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GENETIC DIVERSITY OF *MELICA TRANSSILVANICA* SCHUR (POACEAE) AT ITS NORTHERN RANGE LIMIT

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Geographically marginal populations are expected to have low genetic variability, which potentially can affect their viability. In Poland *Melica transsilvanica* Schur reaches the northern limit of its continuous geographical range. Genetic diversity and population genetic structure were analyzed in 15 of its marginal and more central populations using AFLPs. Overall, genetic diversity parameters did not differ significantly, and comparable patterns of genetic variation were found in central and marginal populations. All AFLP phenotypes were unique to particular populations. Unique alleles were fixed in some central and some marginal populations. The percentage of polymorphic loci varied from 1.30 to 5.19 (3.24 average) in central populations and from 0.43 to 5.63 (2.36 average) in marginal ones. Hierarchical analyses of molecular variance (AMOVA) for each species/region combination revealed highly significant differentiation between populations and showed similar partitioning of molecular variance in marginal and central populations of *M. transsilvanica* (diversity between populations: 93.24% and 93.18%, $p < 0.001$, respectively). The scattered distribution of suitable species habitats and the predominant selfing breeding system of the species strengthen the effect of selection pressure on fixation of unique loci in individual populations. Marginal populations of *M. transsilvanica* with unique alleles considerably expand the genetic variation of the species and are therefore valuable for conservation of genetic diversity.

Key words: AFLP, genetic diversity, marginal populations, *Melica transsilvanica*, range limit.

INTRODUCTION

Marginal populations are commonly expected to be less genetically variable than populations in the central range, which can make them less viable than the latter (Lesica and Allendorf, 1995). The degree of environmental and geographical marginality influences the genetic structure of populations (Johannesson and André, 2006). According to Mayr's (1970) model, the isolation of marginal populations is accompanied by a genotype change resulting from natural selection or genetic drift. Studies provide empirical evidence that genetic erosion, genetic drift and accumulation of deleterious alleles in small and isolated populations can reduce species viability (Lynch et al., 1995; Rasmussen and Kollmann, 2004; Johannesson and André, 2006). Both marginal and central-range populations effectively isolated over a long period have been found to be characterized by high interpopulational variability and diverge from the typical variability pattern formed by random breeding (Mitka, 1997).

Many studies, however, have not confirmed the predicted consequences of population isolation by habitat fragmentation, or have even found evidence to the contrary (Llorens et al., 2004). Genetic diversity within the endangered shrub *Grevillea caleyi* (Proteaceae) did not vary with population size or degree of isolation, and the current genetic structure of populations may be almost entirely the result of historical events (Llorens et al., 2004). The consequences of habitat fragmentation for the genetic diversity of *Anthyllis vulneraria* (Fabaceae) were shown to be relatively minor in comparison with historical high levels of seed exchange between habitat fragments. Hence, seed flow is responsible for conservation of the relatively high genetic diversity of this species even within populations in small fragments (Honnay et al., 2006).

Melica transsilvanica Schur is a submediterranean-continental, mainly steppe and steppe-forest species (Hempel, 1970). Its main geographical range covers Central Asia, the Middle East, eastern Siberia, the Caucasus, Western Asia, China, Eastern and Central Europe, reaching southern France and northern Italy in the west (Hultén and Fries, 1986). In the Polish flora it represents the Sub-Irano-Turanian geographical element (Zając and Zając,

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TABLE 1. Origin of studied population samples

Population	Location	Coordinates
Melica transsilvanica		
RUS	Russia, Volgograd	N48°48' E44°35'
ROM	Romania, near Cluj-Napoca, Cojocna	N46°45' E23°50'
SLO	Slovakia, Pieniny Mts., Červený Kláštor, on Dunajec River	N49°23' E20°25'
AUS	Austria, Niederösterreich, Waldviertel, Umlaufberg hill	N48°43' E15°50'
CZE-1	Czech Republic, Pozorice	N49°12' E16°47'
CZE-1	Czech Republic, Hodonin	N49°30' E16°25'
PIE-1	Poland, Pieniny Mts., Małe Pieniny range, Biała Woda Reserve	N49°23' E20°35'
PIE-2	Poland, Pieniny Mts., Małe Pieniny range, Szczawnica, Góra Szafranówka Mt.	N49°25' E20°28'
PIE-3	Poland, Pieniny Mts., Pieniński Pas Skałkowy (Pieniny klippen belt), Falsztyn	N49°27' E20°17'
KCU-1	Poland, Kraków-Częstochowa Upland, Dolina Bolechowicka Reserve, Bolechowice, Wąwóz Bolechowicki ravine	N50°07' E19°46'
KCU-2	Poland, Kraków-Częstochowa Upland, Dolina Szklarki Reserve, Szklary, Słoneczne Skały cliffs	N50°11' E19°43'
SUD-1	Poland, Sudetes Mts., Przedgórze Sudeckie foothills, Wzgórza Strzegomskie hills, Strzegom, Góra Krzyżowa Mt.	N50°59' E16°20'
SUD-2	Poland, Sudetes Mts., Przedgórze Sudeckie foothills, Wzgórza Strzegomskie hills, Strzegom, Góra Św. Jerzego Mt.	N50°59' E16°20'
SUD-3	Poland, Sudetes Mts., Pogórze Bolkowsko-Wałbrzyskie foothills, Dobromierz, Góra Dębowa Mt.	N50°55' E16°15'
SUD-4	Poland, Sudetes Mts., Góry Kaczawskie range, Mysłów-Sobocin, Wapienna Góra Mt.	N50°59' E15°59'
Melica ciliata		
GER-C	Germany, Thuringia district, near Magdala	N50°54' E11°27'

