due to both its potential for ornamentation and the degradation of its habitat due to anthropic action (Machado *et al.* 2005; Santos 2013). In particular, at least 12 species of *Discocactus* are considered to be at-risk in some IUCN categories (2017). However, no subspecific taxa (as in, subspecies) have been included in this at-risk categorization despite being commonly recognized as such within the genus (Machado 2004; IUCN 2017).

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Discocactus zehntneri subsp. *petr-halfari* represents one of these threatened taxa and is listed as critically endangered (IUCN 2017). Zachar (2008) originally described it as a single species based on differences in the stem size, flowers and number of spines when compared to *D. bahiensis* and *D. zehntneri*; in the same year, this taxon was described as a subspecies of *D. bahiensis* due to the morphological characters of its seeds (Braun & Esteves 2008). The taxon was recently transferred to the *D. zehntneri* subspecies based on combined molecular and morphological data (Santos 2013; Santos *et al.* 2015). The incongruences between these classifications might suggest a hybrid origin of this taxon, between *D. zehntneri* and *D. bahiensis* subsp. *subviridigriseus* (Zachar 2008).

The subspecies is currently represented by a single population from Juazeiro in Bahia state, Brazil (Zachar 2008; Braun & Esteves 2008; Conceição 2013; Santos *et al.* 2015). The individuals of this subspecies occur alongside shrub vegetation in an open, stony area of the Caatinga biome (Zachar 2008; Braun & Esteves 2008; Conceição 2013) (Fig. 1A-C). These individuals exist in hierarchical clumps, which may contain clonal individuals (ramets) or non-clonal individuals (genets), similar to what happens in other species of Cactaceae (Harper 1977; Nobel 2002) (Fig. 1B). The plants are characterized by the presence of globular, usually solitary stems that are 5 cm high and 10 to 11 cm in diameter. Furthermore, the plants possess marginal and central spines of 25 x 1.5 mm, small floral buds (white flowers) approximately 4 x 3 cm large, and red elongated fruits with approximately 100 small seeds at 1 x 1 mm (Santos *et al.* 2015; Zappi & Taylor 2018). Its flowering period occurs between November and March (Santos *et al.* 2015).

The population of *D. zehntneri* subsp. *petr-halfari* has resisted extinction even under recent indiscriminate anthropic actions (Leal *et al.* 2003; Conceição 2013; Meiado *et al.* 2015), such as the expansion of a highway in its area of occurrence, which have destroyed approximately 50 % of the plant's population (Santos *et al.* 2015). Despite this decrease, there is no evidence of possible steps being taken to promote the maintenance of this taxon.

According to some authors, plants found in devastated areas seem to invest in clonal reproduction due to the instability of the climate (Clark-Tapia *et al.* 2005b; Honnay & Jacquemyn 2008; Mandujano *et al.* 2010; Ortega-Baes

