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Research Article

RAD sequencing, morphometry and synecology clarify the taxonomy of the *Melica ciliata* (Poaceae) complex in France and PolandSergio Castro^{1*} , Audrey Muratet¹, Magdalena Szczepaniak² , Julie Nguefack³, and Laurent Hardion^{1*}¹Laboratoire Image Ville Environnement (LIVE), CNRS, University of Strasbourg, Strasbourg, France²W. Szafer Institute of Botany, Polish Academy of Sciences, Lubicz 4631-512 Kraków, Poland³Conservatoire Botanique d'Alsace-Lorraine, Erstein, France

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Abstract *Melica* (Poaceae) consist of about 92 species distributed across temperate regions of the world. Within section *Dalycum*, *Melica ciliata* sensu lato forms a taxonomic complex of several species and subspecies with clinal morphological variation causing conflicting identifications. To resolve taxonomic confusion, we used three complementary approaches, through molecular, morphological, and phytoecological analyses. The double-digest restriction-associated DNA markers significantly support the monophyly of three taxa: (i) the Mediterranean *Melica magnolii*, (ii) the Eurasian *Melica transsilvanica* subsp. *transsilvanica*, and (iii) the west-European *M. ciliata* subsp. *glauca*. This differentiation is corroborated by the analysis of 22 morphometric variables. Furthermore, phytoecological analysis of 221 floristic inventories revealed habitat distinctions among these taxa. Our approach of integrative taxonomy argues for a specific distinction for these three taxa, and we include a key to separate these forms. These new molecular data on the section *Dalycum*, subsection *Ciliatae*, call for further phylogenetic analyses including samples of *M. ciliata* subsp. *ciliata* and other East-Mediterranean and South African taxa.

Key words: double-digest restriction site-associated DNA sequencing (ddRAD), *Melica ciliata*, morphology, species delimitation, synecology, taxonomy.

1 Introduction

Biological systematics is the study of the historical diversification of living forms and the resulting phylogenetic relationships, with the goal of creating a formal classification that reflects this history and that would be accepted by future systematists for its biological and historical relevance (Maturana-Romesin & Mpodozis, 2000; Kellogg, 2001). However, species boundaries are sometimes difficult to define, especially in young complexes, where there are few morphological characters to differentiate taxa.

The genus *Melica* L. (Poaceae) includes about 92 species growing in the temperate zones of the world, except for Australia (Soreng et al., 2015, 2017, 2022). Among the five sections of the subgenus *Melica*, the xerophyte section *Dalycum* Dumort. from the Mediterranean basin represents a highly polymorphic group with a challenging taxonomy (Hempel, 1970, 2011). Within section *Dalycum*, the taxonomy of the subsection *Ciliatae* (Lavrenko) Bor., including *Melica ciliata* L. and Eurasian melics with regular ciliation on the two outer veins of the lemma, remains problematic due to

several morphotypes with clinal rather than discrete morphological characters, and therefore form a taxonomic complex.

As reported by Hempel (2011), systematic studies of the subsection *Ciliatae* started in 1845 with the description of *Melica nebrodensis* Parl, having unilateral panicles. In 1856, *Melica magnolii* Godr. & Gren. was described based on having long, branched, and interrupted panicles. Schultz (1862) described *Melica glauca* F.W. Schultz. in contrast with *M. ciliata* sensu Parlatore, Godron and Grenier, the latter name actually corresponding to *Melica transsilvanica* Schur. (Coste, 1937; Hempel, 2011). *Melica transsilvanica* was described by Schur (1866), based on having short, compact, and cylindrical panicles. This and other taxonomic inconsistencies led Rouy (1882) to investigate the Linnean type of *M. ciliata*, distinguish *M. transsilvanica*, and place several other species in synonymy based on morphological characters, that is, shape of the leaf blade, leaf sheath pilosity, and plant color (Husnot, 1898; Hempel, 1970). In the latest monography of *Melica*, subsection *Ciliatae* includes four species: (i) *M. ciliata*, comprising four subspecies *M. ciliata* subsp. *ciliata*, *M. ciliata*

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subsp. *magnolii* (Gren.) K. Richt., *M. ciliata* subsp. *glauca* (F.W. Schultz) Nyman, and *M. ciliata* subsp. *taurica* (K. Koch) Tzvelev. (hereafter referred to as *ciliata*, *magnolii*, *glauca*, and *taurica*, respectively), (ii) *M. transsilvanica*, with two subspecies *M. transsilvanica* subsp. *transsilvanica* and *M. transsilvanica* subsp. *klokovii* Tzvelev. (hereafter referred to as *transsilvanica* and *klokovii*), (iii) *Melica cretica* Boiss. & Heldr., and (iv) *Melica racemosa* Thunb. from South Africa (Fig. 1). Phylogenetic relationships of this complex and the monophyly of subsection *Ciliatae* remain uncertain (Hempel, 2011).

Phylogenetic studies using different molecular markers have provided insights into the *M. ciliata* complex (Tyler, 2004; Szczepaniak & Cieślak, 2006, 2007, 2009, 2011; Szczepaniak, 2013). Based on allozyme polymorphism, Tyler (2004) showed a high genetic variability, mostly among populations, delineating geographic groups with poor taxonomic support, except for Mediterranean *magnolii* and Eurasian *transsilvanica*. Based on the *trnL-trnF* markers from all European *Ciliatae* taxa, except *M. cretica*, Szczepaniak (2013) found low genetic diversity restricted to Mediterranean peninsulas, possibly acting as interglacial refugia, and only one haplotype among different taxa in temperate Europe. The distribution of these haplotypes does not support current taxonomy, although it appears that some haplotypes could be the Mediterranean *magnolii* in the Iberian Peninsula. Based on amplified fragment length polymorphism (AFLP) fingerprints, Szczepaniak & Cieślak (2011) found *transsilvanica* and *ciliata* to be genetically distinct (including *glauca* in synonymy) and this was supported

by a morphometric analysis. This and other AFLP-based studies found genetic patterns of high variation among populations and low diversity within populations, which are explained by habitat fragmentation, self-fertilization, and the resulting low gene flow (Szczepaniak & Cieślak, 2006, 2007, 2009). However, the shared occurrence of rare AFLP alleles among geographically distant populations (Szczepaniak & Cieślak, 2009) and the wide distribution of some allozyme alleles (Tyler, 2004) suggest that gene flow was effective in the past.

Other studies focusing on soil solution chemistry and phytosociology highlight ecological and habitat characteristics specific to some taxa in the *M. ciliata* complex (Tyler, 1993; Szczepaniak, 2007). They often belong to xerothermic plant communities growing on outcrops of limestone and ultrabasic bedrock, or to steppe communities in the eastern range of their distribution (Hempel, 2011; Szczepaniak, 2013). These types of floristic assemblages have a scattered distribution in Europe (Pärtel et al., 2005) and they are threatened by contemporary changes in agropastoral practices (i.e., crop and pasture abandonment; Pärtel et al., 2005; Palmero-Iniesta et al., 2020). Both *glauca* and *transsilvanica* are listed as vulnerable, endangered, or protected in different regions of France (e.g., Vangendt et al., 2014).

