

## RESEARCH ARTICLE

# Diversification of fescues (*Festuca* sect. *Eskia*, Poaceae), a key-component of southern European mountainous grasslands

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DOI <https://doi.org/10.1002/tax.13278>

**Abstract** Grasslands cover approximately 30% of all land area and represent one of the most extensive and diverse ecosystems of the world. One of the largest globally distributed genera dominating various types of grasslands is *Festuca* (Loliinae, Poaceae). In this study, we disentangle the origin and diversification of early divergent lineages within fine-leaved (FL) Loliinae, namely *F.* sect. *Eskia* and *F.* sect. *Dimorphae* (*Eskia*-*Dimorpha* Group). We inferred phylogenetic relationships among 218 populations of 129 Loliinae taxa using nuclear ribosomal ITS and plastid *trnT-trnF* sequences. Relative genome size (RGS) and ploidy level estimations revealed multiple polyploidisations within *F.* sect. *Eskia*. We uncovered previously unknown tetraploid and hexaploid populations of different species from the Balkan Peninsula. The inferred phylogenies revealed the sequential divergence of *Eskia*-*Dimorpha* Group lineages in the early evolution of FL Loliinae, but also incongruences in the position as well as the composition of different clades between the nuclear and plastid-based trees. Despite incongruences, some well-supported and geographically segregated clades of single or closely related species from the Alps, the Pyrenees or the Balkan Peninsula were resolved. The most pronounced discordance between the plastid and nuclear data were among several taxa endemic to the Balkan Peninsula. Our data suggest that genome downsizing followed polyploidisation and that evolutionary dynamics of RGS-ups and downs within *F.* sect. *Eskia* were strongly phylogenetically correlated. Overall, our study underscores the intricate evolutionary history and differentiation of species within the *Eskia*-*Dimorpha* Group, emphasizing the need for further research to refine phylogenetic hypotheses and taxonomic circumscriptions, thereby enhancing our understanding of grassland biodiversity and evolution.

**Keywords** Balkan Peninsula; fine-leaved Loliinae; genome size; ITS; phylogenetic analyses; *trnT-trnF*

**Supporting Information** may be found online in the Supporting Information section at the end of the article.

## ■ INTRODUCTION

Grasslands cover approximately 30% of all land area and represent one of the most diverse ecosystems (Lyons & al., 2023); furthermore, they are key habitats for many species. They provide diverse ecosystem services such as water supply, carbon storage, erosion control and climate mitigation and have a crucial role in agriculture (Bengtsson & al., 2019). The European grassland flora is particularly remarkable, harbouring a large number of rare and endangered species (Poschlod & WallisDeVries, 2002; Hobohm & Bruchmann, 2009; Pipenbahr & al., 2013). Despite their high diversity, European grasslands are under constant decline and belong to the most threatened habitats (Poschlod & WallisDeVries, 2002). Therefore, species-rich grasslands have been identified as habitats of high conservation value within the European Union (Council Directive, 2013). One of the key components of Eurasian temperate grasslands are

various fescue species (*Festuca* L.; Poaceae; Catalán, 2006; Minaya & al., 2017).

*Festuca* is the largest genus with nearly cosmopolitan distribution within subtribe Loliinae, including 681 species (Catalán, 2006; WFO, 2023). Within this subtribe, two main clades were resolved by plastid data and were named broad-leaved (BL) and fine-leaved (FL) Loliinae, and a smaller clade referred to as the intermediate Loliinae (IL; Catalán & al., 2004, 2007; Inda & al., 2008; Minaya & al., 2017; Moreno-Aguilar & al., 2020, 2022). The two main clades diverged in the Miocene (13–15 million years ago) and both diversified extensively in the Mediterranean Basin and adjacent south-west Asia. On the other hand, nuclear ITS sequences inferred BL Loliinae as paraphyletic, with monophyletic FL Loliinae nested within (Inda & al., 2014; Minaya & al., 2017; Moreno-Aguilar & al., 2020). The FL Loliinae include the largest of 11 subgenera recognised within *Festuca*, that is *Festuca* subg. *Festuca*, along with the much

**Article history:** Received: 26 Feb 2024 | returned for (first) revision: 5 Jul 2024 | (last) revision received: 2 Sep 2024 | accepted: 5 Sep 2024

**Associate Editor:** Gulzar Khan | © 2024 International Association for Plant Taxonomy.

smaller *F.* subg. *Helleria* E.B.Alexeev and 10 other genera, e.g., *Psilurus* Trin., *Vulpia* C.C.Gmel. and *Wangenheimia* Moench. (Moreno-Aguilar & al., 2020). The remaining nine subgenera of *Festuca*, along with *Lolium* L., *Micropropraxis* Romero Zarco & Cabezudo, and *Pseudobromus* K.Schum constitute the BL Loliinae (Catalán, 2006; Minaya & al., 2017; Moreno-Aguilar & al., 2020).

The Eurasian members of *Festuca* subg. *Festuca* have traditionally been classified in several sections, e.g., *F.* sect. *Festuca* (informal *F. ovina* group), *F.* sect. *Aulaxyper* Dumort. (*F. rubra* group), *F.* sect. *Eskia* Willk. (*F. varia* group) and *F.* sect. *Dimorphae* Joch.Müll. & Catalán (= *F.* sect. *Amphigenes* (Janka) Tzvelev; Torrecilla & al., 2003; Catalán, 2006). However, phylogenetic studies pointed to non-monophyly of some of the sections; therefore, Minaya & al. (2017) introduced informal names for the main lineages. One of them is the *Eskia*-*Dimorpha* Group, which includes several lineages that diverged early in the evolution of FL Loliinae. These lineages comprising species of the Eurasian *F.* sect. *Eskia* and *F.* sect. *Dimorphae* were in a polytomy with other *Festuca* groups (Minaya & al., 2017). Most members of the *Eskia*-*Dimorpha* Group are distributed in grasslands of the southern and central European mountain ranges, but extending their distribution to north-west Africa (*F. gautieri* subsp. *scoparia* (A.Kern. & Hack.) Kerguelen), the Caucasus (*F. woronowii* Hack.) and Anatolia (*F. anatolica* Markgr.-Dann.; Markgraf-Dannenberg, 1980, 1985; Lakušić, 1999; Aeschimann & al., 2004; Foggi & Müller, 2009–). As most FL Loliinae, members of the *Eskia*-*Dimorpha* Group have leaves less than 3 mm wide; they are rigid and often pungent in *F.* sect. *Eskia*, which is also characterised by a dense, caespitose growth and lax, nodding panicles (Markgraf-Dannenberg, 1980; Lakušić, 1999). For Europe, 24 species were listed by Markgraf-Dannenberg (1980); most of them have large distributions, whereas some are endemic to small areas, especially in the Balkan Peninsula (Markgraf-Dannenberg, 1985). On the other hand, *F.* sect. *Dimorphae*, phylogenetically nested within *F.* sect. *Eskia* (Minaya & al., 2017), only includes seven species distributed in the mountains of southern and central Europe, characterised by extravaginal non-flowering shoots and stolons with scale-like sheaths (Markgraf-Dannenberg, 1980).

The mountain ranges of south-east Europe and the Eastern Alps represent diversification centres of *Festuca* sect. *Eskia* and several taxa from this area are important constituents of different grassland associations (Lakušić, 1999; Torrecilla & al., 2003). Several species are sympatric and morphologically similar, often growing in comparable habitats (Markgraf-Dannenberg, 1980), which makes the group taxonomically notorious. In addition, common interspecific hybridisations and polyploidisations make species delimitations difficult (Torrecilla & al., 2013). In general, polyploidisation is frequent in *Festuca*, in which 70% of the species are polyploid, with ploidy levels ranging to dodecaploids (Šmarda & al., 2008). Within *F.* sect. *Eskia*, di-, tetra- and hexaploids have been recorded, with polyploids restricted to the Iberian

Peninsula and the Alps (Loureiro & al., 2007; Fischer & al., 2008; Šmarda & al., 2008; Garnatje & al., 2023). In the Balkan Peninsula, only diploids with  $2n = 14$  have been recorded, namely for *F. bosniaca* subsp. *pirinensis* (Acht.) Markgr.-Dann., *F. graeca* subsp. *pawlowskiana* Markgr.-Dann., and *F. penzesii* (Acht.) Markgr.-Dann. (Kožuharov & Kuzmanov, 1970; Strid & Franzen, 1981; Strid & Anderson, 1985; Petrova & Stoyanova, 1997).

Despite high species diversity and ecological importance, the taxa of *Festuca* sect. *Eskia* were largely neglected in phylogenetic studies. Only selected species like *F. bosniaca* Kumm. & Sendtn., *F. burnatii* St.-Yves, *F. eskia* Ramond ex DC., *F. gautieri* (Hack.) K.Richt. and *F. elegans* Boiss., with focus on the Iberian Peninsula, have been included in phylogenetic studies thus far (Minaya & al., 2013, 2017; Moreno-Aguilar & al., 2020). The Iberian representatives have been studied in more detail also morphologically, cytologically and ecologically (Ortúñez & de la Fuente, 2004, 2010; Torrecilla & al., 2013; Marques & al., 2016; Garnatje & al., 2023), whereas the Alpine and the Balkan taxa received less attention (but see Lakušić, 1999; Wallossek, 1999, 2000).

To infer the evolutionary origin of and disentangle relationships among different species of the *Eskia*-*Dimorpha* Group, with geographic focus on the Balkan and Apennine peninsulas and the Alps, we performed phylogenetic analyses using the nuclear ribosomal ITS and the plastid *trnT-trnF* region. We sampled a total of 22 out of 34 species from the *Eskia*-*Dimorpha* Group, and complemented the dataset with numerous other species of FL Loliinae. Although the primary focus of this study was the *Eskia*-*Dimorpha* Group, we also inferred the phylogenetic position for several Balkan fescues from other sections. We revealed incidence of polyploids through relative genome size (RGS) estimation and inferred evolutionary dynamics of RGS up- and downsizings along different lineages of *F.* sect. *Eskia*.

## ■ MATERIALS AND METHODS

**Sampling of plant material.** — We generated sequences from plant material sampled in the field as well as for herbarium specimens gathered from different herbaria, complemented with published sequences from the GenBank (Appendix 1, suppl. Table S1). In total, we generated sequences from 142 populations belonging to 60 taxa, and included 267 sequences of 90 taxa from the GenBank. Out of 142 populations, we sampled 83 populations (1–6 individuals per population, average 3.4) of 28 taxa in the Balkan Peninsula (Albania, Bosnia and Herzegovina, Bulgaria, Croatia, Greece, Montenegro, North Macedonia, Serbia, Slovenia), the Apennines (Italy), the Alps (Austria, Italy, Slovenia, Switzerland), the Iberian Peninsula (Andorra, France, Spain), and the Caucasus (Armenia, Georgia) (Appendix 1, suppl. Table S1) between 2019 and 2021. Additionally, we retrieved 59 populations from 40 taxa from the herbaria BEOU, IB, Z, ZAGR, ZAHO, and ZT. Out of the 142 populations

sequenced within this study, 87 populations belong to 20 species of the *Eskia*-*Dimorpha* Group (77 field material, 10 herbarium material; suppl. Table S1), 35 populations to 23 species of the *Festuca*-*Wangenheimia* Group, and the remaining 20 populations to 11 species within FL Loliinae, BL Loliinae and outgroup taxa. We also sampled 75 populations from 20 taxa of *Festuca* sect. *Eskia* (partially overlapping with the sequenced populations) for RGS and ploidy level estimation (suppl. Table S1). The nomenclature used is based on *Flora Europaea* (Markgraf-Dannenberg, 1980). Additionally, we used the treatment of Fischer & al. (2008) for subspecies of *F. varia* Haenke, and included *F. adamovicii* subsp. *bistrae* Micevski & Kostad. collected at its locus classicus.

As the focus of this study was resolving relationships within the *Eskia*-*Dimorpha* Group and other FL Loliinae (ingroup hereafter), we refer to the taxa of BL and IL Loliinae as well as more distant representatives of Poeae and Triticeae as outgroup. For readability, we omit species authorities (incl. subspecies) hereafter; full names are listed in Appendix 1.

**DNA extraction, PCR amplification and aligning of sequences.** — Genomic DNA (approx. 30 mg of leaf tissue) was isolated with GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, Missouri, U.S.A.) using the manufacturer's instructions. We amplified nuclear ITS and plastid *trnT-trnF* regions. For the latter, two separate PCRs were performed, corresponding to *trnT-trnL* and *trnL-trnF* (details see in Minaya & al., 2017). PCR products were purified with Exosap-IT (ThermoFisher Scientific, Waltham, Massachusetts, U.S.A.) and sequenced by Macrogen (Amsterdam, The Netherlands) with the same primers as used for amplification (listed in Minaya & al., 2017). We sequenced 139 ITS, 135 *trnT-trnL* and 131 *trnL-trnF* sequences (one individual per population) to which we added GenBank accessions for each region (see Appendix 1 or suppl. Table S1 for GenBank numbers and voucher information of here sequenced samples). Complementary strands were assembled using DNA Baser Assembler v.5.15.0 (Heracle Bio-Soft SRL, Romania) and Geneious v.10.2.6 (<https://www.geneious.com>). Base polymorphisms in ITS sequences were coded using NC-IUPAC ambiguity codes. Two separately sequenced plastid regions (*trnT-trnL*, *trnL-trnF*) were concatenated in Mesquite v.3.7 (Maddison & Maddison, 2015) and the final alignments were adjusted manually in AliView v.1.28 (Larsson, 2014). The final ITS dataset included 230 sequences belonging to 146 taxa and was 614 bp long, whereas plastid dataset had 228 sequences belonging to 145 taxa and was 1984 bp long (*trnT-trnL*: 927 bp; *trnL-trnF*: 1057 bp).

**Phylogenetic analyses.** — The stationarity/homogeneity symtest performed with IQ-TREE v.2.1.3 and topology AU test (Naser-Khdour & al., 2019) rejected a concatenation of nuclear and plastid regions (SymPvalue = 0.00025; topologies of both regions were significantly different). Therefore, the two datasets were analysed separately.

We performed Bayesian inference (BI) of both datasets using MrBayes v.3.2.7a (Ronquist & al., 2012), and applying

a GTR substitution model, using  $\Gamma$  distribution with four rate categories that best correspond to models selected using ModelFinder v.3.1.1 (Kalyaanamoorthy & al., 2017; see below), and by estimating the proportion of invariant sites. We ran four simultaneous Markov chain Monte Carlo (MCMC) simulations, each composed of one cold and three heated chains, for a total of 50 million generations with a sampling frequency of one thousand generations. Log files were analysed using Tracer v.1.4 (Rambaut & Drummond, 2007) to assess convergence and ensure that the effective sample size (ESS) for all parameters was >200. The burn-in was 25% of the sampled posterior distributions.

