Isolation and Characterization of Microsatellite Markers for Hypochaeris incana (Asteraceae) and Close Relatives

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PRIMER NOTE

ISOLATION AND CHARACTERIZATION OF MICROSATELLITE MARKERS FOR *HYPOCHAERIS INCANA* (ASTERACEAE) AND CLOSE RELATIVES[1]

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- *Premise of the study:* We developed microsatellite markers to study clonal growth and interspecific hybridization in the Patagonian and subantarctic plant *Hypochaeris incana* (Asteraceae) and its closest relatives.
- *Methods and Results:* We developed primers for microsatellite loci from 454 sequence reads of genomic DNA of *H. incana*. We tested them on individuals of *H. acaulis*, *H. hookeri*, *H. incana*, *H. palustris*, and *H. tenuifolia*. We selected 15 polymorphic microsatellite loci, which delivered clearly scorable fragments in most or all species. With mean values between 0.7 and 0.8, the expected heterozygosity in populations of *H. incana* is high.
- *Conclusions:* Due to high levels of polymorphism, the developed markers make it possible to distinguish between genets and ramets in *H. incana*. In some markers, null alleles complicate the scoring of genotypes in tetraploids. All of the developed markers are suitable to study interspecific hybridization among this group of closely related species.

Key words: Asteraceae; clonal growth; hybridization; *Hypochaeris incana*; perennial herb; polyploidy; South America.

Hypochaeris incana (Hook. & Arn.) Macloskie (Asteraceae, Cichorieae) is a rosulate perennial herb that may propagate by underground stolons. It inhabits the Patagonian steppe of southern South America and extends its range to the subantarctic southernmost part of the continent in Tierra del Fuego. The species includes diploid, triploid, and tetraploid cytotypes. Peculiarly, diploids occur in the southern part of its range and tetraploids in the northern part of its range, but *H. incana* seems to have originated in the north (Tremetsberger et al., 2009). Tremetsberger et al. (2009) suggested that tetraploids may have repeatedly replaced their diploid progenitors in the northern part of the range. The factors involved in the establishment of polyploid cytotypes, however, are still poorly understood. We developed microsatellite primers for *H. incana* to investigate the competitive abilities of diploids and tetraploids in terms of their clonal growth strategies (discrimination between genets and ramets). We also tested the primers in the close relatives *H. acaulis* (J. Rémy) Britton, *H. hookeri* Phil., *H. palustris* (Phil.) De Wild., and *H. tenuifolia* (Hook. & Arn.) Griseb. to study the possible relationship between interspecific gene flow and the origin of the polyploid cytotypes.

METHODS AND RESULTS

We extracted genomic DNA from leaf material of *H. incana* and related species dried on silica gel in the field with the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany; Appendix 1). The ploidy level of all individuals of the Cerro La Buitrera population of *H. incana* and of a few other populations was determined by flow cytometry (C. König, unpublished data; see Appendix 1). The ploidy level of the remaining populations was retrieved from Weiss et al. (2003), Weiss-Schneeweiss et al. (2007), and Tremetsberger et al. (2009) and/or inferred from microsatellite peak patterns. One diploid individual of the Cerro La Buitrera population of *H. incana* was sequenced on a GS FLX Titanium sequencer (454 Life Sciences, a Roche Company, Branford, Connecticut, USA) at LGC Genomics (Berlin, Germany). The mean length obtained for the 180,338 sequences was 1048 bp (range = 50–1780 bp; National Center for Biotechnology Information [NCBI] Sequence Read Archive BioProject no. PRJNA314301). The methodology for primer development followed Böckelmann et al. (2015) with slight modifications as outlined below. MSATCOMMANDER version 0.8.2 (Faircloth, 2008) identified 2466 sequences with microsatellite motifs with the following options: di-, tri-, and tetranucleotide repeats ≥6 repeat units, combine multiple arrays within a sequence if within 50 bp distance. Primers for a total of 838 microsatellite loci were designed using Primer3 implemented in MSATCOMMANDER (Rozen and Skaletsky, 1999). A CAG or M13R tail (CAG: 5′-CAGTCGGGCGTCATCA-3′; M13R: 5′-GGAAACAGCTATGACCAT-3′) was added to the 5′ end of one primer (Schuelke, 2000) and a GTTT PIG-tail was

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TABLE 1. Characteristics of the 15 polymorphic microsatellite markers developed for *Hypochaeris incana* and related species.[a]