Since the taxonomic clarification of the subsection *Ciliatae* by Hempel (2011), phylogenetic relationships among the lineages have not been resolved. Plant systematics has benefited from rapid advances in DNA sequencing, making it possible to obtain billions of base pairs of sequences in a short time and at a low cost (Soltis et al., 2013). The

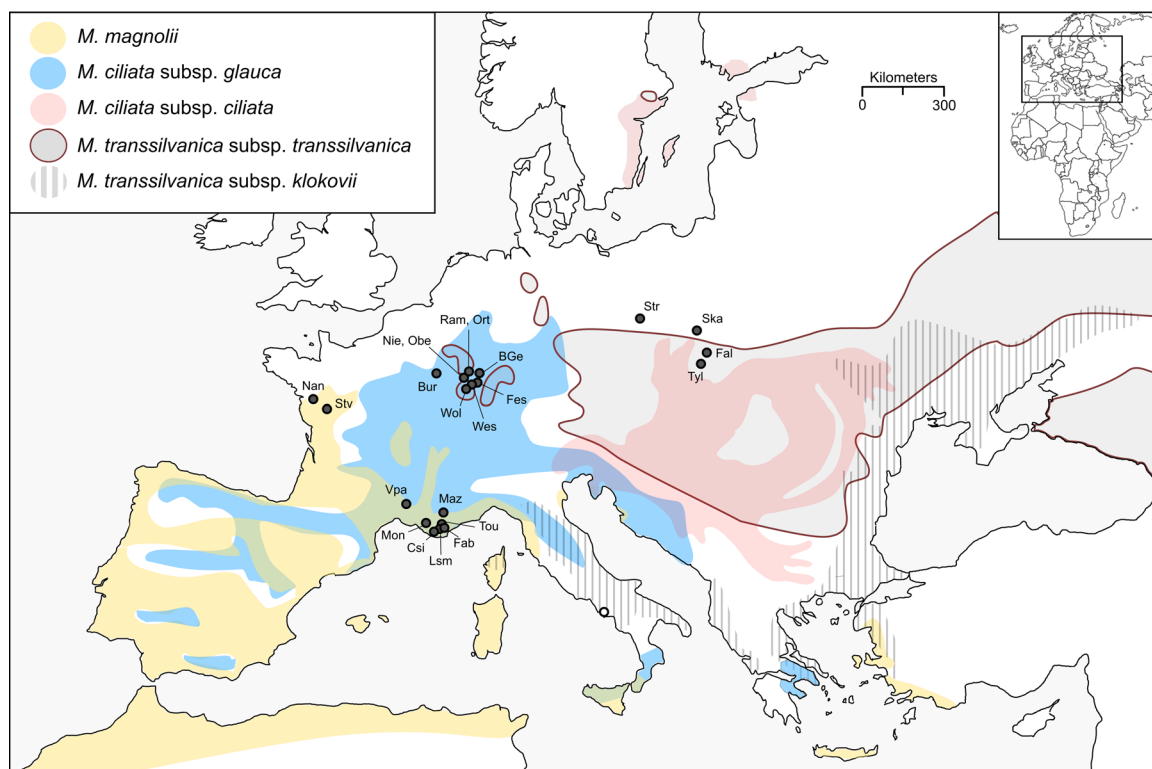


Fig. 1. Distribution ranges (based on Hempel, 2011) of *Melica ciliata* complex taxa and sampling locations. Black dots show locations analyzed in RAD-seq and morphometry, the open dot only in morphometry. In the rest of the subsection *Ciliatae*, the distributions of *M. ciliata* subsp. *taurica*, *Melica cretica*, and *Melica racemosa* are not shown.

restriction-site-associated DNA sequencing (RAD-seq) method, through the massive single-nucleotide polymorphism (SNP) discovery, has become a powerful tool for inferring well-resolved phylogenies once unresolved in the light of Sanger sequencing (Paetzold et al., 2019; Appelhans et al., 2020; Mu et al., 2020). Also, combined morphology cluster analyses and next-generation sequencing techniques have proven to be of high value in delimiting species from different plant families (Ding et al., 2018; Wang et al., 2020) and in searching for intraspecific divisions (Avendaño-González et al., 2019; Frajman et al., 2019), as the two approaches usually yield congruent results.

To resolve the taxonomy and establish boundaries among three taxa of subsection *Ciliatae*, two subspecies of *M. ciliata*, Mediterranean *magnolii* and Western-European *glauca*, and *M. transsilvanica*'s autonym, Eurasian *transsilvanica*, we developed an integrative approach based on (i) thousands of SNPs found with double-digest RAD-seq, (ii) morphological characters, quantitative and discrete, and (iii) habitat distinction based on phytoecological analyses. These results were combined to test Hempel's (2011) taxonomic assumptions on the *M. ciliata* complex and to confirm patterns of genetic differentiation among populations.

2 Material and Methods

2.1 Plant material

One to six individuals were collected for each of the 22 populations of *glauca*, *magnolii*, and *transsilvanica* from NW, NE, and SE France and South Poland (Fig. 1; Table S1). In addition, *Melica uniflora* Retz., *Melica minuta* L., *Melica nutans* L., and *Melica amethystina* Pourr. were sampled as putative outgroups. To collect material in the wild, we used species distribution data from the SILENE database and from the database of the Société Botanique d'Alsace. Voucher specimens were deposited at the Herbarium of the University of Strasbourg (STR). For morphometric analyses, we included an additional specimen identified as *klokovii* from Herbarium collections and excluded one population from RAD-seq analyses (Saint-Varent, "Stv," Fig. 1; Table S1) because of damaged vegetative and floral structures. As a prior taxonomic assumption, we reidentified every sample and herbarium material based on the determination key of Hempel (2011) for *glauca*, *magnolii*, *transsilvanica*, and *klokovii*.

2.2 DNA extraction, library preparation, and sequencing

Approximately 40 mg of silica dried leaf material was selected for DNA extraction using the Qiagen Dneasy Plant Mini Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. The DNA concentration was measured using the Qubit fluorometer with the dsDNA HS Assay Kit (Thermo Fisher Scientific, Darmstadt, Germany), and then adjusted to 30 ng/μL. Microsynth Ecogenics (Balgach, Switzerland) generated RAD libraries using the *EcoRI*-*MseI* enzyme combination using a method following Peterson et al. (2011) including the use of sample-specific barcodes. Samples were sequenced on an Illumina NextSeq platform to produce 75-bp single-end reads. We used the SimRAD R-package version 4.0.2 (Lepais & Weir, 2014) to estimate the total number of reads necessary to achieve a mean sequencing

depth of 12 reads per locus per sample. With a simulated haploid genome of 3969.58 Mbp and 46.5% of GC content as documented for *Melica ciliata* (Šmarda et al., 2019), we calculated the number of fragments produced by performing an *in silico* enzyme digestion using the function `insilico.digest()`. Then, the function `size.select()` was used to simulate the size selection step performed in double-digestion RAD-seq procedures. This estimation enabled us to sequence about 400 000 000 reads for this project.

