

Genetic and morphological differentiation between *Melica ciliata* L. and *M. transsilvanica* Schur (Poaceae) in Europe reveals the non-presence of *M. ciliata* in the Polish flora

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Abstract

A good knowledge of species delimitation is crucial for the biodiversity protection and the conservation of wild species. We studied the efficiency of AFLP markers and morphological characters to assist species determination for *Melica ciliata* L. and *M. transsilvanica* Schur within European range of distribution, including isolated and range-limit populations of “*M. ciliata*” (i.e. *M. cf. ciliata*) from the Polish Sudetes, where it is regarded as critically endangered. AFLP markers were found to be more effective than morphological characters (more or less continuous) in distinguishing the both studied species. AMOVA revealed very low genetic diversity within populations and high differentiation among populations of *M. ciliata* and *M. transsilvanica* ($F_{ST} = 0.89$ and 0.95 , respectively). The species-diagnostic AFLP markers of *M. transsilvanica* shared with “*M. ciliata*” from the Sudetes were detected. On the other hand, no species-diagnostic genetic markers of *M. ciliata* or hybrid-diagnostic markers of *M. × thuringiaca* were found within “*M. ciliata*”. PCoA and NJ showed an overlapping genetic diversity of “*M. ciliata*” and *M. transsilvanica*. Hierarchical AMOVA supported the absence of a significant genotypic distinction between “*M. ciliata*” and *M. transsilvanica*. ANOVA showed that the length ratio of lower to upper glumes was the best morphological character to discriminate between *M. ciliata* and *M. transsilvanica*. Combined morphological and genetic data show that *M. ciliata* is not currently present in Poland as its putative Polish populations represent *M. transsilvanica*. A significant decrease in genetic variability that could influence viability was not observed in the Sudetian populations of *M. transsilvanica*. However, the population size changes significantly as a result of plant succession. Correction of the northern limit of the continuous distribution of *M. ciliata* L. in Central Europe is presented.

Keywords: AFLP, genetic diversity, *Melica ciliata*, *Melica transsilvanica*, morphological variation, Europe

Introduction

Morphological characters that identify and describe living organisms have been a major practical criterion used in plant and animal systematics since morphological data form the basis of virtually all systematic descriptions [1]. However, results of the combined molecular and morphological research techniques, give insight into speciation processes and are fundamental to species-level taxonomy. They are successfully used in phylogeny reconstruction, to examine the causes of variability, to construct natural classification systems, and to define taxonomic borders [2]. Genetic studies of species complexes, within which taxonomic borders based on morphology are usually blurred, often permit to assess the level of inter-species distinction and, on the other hand, their relationships [3,4].

Phylogenetic and taxonomical relationships between *Melica ciliata* L. and *M. transsilvanica* Schur (Poaceae) have not been exhaustively explained and established [5-7]. *M. ciliata* was described by Linnaeus [8] as a species that occurs in rocky and infertile hills of Europe but the exact place of its collection is unknown. *M. transsilvanica*, distinguished by very unequal glumes and pubescent lower leaf-sheaths, was described by Schur [9] from the vicinity of the Sibiu town in the Transylvanian Plateau in Romania. An intricate infraspecific variability and some morphological overlap between species makes them taxonomically problematic [5,6]. *M. transsilvanica* has for a long time been regarded either as a subspecies or as a variety of *M. ciliata* in many European floras [10-15]. However, Papp [16] pointed out that *M. transsilvanica* is a separate species distinguished from *M. ciliata* by several characters, including a dense inflorescence, flatter leaves and details of leaf-sheath pubescence.

Melica ciliata L. is a sub-Mediterranean species whose main continuous geographical range covers the area from the Atlantic and Mediterranean region, Central Europe, to southern Ukraine and the Crimea (Fig. 1). It also occurs in the southern part of the Scandinavian Peninsula and in north Africa. Single scattered records have been reported from the Middle East [17]. *M. transsilvanica* Schur is a sub-Mediterranean-continental, mostly steppe and steppe-forest species. Its main

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geographical range covers Central Asia, the Middle East, the Caucasus, Western Asia, Eastern and Central Europe, reaching southern France as well as northern and central Italy in the west (Fig. 1). According to Hultén and Fries [17], *M. ciliata* and *M. transsilvanica* reach the northern limit of their continuous geographical range among others in Poland. The occurrence of *M. ciliata* in Poland, seems, however, questionable based on literature and herbarium records as well as on the results of the morphological and genetic studies presented below.

Studies on *Melica ciliata*/*M. transsilvanica* in Poland

Melica ciliata s. l. has been reported from Lower Silesia in south-western Poland by German botanists since the late 19th century (e.g. [18-23]). Further, it is unknown to which contemporary populations in south-western Poland the vague information on the occurrence of *M. ciliata* L. var. *nebrodensis* Coss. (= *M. nebrodensis* Parl. pro spec.) reported by Szafer [11] refers. Additionally, Szafer [11] cited the occurrence of *M. ciliata* L. var. *transsilvanica* (= *M. transsilvanica* Schur pro spec.) in the Podkarpacie, the Carpathian Mts. and the Wyżyna Małopolska upland. The first record clearly referring to Sudetian *Melica* populations as *M. ciliata* s. str. is that of *M. ciliata* var. *linnaei* by Papp [16] reported only from one location, i.e. Nowa Wieś Kłodzka, which was not confirmed at present [24]. Papp [16] also cited the occurrence of *M. transsilvanica* var. *bourgaei* (Gris.) Asch. et Graeb. from Bardo near Kłodzko, currently located in the Polish Sudetes. This locality exists at present

[24]. However, Podpěra [25], who distinguished between *M. ciliata* s. str. and *M. transsilvanica* s. str., already decided on the identity of both species in “German Silesia”, comprising the present area of Lower Silesia in Poland. Referring to Schube [19], Podpěra [25] unambiguously attributed all Silesian populations in “refuge” habitats in deep valleys of the Nysa and the Kaczawa rivers to *M. transsilvanica* s. str.

It should be pointed that the 19th and some early 20th century records of *Melica* from the Pieniny Mts. and from the Ojców National Park in south Poland were also attributed to *M. ciliata* s. l. (e.g. [26,27]). Probably the first taxon named *M. ciliata* subsp. *transsilvanica* Hackel. was reported from the Dolina Ojcowska valley, the Pieniny Mts. and from the Beskid Sądecki by Zapałowicz [28]. *M. transsilvanica* was simply not distinguished from *M. ciliata* not only in the Polish but also in other European floras (e.g. [19,29,30]) at the time, which may partly be accounted for by the fact that *M. transsilvanica* was distinguished by Schur as a distinct species only in 1866 [9].

Solely *M. ciliata* was again reported in floristic studies conducted in the Sudetes and the Przedgórze Sudeckie foothills in the second half of the 20th century [31-38]. Having only few localities, *M. ciliata* L. was included in the first List of threatened plants in Poland in the category of indeterminate threat [39]. Research into the distribution of *M. ciliata* and the condition of its populations in Poland was later used to reclassify the species as critically endangered [40]. This threat category was recently maintained [41].