Locus	Primer sequences (5′–3′)[b]	Repeat motif	Allele size range (bp)[c]	Fluorescent dye[d] (PCR multiplex set)	GenBank accession no.
Hypinc_05	F: AGTCAGATTTACTTCGCCACC R: GTTTCTCACACGCACCTCTTTGG	$(AG)_{12}$	198–392	ATTO 532 (1)	KY111439
Hypinc_10	F: GTTTAAGTCTTGCCAACAGCTCC R: TCTTGGCACCCATTTCACC	$(AG)_{17}$	229–271	ATTO 565 (1)	KY111440
Hypinc_14	F: AACAGCTCGCAATCTCAGG R: GTTTACCCTTGATCCTTGATTGATACTTC	$(GT)_{10}$	276–300	ATTO 565 (2)	KY111441
Hypinc_16	F: TCCCATAGCCTCATGCCAG R: GTTTCCCTATCACACTCGGTCAGG	$(AC)_{10}$	320–348	ATTO 550 (2)	KY111442
Hypinc_17	F: CTGGTGCCCAGAACTCCAC R: GTTTGTGCAATAGAAGGGCGATGG	$(AG)_{10}$	355–390	ATTO 532 (2)	KY111443
Hypinc_24	F: GTTTCACTGTGTAACCGGCTCCC R: GCCTCGCCAAACATCGAC	$(AC)_{18}$	134–211	ATTO 532 (3)	KY111444
Hypinc_26	F: CCGGCATTTCTTAGGGCAAG R: GTTTGCAAGGTGAACCTGGTCGG	$(AG)_{11}$	248–300	FAM (1)	KY111445
Hypinc_28	F: ACGGAATTTGCAAGCCAAC R: GTTTCACTTTGCATCACCCACCG	$(GAT)_9$	409–460	FAM (2)	KY111446
Hypinc_33	F: GTTTCGATCGAGCATCCAACCC R: AAGTTTGACGGCGGTTGAC	$(AG)_{14}$	272–322	ATTO 550 (1)	KY111447
Hypinc_41	F: ATTCATGGCCTTCCGGGTC R: GTTTCTATCGAAGCTATTGATTTCCAG	$(AC)_{11}$	155–173	ATTO 550 (4)	KY111448
Hypinc_42	F: GTTTATCCGGTGGAGCATCAGTC R: ACGACGCCATACTCTCGTG	$(AAT)_8$	420–438	FAM (3)	KY111449
Hypinc_49	F: CGTCAGCGCTTAGACTGTAG R: GTTTACCTCGATTCGTTCTCCAC	$(GGT)_8$	321–342	ATTO 550 (3)	KY111450
Hypinc_53	F: TGGAAGCTCTTGATGAAACTCG R: GTTTCTCCTCTTATGCTCACGGG	$(GT)_8$	235–245	ATTO 565 (3)	KY111451
Hypinc_56	F: TCGGCCACCATTAACCCTC R: GTTTGTGCGTGATATGTGCCCTTC	$(CT)_8$	290–326	ATTO 565 (4)	KY111452
Hypinc_59	F: GTTTACCCACAACAATCTCAGTTAGC R: TCTACTTAACCAACGGATGAGC	$(AC)_9$	165–207	ATTO 532 (4)	KY111453

[a]Touchdown PCR was used for all loci.

[b]GTTT PIG-tails (Brownstein et al., 1996) added to the 5′ end of one primer are underlined. CAG or M13R tails added to the 5′ end of the other primer are not shown.

[c]Refers to *H. incana* only.

[d]Added to the 5′ end of the primers without PIG-tail.

added to the 5′ end of the other primer (Brownstein et al., 1996). OLIGO 7 (Rychlik, 2007) was used to reevaluate the quality of primers, and 75 primer pairs were selected for the subsequent preliminary trial on seven individuals of *H. incana* and eight individuals from the congeneric species (three individuals of *H. hookeri*, three individuals of *H. tenuifolia*, and two individuals of *H. palustris*; Appendix 1). The PCR mix for amplification (total volume 12.5 µL) contained: 6.25 µL of JumpStart REDTaq ReadyMix (Sigma-Aldrich, St. Louis, Missouri, USA), 0.25 µL of GTTT-tailed primer, 0.05 µL of CAG- or M13R-tailed primer, 0.25 µL of 5′ FAM-labeled universal CAG or M13R primer, and 0.5 µL of diluted DNA extract. The concentration of the primers was 10 pmol/µL (10 µM). A touchdown PCR protocol was used. The cycling conditions were: 95°C for 5 min (initial denaturation); 17 cycles with 95°C for 45 s (denaturation), 58–50°C for 90 s

