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TECHNICAL NOTE

# Isolation and characterization of microsatellite loci in *Pulsatilla patens* (L.) Mill. (Ranunculaceae) a rare and endangered plant species in Europe

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**Abstract** *Pulsatilla patens* is a rare and endangered plant species in many areas of Europe and protected under the Bern Convention and it is listed in Annex II and Annex IV to the Habitats Directive. In this study we developed 12 novel microsatellite loci using via 454 sequencing. We determined 11,220 contigs with a length of 156–11,384 bp. Within this dataset, we identified 319 SSR motifs in 301 contigs. All markers were genotyped on 56 individuals from three populations located in Poland. The number of alleles and expected heterozygosity were 2–12 (mean 3.7) and 0.142–0.820 (0.541 on average) respectively. The markers described in this study will be useful for evaluating genetic diversity of *P. patens* populations, could be applied to investigate the biological aspects and to develop effective conservation programs for the European populations of this species.

**Keywords** *Pulsatilla patens* · SSR markers · 454 Sequencing

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*Pulsatilla patens* (L.) Mill. (Ranunculaceae) is a long-lived perennial herb. It is a lowland species with a circumpolar range, found in all three continents in the northern hemisphere (Hulten and Fries 1986). *Pulsatilla patens* is widespread in Central and Eastern Europe, with its western range extending to Sweden and Germany (Akeroyd 1993). In North America, the species occurs mostly in the Central and Western United States, in Central and Northwestern Canada and in Eastern Alaska. *Pulsatilla patens* shows a preference for dry, sun-exposed sites. In Europe, it can be found in thermophilous grasslands and coniferous forest. Within its range in Europe, *P. patens* is considered to be critically endangered in many areas. The species is protected under the *Bern Convention*, and it is listed in Annex II and Annex IV to the Habitats Directive (Council of Europe 1979; European Communities 2004) due to a small number of localities, low abundance and the gradual disappearance of populations. The species decline is related to changes in land use, especially in forestry practices where efficient wildfire prevention and termination of cattle grazing in forests have led to the formation of a continuous moss layer or strongly grass dominated vegetation, which severely hinders the regeneration of *P. patens* (Kalamees et al. 2005). Long-term protection and management plans aimed at preserving *P. patens* populations should involve habitat and environmental monitoring as well as the quantification of genetic diversity within and among populations. Microsatellite markers, also referred to as simple sequence repeat (SSR) markers, are widely used in ecological studies and can also be employed to investigate the genetic diversity of populations. In this study, we developed nuclear microsatellite markers for *P. patens* with the involvement of GS Junior next generation sequencing (Roche 454 Life Sciences, Branford, CT, USA).