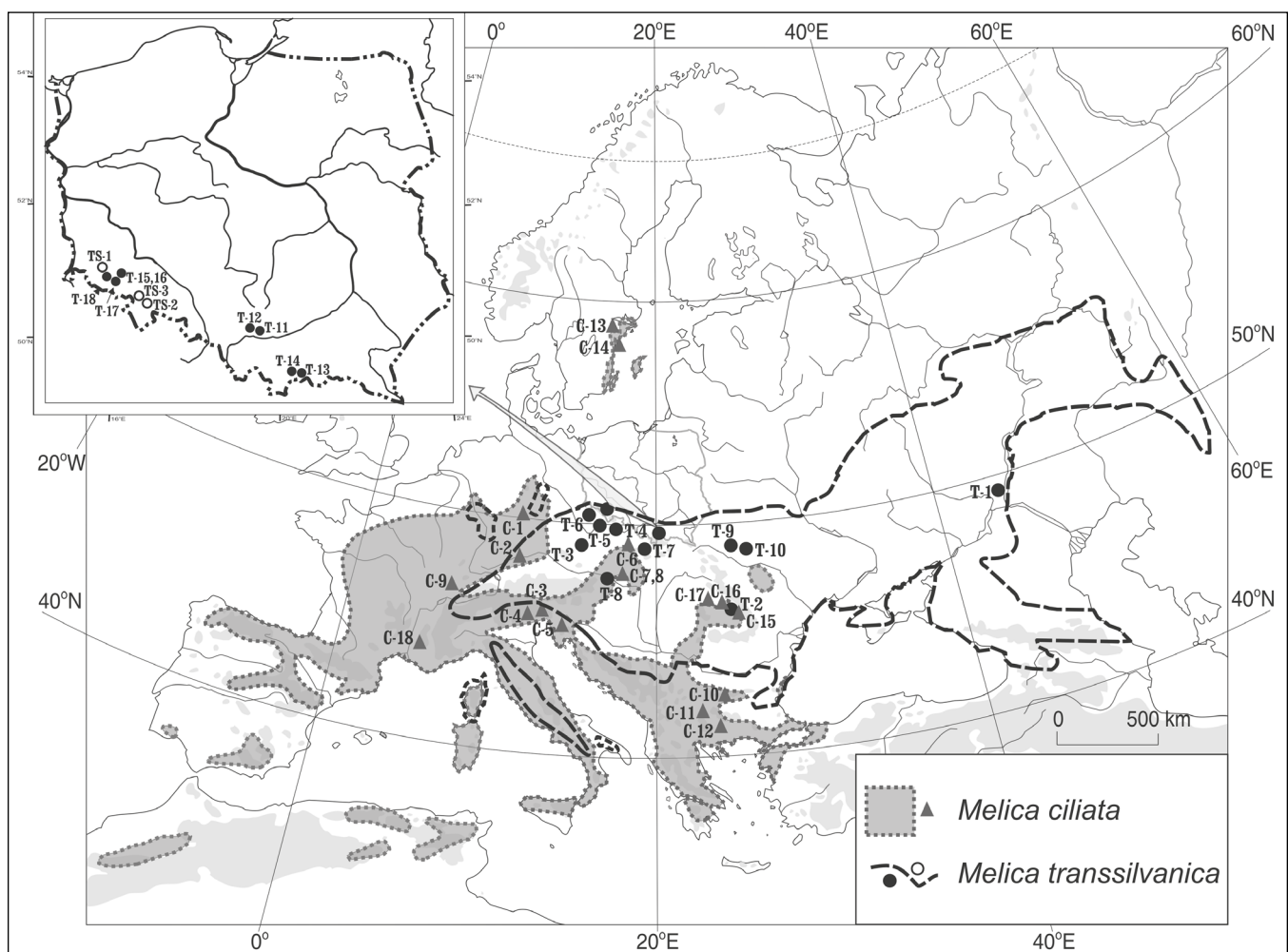


Fig. 1 Distribution range of *M. ciliata* L. and *M. transsilvanica* Schur (European part), and locations of populations sampled. The map is compilation based on Hempel [5], Zángheri [94] and Hultén and Fries [17] that was revised and modified. For explanation of abbreviations, see Tab. 1.

Morphological discrimination between *M. ciliata* and *M. transsilvanica* is sometimes problematic due to a large variation of both species as well as inter-specific continuity of some characters, often make impossible unequivocal identification of specimens. Additionally, both species are able to hybridize in natural habitats that can blur inter-specific morphological differences [42]. However, the previous genetic analyses showed that these species are clearly genetically distinct [43,44]. Thus, we were interested in determining whether combined molecular and morphological data would be effective in distinguishing *M. ciliata* and *M. transsilvanica*.

Based on the morphological analysis of *Melica* collected in Lower Silesia, Szczęśniak [24,45] recently argued for the presence of individuals of *M. ciliata*, *M. transsilvanica* and their interspecific hybrid, *M. × thuringiaca* Rauschert. Kwiatkowski ([46] and references therein) reported few localities of *M. ciliata* and *M. transsilvanica* from the Góry Kaczawskie Mts. and the Pogórze Kaczawskie with notes concerning the presence of untypical characters of some individuals. However, in the preliminary genetic study of *Melica* we showed that some “problematic” Sudetian populations (regarded primarily as *M. ciliata* or its hybrids) were unexpectedly located within *M. transsilvanica* group [43,47,48]. Then, our molecular evidences [43,47,48] were basis to state that only *M. transsilvanica* is present in the Polish flora currently [49], however this statement was developed without strong evidences and comparison with typical specimens of *M. transsilvanica* and *M. ciliata* from outside Poland. Therefore, we wished to provide a contemporary morphological and molecular assessment of the taxonomical classification of Sudetian populations in context of *M. ciliata* and *M. transsilvanica* variation within European range. Solution of this problem was also needed with regard to the protection of *M. ciliata* as the critically endangered species in the Polish flora [40]. If it is accepted that both species were present in the Polish Sudetes, then it could be expected that natural hybridization and subsequent introgression between *M. ciliata* and *M. transsilvanica* may have taken place. These processes cause an impoverishment of biodiversity and lead to local extinction of one or both parental species, especially in small and isolated populations [50]. For the sake of clarity, doubtful populations from the Sudetes are preliminarily defined as “*M. ciliata*” further in this paper to distinguish them from population samples of *M. ciliata* s. str. collected from other European localities.

The main aims of our studies were: (i) to evaluate the efficiency of the morphological characters and AFLP markers for distinguishing between *M. ciliata* L. and *M. transsilvanica* Schur, (ii) to assess levels of genetic and morphological variation of isolated populations of “*M. ciliata*” from the Sudetes in comparison with populations of *M. ciliata* L. and *M. transsilvanica* Schur from the continuous distribution range of species, and in result to discuss the taxonomical position of “*M. ciliata*”, and (iii) to correct the northern limit of the continuous geographical range of *M. ciliata* L. in Central Europe.

