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REGULAR PAPER

Phylogenetic relationships of the '*Briza* complex' to other members of the subfamily Pooideae (Poaceae)

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Background and aims – The species of the '*Briza* complex' (Pooideae, Poaceae) are distributed in South America and Eurasia. They are relatively well-studied morphologically and have a complex taxonomic history, but only a few phylogenetic studies have been conducted using molecular data. Monophyly of the complex, which is based on presence of 'brizoid' spikelets, has not been questioned and sampling strategies in previous studies have prevented assessments thereof.
Methods – We investigate phylogeny and node ages in the *Briza* complex and test monophyly of the group using nuclear and chloroplast data. Extensive sampling from the *Briza* complex and putatively related species in the subfamily Pooideae is employed.
Key results – Despite morphological similarity among species, the *Briza* complex is polyphyletic. Members were found in three different clades, showing the South American species, the Eurasian species and *Briza humilis* to be distinct groups. The South American and the Eurasian clades originated about 11 and 13 million years ago, respectively. *Briza humilis* diverged from *Phleum* (or a related genus) about 10 million years ago, whereas its crown clade is from the Pliocene-Pleistocene border. The almost simultaneous origins of these clades in the mid-Miocene coincide with temporal estimates of major diversification in grasses and formation of grassland habitats.
Conclusions – Based on our results, we support the names *Chascolytrum* for the South American clade and *Briza* for the Eurasian clade. For the *Briza humilis* clade, we propose the name *Brizochloa.* The parallel evolution of (seemingly) similar 'brizoid' spikelets in the Pooideae is surprising; however, studies have shown that floral morphology can alter dramatically by one-step mutations, causing evolutionarily distantly related species to have similar appearance. Our findings may hopefully inspire new morphological investigations of the species of the former *Briza* complex, as well as other poorly studied and potentially polyphyletic genera, such as *Deschampsia* and *Echinopogon*.

Key words – Bayesian analysis, birth-death prior, *Briza*, *Brizochloa*, *Chascolytrum*, classification, model selection, node ages, Pooideae, Yule prior.

INTRODUCTION

Ever since the birth of modern systematics, taxonomic groups have been founded on shared specific similarities (synapomorphies) (Hennig 1965, Wiley et al. 1991). Early studies utilised morphological data (e.g. Crane 1985), but in recent decades there has been a remarkable progress in molecular methods and the use of DNA sequences to determine relationships. Today, molecular methods are often considered a necessary complement to morphological studies (Simpson 2010). The grass family (Poaceae) represents one example of a group where analyses based on morphology as well as molecular data confirm its monophyly (Barker et al. 2001). The clade can be traced to the late Cretaceous (Maastrichtian) based on fossil pollen (Linder 1987, Friis et al. 2011) and the discovery of grass phytoliths in dinosaur remains (Prasad et al. 2005). Grass-dominated ecosystems do, however, not appear until much later, beginning in South America during the Oligocene and in the Miocene in other continents (Friis et al. 2011, Stromberg 2011). Today the about 11,000 members of the Poaceae are spread all over the world and occupy more than a third of the land surface of the Earth (Gibson 2009, Stromberg 2011).

The '*Briza* complex' of the subfamily Pooideae is a relatively small group of grasses, characterized by 'brizoid'

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spikelets, which are laterally compressed with closely overlapping, heart-shaped lemmas (Matthei 1975, Bayón 1998). The complex has a natural distribution in South America and western Eurasia, although recent anthropogenic activity has brought a few species to other parts of the world, e.g. North America (Hitchcock 1951). Several authors have classified them into a Eurasian and a South American clade, based both on morphology, karyotype data and molecular data (Murray 1975, King 1986, Essi et al. 2008, de Pelegrin et al. 2009). There are, however, taxonomic disagreements, in particular regarding the South American species and whether they should be called *Briza* or if they should be assigned to one or several other genera. Based on morphological data, Matthei (1975) recognised four genera within the *Briza* complex: *Briza*, *Calotheca*, *Chascolytrum* and *Poidium*, but Nicora & Rúgolo de Agrasar (1981) and Bayón (1998) recognised five genera: *Briza*, *Calotheca*, *Microbriza*, *Poidium* and *Rhombolytrum*. Clayton & Renvoize (1986) recognised two genera: *Briza* and *Microbriza.* Based on molecular data, Essi et al. (2008) also recognised two genera within the *Briza* complex: *Briza* and *Chascolytrum*, and in their subsequent articles (Essi et al. 2010, 2011) the South American species were transferred to *Chascolytrum*, which is now an accepted classification (WCSP 2014). Today, *Chascolytrum* also includes species never assigned to *Briza*, e.g. *C. koelerioides*. In contrast, *Desmazeria* and *Eragrostis* are examples of genera that include species that were previously assigned to *Briza* (WCSP 2014).

Previous studies of the *Briza* complex have, however, focused exclusively on the phylogeny within the group itself, i.e. a taxonomically narrow but extensive sampling within the complex (King 1986, Bayón 1998, Essi et al. 2008, de Pelegrin et al. 2009). Other studies have addressed relationships within the subfamily Pooideae, using a taxonomically wide sampling but with few species representing each genus (Döring et al. 2007, Quintanar et al. 2007, Schneider et al. 2009). Thus, due to these sampling strategies, no study has so far been able to rigorously test for monophyly of the *Briza* complex. For example, although Essi et al. (2008) used a generous sampling of *Briza*, they only included three outgroup terminals (*Amphibromus*, *Bromus* and *Poa*). Further, in contrast with morphological studies of the *Briza* complex, which have generated well-resolved trees, the molecular study by Essi et al. (2008) was partly unresolved.

