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

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

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- 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* is a critically endangered species of the southern Brazilian grasslands with a range of <100 km², 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of populations, and reproduction of *H. zebrina* is sparse (C. Forgiarini, Universidade Federal do Rio Grande do Sul, unpublished manuscript).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* 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...
cetyltrimethylammonium bromide (CTAB) protocol developed by Doyle and Doyle (1987), with modifications to the quantity of dried leaves used (10–50 mg) and microcentrifugation (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 RsaI (Invitrogen, Carlsbad, California, USA) and ligated to the adapters M28 (5′-CTCGTTGATATGCAGGGTATGATCTTCTAATCT-3′) and M29 (5′-TATCCGGAATTTCCAGAGACGCACA-3′) using T4 DNA ligase. Linker-adapted fragments were then enriched by hybridization with 5′ biotin (GT) and (CT) biotin-linked probes followed by purification with paramagnetic beads (Streptavidin MagneSphere Paramagnetic Particles; Promega Corporation, Madison, Wisconsin, USA). Of the 47 primer pairs developed from the two libraries, 33 primer pairs resulted using Illumina MiSeq. The amplifications were confirmed by gel electrophoresis 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 \textit{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 \textit{Herbertia zebrina}.

| Locus | Primer sequences (5′–3′) | Repeat motif | Allele size (bp) | Fluorescent dye | \( T_a (°C) \) | GenBank accession no. |
|-------|---------------------------|--------------|-----------------|-----------------|-----------------|-----------------------|
| HZ2\(^a\) | F: GCCAGTCCTCAAGGGAATAAG | (GA)\(^a\) | 208 | VIC | 60 | KY775362 |
|       | R: GTGTGGGCTCAATATGCGTTT |            |            |                |                |                       |
| HZ3\(^b\) | F: ACATAAAACCCGAGGGGACA | (CA)\(^b\) TGT(AC)\(^b\) | 180 | NED | 60 | KY775363 |
|       | R: AAACCTGATGGTGGACATGTGTA |            |            |                |                |                       |
| HZ4\(^b\) | F: TAGGGCCCAACAGGTATAGA | (GA)\(^b\) | 202 | PET | 60 | KY775364 |
|       | R: AAGCTGATGGTGGACATGTGTA |            |            |                |                |                       |
| HZ5\(^c\) | F: TGGTGATGGTGGACATGTGTA | (GC)\(^c\) (AC)\(^c\) | 253 | FAM | 60 | KY775365 |
|       | R: TCCATGCTACATCCTTCTGTA |            |            |                |                |                       |
| HZ6\(^b\) | F: AATCGGCTTGGCATTTCTGTA | (GT)\(^b\) GG(CT)\(^b\) | 158 | VIC | 60 | KY775366 |
|       | R: GTGGCTATGCGCGAACACTGAC |            |            |                |                |                       |
| HZ7\(^b\) | F: TGAAAGCTAGTACATGAGA | (GT)\(^b\) (AG)\(^b\) AT | 162 | FAM | 60 | KY775367 |
|       | R: AGGCTGATGGTGGACATGTGTA |            |            |                |                |                       |
| HZ8\(^a\) | F: TCGAGGGTTAGGTTGGTA | (GAA)\(^a\) | 174 | FAM | 60 | KY775368 |
|       | R: CAAGCTTCTCTCCAGAACGCTAT |            |            |                |                |                       |
| HZ9\(^b\) | F: GAAGAGATATTGTGGCCCA | (CAA)\(^b\) | 153 | PET | 60 | KY775369 |
|       | R: GCCAACCTGGGTTAAGCCTAT |            |            |                |                |                       |
| HZ10\(^b\) | F: GACGTGATATATTCAGCCATC | (GAGCC)\(^b\) | 151 | NED | 60 | KY775370 |
|       | R: AATGCTGATGGTGGACATGTGTA |            |            |                |                |                       |
| HZ10E\(^a\) | F: TTTTGATCGAAGGGACAGCA | (TG)\(^a\) | 207 | FAM | 58 | KY781890 |
|       | R: CACCAATGCTACACCATCCGTA |            |            |                |                |                       |
| HZ11\(^b\) | F: TTTTGATCGAAGGGACAGCA | (GAAAGA)\(^b\) | 177 | PET | 60 | KY775371 |
|       | R: TCTCAGGCTGAGGAGCTATCCT |            |            |                |                |                       |
| HZ12\(^b\) | F: CAATCTGCTAGGACATTTCCATA | (TA)\(^b\) | 109 | NED | 60 | KY775372 |
|       | R: TGTTGAGCTGACATTTCCATA |            |            |                |                |                       |
| HZ13\(^b\) | F: GGTGCTCTAGGACATTTCCATA | (AT)\(^b\) GT | 116 | FAM | 60 | KY775373 |
|       | R: CAGATGACTAGGACATTTCCATA |            |            |                |                |                       |
| HZ14\(^b\) | F: AGGTGATTGCACTTTGAGGA | (GAA)\(^b\) | 100 | NED | 60 | KY775374 |
|       | R: CATCTCATGCTAGTATGCTGG |            |            |                |                |                       |
| HZ15\(^b\) | F: CCAAGCTTCTCAGGAGGAAAT | (GTT)\(^b\) | 100 | PET | 60 | KY775375 |
|       | R: TGACTACTTACACAGAGGCA |            |            |                |                |                       |

