Natural Hybrid Zone of *Aconitum* Species in the Western Carpathians: Linnaean Taxonomy and ISSR Fingerprinting

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Aconitum lasiocarpum (Carpathian endemic) and *A. variegatum* (European endemic) occur sympatrically in the Polish Western Carpathians. Here their taxonomic hybrid *A. ×pawlowskii* occurs. The aim of this study was to determine the relationship between the taxonomic (Linnaean approach) and genetic structure (PCR-ISSR analysis) of the populations and individuals in two allopatric and four sympatric populations. We determined 309 individuals (OTUs) to species, subspecies and nothospecies using the Linnaean system of classification, and then genetically fingerprinted 39 randomly chosen OTUs. Comparison of the Nei and Li distances obtained from ISSR and morphological matrices using the Mantel test indicated a significant correlation (n = 39, r = 0.53, P = 0.001). Genetic analysis (NEWHYBRIDS) pointed to 7 OTUs as being later-generation hybrids (B1 introgessants) in the sympatric area. Five of them belong to *A. variegatum*, indicating cryptic introgression, and two belong to *A. ×pawlowskii*. Nonmetric multidimensional scaling (NDMS) showed gene flow between *A. lasiocarpum* and *A. ×pawlowskii*. Allopatric, morphologically pure *A. lasiocarpum* and *A. variegatum* populations differed significantly in their ISSR profiles (Fischer's R×C test, P < 0.0001). Expected heterozygosity (*Hj*) was significantly (p=0.05) lower in allopatric (0.1261–0.1268) than in sympatric populations (0.1348–0.1509), indicating a genetic melting pot in sympatry. The results support the existence of a natural interspecific hybrid swarm zone in the sympatric area of occurrence of *Aconitum*, and the taxonomic circumscription of the nothospecies within the Linnaean taxonomic system.

Key words: Beskid Niski Mts., cryptic introgression, genetic melting pot, geographical range limit, hybridization, nothotaxa, postglacial migrations.

INTRODUCTION

Identification of hybrids is of taxonomic and biogeographical interest, and recognition of hybrid taxa (nothotaxa) often clarifies a taxonomic system, although in genetic terms their hybrid status remains hypothetical (Mráz et al., 2005). This is the case in the genus *Aconitum* (monkshood, Ranunculaceae), in which hybrids are often noted (Kadota, 1987; Park et al., 1997; Luo et al., 2005). For example, in the Western Carpathians nearly 50% of the *Aconitum* taxa are presumptive hybrids, ancient and recent (Mitka, 2003; Mitka et al., 2007). Nowadays, hybridization is enhanced by anthropogenic breaking of ecological barriers. Historically, hybrids might have arisen naturally by secondary contact between two postglacial migrations from allopatric refugia (Barton and Charlesworth, 1984), as probably was the case in the study species, *Aconitum lasiocarpum* (Rchb.) Gáyer and *A. variegatum* L. (Joachimiak et al., 1999; Ilnicki and Mitka, 2009). Their ranges meet in the Western Carpathians in a sympatric area (Fig. 1b), where the presumed hybrid *A. ×pawlowskii* Mitka & Starmühl. occurs (*A. lasiocarpum* × *A. variegatum*). The aim of this study was to verify the existence of a hybrid zone in the sympatric area of *Aconitum variegatum* and *A. lasiocarpum* subsp.

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kotulae in the Western Carpathians. *A. lasiocarpum* and *A. variegatum* occur here in the Beskid Niski Mts. and the Doły Jasielsko-Sanockie depression (Mitka, 2003; Fig. 1a,b). Those two areas are at the border between the Eastern and Western Carpathians, where the two Holocene migratory elements met from the west and southeast (Konnert and Bergmann, 1995; Mráz and Szeląg, 2004; Mráz et al., 2007).

The genus *Aconitum* is characterized by high endemicity (Dhar and Samant, 1993), morphological plasticity and hybridism. In effect, its morphological variability is exceedingly high and taxonomic classification difficult. There are numerous reports on the occurrence of hybrids in *Aconitum* (Gáyer, 1909; Götz, 1967; Seitz, 1969), and interspecific hybrids are distinguished in recent taxonomic treatments of the genus (Kadota, 1981, 1986, 1987; Chung and Park, 2000; Ilnicki and Mitka, 2009), some between diploid and tetraploid taxa (Zieliński, 1982a,b; Mitka, 2003). Taxonomic treatments are supported by experimental crossings. Oh and Park (1998) carried out artificial crossings in the *A. jaluense* complex from Korea. The crossing program employed 103 parental plants in 337 crosses, including reciprocal cross-pollinations for all possible pairwise combinations among four species. Crosses between diploid and tetraploid species yielded no fruit-set or seed-set, but among the remaining tetraploid species there were various levels of crossability, enabling two species complexes to be distinguished. These groupings parallel the major entities recognized previously on the basis of flavonoid chemistry (Lim et al., 1999).

We used a PCR-ISSR protocol and a population genetics approach to assess hybridization in taxonomically mixed populations in the sympatric area, versus taxonomically pure allopatric populations. Inter-simple sequence repeat (ISSR) markers are said to offer high resolution of species relationships and introgression patterns (Wolfe et al., 1998). Of the various analytic methods, genetic fingerprinting with hypervariable PCR markers is the most appropriate technique in plant population genetics studies (Bonin et al., 2007). ISSRs have been employed to detect hybrids in the Tasmanian endemics *Eucalyptus richardsonii* and *E. amygdalina* (Sale et al., 1996), to study two closely related Mexican oaks with partially overlapping distributions (González-Rodriguez et al., 2004), and to analyze black and red spruces and their hybrids (Nkongolo et al., 2003). Hybrids between closely related species, and hybrid zones, may be recognized based on morphological markers, but though very helpful they are not suited for detailed analyses (Minder et al., 2007).

In this study we sought to determine (i) whether (*A. lasiocarpum* and *A. variegatum* are genetically 'good' species, that is, whether they represent different and extreme genetic PCR-ISSR profiles; (ii) whether the sympatric, taxonomically mixed populations are genetically intermediate between the taxonomically pure, allopatric populations; and (iii) the genetic status of individuals of particular taxa, based on the expectation that genetic hybrids will be confined to the taxonomic hybrid *A.* ×*pawlowskii*. Verification of the existence of a hybrid zone in the Western Carpathians will aid future studies on postglacial migrations of forest plants along mountain forelands. This is the first study on a putative plant hybrid zone in the Polish Western Carpathians (see Lihová et al., 2007).

