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PRIMER NOTE

DEVELOPMENT OF SSR MARKERS FOR *ENCHOLIRIUM HORRIDUM* (BROMELIACEAE) AND TRANSFERABILITY TO OTHER PITCAIRNIOIDEAE[1]

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- *Premise of the study:* We developed a set of primers for *Encholirium horridum*, a species closely associated with inselbergs of the Atlantic Forest, to assess genetic diversity, genetic structure, and gene flow between populations of this species.
- *Methods and Results:* From an enriched genomic library, 10 primer pairs for polymorphic microsatellite regions were developed. The average number of alleles ranged from eight to 20, and the observed and expected heterozygosities ranged from 0.000 to 1.000, and from 0.000 to 0.929, respectively, across the populations.
- *Conclusions:* These markers will be useful in evaluating genetic diversity, spatial genetic structure, analysis of gene flow by paternity, and characterization of mating system of *E. horridum*.

Key words: Atlantic Forest; Bromeliaceae; cross-amplification; *Encholirium horridum*; inselberg; microsatellites.

Encholirium Mart. ex Schult. & Schult. f., together with *Deuterocohnia* Mez, *Dyckia* Schult. & Schult. f., *Fosterella* L. B. Sm., and *Pitcairnia* L'Hér., belongs to Bromeliaceae, subfamily Pitcairnioideae (Givnish et al., 2011). *Encholirium* is an exclusively Brazilian genus, with a distribution closely associated with inselbergs, in caatinga, cerrado, and Atlantic Forest phytogeographic domains. Of the 27 species described in this genus, only *E. horridum* L. B. Sm. and *E. gracile* L. B. Sm. are endemic to the Atlantic Forest (Forzza et al., 2013). *Encholirium horridum* has large populations distributed mainly in Espírito Santo State, extending to southern Bahia, eastern Minas Gerais, and northern Rio de Janeiro (Forzza et al., 2013). In 2008, the species was indicated as Data Deficient in the Official List of the Endangered Species of the Brazilian Flora (Ministério do Meio Ambiente, 2008).

Inselbergs are isolated rock outcrops that can be considered terrestrial islands (Porembski and Barthlott, 2000), where the species usually exhibit spatial isolation and restricted gene flow (Barbará et al., 2008; Boisselier-Dubayle et al., 2010). These features may allow *E. horridum* populations from these habitats to evolve separately, favoring long-term processes of speciation. To understand possible genetic patterns of these particular habitats, microsatellite markers were developed to assess genetic diversity, genetic structure, and gene flow between populations of *E. horridum* from different inselbergs.

METHODS AND RESULTS

A microsatellite-enriched library was constructed according to the method described by Billotte et al. (1999). For this purpose, genomic DNA from fresh leaves of an individual of *E. horridum* (Appendix 1) was extracted following Doyle and Doyle (1990). The DNA sample was digested with the *Rsa*I restriction enzyme (Invitrogen, Carlsbad, California, USA), and the fragments were then linked to *Rsa*I adapters. The library was enriched for dinucleotide sequences, using biotinylated $(CT)_8$ and $(GT)_8$ bound to Streptavidin MagneSphere Paramagnetic Particles (Promega Corporation, Fitchburg, Wisconsin, USA). Enriched fragments were amplified in 100-µL final volume containing 20 µL of selected fragments, 1× *Taq* buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 4 pmol of primer Rsa21, and 2.5 U of *Taq* DNA polymerase. A PTC-100 thermal cycler (MJ Research, Waltham, Massachusetts, USA) was used for PCR with the following program: 95°C for 1 min, followed by 25 cycles of denaturation at 94°C for 40 s, 60°C for 1 min, extension of 72°C for 2 min, and a final extension of 72°C for 5 min. The amplicons were cloned into a pGEM-T Easy Vector (Promega Corporation) and transformed into *Escherichia coli* XL1-Blue competent cells. Positive clones were selected by the expression of β-galactosidase gene and sequenced in an automated ABI 377 sequencer (Applied Biosystems, Foster City, California, USA) using T7 and SP6 primers and the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems). Chromatograms were assembled and edited using SEQUENCHER 4.9 (Gene Codes Corporation, Ann Arbor, Michigan, USA). Microsatellites were identified using the Simple Sequence Repeat Identification Tool (SSRIT; Temnykh

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TABLE 1. Characteristics of 10 polymorphic microsatellite markers developed for *Encholirium horridum*.