Material and methods

Plant material

For AFLPs studies, we typically collected 5-8 (rarely 4 or 10) plants per population, with 32 populations of *M. ciliata*/*M. transsilvanica*, and 203 specimens were sampled

in total (Tab. 1). *M. ciliata* L. and *M. transsilvanica* Schur both from 14 European populations, one cultivar population of *M. × thuringiaca* and “*M. ciliata*” from the three localities in the Polish Sudetes (i.e.: Nowa Ruda-Dzikowiec, Ożary and Góra Grodzik Mt. near Mysłów; Szczęśniak [40]) were analysed. Four localities (Miłek Mt., Połom Mt., “Wąwóz Lipa” reserve near Nowa Wieś Wielka and “Wąwóz Myśluborski” reserve near Myślubórz) of “*M. ciliata*” reported by Kwiatkowski [46,51] did not were confirm during our fieldwork conducted in 2011 as well as herbarium specimens were unavailable, so we were not able to include samples from the Góry Kaczawskie Mts. into our studies. In addition, *M. ciliata* subsp. *magnoliae* population (signed as M-1 from Spain, Province Cádiz, Bernalup, 36°25'N, 05°45'W) served as an outgroup. Young and fresh leaves were collected from randomly chosen specimens from tufts spaced at ca. 5-6 m intervals. Leaves were dried and stored in silica gel to preserve genetic material for extraction. Sampling strategy that aims to analyze many populations but lower number of individuals per population was considered, because no significant correlations between the population size and the genetic diversity indices were found (*M. Szczepaniak* unpublished data). This strategy is congruent with previous studies which showed that genetic diversity of self-compatible species is less affected by decreasing population size than that of mainly outcrossing species [52,53].

Morphometric analyses included the major part of individuals used in AFLPs. In supplement, measurements were taken for plants not analyzed genetically but representing *M. ciliata* L. and *M. transsilvanica* Schur from different parts of European geographical ranges. Only well developed and not disarticulating panicles were chosen for measurements. A total of 156 plants were surveyed, including individuals of *M. ciliata* from 13 populations, *M. transsilvanica* from 10 populations and specimens of “*M. ciliata*” from three Sudetian localities (Tab. 1). Voucher specimens were deposited in the herbarium of the Institute of Botany, Polish Academy of Sciences, Kraków (KRAM).

DNA extraction and AFLP fingerprinting

Total genomic DNA was extracted from 20 mg of dried leaf tissue using the DNeasy Plant Mini Kit (Qiagen) following the protocol of the manufacturer. DNA quality and concentration were estimated against λ -DNA on 1% agarose gel stained with ethidium bromide. AFLP analysis was performed according to the procedure described by Vos et al. [54] with some modifications [55]. After initial screening of 16 selective primer pair combinations, four combinations were selected that gave the highest polymorphism and reliability of AFLP profiles: *EcoRI*-ACG/*MseI*-CAG, *EcoRI*-AGA/*MseI*-CGT, *EcoRI*-AAT/*MseI*-CGC and *EcoRI*-ATC/*MseI*-CAT. Products of selective amplification were separated on the POP 4 polymer with an internal size standard GeneScan-500 [ROX] on an automated sequencer ABI 3100-Avant (Applied Biosystems, USA). DNA extracts from three individuals double-collected in the field from each population were analyzed to test the reproducibility of AFLP profiles [56].

Genetic analyses

AFLP profiles were manually analyzed using GeneScan (ver. 3.7, Applied Biosystems) and GenoGrapher (ver. 1.6.0, Montana State University 1999). AFLP fragments were scored in the range between 50-500 bp for the presence (1) or absence (0) of bands and assembled as a binary matrix. Only

Tab. 1 Geographic origin and size of *M. ciliata*/*M. transsilvanica* population samples.

Population code	N AFLPs	N morphol.	Locality	Lat. (°N)/long. (°E)
<i>Melica ciliata</i> L.				
C-1	8	8	Germany, near Magdala	50°54'/11°27'
C-2	5	5	Germany, Langenthalheim	48°53'/10°55'
C-3	8	–	Slovenia, Ljubljana, Polhograjska Grmada Mt.	46°05'/14°20'
C-4	–	5	Slovenia, Postojna	45°46'/14°12'
C-5	4	5	Croatia, Pasjak	45°29'/14°14'
C-6	5	5	Slovakia, near Vrútky	49°07'/18°55'
C-7	6	6	Hungary, Pilisszanto, Pilis Mts.	47°39'/18°53'
C-8	–	7	Hungary, Budaórs, Odvas hegy hill	47°28'/18°56'
C-9	5	5	Switzerland, Crésuz near Bulle,	46°37'/07°08'
C-10	5	–	Bulgaria, Shipka monument	42°44'/25°19'
C-11	–	5	Bulgaria, Struma valey between Boboševo and Pastuch	42°12'/22°54'
C-12	–	5	Bulgaria, Velingrad	42°05'/24°07'
C-13	5	–	Sweden, Marmorbruket	58°40'/16°24'
C-14	5	–	Sweden, Högsrum	56°40'/16°35'
C-15	6	5	Romania, near Bogata	45°59'/25°18'
C-16	6	5	Romania, Bucea	46°57'/22°42'
C-17	5	5	Romania, near Borod	46°59'/22°38'
C-18	6	–	France, Serignon	44°00'/05°10'
Total	79	71		
<i>Melica transsilvanica</i> Schur				
T-1	7	–	Russia, Volgograd	48°48'/44°35'
T-2	8	8	Romania, Cojocna	46°45'/23°50'
T-3	8	–	Austria, Waldviertel, Umlaufberg hill	48°43'/15°50'
T-4	5	–	Czech Republic, Pozorice	49°12'/16°47'
T-5	5	–	Czech Republic, Hodonin	49°30'/16°25'
T-6	–	5	Czech Republic, Znojmo	48°51'/15°51'
T-7	7	7	Slovakia, Červený Kláštor	49°23'/20°25'
T-8	–	6	Hungary, SE Márko	47°06'/17°49'
T-9	–	5	Ukraine, Kam'änets' Podil's'kij	48°40'/26°33'
T-10	–	6	Ukraine, E Ustâ, riverside of the Dniester	48°33'/26°41'
T-11	5	5	Poland, Bolechowice, Wąwóz Bolechowicki Ravine	50°07'/19°46'
T-12	5	–	Poland, Szklary, Słoneczne Skały rocks	50°11'/19°43'
T-13	5	–	Poland, Biała Woda reserve	49°23'/20°35'
T-14	7	7	Poland, Falsztyn	49°27'/20°17'
T-15	8	–	Poland, Strzegom, Góra Krzyżowa Mt.	50°59'/16°20'
T-16	7	7	Poland, Strzegom, Góra Św. Jerzego Mt.	50°59'/16°20'
T-17	8	6	Poland, Dobromierz, Góra Dębowa Mt.	50°55'/16°15'
T-18	8	–	Poland, Mysłów-Sobocin, Wapienna Góra Mt.	50°59'/15°59'
Total	93	62		
“<i>Melica ciliata</i>”				
TS-1	7	7	Poland, Mysłów, Góra Grodzik Mt.	50°59'/15°59'
TS-2	6	6	Poland, Ożary	50°30'/16°50'
TS-3	10	10	Poland, Nowa Ruda-Dzikowiec	50°34'/16°35'
Total	23	23		
<i>Melica</i> × <i>thuringiaca</i> Rauschert				
× thu	8	–	cultivar	
Altogether	203	156		

The numbers of plants from each site, analyzed with AFLP and for morphological variation are indicated in columns 2 and 3. *N* – numbers of plants.

reproducible, well-separated and unambiguous AFLP bands were considered in further analyses. Genetic diversity in populations was estimated with the approach of Lynch and Milligan [57] and with using AFLP-SURV ver. 1.0 [58]. Allelic frequencies at AFLP loci were computed from the frequencies of amplified fragments using the Bayesian approach with the non-uniform prior distribution of allele frequencies proposed by Zhivotovsky [59] for diploid species. Additionally, deviation from Hardy-Weinberg genotypic equilibrium was assumed in our computations ($F_{is} = 0.9$), as derived from allozyme data [7].