2.3 RAD locus assembly and SNP calling

The program *ipyrad* v.0.7.21 (Eaton, 2014) was used to demultiplex raw reads, remove adapter sequences, trim bases with Phred scores < 31, and remove reads shorter than 50 bp after trimming. Some samples were removed based on their limited number of reads. Trimmed reads were assembled *de novo* using a cluster threshold of 90, a minimum of four samples per locus, and a maximum number of two alleles per locus for consensus reads. These analyses were conducted at the high-performance computing center of the University of Strasbourg.

After several preliminary tests, four assemblies were generated with the already described parameters but differing in the number of samples and taxa included (Table 1). The first assembly (**a1**) contained a reduced number of samples per population to focus on *M. ciliata* complex taxa phylogenetic support, with *M. minuta* and *M. nutans* as the outgroup. A second assembly (**a2**) included samples of *Melica amethystina* to verify its hybrid origin between *glauca* and *M. minuta*, as suggested by our preliminary observations and literature (Hempel, 2011). The last two assemblies included all available individuals from all populations of the *M. ciliata* complex (**a3**) or from *glauca* populations only (**a4**) to analyze taxa molecular differentiation and among-population genetic differentiation, respectively. In assemblies (**a2**), (**a3**), and (**a4**), a final filtering was performed by first removing individuals with >90% missing data and then SNPs with >5% missing data to avoid biased results when analyzing allele frequencies (Table 1). This last filtering was performed using the `vcfR` R-package version 4.0.2 (Knaus & Grunwald, 2017).

2.4 RAD-seq phylogenetics, genetic structure, and admixture analyses

Maximum likelihood (ML) analyses on assembly (**a1**) were conducted using IQTREE with 10 000 bootstrap replicates and the nucleotide substitution model TIM2 + I + G. Species genetic structure and admixture were inferred based on individual ancestry coefficients, and the choice of the most suitable number of ancestral populations (K) was based on the cross-entropy criterion, using assembly (**a2**). To evaluate molecular distinction of the three taxa from the *M. ciliata* complex and the genetic differentiation among *glauca* populations, we conducted analyses of molecular variance (AMOVA) with assemblies (**a3**) and (**a4**), respectively. Analyses were performed using `poppr` (Kamvar et al., 2014), `ade4` (Dray & Dufour, 2007), `LEA` (Frichot & François, 2015), and `adegenet` (Jombart & Ahmed, 2011) R-packages version 4.0.2.

Table 1 Taxonomic composition of assemblies, number of retained SNPs, individuals, and percentage of missing data before and after final filtering

Assembly	Taxa	Before filtering			After filtering		
		Individuals	SNPs	Missing data	Individuals	SNPs	Missing data
a1	<i>M. magnolii</i> <i>M. ciliata</i> subsp. <i>glauca</i> <i>M. transsilvanica</i> subsp. <i>transsilvanica</i> <i>M. minuta</i> <i>M. nutans</i>	25	294 231	62.40%	-	-	-
a2	<i>M. magnolii</i> <i>M. ciliata</i> subsp. <i>glauca</i> <i>M. transsilvanica</i> subsp. <i>transsilvanica</i> <i>M. minuta</i> <i>M. amethystina</i>	23	234 316	55.02%	23	4159	0.69%
a3	<i>M. magnolii</i> <i>M. ciliata</i> subsp. <i>glauca</i> <i>M. transsilvanica</i> subsp. <i>transsilvanica</i>	60	333 039	69.49%	58	7375	0.65%
a4	<i>M. ciliata</i> subsp. <i>glauca</i>	30	96 302	43.73%	30	23 044	0.86%

Assembly (a1) was not submitted to additional filtering in order to keep a maximum of polymorphisms in phylogenetic analyses; SNP, single-nucleotide polymorphism.

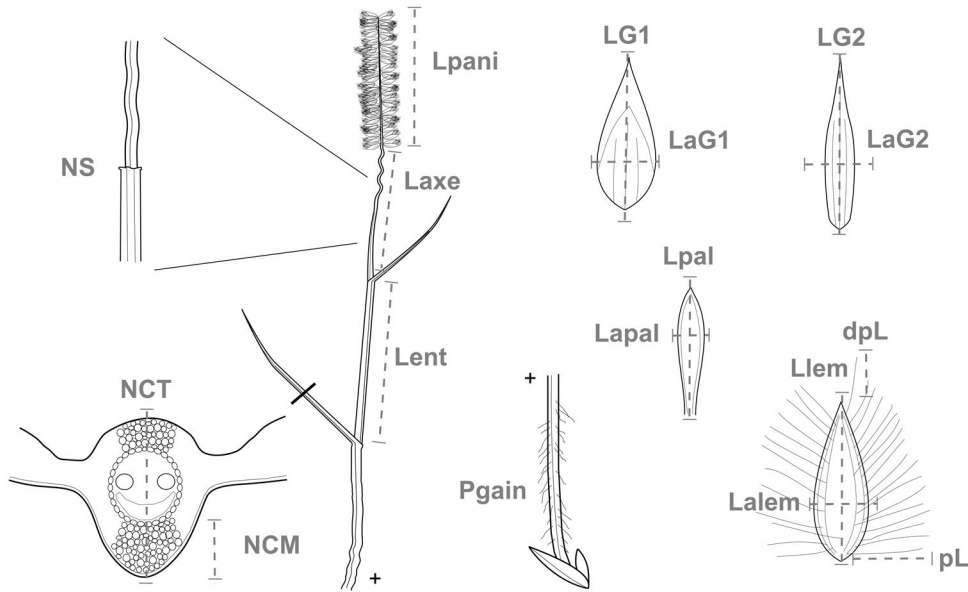


Fig. 2. Description of morphometric quantitative and qualitative variables measured. Quantitative variables: length of the lower glume (LG1), width of the lower glume (LaG1), length of the upper glume (LG2), width of the upper glume (LaG2), length of the lemma (Llem), width of the lemma (Lalem), length of lemma lateral hairs (pL), length of lemma apical hairs (dpL), length of the palea (Lpal), width of the palea (Lapal), length of the panicle (Lpani), length of the axis (Laxe), length of the first internode (Lent), number of sterile nodes on the axis (NS), distance between the mestome sheath and the abaxial leaf side (NCM), and leaf midrib width (NCT). Qualitative variables: lower leaf sheath hairiness (Pgain); leaf adaxial side hairiness (Pfeui) is not shown. Ratios: LG1/LG2, LG1/Llem, Lpani/Laxe, NCM/NCT.

