Cytotype distribution and colonization history of the steppe plant *Iris aphylla*

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The polyploid origin and colonization history of *Iris aphylla* in central and southeastern Europe were investigated by means of karyological and AFLP analyses. The study indicated two chromosome races within *I. aphylla* based on chromosomal numbers 2n = 24 and 2n = 48. The overall chromosome counts and determined ploidy suggest tetraploidy as the most common ploidy level for this species in Europe. Only one diploid *I. aphylla* population was found, with a restricted distribution area in the Slovak Karst. The analyses showed that tetraploids arose independently at least three or more times in the past. The karyological results were in agreement with the AFLP data and strongly suggested that migration between the two Carpathian Mts. sites occurred recently *via* the Dukielska Pass. The low number of AFLP fragments unique to each population supports the hypothesis of recent range expansion and colonization of different environments in central Europe by *I. aphylla*. All populations were genetically depauperated and had 0–3 unique bands, indicating that not enough time elapsed for many unique fragments to form through mutation. We also observed relatively high and significant differentiation between the one diploid and all investigated tetraploid *I. aphylla* populations (Φ_{ST} = 0.470, $p < 0.001$). The high and significant values of genetic differentiation can be explained by several factors such as anthropopression and natural succession, which have fragmented the open calcareous habitats in central Europe.

Key words: AFLP, autopolyploid, endangered species, karyotypes, phylogeography, Iridaceae

Introduction

Past and current polyploidization processes play a crucial role in determining the evolution and phylogeography of plant species. Polyploid taxa often have a broader ecological amplitude, and tend to be weedy or widespread relative to congeneric or conspecific diploids (Doyle *et al*.

1999, Soltis & Soltis 2000). The evolutionary success of polyploid as compared with that of diploid plants has often been attributed to the consequences of increased heterozygosity, allelic diversity and enzyme multiplicity. Their establishment and spread in nature may be favored particularly by vegetative multiplication. Recent works on polyploid taxa have stated that multiple origins of polyploids over the species range are the rule (Soltis & Soltis 1993, 1995, Soltis *et al*. 2004). Genomic changes can occur rapidly during the formation of polyploids, potentially producing genotypes different from diploid progenitors or causing chromosomal rearrangements (Aggerwal *et al*. 1997, Friesen *et al*. 1997). In this case, mixed cytotypes may often contrast in geographical distribution (Stebbins & Dawe 1987, Lumaret 1988). This makes the evolution of autopolyploids and allopolyploids more complex than previously thought.

Studies of *Iris aphylla*, a long-lived rhizomatous perennial, have documented the distribution of autotetraploids with 2n = 48 somatic chromosomes (Pólya 1949, 1950, Wcisło 1964, Dostál 1989, Taylor 2000) across Poland, Slovakia, the Czech Republic, Hungary, Germany, Romania and Belarus. Dostál (1989) reported different numbers of chromosomes: 2n = 24 and 40. *Iris aphylla* is listed as rare and endangered in the countries mentioned above (Maglocký & Feráková 1993, Ludwig & Schnittler 1996, Sándor 1999, Holub & Procházka 2000, Kaźmierczakowa & Zarzycki 2001). In Ukraine and Russia, it is widespread and not under protection. Dostál (1989) proposed a subspecies status for *I. aphylla* forms described on the basis of morphological characters. In Poland, *I. aphylla* was classified as var. *typica*, a synonym for subsp. *bohemica* (Medwecka-Kornaś 1959). In Slovakia and Hungary, the form of this species is recognized as subsp. *hungarica*. The diagnostic morphological characters provided by Dostál (1989) and the division of *I. aphylla* into subspecies are controversial. One of the most important characters is the flower bud, slightly leaning before flowering in subsp. *hungarica*, and straight in subsp. *bohemica* (= var. *typica*). Observations have shown these features to greatly vary between years in Polish and Slovak populations, and to depend on seasonal changes (A. Wróblewska unpubl. data). Since the identification of clear

morphological characters to determine subspecies is so highly problematic, in this paper we consider all investigated populations as *I. aphylla*.