MATERIALS AND METHODS

STUDY SPECIES

Aconitum variegatum L. subsp. *variegatum* (*A.* subgen. *Aconitum* sect. *Cammarum*) is a European temperate montane species of Alpine-Central European distributional type (Zając and Zając, 2009), representing the mountain element in the lowlands (Zając, 1996). It is a forest species growing on lowlands in wet broadleaved forest and thickets of *Querco-Fagetea* along torrents and rivers, in mountains in montane alderwood *Alnetum incanae* and in tall-herb community *Arunco-Doronicetum*. The center of occurrence of subsp. *variegatum* in Central Europe is in the Western Carpathians, where in the Beskid Niski Mts. it reaches the eastern limit of its geographical distribution in the Carpathians (Fig. 1a,b). *A. variegatum* subsp. *nasutum* (Fisch. ex Rchb.) Götz occurs in Transylvania (Romania), in the Eastern and Southern Carpathians, in the Balkans in Stara Planina and Rodopes, and in the Eastern Alps (Götz, 1967; Mucher 1991). According to Luferov (2000), *A. nasutum* from the Caucasus belongs to the Asian section *Catenata*. Thus, European *A. variegatum* subsp. *nasutum* is a critical taxon and needs further taxononomic study.

Aconitum lasiocarpum (Rchb.) Gáyer is a European temperate montane species of the Carpathian distributional type (Zając and Zając, 2009). Its typical form is endemic to the Eastern Carpathians; *A. l.* subsp. *kotulae* (Pawł.) Starmühl. et Mitka (Mitka and Starmühler, 2000) has two centers of occurrence: the Polish and Slovak Western Carpathians, including the Tatra Mts., Beskid Niski Mts., Bieszczady Mts. (Fig. 1b), Nizké Tatry Mts. and Podolia in Ukraine. In the mountains it grows in subalpine rowan-green alder thickets. In the montane zone it occurs in beechwood and montane alderwood, also along rivers and in wet meadows, and in forelands it grows in submontane ash carr.

Aconitum ×*pawlowskii* Mitka & Starmühl. (*A. lasiocarpum* × *A. variegatum*; Mitka and Starmühler, 2000; http://www.theplantlist.org/tpl/record/kew-2619335) is a nothospecies of the Carpathian distributional type (Zając and Zając, 2009), endemic to the Western Carpathians. It occurs in the sympatric areas of the parental taxa, with two centers of occurrence: in the Tatra Mts. and in the Beskid Niski Mts. (Fig. 1b).

The species differ in the indumentum: in *A. variegatum* it is glabrous and in *A. lasiocarpum* glandular-pubescent. The subspecies *kotulae* differs from the typical form in having glabrous pedicels below the bracteoles and an admixture of non-glandular hairs. Their putative hybrid *A.* ×*pawlowskii* is easily recognized by hairiness on the inflorescence, as in *A. lasiocarpum*, and by carpels pilose on the suture, as in *A. variegatum* (Mitka and Starmühler, 2000). We qualitatively assessed taxonomically relevant morphological traits in 39 randomly chosen OTUs to calculate phenetic distances scored as follows:

1. Pedicel pubescence below bracteoles (0 – no, 1 – moderate, 2 – extreme)
2. Pedicel pubescence above bracteoles (0 – no, 1 – moderate, 2 – extreme)
3. Type of helmet pubescence (0 – no, 1 – glandular, 2 – eglandular)
4. Type of carpel pubescence (0 – no, 1 – overall, 2 – on suture)
5. Intensity of helmet pubescence (0 – no, 1 – moderate, 2 – extreme)
6. Intensity of carpel pubescence (0 – no, 1 moderate, 3 – extreme)
7. Hairiness of lateral tepals (0 – no, 1 – yes)
8. Hairiness of filaments (0 – no, 1 – yes)
9. Shape of bracteoles (1 – minute-acicular, 2 – linear, 3 – lanceolate, 4 – spatulate, 5 – ovate, 6 – lobate).

All the species are diploids with 2n = 16 (Joachimiak et al., 1999; Ilnicki and Mitka, 2011). Taxonomic identification was done by (JM) using the key for *Aconitum* species determination by Mitka (2003).

SAMPLE COLLECTION AND DNA EXTRACTION

The specimens (OTUs) for genetic and taxonomic studies originated from three populations in the Western Carpathians (Beskid Niski Mts.: Mt. Cergowa, CS; Mt. Kornuty, KS; Stasiany, SS) and one population from the Doły Jasielsko-Sanockie Depression (Targowiska, TS) (Fig. 1c). Additionally, two reference allopatric populations from distant localities were sampled: one from the Eastern Carpathians (Western Bieszczady: Sianki, SA) for *A. lasiocarpum* and the other from the Silesian Upland (Dąbrowa Górnicza, DA) for *A. variegatum*
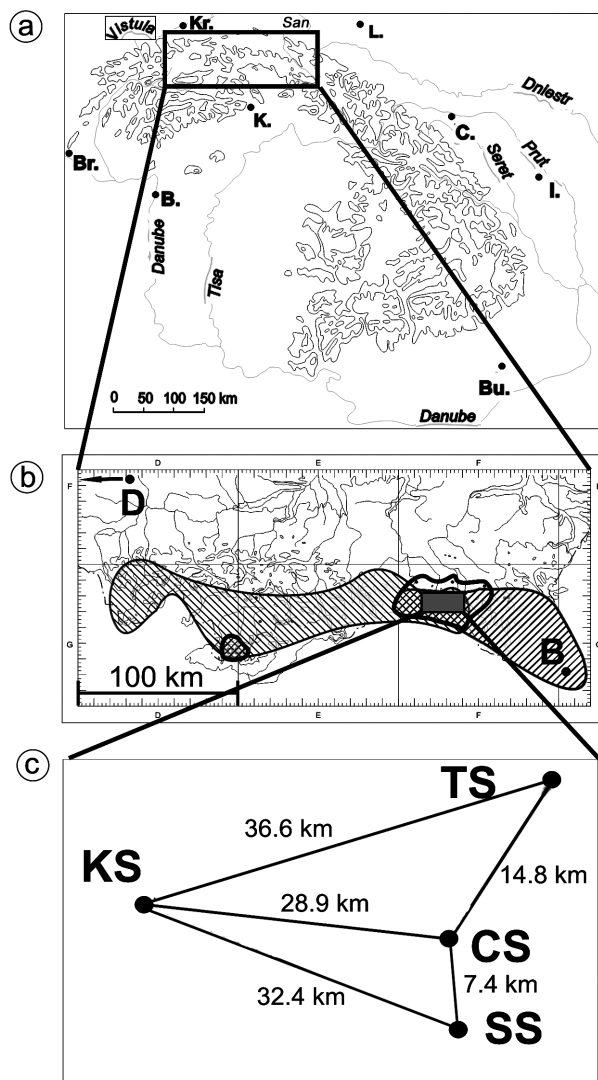