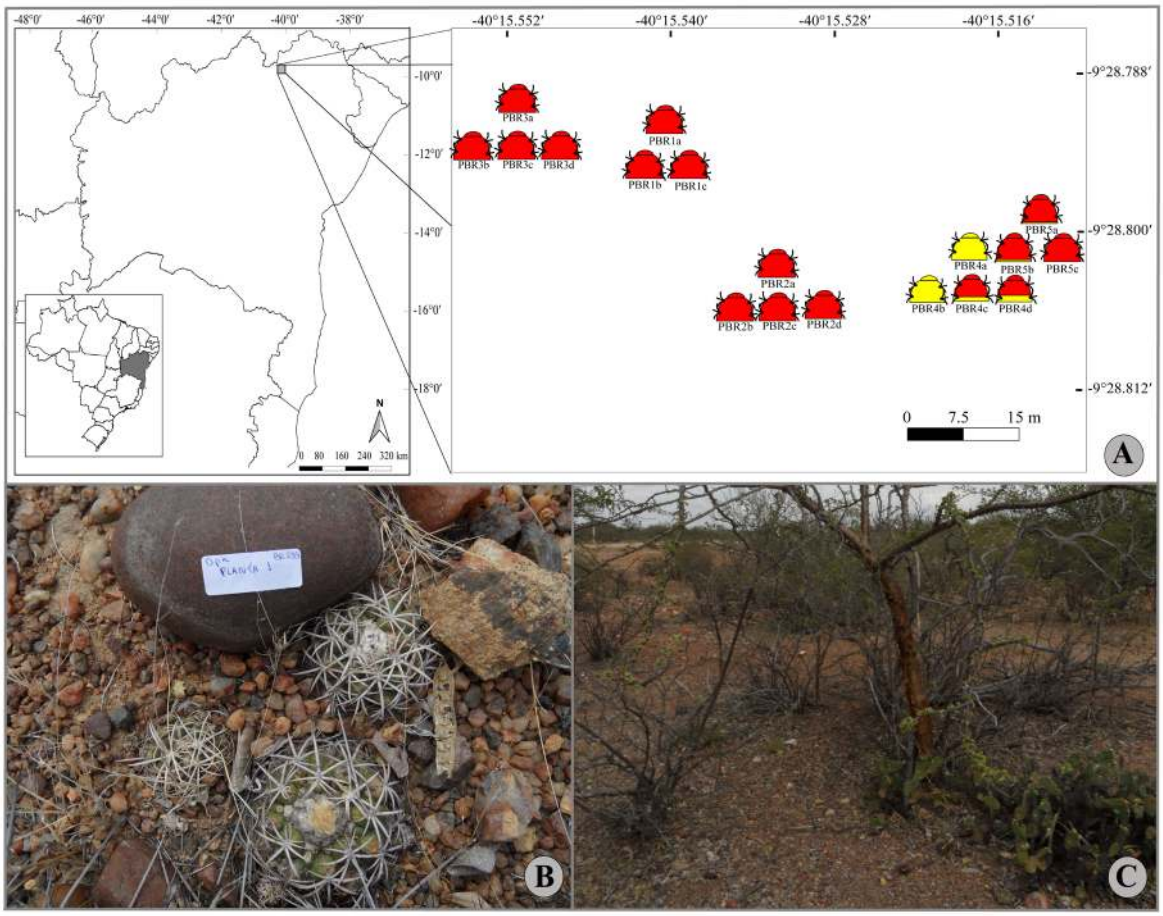

Figure 1. Distribution of *D. zehntneri* subsp. *petr-halfari* in Juazeiro, Bahia, Brazil. **A.** Population locality in Bahia and clumps within the population. **B.** Organization of clumps. **C.** Caatinga area were the population is found. Colors in the individuals represent the results of the Structure Analysis (see Fig. 3A). For the legend of the individuals see Table 1.

& Gorostiague 2013; Fukui & Araki 2014; Blambert *et al.* 2016). This type of reproduction is frequently associated with population maintenance due to its high regeneration capacity and its preservation of successful genotypes (Abrahamson 1980; Caswell 1985; Cook 1985). In this sense, studies on reproductive patterns can be important both to understand population dynamics and to develop conservation strategies, especially for a population's taxa (Kearns & Inouye 1997) as small as *D. zehntneri* subsp. *petr-halfari*.

Reproductive patterns can be assessed by evaluating the population's diversity and genetic structure via molecular markers (Eriksson & Bremer 1993; Clark-Tapia *et al.* 2005a). The Inter Simple Sequence Repeats (ISSR) markers, for example, constitute an interesting and inexpensive tool for this matter because, in addition to being dominant, are abundant throughout the genome, have knowing anchorage and present a high polymorphism rate and not require a prior information for their use (Gupta *et al.* 1994; Zietkiewicz *et al.* 1994; Wolfe *et al.* 1998; Wolfe 2005; Grover & Sharma 2014).

For these reasons, and to access the reproductive strategy and preferential reproduction mode of *D. zehntneri* subsp. *petr-halfari*, the aim of this study was to evaluate the genetic diversity and structure of this subspecies using ISSR markers. We also intend to provide information for the implementation of both *ex situ* and *in situ* conservation strategies for this subspecies.

