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Development and characterization of microsatellite markers for *Echinopsis rhodotricha* and cross-amplification in other species of Cactaceae

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ABSTRACT

Located in central South America, the Chaco is a large subtropical dry forest characterized by plants with xeromorphic features, including numerous cacti. In the Brazilian Chaco at the northeasternmost part of the Chaquenian region, *Echinopsis rhodotricha* (Cactaceae, Trichocereeae) is one of the species threatened by intense deforestation caused by expanding pastureland. This study characterizes the microsatellite loci isolated from *E. rhodotricha* and cross-amplification in thirteen other cactus species. Twelve microsatellite loci were developed from an enriched genomic library. Eight of these were polymorphic and characterized in 48 individuals from three *E. rhodotricha* populations. The loci showed a mean of 3.2 alleles per locus and overall levels of expected and observed heterozygosities ranging from 0.00 to 0.83 and 0.00 to 0.66, respectively. Five loci showed significant departures from the Hardy–Weinberg equilibrium and also exhibited signs of null alleles. Cross-amplification in other Cactaceae species was successful, ranging from one (*Ferocactus latispinus*, Cacteae and *Harrisia adscendens*, Trichocereeae) to twelve loci (*Echinopsis calochlora*, Trichocereeae). The development of these microsatellite markers will contribute to investigations of population structure, genetic diversity, and gene flow in *E. rhodotricha* populations, as well as in other cactus species, providing information useful for the creation and delimitation of conservation areas in the Brazilian Chaco region.

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1. Introduction

The Chaco, or "Gran Chaco," is a subtropical dry forest that covers a wide plain (ca. 800,000 km^2) in Argentina, Bolivia, Paraguay and Brazil. The Brazilian Chaco (Fig. S1) is restricted to a narrow strip (ca. 6700 km^2 of natural vegetation — Stepic Savanna, ZEE-MS, 2015) parallel to the Paraguay River (Prado, 1993). It occupies a transitional area between the Pantanal wetlands and the Brazilian Savanna (Cerrado). Its vegetation is usually associated with saline soil and includes a great diversity of Leguminosae, Bromeliaceae and Cactaceae species (Pennington et al., 2000; Silva et al., 2000).

Cactaceae are represented by 260 species in Brazil, out of which only twelve are found in the Chaco area (Eggli, 2002; Freitas et al., 2013; Zappi et al., 2015). Studies concerning floristic, physiological, taxonomic and ecological aspects of cacti in the Brazilian Chaco are scarce (Gomes and Araujo, 2015), and there are no studies evaluating its populational genetic diversity. The *Echinopsis* Zucc. (Trichocereeae) genus comprises 128 species that vary from large tree-like to small globose plants (Anderson, 2001) and is one of the least understood groups of cacti. *Echinopsis rhodotricha* K. Schum. occurs in northern Argentina, Paraguay, Uruguay, as well as Brazilian Chaco formations (Anderson, 2001; Eggli, 2002), and have funnel-shaped white flowers that provide pollen and nectar as floral rewards, open at night and remain open until the next morning (Gomes and Araujo, 2015). The reproductive system of this species is still unknown.

In Brazil, populations of *E. rhodotricha* have been threatened by intensive deforestation caused by expanding pastureland (Silva et al., 2011), thus threatening the few remaining areas of Chaco. Despite being considered a high priority region for conservation (Tálamo and Caziani, 2003), no conservation units are found in the Brazilian Chaco. This can be attributed to the lack of basic information about the total diversity of species, as well as details of their geographical distribution and aspects of their taxonomy, ecology and genetics.

Molecular markers have been revealing patterns of gene flow and population structure in a wide variety of species. This has resulted in uncovering genetic data applicable to such conservation purposes as definition of management units and the detection of inbreeding signals (Frankham et al., 2004). Simple sequence repeat markers (SSRs), commonly termed microsatellite markers, are tandem repeats of short DNA motifs, typically 1–6 bases in length. Such microsatellites are highly variable, and by allowing individual genotyping through noninvasive sampling, they have advantages over other molecular markers that measure genetic diversity. Although microsatellites can 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).

The use of SSR markers in genetic variation studies of Cactaceae species is relatively restricted. In a survey of the Web of Science® (Institute of Scientific Information, Thomson Scientific) using the keywords 'Cactaceae' and 'microsatellite', we found only 29 studies (Table S1) that characterized new microsatellite markers (65%) and/or described the genetic diversity of cactus species using cross-amplification loci (35%). Considering all studies, only five were developed in Brazil, mainly with the *Pilosocereus* genus and using heterologous microsatellite markers (Table S1). This represents ca. 0.02% of all Brazilian species. Therefore, to assess the genetic diversity of a typical chaquenian species, we described twelve new microsatellite loci for *E. rhodotricha* and their cross-amplification in thirteen species.

2. Materials and methods

To identify and characterize microsatellites, genomic DNA was extracted from root tip fragments of one *E. rhodotricha* individual (−21.68S; −57.77W), following the CTAB protocol described in Roy et al. (1992) with modification (see Protocol S1, Supplementary Material). A microsatellite-enriched library was then constructed according to Billote et al. (1999) with modifications described in Zanella et al. (2012). Sixty positive clones were bidirectionally sequenced with T7 and SP6 primers in an ABI 377 automated sequencer (Perkin–Elmer, Applied Biosystems) using the BigDye® Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems). Sequences were aligned and edited using Chromas (Technelysium) and Chromatogram Explorer (Heracle Biosoft). WebSat software (Martins et al., 2009) was used to design 20 primer pairs.

The amplifications were performed in a Veriti Thermal Cycler (Applied Biosystems) in 10 μL reactions containing ca. 10 ng of DNA template, 1× Taq buffer, 2 mM $MgCl_2$, 0.2 mM of dNTP set, 4 pmol forward primer, 4 pmol reverse primer, and 0.5 U GoTaq® Flexi DNA Polymerase (Promega, Madison, WI, USA). A touchdown cycling was used: 95 °C for 3 min, 10 cycles of 94 °C for 30 s, 58 °C decreasing to 48 °C at 1 °C per cycle for 30 s, 72 °C for 30 s, followed by 30 cycles of 94 °C for 30 s, 48 °C for 30 s, and 72 °C for 30 s, concluding with a 10 min extension at 72 °C. Amplification products were verified by electrophoresis in 2% agarose gel (Promega, Fitchburg, WI, USA) stained with GelRed™ (Biotium, Hayward, CA, USA) and visualized under ultraviolet light. A locus was considered successfully amplified when one band of the expected size was visualized. A 100-bp DNA Ladder (Promega, Fitchburg, WI, USA) was used as a molecular size marker.

To assess the polymorphism levels, 48 individuals from three *E. rhodotricha* populations (16 per population) were genotyped. All of them were collected in patches of wooded Chaco located in Mato Grosso do Sul State, Brazil. For each designed microsatellite marker, the forward primers were synthesized with a 19-bp M13 tail (5′- CAC GAC GTT GTA AAA CGA C -3′), following the method of Schuelke (2000).

All PCR amplifications were performed in a Veriti Thermal Cycler (Applied Biosystems) in 10 μL reactions following the protocol described in Faggioni et al. (2014), containing ca. 10 ng of DNA template, 1× Taq buffer, 1.5 mM $MgCl_2$, 0.4 mM dNTP set, 0.1 μM forward primer, 0.1 μM reverse primer, 0.3 μM universal fluorescent M13 primer (6-FAM, NED, PET or VIC), and 0.25 U GoTaq® Flexi DNA Polymerase (Promega, Fitchburg, WI, USA). We used the same touchdown cycling program described above.