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THE ALLOTETRAPLOID INVASIVE WEED *BROMUS HORDEACEUS* L. (*POACEAE*): GENETIC DIVERSITY, ORIGIN AND MOLECULAR EVOLUTION

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Abstract: *Bromus hordeaceus* (section *Bromus*, *Poaceae*), a predominantly self-fertilizing tetraploid (2n=28), is an annual weed native to the Mediterranean Basin, which now has a world-wide distribution. High morphological variation led to the recognition of four subspecies, three of which correlated with habitat-type. We examined genetic diversity at enzyme loci in 15 populations from the Mediterranean and the Atlantic region. Although sampled over a larger range of ecological and geographical conditions, the North-African populations appeared less genetically differentiated than populations from Brittany, suggesting higher levels of gene flow among the first ones (*Nm* = 3.756 and 1.066 respectively). No genetic differentiation was encountered among the four subspecies. The populations were homozygous at homologous loci, suggesting high rates of selfing, but they frequently exhibited fixed intergenomic heterozygosity. The meiotic chromosome behaviour and disomic inheritance encountered are in accordance with the previously proposed allopolyploid origin of the species. The diploids *B. arvensis* and *B. scoparius* have been previously implicated in the parentage of *B. hordeaceus* on the basis of morphology and serology. We compared *B. hordeaceus* with related diploid species belonging to the same section (section *Bromus*) using different sources of data (flow cytometry, karyotypes, RAPD and DNA sequences). Molecular phylogeny based on internal transcribed spacer sequences of nuclear ribosomal genes provided the first clear scheme of relationships among monogenomic species of the section. A new hypothesis is proposed concerning the origin of *B. hordeaceus*: We found that it diverged earlier than all other species of section *Bromus* excluding the diploid *B. caroli-henrici* which is basal in this group. The 13 autapomorphies accumulated by *B. hordeaceus*, and the absence of intra-individual sequence heterogeneity are also consistent with the relatively ancient origin of the species within the section.

INTRODUCTION

Human-caused dispersal of species and disturbances have dramatically increased in this century, and have given rise to the expansion of new well-adapted weedy species. Good examples are encountered in the taxonomically complex genus, *Bromus* L. (*Poaceae*), which arose during the Pliocene (STEBBINS 1981). Several species have colonized different continents since the beginning of this century (ROY et al. 1991). Six sections are usually recognized within the genus, comprising perennial, annual and biennial species (SMITH 1970). The annual Mediterranean species belonging to sections *Genea* DUMORT. and *Bromus* (as defined by SMITH 1970) are considered as the most evolutionarily advanced, having arisen during the

Pleistocene (SMITH 1986). Most of the extant species have evolved together with grazing and agriculture. They represent most of the brome invaders in the New World (PAVLICK 1995). Our interest has been particularly focused on species variability and evolution within section *Bromus* (AINOUCHE 1993, AINOUCHE et al. 1995, 1996, AINOUCHE & BAYER 1997). Two ploidy levels are encountered in this section: diploid (2n=14) and tetraploid (2n=28). Most of the tetraploids are considered to be of hybrid origin (allopolyploid) according to meiotic chromosome behaviour and allozyme segregation (STEBBINS 1981, ARMSTRONG 1991, AINOUCHE et al. 1995). In the Mediterranean region, the tetraploid species are frequently more widely distributed than the diploids (AINOUCHE 1993). One of them, *Bromus hordeaceus* L. is a predominantly self-fertilizing annual weed, native to the Eastern Mediterranean region, and now having a world-wide distribution. Expansion and evolution of this aggressive ruderal has been linked to the development of human habitats. Considerable morphological variation and plasticity have been previously reported for this species where four subspecies are recognized (SMITH 1980): subsp. *molliformis* (LLOYD) MAIRE et WEILLER is considered as the most primitive Mediterranean subspecies which is well-adapted to dry conditions, whereas subsp. *thominii* (HARDOUIN) MAIRE et WEILLER and *ferronii* (MABILLE) P.M. SM. are considered coastal ecotypes from sand dunes and cliffs in West Europe (SMITH 1981, 1983). In contrast, subsp. *hordeaceus* (syn. *Bromus mollis* L.) occurs in a large range of ecological and geographical conditions within Eurasia, Africa, the New World and Australia (ROY et al. 1991).