In this study, we used chromosomal analysis together with AFLP to describe and discuss (i) origins of polyploids and chromosomal variation across marginal *I. aphylla* populations as well as populations within the continuous range, (ii) the geographical structure of polyploidy and the colonization history of this species in Europe, and (iii) taxonomy within *I. aphylla*. This survey of *I. aphylla* covers some previously sampled European populations, including northern, southeastern and central ones (Wróblewska 2008).

Material and methods

Plant material

Fifteen populations of *Iris aphylla* on the periphery and within its continuous geographical range in northern, southeastern and central Europe were studied, from different habitat types and altitudes (Table 1 and Fig. 1). The plant material for karyological and genetic analyses was collected from eight populations in Poland (Małopolska Upland, Lublin Upland, Biebrza Valley), two populations in Slovakia (Slovak Karst) and five populations in Ukraine (Volhynian and Podolian Uplands; Fig. 1). *Iris aphylla* is under protection and placed in the highest threat category in central Europe, and is listed as endangered on the national scale in a number of countries (*see* Introduction). In order to conserve the few collections of rhizome fragments, only one rhizome fragment from each Polish, Slovak and Ukrainian population was used for karyological analysis. For genetic analysis, ten leaf samples in each population were taken randomly, > 2–3 m apart in order to avoid sampling the same genotype more than once (Wróblewska *et al*. 2003); 150 samples were analysed by AFLP.

Chromosome preparation and ploidy level determination

Root tips were obtained by hydroponic techniques. Root apices 1.5-cm long were immersed

Table 1. Characteristics of *Iris aphylla* populations (region, site code, ploidy level, type of chromosome, unique AFLP fragments, coordinates, elevation and habitats). * centromeric index of arm ratio values based on Levan *et al.* (1964): m = metacentric, sm = submetacentric, sa = subacrocentric.

| Region/ population	Site code	Ploidy level	(Type of chromosome)/*	Unique AFLP fragments	Lat. N	Long. E	Elevation (m a.s.l.)	Habitat
North Europe (**Biebrza River Valley, Malopolska and Lublin Upland**)								
Kapice	P1	48 (4x)	(E) 14m/sm, 10sa	0	53°43'	22°44'	150	Mineral island, with *Quercus robur* and *Calamagrostis epigejos*
Tunel	P2	48 (4x)	(E) 14m/sm, 10sa	0	50°27'	19°58'	370	Patch of *Cirsio–Brachypodion* community
Kazimierz Dolny	P3	48 (4x)	(E) 14m/sm, 10sa	1	51°19'	21°55'	300	Loess slope (85° inclination); species from *Festuco-Brometea* and *Trifolio–Geranietea sanguinei* communities
Gościeradów	P4	48 (4x)	(E) 14m/sm, 10sa	1	50°49'	21°59'	200	High limestone rubble; species from *Festuco-Brometea*
Sobianowice	P5	48 (4x)	(B) 16m/sm, 8sa	0	51°17'	22°40'	200	Loess hill (50°–60° inclination), species from *Festuco-Brometea*
Zawadówka	P6	48 (4x)	(E) 14m/sm, 10sa	1	51°07'	23°23'	200	Mineral island, species from *Festuco–Brometea*, shrubs *Rhamnus cathartica*
Tarnogóra	P7	48 (4x)	(E) 14m/sm, 10sa	0	50°53'	23°06'	200	Mosaic of communities *Cirsio–Brachypodion* and *Festuco–Brometea*
Czumów	P8	48 (4x)	(B) 16m/sm, 8sa	0	50°47'	23°56'	400	Loess slope, the calcareous grassland communities
Slovak Karst								
Dlha Hora	S1	48 (4x)	(D) 15m/sm, 9sa	1	48°31'	21°47'	400	Calcareous grassland communities from *Festucetalia valesiacae*
Krkavčie Skaly	S2	24 (2x)	(A) 8 m/sm, 4sa	3	48°38'	20°51'	800	Limestone rock-fall, species from *Festucetalia valesiacae*
Volhynian and Podolian Upland								
Suchovolija	U1	48 (4x)	(C) 15m/sm, 9sa	1	50°02'	25°15'	350	Loess hill, species from *Inuletum ensifoliae*
Bilyjkamin	U2	48 (4x)	(F) 13m/sm, 11sa	1	49°52'	24°50'	400	Narrow (5 m) margin of *Pinus sylvestris* forest
Zoločiv	U3	48 (4x)	(F) 13m/sm, 11sa	1	49°48'	24°42'	400	The chernozem, species from *Inuletum ensifoliae*
Babuchiv	U4	48 (4x)	(F) 13m/sm, 11sa	1	49°20'	24°39'	450	Margin of limestone steep, species from *Festucetalia valesiacae*
Burstin	U5	48 (4x)	(F) 13m/sm, 11sa	1	49°13'	24°41'	500	Loess hill (with 50° inclination), species from *Inuletum ensifoliae*
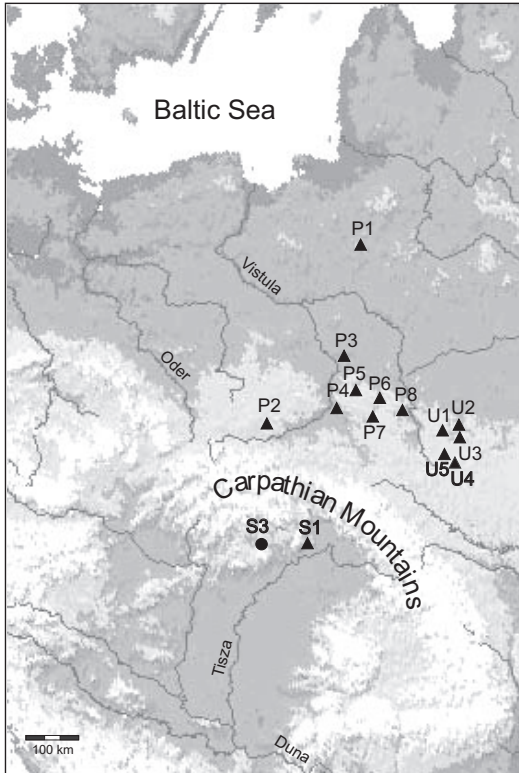
Fig. 1. Location of *Iris aphylla* populations in North Europe, the Slovak Karst and the Podolian and Volhynian Upland (description of populations is given in Table 1). ▲ = tetraploid population, ● = diploid population.