*Note: \( T_a \) is annealing temperature.

\(^{a}\)Monomorphic markers.

\(^{b}\)Tested for polymorphism.

\(^{c}\)Loci developed using method of Billote et al. (1999).

http://www.bioone.org/loi/apps
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). Hs was 0.00–0.95, and He was 0.18–0.89. Overall, H0 was lower than Hs 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 C. crocoides and five for C. xerophyticus (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 congenic 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 2. Genetic characterization of 12 newly developed polymorphic microsatellites of *Herbertia zebrina*.a

| Locus  | Cachoeira, Brazil (n = 20) | Santana, Brazil (n = 15) | Encruzilhada, Brazil (n = 15) |
|--------|--------------------------|--------------------------|-----------------------------|
|        | A | Hs | He | HWE                  | A | Hs | He | HWE                  | A | Hs | He | HWE                  |
| HZ4    | 13 | 0.65 | 0.88 | 0.000*               | 6 | 0.53 | 0.71 | 0.028                  | 3 | 0.13 | 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   |   | 0.37 | 0.69 |—                     | 6.83 | 0.34 | 0.66 |—                     | 6.5 | 0.30 | 0.59 |—                     |

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

See Appendix 1 for locality and voucher information for all populations sampled.
aSignificant 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.

Table 3. Amplification of 12 polymorphic microsatellite loci developed for *Herbertia zebrina* for one congenic 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 | Voucher no. |
|-----------------------|------------------------------|----|------------------------|-------------|
| *Herbertia zebrina*   | Deble Santana da Boa Vista/RS| 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       |
|                       | Encruzhilhada do Sul/RS, Brazil | 15 | 30°23’45.18”S, 52°38’22.16”W | CF109       |
|                       | Encruzhilhada do Sul/RS, Brazil | 1  | 30°46’20.56”S, 53°08’17.10”W | CF115c      |
|                       | Encruzhilhada do Sul/RS, Brazil | 1  | 30°31’3.9”S, 52°41’48.9”W   | ESC421      |
| *Herbertia darwinii*  | Raotman & J. A. Castillo     | 5  | 30°52’28.95”S, 55°28’54.02”W | ESC502      |
| *Calydorea crocoides*| Ravenna                      | 5  | 28°28’53.23”S, 50°19’48.67”W | ESC684      |

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

- Datum: World Geodetic System 1984 (WGS84).
- All vouchers were deposited in the Herbarium of the Institute of Natural Sciences (ICN), Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.
- Sample used to construct the Illumina library.