We also analysed both datasets with maximum likelihood (ML) in IQ-TREE v.2.1.3 (Nguyen & al., 2015) and applying 500 runs. Prior to ML analysis the best-fitting substitution model was selected using ModelFinder v.3.1.1 (Kalyaanamoorthy & al., 2017), applying the Akaike information criterion corrected for small sample sizes (AICc). For ITS the selected model was SYM + I + I + R3, and for *trnT-trnF* (both regions were analysed separately) TVM + F + I + I + R3. The datasets were rooted with *Brachypodium distachyon* and *Oryza sativa* based on previous studies (Catalán, 2006; Minaya & al., 2017; Moreno-Aguilar & al., 2020) and both BI and ML analyses were run on HTC computing server Isabella (University of Zagreb, University Computing Centre-SRCE). All used IQ-TREE and MrBayes scripts and alignments are available on zenodo: <https://zenodo.org/records/7929078>. Both BI and ML trees were visualised in FigTree v.1.4.4. (Rambaut, 2010).

In addition, we constructed an ITS NeighbourNet of the *Eskia*-*Dimorpha* Group and a few selected FL Loliinae using SplitsTree v.4.12 (Huson, 1998) and applying Uncorrected P distances. Ambiguous base codes were treated as missing states. We also constructed a plastid haplotype network for the same group, using TCS v.1.6 (Clement & al., 2000) as implemented in PopART v.1.7.2 (Leigh & Bryant, 2015). Indels longer than 1 bp were reduced to single base pair columns allowing those structural mutations to be counted as single base pair mutations only. The accessions from populations 29, 30, 37, 85, 165 and those of *Festuca laxa* SI and *F. dimorpha* FR from GenBank were excluded from the latter analysis due to the high amount of missing data.

**Relative genome size and ploidy level estimation within *Festuca* sect. *Eskia*.** — Silica gel-dried leaves of 258 individuals from 75 populations belonging to 20 taxa of *Festuca* sect. *Eskia* (suppl. Table S1) were analysed using flow cytometry (FCM) of 4',6-diamidino-2-phenylindole (DAPI; final concentration 0.036 M) stained nuclei (Suda & Trávníček, 2006) to estimate RGS and DNA-ploidy of sampled populations. We used *Pisum sativum* L. 'Kleine Rheinländerin' (2C = 8.84 pg; Greilhuber & Ebert, 1994) as the primary internal standard for diploid samples and *Bellis perennis* L. (2C = 3.38 pg; Schönswetter & al., 2007) as secondary standard for polyploid samples, in which the sample peak overlapped with that of *Pisum sativum*. In such cases, we first measured the RGS of both standards together, and

then that of the secondary standard and sample. We then recalculated the RGS of the sample and displayed it in relation to the primary standard. Desiccated leaf tissue (c. 0.5 cm<sup>2</sup>) of each individual was co-chopped with an appropriate amount of fresh reference standard using a sharp razor blade in a plastic Petri dish, and processed as described in Suda & al. (2007). The relative fluorescence intensity of 3000 nuclei was recorded using a Partec CyFlow Space flow cytometer (Sysmex Partec, Münster, Germany). We used Partec FloMax software to evaluate histograms and to calculate coefficients of variation (CV) of the standard and sample peaks. We calculated RGS as the ratio between the mean relative fluorescence of sample and standard. Samples with CV > 5% were excluded from further analyses (details see suppl. Table S1). To test the differences in holoploid and monoploid RGS among ploidy levels, we used Kruskal-Wallis tests followed by Dunn's pairwise comparisons and Bonferroni correction as employed in R v.4.2.3 (R Core Team, 2021) and visualised in ggplot2 (Wickham, 2016).

#### Inferring RGS evolution within *Festuca* sect. *Eskia*. —

We reconstructed evolutionary changes in RGS among the members of *Festuca* sect. *Eskia*. To do this, we pruned the ITS dataset to include only accessions from 72 populations (20 taxa) with available RGS data. Bayesian phylogenetic analyses were performed as described above for the complete dataset, with only difference in running the analysis for a total of 100 million generations. The evolution of RGS was mapped onto the Bayesian consensus phylogram using the *contMap* method from the R package *phytools* v.2.2.0 (Revell, 2012). We tested for the presence of phylogenetic signals to determine if the distribution of RGS is predicted by our phylogenetic hypothesis ( $\lambda > 0$ ) or distributed randomly ( $\lambda = 0$ ), applying “K” (Blomberg & al., 2003) and “lambda” tests (Pagel, 1999) in *phylosig* (part of the *phytools* package).

## ■ RESULTS

**Phylogenetic relationships within Loliinae based on ITS and plastid *trnT-trnF* sequences.** — The relationships within the outgroup including BL Loliinae (suppl. Figs. S1, S2) were congruent with previous studies (Catalán & al., 2004, 2007; Inda & al., 2008; Minaya & al., 2017). ITS data resolved BL Loliinae as paraphyletic, as monophyletic FL Loliinae (Bayesian posterior probability, PP/ML bootstrap, BS = 1/100) as well as IL Loliinae (1/100) were positioned within. In addition, a clade (1/100) containing *Cynosurus*, *Sphenopus*, *Parapholis*, *Desmazeria* and *Catapodium*, as well as one (1/100) containing *Dactylis* and *Lamarckia*, was nested within BL Loliinae. On the other hand, in the plastid tree (suppl. Figs. S3, S4) both BL and FL Loliinae were monophyletic (1/99 and 0.97/96, respectively); *F. contracta* was strongly divergent and sister to the rest (1/100) of FL Loliinae.

Within FL Loliinae the *Eskia*-*Dimorpha* Group was paraphyletic in the ITS tree (Fig. 1A,B) and its taxa formed a

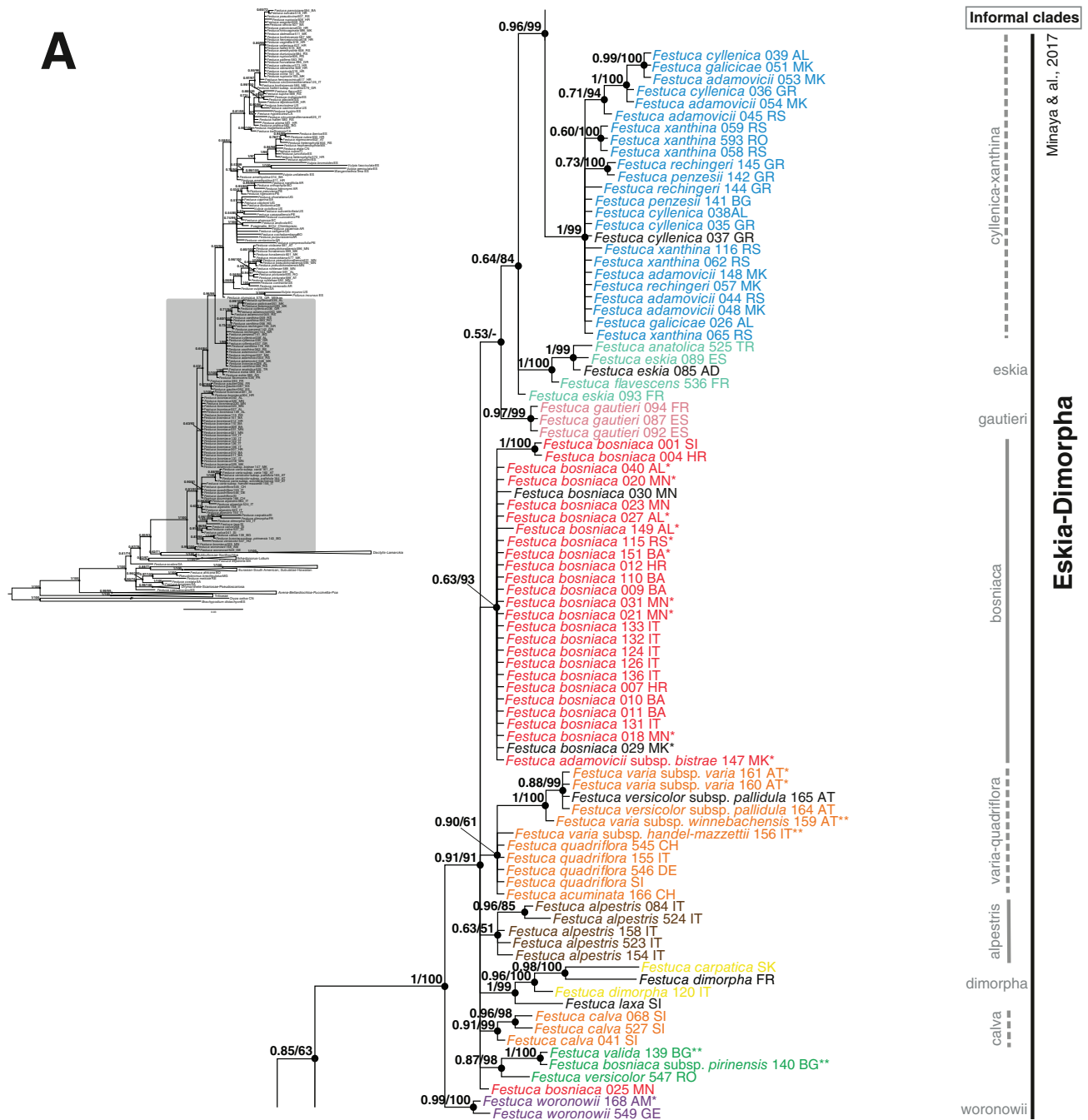
succession of divergent lineages (some with poor support), whereas in the plastid tree it was polyphyletic (Fig. 2A). In the ITS tree the Caucasian *Festuca woronowii* (0.99/100) was most divergent; it was resolved as sister to all other FL Loliinae (0.91/91). Within the latter clade, there was a basal polytomy including different *Eskia*-*Dimorpha* lineages: the Carpathian *F. versicolor* + eastern Balkan *F. bosniaca* subsp. *pirinensis* and *F. valida* (0.87/98); the Alpine *F. calva* (0.91/99); species of *F. sect. Dimorphae* (*F. carpatica*, *F. dimorpha*, *F. laxa*; 1/99); the Alpine *F. alpestris* (0.63/51); the Alpine *F. acuminata* + *F. quadriflora* + *F. varia* subsp. *handel-mazzettii* + a clade (1/100) composed of the Alpine *F. versicolor* subsp. *pallidula* + *F. varia* subsp. *varia* + *F. varia* subsp. *winnebachensis* (0.90/61). Balkan-Apennine *F. bosniaca* + *F. adamovicii* subsp. *bistrae* (0.63/93); the Pyrenean *F. gautieri* (0.97/99). The most diverse clade (0.64/84) in this basal polytomy included the Anatolian *F. anatolica* + western Mediterranean *F. eskia* and *F. flavescens* (1/100), accession 093 of *F. eskia*, and a strongly supported clade (0.96/99) including all other FL Loliinae. Within this last clade the remaining species of *F. sect. Eskia* from the south-eastern parts of the Balkan Peninsula and adjacent southern Carpathians (*F. adamovicii*, *F. cyllenica*, *F. galicicae*, *F. penzesii*, *F. rechingeri*, *F. xanthina*) formed a clade (1/99) sister to a clade (0.99/90) containing all other species of FL Loliinae not belonging to the *Eskia*-*Dimorpha* Group (Core FL Loliinae hereafter). On the other hand, the most early-diverging lineage (0.98/99) in the plastid tree comprised the Alpine *F. acuminata* + *F. quadriflora* + *F. varia* + *F. versicolor* subsp. *pallidula* + a clade (0.76/94) corresponding to *F. sect. Dimorphae* (*F. carpatica* + *F. dimorpha* + *F. laxa*). The remaining species of the *Eskia*-*Dimorpha* Group formed a clade (0.62/96) sister (0.96/98) to the rest of the FL Loliinae (1/99). Within the *Eskia*-*Dimorpha* clade several well-supported clades and some individual accessions were in a polytomy. Only two species, the Caucasian *F. woronowii* (1/99) and the Alpine *F. alpestris* (1/100), were monophyletic, whereas all the other clades included multiple species. In addition, a group of three American species not belonging to the *Eskia*-*Dimorpha* Group (*F. purpurascens* + *F. glumosa* + *F. vaginalis*; 0.99/98) was included in the polytomy.

Within the Core FL Loliinae the main relationships in the ITS tree (Fig. 1B) and the plastid tree (Fig. 2B) based on GenBank sequences, e.g., those of *Vulpia*, *Psilurus*, *Festuca* sect. *Aulaxyper*, various Afroalpine and American *Festuca* species corresponded to previous studies (Minaya & al., 2017). In the ITS tree the majority of the newly sequenced Balkan species were included in (1) the *F. violacea* group (0.59/75) along with the previously sequenced *F. contracta* + *F. nemoralis* + *F. vulpioides* (0.56/64), (2) within the assemblage of the *Aulaxyper*-*Vulpia* + *Exaratae*-*Loretia* + *Wangenheimia* groups (0.75/90) and mostly corresponding to the *F. rubra* group (0.96/98), and (3) within *F. sect. Festuca* (0.81/59) including a polytomy of single accessions and clades having various support values and mostly corresponding to the

*F. ovina* group (0.80/99). On the other hand, in the plastid tree the newly sequenced Balkan species were included in three different clades. (1) Those of the *F. violacea* group belong to the clade (0.92/97) including American *Vulpia*-Pampas + Exaratae-Loretia + Subulatae-Hawaiian; (2) the species of the *F. rubra* group were nested in the Aulaxyper-Vulpia clade

(1/100); (3) all other species, mostly belonging to the *F. ovina* group were positioned in a polytomy (1/100) including previously sequenced accessions of *Festuca*, *Wangenheimia* and the Exaratae group.

