On the origins of the tetraploid *Bromus* species (section *Bromus*, Poaceae): insights from internal transcribed spacer sequences of nuclear ribosomal DNA

Malika L. Ainouche and Randall J. Bayer

Abstract: The internal transcribed spacer (ITS) region of nuclear ribosomal DNA from 22 diploid and tetraploid annual *Bromus* species of section *Bromus* (Poaceae) and three species belonging to other *Bromus* sections, *Bromus catharticus* (section *Ceratochloa*), *Bromus anomalus* (section *Pnigma*), and *Bromus sterilis* (section *Genea*), were investigated by PCR amplification and direct sequencing. The length of the ITS-1 region varied from 215 to 218 bp, and that of the ITS-2 region from 215 to 216 bp, in the species analyzed. ITS-1 was more variable and provided more informative sites (49) than ITS-2 (32). No variation was encountered within species. In pairwise comparison among species of section *Bromus*, sequence divergence ranged from 0.0 to 8.0% for the combined ITS-1 and ITS-2 regions. Parsimony analysis using *Avena longiglumis* and *Hordeum vulgare* as outgroups resulted in well-resolved phylogenetic trees and showed that section *Bromus* is monophyletic according to the species analyzed outside of the section. The analysis clarified the phylogenetic relationships among monogenomic (diploid) species. Introduction of the allotetraploid species did not change the general topology of the trees obtained using only the diploid species. Although some tetraploid–diploid species relationships will have to be clarified with faster evolving markers, the ITS sequences are shown to be useful for assessing evolutionary relationships among closely related *Bromus* species, as well as for clarifying taxonomic problems in previously controversial cases (e.g., *Bromus alopecuros* and *Bromus caroli–henrici*). New hypotheses are proposed concerning the origin of several allotetraploid species. For example, it is shown that the tetraploid *Bromus hordeaceus* diverged earlier than all other species of section *Bromus*, excluding the diploid *B. caroli–henrici*, which is found to be basal in this group. The tetraploid *Bromus arenarius,* which was considered a hybrid between sections *Bromus* and *Genea*, and the tetraploid *Bromus adoensis* are sister taxa within section *Bromus*; they belong in a weakly differentiated clade with the diploids *Bromus brachystachys*, *Bromus japonicus*, *Bromus squarrosus*, *Bromus arvensis*, and *Bromus intermedius*.

Key words: *Bromus*, allopolyploidy, ITS, ribosomal DNA, phylogeny.

Résumé : Les espaceurs internes transcrits (ITS) de l'ADN ribosomique provenant de 22 espèces annuelles diploïdes et tétraploïdes du genre *Bromus* (section *Bromus*, Poaceae) et de 3 espèces provenant d'autres sections du genre, *Bromus catharticus* (sect. *Ceratochloa*), *Bromus anomalus* (sect. *Pnigma*) et *Bromus sterilis* (sect. *Genea*), ont été analysés par amplification (PCR) et séquençage direct. La longueur de la région ITS-1 varie de 215 à 218 pb, tandis que la région ITS-2 varie de 215 à 216 pb chez les espèces analysées. La région ITS-1 s'avère plus variable et comporte 49 sites informatifs, contre 39 pour la région ITS-2. Les séquences ne varient pas à l'intérieur des espèces. Les distances entre espèces estimées à partir des taux de substitution nucléotidique montrent une divergence allant de 0.0 à 8.0% pour l'ensemble de la région ITS. Les analyses de parcimonie utilisant *Avena longiglumis* et *Hordeum vulgare* comme extra-groupe ont fourni une bonne résolution des liens phylétiques entre taxa, et montrent que, sur la base des espèces analysées, la section *Bromus* est monophylétique. Ces analyses ont permis de clarifier l'évolution des taxa monogénomiques (diploïdes). L'introduction des espèces allotétraploïdes dans les analyses ne change pas la topologie générale des arbres obtenue avec les espèces diploïdes. Bien que les relations entre certaines espèces diploides et tétraploides restent à éclaircir par l'utilisation de marqueurs évoluant plus rapidement, les séquences d'ITS se révèlent utiles pour déterminer les liens évolutifs entre taxons proches au sein de la même section et pour clarifier la taxonomie de cas critiques ayant fait antérieurement l'objet de controverses (par exemple, *Bromus alopecuros* et *Bromus caroli–henrici*). De nouvelles hypothèses concernant l'origine de certaines espèces allotétraploïdes sont proposées. Il est notamment démontré que l'allotétraploïde *Bromus hordeaceus*, comme la diploïde

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M.L. Ainouche[1] and R.J. Bayer. Department of Biological Sciences, University of Alberta, Edmonton, AB T6G 2E9, Canada.

[1] Author to whom all correspondence should be sent at the following address: Service de biosystématique et génétique des populations végétales, Unité mixte de recherche, Centre national de la recherche scientifique, Ecobio 6553, Université de Rennes I, Campus scientifique de Beaulieu, 35 042 Rennes Cédex, France (e-mail: Malika.Ainouche@univ-rennes1.fr).

B. caroli–henrici, s'est différenciée trés tôt au sein de la section, bien avant les autres espèces actuelles. *Bromus arenarius*, tétraploïde considérée dans la littérature comme résultant d'un hybride entre un membre de la section *Bromus* et un membre de la section *Genea*, se révèle avoir la même origine, au sein de la section *Bromus*, que la tétraploïde *B. arenarius*. Ces espèces appartiennent au même clade terminal, encore peu différencié.

Mots clés : *Bromus*, allopolyploïdie, ITS, ADN ribosomique, phylogénie.

Introduction

Polyploidy has long been known to play a major role in plant evolution. This phenomenon occurs in about 50% of angiosperms and in more than 80% of the grass family (Stebbins 1987). Recent advances in molecular techniques have greatly improved the understanding of species relationships in polyploid complexes, the multiple versus unique origin of polyploids, and the molecular and adaptative consequences of polyploidy (Soltis and Soltis 1993). The genus *Bromus* presents many fine opportunities for learning about the evolution of grasses (Stebbins 1981). It contains species of different ploidy levels, and has long been known for interspecific hybridization, variability, and taxonomic complexity.

Within *Bromus*, section *Bromus* P.M. Sm. is a morphologically well-defined group (Smith 1970) with pronounced genome differentiation (Armstrong 1991), and is reproductively isolated from other sections by genetic barriers (Knowles 1944). Polyploidy occurs in more than 50% of the species of the section, but is restricted to the tetraploid level ($4x = 28$). Most of the tetraploids are considered to be of hybrid origin based on meiotic chromosome behaviour and allozyme segregation (Stebbins 1981; Armstrong 1991; Ainouche 1993; Ainouche et al. 1995).

Section *Bromus* is considered to be the most advanced section in the genus and probably arose during the Pleistocene in southwestern Asia and the Mediterranean area (Stebbins 1981). This section represents a taxonomically complex group with lengthy synonymy. Many of the species are difficult to identify and the number of recognized species varies among authors, because of the existence of several species complexes and differing nomenclatures (Smith 1968, 1973; Scholz 1970). Smith (1972) described 28 taxa, some of which have now been reduced to subspecies (e.g., *Bromus hordeaceus* ssp. *mollis* and *Bromus hordeaceus* ssp. *molliformis*) or extirpated in nature (e.g., *Bromus bromoideus*), and which are now only available in botanical gardens (Tutin et al. 1980). All species of section *Bromus* are annual or biennial and predominantly self-fertilizing, with varied outcrossing potential (Ainouche 1993; Ainouche et al. 1995). Several species have colonized different continents (Roy et al. 1991), and in the New World, most of the brome invaders belong to section *Bromus* (Pavlick 1995). They are frequently ruderals and are found in field margins, roadsides, and disturbed habitats. The activities of humankind have played a determinant role in the evolution of this group (Stebbins 1981; Smith 1986).

During biosystematic investigations of annual bromes of section *Bromus* (Ainouche 1993), a reduction in the number of populations of the diploid species in the Mediterranean area was noted, particularly in North Africa where they are limited to natural mountainous habitats. In contrast, tetraploids *B. hordeaceus* and *Bromus lanceolatus* are widespread in disturbed habitats, roadsides, and field margins, where they seem to be well adapted to human disturbance. In France, the Eurasian diploid *Bromus arvensis* is becoming extinct (Jauzein and Montagu 1983; M. Ainouche, personal observations).

