MASSIVE GENETIC INTROGRESSION AMONG *ACONITUM* (RANUNCULACEAE) SPECIES IN A MOUNTAIN HYBRID ZONE

Agnieszka Sutkowska[1], Piotr Boroń[2], Tomasz Warzecha[1], and Józef Mitka[*3]

[1] *Department of Physiology, Plant Breeding and Seed Science, University of Agriculture, ul. Podłużna 3, 30-239 Cracow, Poland*

[2] *Department of Forest Phytopathology, University of Agriculture, Al. 29 Listopada 46, 31-425 Cracow, Poland*

[3] *Institute of Botany, Department of Plant Taxonomy, Phytogeography, and Palaeobotany, ul. Gronostajowa 9, PL-30-387 Kraków, Poland*

* corresponding author: E-mail j.mitka@uj.edu.pl

Abstract

Hybridization in Aconitum is a widespread phenomenon. However, the introgression between the diploid and tetraploid species is rare. We studied species of different ploidy in a mountain hybrid zone in the Tatra Mts (West Carpathians). In this paper, we aimed to identify the extent of introgressive hybridization using PCR-ISSR fingerprinting and chloroplast DNA (cpDNA) sequencing. The diploid and tetraploid species had different cpDNA haplotypes, and the triploid hybrid was stabilized on the tetraploid level. ISSR markers showed the locally pervasive introgression, but genetic introgressants were not morphologically changed. Principal Coordinates Analysis, NeighborNet clustering, and Bayesian inference showed close genetic links between individuals in the same population but populations were significantly genetically differentiated (FST = 0.129, $p < 0.001$). A genetic model of introgression via the triploid bridge was proposed. Genetic introgression does not seemingly pose a threat to species taxonomic identity. The genetic differences between the same species in different populations advocate for a web-of-life than a biological species concept. Genetic introgressants in the isolated mountain populations make each of them genetically unique.

Key words: hybrid zone, introgressive hybridization, ISSR, local genetic pool, biodiversity conservation

Introduction

The genus monkshood *Aconitum* belongs to the evolutionary old family Ranunculaceae, order Ranunculales that emerged c. 115 million years ago (Magallon et al. 2015). Its phylogenetic history in Europe could be dated to the Late Tertiary (Boroń et al. 2020).

Hybridization is a phenomenon common in plants that plays an important role as a creative evolutionary stimulus (Anderson and Stebbins 1954; Soltis and Soltis 2009, von Holdt et al. 2017), especially in phylogenetically young taxa (Baack and Rieseberg 2007). In this context, *Aconitum* breaks the rule because hybridization in this ancient genus is very often. It concerns both present hybrid zones (Sutkowska et al. 2013a; Ilnicki 2014), and historical reticulate evolution (Mitka et al. 2007) in effect of secondary contacts during Quaternary glaciations (Stebbins 1984; Durand et al. 2000; Adams 2015).

A special case of hybridization is the gene introgression from the species with a lower ploidy to the species with a higher ploidy level (Maceira et al. 1993; Petit et al. 1999; Henry et al. 2005) *via* the so-called triploid bridge (Buerkle et al. 2000; Husband 2004; Rieseberg and Willis 2007; Mallet 2007). It seems that a female tetraploid bridge *via* unreduced egg cells is the major pathway to the formation of polyploids, as in *Ranununculus kuepferi* (Schinkel et al. 2017), and other species (Mallet 2007; Rieseberg and Willis 2007; Nemorin et al. 2013). The unreduced gametes may have had a major rule in the evolution of polyploids (Tayalé and Parisod 2013).

The other consequence of the process could be gene introgression from tetraploid to the diploid line (reverse triploid bridge). It was described in *Aconitum* (Sutkowska et al. 2017a) and in other species (Thórsson et al. 2001; Ståhlberg 2009; Bendiksby et al. 2011; Naczk et al. 2015). Hybrids between species of *Aconitum* species have combinations of morphological features of the parental species (Luo et al. 2005; Mitka and Starmühler 2000, Sutkowska et al. 2013a). In the Carpathians, the hybrids between diploids (e.g., *A.* ×*pawlowskii*, i.e., *A. variegatum* × *A. lasiocarpum*), tetraploids (e.g., *A.* ×*nanum* (*A. fissurae* × *A. bucovinense*)), and between diploids and tetraploids (e.g., *A.* ×*berdaui*, (*A. firmum* × *A. variegatum*)) were described, see www.ipni.org and Mitka (2003).

The genetic diversity of natural hybrids does not demonstrate any uniform pattern. For some of the hybrids, the variability is lower than for their parental forms, while for others it is much higher (Nielsen 2000; Sutkowska et al. 2013b). For example, the decreased diversity in natural hybrids formed in northern France between the allotetraploid *Bromus benekenii* and the allohexaploid *B. ramosus* was noted (Sutkowska et al. 2015a).

Genetic introgression among diploid *Aconitum* species seems a widespread phenomenon. An example is a natural hybrid zone in the Beskid Niski Mts (Western Carpathians), where diploids *A. lasiocarpum* and *A. variegatum* reach the limits of the geographical distribution (Sutkowska et al. 2013b). In the sympatric zone, a spontaneous hybrid *A. ×pawlowskii* originates (Mitka and Starmühler 2000). The parental species had distinct genetic ISSR profiles in allopatric populations. In the taxonomically mixed populations, many introgressive individuals of *Aconitum* were found. This process did not pose a substantial risk of extinction via genetic erosion, as in local endemics (Čertner et al. 2015).

The introgression between diploid and tetraploid species in nature occurs rather sporadically. However, it is a relatively common process for *Aconitum* in the Tatra's valleys, detected in hypervariable ISSR fingerprinting and cytogenetic analysis (Zieliński 1982, Sutkowska et al. 2017a, present study). In a previous study (Sutkowska et al. 2017a), we found that the cpDNA gene was transferred between tetraploid and diploids, the case we did not find in the present paper. Instead, we noted massive gene introgression based on nuclear DNA markers. The ISSR markers surprisingly revealed the greater genetic distance between two valleys than between particular taxa. One should expect that the representatives of the same taxon sampled at two different sites should be phylogenetically (genetically) closer to each other than the representatives belonging to different taxa. In our opinion, the result is a consequence of the various histories of the population at the ecological time and the spatial gene flow barriers. ISSR fingerprinting is a useful tool in resolving intra- and inter-population diversity of *Aconitum* species.

Some *Aconitum* species can be ancient hybrids originated in the Quaternary (Mitka et al. 2007) or even in the Neogene (Boroń et al. 2020).

In this paper, we present the results of the molecular analysis of two isolated populations formed by three species of *Aconitum*: *A. variegatum*, *A. lasiocarpum, A. firmum*, and hybrids: *A. ×berdaui* and *A. ×pawlowskii*. They are localized in the Tatra Mountains (Western Carpathians). The study aimed to check the extent of gene flow between isolated populations located in adjacent mountain valleys. We expect that the gene flow was hampered between the valleys due to a topographic barrier. An attempt was also made to determine the potential introgression and identification of cryptic hybrids in the mountain populations of *Aconitum*.

