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From the center to the margins of geographical range: molecular history of steppe plant *Iris aphylla* L. in Europe

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Summary. The steppe plant *Iris aphylla* was chosen to describe the genetic diversity patterns and postglacial expansion over the whole geographical range. By studying 29 populations, both in the centre and at the periphery of the geographical range, a moderate level of genetic diversity ($P_{\%} = 32.5\%$; $H = 0.105$), a low level of linkage disequilibria and a low percentage of fixed loci (LD = 2.8%; $F_{UL} = 2.1\%$) were detected. The intermediate level of *I. aphylla* genetic diversity is rather close to plant species with a limited geographical range as well as rare and/or endangered species. It could also be explained by common processes of vegetative reproduction, the occasional or absent recruitments as well as the recent history of *I. aphylla* populations. The lack of significant genetic differences between central and marginal populations (AMOVA, 1.52%; $P = 0.112$) and the low number (1–3 per population) or lack of unique bands confirmed that the populations in both cases were recently established.

Keywords: AFLP; colonization routes; endangered species; steppe plant

Introduction

The survey of genetic diversity across the whole species' range provides an opportunity for revealing evolutionary changes and processes at different geographical scales. Genetic diversity of species has consequences in different processes such as colonization, life history traits (i.e. reproduction, dispersal and life cycle), historical and present population size, impact of environmental factors and anthropogenic disturbances as well as hybridization and polyploidization events. These factors were frequently mentioned by many authors such as Loveless and Hamrick (1984); Hamrick and Godt (1989); Taberlet et al. (1989); Hewitt (1999); Nybom and Bartish (2000) and Nybom (2004). From the evolutionary and historical perspectives, the peripheral populations situated at the edges of the species' range seem to be of great interest. Theoretical studies have supported that the marginal populations are more sensitive to genetic drift and/or strong directional selection (Barrett and Husband 1990). Therefore, the genetic diversity should be depleted within populations that are located at the margins of the range and that are repeatedly isolated. Empirical studies across the species' range partially supported these results and showed that peripheral populations could have a lower genetic diversity (Lammi et al. 1999;

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Després et al. 2002; Tyler 2002; Schönswetter et al. 2003), similar (Schiemann et al. 2000; Van Rossum and Prentice 2003) or a higher rather than central populations (Tremblay and Simon 1989; Lagercrantz and Ryman 1990). The wide scale of investigation that included, the recent colonized area (periphery populations), as well as their corresponding source populations (centre of distribution range), permitted the determination of a global view of the species' history. Many authors have reviewed the various patterns for alpine, arctic or tree plant species in Europe by comparing their current phylogeographical structure and with the use of DNA markers (Demesure et al. 1996; King and Ferris 1998; Grivet and Petit 2002; Petit et al. 2002; Schönswetter et al. 2003). Because of the insufficient pollen data, which we can interpret from peat layers, relatively little is known about the colonization history of most steppe plants, the mechanisms of their expansion and major refugial regions. It is know that the thermophilous plant species were more affected by the cold phase of glacial cycles than cold-tolerant species and therefore, changes in vegetation and flora associated with climatic fluctuation altered the range of woodland and steppe species in opposite ways. During the Younger Dryas, the last period of the Pleistocene (around 11,000 BP), the climate became colder and the northern advance was sharply reversed. Many steppe plants, preferring open habitats, spread from the southern glacial refugia such as the Balkans and possibly near the Caucasus and the Caspian Sea, to northern, western and southern Europe, before the climate warmed and vegetation advanced rapidly throughout Europe (Szafer 1983; Taberlet et al. 1998; Hewitt 1999; Schmitt and Seitz 2001). Only a relatively few steppe or thermophilous herbs are present in the peat layers and the palynological data, referring to *Artemisia* or species from the Chenopodiaceae are described in detail in Europe (Huntley and Birks 1983) or in Poland (Rafalska-Jasiewiczowa et al. 2004). The latter author noted, that at the beginning of the Younger Dryas, the steppe communities containing species from *Artemisia* and Chenopodiaceae expanded, arriving originally from south-eastern Europe. In many cases, the mountains were physical barriers to the colonization by steppe plants of other European regions and the main waves of expansion could have been via mountain passes (i.e. through the Moravian and the Iron Gap as well as the Dukielska Pass; Szafer 1983).

One of the steppe plant species, for which the pattern of genetic diversity and colonization has been demonstrated, is *Iris aphylla* (Wróblewska et al. 2003; Wróblewska and Brzosko 2006). It is a long-living, rhizomatous perennial that belongs to the group of Pontian plants. A subspecies status has been proposed for *I. aphylla* forms, which were described only on the basis of morphological and cytogenetical characteristics (Dostál 1989). The diagnostical characters provided by Dostál (1989) and division of *I. aphylla* into subspecies, are rather speculative at the present. This species is also classified as extremely rare and endangered in red data books and red data lists in central Europe (Maglocký and Ferákowá 1993; Ludwig and Schnittler 1996; Sándor 1999; Holub and Procházka 2000; Kaźmierczakowa and Zarzycki 2001). According to Pólya (1949, 1950) and Wcisło (1964) the investigated *I. aphylla* populations are autotetraploid with the number $2n = 48$ of somatic chromosomes. In previous study with using RAPDs, the genetic diversity of the northern marginal *I. aphylla* populations was determined (Wróblewska et al. 2003; Wróblewska and Brzosko 2006). The results demonstrated a moderate genetic diversity at the species and population level, which is a result of a low sexual reproduction and the recent history of marginal *I. aphylla* populations in the study area. We also suggested that the studied marginal populations could have originated from a single colonizing source. The purposes of the present survey are to examine the genetic structure and colonization patterns of European populations of the leafless iris (*I. aphylla*) across the whole geographical range using AFLP markers. The following main questions were addressed:

1. Is the genetic diversity gradient less in populations situated in the centre of the

geographical range than the more distant populations at the border? Do populations from various phytogeographical regions across species distribution show significant differences in genetic diversity?

2. Are there any relationships between genetic and geographical distances despite the presence of the physical barrier (Carpathian Mountains)? It other words, "do genetic data suggest long-distance dispersal or continuous colonization throughout the distribution range"?