2001b). It reaches the northern limit of its continuous geographical range in Poland, where it is a species of uplands and lower mountain sites. The distribution limit of *M. transsilvanica* extends from the Pieniny Mts. and the southeastern fringes of the Gorce Mts., crossing the southern part of the Kraków-Częstochowa Upland to the Sudetes Mts. (Szczęśniak, 2001; Zając and Zając, 2001a). It is a locally frequent species in the Pieniny Mts. and the Kraków-Częstochowa Upland (Grodzińska, 1976; Michalik, 1979; Zarzycki, 1981), but occurs at only 12 localities in the Sudetes and is considered a vulnerable species (Szczęśniak, 2001; Kącki et al., 2003). The ecological tolerance of *M. transsilvanica* is relatively broad. It grows in grasslands of the class *Festuco-Brometea*, both on dry, exposed outcrops in pioneer rupicolous grasslands and in xerothermic grasslands with a high participation of dicotyledonous perennials, as well as in xerothermophilous scrub formations, on basic or neutral soils formed on shale or limestone, sometimes rich in nitrogen compounds (Oberdorfer, 2001).

Recent studies have shown that the low level of genetic variation of *Melica transsilvanica* was due to the geographical isolation of populations and the absence or sporadicity of gene flow between populations, which preserve the dominant self-mating breeding system (Szczepaniak and Cieślak, 2006, 2007). This study examines the relationship between the patterns of genetic variation and the location of *M. transsilvanica* populations in the species distribution range. We hypothesized that genetic diversity and population genetic structure would be significantly lower in marginal populations than in those more centrally located in the species range. The amplified fragment length polymorphisms method (AFLPs; Vos et al., 1995) was used to test the hypothesis.

MATERIAL AND METHODS

PLANT MATERIAL

A total 97 plants were sampled from 15 natural populations of *Melica transsilvanica* in the European part of the species distribution, including Polish populations from the geographical range margin (Tab. 1). Plants were randomly collected across populations. Leaves were dried in the field in plastic bags with silica gel. Voucher specimens are deposited in the herbarium of the W. Szafer Institute of Botany, Polish Academy of Sciences in Cracow – KRAM. A few (5–8) plants from many populations were used in the analyses, since the level of intrapopulation variation was expected to be low between individuals of the *Melica ciliata* complex (including *M. transsilvanica* s. str.), based on the results of previous genetic studies of these species (c.f. Tyler, 2004; Szczepaniak and Cieślak, 2006, 2007).

DNA EXTRACTION AND AFLP FINGERPRINTING

DNA was isolated from ~20 mg dried leaves using the DNeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer's protocol. DNA quality and concentration were estimated from electrophoresed samples on 1% agarose gels stained with ethidium bromide, against a concentration gradient of λ-DNA.

AFLP analysis was performed according to the procedure described by Vos et al. (1995). PCR preselective amplification was performed using primers with single selective nucleotides: *Eco*RI+A and *Mse*I+C. Products were verified on 1.5% agarose gels and diluted 1:10 with sterile ddH_2O. Selective PCR employed primers with three selective nucleotides (*Eco*RI primers were fluorescence-labelled with FAM-6). After initial screening of 16 primer pair combinations, four combinations were selected: *Eco*RI-ACG/*Mse*I-CAG, *Eco*RI-AGA/*Mse*I-CGT, *Eco*RI-AAT/*Mse*I-CGC and *Eco*RI-ATC/*Mse*I-CAT. Products of selective amplification were separated on POP 4 polymer with GeneScan-500 [ROX] internal size standard on an ABI Prism 3100-*Avant* automated sequencer (Applied Biosystems, USA). Duplicates of three individuals double-collected from each population were analyzed to test the reproducibility of AFLP profiles (Bonin et al., 2004).

DATA ANALYSIS

Data were analyzed using GeneScan ver. 3.7 (Applied Biosystems) and further examined using GenoGrapher ver. 1.6.0 (Montana State University, 1999; http://hordeum.oscs.montana.edu/genographer). AFLP fragments were scored in the 50–500 bp range for the presence (1) or absence (0) of bands and assembled as a binary matrix. Only reproducible, well-separated and unambiguous AFLP bands were considered in further analyses. Unless otherwise specified, all analyses of genetic diversity and population genetic structure were performed using AFLP-SURV ver. 1.0 (Vekemans et al., 2002). This program estimates allelic frequencies at each marker locus assuming they are dominant and have only two alleles (a dominant marker allele coding for the presence of a band at a given position and a recessive null allele coding for the absence of the band).

