Systematic relationships among four taxa of genus *Melica* (*Poaceae*) in Bulgaria: isoenzymatic survey

Georgi. B. Angelov

Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., bl. 23, 1113 Sofia, Bulgaria, e-mail: gbangv@bio.bas.bg

Received: January 28, 2015 ▷ Accepted: May 8, 2015

Abstract. Polyacrylamide gel electrophoresis (PAGE) was employed to examine the variation of six isoenzymes, in an attempt to clarify the systematic relationships among *Melica* transsilvanica, *M. ciliata*, *M. nutans*, and *M. uniflora*. Their affinities were assessed by calculating the coefficient of divergence D. Averaged over the six surveyed enzymes, the mean values of coefficient D demonstrated that the species *M. uniflora* was equidistantly and far positioned from the species pairs *M. ciliata* (D = 0.56) and *M. transsilvanica* (D = 0.57), while the last two species were much close to each other (D = 0.33), as compared to *M. uniflora*. On the other hand, due to the number of species-specific isoforms, the species *M. transsilvanica* seems to be a different genetic entity, as compared to *M. ciliata*. It was concluded that *M. transilvanica* and *M. ciliata* were closely related but genetically different entities within genus *Melica*. These two species were almost equidistantly and far positioned from both *M. uniflora* and *M. nutans*. Results of the present study are in concordance with the main morphological features of the examined taxa of genus *Melica*.

Key words: isoenzymes, *Melica*, PAGE, systematic relationships

Introduction

The present study includes *Melica ciliata* L., *M. transsilvanica* Schur., *M. nutans* L., and *M. uniflora* Retz. The *Melica ciliata–M. transsilvanica* complex encompasses a group of Sub-Mediterranean-Continental species (Tutin 1980). These taxa are caespitose perennials, diploids with chromosome number 2n = 18. *M. transsilvanica* is distinguished from *M. ciliata* by a set of several characters, including dense inflorescence, flatter leaves, very unequal glumes, and pubescent lower leaf-sheaths. However, the intricate morphological variability and overlapping traits make the *Melica ciliata–M. transsilvanica* complex a taxonomically problematic group (Hempel 1970).

Melica uniflora is a rhizomatous, perennial and diploid (2n = 18) grass. Its spikelets are 3–7 mm long, erect, with one fertile floret. It occurs mainly in shady places. Its range of distribution in Europe goes northwards to Scotland, and eastwards to Moldavia (Tutin 1980).

The species *Melica nutans* is shortly-rhizomatous, long-living perennial grass, diploid, with chromosome number 2n = 18. Its spikelets are 6–8 mm long, eventually nodding, with 2–3 fertile florets, falling together when ripe. This species occupies shady and often rocky places. It is distributed in most of Europe, but seldom in the Mediterranean region and its islands (Tutin 1980).

Phylogenetic and taxonomical relationships between the taxa belonging to the *Melica ciliata–M. transsilvanica* complex have not been exhaustively explained and verified yet (Hempel 1970). A monographic revision of the genus *Melica* in Eurasia based mainly on morphology and anatomy was done by Hempel (2011). According to his taxonomic treatment, *M. ciliata and M. transsilvanica* are included in the section *Dalycum,* whereas *M. nutans* and *M. uni-*

flora belong to the sections *Melica* and *Husnotchloa*, respectively. The level of genetic variation and structure in natural populations of *M. ciliata* and *M. transsilvanica* were studied by DNA markers (Szczepaniak & Cieślak 2006). The genetic and morphological differentiation between *M. ciliata* and *M. transsilvanica* were also studied (Szczepaniak & Cieślak 2011).

Studies of isoenzyme variation and genetic structure of the natural populations in other *Melica* species are rather scarce. Large-scale geographic patterns in the widespread Eurasian woodland grass *M. nutans* were studied by sets of isoenzyme systems (Tyler 2002a,b; 2004). Electrophoretic patterns of six isoenzymes in *M. uniflora* were also examined, in order to reveal its isoenzyme variation (Angelov 2012). An isophenotype was found to be dominant in each of the surveyed enzymes and the third isophenotype was rare. A substantial portion of isoforms occurred across all studied individuals of *M. uniflora* and were monomorphically fixed – an indication of the low level of genetic variation.

