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MICROSATELLITE LOCI FOR ENDANGERED MOUNTAIN PLANT: TRANSFER FROM TETRAPLOID TO DIPLOID SPECIES IN SUBGENUS *PULSATILLA* (RANUNCULACEAE)

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Abstract

Mountain plants adapted to specific environments are increasingly threatened by global warming. A major concern is forced altitudinal shift, habitat fragmentation, disruption of plant-pollinator interactions, or challenges from competitors and invasive species. Numerous studies shed light on these complex dynamics and highlight the need for immediate conservation action, with maintaining the greatest possible genetic diversity in populations as one of the main goals. One such species threatened by climate change that is on the IUCN World Red List is the spring pasqueflower *Pulsatilla vernalis* (L.) Mill (Ranunculaceae). Our study describes recent advances and applications of nuclear microsatellite markers in the *Pulsatilla* subgenus, which includes both diploid and tetraploid species. To date, few studies have been conducted on the genetic structure of *P. vernalis* populations, and no specific markers have been developed for this purpose. In the present study, we attempted to amplify microsatellite loci for diploid *P. vernalis* using a cross-species procedure originally developed for tetraploid *P. vulgaris*. Using this procedure, eight polymorphic markers were successfully transferred to *P. vernalis*. The selected loci were tested on samples collected from relict lowland populations that are nearly extinct in Central Europe. Analysis of 82 individuals from three Polish lowland populations revealed that these markers were highly polymorphic, with two to 13 alleles per locus and observed heterozygosity ranging from 0.037 to 0.654. FIS coefficient values were not statistically significant for any of the loci (p>0.05) and ranged from -0.200 to 0.696. We tested how informative cross-amplified loci distinguished individuals and which loci deviated from Hardy-Weinberg Equilibrium. We conclude that the microsatellite markers tested in this study can be successfully used for population genetic studies of *P. vernalis*.

Key words: *Pulsatilla vernalis*; Genetic diversity; SSR markers; Polymorphism.

Introduction

Mountain ecosystems, characterized by unique biodiversity and complicated ecological processes, are particularly vulnerable to the effects of climate warming (Elsen & Tingley, 2015). Rising temperatures can cause a shift in altitudinal zones, forcing plant and animal species to migrate or become extinct. Mountain plants adapted to certain cold environments are not only forced to move at altitude, but also to fragment their habitat, disrupt plant-pollinator interactions, or face challenges from competitors and invasive species (Engler *et al*., 2011).

One such species threatened by climate change that is on the IUCN World Red List is the spring pasqueflower *Pulsatilla vernalis* (L.) Mill (Ranunculaceae) (Chappuis, 2014). *Pulsatilla vernalis* is a species endemic to Europe, typically found in the mountains of western and central Europe and in the Scandinavian chains. It also occurs in lowland areas in the central and northern European plains, where it is referred to as a glacial relict (Ronikier *et al*., 2008; Kiedrzyński *et al*., 2017). Lowland populations are most at risk of extinction; they are highly unstable and often consist of only a small number of individuals (Grzyl & Ronikier, 2011). In Poland, the population of *P. vernalis* has experienced a drastic decline in recent decades and is considered one of the rarest plants in the national flora (Grzyl & Ronikier, 2011; Ronikier, Grzyl & Wójtowicz, 2014). In Germany, only a few relict populations are known from the Bavarian region, which are threatened with extinction (Scheuerer, 1996). Numerous lowland populations are still reported from the northern European lowlands in Scandinavia and Russia, while others in the Czech Republic and Belarus are considered to have disappeared (Grzyl & Ronikier, 2011; Chappuis, 2014; Ronikier, Grzyl & Wójtowicz, 2014).

Many studies shed light on the complex dynamics of mountain biota and emphasize the need for immediate conservation action, with maintaining the greatest possible genetic diversity in populations as one of the main goals (Qian *et al*., 2021). Genetic diversity is the basis for species adaptability and enables populations to cope with environmental changes and various stressors (Hohenlohe *et al*., 2021). Therefore, conservation strategies must prioritize genetic diversity as an integral component and recognize its critical role in conserving species and ecosystems. Widespread interest in endangered *Pulsatilla* species throughout the Northern Hemisphere is prompting researchers to develop and compare different genetic tools, including traditional neutral SSR (single sequence repeats) markers and adaptive ISJ (intron-exon splice junctions) markers (example for *P. patens* from Bilska & Szczecińska (2016)). In addition, recent advances in sequencing technology enable the description of *Pulsatilla* chloroplast and mitochondrial genomes (Szczecińska & Sawicki, 2015; Szandar *et al*., 2022), and even more, performing a comparative analysis of organellar genomes enables the evaluation of their effectiveness in studies of population variability and genetic structure (Szandar *et al*., 2023).