Allelic frequencies at AFLP loci were computed from the observed frequencies of fragments using the Bayesian approach with non-uniform prior distribution of allele frequencies proposed by Zhivotovsky (1999) for diploid species, assuming some deviation from Hardy-Weinberg equilibrium. The frequency of the null allele at each locus is computed from two numbers: sample size, and the number of individuals in the sample that lack the AFLP fragment. It uses the Bayesian method, which also estimates the distribution of allele frequencies based on the variation of the frequencies of AFLP fragments over loci in the sample. This method is thought to give the most accurate and authentic results from dominant markers (Zhivotovsky, 1999; Bonin et al., 2007).

Based on these computations of allelic frequencies, genetic diversity and population genetic structure were estimated using the approach of Lynch and Milligan (1994). The following measures of genetic diversity were calculated for each population: the number (NPL) and proportion of polymorphic loci (PPL) at the 5% level, that is, loci with allelic frequencies lying within the 0.05–0.95 range, and Nei's gene diversity (= expected heterozygosity) (H_j). Additionally, the number of unique AFLP phenotypes (UPh), unique AFLP fragments (UL) and rare AFLP fragments (RL) with less than 20% frequency were found using ARLEQUIN 2.0 (Schneider et al., 2000). The significance of differences between the genetic diversity indices of central and marginal populations was tested by the nonparametric Mann-Whitney U test for two independent groups, performed with STATISTICA 8.0 (StatSoft, Inc., 1984–2007, Tulsa, OK, USA).

The genetic structure of a population was examined at different levels: for all populations together, between and within central and marginal populations using total gene diversity (H_t), mean gene diversity within populations (H_w), average gene diversity between populations (H_b), and Wright's fixation index (F_{ST}). Because no empirical information on the mating system of *Melica transsilvanica* was known, genetic variation was recalculated for different values (0.5, 0.9) of inbreeding coefficient F_{IS}. The effect of both the mixed mating system (F_{IS} = 0.5 in the calculations) and a high degree of selfing (F_{IS} = 0.9) was of little importance for the results (a difference of ~14%) and did not determine the overall outcome. Hence, we assumed deviation from Hardy-Weinberg genotypic proportions and a selfing rate of 0.9. This assumption was also based on a previous allozyme survey of *M. ciliata* s. l. (including *M. transsilvanica* s. str.) in which almost all studied populations showed a fewer multilocus genotypes than would be expected under free segregation and random mating (Tyler, 2004). The significance of genetic differentiation between populations in the above regional scheme was tested by comparing the observed F_{ST} with the distribution of F_{ST} under the hypothesis of no genetic structure obtained by means of 5000 random permutations of individuals between populations.

The assumption of genetic isolation by distance was verified with the Mantel test (Mantel, 1967) using MANTEL ver. 2.0 (Liedloff, 1999). The Mantel test was performed on a triangular matrix with pairwise geographical distances between populations and a triangular matrix with pairwise F_{ST} values dif-

ferences between populations. Geographical distances were measured as the approximate straight-line distances between populations using the option for distance measure between two locations from GPS. Genetic distances were calculated in AFLP-SURV ver. 1.0 (Vekemans et al., 2002) based on the F_{ST} pairwise differences between populations. For central and marginal populations, the scatter plot of F_{ST} against geographical distance was analyzed to infer the relative influence of gene flow and drift on the distribution of genetic diversity following Hutchison and Templeton (1999). Additionally, the distribution of total genetic variation between and within central and marginal populations of *Melica transsilvanica* was assessed in three-hierarchical AMOVA based on the nonparametric permutation approach and on pairwise squared Euclidean distances between AFLP phenotypes (Excoffier et al., 1992). AMOVAs were done with ARLEQUIN ver. 2.0 (Schneider et al., 2000).

Relationships between *Melica transsilvanica* populations were revealed by UPGMA analysis based on Nei's genetic distance measures (Nei, 1978), using TFPGA ver. 1.3 (Miller, 1997). Five individuals of *M. ciliata* L. from a natural population from Germany (GER-C) were used as an outgroup in the UPGMA (Tab. 1). Support for grouping was assessed by bootstrap analysis with 2000 replications (Felsenstein, 1985).

TABLE 2. Distribution of total and polymorphic AFLP fragments generated from four primer pairs in 97 specimens from 15 populations of *Melica transsilvanica*; NPL – number of polymorphic loci; PPL – proportion of polymorphic loci

Primer combination *Eco*RI.../*Mse*I...	Total	NPL	PPL (%)
E-ACG/M-CAG	80	41	51.25
E-AGA/M-CGT	46	24	52.17
E-AAT/M-CGC	39	20	51.28
E-ATC/M-CAT	67	39	58.21
Total	232	124	53.45

RESULTS

GENETIC DIVERSITY WITHIN POPULATIONS

Analysis of 97 individuals of *Melica transsilvanica* from 15 populations with four AFLP selective primer combinations generated 232 AFLP fragments ranging from 50 to 500 base pairs. The number of polymorphic fragments for each primer pair

TABLE 3. Genetic diversity within 15 populations of *Melica transsilvanica* based on 232 AFLP markers. Population abbreviations according to Table 1. *n* – number of individuals; NPL – number of polymorphic loci; PPL – proportion of polymorphic loci at 5% level; UPh – number of unique AFLP phenotypes; H_j – Nei's gene diversity (= expected heterozygosity); SE (H_j) – standard error of H_j