3. What exactly was the scenario that accurately describes the colonization history of this steppe plant in Europe?

Materials and methods

Population characteristics. Firstly, the scale of the study was defined, in which the genetic diversity parameters and colonization patterns of *Iris aphylla* were analyzed. The samples were collected from 29 populations across the whole species' geographical range, described as the species level. Six of the 29 populations were categorized as central populations located in the continuous geographical distribution. Twenty-three populations were described as peripheral, island populations with disjunctive occurrences. The populations from the Slovak Karst and the Hungarian Lowland were also recognized as marginal because the low number of these local populations consisting of small groups are strongly fragmented and isolated from one another (Fig. 1, Table 1).

Both in the centre and periphery of the geographical range, the genetic structure of populations was investigated in the context of their locations, which indicates an attachment to the phytogeographical provinces of Europe (Matuszkiewicz 1993). This type of division gives us information about the role of the historical and recent vegetation in the shaping of the genetic diversity of studied species. As such, eight groups were distinguished: two in the centre and six at the periphery of *I. aphylla* geographical range (Fig. 1, Table 1). From the centre of the geographical range samples were collected from five populations occupying the Volhynian and Podolian Upland ("U", Ukraine) and one from parts of Kursk city ("R", Russia). From the periphery of the geographical range, samples were taken from four populations situated in the Slovak Karst ("S", Slovakia), five in the Hungarian Lowland ("H", Hungary), eight in northern Europe ("P", Poland), two in the Saale Valley ("G", Germany), one in central Bohemia ("C", Czech Republic), two in the Volga Valley and one in the Caucasus ("R", Russia, respectively, Fig. 1; Table 1). From each population, ten samples were arbitrarily chosen along a transect at 2–3 m intervals, so eliminating the chance of obtaining the same genotype.

DNA isolation and AFLP analysis. The total genomic DNA was extracted from ca 100 mg of dried leaf tissue with a DNeasy Plant Mini Kit, according to the manufacturer's instruction (Qiagen). The AFLP protocol was carried out with a procedure described by Vos et al. (1995) with a modification (PE Applied Biosystem). First, 64 combinations of primer pairs on 20 samples was tested (1 or 2 samples randomly selected from each *I. aphylla* population). From this analysis, four primer combinations were chosen: *Eco*RI-ACC/*Mse*I-CAC, *Eco*RI-ACC/*Mse*I-CAT, *Eco*RI-AGC/*Mse*I-CTG, *Eco*RI-ACA/*Mse*I-CTA which gave the clear, reproducible and homogeneous intensity bands showing the variation among individuals and the discriminated clones within populations. The fluorescence-labeled selective amplification products were separated on 36 cm denaturing polyacrylamide gels (4.5%), in a 1 × TBE buffer with a labeled size standard ROX-500 (Applied Biosystem) on an automated sequencer (ABI PRISM 377). The detection of amplification products took on GeneScan version 3.1 (Applied Biosystem). The presence or absence of each fragment within each individual was scored as a binary matrix in a GENOTYPER version 2.1 (Applied Biosystem).

Data analysis. Genetic analysis was performed using a procedure recommended by Lynch and Milligan (1994) to reduce bias in estimating the population genetics parameters. The genetic diversity was evaluated at the species level (all 29 investigated populations), both in the central and marginal groups of populations, in the phytogeographical regions and within populations using only polymorphic loci and quantified as the proportion of the polymorphic loci ($P_{\%}$), at the 5% level (AFLPSURV version 1.0; Vekemans 2002) and Nei's gene diversity (H) using the Bayesian approach of Holsinger et al. (2002). The H values were estimated by the program HICKORY version 1.0, with a full model and using noninfromative

Table 1. Characteristic of *Iris aphylla* populations (division into centre and periphery of geographical range, populations located in different phytogeographical provinces and site code)

Group/population	Site code	Coordinate	*N*	$P_{\%}$	*H* (±SD)	$LD_{\%}$	$B_{F\%}$	B_u
Center of distribution								
Volhynian and Podolian Upland								
Suchovolija	U1	50° 02′ N/25°15′ E	~500	47.1	0.111 (±0.004)	4.0	1.8	1
Bilyjkamin	U2	49°52′ N/24°50′ E	~600	25.5	0.086 (±0.003)	1.9	1.4	1
Zoločiv	U3	49°48′ N/24°42′ E	~1,600	25.9	0.094 (±0.003)	2.0	0.6	1
Babuchiv	U4	49°20′ N/24°39′ E	~1,000	26.1	0.101 (±0.003)	1.6	0.0	1
Burstin	U5	49°13′ N/24°41′ E	~2,000	19.4	0.095 (±0.003)	3.1	0.0	1
Overall				28.8	0.097 (±0.003)	2.5	0.8	
Eastern Europe								
Kursk	R1	51°56′ N/36°01′ E	~500	32.1	0.111 (±0.003)	2.4	2.0	1
Periphery of distribution								
Slovak Karst								
Dlha Hora	S1	48°31′ N/21°47′ E	~2,000	36.1	0.109 (±0.003)	2.3	3.0	1
Krkavčie Skaly	S2	48°38′ N/20°51′ E	~500	29.7	0.101 (±0.003)	4.8	1.8	3
Haj	S3	48°38′ N/20°49′ E	~1,200	27.8	0.104 (±0.003)	6.3	4.2	–
Silica	S4	48°34′ N/20°33′ E	~1,400	39.3	0.099 (±0.003)	3.4	0.8	–
Overall				33.2	0.103 (±0.003)	4.2	2.5	
Hungarian Lowland								
Udulóreled	H1	47°27′ N/21°47′ E	~1,000	38.7	0.094 (±0.003)	3.8	2.6	–
Ligetpuszta	H2	47°27′ N/21°53′ E	~600	29.7	0.099 (±0.003)	2.5	2.2	–
Erdőbénye	H3	48°16′ N/21°21′ E	~500	27.8	0.077 (±0.003)	1.3	2.2	–
Tállya	H4	48°12′ N/21°23′ E	~500	35.3	0.092 (±0.003)	2.7	1.0	–
Tarcal	H5	48°08′ N/21°20′ E	~500	23.6	0.093 (±0.003)	2.5	0.4	1
Overall				31.0	0.091 (±0.003)	2.6	1.7	
Northern Europe (Biebrza Valley, Małopolska and Lublin Upland)								
Kapice	P1	53°43′ N/22°44′ E	2,147	29.1	0.098 (±0.003)	3.0	2.6	–
Tunel	P2	50°27′ N/19°58′ E	177	38.1	0.117 (±0.003)	3.3	2.6	–
Kazimierz Dolny	P3	51°19′ N/21°55′ E	228	41.5	0.131 (±0.003)	2.7	5.4	1
Gościeradów	P4	50°49′ N/21°59′ E	129	34.1	0.112 (±0.003)	2.5	5.0	1
Sobianowice	P5	51°17′ N/22°40′ E	905	36.9	0.112 (±0.003)	2.5	5.2	–
Zawadówka	P6	51°07′ N/23°23′ E	135	44.5	0.104 (±0.003)	2.3	2.7	1
Tarnogóra	P7	50°53′ N/23°06′ E	~2,500	37.1	0.123 (±0.003)	3.0	1.2	–
Czumów	P8	50°47′ N/23°56′ E	2,312	26.9	0.099 (±0.003)	2.5	0.2	–
Overall				36.0	0.114 (±0.003)	2.7	3.1	
Western Europe (Bohemia and Saale Valley)								
Karlstejn	C1	49°57′ N/14°09′ E	~200	31.1	0.128 (±0.003)	3.2	0.6	1
Freyburg	G1	51°11′ N/11°45′ E	~1,400	36.5	0.114 (±0.003)	2.5	1.3	1
Huysburg	G2	51°53′ N/11°03′ E	~1,500	28.9	0.112 (±0.003)	1.7	0.4	1
Overall				32.1	0.118 (±0.003)	2.5	0.8	
Volga Valley								
Nizhniy Novgorod-1	R2	56°30′ N/44°27′ E	~1,000	29.1	0.106 (±0.003)	2.4	0.0	2
Nizhniy Novgorod-2	R3	56°46′ N/45°00′ E	~1,000	39.3	0.126 (±0.003)	2.7	1.8	3
Overall					0.116 (±0.003)	2.6	0.9	
Caucasus								
Stavropol	R4	45°12′ N/42°01′ E	~500	25.3	0.090 (±0.003)	1.5	2.7	1