The monkshoods are mainly pollinated by bumblebees. For example, Klepacz-Baniak (2011) has shown that bumblebees in the mountains move only over short distances (200 -

300 m from the nest). The plants are self-compatible and pollinated by geitonogamous pollen (Utelli and Roy 2000). All of these may prevent the transfer of alleles between even closely located populations. In the mountains, species of *Aconitum* usually occur along streams in deep valleys where populations are additionally isolated by ridges and mountain peaks. In effect, we expected that the two populations could have been characterized by different genetic profiles. The other question was to reveal the genetic identity of the particular species using two markers: microsatellite ISSR and plastid cpDNA *trn*L$^{(UAG)}$–*ndh*F. The knowledge of the population structure is essential to rare and endangered species and aids their protection measures.

The nomenclature and taxonomy of Aconitum follow: http://www.ipni.org; Starmühler and Mitka 2001; Mitka 2003; Novikoff and Mitka 2011.

Materials and Methods

Study species

Species of *Aconitum* are self-compatible, perennial plants pollinated by bumblebees (Utelli et al. 2000). *Aconitum* subgen. *Aconitum* in the Tatras consists of the diploids (2n = 16): *A. variegatum* L., *A. lasiocarpum* Gáyer subsp. *kotulae* (Pawł.) Starm. & Mitka, and the tetraploid (2n = 32) *A. firmum* Rchb. The crossings *A. firmum* and *A. variegatum* form a partially sterile hybrid *A. ×berdaui* Zapał. (2n = 24, see Zieliński 1982, Wacławska-Ćwiertnia, Mitka 2016), and *A. lasiocarpum* and *A. variegatum* form a fertile hybrid *A. ×pawlowskii* Mitka & Starmühl. (Mitka and Starmühler 2000, Mitka 2003). Both diploids and their hybrids are forest - lower montane zone species, the tetraploid *Aconitum firmum* is a high-mountain species that occurs in the subalpine and alpine zones. *A. firmum* occasionally descends to forest montane zones along torrents (Mitka 2003). Here it inhabits man-made glades and tall-grass communities mostly linked to historical pastoral husbandry.

Study area

The ML valley is one of the valleys in the Western Tatra Mts (Western Carpathians), located in Poland (coordinates: N49.258889/E19.905) (Fig. 1). It is the only valley in Tatra Mts, which as a whole has been grooved in sedimentary rocks. Only a few ridges are covered with crystalline rocks (Radwańska-Paryska and Paryski 2004). This valley, except the lower part, has been shaped by glaciers. The stream appears only in the lower part of the valley (Małołącki stream). In the middle and upper valleys, no surface runoff exists. The central part

of the ML valley was formed on the site of a former glacial lake, which filled the recess caused by the terminal moraine. The lake was then buried by debris cones (Nyka 2003).

The northern part of the valley is covered by forests, and the middle part by spruce trees and meadows, which form subalpine forest vegetation. The southern part of the valley is mostly overgrown with dwarf pine *Pinus mugo* L.

The DM valley (coordinates: N49.255833/E19.893889) is located near the ML valley (Fig. 1) and consists of segments: Kobylarz (coordinates: N49.245833/E19.904167), Kobylarzowy Żleb (coordinates: N49.243056/E 19.909444) and Niżnia Miętusia Rówień (coordinates: N49.258056/E19.893611). Almost the entire DM valley is made of sedimentary rocks - dolomite and limestone. In such a bedrock, typical karst phenomena develop, including underground water flow slots, the disappearance of water, and numerous caves. The lowest part is a typical river valley. Starting from the Niżnia Miętusia Rówień (subalpine forest glade), it forms a typical glacial valley (Cywiński 1996).

Kobylarz is a meadow at an altitude of about 1430 m. a.s.l. (above sea level), located on the eastern slopes of the DM valley. It is in a limestone area covered with rich calciphilous flora (Mirek and Piękoś-Mirkowa 2008; Radwańska-Paryska and Paryski 2004).

The Kobylarzowy Żleb couloir is a deep gorge with steep walls built of limestone rocks, cut in the past by the glacier. There are numerous typical mountain plants here, some of which grow only in the Tatra Mts (Mirek and Piękoś-Mirkowa 2008; Radwańska-Paryska and Paryski 2004) (Fig. 1).

Sample collection

Random samples of *Aconitum* (fresh leaves stored in silica gel) were collected from 1010 m to 1400 m above sea level from individuals scattered along the ML and DM valleys (Fig. 1) in August of 2014. Altogether, 27 samples from the Dolina Małej Łąki valley and 23 samples from the Dolina Miętusia valley were collected (Tab. 1, Fig. 1). They represented the overall number of species in the studied valleys.

DNA isolation and ISSR analyses

The ISSR (Inter Simple Sequence Repeats) method is applied in studies of phylogenetic, inter- and intra-population variation and phylogeographic research because the primers allow for the identification of numerous polymorphic loci in a single PCR reaction (Hansen et al. 2000; Li and Ge 2001; Ilnicki et al. 2011; Sutkowska et al. 2013a). ISSR methodology is based on highly polymorphic sequences of satellite DNA, consisting of

nucleotide sequences tandemly repeated in thousands of copies (microsatellites) (Parker et al., 1998). PCR reaction products are segments of DNA that are located between regions including microsatellite sequences (Stepansky et al. 1999).

DNA was isolated from fully developed leaves without damage symptoms caused by insects and mold. DNA was extracted using Genomic Mini AX Plant (A and A Biotechnology). Six ISSR primers were used. The sequence of primers was taken from Stepansky et al. (1999; Sutkowska et al. 2013b) and are shown in Table 2. Amplification reactions were conducted on a 2720 thermal cycler (Applied Biosystems). Amplification was carried out with a 25 µl reaction mixture: 2.5µl 10-fold concentrated reaction buffer supplied by the Taq DNA polymerase manufacturer (Fermentas), 1.5 mM $MgCl_2$, 0.19 mM of each dNTPs (Fermentas), 27 pmol primer, 100ng template DNA and 1.4 units of Taq polymerase. The annealing temperature for primers ISSR2, ISSR4, ISSR7 was 44°C, and for ISSR1, ISSR3, ISSR5, ISSR6 was 47°C. Optimal conditions for the reaction were as follows: initial denaturation: 94°C – 5 min., 42 amplification cycles: denaturation 94°C – 59 s., annealing 44°C (47°C) – 59 s., polymerization 72°C – 59 s., final polymerization 72°C – 7 min. A negative control reaction without DNA template was included in each amplification. To confirm the results, 50% of the samples were amplified twice.

Products were subjected to electrophoresis in 1.5% agarose gel stained with ethidium bromide (50µl/100ml) at 100V about 1.5 h. Bands were observed and archived with Imagemaster VDS (Pharmacia – Amersham). Original software Liscap Capture ver. 1.0 was also applied.

