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Polymorphic microsatellite markers for the rare and endangered cactus *Uebelmannia pectinifera* (Cactaceae) and its congeneric species

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ABSTRACT. The cactus genus *Uebelmannia* includes 3 narrow endemic species associated with rocky savanna habitats in eastern South America. Because of their rarity and illegal over-collection, all of these species are endangered. Taxonomic uncertainties resulting from dramatic local variation in morphology within *Uebelmannia* species preclude effective conservation efforts, such as the reintroduction or translocation of plants, to restore declining populations. In this study, we developed and characterized 18 perfect, dinucleotide simple-sequence repeat markers for *U. pectinifera*, the most widely distributed species in the genus, and tested the cross-amplification of these markers in the remaining congeneric species and subspecies. All markers were polymorphic in a sample from 2 *U. pectinifera* populations. The effective number of alleles ranged from 1.6 to 8.7, with an average per population of 3.3 (SE ± 0.30) and 4.5 (SE ± 0.50). Expected heterozygosity ranged

from 0.375 to 0.847 and 8-10 loci showed departures from Hardy-Weinberg equilibrium in the analyzed populations. Based on the observed polymorphism level of each marker, as well as the analysis of null allele presence and evidence of amplification of duplicate loci, a subset of 12 loci can be used as reliable markers to investigate the genetic structure, diversity, and species limits of the *Uebelmannia* genus.

Key words: Endangered species; Perfect microsatellite markers; Cactaceae; Rare plants

INTRODUCTION

The neotropical cactus genus *Uebelmannia* includes 3 narrow endemic species associated with rocky savanna habitats extending across an area of approximately 8000 km^2 in eastern South America. All *Uebelmannia* species are categorized as critically endangered or endangered (IUCN, 2013), and since 1992, have been listed in Appendix I of Convention on International Trade and Endangered Species as a direct consequence of their rarity and illegal overcollection, among other threats (Taylor and Zappi, 2004). *Uebelmannia buiningii* Donald consists of a single population on the verge of extinction that is restricted to an unprotected area of approximately 40 km^2 (Schulz and Machado, 2000). *Uebelmannia gummifera* Donald includes 2 subspecies [the nominate form and subsp *meninensis* (Buining) Braun & Esteves] in an area of ~230 km^2, where only some populations of *U. gummifera* subsp *meninensis* are within a conservation unit. *Uebelmannia pectinifera* has the broadest distribution in the genus, present in a mostly unprotected area of ~3000 km^2, and includes 3 subspecies: the nominate form, subsp *flavispina* (Buining & Brederoo) Braun & Esteves, and subsp. *horrida* (Braun) Braun & Esteves. Currently, only populations of *U. pectinifera* subsp *pectinifera* and *U. pectinifera* subsp *horrida* are protected in conservation units.

In addition to threats faced by *Uebelmannia* species, the phylogenetic distinctiveness of *Uebelmannia* is an evolutionary component that increases the need to conserve these taxa (Winter et al., 2013). *Uebelmannia* is morphologically similar to members of the tribe Notocacteae from southern South America, such as in the globose to short columnar habit and bee-pollinated yellow flowers. However, the genus is not phylogenetically closely related to Notocacteae but rather is a sister group to a clade including the tribes Browningeae, Cereeae, and Trichocereeae (Crozier, 2004; Ritz et al., 2007; Hernández-Hernández et al., 2011), which includes several South American columnar or arborescent species. Thus, the phylogenetic position of *Uebelmannia* suggests that it is a relictual lineage, representing an early diverging branch from its nearest relatives.

Uebelmannia species exhibit substantial local variation in morphology, considerably complicating species circumscriptions and taxonomic arrangements (Schulz and Machado, 2000). Furthermore, taxonomic uncertainties within *Uebelmannia* prevent effective conservation efforts such as the reintroduction or translocation of plants such as the restoration of declining populations. Whether variation in morphological traits of the *Uebelmannia* population reflects local adaptations or the presence of non-recognized species, introduction of foreign individuals to increase local populations may lead to harmful genetic effects, such as outbreeding depression and hybridization (Edmands, 2007). Thus, genetic studies aimed at understanding the genetic structure and species delimitation of *Uebelmannia* species are es-

sential for planning conservation efforts for these taxa. However, such studies are dependent upon the availability of suitable molecular markers, such as microsatellite (simple-sequence repeats, SSR) markers, of the target taxa. Therefore, we developed and characterized 18 SSR markers for *U. pectinifera* subsp *pectinifera*, the most widely distributed subspecies in the genus, and tested their effectiveness for amplifying homologous products in the remaining congeneric species and subspecies. These markers will be useful for investigating the population structure and taxa delimitation of the endangered genus *Uebelmannia*.