N the number of shoots within populations (see details in Sect. 2), $P_{\%}$ proportion of polymorphic loci, *H* Nei's gene diversity (±SD), $LD_{\%}$ percent of linkage disequilibrium at the 5% level, $B_{F\%}$ percent of fixed loci (occurring in all analyzed individuals of one population, i.e. with a frequency of 1.0), B_u number of unique bands (fragments of DNA, confirmed to occur in a single population within a given group)

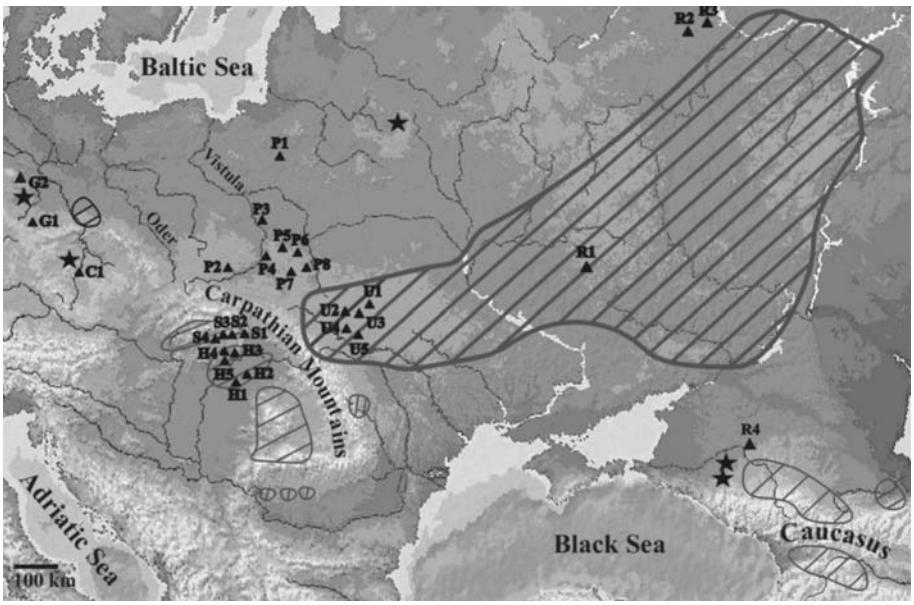

Fig. 1. Geographical distribution of *Iris aphylla* in Europe. The large, *thick lines* indicate the continuous distribution and the *thinner lines* are the groups of few populations distributed close to main range. The *star* indicates marginal population, which was not included into analysis; the *triangles* are the 29 studied populations. Site codes of populations are identified in Table 1

priors for f and θ_B. The data were run with the default parameters (burn-in = 50,000, number of samples = 250,000, thinning factor = 50). Then, the differences within genetic diversity parameters ($P_{\%}$ and H) among the central and marginal groups of populations were estimated by use of the Mann–Whitney U-test and the correlation between the proportion of polymorphic loci ($P_{\%}$) and Nei's gene diversity (H) among populations, with a pairwise Spearman's rank correlation coefficient. The genetic data were also used to asses the association between genetic diversity parameters ($P_{\%}$ and H) and population size using a pairwise Spearman's rank correlation. Short-time observations of population size were completed in all eight Polish populations over four (south and south-eastern Poland) to 6 years (north-eastern Poland) and expressed as the mean number of shoots (N) for 4–6 years. In the remaining central and marginal populations, information about the approximated population size only from 1 year during plant collection for genetic analysis (2002) was included (Table 1). It was also tested linkage disequilibrium (LD) between pairs of loci with the χ^2 test using POPGENE version 1.32 (Yeh et al. 1997). The fixed bands ($B_{F\%}$) were also counted in populations (these occurring in all individuals of one population).

An estimate was made for each phytogeographical group of the number/proportion of private bands (B_P, the fragments of DNA present only within a group), which could be shared among all populations (B_S) within this group or unique bands (B_U) as the fragments of DNA, confirmed to occur in a single population within given group.

