ONLINE RESOURCES

Isolation and characterization of twelve polymorphic microsatellite loci for *Hypochaeris catharinensis* (Asteraceae) and cross-amplification in related species

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Introduction

In this study, we describe the development and screening of an enriched genomic DNA library aiming to identify microsatellite loci in *Hypochaeris catharinensis*, an endemic species from southern Brazil. Twelve polymorphic microsatellite loci tested in a sample of 60 individuals from three different populations of *H. catharinensis*, identified 98 alleles with an average of 8.17 alleles per locus. The average polymorphic information content (PIC) varied from 0.098 to 0.757, with five loci exhibiting significant deviation from Hardy–Weinberg equilibrium (HWE). Transferability test for cross-amplification was done in 10 related *Hypochaeris* species with a rate of success ranging from 12 loci, for *H. petiolaris* and *H. varigata*, to six loci for *H. patagonica*. These microsatellite loci will provide valuable information for studying genetic diversity, population genetic structure, conservation biology and for understanding the past demographic events of *H. catharinensis* and other related species of *Hypochaeris*.

Hypochaeris is considered as a biological model to understand evolutionary processes in the vascular flora of South America, particularly from the temperate portion of the continent. As suggested by molecular clock dating, the South American species of *Hypochaeris* was derived from a common ancestor from northwest Africa that arrived in the continent between 3.5 and 0.25 mya by long-distance dispersal (Tremetsberger *et al.* 2005). Following rapid events of adaptive radiation in the continent, the genus dispersed (between 0.25 and 1.00 mya) into new habitats, developing great deal of morphological variation (Stuessy *et al.* 2003; Tremetsberger *et al.* 2006). Evidence of adaptive radiation, producing ecologically restricted and morphologically distinct taxa, has been documented in plants (Sang *et al.* 1994; Kim *et al.* 2008). For instance, molecular data based on plastid DNA (ITS, *matK*, *trnL-F*) revealed that the genus *Hypochaeris* is monophyletic in South America, although amplified fragment length polymorphism (AFLP) analyses resolved six different independent lineages within the continent (Stuessy *et al.* 2003; Tremetsberger *et al.* 2006).

In Brazil, *Hypochaeris* is represented by eight to ten species, mostly endemic to the southern region where *H. catharinensis* Cabrera can be cited as an example of a species with unique morphological and ecological characteristics. The distribution of *H. catharinensis* comprises the north of Rio Grande do Sul and south of Santa Catarina states, occurring exclusively at altitudes up to 1400 m, in areas where the temperature in winter is below −1°C, with incidence of dry, rocky and shallow soils, characterizing a nutrient-poor environment (Azevêdo-Gonçalves and Matzenbacher 2007). Adaptation to this environment is facilitated by horizontal rhizomes, a feature of taxonomic importance (Azevêdo-Gonçalves and Matzenbacher 2007) that also influences the vegetative spread of the species. Morphologically, *H. catharinensis* has rose-like leaves, forming a rosette that favours the total light incidence, a solitary stem, capitula

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enclosing florets with yellow corolles and beaked achenes (Azevêdo-Gonçalves and Matzenbacher 2007).

Until recently, *H. catharinensis* was only known by its ecology and morphological characters. However, recent reports based on cytogenetic studies included *H. catharinensis* within a chromosomal group described for the South American species of *Hypochaeris*, while AFLP data provided evidence that *H. catharinensis* constitutes a new phylogenetic lineage with the sister-species *H. lutea* (Reck *et al.* 2011). Studies at population level are important to understand the potential for broad and rapid speciation of *Hypochaeris* in South America, but information at population level is still lacking for the species *H. catharinensis*.

Since microsatellite markers (SSR) have become the preferred tool for genetic studies in a variety of organisms, especially as a result of the high level of polymorphism, codominant nature, multiallelism and reproducibility, this paper describes the development and screening of a microsatellite-enriched library for *H. catharinensis*, to study genetic diversity, population genetic structure, conservation biology and to understand past demographic events of *H. catharinensis* and closely-related species. Such studies could test the assumption of a recent evolution and biogeographic emergence of the genus *Hypochaeris* in the South American continent.

Materials and methods

Genomic DNA of *H. catharinensis* and related species was extracted from fresh leaves using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1987). An enriched microsatellite library was constructed using DNA of one individual of *H. catharinensis* by the hybridization based capture method with biotin-labelled $(CT)_8$ and $(GT)_8$ oligonucleotides (Billotte *et al.* 1999). DNA (5 μg) was digested with *Rsa*I (Promega, Madison, USA) and blunt-ended fragments were linked to adapters (*Rsa*-21 and *Rsa*-25). Fragments containing repeats were selected by hybridization with the biotinylated oligonucleotides and recovered by streptavidin-coated magnetic beads (Invitrogen-Dynal, Lillestrøm, Norway). Microsatellite-rich fragments were amplified by PCR with the *Rsa* 21 adapter and cloned into the pGEM-T Easy vector II (Promega). Recombinant clones (98) were randomly selected and double-sequenced with M13 forward and reverse primers using an ABI 3500xl Genetic Analyzer (Applied Biosystems, New York, USA). A total of 36 clones were found possessing motifs with perfect compound and interrupted microsatellite and 20 of them showed flanking regions adequate for primers design (Primer3Plussoftware; Untergasser *et al.* 2007).

