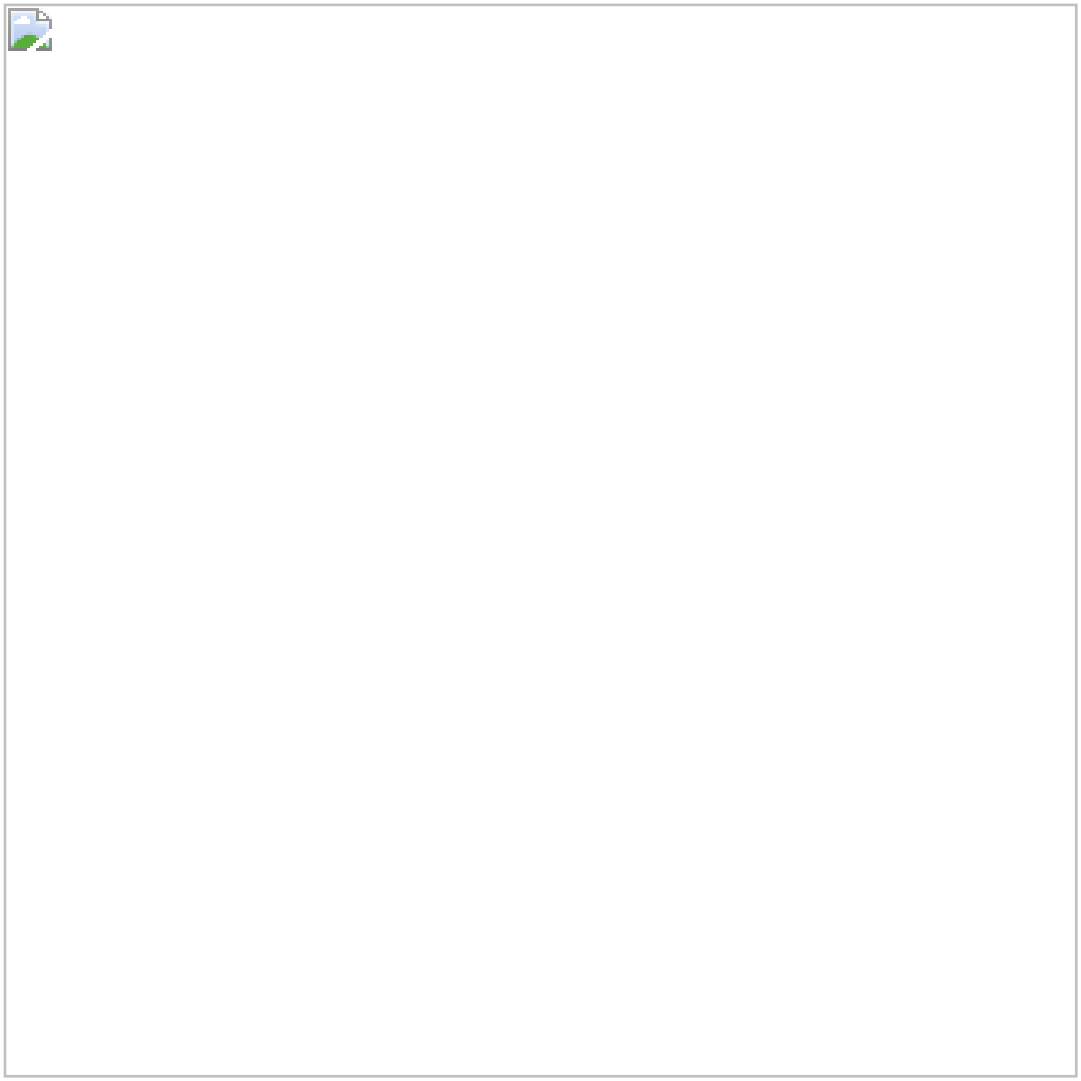

Fifteen Microsatellite Markers for Herbertia zebrina (Iridaceae): An Endangered Species from South American Grasslands

Authors: Forgiarini, Cristiane, Curto, Manuel, Stiehl-Alves, Eudes Maria, Bräuchler, Christian, Kollmann, Johannes, et al.