in 0.02 M 8-hydroxyquinoline for 5 h at room temperature and fixed in a freshly prepared mixture of absolute alcohol and glacial acetic acid (3:1). The material was washed in citrate buffer and macerated in enzyme solution (4% Onozuka R-10 cellulose and 15% pectinase, Sigma) for 60 min. at 37 °C. Squash preparations were made in acetic acid and stained conventionally with aceto-orcein, and fluorescently with DAPI. We applied 2% Giemsa following the procedure of Sharma and Sharma (1994) with modifications for C-banding technique (Chudzińska *et al.* 2005). Chromosomes were counted only from mitotic plates at metaphase or anaphase. At least ten plates were studied for each sample. The best metaphase plates from each population were photographed and ideograms were prepared from enlarged prints by measuring the total length of the chromosomes and of their arms. The ideograms represent average measure-

ments of at least ten plates from each population. The centromeric index (*r*) was calculated as the long:short (q:p) arm ratio, and the chromosomes classified according to Levan *et al.* (1964): m = metacentric (*r* = 1.00–1.69), sm = submetacentric (*r* =1.70–2.99), sa = subacrocentric (*r* = 3.00–6.99).

DNA extraction and AFLP analysis

Total genomic DNA was extracted from ca. 100 mg of dried leaf tissue with the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. The AFLP protocol was carried out as described by Vos *et al.* (1995) with modifications (PE Applied Biosystems). We used four fluorescence-labelled primer combinations: *Eco*RI-ACC/*Mse*I-CAC, *Eco*RI-ACC/*Mse*I-CAT, *Eco*RI-AGC/*Mse*I-CTG, *Eco*RI-ACA/*Mse*I-CTA. They gave clear, reproducible and homogeneous intensity bands showing variation between individuals and discriminated clones within populations (Wróblewska 2008). DNA products were identified using an ABI PRISM 377 sequencer (Applied Biosystems) on 36 cm denaturing polyacrylamide gels (4.5%) in $1 \times$ TBE buffer. 500 TAMRA-labelled size standard was loaded in each lane. ABI GENESCAN software was used for detection of amplification products.

