

# Phylogeny and Evolution of the Genus *Brachypodium*

Pilar Catalan, Diana López-Álvarez, Antonio Díaz-Pérez, Rubén Sancho, and María Luisa López-Herránz

**Abstract** We present an updated review of the phylogenetic and evolutionary studies conducted on the model genus *Brachypodium*. The genus, which contains approximately 20 globally distributed taxa (17 species, 1 variety, and 2 undescribed cytotypes) shows an intermediate evolutionary placement within the grass temperate pooid clade, being closer to the basal than to the recent Pooideae lineages. Our comprehensive molecular phylogenetic survey of all the currently known *Brachypodium* lineages illustrates a complex reticulate scenario of recently evolved diploid and allopolyploid lineages. Haplotypic statistical parsimony networks, multilabelled (multigenic) Minimum Evolution gene tree discordances, and Bayesian dating analysis have provided a testable hypothesis for the reconstruction of the *Brachypodium* species tree and for the estimation of its nodal divergence times. Our results support the early splits of the annual and short-rhizomatose lineages (*B. stacei*, *B. mexicanum*, *B. distachyon*) in the Holarctic region during the early-Middle Miocene (and *B. hybridum* in the Pleistocene), and a profusion of rapid splits for the perennial lineages since the late Miocene to the Pleistocene in the Mediterranean and Eurasian regions, with sporadic colonizations of more remote areas. Several perennial allopolyploid species (*B. boissieri*, *B. retusum*, *B. phoenicoides*, *B. rupestre* 4x, *B. pinnatum* 4x) showed homeologous copies from both ancestral and recent genome donors. More in-depth studies of the species of the *B. distachyon* complex have demonstrated the polyphyletic origin of the allotetraploid *B. hybridum* from bidirectional crosses of its diploid *B. stacei* and

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*B. distachyon* parents. Our niche modeling analysis has also detected distinct adaptations to different ecological tolerances in the diploids and evidence of niche conservatism for *B. hybridum* and each of its parents in their native Mediterranean region. Future perspectives include ongoing comparative genomics, phylogenomic and genotype-based phylogeographic studies of *Brachypodium*.

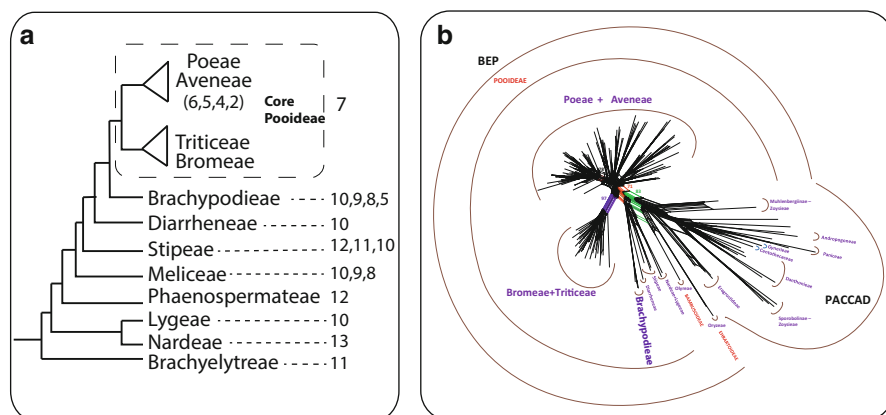
**Keywords:** Annual and perennial *Brachypodium* species • Dated phylogeny • Environmental niche modeling (*B. distachyon* group) • Haplotypic networks • Multigenic Minimum Evolution species tree • (allo)polyploid complexes

## Evolutionary Placement of *Brachypodium* within the Poaceae Tree

The genus *Brachypodium* has received considerable attention since the selection of the annual species *B. distachyon* as model functional plant for temperate cereals and biofuel grasses (IBI 2010; Catalán et al. 2014; Mur et al. 2011). Recently, the three segregated annual species of the *B. distachyon* complex (*B. distachyon*, *B. stacei*, *B. hybridum*; Catalán et al. 2012) have been proposed as a model system for grass polyploid speciation (Catalán et al. 2014) and the whole genus, containing taxa characterized by their small-size and compact genomes (Mur et al. 2011; Betekhtin et al. 2014), is also seen as an ideal candidate for comparative genomics of monocots.

Decades of systematic and phylogenetic studies were necessary, however, to frame its evolutionary position within the grasses. *Brachypodium* is considered today the single representative genus of the monotypic tribe Brachypodieae, which constitutes one of the intermediate diverging lineages of the temperate Pooideae grasses (Catalán et al. 1997; Bouchenak-Khelladi et al. 2008; Schneider et al. 2011) (Fig. 1). Its controversial position was caused by its shared or similar morphological and anatomical traits with distinct pooid groups (Catalán et al. 1995, and references therein). Consequently, it was classified in different tribes, based on the possession of embryo with mesocotyl (Poeae), hairy terminal ovary appendage and long narrow caryopsis and hilum (Bromeae), or spicate to racemose inflorescence and hairy lodicules (Triticeae), until its definitive adscription to its own tribe Brachypodieae (Jacques-Félix 1962; Schippmann 1991; Watson and Dallwitz 1992). Subsequently, its separate tribal treatment was confirmed by a number of private biological (embryo development), biochemical (exclusive seed storage proteins, seed globulins, seed storage polysaccharides and stem and leaf fructosans) (Schippmann 1991), and karyotype (large dispoloidy) (Robertson 1981; Khan 1984) characters.

The most recent phylogenetic works have consistently resolved Brachypodieae as the sister lineage of the recently evolved core pooid clade of temperate cereals and forages [Triticodae (Triticeae + Bromeae)/Poodae (Poeae + Aveneae)] (Fig. 1a). Its intermediate placement between the basal (Brachyelytreae, Lygeae-Nardeae, Phaenospermatæ, Meliceae, Stipeae) and the recently evolved (Triticodae/Poodae) Pooideae lineages has been recovered from both plastid and nuclear based topologies (Catalán et al. 1997; Davis and Soreng 2007; Bouchenak-



**Fig. 1** (a) Summarized plastid phylogeny of the temperate grasses showing the evolutionary placement of *Brachypodium* (Brachypodieae) between the early diverging and the recently evolved Pooideae tribes, and the intermediacy of its chromosome base numbers. (b) NeighborNet partition network tree based on nuclear  $\beta$ -amylase sequences showing the phylogenetic relationships of major tribal and subtribal grass lineages; *Brachypodium* is resolved close to the basal poooids. Pooideae (green), core poooids (red) and Triticeae + Bromeae (purple) splits showing bootstrap support values. Subfigure (a) partially adapted and updated from Catalán et al. (1997; Fig. 4); subfigure (b) adapted from Minaya et al. (2015; Fig. 3)

Khelladi et al. 2008; Schneider et al. 2011) and from combined analysis of molecular and morphological data (GPWG 2001). An intermediate position in the Pooideae tree is also reconstructed for the isolated *Diarrhena* (Diarrheneae) lineage, which apparently split earlier than *Brachypodium* (Catalán et al. 1997; Davis and Soreng 2007; Schneider et al. 2011). Recent phylogenetic studies based on a low copy nuclear gene ( $\beta$ -amylase) showed, however, that *Brachypodium* and *Diarrhena* could be closer to the basal poooids than to the recently evolved core poooid clade (Minaya et al. 2015; Fig. 1b). The two independent and small monogeneric Brachypodieae and Diarrheneae tribes present remarkable embryo features (bambusoid-like in *Diarrhena*, first lateral stem developing from coleoptile in *Brachypodium*), with *Brachypodium* also showing intermediate chromosome base numbers when mapped into the poooid tree (Catalán et al. 1997). A karyotype evolutionary trend of increasing chromosome sizes and decreasing chromosome base numbers is observed in the Pooideae, ranging from basal tribes with small chromosomes and high chromosome base numbers (Brachyelytreae = 11; Lygeae = 10; Nardeae = 13; Phaenospermateae = 12; Meliceae = 10, 9, 8; Stipeae = 12, 11, 10; Diarrheneae = 10), through the intermediate ones of Brachypodieae (10, 9, 8, but also 5), to the large chromosomes and almost constant chromosome base number of  $x = 7$  present in the more recently evolved Triticeae + Pooideae although  $x = 6, 5, 4, 2$  occasionally occur in Aveneae (Pooideae) (Fig. 1a).

The isolated monophyly of *Brachypodium*, close but divergent from the core poooid clade (Fig. 1a, b), corroborates other unique genomic features reported for this genus, like the possession of small genomes with low amounts of repetitive DNA (Shi et al. 1993) and of private repetitive DNA and ribosomal DNA families and nuclear RFLP markers (Catalán et al. 1995). Recent studies have confirmed that *Brachypodium*

combines both genus-specific and core-pooids-type or basal-pooids-BEP-type genomic traits. *Brachypodium* exhibits EST (expressed sequence tag)-contig chromosomal orthology, and similar globulin gene duplication and loci controlling phenotypic traits [e.g., spiking Eps-A (m)1, earliness Mot1 and FtsH4] and pathogen resistance (e.g., stem rust resistance, Rpg1 and Rpg4) responses with the Triticeae; however, it lacks colinearity for several STS (sequence tagged sites) and other stress controlling genes with this tribe (Mur et al. 2011, and references therein). Also, the *Brachypodium* genome shows greater synteny with the more ancestral *Oryza* (Ehrartoideae, early BEP lineage) genome than with the more recently evolved Triticeae genomes, probably due to accelerated genomic rearrangements in the Triticeae (Mur et al. 2011). Despite these findings, the *Brachypodium* genome is more closely related to the core pooid genomes than the rice genome, and, together with its intermediate evolutionary position within the BEP clade (Fig. 1b), is well placed to serve as model plant not only for the temperate cereals and forages but also for tropical PACCMAD grasses including species proposed as biofuel crops (e.g. *Miscanthus*, *Panicum* (switchgrass), *Paspalum*) (Mur et al. 2011; Catalán et al. 2014).