Three different hierarchical levels of molecular variance AMOVA were performed, in the study of the genetic structure of *I. aphylla* populations, with ARLEQUIN version 2.000 (Schneider et al. 2000). Levels of significance for populations were determined using a permutation test (1,000 permutations).

The F_{ST} values between pairs of populations were calculated on a different scale based on Reynolds' distance (Reynolds et al. 1983) with AFLPSURV version 1.0 (Vekemans 2002). The F_{ST} values were also used in assessing correlation between the genetic and geographical distances (isolation by distance, IBD) among populations (Slatkin 1987, 1993; Rousset 1997). The regression of $F_{ST}/(1 - F_{ST})$ estimates on the logarithm of the geographical distance (in km) for population pairs was evaluated by Mantel test with 1,000 permutations, using TFPGA version 1.3 (Miller 1997). The IBD model was performed for all 29 populations as well as within central and marginal

groups. It was predicted that IBD patterns would start to break down due to suppressed migration and thus, the enhanced effects of drift (Hutchinson and Templeton 1999). To test for this, the slopes of the regressions of F_{ST} geographical distance between the central and marginal populations were tested with ANCOVA, where the group comprising the central and marginal populations was the dependent variable, $F_{ST}/(1 - F_{ST})$ independent variable and the logarithm of geographical distances was defined as the covariate (STATSOFT version 5.0 1997).

Neighbor-joining genetic distance analysis was performed among all populations using PHYLIP version 3.6 (Felsenstein 2004). Branches important for discerning associated populations/groups were evaluated by bootstrap approach with 1,000 replicates. Then, we created the unrooted consensus tree based on 29 trees of *I. aphylla*, using CONSENS from the software package PHYLIP, version 3.6 only branches with bootstrap support above 70% were considered (Felsenstein 2004). Principal component analysis (PCA) was used for analysis of genetic differentiation among populations with GENALEX version 6.0 (Peakall and Smouse 2006), for delineating barriers to gene flow and direction of range expansion. Then, first-axis principle component scores (PC 1) together with the geographical coordinates for all 29 populations were included in the kriging interpolation procedure (Journel and Huijbregts 1978). Kriging interpolates based on spatial autocorrelation was performed using SURFER version 8 (GOLDEN Software 2002) and showed as two-dimensional plots. Additionally, Monmonier's (1973) maximum difference algorithm was applied to identify boundaries of abrupt change in the genetic differences, between pairs of populations using program Barrier version 2.2 (Manni et al. 2004). This allowed for the display of the genetic discontinuities existing between different pairs or groups of populations, where genetic differences between pairs of populations were the highest (Manni and Barrai 2001). This analysis links mapped populations into a network using Delaunay triangulation, which is the dual structure of the Voronoï tessellation (Voronoï 1908). Monmonier's algorithm has often been applied in landscape genetics inferring genetic boundaries and enabling a better interpretation of micro-evolutionary processes (gene flow, genetic drift and/or natural selection). These genetical barriers could be the result of different factors (geographical, ecological, etc.; Manni et al. 2004). It should be noted that Monmonier's algorithm provides information that is not captured by classical clustering or multivariate methods. Palmé et al. (2003) pointed out "... In this case, genetic frequencies follow a perfect gradient where no boundaries can be identified. In other words, the pattern of change is so gradual that it becomes impossible to find where a given genetic parameter changes at a different rate in space". At the larger scale of the investigated area, and the complex molecular history of a given species, genetic differences between populations are unlikely to follow an IBD model and therefore, sudden genetic changes are to be expected.

Results

AFLP and genetic diversity patterns. Four selective primer combinations provided 501 fragments, with the length ranging from 35 to 350 bp. After applying the Lynch and Milligan's restrictions (1994) for dominant markers, the number of polymorphic AFLP markers was not reduced. Each sampled individual also exhibited the distinct AFLP multiband genotypes in all data sets.

The proportion of polymorphic loci and Nei's gene diversity at the species level was moderate ($P_{\%} = 32.5\%$, $H = 0.105$). However, the proportion of polymorphic loci between the central (29.3%) and marginal group (33.2%) did not differ significantly (Mann–Whitney test, $U = 44.5$; $P > 0.05$) as well as Nei's gene diversity ($H = 0.099$ and $H = 0.106$, respectively; Mann–Whitney test, $U = 48.0$; $P > 0.05$).

Moreover, the $P_{\%}$ varied between phytogeographical groups from 23.3% (Caucasus) to 36.0% (Northern Europe) and for H values from 0.090 (Caucasus) to 0.114 (Western Europe) (Table 1). The $P_{\%}$ and H values were strongly correlated with each other (Spearman's rank correlation, $R = 0.64$, $P < 0.001$) but not with population size ($R = -0.23$ and $R = -0.04$, respectively, $P > 0.05$) over the whole geographical range.

Linkage disequilibrium examined by χ^2 showed, that the proportion of significant pairs was not higher than expected by chance at the species level (2.8%), within the central (2.5%) and marginal populations (2.9%, Mann–Whitney test, $U = 45.5$; $P > 0.05$), as well as within the

phytogeographical groups (see Table 1). Only in one population from the Slovak Karst (S3, Haj) LD exceed this level, being equal to 6.3%. The percentage of fixed loci also maintained low values at the species level (2.1%), whilst in the central populations this value was twice as low as in the marginal group (0.9 and 2.2%, respectively), although the differences were not significant (Mann–Whitney test, $U = 55.8$; $P > 0.05$).

The participation of private bands was very low within each phytogeographical group both in the center and periphery of the range. The highest frequency of these bands was noted in the group of populations located on the Volhynian and Podolian Upland (9 bands, 1.8% of all polymorphic bands). In the groups from the periphery of the range, the number of private bands ranged from one band (0.2%; Eastern Europe and Caucasus) to five bands (1% of all polymorphic bands, the Volga Valley, Northern Europe, the Hungarian Lowland and the Slovak Karst). In groups of populations no shared bands between all populations from the Slovak Karst, Western Europe and from the Volga Valley were found. On the other hand, 1–2 shared bands in the others were noted (data not showed). In nine of the 29 populations only one unique band and three unique bands in the S2 (Slovak Karst) and R3 population (Volga Valley, Table 1) were found.