Populations	*n*	NPL	PPL	UPh	H_j	SE (H_j)
central						
RUS	7	4	1.73	3	0.0550	0.0028
ROM	8	4	1.73	4	0.0512	0.0031
SLO	8	11	4.76	7	0.0642	0.0046
AUS	8	11	4.76	3	0.0622	0.0035
CZE-1	5	3	1.30	2	0.0768	0.0035
CZE-2	5	12	5.19	3	0.0935	0.0050
Total	41	45	19.40	22		
Mean	6.83	7.50	3.24	3.67		
marginal						
PIE-1	5	3	1.30	4	0.0790	0.0039
PIE-2	5	1	0.43	2	0.0763	0.0034
PIE-3	5	7	3.03	5	0.0847	0.0049
KCU-1	5	2	0.87	2	0.0768	0.0032
KCU-2	5	5	2.16	4	0.0821	0.0038
SUD-1	8	5	2.16	5	0.0605	0.0045
SUD-2	7	3	1.30	4	0.0571	0.0033
SUD-3	8	13	5.63	8	0.0669	0.0052
SUD-4	8	10	4.33	6	0.0610	0.0042
Total	56	49	21.12	40		
Mean	6.22	5.44	2.36	4.44		

TABLE 4. Occurrence of unique (UL) and rare (RL) AFLP fragments in 15 populations of *Melica transsilvanica*. Eight unique AFLP fragments with frequency equal to 100% and 14 rare AFLP fragments with frequency less than 20% were obtained with the use of four selective primer pairs. Unique AFLP fragments are shaded. Population abbreviations according to Table 1

Primer pair *Eco*RI…/ *Mse*I…	AFLP fragments [bp]	Population														
		central						*marginal*								
		RUS	ROM	SLO	AUS	CZE-1	CZE-2	PIE-1	PIE-2	PIE-3	KCU-1	KCU-2	SUD-1	SUD-2	SUD-3	SUD-4
E-ACG/M-CAG	97.65			×				×		×						
	109.28	×					×									
	112.18		×		×										×	×
	188.71			×				×		×						
	208.04	×														
	279.95															×
E-AGA/M-CGT	155.46					×			×			×				
	199.64		×													
	324.94						×		×							
E-AAT/M-CGC	58.62			×				×		×						
	65.65				×				×	×						
	131.01		×								×	×				
	198.10	×	×													
	262.34	×	×	×						×	×				×	×
	318.26															×
	345.92	×														
	403.71		×													
E-ATC/M-CAT	226.83										×	×				
	230.44		×								×	×				
	282.59															×
	305.04		×		×											
	336.57												×			
Total	14 RL	3 RL	6 RL	4 RL	3 RL	1 RL	2 RL	3 RL	3 RL	5 RL	4 RL	4 RL	–	–	2 RL	2 RL
	8 UL	2 UL	2 UL	–	–	–	–	–	–	–	–	–	1 UL	–	–	3 UL

ranged from 20 (51.28%, for E-AAT/M-CGC) to 41 (51.25%, for E-ACG/M-CAG). Altogether, 124 (53.45%) polymorphic AFLP fragments were detected in the data set (Tab. 2).

Overall, AFLP genetic variation was low within all sampled populations of *Melica transsilvanica*. In populations from the central range the percentage of polymorphic loci (PPL) was very low and varied from 1.30 (3 AFLP fragments) in population CZE-1 to 5.19 (12 AFLP fragments) in CZE-2, with a population average of 3.24 (7.50 AFLP fragments). Peripheral populations had PPLs ranging from 0.43 (1 AFLP fragments) in PIE-2 to 5.63 (13 AFLP fragments) in SUD-3, with a slightly lower population average of 2.36 (5.44 AFLP fragments). Per-population Nei's gene diversity (H_j) (= expected heterozygosity), under a model assuming some deviation from Hardy-Weinberg genotypic proportions and a high level of self-fertilization, ranged from 0.0512 to 0.0935 (average H_w = 0.0671) within central populations and from 0.0571 to 0.0847 (average H_w = 0.0716) within marginal populations (Tab. 3). The intrapopulation genetic diversity indices characterized above did not significantly differ ($p > 0.05$) between the central and marginal populations (NPL: U = 19, p = 0.35; PPL: U = 19, p = 0.35; H_j: U = 20, p = 0.44).

Among the 97 samples, 62 unique AFLP phenotypes (UPh) were found, on average 3.67 per central and 4.44 per marginal population (Tab. 3). All AFLP phenotypes were unique to particular populations of *Melica transsilvanica*. However, the number of shared phenotypes between specimens was highest within the central CZE-1 and in the marginal KCU-1 and PIE-2 populations (2 phenotypes per 5 individuals). In contrast, all the individuals in only the marginal PIE-3 and SUD-3 populations had unique phenotypes.

