Microsatellite Markers for the Pilosella alpicola Group (Hieraciinae, Asteraceae) and Their Cross-Amplification in Other Hieraciinae Genera

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Primer Note

Microsatellite markers for the *Pilosella alpicola* group (Hieraciinae, Asteraceae) and their cross-amplification in other Hieraciinae genera[1]

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- *Premise of the study:* Microsatellite markers were developed for the *Pilosella alpicola* group (Asteraceae), comprising four closely related species distributed in subalpine areas of Europe. These species are believed to have diverged recently, but display contrasting cytogeographic patterns and variation in breeding systems, representing a promising model system for studying plant speciation, adaptation, and recent polyploidization.
- *Methods and Results:* We developed 17 microsatellite markers for the *P. alpicola* group using 454 sequencing. Sixteen markers were polymorphic, with the number of alleles per locus ranging from seven to 16 and observed and expected heterozygosity ranging from 0.45 to 0.84 and 0.72 to 0.92, respectively. Ten and five loci amplified in the related species, *P. echioides* and *P. officinarum*, respectively, but only two in *Andryala* and one in *Hieracium* s. str.
- *Conclusions:* The developed microsatellite markers have high potential to become useful tools to study microevolutionary processes in the *P. alpicola* group and related *Pilosella* species.

Key words: hybridization; *Pilosella alpicola*; polyploidy; population genetics; simple sequence repeat (SSR) markers.

The *Pilosella alpicola* group (Asteraceae) comprises four vicariant species of perennial herbs distributed disjunctly in subalpine areas of Europe (the Alps, Carpathians, and Balkan mountains). In this study, we followed the generic circumscriptions outlined by Bräutigam and Greuter (2007) in our treatment of the *P. alpicola* group. Our recent studies (Šingliarová et al., 2011a, b) showed that although the taxa of this group are very closely related and diverged relatively recently, they are morphologically well defined and display contrasting cytogeographic patterns and variation in breeding systems. Two species (*P. ullepitschii* (Błocki) Szeląg from the Carpathians and *P. serbica* (F. W. Schultz & Sch. Bip.) Szeląg from Serbia) are exclusively diploid and outcrossing. The Balkan taxon *P. rhodopea* (Griseb.) Szeląg represents a unique diploid-polyploid complex with up to five cytotypes (diploids to hexaploids) occurring mostly in mixed-ploidy populations. All *P. rhodopea* cytotypes are outcrossing, although polyploids exhibit severely reduced fertility (Šingliarová et al., 2011b). The alpine *P. alpicola* F. W. Schultz & Sch. Bip. (s. str.) has two allopatric cytotypes (tetraploids and pentaploids) with presumably polytopic allopolyploid origins and agamospermic mode of reproduction (Šingliarová et al., 2011a, b). All species of the *P. alpicola* group are rather rare, and their populations are often small and endangered by human activities. Recently, the conservation status of all taxa of the group was evaluated and their inclusion in relevant national Red Lists was proposed (Šingliarová et al., 2013). Interesting distributional patterns and a variety of evolutionary mechanisms playing a role in the differentiation of the *P. alpicola* group suggest it as a promising model system for studying plant speciation, adaptation, and recent polyploidization.

Microsatellite markers have already been developed for the related *P. officinarum* F. W. Schultz & Sch. Bip. (Zini and Komjanc, 2007) and the closely related genus *Hieracium* L. (s. str.) (Jönsson et al., 2010). The cross-amplification of the *P. officinarum* markers in other *Pilosella* Hill species and *Hieracium* s. str. gave unsatisfactory results (P. Trávníček, personal communication; Jönsson et al., 2010). Low cross-amplification success reported so far for the developed markers in *Hieracium* s. str. and *Pilosella* led us to deduce their poor transferability also to the *P. alpicola* group; therefore, a new set of markers is developed here. In addition, other *Pilosella*, *Hieracium* s. str., and *Andryala* L. taxa were used here to evaluate the potential cross-amplification of the developed

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markers in other phylogenetic lineages within the subtribe Hieraciinae (Fehrer et al., 2007; Krak and Mráz, 2008).