**ITS NeighbourNet of the Eския-Dimorpha Group.** — The NeighbourNet of the Eския-Dimorpha Group based on ITS



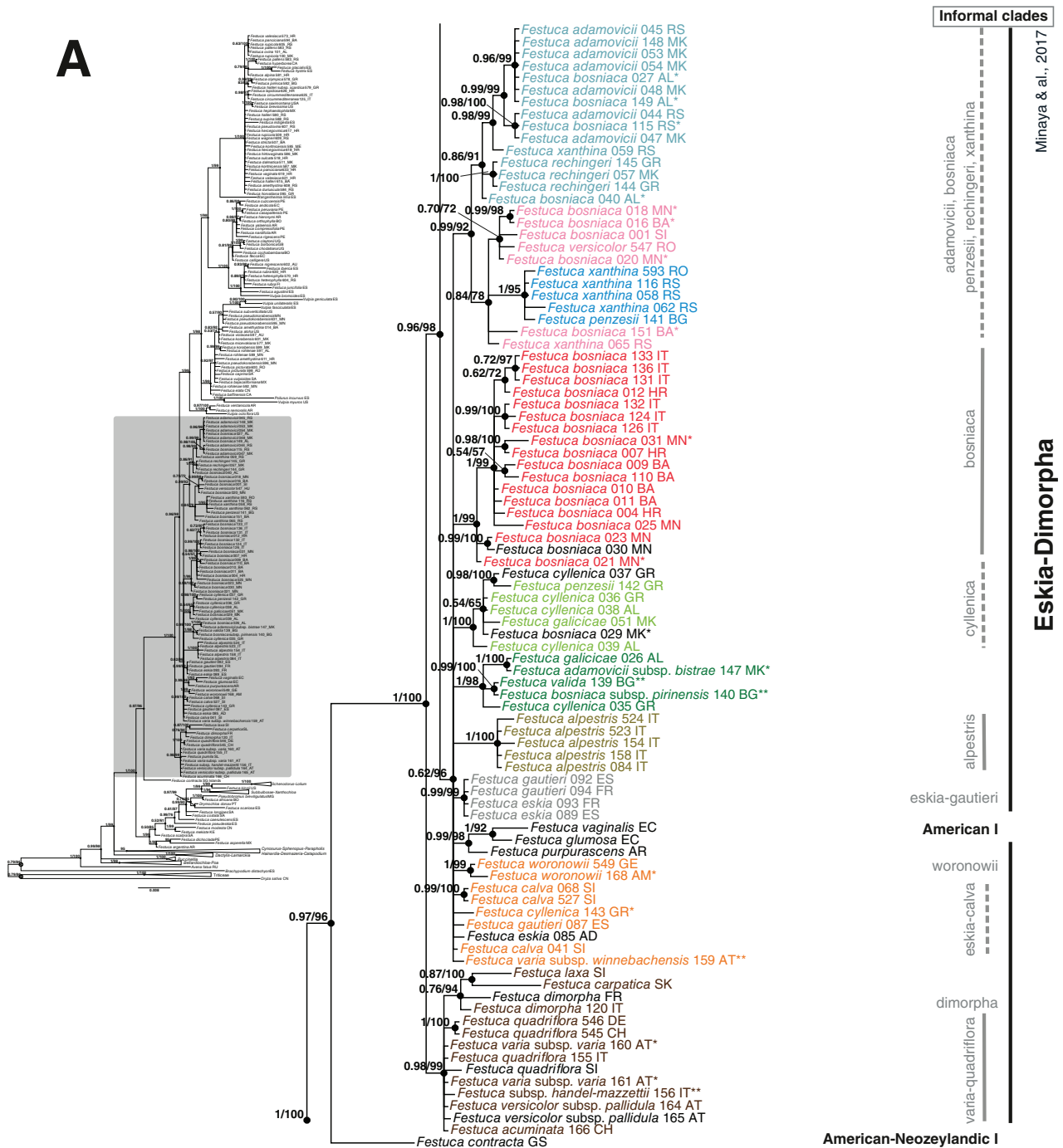
**Fig. 1A.** Bayesian consensus phylogram inferred with internal transcribed spacer sequences, showing relationships among the members of fine-leaved Loliinae (*Festuca* and related genera; the complete tree is in suppl. Fig. S1). Eския-Dimorpha Group of *Festuca* sensu Minaya & al. (2017) indicated in black to the right of the tree; additional groups discussed in this study are indicated in grey. Numbers above branches are posterior probabilities >0.50 and maximum likelihood bootstrap values >50%. The colours correspond to the ribotype groups shown in Fig. 3. Population identifiers following the names are given only for the accessions sequenced in this study and correspond to Appendix 1/suppl. Table S1. All names are followed by a two-letter ISO country code indicating the origin of the samples. Tetra- and hexaploid populations are indicated with one or two asterisks, respectively.



**Fig. 1B.** Bayesian consensus phylogram inferred with internal transcribed spacer sequences, showing relationships among the members of fine-leaved Loliinae (*Festuca* and related genera; the complete tree is in suppl. Fig. S1). Informal groups other than Eския-Dimorpha Group as in Minaya & al. (2017) and indicated in black to the right of the tree; additional groups discussed in this study are indicated in grey. Numbers above branches are posterior probabilities >0.50 and maximum likelihood bootstrap values >50%. Population identifiers following the names are given only for the accessions sequenced in this study and correspond to Appendix 1/suppl. Table S1. All names are followed by a two-letter ISO country code indicating the origin of the samples.

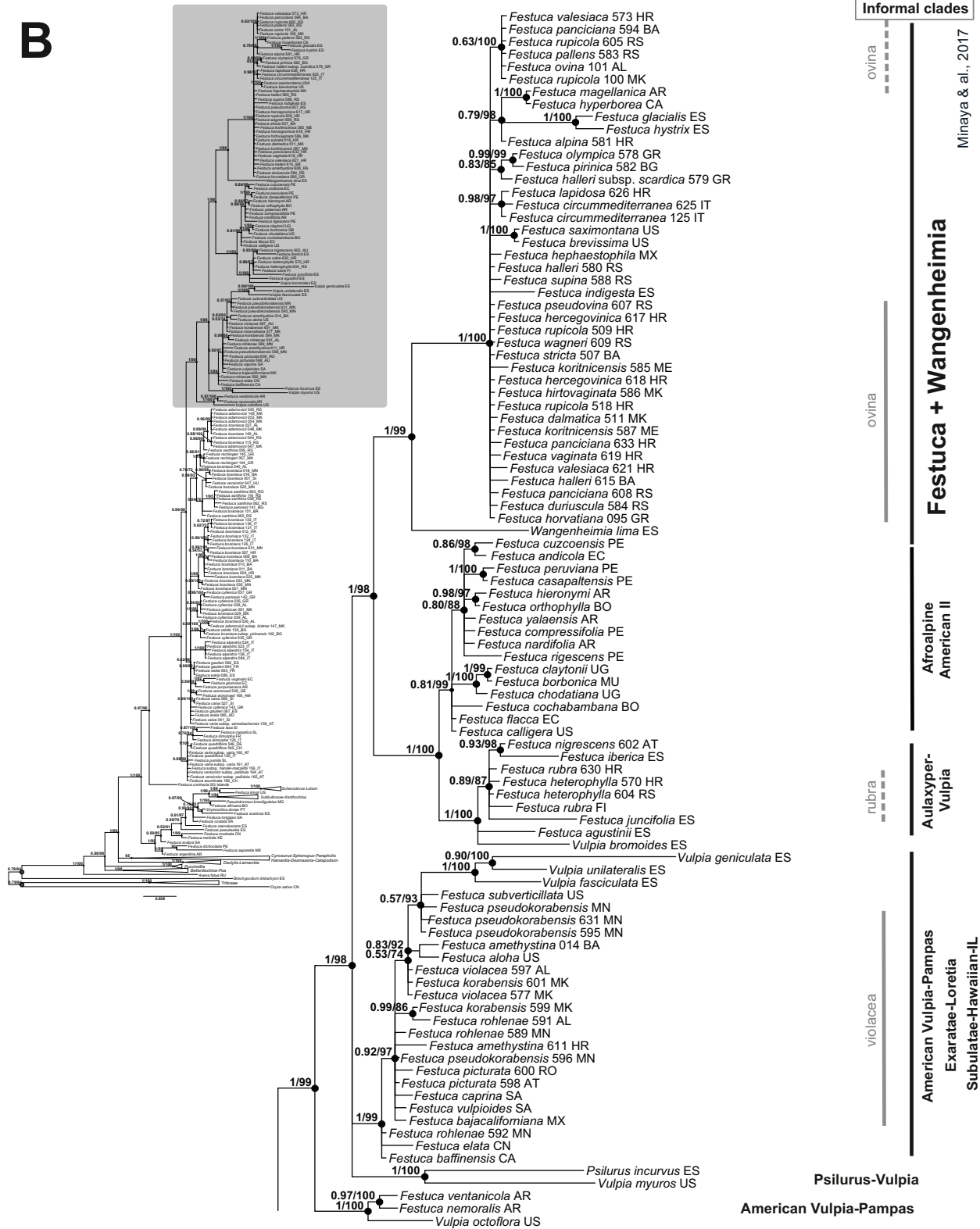
sequences (Fig. 3A) revealed clusters that largely correspond to the clades in the tree (Fig. 1A). The grouping of the clusters that share several common splits mostly reflects the geographic origin of the taxa (Fig. 3B). The most divergent cluster (blue) that shares several common splits with other groups of

FL Loliinae (black; not shown in Fig. 3B) included the species from the south-eastern Balkan Peninsula and adjacent southern Carpathians (*Festuca adamovicii*, *F. cyllenica*, *F. galicicae*, *F. penzesii*, *F. rechingeri*, *F. xanthina*). Another divergent cluster close to these two groups (turquoise) included disjunct



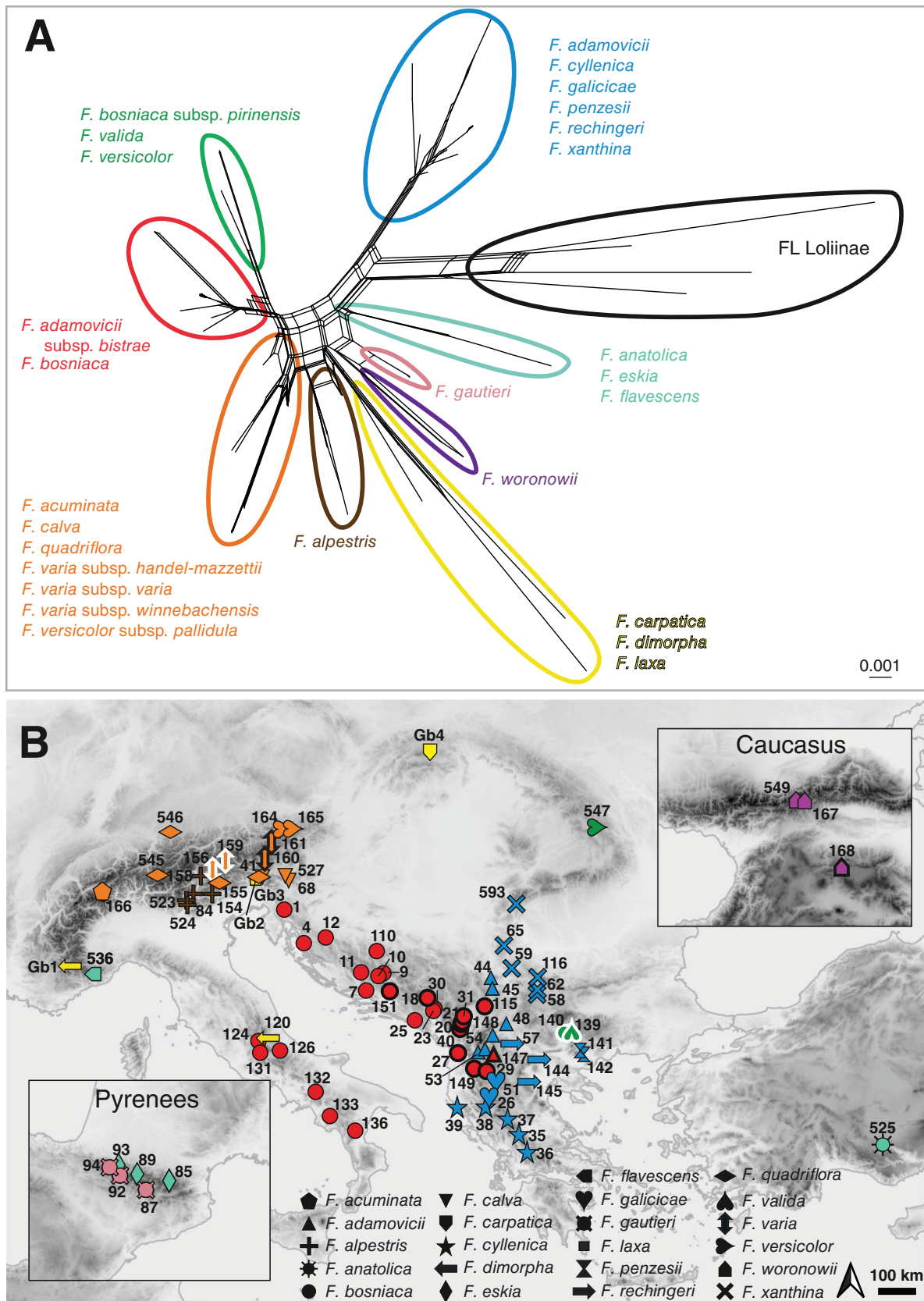
**Fig. 2A.** Bayesian consensus phylogram inferred with *trnT-trnF* sequences, showing relationships among the members of fine-leaved Loliinae (*Festuca* and related genera; the complete tree is in suppl. Fig. S3). *Eския-Dimorpha* Group of *Festuca* sensu Minaya & al. (2017) indicated in black to the right of the tree; additional groups discussed in this study are indicated in grey. Numbers above branches are posterior probabilities >0.50 and maximum likelihood bootstrap values >50%. The colours correspond to the haplotype groups shown in Fig. 4. Population identifiers following the names are given only for the accessions sequenced in this study and correspond to Appendix 1/suppl. Table S1. All names are followed by a two-letter ISO country code indicating the origin of the samples. Tetra- and hexaploid populations are indicated with one or two asterisks, respectively.