In the case of *P. vernalis*, immediate and more intensive monitoring of existing populations is needed. Considering the rapid decline and specific habitat requirements, especially in lowland populations (Zielińska *et al*., 2016), the search for low-cost and effective genetic tools is needed. Apart from dominant markers such as

AFLP (Ronikier, 2002; Betz *et al*., 2013), no codominant have been used to study the genetic structure and diversity of *P. vernalis* populations. Therefore, the present study was conducted to evaluate the potential of previously developed SSR markers in the subgenus *Pulsatilla* for microsatellite loci analysis in *P. vernalis* and to assess their utility for population studies of this endangered species.

Microsatellite markers are an effective tool for studying genetic variation among and within populations. Microsatellites are defined as tandem repeats of short (2 to 6 nucleotides) DNA motifs that form more or less uniform segments up to 100 nucleotides in length. Because these genetic markers are noncoding DNA segments, mutations that may occur in them generally do not result in changes in the fitness of a living organism and are not repaired (Chambers & MacAvoy, 2000). Therefore, the mutation rate of microsatellites is high and consequently such repetitive sequences are polymorphic. As such, they have been used as genetic markers for various purposes, such as genomics and genetic disease detection, paternity determination, forensics, animal breeding monitoring, population genetics research, and reconstruction of gene flow between populations in the recent past (Selkoe & Toonen, 2006). Because of the above properties, microsatellite markers are used for population genetic studies.

Unfortunately, the selection of new SSR markers involves a significant financial and time investment. A much faster and less expensive alternative is the use of cross-amplification, i.e., the use of already evolved loci from related species. However, this approach has its own limitations, since the success of amplification depends mainly on the degree of relatedness of the species under study, and even in the case of successfully amplified loci, their degree of polymorphism may not be satisfactory and they may have monomorphic features. Therefore, there is a great need for preliminary studies using cross-species amplification.

Our study describes recent advances and applications of nuclear microsatellite markers in the subgenus *Pulsatilla*, which includes both diploid and tetraploid species. Results of cross-species amplification of microsatellite markers for the diploid *Pulsatilla vernalis* are presented, using primers originally designed for the tetraploid *Pulsatilla vulgaris* (DiLeo *et al*., 2015).

Materials and Method

Identifying candidate SSR markers: From *Pulsatilla* species, 40 nuclear microsatellite loci have been developed: 12 for *P. patens* (Szczecińska *et al*., 2013) and 28 for *P. vulgaris* (DiLeo *et al*., 2015; Gargiulo *et al*., 2019). Recently, six of the above-mentioned loci (five from *P. patens* and one from *P. vulgaris*) were transferred to *P. cernua* in a phylogeographic study from Japan (Takaishi *et al*., 2019).

The transferability and analysis of markers requires particular care as *P. patens*, *P. cernua* and *P. vernalis* are diploids (2n=2x=16), while *P. vulgaris* is an allotetraploid (2n=4x=32) (Sramkó *et al*., 2019), and these different patterns of inheritance should be taken into consideration (Dufresne *et al*., 2014). Fortunately, as our target species, *P. vernalis*, is diploid, and the source, *P. vulgaris*, is tetraploid, the cross-amplification procedure do not involve as many pitfalls as if the SSR markers has been transferred in the opposite direction.

To determine the genetic/phylogenetic distance between the analysed target species and the source taxa from which nuclear SSR markers were developed and used, a phylogenetic tree was prepared, with the internal transcribed spacer (ITS) as a nuclear marker. Among the barcoding single loci, the ITS marker showed the highest interspecific distances and the highest rate of correct identification in the *Pulsatilla* species (Li *et al*., 2019). The constructed phylogenetic tree displays the current "state-of-the-art" of the SSR markers being developed and used in *Pulsatilla* species (Fig. 1). The tree was created using sequences of chosen *Pulsatilla* species from GenBank Accession numbers, with these presented in the following order on the phylogenetic tree: *Pulsatilla vulgaris* MK341851, *P. integrifolia* MK550993, *P. rubra* MK550991, *P. tatewakii* MK550979, *P. cernua* MK341836, *P. patens* MK550998, *P. vernalis* MN997025, *P. pratensis* MK551003, *Anemone turczaninovii* MK341812, *P. halleri* MK551001, *A. alpine* MK341853. The phylogenetic analyses were performed with MEGA X using maximum likelihood (ML) algorithms, with 1000 bootstrap steps, under the Tamura-Nei model.