METHODS AND RESULTS

Total genomic DNA was extracted from fresh or silica gel–dried leaf material using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA from one diploid individual of *P. rhodopea* was used for 454 sequencing. Genomic library preparation and 1/4 run on the Roche GS FLX platform was performed at GATC Biotech (Konstanz, Germany). MSATCOMMANDER version 0.8.2 (Faircloth, 2008) was used to identify reads containing di-, tri-, tetra-, penta-, and hexanucleotide repeats and to design primers. Primers were successfully designed to 179 microsatellite-containing reads.

Sixty primer pairs were selected and tested for amplification in three diploid individuals, each representing a different species of the studied group. Each reaction contained 1× PCR buffer with KCl (Fermentas, St. Leon, Germany), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of both primers, 0.5 units of *Taq* DNA polymerase (Fermentas), and 20 ng of DNA. The cycling conditions were: 3 min at 95°C; followed by 35 cycles of 95°C for 30 s, locus-specific annealing temperature (see Table 1) for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. PCR products were checked on 2.5% agarose gels. Twenty-three primer pairs that amplified in the three tested species of the *P. alpicola* group were further applied to the complete set of 31 diploid individuals, originating from five distinct and geographically well-separated natural populations (Appendix 1). In this step, the amplification protocol was modified as described in Schuelke (2000) to facilitate fluorescent labeling of the PCR products by 6-FAM (Applied Biosystems, Foster City, California, USA). One microliter of each PCR product was mixed with 0.1 µL of GeneScan 500 LIZ internal size standard (Applied Biosystems) and 12 µL of Hi-Di formamide (Applied Biosystems) and electrophoresed using ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Allele calling was performed using GeneMarker version 2.4.0 (SoftGenetics, State College, Pennsylvania, USA). Six primer pairs amplified three or four alleles in the majority of samples, indicating possible duplications of these loci; these were therefore discarded. One locus, Palpi21, was invariable. Two other loci, Palpi25 and Palpi35, showed multiple peaks in four samples belonging to populations COZ, DOD, and MEN (see Appendix 1 for population localities). For these particular samples, the allele lengths were scored as missing data, and the markers were retained for genetic diversity estimates. For the summary information on the 16 polymorphic loci see Table 1.

The genetic diversity of these 16 loci was then estimated. Number of alleles per locus, expected and observed heterozygosity, and polymorphic information content were calculated based on allele frequencies for each population as well as for the complete data set using CERVUS version 3.0.3 (Field Genetics, London, United Kingdom). All markers showed a relatively high degree of polymorphism (Table 2). The number of detected alleles ranged from seven for Palpi1 to 16 for Palpi18, Palpi34, and Palpi37 (average 11.4). The observed heterozygosity ranged from 0.45 at Palpi32 to 0.84 at Palpi49 (average 0.64). The values of expected heterozygosity varied from 0.72 at Palpi1 to 0.92 at Palpi34 (average 0.85). The values of the polymorphic information content varied from 0.67 at Palpi1 to 0.89 at Palpi34 (average 0.81). The level of variability within the tested populations was comparable for the populations of