Genetic diversity within populations was characterized by: the number (N_{poly}) and proportion ($\%_{poly}$) of polymorphic AFLP fragments at the 5% level (i.e. loci with allelic frequencies lying in the range from 0.05 to 0.95), the number of private AFLP fragments (N_{prt} – a private fragment is defined as a band which is present only in individuals of the given population but not in any individuals not belonging to the current population) and Nei's gene diversity in populations (H_j ; [60]). Assuming that *M. ciliata* s. l. is mainly selfing plant where heterozygotes are infrequent, H_j index should yield accurate estimations also for dominant markers [57].

To estimate the molecular distinctiveness of *M. ciliata*, *M. transsilvanica* and *M. × thuringiaca* the species-diagnostic (private; N_{prt}) AFLP fragments, i.e. the number of fragments

present in analysed individuals of a respective species and absent elsewhere, were sought. Species-diagnostic AFLP fragments of taxa shared with "*M. ciliata*" (N_{shr}) were identified to determine the level of pairwise genetic relationships and to reveal potential hybridization and introgression direction.

To represent overall genetic relationships among populations and species, a dendrogram based on pairwise Nei and Li's [61] genetic distances with applying the neighbour-joining method (NJ) were constructed using TREECON 1.3b [62]. Support for each node was tested by 2000 bootstrap replicates. Principle coordinates analysis (PCoA) was performed in FAMD software 1.25 [63] using Nei and Li's [61] genetic distances (with $r = 6$), in conjunction with STATISTICA ver. 5.1 [64], to illustrate individuals grouped according to the AFLP fragments similarity pattern. The Nei and Li coefficient [61] counts the percentage of shared bands among individuals and gives more weight to those bands that are present in both. It considers that absence has less biological significance, and so this coefficient has complete meaning in terms of DNA similarity.

A hierarchical analysis of molecular variance (AMOVA), based on a matrix of squared Euclidean distances (ARLEQUIN, ver. 3.0; [65]), was performed to quantify the distribution of genetic variation among and within populations of

Tab. 2 Characters used in morphometric analyses of *M. ciliata*, *M. transsilvanica* and Sudetian "*M. ciliata*" populations.

No.	Character	<i>M. ciliata</i> (N = 71)	" <i>M. ciliata</i> " (N = 23)	<i>M. transsilvanica</i> (N = 62)
Quantitative characters (spikelet in the middle part of panicle)				
1	length of lower glume	5.03 (± 0.56) (3.87-6.33)	4.43 (± 0.40) (3.80-5.27)	4.38 (± 0.49) (3.20-5.47)
2	width of lower glume	2.36 (± 0.30) (1.67-3.07)	1.98 (± 0.20) (1.67-2.33)	2.09 (± 0.30) (1.40-2.67)
3	ratio: length/width of lower glume (1/2)	2.15 (± 0.25) (1.71-2.84)	2.25 (± 0.23) (1.88-2.92)	2.14 (± 0.36) (1.60-3.05)
4	length of upper glume	6.13 (± 0.69) (4.53-7.80)	6.81 (± 0.44) (5.73-7.67)	6.58 (± 0.51) (5.53-7.67)
5	width of upper glume	1.87 (± 0.25) (1.40-2.40)	1.66 (± 0.18) (1.33-2.00)	1.65 (± 0.18) (1.13-2.07)
6	ratio: length/width of upper glume (4/5)	3.31 (± 0.40) (2.63-4.41)	4.16 (± 0.56) (3.37-5.33)	4.04 (± 0.55) (3.10-5.88)
7	ratio: length of lower glume/length of upper glume (1/4)	0.82 (± 0.06) (0.68-0.94)	0.65 (± 0.05) (0.56-0.72)	0.67 (± 0.05) (0.54-0.73)
8	length of lemma of the lowest floret	5.23 (± 0.55) (4.00-6.53)	5.70 (± 0.38) (5.20-6.53)	5.44 (± 0.44) (4.67-6.73)
9	ratio: length of lower glume/length of lemma of the lowest floret (1/8)	0.96 (± 0.08) (0.78-1.18)	0.78 (± 0.09) (0.63-0.93)	0.80 (± 0.07) (0.66-0.95)
Qualitative characters				
10	lower leaf-sheaths: glabrous or scabridulous to scabrous with short, stiff hairs directed upwards – 0; pilose with long, soft hairs directed downwards – 1	0-66 (92.96%) 1-5 (7.04%)	0-16 (69.56%) 1-7 (30.44%)	0-0 1-62 (100%)
11	panicle axis: more or less visible, partially lax – 0; invisible, cover up by spikelets – 1	0-71 (100%) 1-0	0-12 (52.17%) 1-11 (47.83%)	0-0 1-62 (100%)

Values are: mean \pm standard deviation, minimum and maximum of quantitative characters and frequency at qualitative characters. All measurements are given in mm. N – numbers of plants.

M. ciliata and *M. transsilvanica* and to test statistical significance of genetic distinction of “*M. ciliata*”. Additionally, we checked whether unbalanced numbers of samples per population affected the results of AMOVA. To investigate this, we selected at random 5 individuals in each population. In all comparisons the effect of number of samples was of little importance for the results (differences between F_{ST} obtained from complete and reduced dataset were <0.02) and did not influence the overall outcome.

Morphometric analyses

In a morphometric analyses, specimens from populations were treated as operational taxonomical units (OTUs; [66]). A set of analysed characters was selected based on previous taxonomical treatments and plant keys [5,16,42,67-72], morphological studies [24,45] and the authors’ field and herbarium observations. A total of 11 morphological characters of panicles, spikelets and leaf-sheaths were found to be the most effective in the data evaluation and distinguishing *M. ciliata* and *M. transsilvanica*. Detailed descriptions of characters are provided in Tab. 2. Plants were studied and characters were measured using a light microscope Nikon Eclipse E600.

The range of morphological variability of *M. ciliata*, *M. transsilvanica* and “*M. ciliata*” populations were examined using univariate statistics (minimum, maximum, arithmetic mean and standard deviation). All quantitative characters followed a normal or log-normal distribution, confirmed by Shapiro-Wilk tests [73]. The morphometric data matrix was standardized, i.e. the variability in each character was scaled between 0 and 1.

Overall patterns of morphological differences and relationships among species were examined with multivariate morphometry. Principal component analysis (PCA) was applied as a ordination method to group population samples and to find those characters that greatly contributed to the differences among groups of populations and species and best explained the existing variation regardless of the taxonomical classification [74]. One-way analysis of variance (one-way ANOVA) was used to assess the significance of differences: (i) among three groups, i.e. morphologically unequivocal *M. ciliata*, *M. transsilvanica* and Sudetian “*M. ciliata*” and (ii) between two groups, i.e. *M. ciliata* and *M. transsilvanica* (including “*M. ciliata*”). Values of F statistic were used to identify characters that contribute to the resulting patterns the most. Then, F values for each character obtained from one-way ANOVA for three groups (i) and for two groups (ii) were compared. The significance of differences between the character’s means was examined using Tukey’s HSD post hoc tests ($P < 0.001$; [75]). A scatter diagram of the two most discriminating characters was plotted to show morphological similarities or differences between *M. ciliata*, *M. transsilvanica* and Sudetian “*M. ciliata*”. Numeric analyses of morphological characters were conducted using STATISTICA ver. 5.1 G [64].

Results

AFLP analyses

AFLP fingerprinting yielded 259 clearly resolved and unambiguously scored fragments, of which 83% were polymorphic, and with an average number of 122 fragments per individual. Overall repeatability of AFLP phenotypes was high (>98%).