Tetraploid bromes displayed more genetic diversity at enzyme loci than the diploids, owing to their intergenomic fixed heterozygosity resulting from a hybrid origin (Ainouche et al. 1995). The relationships of the different polyploid species to diploid progenitors have not been clearly established. Because of the recent origin of the taxa, there has been little genetic differentiation and a number of conflicting interpretations have been made from the analysis of data from different sources (Ainouche 1993; M. Ainouche, A. Defontaine, M.T. Misset, and J.P. Gourret, unpublished data): morphology, chromosomes, enzymes, and randomly amplified polymorphic DNA (RAPDs). This makes understanding the evolutionary patterns within the section and elucidating the parentage of the tetraploid species particularly difficult.

Molecular data, including restriction site variation and DNA sequences, are now widely used to assess the evolutionary relationships among plants. The chloroplast genome (cpDNA) has been intensively investigated and has provided an important source of phylogenetic information at the family level and above (Olmstead and Palmer 1994) . In Poaceae, variation of cpDNA restriction sites showed that *Bromus* (Bromeae) is a sister group of the Triticeae (Soreng et al. 1990). Pillay and Hilu (1995) analyzed the phylogenetic relationships among *Bromus* subgenera using cpDNA restriction sites, but the evolutionary patterns remain obscure for some groups, particularly for the Mediterranean subgenus *Stenobromus* (section *Genea* Dumortier) and the subgenus *Bromus* (section *Bromus* P.M. Sm.).

The relatively slow rate of evolution of the chloroplast genome frequently limits its use at low taxonomic levels, particularly among closely related and recently diverged taxa. Moreover, several studies have revealed that phylogenetic analysis based on cpDNA data below the generic level may be distorted by chloroplast capture resulting from hybridization (Rieseberg and Soltis 1991; Soltis and Kuzoff 1995), and in allopolyploid species, the uniparental inheritance of the organelle genomes may yield misleading results. This is particularly important in the genus *Bromus*, for which polyploidy and reticulate evolution are the main evolutionary features.

For these reasons, an increasing interest has recently been paid to faster evolving sequences from the nuclear genome. The components of the nuclear ribosomal genes (nrDNA) present differential rates of evolution, and they have become the favored markers in evolutionary studies. The internal transcribed spacers (ITSs) of the nrDNA are particularly suitable for phylogenetic analyses of angiosperms (reviewed by Baldwin et al. 1995). The spacers are flanked by highly conservative coding regions, so they can be easily amplified by PCR and sequenced using universal primers (White et al. 1990). Moreover, along with the other components of the nrDNA multigene family, the ITS region is highly repeated in

the plant nuclear genome and undergoes rapid concerted evolution, allowing direct sequencing of pooled nrDNA PCR products for phylogenetic reconstructions. The rate of evolution of ITS sequences is particularly appropriate for studies at the specific and generic levels (Baldwin et al. 1995). There are several examples in different families of ITS sequences providing good resolution among closely related taxa (in *Astralagus*, Wojciechowski et al. 1993; in *Antennaria*, Bayer et al. 1996*a*; in *Gentiana*, Yuan et al. 1996). In Poaceae, ITS sequences have been successfully employed to resolve phylogenetic relationships at the subfamilial (Hsiao et al. 1994, 1995*a*) and tribal (Hsiao et al. 1995*b*) levels; the tribe Bromeae was found to be monophyletic and confirmed as a sister tribe to the Triticeae. In studying evolution within Triticeae, Hsiao et al. (1995*b*) emphasized the need to begin with the analysis of monogenomic species in order to understand evolutionary relationships among polyploid species of hybrid origin. Buckler and Holtsford (1996) found that ribosomal ITS sequences showed substantial divergence among species and even among subspecies of *Zea*.

In spite of the recent development of different molecular tools, little is known about evolution within section *Bromus*. What is known is largely a result of the studies of Scholz (1970), based on morphology, and of Smith (1972, 1983) and Smith and Sales (1993), based on morphology and serology, and the work of Stebbins, based on chromosomes (reviewed in Stebbins 1981). All authors agree about the difficulty of assessing interspecific relationships and phylogeny in *Bromus* with morphological characters, as is typical of many grasses (Kellogg and Watson 1993). Stebbins (1987) highlighted the phylogenetic complexity of the grass family, involving bidirectional character evolution, polyploidy, and hybridization. The phylogenetic relationships among species in *Bromus* section *Bromus* are still poorly understood.

The present work was carried out to provide additional data from rapidly evolving sequences in the nuclear genome that can be used to assess phylogenetic relationships among species of section *Bromus*, with particular focus on the relationships between diploid and tetraploid species. Such a study is particularly critical now, because of the rapidly changing population–species dynamics (depletion of diploids on the one hand and expansion of ruderal polyploid populations on the other) in nature, resulting from the dramatic increase in human disturbance and the evolution of agricultural techniques.

The aim of this paper is (*i*) to explore ITS sequence divergence among closely related species of *Bromus* and therefore to evaluate their phylogenetic utility, (*ii*) to estimate divergence among diploid species, (*iii*) to re-examine diploid–tetraploid species relationships left unresolved by earlier studies, and (*iv*) to analyse congruence between ITS-based phylogeny and interpretations based on other data.

Materials and methods

Plant material and DNA isolation

The sources of the plant material used in this study are given in Table 1. Ploidy levels have been determined for each accession using chromosome counts determined either (or both) in root-tip mitoses or by using flow cytometry analysis (Ainouche 1993; M. Ainouche, J.P. Gourret, and M.T. Misset, unpublished data). Twenty-two taxa of the section *Bromus*, including 10 diploids and 12 tetraploids, were analyzed and both specific and subspecific levels were represented. For *B. hordeaceus* sp. *mollis*, two populations from different ecogeographic localities were analyzed, one from northern Algeria (population 1) and one from northwestern France (population 2), as genetic differentiation was found at enzyme loci in Mediterranean and Atlantic populations of this subspecies (Ainouche et al. 1996).

All diploid species of section *Bromus* described by Smith (1972) are represented, except *Bromus palaestinus*, for which it was not possible to obtain material. Three species belonging to other *Bromus* sections were also analyzed: *Bromus anomalus* (sect. *Pnigma*), *Bromus sterilis* (sect. *Genea*), and *Bromus catharticus* (section *Ceratochloa*). The plant material was either collected from the field or kindly provided by seed banks or botanical gardens (Table 1).

Seeds were sown and plants maintained in a greenhouse. Total genomic DNA was isolated from young fresh leaves, following a modified DNA extraction procedure for small quantities of tissue (Doyle and Doyle 1987). About 10 mg of tissue from a single individual was ground in 250 µL of preheated (65°C) CTAB (cetyltrimethylammonium bromide) grinding buffer (Doyle and Doyle 1987) with 1% mercaptoethanol and incubated for 45 min at 65°C; 250 µL of chloroform – isoamyl alcohol (24:1) solution was added, mixed by inversion, and centrifuged for 10 min. The aqueous phase was transferred to another tube and 350 µL of 95% ice-cold ethanol was added to precipitate the DNA. After 3 h at 4°C, tubes were centrifuged for 3 min at 12 000 × *g*. Pellets were washed with 70% ethanol, dried, and resuspended in 100 µL TE buffer (10 mM Tris–HCl plus 1 mM EDTA, pH 8.0).

Amplification and sequencing

The ITS region was amplified by PCR using primers ITS-4 (White et al. 1990) and ITS-L (Hsiao et al. 1994). The PCR mixture was as previously described (Bayer et al. 1996*b*). The samples were preheated to 94°C for 3 min prior to the addition of Taq DNA polymerase to denature unwanted DNAases and proteases. PCR conditions involved 30 cycles of denaturation of the DNA at 94°C for 1 min, annealing of the primer to the template at 48°C for 1 min, and extension at 70°C for 2 min. A 7-min extension at 72°C followed the completion of the 30 cycles. The PCR products were visualized on 1.2% agarose gels and then purified by centrifuge filtration using Millipore Ultrafree-MC tubes (30 000 NMWL Filter Unit, Cat. UFC 3 LTK00, Millipore Corporation), according to the manufacturer's instructions. All the double-stranded PCR products appeared as single sharp bands.