For analysis of band patterns, GelScan ver. 1.45 (Kucharczyk TE) software was used. The creation of a calibration curve based on the band pattern of markers' length (GeneRuler TM 100 bp - Fermentas) made it possible to determine the molecular weight of the resulting amplification products. ISSR reproducibility tests (Bonin et al. 2004) included within-plate (n = 12) and between-plate (n = 9) replicates independently analyzed from the DNA extracts.

DNA sequencing

The undiluted DNA extracts were used as templates in the amplification of the *trn*L$^{(UAG)}$–*ndh*F region of chloroplast DNA with primers *trn*L(UAG) – 5'-CTGCTTCCTAAGAGCAGCGT-3' and ndhF – 5'-GAAAGGTATKATCCAYGMATATT-3' (Shaw et al. 2007). The reaction was carried out in a total volume of 50 µl containing: 1× DreamTaq Green buffer (ThermoFisher Scientific); 3.5 mM MgCl2; 0.08mM of dNTPs; 0.08µM of both primers; and 1u of DreamTaq DNA polymerase (ThermoFisher Scientific).

Amplifications were run on a T100 Thermal Cycler (Bio-Rad) with the following temperature profile: 5 min. of initial denaturation at 94°C; 25 touchdown cycles composed of 30 s at 94°C; 30 s of decreasing annealing temperature (0.5°/cycle from 67.5°C in the 1st to 55°C in the 25th cycle); 1 min at 72°C; and 20 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C; and 10 min at 72°C for the final extension step. The amplification efficiency was verified by agarose gel electrophoresis and positive PCR products were purified with the Clean-Up DNA purification kit (A&A Biotechnology, Poland). For purified PCR products, the fragment of the *trn*L$^{(UAG)}$–*ndh*F amplicon was sequenced with the internal primer V2_F – 5'-GTTCGCAAAGAACTGAAGTGAC-3' (Boroń et al., unpubl.). Sequencing was performed with a BigDye Terminator v3.1 Cycle Sequencing Kit (Life technologies, USA) on a T100 thermal cycler (Bio-Rad) and a 3500 Series Genetic Analyzer (Life Technologies, USA) using standard protocols. The obtained sequences were processed and aligned with MEGA 6 software (Tamura et al. 2013), followed by manual adjustment.

The sequences we deposited in GenBank with accession numbers KU314873 and KU314868.

Data analysis

A total of 216 amplified DNA fragments were scored as a presence-absence allele matrix of binary data, 108 in the DM valley and 118 in the ML valley. The dominant ISSR data were analyzed by treating each class of genotypes as being effectively haploid alleles. Hardy-Weinberg equilibrium was assumed in the calculations of the indices of genetic diversity. STRUCTURE (Pritchard et al. 2000) was used to identify the genetically homogeneous groups of individuals. The software places individuals in K clusters characterized by a distinct set of allele frequencies at each locus. An admixture ancestry model was used and allele frequencies were correlated; 3×10^5 replicates of Markov chain Monte Carlo (MCMC) with a burn-in of 5×10^4 iterations gave a minimum number of interpretable genetic clusters for K = 3.

The matrix of samples and ISSR products, coded as binary (0-1) data, was used to calculate Nei and Li (1979) distances and the Neighbour Net (NN) ordination with SplitsTree 4.12 software (Huson and Bryant 2006). Bootstrap values (BS) for clusters were determined in 1000 random runs. Additionally, Nei and Li distances were used for Principal Coordinates Analysis (PCoA) ordination with a Minimum Spanning Tree MST (Gower and Ross 1969) superimposed on the PCoA ordination to detect local distortions (Rohlf 1975). Calculations

were performed with the use of the NTSYSpc ver. 2.11 multivariate analysis package (Rohlf 2002).

An AFLP-SURV program (Vekemans et al. 2002) enabled an estimation of the frequency of null alleles based on a Bayesian method with a non-uniform prior distribution of allele frequencies, which reduces the bias of the square method (Zhivotovsky 1999). Statistics of genetic diversity and population genetic structure were then computed strictly following the treatment of Lynch and Milligan (1994): genetic diversity within taxonomic groups was measured by the percentage of polymorphic bands (*PLP*), Nei's (1973) gene diversity, or expected heterozygosity (*h*).

We estimated the number of private bands, defined as markers found only in one taxon. We calculate a genetic divergence index *DW* by computing 'frequency-down-weighted marker values' (Schönswetter and Tribsch 2005). For it, one-way ANOVA and *post-hoc* contrast (Tukey's HSD test) were calculated with STATISTICA ver. 10 (StatSoft, Inc.) and Shannon's index (*I*). The indices excluding Shannon's *I* were calculated with AFLP-SURV, and index *I* with POPGENE ver. 1.32 (Yeh et al. 1999).

Results

PCR-ISSR analysis resulted in 161 polymorphic loci. Data quality tests indicated high repeatability across the ISSR bands, above 97%. The total number of PCR-ISSR products generated by the primers varied from 23 to 28 in ML and from 14 to 30 in DM. The number of bands generated by particular primers per individual varied from (1) 5-6 to 9-11 in ML and from 3-6 to 7-9 in DM. In the ML valley, the median of PCR products generated in individuals across particular primers varied between 6 and 9, and in the DM valley between 5 and 8 (Table 2).

In the ML valley, *A. firmum* (9 specimens), *A. ×pawlowskii* (7), *A. variegatum* (8), and one *A. ×berdaui* occur. In the DM valley, *A. firmum* (1), *A. lasiocarpum* (12), *A. ×pawlowskii* (12), and *A. variegatum* (3) were found (Fig. 1, Table 1). *A. ×pawlowskii* in ML is an orphan hybrid (lack of *A. lasiocarpum*).

The sequencing of the *trnL*$^{(UAG)}$*–ndh*F region of cpDNA revealed the presence of two types of cpDNA haplotypes of *Aconitum* in Europe, described in our previous papers (Mitka et al. 2016, Sutkowska et al. 2017a, Boroń et al. 2020). They are distinguished by two unique indels. cpDNA type I is typical for tetraploids, and cpDNA type II for diploids. The triploid *A. ×berdaui* possesses, alternatively, both haplotypes. In this study, it had a tetraploid type of cpDNA haplotype.

The populations in the two valleys were genetically differentiated (Figs. 2 and 3, Table 3) - F_{ST} index equaled 0.129 ($p < 0.001$). In ML valley, the genetic diversity indices were higher in comparison to DM valley, including Nei's *h* ($p < 0.001$) and *DW* ($p = 0.05$) indices.

The first three Axes of PCO 1 - 3 explained 32.79% of the total ISSR genetic variability. In the ordination diagram, the two valleys DM and ML were separated along the PCoA 1 (Fig. 2). PCoA 2 discriminated *A. lasiocarpum*, and PCoA 3 *A. firmum*. In ML hybrid *A. ×berdaui* (BK1) was placed in an intermediate position between the tetraploid *A. firmum* and the diploid *A. variegatum* along PCO 3. The MST showed the connectivity between *A. ×berdaui* BK1 and a specimen of *A. ×pawlowskii* PK7 (Fig. 2).