The main purpose of our study was to reveal the isoenzyme population variability, in order to assess the systematic relationships among the above-mentioned *Melica* species.

Material and methods

Two natural populations of each *Melica ciliata* (Mt Lozenska, Lozen, N 42°35'/E 23°29; Mt Sredna Gora, Petrich, N 42°36'/E 24°09'), *M. transsilvanica* (Danubian Hilly Plain: Tarnene, N 43°23'/E 24°30'; Brest-Gulyantsi, N 43°37'/E 24°37'), *M. nutans* (Pirin Mts, Banderitsa, N 41°46'/E 23°25'; Mt Lozenska, Dolni Pasarel, N 42°32'/E 23°29'), and *M. uniflora* (Mt Vitosha, Knyazhevo, N 42°39'/E 23°14'; Mt Lyulin, Klisura, N 42°43'/E 23°15') were individually studied. The enzymes cathodal esterase (EST), glutamate oxaloacetate transaminase (GOT), malate dehydrogenase (MDH), NADP-malate dehydrogenase (NADP-MDH), glucose-6-phosphate dehydrogenase (G-6-PDH), and anodal peroxidase (PER) were analyzed. Leaves were grinded in 0.01 M Tris, 0.08 M glycine, 0.005 M cysteine, 20 % sucrose, pH 8.3. Anodally migrating isoforms were resolved on 7.5 % polyacrilamide slab gels as described by Davis (1964). The cathodal EST was run on 7.5 % polyacrylamide slab gels (Reisfeld & al. 1962). The length of gels was 7.5 cm for GOT, MDH, NADP-MDH, G-6-PDH; 6.25 cm for anodal PER; and 6 cm for cathodal EST. The following staining recipes were used: PER, GOT (Przybylska & al. 1982), EST (Schmidt-Stohn & Wehling 1983), G-6-PDH, MDH and NADP-MDH (Shaw & Prasad 1970).

Systematic relationships among the above-mentioned taxa of genus *Melica* were assessed by calculating the coefficient of divergence D (Stuessy 1990), according to the following formula:

$$D = \sqrt{\frac{1}{N}\sum_{i=1}^{N}\left(x_{ij} - x_{ik}\right)^2}$$

where, N is the number of isoforms for each enzyme, and x_{ij} and x_{ik} are the mean frequencies of i^{th} isoform in taxa j and k.

Results and discussion

Cathodal esterase. A total of eleven isoforms of the enzyme were electrophoretically resolved in *Melica transsilvanica, M. ciliata, M. nutans,* and *M. uniflora* (Table 1). Isoforms 16, 22 and 27 were shared by all examined species. Isoforms 19, 31 and 33 were common for *M. transsilvanica* and *M. ciliata.* Isoform 20 was species-specific for *M. uniflora,* while isoform 44 was found in *M. transsilvanica* only. Pairwise comparisons among *M. ciliata*, *M. transsilvanica* and *M. uniflora* resulted in values of coefficient D equal to 0.33 and 0.45, respectively. The value of comparison be-

Table 1. Mean isoform frequencies of cathodal esterase in the studied populations of *Melica transsilvanica, M. ciliata, M. nutans,* **and** *M. uniflora.*

Species	Mean isoform frequencies										
	16	19	20	22	24	27	31	33	35	44	47
M. transsilvanica	0.50	0.12	0.00	1.00	0.12	0.87	0.50	0.05	0.62	0.12	0.38
M. ciliata	0.71	0.75	0.00	0.23	0.08	0.75	0.75	0.12	0.00	0.00	0.04
M. nutans	0.75	0.00	0.24	0.88	0.00	0.62	0.00	0.00	0.00	0.00	0.08
M. uniflora	0.40	0.00	0.60	0.71	0.00	0.10	0.00	0.00	0.06	0.00	0.00

tween *M. ciliata* and *M. transsilvanica* was 0.41. The value of comparison between *M. uniflora* and *M. nutans* was much lower and equalled 0.16.