Double-stranded DNA was sequenced directly by the dideoxy chain termination method (Sanger et al. 1977) using ^{32}P-labelled ITS-1 and ITS-2, and ITS-3 and ITS-4 (White et al. 1990) sequencing primers for the ITS1 and ITS2 regions, respectively.

Fragments were separated in 6% polyacrylamide gels. The gels were fixed in 10% acetic acid for 20 min, washed in distilled water, and air-dried. They were then exposed to Kodak Biomax x-ray films.

Sequence analysis and phylogenetic reconstruction

The ITS sequences were easily aligned visually, as only a few small (1 bp) insertions–deletions were detected. The sequences for the taxa have been submitted to the Genome Sequence Data Base (GSDB accession numbers are sequential from U 82325 to U 82236, and from U 82352 to U 83396). The 5.8S coding sequence separating the ITS-1 and ITS-2 regions was not considered in this study, as no variation was found among the species analyzed.

The data matrix was entered and aligned with MACCLADE (Maddison and Maddison 1992), and phylogenetic reconstruction was performed employing PAUP 3.1.1 (Swofford 1993).

Two diploid outgroup species were used, *Hordeum vulgare* (Triticeae) and *Avena longiglumis* (Avenae), as the Triticeae are known to be a sister group of the Bromeae (Soreng et al. 1990; Kellogg 1992; Hsiao et al. 1994) and the Aveneae belong to an adjacent clade of the Bromeae–Triticeae (Hsiao et al. 1994, 1995*a*). For

Table 1. Chromosome number and origin of the *Bromus* taxa analyzed.

Taxon	2*n*	Geographic origin	Source and accession No.
Section *Bromus*			
Bromus briziformis Fich. & Meyer	14	Former Soviet Union	PI 368 861
Bromus danthoniae Trin.	14	Afghanistan	PI 254 874
Bromus intermedius Guss.	14	Algeria	PC 4-89
Bromus arvensis L.	14	Germany	Stuttgart BG 861
Bromus squarrosus L.	14	Algeria	PC 21-87
Bromus japonicus Thunb.	14	Serbia	PI 362 117
Bromus brachystachys Hornung	14	Iran	PI 229 598
Bromus scoparius L.	14	Corsica	SNES
Bromus alopecuros (Poiret) ssp. *alopecuros* Poiret	14	Algeria	PC 16-89
Bromus alopecuros ssp. *caroli-henrici* (W. Greuter) P.M. Sm.	14	Tunisia	SNES
Bromus adoensis Hoscht.	28	Belgium	PI 202 531
Bromus arenarius Labill.	28	New Zealand	Auckland Museum
Bromus bromoideus (Lej.) Crep.	28	Belgium	Liege BG 1093
Bromus commutatus Schrader	28	Slovakia	PI 34 45 70
Bromus grossus Desf.	28	Belgium	Liege BG 1088
Bromus hordeaceus L. ssp. *mollis* (L.) Maire & Weiller	28	France and Algeria	PC 11-95 and 14-89
Bromus hordeaceus ssp. *molliformis* (Lloyd.) Maire & Weiller	28	Algeria	PC 6-89
Bromus interruptus (Hack.) Druce	28	England	Kew BG 20150
Bromus lanceolatus Roth	28	Algeria	PC 2-88
Bromus racemosus L.	28	Afghanistan	PI 219 999
Bromus secalinus L.	28	Belgium	Liege BG 2000
Bromus oxyodon Schrenk.	28	Turkey	PI 203 451
Section *Pnigma*			
Bromus anomalus Rupr. ex Fourn.	14	Maryland (U.S.A.)	PI 232 200
Section *Ceratochloa*			
Bromus catharticus Vahl.	42	Canberra (Australia)	PC 1-96
Section *Genea*			
Bromus sterilis L.	14	Turkey	PI 204 866

Note: PI, Plant introduction Station, Pullman, Wash., U.S.A.; PC, personal collection of the senior author; BG, botanical garden; SNES, Station nationale d'essai de semences, La Minière, Guyancourtt (France).

these species we used published sequences (GenBank accession numbers Z11759 and Z11758 for *H. vulgare* and *A. longiglumis*, respectively) from Hsiao et al. (1994). These authors also published the ITS sequence of one species (*Bromus briziformis*) belonging to section *Bromus* (Hsiao et al. 1995*a*).

The species belonging to sections *Pnigma* (*B. anomalus*), *Genea* (*B. sterilis*), and *Ceratochloa* (*B. catharticus*) were considered as ingroups in all analyses, to test the monophyly of section *Bromus*.

All characters were considered in phylogenetic analyses. Polymorphisms (i.e., two different nucleotides at the same locus) were encountered in some species. Indel polymorphism results in offset of bands, making the sequence unreadable on the gel from this locus. This problem was resolved by using the reverse terminal sequencing primer instead of the internal primer, and then the remaining (complementary) sequence could be read. However, ambiguities still persisted in the ITS-1 region of *Bromus oxyodon*. A cloning procedure would be more appropriate in this case, and the sequence of this taxon was consequently not included in the subsequent analyses. In the phylogenetic analyses, polymorphisms were considered using the "multistate character" option of PAUP.

The G+C content was determined for each species with the AMPLIFY (Engels 1993)[2] program. The transition–transversion ratio was calculated with MACCLADE (Maddison and Maddison 1992). Pairwise distances between taxa were estimated using the mean distance with PAUP 3.1.1 (Swofford 1993).

Parsimony analyses were conducted on combined ITS-1 and ITS-2 sequences using PAUP 3.1.1. Given the controversy concerning the potential distortion induced by species of hybrid or allopolyploid origin in cladistic analysis (Mc Dade 1995), two kinds of analyses were conducted. First, we considered only diploid species in order to clarify relationships among monogenomic taxa. This analysis was performed on unweighted characters by branch and bound searches computed via stepwise addition of furthest sequences, and topological constraints were not enforced. Branches of zero length were collapsed to yield polytomies. Tetraploid species were introduced in a second analysis and the relationships of tetraploid with diploid species were then examined. Because of the large number of taxa, this analysis was performed using heuristic searches with the TBR branch-swapping and MULPARS options en-forced.

The relative support of the various clades was determined by bootstrap analysis (Felsenstein 1985), and by decay analyses (Bremer 1988; Donoghue et al. 1992) performed using a converse constraint (ENFORCE CONVERSE command) method of Baum et al. (1994). The bootstrap analysis (branch and bound search) was done with 500 replicates for the diploid species. For diploid and tetraploid species analysis (heuristic search), the bootstrap employed 100 replicates with a maximum of

[2] B. Engels. 1993. Amplify 1.2, software for designing, analysing, and simulating experiments involving the polymerase chain reaction (PCR). Available from WREngels@macc.wisc.edu.

1000 trees having a length >200 saved during each bootstrap replicate.

Results

The ITS region in *Bromus*

The length of ITS-1 ranged from 215 to 218 bp and of ITS-2 from 215 to 216 bp in the *Bromus* species surveyed. ITS-1 was more variable than ITS-2 and provided more potentially phylogenetically informative sites (49 and 32 informative sites, respectively).

Twenty-seven sites were found to be polymorphic among several *Bromus* species (Table 2) and were more frequent in the tetraploids *B. lanceolatus*, *Bromus secalinus*, *Bromus grossus*, and *B. bromoideus*. The same polymorphic sites were found when examining different accessions (or different subspecies, as for *B. hordeaceus*) of the same species. In the tetraploids, intraspecific polymorphism could reveal heterogeneity resulting from two different diploid parental genomes. However, a few diploid species (particularly *B. briziformis*, *Bromus japonicus*, and *Bromus intermedius*) also display polymorphic sites. Interpretation in this case (intraspecific polymorphism/heterogeneity) was limited by analyzing repeated DNA sequences obtained from pooled PCR products; cloning of ITS repeat types could contribute to determining the exact sequence of each repeat and its frequency, but would not change the phylogenetic interpretation in this study (see Baldwin et al. 1995 for review).