## Systematics of *Brachypodium*

*Brachypodium* is a relatively small genus that contains ca. 18 species distributed worldwide (Schippmann 1991; Catalán and Olmstead 2000; Catalán et al. 2012) (Table 1; Fig. 2). According to the most recent taxonomic updating (Catalán et al. 2012; Diaz-Pérez et al. unpub. data), 3 of them are annual species and 15 are perennial taxa. It has been recently demonstrated that the three annuals have a large distribution in their native circumMediterranean region (*B. distachyon*, *B. stacei*, *B. hybridum*) (Catalán et al. 2012; López-Alvarez et al. 2012, 2015). Among the perennials, few species show a large native Eurasian (*B. sylvaticum*, *B. pinnatum*, *B. rupestre*) or Mediterranean (*B. retusum*) distribution, whereas the rest have a restrict disjunct distributions in their respective native ranges [W Mediterranean (*B. phoenicoides*), C Mediterranean (*B. genuense*), E Mediterranean—SW Asia (*B. glaucovirens*), S Spain (*B. boissieri*), Canary isles (*B. arbuscula*), South Africa (*B. bolusii*), tropical and South Africa (*B. flexum*), Madagascar (*B. madagascariense*), Taiwan (*B. kawakamii*), SE Asia—New Guinea (*B. sylvaticum* var. *pseudodistachyon*), and America (*B. mexicanum*)] (Schippmann 1991; Diaz-Pérez et al. unpub. data; Fig. 2). Since 1812 two segregated genera were erected, *Trachynia* Link, to cover the annual species, and *Brevipodium* Lovë & Lovë, to accommodate *B. sylvaticum*; however, in almost all modern works neither of these two segregates were recognized (Catalán et al. 1995), and all the newly described species have been subsumed within *Brachypodium* (Schippmann 1991; Catalán and Olmstead 2000; Catalán et al. 2012).

The annual species are characterized by their short life-cycle, ephemeral habit and self-fertility (Catalán and Olmstead 2000; Catalán et al. 2012). Recent analysis of cryptic phenotypic, cytogenetic and molecular traits allowed us to separate the three species (Catalán et al. 2012). By contrast, most of the perennial taxa show long-rhizomes and self-incompatibility (Catalán et al. 1995; Khan and Stace 1999),

**Table 1** List of world *Brachypodium* taxa, cytotypes and ecotypes used in the phylogenetic analysis

Taxon	Code	Geographical distribution (native range)	2n	x	Ploidy	Genome size (pg/2C)
Annuals						
<i>B. distachyon</i> (L.) P. Beauv.	Bdis	circumMediterranean (Mediterranean, SW Asia)	2n = 10	5	2x	0.63
<i>B. stacei</i> Catalán, Joch. Müll., Mur & Langdon	Bsta	circumMediterranean (Mediterranean, Macaronesia, SW Asia)	2n = 20	10	2x	0.56
<i>B. hybridum</i> Catalán, Joch. Müll., Hasterok & Jenkins	Bhyb	circumMediterranean (Mediterranean, Macaronesia, SW Asia)	2n = 30	5 + 10	4x	1.26
Short-rhizomatose perennial						
<i>B. mexicanum</i> (Roem. & Schult.) Link	Bmex	America (from Mexico to N Bolivia)	2n = 40	10?	4x?	?
Long-rhizomatose perennials						
<i>B. arbuscula</i> Gay ex Knoche	Barb	Macaronesia; Canary isles (Spain)	2n = 18	9	2x	0.70
<i>B. boissieri</i> Nym.	Bboi	Spain: Betic mountain ranges (southern Spain)	2n = 42, 46	?	6x–8x?	?
<i>B. bolusii</i> Stapf	Bbol	South Africa	?	?	?	?
<i>B. flexum</i> Nees	Bflex	Tropical Africa and South Africa	?	?	?	?
<i>B. genuense</i> (D.C.) Roem. & Schult.	Bgen	Italy	2n = 18	9	2x	?
<i>B. glaucovirens</i> (Murb.) Sagorski	Bgla	East Mediterranean and SW Asia	2n = 16	8	2x	0.88
<i>B. kawakamii</i> Hayata	Bkaw	Taiwan	?	?	?	?
<i>B. madagascariense</i> Camus & Perrier	Bmad	Madagascar	?	?	?	?
<i>B. phoenicoides</i> (L.) P. Beauv. ex Roem. & Schultes	Bpho	West Mediterranean	2n = 28	5 + 9	4x	1.49
<i>B. pinnatum</i> (L.) P. Beauv. (diploid A)	Bpin2xA	Eurasia	2n = 18	9	2x	0.88
<i>B. pinnatum</i> (L.) P. Beauv. (tetraploid)	Bpin4x	Eurasia	2n = 28	5 + 9	4x	1.57
<i>B. pinnatum</i> (L.) P. Beauv. (diploid B)	Bpin2xB	SW Asia	2n = 16	8	2x	?

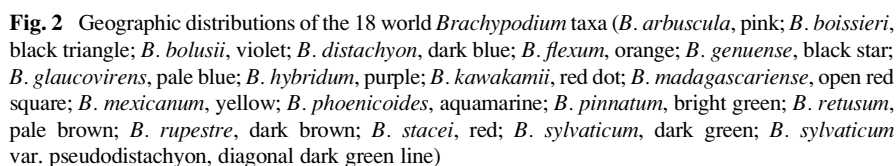
(continued)

Table 1 (continued)

Taxon	Code	Geographical distribution (native range)	2n	x	Ploidy	Genome size (pg/2C)
<i>B. retusum</i> (Pers.) P. Beauv.	Bret	Mediterranean	2n = 36	?	6x?	2.57
<i>B. rupestre</i> (Host) Roem. & Schult. (diploid)	Brup2x	West Eurasia	2n = 18	9	2x	0.84
<i>B. rupestre</i> (Host) Roem. & Schult. (tetraploid)	Brup4x	West Eurasia	2n = 28	5 + 9	4x	?
<i>B. sylvaticum</i> (Huds.) P. Beauv.	Bsyl	panEurasia (Eurasia, Macaronesia)	2n = 18	9	2x	0.87
<i>B. sylvaticum</i> (Huds.) P. Beauv. (Eastern lineage)	BsylEAs	East Asia (China)	?	?	?	?
<i>B. sylvaticum</i> var. <i>pseudodistachyon</i> J. D. Hook.	Bsypse	Malesia and New Guinea	?	?	?	?

Geographical distribution taken from Schippmann (1991), Catalán et al. (1995, 2012), Catalán and Olmstead (2000), López-Alvarez et al. (2012, 2015), 2n, x, ploidy level and genome size taken from Robertson (1981), Khan (1984), Schippmann (1991), Wolny and Hasterok (2009), Catalán et al. (2012), Betekhtin et al. (2014)

Information on geographical distribution in the native range, code, chromosome number (2n), chromosome base number (x), ploidy level and genome size is provided for each taxon. ? = unknown or unclear



except *B. mexicanum* and *B. sylvaticum* that are self-compatible (Khan and Stace 1999; Steinwand et al. 2013). *B. mexicanum* differs from them in its short-rhizomatous habit and self-compatibility (Khan and Stace 1999), taxonomically resembling more the annual than the perennial taxa in those traits and in seed protein contents and RFLP patterns (Khan 1992; Shi et al. 1993). The rhizomatous perennials are separated by their morphoanatomical and karyotypic traits. *B. arbuscula*, *B. retusum* and *B. boissieri* bear branched woody stems and long-lasting innovations. The Canarian *B. arbuscula* possesses top branched buds and dispersed root xylem and phloem, and grows in more humid places, whereas *B. retusum* and *B. boissieri* are adapted to xeric Mediterranean habitats and show strongly inrolled leaves. The narrow endemic *B. boissieri*, previously circumscribed within the broadly spread *B. retusum*, differs from it in its single-spikelet inflorescence, short habit and leaf blade morphology and anatomy (Schippmann 1990); the species is confined to dolomitic mountain ranges of southern Spain. The remaining taxa of the core perennial clade do not bear branched stems. The endemic alpine *B. kawakamii* and *B. bolusii* have a relatively short stature and dense, erect, and glabrous leaves; the inflorescences of *B. bolusii* present more spikelets but with less fertile florets than those of *B. kawakamii*. *B. pinnatum*, *B. rupestre* and *B. phoenicoides* show erect panicles. *B. phoenicoides*, adapted to dry places, is glabrous and presents partially inrolled leaves, semi-patent twisted spikelets and awnless lemmas, whereas the mesic *B. pinnatum* and *B. rupestre* have short awns and bright green colored leaves.

*B. rupestre*, considered until recently a subspecies of *B. pinnatum*, differs from it in its glabrous leaves and spikelets and in leaf epidermal traits (Schippmann 1991). The central Mediterranean endemic *B. genuense*, classified within *B. pinnatum* by some authors (Clayton et al. 2015), departs from it based on its particular karyotype, showing co-localized 5S and 25S rDNA loci in the same chromosome (Betekhtin pers. com.) and minor morphological differences (Valdés and Scholz 2009).

*Brachypodium sylvaticum* is the most distinct and widespread species of the genus. Its native Palearctic area ranges from Macaronesia in the west to New Guinea in the east (Fig. 2). It is characterized by its nodding panicle, densely hairy habit and long-awned lemma. Most of these 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, and from each other in the overall smaller habit of the mountain endemic island species. The ‘*B. sylvaticum*’ complex also includes the eastern Mediterranean—SW Asian endemic *B. glaucovirens*. This taxon, formerly synonymized to *B. sylvaticum*, or even considered a hybrid between this species and *B. pinnatum* (Schippmann 1991), has been recently 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. Furthermore, some of the six infraspecific *B. sylvaticum* taxa described in eastern Asia and Malesia—New Guinea (Schippmann 1991), like *B. sylvaticum* subsp. *pseudodistachyon*, which is characterized by its mountain dwarf habit and stiff leaves, could correspond to independent species.