Statistical analyses were applied to the complete data set restricted according to Lynch and Milligan's (1994) criterion for dominant markers: bands were excluded from analysis when their frequency in the whole sample was higher than $1 - (3/N)$, where *N* is the total number of samples. Genetic diversity was calculated using only polymorphic loci. For each population, the number of unique fragments (U_B; i.e., DNA fragments confirmed to occur in only one population) was assessed. Analyses of molecular variance (AMOVA) were performed to study the genetic structure, using Genotype/Genodive 1.2 (Meirmans & Van Tienderen 2004). AMOVA was used to estimate the partitioning of total genetic diversity among all ploidy levels. Levels of significance for populations were determined using permutation tests (1000 permutations). AMOVA was also applied to calculate pairwise differences as Φ_{ST}, an analogue of F_{ST}, between all populations

differing in ploidy level, and they were tested for significance with a permutation procedure (1000 permutations). A neighbour-joining tree based on Reynold's genetic distances between populations was constructed using PHYLIP 3.6 (Felsenstein 2004). Support for each node was tested by 10 000 bootstrap replicates. Finally, we constructed an unrooted consensus tree based on 15 trees of *I. aphylla* using CONSENS in PHYLIP. Only branches with bootstrap support above 70% were considered (Felsenstein 2004). Genetic relationships between individuals were identified by principal coordinate analysis (PCO) plotted with MVSP 3.0 (Kovach 1999). Euclidian distance was chosen in preference to other distance measures, as it does not classify a common absence of a band as a shared characteristic. We tested the differences in average values of PC1 and PC2 between populations using one-way ANOVA in STATSOFT 5.0 (StatSoft Inc. 1997).

Results

Chromosome number and cytotype distribution

The numbers of mitotic chromosomes were determined in the 15 examined populations (Table 1). The chromosome numbers of all populations were multiples of x = 12. Most samples had the chromosome number 2n = 4x = 48. In one Slovak sample (S2), however, the chromosome number was 2n = 2x = 24.

Iris aphylla karyotypes (morphology and C-banding patterns) showed a disjunct distribution from north to south in the geographical range (Table 1, Figs. 2 and 3). In this survey, two groups of karyotypes were distinguished according to the ratio of the number of meta- and submetacentric to subacrocentric chromosomes, and according to the C-banding patterns. The karyotypes of both diploid and tetraploid cytotypes reported here fall into these two groups. The first group includes the karyotypes A (S2), B (P5, P8) and C (U1), and the second group comprises the karyotypes D (S1), E (P1–P4, P6, P7) and F (U2–U5; Figs. 2 and 3). Arm lengths and C-banding patterns did not differ between homologous chromosomes in the 14 tetra-

ploid populations. Differences were noted only between homologous chromosomes in the one diploid population (2nd, 4th, 5th and 10th pairs of chromosomes), which were heterozygous for the length of arms and C-bands (Figs. 2 and 3).

## AFLP analysis and genetic diversity within populations

The four applied primer combinations generated 501 unambiguously scorable fragments, of which 459 (91.6%) were polymorphic. The length of amplified fragments of DNA ranged from 50 to 350 bp. No identical genotypes were detected in the whole data set; each sample represented a distinct AFLP multiband genotype. In 9 of 14 tetraploid populations, only one unique fragment was detected (Table 1).

Partitioning of total genetic diversity of all *I. aphylla* populations by AMOVA is given in Table 2. The variance components were highly significant at all levels ($p < 0.001$). Variation was the highest within diploid and tetraploid populations (52.93%).