Annealing temperature and amplification consistency for each primer pair were tested in a sample of five individuals of *H. catharinensis.* Twelve of the 20 primer pairs provided clear and consistent amplification pattern and they were applied for genotyping a sample of 60 individuals of *H. catharinensis*, representing three native populations (20 individuals of each) from southern Brazil (two populations from Santa Catarina state: Rancho Queimado —27°38′S, 48°59′W and Caçador-BR153—26°34′S, 51°44′W; one population from Rio Grande do Sul state, Cambará do Sul—29°10′W). Vouchers were deposited at the Herbarium FUEL, Universidade Estadual de Londrina under the identification numbers: FUEL 40696 (Rancho Queimado), FUEL 50926 (Caçador-Br153) and FUEL 42237 (Cambará do Sul). For genotyping, we used indirect fluorescent labelling of fragments with a three-primer system (Schuelke 2000), with the forward primers tagged with M13 sequences (TGTAAAACGACG GCCAGT) at the 5′end. The standard PCR reactions were in a 10 μL volume containing 4.5 μL GoTaq Green Master Mix (Promega), 0.08 μL of M13-tailed forward primer, 0.32 μL of reverse primers (table 1) and 0.32 μL of M13-labelled (6-FAM, HEX or NED) primers, 2 μL (30 ng) of genomic DNA and 2.78 μL of nuclease free water. PCR amplification profiles consisted of an initial denaturation of 4 min at 94°C, followed by 16 touchdown cycles at 94°C for 30 s, 65−50°C (−1°C per cycle) for 30 s, 72°C for 1 min, followed by 30 additional cycles at 94°C for 30 s, 50°C for 30 s and 72°C for 1 min, with a final extension at 72°C for 7 min. The PCR products were run in the ABI 3500-xl Genetic Analyzer (Applied Biosystems).

For characterization of the polymorphic loci, we applied standard population genetic statistics, calculated using Cervus ver 2.0 software (Marshall *et al.* 1998) and GENEPOP ver. 1.2 (Raymond and Rousset 1995). Linkage disequilibrium was tested by sequential Bonferroni correction for multiple comparisons at 5% significance. Presence of null alleles was verified using the software Micro-Checker 2.2.3 (Van-Oosterhout *et al.* 2004).

Results and discussion

The genotyping of 60 individuals of *H. catharinensis* identified a total of 98 alleles, ranging from 152 (Hcat2) to 264 bp (Hcat3), and a moderate level of polymorphism. The number of alleles per locus ranged from three (Hcat3) to 14 (Hcat21), with an average of 8.17 alleles per locus (table 1). PIC ranged from 0.064 (Hcat6) to 0.757 (Hcat14), whereas levels of observed (H_o) and expected (H_e) heterozygosity varied from 0.000 (Hcat3) to 0.804 (Hcat24) and from 0.066 (Hcat6) to 0.792 (Hcat14) with mean values of 0.325 and 0.498, respectively (table 1).

Five loci showed allelic frequencies that significantly deviated from expected HWE proportions, with loci Hcat3, Hcat14, Hcat17 been significant at $P \leq 0.001$ and loci Hcat21 and Hcat24a were significant at $P \leq 0.05$. Pairwise comparisons for multiple tests among the polymorphic loci showed significant linkage disequilibrium only between loci Hcat24a/Hcat24b and Hcat14/Hcat17, while six loci showed significant evidence for the presence of null alleles according

Table 1. Characterization of 12 polymorphic microsatellite loci genotyped in 60 individuals from three populations of *H. catharinensis*.