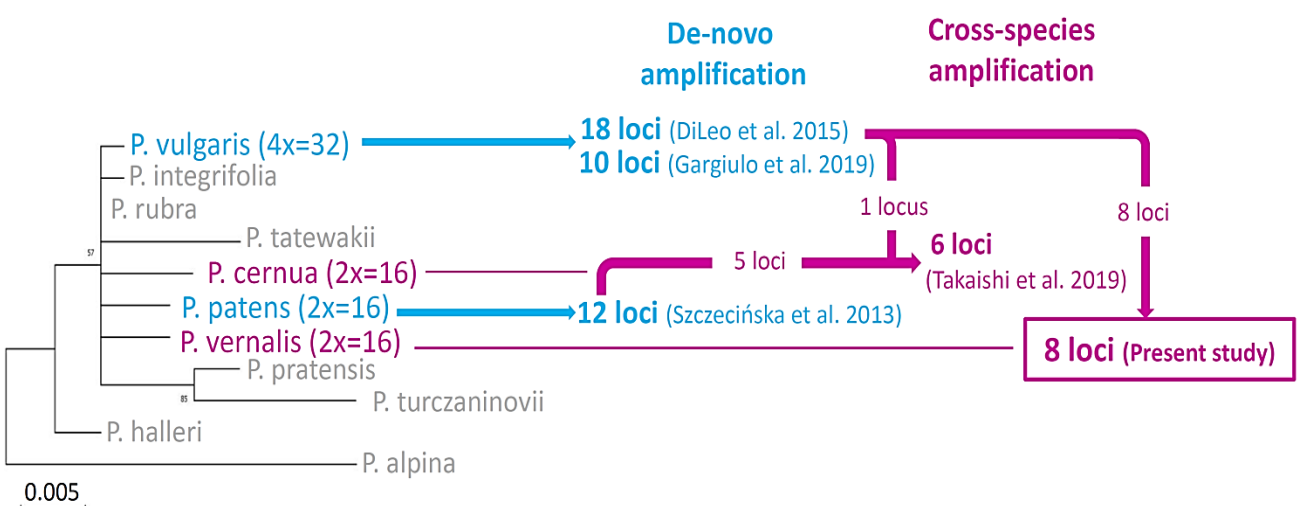

Fig. 1. The current "state-of-the-art" of nuclear SSR markers, in use or in development, present in taxa from *Pulsatilla*, subgenus *Pulsatilla*. The phylogenetic relations between *Pulsatilla* species were conducted according to the ITS region based on the maximum likelihood algorithm, 1000 bootstrap steps and the Tamura-Nei model.

Plant material and DNA extraction: In total, 82 samples were collected from three lowland locations of *P. vernalis* in Poland: Bory Tucholskie (N = 40), Bocheniec (N = 19) and Rogowiec (N = 23). Plant material was immediately dried and stored in silica gel. The samples were collected with the permission given by Polish Minister of Environment and Regional Directorates of Environmental Protection (Bory Tucholskie - RDOŚ-Gd-WZG.6400.122.2018.MM.1; Rogowiec - WPN.64oo.z4.z; Bocheniec – WPN.I.6400.18.2018.BD). For DNA extraction, 2 to 10 gms of dry *P. vernalis* leaves were used. DNA isolation was performed using the NucleoSpin® Plant II (Macherey-Nagel, Germany) kit according to a modified procedure. Briefly, cell lysis was performed in a 600 µl volume of PL2 lysis buffer. The volumes of precipitation buffer after the PL3 lysis step (150 µl) and PC binding buffer (900 µl) were also increased. The final elution of DNA was performed in a volume of 50 µl of elution buffer, after which the isolates were stored at -20°C until further analysis.

Amplification: PCR reactions were performed for all 18 loci developed for *P. vulgaris* (Dileo *et al*., 2015). Of these, 10 loci which provided an undyed PCR product were subjected to further steps to obtain genotypes with dyed primers (Table 1). The markers were amplified in 3 multiplexes (dyes in parentheses): multiplex A – PV32 (VIC), PV52 (VIC), PV54 (6- FAM), PV65 (VIC), multiplex B – PV9 (6- FAM), PV27 (6- FAM), PV44 (PET), PV48 (PET), multiplex C–PV50 (NED), PV59 (PET). PCR reactions were performed under the following conditions: initial denaturation 95°C –15 min, denaturation 94°C–30 s, primer annealing stage, multiplex A at 56°C, multiplex B at 54°C, multiplex C at 60°C – 90 s, extension 72°C – 1 min, final extension 60°C – 30 min. in each case, 33 cycles were used for the reactions. Each PCR reaction was composed of 5 µl of the Multiplex PCR Kit (Qiagen, Germany), 1 µl of each 10 nM primer, 1 µl of PCR water and 2 µl of extracted DNA, which gave a final reaction volume of 10 µl. The PCR products were genotyped on an ABI 3500 capillary sequencer (Applied Biosystems, USA), and the genotypes were read out in GeneMapper ver. 5 (Thermo Fisher Scientific Inc, USA).