Two groups of populations related genetically and geographically were found by the neighbour-joining method (Fig. 4). The first group of tetraploid populations included P1–P4, P6 and P7 (100% bootstrap). The second group showed close a genetic relationship among five populations from the Volhynian and Podolian Uplands. In only one case was there a discrepancy between the phenetic analysis and the geographical distribution: two populations from northern Europe (P5, P8), one from the Slovak Karst (S1), and the single diploid population (S2) grouped together (72% bootstrap) (Fig. 4). The same structure was evident from the separate PCO ordination, including all individuals from the single diploid and 14 tetraploid populations (Fig. 5). For example, six populations (P1–P4, P6, P7) from northern Europe grouped separately from the other analysed populations. The diploid (S2) and two tetraploid northern populations of *I. aphylla* (P5, P8) were not clearly separated from each other, but individuals from these populations were not mixed together on the diagram plots. By PCO analysis the first factor explained 23.0% and the second 10.8% of the
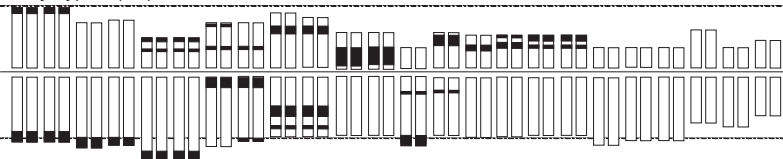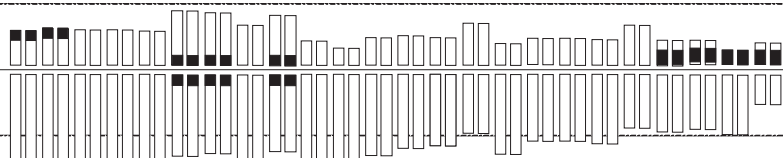
GROUP I

Karyotype A(S2), 2n = 24
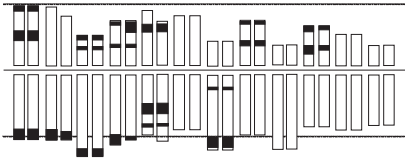
Karyotype B(P5, P8), 2n = 48
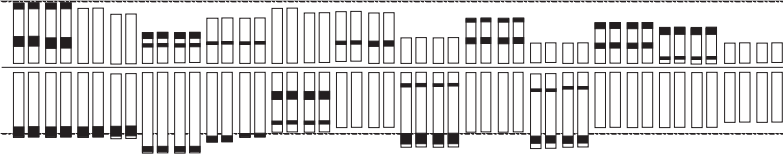
Karyotype C(U1), 2n = 48

GROUP II

Karyotype D(S1), 2n = 48

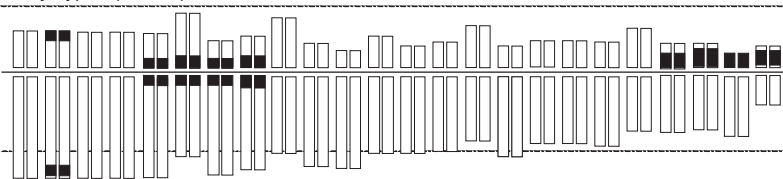Karyotype E(P1–P4, P6, P7), 2n = 48

Karyotype F(U2–U5), 2n = 48

Fig. 2. Idiograms of karyotypes of *Iris aphylla* chromosomes showing Giemsa C-banding patterns. Karyotypes are divided into two groups, similar in ratio of number of metacentric and submetacentric to subacrocentric chromosomes and C-banding patterns. Description of the karyotypes is given in Table 1.

overall variance, but only the PC1 value was significant (one-way ANOVA: $p = 0.009$, Fig. 5).