Within two populations from the southeasternmost section of the range (RUS and ROM), two unique AFLP fragments (UL) per population were found. One and three unique AFLP markers were detected within Sudetan populations SUD-1 and SUD-4, respectively (Tab. 4). The majority of rare AFLP fragments (11 of 14) occurred in all populations of the central and marginal parts of the range. Accordingly, the central and marginal populations

TABLE 5. Genetic differentiation between populations based on 232 AFLP markers found in 97 individuals from 6 central and 9 marginal populations of *Melica transsilvanica*; N – number of populations; H_t – total gene diversity; H_w – average gene diversity within populations; H_b – average gene diversity between populations; SE – standard deviations; F_{ST} – Wright's fixation index, i.e. differentiation between populations; Lower 99% F_{ST} and Upper 99% F_{ST} – critical values at the 99% at the randomization distribution of F_{ST} assuming no genetic differentiation between populations, based on 5000 random permutations

Comparison	N	H_t	H_w	SE (H_w)	H_b	SE (H_b)	F_{ST}	Lower 99% F_{ST}	Upper 99% F_{ST}	p-value
All populations	15	0.1934	0.0698	0.0032	0.1236	0.0100	0.6399	–0.0385	0.0374	<0.0001
Between central and marginal populations	2	0.1675	0.1459	0.0023	0.0216	0.0000	0.1290	–0.0151	0.0332	<0.0001
Between central populations	6	0.1945	0.0671	0.0064	0.1274	0.0121	0.6557	–0.0672	0.0940	<0.0001
Between marginal populations	9	0.1906	0.0716	0.0034	0.1190	0.0000	0.6239	–0.0617	0.0696	<0.0001

TABLE 6. Results of three-hierarchical analysis of molecular variance (AMOVA) of central and marginal populations of *Melica transsilvanica*. The analysis is based on AFLP phenotypes consisting of 232 band states. Levels of significance are based on 1023 iteration steps

Level of variation	d.f.	Sum of squares	Variance components	Percentage of total variation	p
Between central and marginal populations	1	136.842	0.502	2.79	>0.277
Between populations within regions	13	1375.160	16.310	90.61	<0.001
Between individuals within populations	82	97.482	1.189	6.60	
Between central populations	5	581.303	16.963	93.18	<0.001
Between individuals within central populations	35	43.429	1.241	6.82	
Between marginal populations	8	793.857	15.863	93.24	<0.001
Between individuals within marginal populations	47	54.054	1.150	6.76	

were not significantly different in terms of the presence of rare AFLP fragments (RL: $U = 19$, $p = 0.35$).

POPULATION GENETIC STRUCTURE AND DIVERSITY BETWEEN POPULATIONS

We found highly significant genetic differentiation among the *Melica transsilvanica* populations studied here ($F_{ST} = 0.6399$, $p < 0.0001$; Tab. 5). Total gene diversity (H_t) was slightly higher in the central (0.1945) than in the marginal (0.1906) populations, but average within-population gene diversity (H_w) was slightly higher in marginal (0.0716) than in central (0.0671) ones. Genetic differentiation was significant in both groups and was slightly higher between central ($F_{ST} = 0.6557$) than between marginal ($F_{ST} = 0.6239$) populations ($p < 0.0001$, Tab. 5). Genetic differentiation was considerably lower between the central and marginal populations ($F_{ST} = 0.1290$, $p < 0.0001$).

Hierarchical AMOVA revealed that the variance components were significant, and partitioning of molecular variance was similar in the marginal and central populations. In both regions the vast majority of genetic differentiation, with almost the same values, was attributable to variation between populations within the central range (93.18%) and within the peripheries (93.24%) of the species range. The remaining small component of diversity, 6.82% and 6.76%, was among individuals within the central and marginal populations. Only 3% of genetic variation was between regions, but this value was not significant ($p > 0.277$, Tab. 6).

On the whole, the very high genetic distinctness of the *Melica transsilvanica* populations was also confirmed by comparisons of F_{ST} between population pairs. All pairwise F_{ST} values (except F_{ST} between populations KCU-1 and KCU-2) differed significantly at $p < 0.001$. The high F_{ST} between central populations ranged from 0.4763 to 0.7494, and between marginal populations from 0.2221 to 0.7355. In one case only, F_{ST} was zero between adjacent marginal populations KCU-1 and KCU-2, suggesting gene flow between them or else retention of ancestral polymorphisms following initial migration into the region. The Mantel test showed the isolation-by-distance relation between *M. transsilvanica* populations ($r = 0.46$, $p < 0.01$;

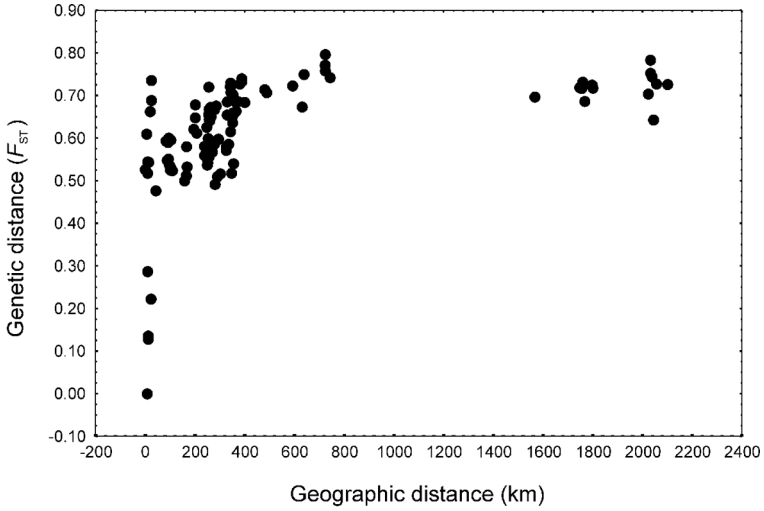

Fig. 1. Relationship between pairwise genetic differentiation (measured as F_{ST} distance) and geographic distance among 15 populations of *Melica transsilvanica*. The significant isolation-by-distance relation was displayed by the Mantel test ($r = 0.46$, $p < 0.01$).

