

## PERMANENT GENETIC RESOURCES

# Characterization of microsatellite loci for the critically endangered cactus *Ariocarpus bravoanus*

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## Abstract

*Ariocarpus bravoanus* is common in trade but critically endangered in its natural habitat. With the ultimate aim of developing a certification scheme to aid in the conservation of this species, we have isolated *A. bravoanus* microsatellites from a nonenriched library. Fifty-four sequences contained a microsatellite array, of which eight were polymorphic among 23 individuals, 20 from one population and three plants from trade.

**Keywords:** *Ariocarpus*, Cactaceae, conservation, microsatellites, polymorphism, SSR

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*Ariocarpus bravoanus* (Cactaceae) was discovered in 1992 and is only known from a small area within the state of San Luis Potosí, Mexico (Hernández & Anderson 1992). It became one of the most sought-after of Mexican plants, and despite Mexican law to prevent export of the species, the site has been plundered and many plants seen on the international market.

Microsatellite markers have recently been developed in the family Cactaceae (Otero-Arnaiz *et al.* 2004; Otero-Arnaiz *et al.* 2005; Terry *et al.* 2006; Hardesty *et al.* 2008). However, microsatellite markers have not been described for any *Ariocarpus* species. Here, we characterize eight polymorphic microsatellite loci for *A. bravoanus*.

To construct the genomic library, DNA was extracted from an *A. bravoanus* individual using a cetyltrimethyl ammonium bromide (CTAB)-based method (Doyle & Doyle 1987). Tissue was collected and dried in silica before DNA extraction and stored at ambient temperatures. A nonenriched genomic library was constructed following the protocol described by Croxford *et al.* (2006) and Hardesty *et al.* (2008). We digested DNA with *Sau3AI* (Promega) and *HindIII* (Gibco-BRL) enzymes which were fractionated by electrophoresis on a 1% w/v agarose gel. Fragments of ~300–1000 bp in length were excised,

extracted and purified using NucleoSpin Extract column protocol following manufacturer's instructions (Machery-Nagel). DNA fragments were ligated into pGEM-3Z vector (Promega) cut with *HindIII* and *BamHI* (Promega). Positive bacterial clones, containing a microsatellite of more than five repeat units, were identified as described by Croxford *et al.* (2006). This method entails the transformation of the vector into ultracompetent XL1-Blue *Escherichia coli* cells (Stratagene) and the use of a Qpix2 Robotic Colony Picker (Genetix) to select and transfer transformed colonies to 384-well plates containing 60 µL Luria-Bertani/ampicillin broth.

Hybridization probing of the library was carried out as described by Armour *et al.* (1994) using two repeat probe sequences: AG<sub>15</sub> and AAG<sub>8</sub>. The 54 positive bacterial clones identified were sequenced using the M13 forward primer with BigDye Terminators on a 3730xl DNA Analyzer (Applied Biosystems). Of the 54 positive clones, 51 had sufficient flanking sequence for primer design using the PRIMER3 software (Rozen & Skaletsky 2000).

The potential variability of the 51 microsatellite loci was assessed on a pooled sample of 10 individuals using the ligated-pooling strategy described by Cryer *et al.* (2005). DNA was extracted from each individual using the QIAGEN DNeasy Plant Mini Kit and DNA samples were pooled together to a final concentration of 5 ng/µL. PCRs were conducted in 10-µL mixtures comprising 1× PCR

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**Table 1** *Ariocarpus bravoanus* microsatellite loci showing name of locus with fluorescent labels used

| Locus   | EMBL Accession no. | Primer sequence (5'–3')                               | Repeat motif                                                  | $T_a$ (°C) | Population sample and traded plants |              |         | Population sample only |       |       |           |
|---------|--------------------|-------------------------------------------------------|---------------------------------------------------------------|------------|-------------------------------------|--------------|---------|------------------------|-------|-------|-----------|
|         |                    |                                                       |                                                               |            | $I_{apop}$                          | $I_{atrade}$ | ASR     | $N_a$                  | $H_O$ | $H_E$ | $P$ value |
| mAbR05* | EU400588           | F: AATAGGGACAGTGAGGGATT<br>R: TCTTCTCATGAGCCTTGTCG    | (AG) <sub>9</sub>                                             | 53.8       | 20                                  | 3            | 293–300 | 5                      | 0.350 | 0.347 | 0.585     |
| mAbR28* | EU400589           | F: CCATAAGCTGTGGTGGGTCT<br>R: ATTTTAAAGCTCCCCCTCCA    | (AG) <sub>7</sub>                                             | 53.8       | 20                                  | 2            | 207–222 | 6                      | 0.550 | 0.612 | 0.064     |
| mAbR40* | EU400590           | F: TTTAATGCGGAGGAAGTTGG<br>R: GCTCAAAGTCTCTCCGTTAATGA | (TCT) <sub>2</sub> (CT) <sub>3</sub><br>TT(CT) <sub>10</sub>  | 51.4       | 19                                  | 3            | 238–301 | 5                      | 0.737 | 0.575 | 0.005     |
| mAbR42† | EU400591           | F: GGGCAATTCACTATGCACAA<br>R: TTGTCCACCTTCCCTATTG     | (TC) <sub>10</sub>                                            | 53.8       | 19                                  | 2            | 196–217 | 6                      | 0.526 | 0.546 | 0.121     |
| mAbR77† | EU400592           | F: CGGGGAAGGAATAATCCAAG<br>R: ATGTGCCGTTTGCAATCTTT    | (AG) <sub>4</sub> CG(AG) <sub>11</sub>                        | 51.4       | 19                                  | 3            | 346–374 | 4                      | 1.000 | 0.688 | 0.000     |
| mAbR82† | EU400593           | F: TTCCATCTGGAGTTTATGGG<br>R: GAAAACATTTCAAGTGCAACACA | (AG) <sub>20</sub>                                            | 51.4       | 20                                  | 3            | 245–259 | 5                      | 0.750 | 0.617 | 0.045     |
| mAbR86* | EU400594           | F: TTGCAACTTGGGCATCAGTA<br>R: GGCAGCTCAACTCCAGAGAC    | (TC) <sub>11</sub> (TG) <sub>2</sub>                          | 53.8       | 19                                  | 3            | 432–452 | 8                      | 0.211 | 0.201 | 1.000     |
| mAbR91† | EU400595           | F: CGACATCAAGTTGCACCATC<br>R: TCAGGAGTTGCTTGATGGAA    | (CT) <sub>8</sub> CGC(TC) <sub>8</sub><br>GC(TC) <sub>4</sub> | 53.8       | 19                                  | 3            | 329–360 | 6                      | 0.474 | 0.508 | 0.746     |

