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CROSS-AMPLIFICATION OF MICROSATELLITE LOCI IN THE CACTI SPECIES FROM BRAZILIAN CHACO, AND THEIR APPLICATION FOR POPULATION GENETICS AND PHYLOGEOGRAPHIC STUDIES

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Abstract

We tested the cross-amplification of 50 microsatellite loci (27 nuclear; 23 plastidial) in 16 cacti species. We also tested the transferability of 26 loci (19 nuclear; 7 plastidial) in three populations of *Echinopsis rhodotricha*. Twenty-five nuclear loci amplified at least in 10 species, whereas 16 plastid loci amplified in six species. From the 19 successfully amplified nuclear loci in *E. rhodotricha*, 13 were polymorphic, displaying an average allele number of five. From the seven universal plastid loci, six of them were polymorphic, displaying 37 haplotypes. In *E. rhodotricha*, all populations showed significant deviation from HWE, the inbreeding coefficients were high, and we could not discard the presence of null alleles in severall loci. The cross-amplification rates indicate high potential for comparative studies and might help in further investigations on population genetics of cacti species from Brazilian Chaco, an area threatened by the continuous expansion of deforestation.

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Introduction

The Chaco covers about 840,000 km^2 and is located in northern Argentina, western Paraguay, southeastern Bolivia, and western Brazil (Prado et al. 1992; Prado 1993a; Pennington et al. 2000). The Brazilian Chaco comprises ca. 70,000 km^2 as a narrow strip parallel to the Paraguay River, in Mato Grosso do Sul state (Prado 1993b; Sartori et al. 2018). Trees of the genus *Schinopsis*, *Aspidosperma*, *Tabebuia*, *Vachellia* and *Bulnesia* make up the forests of the Chaco (Pennington et al. 2000). There is also a discontinuous shrub layer and a generally sparse stratum of Bromeliaceae and Cactaceae.

Regarding the Cactaceae, 16 species were reported in the Brazilian Chaco (Gomes & Araujo 2015; Gomes et al. 2018). This family is the fifth most threatened among large taxonomic groups, since one-third of all species are under some extinction risk in decorrence of the human actions (Goettsch et al. 2015). In the Brazilian Chaco, the combined impacts of livestock and forestry practices have led to a highly fragmented landscape and an impoverished ecological system (Bucher & Huszar 1999), threatening the few remaining areas (Pott & Pott 2003). Despite being an area of biological interest, few studies have aimed to understand the genetic patterns of plants (Alves et al. 2018a; Alves et al. 2018b; Godoy et al. 2018), mainly cacti species (Fava et al. 2016). Given the rapid rate of devastation allied with the lack of conservation units, the efforts to understand the biology of the species, specifically regarding the genetic diversity, is paramount as they might serve as a basis for decision-making on the conservation and management of this region.

Here, we tested the cross-amplification of 50 microsatellite loci in all cacti species reported in the Brazilian Chaco. Cross-amplification is a good alternative to avoid expenses in generating new markers, besides being less time-consuming. Furthermore we tested the transferability of 26 loci (19 nuclear and 7 plastidial) in three populations of *Echinopsis rhodotricha*. We believe that this study will help in further investigations concerned with the relationship between geographic isolation and population genetic differentiation of this species and guide studies addressing other cactus species occurring in the Brazilian Chaco.

Material and Methods

We sampled root tip fragments of 16 cacti species from Brazilian Chaco, and stored them in ethanol 70%. We extracted total DNA following the CTAB protocol described by Fava et al. (2016). We tested the cross-amplification of 50 microsatellite (simple sequence repeat, SSR) loci (27 nuclear and 23 plastidial). We used nuclear SSR loci developed for *Echinopsis chiloensis* (Ossa et al. 2016) and *E. rhodotricha* (Fava et al. 2016). For the plastid SSR, we used 10 loci developed for *Pinus* spp. (Vendramin et al. 1996) and 13 universal chloroplast SSR loci (Weising & Gardner 1999). We performed all PCR amplifications following the protocols described by Fava et al. (2016). We analyzed the amplification products by electrophoresis on 2% agarose gel stained with GelRed, considering the loci successfully amplified when we clearly visualized a band of the expected size.

To assess the polymorphism levels, we selected 48 individuals of *E. rhodotricha* from three populations (16 per population). For each successfully amplified microsatellite marker (29 loci, 13 nuclear and 6 plastidial) we synthesized the forward primers with a 19-bp M13 tail, following Schuelke's (2000) method. We performed all PCR amplifications and genotyping as described by Fava et al. (2016). Microsatellite allele genotyping was performed by Macrogen (Seoul, South Korea), using an ABI 3730XL Genetic Analyzer (Applied Biosystems). We determined the fragment size with GENEMARKER v1.95 software and GS500 LIZ as the molecular size standard (Applied Biosystems).

Nuclear SSR data analyses: For each population and locus, we estimated the number of alleles, observed and expected heterozygosities according to the Hardy–Weinberg equilibrium (HWE), using MSA 4.00 software (Dieringer & Schlotterer 2003). We accessed the within-population inbreeding coefficient, and genotypic disequilibrium between all pairs of loci in each populationusing FSTAT (Goudet 1995). We evaluated deviations from HWE using exact tests, as implemented in GENEPOP on the Web (Raymond & Rousset 1995). We calculated the frequency of null alleles following Brookfield (1996) for each population using Micro-Checker version 2.2.3 (Van Oosterhout et al. 2004).

Plastidial SSR data analyses: We used the fragment sizes obtained from the amplification of plastid DNA to determine haplotypes found in all individuals. We characterized the genetic diversity of each population based on the number of haplotypes found, the genetic diversity (Nei 1978) and allelic richness (El Mousadik & Petit 1996), which were estimated using Contrib 1.02 software (Petit et al. 1998). We performed the AMOVA in Arlequin 3.5 software to assess the distribution of intraspecific genetic variation.