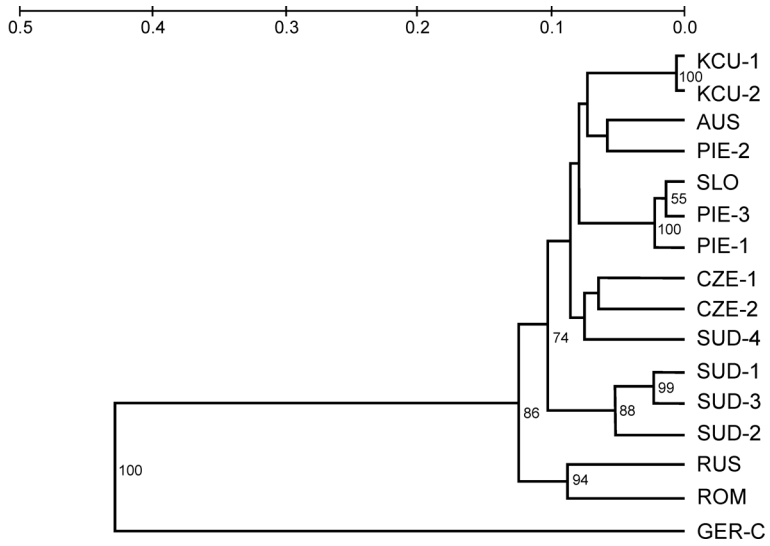

Fig. 2. UPGMA dendrogram of 15 populations of *Melica transsilvanica* and one population of *M. ciliata* (GER-C) as the outgroup, based on Nei's (1978) genetic distance and 232 AFLP markers, obtained with four primer combinations. Population labels correspond to abbreviations given in Table 1. Bootstrap values > 50% are marked on the tree and were assessed with 2000 replications.

Fig. 1). The scatter plot (Fig. 1) suggests a lack of regional equilibrium, and that gene flow is relatively more important at shorter distances while drift is more important at greater distances, in line with the theoretical relationships put forward by Hutchison and Templeton (1999). Alternatively, our results could suggest that the more recently established populations at the periphery retain their initial ancestral polymorphisms.

In cluster analysis the location of populations in the central or peripheral areas was not reflected (Fig. 2). No population formed a distinct group reflecting intraspecific genetic division, and the high bootstrap value (86%) supports high genetic similarity among populations from the whole distribution range. However, neighboring populations of *Melica transsilvanica* from specific regions in Poland (Pieniny Mts., Kraków-Częstochowa Upland, Sudetes Mts.) were clustered with high bootstrap support, which suggests possible gene flow or maintenance of early shared alleles following founder events (Fig. 2).

DISCUSSION

The obtained level of genetic variation within populations of *Melica transsilvanica* from the northern margin of the species distribution range in Poland was comparable to that in the central part of the range. Many factors influence levels of genetic variability of plants: historical population bottlenecks, past and current migrations, and genetic drift. We initially assumed that genetic variation in more isolated populations at the limit of the distribution range should be considerably less than in core populations. We did not find a significant effect of geographical location of *M. transsilvanica* populations on genetic differentiation. Average gene diversity over all AFLP fragments was very low in all analyzed populations (H_w = 0.0698, assuming F_{IS} = 0.9) and both in central (H_w = 0.0671) and marginal (H_w = 0.0716) populations. There are no empirical studies on the biology and mating system of *M. transsilvanica*; however, allozyme studies have revealed significant deviations from Hardy-Weinberg equilibrium, indicating selfing and/or facultative apomixis with high probability (Tyler, 2004). Our results are comparable to those reported for *Typha latifolia*, a predominantly selfing wetland plant (gene diversity ranging from 0.095 to 0.100, also derived from AFLP markers; Lamote et al., 2005). The genetic diversity of *M. transsilvanica* is lower than in other facultative self-pollinating grass species such as *Elymus caninus* (Diaz et al., 1999) or *Elymus alaskanus* (Sun et al., 2002), but comparable to the level found in obligate self-pollinators including *Bromus tectorum* (Bartlett et al., 2002; Ramakrishnan et al., 2004) and *Calamagrostis porteri* subsp. *insperata* (Esselman et al., 1999). The very low and comparable levels of polymorphism we found in central (21%) and marginal (19%) populations indicate that even populations that often occurred in small fragments at the range limit did not sustain strong genetic depletion. Partitioning of genetic variability with the prevailing component being between populations was characteristic of the central (93.18%) as well as the marginal (93.24%) populations, and is consistent with the life history traits of *M. transsilvanica*. Large reduction of genetic variability within populations and increased differentiation between populations have also been observed in other self-fertilizing grasses (Price et al., 1984; Larson et al., 2001). The non-random mating breeding system seems to be the main factor determining the genetic diversity and genetic structure of *M. transsilvanica* (Tyler, 2004; Szczepaniak and Cieślak, 2006, 2007).