Materials and methods

Taxon sampling

The only known population of *Discocactus zehntneri* subsp. *petr-halfari* (M.Zachar) M.R.Santos & M.C.Machado occurs in the municipality of Juazeiro, Bahia, Brazil on the margins of highway BR 235 and at an altitude of 350-550 m (9°28'47.6"S 40°15'33.01" W) (Fig. 1A). Considering the existence of a single population we reduced our sampling. We collected 18 entire small juvenile individuals (Voucher 13514 HVASF), which were selected from five separate clumps to avoid genetically similar clumps (Tab. 1 for clumps acronyms, Fig. 1A-B). From each clump, three to four samples were collected, one of which was connected to the parental through the roots (branches) (Tab. 1).

Table 1. Information on the five clumps of *D. zehntneri* subsp. *petr-halfari* collected in Juazeiro, Bahia, Brazil and analyzed in the present study. For the location of the clumps see Figure 1A.

Clumps	Sample size	Latitude	Longitude
PBR1	3	9°28'47.6"S	40°15'32.62"W
PBR2	4	9°28'47.54"S	40°15'33.23"W
PBR3	4	9°28'48.25"S	40°15'31.86"W
PBR4	4	9°28'48.09"S	40°15'30.92"W
PBR5	3	9°28'48.03"S	40°15'30.76"W

DNA extraction and amplification

All individuals were previously dehydrated in a heater at 60 ºC to facilitate maceration, and DNA extraction was performed on the epidermis via the 2 % CTAB (Cetyl-trimethyl-ammonium bromide) protocol (Doyle & Doyle 1987), which was modified for microtubes as proposed by Conceição (2013). Protocols using root fragments, which would avoid the death of the individuals, were not used due to difficulties with bacteria in biological soil disinfestation and the use of specific, high-cost kits. The extracted DNA was quantified in an L-Quant spectrophotometer, analyzed in agarose gel (1 %) with 1x TAE buffer, stained with ethidium bromide and visualized under ultraviolet light.

Amplifications were performed through the Polymerase Chain Reaction (PCR) for five ISSR markers, which are recognized as polymorphic for this subspecies (Conceição 2013) (Tab. 2). For the PCR, we used the TopTaq Master Mix Kit (QIAGEN), following the manufacturer's protocol and adding 6 μM of primer and approximately 50 ng of DNA template. The amplification reactions were performed in the Veriti Thermal Cycler (Applied Biosystems), beginning with an initial denaturing at 94 °C for 1.5 min, followed by 35 cycles of denaturing at 94 °C for 40 s, annealing at 45 °C for 45 s with an extension at 72 °C for 1.5 min, then one cycle of denaturing at 94 °C for 45 s, annealing at 44 °C for 45 s, and then a final extension at 72 °C for 7 min. The PCR products were analyzed in 1.4 % agarose gel with 1x TAE buffer, stained with ethidium bromide and visualized under ultraviolet light. The band profiles were read manually and encoded in a presence (1) or absence (0) data matrix.

Data analysis

The genetic diversity of the individuals was calculated using GenAlEx 6.5 software (Peakall & Smouse 2012), estimating the number of loci (N), the mean expected heterozygosity (He) and the percentage of polymorphic loci (P). The genetic identities between and within the clumps were identified using Nei's unbiased genetic identity measure (Nei 1978) also through software GenALEx 6.5. To infer the genetic relationship between the individuals, a cluster analysis was performed using the Jaccard similarity coefficient and a pairwise analysis with Ward's method (Jaccard 1908; Ward 1963); both analyses were run with the PAST 3.07 (PAleontological STatistics) program (Hammer *et al.* 2001).

To calculate the number of genetic groups (K), two Bayesian Inference was performed, using Structure 2.3.3 (Falush *et al.* 2003) and Geneland v. 4.0.8 (Guillot *et al.* 2005). In the first analysis the admixture model and the correlated allele frequencies were used due to the dominant nature of the marker. We used 10 independent runs with 750,000 Markov Chain Monte Carlo (MCMC) repetitions and a 250,000 burn-in for each value of K, which ranged

from 1 to 4. The results were then analyzed using Structure Harvester 0.6.94 (Earl & Holdt 2012), where the graphs of the estimated parameters were recovered and the most probable K was confirmed (Pritchard *et al.* 2000; Evanno *et al.* 2005). For the analysis on the Geneland software, in turn, a spatial grouping method was implemented in the R environment 3.4.1 (R Development Core Team 2017). We used 1,000,000 Markov Chain Monte Carlo (MCMC) repetitions, saving every 5,000 interactions and the burn-in period of 200. Was realized 10 independent runs were performed, testing from one to five groups (K) in each run. The best parameter of analyze was chosen based on the highest posterior probabilities for clusters