Fig. 1. Geographic localization of the 6 sampled populations of *Aconitum* in southern Poland.(**a**) the Carpathians between Vistula and Dniester rivers, Br. – Bratislava, B – Budapest, Bu – Bucureæti, C – Chernivtsi, I – Iassy, Kr. – Kraków, L. – Lviv, (**b**) Distribution of *A. variegatum* (▨) and *A. lasiocarpum* (▧) populations, with the sympatric areas of *A.* ×*pawlowskii* occurrence (line), D – Dąbrowa Górnicza (Silesian Upland, DA), B – Western Bieszczady (E Carpathians, Sianki SA), (**c**) Sympatric area in the Polish Western Carpathians. CS – Mt. Cergowa (W Carpathians), KS – Mt. Kornuty (W Carpathians), SS – Stasiana (W Carpathians), TS – Targowiska (W Carpathians), see Table 2 for details, Taxonomic nomenclature after Mitka (2003). Distribution maps courtesy of ATPOL (Zając and Zając, 2001).

(Fig. 1b, denoted B and D, respectively). All the populations formed distinct demes except for the population in Stasiany, which was scattered along a river and was sampled along a 500 m transect. The extent of their occurrence in the sympatric populations was

TABLE 1. Primers used in ISSR analysis (after Stepansky et al., 1999) and banding characteristics of *Aconitum* samples from the Eastern and Western Carpathians and the Silesian Upland (S and SE Poland)

Primer	Size (pb)	Sequence 5' – 3'	Maximum no. of bands/ind.	No. of bands/ind.	
				Mean	Median
ISSR 2	160 – 810	$(AG)_8T$	19	5.8	4
ISSR 3	160 – 900	$(GGGTG)_3$	15	5.4	5
ISSR 4	170 – 890	$(ATG)_6$	17	5.0	4
ISSR 5	170 – 880	$(TG)_8G$	21	5.5	4
ISSR 6	160 – 1120	$(AC)_8G$	11	4.9	5

TABLE 2. Taxonomic structure of *Aconitum* populations in S and SE Poland. Total sample size equals size of population, excluding Mt. Kornuty, where the *A. variegatum* population contains ~ 500 individuals. Number of individuals randomly sampled for PCR-ISSR analysis is given in parentheses

Locality (Population)	Area	Region	Altitude (m)	Coordinates North/East	*A. variegatum*	*A. lasiocarpum*	*A. × pawlowskii*	Total
Sianki (SA)	A	E Carpathians	760	49°02'/22°52'	0 (0)	30 (5)	0 (0)	30 (5)
Dąbrowa Górnicza (DA)	A	Silesian Upland	250	50°21'/19°14'	30 (5)	0 (0)	0 (0)	30 (5)
Mt Cergowa Góra (CS)	S	W Carpathians	690	49°31'/21°43'	0 (0)	37 (4)	3 (0)	40 (4)
Mt Kornuty (KS)	S	W Carpathians	700	49°34'/21°22'	35 (9)	0 (0)	0 (0)	35 (9)
Stasiana (SS)	S	W Carpathians	350	49°27'/21°43'	8 (2)	29 (0)	14 (4)	51 (6)
Targowiska (TS)	S	W Carpathians	290	49°38'/21°50'	42 (3)	49 (5)	32 (2)	123 (10)
Total					115 (19)	145 (14)	49 (6)	309 (39)

Area: A – allopatric; S – sympatric.

within 1–3 ha, and with abundance close to the number of individuals sampled (Tab. 2); only the population on Mt. Kornuty had < 500 mature individuals. The allopatric populations were taxonomically pure, with abundance of < 500 mature individuals, collected in undisturbed forest communities of beech-sycamore forest above 600 m a.s.l. (Sianki) and oak-hornbeam forest below 600 m a.s.l. (Dąbrowa Górnicza). The sympatric population in Targowiska grows in a submontane ash carr.

We determined 309 *Aconitum* individuals to species, subspecies and nothospecies to assess the taxonomic structure of the populations under study (Tab. 2). Herbarium material (inflorescence fragments) is deposited in the Herbarium of the Institute of Botany of the Jagiellonian University KRA: *A. lasiocarpum* subsp. *kotulae* – voucher nos. 0388684–0388677, 0388807, 0388826–0388841, 0388866–0388879; *A. variegatum* – 0388685–0388703, 0388823–0388825, 0389498–0389510; *A. ×pawlowskii* – 0388488–0388497, 0388704–0388711, 0388808–0388822, 0388858–0388865, 0388954.

In the ISSR analyses we used 39 randomly selected individuals representing all the taxa studied (Tab. 1). Genomic DNA was isolated from fully developed laminas of healthy leaves showing no signs of damage from insects, mold or other pathogens that might cause contamination. The plant material was washed in distilled water.

DNA was isolated from samples with DNA-zol reagent (Invitrogen) according to the manufacturer's instructions. After preparation the DNA samples were additionally electrophoresed with 1% LMP (LMP – low melting point GIBCO-BRL) agarose with ethidium bromide in TAE buffer at 15 V for 3–4 h. Gel smug analysis intensity is used to estimate the DNA concentration and degradation level. After electrophoresis the purified DNA was cut out in gel blocks.

ISSR-PCR AMPLIFICATION

Five ISSR primers (ISSR2, ISSR3, ISSR4, ISSR5, ISSR6; Stepansky et al., 1999) were used as a single primer in DNA amplification (Tab. 1). PCR reactions were performed in a total volume of 25 µl consisting of 2.5 µl reaction buffer (Taq polymerase supplied), 1.5 mM $MgCl_2$, 0.19 mM of each dNTPs, 27 pmol primer, 10 ng template DNA and 1.4 U Taq DNA polymerase (Invitrogen). The reaction was carried out in a GeneAmp 2400 thermal cycler (Applied Biosystems) with the following amplification profile: 5 min initial denaturation at 94°C followed by 40

cycles of 30 sec at 94°C, 30 sec at 44°C (primers ISSR2 and ISSR4) or 47°C (primers ISSR3, ISSR5 and ISSR6) and 30 sec at 72°C, with final elongation of 7 min at 72°C. The amplification products were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide. Band size was estimated by comparison with a comigrating 100 bp DNA ladder (Fermentas).