In Central and Western Europe, *Melica transsilvanica* occurs mainly in secondary anthropogenic grasslands of the class *Festuco-Brometea*, originating from clearing of forests and maintained by grazing and mowing. Hence, habitats suitable for *M. transsilvanica* are fragmented in this part of the species distribution, which limits the current gene flow between populations (Tyler, 2004; Szczepaniak and Cieślak, 2007). Genetically distinct groups of core and peripheral populations were not reflected in UPGMA. On the other hand, high genetic similarity between populations of the entire species range was displayed with high bootstrap value support. On the small geographical scale, cluster analysis showed subclusters corresponding to populations from particular regions in Poland: the Pieniny Mts., Kraków-Częstochowa Upland and Sudetes Mts. These results are consistent with evidence from our previous studies demonstrating significant genetic differentiation between populations from the Kraków-Częstochowa Upland and the Pieniny Mts., based on the occurrence of unique and region-specific AFLP fragments (Szczepaniak and Cieślak, 2007). Hence, on the microgeographical scale of Poland there is spatial isolation between range-limit populations (Szczepaniak and Cieślak, 2007). Dominant self-fertilizing species with a fragmented landscape distribution tend to have strongly differentiated populations (Hamrick and Godt, 1990).

The relative influences of gene flow and random genetic drift on the formation of population structure both between and within regions can be assessed based on the significance of the association between genetic (F_{ST}) and geographical distances (Hutchison and Templeton, 1999). We found a significant correlation between these two distance matrices, suggesting that the migration rate is not high enough and that isolation by distance may substantially contribute to structuring, especially on a larger scale. Theoretical models have assumed equilibrium or disequilibrium between gene flow and genetic drift. A pattern of isolation by distance (Wright, 1931) requires that genetic drift and gene flow should be in equilibrium. However, the assumption of equilibrium conditions presumed in the isolation-by-distance model is often not met in natural populations. Expected patterns under disequilibrium conditions are affected by historical effects, that is, the period of time a region has been occupied, and contemporary effects such as the level of regional dispersal (Eckstein et al., 2006). In some pairwise comparisons between *Melica transsilvanica* populations, gene flow was more important than drift on a shorter scale (F_{ST} = 0), but this is not a rule. For example, populations 200 m apart were highly genetically distinct (F_{ST} = 0.53). Moreover, distinct sets of multilocus genotypes were found in most populations of *M. transsilvanica*, indicating a strong structure in the central (F_{ST} = 0.6557) and marginal (F_{ST} = 0.6239) populations ($p < 0.0001$). However, regional differences between the center

and periphery were considerably lower (F_{ST} = 0.1290). The strong structure of populations at the distribution limit, similar to that observed in central populations on a much larger scale, suggests that population bottlenecks in this selfing species, resulting in smaller effective population size, may be a sufficient explanation for the structuring between populations.

Geographical fragmentation of genetic variation is also connected with the occurrence of unique genetic markers. The easternmost populations of *Melica transsilvanica* formed a separate subcluster in cluster analysis (UPGMA) with a high bootstrap value, confirming their remarkable genetic distinctness. Populations at the range limit experienced a relatively high decline in population size, particularly conspicuous in the Sudetan populations (Szczęśniak, 2003). The SUD-4 population from the Góry Kaczawskie range is very small and consists of about ten tufts, representing morphologically differentiated individuals (Szczepaniak and Cieślak, 2006). Despite the population size, however, our results show the relatively high genetic distinctness of this population, with three unique AFLP fragments. Population SUD-1 from Góra Krzyżowa Mt. near Strzegom is formed by about 30 tufts and characterized by one unique marker. The genetic identity of populations depends on the time of isolation, when old bottlenecked populations can accumulate unique markers (Tribsch et al., 2002). Our results suggest that some small Sudetan populations have been isolated for a long period and are highly inbred, which results in their genetic distinctness. It has been shown that self-compatibility and moderate inbreeding depression can maintain a very small population for some time (Fischer and Matthies, 1997). Generally the populations of *M. transsilvanica* are characterized by isolated gene pools differing by a small number of loci regardless of the collection site within the species distribution range.

Past migration and the historical gene flow may have significantly influenced the current level of genetic diversity of *Melica transsilvanica*. This suggestion is supported by some rare AFLP markers distributed throughout the studied populations, from the eastern to the western part of the species distribution. *M. transsilvanica* is a species of steppe communities in the eastern part of its range (Hempel, 1970). The shared occurrence of rare AFLP markers between geographically often very distant populations may indicate more effective gene flow in the Holocene, when steppe communities occupied vast and continuous areas. The wide distribution of allozyme alleles in *Melica ciliata* s. l. (including *M. transsilvanica* s. str.) also supports this idea (Tyler, 2004). At the end of the Eocene, open grassland vegetation began to develop due to dry conditions in the Eurasian landscape, previously almost completely forested (Pott, 1995). Because of the disjunct distribution of xerothermic grassland vegetation in Western and Central Europe, the great distance between typical eastern Eurasian steppe and Western and Central European xerothermic grassland, and important climatic differences between these regions, it is now almost impossible for eastern steppe taxa to migrate to Central Europe (Bredenkamp et al., 2002). In the Holocene, especially during the hypsithermal 5000–6000 years ago when climates were considerably warmer and drier, many xerothermic grassland species expanded their ranges in Eastern and Central Europe. The disjunct distribution supports the idea that xerothermic grasslands in Western and Central Europe are postglacial relicts (Pott, 1995). These grasslands must be regarded as remnants of a once much larger territory connecting Western and Central Europe to the southern and eastern Eurasian regions during subarctic times (Pott, 1995).