TABLE 1. Characteristics of 17 newly developed microsatellite markers for the *Pilosella alpicola* group. Locus Palpi21 is monomorphic; the 16 other loci are polymorphic.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	Cross-species amplification[a]	GenBank accession no.
Palpi1	F: TGCATTCCTTCGGTCCCAC	$(AT)_{13}$	232–247	55	—	KJ415260
	R: TGAAGAACATCGACATAATGCAC					
Palpi5	F: TGCATTCCTTCGGTCCCAC	$(AC)_{14}$	216–230	55	Po, Pe	KJ415261
	R: TGAAGAACATCGACATAATGCAC					
Palpi8	F: AGGCAATATACTATGTGTTGACCTG	$(AC)_{14}$	458–474	57	Po, Pe	KJ415262
	R: TCAGTGGCCCTCTATTTATACCC					
Palpi18	F: GCACGCATCTTACCAGACG	$(ATT)_{23}$	182–257	53	—	KJ415263
	R: TCGGTTAATCCTCTGGCGG					
Palpi19	F: TGTAATTCCGCTGCAAATCG	$(AAT)_{19}$	253–292	55	—	KJ415264
	R: TCACGAGTGGTTTCCTTACTC					
Palpi20	F: TGTTTGATGGCGATTCACGAG	$(AAT)_{20}$	331–383	53	—	KJ415265
	R: GCCCAGTTCCTTTGAACCC					
Palpi21[b]	F: GCCACAGCTTACGAGCATC	$(CT)_{12}$	220	55	NA	KR862872
	R: TTGCGGTGAAGACGGTG					
Palpi25	F: TCCCGCTGGTTCGAAAGAC	$(GT)_{12}$	142–176	55	Po, Pe	KJ415266
	R: GCCCTCAAGGCCTCAACC					
Palpi26	F: CCCATCTGGTTAAAGCGCC	$(AC)_{12}$	192–230	53	—	KJ415267
	R: TCGAAGTTGTTACGAGTTGCAG					
Palpi29	F: CTGCCATTGTTGGTGGCTC	$(AT)_{12}$	377–396	55	Po, Pe, Hu, Hs, Al, Ai	KJ415268
	R: AGGGATAGCACTTGCTGGG					
Palpi32	F: CTGTGAGGTACATACGTGGC	$(ATT)_{13}$	377–413	55	Pe	KJ415269
	R: TTTGTGGTTTGAGTTCACCG					
Palpi33	F: TCACTCATGAAATCTGTAACATCCG	$(AAT)_{11}$	224–273	56	Pe	KJ415270
	R: CCATAAAGGCAACTTTAGTCCC					
Palpi34	F: TGGTTAACGGTGGGCAGTC	$(AAC)_{12}$	406–436	52	Pe	KJ415271
	R: CTAGCAGGTGGCCTTGACC					
Palpi35	F: AAGTCGACACCTTTAGTCCC	$(ATT)_{17}$	243–274	54	—	KJ415272
	R: GCTGCTAATCCGTGGTCAG					
Palpi37	F: TCCTCTAAGTGGGTTCTTTGC	$(CTT)_{11}$	451–505	52	Po, Pe	KJ415273
	R: ATGCCTCACGAGACGTTCC					
Palpi45	F: GGCAGAAGGCTAAGAACTGC	$(AC)_{11}$	277–319	55	Pe	KJ415274
	R: GGAAGAATTTGATGGGTTGAC					
Palpi49	F: GGGCTGCCTATTCCCGTAG	$(AG)_{10}$	240–265	55	Pe, Ai	KJ415275
	R: GGGTGTATGACGACGAGTTTG					

Note: T_a = annealing temperature.

[a]Loci that were successfully cross-amplified in other Hieraciinae species: *Pilosella officinarum* (Po), *P. echioides* (Pe), *Hieracium umbellatum* (Hu), *H. stelligerum* (Hs), *Andryala laevitomentosa* (Al), and *A. integrifolia* (Ai); NA = information not available.

[b]Monomorphic locus.

TABLE 2. Genetic diversity parameters estimated for 16 polymorphic microsatellite loci for each analyzed population and the complete data set of the *Pilosella alpicola* group.