Results

Population genetic diversity

The results revealed the presence of 39 loci within the population (see Tab. 1 for clumps acronyms), 30 of which were polymorphic (79.93 %), and a mean value of approximately 7.8 loci per primer (results not shown). The mean expected heterozygosity found for the population was 0.080. The analysis of Nei's unbiased genetic identity measure revealed moderate to high values of genetic similarity for the studied population, with an average of 0.809 due to PBR2 and PBR3, which were the most related clumps (0.951) (Tab. 2). Within the PBR1 and PBR 3 clumps, the values of genetic identity were 1.000 for all individuals. Already within PBR2, PBR4 and PBR5 clumps, the values of genetic identity were 0.871, 0.696 and 0.812, respectively (Tab. 2), and we found individuals with high (0.846-1.000) and moderate to low rates of genetic similarity (0.795-0.538) (Tab. 2).

Finally, the cluster analysis revealed that the previously selected clumps did not necessarily correspond to the genetic groups for both the clonal (PBR1 and PBR3) and non-clonal (PBR2, PBR4 and PBR5) clusters; there were differences in genetic relation presented by both Jaccard's similarity coefficient and Ward's method (Fig. 2A-B).

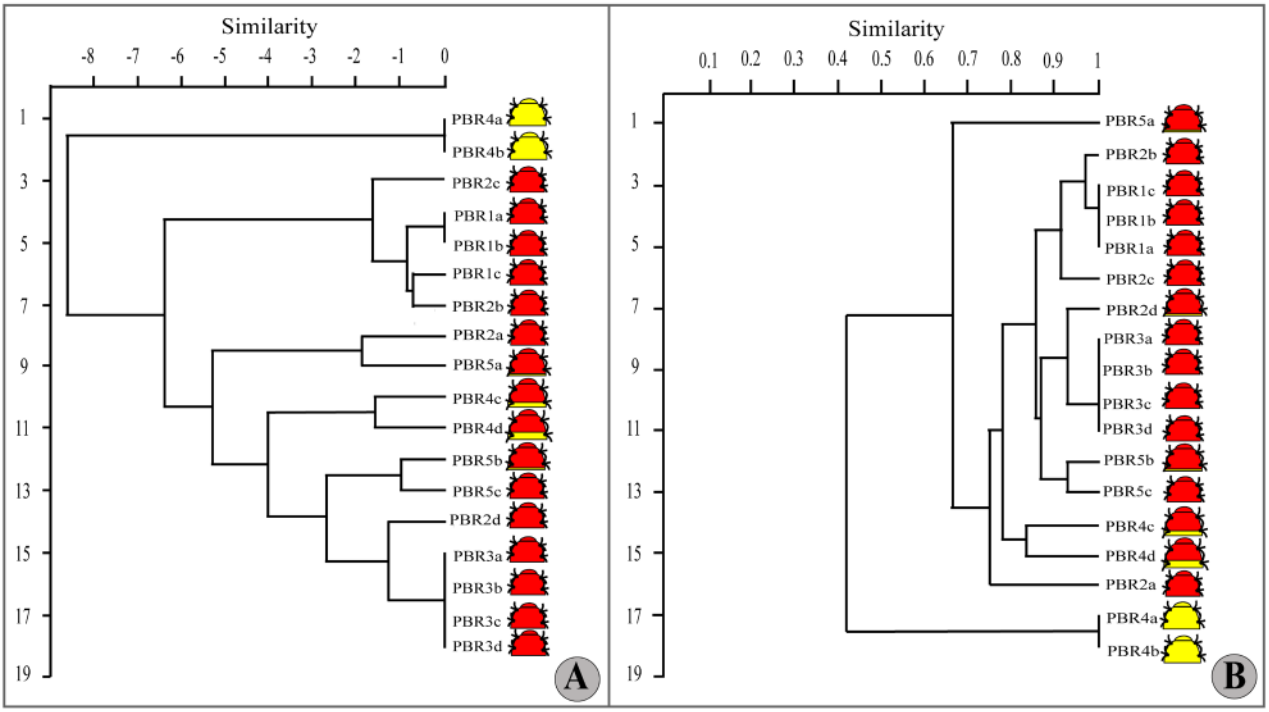

Figure 2. Sumary of the populational genetic diversity. **A.** Jaccard's Coefficient of Similarity. **B.** Ward's Method. Colors in the individuals represent the results of the Bayesian analysis through Structure (see Fig. 3A). For the legend of the individuals see Table 1.