The different *Bromus* species examined all have fairly equivalent G+C contents in the ITS region, 56%, a slightly lower value than for other Poaceae (56–61%; Hsiao et al. 1994) but similar to most other angiosperms (Baldwin et al. 1995).

The overall transition–transversion ratio was 1.256, ranging from 0.00 to 1.84 within *Bromus* and from 0.00 to 2.46 between *H. vulgare* and *B. anomalus*. No substantial substitution rate heterogeneity was found within the genus *Bromus*. However, the overall transition–transversion ratio was higher in ITS-2 (2.39) than in ITS-1 (0.89).

Pairwise divergence among taxa ranged from 0.0 to 8.0% within section *Bromus* and from 0.0 to 10.6% when species from other sections were included. There was no sequence variation between the populations of *B. hordeaceus* ssp. *mollis* that originated from different geographic origins, or between the subspecies *mollis* and *molliformis*. Intraspecific variation was also absent among different accessions of *B. intermedius*, *Bromus squarrosus*, *B. lanceolatus*, and *Bromus scoparius*. In contrast, 7.6% divergence was encountered between *Bromus alopecuros* ssp. *alopecuros* and *Bromus alopecuros* ssp. *caroli-henrici*.

Phylogenetic analysis

ITS based phylogeny of the diploid species

Phylogenetic analysis of the diploid species using the branch and bound search yielded two most parsimonious trees (Fig. 1), which required 221 steps and had low homoplasy (consistency index (CI) = 0.891, retention index (RI) = 0.855). Section *Bromus* appears to be monophyletic (bootstap value 83), with the species of other sections grouped into a sister clade; however, the section *Bromus* clade decays in trees 2 steps longer than the most parsimonious. Within section *Bromus*, *B. alopecuros* ssp. *alopecuros* diverged early, with 14 autapomorphies (Fig. 1); this taxon represents the sister group of a strongly supported monophyletic clade (17 synapomorphies, bootstrap value of 100 and decay index of 15) consisting of all the other taxa of section *Bromus*. These remaining species of section *Bromus* are distributed into three clades (Fig. 2). The first one contains *B. briziformis* and *Bromus danthoniae*, the second one groups *B. scoparius* and *B. alopecuros* (which have the same ITS sequence), and the third group is composed of *Bromus brachystachys* with two subclades containing *B. squarrosus* with *B. japonicus* and *B. intermedius* with *B. arvensis* (Fig. 2). The position of this third group is not well resolved (Fig. 1). In the first tree, it is sister to the first clade (*B. briziformis* and *B. danthoniae*), whereas in the second tree, it is sister to the second clade (*B. scoparius* and *B. alopecuros* ssp. *alopecuros*), and this results in a polytomy in the strict consensus tree (Fig. 2). Excepting *B. alopecuros* ssp. *caroli-henrici*, short terminal branches are the main phylogenetic feature of the taxa in section *Bromus*, indicating their probable recent divergence.

Diploid–tetraploid species relationships

The phylogenetic analysis (heuristic search) of both diploid and tetraploid species provided 1620 most parsimonious trees of 282 steps (CI = 0.876, RI = 0.860).

The strict consensus tree (Fig. 3) shows that the general topological relationships among the diploid species, after introducing the tetraploid species, is the same as in the first analysis without the polyploids. Section *Bromus* remains monophyletic and the diploid *B. alopecuros* ssp. *caroli-henrici* is clearly differentiated from the other taxa of the section (Fig. 3). In the majority-rule tree, three clades appear to be well supported with high bootstrap values (Fig. 4). The first one contains the tetraploids *Bromus interruptus* and *B. hordeaceus* (three different accessions corresponding to *B. hordeaceus* ssp. *molliformis*, *B. hordeaceus* ssp. *mollis* 1 from Algeria, and *B. hordeaceus* ssp. *mollis* 2 from Brittany), supported by 13 synapomorphies, and is the sister group to the other taxa. The second clade comprises the diploids *B. scoparius* and *B. alopecuros* ssp. *alopecuros*. The remaining diploid and tetraploid taxa are grouped into an unresolved clade, with one resolved monophyletic subclade containing *B. japonicus* with *B. squarrosus* and one containing *Bromus arenarius* with *Bromus adoensis*. The other relationships within this clade are weakly supported by both bootstrap and decay values (Fig. 4).

Discussion

Phylogenetic relationships among monogenomic (diploid) species

The ITS region of nuclear rDNA is shown here to be a suitable marker for phylogenetic analysis at the species level in *Bromus*, although low sequence divergence was found in some cases. Percent pairwise divergence encountered within section *Bromus* (0.00–8.00%) is consistent with levels reported for other closely related species (Baldwin et al. 1995). Hsiao et al. (1995*b*) reported 0.00–6.60% divergence among diploid annual species of Triticeae.

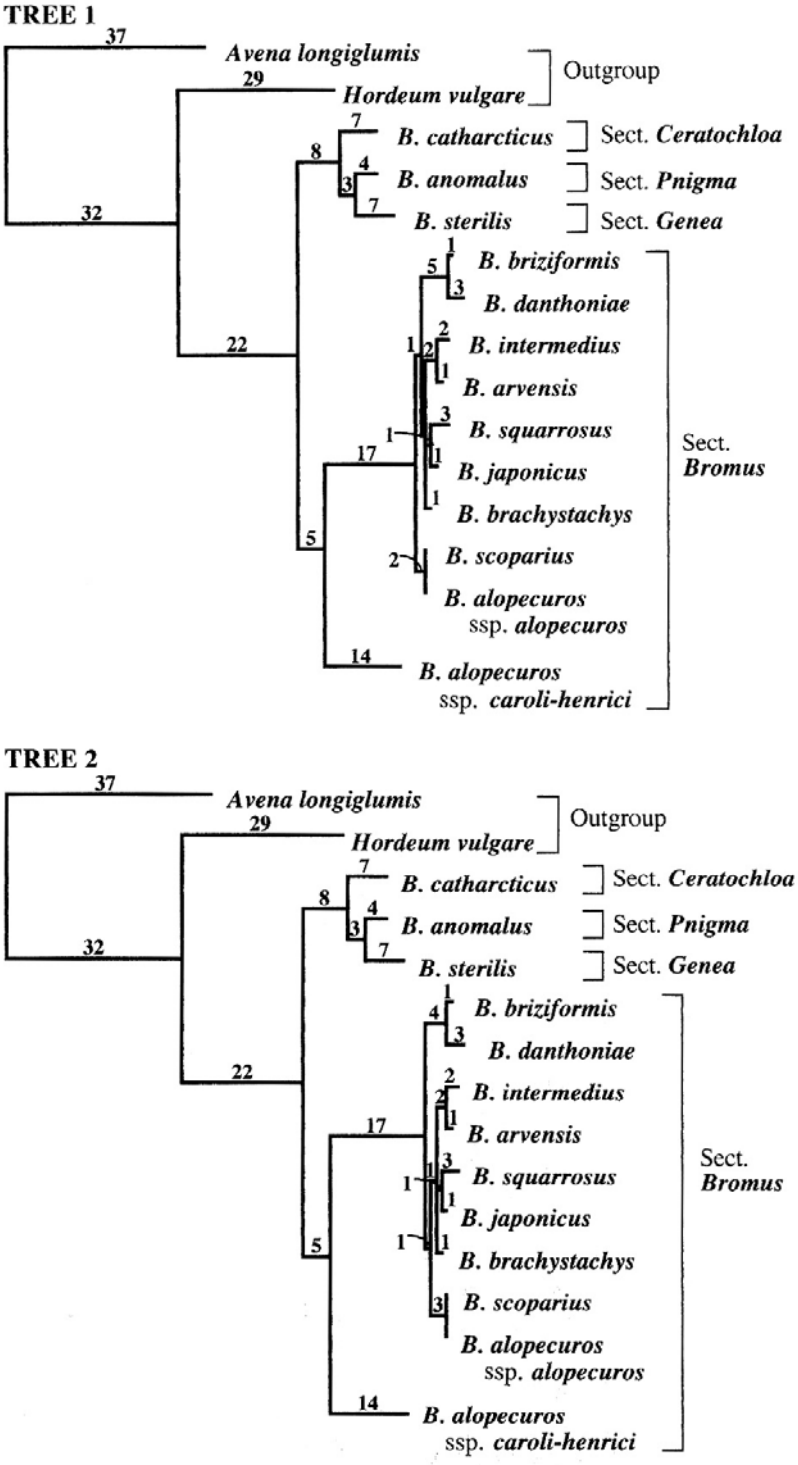
Section *Bromus* is found to be monophyletic, which is

Table 2. Polymorphic sites in the ITS1 and ITS2 regions of the *Bromus* species studied.