A negative control reaction without DNA template was included in each amplification. Products were subjected to electrophoresis on 1.5% agarose gel stained with ethidium bromide (50 µl/100ml) at 100V for ~1.5 h. The electrophoresis results were captured using Imagemaster VDS (Pharmacia-Amerscham) with Liscap Capture ver. 1.0 and archived as digital images. Analysis of band patterns employed GelScan ver. 1.45 (Kucharczyk TE; (http://www.webstatsdomain.com/tags/gelscan/). The molecular weight of the resulting amplification products was determined versus a calibration curve based on the band pattern of the molecular length marker (GeneRuler^{TM} 100 bp, Fermentas). ISSR reproducibility tests (Bonin et al. 2004) included within-plate (n = 12) and between-plate (n = 9) replicates independently analyzed from the DNA extracts.

DATA ANALYSIS

At the population level we analyzed PCR-ISSR polymorphism as alleles, under three assumptions: ISSR products segregate as dominant alleles in Mendelian fashion, genotype frequencies at ISSR loci are in Hardy-Weinberg equilibrium, and comigrating fragments are considered homologous loci (Apostol et al., 1996). Despite the strong assumptions, the high number of ISSR fragments generated from the whole genome can recover useful discriminatory information for phylogenetic and systematic studies of closely related species (Bussell et al., 2005) and can provide a reliable estimation of genetic variability at the population level (Sica et al., 2005).

A total of 199 amplified DNA fragments were scored as a presence-absence primary matrix of binary data. The dominant ISSR data were analyzed by treating each class of genotypes as being, effectively, haploid alleles. Hardy-Weinberg equilibrium was assumed in most calculations. An exact test was carried out using the contingency table approach (Fischer's R×C test) for groups of populations, based on a Markov chain Monte Carlo approach (MCMC, Raymond and Rousset, 1995). Additionally, Fisher's combined probability test (Fischer, 1954; Manly, 1991; Sokal and Rohlf, 1995) was employed as a global test over loci to determine overall significance between groups of taxonomically pure and mixed populations. TFPGA ver. 1.3 (Miller, 1997) was used for the calculations.

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 stable priors and the optimum likelihood for K = 4. The numbers of K from 2 to 5 were tested with ten replicates per K. In the LOCPRIOR model (Hubisz et al., 2009) the sampling location for each individual was specified, with the expectation that the sampling locations may be informative about ancestry. The model is recommended when the amount of available data is very limited.

The genetic distances (Reynolds et al., 1983) were calculated for each pair of the six populations and then used to construct a tree based on an unweighted pair-group method with arithmetic mean (UPGMA) algorithm (Sokal and Michener, 1958) implemented in SPLITSTREE4 (Huson and Bryant, 2006). Nei's D genetic distance (Nei, 1972) obtained with AFLP-SURV (Vekemans, 2002) gave the same tree topology with slightly lower bootstrap values. Bootstraps generated in AFLP-SURV based on 1000 runs produced 1000 random trees to obtain a consensus network (displayed in phylogram form) with the help of SPLITSTREE4 (Huson and Bryant, 2006). Allele frequencies were estimated based on a Bayesian method with non-uniform prior distribution of allele frequencies, which efficiently reduces the bias of the square method (Zhivotovsky, 1999), with a predefined value of Fis = 0.1 because of presumed inbreeding due to geitonogamy (see Utelli et al., 1999). The distribution of allele frequencies was estimated separately for each population. Statistics of genetic diversity and population genetic structure were then computed strictly following the treatment of Lynch and Milligan (1994).

Additionally, Nei and Li (1979) distances among 39 *Aconitum* OTUs were calculated for ISSR data and then used for nonmetric multidimensional scaling analysis (NMDS – Kruskal, 1964). A Mantel (1967) test to calculate the correlation between ISSR and phenetic matrices based on Nei and Li distances was carried out based on 999 permutations. Both analyses were performed with the use of the NTSYSpc ver. 2.11 multivariate analysis package (Rohlf, 2002).

We used the NEWHYBRIDS ver. 1.1 beta Bayesian assignment program, which implements a multilocus allele frequency model-based method for determining hybrid status (Anderson and Thompson, 2002). This method performs individual clustering without any a priori knowledge of parental allele frequencies, and has the advantage of
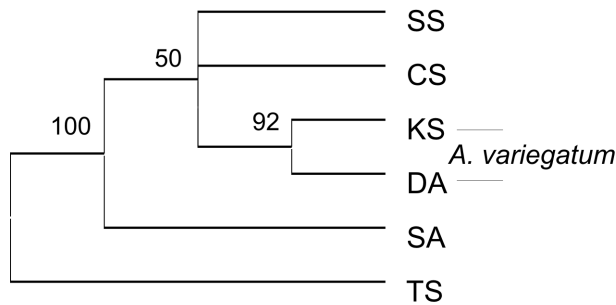
Fig. 2. UPGMA majority consensus tree based on bootstrapped (n = 1000) Reynolds's genetic distances. CS – Mt. Cergowa (W Carpathians); DA – Dąbrowa Górnicza (Silesian Upland); KS – Mt. Kornuty (W Carpathians); SA – Sianki (E Carpathians); SS – Stasiana (W Carpathians); TS – Targowiska (W Carpathians).

Fig. 3. Consensus network based on bootstrapped (n = 1000) Reynolds's distances. For population abbreviations see Figure 2 and Table 2.

specifically assuming a mixture of parental and various hybrid classes in its probability model. Six genotype categories corresponding to parental (pure *Aconitum lasiocarpum* and pure *Aconitum variegatum*), F1, F2 and later generation hybrids B1 and B2 (B1 – *Aconitum lasiocarpum* backcross, i.e., F1 × *A. lasiocarpum* and B2 – *A. variegatum* backcross, i.e., F1 × *A. variegatum*), were considered. Individual posterior probabilities belonging to each hybrid category were estimated using the MCMC method in a Bayesian framework. Calculations were run using Jeffreys-type priors and a burn-in period of 100,000 iterations followed by 50,000 sweeps for sampling from the posterior distribution.