Table 2. Nei Unbiased Genetic Identity (Nei 1978) estimated based on the ISSR patterns among and within *D. zehntneri* subsp. *petr-halfari* and clumps. For the legend of the clumps see Table 1 and Figure 1A.

	PBR1	PBR2	PBR3	PBR4	PBR5		PBR2a	PBR2b	PBR2c	PBR2d		PBR4a	PBR4b	PBR4c	PBR4d		PBR5a	PBR5b	PBR5c
PBR1	-----	------	-----	-----	-----	PBR2a	-----	-----	-----	-----	PBR4a	-----	-----	-----	-----	PBR5a	-----	-----	-----
PBR2	0.924	-----	-----	-----	-----	PBR2b	0.795	-----	-----	-----	PBR4b	1.000	-----	-----	-----	PBR5b	0.846	-----	-----
PBR3	0.872	0.951	-----	-----	-----	PBR2c	0.846	0.949	-----	-----	PBR4c	0.538	0.538	-----	-----	PBR5c	0.795	0.795	-----
PBR4	0.686	0.678	0.698	-----	-----	PBR2d	0.795	0.949	0.897	-----	PBR4d	0.615	0.615	0.872	-----				
PBR5	0.812	0.921	0.871	0.679	-----														

Populational genetic structure

The same numbers of genetic cluster within the population (K=2) was chosen in both Bayesian inferences (Figs. 1A, 3A-C) (Fig. S1 in supplementary material). Bayesian analysis through Structure indicated that the individuals of the clumps PBR1, PBR2, PBR3 and PBR5 presents an only genetic pool (Figs. 1A, 3A). On the other hand, the clump PBR4 presented two individuals are from a different genetic pool (PBR4a and PBR4b) and two others (PBR4c and PBR4d), a mixture between the two genetic pools found (Figs. 1A, 3A. In addition, it is possible to observe that, although individuals of PBR5 are composed exclusively of a single genetic pool, PBR5a and PBR5b have a slight mixture with the genetic pool of PBR4.The Bayesian analysis used the Geneland software confirm the previous analysis from the Structure showing that the individuals from the PBR1, PBR2, PBR3 and PBR5 clusters belong to the same genetic pool (Fig. 3B), while the individuals from PBR4 compose a different genetic pool (Fig. 3C).