TABLE 2. Genetic variation of the 15 polymorphic microsatellite markers in three populations of *Hypochaeris incana*.[a]

	Magallanes ($N = 27$)						Tierra del Fuego ($N = 26$)						Cerro La Buitrera ($N = 28$)					
Locus	Null[b]	A	N_{Geno}/N_{Ind}	H_e	H_o	F_{IS}	Null[b]	A	N_{Geno}/N_{Ind}	H_e	H_o	F_{IS}	Null[b]	A	N_{Geno}/N_{Ind}	H_e	H_o	F_{IS}
Hypinc_05	No	21	24/27	0.928	1.000	–0.079	No	17	20/26	0.916	1.000	–0.093	No	21	26/28	0.952	0.940	0.011
Hypinc_10	No	13	23/27	0.905	0.889	0.018	Yes	14	17/26	0.900	0.731	0.191	No	16	26/28	0.918	0.833	0.065
Hypinc_14	No	10	17/24	0.874	0.875	–0.001	No	6	13/25	0.819	0.760	0.073	No	11	19/28	0.788	0.750	–0.001
Hypinc_16	No	8	13/27	0.729	0.704	0.035	No	7	10/26	0.717	0.731	–0.019	No	10	21/28	0.753	0.827	–0.091
Hypinc_17	No	10	13/27	0.662	0.556	0.163	No	7	9/26	0.727	0.846	–0.168	No	13	23/28	0.858	0.815	0.029
Hypinc_24	Yes	13	20/26	0.913	0.462	0.499	Yes	12	19/25	0.903	0.640	0.295	No	29	24/26	0.959	0.872	0.084
Hypinc_26	No	14	16/21	0.862	0.762	0.118	No	11	12/25	0.754	0.680	0.100	No	16	20/28	0.786	0.458	0.373
Hypinc_28	No	5	8/27	0.568	0.593	–0.044	No	7	8/26	0.694	0.769	–0.111	No	19	24/28	0.920	0.863	0.059
Hypinc_33	Yes	11	16/25	0.861	NA	NA	Yes	11	12/25	0.849	NA	NA	Yes	17	21/26	0.911	NA	NA
Hypinc_41	Yes	6	9/27	0.722	NA	NA	Yes	6	9/26	0.702	NA	NA	Yes	8	13/28	0.729	NA	NA
Hypinc_42	No	5	8/27	0.657	0.481	0.271	No	6	11/26	0.755	0.846	–0.124	No	7	15/28	0.591	0.637	–0.045
Hypinc_49	Yes	5	9/27	0.746	0.519	0.309	No	7	11/26	0.793	0.846	–0.069	No	6	10/28	0.382	0.363	0.053
Hypinc_53	Yes	5	6/27	0.566	0.296	0.481	No	2	3/26	0.382	0.346	0.096	No	5	10/28	0.438	0.494	–0.068
Hypinc_56	Yes	5	7/19	0.765	NA	NA	Yes	9	12/24	0.749	NA	NA	Yes	8	19/28	0.760	NA	NA
Hypinc_59	Yes	11	16/27	0.831	0.519	0.380	No	7	9/26	0.631	0.615	0.026	No	14	21/28	0.832	0.708	0.157
Mean		9.5		0.773	0.638	0.179		8.6		0.753	0.734	0.016		13.3		0.772	0.713	0.052

Note: A = number of alleles; F_{IS} = inbreeding coefficient; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals used; N_{Geno} = number of genotypes; N_{Ind} = number of successfully scored individuals; NA = not applicable.

[a]Locality and voucher information are provided in Appendix 1.

[b]Significant evidence for the presence of a null allele.

TABLE 3. Cross-species amplification of the 15 polymorphic microsatellite markers developed for *Hypochaeris incana* in four related species.[a]