Taxonomic uncertainty still persists among some poorly known extra-European taxa and within some Eurasian cryptic complex taxa (Schippmann 1991; Catalán and Olmstead 2000). Among the less known extra-European taxa, up to 5 different species have been described in America, 11 in Africa and 15 in Asia; however, most of them could probably be synonymized to currently recognized species from those regions (cf. Schippmann 1991). Regarding the Eurasian cryptic taxa, they correspond to ploidy complexes of putative diploid parents and their derived allopolyploids, involving different cytotypes of *B. pinnatum* (2x, 4x) and *B. rupestre* (2x, 4x) (Khan and Stace 1999; Wolny and Hasterok 2009; Betekhtin et al. 2014, and references therein). The intraspecific cytotypes could hardly be differentiated based on morphological traits; however, cytogenetic studies using Comparative Chromosome Painting (CCP) approaches suggest that the allopolyploids derive from interspecific crosses of distinct diploid progenitors, involving their respective diploid perennial counterparts (Wolny and Hasterok 2009; Idziak et al. 2014), or even those perennials and the annual *B. distachyon* (Wolny and Hasterok 2009; Betekhtin et al. 2014). The genus shows a remarkable dispoloidy, with chromosome base numbers of diploids ranging from the presumably more ancestral  $x = 10$  (*B. stacei*), through  $x = 9$  (*B. arbuscula*, *B. sylvaticum*, *B. pinnatum*, *B. rupestre*) and  $x = 8$  (*B. glaucovirens*), to  $x = 5$  (*B. distachyon*) (Robertson 1981; Betekhtin et al. 2014). Noticeably, the ‘recently evolved’ chromosome base number  $x = 7$  (Robertson 1981), which is almost fixed in most species of the large and young core pooid clade (Catalán et al. 1997), is apparently absent in *Brachypodium*, where



tetraploid species with  $2n = 28$  chromosomes have been found to be hybrid allopolyploids, potentially derived from diploid  $2n = 18$  ( $x = 9$ ) and  $2n = 10$  ( $x = 5$ ) progenitors (Khan and Stace 1999; Wolny and Hasterok 2009; Betekhtin et al. 2014). Betekhtin et al. (2014) proposed two alternative hypotheses for karyotype evolution in *Brachypodium*, continuous descendant dispoloidy ( $x = 10$  to  $x = 9$ , 8 to  $x = 5$ ) vs. descendant + ascendant dispoloidy ( $x = 10$  to  $x = 5$  to  $x = 9$ , 8), with allotetraploid  $2n = 28$  species originating always in a later stage.

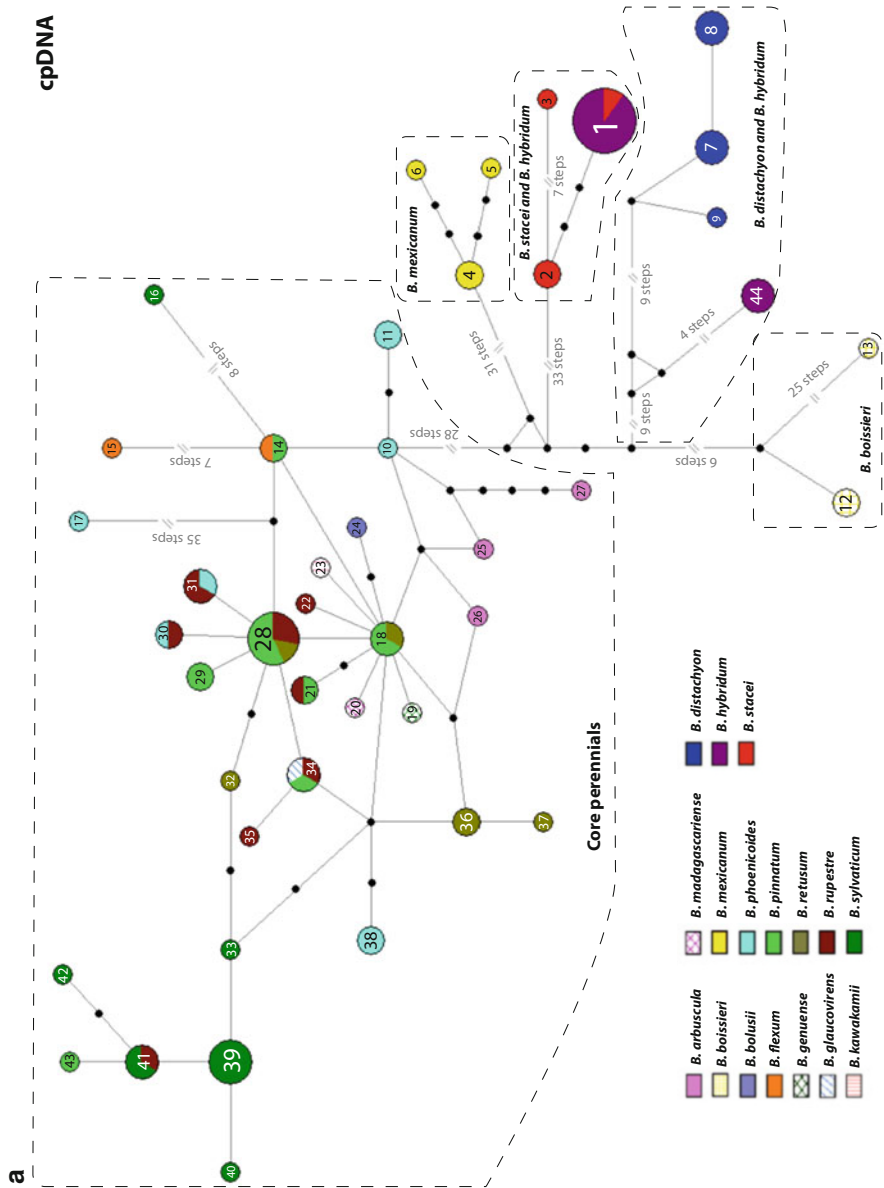
The taxonomic identity of these allotetraploid cytotypes is still unclear, though they might constitute separate species, paralleling the case of the segregated annual species of the diploid-allopolyploid *B. distachyon* complex (Catalán et al. 2012). Overall, *Brachypodium* constitutes a small isolated genus of approximately 20 species, with native ranges distributed in five continents. Two of its species, the annual *B. hybridum* and the perennial *B. sylvaticum*, are invasive plants. *B. sylvaticum* has been introduced and is spread in western N America and in Australia, and *B. hybridum* has successfully colonized C Europe, western N America (California), S America (Uruguay, Argentina), South Africa and Oceania (Australia, New Zealand) (Jenkins et al. 2003; Garvin et al. 2008; Bakker et al. 2009; Catalán et al. 2012).

## Phylogeny of *Brachypodium*

All phylogenetic studies conducted on *Brachypodium* support a rapid and relatively recent radiation of its crown ancestor, after a long time span from the earlier split of the stem ancestor and the recent split of the crown clade (Catalán and Olmstead 2000; Catalán et al. 1995, 2012; Diaz-Perez et al. unpub. data). This long isolation, followed by recent divergence, is corroborated by its exclusive nuclear genomic families (Catalán et al. 1995; Mur et al. 2011) and by its confounding assorted lineages (Catalán and Olmstead 2000; Wolny et al. 2011; Catalán et al. 2012). Successive phylogenetic works based mostly on analysis of plastid and nuclear rDNA sequences and on nuclear (RAPD) markers, including approximately half of the species of *Brachypodium*, recovered a congruent evolutionary framework for the genus (Catalán et al. 1995, 2012; Catalán and Olmstead 2000). Dated phylogenies based on combined analysis of nuclear ribosomal genes and plastid genes have estimated the origin of the common ancestor of *Brachypodium* in the mid Miocene, showing the early successive divergences of *B. boissieri*, *B. stacei*, and *B. mexicanum*, a later Pliocene split of *B. distachyon* and the recent Pliocene-Pleistocene radiation of the core perennial clade (Catalán et al. 2012). Within the latter group, a congruent trend was observed in the early divergence of *B. arbuscula*, followed by that of *B. retusum*, though uncertainty affected the rapid splits of the most recent nodes, ending in an unresolved scenario for the divergence of the *B. pinnatum*, *B. rupestre*, *B. phoenicoides*, *B. glaucovirens* and *B. sylvaticum* lineages (Catalán et al. 1995, 2012; Catalán and Olmstead 2000; Wolny et al. 2011). Phylogenetic trees reconstructed from low copy nuclear genes concurred with this hypothesis, but also showed basal homeologous copies in one allopolyploid member of the core perennial clade (*B. retusum*; Wolny et al. 2011; Catalán et al. 2012).

Deep evolutionary analysis of the perennial *Brachypodium* genomes has been hampered, however, by the intricate reticulate nature of the species in this core clade, which shows a prevalence of allopolyploid taxa, and by their explosive radiation, manifested in the mostly unresolved or weakly supported topologies (Catalán et al. 2012). Recently, a thorough taxonomic and geographic sampling of all the currently recognized species of the genus allowed us to conduct the largest and most comprehensive phylogenetic study of *Brachypodium* to date (Díaz-Pérez et al. unpub. data described below). A total of 110 samples representing the 17 recognized species plus one geographically isolated infraspecific taxon (*B. sylvaticum* var. *pseudodistachyon*) were included in the study (Table 1; Fig. 2). Six taxa (35.3 % of the total taxonomic diversity) were studied molecularly for the first time (*B. bolusii*, *B. flexum*, *B. genuense*, *B. kawakamii*, *B. madagascariense*, *B. sylvaticum* var. *pseudodistachyon*). Our study also included representatives of both diploid and allotetraploid cytotypes of the perennial *B. pinnatum* and *B. rupestre* species. Chromosomal, genome size and ploidy data information was collected for all samples except for some poorly known taxa which have not been karyotyped yet (Table 1). One thousand one hundred fifty-four DNA sequences from three nuclear (ETS, ITS, GI) and two plastid (*ndhF*, *trnLF*) loci were used to reconstruct the phylogeny of *Brachypodium*. The non-recombinant plastid *ndhF*+*trnLF* sequences were concatenated into a combined (cpDNA) data set and provided information about the maternal genomic inheritance in the hybrids, and the cloned sequences of the nuclear loci retrieved homeologous copies in the allopolyploids.