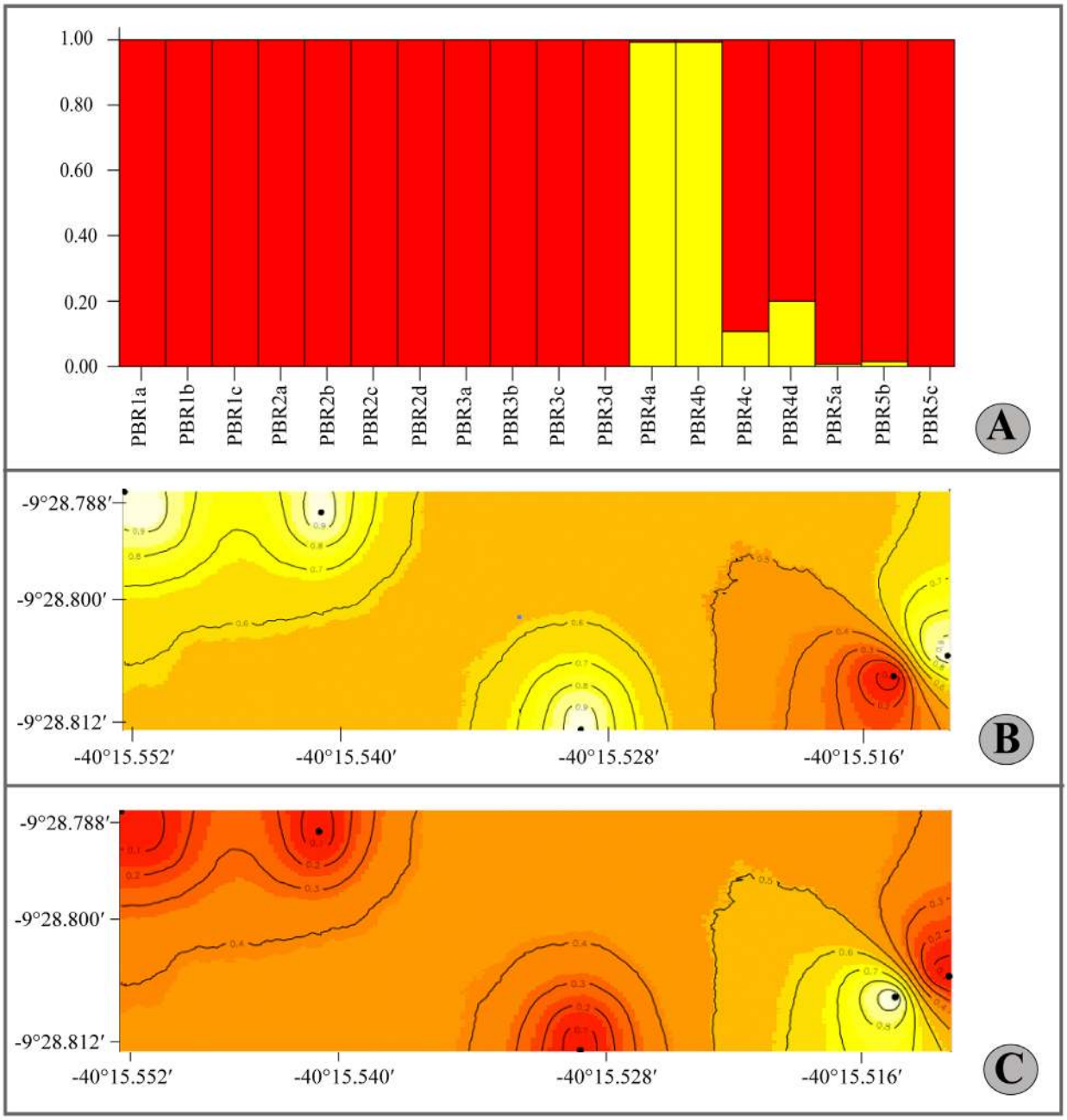

Figure 3. Population genetic structure based on Bayesian Inference. **A.** Results of the clustering analysis by Structure for K = 2. **B.** Spatial distribution of cluster 1 defined by Geneland for K = 2. **C.** Spatial distribution of cluster 2 defined by Geneland for K = 2. Yellow color in B and C indicate high likelihood rates for individuals from the same cluster. The red color indicates a smaller probability of the individuals belonging to that cluster.

Discussion

Discocactus zehntneri subsp. *petr-halfari* genetic characterization

The results of this study revealed that three of the analyzed *D. zehntneri* subsp. *petr-halfari* clumps (clumps PBR2, PBR4 and PBR5) presented low to moderate rates of genetic similarity with individuals of the same clumps and high rates with individuals of different clusters, except to PBR4. The clumps also exhibited relatively moderate to high rates in relation to the percentage of polymorphic loci when compared to values commonly found in other cacti species (Figueredo *et al.* 2010).

The cluster analyses showed that individuals from different clusters are genetically similar. These results suggest that the clumps selected for this study are not genetically supported and, surprisingly, that half of the individuals analyzed are genetically different. Furthermore, the analyses of genetic similarity reveal that two of the study clumps consisted exclusively of clonal individuals. The Bayesian analysis showed that the population is genetically homogeneous, with a group presented a different genetic pool composition, indicating that this subspecies can carry out both sexual and clonal reproduction.

Similar reproductive patterns were found by Ribeiro *et al.* (2015) when they studied six species of cacti (*Arrojadoa rhodantha*, *Pilosocereus gounellei* subsp. *gounellei*, *Melocactus zehntneri*, *Cereus jamacaru* subsp. *jamacaru*, *Tacinga inamoena* and *Tacinga palmadora)*; their study illustrated that these six species exhibit dynamic between sexual and clonal reproduction. Interestingly, three of the studied species (*A. rhodantha*, *P. gounellei* subsp. *gounellei* and *M. zehntneri*) are phylogenetically related to *Discocactus* (Hernández-Hernández *et al.* 2011), which provides an explanation for the similar pattern found by our results.