AMOVA. The partitioning of total genetic diversity by AMOVA is present in Table 2. In all cases at the species level, groups of central and marginal populations and in phytogeographical groups, the largest variation was detected within populations ($P < 0.001$, Table 2). The molecular variation with division into phytogeographical groups explains only 7.3% of the total variation, although this was statistically significant ($P < 0.001$). On the contrary, the part of the variance of genetic diversity between the central and marginal group of populations was negligible (Table 2).

Genetic diversity among populations and IBD. The total genetic differentiation among all 29 *I. aphylla* populations was moderate $F_{ST} = 0.208$ and highly significant (permutation test, $P < 0.001$). The F_{ST} among the central populations (0.147) differed twice in comparison to the marginal ones (0.269). At the scale of phytogeographical groups, the lowest genetic differentiation was noted in the Volhynian and Podolian Upland, located in the center of distribution (0.083) and the highest at the

Table 2. Hierarchical analysis of molecular variance (AMOVA) with division into central and periphery of *Iris aphylla* geographical range (a) as well as without (b) and with (c) division into eight phytogeographical groups

Source of variation	*Df*	Sum of s quares	Variance components	Percentage of variation	*P*
(a) Center versus periphery of species' range					
Among groups	1	32.27	0.70	1.52	0.112
Among populations within group	27	6,257.07	21.28	46.08	0.001
Within populations	282	6,147.98	24.20	52.40	0.001
(b) Without division into 8 phytogeographical groups					
Among populations	29	6,643.34	21.070	46.24	0.001
Within populations	261	6,346.21	24.502	53.76	0.001
(c) With division into 8 phytogeographical groups					
Among groups	7	2,126.34	4.534	7.25	0.001
Among populations within group	20	3,785.95	17.400	40.38	0.001
Within populations	262	5,923.88	24.378	52.37	0.001

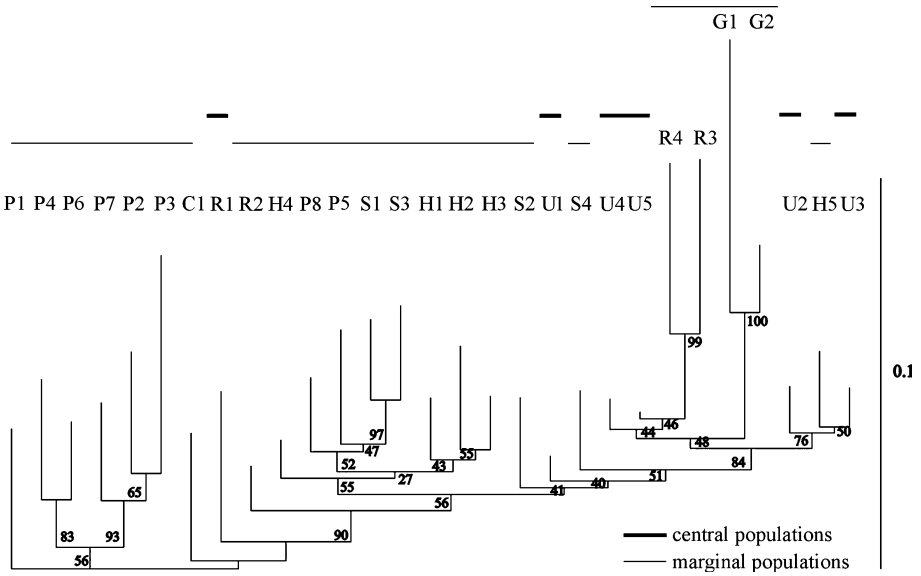

Fig. 2. Neighbour-joining tree based on Reynold's distance among 29 *Iris aphylla* central and marginal populations. Bootstrap values (1,000 replicates) are presented for each node

periphery in Western Europe (0.391). The moderate and similar values were observed within the Slovak Karst (0.178), and Hungarian Lowland (0.172), while F_{ST} in Northern Europe and the Volga Valley group equalled 0.245 and 0.241, respectively. However, the Western Europe appeared to be the more genetic differentiated. The F_{ST} values between populations particularly from the Saale Valley, compared with other central and marginal populations exceeded 0.500 ("Appendix 1").

Positive and significant associations were observed between genetic differentiation and the log of geographical distances (in km) at the species level ($r^2 = 0.17$, $P = 0.001$) and among populations in the center ($r^2 = 0.94$, $P = 0.001$) and at the periphery of the range ($r^2 = 0.39$, $P = 0.001$). Non-significant associations between genetic differentiation and geographical distances were detected, when we compared the populations within each phytogeographical region ($P > 0.05$, data not shown). An ANCOVA between the central and marginal populations, with F_{ST} as the independent variable and geographical distance as the covariate, highlighted a significant difference between the slopes of the regressions ($df = 1$, $F = 13.22$, $P < 0.001$).

Spatial genetic analysis. A weak geographical structure could be observed in the consensus tree of *I. aphylla*, with a few branches, which were supported by high bootstrap values (70–100%, Fig. 2). However, it seems that populations in a few cases tended to cluster independently to their geographical origin. The tree was differentiated into two groups. The first one constituted two well-supported groups of six populations from Northern Europe (56–93% bootstrap support). The second one was formed from the other studied populations (Fig. 2). In the second cluster three pairs of populations: two from the Saale Valley and Slovak Karst as well as one from the Volga Valley, together with a population from the Caucasus were highly supported (97–100%).