Locus	Primer sequences (5′–3′)	Fluorescent dye	Repeat motif	No. of alleles	Allele size (bp)	T_a (°C)	GenBank accession no.
Eh1G03	F: TTGTCGTTGTGACCTTCGTT	6FAM	$(TG)_{10}$	15	213–257	58	JX183384
	R: CTGTCTGCTTTTTGTTCCAGTG						
Eh2A07	F: ACTACTGCCCGGACTACCAA	6FAM	$(GA)_{16}N(AG)_9$	18	215–259	60	JX183385
	R: GGCATGTTAGGGTCCTTCAA						
Eh2A08	F: CTCCTTCAATTCACCCTTCG	VIC	$(CA)_6(CT)_8$	8	105–123	60	JX183386
	R: GTAGATCCAAACCGCATCG						
Eh2A11	F: AAAGTTGCCACCATGCAG	NED	$(GA)_{16}$	16	83–115	59	JX183387
	R: GGGACATGTTGGTGATCTTG						
Eh2B09	F: GCAGTTAGGCCCATACCAAG	VIC	$(GA)_{17}$	15	162–190	59	JX183388
	R: TACAGAGAAGGCGATGTCCA						
Eh2C03	F: CGCATGAAAGGAGTAGGATG	PET	$(GT)_{20}$	20	245–283	58	JX183389
	R: AGCACTCACAAAACGGAGAG						
Eh2E01	F: GCACTCTAAAGGCGACCAC	VIC	$(TCT)_5NN(TC)_{19}$	20	185–248	60	JX183390
	R: CGATCATCTGCTCCTCCTC						
Eh2E02	F: ATGCACCAAAAGGGACCA	NED	$(TG)_{10}$	9	241–261	58	JX183391
	R: TCGATTGTGACTCGACCAAC						
Eh2E11	F: GGCTTTTGAGGGTGAAGG	NED	$(GA)_{11}$	9	179–195	58	JX183393
	R: GGAGAGAGAAGAGAGAGGGAGA						
Eh2G07	F: GATGAACTCCAGTGCCTTCC	PET	$(AG)_{20}$	17	162–202	56	JX183394
	R: CCACCCTTATTGCTCTCACC						

Note: T_a = annealing temperature.

et al., 2001). Of the 96 clones sequenced, 34 contained simple sequence repeat (SSR) motifs. It was not possible to design suitable primers harboring SSR regions for four of these clones, because these regions were either at the beginning or end of the sequences. Primer pairs were designed using Primer3Plus software (Untergasser et al., 2007).

Evaluation of 30 primer pairs was performed by PCR in a Veriti Thermal Cycler (Applied Biosystems), in 10-µL final volume containing 10 ng of template DNA, 1× *Taq* buffer (Bioline, Tauton, Massachusetts, USA), 2.0 mM $MgCl_2$ (Bioline), 100 µM dNTPs (Fermentas, Glen Burnie, Maryland, USA), 2 pmol of each forward and reverse primer, and 0.25 U of *Taq* DNA polymerase (Bioline). The following program was used: 95°C for 3 min, followed by a first step consisting of 10 cycles of denaturation at 94°C for 30 s, at the specific annealing temperature (T_a; Table 1) for 30 s, extension at 72°C for 30 s, and a second step consisting of 20 cycles of denaturation at 90°C for 30 s, at the T_a (Table 1) for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 60 min. Eight samples from four populations located at the outermost geographic boundaries of species distribution were used to evaluate amplification success and loci polymorphism, respectively, on 1.5% and 3.0% agarose gels stained with GelRed (Biotium, Hayward, California, USA). Those loci with detected polymorphism were reamplified using forward primers labeled with fluorescence (FAM, NED, VIC, PET; Applied Biosystems; Table 1) and were resolved in an ABI 3500xL Genetic Analyzer (Applied Biosystems) using GSLIZ600 as the size standard (Applied Biosystems). Loci amplifications were made in single PCRs and were then multiplexed for fragment analysis. Fragment size and allele identification were determined using GeneMapper version 4.1 software (Applied Biosystems). For polymorphism evaluation, we used 89 individuals from 11 populations located along the species distribution (Table 2). These samples were dried in silica gel, and the genomic DNA was extracted using a NucleoSpin Plant II Kit (Macherey-Nagel, Düren, Germany). Genetic diversity parameters were estimated using FSTAT version 2.9.3.2 (Goudet, 2002). Out of the 30 microsatellite loci isolated, 18 were successfully amplified by PCR, of which 10 were polymorphic in the tested samples (Table 1). The number of alleles for the polymorphic loci ranged from eight to 20, with an average of 14.7. The observed and expected heterozygosities ranged from 0.000 to 1.000, and from 0.000 to 0.929, respectively, across the populations, with an average of 0.345 and 0.433 (Table 2).

