



Phylogenetics of the Palearctic model grass *Brachypodium sylvaticum* uncovers two divergent oriental and occidental micro-taxa lineages

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ABSTRACT

Brachypodium sylvaticum has been selected as a model for perennial grasses, and considerable genomic resources have been generated and a reference genome and several resequenced pangenome accessions are available for this species. Despite these genomic advances, the evolution and systematics of diploid *B. sylvaticum* s. l. is almost unknown. The *B. sylvaticum* complex is formed by up to seven taxonomically close micro-taxa which differentiate from typical *B. sylvaticum* s. s. based on a few morphological features. Moreover, some of them show some largely disjunct geographic distributions on both sides of their native Palearctic region. In this study, we used a phylogenomic approach including representative populations from the oriental and occidental distribution range of *B. sylvaticum* micro-taxa to elucidate their evolutionary relationships and assess the systematic value of the morphological features that separate them. A combined plastome and nuclear phylogenetic tree supports an early split and high divergence of the oriental lineage, showing the close relationship of the Himalayan *B. sylvaticum* var. *breviglume* lineages to the Pacific *B. miserum* / *B. kurilense* clade, and the contrasting large homogeneity and low divergence of the occidental European, N African and SW and C Asian lineage, with several *B. sylvaticum* s. s., *B. spryginii*, and *B. glaucovirens* samples showing identical or similar sequences. Divergence time estimate analysis suggests that the oriental lineage diverged from the common ancestor in the early Pleistocene (2.0 Ma), followed by subsequent colonization and isolations in the Himalayas (2.0 – 1.7 Ma) and the Far East (0.36 Ma) in more recent times, while the occidental lineage split in the Mid-Late Pleistocene (0.97 Ma), followed by rapid radiation and postglacial spread in the western Palearctic during the last thousand years.

Keywords: *Brachypodium sylvaticum* complex, diploid populations, discriminant morphological traits, eastern and western Eurasian micro-taxa, phylogenetics, plastomes, rDNA 35S gene, systematics

РЕЗЮМЕ

Каталан П., Ангелес Децена М., Санчо Р., Вируел Х., Перес-Колазос Э., Пробатова Н.С. Филогенетика палеарктического модельного злака *Brachypodium sylvaticum* показывает две дивергентные восточную и западную линии микротаксонов. *Brachypodium sylvaticum* был выбран в качестве модели для многолетних трав, и для этого вида были созданы значительные геномные ресурсы и доступны эталонный геном и несколько повторно секвенированных пангеномных доступов. Несмотря на эти геномные достижения, эволюция и систематика диплоидного *B. sylvaticum* s. l. практически неизвестна. Комплекс *B. sylvaticum* образуют до семи таксономически близких микротаксонов, которые отличаются от типичного *B. sylvaticum* s. s. по нескольким морфологическим признакам. Более того, некоторые из них демонстрируют в значительной степени дисъюнктивное географическое распространение по обе стороны их родного Палеарктического региона. В данном исследовании мы использовали филогеномный подход, включая репрезентативные популяции из восточного и западного ареалов распространения микротаксонов *B. sylvaticum*, чтобы выяснить их эволюционные отношения и оценить систематическое значение разделяющих их морфологических признаков. Объединенное филогенетическое древо, построенное на основе пластома и ядерной ДНК, подтверждает раннее разделение и высокую дивергенцию восточной линии, показывая близость гималайской линии *B. sylvaticum* var. *breviglume* с тихоокеанской линией *B. miserum* / *B. kurilense*, и контрастирующую большую однородность и низкую дивергенцию восточно-европейской, североафриканской и южно- и среднеазиатской линии, с несколькими видами *B. sylvaticum* s. s., *B. spryginii* и *B. glaucovirens* демонстрируют идентичные или похожие последовательности. Анализ временной оценки дивергенции позволяет предположить, что восточная линия отделилась от общего предка в раннем плейстоцене (2,0 млн лет назад), после чего последовали колонизация и изоляция в Гималаях (2,0 – 1,7 млн лет назад) и на Дальнем Востоке (0,36 млн лет назад) в более поздние времена, в то время как окцидентальная линия отделилась в среднем-позднем плейстоцене (0,97 млн лет назад), после чего последовали быстрая радиация и последующее распространение в западной Палеарктике в течение последней тысячи лет.

Ключевые слова: комплекс *Brachypodium sylvaticum*, диплоидные популяции, дискриминантные морфологические признаки, микротаксоны восточной и западной Евразии, филогенетика, пластома, ген 35S рДНК, систематика

Переведено редколлегией

Since its proposal as a model perennial grass system (Steinwand et al. 2013, Gordon et al. 2016), *Brachypodium sylvaticum* has attracted the attention of the scientific community as a suitable model plant for understanding the molecular mechanisms that caused the transitions between perennial-annual plants (Hu et al. 2011, Scholthof et al. 2018, Friedman 2020), and to design strategies for improving perennial energy crops (Carroll & Somerville 2009, Dohleman & Long 2009) and develop perennial grain crops (Glover et al. 2010, Gordon et al. 2016). This conceptual framework has conveyed the generation of large genomic and biological resources (e.g., transformation and inbred lines, Steinwand et al. 2013; transcriptome atlases, Fox et al. 2013; salt tolerance responses, Sade et al. 2018) for *B. sylvaticum*, paralleling those of its well-known annual congener *B. distachyon* (Scholthof et al. 2018). A reference genome has been assembled for one *Brachypodium sylvaticum* accession (*B. sylvaticum*-Ain1; Phytozome https://phytozome-next.jgi.doe.gov/info/BsylvaticumAin_1_v2_1) and current research on the genomic resequencing of other *B. sylvaticum* lines aims to generate a pangenome for this model perennial grass (Joint Genome Institute, Community Science Program; <https://jgi.doe.gov/brachypodium-model-grass-genus-bioenergy/>).

