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Genetic variation and structure in natural populations of *Melica ciliata* and *M. transsilvanica* (Poaceae) as indicated by AFLP markers

Magdalena Szczepaniak & Elżbieta Cieślak

Department of Vascular Plants Systematics, W. Szafer Institute of Botany, Polish Academy of Sciences, Lubicz 46, 31-512 Kraków, Poland, e-mail: Magdalena.Szczepaniak@ib-pan.krakow.pl

Abstract: *Melica ciliata* L. and *M. transsilvanica* Schur are rare species in the Polish flora and reach here northern limit of their continuous range. A fluorescence-labeled Amplified Fragment Length Polymorphisms (AFLPs) DNA profiling method was used to measure genetic diversity and relationships between and within natural populations of *M. ciliata* and *M. transsilvanica* from central Europe. Analysis of molecular variance (AMOVA) revealed the significant genetic diversity (70.77%, $p<0.002$) between these species. At the intraspecific level, most genetic diversity resided between populations of both *M. ciliata* and *M. transsilvanica* (F_{ST}=0.84 and 0.94, respectively), that indicates a high level of inbreeding. The obtained low level of AFLP variation and the high F_{ST} values suggest the weakness or absence of gene flow among populations of these species, and maintenance the separate, local gene pool in each population. Fragmented landscapes, the putative self-pollinated breeding system and genetic drift, particularly in small size populations, may perform an important role in shaping the genetic diversity of the populations and the genetic structure of *M. ciliata* and *M. transsilvanica*.

Key words: *Melica ciliata*, *Melica transsilvanica*, Poaceae, genetic variation, population structure, AFLP, habitat fragmentation

1. Introduction

Melica ciliata L., in which four subspecies are distinguished (Tutin 1980), is the most polymorphic species of the genus *Melica*. This species is included in a group of the Sub-Mediterranean geographical element, and occurs mainly in western, south and central Europe, with disjunct area in the Scandinavian Peninsula and in North Africa. Single locations are reported from the Middle East (Meusel *et al*. 1965; Hultén & Fries 1986).

Melica transsilvanica Schur is closely related to *M. ciliata* and sometimes treated as its subspecies (Hempel 1970; Beldie 1979; Tyler 2004). This species represents Irano-Turanian geographical element and ranges from the western Europe through the central Europe and the Middle East to central Asia (Hultén & Fries 1986).

Both *Melica ciliata* and *M. transsilvanica* are rare species in Poland, where they reach northern limit of their continuous geographic range. *M. ciliata* is classified here among the critically endangered plants and is included in the *Polish Red Data Book of Plants* (Szczęśniak 2001a). Populations of *M. ciliata* consist from several to tens of tufts and they grow only in the Sudety Mts. and their foothills (Przedgórze Sudeckie), but the number and the size of these populations are regularly decreasing (Kwiatkowski 1997; Szczęśniak 2001b). Currently, only three localities of *M. ciliata* are known and confirmed from the Sudety Mts. (Szczęśniak 2001a, 2001b). It is the characteristic species of the rupicolous calcareous grasslands of the *Festucion pallentis* alliance, and also occurring in pioneer rupicolous grasslands of the *Alysso-Sedion* alliance. Competitive weakness and transformations of the species' habitat – xerothermic communities from the *Festuco-Brometea* class – are the main factors causing disappearing of this plant (Kwiatkowski 1997; Szczęśniak 2001a, 2001b).

Melica transsilvanica is a locally frequent species in Poland: in the Pieniny Mts. and in the Kraków-

Częstochowa Upland (Grodzińska 1975; Zając & Zając 2001), whereas in the Sudety Mts. it is a rare species, which occurs only in several localities (Szczęśniak 2001b). The number of tufts in particular *M. transsilvanica* populations is very diverse: from several in the mountain localities to several thousand in the lower locations (Szczęśniak 2001b). *M. transsilvanica* displays wider ecological tolerance than *M. ciliata*; it grows in pioneer rupicolous grasslands of *Seslerio-Festucion duriusculae* alliance, and also in xerothermic grasslands of *Cirsio-Brachypodion pinnati* alliance of the *Festuco-Brometea* class, as well as in thermophilous thickets from *Berberidion* alliance.

