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MICROSATELLITE LETTERS

Development and characterization of microsatellite loci for the primrose *Primula vulgaris* and successful cross-amplification in the congeneric *P. elatior* and *P. veris*

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Abstract The perennial herb *Primula vulgaris* (Primulaceae) faces severe fragmentation of its natural forest habitat in the northern border of its distribution due to intensive agricultural practices and increased urbanisation. In Belgium and northern France, *P. vulgaris* populations show dramatic declines that could lead to local extinctions. Here we describe 12 novel microsatellite loci obtained by 454-pyrosequencing of a microsatellite-enriched library. Number of alleles per polymorphic locus varied between 2 and 6, and observed heterozygosity ranged from 0.16 to 0.73. Furthermore, sets of 8 loci successfully cross-amplified in the congeneric *P. elatior* and *P. veris*. These markers will be of great value for genetic diversity and conservation studies.

Keywords 454-pyrosequencing · Fragmentation · Microsatellites · *Primula elatior* · *Primula veris* · *Primula vulgaris*

Primula vulgaris Huds. (=*Primula acaulis* (L.) L.) is a diploid, self-incompatible (distylous), long-lived perennial herb that flowers in early spring. It has a North-Atlantic and Mediterranean European distribution, growing in humid, shaded habitats in woodlands and open grasslands (Jacquemyn et al. 2009). In west-central Europe, *P. vulgaris* is a rare, declining species, with highly fragmented populations due to habitat destruction as a consequence of urbanisation and intensive agricultural practices (Brys et al. 2004). Here we characterize 12 novel microsatellite loci for this species that, in combination with the 3 loci previously reported by Van Geert et al. (2006), will contribute to gain insights into population genetic diversity and structure which can help to evaluate the species' conservation status. Most of these microsatellites successfully amplified in the related species *Primula elatior* (L.) L., which is threatened in the southern margin of its distribution range; and *Primula veris* L., for which these microsatellites will complement others previously developed by 454 pyrosequencing (Bickler et al. 2013).

Genomic DNA was extracted from dried leaf samples using either DNeasy Plant Mini Kit (Qiagen) or Invisorb Spin Plant Mini Kit (Stratec Molecular). Microsatellite loci were isolated by Genoscreen (Lille, France) using a microsatellite enrichment method coupled to 454 GS-FLX Titanium next-generation sequencing (Malausa et al. 2011). Sequences of the oligonucleotide probes used were: TG, TC, AAC, AAG, AGG, ACG, ACAT and ACTC. A total of 10,598 sequence reads were obtained, from which 154 primer pairs were designed for potential microsatellite loci. We selected a set of 72 primers for amplification trials on 15 *P. vulgaris* individuals from four populations. For amplification trials, PCR reactions were conducted separately for each marker. Forward primers were attached with a 'M13-tail' (5′-CAGTCGGGCGTCATCA-3′) to allow fluorescent labelling. PCR reactions (20 μL) contained c.100 ng of template DNA, 1× BSA, 1× reaction buffer, 3.5 mM $MgCl_2$, 0.3 μM of each forward, reverse and universal 'M13' primers tagged with one of FAM, VIC, NED or PET, 0.25 mM dNTP and 0.5 U *Taq* DNA polymerase. PCR conditions used were: 95 °C for 3 min, 19 cycles of 95 °C for 1 min, 55 °C for 30 s with a decrease

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Table 1 Characteristics of 12 microsatellite loci developed for *P. vulgaris*

Locus	Genbank accession number	Primer sequence (5′–3′)	Motif
Paca-3	KJ134997	F: ACGTTGATTAGTTTTACCGGTACTT	$(TC)_4$
		R: CCAGACAAATCCAAAATGGG	
Paca-11	KJ134992	F: TTCGTGATGAAGTTGACTTTTATG	$(TC)_{12}$
		R: AAACAGCAATATCAGAGTCCAGA	
Paca-22	KJ134993	F: CCCTTAGATACCAACAAAGGTCA	$(GACTAC)_6$
		R: GGAGTCATCTTCCATCAAA	
Paca-38	KJ134995	F: ACATTCAGCGAGTGATTTGG	$(AAC)_5$
		R: GCAGAGCGTGGTCTATGTT	
Paca-44	KJ134996	F: CCTTCCGGCGGATTTAGT	$(TC)_6$
		R: AGAATTATACGATTCAACTTGGAAA	
Paca-45	KJ135000	F: AAAGCAGTAATCAATAACTGAAGAAA	$(AG)_8$
		R: CACTCAATAGTCAATGTGCATAGG	
Paca-74	KJ134998	F: GAGCTTTTAACCCATATCCCA	$(AC)_{13}$
		R: GGCCAAATAATGAAATAAGCTAGA	
Paca-78	KJ135002	F: TGTGCGACTGCCTCTATCTC	$(CT)_{11}$
		R: GACTGAGAAGACATATGTTGAAAGA	
Paca-129	KJ135001	F: ATTATATTTTCGCTTCGTACAATTAAA	$(GAG)_5$
		R: GAATCCAGCAACTTTTCTCCA	
Paca-150	KJ134999	F: GATGCTTCTCAGTTCCAGCA	$(AAG)_6$
		R: CAAAGAACCTCCTCTTACTTCA	
Paca-218	KJ134994	F: CATAATTTGGGTTTTAATGGAATTT	$(AG)_8$
		R: AGAATCATGGGTCCAAGTCG	
Paca-404	KJ135003	F: GAAGACAAAACAGGCGGAGA	$(CT)_{11}$
		R: GTGGGAGAAAAGCATGGAAA	