Discussion

Karyotype variability and polyploid origins of *Iris aphylla*

Our observations suggest that there are two chromosome races within *I. aphylla* based on the chromosome number x = 12. The tetraploid *I. aphylla* (2n = 48) was the most common occurring in northern, central and southern Europe and was present across 14 investigated populations. The one diploid *I. aphylla* population (2n = 24) had a restricted distribution range in the Slovak Karst. These results support the earlier work of Pólya (1950), Mitra (1956), Löve and Löve (1961), Wcisło (1964) and Taylor (2000), who
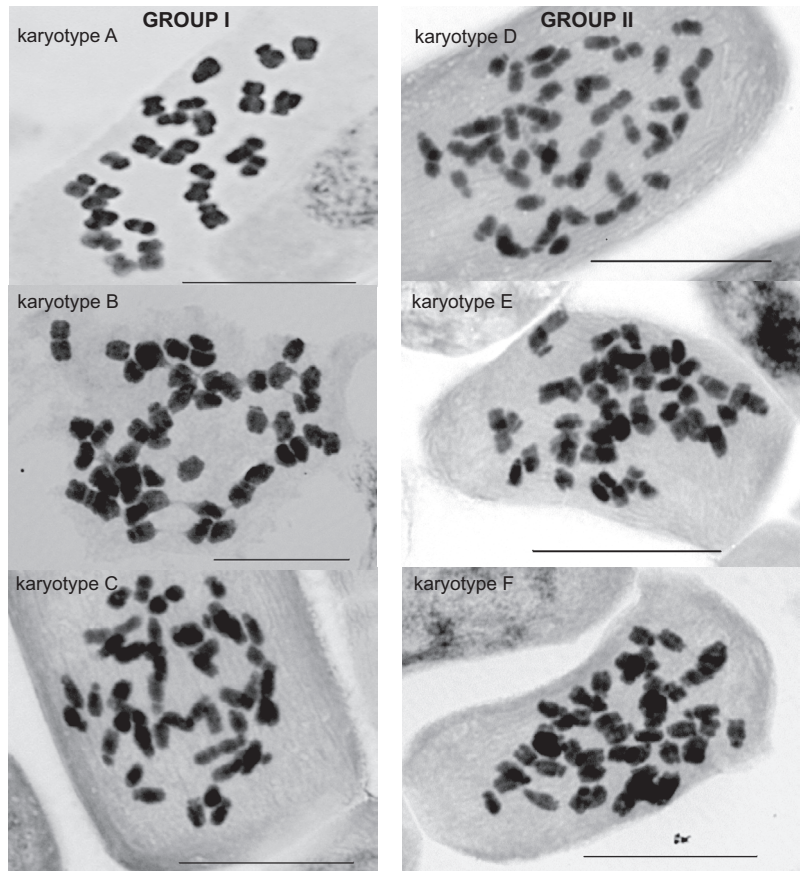
Fig. 3. Metaphase chromosomes of diploid and tetraploid *Iris aphylla* cytotypes. The first group includes karyotype 2n = 24: A (S2) and karyotype of 2n = 48: B (P5, P8) and C (U1). The second group includes karyotypes 2n = 48: D (S1), E (P1–P4, P6, P7), F (U2–U5). Scale bar = 20 μm. Site codes for populations are given in Table 1.

found 2n = 48; Dostál (1989) reported 24 and also 48 somatic chromosomes.

This is the first report showing polymorphism in karyotypes among *I. aphylla* populations. C-banding technique successfully identified chromosomes in diploids and tetraploids and it should prove to be a useful method for comparing genetic relationships within *I. aphylla*. We observed no differences in arm lengths and C-banding patterns between homologous chromosomes in any of the 14 tetraploid populations. Only in the single diploid population (S2) did we find asymmetry between homologous chromo-

somes, in long-arm lengths and heterochromatic band positions. This asymmetric karyotype seems to result from hybridization or mutation (Cai & Chinnappa 1987, Masoud *et al.* 1991, Cai & Bullen 1991). Further chromosome C-banding studies should shed light on the evolution of diploid populations within this species.

Mitra (1956) hypothesized an autotetraploid origin for *I. aphylla*, based on analysis of the chromosome morphology of the somatic complement. Our survey points to an autotetraploid origin of this species by comparative karyotype analysis of, for example, two tetraploid popula-

Table 2. Analysis of molecular variance (AMOVA) among diploid and tetraploid *Iris aphylla* populations. * $p < 0.001$.