**Table 1** Characteristics of 12 compound microsatellite loci for *P. patens*

| Locus | Repeat motif | Size range (bp) | Primer sequence | Ta (°C) | No. of total alleles | Gen bank accession no. |
|---|---|---|---|---|---|---|
| Pul01 | $(GCT)_4$ | 136–139 | F-CACCTTGTCCACGGTTCTG | 53 | 4 | JX847585 |
| | | | R-ACCAGGTCAGAGAGCTCAAC | | | |
| Pul02 | $(AC)_6$ | 325–330 | F-TGAGTTCTTGCACTTCAGGG | 51 | 4 | JX847586 |
| | | | R-AATCCCACGAGTTAGTGCC | | | |
| Pul03 | $(GAT)_5$ | 169–173 | F-AGGTTGGAGGAAGCTTTAATGG | 50 | 4 | JX847587 |
| | | | R-TCCGGTGAACTCGAAGC | | | |
| Pul04 | $(CT)_6$ | 249–260 | F-ACCGTTACTGTCCAACGGG | 53 | 11 | JX847588 |
| | | | R-CCTGTATGAATGCAACTTGACG | | | |
| Pul05 | $(CT)_8$ | 270–276 | F-GATTAATGGCGGGCGACAG | 54 | 6 | JX847589 |
| | | | R-TGGGTGTCGCTAATCGAGG | | | |
| Pul06 | $(ATT)_4$ | 184–189 | F-TGGCATTCCTAGTTGAGGATGG | 55 | 5 | JX847590 |
| | | | R-GCTAGACAAACAAGAATCCCTGC | | | |
| Pul07 | $(AG)_6$ | 334–336 | F-ATCCCGAGGGAGAATGCAC | 51 | 2 | JX847591 |
| | | | R-AAGCATGAGGTGTCTTGGC | | | |
| Pul08 | $(GAT)_4$ | 353–365 | F-AGGTGTCTGATTCCATACGGG | 54 | 3 | JX847592 |
| | | | R-GATCTTCTTCACGGAGCCAC | | | |
| Pul09 | $(CTT)_4$ | 387–392 | F-TGCACCTCCCGTCCAATTC | 53 | 5 | JX847593 |
| | | | R-GGTCCCTCGGTGGTCATAC | | | |
| Pul10 | $(ATC)_4$ | 108–111 | F-TTCCAAGCTCCGGCCAC | 52 | 4 | JX847594 |
| | | | R-ACCGGTTGAGACACCCAAG | | | |
| Pul11 | $(CTT)_5$ | 306–312 | F-TCAATCAACCGCATGTAGAGC | 52 | 5 | JX847595 |
| | | | R-CACGTGTATTCGGCAGTCAG | | | |
| Pul12 | $(AT)_6$ | 222–228 | F-GGGACCGGCAATGCAAAC | 54 | 7 | JX847596 |
| | | | R-CTAGTCGCTCCCAAGCCC | | | |

High-throughput next generation sequencing enables the identification of even several hundred polymorphic loci in a single run (Parchman et al. 2010). To date, this technique has been successfully applied in animal, plant and bryophyte research (Abderkim et al. 2009; Szczecińska et al. 2012; Sawicki et al. 2012). Twelve microsatellite markers developed for *P. patens* are characterized in this paper.

Total genomic DNA was extracted from the leaf tissue of 56 individuals from 3 populations located in Poland (Supraśl n = 27, Rudne n = 16 and Orzysz n = 13), respectively using the DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany). The library preparation and 454 sequencing was the same as described in our previous studies (Szczecińska et al. 2012; Sawicki et al. 2012). A two-step assembly was performed. First, the obtained sequences were assembled using the chloroplast genome of *Ranunculus macranthus* (NC008796) to separate chloroplast reads from nuclear reads. 3,703 out of 144,182 reads were assembled to chloroplast genome. The remaining reads were *de novo* assembled into 11,220 contigs with a length of 156–11,384 bp using the GS Newbler *de novo* assembler (Roche/454 Life Sciences).

Analysis of the obtained contigs with the MSTAT-COMMANDER software (Faircloth 2008) identified 319 SSR motifs in 301 contigs. Di- (92) and tri-nucleotide (210) repeats dominated among the discovered microsatellite motifs. Longer repeat motifs included seven tetra-, two penta- and eight hexa-nucleotide motifs. Among identified SSR motifs we designed primers for 60 of them.

Amplification, PCR conditions and electrophoresis followed Szczecińska et al. (2012). Genetic diversity measures were estimated using GenAlEx 6.41 software (Peakall and Smouse 2006). Deviations from the Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium between loci were tested using FSTAT software version 2.9.3 (Goudet 1995). Significance levels were adjusted using Bonferroni correction for multiple testing. The sequences of the SSR fragments were deposited in the GenBank (Table 1).

The 12 microsatellite loci identified in the study showed a clear, single peak for each allele. These 12 loci were subsequently used to screen 56 individuals collected from three polish populations of *P. patens*. In the studied populations all loci showed polymorphism (Table 1).