Exploratory phylogenetic and haplotypic network analyses were conducted with the respective sets of sequences (Fig. 3a–e). Phylogenetic analyses based on Maximum Likelihood (ML; RAxML) and Bayesian Inference (BI; MrBAYES) methods recovered the evolutionary relationships among the *Brachypodium* lineages, using other pooid representatives and *Oryza* (Ehrartoideae) as outgroups (Fig. 3e). Haplotypic networks were constructed to infer the genealogical relationships of the *Brachypodium* haplotypes (species and samples) obtained from each separate data set using statistical parsimony approaches (NETWORK) (Fig. 3a–d). The maternally inherited plastid haplotypic network consisted of 43 haplotypes (Fig. 3a) and was relatively well resolved for the early divergences of the monophyletic *B. boissieri*, *B. stacei*, *B. mexicanum* and *B. distachyon* clusters, each separated by a number of mutational steps in a star-like net (with highly supported divergences in the phylogenetic tree; Fig. 3e). The *B. hybridum* haplotypes were shared with its *B. stacei* and *B. distachyon* parents, though more frequently with the former. However, the cluster of the recently evolved core perennial species showed a lack of genealogical and taxonomic structure (Fig. 3a), denoted by the high number of interspecific shared haplotypes (with some haplotypes shared by up to three species; e.g., h. 28: *B. pinnatum*, *B. retusum*, *B. rupestre*), and an ambiguous resolution, manifested in the high number of internal loops and few internal mutational steps. The high number of interspecific shared maternal haplotypes reflects a history of repeated introgressions among lineages of the core perennial



**Fig. 3** Haplotype statistical parsimony networks (a–d) and summarized Bayesian phylogenetic trees (e) of *Brachypodium* reconstructed from the four independent studied loci. (a) cpDNA; (b) ITS; (c) ETS; (d) ETS. Species colors are indicated in the charts. Numbers inside the circles indicate haplotype number. Size of the circles is correlated with number of samples showing the haplotype

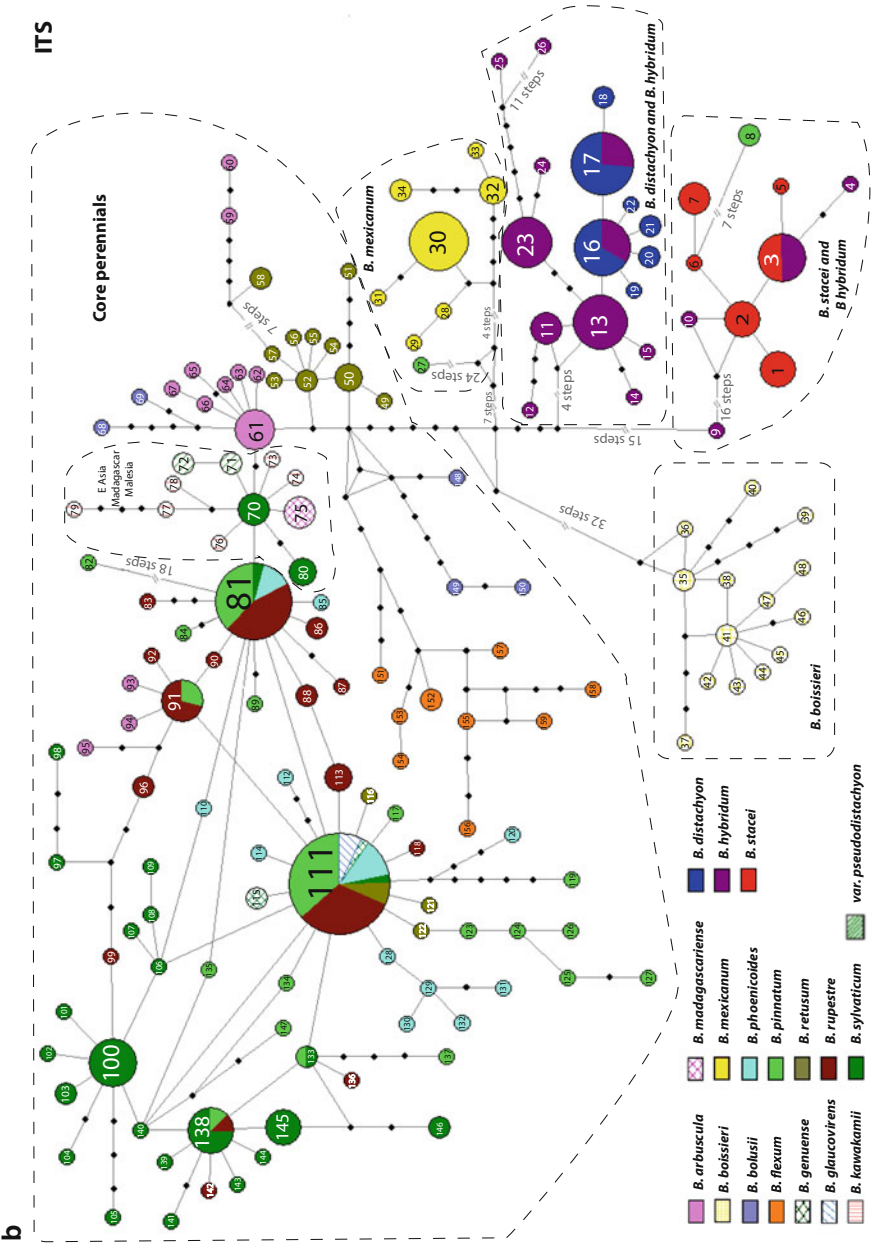
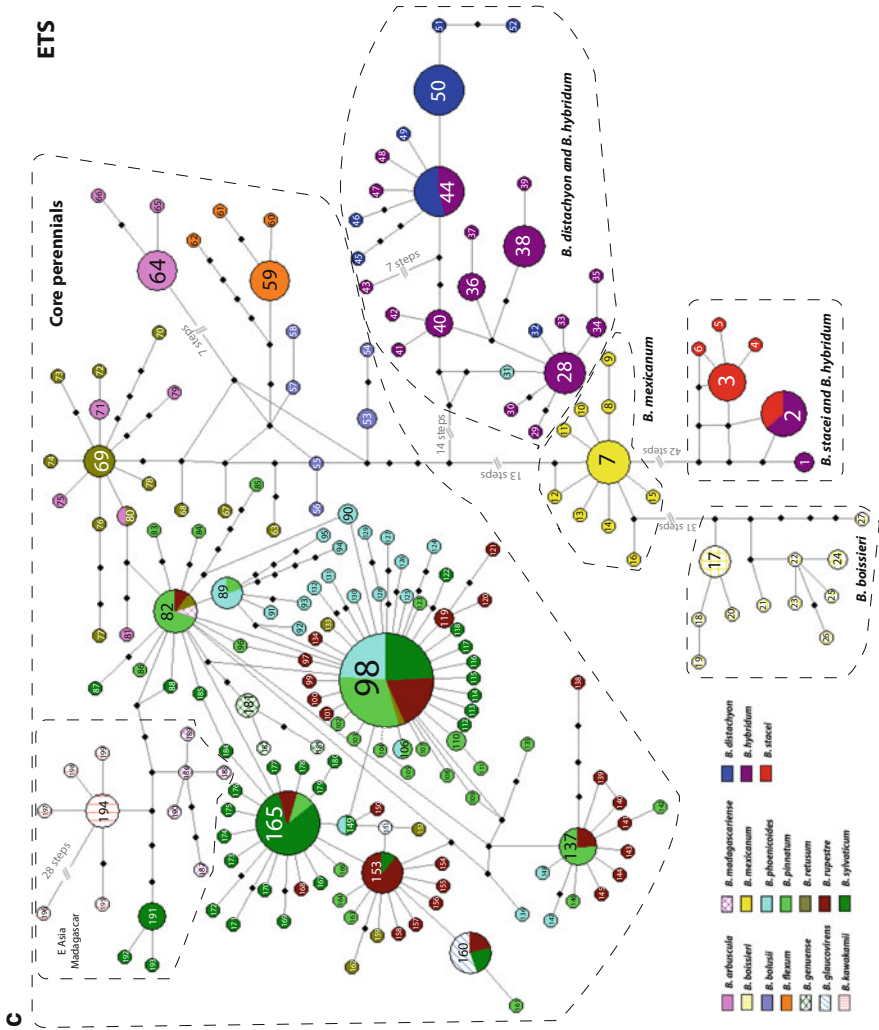


Fig. 3 (continued)