The contour maps based on AFLPs displayed an irregular gradient with a south-western or north orientation across Europe (Fig. 3a). The concentric lines forming high-gradient regions around Northern Europe and from the Saale Valley

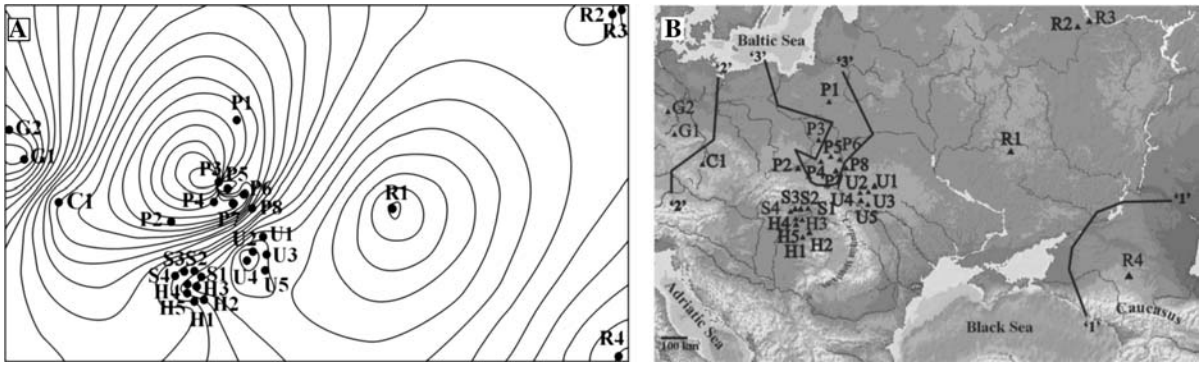

Fig. 3. Maps of *Iris aphylla* obtaining from: the kriging procedure **A** and Monmonier's maximum difference algorithm **B**. On the first map **A** the *lines* represent first the axis of PCA. On the second map the locations of studied *Iris aphylla* populations in Europe with genetical boundaries (*bold lines*) as "1", "2" and "3" are shown. Populations are indicated according to their site codes given in Table 1

populations, suggested that these areas represented barriers to gene dispersal as well as the recent range expansion into these regions. Monmonier's maximum difference algorithm identified three main boundaries (Fig. 3b) that were only partially delineated by kriging analyses. The first putative barrier (1), which was not confirmed by the above-mentioned analysis, isolated one R3 (Caucasus) from the remaining 28 populations. The second barrier (2) supported the distinctiveness of the populations from the Saale Valley from the others whilst the third one (3) separated the five northern populations.

Discussion

Genetic diversity across geographical range. The genetic diversity of *I. aphylla* ($P_{\%} = 32.5\%$; $H = 0.105$; LD = 2.8% and $F_{\mathrm{L}} = 2.1\%$) is maintained on a moderate level not typical for long-lived, outcrossing and autopolyploid perennials (Hamrick and Godt 1989). The species biologically comparable to *I. aphylla* but with a wider geographical range, i.e. *Trollius europaeus* (Després et al. 2002), were characterized by higher genetic diversity than the analyzed steppe plants. On the other hand, within a few endangered, diploid plants such as *I. aphylla*, relatively similar values of genetic diversity parameters were obtained (*Astragalus*, Travis et al. 1996; *Moringa oleifera*, Muluvi et al. 1999; *Carex digitata*, Tyler 2002). The intermediate level of *I. aphylla* genetic diversity is rather close to plant species with a limited geographical range (Karron 1997; Gitzendanner and Soltis 2000) as well as rare and/or endangered species (Hamrick and Godt 1989). The study of genetic diversity can also show the regularities and mechanisms shaping this diversity at the different geographical scales. In most cases, the theoretical expectations and experimental studies illustrate that the populations near to the center of the species' range are genetically diverse, whereas marginal isolated populations are less genetically variable (Hoffmann and Blows 1994). Contrary to these expectations, there are also examples where the comparable values of genetic diversity in central and marginal populations are noted (Scheiner and Goodnight 1984; Schiemann et al. 2000; Van Rossum and Prentice 2003). Nevertheless, in the case of *I. aphylla*, the genetic difference between the central and marginal group was not statistically significant. Additionally, AFLP diversity values (percent of polymorphic loci and Nei's estimate) were approximate for phytogeographical regions both in the center and at the edge of the species' distribution. Obviously, it does not mean that the studied populations do not differ from one another. These differences mainly refer to fixed loci particularly at the level of phytogeographical

regions. Relatively higher amounts of them were observed within groups at the periphery ($F_L = 0.2–5.4\%$), but were low or indeed absent ($F_L = 0–1.8\%$) in the continuous range. The low but differentiated frequency of fixed loci and also LD within all *I. aphylla* populations could also be explained by intense processes of vegetative reproduction, maintaining the observed values of these parameters as well as the recent history of populations and the occasional presence or absence of juveniles within populations (Wróblewska and Brzosko 2006). The values of fixed loci are one of the important factors in detecting genetic drift within populations with AFLP markers. Within *I. aphylla* the mean amount of fixed loci totaled 2.1% although this value was lower than in other plant species (Miyashita et al. 1999; Lamour and Hausbeck 2001). The amount of LD of this steppe species also appeared to be sporadic (2.8%) and seems to be the lower value estimated by using dominant markers (Tero et al. 2003). The genetic linkage analysis of autotetraploids remains still a theoretical and methodological problem to be solved and therefore, it was more often estimated for diploid than polyploid species (Luo et al. 2004). Innan et al. (1999), McVean (2002) and Tero et al. (2003) stated, that the LD using AFLP markers has only rarely been calculated, although the presence of LD is generally common in plant populations with a low level of generative reproduction.

Moreover, a non-significant correlation between population size and genetic diversity within *I. aphylla* populations was found and therefore, the moderate levels of genetic diversity cannot be explained based only on the number of shoots. In many studies of clonal plants the population size based on the shoot number only is not significantly correlated with observed intra-population genetic diversity (Prentice and White 1988; Lannér-Herrera et al. 1996; Schmidt and Jensen 2000; Arafeh et al. 2002; Tero et al. 2003). I would rather suggest that the role of vegetative reproduction should be highlighted as it undoubtedly plays the most important role in the dynamics of *I. aphylla* populations (Wróblewska 2003; Wróblewska et al. 2003; Wróblewska and Brzosko 2006). On the other hand, the long-lived clones and the disintegration of long rhizomes of individuals could maintain the genetic diversity and protect the elimination of individuals. The fact that this species is an autopolyploid is also considered as crucial in buffering against the genetic drift (Soltis and Soltis 1995).