	H. hookeri (*N* = 8)			*H. tenuifolia* (*N* = 10)			*H. palustris* (*N* = 7)			*H. acaulis* (*N* = 7)		
Locus	Success	*A*	Allele size range (bp)	Success	*A*	Allele size range (bp)	Success	*A*	Allele size range (bp)	Success	*A*	Allele size range (bp)
Hypinc_05	++	4	200–206	++	12	216–238	++	4	208–230	++	2	202–204
Hypinc_10	++	9	251–267	++	11	239–269	++	3	249–261	++	2	275–281
Hypinc_14	++	3	276–292	+	5	284–292	++	2	292–294	++	3	288–292
Hypinc_16	++	2	326–328	++	7	320–332	++	3	322–328	++	1	328
Hypinc_17	++	4	358–370	++	10	360–388	++	3	366–378	++	3	374–378
Hypinc_24	++	5	159–169	++	9	134–198	++	2	134–150	—	NA	NA
Hypinc_26	++	8	258–276	+	3	266–272	++	2	244–250	++	1	250
Hypinc_28	++	9	412–438	++	7	412–454	++	3	424–436	++	2	433–445
Hypinc_33	++	8	260–282	++	12	264–306	—	NA	NA	—	NA	NA
Hypinc_41	++	6	161–171	++	4	159–165	++	2	159–161	++	1	167
Hypinc_42	—	NA	NA	+	2	435–438	++	1	420	—	NA	NA
Hypinc_49	++	2	322–327	+	3	327–336	++	3	333–348	+	1	339
Hypinc_53	—	NA	NA	—	NA	NA	—	NA	NA	—	NA	NA
Hypinc_56	++	5	310–316	++	6	308–322	+	1	320	+	1	302
Hypinc_59	—	NA	NA	—	NA	NA	++	2	180–185	+	1	178
Mean		5.4			7.0			2.4			1.6	

Note: ++ = successful amplification and scoring of all individuals; + = successful amplification and scoring of some individuals; — = failed amplification or ambiguous genotypes; *A* = number of alleles; *N* = number of individuals used; NA = not applicable.

[a]Locality and voucher information are provided in Appendix 1.

(annealing with a 0.5°C decrease per cycle), and 72°C for 60 s (extension); 20 cycles with 95°C for 45 s, 50°C for 90 s, and 72°C for 60 s; and 72°C for 5 min and 60°C for 30 min (final extension). PCR products were separated on a 3730xL DNA Analyzer (Applied Biosystems, Foster City, California, USA) at Microsynth (Balgach, Switzerland), and fragment sizes were estimated with GeneMarker 2.4 (SoftGenetics, State College, Pennsylvania, USA). Of the 75 microsatellite loci tested, 15 were clearly interpretable and polymorphic and were therefore selected for further study. The primers without the GTTT PIG-tails were labeled with a fluorescent dye at their 5′ end rather than with the previously used CAG or M13R tail and were used in multiplex PCR reactions (Table 1) to amplify a larger number of individuals of the five species. PCR was performed in a total volume of 20 μL containing 10 μL of JumpStart REDTaq ReadyMix, 0.4 μL of forward primer and 0.4 μL of reverse primer (each at a concentration of 10 μM) of each primer pair entering in the multiplex reaction, and 1 μL of diluted DNA extract, using the same cycling protocol described above. The PCR products were analyzed and scored as described above. In most cases, genotype assignment was unambiguous for diploid, triploid, and tetraploid cytotypes (Tables 2, 3).

We checked for the presence of null alleles in the two purely diploid populations as well as in the diploids of the mixed ploidy population (*N* = 14) of *H. incana* using the software MICRO-CHECKER version 2.2 with default settings (van Oosterhout et al., 2004). Three loci showed significant evidence of the presence of a null allele in all three populations (Table 2); for these loci, we adjusted diploid homozygous genotypes of *H. incana* by setting the state of the second allele to missing and adjusted tetraploid homozygous genotypes by setting the states of the third and fourth alleles to missing. One heterozygous triploid and one heterozygous tetraploid genotype demonstrated the suspected presence of a null allele based on peak heights; these were adjusted by setting one allele as missing in each case. Observed heterozygosity (H_o) and inbreeding coefficient (F_{IS}) are not reported for these loci. The number of alleles per locus, H_o, expected heterozygosity (H_e), and F_{IS} were calculated using SPAGeDi 1.5 (Hardy and Vekemans, 2002) by entering all (i.e., diploid, triploid, and tetraploid) individuals. All of the 15 microsatellite loci showed polymorphisms among the three populations of *H. incana* (Table 2). The number of alleles per locus and population ranged from two to 29. H_e and H_o ranged from 0.382 to 0.959 and 0.296 to 1.000, respectively. F_{IS} ranged from –0.168 to 0.499. Most of the 15 newly developed markers were successfully amplified and scored in the four congeneric species (Table 3). To assess the power of the markers to discriminate among species, we produced a Neighbor-Net split network based on a matrix of Rousset's (2000) interindividual differentiation with the software SplitsTree4 version 4.14.5 (Huson and Bryant, 2006) and performed a Bayesian admixture clustering analysis using the software Structure version 2.3.4 (Pritchard et al., 2000) assuming independent allele frequencies among populations. For each *K* from 2 to 13, we requested five independent runs with a burn-in period of 100,000 and 500,000 subsequent repetitions of the simulation. A typical run with *K* = 6 perfectly distinguished among species as well as between the two southern populations and the northern population of *H. incana*, with some indication of admixture in *H. tenuifolia* (Appendix S1).