	P. rhodopea (population COZ; $n = 6$)				*P. rhodopea* (population DOD; $n = 5$)				*P. ullepitschii* (population MEN; $n = 7$)				*P. ullepitschii* (population SMARE; $n = 7$)				*P. serbica* (population KOP; $n = 6$)				Overall ($n = 31$)			
Locus	*A*	H_o	H_e	PIC	*A*	H_o	H_e	PIC	*A*	H_o	H_e	PIC	*A*	H_o	H_e	PIC	*A*	H_o	H_e	PIC	*A*	H_o	H_e	PIC
Palpi1	2	1	0.545	0.375	4	0.8	0.78	0.65	3	0.86	0.58	0.45	2	1	0.54	0.38	3	0.33	0.59	0.46	7	0.81	0.72	0.67
Palpi5	4	0.67	0.697	0.569	6	1	0.89	0.77	3	0.71	0.6	0.47	2	0	0.26	0.22	4	0.6	0.53	0.45	9	0.57	0.77	0.73
Palpi8	4	0.67	0.682	0.559	4	0.4	0.71	0.58	6	0.86	0.87	0.78	3	0.57	0.6	0.47	6	1	0.8	0.7	10	0.71	0.85	0.82
Palpi18	4	0.67	0.803	0.687	6	0.75	0.93	0.77	6	0.27	0.84	0.74	2	0	0.3	0.28	8	0.83	0.89	0.8	16	0.48	0.9	0.87
Palpi19	7	0.83	0.924	0.828	3	0	0.71	0.56	9	1	0.9	0.82	3	0.71	0.54	0.43	5	0.67	0.8	0.69	15	0.7	0.9	0.88
Palpi20	3	0.33	0.439	0.363	4	1	0.78	0.65	6	0.86	0.86	0.77	3	0.14	0.47	0.39	3	0.6	0.71	0.56	11	0.57	0.83	0.79
Palpi25	3	0.6	0.711	0.563	2	0.33	0.33	0.24	3	0.5	0.44	0.36	3	0.57	0.56	0.46	4	0.67	0.65	0.55	9	0.56	0.85	0.81
Palpi26	7	1	0.894	0.796	5	0.8	0.84	0.72	3	0.71	0.6	0.47	2	0	0.3	0.24	7	0.6	0.91	0.8	8	0.62	0.81	0.77
Palpi29	4	0.83	0.712	0.599	4	0.6	0.73	0.61	6	0.86	0.79	0.7	4	0.57	0.69	0.59	4	1	0.71	0.6	9	0.77	0.83	0.79
Palpi32	4	0.33	0.636	0.53	3	0.2	0.51	0.41	6	0.86	0.81	0.72	2	0.14	0.14	0.12	5	0.67	0.79	0.68	11	0.45	0.89	0.86
Palpi33	6	1	0.848	0.746	3	1	0.71	0.56	5	0.71	0.74	0.65	1	0	0	0	2	0.33	0.3	0.24	12	0.58	0.87	0.84
Palpi34	6	0.67	0.894	0.794	5	0.8	0.76	0.64	6	0.86	0.82	0.73	4	0.57	0.71	0.62	4	0.8	0.79	0.65	16	0.73	0.92	0.89
Palpi35	5	0.6	0.822	0.701	4	0.67	0.87	0.67	5	0.5	0.74	0.64	4	0.27	0.71	0.6	3	0.67	0.62	0.51	10	0.52	0.9	0.87
Palpi37	5	0.83	0.758	0.644	6	1	0.89	0.77	6	0.43	0.88	0.79	4	0.43	0.69	0.59	6	0.67	0.76	0.67	16	0.65	0.88	0.86
Palpi45	4	0.67	0.561	0.476	2	1	0.56	0.38	4	0.83	0.77	0.65	3	0.14	0.28	0.24	4	0.83	0.77	0.66	13	0.67	0.81	0.77
Palpi49	9	0.83	0.939	0.847	4	0.8	0.8	0.67	7	0.57	0.89	0.8	2	1	0.54	0.38	4	1	0.74	0.62	10	0.84	0.84	0.8
Average	4.18	0.72	0.74	0.63	4.06	0.7	0.74	0.6	5.3	0.71	0.76	0.67	2.8	0.38	0.46	0.38	4.5	0.7	0.71	0.6	11.4	0.64	0.85	0.81

Note: A = number of alleles per locus; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals; PIC = polymorphic information content.

P. rhodopea, *P. serbica*, and one population of *P. ullepitschii* (population MEN). The other population of *P. ullepitschii* (SMARE) showed lower values for all estimated parameters (see Table 2 for details).

To evaluate the potential utility of the newly developed markers in other phylogenetic lineages of the Asteraceae subtribe Hieraciinae (Fehrer et al., 2007; Krak and Mráz, 2008), cross-amplification experiments were performed in one individual of *P. echioides* F. W. Schultz & Sch. Bip., *P. officinarum*, *Hieracium umbellatum* L., *H. stelligerum* Froel., *Andryala laevitomentosa* (Nyár. ex Sennikov) Greuter, and *A. integrifolia* L. The results of cross-amplification tests are summarized in Table 1. Higher cross-amplification success was reached within the genus *Pilosella*, where 10 markers could be cross-amplified in *P. echioides* and five in *P. officinarum*. Only one marker (Palpi29) was cross-amplified in all tested taxa. No other marker gave a positive result in the three *Hieracium* s. str. species and *A. laevitomentosa*. In *A. integrifolia*, cross-amplification of Palpi49 was positive as well.