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Primer Note

Fifteen microsatellite markers for *Herbertia zebrina* (Iridaceae): An endangered species from South American grasslands[1]

Cristiane Forgiarini[2,5], Manuel Curto[3], Eudes Maria Stiehl-Alves[2], Christian Bräuchler[4], Johannes Kollmann[4], Harald Meimberg[3], and Tatiana Teixeira de Souza-Chies[2]

[2]Department of Biosciences, Programa de Pós-Graduação em Botânica, Universidade Federal do Rio Grande do Sul, Brazil; [3]Institute of Integrative Nature Conservation Research, University of Natural Resources and Life Science, Vienna, Austria; and [4]Department of Ecology and Ecosystem Management, Chair of Restoration Ecology, Technical University of Munich, Munich, Germany

- *Premise of the study:* Polymorphic microsatellite loci were developed and used to genotype individuals of *Herbertia zebrina* (Iridaceae) as a first step for assessment of intraspecific genetic diversity.
- *Methods and Results:* Primer pairs for 47 markers were developed: 20 from a microsatellite-enriched library and 27 from a next-generation sequencing run using the Illumina MiSeq platform. Of those, 15 loci were considered successful, of which 12 were polymorphic and three were monomorphic. The primers were tested in 50 individuals from three populations of *H. zebrina*. Two to 14 alleles per locus were identified, and observed and expected heterozygosity were 0.00–0.95 and 0.18–0.89, respectively. Tests of cross-amplification to evaluate the applicability of these markers showed positive results in one congeneric species, *H. darwinii*, and in a phylogenetically closely related species, *Calydorea crocoides*.
- *Conclusions:* These microsatellite markers can be used for studies of genetic variation and genetic population structure, as well as to support conservation efforts.

Key words: *Calydorea crocoides*; *Herbertia darwinii*; *Herbertia zebrina*; Illumina MiSeq; Iridaceae; next-generation sequencing; simple sequence repeat (SSR) marker.

Herbertia zebrina Deble (Iridaceae) is a critically endangered species of the southern Brazilian grasslands with a range of <100 km^2, high fragmentation, and declining habitat quality (International Union for Conservation of Nature [IUCN] criterion B1ab[iii,v]). The populations are restricted to a mountainous region with granitic soils, and it was recognized as a distinct species only recently (Deble, 2010). Information on distribution, number of populations, and reproduction of *H. zebrina* is sparse (C. Forgiarini, Universidade Federal do Rio Grande do Sul, unpublished manuscript). All known populations are located within an area that has changed substantially during the past 10 years and is severely threatened by monocultures (Roesch et al., 2009). The genus *Herbertia* Sweet is of recent origin (Goldblatt et al., 2008), and its radiation was probably linked to pollinator shifts that occur frequently in Iridaceae (Chauveau et al., 2012). Most *Herbertia* species, with the exception of the widespread *H. lahue* (Molina) Goldblatt, are restricted to South American grasslands. *Herbertia zebrina* is thus a suitable model to understand the mechanisms that lead to the high level of endemism in that region, and to study the effects of land-use changes threatening this diversity.

Microsatellite markers (simple sequence repeats [SSRs]) are a well-established approach to evaluate genetic diversity of populations for conservation planning of threatened species (Wan et al., 2014). Thus, we developed markers for *H. zebrina* using two methods of microsatellite development. In the future, we expect that these markers can be used to analyze the genetic structure of the species. We also present the conditions for amplification, primer sequences, size range, heterozygosity, Hardy–Weinberg equilibrium (HWE), null alleles, and linkage disequilibrium. To evaluate the applicability of these markers, cross-amplification was tested for the congeneric species *H. darwinii* Roitman & J. A. Castillo and for a species of another closely related genus, *Calydorea crocoides* Ravenna.

METHODS AND RESULTS

Total genomic DNA was extracted from silica gel–dried leaves of 50 individuals from three populations of *H. zebrina* (Appendix 1) using the

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[5]Author for correspondence: crisforgiarini@gmail.com