CONCLUSIONS

We developed 15 polymorphic microsatellite markers for *H. incana*, which also worked well in some of the analyzed congeneric species. These 15 primer pairs will be suitable for studying the population clonal structure, genetic diversity, phylogenetic relationships, and interspecific hybridization in *H. incana* and its closest relatives.

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Appendix 1. Voucher information for *Hypochaeris* populations used in this study.

Species	Collectors and number/year (Herbaria)[a]	Collection locality (Geographic coordinates)	*N*	Ploidy level
Hypochaeris acaulis (J. Rémy) Britton	T. F. Stuessy & C. M. Baeza 15565/1999 (CONC, WU)[e]	Chile, Región VIII, Termas de Chillán, Valle da las Nieblas	4	2*x*[f,i]
Hypochaeris acaulis	T. F. Stuessy & C. M. Baeza 15571/1999 (CONC, WU)[e]	Chile, Región VII, Laguna del Maule	3	2*x*[j]
Hypochaeris hookeri Phil.	T. F. Stuessy, E. Urtubey & K. Tremetsberger 18019/2002 (LP, WU)[c,e]	Argentina, Prov. Río Negro, SE of Bariloche (41.20°S, 71.15°W)	1	2*x*[g]
Hypochaeris hookeri	T. F. Stuessy, E. Urtubey & K. Tremetsberger 18044/2002 (LP, WU)[c,e]	Argentina, Prov. Río Negro, Estancia Rayhuao S of Pilcaniyeu (41.29°S, 70.74°W)	7	2*x*[g]
Hypochaeris incana (Hook. & Arn.) Macloskie	A. Terrab & C. M. Baeza 31/2006 (SEV)[d]	Chile, Región XII, Provincia Magallanes (52.80°S, 71.17°W)	27	2*x*[h,i]
Hypochaeris incana	A. Terrab & C. M. Baeza 53/2006 (SEV)[d]	Chile, Región XII, Provincia Tierra del Fuego (53.27°S, 68.70°W)	26	2*x*[h]
Hypochaeris incana	E. Urtubey & K. Tremetsberger 454/2010, 454/2012 (LP, WHB)[b,c,d]	Argentina, Prov. Río Negro, Cerro La Buitrera SE of Bariloche (41.30°S, 71.14°W)	28	2*x* (*N* = 14), 3*x* (*N* = 2), 4*x* (*N* = 12)[i]
Hypochaeris palustris (Phil.) De Wild.	A. Terrab & C. M. Baeza 1/2006 (SEV)[c,e]	Chile, Región X, Volcán Hornopirén (41.88°S, 72.42°W)	4	2*x*[j]
Hypochaeris palustris	A. Terrab & C. M. Baeza 5/2006 (SEV)[c,e]	Chile, Región X, Volcán Rayhuen, Cerro Mirador (40.78°S, 72.18°W)	3	2*x*[j]
Hypochaeris tenuifolia (Hook. & Arn.) Griseb.	T. F. Stuessy & C. M. Baeza 15558/1999 (CONC, WU)[c,e]	Chile, Región VIII, Termas de Chillán	2	2*x*[j]
Hypochaeris tenuifolia	T. F. Stuessy & C. M. Baeza 15563/1999 (CONC, WU)[c,e]	Chile, Región VIII, Termas de Chillán	1	4*x*[i]
Hypochaeris tenuifolia	T. F. Stuessy & C. M. Baeza 15812/2000 (CONC, WU)[e]	Chile, Región IX, Volcán Lonquimay	5	2*x*[j]
Hypochaeris tenuifolia	T. F. Stuessy & C. M. Baeza 15823/2000 (CONC, WU)[c,e]	Chile, Región IX, Volcán Llaima	2	2*x*[j]

Note: N = number of individuals used.
[a] Herbarium code according to Index Herbariorum.
[b] Used for NGS run.
[c] Test individuals for screening of primer pairs.
[d] Test populations for assessment of genetic diversity in *H. incana*.
[e] Test populations for assessment of cross-amplification in related species.
[f] Weiss et al. (2003).
[g] Weiss-Schneeweiss et al. (2007).
[h] Tremetsberger et al. (2009).
[i] Determined by flow cytometry (C. König, unpublished data).
[j] Inferred from microsatellite peak patterns.