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RETICULATE EVOLUTION OF HIGH-ALPINE *ACONITUM* (RANUNCULACEAE) IN THE EASTERN SUDETES AND WESTERN CARPATHIANS (CENTRAL EUROPE)

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The chromosomal and molecular PCR-ISSR+RAPD pattern of high-mountain *Aconitum* sect. *Aconitum* in the Sudetes and Carpathians were analyzed to test whether the taxon has common markers in these two mountain systems. In the Sudetes the taxon forms an autopolyploid chromosome complex (2n = 32), and allopolyploid in the neighboring Western Carpathians. The chromosome Giemsa C banding pattern of the allotetraploid Carpathian *A. firmum* was found to be common to both Sudetic autopolyploid *A. plicatum* and diploid *A. variegatum*. The Sudetes are geologically older than the Carpathians, and it is argued that an ancient Sudetic taxon may have contributed to the genome of the Carpathian taxon. The Quaternary glaciations and corresponding range extensions of alpine floras may have facilitated their secondary contact(s). This is supported by a molecular ISSR+RAPD pattern that points to introgression between the Sudetic *A. plicatum* subsp. *sudeticum* and Carpathian *A. firmum* subsp. *maninense*.

Key words: C-banding, heterochromatin, historical biogeography, hybridization, ISSR, phenetics, polyploidy, RAPD.

INTRODUCTION

Several cytological studies have been revealed that high-mountain *Aconitum* L. sect. *Aconitum* in Europe forms a tetraploid complex (e.g., Schafer and La Cour 1934; Leszczak 1950; Zieliński 1982; Okada 1991). In the Carpathians and Sudetes it is represented by two distinct endemics, *A. firmum* Rchb. (Starmühler and Mitka, 2001) and *A. plicatum* Koehl. (Mitka 2003). Each of them has geographical races at the rank of subspecies (Mitka, 2003). Two of them – the narrow endemics *A. plicatum* subsp. sudeticum Mitka and *A. firmum* subsp. *maninense* (Skalický) Starmühl. – show intriguing Sudetic-Carpathian affinities. They share a character unusual in *A.* sect. *Aconitum*, glandular-hairy indumentum and carpels (Starmühler and Mitka, 2001). Type of indumentum hairiness has proven to be a key character in recent Linnaean taxonomic treatments of *Aconitum* (Kadota, 1987, Kita et al., 1995; Yang, 1999; Mitka and Starmühler, 2000; Mitka, 2003; Luo and Yang, 2005). Have historical events contributed to such a morphological pattern?

Recently, several attempts have been made to understand the molecular phylogeny and phylogeography of *Aconitum* by sequencing the internal transcribed spacer regions of nuclear ribosomal DNA (ITS) or noncoding regions of chloroplast DNA (cpDNA) (Kita et al., 1995; Kita and Ito, 2000; Utelli et al., 2000; Luo et al., 2005). It has been shown that many taxa that are morphologically well-defined or geographically disjunct possess identical or nearly identical ITS and cpDNA sequences. Neither ITS nor cpDNA sequencing have revealed much information on hybridization and introgression within the analyzed groups of taxa. Hence, hypervariable, arbitrarily amplified dominant markers (AFLP, ISSR, RAPD) seem to provide more useful data to generate phylogenies (Archibald et al., 2006), including potential hybridization and introgression events

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(e.g., Bartish et al., 2000; Cole and Kuchenreuther, 2001, Rieseberg et al., 2000). For example, RAPD analysis has proven to be a useful tool for clear systematic separation of taxa belonging to subgen. *Aconitum* sect. *Aconitum*, sect. *Cammarum*, and A. subgen. *Lycoctonum* from the Italian Alps (Fico et al., 2003), and for analyzing the genetic differentiation and taxonomy of the *A. delavayi* Franch. complex in the Hengduan Mts. of China (Zhang et al., 2005).

The historical-biogeographical affinities between the two bordering mountain systems could reach far back in time. It is known from the palaeogeological record that parts of the Eastern Carpathians and Western Carpathians were formed at the Oligocene/Miocene boundary (26–22 myr BP), that the whole of the Carpathians were united some 14 myr BP (in the Middle Miocene), and that this event was accompanied by regression of the sea from the Alpine-Carpathian Foredeep (Rögl, 1998; Golonka et al., 2006). Following uplift of the mountain range it could be colonized from the adjacent Sudetic, Paleogene flora (Syabryay and Stuchlik, 1994).

The floristic relationships between the Carpathians and Sudetes could also be of more recent age. Palaeobotanical, palaeoenvironmental and molecular phylogeographical studies support the idea that even during the most severe glacial maxima the alpine flora of Central Europe persisted in peripheral periglacial refugia in intermountain lowlands or restricted to a few ice-free mountain tops – nunataks (Starkel, 1988; Abbott et al., 1995; Obidowicz, 1996; Stehlik, 2000; Stehlik et al., 2002; Holderegger et al., 2002; Kropf et al., 2003; Valero-Garces et al., 2004, Schönswetter et al., 2004, 2005). Moreover, cooling episodes, pushing out part of the high-mountain flora to peripheral refugia, could facilitate secondary contacts between otherwise isolated genetic stocks and thus originate hybridogenous taxa.

The aim of our study was to investigate cytogenetic (Giemsa C-banding) and molecular ISSR and RAPD markers in Sudetic and Western Carpathian high-mountain *Aconitum* sect. *Aconitum*, and to compare those data with predictions of species relationships based on biogeographical evidence. In the European Alps, molecular studies have shed some light on the origin of high-mountain flora (Stehlik et al., 2002, Comes and Kadereit, 2003, Schönswetter et al., 2005), but hardly any robust phylogenetic analyses have been performed for other European mountain systems such as the Sudetes and Carpathians. Here, we sampled biogeographically representative areas of species occurrence. Their taxonomic treatment is discussed in terms the phylogeographic scenario adopted.