Data analysis: To determine if loci are in Hardy-Weinberg equilibrium (HWE) according to populations, we used *pegas* R package (Paradis, 2010). Function hw. Test () computes the $\chi 2$ statistic and p-values, derived from 1000 permutations. We visualized results by locus and population as a heatmap in *lattice* R package (Sarkar, 2008). We defined significant p-value as $\alpha<0.05$ to detect candidate loci where we might reject the hypothesis of HWE.

Genotype SSR data were imported into *poppr* R package (Kamvar *et al*., 2014). To assess how much power we had to discriminate between unique individuals given a random sample of nn loci, a genotype accumulation curve was conducted. We specified sample = 1000 in function call. This means that nn loci were randomly sampled 1000 times to create the distribution visualized in boxplots.

For the amplified locus, allele size, the number of alleles (Na), allelic richness (R), heterozygosity observed (H_O), and expected (H_E) and inbreeding (F_{IS}) coefficient were estimated. All calculations were done in FSTAT ver. 2.9.3.2 (Goudet, 1995), and GeneAlEx ver. 6.5 (Peakall & Smouse, 2012) software.

Results

Ten out of the initially tested 18 loci yielded amplified PCR products (Table 1). Two of the loci (PV44, PV48) demonstrated monomorphic alleles, and these were excluded from further statistical analysis. The remaining 8 loci proved to be polymorphic in *Pulsatilla vernalis* but with a highly variable number of alleles (from 2 to 13, for 82 individuals) (Table 2).

We identified candidate cross-amplified loci that might be rejecting the hypothesis of HWE according to studied populations of *P. vernalis* (Fig. 2). Two loci (PV65 and PV27) are suspected to be in HWE ($p \leq 0.05$) in all studied populations. However, we have any population which is being in HWE according to all loci analyzed.

In the analysis of genotype accumulation curve, we confirmed that for the used loci a plateau has been reached at eight loci, hence we have sampled enough loci in the analysis necessary to discriminate individuals (Fig. 3).

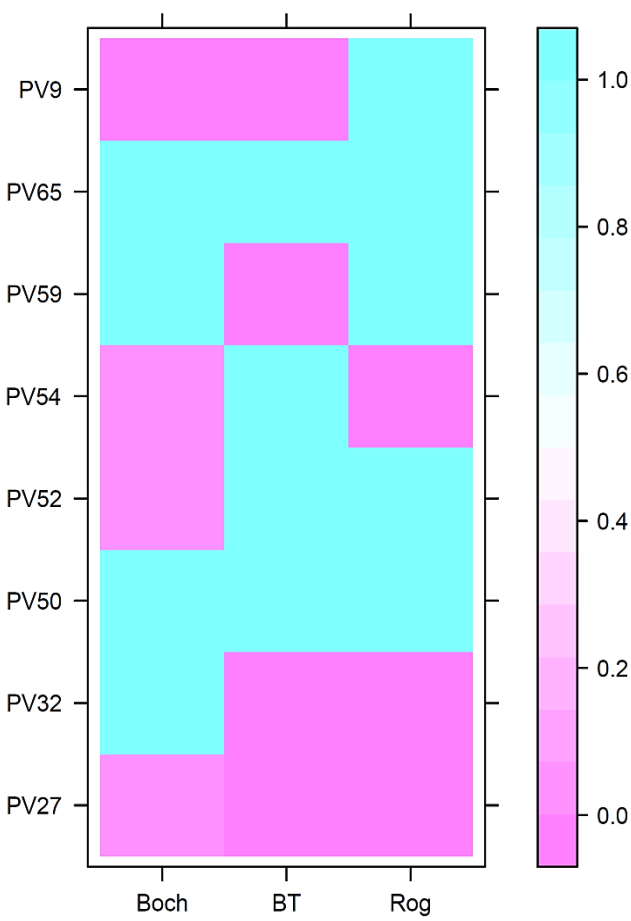

Fig. 2. Candidate cross-amplified loci where might be rejecting the hypothesis of Hardy-Weinberg equilibrium (HWE) according to studied populations of *Pulsatilla vernalis*. Loci shown in pink(darker) are suspected of not being in HWE with $p \leq 0.05$. Populations abbreviations: Boch – Bocheniec, BT – Bory Tucholskie, Rog – Rogowiec.

The allelic richness across eight loci ranged from 1.406 to 9.568, which correlated with the total number of alleles obtained. Observed (H_O) and expected (H_E) heterozygosity ranged from 0.037 to 0.654 and from 0.036 to 0.878, respectively. The F_{IS} coefficient ranged from -0.200 to 0.696, and none of its values were statistically significant (p>0.05; Table 2).