The different genetic stocks in the two valleys were also confirmed by a main split (BS 70.2%) in the NN ordination (Fig. 3). In ML valley, hybrid *A. ×berdaui* BK1 was genetically close to *A. ×pawlowskii* PK7 (BS 50.3%). *Aconitum lasiocarpum* and *A. ×pawlowskii* formed separate genetic groups in both mountain valleys.

STRUCTURE analysis was conducted separately for the valleys under study (Fig. 4A, B). In DM (Fig. 4A) genetic hybrids were: *A. ×berdaui, A. firmum,* and *A. variegatum* at 1450-1800 m. *A. ×pawlowskii* and *A. variegatum* formed distinct genetic groups. *Aconitum variegatum* VK6 shared the genetic group of *A. ×pawlowskii*, and *A. variegatum* VK7 shared *A. firmum*. *A. firmum* FK7 and FK8 were genetically introgressed with *A. ×pawlowskii*.

In ML (Fig. 4B), three genetic populations were distributed among four taxa. The specimen of *A. firmum* FM1 was introgressed with *A. lasiocarpum* genetic group, and three specimens of *A. variegatum* VM1-VM3 with *A. firmum*, similarly as *A. lasiocarpum* LM1 and LM3. *Aconitum variegatum* VM3 seems introgressed both with *A. firmum* and *A. ×pawlowskii*.

Ten specific PCR-ISSR products were found only in *A. lasiocarpum*.

Discussion

General remarks

Genetic ISSR fingerprinting and its statistical analyses showed a clear difference between the examined populations in two adjacent mountain valleys. It could be seen both on the NN clustering dendrogram, where the distinction is supported by a high BS (70.2%), and in the PCoA ordination analysis along axis 1. A restricted or no gene flow between the valleys is the effect of the topographical barrier. Additionally, the bumblebees could not carry *Aconitum* pollen in the mountains for longer distances (see Introduction). In mountain valleys,

extrinsic dynamic processes, such as fluctuations in size including bottlenecks, invasions or founding events, ecological succession (Gray 1996), and today tourism and climate global change affect species' populations. In the mountains, spatial barriers to gene flow have also great importance. For example, while studying the genetic structure of the marginal populations of *Aconitum bucovinense* in the W Bieszczady Mts (Polish Eastern Carpathians), two relict populations of the species, lying seven kilometers each of the others were genetically differentiated (p=0.024, AMOVA). It has practical significance in the formulation of protection guidelines for rare and endangered *Aconitum* species (Boroń et al. 2011).

In both populations, besides the pure species, hybrid specimens existed: *A.* ×*pawlowskii* and *A.* ×*berdaui*. Their hybridogenous status is proved by their key morphological characters (Mitka and Starmühler 2000, Mitka 2003), as well as ISSR markers. However, some of the taxonomically pure specimens seem to be cryptic (with the genetic admixture of alien ISSR *loci*) introgressants (Labate and Robertson 2012; McIntosh et al. 2014; Beatty et al. 2015).

In our previous study on two endemic *Aconitum* species and their hybrids carried out in the whole Carpathians (Sutkowska et al. 2017b), an Analysis of Molecular Variance (AMOVA) revealed the highest percentage of variation attributed to the ISSR genetic group (22.6%), than to the three taxa (15.6%, all $p < 0.001$). In that case, ancient reticulations may conceal species' genetic boundaries. Interestingly, the hybrid species (*A.* ×*nanum*) had a distinct genetic profile, similarly as *A.* ×*pawlowskii* in the present study. Among the examined species of *Aconitum* in another valley of the Tatra Mts, the highest variability was found in *A.* ×*pawlowskii* (Sutkowska et al. 2017a).

Genetic introgressions

As was shown in our previous studies, *A.* ×*pawlowskii* originates in the contact zone of the parental species (Sutkowska et al. 2013b). The hybrid nature of *A.* ×*pawlowskii* in DM valley could be indicated by its close position to *A. variegatum* in the PCO and NN analyses. STRUCTURE analysis showed genetically specific groups of *A. variegatum*, *A. firmum*, *A.* ×*pawlowskii*, and cryptic genetic hybrids. These cryptic hybrids in DM did not differ morphologically from the pure species. The degree of introgression in populations could be not reflected in the species morphology (Čertner et al. 2020).

The individuals FK7 and FK8 of *A. firmum* have fragments of the nuclear genome of *A.* ×*pawlowskii*. Moreover, FK7, in addition to the genomes of *A. firmum* and *A.*

341 ×*pawlowskii,* has part of the nuclear genome of *A. variegatum*. Similarly, *A. variegatum*
342 (VK7) has ISSR fragments common to *A. firmum* and *A. ×pawlowskii*.

343 This result suggests that there were mixing of genomes, and one of the individuals has
344 a genome composed of fragments from different ploidy levels.

345 At the molecular level (PCO and NN), *A. ×berdaui* BK1 is a hybrid between *A.*
346 *firmum* and *A. ×pawlowskii*, with the PCR-ISSR introgression directed to the latter
347 taxon. According to the Linnaean taxonomy, *Aconitum ×berdaui* was described as the hybrid
348 between the tetraploid *A. firmum* and diploid *A. variegatum* (Wacławska-Ćwiertnia and Mitka
349 2016). The present result shows that not only *A. variegatum,* but other diploid species, such
350 as *A. ×pawlowskii*, when crossed with *A. firmum*, can produce a morphological form of *A.*
351 ×*berdaui*. Both NN and PCO analyses showed that *A. ×berdaui* BK1 and *A.*
352 ×*pawlowskii* PK7 had similar nuclear genome composition. Seemingly, PK7 could have been
353 one of the parental species to the hybridogenous *A. ×berdaui* (see Figs. 2 and 3).

354 Likely, *A. variegatum* VK7, *A. firmum* FK7 and FK8, are backrossed introgressants.
355 The diploid *A. variegatum* VK7 individual was introgressed mainly from *A. firmum* (similarly
356 as VM1-VM3, LM1, and LM3). The tetraploid *A. firmum* FK7 and FK8 was introgressed
357 from *A. ×pawlowskii*, similarly as FM1 probably was introgressed by *A. lasiocarpum*. In both
358 cases, an intermediate triploid form must have been created that allows transferring alleles
359 between various ploidy levels. In VK7, and also in VM1 – VM3 LM1, LM3 in ML, the gene
360 flow was from the tetraploid to diploid species via the triploid (scenario “c”, Fig. 5).