		ITS 1														ITS 2													No. of polymorphic sites
Species	Ploidy	1	2[a]	3	4	5[a]	6	7[a]	8[a]	9[a]	10	11	12	13[a]	14[a]	15[a]	16[a]	17	18[a]	19	20	21	22[a]	23[a]	24	25	26[a]	27	
Avena longiglumis	2*x*	T	A	C	G	G	C	A	T	G	—	G	C	T	G	C	C	A	T	—	T	G	T	G	G	G	T	G	0
Hordeum vulgare	2*x*	C	G	C	G	G	C	A	T	G	—	G	T	T	G	C	C	A	C	G	G	G	T	G	T	G	C	G	0
Bromus catharticus	6*x*	C	A	C	G	T	C	A	T	G	—	G	T	T	G	C	C	A	C	G	G	G	T	A	G	G	T	G	0
Bromus anomalus	2*x*	C	A	C	G	T	C	A	T	G	—	G	T	T	G	C	C	A	C	G	G	G	T	A	A	G	T	G	0
Bromus sterilis	2*x*	C	A	C	G	T	C	A	T	G	—	G	T	T	G	C	C	A	C	G	G	G	T	A	G	G	T	G	0
Bromus briziformis	2*x*	C	T	C	G	—	C	T	C	G	—	G	T	C	A+G	C	C	A	C	G	G	G	T	A+G	A+G	G	C+T	G	4
Bromus japonicus	2*x*	C	A	C	C+G	—	C	A	C	A	C	G	T	C	G	T	C	A	C	G+T	G+T	G	C	A	G	G	T	G	3
Bromus danthoniae	2*x*	C	T	C	G	—	G	T	A+C	G	—	G	T	C	G	C	C	A	C	G	G	G	T	A	G	G	C	G	1
Bromus intermedius	2*x*	C	A	C	G	—	C+G	A	C	A	—	G	A+T	C	G	C	T	A	A	G	G	G	C	A	G	A+G	T	G	3
Bromus squarrosus	2*x*	C	A	C	G	—	C	A	C	A	C	G	T	C	G	T	C	G	C	G	G	G	C	A	G	G	T	G	0
Bromus scoparius	2*x*	C	A	C	G	—	C	A	C	G	—	G	T	T	G	C	C	A	C	G	G	G	C	A	G	G	T	G	0
Bromus arvensis	2*x*	C	A	C	G	—	C	A	C	A	—	G	T	C	A	C	T	A	A	G	G	G	C	A	G	G	T	G	0
Bromus brachystachys	2*x*	C	A	C	G	—	C	A	C	A	C+—	A+G	T	C	G	C	C	A	C	G	G	G	C	A	G	G	T	G	2
Bromus alopecuros ssp. *alopecuros*	2*x*	C	A	C	G	—	C	A	C	G	—	G	T	T	G	C	C	A	C	G	G	G	C	A	G	G	T	G	0
Bromus alopecuros ssp. *caroli-henrici*	2*x*	C	A	C	G	—	C	A	T	G	—	G	T	T	G	C	C	A	C	G	G	G	T	A	G	G	T	G	0
Bromus arenarius	4*x*	C	A	C	G	—	C	A	C	A	C	G	T	C	G	C	C	A	C	G	G	G	C	A	G	G	T	G	0
Bromus racemosus	4*x*	C	T	C	G+T	—	C	T	A+C	G	—	G	T	C	G	C	C	A	C	G	G	G	T	A	G	G	T	G	2
Bromus adoensis	4*x*	C	A	C	G	—	C	A	C	A	C	G	T	C+T	G	C+T	C	A	C	G	G	G	C	A	G	G	T	G	2
Bromus hordeaceus ssp. *mollis*	4*x*	C	A	C	G	—	C	A	T	G	—	G	T	A	G	C	C	A	C	G	G	C+G	C	T	G	G	T	G	1
Bromus hordeaceus ssp. *molliformis*	4*x*	C	A	C	G	—	C	A	T	G	—	G	T	A	G	C	C	A	C	G	G	C+G	C	T	G	G	T	G	1
Bromus lanceolatus	4*x*	C	A+T	C	G	—	C	A+T	C	A+G	C+—	G	T	C	G	C+T	C	A+G	C	G	G	G	C+T	A	G	G	T	G	7
Bromus commutatus	4*x*	A+C	A	C	G	—	C	A	C	A	—	G	T	C	A	C	T	A	A+C	G+T	G	G	C	A	G	G	T	A	3
Bromus secalinus	4*x*	C	A+T	C+G	G	—+?	C	A	C	A	C+—	G	T	C	A+G	C	C+T	A	A+C	G+T	G	G	C	A	G	G	T	G	8
Bromus interruptus	4*x*	C	A	C	G	—	C	A	T	G	—	G	T	A	G	C	C	A	C	G	G	C+G	A+C	T	G	G	T	G	2
Bromus bromoideus	4*x*	C	A+T	C+G	G	—+?	C	A	C	A	C+—	G	T	C	A+G	C	C+T	A	A+C	G+T	G+T	G	C	A	G	G	T	A+G	10
Bromus grossus	4*x*	C	A+T	C+G	G	—+?	C	A	C	G	C+—	G	T	C	A+G	C	C+T	A	A+C	G+T	G+T	G	C	A	G	G	T	A+G	10

Note: "—" indicates a gap.
[a]Informative site.

Fig. 1. Two equally most parsimonious trees resulting from phylogenetic analysis considering only the diploid taxa of section *Bromus*. The length of the branches is proportional to the number of unambiguous base pair changes, which are indicated above each branch.



consistent with chromosome data (Armstrong 1991). Restriction site mapping of rDNA genes in *Bromus* (Pillay 1996) also showed that 2 species belonging to section *Bromus* (*B. hordeaceus* ssp. *mollis* and *B. arvensis*) occupy a position isolated from other sections of the genus.

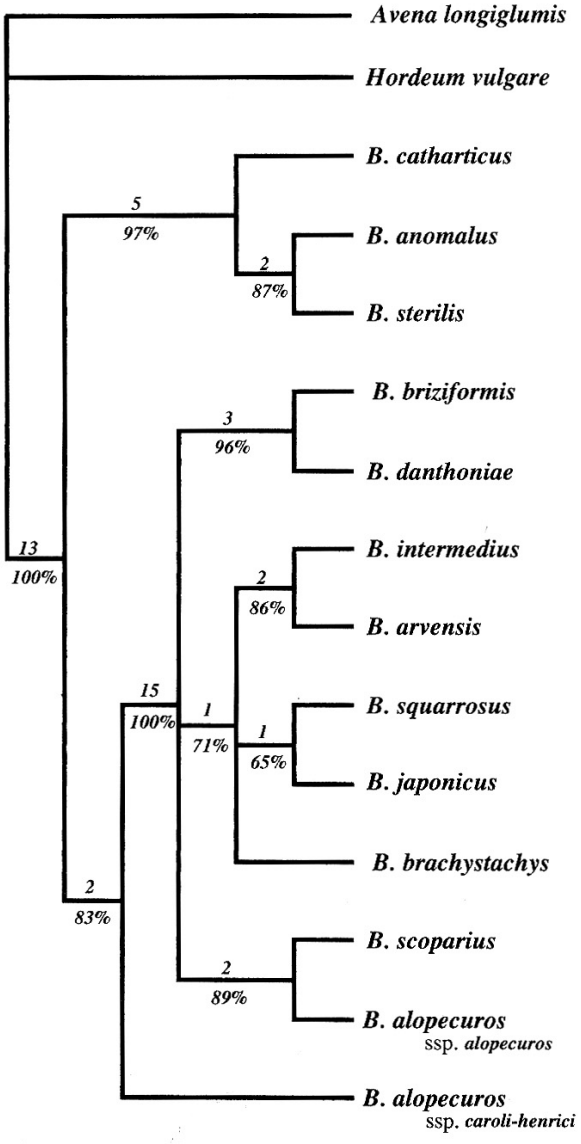
Section *Bromus* is believed to have originated during the Pleistocene and to have evolved in adaptation to conditions produced by human agriculture, particularly the grazing of livestock (Stebbins 1981). The ITS phylogeny suggests that the monogenomic species (Figs. 1 and 2) of section *Bromus* diverged into four lineages: the first lineage is represented by *B. alopecuros* ssp. *caroli-henrici*, which is basal in the section; the second by *B. scoparius* and *B. alopecuros* ssp. *alopecuros*; the third by *B. briziformis* and *B. danthoniae*; and *B. brachystachys*, *B. japonicus*, *B. squarrosus*, *B. arvensis*, and *B. intermedius* constitute the fourth (Fig. 2).