Brachypodium sylvaticum has also been the subject of large taxonomic, cytogenetic, evolutionary and ecological research. This perennial grass presents the broadest native distribution of all worldwide *Brachypodium* species, covering the entire Palearctic region, from Macaronesia (West) to New Guinea (East) and from Scandinavia and Siberia (North) to northern Africa and Malaysia (South) (Catalán et al. 2016). It is also the only known invasive perennial *Brachypodium* taxon in the New World (Rosenthal et al. 2008, POWO <https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:393196-1>, accessed 16 April 2023). Morphologically, *B. sylvaticum* separates from other *Brachypodium* congeners based on the possession of short and slender rhizomes, nodding panicle, densely hairy habit and long-awned lemma (Schippmann 1991). It is a self-compatible perennial species (Khan & Stace 1999, Steinwand et al. 2013), adapted to mesic and nemoral habitats of humid forest (Schippmann 1991). Some of its features are also shared by the tropical and South African *B. flexum* and the Malagasy *B. madagascariense*, though they differ from the former in their shorter panicles, spikelets and awns (Catalán et al. 2016). The *B. sylvaticum* complex includes up to seven micro-taxa; *B. sylvaticum* s. s., described from England, is the most widespread species in Europe, N Africa, and SW and C Asia (Schippmann 1991). The remaining six cryptic micro-taxa were described from different regions of eastern Europe, Asia, and Malesia. They were formerly synonymized with *B. sylvaticum*, although some of them were later disintegrated from it (*B. sylvaticum* var. *breviglume*, *B. sylvaticum* var. *pseudodistachyon*, *B. kuirilense*, *B. miserum*, *B. pubescens*, *B. spryginii*; Keng 1982, Tzvelev 1983, 2015, Probatova & Skolovskaya 1985, Tzvelev & Probatova 2019) based on features related to the length of glumes, plant pubescence and height of the plant. All the *B. sylvaticum* complex taxa are further characterized by a constant diploid chromosome number of $2n = 2x = 18$ and a chromosome base number of

$x = 9$ (Wolny & Hasterok 2009, Catalán et al. 2016, Tzvelev & Probatova 2019, Decena et al. 2023). A close taxon to the *B. sylvaticum* complex is the eastern Mediterranean – SW Asian endemic *B. glaucovirens*, which is also a diploid species but showing a smaller chromosome number and chromosome base number ($2n = 2x = 16$; $x = 8$) than *B. sylvaticum* s. s. (Wolny & Hasterok 2009, Catalán et al. 2016).

Phylogenetic studies of *Brachypodium* based on plastid and nuclear loci have consistently reconstructed *B. sylvaticum* as a main lineage of the recently evolved core-perennial *Brachypodium* clade (Catalán & Olmstead 2000, Catalán et al. 2016, Díaz-Pérez et al. 2018). Molecular dating analysis inferred a recent Mid-Late Pleistocene origin for the *B. sylvaticum* s. l. lineages (1.2–0.2 Ma; Díaz-Pérez et al. 2018). A phylogenomic survey based on transcriptome data also supported a recent split of *B. sylvaticum* s. s. from its close *B. pinnatum* sister lineage in the Pleistocene (1.4 Ma; Sancho et al. 2022). Fluorescent In Situ Hybridization (FISH)-based comparative chromosome barcoding (CCB) analysis characterized the karyotypic profiles of *B. sylvaticum* s. s. ($x = 9$) and of *B. glaucovirens* ($x = 8$) and hypothesized their derived origins from either ancestral $x = 10$ or intermediate-ancestral $x = 9$ *Brachypodium* karyotypes through descendant disploidy (Lusinska et al. 2019, Sancho et al. 2022); however, the *B. sylvaticum* s. s. barcoded karyotype could not be differentiated from those of other close diploid core perennial $x = 9$ species (*B. arbuscula*, *B. pinnatum*) probably due to their recent divergences from the common ancestor (Sancho et al. 2022).