F forward primer, *R* reverse primer

Table 2 Population statistics of 12 microsatellite loci developed for *P. vulgaris* ('La Cabrilla', Sierra de Cazorla, Spain, N37°55′42.2″, W2°46′57.6″) and successfully cross-amplified in *P. elatior* ('Aalsbos', Belgium, N50°43′57.0″, E5°48′36.0″) and *P. veris* ('Welberg', Belgium, N50°44′19.5″, E5°50′55.5″)

Locus	*P. vulgaris*					*P. elatior*					*P. veris*				
	N	Size range (bp)	N_A	H_O	H_E	N	Size range (bp)	N_A	H_O	H_E	N	Size range (bp)	N_A	H_O	H_E
Paca-3	30	105–105	1	0.000	0.000	0	–	0	–	–	0	–	0	–	–
Paca-11	30	94–102	5	0.733	0.771	17	94–98	2	0.471	0.428	19	94–104	2	0.158	0.145
Paca-22	30	105–129	4	0.300	0.295	9	113–113	1[a]	0.000	0.513	0	–	0	–	–
Paca-38	30	197–215	4	0.667	0.699	17	205–207	2	0.412	0.515	19	205–207	2	0.211	0.332
Paca-44	30	296–300	3	0.267	0.352	17	294–294	1	0.000	0.000	19	294–298	2[a.b]	0.000	0.332
Paca-45	30	132–154	6	0.500	0.594	0	–	0	–	–	0	–	0	–	–
Paca-74	30	161–163	2	0.333	0.499	0	–	0	–	–	14	161–165	3[a]	0.429	0.426
Paca-78	30	132–138	4	0.233	0.245	16	145–147	2	0.375	0.315	18	121–123	2	0.667	0.475
Paca-129	30	184–199	3	0.533	0.505	17	187–187	1	0.000	0.000	18	187–187	1[a]	0.000	0.000
Paca-150	30	179–185	3	0.333	0.402	17	174–174	1	0.000	0.000	19	173–185	4	0.789	0.716
Paca-218	30	165–167	2	0.467	0.508	0	–	0	–	–	0	–	0	–	–
Paca-404	30	168–179	3	0.500	0.569	17	165–169	3	0.765	0.551	16	165–182	4[a]	0.438	0.605

N_A number of alleles, H_O observed heterozygosity, H_E expected heterozygosity

[a] Significant frequency of null alleles

[b] Significantly deviated from Hardy-Weinberg equilibrium

of 0.5 °C every cycle, 72 °C for 30 s, and 19 cycles of 95 °C for 1 min, 45 °C for 30 s and 72 °C for 30 s, with a final extension step of 72 °C for 10 min.

Fifty-six loci amplified products of expected size, 41 % of which were polymorphic. We selected 12 polymorphic loci which allowed clear scoring (Table 1) and further genotyped 30 *P. vulgaris* individuals from a single site to determine population statistics (Table 2). Two multiplex PCR reactions using fluorescently labelled forward primers were optimized. We adopted the same method to test cross-amplification in *P. elatior* and *P. veris*. Multiplex PCR (20 μL) contained: c.120 ng of template DNA, 1× BSA, 1× reaction buffer, 3.5 mM $MgCl_2$, 0.2 μM of forward primer, 0.2 μM of reverse primer, 0.25 mM dNTP and 2 U *Taq* DNA polymerase. Conditions for multiplex PCR were: 95 °C for 3 min, 38 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min and a final extension of 72 °C for 3 min. PCR products were analysed in an ABI PRISM 3130xl sequencer, sized using 500 LIZ size standard and scored using GENEMAPPER v4.0 (Applied Biosystems).

Across the three species, number of alleles per polymorphic locus varied between 2 and 6 (mean = 3). *Paca-3* revealed polymorphism in the amplification trials but appeared to be monomorphic in the sampled population. Observed heterozygosity in the polymorphic loci varied between 0.158 and 0.789, and expected heterozygosity between 0.145–0.771. Only *Paca-44* deviated from Hardy-Weinberg equilibrium in *P. veris*, which can be attributed to the presence of null alleles with an estimated probability (p) of 0.26. Other loci were also expected to present null alleles in *P. veris*: *Paca-74* ($p = 0.22$), *Paca-129* ($p = 0.22$) and *Paca-404* ($p = 0.23$); and in *P. elatior*, *Paca-22* ($p = 0.65$). None of the loci pairs showed evidence of linkage disequilibrium in either species.

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