The basal position of *B. alopecuros* ssp. *caroli-henrici* and its strong divergence from *B. alopecuros* ssp. *alopecuros* (Fig. 2) raises the question of their taxonomic status. In the literature, the position of *B. alopecuros* ssp. *caroli-henrici* has been controversial. Maire (1955) described *Bromus lanceolatus* ssp. *biaristulatus*, a North African endemic, and highlighted its morphological similarity to *B. alopecuros*. Greuter (1971) described *Bromus caroli-henrici* as a new species from the eastern Mediterranean region, and Scholz (1974) proposed that the North African endemic be classified as *Bromus caroli-henrici* ssp. *biaristulatus*. However, Smith (1981), considering the common features of these taxa with *B. alopecuros*, proposed a subspecific rank within *B. alopecuros*, and suggested that the morphological differences between *B. alopecuros* ssp. *caroli-henrici* and *B. alopecuros* ssp. *alopecuros* (i.e., solitary larger spikelets at the nodes of the panicle, and acuminate lemma teeth in the former) are probably due to ecotypic differentiation. This proposition has been retained in Flora Europaea (Tutin et al. 1980), and we have followed this nomenclature, although some authors recognize the specific status of *B. caroli-henrici* (Portal 1996).

The sample of *B. alopecuros* ssp. *caroli-henrici* we analyzed was collected from Tunisia in 1987 (Table 1) and was cultivated in an experimental garden for several generations. Its morphology displays characteristic features of *B. alopecuros* ssp. *caroli-henrici* (single spikelets at the nodes, acuminate lemma teeth), although this taxon has not previously been recorded in North Africa. However, it also displays other unusual characters, such as narrower 7-nerved (instead of 9-nerved) lemmas and a unique inflorescence shape (H. Scholz, personal observations). On the other hand, it seems different from the North African endemic *B. caroli-henrici* ssp. *biaristulatus* (11-nerved lemmas with pronounced apical teeth forming small awns).

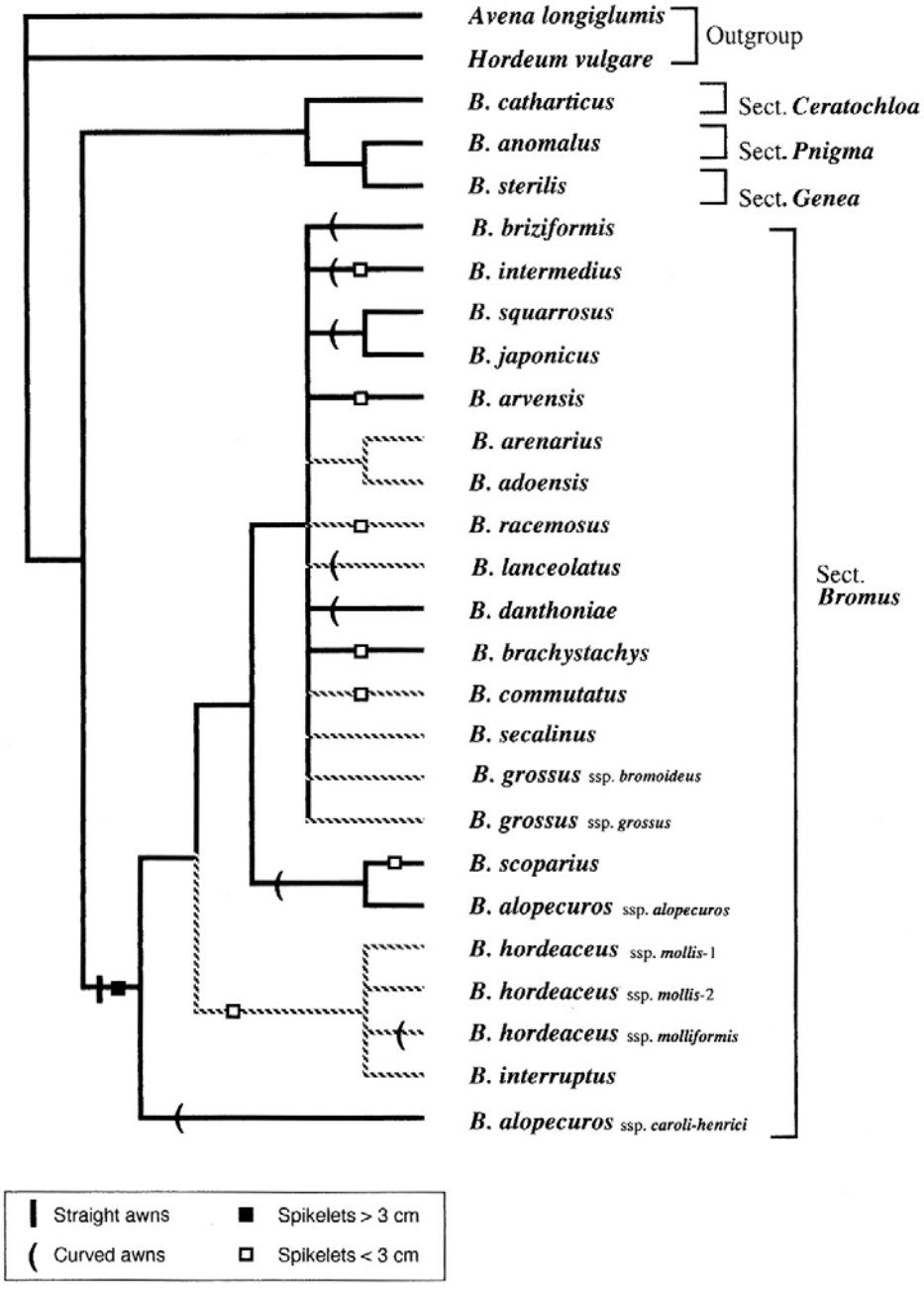
In previous studies, we pointed out the ambiguous situation of this accession with respect to its chromosomes and the morphometric analyses of quantitative morphological data (Ainouche 1993). Additional investigations analyzing different natural populations are needed to determine whether this sample is a morphological variant of what we could describe as "the *B. caroli-henrici* complex," or as a new undescribed *Bromus* species. In either case, our results from the ITS sequences clearly show that our sample, determined as

Fig. 2. Strict consensus tree resulting from phylogenetic analysis considering only the diploid taxa of section *Bromus*. Bootstrap values are given as percentages below each branch, and decay index values are indicated above the branches.