361 Genetic introgression in *Aconitum* between various ploidies (diploid and tetraploid) in
362 mountain hybrid zones was previously detected. For example, in another study carried out in
363 the Tatra Mts we found a tetraploid cpDNA marker among diploids pointing to the reverse
364 triploid bridge, i.e. introgression from tetraploid to diploids (Sutkowska et al. 2017a). The
365 initial donor of the maternally inherited plastid DNA in a hybrid triploid was tetraploid *A.*
366 *firmum*. The subsequent backcrossing involved monoploid (n = *x*) maternal gametes from a
367 triploid (3*x*) line and normal paternal pollen of a diploid (2*x*) line (*A. lasiocarpum*, *A.*
368 *variegatum* or *A. × pawlowskii*). In the second step, maternally inherited cpDNA was
369 introgressed from the tetraploid to diploid species following recurrent backcrossing of the
370 triploid (1*x* ♀ gamete) with the parental diploid (1*x*♂gamete). In this way, the tetraploid
371 specific cpDNA gene could be introgressed from the tetraploid to diploid species. This is a
372 mechanism of the reverse triploid bridge (Sutkowska et al. 2017a).

Similarly, in this study we adopt the hypothesis that at first a triploid is formed through the union of gametes of a diploid and tetraploid species, using unreduced gametes of the diploid. Triploids produce an array of gametes with variable ploidy. In this respect, it is important that gamete ploidy (diploid or tetraploid) drives the subsequent origin and introgression of the diploid or tetraploid line. The subsequent backcrossing involved monoploid (n = 1*x*) gametes from a triploid line and regular gametes (n = 1*x*) of a diploid (scenario "c", Fig. 5). In this respect, the introgressed specimens are represented by *A. variegatum*: VM1, VM2, VM3, and *A. lasiocarpum*: LM1, LM3 (see also Fig. 4B).-

We did not find, as in the previous study (Sutkowska et al. 2017a), the introgression of cpDNA, maternally inherited. Thus, probably the triploid was only a donor of pollen. It was postulated in a study carried out in the Tatra Mts by Zieliński (1982). According to the author triploid *A. ×berdaui* can produce in 13% viable pollen. Such the triploid pollen could backcross both to diploid and tetraploid lines (Ramsey and Schemske 1998).

In the present study, the introgression was found among *A. firmum/A. lasiocarpum/A. variegatum*, or *A. firmum/variegatum/A.×pawlowskii*, in dependence on the local species pool. Because the tetraploid cpDNA marker was found in the triploid *A. ×berdaui,* we infer that the hybrid formed probably a female triploid bridge with a triploid (3*x* ♀ gamete). In the second step, it could be backcrossed with diploid pollen (1*x* ♂ gamete) (Schinkel et al. 2017). In this way, introgression via the triploid bridge was from diploid to tetraploid (scenario "b" in Fig. 5). Similarly, also in ML valley "a cryptic" triploid bridge could be postulated: cpDNA was transferred from a tetraploid individual to another tetraploid individual (Fig. 4 B) along with a part of the nuclear DNA from a diploid species (scenario "b", Fig. 5)

The interesting is *A. variegatum* VM3 possessed three genetic groups. It could be later-generation hybrid.

Genetic diversity in populations

The ML valley population was genetically richer than the neighboring population DM. It was probably affected by the presence of the genetically distinct *A. lasiocarpum*. It was the only species that had private *loci* and thus the best genetically discriminated. A hybrid *A. ×pawlowskii* had not additive ISSR *loci*. They were found, for example, in natural hybrids between the forest grass species *Bromus benekenii* and *B. ramosus* (Sutkowska et al. 2015a) or *Phoenix canarienis* and *Ph. dactylifera* (Gonzalez-Perez et al. 2004). The lack of additive genetic effects could be the result of the asymmetric introgression between the parental

406species, and such an explanation has been postulated in a study on the hybrid zone of *A.*
407*lasiocarpum* and *A. variegatum* in the Western Carpathians (Sutkowska et al. 2013b).
408Additionally, STRUCTURE outcomes formed separated genetic groups of *A.*
409*lasiocarpum* and *A. ×pawlowskii*. Its distinct genetic profile and lack of ISSR genetic
410additivity suggests the existence of a mechanism that stabilizes the genetic constitution of the
411hybrid species. The similar result we found while studying the populations of *Aconitum* in
412another mountain valley in the Tatra Mts (Sutkowska et al. 2017a), and a natural hybrid zone
413in the Niski Beskids (Polish Western Carpathians, Sutkowska et al 2013b). This could be a
414result of the rearrangement of the hybrid genomes associated with their stabilization (Feldman
415et al. 1997; Warzecha et al. 2014). According to Hamilton and Miller (2018), recombinant
416introgressed genotypes may have direct implications in terms of species' evolutionary
417potential and provide additional adaptive capacity.

418 Among the examined populations, the ML valley had the highest genetic diversity. The
419high within-population variability (*PLP*) and the high values of the genetic disparity index
420*DW* in ML could point to the presence of genetically distinct *A. lasiocarpum* due to its relic
421status or origin from different sources. Relatively low variability is characteristic of the DM
422population could be a result of the founder effect or genetic bottleneck. Some theoretical and
423empirical studies seem to suggest that the founder effects and bottlenecks can be both strong
424and frequent (Avise and Hamrick 1996) and can consequently determine the fate of species
425colonization in a new habitat (Barton and Charlesworth 1984; Carson and Templeton 1984,
426Sutkowska et al. 2015b). Furthermore, the occurrence on the edge of the geographical
427distribution could affect the population's genetic structure. For example, two marginal
428populations of an endemic to the Eastern-Southern Carpathians *A. bucovinense* Zapał. in the
429Western Bieszczady Mts. (Polish Eastern Carpathians), showed high genetic taxonomic
430divergence ($p = 0.024$), accompanied by the loss of genetic diversity indices (Shannon
431diversity index *I* and genetic divergence index *DW*), in comparison to the core population
432(Boroń et al. 2011).

433

434**Conclusion**

435The crossing between species possessing different ploidy levels and, additionally, occurring
436in different mountain zones, is rarely observed (Sutkowska et al. 2017a). Crucial is the origin
437of the triploid species *A. ×berdaui*. This and previous (Sutkowska 2017a) studies showed that
438the hybrid triploid could be both a donor of male and female gametes, however the latter
439seems rather a rare phenomenon. Taxonomically pure individuals in both Tatra's valleys were

often genetic chimeras. More importantly, the populations differed significantly in adjacent valleys. It could be probably a compound effect of various genetic mechanisms including the founder effect, bottlenecks, and within-population gene transfer. As the amount of genetic information and species' genetic profiles vary between valleys, we need a proper schedule for species conservation. In this case, it seems that species management and protection plans should be relayed rather within a web-of-life than a biological species concept (vonHoldt et al. 2017) and consider the extant genetic structure of the populations (Gray 1996).

Mosaic ancestry seems to be a pathway provides genetic variation for rapid adaptive evolution of a species in response to rapidly changing environments (Thomson et al. 2010, Hamilton and Miller 2016, Hiraschiki et al. 2021).

We found that in the triploid formation *A. ×pawlowskii* was involved, extending in this way the array of putative parental diploids (*A. variegatum*), and supplementing the nothospecies taxonomic diagnosis (Mitka and Starmühler 2000).