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cetyltrimethylammonium bromide (CTAB) protocol developed by Doyle and Doyle (1987), with modifications to the quantity of dried leaves used (10–50 mg) and microcentrifuge tube size (2-mL tubes). Two types of libraries were prepared, one using the method of Billote et al. (1999) and another using two partial (2%) Illumina MiSeq paired-end runs with read length of 300 bp (Illumina, San Diego, California, USA). For the first library, 20 primer pairs were designed from a single individual (voucher no. ESC421, Herbarium of the Universidade Federal do Rio Grande do Sul [ICN], Porto Alegre, Rio Grande do Sul, Brazil; Appendix 1). Total DNA was digested with *Rsa*I (Invitrogen, Carlsbad, California, USA) and ligated to the adapters M28 (5′-CTCTTGCTTGAATTCGGACTA-3′) and M29 (5′-TAGTCCGAATTCAAGCAAGAGCACA-3′) using T4 DNA ligase. Linker-adapted fragments were then enriched by hybridization with 5′ biotin $(GT)_8$ and $(CT)_8$ biotin-linked probes followed by purification with paramagnetic beads (Streptavidin MagneSphere Paramagnetic Particles; Promega Corporation, Madison, Wisconsin, USA). After the process described above, the enriched genomic DNA fragments were cloned into plasmid (pGEM-T Easy Vector, Promega Corporation) and single colonies containing microsatellite markers were identified by dot blot hybridization. Inserts were amplified with universal primer M13, treated with exonuclease I and shrimp alkaline phosphatase (New England Biolabs, Ipswich, Massachusetts, USA), and sequenced using the ABI 3500xL sequencer (Life Technologies/Applied Biosystems, Foster City, California, USA). Primers were designed using Primer3 (Untergasser et al., 2012), according to the following criteria: (i) size of primers 18–22 bp, (ii) melting temperature (T_m) 45–60°C, (iii) T_m difference between primer pairs no higher than 3°C, (iv) GC content 40–60%, (v) no complementarity between primer pairs, and (vi) amplified product length 100–300 bp.

To increase the number of polymorphic loci, we also used one sample of *H. zebrina* (voucher no. CF115 [ICN]; Appendix 1) to construct an Illumina library and identify microsatellites, from which 27 primer pairs resulted. The library was sequenced twice on a MiSeq run in five steps: DNA fragmentation, end repair, dA-tailing, Y-adapter ligation, and index PCR and bioinformatics analyses according to Deck et al. (2016). This process was developed at the Institute for Integrative Nature Conservation Research, University of Natural Resources and Life Sciences (Vienna). The Illumina run was done by the Genomics Service Unit from Ludwig-Maximilians-University (Munich). Primers were designed using Primer3Plus (Untergasser et al., 2007). Fluorescent dyes were added to the primers using the M13-tailed primer method (Schuelke, 2000). Four tail primers were used, and each one was tagged with a unique fluorescent dye: 6-FAM (TGTAAAACGACGGCCAGT), VIC (TAATACGACTCACTATAGGG), NED (TTTCCCAGTCACGACGTTG), and PET (GATAACAATTTCACACAGG). The amplifications were done by multiplex, with a combination of two to four primers using HotStarTaq *Plus* Master Mix Kit (QIAGEN, Hilden, Germany), following the protocol described in Deck et al. (2016).

The conditions of PCR amplification were identical in both techniques, i.e., an initial denaturation at 95°C for 15 min; followed by 10 cycles of 95°C for 30 s, annealing temperature (with a touchdown of 65–60/62–58°C, –0.5°C per cycle) for 45 s, and 72°C for 30 s; 35 cycles at 95°C for 30 s, annealing temperature (58–60°C) for 45 s, and 72°C for 30 s; and a final extension at 72°C for 10 min. Of the 47 primer pairs developed from the two libraries, 33 primer pairs resulted in PCR-amplified products, six using the method of Billote et al. (1999) and 27 using Illumina MiSeq. The amplifications were confirmed by gel electrophoresis (1.5%). One microliter of fluorescent PCR product was added into the mixture with 11 µL of formamide and 0.11 µL of GeneScan 500 LIZ Size Standard (Applied Biosystems/Life Technologies, Waltham, Massachusetts, USA). The material was sent to the Genomics Service Unit (Ludwig-Maximilians-University) for genotyping. The genotypes were analyzed using the program GeneMarker 1.75 (SoftGenetics, State College, Pennsylvania, USA). Of the 33 markers, 12 were considered polymorphic, three monomorphic (Table 1), and 18 presented poor amplification and were not included here.