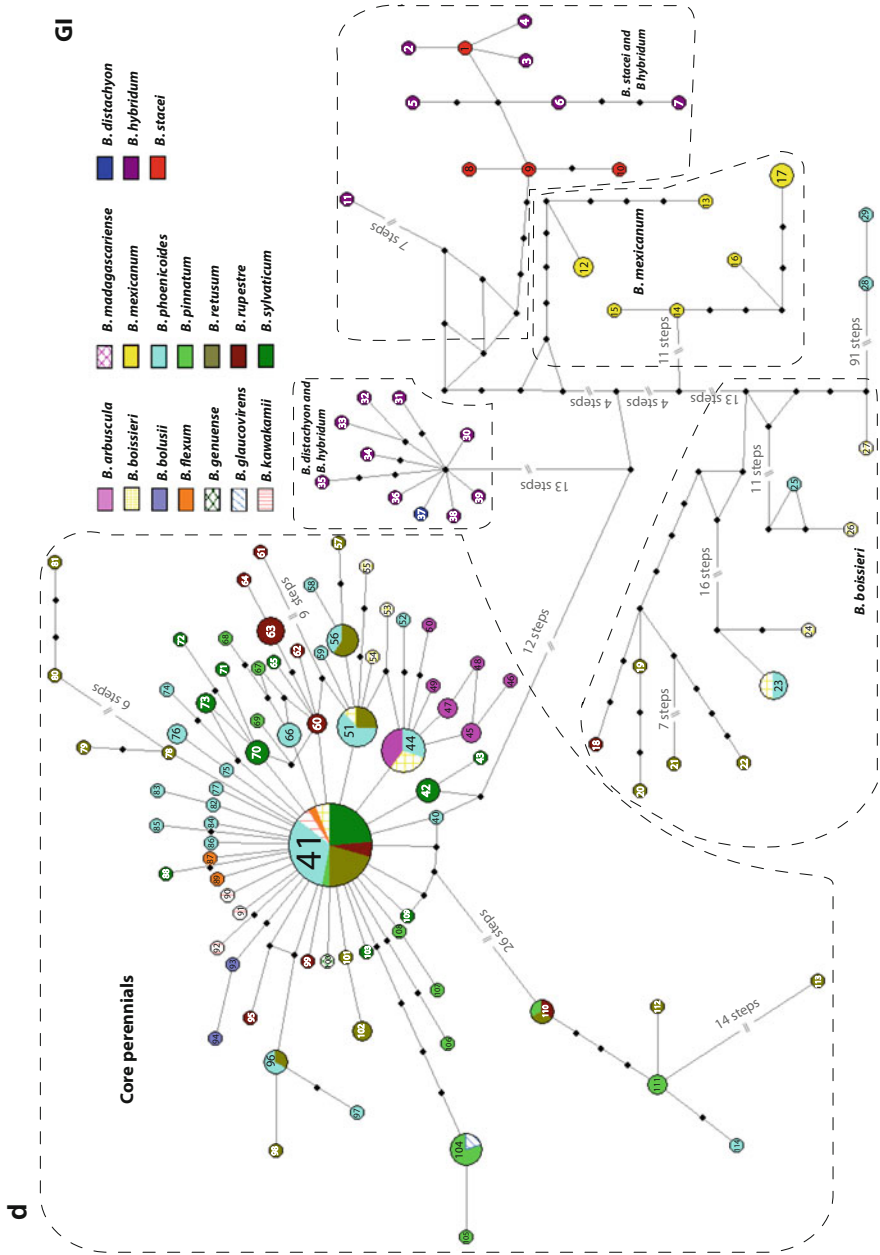


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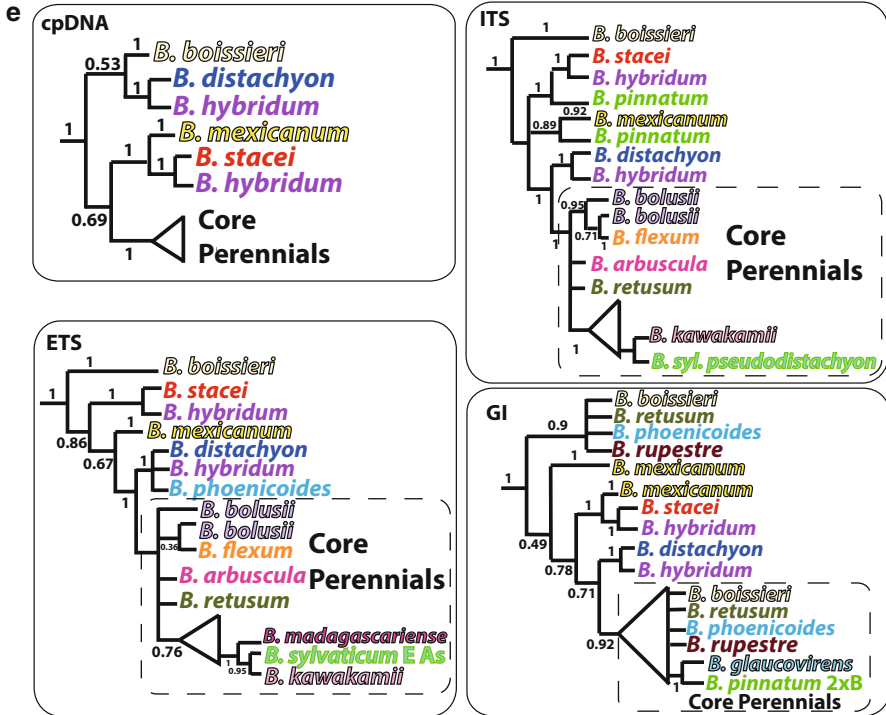


Fig. 3 (continued)

group, whereas the lack of resolution of the network (and the phylogenetic tree; Fig. 3e) indicates a high level of homoplasy in the data.

The biparentally-inherited and more variable ITS and ETS loci were overall congruent with the plastid data but reconstructed more resolved trees and networks for the earlier splits within the core perennials clade and for some geographical groupings. These loci also detected ‘ancestral’ homeologous ribotypic copies in some core perennials allopolyploids. The ITS and ETS phylogenies and haplotypic networks (Fig. 3b, c, e) constructed, respectively, with 159 and 199 haplotypes, were congruent in the separate basal divergences of the *B. boissieri*, *B. stacei*, *B. mexicanum* and *B. distachyon* lineages and in the complex reticulate structure of the core perennials group. They further detected the early divergences of the *B. bolusii*/*B. flexum*, *B. arbuscula* and *B. retusum* lineages within the core perennials clade, and the clustering of endemic East Asia (*B. sylvaticum* (China)/*B. kawakamii*)—Madagascar (*B. madagascariense*)—New Guinea (*B. sylvaticum* var. *pseudodistachyon*) haplotypes in their respective regional subnetworks (Fig. 3b, c, e). The introgression and homoplasy levels detected by these loci were much higher than those detected by the plastid data within the core perennial cluster, and mostly affected the Eurasian and Mediterranean species. Thus, the commonest ITS haplotype (h. 111) was shared by seven species (*B. genuense*, *B. glaucovirens*,



*B. phoenicoides*, *B. pinnatum*, *B. retusum*, *B. rupestre*, *B. sylvaticum*; Fig. 3b) and the commonest ETS haplotype (h. 98) by five species (*B. phoenicoides*, *B. pinnatum*, *B. retusum*, *B. rupestre*, *B. sylvaticum*; Fig. 3c). Both loci detected co-inherited *B. stacei*-type and *B. distachyon*-type parental ribotypes in *B. hybridum*, being more frequent those from the later parent, and a few ancestral haplotypes in individual samples of some core perennials allopolyploid species (a *B. stacei*-type and a *B. mexicanum*-type ITS ribotypes in tetraploid *B. pinnatum* samples, and a *B. distachyon*-type ETS ribotype in *B. phoenicoides*; Fig. 3b, c).

The highly variable and bi-parentally inherited low copy nuclear gene GI which, in contrast to the nuclear multicopy ribosomal ITS and ETS loci, is not subjected to concerted evolution, was also congruent with the main evolutionary patterns reconstructed by the plastid and ribosomal genes for *Brachypodium*. Additionally, this locus provided new data about the putative origins of several allopolyploid perennial species. The GI phylogeny and haplotypic network (Fig. 3d, e), constructed with 114 haplotypes, also supported the early divergence of the basal lineages and the reticulation of the recent core perennials clade, though relationships were less resolved within the last group and varied slightly with respect to the successive basal divergences of the *B. boissieri*, *B. mexicanum*, *B. stacei* and *B. distachyon* lineages. The level of potential introgression detected by the GI network was apparently very high, the most common haplotype (h. 41) was shared by samples from eight perennial species (*B. boissieri*, *B. flexum*, *B. kawakamii*, *B. phoenicoides*, *B. pinnatum*, *B. retusum*, *B. rupestre*, *B. sylvaticum*). In contrast, the GI clones detected the highest number of homeologous copies among the perennial *Brachypodium* allopolyploid species. Most interestingly, highly divergent GI sequences of *B. boissieri*, *B. retusum*, *B. phoenicoides* and *B. rupestre* 4x were nested within both the basal '*B. boissieri*' cluster and the recent core perennial cluster. The *B. hybridum* individuals showed homeologous copies from each *B. stacei* and *B. distachyon* parent. The analyses also recovered two close but separate homeologous lineages within *B. mexicanum* (Fig. 3d, e).

Our new findings provide new insights into the evolutionary history of *Brachypodium* (Díaz-Pérez et al. unpub. data, Fig. 3). All the analyzed plastid and nuclear loci agree with previous studies (Catalán et al. 2012; Catalán et al. 2014) in the more ancestral divergences of the annual *B. stacei* and the shortly-rhizomatose *B. mexicanum*, and in the sister relationship of the annual *B. distachyon* to the recentmost core perennial clade (Fig. 3a–e). However, the homeologous 'ancestral' and 'recently evolved' copies detected in several perennial species at the GI locus and, to a lesser extent, at the ITS and ETS loci, provide a new scenario for the likely allopolyploid origins of these perennial plants. Our results indicate that *B. boissieri* is not an early divergent lineage within the *Brachypodium* clade but, most probably, an allopolyploid species originated from the cross of at least one ancestral genome donor and one recent perennial-core genome donor (Fig. 3d, e). Similarly, *B. retusum* and *B. rupestre* 4x (GI network, Fig. 3d), *B. phoenicoides* (ETS and GI networks, Fig. 3c, d) and *B. pinnatum* 4x (ITS network, Fig. 3b) would have also resulted from different crosses of at least one ancestral genome donor and one recent perennial-core genome donor. Wolny et al. (2011) and Catalán et al. (2012) also detected both ancestral and

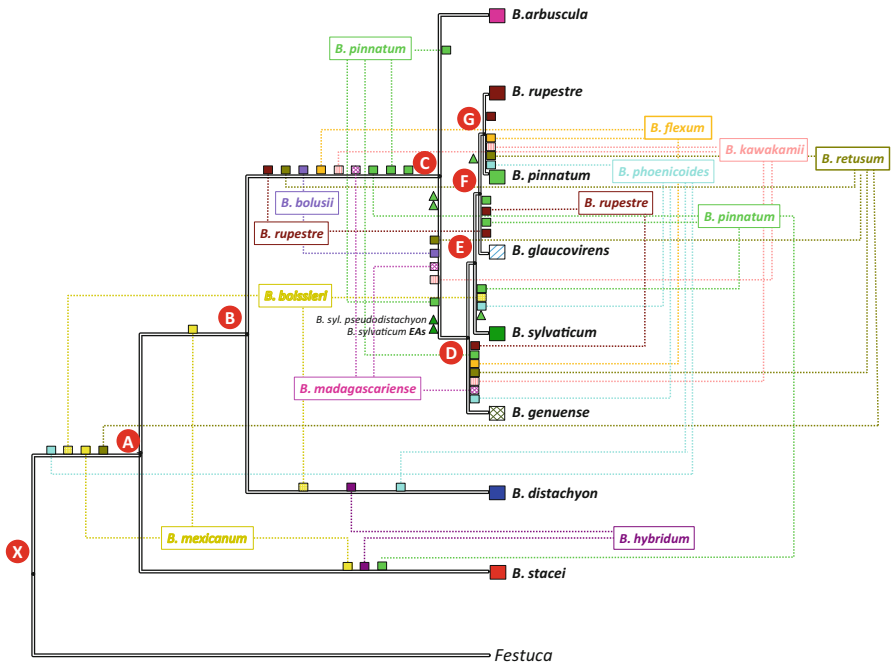


recent homeologous copies in the purported allohexaploid *B. retusum* for the low copy nuclear genes *GI*, *CAL* and *STT3A*, and *CAL*, *DGAT* and *GI*, respectively; and Catalán et al. (2012) detected two close homeologous genomes in *B. mexicanum* also for *CAL*. Betekhtin et al. (2014) hypothesized about the potential origins of the allotetraploids *B. pinnatum*, *B. rupestre* and *B. phoenicoides* from a cross of a *B. distachyon*-type diploid genome ( $x = 5$ ) and a perennial-core-type diploid genome ( $x = 9$ ). Their CCP study used BAC libraries from the *B. distachyon* genome, which is more closely related to the core perennials' genomes (Fig. 3); however, no other CCP analyses have been conducted yet with BAC clones derived from other *Brachypodium* genomes. Noticeably, this cytogenetic analysis revealed the absence of CCP hybridization signals of the *B. distachyon* BAC probes in approximately half of the chromosomes of the basal *B. stacei* and *B. mexicanum*, and in some chromosomes of *B. retusum* (*B. boissieri* was not included in the analysis; Betekhtin et al. 2014, pers. com.). These phylogenetic and cytogenetic evidences support the existence of an ancestral-type *Brachypodium* genome, divergent from the recently evolved *B. distachyon* and core perennial genomes, in the nuclear genomes of those species.