Genetic differentiation and isolation of populations. One of the assumptions of isolation by distance model (IBD) is the equilibrium between gene flow and genetic drift among populations (Kimura and Weiss 1964; Slatkin 1993; Hutchinson and Templeton 1999). The other studies have also reported that significant IBD patterns could exist, despite the presence of physical barriers limiting gene flow (Latta and Mitton 1997; Pogson et al. 2001). In the case of *I. aphylla*, it was noted that significant genetic differentiation exists among all populations, as well as significant IBD patterns in the whole geographical range. The low, albeit significant regression coefficient (0.17, $P < 0.001$), has been reported in plant (Sharbel et al. 2000; Bockelmann et al. 2003; Schönswetter et al. 2003) and animal species (Hellberg 1995; Johnson and Black 1998; Ehrich and Stenseth 2001). These authors showed that ongoing isolation among populations and habitat fragmentation at the periphery of the species' range could contribute to this low value. The confirmation of this fact was an analysis of covariance, which revealed the significant differences between the slopes of the regressions between the central and marginal *I. aphylla* populations. If the correlations between genetic and geographical distances are weaker at the edge of the range ($r^2 = 0.39$; $P < 0.001$) than in the center of distribution ($r^2 = 0.94$; $P < 0.001$) but in both cases statistically significant, this phenomenon could be explained by the domination of a certain level of genetic drift over gene flow in the fragmented marginal populations (Sumner et al. 2004). The influence of microevolutionary events shaping IBD patterns within plant species are still insufficient (Sharbel et al. 2000; Bockelmann et al. 2003; Tero et al. 2003; Olsen et al. 2004), but quite well described in invertebrate animals (Peterson 1995, 1996; Bossart and Powell 1998; Hutchinson and Templeton 199l; Pogson et al. 2001; Garnier et al.

2004). The research of adaptation for dispersal, explained how the plant diaspores fill the new niche but still do not clarify how they colonize the new habitats. Most plant seeds disperse at once, whilst a few rarely exceed 100 m and the relations between genetic and geographical distances were also included with an interpretation of many metapopulation models, distorting the patterns of colonization (Cain et al. 1998). Taking into consideration the limited dispersal capabilities of the *I. aphylla* seeds particularly over long distances (lack of dispersal mechanisms), it can be assumed that the gene flow appeared mainly between neighboring populations via the stepping stone model. In a few cases, such as the populations from the Saale Valley or populations from Northern Europe, the concentric rings around them detected in the kriging analysis, are not the only signals of a later colonization history of these areas, but also accidentally long-distance dispersal of *I. aphylla* diaspores. The positive and significant correlation between genetic and geographical distances also exists, despite physical barriers such as the Carpathian Mountains. It verified the fact, that mountains were not a strong barrier to gene flow. Despite the geographical isolation of populations and habitat fragmentation by humans or the considerable influence of natural succession in communities, another factor could limit gene flow among *I. aphylla* populations, e.g. the flowering time at different latitudes. The flowering time in all northern populations is differentiated and begins from the first ten days of May in the Tunel population on the Małopolska Upland and the seventeenth to the twenty fourth of May in populations on the Lublin Upland, and the first ten days of June in the Biebrza Valley (Wróblewska 2003). The variable flowering time in connection with geographical distances between populations and the behavior of the main pollinators (*Bombus*), suggest that the gene flow among populations is influenced by the seeds rather that by pollens.

The historical gene flow between central and marginal populations is quite adequately described by analysis of molecular variance. The lack of significant differences between groups of central and marginal populations, confirmed that the populations in both cases were recently established. It also appeared that the main genetic variations were partitioned within populations not among them. Results of this study support the fact that the gene flow via pollen and/or seed dispersal happens generally within populations, which were reported for long-lived outcrossing perennials with a low amount of seedling establishment and extensive vegetative reproduction within populations (Fischer et al. 2000; Schmidt and Jensen 2000; Lindqvist-Kreuze et al. 2003). On the other hand, when genetic variation is examined at the level of phytogeographical region, each of them preserved the integrity (7.25%, $P < 0.001$).

Immigration patterns and colonization routes. AFLP markers were widely used to reconstruct molecular history and colonization routes of many plant species (Schaal et al. 1998; Després et al. 2002; Abbott and Brochmann 2003; Schönswetter et al. 2003). This study is the first illustrating postglacial expansion of the *Iris aphylla* in Europe, in which the results of the phenetic, kriging and Monmonier's analysis, allow us to partially confirm earlier biogeographical approach. Certainly, the European communities, both in the present central distribution and at the edge of the *I. aphylla* geographical range, were colonized recently by diaspores of this plant. The fact that all populations are genetically depauperated is confirmed by the low number (1–3 per population) or lack of unique bands, which are characteristic for young (recently established) rather than older populations. On the other hand, the number of private bands characterizing each phytogeographical group, was twice as high in the continuous distribution of this species (the Volhynian and Podolian Upland). It seems that these steppe-forest areas in South-Eastern Europe could have been colonized somewhat slightly earlier in the postglacial history, than other analyzed areas of the geographical range. The recent colonization of European biota by the steppe plants, was also confirmed by Pawłowski (2003) and Guiter et al. (2003), who reported that the main wave of expansion of this plant group

occurred in the Younger Dryas (12.5 ky BP), and ended in the Earlier Holocene (10–9 ky BP) when the open habitats were still unforested. The Younger Dryas is considered as the most important in the spread of xerothermophilous flora and the colonization occurred via a few routes throughout the loess and calcareous territories (chalk, gypsum or marl), and occasionally through the areas of the Carpathian Mountains poorer in precipitation, [i.e. places with a so-called "intramontane calms climate", could also be the harbors of these plants (Cyunel 1959)]. It has been assumed that the Carpathian Mountains remained outside of the main fluctuations of steppe expansion and the other xerothermic species, because of the different climatic and edaphic condition of their habitats. Therefore, this massif was only the scene of secondary migration (Kornaś 1955; Cyunel 1959). Although the early history of *I. aphylla* in Europe can be supported, there is no evidence for glacial refugia of this steppe species in the Caucasus. In phytogeographical data referring to Pontian species, many authors pointed out that the Caucasus might be the refugium for this plant group (Szafer 1983). Notwithstanding, this analysis did not confirm this fact because of the low number of studied populations from this region or the type of DNA markers, which was used in the analysis. Tribsch et al. (2002) and Schönswetter et al. (2003), stressed that the refugial regions or populations generated the highest number of private or unique markers in the Alps, contrary to recent established populations in the Carpathian Mountains or Central Asia. At this stage of the investigation, I favor the view that the colonization to the present western, southern and northern *I. aphylla* populations in Europe was extended from the Volhynian and Podolian Upland as well as from populations surrounding this area. Therefore, the significant range expansion of *I. aphylla* occurred rather from east and/or southeast to south, southwest and north directions. From the Volhynian and Podolian Upland, three main colonization routes could be distinguished. The first of them was directed from the Volhynian and Podolian Upland into the north-eastern Hungarian Lowland and the second into the Slovak Karst both via the Dukielska Pass. The third direction expanded into south-eastern Poland (the Lublin Upland) and from this place the diaspores migrated in a westerly direction into the Małopolska Upland and into northern Poland. I do not exclude the migration between the southern and northern populations located on both of two sites of the Carpathian Mountains also via the Dukielska Pass. It is most likely that the strongly isolated populations from the Saale Valley and from Bohemia are derived rather from the group located in the Panonian Basin than from the group of populations located north of the Carpathian Mountains (Northern Europe). Moreover, the phytogeographical similarity of Bohemia and the Saale Valley was stressed by Knobloch (1984) and Knobloch and Konzalová (1998) illustrating the common floristic elements between these two regions. The origin of the north-eastern populations (Volga Valley) is also still problematic. In the present day, there is no evidence that these places were colonized by diaspores coming from the continuous distribution of *I. aphylla*. Alternatively, the pattern of colonization can also be explained including collections from these areas insufficiently represented in the available analysis.