The number of alleles per locus ranged from two to eleven, with an average of 4.4 in the Supraśl population, 3.6 in the Rudne population and 3.1 in the Orzysz population. The expected ($H_E$) and observed ($H_O$) heterozygosities

**Table 2** Results of initial simple sequence repeats (SSR) polymorphism analysis in three populations of *P. patens*

| Locus | Supraśl N = 27 | | | | Rudne N = 16 | | | | Orzysz N = 13 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $N_A$ | $H_O$ | $H_E$ | F | $N_A$ | $H_O$ | $H_E$ | F | $N_A$ | $H_O$ | $H_E$ | F |
| Pul01 | 2 | 0.000 | 0.466 | 1.000 | 2 | 0.000 | 0.375 | 1.000 | 3 | 0.000 | 0.651 | 1.000 |
| Pul02 | 4 | 0.000 | 0.513 | 1.000 | 2 | 0.000 | 0.469 | 1.000 | 2 | 0.000 | 0.142 | 1.000 |
| Pul03 | 4 | 0.259 | 0.558 | 0.535 | 3 | 0.250 | 0.404* | 0.382 | 3 | 0.154 | 0.568 | 0.729 |
| Pul04 | 9 | 0.185 | 0.820 | 0.774 | 8 | 0.438 | 0.834 | 0.475 | 5 | 0.154 | 0.669 | 0.770 |
| Pul05 | 5 | 0.111 | 0.560 | 0.802 | 4 | 0.000 | 0.484 | 1.000 | 2 | 0.000 | 0.473 | 1.000 |
| Pul06 | 5 | 0.000 | 0.590 | 1.000 | 4 | 0.000 | 0.602 | 1.000 | 3 | 0.000 | 0.272 | 1.000 |
| Pul07 | 2 | 0.000 | 0.346 | 1.000 | 2 | 0.000 | 0.305 | 1.000 | 2 | 0.000 | 0.426 | 1.000 |
| Pul08 | 3 | 0.000 | 0.532 | 1.000 | 3 | 0.000 | 0.320 | 1.000 | 3 | 0.000 | 0.556 | 1.000 |
| Pul09 | 5 | 0.000 | 0.628 | 1.000 | 4 | 0.000 | 0.648 | 1.000 | 4 | 0.000 | 0.556 | 1.000 |
| Pul10 | 3 | 0.000 | 0.631 | 1.000 | 4 | 0.000 | 0.688 | 1.000 | 3 | 0.000 | 0.639 | 1.000 |
| Pul11 | 5 | 0.000 | 0.601 | 1.000 | 2 | 0.000 | 0.469 | 1.000 | 3 | 0.000 | 0.604 | 1.000 |
| Pul12 | 6 | 0.222 | 0.680 | 0.673 | 6 | 0.313 | 0.787 | 0.603 | 5 | 0.385 | 0.618 | 0.378 |
| Mean | 4.4 | 0.065 | 0.577 | 0.899 | 3.66 | 0.029 | 0.083 | 0.532 | 3.16 | 0.058 | 0.515 | 0.906 |

$N_A$ number of alleles, $H_O$ observed heterozygosites, $H_E$ expected heterozygosites, $F$ fixation index

A significant deviation from Hardy–Weinberg equilibrium expectations * ($p < 0.05$)

ranged from 0.142 to 0.820 (0.541 on average), and from 0.000 to 0.438 (0.069 on average), respectively. The values of coefficients $H_E$ and $H_O$ were similar in the three analyzed populations (Table 2). Significant deviations ($P < 0.05$) from HWE were detected for locus *Pul03* in the Rudne population, which suggests the presence of null alleles. Significant LD were noted between two pairs of loci: Pul07/Pul11 and Pul04/Pul05.

The microsatellite markers presented here should be useful for measuring genetic diversity with in and between populations, and gene flow between *P. patens* populations. These markers could be also applied to investigate the biological aspects of *P. patens*, and will be useful for conservation genetic studies this rare and endangered species.

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