2.5 Morphometric analyses

A set of 16 quantitative traits, two qualitative traits, and four ratios (Fig. 2; Table S3) were selected based on plant keys (Husnot, 1898; Coste, 1937; Hempel, 2011; Tison & De Foucault,

2014; Jäger, 2017), morphological studies (Szczęśniak, 2007; Szczepaniak & Cieślak, 2011), and herbarium observations. Sizes of vegetative and reproductive structures were measured on dry material from 56 individuals with a micrometer using a light

microscope or a ruler. Three spikelets per panicle were measured for quantitative variables of the reproductive organs. Leaf cross sections were made on the second uppermost leaf blade. The range of morphological variability of *glauca*, *magnolii*, and *transsilvanica* populations was examined using univariate statistics (minimum, maximum, first quartile, third quartile, and arithmetic mean).

One-way analysis of variance (one-way ANOVA) was used to assess the significance of differences among the three taxa. If Shapiro–Wilk tests did not confirm residuals of a normal distribution, the nonparametric Kruskal–Wallis test was used. Bonferroni correction and Wilcoxon tests were used to identify significant differences among the three taxa. Morphometric affinities were represented in a principal component analysis (PCA) to group population samples without *a priori* taxonomic assignment. Then, a discriminant analysis (DA) was carried out to identify the most explicative variables of our *a priori* taxonomic assumption by correlating them with coordinates (Hardion et al., 2012). Model accuracy of DA and the κ coefficient were established with a confusion matrix using the function `confusionMatrix()` of *caret* R-package (Kuhn, 2008). Herbarium accession assigned as *klokovii* was included only in the PCA. All these analyses were performed using *vegan* (Oksanen et al., 2016), *ade4* (Dray & Dufour, 2007), and *caret* (Kuhn, 2008) R-packages version 4.0.2.

2.6 Phytoecological analyses

Ecological distinctions among the three taxa studied were assessed by regarding the beta diversity of 221 phytosociological inventories (162 for *glauca*, 27 for *magnolii*, and 32 for *transsilvanica*) from France National Botanical Conservatories (“Mediterranean,” “Nord-Est,” and “Bassin Parisien”) and from Pieniny and Ojcow National Parks studies (Kaźmierczakowa, 2004; Sołtys-Lelek, 2009; Janicka, 2013). We analyzed 1077 species (895 associated with *glauca*, 296 associated with *magnolii*, and 237 associated with *transsilvanica*). All species synonyms were checked using the French taxonomic reference source TAXREF v.14.0. (Gargominy et al., 2021).

First, to assess taxonomic differences among *glauca*, *magnolii*, and *transsilvanica* communities, a nonmetric multidimensional scaling (nMDS) was performed based on the Jaccard similarity matrix of presence/absence data. Only similarities among communities located in the same geographic region (Continental or Mediterranean) were studied to depict differences associated with habitat conditions rather than the geographic distribution of species. Significance of observed grouping patterns was determined using the nonparametric analysis of similarities test (ANOSIM). Species contributing most to the dissimilarity among ordination clusters of communities were investigated using Similarity percentage analysis (SIMPER).

Then, we characterized *glauca*, *magnolii*, and *transsilvanica* habitats by calculating their community species mean Ellenberg’s indicator for Light, Moisture, pH, Temperature, and Nitrogen together with the community mean species maximum height and the proportion of species presenting a given biological trait (autogamy, pluriannual life span, and biotic-mediated pollination). Those nine variables were subjected to PCA and DA. Kruskal–Wallis and Wilcoxon tests were used to assess the significance between groups of surveys for each variable. R-package TR8 version 4.0.2

(Kattge et al., 2011) was used to obtain species biological and ecological data and *vegan* (Oksanen et al., 2016), *ade4* (Dray & Dufour, 2007), and *caret* (Kuhn, 2008) were used to perform statistical analyses.

3 Results

3.1 Raw data and assembly

Discarding underrepresented individuals, Illumina sequencing provided between 1.5 and 9.6 M reads per sample, with an average of 5.24 M reads per sample. After quality filtering, the number of loci and SNPs retained in the different assemblies were 111 887, 294 231 (**a1**); 77 265, 234 316 (**a2**); 149 854, 333 039 (**a3**); and 99 470, 96 302 (**a4**). The number of SNPs and the percentage of missing data decreased considerably after final filtering (Table 1).

3.2 RAD-seq phylogenetics, genetic structure, and admixture analyses

The ML tree fully supported three clades (100% BS), each one corresponding to one monophyletic taxon (Fig. 3A). The Western European *glauca* and the Eurasian *transsilvanica* are sister, while the Mediterranean *magnolii* diverged earlier. In hierarchical AMOVA, 57.43% of the total variation was assigned to the differentiation among *glauca*, *transsilvanica*, and *magnolii* ($F_{CT} = 0.57$, $P < 0.01$). The second AMOVA revealed strong differentiation among *glauca*’s populations ($F_{ST} = 0.61$, $P < 0.01$) (Table S2). Admixture structure analysis distinguished four clusters based on the cross-entropy criterion (Fig. 3B), three of them corresponding to *glauca*, *transsilvanica*, and *magnolii*, and one to *Melica minuta*. The four individuals belonging to *Melica amethystina* showed considerable admixture between *glauca* and *M. minuta* (Fig. 3C).

3.3 Morphometric analyses

Among the 16 quantitative morphometric variables, 12 showed significant differences among at least two taxa and the four ratios too. The main differences were found in *magnolii* presenting narrow floral pieces, longer panicles, longer lemma apical hairs, and lacking sterile nodes under the panicle. Also, equal to slightly unequal glumes were a characteristic trait of *glauca* and only *transsilvanica* presented hairy basal leaf sheaths and prominent leaf midrib protrusion (Fig. S2).

The first two axes of the PCA (25.2% and 19.4% of inertia) distinguish three groups with a slight overlap in the multivariate space (Fig. 4A). The first axis separated *magnolii* from *transsilvanica* and was mainly correlated to the width of the upper glume (LaG2; $r = 0.72$), the width of the lower glume (LaG1; $r = 0.62$), and the width of the lemma (Lalem; $r = 0.55$). The second axis separated *glauca* from the other two taxa and was mainly correlated with the ratio of glume lengths (LG1/LG2; $r = 0.73$). The sample previously identified as *klokovii* was placed within the *transsilvanica* variation range. In the DA, leaf midvein protrusion (NCM/NCT; $r = -0.83$) and leaf sheath hairiness (Pgain; $r = -0.78$) were the most correlated variables in the first axis, while the length of lemma apical hairs (dpL; $r = -0.79$) and the number of sterile nodes on the panicle axis (NS; $r = 0.74$) were the most correlated variables in the second axis (Fig. 4B). Model