Our results point towards to alternative sources of ancestral genome donors for the perennial allopolyploids (e.g., *B. stacei*-type or *B. mexicanum*-type for *B. pinnatum* 4x, *B. distachyon*-type for *B. phoenicoides*, '*B. boissieri*' ancestral-type for *B. boissieri*, *B. retusum* and *B. phoenicoides*; Fig. 3b, c, d). Conversely, the low and ambiguous resolutions obtained for the core perennial group in all assayed loci preclude the identification of the recent core perennial genome donors. Regarding the morphologically close high allopolyploid Mediterranean *B. boissieri* (cf. 6x–8x) and *B. retusum* (6x) species, it could be hypothesized that convergent evolution has apparently converted the *B. boissieri* ITS and ETS copies into the ancestral '*B. boissieri*'-ancestral-type ribotypes and the *B. retusum* copies into the recent core perennial-type ribotypes (Fig. 3b, c). This might be also the reason for the low number of 'ancestral' ITS and ETS ribotypes found in the *B. pinnatum* 4x and *B. phoenicoides* samples (convergence towards the recent core perennial types). These plausible hypotheses on alternative origins of the allopolyploids could not be confirmed, however, with the current data. Low copy nuclear genes are prone to several phylogenetic distorting events, such as paralogy (duplication), losses, recombination and pseudogenization (Díaz-Pérez et al. 2014; Minaya et al. 2015), and multicopy nuclear ribosomal genes are constrained by convergent evolution. These recently diverged species could also have experienced a number of additional confounding evolutionary events, apart from hybridization, such as incomplete lineage sorting and vector-mediated or other horizontal gene transfers (Minaya et al. 2013). A larger genomic coverage would be necessary to dissect the evolutionary history of the *Brachypodium* allopolyploids. Ongoing phylogenomic studies are under way to target it.

Phylogenetic studies of highly reticulate polyploid groups confront the difficulty or impossibility of reconstructing bifurcate tree-like topologies from genome-mergers and genome-doubled species, which render network-like phylogenies (Jones et al. 2013; Marcussen et al. 2015). In our attempt to construct a robust explicit phylogenetic framework for *Brachypodium*, we used comparative statistical analysis of diploid/polyploid multiple gene tree discordances (Cai et al. 2012) to

build the species tree of this highly reticulate and allopolyploid genus. In order to discard spurious variation generated from PCR or cloning artifacts, intraindividual consensus sequences were generated collapsing closely related sequences that showed a p-distance value lower than 0.01 (cf. Diaz-Pérez et al. 2014). Multilabelled gene phylogenies were constructed for each diploid and diploid + polyploid data set through ML and BI methods. The resulting topologies were used as reference trees to select the best consensus sequences and to build the respective species trees. We followed the method of Cai et al. (2012), based on Minimum Evolution (FASTME) analysis, to calculate the internodal distances (NJst) among tips in each multilabelled gene tree for every polyploid consensus allele, using averaged pairwise distances for all the diploid and diploid-polyploid combinations. Integrated data matrices of averaged distances from the four loci were constructed for all diploid species and for one polyploid allele each time, generating as many integrated data matrices as polyploid alleles. The unrooted species tree of all diploid *Brachypodium* taxa had 15 branches. To estimate the optimal placement(s) of the polyploid alleles in the diploid skeleton tree (Fig. 4),



**Fig. 4** Multilabelled Nst-FastME species tree of *Brachypodium* obtained from a four gene-tree-discordance Minimum Evolution approach. Insertion of the allelic copies of the allopolyploids (discontinuous lines) on the branches of the diploid skeleton tree (solid lines) was done according to their positions in the selected trees (minimum tree values) for each polyploidy allele per locus. Species colors correspond to those indicated in Fig. 3

each polyploid allele was inserted in any potential branch, rendering 15 species trees per allele. The lengths of the trees were calculated according to the minimum evolution method, using the integrated distance matrices and the 15 species trees per polyploid allele, and selecting the minimum tree length value each time as indicator of the optimal placement of each polyploid allele in a particular branch of the diploid tree.

Our diploid skeleton tree included only *Brachypodium* species of confirmed diploid nature (Fig. 4). According to this multigene tree, the lineage of the annual *B. stacei* diverged first from the common ancestor, followed by that of the annual *B. distachyon* and then by the clade of core perennial taxa, which showed the successive divergences of the *B. arbuscula*, *B. genuense*, *B. sylvaticum*, *B. glaucovirens*, and the *B. pinnatum* 2x/*B. rupestre* 2x lineages. The FASTME reconstruction placed the multigene alleles of *B. mexicanum*, and therefore the potential origins of its genome donors, in three basal and subbasal positions of the tree (stem branch of *Brachypodium*, stem branch of *B. distachyon*/core perennials clade, terminal branch of *B. stacei*), those of *B. hybridum* along each terminal branch of its *B. stacei* and *B. distachyon* diploid parents, and those of *B. boissieri*, *B. retusum*, *B. phoenicoides* and *B. pinnatum* 4x in basal and subbasal branches of the tree but also in more recent branches of the core perennial subtree and in its stem branch (Fig. 4). The topological placement of the allelic copies of the remaining polyploid or unknown ploidy *Brachypodium* species was restricted to the recent stem branch and internal branches of the core perennial clade. Among them, *B. bolusii* showed a more ancestral putative origin, whereas a highly reticulate scenario of multiple potential crosses was reconstructed for the respective origins of the other species (Fig. 4). Though some of these reconstructions might have been affected by homoplasy, the evolutionary trends depicted in this ME species tree explain the reproductive biology and hybridization capability observed in the *Brachypodium* species. Artificial crosses involving five perennial species (*B. glaucovirens*, *B. phoenicoides*, *B. pinnatum*, *B. retusum*, *B. sylvaticum*) demonstrated the ability of these taxa to interbreed and produce viable F<sub>1</sub> hybrids in all directional crosses (Khan 1984; Khan and Stace 1999). Furthermore, all the obtained F<sub>1</sub> hybrids exhibited some degree of fertility. Successful hybridisations were also performed between those perennials and the annual *B. hybridum* ("*B. distachyon*" 2n = 30); however, despite the fact that hybrids were vigorous and long-lived, they were sterile. By contrast, no hybrids could be raised from crosses of these perennials and *B. hybridum* with *B. mexicanum* (Khan and Stace 1999). Recent attempts to cross current *B. stacei* and *B. distachyon* individuals and to produce a synthetic artificial *B. hybridum* plant from bidirectional crosses of these parental-genomes species have also failed (López-Alvarez et al. 2012) though Boulos Chalhoub and Vinh-Ha Dinh-Thi made a viable but sterile F1 (Chalhoub pers. com.). These experimental evidences support the reproductive isolation of the ancestral *B. stacei* and *B. mexicanum* lineages (and genomes), the reproductive compatibility of the more recently diverged *B. hybridum* (*B. distachyon* genome) with the perennials, and the high reproductive success of the recentmost perennial

lineages in all interspecific crosses. All these events fit well the reticulate scenario recovered in our evolutionary study (Fig. 4).

Divergence time estimations for the nodal splits of the *Brachypodium* species tree were obtained from a multigene diploid + polyploid data set using a Bayesian dating approach (BEAST). A consensus topology derived from the diploid skeleton tree (Fig. 4) was enforced for the placement of the unknown-ploidy and allopolyploid species, considering subtree resolutions supported by one or more loci (Fig. 3b–e) and the topology of the multigene ME species tree (Fig. 4). It was based on the premise that the allopolyploid hybrid lineages could never have originated before its genome donor lineages (Fig. 4). Nonetheless, due to the absence of a current diploid ‘ancestral’ lineage (e.g. ‘ancestral’-type genome), a basal *B. boissieri* ‘ancestral’-type allele was also included in the analysis in order to estimate the divergence time of the potential earliest split of the *Brachypodium* crown node. We calibrated the crown node of the BEP clade using a secondary calibration (mean age  $54.9 \pm 5.7$  Ma), according to Bouchenak-Khelladi et al. (2010), and the crown node of the Pooideae clade using a fossil calibration ( $48.4 \pm 4.0$  Ma), based on a pooid-type phytolith from the Middle-Eocene (Zucol et al. 2010). Our dating analysis (Diaz-Perez et al. unpub. data) indicated that the *Brachypodium* lineage branched off from its stem node in the Late Eocene (38.8 Ma) and that the split of the crown node could have occurred earlier (21.6 Ma) or later (16.2 Ma) in the early Miocene, considering or not the basal divergence of the ‘ancestral’-type *B. boissieri* genome. The long time elapsed between the stem and crown splits (17.2–22.6 Ma) would explain the evolutionary and genomic isolation of *Brachypodium* from its closest pooid relatives. Our analysis also showed successive early and Mid-Miocene divergences for the basalmost currently extant *Brachypodium* lineages (*B. stacei*, 16.2 Ma; *B. mexicanum*, 14.0 Ma; *B. distachyon*, 10.6 Ma), followed by a rapid radiation of the core perennial lineages since the end of the Miocene (6.1 Ma) and through the Pliocene and the Pleistocene, with the annual allotetraploid *B. hybridum* also arisen in the Pleistocene. Our nodal datings were older than those proposed by Catalán et al. (2012) though both studies showed overlapping values in their respective 95 % highest posterior density (HPD) intervals for the divergence time estimates.

## **Evolutionary History and Environmental Niche Variation of the Model *B. distachyon* Complex Species (*B. distachyon*, *B. stacei*, *B. hybridum*)**

The most exhaustively studied species of *Brachypodium* are those of the annual *B. distachyon* complex, which include the model grass plant *B. distachyon* and its close allies *B. stacei* and *B. hybridum* (which show, respectively,  $2n = 10$ , 20 and 30 chromosomes; Catalán et al. 2012; Catalán et al. 2014). The three cytotypes were previously attributed to different ploidy levels of the same taxon *B. distachyon* s. l.