**B**

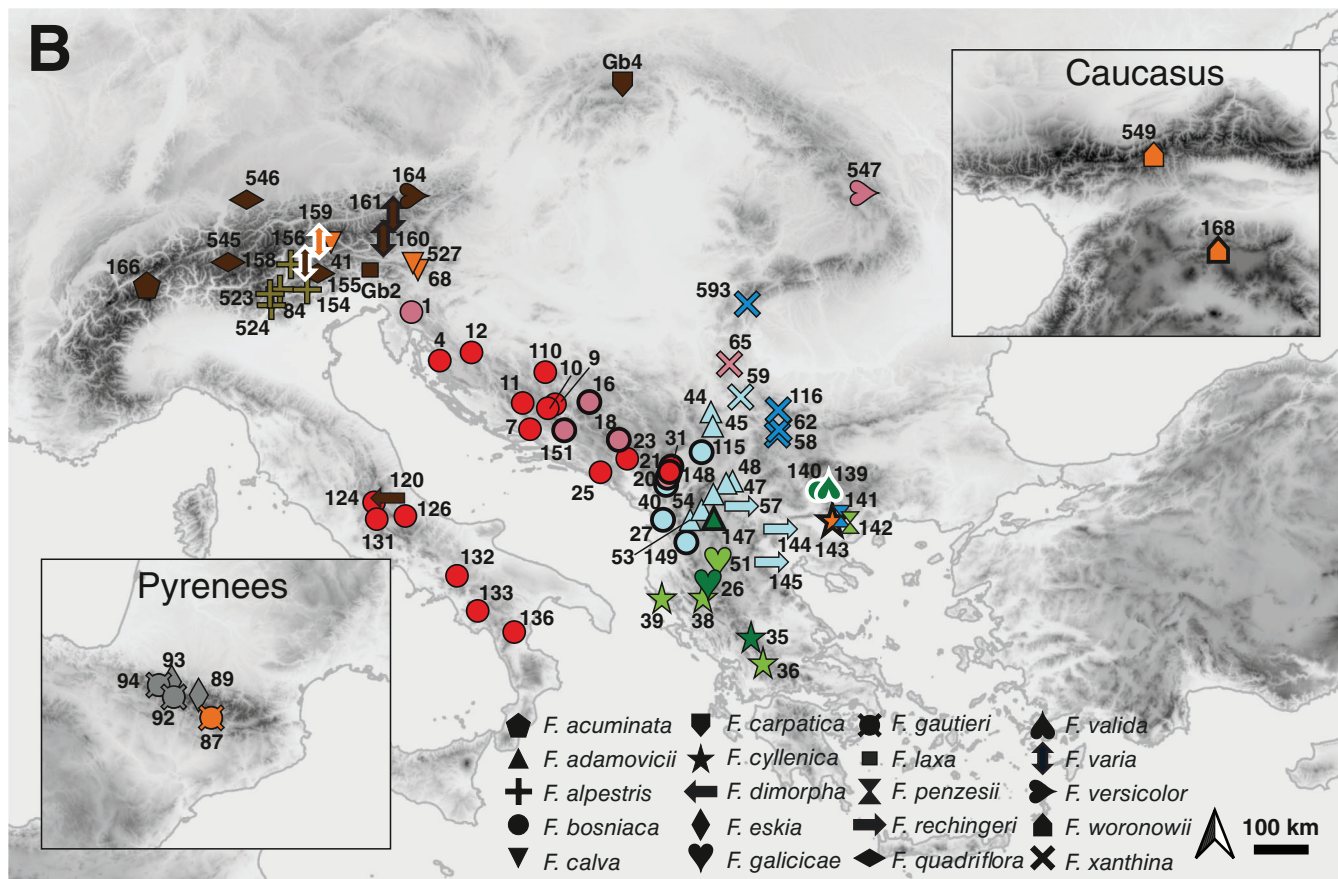
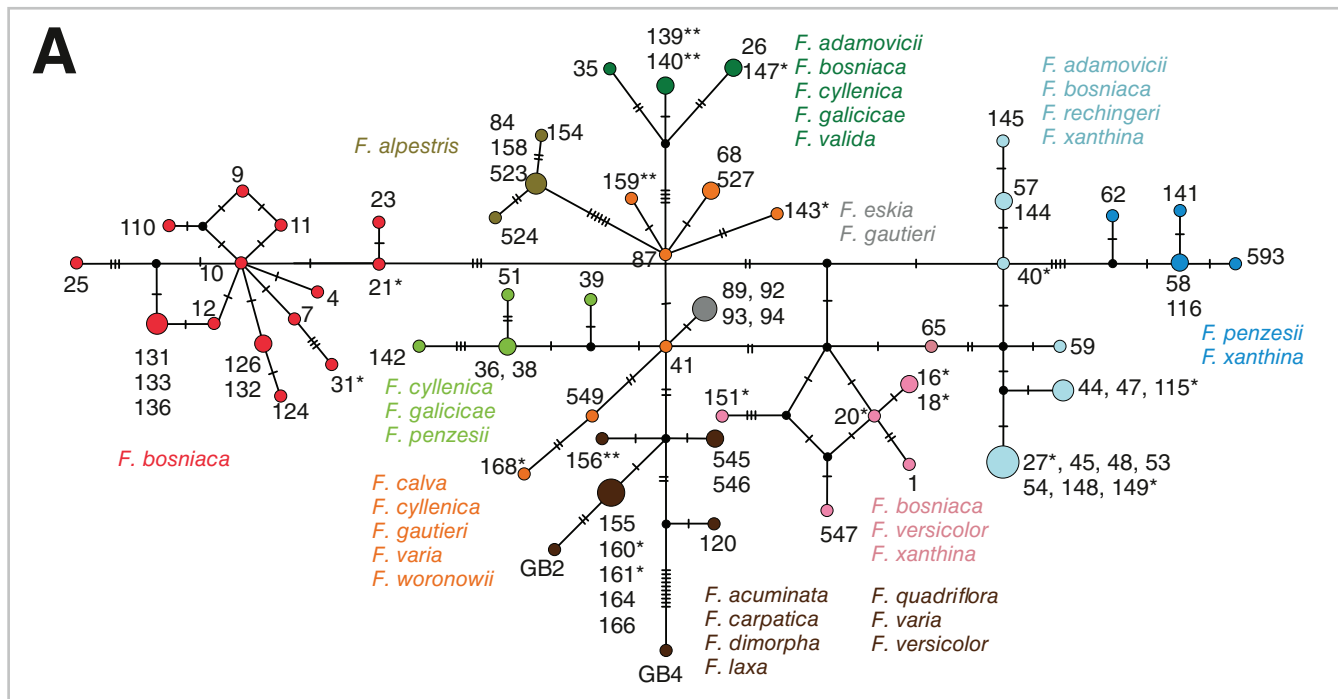


**Fig. 2B.** Bayesian consensus phylogram inferred with *trnT-trnF* sequences, showing relationships among the members of fine-leaved Loliinae (*Festuca* and related genera; complete tree is in suppl. Fig. S3). Informal groups other than *Eskia-Dimorpha* Group as in Minaya & al. (2017) and indicated in black to the right of the tree; additional groups discussed in this study are indicated in grey. Numbers above branches are posterior probabilities >0.50 and maximum likelihood bootstrap values >50%. Population identifiers following the names are given only for the accessions sequenced in this study and correspond to Appendix 1/suppl. Table S1. All names are followed by a two-letter ISO country code indicating the origin of the samples.





**Fig. 3.** Internal transcribed spacer (ITS) variation in the Eskia-Dimorpha Group of *Festuca*. **A**, NeighbourNet; **B**, Geographical distribution of ribotype groups. Population numbers correspond to Appendix 1/suppl. Table S1 and the colours to the ribotype groups in A. Tetraploid populations are indicated with thick black outline, and hexaploid with thick white outline.



**Fig. 4.** Plastid *trnT-trnF* sequence variation in the Eския-Dimorpha Group of fine-leaved *Festuca*. Population numbers correspond to Appendix 1/ suppl. Table S1. **A**, Parsimony haplotype network. Tetra- and hexaploid populations are indicated with one or two asterisks, respectively. **B**, Geographical distribution of haplotypes. Tetra- and hexaploid populations are indicated with thick black or white outlines, respectively. The colours correspond to haplotype groups in A.

Anatolian *F. anatolica*, Pyrenean *F. eskia* and Alpine *F. flavescens*. The Carpathian *F. versicolor* and the eastern Balkan *F. bosniaca* subsp. *pirinensis* and *F. valida* (green) shared several unique splits with amphi-Adriatic *F. bosniaca*, including one population of *F. adamovicii* subsp. *bistrae* (red). The remaining clusters shared several splits and correspond to Alpine *F. acuminata*, *F. calva*, *F. quadriflora*, *F. varia* and *F. versicolor* (orange), Alpine *F. alpestris* (brown), disjunct *F. carpatica*, *F. dimorpha* and *F. laxa* (*F. sect. Dimorphae*; yellow), Caucasian *F. woronowii* (violet), and Pyrenean *F. gautieri* (pink).

#### Haplotype distribution within *Eskia-Dimorpha* Group.

— Plastid haplotype analysis of the *Eskia-Dimorpha* Group revealed 54 haplotypes (Fig. 4A). Most of the haplotypes (38) were limited to single accessions, but there were a few present in up to seven populations, such as the shared haplotype between *Festuca adamovicii* and *F. bosniaca* from central parts of the Balkan Peninsula (populations 27, 45, 48, 53, 54, 148, 149; Fig. 4B). The second most common haplotype was found in five populations of *F. acuminata*, *F. quadriflora*, *F. varia* and *F. versicolor* from the Alps (populations 155, 160, 161, 164, 166).

Based on the combination of genetic distance (number of mutational steps), support values in the phylogenetic tree (Fig. 2A) and geography, we depicted ten haplotype groups (different colours in Fig. 4) that partly correspond to the clades inferred in the plastid tree (Fig. 2A). Genetically, geographically and taxonomically most coherent is the red group, which includes several Balkan and all Apennine populations of *Festuca bosniaca*; this group was also strongly supported in the plastid tree (1/99). Another such coherent group (dark olive) included all populations of the Alpine *F. alpestris*, which was monophyletic (1/100) also in the plastid tree. Although taxonomically heterogeneous, the dark blue group (*F. penzesii* and *F. xanthina* from the eastern Balkan Peninsula and southern Carpathians; support 1/95) and the dark green group (*F. adamovicii* subsp. *bistrae*, *F. bosniaca* subsp. *pirinensis*, *F. cyllenica*, *F. galicicae* and *F. valida* from the south-eastern Balkan Peninsula; support 1/98) are also genetically clearly differentiated. Other groups of haplotypes are genetically less distinct and taxonomically and geographically very heterogeneous. The orange group of haplotypes is the most heterogeneous and geographically widespread. It includes five taxa (*F. calva*, *F. cyllenica*, *F. gautieri*, *F. varia*, *F. woronowii*), which are distributed from the Pyrenees, across the Alps and the Balkan Peninsula to the Caucasus. Members of this group formed several distinct clades in the plastid tree. Of all the species, *F. bosniaca* is genetically most diverse, harbouring haplotypes from four haplotype groups (red, light blue, dark green, pink); it is also present in different lineages in the tree. The haplotypes of *F. cyllenica* and *F. xanthina* are found in three haplotype groups each, *F. adamovicii*, *F. galicicae*, *F. gautieri*, *F. penzesii*, *F. varia* and *F. versicolor* are found in two haplotype groups each, while the other taxa bear haplotypes related to individual haplotype groups.

#### Relative genome size and ploidy level variation within *Festuca sect. Eskia*.

— The RGS ranged from 0.426 in *Festuca eskia* (population 089) to 1.358 in *F. valida* (population 139), exhibiting 3.19-fold variation (suppl. Table S1). The RGS values were discretely distributed, corresponding to putatively three ploidy levels: RGS of the diploids (74.7% of the samples) ranged from 0.426 to 0.610, that of the tetraploids (20%) from 0.929 to 1.104, and that of the hexaploids (5.3%) from 1.304 to 1.358. RGS variation was highest in diploids (1.84-fold variation), followed by tetra- (1.19-fold) and hexaploids (1.04-fold). There was no ploidy variation within populations. *Festuca bosniaca* included populations of all three ploidies, *F. adamovicii*, *F. cyllenica* and *F. woronowii* each had diploid and tetraploid populations, *F. varia* had tetraploid and hexaploid populations, *F. valida* was hexaploid, whereas all other species were diploid (Fig. 5A, suppl. Table S1).

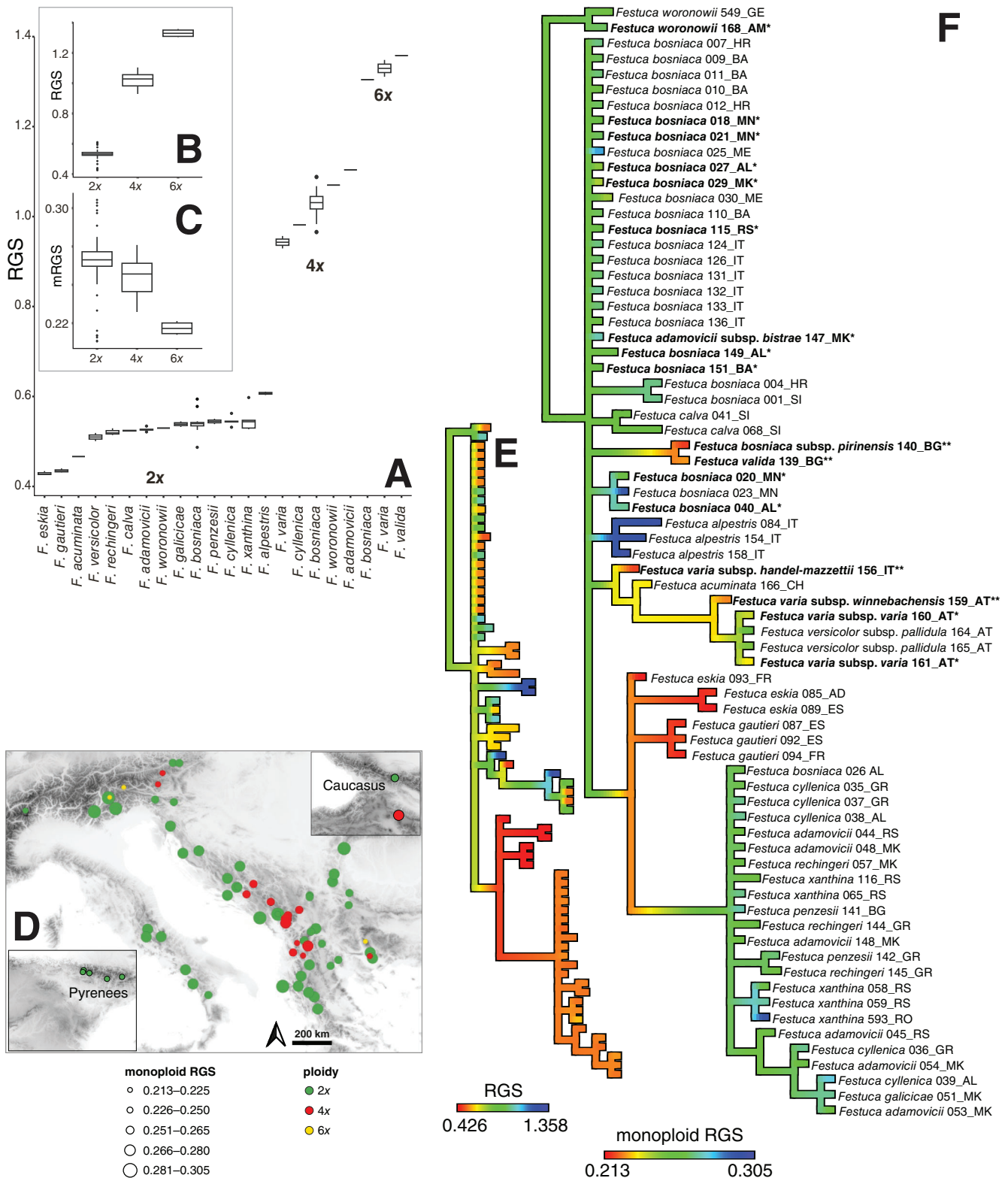
The monoploid RGS of diploids ranged from 0.213 to 0.305 and was significantly different ( $P < 0.01$ ) from that of the hexaploids (0.217–0.226), whereas the differences between di- and tetraploids (0.232–0.276) as well as tetra- and hexaploids were not significant (Fig. 5C). The tetraploids were recorded from the Alps (*F. varia*), the southern Dinaric mountains and the adjacent northern part of the Scardo-Pindic range (*F. adamovicii*, *F. bosniaca*, *F. cyllenica*), as well as the Caucasus (*F. woronowii*), whereas the four hexaploid populations originated from the Alps (*F. varia*) and the central Balkan Peninsula (*F. bosniaca*, *F. valida*; Fig. 5D).

#### Evolution of RGS within *Festuca sect. Eskia*.

— Reconstruction of RGS evolution along the branches of the ITS tree of *Festuca sect. Eskia* (Fig. 5E) revealed phylogenetic clustering of intermediate RGS at the root of the tree, with most decreased values observed within the clade including the Pyrenean *F. eskia-F. gautieri* group and an assemblage of different south-eastern Balkan taxa (e.g., *F. cyllenica* and *F. xanthina*). On the other hand, increased RGS was inferred in lineages leading to the tetra- and hexaploid populations. The Chi-square test and estimated  $\lambda = 0.999$  indicated that RGS evolution exhibits a strong phylogenetic signal. Contrary to the holoploid RGS, the monoploid RGS strongly decreased along the branches leading to the polyploid populations (Fig. 5F), indicating genome downsizing. On the other hand, an evident increase was inferred in the lineage of *F. alpestris* and single accessions of *F. bosniaca* and *F. xanthina*. Also, the evolution of the monoploid genome size exhibited a strong phylogenetic signal ( $\lambda = 0.999$ ). Tetraploids originated in different lineages of *F. sect. Eskia*, whereas the hexaploids were found only in two clades (Fig. 5F).