Despite the enormous advances attained in the genomic study of the model perennial grass *B. sylvaticum* s. s., the systematics of the *B. sylvaticum* complex micro-taxa is almost unknown. Taxonomically, the morphological characters used to separate several of these micro-taxa are extremely plastic and variable, and have been considered of doubtful taxonomic relevance in some cases (Tzvelev 2015, Catalán et al. 2016). Nonetheless, some of these taxa show large disjunct geographic distributions in both extremes of Eurasia, which could have led to genetic isolation and evolutionary divergence. Moreover, no evolutionary study has included representative samples of oriental and occidental populations of *B. sylvaticum* s. l. yet. Therefore, the objectives of our study were to conduct a phylogenomic study of the *B. sylvaticum* complex micro-taxa using whole plastid genome (plastome) and nuclear ribosomal DNA (rDNA) 35S gene sequences to elucidate their evolutionary relationships and to assess the systematic value of the morphological features that separate them. Our study also aimed to infer the divergence ages of these lineages and to estimate their respective ancestries.

MATERIAL AND METHODS

Sampling and taxonomic identification

A total of 18 *Brachypodium sylvaticum* s. s. samples collected from different European, N African and Asian populations, plus six samples of close *B. sylvaticum* complex taxa [*B. sylvaticum* var. *breviglume* (2), *B. kuirilense* (1), *B. miserum* (1), *B. glaucovirens* (1), *B. spryginii* (1)], five samples from other congeners [*B. pinnatum* (2), *B. arbuscula* (1), *B. distachyon* (1), *B. stacei* (1)] and three outgroup grass samples (*Triticum*

aestivum, *Oryza sativa*, *Sorghum bicolor*) were included in the evolutionary and systematic study (Fig. 1, Table 1). The taxonomic identification of the studied materials was based on previous taxonomic treatments of *Brachypodium* (Keng 1982, Schippmann 1991, Catalán et al. 2012, Tzvelev 2015, Tzvelev & Probatova 2019). Chromosome and ploidy level information was retrieved from these studies and from a broad cyto-phylogenomic study of *Brachypodium* perennial species performed by Decena et al. (2023, and unpub. data).

Genomic data and phylogenetic analysis

Total genomic DNA isolation and genome skimming sequencing procedures are detailed in Moreno-Aguilar et al. (2020) and Decena et al. (2023). Genomic data for plastome reconstruction and nuclear *rDNA* 35S genes were retrieved from the respective reference genomes of *B. sylvaticum* Ain1,

B. distachyon Bd21 and *B. stacei* ABR114 accessions (Sancho et al. 2018, 2022) and downloaded from Phytozome (<https://phytozome-next.jgi.doe.gov/>). Assembly of plastomes and *rDNA* 35S sequences of the remaining *Brachypodium* samples was performed using Novoplasty v. 2.7.1 (Dierckxsens et al. 2017) and read-mapping strategies to the reference 35S sequences with Geneious Prime 2023, respectively. Multiple sequence alignments (MSA) were performed separately for entire plastomes and nuclear 35S sequences using MAFFT.v7.450 (Katoh & Standley 2013), and removal of low quality regions from each of the MSAs was performed with TrimAl v. 1.2rev59 (Capella-Gutiérrez et al. 2009) followed by manual curation.

Maximum likelihood (ML) phylogenetic trees were reconstructed for each separated data set with IQ-TREE 1.6. imposing the best-fit nucleotide substitution model, ac-

Table 1. List of the *Brachypodium sylvaticum* complex samples (*B. sylvaticum* var. *sylvaticum*, *B. sylvaticum* var. *breviglume*, *B. glaucovirens*, *B. kurilense*, *B. miserum*, *B. spryginii*) and other congeners (*B. pinnatum*, *B. arbuscula*, *B. distachyon*, *B. stacei*) and outgroup taxa (*Triticum aestivum*, *Oryza sativa*, *Sorghum bicolor*) used in the study indicating taxon name and authority, population code, geographic location and sample source [INIA, Spanish National Institute of Agriculture accession; JGI, Joint Genome Institute accession; RIKEN, Japanese Center for Sustainable Resource Science accession; USDA, US Department of Agriculture accession; UZ, University of Zaragoza accession; Herbarium specimens: B (Berlin), M (München), LD (Lund), VLA (Vladivostok)], chromosome number (2n), ploidy level, and data source. All studied *Brachypodium* samples are diploids. Taxonomic identities are based in the taxonomic works of Keng (1982), Probatova (1985), Schippmann (1991), Catalán et al. (2012), Tzvelev (2015), Tzvelev & Probatova (2019).