In this paper, we use different sources of data from cytogenetics (flow cytometry and comparative karyotypes), allozymes, randomly amplified polymorphic DNA (RAPD) and DNA sequence data to address the following questions: First, how is the genetic diversity organized in *B. hordeaceus*, and how genetically differentiated are the traditionally recognized subspecies? Second, what is the origin of the tetraploid *B. hordeaceus*, and how can comparisons with diploid extant species belonging to the same section help to answer this question?

MATERIAL AND METHODS

Plant material

The genetic diversity within *B. hordeaceus* has been examined in 15 populations sampled in both Mediterranean (9 populations) and Atlantic (6 populations) regions, where the four subspecies are represented (Tab. 1). The Mediterranean populations were collected in different ecological conditions of North Algeria from the coast, hills and mountains of the Tellian Atlas, the High Plains, and the Saharian Atlas. Atlantic populations were distributed on coastal and central Massif Armoricain (Brittany, France). Each population sampled consisted of mature individuals, which were at least one meter apart to avoid the neighbour effect. The collected samples were cultivated in a greenhouse under conditions of experimental self-fertilization and natural fertilization. All populations were screened for isozyme variation and chromosome number. Karyotype analysis was performed on the Algerian population from Larhat. Individuals belonging to subsp. *hordeaceus* (from Benchicao, Algeria, and from Rennes, France) and to subsp. *molliformis* (from Oran-Sebkha, Algeria) were used for DNA polymorphism and molecular phylogenetic analyses (see below).

In order to clarify the origin of *B. hordeaceus*, we compared it to ten diploid species belonging to the same section (i.e. section *Bromus*) on the basis of chromosome and molecular

Table 1. Origin of the *B. hordeaceus* populations sampled in the Mediterranean and the Atlantic regions.

Location	Subspecies	Longitude	Latitude
North Africa			
Bouzadjar	*molliformis* (LLOYD) MAIRE et WEILLER)	0°50' W	35°45' N
Oran-Sebkha	*molliformis* (LLOYD) MAIRE et WEILLER)	0°55' W	35°40' N
Ksar Boukhari	*molliformis* (LLOYD) MAIRE et WEILLER)	1°50' W	36°00' N
Biban	*hordeaceus* (syn. *B. mollis* L.)	6°00' E	36°00' N
Chelia	*hordeaceus* (syn. *B. mollis* L.)	6°50' E	35°20' N
Djurdjura	*hordeaceus* (syn. *B. mollis* L.)	4°00' E	36°30' N
Ouricia	*hordeaceus* (syn. *B. mollis* L.)	5°30' E	36°10' N
Sahel	*hordeaceus* (syn. *B. mollis* L.)	5°50' E	36°60' N
Larhat	*hordeaceus* (syn. *B. mollis* L.)	2°10' E	36°60' N
Brittany			
Rennes	*hordeaceus* (syn. *B. mollis* L.)	1°40' W	48°00' N
Mont Dol	*hordeaceus* (syn. *B. mollis* L.)	1°45' W	48°32' N
Mont Saint Michel	*hordeaceus* (syn. *B. mollis* L.)	1°30' W	48°38' N
Hirel	*thominii* (HARDOUIN) MAIRE et WEILLER	1°45' W	48°36' N
Loctudy	*thominii* (HARDOUIN) MAIRE et WEILLER	4°10' W	47°50' N
Pointe du Raz	*ferronii* (MABILLE) P.M. SM.	4°40' W	48°02' N

data. The following species have been collected (personal collections of the first author: PC), or kindly provided by botanical gardens (BG) and seed banks (PI = Plant Introduction Station of Pullman, USA; SNES = Station Nationale d'Essais et de Semences, La Minière, Guyancourt, France): *B. briziformis* FISCH. et C.A. MEY. (PI 368 861); *B. danthoniae* TRIN. (PI 254 874); *B. intermedius* GUSS. (PC 4-89); *B. arvensis* L. (Stuttgart BG 861); *B. squarrosus* L. (PC 21-87); *B. japonicus* THUNB. (PI 362 117); *B. pseudobrachystachys* H. SCHOLZ (PI 229 598; this accession has been sent to us under the name *B. brachystachys* HORNUNG, and was verified by H. SCHOLZ, pers. comm.); *B. scoparius* L. (SNES); *B. alopecuros* POIR. (PC 16-89); *B. caroli-henrici* GREUTER (SNES).