ACKNOWLEDGMENTS

The research was financially supported by the Polish Ministry of Science and Higher Education - Contract No. 0117000000-D127 and a Polish National Science Centre grant no. N303 814440 given to Józef Mitka. We would like to express our gratitude to the anonymous referees for critically reading the manuscript, as well as Iwona Katarzyna Łakomy, M.Sc., for technical support while conducting molecular analysis and Kendra Hutchins from School of Integrative Plant Science, Horticulture Section Program Coordinator, Cornell Annual Flower Trials for her help with language editing. We would also like to thank Dr. Bogusław Binkiewicz for support in the development of graphic materials for this article. The open-access publication of this article was funded by the Priority Research Area BioS under the program "Excellence Initiative – Research University" at the Jagiellonian University in Krakow.

References

Adams RP (2015) Allopatric hybridization between *Juniperus maritima*. In: R. P. Adams and J. Scopulorum Sarg.: evidence from nuclear and cpDNA and leaf terpenoids. Phytologia 97(1): 55–66.

Anderson E, Stebbins GL (1954) Hybridization as an evolutionary stimulus. Evolution 8: 378–388.

Avise JC, Hamrick JL (1996) Conservation Genetics: Case Histories from Nature. New York: Chapman and Hall.

Baack EJ, Rieseberg LH (2007) A genomic view of introgression and hybrid speciation Curr Opin Genet Dev 17: 513–518.

Barton NH, Charlesworth B (1984) Genetic revolutions, founder effects, and speciation. Ann Rev Ecol Syst 15: 133–164.

Beatty GE, Barker L, Chen P-P, Kelleher CT, Provan J (2015) Cryptic introgression into the kidney saxifrage (*Saxifraga hirsuta*) from its more abundant sympatric congener *Saxifraga spathularis*, and the potential risk of genetic assimilation. Ann Bot 115(2): 179-186.

Bendiksby M, Tribsch A, Borgen L, Trávníček P Brysting AK (2011) Alloploid origins of the Galeopsis tetraploids – revisiting Müntzing's classical textbook example using molecular tools. New Phytologist 191: 1150–1167.

Bonin A, Bellemain E, Bronken Eidesen P, Pompanon F, Brochmann C, Taberlet P (2004) How to track and assess genotyping errors in population genetics studies. Mol Ecol 13: 3261–3273.

Boroń P, Zalewska-Gałosz J, Nowak A, Sutkowska A, Zemanek B, Mitka J (2011) *Aconitum bucovinense* Zapał. (Ranunculaceae) at the range margin: spatial population-genetic structure of the Carpathian endemic and its conservation. Acta Soc Bo Pol 80: 315–326.

Boroń P., Wróblewska A., Binkiewicz B., Mitka J. (2020). Phylogeny Aconitum Subgenus Aconitum in Europe. Acta Soc Bot Pol 89: 1-22. doi: 10.5586/asbp.8933.

Buerkle CA, Morris RJ, Asmussen MA, Rieseberg LH (2000) The likelihood of homoploid hybrid speciation. Heredity 84: 441–451.

505 Carson HL, Templeton AR (1984) Genetic revolutions in relation to speciation
506 phenomena: the founding of new populations. Ann Rev Ecol Syst 15: 97–131.
507 Cywiński W (1996) Czerwone Wierchy, część zachodnia. Przewodnik szczegółowy,
508 tom 3. Poronin, Wyd. Górskie, ISBN 83-7104-011-3.
509 Čertner M, Kolář, F, Frajman B, Winkler M, Schönswetter P. 2020. Massive
510 introgression weakens boundaries between a regionally endemic allopolyploid and
511 a widespread congener. Perspectives in Plant Ecology, Evolution and Systematics
512 42: 125502. https://doi.org/10.1016/j.ppees.2019.125502.
513 Čertner M, Kolář, F, Schönswetter P, Frajman B. 2015. Does hybridization with a
514 widespread congener threaten the long-term persistence of the Eastern Alpine rare
515 local endemic *Knautia carinthiaca*? Ecology and Evolution 19: 2045-7758.
516 https://doi.org/10.1002/ece3.1686.
517 Durand JD, Erhan Ü, Doadrio I, Pipoyan S, Templeton A (2000) Origin, radiation,
518 dispersion and allopatric hybridization in the chub *Leuciscus cephalus*. Proc R
519 Soc [Biol] 267(1453): 1687–1697.
520 Feldman M, Liu B, Segal G, Shahal Abbo S, Levy AA, Vega JM (1997) Rapid
521 elimination of low-copy DNA sequences in polyploid wheat: A possible
522 mechanism for differentiation of homoeologous chromosomes. Genetics 147:
523 1381–1387.
524 Gonzalez-Perez MA, Caujape-Castells J, Sosa PA (2004) Molecular evidence of
525 hybridisation between the endemic *Phoenix canariensis* and the widespread *P.*
526 *dactylifera* with Random Amplified Polymorphic DNA (RAPD) markers. Plant
527 Syst Evol 247: 165–175.
528 Gower JC, Ross GJS (1969) Minimum spanning trees and single linkage cluster
529 analysis. App Stat 18: 54–64.
530 Gray A. 1996. Genetic diversity and its conservation in natural populations of plants.
531 Biodiv Letters 3: 71-80.
532 Hansen KT, Elven R, Brochmann C (2000) Molecules and morphology in concert: tests
533 of some hypotheses in arctic *Potentilla* (Rosaceae). Am J Bot 87(10): 1466–1479.
534 Hamilton, J.A. and Miller, J.M. (2016). Adaptive introgression as a resource for
535 management and genetic conservation in a changing climate. Conservation
536 Biology, 30: 33-41. https://doi.org/10.1111/cobi.12574
537 Henry IM, Dilkes BP, Young K, Watson BH Wu, *et al.* (2005) Aneuploidy and genetic 537
538 variation in the *Arabidopsis thaliana* triploid response. Genetics 170: 1979–1988.