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Appendix 1. Pairwise genetic differentiation (F_{ST} – below diagonal) between pairs of *Iris aphylla* populations (all F_{ST} values are statistically significant, $P < 0.001$)

	U1	U2	U3	U4	U5	R1	R2	R3	R4	S1	S2	S3	S4	H1	H2	H3	H4	H5	P1	P2	P3	P4	P5	P6	P7	P8	C1	G1	G2
U1	-																												
U2	0.091	-																											
U3	0.073	0.079	-																										
U4	0.065	0.130	0.109	-																									
U5	0.046	0.107	0.094	0.038	-																								
R1	0.210	0.290	0.256	0.308	0.313	-																							
R2	0.056	0152	0.134	0.148	0.133	0.423	-																						
R3	0.253	0354	0.335	0.322	0.307	0.318	0.241	-																					
R4	0.165	0235	0.230	0.217	0.209	0.219	0.351	0.245	-																				
S1	0.136	0.208	0.184	0.182	0.187	0.193	0.264	0.329	0.233	-																			
S2	0.072	0.147	0.140	0.155	0.144	0.110	0.200	0.317	0.221	0.154	-																		
S3	0.174	0.260	0.238	0.255	0.269	0.227	0.273	0.386	0.267	0.132	0.190	-																	
S4	0.084	0.147	0.141	0.117	0.114	0.130	0.276	0.331	0.229	0.201	0.116	0.241	-																
H1	0.076	0.160	0.139	0.129	0.142	0.119	0.225	0.310	0.225	0.133	0.126	0.163	0.140	-															
H2	0.187	0.241	0.233	0.259	0.280	0.207	0.263	0.417	0.306	0.232	0,181	0.261	0.242	0.157	-														
H3	0.120	0.200	0.184	0.153	0.186	0.143	0.245	0.375	0.276	0.186	0.164	0.255	0.172	0.103	0.174	-													
H4	0.101	0.189	0.179	0.187	0.190	0.135	0.239	0.394	0.270	0.195	0.139	0.224	0.170	0.137	0.195	0.125	-												
H5	0.134	0.152	0.115	0.158	0.166	0.201	0.310	0.389	0.277	0,220	0.198	0.260	0.202	0.171	0.274	0.209	0.233												
P1	0.219	0.330	0.297	0.354	0.351	0.275	0.458	0.348	0.207	0.284	0.225	0.286	0.291	0.254	0.272	0.292	0.265	0.351	-										
P2	0.231	0.315	0.296	0.359	0.346	0.277	0.444	0.339	0.235	0.284	0.232	0.314	0.285	0.254	0.293	0.327	0.281	0.355	0.221	-									
P3	0.187	0.268	0.255	0.294	0.288	0.156	0.390	0.287	0.191	0.236	0.193	0.243	0.240	0.195	0.248	0.235	0.183	0.316	0.314	0.177	-								
P4	0.264	0.324	0.310	0.372	0.375	0.288	0.459	0.343	0.309	0.303	0.286	0.330	0.322	0.267	0.323	0.337	0.297	0.333	0.221	0.218	0.296	-							
P5	0.180	0.245	0.228	0.262	0.267	0.216	0.357	0.229	0.202	0.207	0.197	0.228	0.223	0.191	0.223	0.225	0.201	0.275	0.276	0.221	0.309	0.273	-						
P6	0.299	0.363	0.354	0.378	0.393	0.274	0.463	0.340	0.329	0.298	0.288	0.327	0.340	0.279	0.334	0.335	0.305	0.365	0.207	0.140	0.203	0.141	0.218	-					
P7	0.189	0.260	0.260	0.273	0.296	0.257	0.436	0.315	0.225	0.193	0.219	0.229	0.273	0.185	0.233	0.204	0.182	0.287	0.305	0.185	0.233	0.245	0.303	0.213	-				
P8	0.084	0.157	0.151	0.135	0.138	0.272	0.335	0.211	0.138	0.124	0.135	0,167	0,158	0.104	0.218	0.147	0.124	0.181	0.329	0.224	0.320	0.331	0.188	0.255	0.316	-			
C1	0.155	0.233	0.207	0.264	0.257	0.206	0.378	0.276	0.152	0.221	0.179	0.248	0.213	0.198	0.232	0.213	0.188	0.271	0.196	0.209	0.167	0.241	0.190	0.275	0.218	0.190	-		
G1	0.400	0.445	0.418	0.399	0.272	0.416	0.412	0.455	0.462	0.476	0.459	0.455	0.387	0.495	0.465	0.500	0.418	0.426	0.502	0.479	0.513	0.418	0.484	0.598	0.561	0.562	0.509	-	
G2	0.359	0.358	0.328	0.299	0.205	0.477	0.345	0.523	0.460	0.540	0.435	0.423	0.329	0.459	0.504	0.433	0.375	0.311	0.463	0.455	0.501	0.440	0.458	0.555	0.508	0.508	0.460	0.205	-

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