Discussion

The main goal of this study was to determine the suitability of genetic markers for population studies of *P. vernalis*, while minimizing the cost and time of developing new microsatellite markers. Promising results were obtained for eight loci that were cross amplified for *P. vernalis*. Although it is suggested that a minimum of ten microsatellite markers are appropriate in population studies (Koskinen *et al*., 2004), analyses based on smaller numbers of markers have also yielded satisfactory results (Bilska & Szczecińska, 2016; Luo *et al*., 2016; Takaishi *et al*., 2019). Moreover, our analysis of genotype accumulation curve, confirms that we have sampled enough loci in the analysis necessary to discriminate individuals.

Of 18 polymorphic loci originally developed for *Pulsatilla vulgaris* (Dileo *et al*., 2015)., ten have been successfully amplified for *P. vernalis.* Two of the loci (PV44, PV48) showed monomorphic alleles in both the species *P. vernalis* study and in *P. vulgaris*, the target species (DiLeo *et al*., 2015).

The observed heterozygosity for *Pulsatilla vulgaris* differed from previously obtained values (Dileo *et al*., 2015); for example, the heterozygosity of locus PV27 was found to be H_O= 0.83 for n = 41), and lower values were obtained also for locus PV52 (H_O= 0.146 vs H_O= 0.75 for n=41). Only locus PV65 demonstrated a similar level of heterozygosity to those observed in German populations of *P. vulgaris* (Dileo *et al*., 2015).

Although *Pulsatilla vulgaris* is an allotetraploid species, some markers showed behavior characteristic of disomic inheritance and were labeled as “diploid” markers; others demonstrated more than two alleles for each individual, and these were called “tetraploid” markers, as this was suspected to be indicative of tetrasomic inheritance (Dileo *et al*., 2015). However, in the case of *P. vernalis*, no such phenomenon was noted and typically two alleles were observed for each specimen.

Our successful amplification of eight microsatellite loci in *P. vernalis* demonstrates that such cross-amplification between closely related species offers accurate results while avoiding costly and time-consuming procedures. The threats of the spring pasqueflower populations bring with them a growing need for the development of new research methods whose results can be applied to conservation initiatives. Our findings provide new, easy-to-apply, and relatively inexpensive tools for better understanding the intraspecific genetic diversity of *P. vernalis* – species of high conservation importance.