B. alopecuros ssp. *caroli-henrici* sensu Tutin et al. (1980), diverged earlier than the other species of section *Bromus* and should definitely be considered as a different species, not as part of *B. alopecuros*. On the other hand, *B. alopecuros* ssp. *alopecuros* displayed the same ITS sequence as the diploid *B. scoparius*. They also share an identical total nuclear DNA amount (M. Ainouche, J.P. Gourret, and M.T. Misset, unpublished data). These taxa are differentiated in the floras primarily by their spikelet number and size, but intermediate sizes can be encountered in the field, making their identification difficult (M. Ainouche, personal observations). A more detailed biosystematic study testing the genetic barriers among natural

Fig. 3. *Bromus* section *Bromus* phylogeny based on ITS nrDNA sequences. Strict consensus tree resulting from phylogenetic analysis considering both diploid and tetraploid taxa of section *Bromus*, with two morphological characters (spikelet size and awn shape) mapped on the tree. Hatched lines refer to tetraploid species.

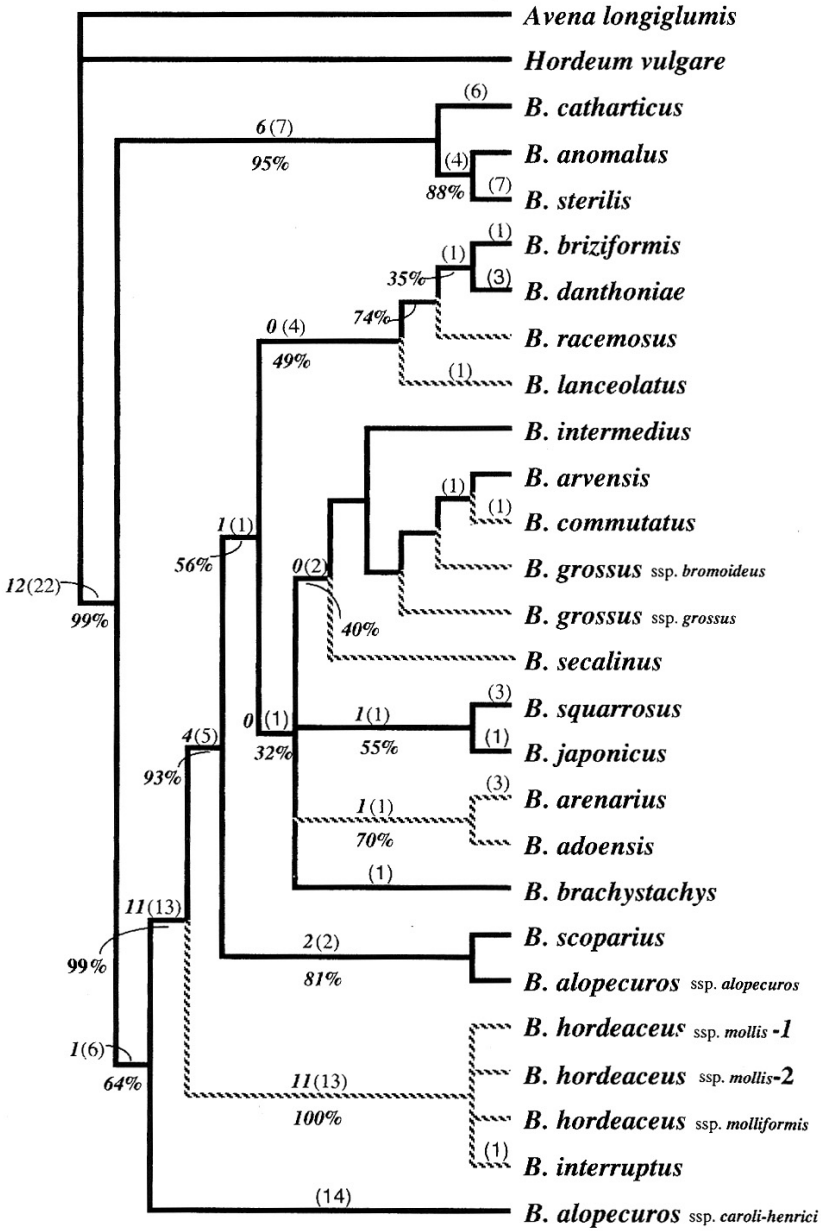


populations of these two taxa would be needed to determine whether they should actually be recognized as distinct species.

ITS sequence evolution in polyploids

Interpreting polyploid sequences from species of hybrid origin (allopolyploids) and considering them in phylogenetic approaches poses a series of problems (reviewed in Mc Dade 1995). Analyzing repeated sequences raises two questions: (*i*) did concerted evolution homogenize the parental sequences, and (*ii*) in what direction did the homogenization occur? This depends on the initial parental genome divergence and on the time that has elapsed since the polyploidization event (Soltis

Fig. 4. A 50% majority-rule consensus tree generated from phylogenetic analysis considering both diploid and tetraploid taxa of section *Bromus*. Above the branches, unambiguous base pair changes are indicated in parentheses and decay index values in bold italic type. Percentages of bootstrap values are given under the branches. Hatched lines refer to tetraploid species.



and Soltis 1993). Recent hybrids are expected to have maintained both parental types, as found in the allopolyploids *Tragopogon* (Soltis and Soltis 1991) and *Senecio* (Lowe and Abbot 1996) and in recent diploid *Helianthus* hybrids (Riesberg et al. 1990). In contrast, Wendel et al. (1995) showed that concerted evolution has been bidirectional in four allopolyploid *Gossypium* species that date from the mid-Pleistocene, the rDNA sequences being homogenized into either parental type. However, homogenization of rDNA repeats was not found in the few thousand year old allotetraploids of the *Brassica* triangle (Waters and Schaal 1996), suggesting that the rate of the concerted evolution process may vary among lineages.

In the allotetraploid *Bromus* species analyzed, we found no evidence of significant sequence heterogeneity within the same plant, except for the polymorphism described above in some species. We should note, however, that most of the diploid species seem to have diverged relatively recently (Fig. 1) and are not highly differentiated, so their allotetraploid

derivatives would not be expected to display significant sequence heterogeneity. Interestingly, we found no polymorphism in the allotetraploid *B. hordeaceus*, which probably diverged early within the section (with 13 autapomorphies), whereas allotetraploids, such as *B. lanceolatus* or *B. secalinus*, belonging to relatively younger clades, displayed more intraspecific polymorphisms, as their sequences are probably not yet fully homogenized by concerted evolution.

In phylogenetic analysis, concerted evolution, resulting in silencing the contribution of one parent's genome, leads to incongruence between organismal and gene phylogenies (Wendel et al. 1995). Moreover, it has been argued that reticulate patterns of evolution in allopolyploids (and diploid hybrids) are unlikely to be depicted through cladistic analysis, as they increase levels of homoplasy and may distort phylogenetic relationships of nonhybrid species (McDade 1995). However, Rieseberg and Morefield (1995) demonstrated that inclusion of known hybrid *Helianthus* species did not affect the tree topology for other species. They concluded that reticulate evolution should not be considered a major impediment for phylogenetic study of hybrid taxa, particularly when the parents are closely related.

In our study, we found that inclusion of allotetraploid *Bromus* species did not change the topology obtained with diploid species, even though some diploid–tetraploid species relationships were not well resolved. A higher homoplasy index was found when including tetraploid species (0.309) compared with the level of homoplasy obtained with diploid species (0.163). We should note, however, that increasing homoplasy could also result from the introduction of more species into the analysis.

Origin of the tetraploid species of section *Bromus*

The tetraploid *B. interruptus* is closely related to *B. hordeaceus*, with only a one base-pair difference in the ITS-2 region. These taxa are very similar morphologically and were found to have identical serological patterns (Smith 1972). *Bromus interruptus* is a British endemic, now apparently extirpated in nature (Stace 1992), that was associated with *Onobrychys–Trifolium* cultivation and has been considered to be a compact-inflorescence mutant from *B. hordeaceus* (Scholz 1970; Smith 1972). The results from our ITS-sequence analyses fully agree with this interpretation. In contrast, our results concerning the relationships between *B. hordeaceus* and the other species of section *Bromus* lead to new unexpected conclusions. On the basis of serological affinities, Smith (1972) proposed that the ancestors of the allotetraploid *B. hordeaceus* were the diploids *B. arvensis* and *B. scoparius*. Morphological, isozyme, and RAPD data (Ainouche 1993; M. Ainouche, A. Defontaine, M.T. Misset, and J.P. Gourret, unpublished data) did not refute this hypothesis. The ITS sequences clearly demonstrate that *B. hordeaceus* diverged earlier than all other species of section *Bromus* (excluding *B. alopecuros* ssp. *caroli-henrici*), and that subsequently, at least one of its diploid ancestors might have been a more unspecialized extinct or undiscovered species, perhaps related to *B. alopecuros* ssp. *caroli-henrici*, or belonging to another section of the genus *Bromus*. Thus, the hypothesis that *B. hordeaceus* originated from both *B. arvensis* and *B. scoparius* can be rejected.