Phylogenetic and taxonomic relationships of *Melica ciliata* and *M. transsilvanica* are problematic and up to the present not fully elucidated (Hempel 1970; Tutin 1980; Tyler 2004). Tyler (2004) treated the both species and related taxa as *M. ciliata* s. lat. Studied species have similar ranges of distribution (Meusel *et al.* 1965) and habitat requirements. They were sometimes taxonomically misclassified, because of the similarity of diagnostic characters of species and the existence of a hybrid taxon, *M. ×thuringiaca* Rauschert (Rauschert 1963).

The main purpose of the present study was an assessment of the genetic variation of *Melica ciliata* and *M. transsilvanica* on intra- and interpopulation level and establishing the spatial relations of this variability within particular populations, taking into account the degree of isolation of the neighbouring populations.

2. Material and methods

Young, fresh leaves were collected from 2-15 randomly chosen individuals (188 altogether) from tufts about 5-6 m distant one from another, derived from 12 Polish and 6 central European natural populations of *Melica ciliata* s. lat. (Table 1). Leaves were harvested and immediately, in the field, dried in polyethylene bags with silica gel and stored in them prior to DNA extraction.

Table 1. Sample sizes (N) and locations of *Melica ciliata* s. lat. accessions analyzed for AFLP markers

Populations	N	Locations	Position	Altitude a. s. l.
T-1	12	Poland, Kraków-Częstochowa Upland, Dolina Bolechowicka reserve, Bolechowice, Wąwóz Bolechowicki ravine	N50°07' E19°46'	400-450 m
T-2	10	Poland, Kraków-Częstochowa Upland, Dolina Szklarki reserve, Szklary, Słoneczne Skały rocks	N50°11' E19°43'	400-450 m
T-3	12	Poland, Pieniny Mts., Pieniński Pas Skałkowy (Pieniny klippen belt), mount Obłazowa	N49°25' E20°07'	670 m
T-4	12	Poland, Pieniny Mts., Małe Pieniny range, Biała Woda reserve	N49°23' E20°35'	ca 800-850 m
T-5	10	Poland, Pieniny Mts., Małe Pieniny range, Szczawnica, mount Góra Szafranówka	N49°25' E20°28'	743 m
T-6	15	Poland, Sudety Mts., Przedgórze Sudeckie foothills, Wzgórza Strzegomskie hills, Strzegom, mount Góra Krzyżowa	N50°59' E16°20'	354 m
T-7	2	Poland, Sudety Mts., Przedgórze Sudeckie foothills, Wzgórza Strzegomskie hills, Strzegom, mount Góra Św. Jerzego	N50°59' E16°20'	ca 350 m
T-8	15	Poland, Sudety Mts., Pogórze Bolkowsko-Wałbrzyskie foothills, Dobromierz, mount Góra Dębowa	N50°55' E16°15'	400 m
T-9	3	Poland, Sudety Mts., Góry Kaczawskie range, Mysłów-Sobocin, mount Wapienna Góra	N50°59' E15°59'	ca 600 m
C-1	7	Poland, Sudety Mts., Góry Kaczawskie range, Mysłów, mount Góra Grodzik	N50°59' E15°59'	615 m
C-2	6	Poland, Sudety Mts., Przedgórze Paczkowskie foothills, Ożary	N50°30' E16°50'	270 m
C-3	15	Poland, Sudety Mts., Obniżenie Noworudzkie depression, Nowa Ruda-Dzikowiec	N50°34' E16°35'	530 m
TSK-1	11	Slovakia, Pieniny Mts., Červený Kláštor, on the Dunajec river	N49°23' E20°25'	500-550 m
TAU	10	Austria, Niederösterreich, Waldviertel, Umlaufberg hill	N48°43' E15°50'	330-370 m
TRO-1	12	Romania, near Cluj-Napoca, Cojocna	N46°45' E23°50'	
CDE-1	12	Germany, Thuringia district, near Magdala	N50°54' E11°27'	
CSL-1	12	Slovenia, Ljubljana, Topol, mount Polhograjska Grmada	N46°05' E14°20'	898 m
CHR-1	12	Croatia, the Krk island, Silo	N45°12' E14°35'	
Mean	10.4			
Total	188			