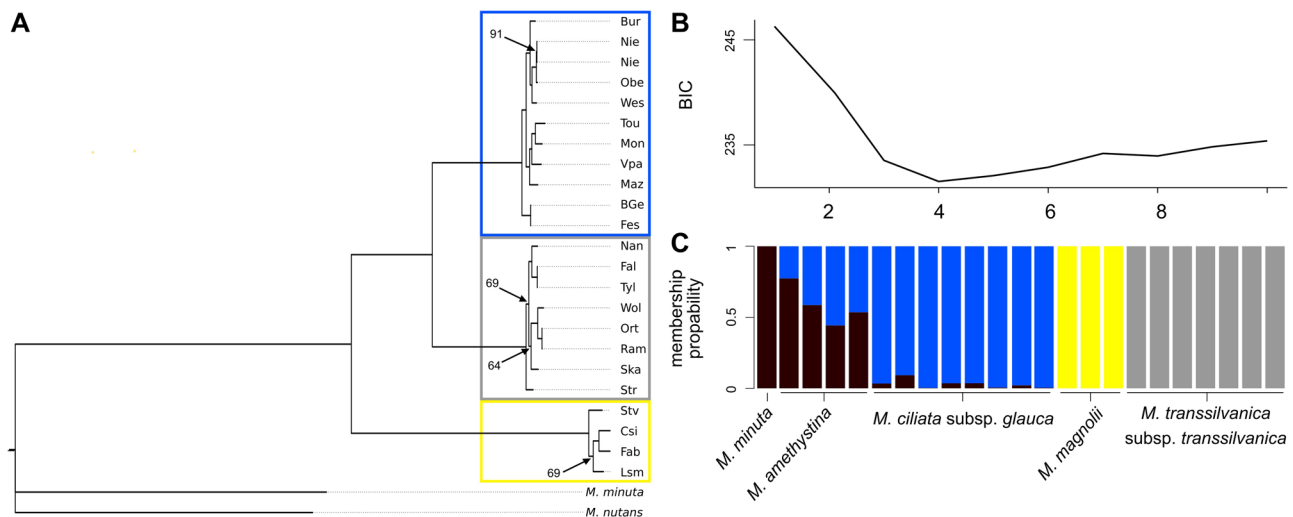


Fig. 3. Phylogenetic relationships, genetic structure, and admixture of the *Melica ciliata* complex resolved by double-digest RAD-seq data analyses. **A**, Molecular phylogenetic inference by maximum likelihood based on 111 887 loci and 294 231 SNPs from assembly (a1). Locality codes are shown at the tree tips. Nodes where bootstrap values (BS) are not shown represent clades with maximum support. Blue box, *M. ciliata* subsp. *glauca* populations; gray box, *Melica transsilvanica* subsp. *transsilvanica* populations; and yellow box, *Melica magnolii* populations (Table S1). **B**, Determination of the number of ancestral populations ($K = 4$, X axis) that best explains genomic data by means of the cross-entropy criterion (Y axis). **C**, Admixture clustering analysis based on 23 individuals and 4159 SNPs from assembly (a2).

accuracy and the κ coefficient were maximal (model accuracy: 1; κ coefficient: 1) and thus our taxonomic assignments were validated by the model.

3.4 Phytoecological analyses

nMDS showed clear taxonomic grouping patterns (Fig. S3), confirmed by the ANOSIM test, with *glauca* communities dissociated from *transsilvanica* communities in the continental region (global $R = 0.303$; $P = 0.001$), and from *magnolii* communities in the Mediterranean region (global $R = 0.381$; $P = 0.001$). There were significant differences among the three groups of plant assemblies for all Ellenberg values and in the percentage of species with biotic pollination. The *magnolii* communities were significantly different from the other two groups in species maximal height, percentage of pluriannual species, and percentage of autogamy, and had higher Ellenberg values of temperature and light (Fig. S4). Also, *transsilvanica* communities had higher mean values of moisture and nitrogen, while *glauca* communities were the driest and had the lowest values of nitrogen.

Multivariate analyses of habitat distinction showed segregation among the three taxa communities (Fig. 5A). The first PCA axis (PC1; 36.3% of inertia) was primarily correlated with the Ellenberg light index ($r = 0.82$) and the Ellenberg moisture index ($r = 0.58$), while the second axis was correlated with the percentage of pluriannual species ($r = 0.56$) and the percentage of autogamous species ($r = 0.52$). In the DA, many *glauca* communities are intermediate between *magnolii* and *transsilvanica* variability range (Fig. 5B). The first axis reflects habitat exposition, as it is mainly correlated with light ($r = -0.71$) and temperature ($r = -0.88$), while the second axis reflects soil conditions: soil nitrogen ($r = -0.73$), pH ($r = 0.66$), and moisture ($r = -0.65$)

(Fig. 5B). Model accuracy and the κ coefficient were high (model accuracy: 0.88; κ coefficient: 0.73), but 26 communities were wrongly assigned to groups.

4 Discussion

4.1 Taxonomic implications

The taxonomy of subsection *Ciliatae* has always been a difficult issue. In addition to earlier synonymies and conflicting opinions (Rouy, 1882, 1885; Hempel, 1970), molecular studies with geographically broad sampling raise more questions than answers. Geographic distributions of allozyme variability outline genetic groups that were not congruent with taxa distributions, except for *magnolii* and *transsilvanica* (Tyler, 2004). Subsequently, Hempel (2011) identified two of these new genetic groups as *klokovii* and *glauca*, changing the taxonomic treatment of *glauca* from *ciliata*'s synonym to subspecies, arguing morphological and biogeographic distinctions (see Section 4.2). In contrast, this distinction was not congruent with further morphometric analyses and AFLP genotypes clustering as individuals from populations within the geographic range of *glauca* and *ciliata* did not particularly dissociate (Szczepaniak & Cieślak, 2011). Our sampling did not include individuals of *Melica ciliata* subsp. *ciliata* from SE Sweden and Eastern Europe. Therefore, we must refer to the last updated taxonomic study considering *glauca* and *ciliata* as subspecies of *M. ciliata* (Hempel, 2011). Additional studies are needed to verify their phylogenetic support and the degree of differentiation between these two taxa.