Taxon	Code	Location, herbarium acronym	2n	Ploidy	Source
<i>B. sylvaticum</i> (Huds.) P. Beauv. var. <i>sylvaticum</i>	Bsylvaticum_Ain1	Tunisia: Ain Draham, JGI	18	2x	Sancho et al. 2022
	Bsylvaticum_Sin1	Turkey: Sinop, JGI	18	2x	Decena et al. 2023
	Bsylvaticum29	Greece: Evrytania, B100281411	18	2x	Decena et al. 2023
	Bsylvaticum30	Denmark: Zealand, B100566906	18	2x	Decena et al. 2023
	Bsylvaticum31	Germany: Ober Bayern, M0177011	18	2x	Decena et al. 2023
	Bsylvaticum32	Russia: Kaluga, M01769847	18	2x	Decena et al. 2023
	Bsylvaticum54	Morocco: Rif Mountains, UZ	18	2x	Decena et al. 2023
	Bsylvaticum63	Morocco: Mulay-Idriss, UZ	18	2x	Decena et al. 2023
	Bsylvaticum434	Ukraine: Krym: USDA PI639821	18	2x	Decena et al. 2023
	Bsylvaticum446	Iran: Ardebil, USDA PI380758	18	2x	Decena et al. 2023
	Bsylvaticum467	Spain: Huesca, Bessen, UZ	18	2x	Decena et al. 2023
	Bsylvaticum470	Spain: Guipuzcoa: San Sebastian, UZ	18	2x	Decena et al. 2023
	Bsylvaticum476	France: Hautes Pyrenees, Barbazan, UZ	18	2x	Decena et al. 2023
	Bsylvaticum501	France: Alpes Maritime. Roquefort les Pins, UZ	18	2x	Decena et al. 2023
	Bsylvaticum506	France: Hérault, Saint Jean de Fos, UZ	18	2x	Decena et al. 2023
	Bsylvaticum508	France: Aude, Villesèque des Corbières, UZ	18	2x	Decena et al. 2023
	Bsylvaticum552	Spain: Cadiz, Tarifa, UZ	18	2x	Decena et al. 2023
Bsylvaticum554	Spain: Malaga, Canillas, UZ	18	2x	Decena et al. 2023	
<i>B. sylvaticum</i> var. <i>breviglume</i> Keng	Bbreviglume33	Tibet: Gongbogyamda, LD135398	18	2x	Mo et al. 2023
	Bbreviglume34	Pakistan: Hazara, Paras-Shogran, M0175636	18	2x	Decena et al. 2023
<i>B. glaucovirens</i> (Murb.) T. Durand & B.D. Jacks	Bglaucovirens	Greece, Crete, B3151	16	2x	Decena et al. 2023
<i>B. kurilense</i> (Prob.) Prob.	Bkurilense9	Russia: Kuril Islands, Iturup, VLA 1625	18	2x	Tzvelev & Probatova 2019, Decena et al. 2023
<i>B. miserum</i> (Thunb.) Koidz.	Bmiserum67	Japan: Honshu, Aramakiob, RIKEN	18	2x	Tzvelev & Probatova 2019
<i>B. spryginii</i> Tzvelev	B spryginii 29	Russia: Krasnodarskii Krai, VLA 11652	18	2x	Tzvelev & Probatova 2019, Decena et al. 2023
<i>B. pinnatum</i> (L.) P. Beauv.	Bpinnatum505	Norway: USDA PI345964	18	2x	Decena et al. 2023
	Bpinnatum515	Kazakhstan: USDA PI440176	18	2x	
<i>B. arbuscula</i> Gay ex Knoche	Barbuscula502	Spain: Canary Islands, La Gomera, INIA	18	2x	Sancho et al. 2022, Decena et al. 2023
<i>B. distachyon</i> (L.) P. Beauv.	Bdistachyon_Bd21	Irak: Salakkudin, JGI	10	2x	Catalán et al. 2012
<i>B. stacei</i> Catalán, Joch. Müll., L.A.J. Mur & T. Langdon	Bstacei_ABR114	Spain: Balearic islands, Formentera, JGI	20	2x	Catalán et al. 2012
<i>Triticum aestivum</i> L.		cultivar	42	6x	Genbank
<i>Oryza sativa</i> L.		cultivar	24	2x	Genbank
<i>Sorghum bicolor</i> (L.) Moench		cultivar	20	2x	Genbank