Tab. 3 Estimates of genetic diversity within 14 populations of *M. ciliata*, 3 populations of Sudetian “*M. ciliata*”, 14 populations of *M. transsilvanica* and one population of *M. × thuringiaca* based on 259 AFLP fragments and assessed with the assumption of Hardy-Weinberg disequilibrium ($F_{IS} = 0.9$).

Population code	N_{poly}	% _{poly}	N_{prt}	H_j	SD (H_j)
<i>M. ciliata</i>					
C-1	21	8.11	1	0.055	0.005
C-2	10	3.86	0	0.061	0.006
C-3	4	1.54	2	0.035	0.004
C-5	5	1.93	0	0.057	0.005
C-6	0	0.00	3	0.029	0.002
C-7	17	6.56	2	0.066	0.007
C-9	6	2.32	1	0.052	0.005
C-10	2	0.77	3	0.042	0.004
C-13	8	3.09	0	0.056	0.006
C-14	7	2.70	0	0.052	0.004
C-15	14	5.41	1	0.059	0.007
C-16	6	2.32	0	0.045	0.004
C-17	3	1.16	0	0.048	0.004
C-18	30	11.58	1	0.087	0.009
<i>M. transsilvanica</i>					
T-1	4	1.54	2	0.038	0.003
T-2	4	1.54	3	0.036	0.003
T-3	4	1.54	0	0.035	0.003
T-4	2	0.77	0	0.051	0.004
T-5	6	2.32	0	0.058	0.005
T-7	6	2.32	0	0.042	0.004
T-11	1	0.39	0	0.049	0.003
T-12	0	0.00	0	0.033	0.002
T-13	0	0.00	0	0.032	0.002
T-14	3	1.16	0	0.039	0.003
T-15	1	0.39	0	0.034	0.002
T-16	0	0.00	0	0.025	0.002
T-17	3	1.16	0	0.039	0.004
T-18	4	1.54	0	0.037	0.003
“<i>M. ciliata</i>”					
TS-1	10	3.86	0	0.048	0.004
TS-2	0	0.00	0	0.029	0.002
TS-3	6	2.32	0	0.032	0.002
<i>M. × thuringiaca</i>					
× thu	3	1.16	2	0.033	0.002

Names of populations refer to Tab. 1. %_{poly} – proportion of polymorphic fragments; H_j – Nei’s gene diversity; N_{poly} – number of polymorphic AFLP fragments; N_{prt} – number of private AFLP fragments, present only in individuals of the respective population but absent in any individuals of other populations; SD – standard deviation.

The number of polymorphic AFLP fragments characterizing intrapopulation variation of *M. ciliata* ranged from 0 in population C-6 to 30 (11.58%) in population C-18, with an average of 9.5 (3.67%; Tab. 3). Populations of *M. transsilvanica* showed lower genetic variation than that of *M. ciliata* and the number of polymorphic AFLP fragments varied from 0 in populations T-12, T-13 and T-16 to 6 (2.32%) in populations

T-5 and T-7, with an average of 2.7 (1.05%). On average, 5.3 (2.06%) polymorphic AFLP fragments were detected within “*M. ciliata*” populations. Within-population gene diversity of *M. ciliata* ranged from 0.029 to 0.087 (average 0.053 ± 0.004). In *M. transsilvanica* populations H_j varied from 0.025 to 0.058 (0.039 ± 0.002), and in “*M. ciliata*” populations H_j varied from 0.029 to 0.048 (0.036 ± 0.006).

A total of 88 and 55 species-diagnostic AFLP fragments were detected for *M. ciliata* and *M. transsilvanica*, respectively (Tab. 4). Furthermore, 41 (74%) species-diagnostic fragments of *M. transsilvanica* and no species-diagnostic fragments of *M. ciliata* were present in “*M. ciliata*” specimens. None of two diagnostic AFLP fragments were shared between hybrid *M. × thuringiaca* and “*M. ciliata*”. For “*M. ciliata*” one private AFLP fragment was detected.

PCoA revealed two well-separated main groups of individuals representing two species, *M. ciliata* and *M. transsilvanica* (Fig. 2). The first three coordinates account for, respectively, 82.06%, 3.98% and 2.85% of the total genetic variation. The scatter diagram clearly shows that specimens from the Sudetian

Tab. 4 Number of species-diagnostic private AFLP fragments for *M. ciliata*, *M. transsilvanica* and *M. × thuringiaca*, and number of private AFLP fragments shared between these taxa and “*M. ciliata*”.

	N_{prt}	N_{shr}
<i>M. ciliata</i>	88	0
<i>M. transsilvanica</i>	55	41
<i>M. × thuringiaca</i>	2	0
“ <i>M. ciliata</i> ”	1	–

N_{prt} – number of private AFLP fragments; N_{shr} – number of private AFLP fragments shared between these taxa and “*M. ciliata*”.

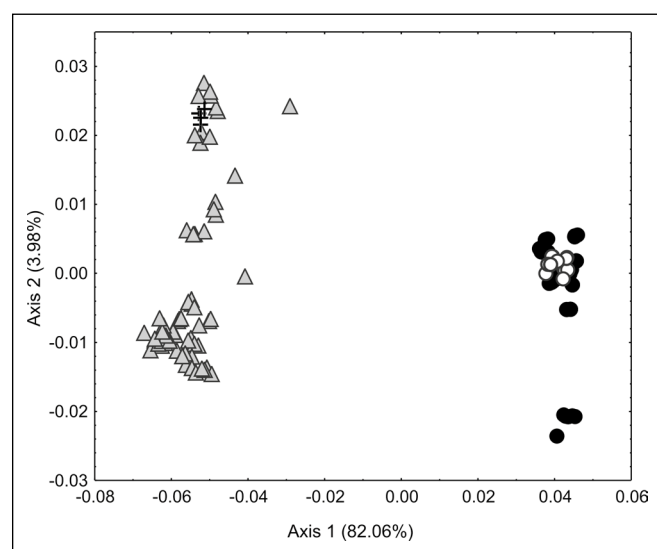


Fig. 2 Principal coordinate analysis (PCoA) of AFLP data from 203 investigated individuals of *M. ciliata* (grey triangles), *M. transsilvanica* (black circles), *M. × thuringiaca* (cross) and Sudetian “*M. ciliata*” (open circles), based on the pairwise Nei and Li’s [61] genetic distances.

“*M. ciliata*” populations were placed within the *M. transsilvanica* group. A similar pattern of genetic diversity was shown by NJ based on pairwise genetic distances [61] among populations (Fig. 3). NJ tree revealed two well-supported (bootstrap value of 100%) clusters corresponding to two main groups in PCoA, i.e. groups of *M. ciliata* and *M. transsilvanica*. All populations of “*M. ciliata*” were positioned within the *M. transsilvanica* cluster. Several subclusters within the *M. transsilvanica* group, mostly representing groups of populations from particular parts of the species occurrence range, were shown, reflecting some genetic distinction related to geographical isolation of populations. The geographical subdivision of *M. transsilvanica* had high bootstrap support ($\geq 99\%$). Interestingly, population TS-1 of “*M. ciliata*” was clustered with an adjacent Sudetian population T-18 of *M. transsilvanica*, whereas TS-2 and TS-3 populations were grouped with remaining Sudetian populations T-15, T-16 and T-17, both with high bootstrap support ($\geq 99\%$). There was no clear geographical structure within the *M. ciliata* cluster, grouped populations C-13 and C-14 from disjunct parts of the species distribution from Sweden and C-15, C-16, C-17 populations from Romania (100% bootstrap) being the only exceptions (Fig. 3).