Our results reinforce the recognition of three species with secondary properties that confirm lineage separation: reciprocal monophyly (Fig. 3A), phenetic distinguishability

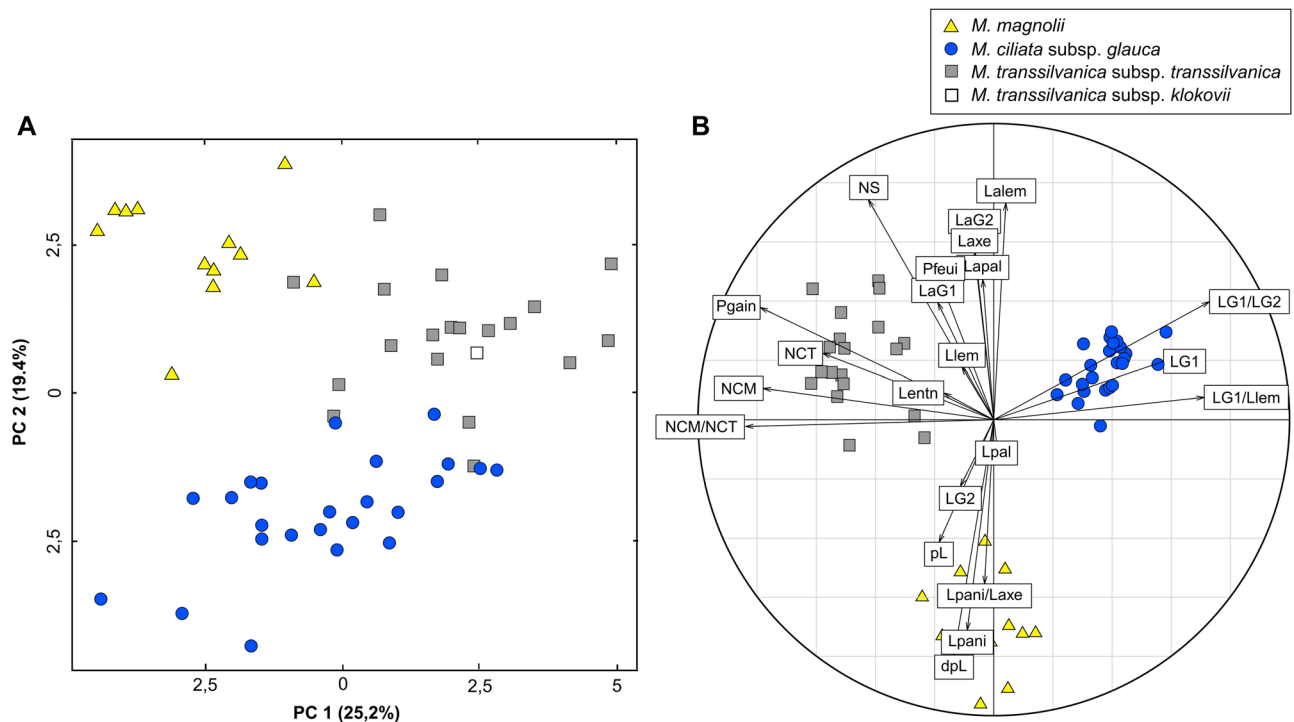


Fig. 4. Morphological differentiation among *Melica ciliata* complex taxa based on 18 morphometric variables and four ratios. **A**, First plan of the principal component analysis showing the distribution of the 56 individuals in the multivariate space. **B**, Discriminant Analysis determining the best explicative variables for our taxonomic hypotheses (Model accuracy: 1; κ coefficient: 1). dpL, length of lemma apical hairs; LaG1, width of the lower glume; LaG2 width of the upper glume; Lapal, width of the palea; Lpal, length of the palea; Lpani, length of the panicle; LG1, length of the lower glume; LG2, length of the upper glume; Lalem, width of the lemma; Llem, length of the lemma; Laxe, length of axis; Lentn, length of the first internode; NCM, distance between the mestome sheath and the abaxial leaf side; NCT, leaf midrib width; NS, number of sterile nodes on the axis; Pfeui, hairy leaf abaxial side; Pgain, hairy lower leaf sheath; pL, length of lemma lateral hairs. Blue circles, *M. ciliata* subsp. *glauca*; gray squares, *Melica transsilvanica* subsp. *transsilvanica*; yellow triangles, *Melica magnolii*; white square, *M. transsilvanica* subsp. *klokovii*.

(Fig. 4), and ecological differences (Fig. 5) (de Queiroz, 2013). Consequently, the species status of *Melica transsilvanica* is supported in our study and, contrary to Hempel's taxonomic treatment, *magnolii* also seems appropriate for recognition as a species.

The origin of *Melica amethystina* was suggested by Hempel (2011) to be the result of the hybridization of *Melica minuta* (section *Melicella*) with *glauca*, morphologically distinct in having the ciliate outer vein of the lemma, as used to differentiate members in subsection *Ciliatae*. Our results support this hybridization hypothesis between two different sections of subgenus *Melica* (Fig. 3C). In addition, *M. amethystina* is the only European tetraploid with a chromosomal number of $2n = 36$ similar to South American *Melica violacea* Cav. (CCDB database, Rice et al., 2014).

4.2 Morphological differentiation and Hempel's phylogenetic hypothesis

Several determinant morphological traits that are taxon-specific could be found in other taxa (Szczęśniak, 2007; Szczepaniak & Cieślak, 2011). This is the case for leaf sheath pilosity, occurring in *transsilvanica*'s Central European distribution, but lacking in the

remaining range (Eastern Europe and Western Asia), curiously when *ciliata* is absent (Hempel, 2011). Most of our *transsilvanica* samples (15 of 19, 79%) had a partially pilose leaf sheath even if this character was unobservable on herbarium samples (e.g., hair disarticulation with age). The unique *klokovii* sample has densely hairy leaf sheaths, while both *magnolii* and *glauca* have glabrous leaf sheaths. The polymorphism of this trait was already reported for *transsilvanica* in Poland (Szczęśniak, 2007). Also, a prominent leaf midrib is a characteristic feature of *transsilvanica* (Szczęśniak, 2007; Hempel, 2011), which is confirmed in our study (Figs. 4B, S2).

The glume length ratio is historically cited in European floras as a crucial characteristic in section *Dalycum* (Husnot, 1898; Coste, 1937; Tison & De Foucault, 2014). Hempel reported that this character could be variable within species between regions, with a progressive reduction of the lower glume length along a west to east gradient in all *M. ciliata* complex species. Based on our sampling, we showed a clear glume ratio distinction for *glauca*, presenting almost equal lower and upper glume lengths, while *transsilvanica* and *magnolii* glumes are clearly unequal (Fig. S2).

The presence of nodes under the panicle and other panicle reductions could be considered as derived in the *M. ciliata*