A few studies on the Pooideae have in fact indicated a possible polyphyly of the *Briza* complex. A maximum parsimony analysis based on chloroplast restriction sites found a group including *Briza minor* and *Chascolytrum erectum*, where *B. minor* was sister to a clade comprising *C. erectum*, *Deschampsia cespitosa* and *Torreyochloa erecta* (Soreng et al. 1990). With the same method of analysis, but including four different chloroplast regions, Davis & Soreng (2007) found a clade including *Briza minor*, *Chascolytrum subaristatum* and *Calotheca brizoides*, where *C. subaristatum* and *C. brizoides* were closest relatives but separated from *B. minor* by *Echinopogon caespitosus*. Using Bayesian inference and the chloroplast *matK* gene, Döring et al. (2007) found a well-supported clade including *Chascolytrum erectum* and *C. subaristatum*, and a few other genera such as *Agrostis* and *Polypogon*, but *Briza media* was placed outside of this clade. The extensive study based on nuclear ITS data by Quintanar et al. (2007) on the tribe Aveneae included *Briza media* and *B. minor*, as well as *Gymnachne koelerioides* (which is now assigned to *Chascolytrum*). Based on Bayesian inference they found that *B. media* and *B. minor* were placed as closest relatives with good support and separated from *G. koelerioides* by *Agrostis* spp., *Gastridium ventricosum* and *Polypogon maritimus*. Only a few authors mention this indicative polyphyly (Soreng et al. 1990, Davis & Soreng 2007, Döring 2009), and no study has so far specifically addressed the issue using relevant and sufficient sampling.

Therefore, the aim of the present study is to, for the first time, test the monophyly of the *Briza* complex using a relevant sampling, and assess divergence times and relationships of the members of the *Briza* complex to other members of the Pooideae.

MATERIAL AND METHODS

Taxon sampling and DNA sequencing

Forty-nine specimens, representing 23 species that historically have been included in the *Briza* complex (see summary in table 1 in Essi et al. 2008) were selected for the present study. Several specimens of each species were included whenever possible. We also included a comprehensive set of potentially related genera from the subfamily Pooideae. The monophyly of the Pooideae has been shown in several previous studies using different nuclear and plastid regions (Hsiao et al. 1999, Barker et al. 2001). Earlier molecular studies using only a few *Briza* species have found them well nested into the tribe complex Aveneae/Poeae of the Pooideae (Döring et al. 2007, Quintanar et al. 2007, Schneider et al. 2009). Based on results in these studies, taxa outside of the *Briza* complex were selected from the sister tribe complexes Aveneae/Poeae and Triticeae/Bromeae. The outgroup species were selected from similar natural distribution areas as occupied by *Briza* whenever possible, i.e. South America, western Eurasia and the Mediterranean region including northern Africa (electronic appendix 1). The exception is *Echinopogon*, which is clearly relevant to include even though it has a distribution limited to Oceania. Trees were rooted with species of *Glyceria* and *Melica* of the tribe Meliceae using the outgroup criterion (Farris 1972).

The majority of the sampling was done from herbarium specimens at the National Herbaria of Stockholm (S) and Uppsala (UPS) in Sweden (acronyms following Thiers 2015). In addition, fresh plant material was collected in Messinia, Greece, in May 2014 and in Sweden in June to September 2014. To increase the data set even further, sequences from relevant species were downloaded from GenBank (electronic appendix 2).

Total DNA content was extracted using a modified version of the CTAB method (Doyle 1991), following the manufacturer's instructions. Three DNA regions were amplified using standard procedures: the nuclear internal transcribed spacer of the ribosomal DNA (ITS1-5.8S gene-ITS2; nrITS) and the granule bound starch synthase I gene (GBSSI) and the chloroplast *matK* gene-3'*trnK* exon. Potentially suitable primers for the three regions were taken from the literature

Table 1 – Primers used for PCR and sequencing.

DNA region	Primer name	Sequence 5'-3'	Reference
GBSSI gene	F-for	TGC GAG CTC GAC AAC ATC ATG CG	Mason-Gamer et al. (1998)
	K-bac	GCA GGG CTC GAA GCG GCT GG	Mason-Gamer et al. (1998)
	M-bac	GGC GAG CGG CGC GAT CCC TCG CC	Mason-Gamer et al. (1998)
ITS1-5.8S gene-ITS2	LEU1	GTC CAC TGA ACC TTA TCA TTT	Vargas et al. (1998)
	ITS4	TCC TCC GCT TAT TGA TAT GC	White et al. (1990)
matK gene-3'*trnK* exon	W	TAC CCT ATC CTA TCC AT	Hilu et al. (1999)
	PO-matK 860F	CAT TAT GTT CGA TAT CAA GG	Schneider et al. (2009)
	9R	TAC GAG CTA AAG TTC TAG C	Hilu et al. (1999)
	trnK-2R	AAC TAG TCG GAT GGA GTA G	Johnson & Soltis (1995)

(table 1) and digitally tested against existing *Briza* sequences from GenBank using the software Amplify3x version 3.1.4 (Engels 2005). Sequencing was performed by Macrogen sequencing service in the Netherlands, using the same primers used for PCR. Sequences obtained from Macrogen were assembled using the Staden Package (Staden 1996).

Alignment and phylogenetic analysis

Alignment of each region was first performed automatically using MUSCLE (Edgar 2004) as implemented in AliView version 1.14 (Larsson 2014) with subsequent manual adjustments of codon positions (GBSSI and *matK*) using GenBank sequences of *Bromus tectorum* (GenBank accession number AY362757, Mason-Gamer 2004) and *Agrostis castellana* (GenBank accession number DQ146799, Reichman et al. 2006) as templates. Gaps in the alignment were treated as missing data.