RESULTS

PCR-ISSR analysis resulted in 199 polymorphic markers. The maximum number of bands generated by individual primers per individual varied from to 11 to 31, with a mean of 4.9–5.8 and median of 4 and 5 (Tab. 1). Data quality tests indicated high repeatability across of the ISSR bands, above 97%.

Two of the four populations in the sympatric zone were taxonomically uniform; they both were located in mountain top conditions. The two remote allopatric populations were also taxonomically uniform (Tab. 2). The Bayesian analyses (STRUCTURE, NEWHYBRIDS, see below) showed that the allopatric population of *Aconitum variegatum* in the Silesian Upland (DA) and the sympatric population of the same species on Mt. Kornuty in the Western Carpathians (locality KS, see Fig. 1b,c) represent the same genetic profile.

The percentage of polymorphic loci varied from 26.1 to 50.8% in the populations, with no consistent trend in taxonomically uniform vs. mixed or allopatric vs. sympatric areas, similarly to the case
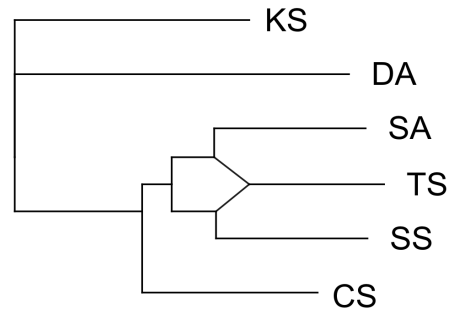
of the remaining genetic indices calculated. The allopatric populations had lower expected heterozygosity Hj (mean 0.126405 ± 0.000488) than sympatric populations (mean 0.143663 ± 0.008338, t-test, P = 0.05) (Tab. 3).

The UPGMA tree clearly revealed that the two taxonomically pure but geographically distant populations of *A. variegatum* in Mt. Kornuty KS and Dąbrowa Górnicza DA had high genetic support (bootstrap 92%). The remaining sympatric populations were unresolved: *A. lasiocarpum* from Mt. Cergowa CS and taxonomically mixed Stasiany SA (Fig. 2). The consensus network gave an additional insight: the sympatric TS population has an intermediate position between the allopatric population SA from the Bieszczady Mts. and the sympatric population SS (Fig. 3). The genetic distances were highest (Reynolds's 0.3725 and Nei's D 0.0685) between the pure population of *A. variegatum* in the allopatric Dąbrowa Górnicza DA locality and *A. lasiocarpum* in the sympatric Mt. Cergowa CS locality. Most similar in this respect were sympatric populations Mt. Kornuty KS and Tylawa TS (Reynolds's 0.1681) and also allopatric Sianki SA and Tylawa TS (Nei's D 0.0259) (Tab. 4).

STRUCTURE estimated the number of distinct genetic clusters for K = 4 distributed in six geographic populations (Fig. 4). The Mt. Kornuty KS and Dąbrowa Górnicza DA populations, though geographically distant, formed one distinct *A. variegatum* cluster. The second cluster is formed by allopatric *A. lasiocarpum* in Sianki SA and consists of two genetic clusters. The two sympatric populations Mt. Cergowa CS and Stasiany SS possess their own genetic identity. The most highly differentiated is population Tylawa TS, where all the recognized genetic clusters occur, and where the taxonomic structure of the individuals was most varied (Tab. 2, Figs. 3, 4). It is also seen that *A. variegatum* from Kornuty KS contributed to Targowiska TS and Stasiany SA, and not to isolated Mt. Cergowa CS (Figs. 1c, 4).

TABLE 3. Genetic variation in taxonomically pure and mixed populations of *Aconitum* analyzed at 199 ISSR loci with AFLP-SURV (Vekemans et al. 2002). Notation follows Lynch and Milligan (1994). For population abbreviations see Table 2

Population	Taxonomic status	Area	n	PLP	H_j	S.E.(H_j)	VarI%	VarL(H_j)	VarL%
SA	uniform	A	5	28.6	0.12675	0.01131	26.7	0.000094	73.3
DA	uniform	A	5	32.2	0.12606	0.01145	31.1	0.000090	68.9
CS	mixed	S	4	26.1	0.15062	0.01240	37.3	0.000096	62.7
KS	uniform	S	9	50.3	0.13476	0.00988	28.5	0.000070	71.5
SS	mixed	S	6	41.2	0.15092	0.01056	36.4	0.000071	63.6
TS	mixed	S	10	50.8	0.13835	0.00937	28.8	0.000063	71.2

Area: A – allopatric; S – sympatric; n – sample size; PLP – proportion of polymorphic loci (95% criterion); H_j – expected heterozygosity under H-W genotypic proportions (Nei's gene diversity); S.E.(H_j) – standard error of H_j; VarI% – proportion of variance H_j due to sampling of individuals, VarL(H_j) – variance component of H_j due to sampling of loci, VarL% – proportion of Var(H_j) due to sampling of loci.

TABLE 4. Genetic distances between populations. Reynolds's distances (below diagonal), Nei's D distances (above diagonal). For population abbreviations see Table 2. Maximum values in bold, minimum values in italics.

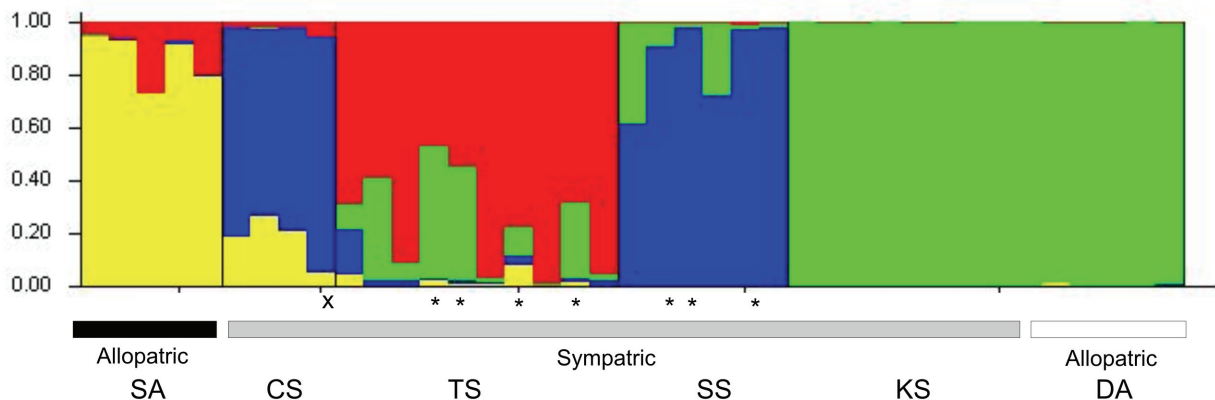|	SA	DA	CS	KS	SS	TS
SA	-	0.0474	0.0426	0.0313	0.0351	*0.0259*
DA	0.2969	-	**0.0685**	0.0457	0.0546	0.0433
CS	0.2619	**0.3725**	-	0.0407	0.0361	0.0425
KS	0.2031	0.2700	0.2362	-	0.0403	0.0264
SS	0.2185	0.3058	0.2069	0.2316	-	-
TS	0.1769	0.2660	0.2528	*0.1681*	0.1904	0.0000