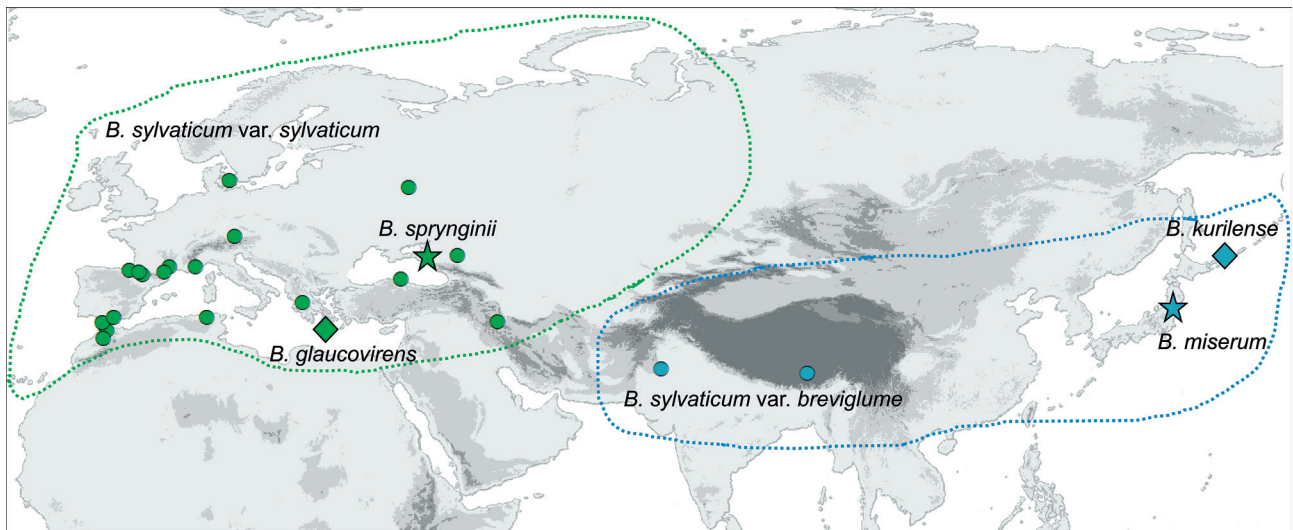


Figure 1 Geographical distribution of the studied samples of the *Brachypodium sylvaticum* complex micro-taxa. Western taxa (green region): *B. sylvaticum* s. s. (green circles), *B. sprynginii* (green star), *B. glaucovirens* (green diamond); Eastern taxa (blue region): *B. sylvaticum* var. *breviglume* (blue circles), *B. miserum* (blue star), *B. kurilense* (blue diamond) (see Table 1 for more details)

according to the Bayesian Information Criterion (BIC), and estimating 1000 ultrafast bootstrap replicates (BS) and SH-*alrt* test with 1000 replicates for the branch support of the best tree (Nguyen et al. 2015). Due to the overall congruence of the plastome and the *rDNA* topologies, both data sets were concatenated into a combined data matrix and used to compute a combined ML plastome+35S tree. Ancestral divergence times of the *Brachypodium* lineages were estimated with BEAST 2 (Bouckaert et al. 2014) imposing independent site substitution models for each partition, log-normal relaxed clock and Yule tree linked models, a broad uniform distribution prior for the uncorrelated lognormal distribution (ucl) mean (lower = 1.0E-6; upper = 0.1) and an exponential prior for ucl standard deviation (SD). Five nodes of the tree were calibrated using secondary age constraints for the crown node of grasses (BOP + PACMAD clade; normal prior mean = 55.0 Ma, SD = 0.5 Ma), the BOP clade (*Brachypodium* + *Oryza*; normal prior mean = 51.7 Ma, SD = 1.9 Ma), the *Pooideae* clade (*Brachypodium* + *Triticum*; normal prior mean = 33.2 Ma, SD = 9.52 Ma), the *Brachypodium* clade (normal prior mean = 32.2 Ma, SD = 9.5 Ma) and the *Brachypodium* core-perennial clade (normal prior mean = 2.95 Ma, SD = 1.49 Ma), following the grass-wide dating analysis of Sancho et al. (2018). We ran 600 million Markov chain Monte Carlo (MCMC) generations in BEAST2 with a sampling frequency of 1,000 generations. The adequacy of parameters was checked using TRACER v. 1.66 with all the parameters showing Effective Sample Size (ESS) > 200. A Maximum clade credibility (MCC) tree was computed after discarding 10 % of the respective saved trees as burn-in.

RESULTS

Plastome and nuclear *rDNA* 35S phylogenies

The full *Brachypodium* plastome data set included 136,380 filtered positions and the 35S data set 6,237 positions. Whole plastome sequences of the *Brachypodium sylvaticum* complex species were highly conserved in terms of synteny and gene

number: they contained a total of 133 genes (76 protein coding genes, 20 non-redundant rRNAs, four rRNAs in both inverted repeats, four pseudogenes, and two hypothetical open reading frames) and a structure and length similar to that of the *B. stacei* plastome (Sancho et al. 2018). The 35S showed a conserved structure and similar average lengths along its aligned transcriptional unit in all the samples studied (5'-external transcribed spacer (ETS) (~500 bp), 18S gene (~1,818 bp), internal transcribed spacers and 5.8S gene (ITS1-5.8S-ITS2) (~590 bp), 25S gene (~3,350 bp).

The best ML plastome tree retrieved a strong to well-supported topology for most *Brachypodium* lineages (100–79 % bootstrap support (BS), Fig. 2A). The earliest splits were those of the annual *B. stacei* and *B. distachyon* lineages, followed by the divergence of the *Brachypodium* core-perennial clade lineages. Within it, the Canarian *B. arbuscula* lineage branched off first, and the next divergences corresponded to the *B. sylvaticum*-complex oriental clade and then that of the sister *B. sylvaticum*-complex occidental clade and *B. pinnatum* clade lineages. The oriental clade showed in turn the successive splits of the strongly supported Himalayan *B. sylvaticum* var. *breviglume* lineages and Pacific *B. miserum* / *B. kurilense* lineages, while the *B. sylvaticum* occidental clade reconstructed the nesting of *B. glaucovirens* and *B. sprynginii* within this broadly homogeneous and non-geographically structured lineage, with several tips collapsing in polytomies (Fig. 2A).