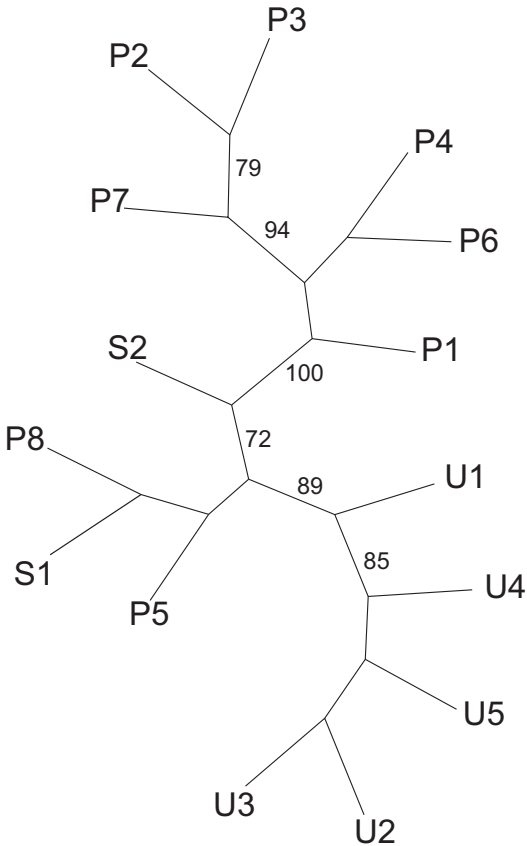| Source of variation	Sum of squares	df	Variance components	Percentage of variation	p	Φ_{ST}
Among 2x and 4x populations	6559.34	28	21.52	47.07	0.001	0.470*
Within populations	6147.98	289	24.20	52.93	0.001	

Fig 4. Neighbour-joining tree based on Reynold's distance among 15 *Iris aphylla* populations. Bootstrap values (1000 replicates) are shown only > 70%.

tions from the Lublin Upland (karyotype B) with the single diploid population located in the Slovak Karst (karyotype A). In the phenetic and PCO results, these two tetraploid populations are also close to the diploid one. The similarity of these two karyotypes (A and B) suggests that tetraploidy was established from a similar diploid population, with subsequent migration to invade new territory. Alternatively, it may be suggested that the existing tetraploid populations evolved from already differentiated diploids, most of which became extinct or are so rare that they have not been collected. It is likely that the tetraploids, with their wider spectrum of tolerance, adapted to ecological conditions after glaciations in different parts of Europe not suitable for diploids (Levin 1983, Trewick *et al.* 2002). Our cytological and AFLP data provide further evidence that tetraploids arose independently three or more times in the past, probably *via* genome duplication. In general, *I. aphylla* showed geographical concordance in distribution of karyotypes in Europe. The distribution of some karyotypes was restricted to a single population (karyotypes: A, C and D). Others were found over a wider territory (karyotypes: B, E and F).

Colonization history of *Iris aphylla*

The cytological history of the *I. aphylla* populations strongly suggests that migration between the southern and northern populations located
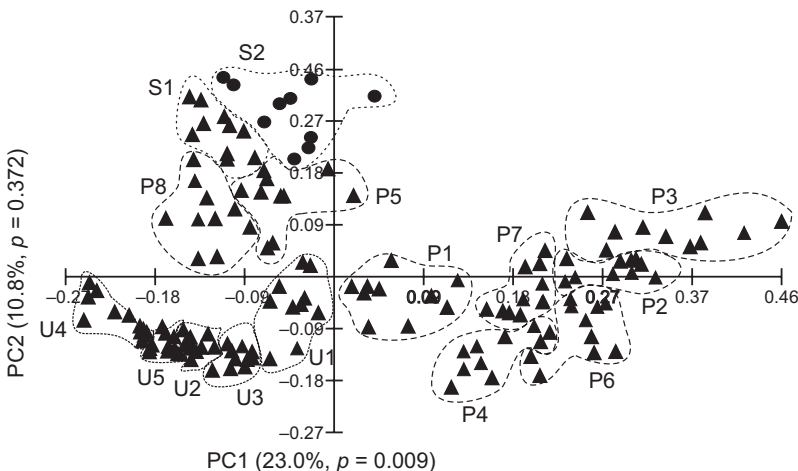

Fig. 5. PCO plot for *Iris aphylla* individuals belonging to 15 populations located in three regions of Europe. ▲ = individuals from tetraploid population; ● = individuals from diploid population; line, one population. Site codes for populations are given in Table 1.

on both sides of the Carpathians occurred *via* the Dukielska Pass (Wróblewska 2008). This mode of plant and animal colonization was previously demonstrated by Petit *et al.* (2002) and Babik *et al.* (2004). In earlier AFLP studies, we suggested that the northern habitats were colonized by steppe plants more intensively during the Younger Dryas or earlier Holocene, when woodland receded and open, tundra-steppe communities developed (Wróblewska *et al.* 2003, Wróblewska & Brzosko 2006). Colonization may also have come from the eastern part of Europe (Volhynian and Podolian Uplands) along loess and calcareous habitats in the direction of the present Lublin and Małopolska Uplands.