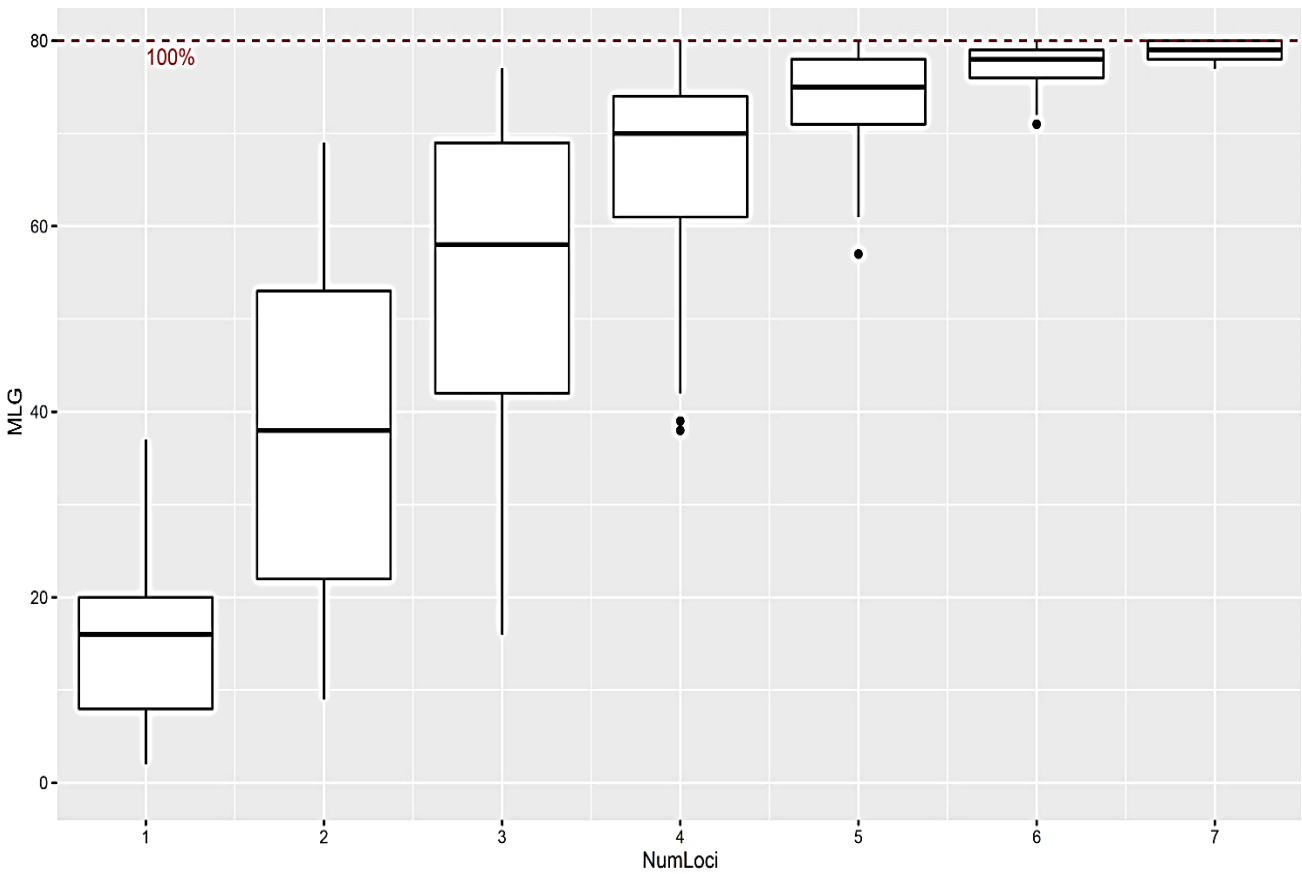

Fig. 3. The genotype accumulation curve through increasing number of nuclear SSR loci used in the analysis of *Pulsatilla vernalis* accessions.

Table 1. Characteristics of ten microsatellite markers developed for *P. vulgaris* (2x=32) according to Dileo *et al*., (2015), and conditions of their cross-amplification to *P. vernalis* (4x=16); T_a = annealing temperature; D – conditions according to Dileo *et al*., (2015); M – modified conditions.

Lo-cus	Primer sequences (5′–3′)	Repeat motif	Cond. of cros-amp.	T_a (°C)	Multi-plex	Fluorescent dye	Marker ploidy	GenBank accession no.
PV 9	F: GAACTAACCTGCTTCCGTGC R: GCAAGCCAAAGTCCACTCTG	$(AG)_{11}$	D M	55 54	single B	HEX 6-FAM	tetraploid	KP885679
PV 27	F: AACCCTTGCACACCAACTTG R: AATCTTATCTGGGCGGGAGG	$(AG)_8$	D M	55 54	A B	6-FAM	diploid	KP885682
PV 32	F: CATGCCTTTGTACCCTGCTG R: ACGACCTTTGTTTGACCGTC	$(AAG)_9$	D M	55 56	single A	6-FAM VIC	tetraploid	KP885683
PV 44	F: GTATGTGTGTGCCAAGGGTC R: TGCTTAAGAGTAGCATGCCG	$(ATC)_{10}$	D M	55 54	single B	HEX PET	—	KR109213
PV 48	F: CGGGCTGTAACTGATGCTTC R: GCAAGTGAGCCAGTTCCATC	$(AG)_{10}$	D M	55 54	single B	HEX PET	—	KR109214
PV 50	F: GATGGTGATGAGGGTTTGGG R: TGCCACCTACTTTCCACACC	$(AAT)_{12}$	D M	55 60	single C	HEX NED	tetraploid	KP885685
PV 52	F: TTGGTCAAATGGTCGCAACG R: GGTGCTCAAGATTATCGGGC	$(AG)_{10}$	D M	55 56	A A	6-FAM VIC	diploid	KP885686
PV 54	F: TACTCGCGACTGACAAGCTC R: TTGTGGGAGTGGAAGGAACC	$(AT)_{10}$	D M	55 56	single A	6-FAM	—	KR109215
PV 59	F: TGGACAACGTACCTTACACAG R: AGCTTCCACACCTCAACTGG	$(AG)_{10}$	D M	55 60	single C	HEX PET	—	KR109216
PV 65	F: ACGGACGCAAATCTTCTGAC R: GAGAACGAACGCCATGACAG	$(AG)_{10}$	D M	55 56	A A	6-FAM VIC	diploid	KP885690

Table 2. Statistics for eight cross-amplified microsatellite loci for 82 samples collected from three lowland populations of *P. vernalis*. Na – number of alleles, R – allelic richness, H_O – observed heterozygosity, H_E – expected heterozygosity, F_{IS} – fixation index. None of F_{IS} values were statistically significant (p>0.05).

Locus	Allele range (size)	Na	R	H_O	H_E	F_{IS}
PV9	283-299	5	4.589	0.333	0.671	0.504
PV27	395-425	10	6.000	0.224	0.737	0.696
PV32	366-414	8	5.944	0.418	0.745	0.439
PV50	405-417	2	1.406	0.037	0.036	-0.019
PV52	266-308	6	2.813	0.146	0.182	0.196
PV54	203-207	3	2.916	0.654	0.545	-0.200
PV59	150-204	9	7.481	0.456	0.828	0.449
PV65	140-174	13	9.568	0.650	0.878	0.260
Mean	-	7.00	5.089	0.365	0.578	0.291

References

Betz, C., M. Scheuerer and C. Reisch. 2013. Population reinforcement – A glimmer of hope for the conservation of the highly endangered spring pasqueflower (*Pulsatilla vernalis*). *Biol. Conser*., 168: 161-167. https://doi.org/10.1016/j.biocon.2013.10.004.