In three-level AMOVA, 71.33% ($P < 0.001$; Tab. 5) of total genetic variation was assigned to differentiation between *M. ciliata* and *M. transsilvanica*, which confirmed grouping of the individuals into distinct species obtained in PCoA and NJ. The existence of the strong genetic structure and similar partitioning of molecular variance in *M. ciliata* and

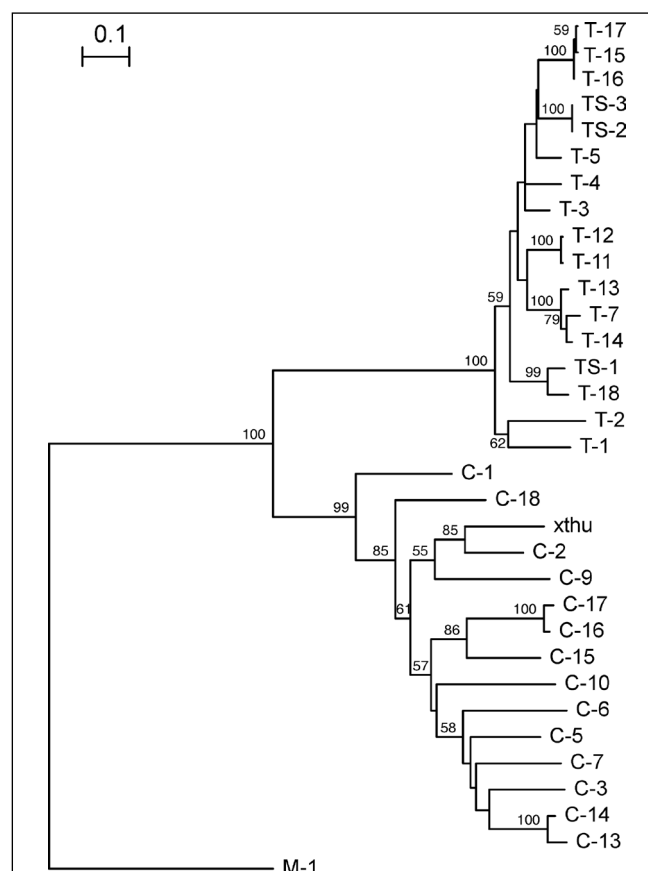


Fig. 3 Neighbour-joining analysis of AFLP data from *M. ciliata*, *M. transsilvanica*, Sudetian “*M. ciliata*” and *M. × thuringiaca* populations, based on the pairwise Nei and Li’s [61] genetic distances. Bootstrap values above 50% are given at nodes. Names of populations refer to Tab. 1 and “Material and methods”.

Tab. 5 Analysis of molecular variance (AMOVA) of the 28 studied populations of *M. ciliata*, *M. transsilvanica* and 3 uncertain populations of “*M. ciliata*” from the Sudetes.

Comparison	d.f.	Variance components	% of total variation	F statistics
<i>M. ciliata</i>				
among populations	13	18.38	89.41***	$F_{ST} = 0.89$
within populations	65	2.18	10.59	
<i>M. transsilvanica</i>				
among populations	13	10.36	95.20***	$F_{ST} = 0.95$
within populations	79	0.52	4.80	
<i>M. ciliata</i> vs. <i>M. transsilvanica</i>				
among species	1	38.13	71.33***	$F_{CT} = 0.71$
among populations	26	14.05	26.30***	$F_{SC} = 0.92$
within populations	144	1.27	2.38	$F_{ST} = 0.98$
“<i>M. ciliata</i>” vs. <i>M. ciliata</i>				
among groups	1	38.40	67.73***	$F_{CT} = 0.68$
among populations	15	16.45	29.01***	$F_{SC} = 0.90$
within populations	85	1.85	3.26	$F_{ST} = 0.97$
“<i>M. ciliata</i>” vs. <i>M. transsilvanica</i>				
among groups	1	1.00	8.76 ^{NS}	$F_{CT} = 0.09$
among populations	15	9.83	86.20***	$F_{SC} = 0.94$
within populations	99	0.57	5.04	$F_{ST} = 0.95$
“<i>M. ciliata</i>” vs. <i>M. transsilvanica</i> from the Sudetes				
among groups	1	1.83	19.67 ^{NS}	$F_{CT} = 0.20$
among populations	5	6.93	74.28***	$F_{SC} = 0.92$
within populations	47	0.56	6.05	$F_{ST} = 0.94$

The analysis is based on AFLP phenotypes consisting of 259 band states. Levels of significance are based on 1023 iteration steps. ^{NS} – non-significant. d.f. – degrees of freedom. *** $P < 0.001$.

M. transsilvanica ($F_{ST} = 0.89$ and $F_{ST} = 0.95$, respectively; $P < 0.001$) were revealed. Hierarchical AMOVA showed highly significant molecular distinction among “*M. ciliata*” and *M. ciliata* ($F_{CT} = 0.68$, $P < 0.001$). On the other hand, “*M. ciliata*” did not differ genetically from *M. transsilvanica* ($F_{CT} = 0.09$, $P = 0.121$). Additionally, a comparison of “*M. ciliata*” with *M. transsilvanica* from the Sudetes also displayed the absence of genetic differences between them ($F_{CT} = 0.20$, $P = 0.207$).

Morphometric analyses

Two groups corresponding to *M. ciliata* and *M. transsilvanica* were showed in the PCA using 11 morphological characters (Fig. 4). The groupings of the species were revealed along PC 1 which separated individuals with lower glume up to 3/4 of lemma length of the lowest flower (9; character loading $r = 0.91$), clearly unequal glumes (7; $r = 0.91$), dense panicles (11; $r = -0.81$) and pilose lower leaf-sheaths with soft, long and downwards directed hairs (10; $r = -0.77$), i.e. diagnostic characters of *M. transsilvanica* (Tab. 2). The second separating group of individuals referred to *M. ciliata* and distinguished by almost equal glumes that nearly covering the lemma of the lowest floret, lax panicles and glabrous or scabridulous to scabrous lower leaf-sheaths with short, stiff hairs directed upwards (Tab. 2). PCA showed that the vast majority of the Sudetian “*M. ciliata*” specimens were scattered within the entire range of

the *M. transsilvanica* variability (open circles; Fig. 4) but several ones were located at the edge of the morphological variability of *M. ciliata*. PC 2 was correlated with the length of the upper glume (4; $r = 0.95$) and the length of the lemma (8; $r = 0.91$), and PC 3 was negatively correlated with the upper glume shape (6; $r = -0.82$), but these characters did not distinguish populations of *M. ciliata* and *M. transsilvanica* (graph not shown). The first three principal components explained 42.26%, 22.45% and 17.85% of total variation in the data set.