Within *B. hordeaceus*, the two morphologically distinct subspecies *mollis* and *molliformis* have the same ITS sequence, as do the Mediterranean and Atlantic populations of *B. hordeaceus* ssp. *mollis* that differ at enzyme loci (Ainouche et al. 1996). This would indicate that the ITS sequences are not evolving fast enough to reveal recent infraspecific divergence in *B. hordeaceus*.

The phylogenetic affinities between the tetraploids *B. arenarius* and *B. adoensis* are well supported by the ITS sequences. These species also share identical serological patterns (Smith 1972). *Bromus adoensis* (syn. *Bromus pectinatus* Thunb.) has an African distribution, from Abyssinia to South Africa, whereas *B. arenarius* has been considered to be an Australian and northern New Zealand endemic. Stebbins (1981) considered *B. arenarius* a hybrid of sections *Bromus* and *Genea*. Its floral morphology actually displays intermediate features. However, this hypothesis is not supported by the ITS-based phylogeny, as *B. adoensis* does not display much sequence affinity with *B. sterilis* of section *Genea*. *Bromus adoensis and B. arenarius* are sister taxa having a common origin within section *Bromus*. *Bromus arenarius* has a more derived ITS sequence than *B. adoensis*, because it displays three more autapomorphies. Their closest diploid relatives appear to be *B. brachystachys*, *B. japonicus*, *B. squarrosus*, *B. arvensis*, and *B. intermedius*.

Bromus secalinus, *B. grossus*, and *B. bromoideus* have the same ITS sequence. These taxa have long been considered to be closely related on the basis of morphology and cross compatibility (Cugnac and Camus 1936; Tournay 1968). *Bromus bromoideus* and *B. grossus* also share identical serological patterns, whereas that of *B. secalinus* was found to be slightly different (Smith 1983). All three species are cereal-field contaminants, *B. secalinus* having a wider Eurasian distribution near rye and wheat fields, whereas *B. bromoideus* and *B. grossus*, now extirpated in nature, were mainly found in the Ardennes (France–Belgium) spelt (*Triticum spelta*) fields. Evolution of these crop-mimic species has been closely associated with traditional human selection within cereal fields. *Bromus bromoideus* was considered a mutant of *B. grossus* (Scholz 1970) and was also circumscribed as *B. grossus* ssp. *eburonensis* (Tournay 1968; Kerguelen 1975). These taxa were undoubtedly derived from the same genetic pool as *B. secalinus*, which is relatively widespread and more diverse. Our results reinforce the opinion that *B. bromoideus* and *B. grossus* should be considered variants of *B. secalinus* resulting from accidental human selection (Jauzein 1995). Smith (1972) suggested that the original allotetraploid from which they developed may have ressembled *Bromus commutatus* or *Bromus racemosus*. We found that these taxa are instead closely related to *B. commutatus* (Fig. 4). All these allotetraploids are related to the Eurasian–Mediterranean diploids *B. arvensis* and *B. intermedius* (Fig. 4).

The allotetraploids *B. lanceolatus* and *B. racemosus* belong to the same clade as the diploids *B. danthoniae* and *B. briziformis* (Fig. 4). Although this clade was weakly supported, we should note that *B. lanceolatus*, a Mediterranean species, is morphologically similar to *B. danthoniae*, a southwestern Asian – eastern Mediterranean species. In fact, the latter was considered to be a possible diploid ancestor of *B. lanceolatus* (Smith 1972). The position of *B. racemosus*

in this clade (Fig. 4) is unexpected, since this species is morphologically very similar to *B. commutatus*. They display overlapping patterns of morphological variation, suggesting introgressive hybridization (Ammann 1981). Stace (1975, 1992) pointed out that these species hybridize frequently in western Europe in lowland water meadows. *Bromus commutatus* has a more ruderal behavior and is distributed over Europe and North Africa, whereas *B. racemosus* has a northern distribution and seems more frequent in damp meadows (Jauzein and Montagu 1983). Several authors group them together as a species (Vivant 1964; Ammann 1981; Kerguelen et al. 1987; Jauzein 1995), whereas others consider them to be different species (Scholz 1970; Smith 1973; Portal 1996). The latter interpretation is better supported by our results, as the ITS phylogeny suggests possibly different origins for *B. racemosus* and *B. commutatus*.

Molecular versus morphological information in bromes

Assessing phylogenetic relationships from morphology is particularly difficult within section *Bromus*, where the characters are often only subtly different and are frequently associated with phenotypic plasticity (Smith and Sales 1993). Several taxonomic characters are considered to result from convergent or parallel evolution in separate lineages. This is confirmed in our analyses (Fig. 4) of two characters usually considered in species diagnosis: the reduction of spikelet and floral size and the shape (straight or curved) of the awn. These characters are regarded as adaptative, resulting from evolution in open habitats in postglacial Eurasia. Small spikelets are associated with rapid production of a maximum number of propagules, which characterizes evolved opportunist annual species (Smith and Sales 1993). Curved awns (more marked in dry habitats) are also considered to be more advanced than straight ones.

When comparing the ITS sequence based phylogeny with previous interpretations based on morphology, we find different situations. In some cases, molecular data confirm morphological similarities: as for example, *B. interruptus* with *B. hordeaceus*; *B. secalinus* – *B. grossus* ssp. *grossus* – *B. grossus* ssp. *bromoideus* with *B. commutatus*; and *B. lanceolatus* with *B. danthoniae*. In contrast, morphological affinities between *B. hordeaceus* and *B. arvensis* – *B. scoparius*, between *B. commutatus* and *B. racemosus*, and between *B. arenarius* and section *Genea* were not supported by ITS sequences.

The ITS sequences of the nrDNA greatly improve the treatment of taxa at the species and subspecies levels in this group of bromes considered to be a "taxonomic nightmare" (Smith 1983). The changing systematics of *Bromus* are the result of the advent of different approaches that reflect the introduction and interpretation of new evolutionary concepts in plant speciation over the past century (e.g., the abundance of names and synonyms as a result of early classical studies that neglected the high populational variability of taxa, or more recently, the inflated role attributed to ecotypic variation for assigning specific versus subspecific ranks). A major dilemma in recently diverged taxa, as we find in *Bromus*, is how to consider the morphological characters that have been either overlooked (e.g., panicle- and lemma-shape differences between *B. alopecuros* ssp. *caroli-henrici* and *B. alopecuros* ssp. *alopecuros*) or overweighted (e.g., spikelet size differences between *B. scoparius* and *B. alopecuros*). It is in such ambiguously or erroneously interpreted cases that molecular data are most useful. Problems encountered in species delimitation when applying the biological species concept to plants and (or) the molecular phylogenetic approach to reconstructing the phylogenetic history of taxa (Davis 1995) are well illustrated among annual bromes. A good example is provided by *B. racemosus* and *B. commutatus*, which hybridize in nature, in spite of the indication in our analyses that they probably do not share a recent common ancestor (Fig. 4).

Conclusion

The ITS sequences have been shown to be good markers for phylogenetic studies among closely related annual brome species. Section *Bromus* is monophyletic, and a good resolution of relationships was obtained among the diploid species. No variation was encountered within species or among different populations and subspecies of *B. hordeaceus*. The ITS data lead to new hypotheses concerning the origin of the allotetraploids. However, these hypotheses should be verified with additional molecular markers in order to reconstruct a robust phylogeny of the group and to confirm some weakly supported diploid–tetraploid species relationships suggested by the ITS sequences. In this respect, particular attention will be paid in the future to the potential usefulness of other quickly evolving sequences for elucidation of the origins of polyploids.

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