MATERIAL AND METHODS

To isolate SSR markers and validate polymorphisms, we used gel-dried root tissue of individuals from 2 populations of *U. pectinifera* subsp *pectinifera* sampled at 2 municipalities in Diamantina, Minas Gerais, Brazil, Inhai (18°02'S, 43°33'W; N = 22) and Mendanha (18°07'S, 43°31'W; N = 20). We also used root tissue from 2 individuals of each remaining species and subspecies of this genus for cross-species amplification tests: *U. pectinifera* subsp flavispina (18°17'S, 43°44'W and 18°11'S, 43°43'W), *U. pectinifera* subsp *horrida* (17°27'S, 43°47'W and 17°30'S, 43°45'W), *U. buiningii* (18°00'S, 42°55'W), *U. gummifera* subsp *gummifera* (18°01'S, 42°57'W), and *U. gummifera* subsp *meninensis* (18°08'S, 43°02'W and 18°05'S, 43°04'W). Total genomic DNA from each individual was isolated using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). DNA quality was evaluated on 1.5% agarose gels, and DNA concentrations were measured using a NanoVue spectrophotometer (GE Healthcare, Little Chalfont, UK).

An SSR-enriched library from the genome of an individual of *U. pectinifera* subsp. *pectinifera* was obtained using protocols adapted from Billotte et al. (1999). Briefly, approximately 2 µg genomic DNA was digested with *Rsa*I enzyme, and the resulting fragments were ligated to the *Rsa* 21 (5'-CTCTTGCTTACGCGTGGACTA-3') and *Rsa* 25 (5'-TAGTCCACG CGTAAGCAAGAGCACA-3') adapters. Enrichment was performed by hybridization-based capture using $(TC)_{10}$ and $(CA)_{10}$ biotin-linked motifs and streptavidin magnetic-coated beads (Promega, Madison, WI, USA). SSR-enriched fragments were polymerase chain reaction (PCR)-amplified using the *Rsa* 21 primer and then cloned into plasmids using the TOPO TA cloning kit (Life Technologies, Carlsbad, CA, USA). A total of 250 positive clones, as determined by blue/white β-galactosidase selection, were grown overnight with ampicillin and then tested by PCR to confirm the presence of inserts. A total of 192 clones selected were purified and bidirectionally sequenced in an ABI PRISM 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using universal M13F and M13R primers. The sequences were assembled and edited with the ChromasPro v. 1.5 software (Technelysium Pty., Ltd., South Brisbane, Australia). The Microsat software (A.M. Riesterucci, CIRAD, pers. com.) was used to eliminate adaptors and restriction sites from the sequences.

The trimmed sequences were screened using the SSRIT software (Temnykh et al., 2001) to identify all possible perfect SSR di-, tri-, and tetranucleotide repeats (i.e., unique repeats uninterrupted by other bases) with a minimum of 8, 5, and 3 subunits, respectively. We prioritized perfect repeats because this type of SSR loci is expected to be more polymorphic (Ellegren, 2004) and exhibit less allele size homoplasy (Estoup et al., 2002) than degenerate loci. A total of 53 sequences contained di- (49), tri- (2), or tetranucleotide (2) SSR motifs. After removing loci with imperfect repeats and with insufficiently long sequences flanking the SSR motifs, we designed primer pairs for 23 SSR loci using the Primer3Plus software (Untergasser et al., 2007) according to the following criteria: minimum annealing temperature

(Ta) of 46°C, maximum Ta difference of 3°C between primers, GC content ranging from 40 to 60%, and PCR product size ranging from 100 to 300 base pairs.

PCRs were performed in a 20-µL volume containing ~20 ng genomic DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 µM of each primer, 0.16 mM dNTPs, and 1 U *Taq* DNA polymerase (Promega). All primer pairs were initially evaluated in a touchdown program described by Laborda et al. (2009), which consisted of an initial denaturing step of 2 min at 94°C; 2X [10 cycles of 94°C for 1 min, 65°C (-1°C/cycle) for 1 min, and 72°C for 2 min]; 18 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C; and a final extension of 5 min at 72°C. When it was necessary to set a specific annealing temperature for the primer pair, PCR was performed in a gradient temperature program consisting of an initial denaturing step at 94°C for 2 min, 30 cycles at 94°C for 1 min, 54°-62°C for 1 min (optimized annealing temperatures in Table 1), and 72°C for 2 min, with a final elongation step at 72°C for 5 min. Amplification products were visualized on 3% agarose gels and then separated on a Fragment Analyzer Automated CE System (Advanced Analytical Technologies, Ames, IA, USA) using the 35-500-bp dsDNA Reagent Kit (Advanced Analytical Technologies). Allele scoring was carried out using the PROSize v. 2.0 software, available from the same company.