The optimal ML 35S tree was less resolved than the plastome tree (Fig. 2B). This phylogeny also recovered the successive divergences of the early splitting *B. stacei*, *B. distachyon* and *B. arbuscula* lineages, followed by those of the more recently evolved core-perennials. Within the recentmost in-core group, all members of the *B. sylvaticum*-complex oriental lineages plus a few members of the occidental lineage (*B. sprynginii*, and *B. sylvaticum* s. s. samples from eastern, central, and SW Europe) formed a moderately supported clade (76 % BS) with low internal resolution except for the strongly supported Pacific *B. miserum* / *B. kurilense* clade (96 % BS). The remaining members of the occidental clade collapsed

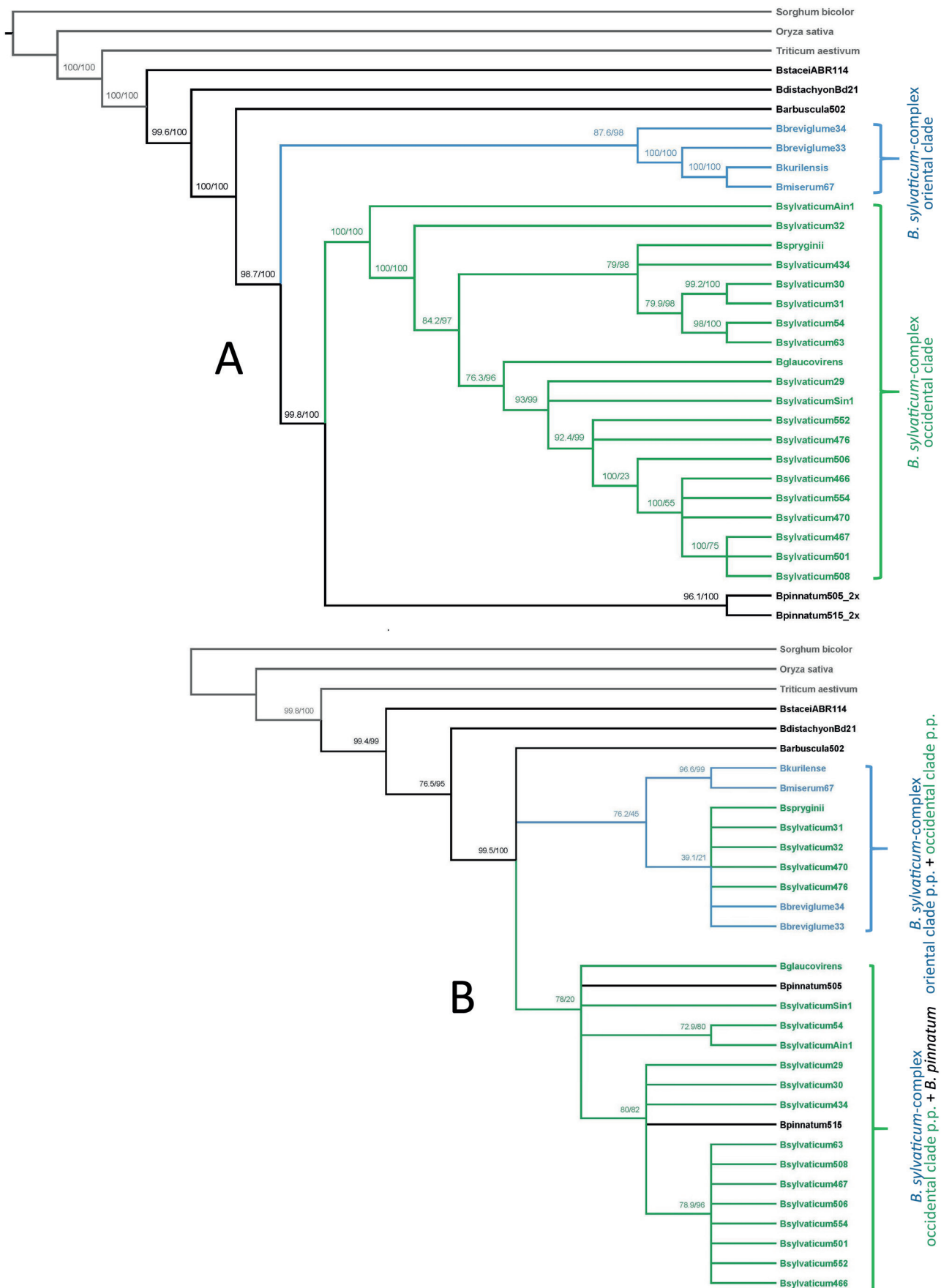


Figure 2 Maximum Likelihood phylogenomic trees of the *Brachypodium sylvaticum* complex taxa studied and other representative species of the genus. A – plastome tree cladogram. B – nuclear rDNA 35S gene tree cladogram. Ultrafast bootstrap / SH-*alrt* support values are indicated above the branches. *Sorghum bicolor*, *Oryza sativa* and *Triticum aestivum* outgroups were used to root the trees. *Brachypodium sylvaticum* complex oriental and occidental lineages are shown in blue and green colors, respectively

in a series of nested polytomies in a moderately supported clade that also included the *B. pinnatum* samples (78 % BS); part of these lineages formed another moderately supported *B. sylvaticum* s. s. pro partim subclade (79 %) without clear geographic structure (but with most samples from the western Mediterranean region) (Fig. 2B).