Glutamate oxaloacetate transaminase. Eight isoforms of the enzyme were found in the studied populations of *Melica* species (Table 2). Two monomorphically-fixed isoforms (24 and 44) were characteristic of *M. ciliata* only. Isoform 39 was invariant across the studied group of species. Beside isoform 39, *M. uniflora* was monomorphic in respect to isoforms 21 and 49. Isoform 46 was species-specific for *M. transsilvanica*. *Melica transsilvanica* and *M. ciliata* proved to be very closely related (D = 0.16), while *M. uniflora* was nearly equidistant from *M. ciliata* (D = 0.76) and *M. transsilvanica,* with coefficient D equal to 0.65. The value of coefficient D in comparison between *M. uniflora* and *M. nutans* was equal to 0.16.

Table 2. Mean isoform frequencies of glutamate oxaloacetate transaminase in the studied populations of *Melica transsilvanica, M. ciliata, M. nutans,* **and** *M. uniflora.*

Species	Mean isoform frequencies							
	15	18	21	24	39	44	46	49
M. transilvanica	0.00	0.25	0.25	1.00	1.00	0.87	0.13	0.00
M. ciliata	0.00	0.00	0.00	1.00	1.00	1.00	0.00	0.00
M. nutans	0.44	0.56	0.92	0.64	1.00	0.00	0.00	0.78
M. uniflora	0.06	0.06	1.00	0.00	1.00	0.00	0.00	1.00

Malate dehydrogenase. Seven isoforms of malate dehydrogenase were detected in *Melica transsilvanica, M. ciliata, M. nutans,* and *M. uniflora* (Table 3). Two monomorphically-fixed isoforms (34, 41) were common for the entire species group. An invariant isoform (29) was species-specific for *M. uniflora.* The latter species was almost equidistant from both *M. transsilvanica* (D = 0.62) and *M. ciliata* (D = 0.67), whereas the value of coefficient D was 0.46, when the latter two species were compared. The value of coefficient D when compared between *M. uniflora* and *M. nutans* equalled 0.20.

Table 3. Mean isoform frequencies of malate dehydrogenase in the studied populations of *Melica transsilvanica, M. ciliata, M. nutans,* **and** *M. uniflora.*

Species	Mean isoform frequencies						
	17	22	25	29	31	34	41
M. transsilvanica	0.63	0.00	0.00	0.00	0.75	1.00	1.00
M. ciliata	0.05	0.00	0.00	0.00	0.05	1.00	1.00
M. nutans	0.00	0.10	0.16	0.00	0.70	1.00	1.00
M. uniflora	0.00	0.50	1.00	1.00	1.00	1.00	1.00

NADP-malate dehydrogenase. A total of eleven isoforms of the enzyme were electrophoretically resolved in the populations of studied *Melica* taxa (Table 4). Isoform 12 was shared by all studied species. Isoform 19 was common for *M. ciliata* and *M. nutans*, while isoform 41 was shared by the latter species and *M. uniflora.* Two isoforms (17, 29) were characteristic for *M. uniflora.* The species *M. uniflora* was equidistant from both *Melica transsilvanica* (D = 0.49) and *M. ciliata* (D = 0.50), whereas the respective value of the comparison between the two latter species equalled 0.26. A pairwise comparison between *M. uniflora* and *M. nutans* resulted in coefficient D value of 0.21.