It is conceivable that since the initial range expansion *Melica transsilvanica* has experienced dynamic "metapopulation" phases with intermittent establishment of populations through short-distance founder events and occasional population declines or local extirpations, with seed dispersal between intervening populations throughout that time interval. Although the species may be predominantly selfing, sporadic contributions to genetic diversity by random founder events over shorter distances on the landscape, and the effects of drift or local bottlenecks, would maintain diversity over much of the species range. The requirement of many rare species for periodic local disturbance (whether as natural regimes or human-mediated) may also exist for *M. transsilvanica* and may also play a role in gene flow through seed dispersal.

The northern range limit of *Melica transsilvanica* in Poland is connected with the latitudinal position of the Carpathian and Sudetan ranges and a similar belt-like arrangement of uplands in Poland. Consequently, the range limit of mountain and meridional species is located here. As a species representing the Sub-Irano-Turanian geographical element, *M. transsilvanica* is one of the warmest species of the flora that may have migrated to Poland as late as in the post-glacial period (Zając and Zając, 2002). It probably arrived here directly from the south across the Beskidy Mts. from Spisz through the gorge valleys of the Dunajec and Poprad Rivers up to the Sądecki region (Pawłowski, 1925). Cluster analysis revealed genetic relationships between the population from the Slovak Pieniny Mts. and adjacent populations from the Polish Pieniny Mts. On the other hand, *M. transsilvanica* may have arrived with xeric flora in the Sudetes Mts. from Central Europe through

the Moravian Gate, using the valley of the Oder River (Kwiatkowski, 2008).

Melica transsilvanica is a rare and potentially endangered species in Poland, scattered in restricted geographical areas and habitats (Zarzycki and Szeląg, 2006). The status and ecological properties of the species as well as threat factors must be established so that its genetic resources may be protected successfully. Threats to this species result mostly from threats to the xerothermic grassland communities of the class *Festuco-Brometea* where *M. transsilvanica* grows. The first stage in the rapid shrinking of populations is connected with the expansion of forest and shrub communities and those formed by tall perennials (Michalik, 1993). In the Pieniny Mts. and in the Kraków-Częstochowa Upland, *M. transsilvanica* populations are in relatively good condition, being located on outcrops of steep and inaccessible sandstone or limestone rocky hillsides that have never been forested and have preserved their natural xerothermic vegetation (our field observations). Sections of suitable habitats naturally resistant to succession can ensure the survival of a species (Booy et al., 2000). However, studies in Ojców National Park show a significant reduction in the number of *M. transsilvanica* localities over 20 years, as well as impoverishment of the existing populations (Michalik, 1993). *M. transsilvanica* is also a vulnerable species in the Sudetes Mts. due to habitat degradation and vegetation succession (Szczęśniak, 2003). There are relatively few areas with limestone substrate in the Sudetes Mts. (Góry Kaczawskie Mts., Masyw Śnieżnika range, Góry Złote Mts., Krowiarki and locally in the Góry Stołowe Mts. and Góry Bystrzyckie Mts.), and this means a smaller number of potential and natural species localities. Intensive exploitation of limestone in Lower Silesia in many quarries, most of them now closed, has altered the natural habitats. These factors weaken the condition of the Sudetan populations of *M. transsilvanica* by reducing population size. Even if the plant is not endangered within its entire distribution area, it has been rapidly declining locally in seminatural habitats in Europe, and species conservation should concentrate on managing succession by implementing moderate grazing or mowing and haymaking (see also Schmidt and Jensen, 2000). Peripheral populations of *M. transsilvanica*, in some cases genetically and morphologically divergent from core populations, can be important sites of future speciation events (Lesica and Allendorf, 1995; Szczepaniak and Cieślak, 2006). Indeed, marginal populations of *M. transsilvanica* have contributed significantly to genetic variation and contain unique markers; they are of great value for conservation of genetic diversity in this species.

CONCLUSIONS

Our study showed the absence of significant differences in genotypes and allele frequencies between populations of *Melica transsilvanica* from the central and marginal parts of the species range. The same pattern was seen in the partitioning of genetic diversity, with the majority of genetic variation occurring between populations within the central and within the marginal areas. Our observations of shared polymorphisms between distant locations indicates continuous input of migration and/or that the population was large at founding, with sharing of ancestral polymorphisms. The presence of an isolation-by-distance relation shows that gene flow is related to the geographical distance between populations. Low values of F_{ST} between some adjacent populations indicate a lack of regional equilibrium, where gene flow is more important at shorter distances than genetic drift (Hutchison and Templeton, 1999). However, landscape fragmentation, especially at greater distances, results in isolation of populations, and genetic drift plays an important role in shaping the level of genetic diversity of *M. transsilvanica*.

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