MATERIALS AND METHODS

Tetraploid *Aconitum* sect. *Aconitum* (former Napellus group, Seitz, 1969) in Europe encompasses mostly high-mountain taxa, which are all endemic and often restricted to narrow geographical ranges. In the Western Carpathians and Sudetes, the Carpathian *A. firmum* and the Sudetic-Hercynian *A. plicatum* belong to the section (Starmühler, 2001; Starmühler and Mitka, 2001; Mitka, 2003; Tab. 1, Fig. 1a).

In the Sudetes and Carpathians, two endemic forms are worthy of mention: *A. p.* subsp. *sudeticum* and *A. f.* subsp. *maninense*. The former is endemic to the Eastern Sudetes (Králický Snežnik, Czech Republic; Śnieżnik Mt., Poland; Hrubý Jeseník Mts., Czech Republic), and the latter is a Western Carpathian endemic restricted to two regions: the Stražovské vrchy Mts. in Slovakia, and the High Tatras and adjacent Rów Podtarzański trench in Poland (Mitka, 2003; Tab. 1, Fig. 1a).

The basal chromosome set of *Aconitum* is bimodal (2 long and 6 short chromosomes), and evolutionarily stable with respect to general chromosome morphology but not to heterochromatin banding patterns (Okada, 1991, Joachimiak et al., 1999). Different C-banding chromosome variants appeared to be of diagnostic value in analysis of *Aconitum* species and hybrids (Joachimiak et al., 1999, Mitka and Starmühler, 2000; Ilnicki, 2005).

PLANT MATERIAL

Thirty randomly chosen specimens of *Aconitum* sect. *Aconitum* were collected in the Sudetes and Western Carpathians from late July to early September 2002–2003, and kept in the Botanical Garden of the Jagiellonian University in Cracow. The sample size was limited by the scarcity of most taxa. DNA from fresh leaves of plants under cultivation was extracted in 2004 just before flowering. The geographic localities and information on the populations sampled are given in Figure 1b and Table 1.

KARYOTYPE ANALYSIS

In the summer of 2005, all planted specimens were dug up, transferred to the laboratory and kept in pots, and root tips were cut for cytological analysis. Chromosomes were studied in squash preparations made according to Grabowska-Joachimiak and Joachimiak (2002) and stained according to the C-banding method of Schwarzacher et al. (1980). From each plant, three complete metaphase plates showing maximum banding response were selected for detailed analysis. An additional sample of *A. variegatum* (*A. sect. Cammarum* DC.) from the Slovakian and Polish Carpathians was also cytogenetically studied (Ilnicki, 2005).

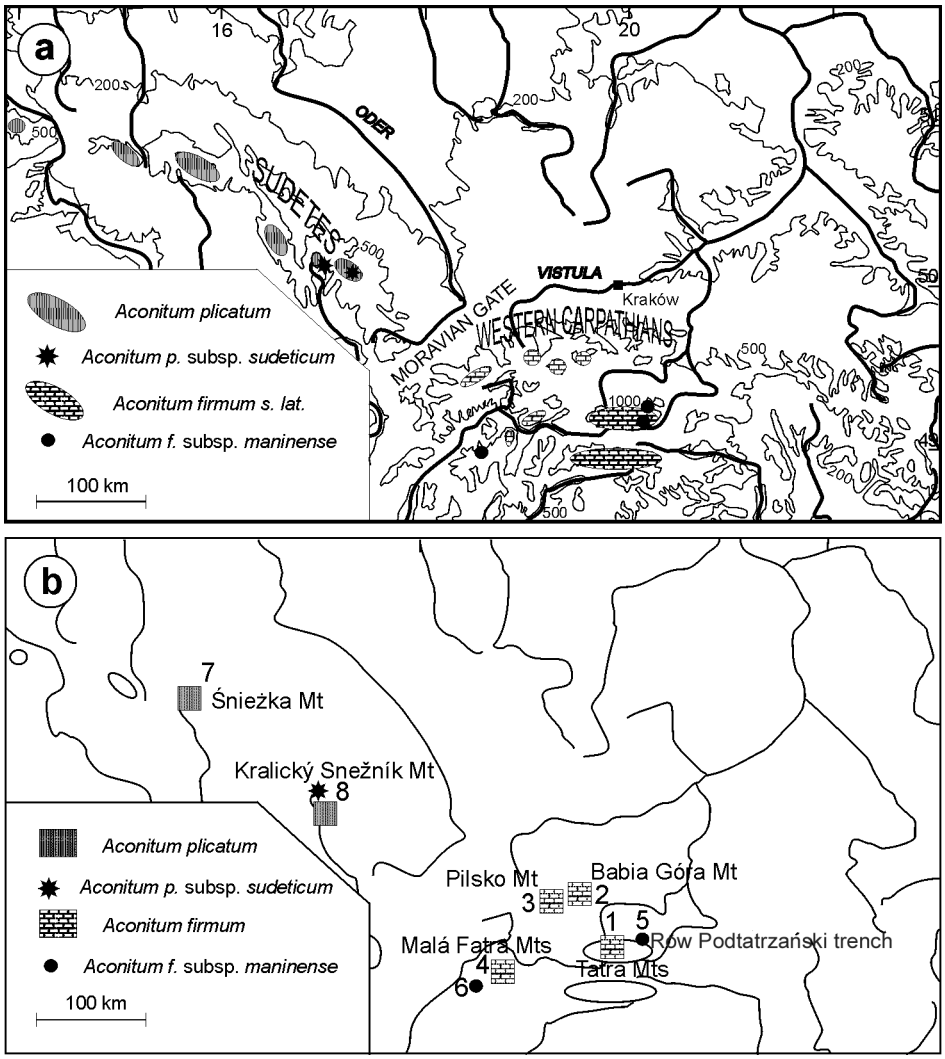

Fig 1. (**a**) Geographic distribution of *A. firmum* and *A. plicatum* in Central Europe, modified from Mitka (2003), (**b**) Geographic locations of studied populations (see Tab. 1).