2.1. DNA extraction and AFLP analyses

Total genomic DNA was extracted from 20 mg dried leaf tissue following DNeasy Plant Mini Kit protocol of the manufacturer (Qiagen).

Genetic variation and population structure were assessed using Amplified Fragment Length Polymorphism (AFLP) method (Vos *et al.* 1995 with minor modifications). Genomic DNA was digested with two restriction enzymes – *Eco*RI and *Mse*I (New England Biolabs, Inc.). The resulting fragments were ligated to end-specific *Eco*RI and *Mse*I with double standard adaptors. The PCR preselective amplification was performed using primers with a single selective nucleotide: *Eco*RI+A and *Mse*I+C. The final selective amplification was performed using *Eco*RI and *Mse*I primers with two pairs of selective nucleotides, EAAT/MCAC and EAGA/MCTG, which had revealed the largest number of polymorphic, well-separated and reproducible bands. The *Eco*RI primers in this reaction were fluorescence-labeled. The amplified restriction products were separated electrophoretically on the ABI Prism automated sequencer together with the internal size standard GeneScan 500 Rox (Applied Biosystems).

2.2. Data analyses

Raw data were collected and aligned with the size standard using GeneScan Analysis Software (ver. 3.7, Applied Biosystems). Peaks (AFLP fragments) were scored in range of 50-500 bp and assembled as a binary (present – 1 and absent – 0) matrix for further data analysis.

Genetic diversity within each population was evaluated as the number of polymorphic loci, the percentage of polymorphic loci, the number of AFLP phenotypes, and the genetic similarities between individuals within population (Nei & Li 1979). Genetic parameters were computed using POPGENE ver. 1.31 (Yeh *et al.* 1997).

Similarities between all pairs of AFLP phenotypes, showing genetic relationships between individuals, populations and species, were estimated through the application of the Nei and Li's similarity coefficients (Nei & Li 1979) with neighbour-joining (NJ) method. Analysis of molecular variance (AMOVA), based on nonparametric permutation approach and on pairwise squared Euclidean distances between AFLP phenotypes (Excoffier *et al.* 1992), was performed in ARLEQUIN ver. 2.0 (Schneider *et al.* 2000) to estimate the partition of the total genetic variation at each hierarchical level. The variance components were used to calculate the fixation indices (F_{ST}) and their significance at each level of hierarchical genetic structure.

3. Results and discussion

3.1. Genetic variation of populations

The two pairs of AFLP primers, EAAT/MCAC and EAGA/MCTG, generated a total of 127 amplification AFLP fragments, of which 83 (65.35%) were polymorphic between studied populations of *Melica ciliata* and *M. transsilvanica*.

The genetic diversity was generally low and slightly varied between and within species. A total of 42 (33.07%) polymorphic loci in *Melica transsilvanica* were detected. Within populations of *M. transsilvanica* the largest number of polymorphic AFLP fragments and the AFLP phenotypes (7 i.e. 5.51% and 7, respectively)