## DISCUSSION

The present study confirms the paraphyletic nature of *Festuca* and infers relationships within Loliinae that are largely in line with previous studies (Catalán & al., 2004, 2007; Inda & al., 2008; Minaya & al., 2017; Moreno-Aguilar & al., 2020,



**Fig. 5.** Relative genome size (RGS) variation within *Festuca* sect. *Eския*. **A**, RGS boxplots of different taxa. **B & C**, RGS (**B**) and monoplloid RGS (**C**) of di-, tetra- and hexaploids. Boxes define 25 and 75 percentiles; horizontal lines indicate medians, whiskers are from 5 to 95 percentiles, circles indicate outliers. **D**, Geographical distribution of monoplloid RGS. The size of a dot is proportional to the mean monoplloid RGS of the corresponding population, colours denote ploidies. **E & F**, Reconstruction of holoplloid (**E**) and monoplloid (**F**) RGS evolution along the branches of the Bayesian phylogram of ITS sequences. The polyploid populations are marked in bold and indicated with one (tetraploids) or two (hexaploids) asterisks. Population identifiers following the names are given only for the accessions sequenced in this study and correspond to Appendix 1/suppl. Table S1. All names are followed by a two-letter ISO country code indicating the origin of the samples.

2022), but provides new insights into the diversification of FL Loliinae, in particular of the *Eskia*-*Dimorpha* Group. Our data revealed incongruences in relation to the positions of FL and BL Loliinae based on nuclear ITS and plastid data. Whereas the plastid data clearly infer both groups as monophyletic, in the ITS dataset the BL Loliinae are paraphyletic, with monophyletic FL Loliinae nested within (suppl. Figs. S1–S4).

**Interspecific relationships within the *Eskia*-*Dimorpha* Group.** — The species of the *Eskia*-*Dimorpha* Group formed a sequence of early-diverged lineages within the FL Loliinae in both datasets (Figs. 1, 3). However, there were incongruences both in the position as well as in the composition of different clades between both datasets. Whereas *Festuca woronowii* from the Caucasus that included one diploid and one putatively tetraploid population, was sister to all other FL Loliinae in the ITS tree (Fig. 1A), the plastid data (Fig. 2A) inferred the Alpine species *F. acuminata*, *F. quadriflora*, *F. varia*, and *F. versicolor* subsp. *pallidula* (i.e., *F. varia*-*F. quadriflora* group), including the taxa of *F. sect. Dimorphae* (*F. carpatica*, *F. dimorpha*, *F. laxa*), as sister to all other FL Loliinae. On the other hand, in the ITS tree the aforementioned Alpine species and those of *F. sect. Dimorphae* formed two independent clades in the basal polytomy of FL Loliinae (if not considering *F. woronowii*).

Despite strong morphological divergence of *Festuca* sect. *Dimorphae* (see Introduction), our study corroborates its origin in the early evolution of FL Loliinae (Torrecilla & al., 2003, 2013; Catalán & al., 2004, 2007; Catalán, 2006; Inda & al., 2008; Minaya & al., 2017; Moreno-Aguilar & al., 2020) and indicates that it originated in the Alps, based on their close relationship to the Alpine members of the *F. varia*-*F. quadriflora* group. The Alpine origin was also inferred for *Sesleria* (Kuzmanović & al., 2017), which, along with species of *F. sect. Eskia*, often dominates alpine and subalpine grasslands over calcareous and ultramafic bedrock in the central and southern European mountain ranges (vegetation classes Festuco-Seslerietea Barbero & Bonin 1969, Daphno-Festucetea Quézel 1964).

Whereas the species of the *Festuca varia* group form dense tussocks in dry (sub)alpine grasslands, the members of *F. sect. Dimorphae* grow in screes and have stolons (Markgraf-Dannenbergh, 1980), which is a typical adaptation of scree species (Körner, 1999). Along with the species from *F. sect. Eskia* they have a key phytosociological role in different scree vegetation types (Valachovič & al., 1997; Mucina & al., 2016). Therefore, a shift in ecology has likely triggered strong morphological divergence of *F. sect. Dimorphae* and consequently its treatment as an independent section.

The *Festuca varia*-*F. quadriflora* group is widespread across the Alps, with most taxa endemic to various parts of the Eastern Alps. In addition, two other Alpine species, *F. alpestris* and *F. calva*, endemic to the western and eastern areas of the Southern Alps, respectively (Wallosek, 1999; Aeschmann & al., 2004), were resolved as monophyletic by ITS data, the former also by plastid data. Whereas the position of *F. quadriflora* close to *F. varia* is surprising, as the former is

not part of *F. varia* agg. due to its divergent morphology (Fischer & al., 2008; but see Wallossek, 1999), both *F. alpestris* and *F. calva* are distinct species within *F. varia* agg. The Alpine members were traditionally divided into acidophilous and basiphilous taxa (Wallosek, 1999), but this is not reflected in phylogenetic relationships. Despite our modest sampling in the Alps, we confirmed the presence of tetra- and hexaploid populations within *F. varia* (Wallosek, 1999; Fischer & al., 2008). Our ITS data suggest origin of tetraploid *F. varia* subsp. *varia* from diploid Alpine *F. versicolor* subsp. *pallidula*. The latter is strongly divergent from the Carpathian *F. versicolor* in both trees and should preferably be treated as a species independent of *F. versicolor*. *Festuca acuminata* has likely been involved in the origin of hexaploid *F. varia* subsp. *handel-mazzettii*, whereas hexaploid *F. varia* subsp. *winnebachensis* probably had geographically close *F. calva* as its maternal parent based on the plastid data. Further studies with denser geographic sampling are needed to resolve the relationships among the Alpine taxa of *F. sect. Eskia*.

Whereas the ITS data inferred a strong divergence between the Pyrenean monophyletic *Festuca gautieri* and non-monophyletic *F. eskia* that appeared closely related to the western Alpine *F. flavescens* and Anatolian *F. anatolica* (Fig. 1A), in the plastid tree two populations of *F. gautieri* were in a strongly supported clade with two populations of *F. eskia* (Fig. 2A). This is possibly due to hybridisation between populations of these geographically overlapping taxa in the Pyrenees, detected also in previous studies for *F. sect. Eskia* (Torrecilla & al., 2013; Marques & al., 2016). In addition, the close relationship between *F. eskia* and *F. flavescens* is not surprising as they have adjacent distributions spanning from the Iberian Peninsula across the French Massif Central towards the Western Alps. Phylogeographic links between the Western Alps and the Pyrenees, sometimes including also Massif Central, have been inferred in several plants (e.g., *Phyteuma globulariifolium* Sternb. & Hoppe, Schönswetter & al., 2002; *Kernera saxatilis* (L.) Sweet, *Silene rupestris* L., Kropf & al., 2006; *Heliosperma pusillum* (Waldst. & Kit.) Rchb., Frajman & Oxelman, 2007), whereas the phylogenetic position of *F. anatolica* close to both western Mediterranean species is more surprising.

For the species of *Festuca* sect. *Eskia* from the Balkan Peninsula, topological incongruences between the phylogenetic trees based on nuclear (Fig. 1A) and plastid data (Fig. 2A) suggest a complex evolutionary history and differentiation. Whereas in the ITS tree they were included in two highly supported clades (i.e., *F. cyllenica*-*F. xanthina* group and *F. bosniaca* group), in the plastid tree four distinct clades were resolved. The phylogenetic diversification of the Balkan species is accompanied by a pronounced phytosociological differentiation as the species of *F. sect. Eskia* play a key role in syntaxonomy. For instance, they differentiate Festucion bosniacae Horvat 1930 (subalpine calcicolous tussock grasslands on steep terraced slopes of the Northern Dinarides), Seslerio-Festucion xanthinae Horvat in Horvat & al. 1974 (subalpine fescue grasslands on shallow skeletal soils over

limestone of eastern Serbia and Bulgaria) and Onobrychido-Festucion Horvat 1960 (subalpine calcicolous tussock grasslands of the southern and central Balkans; Mucina & al., 2016.)

The *Festuca bosniaca* group based on the ITS data constitutes the majority of the *F. bosniaca* populations that exhibit an amphi-Adriatic distribution, characteristic for many species and species groups (Frajman & Schönswetter, 2017; Španiel & Rešetnik, 2022). Since all populations in the Apennine Peninsula are diploid (Fig. 3B), they likely originated from the diploid populations of *F. bosniaca* in the Dinaric mountains. On the other hand, the south-eastern populations of *F. bosniaca*, as well as two populations from the central Dinaric mountains, are tetraploid and were included in two different clades including diploid accessions in the plastid tree suggesting their separate origins via autopolyploidisation. Alternatively, the geographically close species of the *F. cyllenica-F. xanthina* group such as *F. adamovicii* or *F. rechingeri* were involved in the origin of southern tetraploids of *F. bosniaca*, as they are included in the same clades in the plastid tree (Fig. 2A). Their distribution area, the southern Dinaric range and the northern Scardo-Pindic mountains are known regions of high genetic diversity and intersection of genetic lineages within many Balkan taxa (Španiel & Rešetnik, 2022).

A single population of *Festuca adamovicii* subsp. *bistrae* from the Scardo-Pindic mountains in North Macedonia was tetraploid and included in the *F. bosniaca* group in the ITS data, whereas in the plastid data it was sister to the diploid population 026 of *F. galicicae* from Mt. Ostrovica in southern Albania, which was, however, phylogenetically divergent in the ITS tree and included in the *F. cyllenica-F. xanthina* group. It is thus likely that the lineage of *F. galicicae* (026) acted as maternal parent, whereas the geographically close tetraploid populations of *F. bosniaca* as paternal parent in the origin of *F. adamovicii* subsp. *bistrae*. Since, however, all other populations of *F. adamovicii* were diploid and phylogenetically divergent both in the ITS and plastid trees, *F. adamovicii* subsp. *bistrae* should rather be considered a subspecies of *F. bosniaca*. Our data thus indicate that in addition to the morphologically manifested interspecific hybridisation among co-occurring *Festuca* species (Inda & al., 2008; Torrecilla & al., 2013; Marques & al., 2016), also “cryptic hybridisation” appears in this genus, a phenomenon where genetic interspecific introgression is not displayed at the morphological level. In such cases, the morphotype of one species can carry a significant part of the genome of another species. Similar scenarios have been reported for several other genera such as pines (Godbout & al., 2012; Petrova & al., 2018), birch (Thórsson & al., 2001) or proteas (Mitchell & Holsinger, 2018) and are likely more common in wind-pollinated plant groups.

Another case of morphological and phylogenetic incongruence is *Festuca bosniaca* subsp. *pirinensis*, that is not closely related to *F. bosniaca* neither based on the ITS nor the plastid data. This taxon, endemic to Pirin Mountains in Bulgaria, is, based on our phylogenetic data, most closely

related with a sympatric hexaploid population of *F. valida*, which is restricted to siliceous mountains of western Bulgaria, south-eastern Serbia, North Macedonia and northern Greece, and differs from *F. bosniaca* subsp. *pirinensis* in having very tall (up to 105 cm) and thick (up to 4 mm) stems, very long panicles (up to 13 cm), greenish or slightly variegated spikelets and oblong-lanceolate lemmas with short awn (up to 2 mm). Based on our RGS data, both taxa are putative hexaploids, although for *F. bosniaca* subsp. *pirinensis* a diploid chromosome count was published (Kožuharov & Kuzmanov, 1970; Petrova & Stoyanova, 1997) and *F. valida* is supposed to be tetraploid (Šmarda & al., 2008), which suggests a more complex cytogenetic differentiation than previously assumed. Both hexaploid populations show affinities to Carpathian *F. versicolor* in the ITS data and to diploid *F. galicicae* (026) and *F. cyllenica* (039), as well as tetraploid *F. adamovicii* subsp. *bistrae* (147), in the plastid data.

Whereas the Rila-Pirin mountain system in Bulgaria shares many species with other mountains of the Balkan Peninsula, there are also well-known biogeographic links with the Southern Carpathians (Stojanov & Stefanov, 1922; Mráz & Ronikier, 2016; Španiel & Rešetnik, 2022). It remains unclear whether both taxa from Pirin had independent polyploid origins; we consider it more likely that the clear morphological divergence between them developed from a common hexaploid ancestor and was enhanced by adaptation to different substrates. Indeed, *Festuca bosniaca* subsp. *pirinensis* grows in subalpine-alpine calcareous open rocks and grasslands, whereas *F. valida* thrives in subalpine-alpine siliceous rocks, grasslands and open pinewoods. Based on our phylogenetic and RGS data, *F. bosniaca* subsp. *pirinensis* should rather be treated as an independent species, under the name *F. pirinensis* (Acht.) Acht. as currently done in the Flora of Bulgaria (Velev, 1963).

Finally, the *Festuca cyllenica-F. xanthina* group is taxonomically most diverse and phylogenetically youngest within the *Eskia-Dimorpha* Group. In the ITS tree (suppl. Fig. S1) it forms a clade sister to all remaining FL Loliinae. The species of this group (*F. adamovicii*, *F. cyllenica*, *F. galicicae*, *F. penzesii*, *F. rechingeri*, *F. xanthina*) are distributed in the eastern and southern parts of the Balkan Peninsula and the adjacent Southern Carpathians (*F. xanthina*; Fig. 3). On the other hand, populations of these taxa were included in several clades in the plastid tree (Fig. 2A), often along with populations of other Balkan taxa. Interspecific hybridisation is likely the cause for the incongruences; the ranges of several species overlap or they even grow in close vicinity, allowing wind pollination to facilitate hybridisation and introgression (Inda & al., 2008 and references therein). Given the unclear phylogenetic differentiation among the species of the *F. cyllenica-F. xanthina* group, which is accompanied by partly weak morphological divergence among the taxa, it might be more suitable to treat some as subspecies of *F. xanthina* or *F. cyllenica*; highly resolving genomic RAD sequencing data (Mucko & al., unpub.) will hopefully provide a better basis

for a taxonomic revision of this group and other members of *F. sect. Eския*.

**Ploidy level and genome size evolution in *Festuca sect. Eския*.**

***Eския*.** — The ploidy level estimation revealed the presence of di-, tetra-, and hexaploid populations in *Festuca sect. Eския*, with the first indications of polyploids from the Balkan Peninsula and the Caucasus. Polyploid populations within *F. sect. Eския* were previously known only from the Iberian Peninsula and the Alps (Wallossek, 1999; Garnatje & al., 2023). Nevertheless, diploids prevail not only in the Iberian Peninsula and the Alps, but also in the Balkan Peninsula and the Caucasus. Tetraploids were limited to *F. bosniaca* from the central and southern Dinaric mountains and adjacent northern parts of the Scardo-Pindic range, where also a single tetraploid population of *F. adamovicii* subsp. *bistrae* (147) was collected, whereas the tetraploid population of *F. cyllenica* (143) was collected on Mt. Vrontous in north-eastern Greece, and tetraploid *F. woronowii* (168) was found in the Armenian Lesser Caucasus. Hexaploids are limited to *F. bosniaca* subsp. *pirinensis* and *F. valida* from Pirin in Bulgaria.