The phylogeographical pattern of *I. aphylla* demonstrates that present-day geographical distance is not always a good indicator of genetic relatedness. Therefore, there is discrepancy at the level of genetic differentiation among populations as assessed by AFLP markers and chromosome variation. Tetraploid populations (P5, P8 and S1) from distinct geographical regions co-occurred in the phenetic analysis, while the karyotypes of these populations diverged strongly. In such a case the real impact of karyotypic differences on the genetic relationships among populations is often particularly difficult to evaluate (Rieseberg 2001). It would suggest that DNA and chromosomal evolution are largely independent processes (Taberlet *et al.* 1994, Ruedi 1998). The available data also illustrated that the chromosome polymorphism in this group is quite a recent phenomenon. Thus, within *I. aphylla* these extremes may rather demonstrate recent range expansion, which also finds support in the low number of AFLP fragments unique to each population. There tend to be fewer markers of this type in the youngest (most recently established) populations than in older ones (Després *et al.* 2002, Tribsch *et al.* 2002). All the analysed populations are genetically depauperated and have 0–3 unique bands, indicating that not enough time elapsed for many unique fragments to form through mutation. The relatively high and significant differentiation between the one diploid and all investigated tetraploid *I. aphylla* populations ($\Phi_{ST} = 0.470$) can be explained by a few factors such as fragmentation of natural open habitats as a result of anthropopression and

natural succession. Another factor is the limited distribution of suitable open communities of calcareous grassland at the margin of the species range and the differences in the flowering time of *I. aphylla* populations (Wróblewska 2003, 2008).

Conservation implications

The fragmented distribution of the *I. aphylla* diploid and tetraploid populations in northern, central and southern Europe does not support the common notion that polyploidy should allow a species to expand its geographical range. This species seems to be in regression. Some of the populations are in protected areas, but many are undergoing natural succession leading to extensive overgrowing by shrubs and trees. Overcollecting also poses a threat. *Iris aphylla* populations most likely were more frequent in the past when calcareous and loess habitats were more common, for example in southern Poland (Silesia and Lublin Uplands; Kaźmierczakowa & Zarzycki 2001). Conservation programmes should include habitat restoration and *in situ* as well as *ex situ* strategies involving demographic and genetic monitoring of populations and the establishment of germplasm banks. Evidence from karyological and AFLP data analysis suggests ongoing genetic divergence between the diploid and tetraploid lineages; this phenomenon should be considered in planning conservation measures. Given the differences in genetic structure and karyotypes among diploid or tetraploid populations, it is not advisable to introduce or mix individuals or seeds from other distinct populations. Increasing the size or the effective size of populations would reduce the chance of extinction through stochastic processes, improve reproductive performance, and increase genetic variation to buffer future environmental change.

Taxonomic status

Karyotype and AFLP data supports the conclusion that the different *I. aphylla* subspecies should be regarded as a single species. The main character distinguishing the subspecies *bohemica* (= var. *typica*) from *hungarica* is the flower bud,

which leans slightly before flowering in subsp. *hungarica*, while in subsp. *bohemica* (= var. *typica*) the flower bud is straight. Another feature used to separate the subspecies is the relative height of the generative shoot versus the longest leaf (Raciborski & Szafer 1919, Medwecka-Kornaś 1959, Dostál 1989). Observations of these features, particularly for the Polish and also for the Slovak populations, showed that they vary greatly year by year and that they are associated with seasonal changes (Wróblewska 2003, Wróblewska & Brzosko 2006, A. Wróblewska unpubl. data). We suggest that different chromosomal races or variants should rather be distinguished, whose status can be confirmed by AFLP analysis. Karyological analysis and AFLP data make it clear that *I. aphylla* needs further taxonomic revision. Obviously, AFLP markers are more commonly used to examine genetic differentiation than to make inferences about phylogenetics, because they are highly homoplasious markers (Després *et al.* 2003). Therefore, AFLP data and karyological analysis should be verified in future using the chloroplast DNA markers, on which traditional molecular phylogeny and phylogeography are based.

Acknowledgements

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