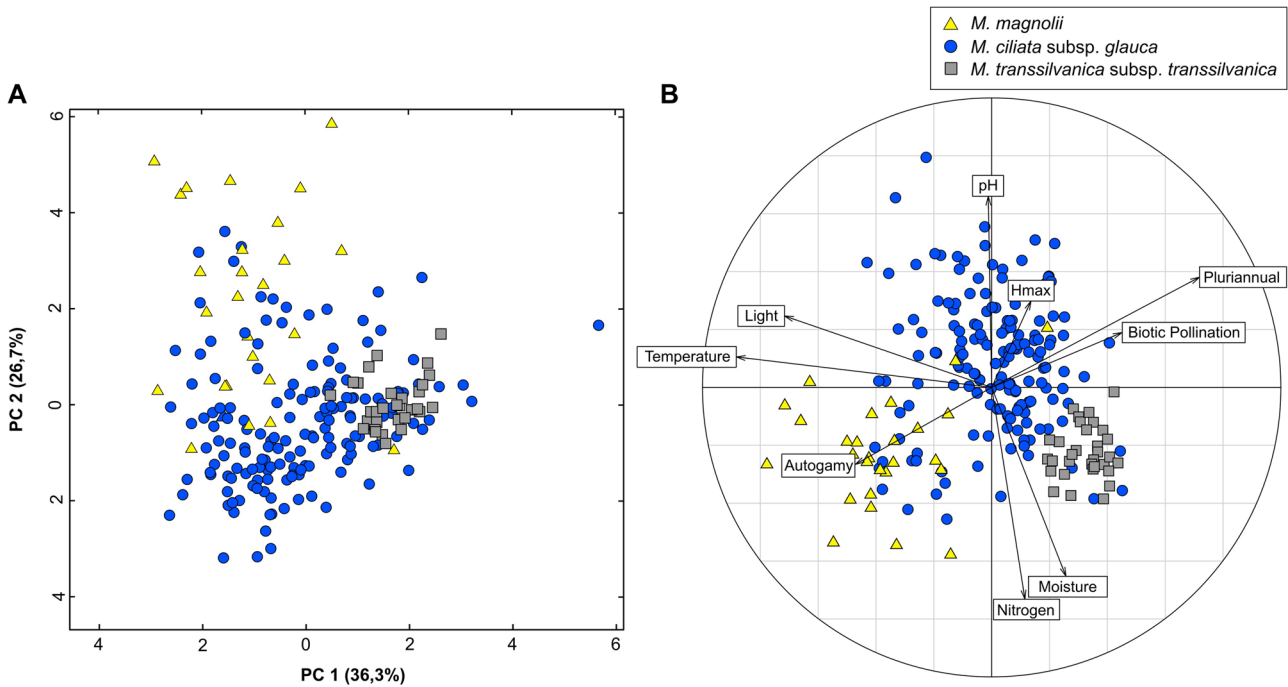


Fig. 5. Habitat distinction among *Melica ciliata* complex taxa. **A**, First plan of the principal component analysis showing the distribution of the 221 communities in the multivariate space. **B**, Discriminant analysis determining the best explicative variables for communities habitat distinction (model accuracy: 0.88; κ coefficient: 0.73). Moisture, Ellenberg indicator for Moisture; pH, Ellenberg indicator for pH; Light, Ellenberg indicator for Light; Nitrogen, Ellenberg indicator for Nitrogen; Temperature, Ellenberg indicator for Temperature; Hmax, species mean maximal height; Pluriannual, community percentage of pluriannual species; Biotic Pollination, community percentage of species with biotic pollination; and Autogamy, community percentage of autogamous species. Blue circles, *M. ciliata* subsp. *glauca*; gray squares, *Melica transsilvanica* subsp. *transsilvanica*; and yellow triangles, *Melica magnolii*.

complex (Hempel, 1970, 2011). In our sampling, only *magnolii* lacked nodes on the panicle axis and had longer panicles (Fig. S2). This result agrees with Hempel's statement of *magnolii* as the ancestral state within the clade, which is congruent with its early divergence in our phylogenetic tree (Fig. 3A). However, some individuals from *transsilvanica*'s Central European populations occasionally lack nodes on the axis (Hempel, 2011) as well as from France's easternmost *glauca* populations (author observations). Other morphological characteristics proper of this ancestral state are a straight panicle axis in contrast to the derived "wavy" panicle axis, and long, branched panicles without reductions in spikelet abundance (Hempel, 2011).

Our morphometric results coincide with Hempel's taxonomy, but our phylogeny does not support his phylogenetic hypothesis. He hypothesized two separate lineages favoring a biogeographical scenario of independent and different northern interglacial migrations: a central European lineage composed of Baltic and Balkan populations of *ciliata* closely related to *transsilvanica*, both taxa showing morphological affinities such as prominent leaf midrib protrusion, unequal glume length, and glume coloration (not studied here), dissimilar to *magnolii* and *glauca*, with the leaf midrib not protruding and equal glumes, both forming a Mediterranean–Western European lineage. In contrast, our results indicate that *magnolii* and *glauca* have different glume ratios and different degrees of leaf midrib protrusion

(Fig. S2). Furthermore, our phylogenetic tree suggests that *glauca* and *transsilvanica* are sister, although this relation could vary with the addition of populations from the remaining *Ciliatae* taxa.

Hempel's monograph of *Melica* (2011) constitutes the most complete and therefore relevant morphological, bibliographical, and naturalistic work, and we disapprove of its poor consideration in current taxonomy. The outstanding number of taxa described in and evolutionary hypotheses cited including distribution ranges and morphological keys should serve as a basis for future taxonomic research on Eurasian *Melica* taxa (Fig. S1).

4.3 Habitat distinction among *magnolii*, *glauca*, and *transsilvanica*

Within subsection *Ciliatae*, most taxa are fully xeromorphic, growing in early succession soils, often associated with xerothermic communities of the *Festuco-Brometea* class (Hempel, 2011). Alternatively, other taxa like *transsilvanica* present mesomorphic related structures and inhabit grasslands (e.g., the *Sedo-Scleranthetalia* order), thermophilous fringes (e.g., the *Trifolio-Geranietea* class), and shrub communities (e.g., the *Rhamno-Prunetea* class) [Szcześniak, 2007]. Habitat distinctions are common among the members of the *M. ciliata* complex.

In the three-fold comparison, the habitat of *magnolii* communities was the warmest and most exposed to light.

Furthermore, *magnolii* communities were characterized by the presence of low-height, autogamous species, together with the lower presence of pluriannuals (Fig. S3). High temperatures and short life span, low biomass investment, and autogamy are features common to species that occupy disturbed habitats and ruderal sites (Grime, 1977; Razanajato et al., 2016). Consequently, our results are in agreement with previous observations on *magnolii* growing in ruderal and urban environments (Szczepaniak, 2013). Concerning differences in taxonomic composition, *glauca* and *magnolii* communities are indicative of a segregation pattern (Fig. S3), with some *magnolii*-associated species like *Lobularia maritima* (L.) Desv. and *Ephedra distachya* L. (Table S4) in agreement with the predominant coastal distribution.

Habitat features for *glauca* communities are low soil moisture and nitrogen (Fig. S3). This reflects drier conditions and lower organic matter in soils as the habitat for *glauca* is restricted to early succession stage soils, that is, screes, gravel, and rocky sites (Hempel, 2011). The wider ecological amplitude observed for *glauca* in the DA may be caused by erroneous taxonomic identifications of *glauca*, *magnolii*, and *transsilvanica* in some inventories, thus producing an intermediate pattern in the multivariate space (Fig. 5B).

Finally, *transsilvanica*'s habitat has the highest amounts of soil moisture and nitrogen, and low temperature and light exposure (Fig. S4), all of this in agreement with its association with secondary communities. Contrary to our expectations, species associated with *transsilvanica* were not the tallest. Because of the diversity of environments found in natural outcrops, *transsilvanica* occurs in phytocoenoses of different natural successions communities, but with low frequency in extremely hot and dry habitats or in competitive grasslands (Szczepaniak, 2007). In thermophilous secondary herbaceous and shrub communities, it has been reported that *transsilvanica* develops until its abundance decreases due to increased shrub density (Szczepaniak, 2007).