Pairwise comparisons using ANOVA showed that “*M. ciliata*” was very similar to *M. transsilvanica* and, on the other hand, it was significantly differ from *M. ciliata* with respect to the majority of the 11 morphological characters (RIR Tukey test, $P < 0.05$). When the “*M. ciliata*” group was included in the *M. transsilvanica* group, F statistics of diagnostic characters considerably increased, that supported morphological similarity between “*M. ciliata*” and *M. transsilvanica*. In the final results of ANOVA, the length ratio of lower to upper glume ($F = 343.85$) and the length ratio of lower glume to lemma of the lowest floret ($F = 177.73$) were the best characters to discriminate between *M. ciliata* and *M. transsilvanica* (critical $F_{0.05;2;\infty} = 4.60$). The scatter diagram drawn on the basis of two characters with the highest values of F statistics obtained from ANOVA, showed discontinuity between *M. ciliata* and *M. transsilvanica* (Fig. 5). It was also evident that “*M. ciliata*”

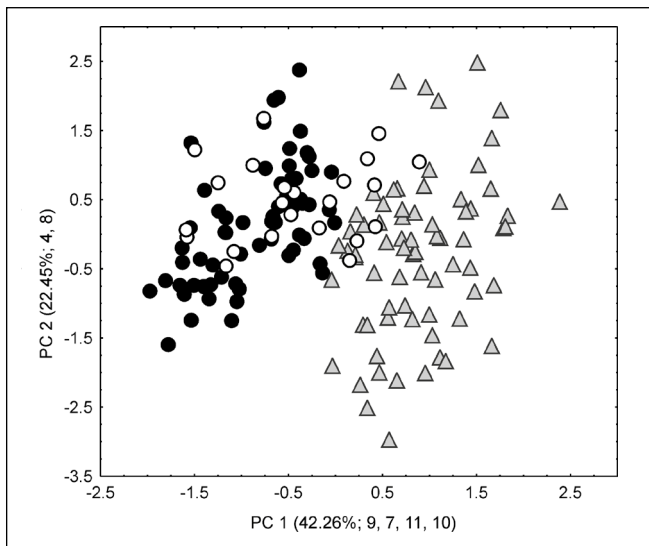


Fig. 4 Principal component analysis (PCA) of *M. ciliata* (grey triangles), *M. transsilvanica* (black circles) and Sudetian “*M. ciliata*” (open circles), based on 11 morphological characters. The characters (i.e. the numbers representing them) highly correlated with PC 1 and PC 2 are given in parentheses; detailed descriptions of characters are provided in Tab. 2 and in the text.

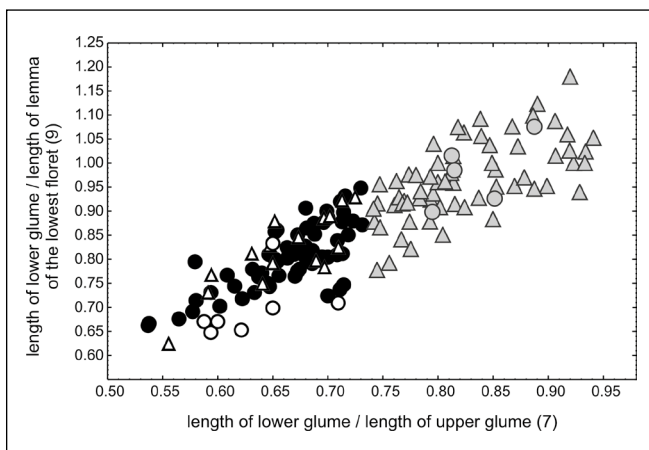


Fig. 5 Scatter plot of the two most important characters (7 and 9) for distinguishing *M. ciliata* (grey filled symbols), *M. transsilvanica* (black filled symbols) and “*M. ciliata*” (open symbols) as revealed by ANOVA. The type of pubescence of the lower leaf-sheaths (10) as the categorical variable was used; specimens with the lower leaf-sheaths glabrous, scabridulous to scabrous with sparse, short and stiff hairs directed upwards – triangles; specimens with the lower leaf-sheaths pilose with long, soft hairs directed downwards – circles. Detailed descriptions of characters are provided in Tab. 2.

specimens were intermingled with *M. transsilvanica* within the entire range of its characters variability. Additionally, individuals of “*M. ciliata*” but also some *M. ciliata* were diverse with respect to the level of leaf-sheath pubescence – from glabrous to pilose (Fig. 5). In our study, plants in the Sudetian populations of “*M. ciliata*” had the mostly glabrous, scabridulous or scabrous lower leaf-sheaths (16 specimens – 70%) and several individuals (7 – 30%) had pilose leaf-sheaths. Collective morphometric results for *M. ciliata*, *M. transsilvanica* and Sudetian “*M. ciliata*” are presented in Tab. 2.

Discussion

Genetic distinction and diversity of *Melica ciliata* and *M. transsilvanica*

Our results of the AFLP fingerprinting and multivariate morphometric analyses provided congruent support for recognition of *M. ciliata* and *M. transsilvanica* as a distinct taxa. However, genetic markers showed clearly higher efficiency for species identification than morphological characters (see PCoA and PCA results). Both studied species are sometimes included within *M. ciliata* complex, taxonomically problematic group consisting of morphotypes or races with pronounced, but mainly clinal or indiscrete, morphological variation [5,7]. It appears that a comprehensive molecular phylogeny of *M. ciliata* complex is still not available, but our results indicate that the AFLPs survey of representative population accessions for *M. ciliata* s. str. and *M. transsilvanica* s. str., based on overall genome dissimilarity, could be considered as conclusive references in future study of phylogeographic and taxonomic patterns in *M. ciliata* complex. Recently, the usefulness of AFLP markers for plant natural systematic in *Hordeum murinum* complex [76] or in *Festuca brachyphylla* complex [3] was found.

Many factors shape the levels of the entire genetic diversity of *M. ciliata* complex, e.g. reproduction mode, bottleneck and genetic drift in small and/or isolated populations, hybridization extent and natural selection [50,77]. The mating system is generally regarded to be the main factor affecting genetic variability within plant populations. *M. ciliata* and *M. transsilvanica* are diploids ($2n = 18$) with predominant selfing [7]. The low intra-population and high inter-population genetic variation are expected for self-pollinators, at which the non-random mating system usually results in high level of inbreeding in populations [78]. Self-pollinators tend to form homogenous populations that, however, greatly differ from one another, even for very small distances, as the genetic exchange between them is very low or entirely absent [66]. We found extraordinarily low gene diversity within populations (on average $H_j = 0.053$ for *M. ciliata* and $H_j = 0.039$ for *M. transsilvanica*) in comparison with other selfing plant species (RAPD-derived $H_s = 0.12$; [52]). The proportion of the gene diversity that was distributed among populations ($F_{ST} = 0.89$ for *M. ciliata* and $F_{ST} = 0.95$ for *M. transsilvanica*) was considerably higher than that commonly reported for selfing species (RAPD-derived $F_{ST} = 0.65$; [52]). Our results indicate a predominant non-random mating that can effect in allelic fixation at many loci within populations and their strong genetic distinctness [79]. Moreover, our findings are congruent with those of previous allozyme study [7] showing that the proportion of the genetic diversity that resides between populations ($G_{ST} = 0.53$) was far higher than reported for most diploid plants and were only comparable with the mean G_{ST} reported for obligatory selfing species [78]. For example, a large reduction of genetic variability within populations and increased differentiation between populations have been observed in obligatory selfing grass *Bromus tectorum* [80] or in predominantly selfing grass *Nassella pulchra* [81] and in wetland species *Typha latifolia* [82].