Table 1. Characterization of 18 polymorphic SSR loci for *Uebelmannia pectinifera* subsp *pectinifera*. For each locus, the repeat motifs, forward (F) and reverse (R) primer sequences, optimized annealing temperatures (Ta), size ranges of PCR products, and GenBank accession numbers are shown.

Locus	Repeat motif	Primer sequence (5'-3')	Ta (°C)	Size (bp)	GenBank
Upec112	$(TG)_7$	F: GGTGAACTTGAGGGTGATAG R: CAAGGCTAGTCAATGCTTTG	57	112-120	KJ617063
Upec123	$(CT)_{16}$	F: GAATGAGGACCCTAATAGCC R: TAATGGCGGATGAGGATAG	62	109-134	KJ617064
Upec126	$(CT)_{17}$	F: GGATGATACTTCCCTCCTTC R: AGTTTATAGCTCCAAGGTGG	55	119-139	KJ617065
Upec132	$(GA)_{12}$	F: CTTGGTATGGCAAGGATAGG R: AGTCGATCCATTAAGCGATG	56	130-134	KJ617066
Upec138	$(CT)_{19}$	F: AGACTTCTAGCTACTCGGAG R: GTGTAGATAAGGACGGTGAG	54	119-149	KJ617067
Upec148	$(AG)_{22}$	F: CTTAAGATGGAAAGAGAGATGC R: TCTTCTTCGCTAAACCTCAC	TD	120-138	KJ617068
Upec153	$(AG)_{15}$	F: TGGGCTCTGAAGAAAAGTAG R: ACCATCTTTTCCCTCTCTT	TD	131-151	KJ617069
Upec182	$(AC)_7$	F: GCAATCCAACCTAGAACAAG R: AATGAATCCTTCACAGTTGG	59	160-180	KJ617070
Upec196	$(AG)_{11}$	F: GACTTTAGCTGCGAGAAAAC R: TCCAGGATTTGTAGCTGAAG	55	166-196	KJ617071
Upec201	$(GA)_{23}$	F: AACAGAACTGAGCCACAATC R: GGGCATTTTGGAAACTATGC	57	174-190	KJ617072
Upec214	$(CT)_{21}$	F: AACCATCACGTCCCGTAC R: ATTTTCGGATGTGAAGCAAC	57	176-210	KJ617073
Upec221	$(TC)_{22}$	F: ATACCTCCGTCATGGCTAC R: TCAGGGCTTACCACCTTC	59	193-215	KJ617074
Upec223	$(TC)_9$	F: GTCAAATTTCCAAGTGAACC R: CAGAAGAGAAACCCTAATCG	TD	213-231	KJ617075
Upec226	$(AG)_{17}$	F: CCCTTAGGACCGAGATTAGT R: GTGTTCTTCCAAACTCAAGG	TD	213-231	KJ617076
Upec235	$(TC)_{14}$	F: TGTCCAATTAGGAATTCAGG R: AGTGGGTCTTAGAGGTTTGC	58	225-251	KJ617077
Upec241	$(CT)_{16}$	F: GAACAACTTTCTTGCAAAGG R: AGTAGTGCTGAAAGGTGGTG	56	230-262	KJ617078
Upec242	$(AG)_{24}$	F: GTTCACACACTTGGGAAATC R: TAAAAGACCAGCCACTTCTC	56	216-240	KJ617079
Upec267	$(AC)_7$	F: GACAATGACGACTCCAAAAC R: CATACGACATGTGAGCTTTG	61	271-281	KJ617080

TD = Ta ranging from 65°-55°C in the touchdown PCR program.

To assess the genetic variation of each analyzed locus, we used the GenAlEx v. 6.501 software (Peakall and Smouse, 2012) to estimate the number of different alleles (N_A), effective number of alleles (N_E), and observed (H_O) and expected (H_E) heterozygosities. Exact tests for departure from Hardy-Weinberg equilibrium (HWE) using the Markov-chain test and tests for linkage disequilibrium (LD) using a likelihood-ratio test were performed using the Arlequin v. 3.5.1.3 software (Excoffier and Lischer, 2010) and corrected for multiple comparisons using a sequential Bonferroni correction. The presence of null alleles was determined using the Micro-checker v. 2.2.3 (Van Oosterhout et al., 2004).