Additionally, this dynamic between both types of reproduction (sexual and asexual) might be important for the dominance of these species in Caatinga environments since the rapid formation of new individuals by clonal reproduction can facilitate their permanence in stressful environments (Mandujano *et al.* 1998; Pimienta-Barrios & Castillo 2002); sexual reproduction through cross-breeding, for example, can maintain genetic diversity and enable multiple biological interactions (Abrahamson 1980; Stearns 1987). A similar situation was discovered by Lenzi & Orth (2012) for the genus *Opuntia*, in which the high frequency of both types of reproduction also seems important for maintaining the population (Rebman & Pinkava 2001; Lenzi & Orth 2012).

In relation to rates of clonal reproduction, despite the lower values found in the present study, our results might indicate that *D. zentneri* subsp. *petr-halfari* invests in this type of reproduction as a response to environmental conditions, with long periods of high temperatures and sporadic rainfall (Alés *et al.* 1993; Polis 1991) and anthropic impacts in their area (Ayyad 2003; McNeely 2003). These events may affect seedling establishment and the reduction of both partners and pollinators, which are essential for successful sexual reproduction (Honnay & Jacquemyn 2008; Mandujano *et al.* 2010; Ortega-Baes & Gorostiague 2013; Fukui & Araki 2014; Blambert *et al.* 2016). Similary, studies with Cactaceae species like *Stenocereus eruca* and *Tacinga palmadora* also demonstrate a high investment in clonal reproduction probably associated with habitat degradation (Clark-Tapia *et al.* 2005b; Lenzi & Orth 2012; Meiado 2012).

The clonal reproduction could also represent an alternative of growing in polyploid species, which often have sterility problems (Eckert 2002). *Opuntia monacantha,* recently indicated as a plolyploid (2n = 44) by Realini *et al.* (2014). However, according to other studies, neither *O. monacantha* or *S. eruca* constitute polyploid species (2n = 22 in both) (Molina-Freaner & Clark-Tapia 2005; Peñas *et al.* 2017), or even, *D. zenthneri* (2n = 22) (Assis *et al.* 2014), suggesting the same situation for *D. zentneri* subsp. *petr-halfari*. Considering this information, it is probable that the clonal reproduction in *D. zentneri* subsp. *petr-halfari* represents a strategy for the population maintenance in a changeable environment.

In addition, *D. zehntneri* subsp. *petr-halfari* has a low self-pollination potential (4.2 %), which associated with the decrease of pollinators (common in fragmented environments) could lead to an increase in clonal reproduction rates (Rathcke & Jules 1993; Wilcock & Neiland 2002; Martínez-Peralta & Mandujano 2011).

Furthermore, our results revealed that the genetic diversity of this subspecies could be explained by both sexual reproduction and hybridization, since *D. zenhtneri* subsp. *petr-halfari* presents higher diversity rates when compared to other taxa of the genus (Zachar 2008; Conceição 2013). As demonstrated by other studies, species with a hybrid origin tend to have higher levels of genetic diversity due to the high rates of gene flow through new gene recombinations (Lewontin & Birch 1966; Suehs *et al.* 2004; Qian *et al.* 2006; Abbott *et al.* 2013; Chunco 2014). This process can promote the acquisition alleles which might facilitate adaptive evolution (Lewontin & Birch 1966; Abbott *et al.* 2013; Chunco 2014).

Reproductive patterns in *D. zehntneri* subsp. *petr-halfari*

The formation of ramets in *D. zehntneri* subsp. *petr-halfari* appears to occur through rooting, as is the case for other cacti species, such as *Cylindropuntia imbricata*, *Opuntia macrocentra*, *O. rastrera* and *Cylindropuntia leptocaulis* (Allen *et al.* 1991; Mandujano *et al.* 1998; 2007; Flores-Torres & Montaña 2012). However, in extreme climatic conditions and high solar incidences, such as in the Caatinga biome, vegetative propagation by rooting can be highly affected,

as observed for the *Cylindropuntia leptocaulis* cactus (Flores-Torres & Montaña 2012). Though, in more stable environments with periodic rainfall, cacti species with ramet formation present higher rates of asexual reproduction (Flores-Torres & Montaña 2012), which may reveal changes in asexual reproduction rates in different environmental conditions through the year.