The aligned sequences were analysed in three different ways: (1) nuclear data alone, (2) plastid data alone and (3) all regions in a combined dataset. Bayesian analyses were carried out in MrBayes version 3.2.3 (Ronquist et al. 2012) at the CIPRES Science Gateway (Miller et al. 2010). For the combined analysis nine unlinked partitions were specified, separating regions, codon positions and introns, according to results found using software PartitionFinder (Lanfear et al. 2012). Single gene matrices were partitioned into codon positions and introns only, again according to results found using PartitionFinder. All data sets were analysed under a mixed model, which utilises a Reversible Jump Markov Chain Monte Carlo algorithm that allows the MCMC chain to sample from all time-reversible models while taking the model uncertainty into account (Huelsenbeck et al. 2004). The Metropolis Coupled Markov Chain Monte Carlo algorithm (MC^3) (Geyer 1991) was used, including one cold chain and three heated chains for each of four runs. A flat Dirichlet prior probability of nucleotide frequencies was specified (all values set to 1.0) and the prior probability of the proportion of invariable sites was uniformly distributed on the interval (0.0, 1.0). Sampling from the chain occurred every 1000^{th} generation and burn-in was set to 20% of the total number of samples from the chain. In order to reach convergence, the analyses had to be run for 5 million generations for *matK* (plastid dataset), 50 million generations for nrITS and GBSSI in combination (nuclear dataset) and 80 million generations for nrITS, GBSSI and *matK* in combination (combined dataset). Four criteria had to be met in order to accept the resulting tree of an analysis: the standard deviation of split frequencies was below 0.01, the chain swap was between 20 and 80% (McGuire et al. 2007), no trend was seen in the overlay plot and the Potential Scale Reduction Factor (PSRF) (Gelman & Rubin 1992) values had reached 1.0 for all parameters. The resulting trees were inspected using FigTree version 1.4.2 (Rambaut 2014) and the layouts of the final trees were edited using Adobe Photoshop CS6.

Parsimony bootstrap analyses were performed in PAUP* (Swofford 2002) (1000 bootstrap replicates, 10 random sequence additions in each). Maximum likelihood analyses were performed in PhyML using the general time reversible model (Tavare 1986) with substitution rates drawn from a gamma distribution, the subtree pruning and regrafting tree searching approach (Evans & Winter 2006) and a parsimony tree as a starting tree. The combined dataset was partitioned into nuclear data and chloroplast data. Statistical support (a Bayesian-like transformation of an approximate likelihood ratio test) was obtained using a fast likelihood-based method (aBayes) (Anisimova & Gascuel 2006, Anisimova et al. 2011).

Analysis of divergence times

Estimates of divergence times of clades were produced using BEAST version 1.8.2 (Drummond et al. 2012) at the CIPRES Science Gateway (Miller et al. 2010) on the combined dataset. Clades were constrained as monophyletic based on the results of the phylogenetic analysis retrieved from MrBayes

(described in the result section). Two tree priors were tested: a birth-death process (Kendall 1948) and a pure birth process (Yule 1925). In addition, two clock models were tested: strict clock and relaxed (uncorrelated lognormal) clock. For the latter, which was used in final analyses, an exponentially distributed prior was specified (ucld.stdev: initial value 0.33, mean 0.33, offset 0.0; ucld.mean: initial value 5.0, mean 5.0, offset 0.0). In order to test the fit of each model to the data, path sampling and stepping-stone sampling (Baele et al. 2012, 2013) were performed in BEAST, and significance was assessed following Kass & Raftery (1995), i.e. a difference in log marginal likelihood values greater than three between two approaches should be seen as significant.

Calibration to absolute ages was, in the absence of reliably placed fossils within the group, accomplished by specifying normally distributed age priors for two nodes based on results in Bouchenak-Khelladi et al. (2010). Mean root height was set to 40.5 million years (mya), with a standard deviation of 5.0. Mean age of the ingroup (i.e. excluding *Glyceria*, *Melica* and *Bromus*) was set to 33.5 mya, with a standard deviation of 5.0. Runs without the data were performed to ensure that the priors did not interact with each other and/or the tree prior to produce unacceptable effective settings. Analyses were run for 150 million generations under the GTR-Γ site model and estimated base frequencies, with sampling every 1000th generation. Convergence of the runs was evaluated in Tracer version 1.6 (Rambaut et al. 2014). Of the 150 000 trees obtained in the analysis, the first 37 500 (25%) were removed as burnin, and the remaining trees were assembled using TreeAnnotator of the BEAST package. The resulting tree was inspected using FigTree and the layout was edited using Adobe CS6.

RESULTS

Data description

In total, 147 new sequences representing 79 specimens were produced for the present study and analysed together with sequences from GenBank. All species of the *Briza* complex were represented by at least one of the three molecular markers used in the present study (electronic appendix 2). Alignment of, in total, 102 sequences of the nrITS region revealed a number of short indels, ranging from 1 to 11 base pairs in length, resulting in a total length of 737 alignment positions. Of these, 390 were variable whereof 310 were phylogenetically informative (79%). Codon alignment of 39 sequences of the GBSSI gene revealed several indels ranging from 1 to 120 base pairs in length, resulting in a total length of 1426 alignment positions. Of these, 708 were variable whereof 276 were phylogenetically informative (39%). As reported by Davis & Soreng (2007), intron 10 in GBSSI was missing for the Aveneae/Poeae species. Codon alignment of 59 sequences of the *matK* region revealed a number of indels ranging from 1 to 16 base pairs, resulting in a total length of 1872 alignment positions. Of these, 471 were variable whereof 262 were phylogenetically informative (56%). The specimens corresponding to "Clade 1" in Döring et al. (2007) and in Schneider et al. (2009) had an insertion of four base pairs in the 3'*trnK* exon, as reported by the latter authors. Otherwise, the vast majority of the indels in all three regions appeared to be species specific or clade-independently variable.

Phylogenetic results

Results found in the Bayesian analyses based on the nuclear dataset and the plastid dataset were overall similar, although the topologies differed slightly (see below). Topological incongruences were however not supported (statistical support here defined as a posterior probability [pp] of ≥ 0.95, a likelihood ratio [lr] or parsimony bootstrap [pb] of ≥ 0.70), with one exception, the placement of a single *Briza minor* specimen (from the Juan Fernandez Islands outside of the coast of Chile), which was included in clade A based on plastid data (pp 1) and in clade B based on nuclear data (pp 1). Two other noteworthy differences (although not highly supported) were the exclusions of *Calamagrostis* spp. and *Ammophila arenaria* from clade A and *Briza maxima* from clade B in the nuclear analysis.