4.4 Genetic and morphological consequences of ecological strategy, conservation and management

In the *M. ciliata* complex, low genetic diversity within populations and high differentiation among them were determined (Tyler, 2004; Szczepaniak & Cieślak, 2006, 2007, 2009), and this is confirmed here by high F_{ST} values (Table S2). Consequently, facultative apomixis or even a predominantly autogamous reproduction mode has been suggested for the *M. ciliata* complex (Tyler, 2004; Szczepaniak & Cieślak, 2007), the latter also supported by features in floral structures, that is, spikelets comprising one fertile floret and two or three diminished florets at the apex, small anthers, and cleistogamous flowers (Szczepaniak & Cieślak, 2007).

Selfing and cleistogamy are both common syndromes in grasses than in any angiosperms, with considerable occurrence in stress-tolerant taxa (Campbell et al., 1983). In this ecological context, both syndromes are pointed to co-evolve with reductions in reproductive traits and thus favoring efficient resource allocations (Campbell et al., 1983; Vallejo-Marín & Barrett, 2009; Mazer et al., 2020). Likewise, the high genetic differentiation among the *M. ciliata* complex populations, driven by selfing, coupled with

derived morphological traits such as narrow, short panicles with few spikelets, appear to be adaptations to xeric habitats.

Our results provide critical information for the morphological recognition of *glauca*, *magnolii*, and *transsilvanica* as species and additional elements on their ecological differentiation. In France, main conservation concerns and efforts should focus on *transsilvanica*'s westernmost populations as they are scarce, small, and highly isolated. The discovery of a new record for *transsilvanica* in an anthropized habitat far west from its continuous range (Nantes, "Nan," Fig. 1; Table S1) probably constitutes an exotic introduction of horticultural origin.

4.5 Conclusions

This study provides new information about the taxonomy of section *Dalycum*, subsection *Ciliatae*. To the best of our knowledge, useful morphological characteristics for taxa recognition like length of lemma apical hairs and floral piece width are cited here for the first time, and some others are uncommon in determination keys, that is, number of nodes on the panicle axis and leaf blade midvein protrusion. We propose a species status for *magnolii* and *transsilvanica* based on genetic, morphological, and ecological distinctions. The phylogenetic analysis of RAD-seq data suggests a closer relation between *glauca* and *transsilvanica*; however, to clarify the phylogenetic relations of subsection *Ciliatae* and to confirm its monophyly further analyses are required. The taxa from the *M. ciliata* complex harbor low intrapopulation genetic diversity and restricted interpopulation gene flow, probably explained by their autogamy, the fragmentation of their habitat, or their evolutionary divergence.

5 Key to Separate *Melica magnolii*, *Melica ciliata* subsp. *glauca*, and *Melica transsilvanica* subsp. *transsilvanica* in France and Poland

- 1a. Panicle axes with 1–3(–4) lower nodes unbranched, panicles 5.3–8.7 cm long; hairs on the apex of the lemma ≤ 1 mm..... 2
- 1b. Panicle axes with ≥ 1 lower nodes unbranched, panicles 11.3–13.5 cm long; hairs on the apex of the lemma > 1 mm..... *M. magnolii* Godr. & Gren.
- 2a. Lower glume 2/3 as long as the upper glume; leaf blade midvein prominent; panicles cylindrical and dense, basal leaf sheaths hairy..... *M. transsilvanica* subsp. *transsilvanica* Schur.
- 2b. Lower glumes equal to subequal in length; leaf blade midvein not prominent; panicles loosely flowered, sometimes secund; basal leaf sheaths glabrous..... *M. ciliata* subsp. *glauca* (F.W. Schultz) Nyman.

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Supplementary Material

The following supplementary material is available online for this article at <http://onlinelibrary.wiley.com/doi/10.1111/jse.12940/supinfo>:

Fig. S1. Phylogenetic relationships of *Melica*. **A**, Hypothesized phylogenetic relationships among *Melica*'s sections proposed by Hempel (2011). Rectangles on branches represent the appearance of xeromorphic structures. **B**, Bayesian Inference consensus tree of *Melica* based on the internal transcriber spacer (ITS). Colors represent the current species circumscription to a given section. Bayesian posterior probabilities (PP) are shown next to the branches. PP values of 1.0 are shown as asterisks (*). Genbank accession codes are shown when they correspond.

Fig. S2. Box-and-whiskers plots for morphological variables and ratios measured on 24 samples of *glauca* (blue circles), 12 samples of *magnolii* (yellow triangles), and 19 samples of *transsilvanica* (gray squares). Units are millimeters (mm), except for NCM and NCT, which are measured in micrometers (μm). Significant differences (ANOVA and Kruskal–Wallis test, Bonferroni correction, and Wilcoxon test, respectively) are indicated by different letters above the boxes. dpL, length of apical lemma hairs; LaG1, width of lower glume; LaG2, width of upper glume; Lapal, width of the palea; LG1, length of lower glume; LG2, length of upper glume; Lalem, width of lemma; Laxe, length of the axis; Lent, length of the first internode; Llem, length of lemma; Lpal, length of the palea; Lpani, length of the panicle; NCM, distance between the mestome sheath and the abaxial leaf side; NCT, leaf

midrib width; NS, number of sterile nodes on the axis; pL, length of lemma lateral hairs.

Fig. S3. Nonmetric multidimensional scaling (nMDS) plot comparing the taxonomic composition of: **(A)** Mediterranean *glauca* and *magnolii* communities, **(B)** continental *glauca* and *transsilvanica* communities.

Fig. S4. Box-and-whiskers plots for mean Ellenberg's indicators and proportion of biological traits on 221 communities. Significant differences (Kruskal–Wallis and Wilcoxon test) are marked by different letters above the boxes. Blue circles, *glauca* communities ($n = 161$); Yellow triangles, *magnolii* communities ($n = 27$); and Gray squares, *transsilvanica* communities ($n = 32$). Ellenberg indicator for Moisture, Moisture; Ellenberg indicator for pH, pH; Ellenberg indicator for Light, Light; Ellenberg indicator for Nitrogen, Nitrogen; Ellenberg indicator for Temperature, Temperature; Mean maximal height in millimeters (mm), Hmax; Community percentage of pluriannual species, Pluriannual; Community percentage of species with biotic pollination, Biotic Pollination; and Community percentage of autogamous species, Autogamy.

Table S1. Plant sampling information.

Table S2. Analysis of molecular variance (AMOVA) of all populations (a3) and *glauca* populations (a4). Statistical significance: $P < 0.01^*$.

Table S3. Morphologic values represented on Box-and-Whiskers plots.

Table S4. SIMPER analysis describing taxonomic assemblies dissimilarities among *Melica ciliata* complex communities. AD, average dissimilarity.