539 Hirashiki C, Kareiva P, Marvier M. 2021. Concern over hybridization risks should not
540 preclude conservation interventions. Conservation Science and
541 Practice; 3:e424. https://doi.org/10.1111/csp2.424
542 Huson DH, Bryant D (2006) Application of Phylogenetic Networks in Evolutionary
543 Studies. Mol Biol Evol 23(2): 254–267.
544 Husband BC (2004) The role of triploid hybrids in the evolutionary dynamics of mixed-
545 ploidy populations. Biol J Linn Soc 82(4): 537–546.
546 Ilnicki T (2014) Plant biosystematics with the help of cytology and cytogenetics.
547 Caryologia 67(3): 199-208.
548 Ilnicki T, Joachimiak A, Sutkowska A, Mitka J (2011) Cytotypes distribution of
549 *Aconitum variegatum* L. in Central Europe, In: Zemanek B (Ed.) Geobotanist and
550 Taxonomist. A volume dedicated to Professor A. Zając on the 70th anniversary of
551 his birth, Institute of Botany, Jagiellonian University, Cracow, pp 169–192.
552 Klepacz-Baniak J (2011) Owady zapylające w sadzie. Info Sad 1: 8–9.
553 Labate JA, Robertson LD (2012) Evidence of cryptic introgression in tomato (*Solanum*
554 *lycopersicum* L.) based on wild tomato species alleles. BMC Plant Biol 12: 133.
555 Li A, S Ge (2001) Genetic variation and clonal diversity of *Psammochloa villosa*
556 (Poaceae) detected by ISSR markers. Ann Bot 87: 585–590.
557 Luo Y, Zhang F, Yang Q (2005) Phylogeny of *Aconitum* subgenus Aconitum
558 (Ranunculaceae) inferred ITS sequences. Plant Syst Evol 252: 11–25.
559 Lynch M, Milligan B (1994) Analysis of population-genetic structure using RAPD
560 markers. Mol Ecol 3: 91–99.
561 Maceira NO, Jacquard P, Lumaret R (1993) Competition between diploid and derivative
562 autotetraploid *Dactylis glomerata* L. from Galicia. Implications for the
563 establishment of novel polyploid population ns, New Phytol 124: 321–328.
564 Magallon S, Gomez-Acevedo S, Sanchez-Reyes LL, T Hernandez-Hernandez (2015) A
565 metacalibrated time-tree documents the early rise of flowering plant phylogenetic
566 diversity. New Phytol 207: 437–453.
567 Mallet J (2007) Hybrid speciation. Nature 446: 279–283.
568
569 McIntosh EJ, Rosetto M, Weston PH, Wardle GM (2014) Maintenance of strong
570 morphological differentiation despite ongoing natural hybridization between
571 sympatric species of *Lomatia* (Proteaceae). Ann Bot 113(5): 861–872.

572 Mirek Z, Piękoś-Mirkowa H (2008) Czerwona Księga Karpat Polskich. Rośliny
573 Naczyniowe. Instytut Botaniki im. W. Szafera PAN, Kraków, pp 615.
574 Mitka J (2003) The genus *Aconitum* L. (Ranunculaceae) in Poland and adjacent
575 countries. Institute of Botany, Jagiellonian University, Kraków.
576 Mitka J, Boroń P., Novikoff A, Wróblewska A, Binkiewicz B (2016) Two major groups
577 of chloroplast DNA haplotypes in diploid and tetraploid Aconitum subgen :
578 Aconitum (Ranunculaceae) in the Carpathians. Mod Phytomorphol 9 Suppl.: 5-15.
579 Mitka J, Sutkowska A, Ilnicki T, Joachimiak A (2007). Reticulate evolution of high-
580 alpine *Aconitum* (Ranunculaceae) in the Estern Sudetes and Western Carpathians
581 (Central Europe). Acta Biol Cracov Ser. Biol 49(2): 15–26.
582 Mitka J, Starmühler W (2000) Phenetic variability of *Aconitum lasiocarpum* (Rchb.)
583 Gáyer (Ranunculaceae): extension of taxonomic and geographic borders. Acta
584 Soc Bot Pol 69 (2): 145–155.
585 Naczk AM, Górniak M, Szlachetko DM, Ziętara MS (2015) Plastid DNA haplotype
586 diversity and morphological variation in the *Dactylorhiza incarnata/maculata*
587 complex (Orchidaceae) in northern Poland. Bot J Lin Soc 178: 121–137.
588 Nei M (1973) Analysis of gene diversity in subdivided populations. PNAS USA 70:
589 3321– 3323.
590 Nei M, Li WH (1979) Mathematical model for studying genetic variation in terms of
591 restriction endonucleases. PNAS USA 76: 5269–5273.
592 Nemorin A, David J, Maledon E, Nudol E, Dalon J, Arnau G (2013) Microsatellite and
593 flow cytometry analysis to help understand the origin of *Dioscorea alata*
594 polyploids. Ann Bot 112: 811–819.
595 Nielsen LR (2000) Natural hybridization between *Vanilla claviculata* (W. Wright) Sw.
596 and V. barbellata Rchb.f. (Orchidaceae): genetic, morphological, and pollination
597 experimental data. Bot J Lin Soc 133: 285–302.
598 Novikoff A, Mitka J (2011) Taxonomy and ecology of the genus *Aconitum* L. in the
599 Ukrainian Carpathians. Wulfenia 18: 37-61.
600 Nyka J (2003) Tatry polskie. Przewodnik. Wyd. XIII. Latchorzew: Wyd. Trawers,
601 ISBN 83-915859-1-3.
602 Parker PG, Snow AA, Schug MD, Booton GC, Fuerst PA (1998) What molecules can
603 tell us about populations: choosing and using a molecular marker. Ecology 79(2):
604 361–382.

Petit C, Bretagnolle F, Felber F. 1999. Evolutionary consequences of diploid-polyploid hybrid zones in wild species. Trends Ecol Evol 14: 306-311. DOI: 10.1016/S0169-5347(99)01608-0

Pritchard JK, Stephens M, Donelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155: 945–959.

Radwańska-Paryska Z, Paryski WH (2004) Wielka encyklopedia tatrzańska. Poronin: Wydawnictwo Górskie. ISBN 83-7104-009-1.

Ramsey J, Schemske DW. 1998. Pathways, mechanisms, and rates of polyploid formation in flowering plants. Ann Rev Ecol Syst 29: 467-501.

Rieseberg LH, Willis JH (2007) Plant speciation. Science 317 (5840):910-914.

Rolhf FJ (1975) Geeralization of the gap test for the detection of multivariate outliers. Biometrics 31: 93-101.

Rohlf FJ (2002) NTSYS-pc. Numerical taxonomy and multivariate analysis, version 2.1 — Exeter Software, Setauket, New York, USA.

Schönswetter P, Tribsch A (2005) Vicariance and dispersal in the alpine perennial *Bupleurum stellatum* L. (Apiaceae). Taxon 54: 725–732.

Shaw J, Lickey EB, Schilling EE, Small RL (2007) Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in Angiosperms: the tortoise and the hare III. Am J Bot 94 (3): 275–288.

Schinkel CF, Kirchheimer B, Dullinger S, Geelen D, De Storm N, Hörandl E (2017) Pathways to polyploidy: indications of a female triploid bridge in the alpine species *Ranunculus kuepferi* (Ranunculaceae). Plant Syst Evol 303(8): 1093–1108.

Soltis PS, Soltis DE (2009) The role of hybridization in plant speciation. Annu Rev Plant Biol 60: 561–588.

Ståhlberg D (2009) Habitat differentiation, hybridization and gene flow patterns in mixed populations of diploid and autotetraploid *Dactylorhiza maculata* s.l. (Orchidaceae). Evol Ecol 23: 295–328.

Starmühler W, Mitka J (2001) Systematics and chorology of *Aconitum* sect. *Napellus* (Ranunculaceae) and its hybrids in the Northern Carpathians and Forest Carpathians. Thaiszia – Journal of Botany, Košice 10: 115–136.

Stebbins G (1984) Polyploidy and the distribution of the arctic–alpine flora: new evidence and a new approach. Bot Helv 94: 1–13.