Cross-species amplification was evaluated in the five genera of subfamily Pitcairnioideae (Appendix 1). Genomic DNA from dried leaves in silica gel was extracted using a DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA). The PCR protocol used was the same as described above. In general, cross-amplification was high (Table 3). All of the 10 primer pairs amplified successfully in *E. gracile*, while for the other four species the success of amplification varied from 40% to 60%.

CONCLUSIONS

The markers presented here will be useful in evaluating genetic diversity, spatial genetic structure, analysis of gene flow by paternity, and characterization of mating system of *E. horridum*. Results of cross-amplification indicate that this set of primers could be promising chiefly in studies with other species of *Encholirium*, but also with other Pitcairnioideae.

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TABLE 2. Results for primer screening of polymorphic loci in samples from 11 populations of *Encholirium horridum*.[a]

	Guaratinga (n = 8)			Carlos Chagas (n = 8)			Ecoporanga (n = 8)			Água Doce do Norte (n = 8)			Vila Pavão (n = 9)			Pancas (n = 8)		
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
Eh1G03	2	0.375	0.321	8	0.875	0.857	1	0.000	0.000	5	0.376	0.545	4	0.334	0.653	3	0.625	0.589
Eh2A07	1	0.000	0.000	4	0.125	0.679	2	0.125	0.339	5	0.876	0.813	6	0.666	0.833	6	0.749	0.830
Eh2A08	1	0.000	0.000	2	0.000	0.429	1	0.000	0.000	3	0.500	0.545	4	0.286	0.679	4	0.000	0.750
Eh2A11	—	—	—	—	—	—	2	0.125	0.125	4	0.501	0.438	7	0.667	0.688	5	0.999	0.821
Eh2B09	2	0.375	0.464	6	0.714	0.869	2	0.250	0.232	5	0.500	0.683	7	1.000	0.868	4	0.750	0.750
Eh2C03	2	0.250	0.583	7	0.857	0.881	2	0.125	0.482	6	0.143	0.929	6	0.428	0.762	7	0.833	0.883
Eh2E01	2	0.000	0.000	2	0.125	0.125	1	0.000	0.000	3	0.250	0.509	7	0.445	0.875	5	0.715	0.798
Eh2E02	1	0.000	0.000	7	0.750	0.875	1	0.000	0.000	3	0.000	0.800	5	0.500	0.750	4	0.429	0.738
Eh2E11	1	0.000	0.000	5	0.751	0.777	1	0.000	0.000	5	0.750	0.795	5	0.626	0.795	2	0.250	0.411
Eh2G07	1	0.000	0.000	7	0.375	0.911	2	0.125	0.125	6	0.750	0.839	8	0.889	0.910	7	0.751	0.911