Results and Discussion

Nuclear Genetic Diversity: From the 27 nuclear SSR primers tested in this study, all of them amplified, but the success rate ranged from five to 14 species, and on average, each primer could be amplified in 11 species. Considering the proportion of amplification success among species, 13 of the 16 species had at least 18 amplified primers, showing that the heterologous amplification from *Echinopsis* spp. markers is a great starting point for the genetic studies with cacti species from Brazilian Chaco.

From the 19 successfully amplified loci in *Echinopsis rhodotricha*, 13 of them were polymorphic, displaying an average allele number of 5, ranging from 1 to 14 (Table 1). Among the 13 analyzed loci, eight loci in Population 1, six in Population 2 and eight in Population 3 showed significant deviation from HWE due to heterozygous deficiency. All populations showed significant deviation from HWE due to heterozygous deficiency, and the inbreeding coefficients were high in the three populations. The excess of homozygous found in this study might be due to inbreeding and/or Wahlund effect, although we could not discard the presence of null alleles in several loci. Significant linkage disequilibrium was found for two loci.

Genetic Diversity of Plastid Microsatellites: From the 23 plastid SSR primers, three of them failed to show amplification products, and the success rate of the 20 remained primers ranged from two to 14 species, and on average, each primer could be amplified in 7 species. The proportion of amplification success among species was lower than the nuclear SSR primers: only six of the 16 species had at least 16 amplified primers.

We found 37 haplotypes in three populations of *E. rhodotricha* (Pop 1 = 13 haplotypes; Pop 2 and Pop 3 = 14 haplotypes each). Populations showed high levels of genetic diversity that ranged from 0.967 to 0.983. Allelic richness ranged from 6.167 to 6.533 (Table 1). From the 37 haplotypes, none of then were shared among all populations. Only four haplotypes were shared between Populations 1 and 2. All haplotypes from Population 3 were exclusive. AMOVA showed that the lowest proportion of genetic variation (20.43%) could be attributed to variation among populations; most variation occurs within populations (79.57%).

Most of the species (13) that occur in the Brazilian Chaco belong to the subfamily Cactoideae and, except for the species in which only one (*Hylocereus setaceus*) or no nuclear microsatellite locus was amplified (*Rhipsalis baccifera* and *Praecereus saxicola*), the other 10 species had on average 24 amplified loci. Although microsatellites may be limited by the need to develop primers for each species, primers from closely related species will often work (Frankham et al. 2004; Barbará et al. 2007; Fava et al. 2016). However, even more phylogenetically distant species, e.g. *Frailea* spp., *Opuntia* spp. and *Pereskia sacharosa*, have shown similar amplification success to those species which are closely related to *Echinopsis*, e.g. (*Cereus bicolor*, *Echinopsis rhodotricha*, *Harrisia balansae*, *Harrisia tortuosa* and *Stetsonia coryne*). Thus, in face to the rapid environmental degradation that the Chaco is subject to (Silva et al. 2011), we highlight that the lack of genetic studies of the cacti species can be overcome by using the markers tested here. Such markers facilitate comparisons among multiple co-occurring species for studying how patterns of diversity at genetic and community levels interact (Noor & Feder 2006; Whitham et al. 2006; Barbará et al. 2007).

Conclusion

Although we have been able to amplify universal plastid markers in the three subfamilies of cacti occurring in the Brazilian Chaco, not only the amplification rate was lower, but the number of polymorphic loci was also inferior to the nuclear loci. Therefore, the use of universal microsatellite markers, although they may overcome the limitations of specificity, for cacti species in the Chaco would not be a viable alternative, causing the development of specific primers, or the use of primers from more related species (or at least of the same family) is required.

In the last 10 years (2008-2018), at least 44 studies using microsatellite markers were published with Cactaceae species, and the main genera studied were *Pilosocereus*, *Opuntia* and *Mammillaria*. Of all these

studies, 43% (19) used the cross-amplification method in their analysis, which evidences the increasing use of this technique as an alternative way for genetic studies without the necessity of the development of new molecular markers that are species-specific. However, of the cacti species that occur in the Brazilian Chaco, only five have representatives of the same genus with previously described microsatellite loci (*Cereus bicolor*, *Echinopsis rhodotricha*, *Hylocereus setaceus*, *Opuntia elata* and *Opuntia retrorsa*). It is worth noting that the two species that are listed in the IUCN Red List of Threatened Species as near threatened (*Frailea cataphracta*) and vulnerable (*Frailea schilinzkyana*) do not have congeneric representatives with described primers (Gomes et al. 2018). Thus, considering the heterologous amplification success of the nuclear microsatellite loci we find, our study represents a starting point for future studies of the species of *Frailea* in the Brazilian Chaco.

Table 1. Genetic diversity in populations of *Echinopsis rhodotricha*, including the number of alleles (A), allelic richness (R_S), observed heterozygosity (H_O), expected heterozygosity (H_E) and inbreeding coefficient (F_{IS}) for nuclear microsatellites and the number of haplotypes (NH) gene diversity (H_E with Standard Error) and allelic richness for plastid microsatellites.

Population	*Nuclear SSR*					*Plastid SSR*		
	A	R_S	H_O	H_E	F_{IS}	NH	H_E(SE)	Allelic richness
Pop 1	69	2.530	0.299	0.663	0.577*	13	0.967 (0.036)	6.167
Pop 2	62	2.300	0.271	0.568	0.544*	14	0.983 (0.028)	6.533
Pop 3	71	2.576	0.328	0.656	0.517*	14	0.983 (0.028)	6.533

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