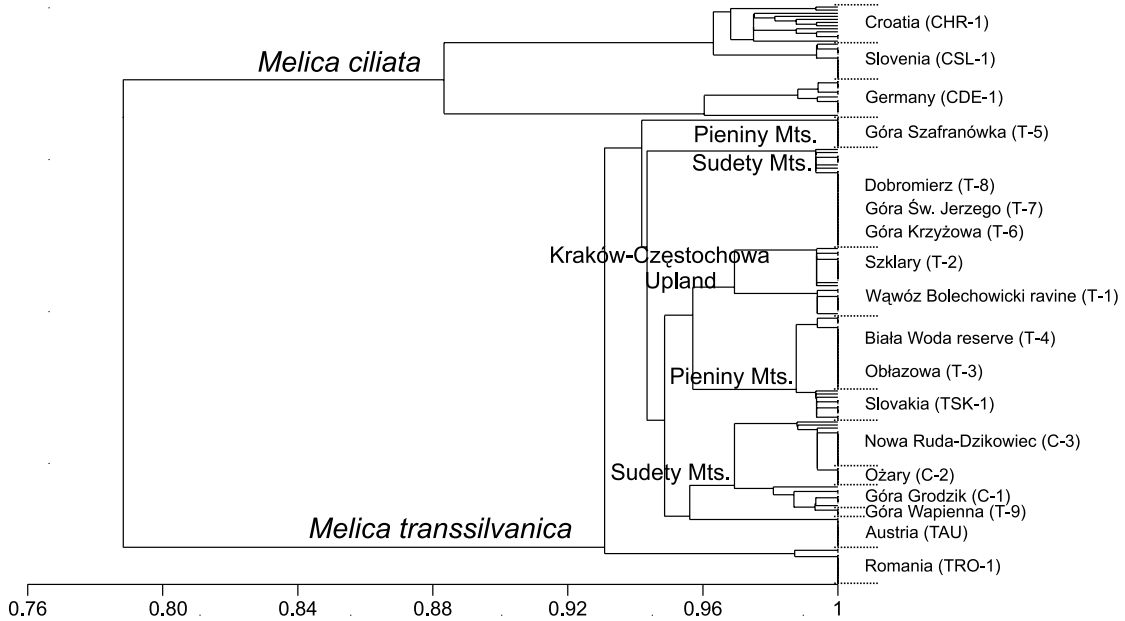

Fig. 1. Neighbour-joining (NJ) dendrogram of *Melica transsilvanica* and *M. ciliata* populations, based on Nei and Li's similarity coefficients (Nei & Li 1979) for 127 AFLP markers, obtained with two primer combinations: EAAT/MCAC and EAGA/MCTG. For abbreviations of particular populations see Table 1

were found in TSK-1 population from the Pieniny Mts., and the analogical parameters equalled zero in TAU, T-5, T-7 and T-9 populations (Table 1), that show their intrapopulational genetic homogeneity. On average, populations of *M. transsilvanica* were characterized by 2.17 (3.41%) of polymorphic AFLP bands and 2.67 multilocus phenotypes per population.

The number of polymorphic loci (41 i.e. 32.28%), found in *Melica ciliata*, was similar to that of *M. transsilvanica*. However, *M. ciliata* displayed slightly higher level of intrapopulational genetic diversity than *M. transsilvanica*. With use of 127 AFLP markers, from 3 to 11 multilocus phenotypes (on average 8.33) were detected within *M. ciliata* populations. Population CHR-1, characterized by 13 polymorphic AFLP loci and 11 phenotypes, was the most variable population within the species (Fig. 1; Table 1). The allozyme variation within *M. ciliata* complex (including *M. transsilvanica*) found in a previous study (Tyler 2004), was somewhat lower than AFLP variation that might be expected with this method; the mean number of allozyme multilocus genotypes within populations found by that author was 3.30 and genetic diversity between populations was 53.00%.

3.2. Genetic structure of populations

Genetic relationships among *Melica ciliata* and *M. transsilvanica* population samples based on the Nei and Li similarity matrix showed the NJ grouping of almost all individuals within their own populations and the clustering of populations within respective species (Fig. 1). In the AMOVA, based on the grouping of populations obtained in the NJ, the significant genetic diversity (70.77%, $p<0.002$) between *M. ciliata* and *M. transsilvanica* was confirmed (Table 2).