Morphological variation and differentiation between *Melica ciliata* and *M. transsilvanica*

The obtained results of the multivariate morphometry analyses showed a great variability within *M. ciliata* and *M. transsilvanica* as well as the partly overlapping morphological variability between these two species. The re-evaluation of

the morphological characters displayed that the length ratio of lower glume to upper glume and the length ratio of lower glume to lemma of the lowest floret were the best characters discriminating between *M. ciliata* and *M. transsilvanica*, and better distinguished these species than type of lower leaf-sheaths pubescence, i.e. character sometimes used in the identification keys [69,70,72]. The remaining studied morphological characters were not found to be effective in distinguishing *M. ciliata* and *M. transsilvanica*. The specification of any infraspecific taxonomic ranks within these taxa does not seem to be justified based on the lack of clear discontinuity of infraspecific morphological variation.

Taxonomic status of '*Melica ciliata*' in Poland

Resuming findings from the previous papers [24,43-49,51,79], own fieldworks conducted in 2005-2011 and currently displayed evidences based on AFLP fingerprinting and morphological data, we can state that *M. ciliata* L. does not occur in the Polish flora. On the contrary, a detailed analysis of the AFLP band patterns of Sudetian "*M. ciliata*" clearly showed its genetic identity with *M. transsilvanica* Schur (see "Results"). In view of these results, morphologically ambiguous specimens of *Melica* from the Sudetes were not genetically distinct from other morphologically typical specimens of *M. transsilvanica*.

Populations of *M. transsilvanica* and *M. ciliata* are sometimes relatively small that is caused by a fragmented distribution area in Europe due to specific xerothermic habitat requirements. Especially, the Polish populations of "*M. ciliata*" are strongly isolated and small; they consist of several to some sixty tufts, and their number and size regularly decrease, often to complete extinction [24,35,51]. They are situated ca. 100 km away from the geographically closest Czech populations of *M. ciliata* s. str. or ca. 400 km away from the closest German populations in a straight line. Contrary to theoretical expectations relating to small and isolated populations, we found no evidence of declining population genetic diversity within small and isolated populations of "*M. ciliata*" under a "stronger effect" of random genetic drift than that observed within other populations of *M. ciliata*/*M. transsilvanica*. Our recent study [79] showed the absence of significant differences in genotypes and allele frequencies between populations of *M. transsilvanica* from the central and marginal parts of the species range. The same pattern was displayed in the partitioning of genetic diversity, with the majority of genetic variation occurring between populations within the central and also within the marginal areas. Present results additionally suggest that stochastic demographic and random environmental factors, limited suitable calcareous habitats as well as natural succession rather than genetic erosion have been proximal causes of the disappearance of "*M. ciliata*" populations.

Hybridization with or without introgression may threaten a rare species' existence [83]. A potential hazard from interspecific matings is genetic assimilation of a rare taxon by a more common closely related taxon. Genetic assimilation involves the loss of the genotypes or phenotypes of the rare taxon through asymmetric gene flow from the more numerous taxon [84]. Assuming after Papp [16] that *M. ciliata* and *M. transsilvanica* occurred in Poland, hybridization and subsequent unidirectional introgression may have led to the elimination of competitively weak *M. ciliata* s. str. in Poland. This scenario, however, is only hypothetical and based on the assumption that *M. ciliata* s. str. did really occur in Poland. In present studies, all accessions of "*M. ciliata*" were clearly placed within the

range of genetic diversity of *M. transsilvanica*. Additionally, no species-diagnostic AFLP markers of *M. ciliata* inherited by "*M. ciliata*" or diagnostic markers of *M. × thuringiaca* shared with "*M. ciliata*" were found in our study. Therefore, despite the presence of intermediate morphological characters in some specimens in the Sudetian populations, AFLP results do not provide any indication supporting hypothesis about their hybrid origin.

Concurrently, also the comparative morphometric analysis of European populations exhibited that only *M. transsilvanica* is present in the Sudetes. Moreover, in the Sudetian populations of "*M. ciliata*" morphologically identifiable specimens of *M. transsilvanica* as well as a few individuals characterized by intermediate characters, were recorded. The panicles of the latter plants were slightly more lax, which may be due to strong shading of the localities (*M. Szczepaniak* personal observation). A previous study from Lower Silesia showed that the great humidity and shading of habitats may have also influenced the type of lower leaf-sheaths pubescence of *M. transsilvanica* resembling those in *M. ciliata* [45,49].

Re-considering the historical records of *M. ciliata* from the Sudetes, it is noteworthy that already Schube [20] provided a general description of plants from Sudetian localities as having dense spike-shaped panicles, i.e. one of discriminating characters of *M. transsilvanica* Schur according to present taxonomical approaches [5,67,68,71]. Recapitulating, it is likely that contemporary non distinguishing between *M. ciliata* s. str. and *M. transsilvanica* s. str. in the Sudetes in many studies (see "Introduction") resulted from referring to the historical floristic records and historical morphological delineation that had been treated *M. ciliata* in the wider sense, i.e. in sensu lato.

The northern limit of the continuous geographic range of *Melica ciliata* L. in Central Europe

Our results indicate that the current northern limit of the continuous distribution of *M. ciliata* in Central Europe should be corrected. Accordingly, the northernmost localities of *M. ciliata* in Central Europe are in Slovakia, spanning the area from the east in the Spiš-Gemer Karst, the Slovenské stredohoří, the Strážov Mountains, the Považský Inovec Mountains to the Little Carpathian Mts. in the west [85,86]. In the Czech Republic *M. ciliata* occurs only in Moravia in the Vysočina and on the Palava hills [85-87], where it is a threatened taxon [88]. In Germany, *M. ciliata* is scattered throughout the southern part of the country. It mostly grows in central, north-western and southern Bavaria, in Baden-Württemberg and in the Rhineland-Palatinate, in the Hesse in the south and south-west as well as in Thuringia [89-91]. The northern limit of its range in Germany roughly runs along the line determined by the following cities: Könnern, Eisleben and Düsseldorf, and the species does not occur in Saxony [70]. *M. ciliata* is not a threatened species in Germany [92].

Conclusions

The essential complementing between morphological and genetic data sets suggest usefulness of combined approach to unravel taxonomic relationships and opens an interesting perspective of planned study of phylogeographic patterns in *M. ciliata* complex. Our results clarified the taxonomic boundary between *M. ciliata* and *M. transsilvanica* and provided morphological and genetic support for the specific recognition

of these taxa.

In view of the current taxonomical division of *M. ciliata* and *M. transsilvanica*, and the pattern of genetic and morphological variation revealed in this study, it should be accepted that only *M. transsilvanica* Schur occurs in Poland. It is present in the Sudetes, the Pieniny Mts., the Kraków-Częstochowa upland, the Gorce Mts., the Beskid Wyspowy Mts. and the Beskid Sądecki Mts. [24,93]. Based on the analyses of morphological characters, some individuals of *M. transsilvanica* from the Sudetes (signed as "*M. ciliata*"; cp. Fig. 5) should be classified as *M. transsilvanica* Schur var. *glabrata* Čelak. ex Lavr. 1940, which is characterized by glabrous leaf-sheaths and is widespread in Ukraine [68].

The condition of Sudetian populations of *M. transsilvanica*, especially those previously believed to be *M. ciliata* and studied by us, is currently very bad and their abundance is low. Competitive weakness and transformations of xerothermic communities from the *Festuco-Brometea* class are the main factors that cause the disappearance of this species in Poland [24,35,40,49]. The recently detected occurrence of unique genetic markers in marginal populations of *M. transsilvanica* considerably expands the genetic variation of the species [79] and confirms that it should be effectively preserved in situ in its whole area of occupancy in Poland.

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