Bayesian dated tree

We applied a total-evidence approach to retrieve a consensus phylogeny for this diploid *Brachypodium* tree and to infer the nodal ages of the *B. sylvaticum*-complex lineages (Fig. 3). The resolution of the combined plastome+35S tree reflected the matrilineal plastome topology, and the Bayesian dating analysis of its maximum clade credibility tree inferred a Mid-Miocene (9.04 Ma) origin for the MRCA of *Brachypodium* and a Late-Pliocene (2.71 Ma) origin for that of the core-perennial clade. The splits of the crown ancestors of the *B. sylvaticum*-oriental (2.0 Ma) and *B. sylvaticum*-occidental (0.97 Ma) ancestors were estimated to have occurred during the early Pleistocene, while the subsequent splits spanned the Late-Quaternary (Fig. 3). The dating analysis indicated that the *B. sylvaticum* var. *breviglume* ancestors were comparatively older (2.00–1.72 Ma) than that of the young Pacific *B. miserum* / *B. kurilense* group (0.36 Ma), while the western taxa showed recent but different ancestries (*B. spryginii* and close lineages 0.52 Ma; *B. glaucovirens* and close lineages 0.29 Ma) (Fig. 3).

DISCUSSION

Our study has unveiled the existence of two main divergent lineages within the hitherto considered single monophyletic species *B. sylvaticum* or unclearly resolved *B. sylvaticum*-complex taxa (Figs 1, 2, 3; Catalan et al. 2016, Díaz-Pérez et al. 2018). Although *B. sylvaticum* s. l. has been taxonomically split into several minor satellite taxa in its eastern and western distribution ranges (Tzvelev 1983, Scholz 2007, Tzvelev &

Probatova 2019), the evolutionary relationships of both the oriental and occidental taxa and populations of *B. sylvaticum* s. l. were mostly unknown. Our analysis has demonstrated that this cytologically homogeneous diploid species complex is composed of two main lineages which are distributed in two largely disjunct Himalayas–Pacific and EuroMediterranean–Siberian regions (Figs 1, 2). Our plastome-based phylogeny clearly separated the two main *B. sylvaticum* s. l. diploid lineages, placing the oriental taxa within a first diverging clade, and the occidental taxa within a subsequently split *B. sylvaticum* s. s. clade (Fig. 2A), sister to its close *B. pinnatum* relative, a resolution mostly congruent with that of the *rDNA* 35S tree (Fig. 2B). The oriental clade showed the successive divergences of Himalayan *B. sylvaticum* subsp. *breviglume* lineages from their respective ancestors, assumed to have occurred in the Early Pleistocene (Gelasian, 2.0–1.7 Ma), while the two Pacific close species *B. miserum* and *B. kurilense* were inferred to have diverged from their common ancestor in the Middle Pleistocene (Chibabian, ~0.3 Ma; Fig. 3). The purported colonization ages of the Tibet and pan-Himalayan Pakistan mountains agreed with previous findings about the migrations of other cool grass lineages in South-Eastern Asia as well as components of the Himalayan flora (Shen et al. 2017) in the Late Pliocene–Early Pleistocene. Population genetic analyses detected high rates of genetic diversity for Tibetan population of *B. sylvaticum* var. *breviglume* (Mo et al. 2013). The close relationship uncovered for the Far East *B. kurilense* and *B. miserum* lineages concurred with the main contribution of southern Japanese floristic elements to the northern Kurils' flora (Pietsch et al. 2003). The survival and adaptation of these diploid lineages to the cold climate conditions of NE Asia could have been favored by the existence of glacial refugia in western Beringia (Lozhkin et al. 2018).

The highly diversifying history of the oriental *B. sylvaticum* complex group contrast with the large genomic homogeneity of the occidental lineage. The latter group was