RESULTS AND DISCUSSION

Of the 23 SSR loci tested, 18 perfect, dinucleotide motif loci exhibited high-quality PCR products of the expected size (Table 1) and were polymorphic in both *U. pectinifera* populations scored. A summary of the estimated genetic diversity per locus within each population is shown in Table 2. The number of alleles per locus ranged from 2 (*Upec132* and *Upec226*) to 11 (*Upec214*), with a total of 98 alleles across the 42 samples scored and an average of 6.0 (± 0.41) alleles per locus. The loci *Upec138*, *Upec196*, *Upec201*, *Upec214*, *Upec221*, and *Upec241* showed the largest number of alleles (≥ 8 alleles), whereas the loci *Upec112*, *Upec123*, and *Upec132* showed the lowest number of alleles (≤ 3 alleles). The mean effective number of alleles over loci was 3.3 (± 0.30) and 4.5 (± 0.50) in the Inhaí and Mendanha populations, respectively, ranging from 1.6 (*Upec132*) to 6.5 (*Upec241*) in the Inhaí population and from 1.9 (*Upec138*) to 8.7 (*Upec214*) in the Mendanha population.

Table 2. Summary statistics of SSR polymorphism determined by screening 44 *Uebelmannia pectinifera* samples from two populations (Inhaí and Mendanha). Presence of null alleles (Null), number of alleles (*A*), number of effective alleles (A_E), and observed (H_O) and expected (H_E) heterozygosity.

Locus	Inhaí (N = 22)					Mendanha (N = 20)				
	Null	*A*	A_E	H_O	H_E	Null	*A*	A_E	H_O	H_E
Upec112	-	3	2.2	1.000	0.549*	-	3	2.3	1.000	0.558*
Upec123	-	3	2.6	0.571	0.622	-	3	2.4	0.611	0.591
Upec126	+	5	2.6	0.150	0.621*	-	9	3.2	0.737	0.691
Upec132	-	2	1.6	0.400	0.375	+	3	2.1	0.294	0.519
Upec138	-	8	3.8	0.563	0.736	-	4	1.9	0.600	0.479
Upec148	-	6	3.1	0.778	0.676*	-	7	6.2	1.000	0.838*
Upec153	+	3	2.4	0.333	0.591*	-	5	2.5	0.412	0.597*
Upec182	+	5	3.4	0.211	0.705*	+	7	4.6	0.429	0.783
Upec196	+	7	4.7	0.190	0.788*	+	9	7.3	0.526	0.863*
Upec201	-	9	4.9	0.706	0.796	+	7	4.5	0.375	0.775*
Upec214	+	8	3.7	0.529	0.732	-	11	8.7	0.769	0.885
Upec221	+	6	3.4	0.333	0.704	-	9	7.8	0.818	0.872
Upec223	-	5	3.0	0.850	0.666	-	6	4.5	0.714	0.776
Upec226	-	2	2.0	1.000	0.500*	-	6	2.7	0.917	0.632
Upec235	+	3	1.7	0.100	0.395*	+	9	6.1	0.647	0.837*
Upec241	-	10	6.5	0.905	0.847	+	10	6.8	0.611	0.852*
Upec242	+	5	3.2	0.400	0.685*	-	6	3.8	0.643	0.735
Upec267	-	6	4.8	0.737	0.789*	-	6	4.7	0.714	0.786*

- and + absence and presence of null alleles, respectively; *significant departure from Hardy-Weinberg equilibrium after Bonferroni correction at α = 0.05.

Observed heterozygosity ranged from 0.100 to 1.000 in the Inhaí population (mean = 0.542 ± 0.069) and 0.294 to 1.000 in the Mendanha population (mean = 0.657 ± 0.048), whereas the expected heterozygosity ranged from 0.375 to 0.847 in the Inhaí population (mean

= 0.654 ± 0.031) and 0.479 to 0.885 in the Mendanha population (mean = 0.726 ± 0.031). Significant departures from HWE after Bonferroni correction from multiple tests within populations were observed in 10 loci in the Inhaí and 8 loci in the Mendanha population (Table 2). All significant results, except for *Upec112*, *Upec148*, and *Upec226*, resulted from heterozygosity deficiencies. These results relative to heterozygosity deficiencies were consistent with the small size and patchy distribution of *U. pectinifera* populations, facilitating inbreeding, or potentially the presence of breeding groups within populations. The unexpected observation of heterozygous genotypes only at the loci *Upec112*, *Upec148*, and *Upec226* for one or both populations may represent scoring alleles from duplicate loci. This hypothesis primarily holds for the *Upec112* and *Upec226* loci, which showed a low number of alleles (≤ 3 alleles) at approximately equal frequencies (data not shown).