DNA ISOLATION AND PCR AMPLIFICATION

DNA was isolated from fresh leaves using Plant DNAzol Reagent (Invitrogen) according to the manufacturer's instructions. DNA quality and quantity were determined in 1% agarose LMP gel (Invitrogen).

PCR reactions for ISSR and RAPD were performed in 25 µl buffer supplied by the Taq polymerase manufacturer (Invitrogen), 1.5 mM $MgCl_2$, 0.19 mM of each dNTP, 27 pmol primer, 10 ng template DNA, and 1.4 units of Taq polymerase. Six oligonucleotides (according to Stepansky 1999) were used for ISSR DNA amplification; the primers used in RAPD analysis were according to Oxelman (1996) (Tab. 2). Amplifications were carried out in a GeneAmp 2400 thermal cycler (Perkin Elmer) programmed for primers ISSR2, ISSR4 and ISSR7 in an initial cycle of 5 min at 94°C, followed by 40 cycles of 30 sec at 94°C, 30 sec at 44°C and 30 sec at 72°C; and for primers ISSR1, ISSR3 and ISSR6 in an initial cycle of 5 min at 94°, followed by 40 cycles of 30 sec at 94°C, 30 sec at 47°C, and 30 sec at 72°C. For RAPD primers the thermal cycler was programmed for an initial cycle of 5 min at 94°C, followed by 40 cycles of 40 sec at 94°C, 40 sec at 35°C and 40 sec at 72°C. The programs were terminated with an additional step of 7 min at 72°C before cooling down to 4°C.

The amplification products were separated on 2.5% agarose gel by electrophoresis, stained in ethidium bromide and stored with Imagemaster VDS (Pharmacia, Amersham). The gels were recorded with Liscap Capture ver. 1.0. For band determination a 100 bp ladder (Fermentas) was used. For band pattern analysis, GeleScan ver. 1.45 (Kucharczyk Techniki Elektroforetyczne) software was used.

TABLE 1. Collection data from *Aconitum* material sampled for molecular and cytogenetic analyses. Voucher specimens are deposited in KRA. Taxonomy, nomenclature and geographical distribution after Mitka (2003) and Starmühler (2001)

Taxon	Place of origin	Pop. no.	N. lat.	E. long.	Altitude [m a.s.l.]	No. of OTUs	Distribution area Geographical status
A. firmum Rchb. subsp. *firmum*	Tatra Mts.	1					Tatra Mt.s., Żywiecki Beskids (Poland), Malá Fatra, Tatra Mts. (Slovakia), Chornogora (Ukraine), Rodna Mts. (Romania) Carpathian endemic
	Kojsówka	1a	49.333	19.817	930	2	
	Ciemniak Mt.	1b	49.233	19.900	940	2	
	Tomanowa Mt.	1c	49.230	19.883	1550	2	
	Żywiecki Beskids Mts.	2					
	Babia Góra Mt.	2a	49.667	19.567	1180	1	
A. firmum nsubsp. *paxii* Starmühl. (*A. f.* subsp. *maninese* × subsp. *moravicum*)	Żywiecki Beskids Mts.						Tatra Mts., Żywiecki Beskids (Poland) Western Carpathian endemic
	Babia Góra Mt.	2b	49.667	19.567	1180	4	
A. firmum subsp. *moravicum* Skalický	Żywiecki Beskids Mts.						Żywiecki and Śląski Beskids (Poland), Malá Fatra, Tatra Mts. (Slovakia), Moravskoslezské Beskids (Czech Republic) Western Carpathian endemic
	Pilsko Mt.	3	49.550	19.316	1260	1	
	Malá Fatra Mts.						
	Révalov	4	49.150	19.050	1150	1	
A. firmum subsp. *maninense* (Skalický) Starmühl.	Rów Podtatrzański trench						Stražovské vrchy Mts. (Slovakia), Tatra Mts. (Poland and Slovakia) Western Carpathian endemic
	Murzasichle	5	49.317	20.050	940	3	
	Stražovské vrchy Mts.						
	Manin	6	49.233	18.700	400	5	
A. plicatum Koehler *ex* Rchb. subsp. *plicatum*	W Sudetes Mts.						Ostbayerische Grenzgebirge (Germany), Hrubý Jeseník, Jizerské hory, Krkonoše (=Giant) Mts., Kralický Sněžník Mt., Krušné hory, Šumava, Žd'árske vrchy Mts. (Czech Republic), Karkonosze Mts., Masyw Śnieżnika Mt. (Poland) Sudetic-Hercynian endemic
	Śnieżka Mt.	7	50.767	15.767	1270	1	
	E Sudetes Mts.	8					
	Kralický Sněžník Mt	8a	50.183	16.000	750–850	5	
A. plicatum subsp. *sudeticum* Mitka	E Sudetes Mts.						Masyw Śnieżnika Mt. (Poland), Kralický Sněžník Mt., Hrubý Jeseník Mts. (Czech Republic) Eastern Sudetic endemic
	Kralický Sněžník Mt.	8b	50.183	16.000	750–850	3	