Fig. 4. Genetic relationships among populations based on 199 PCR-ISSR bands and STRUCTURE analysis revealed the optimum number of genetic clusters for K = 4. ∗ – genetic B1 hybrids according to NEWHYBRIDS; × – introgressed *A. lasiocarpum* (see Figs. 5, 6).

The band-based NMDS ordination showed a slightly different pattern of population differentiation (Fig. 5). The allopatric population Dąbrowa Górnicza DA and sympatric Mt. Kornuty KS of *A. variegatum* are very close each to other. Similarly, the OTUs of *A. lasiocarpum* are very genetically similar, but three OTUs of *A. ×pawlowskii* found a place in their group. Two OTUs (from Mt. Cergowa CS and Targowiska TS) located in the middle of the diagram, however taxonomically different, are genetically similar. Seven asterisked OTUs, recognized by NEWHYBRIDS as B1 introgressants, were five *A. variegatum* and two *A. ×pawlowskii* (Fig. 5).

NEWHYBRIDS showed the genetic status of each of the individuals: pure *A. lasiocarpum*, pure *A. variegatum*, and later-generation hybrids (intro-
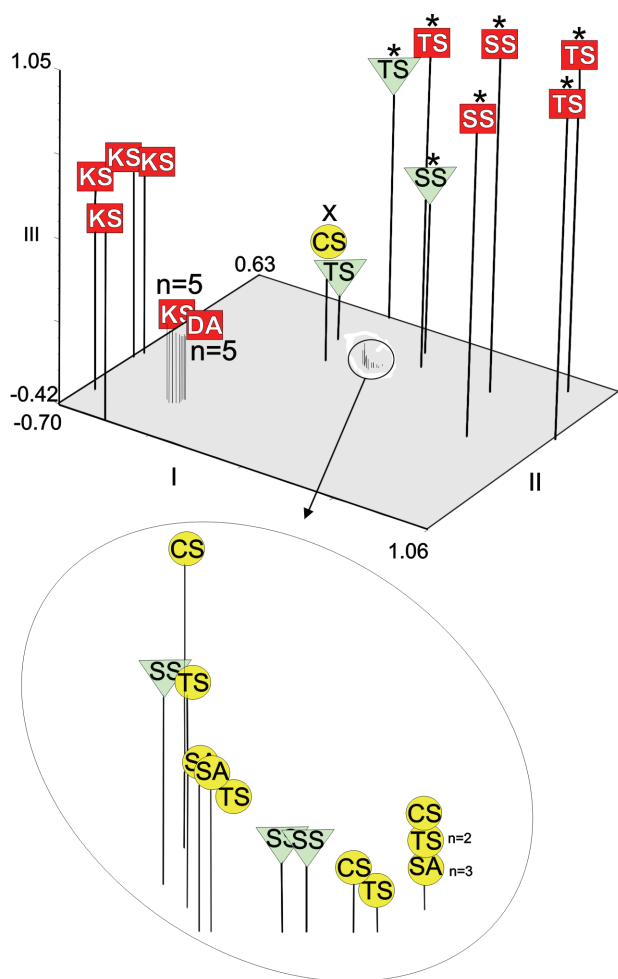
Fig. 5. Nonmetric multidimensional scaling, ∗ – genetic B1 hybrids according to NEWHYBRIDS (see Fig. 6); × – introgressed *A. lasiocarpum*. For population abbreviations see Table 1.

gressants), according to a six-class model implemented in the program (see Materials and Methods). Pure genetic *A. lasiocarpum* was assigned in total to Sianki SA and Mt. Cergowa CS (excluding one OTU) and pure genetic *A. variegatum* in total to Dąbrowa Górnicza DA and Mt. Kornuty KS. Sympatric populations Stasiany SS and Targowiska TS consist of both genetically pure *A. lasiocarpum* and genetic hybrids. Genetic hybrid status was assigned to two individuals of *A. ×pawlowskii* and to five individuals of *A. variegatum*. Three individuals of *A. variegatum* in Targowska TS and two individuals in Stasiana SS were assigned to the genetic hybrid group (Fig. 6). One individual of *A. lasiocarpum* from Mt. Cergowa (CS) has a sign of B1 introgression (Figs. 5, 6).

The Mantel test showed a significant correlation r = 0.53 (P = 0.001) between genetic ISSR and morphological Nei and Li distances obtained from the comparison of 39 *Aconitum* OTUs.