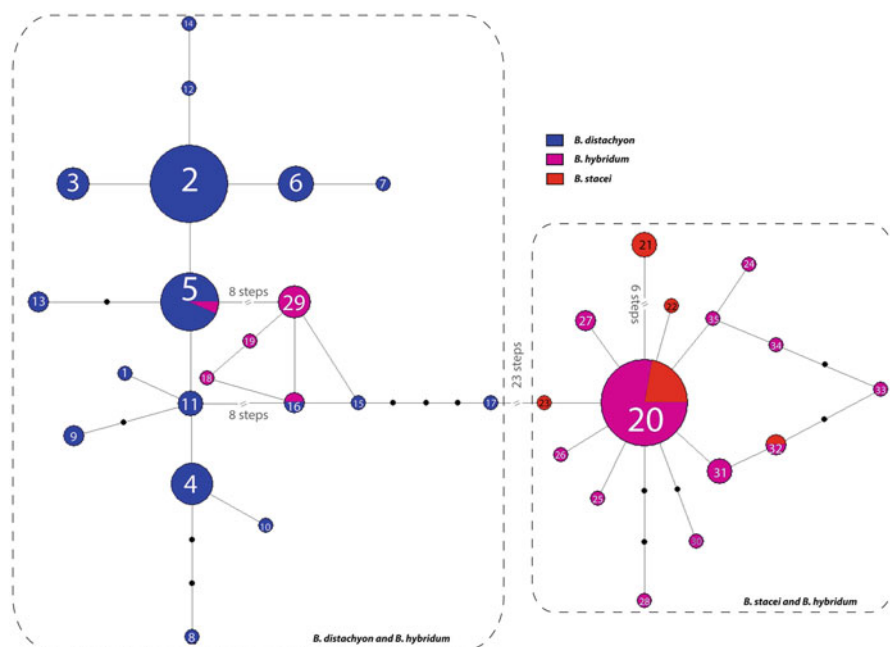
(Robertson 1981); however, phylogenetic, cytogenetic and phenotypic analyses demonstrated that they should be treated as three different species. They consist of two diploids, each with a different chromosome base number [*B. distachyon* ( $x = 5$ ,  $2n = 10$ ); *B. stacei* ( $x = 10$ ,  $2n = 20$ )], and their derived allotetraploid *B. hybridum* ( $x = 5 + 10$ ,  $2n = 30$ ). Phylogenetic analyses of two plastid (*ndhF*, *trnLF*) and five nuclear (ITS, ETS, CAL, GI, DGAT) genes indicated that the more basally-diverging *B. stacei* and the more recently evolved *B. distachyon* emerged from two independent lineages, confirming their contribution as genome donors of *B. hybridum* (Catalán et al. 2012). Further evidence from different molecular sources, like seed protein data (Hammami et al. 2011), nuclear SSRs (Giraldo et al. 2012), DNA barcoding (López-Alvarez et al. 2012), isozymes (Jaaska 2014), and metabolite fingerprinting (López-Alvarez et al. unpub. data) have also confirmed the co-occurrence of progenitor *B. distachyon* and *B. stacei* markers in the *B. hybridum* background.

Statistical analysis of morphometric traits showed that five characters (stomata leaf guard cell length, pollen grain length, upper glume length, lemma length, and awn length) significantly discriminated among the three species when they were grown under controlled greenhouse conditions (Catalán et al. 2012). An enlarged morphological study of both inbred and wild individuals from widespread Mediterranean populations has corroborated the phenotypic differentiation of the three species, with the number of species-specific discriminant traits increasing to 13 when only wild populations are considered (Catalán et al. unpub. data; López-Alvarez et al. unpub. data). Although the three species can be differentiated through several phenotypic and cytogenetic traits, their direct identification is not always straightforward as wild populations show overlapping phenotypic variation for some characters and a similar diploid genome size. This has led to taxonomic uncertainty, or even to taxonomic misclassifications of the model species and its close allies when using currently employed identification methods such as morphology or flow cytometry (López-Alvarez et al. 2012). This is particularly problematic in natural admixed populations, where *B. hybridum* lives in sympatry with one or the other parental species (Fig. 2), and in germplasm collections, where *B. stacei* and *B. hybridum* samples have been found within the *B. distachyon* stocks. Recently, López-Alvarez et al. (2012) provided a reliable method to differentiate the individuals of the three species using a DNA barcoding system that offered a suitable approach to this problem. Three genomic barcoding loci, the plastid *trnLF* region and the nuclear multicopy ribosomal ITS spacer and low copy GI gene successfully discriminated among the three species. Any one of the three assayed loci could unambiguously differentiate the two monophyletic diploid species from direct sequencing of PCR amplicons, whereas the identity of the allotetraploid required combined analysis of direct *trnLF* and direct or cloned ITS sequences or through analysis of cloned GI sequences.

The study also revealed other particularities of the *B. distachyon*, *B. stacei* and *B. hybridum* genomes. Pairwise substitution rates showed high interspecific sequence divergence values and low intraspecific values between and among the diploids *B. distachyon* and *B. stacei*. Regarding *B. hybridum*, the differences

between the intra-parental and inter-parental (*B. distachyon*-like vs. *B. stacei*-like) mean values were equivalent to those found between and within the sequences of the two diploids for the three loci. These results suggested that the original diploid progenitor genomes have remained largely intact in the allotetraploid *B. hybridum* genome and that the time elapsed since the hybridization took part was a brief one. Haplotype networks constructed for each of the separate loci using statistical parsimony methods showed a clear-cut separation between the *B. distachyon*-type and *B. stacei*-type classes of sequences in all cases, corroborating the existence of barcoding gaps between the diploid genomes/subgenomes (Fig. 3). Nonetheless, the detection of some interspecific ITS and GI recombinant sequences in *B. hybridum* (López-Alvarez et al. 2012) pointed towards the occurrence of frequent genomic rearrangements within the hybrid nucleus. This agreed with cytogenetic CCP evidence demonstrating the existence of structural rearrangements in the *B. hybridum* chromosomes with respect to the *B. distachyon* and *B. stacei* ones (Idziak et al. 2011). However, despite the plausible existence of rapid structural changes in the allotetraploid genome, the integrity of the respective subgenomes is still prevalent. The two subgenomes have kept the same or similar signatures as those of the parental genomes, supporting the recent origin of *B. hybridum* in the Pleistocene (Catalan et al. 2012; Diaz-Perez et al. unpub. data).

A striking finding of our study was the demonstration of the existence of different directional crosses that likely gave rise to the new allotetraploid species (López-Alvarez et al. 2012, unpub. data). This was confirmed through the analysis of the maternally inherited plastid haplotypes in the *B. hybridum* sampling. Though the majority of the surveyed *B. hybridum* individuals show the inheritance of a *B. stacei*-like plastid genome, in a few cases some *B. hybridum* individuals have inherited a *B. distachyon*-type plastome (López-Alvarez et al. 2012; Fig. 5). Consequently, it is assumed that the former derived from a cross between maternal *B. stacei* and paternal *B. distachyon* parents whereas the second resulted from a converse cross, with maternal *B. distachyon* and paternal *B. stacei* parents. The fact that *B. hybridum* plants derived from reciprocal crosses occurred in different Mediterranean localities supports the multiple and polytopic origins of the allotetraploid *B. hybridum*. The recurrent formation of allopolyploid plant species has been widely documented in angiosperms (Soltis and Soltis 1999; Soltis et al. 2010), with speciation occurring from bidirectional crosses in some grasses (e.g., *Aegilops*, Meimberg et al. 2009). In some instances, similar directional crosses have even led to distinct allopolyploid species (Meimberg et al. 2009), probably as a result of different genomic rearrangements and losses in the stabilizing allopolyploid genomes. An inspection of the more variable ITS and GI networks and phylogenetic trees also revealed distinct relationships of the *B. hybridum* sequences to different parental geographic haplotypic groups, corroborating the polyphyletic origin of the *B. hybridum* samples. Complementary or unique parental haplotypic clusters have been found for some western and eastern Mediterranean *B. hybridum* groups (López-Alvarez et al. 2012). Nonetheless, all the studied hybrids correspond to what is considered to be the same allopolyploid species.



**Fig. 5** Plastid haplotypic statistical parsimony network of the annual *Brachypodium distachyon*–*B. stacei*–*B. hybridum* complex, based on maternally inherited cpDNA sequences, showing the polyphyletic origin of *B. hybridum*, arisen from bidirectional crosses (♀ *B. stacei* × ♂ *B. distachyon*; ♀ *B. distachyon* × ♂ *B. stacei*) of its progenitors. Species colors are indicated in the charts. Partially adapted from López-Alvarez et al. (2012; Fig. 2a) and unpub. data

Other interesting conclusions about the dispersal capabilities of these species were also drawn from our studies (López-Alvarez et al. 2012, 2015). Despite their abundant distributions in the circumMediterranean region, the intraspecific genetic diversities of the parental *B. distachyon* and *B. stacei* sequences were low. This was manifested in the sharing of their respective most common plastid and nuclear haplotypes by individuals from populations located far apart in disparate parts of the Mediterranean region (Figs. 2 and 5). In contrast, individuals from geographically close populations, or even intraindividual clones, showed different haplotypes. A similar scenario was also recovered for the intra-parental *B. distachyon*-type and *B. stacei*-type sequences found in *B. hybridum*. Because the three annuals are self-fertile plants, the observed pattern could only be explained by long distance dispersal (LDD) of their seeds (Vogel et al. 2009; Mur et al. 2011; López-Alvarez et al. 2012). Selfing species are expected to show low within-population and high among-population genetic diversities (Hamrick and Godt 1996). However, the autogamous *B. distachyon*, *B. stacei* and *B. hybridum* samples show overall low geographical structuring of genetic diversity. This might be a consequence of LDD of seeds coupled with the high capability of these annuals to adapt to different environmental conditions (Manzaneda et al. 2012). Seed dispersal mediated by ants