Genome downsizing, i.e., a decrease in monoploid genome size (Leitch & Bennett, 2004) was observed in *Festuca sect. Eския*, as the monoploid RGS of the hexaploids was significantly smaller than that of the diploids, but not of the tetraploids (Fig. 5C). The RGS variation among species was partly evolutionarily correlated, with a decrease in the monoploid RGS along the branches leading to the diploid Pyrenean and Alpine species of different ploidy levels, as well as in the two hexaploid populations from Pirin (Fig. 5F). On the other hand, a marked increase in monoploid RGS was inferred for the Alpine *F. alpestris*, which has the highest RGS of all investigated species (Fig. 5A). Also the RGS among some other species differed, which corroborates the results of Šmarda & al. (2008) that RGS values can help in taxa delimitation.

As mentioned above, a marked difference in genome size is visible between the Pyrenean *Festuca eskia* and *F. gautieri*, exhibiting the smallest RGS values, and the other species with markedly higher RGS (suppl. Table S1, Fig. 5A). Similarly divergent genome sizes of Iberian lineages in groups with comparable distribution to *F. sect. Eския* were found in the *Euphorbia nicaeensis* All. and *E. verrucosa* L. groups (Caković & al., 2021; Stojilković & al., 2022) as well as in *Knautia sect. Trichera* (Frajman & al., 2015), but the underlying causes remain unknown.

**Relationships among other Balkan fine-leaved fescue species not belonging to *Festuca sect. Eския*.**

— Our phylogenetic data indicate that the clade including all other FL Loliinae (Core FL Loliinae) is included in the *Eския-Dimorpha* Group, either as sister to the predominately Balkan *Festuca cyllenica-F. xanthina* group (ITS data, suppl. Figs. S1, S2), or to the clade composed of most Iberian-Apennine-Balkan members (plastid tree, suppl. Figs. S3, S4). In addition, several early-divergent lineages of the Core FL Loliinae include species from south-eastern Europe, which suggests that the Core FL Loliinae originated in this area and later spread to other regions. It is noteworthy that the American species are

positioned in different clades of FL Loliinae, indicating multiple dispersals across the Atlantic.

The newly sequenced south-eastern European fescues of the Core FL Loliinae belong to several informal species groups sensu Minaya & al. (2017), i.e., the Aulaxyper-Vulpia, American Vulpia + Pampas, Exaratae-Loretia and *Festuca-Wangenheimia* groups (Figs. 1B, 2B, suppl. Figs. S1–S4). Delimitation of these groups (Minaya & al., 2017) was largely based on plastid data, where their members mostly form clades (Fig. 2B, suppl. Figs. S3, S4) whereas in the ITS tree they are intermingled and different clades are composed of members of multiple groups (Fig. 1B, suppl. Figs. S1, S2). The scattered positions of species belonging to the Exaratae-Loretia Group sensu Minaya & al. (2017) in both datasets is not surprising, given that their members did not form a monophyletic group in previous studies (Minaya & al., 2017; Moreno-Aguilar & al., 2020).

One of the early-divergent lineages within the Core FL Loliinae included several species traditionally assigned to the *Festuca violacea* group (*F. violacea* agg.; Pils, 1980; Conert, 1996; Foggi & al., 1999, 2005; Fischer & al., 2008), namely *F. korabensis*, *F. picturata*, *F. pseudokorabensis*, *F. violacea*, and partly *F. amethystina* (see below). In addition, *F. rohlenae* that was recently segregated from the widely circumscribed *F. porcii* (Lakušić, 2010), also belongs to the same clade with largely unresolved interspecific relationships. Pils (1980, 1982) considered the *F. violacea* group as most conservative in terms of morphological, karyological, ecological and chorological characteristics, which is supported by its early divergence within the Core FL Loliinae. On the other hand, *F. porcii* is an intriguing taxon with extravaginal non-flowering shoots and involute to plicate tiller leaves characteristic of FL fescues, with complete sclerenchyma girders and very prominent colourless parenchymatic cells characteristic of BL fescues, therefore several hypotheses about its origin were proposed (Simonkai, 1887; Tzvelev, 1971; Tveretina, 1977; Lakušić, 2010). Only Markgraf-Dannenberg (1980) suggested its close relatedness to *F. amethystina*, which, based on traditional views (e.g., Fischer & al., 2008) and partly our phylogenetic results, also belongs to the *F. violacea* group. Our data, however, indicate that *F. amethystina* is included in the *F. violacea* group by the plastid data (Fig. 2B), and as early-divergent accessions of the Aulaxyper-Vulpia Group based on the ITS data (Fig. 1B). Šmarda & al. (2008) considered the species of the *F. amethystina* alliance as “incertae sedis”, which, along with our results, calls for additional studies to resolve the circumscription as well as the phylogenetic position of both *F. amethystina* and *F. porcii*.

Finally, the evolutionarily most derived and species-rich *Festuca sect. Festuca* included all remaining newly sequenced taxa. This section has been traditionally divided into several species groups based on morphology (Fischer & al., 2008; Šmarda & al., 2008). These groups are only partly reflected in the ITS tree (Fig. 1B), whereas in the plastid tree (Fig. 2B) most accessions are in a polytomy and some internal

clades rather reflect the geographic origin of the samples than their taxonomic position. Nevertheless, in the ITS tree *F. alpina* from Croatia and one accession of *F. halleri* from Serbia, both considered to belong to the *F. halleri* group (Fischer & al., 2008), are positioned with several other species in a weakly supported clade (BS 57%), whereas two other accessions of *F. halleri* from the Balkan Peninsula are divergent, suggesting the need of revising its circumscription. Most of the species of the broadly circumscribed *F. ovina* group (incl. *F. valesiaca* group and *F. pallens* group; Fischer & al., 2008; Šmarda & al., 2008), namely *F. ovina*, *F. pallens*, *F. pseudovina*, *F. rupicola*, *F. vaginata* and *F. valesiaca* were, along with some other species (see above), included in the most species-rich clade of *F.* sect. *Festuca*.

## ■ CONCLUSIONS

Our study reveals complex diversification patterns within FL Loliinae. Incongruences detected between the nuclear and plastid data are likely connected to frequent hybridisation and polyploidisation, characteristic not only for *Festuca*, but also other Poaceae (Ainouche & al., 2004; Kuzmanović & al., 2017; Liu & al., 2017). In addition, incongruent positions of different accessions of allegedly the same species both within *F.* sect. *Eskia* and other FL fescues, can at least partly be connected to the same phenomena, but are also results of insufficient knowledge leading to wrong naming of taxa in different regions. This indicates the necessity of further studies that should resolve the evolutionary relationships among the taxa, but, along with in-depth morphological, anatomical and cytogenetic analyses also provide a basis for revised taxon circumscriptions. In-depth study of the Balkan representatives of *F.* sect. *Eskia* based on high-resolution phylogenomic data (Mucko & al., unpub.) should thus provide further insights into the diversification of the earliest-diverging lineages of the FL fescues.

## ■ AUTHOR CONTRIBUTIONS

IR and MM designed the study. All authors contributed to data collection. DL, SB and NK revised the collected material. MM carried out the bench work, performed phylogenetic analyses, constructed all phylogenetic trees, and created figures under supervision of IR. MD performed RGS measurements. MM wrote the first draft, BF and IR revised the text and added contributions throughout several rounds of revision. IR and BF provided resources for the project and IR conducted project administration.

## ■ ACKNOWLEDGEMENTS

The study was financed by the Croatian Science Foundation (project “Phylogeography and evolution of three ecologically divergent groups of amphi-Adriatic plants – AmphiAdriPlant”, UIP-2017-05-2882) as well as by the bilateral project co-funded by the

Croatian Ministry of Science and Education to IR and the Austrian Agency for International Cooperation in Education and Research (OeAD-GmbH) to BF (project “Diversification of the *Festuca varia* group [Poaceae]”, HR 10/2022). Authors are thankful to A. Terlević for her help with map plotting; M. Magauer, D. Pirkebner and M. Temunović for help with genome size measurements; and T. Wilhelm and L. Schrott-Ehrendorfer for providing distributional data for South Tyrol and Austria, respectively. We also thank the curators of the herbaria for providing the herbarium material for analyses. Peter Schönswetter provided valuable improvements of an earlier version of the manuscript.

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**Appendix 1.** Taxa and GenBank/NCBI accession numbers for sequences (nrITS, *trnL-F*, *trnT-L*) used in this study. Sample code, country of origin, collector(s), coll. date (day.month.year) and herbarium voucher are given only for accessions newly sequenced in this study (marked with \*). A dash indicates missing data.

**OUTGROUP:** *Avena fatua* L., DQ539563, DQ631428, DQ631494; *Bellardiochloa variegata* Lam., Fb060, North Macedonia, *Lakušić, Kuzmanović, Janković, Zbiljić*, 12.7.2018 (BEOU-69442), PP334908\*, PP337564\*, PP337429\*; *Brachypodium distachyon* (L.) P.Beauv., AF303399, AF478500, DQ336855; *Catapodium rigidum* (L.) C.E.Hubb., AF532940, AF533034, EF584986; *Cynosurus echinatus* L., AF532937, AF533031, EF584993; *Dactylis glomerata* subsp. *hispanica* (Roth) Nyman, AF393014, AF533027, EF584994; *Desmazeria sicula* (Jacq.) Dumort., EF584917, EF592948, EF584989; *Lamarckia aurea* (L.) Moench, AF532935, AF533029, EF584995; *Oryza sativa* L., AF478499, AF478537, EF584997; *Parapholis cylindrica* (Willd.) Romero Zarco, AF532941, AF533035, EF584990; *Parapholis incurva* (L.) C.E.Hubb., AF532942, AF533036, EF584991; *Poa alpina* L., AY099005, AY099003, EF584999; *Puccinellia distans* (L.) Parl., AF532934, AF533024, EF584985; *Puccinellia frigida* (Phil.) I.M.Johnst., KF020797, JF904754, JF904753; *Secale cereale* L., AY524829, AY528949, EF584998; *Sphenopus divaricatus* (Gouan) Rchb., AF532939, AF533033, EF584992; *Triticum aestivum* L., KY368800, KY368852, KY368903; **BROAD-LEAVED LOLIINAE:** *Drymochloa donax* (Lowe) H.Scholz & Foggii, EF584935, EF592968, EF585033; *Festuca africana* (Hack.) Clayton, KY368800, KY368852, KY368903; *Festuca argentina* (Speg.) Parodi, EF584923, EF592957, EF585012; *Festuca asperella* E.B.Alexeev, KY368796, KY368848, –; *Festuca costata* Nees, KY368801, KY368853, KY368904; *Festuca dichoclada* Pilg., EF584933, EF592967, EF585031; *Festuca extremorientalis* Ohwi, KY368812, KY368863, KY368912; *Festuca griffithiana* (St.-Yves) Krivot., KY368813, KY368864, KY368913; *Festuca kingii* (S.Watson) Casidy, AF303410, AY099004, EF585058; *Festuca longipes* Stapf, KY368804, KY368856, KY368907; *Festuca mekiste* Clayton, KY368805, KY368857, KY368908; *Festuca modesta* Nees ex Steud., EF584953, EF592985, EF585068; *Festuca pseudeskia* Boiss., AF519979, AY099000, EF585084; *Festuca scabra* Vahl, KY368797, KY368849, KY368900; *Festuca scariosa* Lag. ex Willk., AF519978, AY098999, EF585100; *Lolium giganteum* (L.) Darbysh., AF303416, AF533043, EF379003; *Lolium perenne* L., AF303401, AF478504, EF379024; *Lolium pratense* (Huds.) Darbysh., Fb565, Croatia, *Horvat*, 1945 (ZAHO-69600), PP334909\*, –, –, *Lolium tuberosum* (Romero Zarco & Cabezudo) Banfi, Galasso, Foggii, Kopecký & Ardenghi, JQ972944, AF533037, EF379013; *Patzkea coerulescens* (Desf.) H.Scholz, AF533836, AF533051, EF585027; *Pseudobromus breviligulatus* Stapf ex A.Camus, KY368800, KY368858, KY368909; **INTERMEDIATE LOLIINAE:** *Festuca aloha* Catalán, Soreng & P.M.Peterson, GQ162206, GQ162208, –; *Festuca bacjalforniana* Gonz.-Led. & S.D.Koch, KY368814, KY368858, KY368914; **FINE-LEAVED LOLIINAE:** *Festuca acuminata* Gaudin, Fb166, Switzerland, *Schönswetter*, 20.8.2021 (ZA-77566), PP334910\*, PP337565\*, PP337430\*; *Festuca adamovicii* (St.-Yves) Markgr.-Dann., Fb044, Serbia, *Bogdanović, Doboš, Grgurev, Rešetnik*, 10.7.2019 (ZA-68313), PP334911\*, PP337566\*, PP337431\*; *Festuca adamovicii* (St.-Yves) Markgr.-Dann., Fb045, Serbia, *Bogdanović, Doboš, Grgurev, Rešetnik*, 10.7.2019 (ZA-77509), PP334912\*, PP337567\*, PP337432\*; *Festuca adamovicii* (St.-Yves) Markgr.-Dann., Fb047, North Macedonia, *Bogdanović, Doboš, Grgurev, Rešetnik*, 11.7.2019 (ZA-68358), –, PP337568\*, PP337433\*; *Festuca adamovicii* (St.-Yves) Markgr.-Dann., Fb048, North Macedonia, *Bogdanović, Doboš, Grgurev, Rešetnik*, 11.7.2019 (ZA-77399), PP334913\*, PP337569\*, PP337434\*; *Festuca adamovicii* (St.-Yves) Markgr.-Dann., Fb053, North Macedonia, *Bogdanović, Doboš, Grgurev, Rešetnik*, 13.7.2019 (ZA-77412), PP334914\*, PP337570\*, PP337435\*; *Festuca adamovicii* (St.-Yves) Markgr.-Dann., Fb054, North Macedonia, *Bogdanović, Doboš, Grgurev, Rešetnik*, 13.7.2019 (ZA-68359), PP334915\*, PP337571\*, PP337436\*; *Festuca adamovicii* (St.-Yves) Markgr.-Dann., Fb148, North Macedonia, *Rešetnik, Ljubičić, Doboš*, 21.7.2021 (ZA-68509), PP334916\*, PP337572\*, PP337437\*; *Festuca adamovicii* subsp. *bistrae* Micevski & Kostad., Fb147, North Macedonia, *Rešetnik, Ljubičić, Doboš*, 20.7.2021 (ZA-78663), PP334917\*, PP337573\*, PP337438\*; *Festuca agustini* Lindling., AY099005, AY099003, EF584999; *Festuca alpestris* Roem. & Schult., Fb084, Italy, *Schönswetter*, 2.12.2018 (ZA-69608), PP334918\*, PP337574\*, PP337439\*; *Festuca alpestris* Roem. & Schult., Fb154, Italy, *Schönswetter, Zeni*, 20.7.2021 (ZA-69613), PP334919\*, PP337575\*, PP337440\*; *Festuca alpestris* Roem. & Schult., Fb158, Italy, *Doboš*, 2.8.2021 (ZA-77498), PP334920\*, PP337576\*, PP337441\*; *Festuca alpestris* Roem. & Schult., Fb523, Italy, –, 15.6.1949 (ZT-00191096), PP334921\*, PP337577\*, PP337442\*; *Festuca alpestris* Roem. & Schult., Fb524, Italy, –, (ZT-00191097), PP334922\*, PP337578\*, PP337443\*; *Festuca alpina* Suter, Fb581, Croatia, *Lakušić, Tomović, Kuzmanović, Niketić, Janković, Đurović*, 30.06.2012 (BEOU-38017), PP334923\*, PP337579\*, PP337444\*; *Festuca amethystina* L., Fb611, Croatia, *Bogdanović, Ljubičić*, 14.06.2019 (ZAGR-53683), PP334924\*, PP337580\*, PP337445\*; *Festuca amethystina* L., Fb014, Bosnia and Herzegovina, *Bogdanović, Ljubičić*, 10.7.2018 (ZA-48635), PP334925\*, PP337581\*, PP337446\*; *Festuca anatolica* Markgr.-Dann., Fb525, Turkey, –, 28.8.1958 (Z-000195526), PP334927\*, –, –, *Festuca andicola* Kunth, EF584922, EF592955, EF585009; *Festuca baffinensis* Polunin, EF584925, EF592958, EF585013; *Festuca borbonica* Spreng., KY368840, KY368892, KY368939; *Festuca bosniaca* Kumm. & Sendtn., Fb001, Slovenia, *Rešetnik, Bogdanović, Ljubičić*, 14.6.2018 (ZA-47399), PP334928\*, PP337582\*, PP337448\*; *Festuca bosniaca* Kumm. & Sendtn., Fb004, Croatia, *Rešetnik, Bogdanović, Ljubičić*, 15.6.2018 (ZA-47402), PP334929\*, PP337583\*, PP337449\*; *Festuca bosniaca* Kumm. & Sendtn., Fb007, Croatia, *Rešetnik, Bogdanović, Ljubičić*, 18.6.2018 (ZA-47409), PP334930\*, PP337584\*, PP337450\*; *Festuca bosniaca* Kumm. & Sendtn., Fb010, Bosnia and Herzegovina, *Rešetnik, Bogdanović, Ljubičić*, 20.6.2018 (ZA-47411), PP334931\*, PP337585\*, PP337451\*; *Festuca bosniaca* Kumm. & Sendtn., Fb011, Bosnia and Herzegovina, *Rešetnik, Bogdanović, Ljubičić*, 20.6.2018 (ZA-47412), PP334932\*, PP337586\*, PP337452\*; *Festuca bosniaca* Kumm. & Sendtn., Fb011, Bosnia and Herzegovina, *Rešetnik, Bogdanović, Ljubičić*, 21.6.2018 (ZA-47413), PP334933\*, PP337587\*, PP337453\*; *Festuca bosniaca* Kumm. & Sendtn., Fb012, Croatia, *Rešetnik, Bogdanović, Ljubičić*, 22.6.2018 (ZA-47414), PP334934\*, PP337588\*, PP337454\*; *Festuca bosniaca* Kumm. & Sendtn., Fb016, Bosnia and Herzegovina, *Bogdanović, Ljubičić*, 11.7.2018 (ZA-48637), –, PP337589\*, PP337455\*; *Festuca bosniaca* Kumm. & Sendtn., Fb018, Montenegro, *Bogdanović, Ljubičić*, 13.7.2018