Combined dataset – The Bayesian analysis combining the nuclear (ITS and GBSSI) and plastid (*matK*) data generated a tree with three clades containing species of *Briza* (fig. 1) (here referred to as clades A, B and D). The South American *Briza* species in clade A formed a well-supported group (pp 0.97), but their internal relationships were not resolved with strong statistical support. *Agrostis* spp., *Gastridium* spp., and *Polypogon* spp. (pp 0.95) constituted their sister group (pp 0.97), while *Calamagrostis* spp. and *Ammophila arenaria* (pp 1) formed the sister group to the rest of the species in clade A (pp 0.97). Clade B was well-supported (pp 1) and comprised the Eurasian species *Briza maxima*, *B. marcowiczii*, *B. media* and *B. minor*. The single conflicting *B. minor* specimen (from the Juan Fernandez Islands) was removed from the combined analysis; further studies would be needed to assess the nature of the conflict. Clade B was sister (pp 0.62) to the small clade comprising *Anthoxanthum* spp. and *Hierochloë* spp. (pp 1). Clade C was well-supported (pp 1) and showed two main sister groups; *Avena* spp., *Arrhenatherum* spp. and *Helictotrichon convolutum* in one (pp 1) and *Koeleria* spp., *Trisetum* spp., *Rostraria cristata* and *Lagurus ovatus* in the other (pp 1). In contrast with the nuclear analysis, however, *Briza maxima* was not included in clade C (but in clade B as mentioned above). Finally, *Briza humilis* (pp 1) was placed as sister (pp 0.74) to *Phleum pratense* within clade D (pp 1).

The maximum likelihood and parsimony analyses yielded almost identical topologies as that presented above. No supported conflicts among results using different analytical methods were found and key results were generally well-supported also in the likelihood and parsimony analyses (fig. 1). The exception is clade A, which is present but poorly supported in both likelihood and parsimony analyses (fig. 1: clade A lr 0.52, pb -; clade B lr 0.99, pb 0.89; clade C lr 0.99, pb 0.87; clade D lr 0.98, pb 0.94).

Divergence times – Effective age priors were in all cases consistent with those specified, as assessed by runs without the data. Selecting the Yule model as tree prior generated a phylogeny with similar median node heights as did the birth-death prior, differing only in 2 million years or less at each node. The log marginal likelihoods resulting from the path

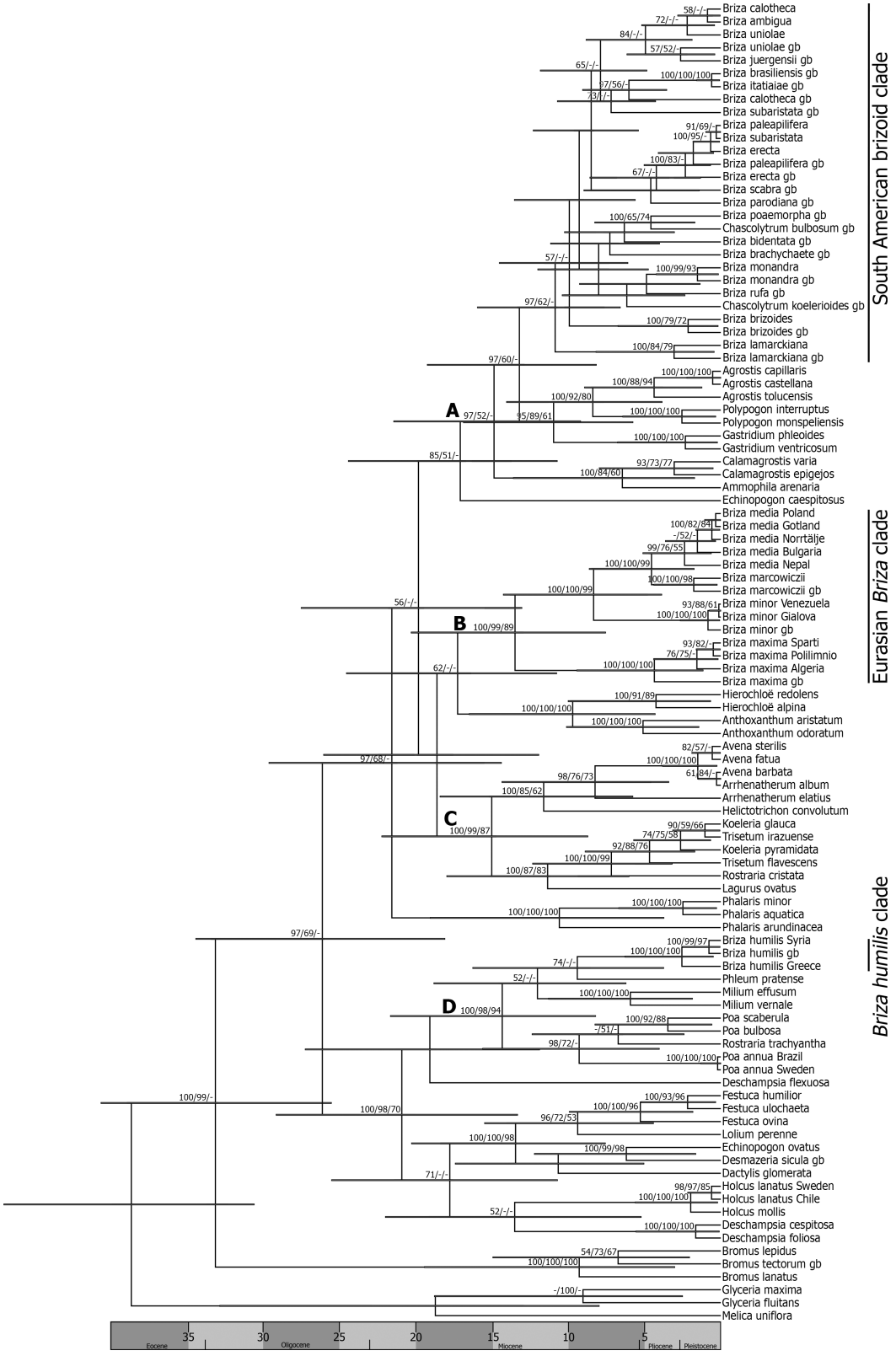


Figure 1 – Time tree produced using a Bayesian approach, as implemented in BEAST, based on a combined dataset of the nrITS region, the GBSSI gene and the plastid *matK* gene (GTR-Γ substitution model; relaxed clock [uncorrelated lognormal]; Yule tree prior; 150 million generations). The topology is identical to that retrieved from the Bayesian analysis performed using MrBayes (mixed model option; 80 million generations). Values above branches indicate posterior probabilities (retrieved from MrBayes), likelihood ratios and parsimony bootstrap values (in %) of clades; dashes (-) indicate support < 50%; letters indicate clades of importance, discussed in the text. gb = GenBank sequence.