NUMERICAL ANALYSIS

The amplified ISSR (177) and RAPD (175) band states were scored as an absence/presence matrix. The original data matrix was reduced to 145 bands allowing the most accurate scoring (38 ISSR, 107 RAPD). From this data matrix of informative bands, Jaccard similarity indices (Jaccard 1908) for all pairs of OTUs were calculated using the DISTANCE procedure based on 1000 bootstrap iterations using PhylTools (http://www.dpw.wau.nl/pv/PUB/pt/, Buntjer, 1997–2001). Relative bootstrap support values were computed with the NEIGHBOR/UPGMA and CONSENSE options in the Phylip package

TABLE 2. Information on ISSR and RAPD marker primers used to distinguish *Aconitum* taxa

Primer	Primer sequence (5'→3')	Total number of informative bands among specimens examined	Total number of band scores among specimens examined	Range of number of bands per specimen
ISSR1	$(TC)_8C$	10	42	2-7
ISSR2	$(AG)_8T$	7	31	0-6
ISSR3	$(GGGTG)_3$	4	24	0-6
ISSR4	$(ATG)_6$	9	58	0-6
ISSR6	$(AC)_8G$	5	18	0-3
ISSR7	$(AC)_8T$	7	46	0-5
OPB01	GTTTCGCTCC	31	168	2-8
OPB02	TGATCCCTGG	19	68	0-6
OPB08	GTCCACACGG	27	119	2-7
OPB14	TCCGCTCTGG	26	116	0-7

(Felsenstein, 2004). Phenetic cluster analysis (unweighted pair-group method using arithmetic averages, UPGMA; Sneath and Sokal, 1973) was constructed with NTSYS-pc version 2.11a (Rohlf, 2002), testing for tied trees.

RESULTS

KARYOTYPE STRUCTURE

Section *Aconitum* in Central Europe is a tetraploid (2n = 4 = 32) taxon with eight well-distinguishable tetrads (groups) of chromosomes. Generally, the distinguished chromosome groups are similar both in morphology (total chromosome length and centromere position) and in C-banding style (data not shown). The most striking feature of karyotypes of the Sudetic *A. plicatum* group is the absence of telomeric heterochromatin on the shorter arm of chromosome 1 (Figs. 2a, 3), so they are homozygous in this respect. The tetraploids from the Carpathians, that is, the *A. firmum* group, showed marked structural heterozygosity in the first chromosome group – two chromosomes are equipped with telomeric heterochromatin and two are not (Figs. 2b, 3). Interestingly, all diploid taxa of the sect. *Cammarum* from the Carpathians and Sudetes analyzed so far invariably show heterochromatin on the shorter arm of chromosome 1 (Fig. 3; Joachimiak et al., 1999; Ilnicki, 2005).

All the representatives of the *A. firmum* group showed structural heterozygosity within the shortest (eighth) chromosome group – two chromosomes of this type possess a large pericentromeric segment of heterochromatin (Fig. 2b). Such a segment was a characteristic feature of the majority of Western Carpathian populations of diploid *A. variegatum* (Ilnicki, 2005; Fig. 3).

NUMERICAL ANALYSES

The UPGMA classification based on molecular ISSR+RAPD data delimited three main groups (Fig. 4). The first consisted of *A. firmum*, *A.* × *paxii* (strong support, 82%), and *A. moravicum* (weak support, 52%). The second included *A. plicatum* (strong support, 80%) and one OTU of A. *maninense* (weak support, 55%). The third group consisted of A. *maninense* and *A. sudeticum* and was only weakly supported (58%), but the bootstrap support for both taxa separately was high, 78% and 80%, respectively. The most conspicuous finding was that of *A. firmum* subsp. *maninense* and *A. plicatum* subsp. *sudeticum* as sister taxa, and the paraphyletic position of A. *maninense*. One specimen of the taxon from the Rów Podtatrzański trench was close to *A. plicatum*, but for this group there was weak support (55%).

DISCUSSION

HISTORICAL AND PHYTOGEOGRAPHICAL BACKGROUND

The high-mountain floras of the Sudetes and Carpathians, separated by the relatively narrow Moravian Gate (Fig. 1) have conspicuously few common floral elements, despite their close geographic proximity (Pawłowski, 1969). This is probably due to biogeographical and historical factors such as the different origins of their floras and the impact of Quaternary glaciations. The Sudetes ranges belong to the old pre-Tertiary Hercynian-Sudetic mountain system of the Waryscynian orogenesis, whereas the Carpathians are part of the younger Late Tertiary alpids. For this reason the geological substrates of the Sudetes and Carpathians also differ. The Sudetic metamorphic and basalt bedrock is general-