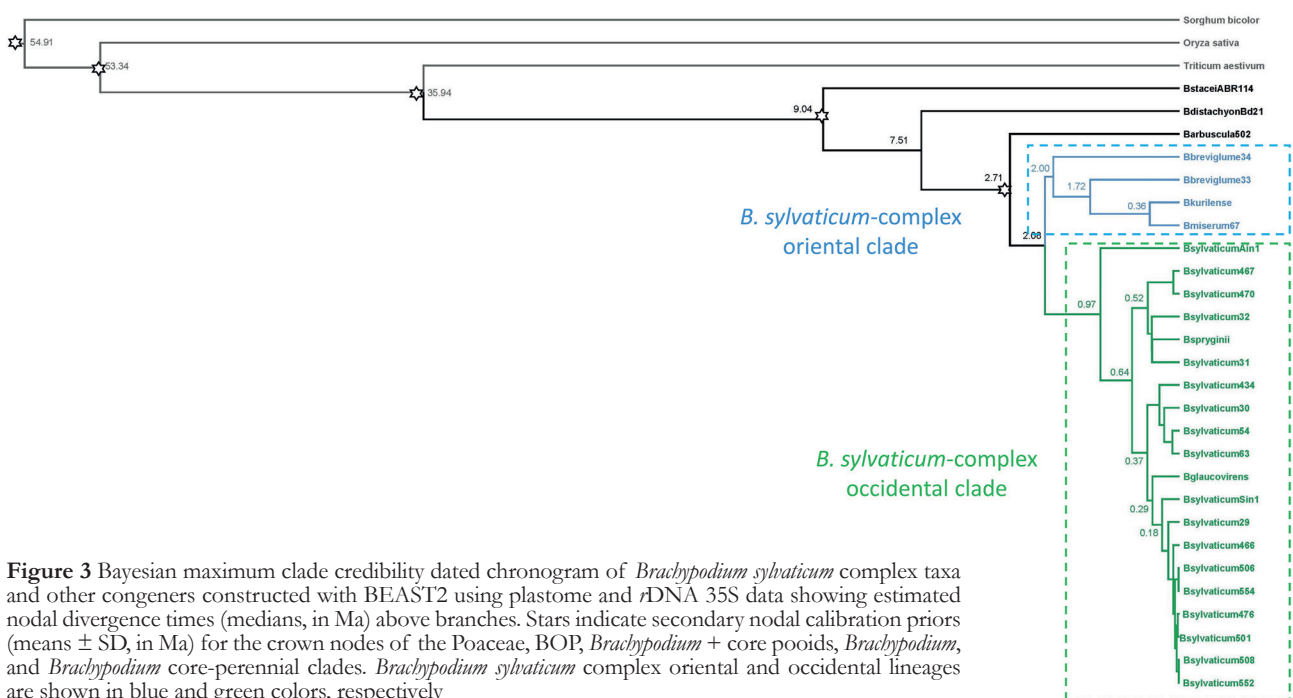


Figure 3 Bayesian maximum clade credibility dated chronogram of *Brachypodium sylvaticum* complex taxa and other congeners constructed with BEAST2 using plastome and *rDNA* 35S data showing estimated nodal divergence times (medians, in Ma) above branches. Stars indicate secondary nodal calibration priors (means \pm SD, in Ma) for the crown nodes of the Poaceae, BOP, *Brachypodium* + core pooids, *Brachypodium*, and *Brachypodium* core-perennial clades. *Brachypodium sylvaticum* complex oriental and occidental lineages are shown in blue and green colors, respectively

composed of European, Asian, and North African *B. sylvaticum* s. s. plus *B. glaucovirens* and *B. spryginii* lineages that did not reveal a strong geographic pattern in any of the phylogenetic trees reconstructed (Fig. 2). Several samples shared the same plastome sequence and collapsed in large polytomies in the plastome-based phylogenetic tree (Fig. 2A), while they split into independent but mostly invariable and unresolved lineages, sometimes mixed with oriental Himalayan and *B. pinnatum* lineages, in the 35S-based phylogenetic trees (Fig. 2B). Their origins from the crown ancestor were dated to more recent times than those of the oriental lineages, having presumably occurred in the last five hundred thousand years (Fig. 3). These results point towards a rapid spread of genomically homogeneous occidental *B. sylvaticum* s. s. lineages across the western Palearctic in late glacial and postglacial times with occasional crosses with oriental lineages (Figs 2, 3). Moreover, some of the characterized *B. sylvaticum* s. s. invasive genotypes in northwestern North America were apparently introduced from occidental European ancestors (Rosenthal et al. 2008).

The taxonomic implications of the phylogenomic results obtained in this study support the oriental micro-taxa *B. sylvaticum* var. *breviglume*, *B. kurilense*, and *B. miserum* as distinct to typical *B. sylvaticum* s. s. based on their short lower glume (more than 1.5 times shorter than the upper glume), which is shorter in *B. sylvaticum* var. *breviglume* (3–5 mm) than in the Pacific taxa (4.5–6 mm) (Keng 1982, Tzvelev 2015). *Brachypodium kurilense*, also subordinated to *B. miserum* in some taxonomic treatments, departs from the latter taxon based on the short prickles and hairiness of its spikelet pedicel and axis (Probatova & Skolovskaya 1985, Tzvelev 2015). These characters, although potentially variable, are in general fixed in the populations and constitute therefore phylogenetic signal traits of systematic value. The studied occidental micro-taxa (*B. glaucovirens*, *B. spryginii*) have distribution ranges that overlap with that of *B. sylvaticum* s. s. in the eastern Mediterranean and SW Asia, and in Ciscaucasia, respectively (Fig 1, Tzvelev 2015). *Brachypodium glaucovirens*, formerly synonymized to *B. sylvaticum*, was later recognized as a separate species (Scholz 2007). Morphologically it shows intermediate features, resembling *B. sylvaticum* in its short rhizome and long awn, and *B. pinnatum* in its bright green leaf color, broad leaf ribs, and erect panicle, and thus being a taxon of purported hybrid origin between the two species (Schippmann 1991). Our phylogenies corroborate this hypothesis and support a *B. sylvaticum* s. s.-type maternal progenitor species for *B. glaucovirens* (Fig. 2). *Brachypodium spryginii* differentiates from *B. sylvaticum* s. s. based on its more abundant plant pubescence and longer hairs (Tzvelev 2015). However, these variable characters are probably plastic and of low systematic value.

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Author contributions: PC designed the study. PC, LAI, EP, and NP collected samples. MD, RS, LAI, and NP developed the experimental work. MD, RS, LAI, EP, JV, and PC analyzed the data and interpreted the results. PC wrote the manuscript. All authors contributed to the article and approved the submitted version.

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