Table 2 – Model fit to data.

Clock	Tree prior	Log marginal likelihood	
		path sampling	stepping stone
relaxed lognormal	Yule	-26474.7	-26466.7
relaxed lognormal	BD	-26485.5	-26485.7
strict	BD	-26761.0	-26761.1
strict	Yule	-26761.0	-26761.2

sampling and stepping stone sampling were however very different, and the approach using a relaxed clock and the Yule prior (fig. 1) had significantly better fit to the data than the other approaches (table 2). The posterior probabilities of clades were in most cases very similar to, and slightly higher than, those generated by the Bayesian analyses in MrBayes (fig. 1).

The median age of the ancestor of the South American *Briza* species in clade A was 10.9 million years (confidence interval [CI] 6.6–16.0 mya) and the ancestor of the European *Briza* clade was 13.5 million years old (CI 7.6–20.3 mya). The most recent common ancestor of clades A, B and C was estimated to have existed 19.8 million years ago (CI 13.0–27.5 mya), and that of clades A, B, C and D 26.1 million years ago (CI 18.1–34.4 mya).

Single genome analyses; nuclear data – The Bayesian analysis of the two nuclear regions in combination generated a tree with four different clades containing species of *Briza* (fig. 2). Even though internally not well-resolved, the South American species formed a well-supported group (pp 0.97) included in clade A (pp 0.99), which also comprised representatives of the genera *Agrostis*, *Gastridium* and *Polypogon*.

Three Eurasian species, *Briza media*, *B. minor* and *B. marcowiczii*, formed a well-supported clade (clade B, pp 1). This clade was sister (pp 0.79) to a group comprising *Anthoxanthum* spp. and *Hierochloë* spp. as sisters (pp 1). The fourth Eurasian species, *Briza maxima*, was found in a low-supported clade (clade C, pp 0.79) together with *Avena* spp. and *Arrhenatherum* spp. (pp 0.64) and to *Koeleria* spp., *Trisetum* spp., *Rostraria cristata*, *Lagurus ovatus* (pp 0.98) and *Helictotrichon convolutum*. The fifth Eurasian species of *Briza*, *B. humilis* (pp 1), was placed as sister (pp 0.88) to *Phleum pratense* in a well-supported clade (clade D, pp 1) also comprising *Poa* spp., *Rostraria trachyantha* and *Milium* spp.

The maximum likelihood and parsimony analyses yielded almost identical topologies as that presented above. No supported conflicts among results using different analytical methods were found. Key results were, however, partly poorly supported both in the likelihood and parsimony analyses (fig. 2: clade A lr -, pb -; clade B lr 0.99, pb 0.97; clade C lr -, pb -; clade D lr 0.77, pb 0.84).

Single genome analyses; chloroplast data – The Bayesian analysis of the chloroplast region (*matK*) generated a tree with three different clades containing species of *Briza* (fig. 3). Clade A (pp 1), containing the South American *Briza* species and representatives of the genera *Agrostis*, *Ammophila*, *Calamagrostis*, *Gastridium* and *Polypogon*, was well-supported although its topology was partly different from that of the nuclear dataset; *Briza lamarckiana* and the specimen of *B. minor* collected on the Juan Fernandez Islands formed a well-supported clade together with *Calamagrostis* spp. (pp 0.91). It was included in a clade (pp 0.79), which also comprised *Ammophila arenaria* and a clade constituting *Agrostis* spp., *Polypogon* spp. and *Gastridium* spp. (pp 0.99). Clade B was well-supported (pp 1) and *B. maxima* was here included in clade B together with the other Eurasian species of *Briza* (except *B. humilis*). Clade C was well-supported (pp 1), including *Avena* spp. and allied taxa, and with *Phalaris arundinacea* as their sister (pp 0.94). Clade D was well-supported (pp 1), showing *Briza humilis* as sister to a low-supported group (pp 0.67) comprising *Milium effusum*, *Phleum pratense* and *Poa annua*. In contrast with results in the combined and nuclear analyses, *Rostraria trachyantha* was not included in this clade.

The maximum likelihood and parsimony analyses yielded almost identical topologies as that presented above. No supported conflicts among results using different analytical methods were found and key results were generally well-supported also in the likelihood and parsimony analyses (fig. 3: clade A lr 0.81, pb 0.63; clade B 1.00 lr, 1.00 pb; clade C 0.97 lr, 0.92 pb; clade D 0.67 lr, - pb).

DISCUSSION

Polyphyly of the *Briza* complex

The species of the *Briza* complex have previously been relatively well studied morphologically (Matthei 1975, Nicora & Rúgolo de Agrasar 1981, Bayón 1998, de Pelegrin et al. 2009). Studies based on molecular data have also been conducted, e.g. Essi et al. (2008), but the monophyly of the complex, which is founded on the presence of the putatively distinct 'brizoid' spikelet, has never been questioned. The present study is the first that explicitly tests monophyly of the complex using an extensive sampling of both the species of the *Briza* complex and of putatively closely related species. The results show with strong statistical support that species of the *Briza* complex are included in at least three clades (fig. 1), making the group polyphyletic. Despite their apparent morphological distinctness, brizoid spikelets must have evolved in parallel several times.