An exact test using a contingency table approach (Fischer's R×C test) for groups of populations, based on a Markov chain Monte Carlo approach, showed that the genetic population of *A. lasiocarpum* was significantly different from the genetic population of *A. variegatum* (chi-square test = 536.71, df = 398, P < 0.0001). The group of
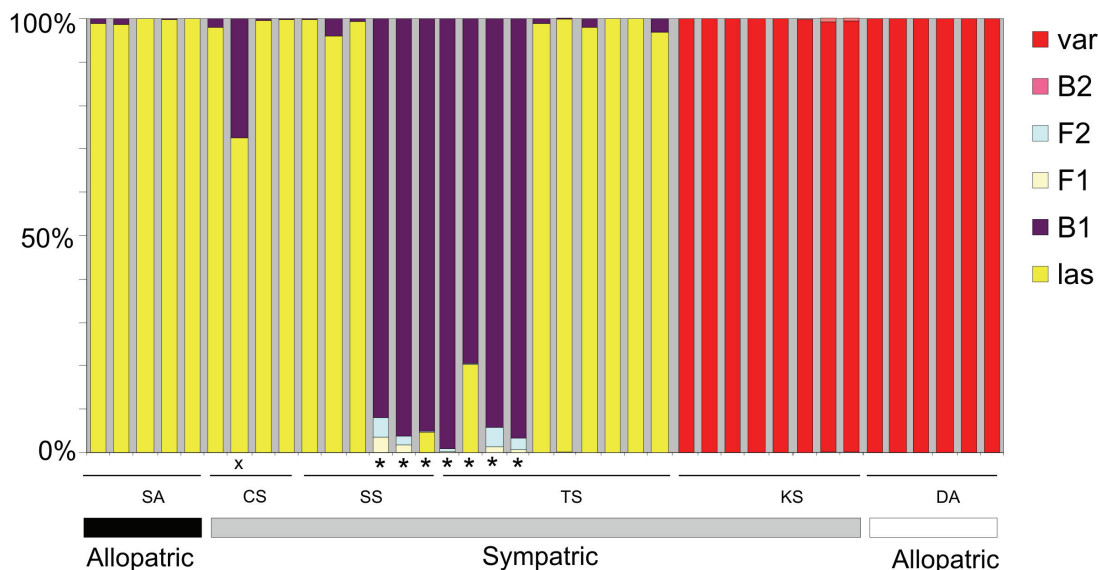

Fig. 6. NEWHYBRIDS (Anderson and Thompson, 2002) classification of *Aconitum* individuals in allopatric and sympatric areas according to Bayesian assignment algorithm to detect gene flow. The seven introgressive individuals representing backcross B1, i.e., F1 × *A. lasiocarpum*, are marked. × – introgressed *A. lasiocarpum*. For population abbreviations see Table 2. las – pure *A. lasiocarpum*; var – pure *A. variegatum*; B1, B2 – later-generation hybrids; F1 – first-generation hybrid; F2 – second-generation hybrid (see Data Analysis).

seven genetic hybrids, as revealed by NEWHYBRIDS, was not significantly different from the two species (*A. lasiocarpum* vs. hybrids, *variegatum*, chi-square test = 282.11, P = 1.00; *A. variegatum* vs. hybrids, chi-square test = 368.32, P = 0.85). In pairwise comparisons, 16 (8%) loci differentiated *A. lasiocarpum* from *A. variegatum*, 12 (6%) hybrids from *A. variegatum*, and only 3 (1.5%) hybrids from *A. lasiocarpum*.

DISCUSSION

HYBRID STATUS OF *ACONITUM* POPULATIONS AND INDIVIDUALS

The meaning of "hybrid" in Linnaean plant taxonomy is not the same as in genetics or zoology. Taxonomic hybrid is a category established within the nothotax-on concept that fulfills ICBN Article H.4.1: "When all the parent taxa can be postulated or are known, a nothotaxon is circumscribed so as to include all individuals recognizably derived from the crossing of representatives of the stated parent taxa (i.e., not only the F1 but subsequent filial generations and also back-crosses and combinations of these)" (Melbourne Code, http://www.iapt-taxon.org/nomen/main.php). In this sense, each designate of the nothotaxon (specimen) is a "genetic hypothesis" based on morphological assessment. So if species hybridize, Linnaean taxonomy has a solution: the concept of nothotaxa. They simply fill the morphological gap (hiatus) between the putative parental species. In fact they stabilize the taxonomic system. Hybridization does not necessarily lead to establishment of a new genetic species, at least within species having the same chromosome number (Mráz et al., 2005). This conceptual background affects the study framework and conclusions.

A hybrid zone is formed when populations of individuals that are distinguishable on the basis of one or more inheritable characters overlap spatially and temporally and cross to form viable and at least partially fertile offspring (Arnold, 1997).

One of the most comprehensive studies on hybridity in *Aconitum* comes from biochemical studies by Zieliński (1982a). He analyzed 636 OTUs: diploid *A. variegatum* and tetraploid *A. firmum* in the Tatra Mts. (Western Carpathians) in 21 taxonomically mixed and uniform populations. Starch-gel analysis based on 6 enzyme systems was employed. The analyzed OTUs mostly possessed enzyme phenotypes corresponding to their taxonomic status, but intermediate phenotypes were found in 4.7% of them. Interestingly, in two F1 triploid hybrids only partial supplementation of the parent phenotypes were found, instead of their simultaneous appearance (Zieliński, 1982b). In one

triploid only peroxidase provided the intermediate heterozygotic phenotype; of the three remaining enzymes, two were characteristic for the tetraploid and one for the diploid species. In the other triploid, none of the differing enzymes gave a phenotype intermediate between the diploid and the tetraploid, although one peroxidase phenotype was unique, representing an example of transgressive segregation (Rieseberg et al., 2000). The remaining 28 tetraploid plants had a peroxidase intermediate phenotype, regarded as introgressive hybridization. The occurrence of introgression between the diploid and the tetraploid aconite was possible, because the triploid hybrids produce 13% viable pollen (Zieliński, 1982a). Apparently it is an example of a "triploid bridge," postulated as one of the most important mechanisms of introgression between organisms with different ploidy (Husband, 2004).

Our results gave an additional insight into hybridization in the sympatric area. The putatively hybridizing species were diploids, making the process common. First, the identity of *A. variegatum* was clearly revealed in a high-elevation population from Mt. Kornuty (KS) and a remote allopatric population from Dąbrowa Górnicza (DA) in the Silesian Upland (Tab. 2, Fig. 4). However, five OTUs of *A. variegatum* in the sympatric lower-elevation populations SA and TS were genetically assigned to the B1 hybrid group (Fig. 5). This means that they form a cryptic hybrid group with the morphological status of *A. variegatum* but with an admixture of genes from *A. lasiocarpum*. On the other hand, only two of six individuals of the taxonomic hybrid *A. ×pawlowskii* were recognized as later-generation B1 hybrids (Fig. 5). The remaining four OTUs of the taxonomic hybrid were genetically close to *A. lasiocarpum* as revealed by NMDS analysis.