To estimate the number of alleles, observed heterozygosity (H_o), expected heterozygosity (H_e), and HWE, we used the package *pegas* (Paradis, 2010) of R software version 3.2.2 (R Development Core Team, 2016). The presence of null

Table 1. Characteristics of 12 polymorphic and three monomorphic loci designed for *Herbertia zebrina*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	Fluorescent dye	T_a (°C)	GenBank accession no.
HZ2[a]	F: GCCATGGTCAAGGGAATAAG	$(GA)_{17}$	208	VIC	60	KY775362
	R: GGTCGCCTTCATATGCTGTT					
HZ3[a]	F: ACATAAAACCGGAGGGAGCA	$(CA)_8TGT(AC)_8$	180	NED	60	KY775363
	R: AACACTGGTGTGTGACATGTGTA					
HZ4[b]	F: TAGGCCCCACCAACGTATAA	$(GA)_{10}$	202	PET	60	KY775364
	R: AACACGTCATCTTCGTCTTCC					
HZ5[b]	F: TTGGGTTGTATCTCGTATCTGG	$(GC)_8(AC)_{12}(AG)_{17}$	253	FAM	60	KY775365
	R: TGCCATGTACATCCCTTAAATC					
HZ6[a]	F: AATGCCTTTGACTGCTGACC	$(GT)_4GG(GT)_8$	158	VIC	60	KY775366
	R: GTTTGTATCGCCGAACTCGT					
HZ7[b]	F: TGAAAGCATGATGCATAGGA	$(GT)_8(AG)_3(AT)_3$	162	FAM	60	KY775367
	R: AGGCTTGTGAATTGGGATTG					
HZ8[b]	F: TCGAGAGGGTTAGGGTTTGA	$(GAA)_7$	174	FAM	60	KY775368
	R: CAAGCTCCTCCAAAGGCTATT					
HZ9[b]	F: GAAGAGAATTTATGGGGCACA	$(CAA)_{16}$	153	PET	60	KY775369
	R: GACCCCACTTGTGGAATATCA					
HZ10[b]	F: GACTCGTTAAGAGAATCGAGCTT	$(GAGCC)_3$	151	NED	60	KY775370
	R: AATGTCATGGCTTCTTTTAGGG					
HZ10E[b,c]	F: TTCGTTGGAGTAACAGAGGACA	$(TG)_6$	207	FAM	58	KY781890
	R: CACCAAATTAGCAACCATCTGA					
HZ11[b]	F: TTTTGAACTGGAGGACCACA	$(GAAAGA)_5$	177	PET	60	KY775371
	R: TTCCAAACCGTAGAGATTCCA					
HZ12[b]	F: CAATTCTGCACCTGTACCCATA	$(TA)_{15}$	109	NED	60	KY775372
	R: TGTGTGCATGCCATTTACCT					
HZ13[b]	F: GGTTTCAGGGTTTAGGTTAGGG	$(AT)_6(GT)_{10}$	116	FAM	60	KY775373
	R: CATGAACGAACTGTCTTAGTTTGG					
HZ14[b]	F: AGGTGGGTTCACCTTAAAAGA	$(GAA)_{10}$	100	NED	60	KY775374
	R: CATCCTATGTGGCTAGTAATGTGG					
HZ15[b]	F: CCAGACCTCACTCGTAGGAAAT	$(GTT)_{11}$	100	PET	60	KY775375
	R: TGTACCATTACCAAGAAGCAAGC					

Note: T_a = annealing temperature.
[a]Monomorphic markers.
[b]Tested for polymorphism.
[c]Loci developed using method of Billote et al. (1999).

Table 2. Genetic characterization of 12 newly developed polymorphic microsatellites of *Herbertia zebrina*.[a]

	Cachoeira, Brazil (n = 20)				Santana, Brazil (n = 15)				Encruzilhada, Brazil (n = 15)			
Locus[b]	A	H_o	H_e	HWE	A	H_o	H_e	HWE	A	H_o	H_e	HWE
HZ4	13	0.65	0.88	0.000*	6	0.53	0.71	0.028	3	013	0.55	0.000*
HZ5	13	0.20	0.80	0.000*	14	0.73	0.89	0.003	14	0.53	0.84	0.000*
HZ7	8	0.95	0.80	0.159	9	0.93	0.83	0.115	11	0.87	0.74	0.087
HZ8	5	0.10	0.62	0.000*	3	0.00	0.59	0.000*	5	0.00	0.74	0.000*
HZ9	8	0.40	0.80	0.000*	4	0.00	0.51	0.000*	6	0.27	0.52	0.000*
HZ10	12	0.20	0.83	0.000*	9	0.27	0.79	0.000*	8	0.27	0.78	0.000*
HZ10E	3	0.45	0.36	0.646	4	0.27	0.66	0.001	3	0.07	0.18	0.038
HZ11	2	0.00	0.38	0.000*	2	0.00	0.23	0.002	3	0.27	0.60	0.001
HZ12	6	0.00	0.65	0.000*	8	0.13	0.61	0.000*	4	0.07	0.24	0.001
HZ13	8	0.45	0.49	0.250	6	0.20	0.52	0.002	8	0.27	0.54	0.003
HZ14	12	0.70	0.86	0.000*	8	0.33	0.83	0.000*	9	0.53	0.76	0.000*
HZ15	10	0.40	0.86	0.000*	9	0.73	0.81	0.335	5	0.33	0.60	0.006
Mean	8.3	0.37	0.69	—	6.83	0.34	0.66	—	6.5	0.30	0.59	—