The South American group (in clade A)

The South American species of the *Briza* complex are strongly supported as monophyletic in Bayesian analyses, with *Agrostis* spp., *Gastridium* spp. and *Polypogon* spp. comprising their sister clade. Relationships within this South

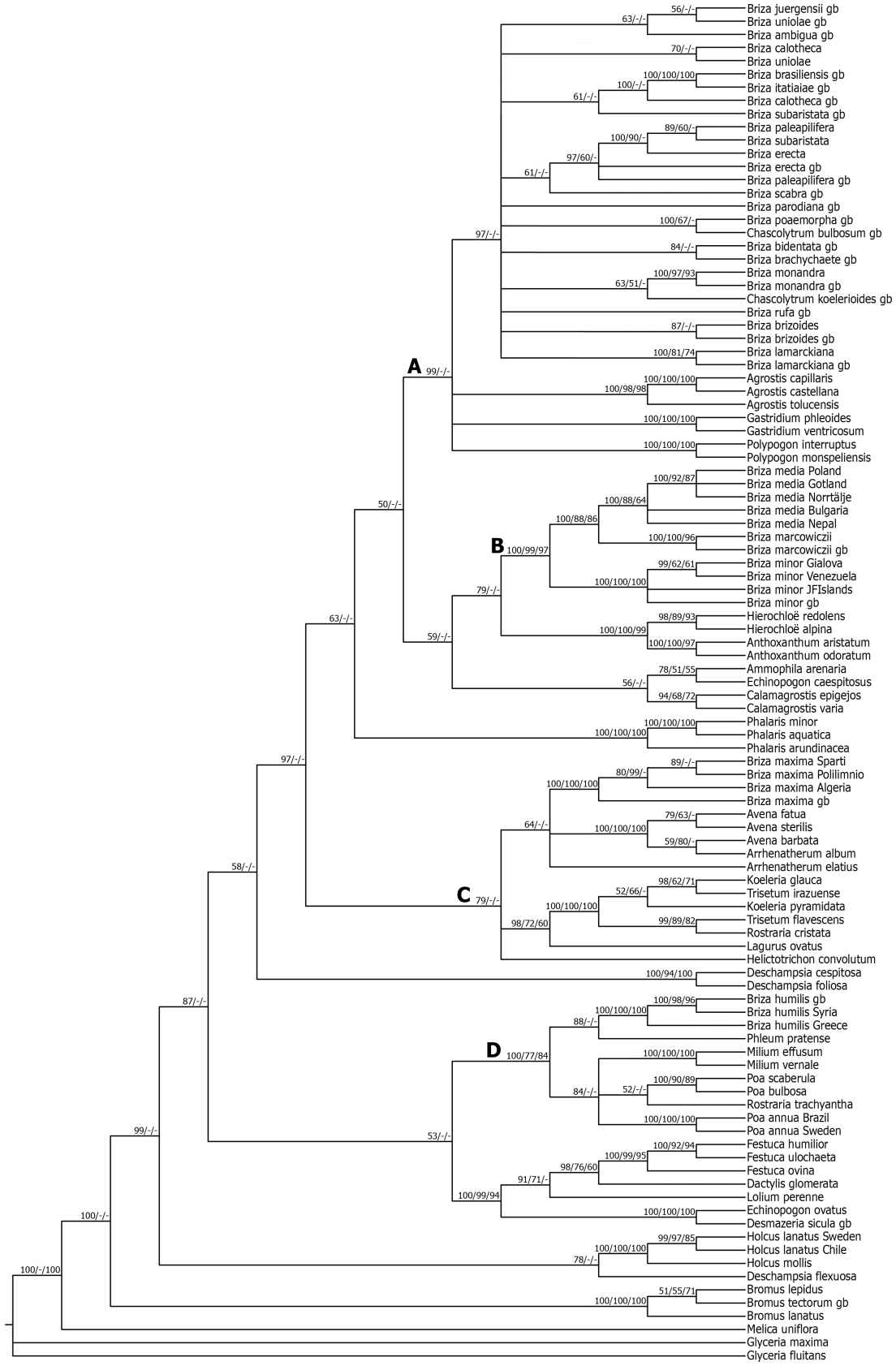


Figure 2 – Phylogenetic results based on a Bayesian analysis (mixed model option, 50 million generations) based on a combined dataset of the nrITS region and the GBSSI gene. Numbers above branches indicate posterior probabilities, likelihood ratios and parsimony bootstrap values (in %) of clades; dashes (-) indicate support <50%; letters indicate clades of importance, discussed in the text. gb = GenBank sequence.

American brizoid group are poorly supported and partly collapsed, but node ages do not indicate that this is due to rapid and recent evolution; the clade is from the mid-Miocene. The single supported topological incongruence between the nuclear and the plastid datasets (i.e. one specimen of the Eurasian species *B. minor* collected on the Juan Fernandez Islands nested in clade A in the plastid analysis but found together with other specimens of the species in clade B in the nuclear analysis) could potentially be due to a single hybridization event, because the displacement in the plastid tree is not true for other included specimens of *B. minor*. It is, however, beyond the scope of the present study to assess the nature of this potential hybrid and whether it was a *Briza*, a *Calamagrostis* or another relative that contributed with the maternal genome.

The Eurasian group (clade B)

The Eurasian species *Briza marcowiczii*, *B. maxima*, *B. minor* and *B. media* formed a monophyletic group (clade B, pp 1). The clade was fully resolved, showing *B. maxima* as sister to the other species and *B. marcowiczii* and *B. media* as closest relatives. This is in accordance with Kim et al. (2009), who (based on neighbour-joining) suggested that *B. marcowiczii* and *B. elatior* (now *B. media*) are closest relatives.

Briza maxima was with low support placed with *Arrhenatherum* ssp. and *Avena* ssp. in clade C in the Bayesian analysis of nuclear data. However, clade C received a high support in all analyses of plastid data as well as in combined analyses, where *B. maxima* always was placed in clade B together with the other Eurasian *Briza* species. Due to the poor support for the conflicting result, we consider the well-supported inclusion found in the combined analysis of *B. maxima* in clade B together with the other Eurasian *Briza* species, reliable.