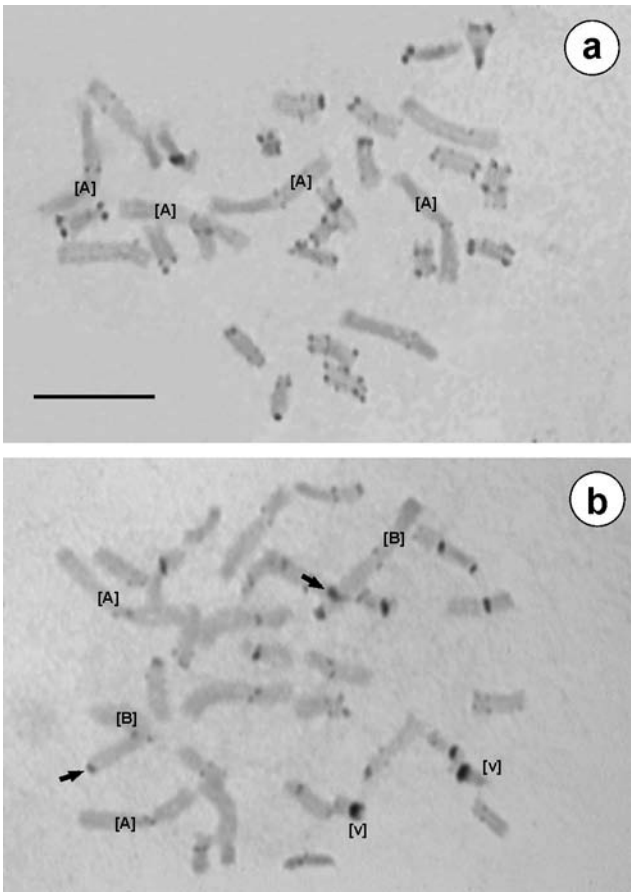

Fig. 2. (**a**) C-banded metaphase plate of a representative of the *A. plicatum* group (*A. plicatum* from Sudetes), (**b**) Representative of the *A. firmum* group (*A. firmum* subsp. firmum from Tatra Mts.). [A] – chromosome 1 without telomeric heterochromatin; [B] – chromosome 1 with telomeric heterochromatin; [v] – chromosome 8 with large pericentromeric heterochromatin segment; arrow – telomeric heterochromatin in chromosome 1. Bar = 10 μm

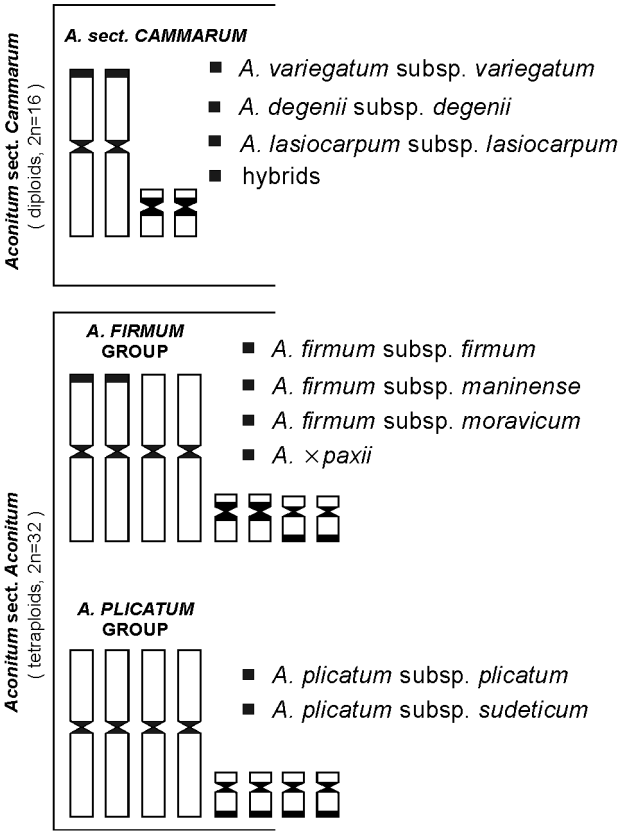

Fig. 3. Schematic presentation of the chromosome 1 and 8 C-banding patterns in Carpathian diploids (*Aconitum* sect. *Cammarum*) and two groups of tetraploids (*A. firmum* and *A. plicatum*).

ly much poorer in calcium carbonate than the Carpathian flysch (Starkel, 1991).

The Sudetes, a part of the Hercynides, were uplifted much earlier at the Cretaceous/Paleocene boundary during the Laramian tectonic phase some 65–60 myr BP. From that time to the present they have formed a stable land mass. Thus, in the regional geological context, the autochthonous Early Tertiary flora of the Hercynides shaped the younger Late Tertiary floras of the Carpathians and Alps (Syabryay, 1995). If this scenario is correct, the Sudetes might be among the oldest sources of genetic diversity of *Aconitum* in Central Europe.

Another important factor contributing to the specific floristic relation between adjoining Central European mountain systems is the influence of the Quaternary glaciations. At least one glacial maximum, the Mindel (48–43 kyr BP), affected the Sudetic flora catastrophically, due to the close proximity of edge of the continental ice sheet (Pawłowski, 1969; Starkel 1991). In the Carpathians the Mindel ice sheet reached their threshold and even pushed across it, leaving behind a line of erratic blocks in the Beskids Mts. (outer Carpathians) at elevations of ~400 m a.s.l. Thus the existence of a close refugium or nunatak in Poland during that time is very unlikely. For at least some of the alpine floras (especially those of the Tatras), such a supposition does not seem to apply (Szafer, 1953; Obidowicz, 1996).

As an effect of the extended Mindel glaciation, the flora of the Sudetes is impoverished in comparison to the Carpathian floristic stock. For example, among ~500 mountain species occurring in the Western Carpathians, only 40%, ~200 species, are found in the Sudetes (Pawłowski, 1969). The most interesting are the Carpathian subendemics occurring in the alpine zone of the Sudetes: *Erigeron macrophyllus* Herbich, *Melampyrum herbichii* Woł., *Sesleria tatrae* (Degen) Deyl and *Thymus carpathicus* Čelak. (nomenclature follows Mirek et al.,