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = P values of exact tests of Hardy–Weinberg equilibrium; n = number of individuals sampled.

[a] See Appendix 1 for locality and voucher information for all populations sampled.

[b] Significant presence of null alleles in HZ4, HZ5, HZ7, HZ9, and HZ15 from Cachoeira; HZ4, HZ9, HZ10, HZ12, and HZ14 from Santana; and HZ4, HZ9, HZ13, and HZ15 from Encruzilhada.

* Locus showed significant deviations from Hardy–Weinberg equilibrium, after Bonferroni correction ($P < 0.001$).

alleles was checked using MICRO-CHECKER 2.2.3 (van Oosterhout et al., 2004), and their statistical significance was assessed using Bonferroni-corrected P values. Linkage disequilibrium was estimated using GENEPOP software version 4.2 (Rousset, 2008). The number of alleles ranged from two to 14 per locus across the three populations (Table 2), H_o was 0.00–0.95, and H_e was 0.18–0.89. Overall, H_o was lower than H_e in the three populations, resulting in deviations from HWE for most markers. Null alleles were observed in nine loci (Table 2). Significant linkage disequilibrium was not detected after Bonferroni correction. Tests of cross-amplification using the same amplification conditions as for *H. zebrina* with the 12 polymorphic markers showed that nine of them amplified for *H. darwinii* and five for *C. crocoides* (Table 3).

CONCLUSIONS

The 15 microsatellites presented here are the first markers developed specifically for *H. zebrina*. Although three of them were determined to be monomorphic, cross-amplification testing showed that those microsatellites amplified not only for a congeneric species but also for a species in a related genus. Thus, they can be considered reliable markers and also a valuable resource for designing appropriate conservation strategies for this South American grassland species.

Table 3. Amplification of 12 polymorphic microsatellite loci developed for *Herbertia zebrina* for one congeneric species and one species from a phylogenetically closely related genus.

Locus	*Herbertia darwinii* (n = 5)	*Calydorea crocoides* (n = 5)
HZ4	+	+
HZ5	+	+
HZ7	+	+
HZ8	+	—
HZ9	+	+
HZ10	+	—
HZ10E	+	—
HZ11	—	—
HZ12	—	—
HZ13	—	+
HZ14	+	—
HZ15	+	—

Note: + = primers successfully amplified; — = primers could not be amplified.

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Appendix 1. Location information for the populations of *Herbertia zebrina*, *H. darwinii*, and *Calydorea crocoides* used in this study.

Species	Locality	*n*	Geographic coordinates[a]	Voucher no.[b]
Herbertia zebrina Deble	Santana da Boa Vista/RS, Brazil	15	30°18′47.44″S, 52°53′24.45″W	CF107
	Cachoeira do Sul/RS, Brazil	20	30°42′44.09″S, 52°58′27.91″W	CF108
	Encruzilhada do Sul/RS, Brazil	15	30°23′45.18″S, 52°38′22.16″W	CF109
	Encruzilhada do Sul/RS, Brazil	1	30°46′20.56″S, 53°08′17.10″W	CF115[c]
	Encruzilhada do Sul/RS, Brazil	1	30°31′3.9″S, 52°41′48.9″W	ESC421
Herbertia darwinii Roitman & J. A. Castillo	Santana do Livramento/RS, Brazil	5	30°52′28.95″S, 55°28′54.02″W	ESC502
Calydorea crocoides Ravenna	Bom Jesus/RS, Brazil	5	28°28′53.23″S, 50°19′48.67″W	ESC684

Note: *n* = number of individuals sampled; RS = Rio Grande do Sul.

[a] Datum: World Geodetic System 1984 (WGS84).

[b] All vouchers were deposited in the Herbarium of the Institute of Natural Sciences (ICN), Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

[c] Sample used to construct the Illumina library.