Table 4. Mean isoform frequencies of NADP-malate dehydrogenase in the studied populations of *Melica transsilvanica, M. ciliata, M. nutans,* **and** *M. uniflora.*

Species	Mean isoform frequencies				
	12	17	19	29	41
M. transsilvanica	0.75	0.00	0.00	0.00	0.00
M. ciliata	0.67	0.00	0.33	0.00	0.00
M. nutans	0.77	0.00	0.42	0.00	0.12
M. uniflora	0.31	0.68	0.00	0.47	0.16

Glucose-6-phosphate dehydrogenase. Altogether, five isoforms of the enzyme were detected in *Melica transsilvanica, M. ciliata, M. nutans,* and *M. uniflora* (Table 5). Isoforms 22 and 26 were common for the entire studied group. Isoforms 18 and 30 occurred with different frequency in most of the studied taxa. Isoform 33 was characteristic for *M. uniflora.* Pairwise comparisons of *M. ciliata* with *M. transsilvanica* and *M. uniflora* resulted in values of coefficient D equal to 0.45 and 0.65, respectively. The value from the comparison between *M. ciliata* and *M. transsilvanica* was 0.28. The value of coefficient D in a comparison between *M. uniflora* and *M. nutans* was lower and equal to 0.21.

Table 5. Mean isoform frequencies of glucose-6-phosphate dehydrogenase in the studied populations of *Melica transsilvanica, M. ciliata, M. nutans,* **and** *M. uniflora.*

Species	Mean isoform frequencies				
	18	22	26	30	33
M. transsilvanica	0.75	1.00	0.87	0.12	0.00
M. ciliata	0.32	0.95	0.53	0.00	0.00
M. nutans	0.22	0.85	0.23	0.12	0.00
M. uniflora	0.00	0.12	0.44	0.57	0.57

Anodal peroxidase. A total of sixteen isoforms of anodal peroxidase were detected in the examined species group of genus *Melica* (Table 6). Most isoforms were

Table 6. Mean isoform frequencies of anodal peroxidase in the studied populations of *Melica transsilvanica, M. ciliata, M. nutans,* and *M. uniflora.*

Species	Mean isoform frequencies															
	11	12	14	16	17	19	22	25	30	32	34	36	37	38	43	47
M. transilvanica	0.87	0.00	0.25	0.14	0.75	0.12	0.75	0.12	0.12	0.62	0.00	0.00	0.12	0.37	0.00	0.00
M. ciliata	0.00	0.03	0.00	0.00	0.03	0.03	0.00	0.00	0.00	0.79	0.24	0.50	0.00	0.00	0.79	0.58
M. nutans	0.00	0.08	0.12	0.28	0.00	0.00	0.48	0.22	0.00	0.12	0.00	0.00	0.00	0.18	0.08	0.06
M. uniflora	0.00	0.93	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.42	0.58	1.00

shared by all studied taxa. Three isoforms, namely, 11, 30 and 37 were unique for *M. transsilvanica,* whereas isoform 36 was observed in the studied populations of *M. ciliata* only. The values of coefficient D from comparisons of the species pairs *M. ciliata – M. transsilvanica* and *M. ciliata – M. uniflora* were 0.48 and 0.51, respectively. A value of 0.60 was obtained, when *M. transsilvanica* and *M. uniflora* were contrasted. The value of coefficient D in the comparison between *M. uniflora* and *M. nutans* equalled a much lower figure of 0.20.

Averaged over the six surveyed enzymes, the mean values of coefficient D demonstrated that the species *M. uniflora* was equidistantly and far positioned from the species pairs *M. ciliata* (D = 0.56) and *M. transsilvanica* (D = 0.57), while the last two species were much closer to each other (D = 0.33), as compared to *M. uniflora.* On the other hand, due to the number of species-specific isoforms, the species *M. transsilvanica* seems to be a different genetic entity, as compared to *M. ciliata.* Multivariate morphometrical analyses revealed a partly overlapping morphological variability between *M. ciliata* and *M. transsilvanica* (Szczepaniak & Cieślak 2011). It was found that the lower-glume-to-upper-glume length ratio was the best discriminating character between the two species. Despite their morphological resemblance, the obtained isozyme data allow to discriminate between *M. ciliata* and *M. transsilvanica.*