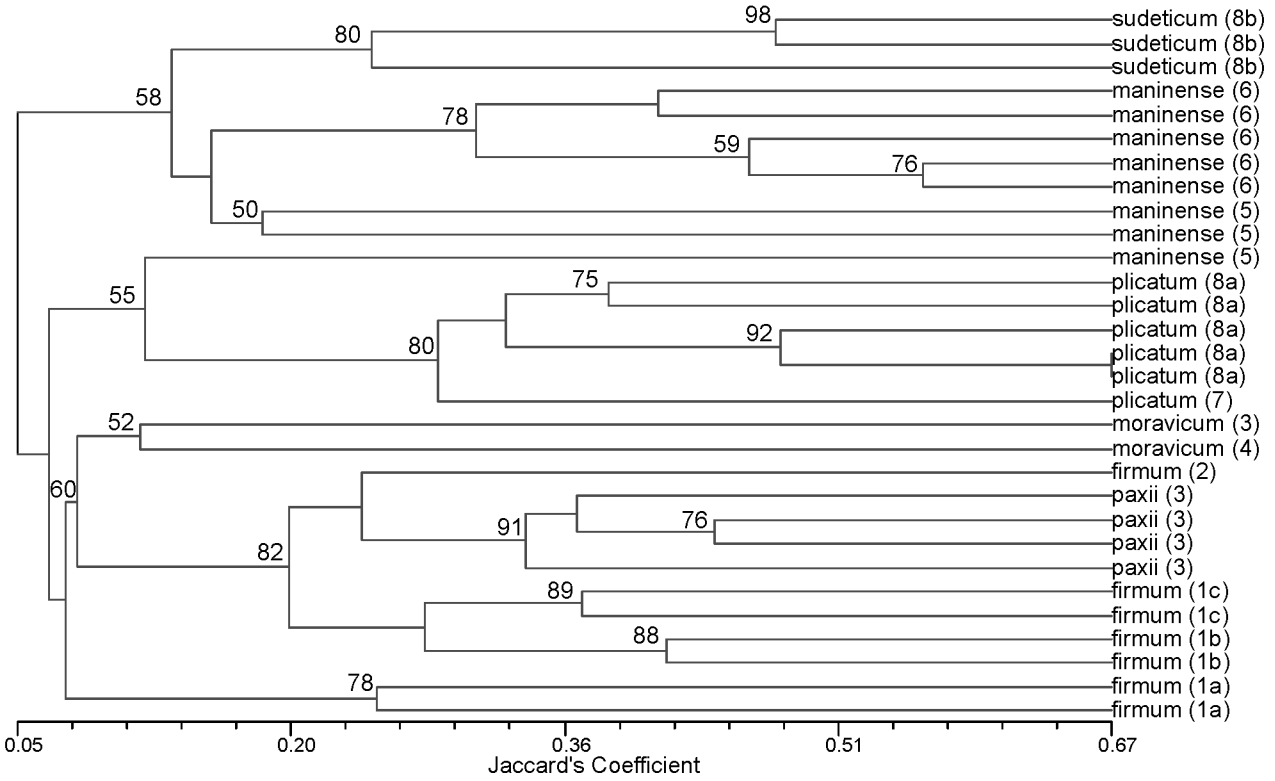

Fig. 4. UPGMA dendrogram of the *Aconitum* OTUs based on PCR-ISSR+RAPD analysis. Bootstrap values greater than 50% are given above the branches. Population no. according to Table 1.

2002). These examples demonstrate that contacts between the Sudetic and Carpathian flora occurred in the past. Especially among the high-mountain flora there may have been dispersal across the open landscape during glacial maxima. Most probably in situ glacial refugia on nunataks did not exist in the Sudetes (as was postulated for the Alps; see Stehlik, 2000). In the Western Carpathians there are a few refugial areas recognized, for example in Poland in the Małe Pieniny Mts. (700 m a.s.l.), where a group of Tatra high-mountain species found shelter (*Crepis jacquini* Tausch, *Dryas octopetala* L. and *Trisetum alpestre* [(Host) P.Beauv.], and in Slovakia at Zadielska rokle in the southern part of Slovensky kras (250 m a.s.l.), where typical high-mountain vegetation with *Sesleria varia* (Jacq.) Wettst. develops (Kornaś, 1958). It is known from the palaeobotanical record (summarized by Mamakowa and Środoń, 1977) that in southern Poland during the last glacial maximum, in the Hengelo and Denekamp intrapleniglacial interstadials a uniform parkland tundra with *Betula nana, Pinus cembra, Ephedra* and *Hippophaë* developed, and the upper forest limit attained ~650 m a.s.l. This means that the alpine heliophytes could extend their geographical ranges far from high-mountain sites to the neighboring foothills at those times.

POTENTIAL PRE-PLEISTOCENE ALLOPOLYPLOIDY OF CARPATHIAN *ACONITUM* TETRAPLOIDS

The clear karyological difference between Sudetic and Carpathian sect. *Aconitum*, the high structural uniformity of the *A. firmum* group, including *A. maninense*, and the lack of extant diploid taxa with chromosome 1 of the *A. plicatum* type (deprived of telomeric heterochromatin), suggest the deep evolutionary history of Central European tetraploids. Our karyotype analysis suggests that the genome of the Sudetic autotetraploid (or a close 2x progenitor of this species) could be ancestral to the Carpathian allotetraploids of the *A. firmum* group. Unfortunately, there are no data on heterochromatin distributions in the other European taxa of *Aconitum* sect. *Aconitum* outside the Carpathians and Sudetes.

Studies by Utelli et al. (2000) also point to the ancient history of European *Aconitum*. One of their investigated species, *A. anthora*, clusters within subgen. *Lycoctonum*. This suggests that there may have been hybridization events between taxa of even

different subgenera. *A. anthora* is known to be genetically isolated in Europe, and no hybrids have been reported between *A. anthora* and any other European taxon (see Utelli et al., 2000 and literature therein). Moreover, the intergenic spacer *psb*A-*trn*H is not identical with any genotype found in subgen. *Lycoctonum*. The authors conclude that none of the European taxa were involved in a possible hybridization event.