\*FAM; †HEX; European Molecular Biology Laboratory (EMBL) Accession number; sequences of forward (F) and reverse (R) primers; repeat motif and melting temperature ( $T_a$ ), the number of individuals successfully amplified from the population sample and from trade ( $I_{apop}$  and  $I_{atrade}$  respectively); allelic size range (ASR) and number of alleles detected ( $N_a$ ). Observed heterozygosities ( $H_O$ ); expected heterozygosities ( $H_E$ ) and  $P$  values are calculated for the field-collected population sample; data from traded plants was excluded from the calculations.

buffer (Bioline, 160 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 670 mM Tris-Cl (pH 8.8), 0.1% Tween 20), 0.3 mM MgCl<sub>2</sub>, 0.4 mM dNTPs, 0.5 µM of each microsatellite primer pair and 1 U of *Taq* DNA polymerase (Bioline). PCRs were performed on a Whatman T-gradient thermal cycler (Biometra) using an initial 94 °C denaturing step for 1 min followed by 35 cycles at 94 °C for 1 min, 1-min annealing temp (see Table 1 for optimized temperatures) and 72-°C extension for 1 min, then a final extension step at 72 °C for 7 min. FAM-labelled M13 universal forward primers were used together with the locus-specific microsatellite reverse primer to amplify each locus from the template pool. Amplicons were separated on an ABI PRISM 3100 capillary sequencer and assessed against a ROX-labelled standard (Applied Biosystems) to determine the number of alleles in the pooled sample using GENEMAPPER (Applied Biosystems).

Eight microsatellite markers amplified two or more alleles in the pooled sample. These were characterized on 20 individuals from a population in central San Luis Potosí, Mexico (Voucher specimen as follows: *R. T. Bárcenas 1610bis*. San Luis Potosí, Mpio. Guadalcázar. 15 de junio del 2006. Lodged in the Universidad Nacional Autónoma de México (MEXU) and the Universidad Autónoma de Querétaro (QMEX) herbaria) and three additional individuals purchased in trade. Traded plants are believed to originate from the type locality of the species; collecting at this site has resulted in severe depletion of plant numbers. DNA

was extracted from each individual using the QIAGEN DNeasy plant mini kit. Where possible, microsatellite loci were multiplexed according to expected amplicon size and annealing temperature (Table 1). Multiplex PCRs were conducted in 10-µL reactions comprising 2× QIAGEN multiplex PCR master mix, 40 ng of DNA template and 10× primer mix (containing a final concentration of 2.0 µM of each primer). PCRs were performed on a Whatman T-gradient thermal cycler (Biometra) using an initial 95 °C denaturing step for 15 min followed by 30 cycles at 94 °C for 30 s, 90-s annealing temp (see Table 1 for optimized temperatures) and 72-°C extension for 1 min, then a final extension step at 60 °C for 30 min. Amplicons were separated on an ABI PRISM 3100 capillary sequencer and assessed against a ROX 500-labelled standard using GENEMAPPER (Applied Biosystems). At least 20 individuals were scored for each primer pair.

Observed and expected heterozygosities and deviations from Hardy–Weinberg equilibrium were assessed for the San Luis Potosí population using CERVUS (Kalinowski *et al.* 2007) and GENEPOP version 3.4 (Raymond & Rousset 1995), respectively. Three loci, mAbR40, mAbR77 and mAbR82 deviated from Hardy–Weinberg equilibrium ( $P < 0.05$ , Table 1). Linkage disequilibrium was assessed for both populations using GENEPOP version 3.4 (Raymond & Rousset 1995). No significant linkage disequilibrium was shown between the pairs of loci indicating the independent behaviour of all loci.

These markers will be useful to describe the genetic structure of *A. bravoanus* populations and to develop a certification scheme to aid in the conservation of wild genotypes by making certified, sustainably managed sources of cacti available for purchase.

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