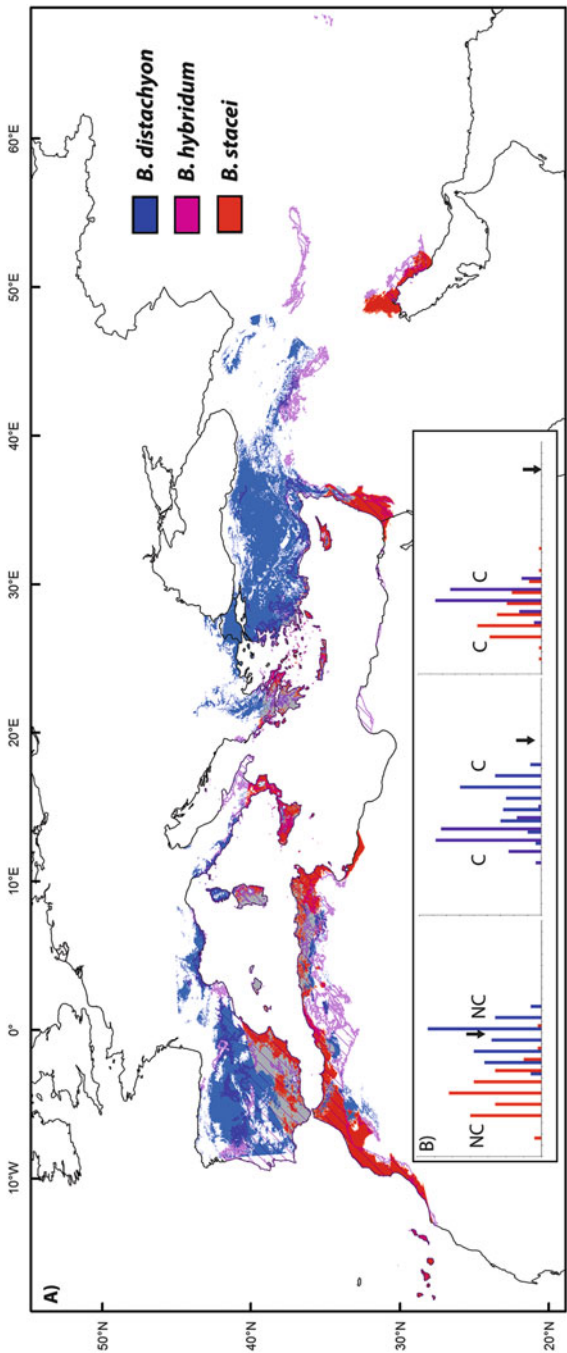


has been observed in *B. distachyon* (Catalán pers. obs.). Ants have been shown to be important seed dispersers in several plant species, though the dispersal distance does not usually exceed 100 m (Gómez and Espadaler 1998). Thus, the suspected LDD of *B. distachyon*, *B. stacei* and *B. hybridum* seeds might have occurred through other dispersal vectors, including anthropogenic mediated dispersal, like the cases documented for the recent introduction of *B. hybridum* in non-native ranges (Bakker et al. 2009; Catalán et al. 2012). Despite the ample distribution of common haplotypes in the native range, the three taxa show, however, some traces of geographic isolation between the western and eastern Mediterranean regions, evidenced by the detection of regional haplotypic clades (e.g. western Mediterranean, Iberian-Balearic, Turkish and Middle East—SW Asian; cf. López-Alvarez et al. 2012). Phylogeographic studies based on next generation genotypic RAD data are currently under way, aiming to dissect the origins and the spatio-temporal colonization routes of populations of the three species in their native circumMediterranean region.

The intriguing question of the potential existence of different environmental-adaptive speciation events among these three annual species was examined through niche overlap tests and niche breadth comparisons (López-Alvarez et al. 2015). Environmental niche modeling (ENM) analysis was conducted for *B. distachyon*, *B. stacei* and *B. hybridum* across their wide circumMediterranean native range as a way to predict the potential distribution of species across time and space and to investigate the roles of geography and environment in the evolutionary diversification of species. A large data set of contrasted occurrence data from 561 accessions and 19 bioclimatic variables plus altitude was used to construct the species' niche models under current and past [e.g., Mid-Holocene (MH), Last Glacial Maximum (LGM), and Last Interglacial (LIG)] climatic scenarios using maximum entropy probability distribution methods (MAXENT). The ENM models obtained under current climate conditions showed a potential distribution of *B. distachyon* in higher altitudinal areas and more northern latitudes, of *B. stacei* in coastal and lowland areas, and of *B. hybridum* in both mountain and lowland and coastal areas, though more commonly in the latter settings (Fig. 6a). A comparative analysis of overlapping areas in these ENMs detected ranges of potential shared occupancy between the two diploids, the allopolyploid and both diploids, and the allopolyploid and one or the other diploid. The environmental data indicate that *B. distachyon* grows in higher, cooler and wetter places than *B. stacei*, which grows in lower, warmer and drier environments, whereas *B. hybridum* grows in zones with intermediate values but also in low altitudinal warmer and drier places, like its *B. stacei* progenitor (López-Alvarez et al. 2015). These results fit well the ecophysiological requirements of the species (e.g., vernalization for most of the *B. distachyon* accessions and lack of it for the *B. hybridum* and *B. stacei* accessions; cf. Vogel et al. 2009, López-Alvarez and Catalán unpubl. data), which are crucial for the germination of seeds and survival of these annual species in their respective Mediterranean niches.

Interestingly, the paleoclimatic MH, LGM and LIG models also showed potential overlapping of environmental niches of the two diploid parents and its hybrid,





**Fig. 6** (a) Environmental niche models of *Brachypodium distachyon*, *B. stacei* and *B. hybridum* in their native circumMediterranean range under current climate conditions showing the overlapping areas of their respective niches. *B. distachyon* (blue), *B. stacei* (red) and *B. hybridum* (purple). (b) Plots of niche divergence and conservatism tests. Overlap values larger than or similar to the null distribution support niche conservatism (C) or are inconclusive (NC), respectively. Adapted from López-Alvarez et al. (2015; Figs. 2, 5)

with larger overlaps in the LGM, more restricted in the LIG and similar to present in the MH (López-Alvarez et al. 2015). These results support the Mediterranean basin and its adjacent areas as long-term refugia for *B. distachyon* and *B. stacei*, suggesting the existence of multiple potential hybrid zones along the Pleistocene and Holocene, which might have favored the recurrent origin of *B. hybridum*. The amount of significant environmental differences found between *B. distachyon* and *B. stacei* for 18 environmental variables, and their divergence in niche equivalence, suggest distinct adaptations to different ecological tolerances in these diploids. However, the reciprocal niche similarity tests were non-significant (López-Alvarez et al. 2015; Fig. 6b), implying that niche divergence was not the major driver of speciation for these species, but rather reproductive isolation or other biological or life-history traits. Surprisingly, despite the apparent recurrent origin of *B. hybridum* in the past, artificial crossing experiments have failed to generate the synthetic hybrid or have only produced sterile F1 hybrids (Chalhoub, pers. com.), confirming the strong reproductive isolation shown by the two diploids today. The pairwise niche similarity comparison tests showed evidence of niche conservatism for *B. hybridum* and each of its parents (Fig. 6b). Most evolutionary ecological studies conducted with sister or with less-related lineages have accumulated evidences of niche conservatism (Warren et al. 2008). In our case, niche conservatism is predicted for the recent allopolyploid, which shares niche occupancy with both progenitors (Fig. 6a) but is also reproductively isolated from them.

A further outcome of our study is that *B. hybridum* shows the largest niche overlap compared to its two diploid progenitors (Fig. 6a), being phenotypically more variable (Catalán et al. 2012; López-Álvarez and Catalán unpubl. data), but a niche breadth smaller than that of *B. distachyon* and only slightly greater than that of *B. stacei* (López-Alvarez et al. 2015). Niche competition with its diploid progenitors could be invoked to explain the observed restricted range distribution and niche breadth of *B. hybridum* in its native area, overlapping with but without displacing them. Conversely, *B. hybridum* is the only species of the complex that has apparently successfully colonized other non-native world regions (Catalán et al. 2012; López-Alvarez et al. 2015). This suggests a greater ecological tolerance of the allotetraploid compared to the diploids. This could be explained by the high potential of highly heterozygous allopolyploids to large genome rearrangements and to genomic and epigenetic expressions, increasing genetic diversity to buffer against inbreeding depression and to boost diversifying selection (Bakker et al. 2009; Meimberg et al. 2009). The environmental success of the young *B. hybridum* colonizers in America, S Africa and Oceania might also be related to rapid shifts in physiological and adaptive traits, such as changes in flowering time related to photoperiod and weediness (cf. Bakker et al. 2009). Yet the underlying factors causing the apparent different adaptive capabilities of *B. hybridum* in its native range and in the allochthonous areas should be tested through more detailed studies. It would also require the corroboration of the apparent but still unconfirmed absence of the diploid *B. distachyon* and *B. stacei* parents in those areas.

## Future Perspectives: Evolutionary Comparative Genomic Studies of *Brachypodium*

The impact of the new model plant *Brachypodium distachyon* on grass genomic research gathered pace since the publication of the full genome sequence of the diploid genotype Bd21 by the International Brachypodium Initiative (IBI 2010). Over the last decade, more than 400 laboratories worldwide have worked on investigating the genomics, transcriptomics and metabolomics of *B. distachyon* (Vain 2011; Mur et al. 2011). *Brachypodium* represents an excellent resource for comparative evolutionary genomic studies (Gordon et al. 2014), and recent work has identified the important role played by hybridization in the history and ecology of its species (Betekhtin et al. 2014; Diaz-Perez et al. unpub. data). The small genome sizes, compact genomes (e.g. low levels of repetitive DNA), diverse ecological tolerances, ready propagation under controlled growth conditions, and considerable existing molecular and genomic resources make this genus an excellent candidate for addressing fundamental questions in ecological and comparative genomics. The new taxonomic and phylogenetic findings, and the advent of inexpensive next generation sequencing technology, has set the stage for high definition investigation of the unusual genomic diversity and evolutionary relationships in *Brachypodium* (Catalán et al. 2014). The demonstration that the model plant was not one but three species (Catalán et al. 2012) opened the way to a thoroughly comparative genomic study of this diploid-polyploid complex. The nuclear and organellar genomes of *B. stacei* and *B. hybridum* are being sequenced and will serve as a model for the origins and consequences of the speciation and polyploidization events that might parallel those of economically important cereals (e.g., wheats; Marcussen et al. 2014). The analysis of the intraspecific diversity in *B. distachyon* is also under way through the nuclear and organellar resequencing of 56 diverse natural accessions (Gordon et al. 2014, unpub. data).

Genomic resources have been also developed for the perennial species *B. sylvaticum* (Steinwand et al. 2013) and the sequencing of other perennial genomes is on the way (John Vogel pers. com.). Comparative genomics of annual vs. perennial species of *Brachypodium* aim to identify the genome donors and the hybridization processes involved in the origin of allopolyploid species (approximately half of the studied taxa) and the switches from perenniality to annuality. A valuable set of metatranscriptomic data would enlarge the scope of the phylogenomic analysis of *Brachypodium*, facilitating the analysis of the transcriptomic contents across the *Brachypodium* phylogeny. Comparative genomic-transcriptomic analysis would allow us to reconstruct the gene content and the non-coding content evolution in *Brachypodium*. It is of particular interest to comparatively examine the evolution of genes and of regulatory elements in non-coding regions and how this might affect the expression of the transcriptomes. The *Brachypodium* data could be contrasted with 970 single or low copy nuclear genes analysed across monocots and a larger number of single or low copy nuclear genes studied in grasses. Since the various *Brachypodium* species and *B. distachyon*

accessions are native to a wide geographic region with varied climates, these new sequence resources will facilitate genome-wide association mapping of genes controlling tolerance to drought and other abiotic stresses (Catalán et al. 2014) and to phenotypic and biological traits that might have triggered different speciation processes.

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