## Appendix 1. Continued.

(ZA-48639), PP334935\*, PP337590\*, PP337456\*; *Festuca bosniaca* Kumm. & Sendtn., Fb020, Montenegro, *Bogdanović, Ljubičić*, 14.7.2018 (ZA-48641), PP334936\*, PP337591\*, PP337457\*; *Festuca bosniaca* Kumm. & Sendtn., Fb021, Montenegro, *Bogdanović, Ljubičić*, 14.7.2018 (ZA-48642), PP334937\*, PP337592\*, PP337458\*; *Festuca bosniaca* Kumm. & Sendtn., Fb023, Montenegro, *Bogdanović, Ljubičić*, 15.7.2018 (ZA-48644), PP334938\*, PP337593\*, PP337459\*; *Festuca bosniaca* Kumm. & Sendtn., Fb025, Montenegro, *Bogdanović, Ljubičić*, 17.7.2018 (ZA-48646), PP334939\*, PP337594\*, PP337460\*; *Festuca bosniaca* Kumm. & Sendtn., Fb027, Albania, *Frajman, Schönschwetter*, 11.7.2018 (ZA-48648), PP334940\*, PP337595\*, PP337461\*; *Festuca bosniaca* Kumm. & Sendtn., Fb029, North Macedonia, *Frajman, Schönschwetter*, 15.7.2018 (ZA-4865), PP334941\*, PP337596\*, PP337462\*; *Festuca bosniaca* Kumm. & Sendtn., Fb030, Montenegro, *Lakusić*, 17.5.2008 (ZA-48671), PP334942\*, –, PP337463\*; *Festuca bosniaca* Kumm. & Sendtn., Fb031, Montenegro, *Lakusić*, 15.7.2010 (ZA-48672), PP334943\*, PP337597\*, PP337464\*; *Festuca bosniaca* Kumm. & Sendtn., Fb040, Albania, *Bogdanović, Ljubičić, Rešetnik, Terlević*, 28.6.2019 (ZA-68315), PP334944\*, PP337598\*, PP337465\*; *Festuca bosniaca* Kumm. & Sendtn., Fb110, Bosnia and Herzegovina, *Doboš*, 27.7.2020 (ZA-68377), PP334945\*, PP337599\*, PP337466\*; *Festuca bosniaca* Kumm. & Sendtn., Fb115, Serbia, *Kuzmanović, Stevanovski*, 27.6.2020 (ZA-77445), PP334946\*, PP337600\*, PP337467\*; *Festuca bosniaca* Kumm. & Sendtn., Fb124, Italy, *Bogdanović, Conti, Rešetnik, Temunović, Terlević*, 01.07.2021 (ZA-68380), PP334947\*, PP337601\*, PP337468\*; *Festuca bosniaca* Kumm. & Sendtn., Fb126, Italy, *Bogdanović, Rešetnik, Temunović*, 02.07.2021 (ZA-68385), PP334948\*, PP337602\*, PP337469\*; *Festuca bosniaca* Kumm. & Sendtn., Fb131, Italy, *Bogdanović, Rešetnik, Temunović, Terlević*, 03.07.2021 (ZA-68492), PP334949\*, PP337603\*, PP337470\*; *Festuca bosniaca* Kumm. & Sendtn., Fb132, Italy, *Bogdanović, Rešetnik, Temunović, Terlević*, 05.07.2021 (ZA-68494), PP334950\*, PP337604\*, PP337471\*; *Festuca bosniaca* Kumm. & Sendtn., Fb133, Italy, *Bogdanović, Rešetnik, Temunović, Terlević*, 07.07.2021 (ZA-68497), PP334951\*, PP337605\*, PP337472\*; *Festuca bosniaca* Kumm. & Sendtn., Fb136, Italy, *Bogdanović, Rešetnik, Temunović, Terlević*, 08.07.2021 (ZA-68503), PP334952\*, PP337606\*, PP337473\*; *Festuca bosniaca* Kumm. & Sendtn., Fb149, Albania, *Rešetnik, Doboš*, 22.7.2021 (ZA-68510), PP334953\*, PP337607\*, PP337474\*; *Festuca bosniaca* Kumm. & Sendtn., Fb151, Bosnia and Herzegovina, *Rešetnik, Ljubičić, Doboš*, 25.7.2021 (ZA-68511), PP334954\*, PP337608\*, PP337475\*; *Festuca bosniaca* subsp. *pirinensis* (Acht.) Markgr.-Dann., Fb140, Bulgaria, *Rešetnik, Ljubičić, Doboš*, 15.7.2021 (ZA-68508), PP334955\*, PP337609\*, PP337476\*; *Festuca brevissima* Jurtzev, EF584928, EF592961, EF585018; *Festuca calligera* (Piper) Rydb., EF584929, EF592962, EF585021; *Festuca calva* (Hack.) K.Richt., Fb041, Slovenia, *Terlević, Ljubičić*, 9.7.2019 (ZA-78664), PP334956\*, PP337610\*, PP337477\*; *Festuca calva* (Hack.) K.Richt., Fb068, Slovenia, *Frajman, Schönschwetter*, 15.9.2018 (ZA-69607), PP334957\*, PP337611\*, PP337478\*; *Festuca calva* (Hack.) K.Richt., Fb527, Slovenia, –, 9.8.1952 (ZT-00191077), PP334958\*, PP337612\*, PP337479\*; *Festuca caprina* Nees, KY368825, KY368877, KY368923; *Festuca carpatica* F.Dietr., AY099006, AY099001, EF585023; *Festuca casapaltensis* Ball, KY368832, KY368884, KY368930; *Festuca chodatiana* (St.-Yves) E.B.Alexeev, KY368842, KY368894, KY368941; *Festuca circummediterranea* Patzke, Fb125, Italy, *Bogdanović, Conti, Rešetnik, Temunović, Terlević*, 01.07.2021 (ZAGR-78749), PP334959\*, PP337613\*, PP337480\*; *Festuca circummediterranea* Patzke, Fb625, Italy, *Bogdanović*, 26.07.2012 (ZAGR-48297), PP334960\*, PP337614\*, PP337481\*; *Festuca claytonii* E.B.Alexeev, KY368843, KY368895, KY368942; *Festuca cochabambana* E.B.Alexeev, EF584931, EF592964, EF585026; *Festuca compressifolia* J.Presl, KY368833, KY368885, KY368931; *Festuca contracta* Kirk, KY368819, KY368870, –; *Festuca cuzcoensis* Stančik & P.M.Peterson, EF584932, EF592966, EF585029; *Festuca cyllenica* Boiss. & Heldr., Fb035, Greece, *Bogdanović, Ljubičić, Rešetnik, Terlević*, 22.6.2019 (ZA-77377), PP334961\*, PP337615\*, PP337482\*; *Festuca cyllenica* Boiss. & Heldr., Fb036, Greece, *Bogdanović, Ljubičić, Rešetnik, Terlević*, 21.6.2019 (ZA-77386), PP334962\*, PP337616\*, PP337483\*; *Festuca cyllenica* Boiss. & Heldr., Fb037, Greece, *Bogdanović, Ljubičić, Rešetnik, Terlević*, 23.6.2019 (ZA-77369), PP334963\*, PP337617\*, PP337484\*; *Festuca cyllenica* Boiss. & Heldr., Fb038, Albania, *Bogdanović, Ljubičić, Rešetnik, Terlević*, 24.6.2019 (ZA-77389), PP334964\*, PP337618\*, PP337485\*; *Festuca cyllenica* Boiss. & Heldr., Fb039, Albania, *Bogdanović, Ljubičić, Rešetnik, Terlević*, 25.6.2019 (ZA-77390, ZA-77392), PP334965\*, PP337619\*, PP337486\*; *Festuca cyllenica* Boiss. & Heldr., Fb143, Greece, *Rešetnik, Ljubičić*, 17.7.2021 (ZA-77482), –, PP337620\*, PP337487\*; *Festuca dalmatica* (Hack.) K.Richt., Fb511, North Macedonia, *Horvat*, 19.6.1948 (ZAHO-69585), PP334966\*, –, PP337488\*; *Festuca dimorpha* Guss., Fb120, Italy, *Bogdanović, Conti, Rešetnik, Terlević*, 30.06.2021 (ZAGR-78750), PP334967\*, PP337621\*, PP337489\*; *Festuca dimorpha* Guss., AF519982, AF519987, EF585032; *Festuca duriuscula* s.l., Fb584, Serbia, *Lakušić, Kuzmanović, Lazarević, Janković, Zbiljić*, 14.07.2018 (BEOU-63953), PP334968\*, –, PP337490\*; *Festuca elata* Keng ex E.B.Alexeev, EF584937, EF592970, EF585037; *Festuca eskia* Ramond ex DC., Fb085, Andorra, *Carnicero*, 20.9.2018 (ZA-77424), PP334969\*, PP337622\*, PP337491\*; *Festuca eskia* Ramond ex DC., Fb089, Spain, *Carnicero*, 25.9.2018 (ZA-77431), PP334970\*, PP337623\*, PP337492\*; *Festuca eskia* Ramond ex DC., Fb093, France, *Carnicero*, 28.9.2018 (ZA-77432), PP334971\*, PP337624\*, PP337493\*; *Festuca flacca* Hack. ex E.B.Alexeev, EF584938, EF592971, EF585041; *Festuca flavescens* Belardi, Fb536, France, –, 26.6.1955 (ZT-00078101), PP334972\*, –, –; *Festuca galicicae* Horvat ex Markgr.-Dann., Fb026, Albania, *Lakusić, Kuzmanović, Janković, Zbiljić*, 11.7.2018 (ZA-48647), PP334973\*, PP337625\*, PP337494\*; *Festuca galicicae* Horvat ex Markgr.-Dann., Fb051, North Macedonia, *Bogdanović, Doboš, Grgurev, Rešetnik*, 12.7.2019 (ZA-78665), PP334974\*, PP337626\*, PP337495\*; *Festuca gautieri* (Hack.) K.Richt., Fb087, Spain, *Carnicero*, 24.9.2018 (ZA-77427), PP334975\*, PP337627\*, PP337496\*; *Festuca gautieri* (Hack.) K.Richt., Fb092, Spain, *Carnicero*, 27.9.2018 (ZA-77436), PP334976\*, PP337628\*, PP337497\*; *Festuca gautieri* (Hack.) K.Richt., Fb094, France, *Carnicero*, 28.9.2018 (ZA-77437), PP334977\*, PP337629\*, PP337498\*; *Festuca glacialis* Miégév. ex Bureau, AF303428, AF478523, EF585045; *Festuca glumosa* Hack. ex E.B.Alexeev, EF584940, EF592973, EF585046; *Festuca halleri* All., Fb580, Serbia, *Kuzmanović, Stevanoski*, 20.07.2020 (BEOU-63457), PP334978\*, PP337630\*, PP337499\*; *Festuca halleri* subsp. *scardica* (Griseb.) Markgr.-Dann., Fb579, Greece, *Lakušić, Kuzmanović, Janković, Zbiljić*, 27.07.2014 (BEOU-46473), PP334980\*, PP337632\*, PP337501\*; *Festuca hephaestophila* Nees (Nees), EF584943, EF592976, EF585048; *Festuca hercegovinica* Markgr.-Dann., Fb617, Croatia, *Bogdanović, Ljubičić, Rešetnik*, 18.06.2018 (ZAGR-51371), PP334982\*, PP337634\*, PP337503\*; *Festuca heterophylla* Lam., Fb570, Croatia, *Horvat*, 3.6.1934 (ZAHO-69603), PP334983\*, PP337635\*, PP337504\*; *Festuca heterophylla* Lam., Fb604, Serbia, *Kuzmanović, Milekić, Zbiljić*, 15.06.2017 (BEOU-52260), PP334984\*, PP337636\*, PP337505\*; *Festuca hieronymi* Hack., KY368835, KY368887, KY368933; *Festuca hirtovaginata* (Acht.) Markgr.-Dann., Fb586, North Macedonia, *Lakušić, Kuzmanović, Janković, Zbiljić*, 13.07.2018 (BEOU-63892), PP334985\*, PP337637\*, PP337506\*; *Festuca horvatica* Markgr.-Dann., Fb095, Greece, *Bogdanović, Grgurev, Rešetnik*, 14.7.2019 (ZAGR-78751), PP334986\*, PP337638\*, PP337507\*; *Festuca hyperborea* Holmen ex Fred., EF584946, EF592978, EF585050; *Festuca hystrix* Boiss., AF478480, AF478520, EF585051; *Festuca iberica* (Hack.) K.Richt., AY118087, AF478516, EF585052; *Festuca indigesta* Boiss., AF303426, AF478519, EF585054; *Festuca juncifolia* Chaub., AF478478, AF478515, EF585057; *Festuca korabensis* (Jáv. ex Markgr.-Dann.) Markgr.-Dann., Fb599, North Macedonia, *Bogdanović, Rešetnik*, 07.2019 (BEOU-69369), PP334987\*, PP337639\*, PP337508\*; *Festuca korabensis* (Jáv. ex Markgr.-Dann.) Markgr.-Dann., Fb601, North Macedonia, *Lakušić, Kuzmanović, Janković*, 22.07.2016 (BEOU-46330), PP334988\*, PP337640\*, PP337509\*; *Festuca koritnicensis* Hayek & Vetter, Fb585, Montenegro, *Lakušić, Kuzmanović, Milekić*, 05.06.2017 (BEOU-52213), PP334989\*, PP337641\*, PP337510\*; *Festuca koritnicensis* Hayek & Vetter, Fb587, Montenegro, *Lakušić, Conti, Bulić, Niketić, Chiasetti, Tomović, Hadžiablahović*, 18.07.2003 (BEOU-24873), PP334990\*, PP337642\*, PP337511\*; *Festuca lapidosa* (Degen) Markgr.-Dann., Fb626, Croatia, *Bogdanović, Ljubičić*, 27.04.2012 (ZAGR-31720), PP334991\*, –, PP337512\*; *Festuca laxa* Host, KY368816, KY368867, KY368917; *Festuca magellanica* Lam., KY368829, KY368881, KY368927; *Festuca nardifolia* Griseb., EF584954, EF592986, EF585070; *Festuca nemoralis* Türpe, KY368821, KY368872, KY368920; *Festuca nigrescens* Lam., Fb602, Austria, *Kuzmanović*, 09.09.2017 (BEOU-496869), PP334992\*, PP337643\*, PP337513\*; *Festuca olympica* Vetter, Fb578, Greece, *Lakušić, Kuzmanović, Janković*, 25.07.2016 (BEOU-46335), PP334993\*, PP337644\*, PP337514\*; *Festuca orthophylla* Pilg., EF584957, EF592989, EF585075; *Festuca ovina* L., Fb101, Albania, *Bogdanović, Ljubičić, Rešetnik, Terlević*, 28.6.2019 (ZAGR-78752), PP334994\*, PP337645\*, PP337515\*; *Festuca pallens* Host, Fb583, Serbia, *Lakušić, Kuzmanović, Lazarević, Janković, Zbiljić*, 14.07.2018 (BEOU-63974), PP334995\*, PP337646\*, PP337516\*; *Festuca panciciana* (Hack.) K.Richt., Fb594, Bosnia and Herzegovina, *Lakušić, Kuzmanović, Janković*, 08.07.2016 (BEOU-46074), PP334996\*, PP337647\*, PP337517\*; *Festuca panciciana* (Hack.) K.Richt., Fb608, Serbia, *Milekić, T., Milekić, B., Milekić, N., Milekić, S., Milekić, L.,*