CONCLUSIONS

In this study, we have developed 17 novel microsatellite markers for the *P. alpicola* group. Although only a relatively small sample set was used to evaluate their variability, 16 markers showed high levels of polymorphism. The transferability of these markers to two additional *Pilosella* species and to the closely related genera *Andryala* and *Hieracium* s. str. is restricted. Five markers were cross-amplified in *P. officinarum* and 10 in *P. echioides*, suggesting high species specificity of these markers. The cross-amplification in the other Hieraciinae genera was even less successful. Only one marker amplified in all four species and an additional one in *A. integrifolia*.

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Appendix 1. Locality and voucher information of the *Pilosella alpicola* group and representatives of other lineages within the subtribe Hieraciinae used for the current study.

Species	Population code	Voucher specimen accession no.	Collection locality[a]	Geographic coordinates	*n*
Pilosella rhodopea (Griseb.) Szeląg	COZ	BS_COZ_6, 10, 12, 13, 19, 20[b]	RO; Cozia Mtns., Mt. Cozia, 1592 m a.s.l.	45°19′04″N, 24°20′17″E	6
	DOD	BS_DOD_1, 6–8, 10[b]	BG; Rila Mtns., Mt. Dodov vrah, 2540 m a.s.l.	42°09′59″N, 23°20′23″E	5
P. serbica (F. W. Schultz & Sch. Bip.) Szeląg	KOP	BS_KOP_1, 2, 4–7[b]	RS; Kopaonik Mtns., Mt. Suvo Rudište, 1917 m a.s.l.	43°16′28″N, 20°48′55″E	6
P. ullepitschii (Błocki) Szeląg	MEN	BS_MEN_12, 14, 18, 19, 27, 29, 30[b]	SK; Vysoké Tatry Mtns., Mengusovská dolina valley, 1800–1875 m a.s.l.	49°09′57″N, 20°03′40″E	7
	SMARE	BS_SMARE_11–15, 17, 18[b]	RO; Nemira Mtns., Mt. Sandru Mare, 1590–1640 m a.s.l.	46°11′57″N, 26°20′21″E	7
P. officinarum F. W. Schultz & Sch. Bip.	1484	PRA_PV_1484[c]	CZ; Prague, Bohnice–Zámky, 205 m a.s.l.	50°08′26″N, 14°23′56″E	1[d]
P. echioides F. W. Schultz & Sch. Bip.	TR608	PRA_PV_TR608[c]	CZ; Beroun, Trubínský vrch hill, 325 m a.s.l.	49°56′37″N, 13°59′47″E	1[d]
Hieracium umbellatum L.	1021	PRA_PV_1021[c]	PL; Województwo pomorskie, Baltic coast, 5 m a.s.l.	54°20′00″N, 19°02′27″E	1[d]
H. stelligerum Froel.	1233	PRA_KK_1233[c]	FR; Vallon Pont d'Arc, along the road D 390, 500 m a.s.l.	44°24′25″N, 04°24′10″E	1[d]
H. intybaceum All.	2509a	PRA_KK_2509a[c]	AT; Tirol, Tuxer Alpen, 3.3 km NE of the village of Navis, 1878 m a.s.l.	47°09′22″N, 11°33′48″E	1[d]
Andryala integrifolia L.	AZ3	PRA_JZ_AZ3[c]	DZ; Alger, town district of Kouba, 90 m a.s.l.	36°43′00″N, 03°05′00″E	1[d]
A. laevitomentosa (Nyár. ex Sennikov) Greuter	D5	PRA_JZ_D5[c]	RO; Bukovina, Mt. Pietrosul Broştenilor, 1780 m a.s.l.	47°22′31″N, 25°32′29″E	1[d]

Note: *n* = number of individuals.

[a]Abbreviations for countries: AT = Austria, BG = Bulgaria, CZ = Czech Republic, DZ = Algeria, FR = France, PL = Poland, RO = Romania, SK = Slovakia, RS = Serbia.

[b]Voucher specimens deposited in the herbarium of the Institute of Botany, Slovak Academy of Sciences (SAV), Bratislava, Slovakia.

[c]Voucher specimens deposited in the herbarium of the Institute of Botany, Czech Academy of Sciences (PRA), Prague, Czech Republic.

[d]Samples used to evaluate the potential cross-amplification of the markers within Hieraciinae.