The genetic variation, residing among populations of both species *Melica ciliata* and *M. transsilvanica*, was on a similar level and equalled 83.95% and 94.17%, respectively (Table 2). The high values of fixation indices (F_{ST}= 0.84 and 0.94; Table 2), which measure inbreeding in relation to the expected genetic variation under random mating in the total population (Keller & Waller 2002), suggest the non-random mating and fixation of unique alleles in particular populations. These populations are more or less isolated by geographical location or fragmentation of suitable habitats (Szczepaniak & Cieślak 2007, in press). Within most of the examined populations of both studied species it was found that some individuals are genetically identical and the coefficients of genetic similarity are very high (0.96-1), which exhibits the high level of inbreeding. The high F_{ST} values can also suggest the weakness or absence of the gene flow among populations. Very important in the interpretation of the F_{ST} value is the knowledge of mating system of particular taxa (Szczepaniak & Cieślak 2007, in press). The recent studies using allozyme and genomic loci have revealed that related species of *M. ciliata* s. lat. with large probability are selfing and facultatively apomictic (Tyler 2004; Szczepaniak & Cieślak 2007, in press).

In NJ grouping within *Melica transsilvanica* a subclaster was distinguished with populations from the Sudety Mts. (C-1, C-2 and C-3; Fig. 1; Table 1). These population samples were collected in localities from which occurrence of *M. ciliata* was reported (Szczęśniak 2001b; Table 1). Preliminary morphological analysis, conducted before genetic analyses, displayed conspicuous morphological variation between individuals in these populations and suggested their putative hybrid origin (see also Szczęśniak 2001b, 2003) or wide range of genetic variation, however within *M. transsilvanica* but not within *M. ciliata*. In this preliminary genetic study it was found that the studied Polish populations shared no AFLP markers with other central European populations of *M. ciliata*. Detailed morphological and genetic analyses will be continued and, as the authors believe,

Table 2. Summary results of the analysis of molecular variance (AMOVA) of *Melica ciliata* and *M. transsilvanica*

Level of variation	d.f.	Variance components	Percentage of variation	Fixation index	*p*
M. ciliata versus *M. transsilvanica*					
among species	1	16.460	70.77	F_{CT} =0.708	<0.002
among populations within species	13	6.183	26.59	F_{SC} =0. 909	<0.001
within populations	145	0.615	2.64	F_{ST} = 0.973	NS
total	159	23.258	100.00		
M. transsilvanica					
among populations	11	5.870	94.17	F_{ST} =0.942	<0.001
within populations	112	0.363	5.83		
total	123	6.233	100.00		
M. ciliata					
among populations	2	7.690	83.95	F_{ST} = 0.839	<0.001
within populations	33	1.470	16.05		
total	35	9.160	100.00		

Explanations: Plants represented 12 populations of *Melica transsilvanica* and 3 populations of *M. ciliata* (taxonomically uncertain populations C-1, C-2 and C-3 were excluded from computations). The analysis is based on AFLP phenotypes consisting of 127 band states. Levels of significance are based on 1023 iteration steps

will permit to define the taxonomic status of the 'problematic' populations from the Sudety Mts.

Populations of *Melica ciliata* and *M. transsilvanica* are generally characterized by the low level of AFLP genetic variation. Fragmented landscapes, the non-random breeding system and genetic drift, particularly in small size populations, may play an important role in shaping the genetic diversity of the populations and the genetic structure of these species. Suitable habitats for *M. ciliata* s. lat. is strongly fragmented and geographic isolation occurs between populations from the Pieniny Mts., southern part of the Kraków-Częstochowa Upland and the Sudety Mts. Also in other part of Europe the collective species has scattered locations in the northern and western parts of its distribution, what limits the gene flow between populations (Tyler 2004; Szczepaniak & Cieślak 2007, in press). Concurrently, the Polish populations of *M. ciliata* s. lat. displayed very low genetic variation, probably due to a founder effect and the border effects that are connected with the location of populations on the edge of the species range (Mitka 1997).

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