## Appendix 1. Continued.

21.06.2017 (BEOU-52298), PP334926\*, –, PP337447\*; *Festuca panciciana* (Hack.) K.Richt., Fb633, Croatia, *Bogdanović, Ljubičić, Rešetnik*, 18.06.2018 (ZAGR-51374), PP334997\*, PP337648\*, PP337518\*; *Festuca penzesii* (Acht.) Markgr.-Dann., Fb141, Bulgaria, *Rešetnik, Ljubičić, Doboš*, 16.7.2021 (ZA-78666), PP334998\*, PP337649\*, PP337519\*; *Festuca penzesii* (Acht.) Markgr.-Dann., Fb142, Greece, *Doboš*, 17.7.2021 (ZA-78667), PP334999\*, PP337650\*, PP337520\*; *Festuca peruviana* Infantes, KY368836, KY368888, KY368934; *Festuca picturata* Pils, Fb598, Austria, *Schönswetter*, 08.09.2017 (BEOU-49688), PP335000\*, PP337651\*, PP337521\*; *Festuca picturata* Pils, Fb600, Romania, *Kuzmanović, Stevanoski*, 17.07.2019 (BEOU-69316), PP335001\*, PP337652\*, PP337522\*; *Festuca pirinica* Horvat ex Markgr.-Dann., Fb582, Bulgaria, *Lakušić, Kuzmanović, Janković*, 13.07.2015 (BEOU-42858), PP335002\*, PP337653\*, PP337523\*; *Festuca pseudokorabensis* D.Lakušić prov. 1999: 99, Fb631, Montenegro, *Bogdanović, Ljubičić*, 13.07.2018 (ZAGR-51956), PP335003\*, PP337654\*, –; *Festuca pseudokorabensis* D.Lakušić prov. 1999: 99, KY368826, KY368878, KY368924; *Festuca pseudokorabensis* D.Lakušić prov. 1999: 99, Fb595, Montenegro, *Lakušić*, 14.08.2017 (BEOU-46642), PP335004\*, PP337655\*, PP337524\*; *Festuca pseudokorabensis* D.Lakušić prov. 1999: 99, Fb596, Montenegro, *Lakušić, Surina, Glasnović, Kuzmanović, Janković, Balant*, 08.07.2017 (BEOU-47143), PP335005\*, PP337656\*, PP337525\*; *Festuca pseudovina* Hack. ex Wiesb., Fb607, Serbia, *Jakovljević, Kuzmanović, Milekić*, 13.06.2017 (BEOU-52244), PP335006\*, PP337657\*, PP337526\*; *Festuca purpurascens* Banks & Sol. ex Hook.f., EF584964, EF592996, EF585087; *Festuca quadriflora* (Honck.), KY368817, KY368868, KY368918; *Festuca quadriflora* Honck., Fb155, Italy, *Schönswetter, Zeni*, 21.7.2021 (ZA-69614), PP335007\*, PP337658\*, PP337527\*; *Festuca quadriflora* Honck., Fb545, Switzerland, –, 18.8.1985 (ZT-00191105), PP335008\*, PP337659\*, PP337528\*; *Festuca quadriflora* Honck., Fb546, Germany, –, 27.7.1990 (ZT-00191108, ZT-00191109), PP335009\*, PP337660\*, PP337529\*; *Festuca rechingeri* Markgr.-Dann., Fb057, North Macedonia, *Bogdanović, Grgurev, Rešetnik*, 17.7.2019 (ZA-68368), PP335010\*, PP337661\*, PP337530\*; *Festuca rechingeri* Markgr.-Dann., Fb144, Greece, *Ljubičić, Doboš*, 18.7.2021 (ZA-78668), PP335011\*, PP337662\*, PP337531\*; *Festuca rechingeri* Markgr.-Dann., Fb145, Greece, *Rešetnik, Ljubičić, Doboš*, 19.7.2021 (ZA-78669), PP335012\*, PP337663\*, PP337532\*; *Festuca rigescens* (J.Presl) Kunth, EF584966, EF592999, KY368936; *Festuca rohlena* D.Lakušić, Fb589, Montenegro, *Lakušić*, 2019 (BEOU-69499), PP335013\*, PP337664\*, PP337533\*; *Festuca rohlena* D.Lakušić, Fb591, Albania, *Frajman, Schoenswetter*, 13.07.2018 (IB-15133), PP335014\*, PP337665\*, PP337534\*; *Festuca rohlena* D.Lakušić, Fb592, Montenegro, *Lakušić*, 10.08.2005 (BEOU-20093), PP335015\*, PP337666\*, PP337535\*; *Festuca rubra* L., Fb630, Croatia, *Bogdanović*, 13.07.2014 (ZAGR-38635), PP335016\*, PP337667\*, –; *Festuca rubra* L., EF584968, EF593001, EF585097; *Festuca rupicola* Heuff., Fb100, North Macedonia, *Bogdanović, Doboš, Grgurev, Rešetnik*, 12.7.2019 (ZAGR-78753), PP335017\*, PP337668\*, PP337536\*; *Festuca rupicola* Heuff., Fb509, Croatia, *Horvat*, 30.5.1935 (ZAHO-69583), PP335018\*, PP337669\*, PP337537\*; *Festuca rupicola* Heuff., Fb518, Croatia, *Horvat*, 5.8.1940 (ZAHO-69586), PP335019\*, PP337670\*, PP337538\*; *Festuca rupicola* Heuff., Fb519, Croatia, *Horvat*, 30.5.1950 (ZAHO-69587), PP335020\*, –, –; *Festuca rupicola* Heuff., Fb605, Serbia, *Lakušić, Kuzmanović, Lazarević, Janković, Zbiljić*, 14.07.2018 (BEOU-63979), PP335021\*, PP337671\*, PP337539\*; *Festuca saximontana* Rydb., EF584969, EF593002, EF585098; *Festuca stenantha* (Hack.) K.Richt., Fb506, Croatia, *Horvat*, 23.7.1929 (ZAHO-69580), PP335022\*, –, –; *Festuca stricta* Host, Fb507, Bosnia and Herzegovina, *Horvat*, 20.7.1956 (ZAHO-69581), PP335023\*, PP337672\*, PP337540\*; *Festuca subverticillata* (Pers.) E.B.Alexeev, EF584974, EF593006, EF585106; *Festuca supina* Schur, Fb588, Serbia, *Kuzmanović, Stevanoski*, 12.07.2020 (BEOU-63327), PP335024\*, PP337673\*, PP337541\*; *Festuca vaginalis* (Benth.) Laegaard, EF584977, EF593010, EF585111; *Festuca vaginata* Waldst. & Kit., Fb619, Croatia, *Bogdanović, Ljubičić*, 11.05.2021 (ZAGR-62851), PP335025\*, PP337674\*, PP337542\*; *Festuca valesiaca* Gaudin, Fb573, Croatia, *Horvat*, 19.8.1933 (ZAHO-69605), PP335026\*, PP337675\*, PP337543\*; *Festuca valesiaca* Gaudin, Fb621, Croatia, *Bogdanović, Rešetnik*, 13.05.2018 (ZAGR-51223), PP335027\*, PP337676\*, PP337544\*; *Festuca valida* (Velen.) Pénzes, Fb139, Bulgaria, *Rešetnik, Ljubičić, Doboš*, 15.7.2021 (ZA-68507), PP335028\*, PP337677\*, PP337545\*; *Festuca varia* Haenke subsp. *varia*, Fb160, Austria, *Doboš, Frajman*, 3.8.2021 (ZA-69618), PP335029\*, PP337678\*, PP337546\*; *Festuca varia* Haenke subsp. *varia*, Fb161, Austria, *Doboš, Frajman*, 3.8.2021 (ZA-69619), PP335030\*, PP337679\*, PP337547\*; *Festuca varia* subsp. *handel-mazzettii* Haenke, Fb156, Italy, *Doboš, Frajman*, 2.8.2021 (ZA-69615), PP335031\*, PP337680\*, PP337548\*; *Festuca varia* subsp. *winnebachensis* Haenke, Fb159, Austria, *Doboš, Frajman*, 2.8.2021 (ZA-69617), PP335032\*, PP337681\*, PP337549\*; *Festuca ventanica* Speg., KY368823, KY368874, KY368922; *Festuca versicolor* J.Presl ex Kunth, Fb547, Romania, –, 26.7.1911 (ZT-00191102), PP335035\*, PP337683\*, PP337552\*; *Festuca versicolor* subsp. *pallidula* (Hack.) Markgr.-Dann., Fb164, Austria, *Doboš, Frajman*, 4.8.2021 (ZA-77515), PP335033\*, PP337682\*, PP337550\*; *Festuca versicolor* subsp. *pallidula* (Hack.) Markgr.-Dann., Fb165, Austria, *Doboš, Frajman*, 4.8.2021 (ZA-77519), PP335034\*, –, PP337551\*; *Festuca violacea* Ser. ex Gaudin, Fb577, North Macedonia, *Lakušić, Kuzmanović, Janković*, 22.07.2016 (BEOU-46385), PP335036\*, PP337684\*, PP337553\*; *Festuca violacea* Ser. ex Gaudin, Fb597, Albania, *Lakušić, Kuzmanović, Janković*, 10.07.2017 (BEOU-47198), PP335037\*, PP337685\*, PP337554\*; *Festuca vulpioides* Steud., KY368828, KY368880, KY368926; *Festuca wagneri* (Degen, Thaisz & Flatt) Krajina, Fb609, Serbia, *Jakovljević, Kuzmanović, Milekić*, 13.06.2017 (BEOU-52224), PP335038\*, PP337686\*, PP337555\*; *Festuca woronowii* Hack., Fb168, Armenia, *Doboš*, 1.9.2021 (ZA-77573), PP335039\*, PP337687\*, PP337556\*; *Festuca woronowii* Hack., Fb549, Georgia, –, 14.7.1975 (ZT-00191071), PP335040\*, PP337688\*, PP337557\*; *Festuca xanthina* Roem. & Schult., Fb058, Serbia, *Bogdanović, Grgurev, Rešetnik*, 18.7.2019 (ZA-68369), PP335041\*, PP337689\*, PP337558\*; *Festuca xanthina* Roem. & Schult., Fb059, Serbia, *Bogdanović, Grgurev, Rešetnik*, 19.7.2019 (ZA-77420), PP335042\*, PP337690\*, PP337559\*; *Festuca xanthina* Roem. & Schult., Fb062, Serbia, *Lakušić*, 5.5.2008 (BEOU-26606), PP335043\*, PP337691\*, PP337560\*; *Festuca xanthina* Roem. & Schult., Fb065, Serbia, *Lakušić, Kuzmanović*, 23.5.2008 (BEOU-27075), PP335044\*, PP337692\*, PP337561\*; *Festuca xanthina* Roem. & Schult., Fb116, Serbia, *Kuzmanović, Stevanoski*, 12.7.2020 (ZA-77449), PP335045\*, PP337693\*, PP337562\*; *Festuca xanthina* Roem. & Schult., Fb593, Romania, *Kuzmanović*, 18.04.2009 (BEOU-29776), PP335046\*, PP337694\*, PP337563\*; *Festuca yalaensis* Joch. Müll. & Catalán, GQ849279, GQ849280, GQ849281; *Psilurus incurvus* (Gouan) Schinz & Thell., JQ972945, AF478533, JQ973013; *Vulpia bromoides* (L.) Gray, AF478485, AF478616, EF585119; *Vulpia fasciculata* (Forsk.) Samp., AF478487, AF478528, EF585121; *Vulpia geniculata* (L.) Link, AF478490, AF478531, EF585123; *Vulpia myuros* (L.) C.C.Gmel., AY118092, AY118103, EF585127; *Vulpia octoflora* (Walter) Rydb., EF584982.1, EF593016, EF585128; *Vulpia unilateralis* (L.) Stace, AY118095, AY118106, EF585130; *Wangenheimia lima* (L.) Trin., AF478498, AF478536, EF585131.