	Colatina (n = 8)			Marliéria (n = 8)			Mutum (n = 8)			Vila Velha (n = 8)			Campos (n = 8)			Total (n = 89)		
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e (SD)
Eh1G03	2	0.500	0.393	1	0.000	0.000	3	0.833	0.650	3	0.625	0.500	1	0.000	0.000	15	0.423	0.410 (0.298)
Eh2A07	2	0.375	0.464	1	0.000	0.000	4	0.375	0.536	2	0.125	0.125	1	0.000	0.000	18	0.307	0.420 (0.346)
Eh2A08	2	0.125	0.125	1	0.000	0.000	6	1.000	0.768	3	0.375	0.589	1	0.000	0.000	8	0.216	0.353 (0.329)
Eh2A11	3	0.250	0.589	1	0.000	0.000	4	0.625	0.589	—	—	—	1	0.000	0.000	16	0.394	0.406 (0.323)
Eh2B09	3	0.500	0.634	2	0.125	0.482	8	0.751	0.911	3	0.749	0.580	1	0.000	0.000	15	0.515	0.588 (0.283)
Eh2C03	4	0.375	0.768	2	0.625	0.518	1	0.000	0.000	5	0.750	0.795	1	0.000	0.000	20	0.397	0.600 (0.332)
Eh2E01	3	0.500	0.420	1	0.000	0.000	5	0.750	0.839	1	0.000	0.000	1	0.000	0.000	20	0.247	0.347 (0.360)
Eh2E02	3	0.375	0.339	1	0.000	0.000	2	0.375	0.464	1	0.000	0.000	1	0.000	0.000	9	0.241	0.361 (0.376)
Eh2E11	1	0.000	0.000	1	0.000	0.000	4	0.625	0.661	2	0.375	0.464	1	0.000	0.000	9	0.299	0.355 (0.361)
Eh2G07	3	0.286	0.274	2	0.000	0.571	7	0.625	0.839	1	0.000	0.000	1	0.000	0.000	17	0.360	0.489 (0.410)

Note: — = unsuccessful amplification; A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity.

[a]Geographic coordinates of the populations: Guaratinga 16°36′S, 39°47′W; Carlos Chagas 17°52′S, 41°03′W; Ecoporanga 18°11′S, 40°40′W; Água Doce do Norte 18°34′S, 40°59′W; Vila Pavão 18°38′S, 40°35′W; Pancas 19°14′S, 40°58′W; Colatina 19°33′S, 40°32′W; Marliéria 19°42′S, 42°42′W; Mutum 19°56′S, 41°29′W; Vila Velha 20°19′S, 40°19′W; Campos 21°20′S, 41°22′W.

TABLE 3. Transferability of 10 microsatellite markers developed for *Encholirium horridum* across species of Bromeliaceae, subfamily Pitcairnioideae.[a]

Species	Eh1G03	Eh2A07	Eh2A08	Eh2A11	Eh2B09	Eh2C03	Eh2E01	Eh2E02	Eh2E11	Eh2G07
Deuterocohnia meziana	* (56°C)	*** (58°C)	*** (58°C)	—	—	—	** (56°C)	—	*** (58°C)	** (56°C)
Dyckia ibiramensis	—	** (56°C)	*** (58°C)	—	—	*** (58°C)	*** (58°C)	—	* (60°C)	—
Encholirium gracile	*** (58°C)	*** (58°C)	*** (58°C)	** (59°C)	*** (62°C)	*** (58°C)	* (60°C)	*** (58°C)	*** (58°C)	** (56°C)
Fosterella windischii	—	** (56°C)	*** (58°C)	—	—	—	* (60°C)	—	*** (58°C)	—
Pitcairnia bradei	—	** (56°C)	* (60°C)	** (59°C)	—	—	* (60°C)	—	*** (58°C)	—

Note: *** = strong intensity amplification with single band visualized; ** = median intensity amplification with single band visualized; * = weak amplification; — = unsuccessful amplification.

[a] Optimum annealing temperature is shown in parentheses.

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APPENDIX 1. Taxa included in the study. Specimens were deposited at Rio de Janeiro Botanical Garden Herbarium (RB). Information presented: taxon, specimen voucher, collection locality.

Deuterocohnia meziana Kuntze ex Mez, Forzza 2253 (RB 382284), Santa Cruz, Bolivia

Dyckia ibiramensis Reitz, Santos-Silva 59 (RB 518922), Ibirama, Santa Catarina, Brazil

Encholirium gracile L. B. Sm., Forzza 5762 (RB 459950), Vila Pavão, Espírito Santo, Brazil

Encholirium horridum L. B. Sm., Forzza 5794 (RB 501400), Vila Pavão, Espírito Santo, Brazil (18°38′S, 40°35′W)

Fosterella windischii L. B. Sm. & Read, Forzza 2370 (RB 382272), La Paz, Bolivia

Pitcairnia bradei Markgr., Forzza 4892 (RB 458381), Botumirim, Minas Gerais, Brazil