Recently, Luo et al. (2005) described the phylogenetic relationships within *A.* subgen. *Aconitum* based on 51 species and one variety from Eastern Asia, North America and Europe. A large part of the taxa sampled (22) originated from the Hengduan Mts., eastern Himalaya, where ~100 *Aconitum* species have been recognized. In spite of their high morphological variation, the Hengduan species have low ITS genetic diversity, probably pointing to a recent radiation. An intriguing finding is the placement of two European species, *A. napellus* (sect. *Aconitum*) and *A. variegatum* (sect. *Cammarum*). They are sister to two Asian species, *A. austrokoreense* Koidz. and *A. monanthum* Nakai [both sect. *Flagellaria* (Steinb.) Nakai]. This could be a sign of deep evolutionary relationships between the presumed Asian cradle of *Aconitum* and its putative secondary center somewhere else, since we exclude long-distance migrations. According to our hypothesis, *Aconitum* in Europe would be a descendant of that unknown secondary center, and the Hercynian-Sudetic mountain system could be a "museum" where the old homotetraploid form persisted.

This line of reasoning has been used several times. For example, *Primula* sect. *Auricula* in the European mountains likely originated from an Asian ancestor in the Late Tertiary (Zhang et al., 2004). The wide-ranging diploid *Primula mistassinica* Michx. may have given rise to the group of European polyploid species (Conti et al., 2000). Also, cytogenetic and morphological data showed that the tetraploid subgenus *Oreogeum* in the genus *Geum* consists of several mountain species (in the Balkans, Corsica, the Alps, Carpathians and Pyrenees), within which *G. montanum* L. represents the remnants of a di- or polypodial Late Tertiary complex (Gajewski, 1957).

The results of the cytogenetic studies presented here led us to reject the most parsimonious hypothesis of common ancestry as a direct mechanism contributing to the similarity between the Sudetic and Carpathian taxa. Instead, we tracked probable palaeopolyploidization and reticulation at the species level. In order to explain some morphological and molecular similarities at the infraspecific level we put forward an additional hypothesis of recent Pleistocene reticulation (introgression) between the Sudetic and Carpathian genetic stocks. In fact, hybridization/introgression in *Aconitum* is common, at both species and population levels (Zieliński, 1982; Kadota, 1987; Youngbae et al., 1997; Oh and Park, 1998; Chung and Park, 2000; Lim and Park, 2001).

The hypothesis of the common ancestry of the Carpathian and Sudetic species, which we reject, involves a vicariant model of evolutionary divergence in two geographically isolated areas. However, this model seems inadequate because the Carpathian and Sudetes are of different ages. The dispersal hypothesis seems more relevant; according to it, the older mountains are the source and the second, the younger, are a sink area. In this scenario hybridization occurred in the younger mountains, and the potential parents originated from the older area. Cytogenetic and molecular studies do not exclude ancient hybridization in the evolution of *Aconitum* (Kita and Ito, 2000; Utelli et al., 2000).

We can also see traces of relatively recent, presumably glacial evolutionary history. The presence of glandular hairs in *A. sudeticum* can be interpreted as an introgression of this character from the Slovak population of *A. maninense*. This interpretation is partially supported by our PCR-ISSR+RAPD study. No other Hercynian-Sudetic taxon of *A.* sect. *Aconitum* possesses any glandular hair on the indument. Thus, the most plausible hypothesis seems to be local gene flow between neighboring populations of the Eastern Sudetes and Western Carpathians, which came into contact in the Moravian Gate during a glacial maximum; that is, recent Pleistocene reticulation.

PROBABLE PLEISTOCENE RETICULATION

Quaternary climatic oscillations are held to be responsible for rapid floristic diversification and extinction of alpine plants. Molecular clock approaches suggest that the floristic diversification did not take place, as previously assumed, in geographical isolation in high-altitude refugia, but rather at low altitudes in geographically isolated glacial refugia (Kadereit et al., 2004). Also, the climatic conditions of this glacial era facilitated the contact of different genetic stocks of alpine plants at mountain foothills. Such contact of the Eastern Sudetic and Western Carpathian genomes of alpine *Aconitum* may have occurred more than once during the Pleistocene. At that time the altitudinal distribution of the alpine flora of European high-mountain plants underwent cyclic advances and contractions (Kropf et al., 2003). Presumably, during one or more glacial maxima the alpine flora of the Sudetes and Carpathians met at lower altitudes, somewhere in the Moravian Gate. Such secondary contact may not only have led to hybrid formation (see Stebbins, 1984, 1985) but also enabled exchange of high-mountain species.

This interpretation is at odds with the present rarity of at least of one of the putative parents, *A. maninense*, in the Western Carpathians. Thus, we have to add an additional premise, such that *A. maninense* must have had a wider occurrence than today. This taxon, held at the rank of subspecies in *A. firmum*, presently grows only in two disjunct localities: in the Stražovské vrchy Mts. in Slovakia (Manin region) and in the High Tatras and their foreland in Poland (Murzasichle region; Skalický, 1985; Starmühler and Mitka, 2001; Fig. 1a). In the Stražovské vrchy Mts. it grows in deep gorges on steep calcareous cliffs, and in the Tatras its ecological center is at lower altitudes in a fissure spring area in a beech forest. Such a relict occurrence may indicate that its glacial or pre-glacial distribution was much wider than today, and that the species was then restricted to narrow ecological zones. In effect the populations have become geographically isolated. This is supported by our ISSR+RAPD analysis, which suggests rather long-term isolation and explains the polyphyletic nature of A. *maninense*. Thus it seems reasonable to adopt the view that the western population of A. *maninense* from the Slovak Carpathians, bordering *A. sudeticum* in the Eastern Sudetes, may have exchanged genes in the past, an event hardly possible between the Polish Tatra and Sudetic populations. On the other hand, there are no visible differences in karyotype structure between these two populations of *A. maninense* and other subspecies of *A. firmum*. All taxa belonging to the *A. firmum* group showed structural heterozygosity within chromosome 1 and the presence of two small marker chromosomes, very characteristic for some populations of diploid *A. variegatum* from Poland (see Fig. 3).