Locus/GenBank accession no.	Primer sequence (5′– 3′)	Repeat motif	T_A	Allele size	K	H_o	H_e	PIC
Hcat1 KF157558	F: ATTGCAACCCTTTGCAACTT R: CATATTTGTGGGATAAAGGTCA	CA_4	59	213–249	5	0.086	0.101	0.098
Hcat2 KF157559	F: CACTTGAAATCGTCCCTCTCA R: AATGTCAAGCCAAGCCAGTC	$(GT)_6GA(GT)_3$	59	154–249	11	0.458	0.416	0.392
Hcat3 KF157560	F: TTCTGCTGCTTTGCTTCTGA R: AAACCCCTACTGCCAGACCT	GA_5	60	260–264	3	0.000**	0.643	0.564
Hcat6 KF157561	F: AATTCGGGAGGTTCCCTTTA R: ACATGAAGGGACGAGTCAGG	$(TTC)_4TTA(TTC)$	60	222–225	4	0.017	0.066	0.064
Hcat7 KF157562	F: TCAAGGGTTCTCCCAACAAC R: GATCAACGTAGCCAGGTGGT	$(CT)_2CCAA(CT)_5$	60	164–246	6	0.183	0.230	0.218
Hcat8 KF157563	F: GAATGTAGCGGGATCGTT R: ATGTCGGGGACGGATCTACT	$(CA)AA(CA)_4$	60	164–216	6	0.621	0.495	0.407
Hcat14 JX418274	F: ATAACCGTCTGATGCCAAGC R: CCCAATCTTGAAGCTGGAAA	$(CTT)_3n(CTA)_3$	60	164–226	12	0.217**	0.792	0.757
Hcat17 JX418277	F: ACACATGAGAAGGGCGATTT R: GGTTCCCTATTGCGTTGAAA	$(TG)_4n(TG)_3$	59	162–226	9	0.217**	0.747	0.707
Hcat19 JX418273	F: AACAAGCAAAACCCAGGATG R: CATCACCACCCCCTCTTCTA	$(TCTG)_6n(GT)_5(TG)_4$	59	213–225	6	0.133	0.218	0.211
Hcat21 JX418272	F: TGCGTGGTTGAATTCTTTGT R: CAAACCAGCACCCTGAAAAT	$(CT)_3TT(CT)_{19}$	59	183–227	14	0.424*	0.787	0.752
Hcat24a JX418275	F: TGGCTGCCCTTTATACTTGC R: CGGGAGTATGTATGCGTGTG	$(GA)_7n(AGG)_5$	60	185–227	11	0.741*	0.749	0.715
Hcat24b JX418276	F: GCCATGCTTTCTCCCTTTCT R: GCTTACGCGTGGACTAGCAT	$(CT)_3n(AC)_{11}$	60	183–227	11	0.804	0.741	0.702

T_A, anneling temperature (°C); allele size indicates the range of observed alleles in bp; K, number of alleles; H_o and H_e, observed and expected heterozygosities, respectively; PIC, polymorphic information content; *significant deviation from with $P \leq 0.05$ and **with $P \leq 0.001$.

Table 2. Cross-amplification test of 12 microsatellite loci throughout 10 species of genus *Hypochaeris*.

Species	Hcat1	Hcat2	Hcat3	Hcat6	Hcat7	Hcat8	Hcat14	Hcat17	Hcat19	Hcat21	Hcat24a	Hcat24b
H. albiflora (Kuntze) Azevêdo-Gonç. & Matzenb	+	+	−	+	+	+	+	+	−	−	+	+
H. angustifolia (Litard. & Maire) Maire	+	+	+	+	+	+	−	−	+	+	+	+
H. argentina Cabrera	+	+	+	+	+	+	+	+	−	+	−	−
H. chillensis (Kunth)	+	+	+	+	+	+	+	+	+	−	+	+
H. lutea (Vell.) Britton	+	+	−	+	+	+	+	+	+	+	+	+
H. megapotamica Cabrera	+	+	+	+	+	+	+	+	+	−	+	+
H. pampasica Cabrera	+	+	−	+	+	+	+	+	+	−	+	+
H. patagonica Cabrera	−	+	−	+	+	+	−	−	−	−	+	+
H. petiolaris (Hook. & Arn.) Griseb.	+	+	+	+	+	+	+	+	+	+	+	+
H. variegata (Lam.) Baker	+	+	+	+	+	+	+	+	+	+	+	+

+, Clear amplification; −, no amplification.

to Bonferroni correction ($P \leq 0.05$), caused probably to homozygosis excess.

The 12 isolated microsatellite loci were tested for cross-amplification in 10 South American representatives of *Hypochaeris* including *H. angustifolia*, the presumed ancestor of this group. Four primer pairs (Hcat2, Hcat6, Hcat7 and Hcat8) showed amplifications in all species tested and in *H. angustifolia*, while the transferability rate of the other loci varied from five to nine species (table 2).

The levels of variation detected for the 12 microsatellite loci revealed the high potential of these markers for applications in genetic diversity, population genetic structure and studies of past demographic events of *H. catharinensis*. High levels of cross-species transferability of microsatellite loci is only expected in closely-related species (Selkoe and Toonen 2006; Souza *et al.* 2009; Rai *et al.* 2013). Therefore, the cross-amplification success of the *H. catharinensis* microsatellites also suggests that these markers can be efficiently applied for

genetic studies of other *Hypochaeris* species, thus providing valuable information to understand the potential for broad and rapid speciation of the genus in South America.

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