638 Stepansky A, Kovalski I, Perl–Treves R (1999) Interspecific classification of melons
639 (*Cucumis melo* L.) in view of their phenotypic and molecular variation. Plant Syst
640 Evol 271: 313–332.
641 Sutkowska A, Boroń P, Mitka J (2013a). Natural hybrid zone of the *Aconitum* species in
642 the Western Carpathians: Linnaean taxonomy and ISSR fingerprinting. Acta Biol
643 Cracov Ser Bot 55(1): 114–126.
644 Sutkowska A, Pasierbiński A, Warzecha T, Mandal A, Mitka J (2013b) Refugial
645 pattern of *Bromus erectus* in Central Europe based on ISSR fingerprinting. Acta
646 Biol Cracov Ser Bot 55(2): 107–119.
647 Sutkowska A, Pasierbiński A, Bąba W, Warzecha T, Mitka J (2015a) Additivity of
648 ISSR markers in natural hybrids of related forest species *Bromus benekenii* and *B.*
649 *ramosus* (Poaceae). Acta Biol Cracov Ser Bot 57(1): 82–94.
650 Sutkowska A, Anamthawat-Jónsson K, Bąba W, Mitka J (2015b) ISSR analysis of two
651 founding plant species on the volcanic island Surtsey, Iceland: grass versus shrub.
652 Surtsey Research 13: 17–30.
653 Sutkowska A, Boroń P, Warzecha T, Dębowski J, Mitka J (2017a) Hybridization and
654 introgression among three *Aconitum* (Ranunculaceae) species of different ploidy
655 levels in the Tatra Mts (Western Carpathians). Plant Spec Biol 32: 292–303.
656 Sutkowska A., Warzecha T., Mitka J. (2017b). Genetic variation of *Aconitum* sect.
657 *Aconitum* (Ranunculaceae) at a macrogeographical scale in the Carpathians. Pol.
658 J. Ecol. 65: 57-68.
659 Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular
660 evolutionary genetics analysis Version 6.0. Mol Biol Evol 30(12) :2725–2729.
661 Tayalé A, Parisod C (2013) Natural pathways to polyploidy in plants and consequences for
662 genome reorganization. Cytogenet. Genome Res 140: 79–96.
663 Thórsson Æ T, Salmela E Anamthawat-Jónsson K (2001) Morphological, cytogenetic,
664 and molecular evidence for introgressive hybridisation in birch. Journal of
665 Heredity 92: 404–408.
666 Utelli A-B, Roy BA (2000) Pollinator abundance and behavior on *Aconitum lycoctonum*
667 (Ranunculaceae): An analysis of the quantity and quality components of
668 pollination. Oikos 89(3): 461-470.
669 Vekemans X, Beauwens T, Lemaire M, Roldan-Ruiz T (2002) Data from amplified
670 fragment length polymorphism (AFLP) markers show indication of size

homoplasy and of a relationship between degree of homoplasy and fragment size. Mol Ecol 11: 139–151.

vonHoldt, B.M., Brzeski, K.E., Wilcove, D.S. and Rutledge, L.Y. (2018), Redefining the Role of Admixture and Genomics in Species Conservation. Conservation Letters, 11: e12371. https://doi.org/10.1111/conl.12371

Wacławska-Ćwiertnia K, Mitka J (2016). Typification of Zapałowicz's names in *Aconitum* section *Aconitum*. PhytoKeys 58: 119-126.

Warzecha T, Sutkowska A, Góral H (2014) Male sterility of triticale lines generated through recombination of triticale and rye maintainers. Span J Agric Res 12(3): 1124–1130.

Yeh FC, Yang RC, Boyle T (1999) POPGENE 32-version 1.31. Population genetics software. http.//www.ualberta.ca/~fyeh/fyeh/.

Zhivotovsky LA (1999) Estimating population structure in diploids with multilocus dominant DNA markers. Mol Ecol 8: 907–913.

Zieliński R (1982) An electrophoretic and cytological study of hybridisation between *Aconitum napellus* ssp. *skerisoare* (2n=32) and *A. variegatum* (2n=16). II Cytological evidence. Acta Soc Bot Pol 51: 465–471.

Tables

Species	Altitude above sea level	Population	Number of specimens	Sample
A. firmum	1180	ML4	1	FM1
A. variegatum	1100 -1150	ML3	3	VM1 – VM3
A. lasiocarpum	850 – 1050	ML1	11	LM1 – LM11
A. ×pawlowskii	950 – 1150	ML2	12	PM1- PM12
Total ML			27	
A. firmum	1450 – 1800	DM3	4	FK5 – FK8
A. firmum	1430	DM2	4	FK1 –FK4
A. ×berdaui	1430	DM2	1	BK1

A. variegatum	1430	DM2	5	VK1 – VK5
A. ×pawlowskii	1430	DM2	5	PK1 – PK5
A. variegatum	1140 – 1200	DM1	2	VK6 – VK7
A. ×pawlowskii	1140 – 1200	DM1	2	PK6 – PK7
Total DM			23	
Grand Total			50	

695

696**Table 1**. *Aconitum* specimens sampled in the Dolina Małej Łąki valley ML and Dolina
697Miętusia valley DM in the Tatra Mts.

698

Primer	Primer sequence	ML			DM		
		Number of PCR products	Range	Median	Number of PCR products	Range	Median
ISSR1	$(TC)_8C$	28	1 - 15	8.0	22	2 - 15	6.5
ISSR2	$(AG)_8T$	26	1 - 20	7.5	27	2 - 23	4.0
ISSR3	$(GGGTG)_3$	23	1 - 24	6.0	30	1 - 18	5.0
ISSR4	$(ATG)_6$	25	1 - 19	4.0	18	1 - 23	2.0
ISSR7	$(AC)_8T$	23	1 - 22	6.0	14	1 - 23	9.0

699

700**Table 2**. The primers used in PCR, total number of reaction products generated by each
701primer, range, and median of PCR products per *Aconitum* individual in Tatra's valley. ML –
702Mała Łąka valley, DM – Miętusia valley.

703

704

Population	*n*	*PLP*	*h*	*h(SD)*	*DW*	*DW(SD)*	F_{ST}
A	23	52.2	0.165	0.012	3.21	0.67	0.129
B	24	62.1	0.201	0.012	3.63	0.74	
I-type error			< 0.001		0.05		< 0,001

705

706**Table 3.** Genetic diversity of *Aconitum* in the Dolina Miętusia (A) valley and Dolina Małej
707Łąki (B) valley (Tatra Mts) based on 161 ISSR loci. *n* – sample size, *PLP* – percentage of
708polymorphic loci, *h* – Nei's gene diversity, *h(SD)* – standard deviation of *h*, *DW* – genetic
709disparity index, *DW(SD)* – standard deviation of DW. Statistical significance for *DW* based
710on one-way ANOVA and Tukey's HDS *post-hoc* tests ($p \leq 0.05$) and for *Hj* on pairwise
711Student-*t* test with Bonferroni correction for the I-type error.

712

713