We found evidence of null alleles at 6 loci in the Inhaí population and 8 loci in the Mendanha population, among which 3 were common to both populations (*Upec182*, *Upec196*, and *Upec235*; Table 2). Because null allele inference approaches assume that populations are in HWE (Van Oosterhout et al., 2006), the increased number of loci showing departures from HWE likely influenced the null allele estimates. Of the 153 possible paired loci tested for LD within each population, 10 loci pairs in the Inhaí population and none in the Mendanha population showed significant LD after Bonferroni's correction for multiple tests. Because all significant LD tests were confined to the Inhaí population, which exhibited lower estimates of genetic diversity than the Mendanha population, these results are likely the result of demographic effects (e.g., bottleneck or population substructure) rather than a linkage relationship between these loci.

We tested the 18 primer pairs for cross-species amplification in the other species and subspecies that belong to *Uebelmannia* using 2 individuals from each taxon. Of the 18 loci tested, only *Upec148* and *Upec214* failed to amplify in *U. buiningii* and *U. gummifera* subsp *meninensis*. All remaining loci successfully amplified the tested taxa after adjusting the PCR annealing temperature and $MgCl_2$ concentration for the loci *Upec123*, *Upec138*, *Upec148*, *Upec214*, *Upec223*, *Upec242*, and *Upec267* (Table 3).

Table 3. Annealing temperatures and $MgCl_2$ concentrations [Ta/($MgCl_2$)] used for cross-species amplification of 18 polymorphic SSR loci developed in *Uebelmannia pectinifera* subsp *pectinifera* across the species and subspecies of *Uebelmannia*.

Locus	*U. buiningii*	*U. gummifera* subsp *meninensis*	*U. gummifera* subsp *gummifera*	*U. pectinifera* subsp *flavispina*	*U. pectinifera* subsp *horrida*
Upec112	=/=	=/=	=/=	=/=	=/=
Upec123	TD/3.0	=/=	59/2.5	=/=	=/=
Upec126	=/=	=/=	=/=	=/=	=/=
Upec132	=/=	=/=	=/=	=/=	=/=
Upec138	=/2.5	=/2.5	=/2.5	=/2.5	=/2.5
Upec148	NA	NA	=/3.0	=/3.0	=/3.0
Upec153	=/=	=/=	=/=	=/=	=/=
Upec182	=/=	=/=	=/=	=/=	=/=
Upec196	=/=	=/=	=/=	=/=	=/=
Upec201	=/=	=/=	=/=	=/=	=/=
Upec214	NA	NA	TD/=	TD/=	TD/=
Upec221	=/=	=/=	=/=	=/=	=/=
Upec223	=/2.5	=/2.5	=/2.5	=/2.5	=/2.5
Upec226	=/=	=/=	=/=	=/=	=/=
Upec235	=/=	=/=	=/=	=/=	=/=
Upec241	=/=	=/=	=/=	=/=	=/=
Upec242	55/2.5	56/=	=/=	=/=	=/=
Upec267	60/2.5	60/2.5	60/2.5	=/=	=/=

"=" successful amplification using the same Ta and/or $MgCl_2$ concentration optimized for *U. pectinifera* subsp. *pectinifera*. TD = Ta ranging from 65°-55°C in the *touchdown* PCR program; NA = no amplification.

CONCLUSIONS

In this study, we report the development and characterization of 18 perfect, dinucleotide SSR loci for *U. pectinifera* subsp *pectinifera* and the cross-amplification of these markers in all other *Uebelmannia* species and subspecies. All markers were polymorphic in a sample of 42 individuals from 2 *U. pectinifera* populations, and most markers exhibited relatively high levels of variation. Although there was evidence of null alleles at several markers, most of these results are likely an effect of Hardy-Weinberg disequilibrium within populations, and only 3 markers exhibited evidence of null alleles in both populations. Three primer pairs amplified duplicate loci. Based on these results, we recommend the markers *Upec123*, *Upec126*, *Upec132*, *Upec138*, *Upec153*, *Upec201*, *Upec214*, *Upec221*, *Upec223*, *Upec241*, *Upec242*, and *Upec267* as reliable for investigating the genetic structure, diversity, and species limits in the *Uebelmannia* genus. Further, this set of markers may be useful for molecular marker-assisted management plans for conserving these endangered cactus species.

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