The proximity of *A. maninense* from the Tatras to *A. plicatum* from the Sudetes, as revealed by ISSR+RAPD data, is most probably an artefact without any evolutionary basis. The only signal from such a result would be the presence of the long-isolated population at Murzasichle (Poland), which has a genetic stock different from that of Manin (Slovakia). Zhang et al. (2005) reported a similar result: two geographically isolated populations of *A. delavayi* did not form their own cluster in a UPGMA dendrogram based on mean character differences estimated from RAPD data, because one of them clustered with an *A. tuguancunense* population initially.

Less likely, but not impossible, is the explanation proposed by Archibald et al. (2006) that such a lack of clustering may be the result of the presence of a suite of ancestral polymorphic markers segregating within populations, referable to more than one species in the current taxonomy of the genus: that is, lineage sorting.

IDENTITY OF THE ANCESTRAL DIPLOID FORM

The exact moment *Aconitum* first appeared in Central Europe cannot be determined, but most probably the high-mountain tetraploid sect. *Aconitum* originated from a hypothetical diploid cytotype. For example, such a diploid ancestor of the Eastern Asian tetraploid complex was recognized in a molecular study by Kita and Ito (2000). It is the Chinese endemic diploid *A. volubile* Pall., with the most primitive type of growth in *Aconitum*, twinning branches (Litvinienko, 1977; Kadota, 1987). This gave strong support to the hypothesis (Kita et al., 1995) that the Asian tetraploid *Aconitum* complex was diversified from an ancestral diploid and non-alpine stock in a short period of time, and then adapted to alpine conditions. This hypothesis can also be used in the Central European context. The presence of *Aconitum* pollen in southern Poland in the Late Tertiary was confirmed in palynological studies by Stuchlik and Shatilova (1987). This undetermined species could be related to a presumably diploid cytotype of an unknown (most probably Asian) origin. Asian origin is indirectly supported by palaeobotanical records showing that the zonal temperate forest biome extended from the Asian Far East to Central Europe, and from western to eastern North America, throughout the Oligocene and Miocene (Mai, 1995; Zastawniak, 1996; Zhilin, 2001). The one diploid species outside Europe, Asian *A. sanyoense* Nakai (Kadota, 1987), possesses telomeric heterochromatin segments on the longest chromosome pair and shows a heterochromatin distribution very similar to that of diploid Carpathian/Sudetic stocks of A. sect. *Cammarum*. There is no published information on the Asiatic forms deprived of this segment or on the karyotype structure of tetraploid taxa from this area. Thus, we cannot rule out that genomes without telomeric heterochromatin in chromosome 1 are characteristic for some Central European taxa.

We did not identify any diploid species that could represent the ancestor of any of our investigated tetraploid representatives of *A.* sect. *Aconitum* in Central Europe. However, one genetic set of the allopolyploid taxon of *A. firmum* from the Carpathians is similar to the heterochromatin-rich genome of diploid *A. variegatum*. This species, most probably the ancestor (or its derivative) to the whole *A.* sect. *Cammarum* in Europe (Mitka, 2003), shows large blocks of heterochromatin on the longest and shortest chromosomes (1 and 8). All the representatives of the tetraploid *A. firmum* group possess both these markers, in the heterozygous state, within the karyotype. This suggests that the genome of *A. variegatum*-type contributed to the emergence of this tetraploid form.

Incongruence between the results of molecular and morphological analyses seems to be the rule rather than the exception in taxonomic studies (Grant, 2003). There is, however, no a priori expectation about which of the molecular or morphological data should prevail in elucidating taxonomical problems. Our karyological results on the relationships in *Aconitum* sect. *Aconitum* in Central Europe are consistent with the hypothesis of ancient hybridization and the palaeopolyploid origin of the younger, Carpathian taxon. The molecular PCR-ISSR+RAPD study was aimed at more recent reticulation. Thus, we propose two reticulation events: one in the deep, most likely pre-Pleistocene evolutionary history, when the Carpathian allotetraploid *A. firmum* group may have originated as an effect of allopolyploidy from the ancient Sudetic *A. plicatum* and diploid *A. variegatum* or its direct ancestor; and recent, presumably in the Pleistocene, when the Slovak population of *A. maninense* came into contact with the East-Sudetic population of *A. plicatum* in the Moravian Gate. As a result, the introgressive *A. plicatum* subsp. *sudeticum* was produced. In cases such as this one, where the species' evolutionary history includes high levels of reticulation (hybridization, also resulting in allopolyploidy and introgression), it is not surprising to find such substantial incongruence between cytogenetic, molecular and morphological markers, and especially the blurring of molecular genetic fingerprints.

Combined analysis of hypervariable PCR-ISSR+RAPD and more conservative cytogenetic markers seems an effective way to elucidate taxonomic problems. Using this approach we came to the conclusion that *A. sudeticum* should be regarded as a Hercynian-Sudetic geographical race (subspecies) of *A. plicatum*, even though its conspicuous morphological character (glandular hairiness) makes it morphologically close to the Carpathian *Aconitum maninense*. All hypotheses put forward in the paper rely on a pilot study and need further support.

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