The separation of the Eurasian species (clade B) from the South American species (in clade A) is strongly supported. Previous studies focusing only on the *Briza* complex have suggested that the South American *Briza* group originated from a Eurasian line (Murray 1976, Essi et al. 2008). Considering that the South American brizoids are tetraploids (Mur-

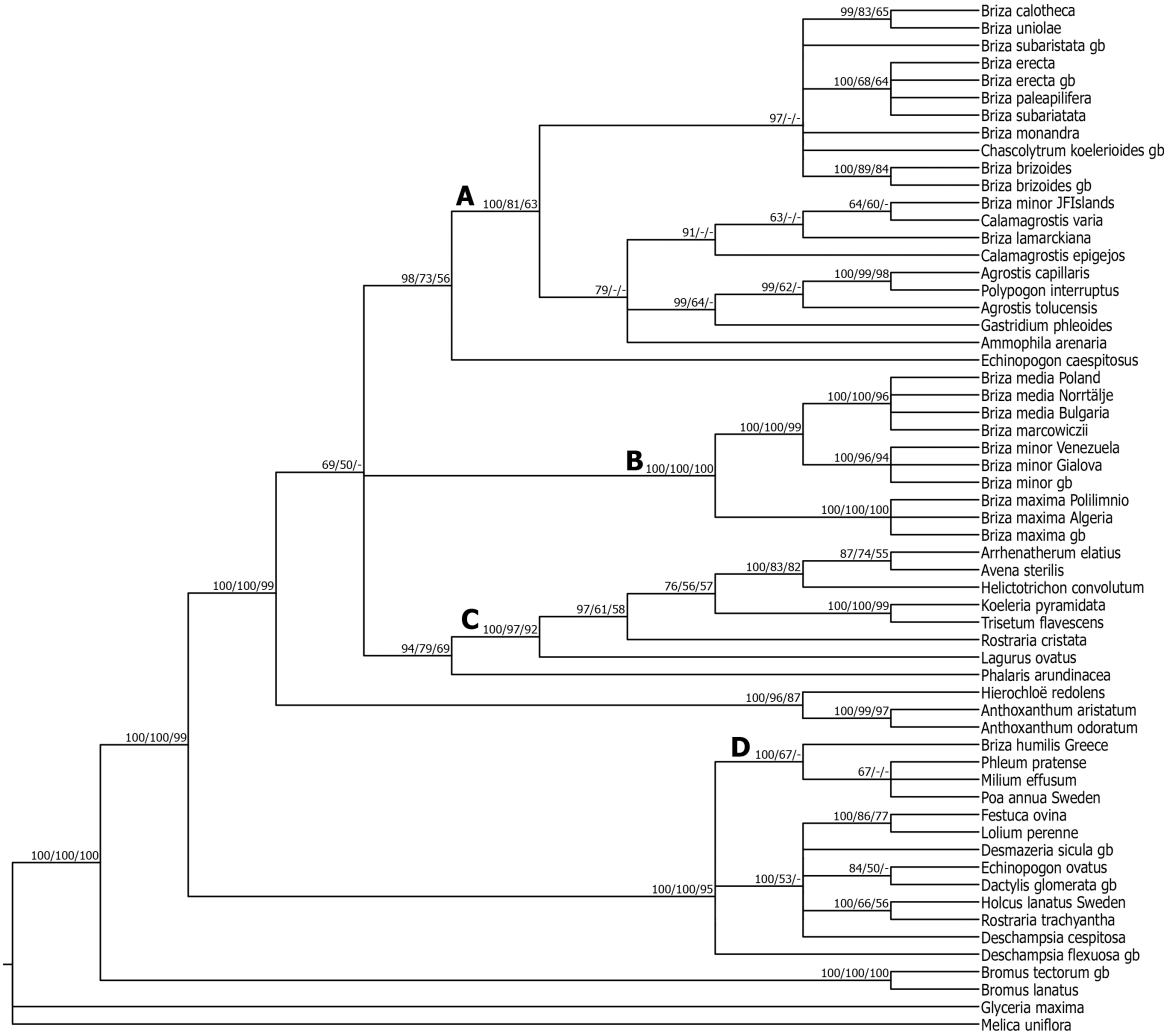


Figure 3 – Phylogenetic results based on Bayesian analysis (mixed model option, 5 million generations) of the plastid *matK* gene. Numbers above branches indicate posterior probabilities, likelihood ratios and parsimony bootstrap values (in %) of clades; dashes (-) indicate support <50%; letters indicate clades of importance, discussed in the text. gb = GenBank sequence.

ray 1975), Essi et al. (2008) speculated that *Briza media* was involved in the origin of the South American clade through hybridization events, due to findings of a tetraploid, eastern European population of *B. media* (Murray 1976). However, based on the here detected polyphyly of the *Briza* complex and the resulting distant relationship between Eurasian *Briza* and South American brizoids, this hypothesis is improbable. Although our results show that the Eurasian *Briza* clade is slightly older than the clade of South American brizoids, the closest relatives of the South American clade are members of other genera (e.g. *Agrostis*, *Calamagrostis*, *Gastridium* and *Polypogon*), not the Eurasian species of *Briza*. A biogeographical analysis could bring more light onto these speculations.

Briza humilis

Briza humilis is distributed in south-eastern Europe to western Asia (WCSP 2014) and was, based on morphological data, placed together with the other Eurasian species by Matthei (1975) and de Pelegrin et al. (2009). In the present study, however, *B. humilis* is with strong support separated from other Eurasian species of *Briza* and instead part of a clade that also comprises *Milium* spp., *Phleum pratense*, *Rostraria trachyantha* and *Poa* spp. (clade D). Even though Essi et al. (2008) used two species of *Poa* as outgroup in their analysis of the *Briza* complex, they could not detect the polyphyly of the complex because they were unable to obtain material from *B. humilis* in their study.

In agreement with our results, a study by Hoffmann et al. (2013) found *B. humilis* to be sister to a clade also comprising members of *Phleum*, *Milium* and *Poa*, as well as genera not included in the present study, for example *Phippsia* and *Puccinellia*. However, although both our results and those of Hoffmann et al. (2013) show with strong support that *B. humilis* is part of the clade corresponding to clade D in the present study (fig. 1), its position within the clade differs and consequently also the stem age of *B. humilis*. According to results in Hoffmann et al (2013), the split between *Briza humilis* and its large sister clade occurred in the early-mid Miocene (Burdigalian), whereas the results of the present study indicate a split between *B. humilis* and *Phleum* in the mid-late Miocene (Tortonian). The (poorly supported) topological difference appears to be the most important explanation for the deviating node ages.