Bilska, K. and M. Szczecińska. 2016. Comparison of the effectiveness of ISJ and SSR markers and detection of outlier loci in conservation genetics of *Pulsatilla patens* populations. *Peer J*., 2016(11): e2504. https://doi.org/10.7717/peerj.2504

Chambers, G.K. and E.S. MacAvoy. 2000. Microsatellites: consensus and controversy. Comparative Biochemistry and Physiology. Part B, *Biochem. Mol. Biol.,* 126(4): 455-476. https://doi.org/10.1016/s0305-0491(00)00233-9

Chappuis, E. 2014. *Pulsatilla vernalis*. The IUCN Red List of Threatened Species 2014*:*

DiLeo, M.F., R. Graf, R. Holderegger, Y. Rico and H.H. Wagner. 2015. Highly polymorphic microsatellite markers in *Pulsatilla vulgaris* (Ranunculaceae) Using Next-Generation Sequencing. *Appl. Plant Sci.,* 3(7): 1500031. https://doi.org/10.3732/apps.1500031.

Dufresne, F., M. Stift, R. Vergilino and B.K. Mable. 2014. Recent progress and challenges in population genetics of polyploid organisms: an overview of current state-of-the-art molecular and statistical tools. *Mol. Ecol*., 23(1): 40-69. https://doi.org/10.1111/mec.12581.

Elsen, P.R. and M.W. Tingley. 2015. Global mountain topography and the fate of mountain species under climate change. *Nat. Clim. Change*, 723(5): 1-5.

Engler, R., C.F. Randin, W. Thuiller, S. Dullinger, N.E. Zimmermann, M.B. Araújo, P.B. Pearman, G. le Lay, C. Piedallu, C.H. Albert, P. Choler, G. Coldea, X. de Lamo, T. Dirnböck, J.C. Gégout, D. Gómez-García, J.A. Grytnes, E. Heegaard, F. Høistad and A. Guisan. 2011. 21st century climate change threatens mountain flora unequally across Europe. *Glob. Change Biol.*, 17(7): 2330-2341. https://doi.org/10.1111/J.1365-2486.2010.02393.X

Gargiulo, R., G. Worswick, C. Arnold, L.J. Pike, R.S. Cowan, K.A. Hardwick, T. Chapman and M.F. Fay. 2019. Conservation of the Threatened Species, *Pulsatilla vulgaris* Mill. (Pasqueflower), is Aided by Reproductive System and Polyploidy. *J. Hered.,* 110(5): 618-628. https://doi.org/10.1093/jhered/esz035

Goudet, J. 1995. FSTAT (Version 1.2): A Computer Program to Calculate F-Statistics. *J. Hered*., 86(6): 485-486.

Grzyl, A. and M. Ronikier. 2011. *Pulsatilla vernalis* (Ranunculaceae) in the Polish lowlands: Current population resources of a declining species. *Pol. Bot. J*., 56(2): 185-194.

Hohenlohe, P.A., W.C. Funk and O.P. Rajora. 2021. Population genomics for wildlife conservation and management. *Mol. Ecol.*, 30(1): 62-82. https://doi.org/10.1111/mec.15720.

Kamvar, Z.N., J.F. Tabima and N.J. Grunwald. 2014. Poppr: An R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. *Peer J.*, 2014(1): 1-14. https://doi.org/10.7717/PEERJ.281/TABLE-6.

Kiedrzyński, M., K.M. Zielińska, E. Kiedrzyńska and A. Rewicz. 2017. Refugial debate: on small sites according to their function and capacity. *Evol. Ecol*., 31(6): 815-827. https://doi.org/10.1007/s10682-017-9913-4.

Koskinen, M.T., H. Hirvonen, P-A. Landry and C.R. Primmer. 2004. The benefits of increasing the number of microsatellites utilized in genetic population studies: an empirical perspective. *Hereditas,* 141(1): 61-67. https://doi.org/10.1111/j.1601-5223.2004.01804.x

Li, Q., X. Wang, J.R. Wang, N. Su, L. Zhang, Y.P. Ma, Z.Y. Chang, L. Zhao and D. Potter. 2019. Efficient Identification of *Pulsatilla* (Ranunculaceae) Using DNA Barcodes and Micro-Morphological Characters. *Front. Plant Sci.,* 10: 1196. https://doi.org/10.3389/fpls.2019.01196.