Mention deserves the fact that the mean value of coefficient D from the comparison between *M. nutans* and *M. uniflora* was 0.19 – an indication of a high similarity of the isoenzyme structure of both taxa. This value is much lower than the values from all pairwise comparisons within the studied species group of genus *Melica*. The result demonstrated that the species *M. nutans* and *M. uniflora* are closer related and more distant from the species pair of *M. ciliata* and *M. transsilvanica.* Both *M. nutans* and *M. uniflora* are rhizomatous, while the species *M. ciliata* and *M. transsilvanica* are caespitose. Thus, the results of the present study are in concordance with the main morphological features of the examined taxa of genus *Melica*.

Summarizing the results of the present study, it could be concluded that the species *M. ciliata* and *M. transsilvanica* are closely related but genetically different entities within genus *Melica*. These two species are almost equidistantly and far positioned from both *M. uniflora* and *M. nutans.*

Acknowledgements. The author is grateful to Dr. M. Szczepaniak for the valuable suggestions and discussions.

References

Angelov, G. 2012. Electrophoretic spectra of six isoenzymes in natural populations of *Melica uniflora* (*Poaceae*) from Bulgaria. – Dokl. Bulg. Akad. Nauk., **65**(8)**:** 1071-1077.

Davis, B. 1964. Disc electrophoresis. I. Method and application to human serum proteins. – Ann. N.Y. Acad. Sci., **121**: 404-427.

Hempel, W. 1970. Taxonomische und chorologische Untersuchungen an Arten von *Melica* L. subgen. *Melica* L. – Feddes Repert., **81:** 131-145.

Hempel, W. 2011. Revision und Phylogenie der Arten der Gatung *Melica* L. (*Poaceae*) in Eurasien und Nordafrika. – Feddes Repert., **122**(1-2)**:** 1-253.

Przybylska, J., Blixt, S., Parzysz, H. & Zimniak-Przybylska, Z. 1982. Isoenzyme variation in the genus *Pisum*. I. Electrophoretic patterns of several enzyme systems. – Genet. Polon., **23**: 103-121.

Reisfeld, R., Lewis, U. & Williams, D. 1962. Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. – Nature, **195**: 281-283.

Schmidt-Stohn, G. & Wehling, P. 1983. Genetic control of esterase isoenzymes in Rye (*Secale cereale* L.). – Theor. Appl. Genet., **64:** 109-115.

Shaw, C. & Prasad, R. 1970. Starch gel electrophoresis – a compilation of recipes. – Biochem. J., **4:** 297-320.

Stuessy, T. 1990. Plant Taxonomy, Columbia Univ. Press, New York.

Szczepaniak, M. & Cieślak, E. 2006. Genetic variation and structure in natural populations of *Melica ciliata* and *M. transsilvanica* (*Poaceae*) as indicated by AFLP markers. – Biodiv. Res. Conserv., **3-4:** 39-43.

Szczepaniak, M. & Cieślak, E. 2011. Genetic and morphological differentiation between *Melica ciliata* L. and *M. transsilvanica* Schur (*Poaceae*) in Europe reveals non-existence of *M. ciliata* in the Polish flora. – Acta Soc. Bot. Pol., **80(4):** 301-313.

Tutin, T. 1980. *Melica* L. – In: **Tutin, T.G. & al.** (eds.), Flora Europaea, Vol. **5**, pp. 178-179, Cambridge Univ. Press, Cambridge.

Tyler, T. 2002a. Geographic variation and dispersal history in Fennoscandian populations of two forest herbs. – Pl. Syst. Evol., **233:** 47-64.

Tyler, T. 2002b. Large-scale geographic patterns of genetic variation in *Melica nutans,* a widespread Eurasian woodland grass. – Pl. Syst. Evol., **236**: 73-87.

Tyler, T. 2004. Studies in the *Melica ciliata* complex: Distribution of allozyme variation within and among individuals, populations and geographic regions. – Pl. Syst. Evol., **248:** 1-30.