Another interesting clonal reproduction strategy reported for Cactaceae involves the formation of clonal propagules, which can be easily dispersed, root and sprout through the stem tissue that surrounds the fruits (Scheinvar 1985; Stevens 2001 onwards, Lenzi *et al*. 2012; Lenzi & Orth 2012). This type of clonal reproduction could also explain, in the current study, the presence of genetically related individuals of both clonal and sexual origin in the same clumps. According to this idea, fruits that originate from clonal propagules can create individuals of sexual origin with their seeds; though, this type of tissue in fruits has never been reported in *D. zehntneri* subsp. *petr-halfari* until the present.

Additionally, the dispersion of cacti seeds has been documented for *Discocactus* and *Melocactus* in favor of ants and lizards, respectively (Fonseca *et al*. 2012; Oliveira 2013; Conceição 2013; Gomes *et al*. 2014). According to some studies, ants can disperse seeds as far as 15.7 meters (Oliveira 2013; Conceição 2013), while lizards usually only carry the fruits at short distance, leaving them just a few meters away from where they were collected (Fonseca *et al*. 2012; Gomes *et al*. 2014). However, this type of dispersal was excludes by Oliveira (2013) for *D. zehntneri* subsp. *boomianus* due to the disposition of the fruits inside the spines, which would make dispersion by vertebrates impossible, according to this author.

The fruits of *D. zehntneri* subsp. *petr-halfari* are situated in the apical portion of the cephalium (above the spines) and are produced one at the time, allowing for dispersion by lizards (Zachar 2008; Braun & Esteves 2008; Santos *et al*. 2015). This type of dispersion could also explain the presence of individuals in different clumps that are genetically related but dissimilar (due to their sexual origin).

Conservation of ***D. zehntneri*** subsp. ***petr-halfari***

Despite the fact that *D. zehntneri* subsp. *petr-halfari* possesses moderate levels of genetic diversity and a large number of exclusive alleles compared to other species of *Discocactus* according Conceição (2013), this subspecies it still faces serious risks of extinction, which is mainly due to the destruction of its habitat by anthropic actions (Santos *et al*. 2015). In this way, actions are essential to preserve and maintain *D. zehntneri* subsp. *petr-halfari* genotypes.

As this taxon has been deemed critically endangered (Santos *et al*. 2015; IUCN 2017), the implementation of specific conservation strategies is needed. While wider strategies occur for the family under the initiative "Plano de Ação Nacional para Conservação das Cactáceas" (PAN Cactáceas) (Assis *et al*. 2011), this subspecies has not been completely covered by this initiative despite the drastic loss of individuals, the increase in anthropic activity in its area of occurrence and the extraction of individuals in sexual maturity (Santos *et al*. 2015).

Considering the drastic habitat loss faced by this subspecies, one of the main conservation strategies should include *in situ* conservation (Santos *et al*. 2015). This action could also protect several populations of *D. bahiensis*, which, despite its high number of individuals and high genetic and morphological diversity, also faces erosion due to anthropic actions (Machado 2004). Previous studies have already suggested creating a conservation unit in this particular area of Caatinga (Machado 2004; Conceição 2013), and it should be considered a priority by government authorities.

Furthermore, it is important to create complementary initiatives, such as *ex situ* conservation (MMA 1992), considering their viability and simplicity to maintain a species (Cohen *et al*. 1991). Some studies have already been carried out for endangered species of cacti examining conservation initiatives such as micropropagation *in vitro* (Marchi 2016; Palacios *et al*. 2016; Civatti *et al*. 2017a; b; Torres-Silva *et al*. 2018), living collections (Hultine *et al*. 2016; Cavalcante *et al*. 2017) and seed storage (Civatti *et al*. 2015; Nascimento & Meiado 2016; Santos *et al*. 2018); these works are crucial to facilitate future studies and to protect the species from extinction.

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