Luo, J.Y., S. Zhang, L. Wang, L.M. Lv, C.Y. Wang, C.H. Li, Z. Xiangzhen, Z.G. Zhou and J.J. Cui. 2016. The Distribution and Host Shifts of Cotton-Melon Aphids in Northern China. *Plos One,* 11: e0152103. https://doi.org/10.1371/journal.pone.0152103

Paradis, E. 2010. Pegas: an R package for population genetics with an integrated–modular approach. *Bioinformatics*, 26: 419-420.

Peakall, R. and P.E. Smouse. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics*, 28(19): 2537-2539.

Qian, H., R.E. Ricklefs and W. Thuiller. 2021. Evolutionary assembly of flowering plants into sky islands. *Nat. Ecol. Evol*., 1-7. https://doi.org/10.1038/s41559-021-01423-1.

Ronikier, M. 2002. The use of AFLP markers in conservation genetics--a case study on *Pulsatilla vernalis* in the Polish lowlands. *Cell. Mol. Biol. Lett*., 7(2B): 677-684.

Ronikier, M., A. Costa, J.F. Aguilar, G.N. Feliner, P. Küpfer and Z. Mirek. 2008. Phylogeography of *Pulsatilla vernalis* (L.) Mill. (Ranunculaceae): chloroplast DNA reveals two evolutionary lineages across central Europe and Scandinavia. *J. Biogeog.*, 35(9): 1650-1664. https://doi.org/ https://doi.org/10.1111/j.1365-2699.2008.01907.x

Ronikier, M., A. Grzyl and W. Wójtowicz. 2014. *Pulsatilla vernalis* (L.) Mill. sasanka wiosenna. In: Kaźmierczakowa R., K. Zarzycki and Z. Mirek. Polska Polish Red Data Book of Plants. Pteridophytes and flowering plants. 3rd edn. *Instytut Ochrony Przyrody PAN, Kraków*, pp. 178-181.

Sarkar, D. 2008. Lattice: Multivariate Data Visualization with R. Springer, New York, http://lmdvr.r-forge.r-project.org

Scheuerer, M. 1996. *Pulsatilla vernalis* Mill. (FrühlingsKuhschelle), auch im Landkreis Kelheim vom Aussterben bedroht. *Hoppea*, 57: 375-394.

Selkoe, K.A. and R.J. Toonen. 2006. Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecol. Lett.,* 9(5): 615-629. https://doi.org/https://doi.org/10.1111/j.1461-0248.2006.00889.x

Sramkó, G., L. Laczkó, P.A. Volkova, R.M. Bateman and J. Mlinarec. 2019. Evolutionary history of the Pasque-flowers (*Pulsatilla*, Ranunculaceae): Molecular phylogenetics, systematics and rDNA evolution. *Mol. Phylogen. Evol.,* 135: 45-61. https://doi.org/10.1016/j.ympev.2019.02.015

Szandar, K., J. Sawicki, Ł. Paukszto, K. Krawczyk and M. Szczecińska. 2023. Are the organellar genomes useful for fine scale population structure analysis of endangered plants?-A Case Study of *Pulsatilla patens* (L.) Mill. *Genes*., 14(1):67. https://doi.org/10.3390/genes14010067

Szandar, K., K. Krawczyk, K. Myszczyński, M. Ślipiko, J. Sawicki and M. Szczecińska. 2022. Breaking the limits-multichromosomal structure of an early eudicot *Pulsatilla patens* mitogenome reveals extensive RNA-editing, longest repeats and chloroplast derived regions among sequenced land plant mitogenomes. *B.M.C. Plant Biol*., 22, 109. https://doi.org/10.1186/s12870-022-03492-1

Szczecińska, M. and J. Sawicki. 2015. Genomic resources of three pulsatilla species reveal evolutionary hotspots, species-specific sites and variable plastid structure in the family Ranunculaceae. *Int. J. Mol. Sci*., 16: 22258-22279. https://doi.org/10.3390/ijms160922258.

Szczecińska, M., M. Kwaśniewski, K. Chwiałkowska and J. Sawicki. 2013. Isolation and characterization of microsatellite loci in *Pulsatilla patens* (L.) Mill. (Ranunculaceae) a rare and endangered plant species in Europe. *Cons. Gen. Resour.,* 5(2): 421-423. https://doi.org/ 10.1007/s12686-012-9818-z

Takaishi, A., A.E. Kozhevnikov, Z.V. Kozhevnikova, H. Ikeda, N. Fujii and A. Soejima. 2019. Phylogeography of *Pulsatilla cernua* (Ranunculaceae), a grassland species, in Japan. *Ecol. Evol.*, 9(12): 7262-7272. https://doi.org/10.1002/ece3.5298

Zielińska, K.M., M. Kiedrzyński, A. Grzyl and A. Rewicz. 2016. Forest roadsides harbour less competitive habitats for a relict mountain plant (*Pulsatilla vernalis*) in lowlands. *Sci. Reports,* 6: 31913. https://doi.org/10.1038/srep31913.

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