Thus, taxonomic/morphological hybrids are not always recognized by ISSR analysis at the individual level. A similar result in general outline was obtained in Zieliński's (1982a) study of *Aconitum* hybrids in the Tatra Mts. based on biochemical starch-gel analysis. These results could be interpreted as a case of cryptic introgression. It means that an individual can appear to be taxonomically pure but in fact be a later-generation introgressant. This can happen when its extended phenotype has arisen out of a chance recombination of differentiating characters. We only found later B1 generation hybrids; that is, backcrossing was asymmetrical, occurring exclusively towards *A. lasiocarpum*. This was indicated by the number of differentiating loci based on Fisher's combined probability test between the genetic hybrids in the sympatric area vs. *A. lasiocarpum* (only 3 differentiating loci), against the 12 loci differentiating the genetic hybrids vs. *A. variegatum*. It could not be explained by differences in phenology (Milne and Abbott, 2008)

because both species flower at the same time, but studies on this are needed. Rusham et al. (2011) reported a similar result in a hybrid swarm of *Geum rivale* and *G. urbanum*, where none of the individuals in the hybrid swarm identified by NEWHYBRIDS were classified as a backcross to *G. urbanum*. Hybrids between *Cardamine enneaphyllos* and *C. glandulifera* were placed by PCR-RFLP analysis in an intermediate position but they also largely overlapped with one of the parental species (Lihová et al., 2007).

If it is not an artifact originating from small sample size, another explanation could be segregation distortion (Rieseberg et al., 2000). Hybrid progeny may receive more alleles from one parent than would be expected under Mendelian rules of segregation, and thus could resemble that parent more closely than these rules would predict. Such asymmetric introgression of alleles presumably would be positively selected in certain environmental conditions (Arnold et al., 2010).

In the sympatric area the population structure of *Aconitum* under study depends on the relief. Here the most isolated mountain top population from Mt. Kornuty (KS) retains its morphological and genetic identity. The high-elevation population from Mt. Cergowa (CS) was probably the source for the lower-lying Stasiana (SA) population (Figs. 1c, 4). The most abundant, lower-elevation population in Targowiska (TS) was intermediate, in both taxonomic and genetic terms (Tab. 2, Figs. 3, 4).

THE HOLOCENE HISTORY OF *ACONITUM* IN THE WESTERN CARPATHIANS

The STRUCTURE and consensus network results indicate that the population in Targowiska (TS) developed as a result of migration from different refugial centers (Figs. 1c, 4). The population is one of the most genetically variable and presumably consists of two migratory elements: *A. variegatum* from the west and *A. lasiocarpum* from the east. It seems to be an example of the genetic melting pot expected in places where different Holocene migratory routes met (Petit et al., 2003). The sympatric *Aconitum* populations had significantly higher (p = 0.05) expected heterozygosity than the allopatric populations, perhaps the result of the proximity of the sympatric populations to the refugial species' areas (Utelli et al., 1999), a hybrid zone wider than the present one, or Holocene migration of *Aconitum lasiocarpum* to the Beskid Niski Mts. from the Eastern Carpathians.

The mixed population in TS seems rather of recent (Holocene) age. The other sympatric and adjacent population, at Mt. Kornuty (KS), is taxonomically and genetically uniform. This could be an effect of its geographical isolation and perhaps a result of its refugial character. For the montane forest species *A. lasiocarpum* the close genetic affinity between the populations from Sianki (SA, E Carpathians) and Targowiska (TS, W Carpathians) suggests an earlier wide hybrid zone where some genetic variants spread further from the present tension zone than others. On the other hand, the existence of a strong phytogeographic barrier (Zemanek, 1999) to dispersal from the Eastern to the Western Carpathians is postulated for alpine (Mráz et al., 2007; Ronikier, 2011) and subalpine (Stachurska-Swakoń et al., 2011) species. The phytogeographic barrier in the lower-lying Beskid Niski Mts. is due to the absence of high-mountain conditions and apparently does not apply to the montane forest species. The genetic relationships we found between the *Aconitum* species in the Eastern and Western Carpathians support this suggestion.

The lack of genetic difference between distant *A. variegatum* populations from the Beskid Niski Mts. (Kornuty) and Polish Uplands (Dąbrowa Górnicza) are in accord with the finding of the presumed refugial Cytotype IV, recognized by cytogenetic C-band analysis of *A. variegatum* in Central Europe (Ilnicki et al., 2011). Such a pattern suggests the existence of cryptic refugia for *A. variegatum* somewhere in the Western Carpathians, from where the species could recolonize the adjacent forelands and uplands in the Holocene. Cryptic glacial forest refugia in the Western and Eastern Carpathians can be postulated as a working hypothesis (Provan and Bennett, 2008). From here, rapid short-distance postglacial recolonization, including the remnants of temperate forest vegetation, might occur.

To verify the hypothesis of rapid short-distance postglacial recolonization of the Beskid Niski Mts. in the Western Carpathians by some forest species, *Aconitum* populations along a NW-SE transect from the Silesian Upland (D) to the Western Bieszczady Mts. in the Eastern Carpathians (B – see Fig. 1b) should be sampled to detect the potential clinal distribution of the genes. A cline may originate when gene flow after secondary contact between two genetically distinct populations leads to continuous changes in allele (or DNA molecular marker) frequencies (Breitenbach-Dorfer et al., 1996; Barton and Hewitt, 1985). In other words, a clinal distribution will confirm the Holocene migrations of some forest species – in opposite directions – in the Western Carpathians from Quaternary forest refugia, as postulated elsewhere (Konnert and Bergmann, 1995). Nei's D genetic distance between sympatric Targowiska TS and allopatric SA Sianki populations, the lowest at 0.0259 (see Figs. 3, 4), also points to this, suggesting migration of *Aconitum lasiocarpum* to the Beskid Niski Mts. from the Eastern Carpathians.

Further studies on the hybrid zone in the Western Carpathians should yield more insight into their postglacial vegetation history.

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CONCLUSIONS

1. PCR-ISSR analysis confirmed a hybrid zone in the Western Carpathians. Genetically hybridogenous specimens were found exclusively within the sympatric range of *A. lasiocarpum* and *A. variegatum*, where their morphological hybrid *A. ×pawlowskii* occurs. Also occurring here were the most genetically variable populations (Nei's gene diversity), a genetic melting pot.

2. Some taxonomically pure species (OTUs) in the sympatric area show mixed ancestry, forming a hybrid cryptic group (cryptic introgression).

3. The presence of the hybrid zone points to the existence of cryptic (unrecognized) glacial refugia for *Aconitum* species in the Western and Eastern Carpathians.

4. The Mantel test revealed a significant correlation (P = 0.001) between the ISSR and morphological diversity of *Aconitum* in the hybrid zone.

5. Taxa and nothotaxa recognized in Linnaean taxonomy may be of use in formulating biogeographical hypotheses.

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