Macromorphology

Concluding that the *Briza* complex is polyphyletic means that the morphological characters used to group these species together have been convergently evolved. Furthermore, having laterally compressed spikelets and closely overlapping lemmas (Bayón 1998) is not unique to the *Briza* complex. Several *Eragrostis* species were in fact previously assigned to *Briza*, i.e. *E. obtusa* and *E. cilianensis* (WCSP 2014), which are similar in spikelet morphology to the Eurasian group, to *B. humilis*, and to *B. calotheca* and *B. uniolae* of the South American group. Today *Eragrostis* has been confirmed to belong to the subfamily Chloridoideae (Peterson et al. 2010).

The present study clearly confirms that the South American brizoid clade is monophyletic; yet it is difficult to characterise morphologically and we find it remarkable that botanists decades ago succeeded in correctly grouping these overall morphologically diverse species into a clade. For example, *Briza brizoides*, *B. erecta* and *B. subaristata* have awned spikelets, but the awns differ considerably in length and their presence was not considered a synapomorphy in the morphological study by Bayón (1998). The long-awned *B. brizoides* was placed in a genus of its own (*Calotheca*), whereas *B. erecta* and *B. subaristata* were placed together with a few other species in *Briza*, characterized by their lemmas with umbo (arched lemmas). Similarly, de Pelegrin et al. (2009) looked at leaf anatomy and proposed very different relationships compared to previous studies.

The morphology of the Eurasian species in clade B is in contrast more uniform; the spikelets are very similar in all species, differing only in size where *B. maxima* has the largest spikelets and *B. minor* the smallest. *Briza marcowiczii* and *B. media* differ mainly in the height of the culm, although the spikelets of *B. marcowiczii* tend to be more purplish (WCSP 2014, N.L. Persson pers. obs.).

The spikelets of *B. humilis* are similar to the South American species *B. calotheca* and *B. uniolae*, although the panicle of *B. humilis* is smaller and contains fewer spikelets. *Briza humilis* and *B. calotheca* also bear some resemblance to *Desmazeria sicula*, which has an old synonym of *Briza disticha* (WCSP 2014) but is sister to *Echinopogon ovatus* in the present study. Thus, convergent evolution of similar morphological structures seems not unique to the *Briza* complex in the Pooideae. Our results provide new phylogenetic information and indication of polyphyly of several poorly studied groups, also outside of the *Briza* complex.

Taxonomic synopsis of the former *Briza* complex

Due to the low resolution within the South American brizoid group, it is neither possible to confirm nor reject the subdivision of the clade into many genera, as proposed by e.g. Matthei (1975), Nicora & Rúgolo de Agrasar (1981) and Bayón (1998). The two species of *Chascolytrum* included in the present study have never been included in the *Briza* complex, but are here placed inside the South American group with good support. Therefore we tentatively agree with the suggestions of Essi et al. (2008, 2011) to assign all South American *Briza* species (those of clade A) to the genus *Chascolytrum*. However, considering the relatively diverse morphology in this clade, future studies may find it relevant to divide the clade into additional genera, as has been done in the past. The Eurasian species retain the name *Briza*, since *B. media* is the type species of the genus.

After digitally examining the type material of *Briza humilis* and reviewing the morphology and phylogenetic placement of the three specimens included in the present study, we conclude that *B. humilis* is clearly not related to other species of *Briza*. There is only one homotypic synonym of this species, *Brizochloa humilis* (WCSP 2014), and we propose this name for the clade. The original publication of *Brizochloa* (see below) separated this genus from *Briza* based on histological differences and the shape of the lemma.

Eurasian clade: *Briza* L. – Original description: Linnaeus (1753: 70).

Type species: *Briza media* L. (Linnaeus 1753: 70).

South American clade: *Chascolytrum* Desv. – Original description: Desvaux (1810: 190).

Type species: *Chascolytrum subaristatum* (Lam.) Desv. (Desvaux 1810: 190)

***Briza humilis* clade:** *Brizochloa* V.Jirásek & Chrtek – Original description: Jirásek & Chrtek (1967: 40).

Type species: *Brizochloa humilis* (M.Bieb) Chrtek & Hadač (Chrtek & Hadač 1969: 170).

Basionym: *Briza humilis* M.Bieb. (von Bieberstein 1808: 66). – Type material: *Bieberstein* s.n (BM).

Heterotypic synonyms: *Briza spicata* Sibth. & Sm. (Sibthorp & Smith 1806: 60), nom. illeg.; *Brizochloa spicata* V.Jirásek & Chrtek (Jirásek & Chrtek 1967: 40).

Concluding remarks

The grass family can be traced back to the Late Cretaceous, but the major diversification within the family and formation of grassland ecosystems occurred much later, in most continents during the Miocene (Gibson 2009, Stromberg 2011). We found an almost simultaneous origin of the three brizoid clades, which coincides with this epoch.

The beauty of the grasses is their almost endless variation of a very fundamental design, but the variation in their bracteate inflorescence is perhaps not so surprising since floral morphology can be drastically changed by one-step mutations (Hilu 1983, Niklas 1997), sometimes compelling taxonomists to assign species to rather distantly related genera while molecular data would reveal a closer relationship. Among brizoids, however, it appears to have been the other way around. Parallel mutations in several groups have apparently led to similar morphology, grouping the *Briza* complex based on convergently evolved morphological characters. The same appears true for several other genera, e.g. *Deschampsia* (see also e.g. Chiapella 2007) and *Echinopogon*. Our results may hopefully inspire further assessment of these genera, as well as new morphological studies of the species formerly assigned to the *Briza* complex.

SUPPLEMENTARY DATA

Supplementary data are available in pdf at *Plant Ecology and Evolution*, Supplementary Data Site (http://www.ingentaconnect.com/content/botbel/plecevo/supp-data), and consist of: (1) natural distribution areas of species included; and (2) information on voucher specimens and GenBank sequence (nrITS, GBSSI, *matK*) accession numbers of species included in this study.

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