Methods

Cytogenetic analysis

Chromosome counts and karyotype analyses have been performed for each species (one population per species) on root tip metaphases, following the procedure described in AINOUCHE (1993). Root tips were pretreated in a saturated solution of α-bromonaphtalene for 9h at 4 °C, then fixed in acetic acid : ethanol (1 : 3), and stored at 4 °C. Before Feulgen staining, the fixed material was washed and hydrolyzed in 1N HCl at 60 °C for 10 min. For each species, idiograms were constructed after measurements on at least five complete cells which presented well-separated chromosomes. Total length, short and long arm length, and the presence of secondary constriction were scored for each chromosome. Centromeric indices (CI) were calculated as long arm / short arm ratios. The karyotype asymmetry was estimated with the parameters proposed by ROMERO-ZARCO (1986): the intrachromosomal asymmetry index $A_1 = 1 - (\Sigma\ b_i/B_i)/n$ and the interchromosomal asymmetry index $A_2 = s/m$ where b_i is the average length of short arms in every homologous chromosome pair, B_i is the average length of long arms in every homologous pair, and n is the number of homologous chromosome pairs. m and s represent the mean chromosome length and the standard deviation, respectively. These two parameters are independent of chromosome size and number.

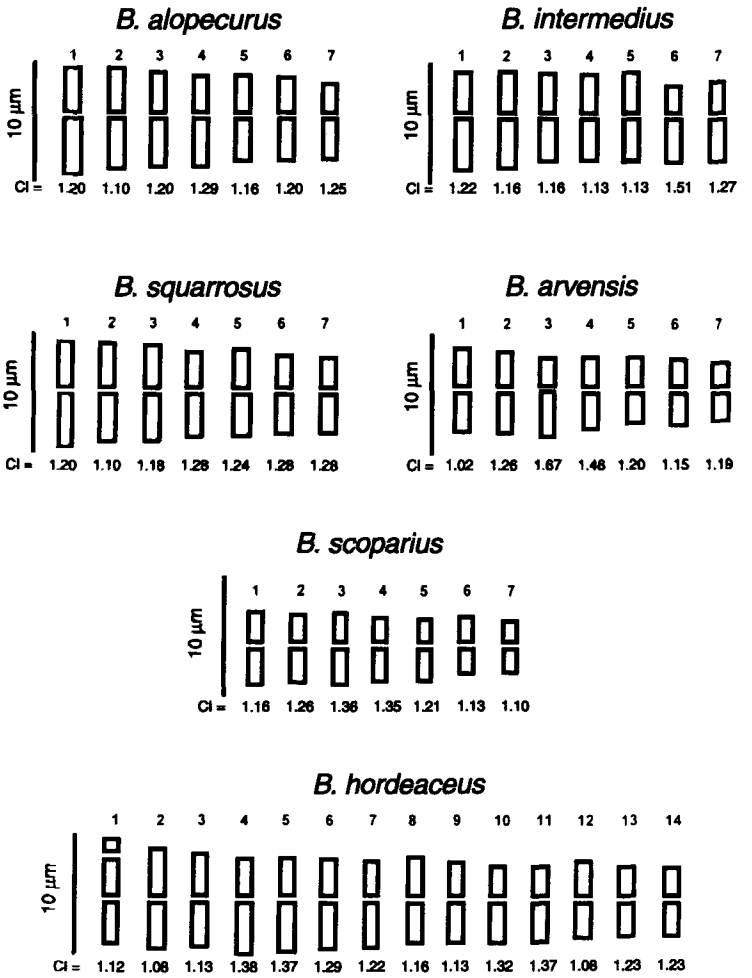

Fig. 1. Idiograms of the tetraploid *B. hordeaceus* (2n = 28) and five diploid species (2n = 14) of section *Bromus*. CI = Centromeric index.

interspecific relationships involving allopolyploid or hybrid taxa with RAPD data, and has proven to be a robust method even for data sets containing non-homologous co-migrating bands (ADAMS & RIESEBERG 1998).

Nuclear DNA sequence analysis

Internal transcribed spacers (ITS) of nuclear ribosomal DNA are known to evolve fast enough to provide informative data in comparisons involving related species, and thus are the most widely used nuclear sequences in phylogenetic studies at the generic level (SOLTIS & DOYLE 1998). Moreover, nuclear markers are very useful for studying reticulate evolution. Uniparentally-inherited cytoplasmic markers are likely to detect the maternal parent of an allopolyploid providing that enough variation can be detected among the putative diploid parents. In *Bromus*, very low sequence variation is encountered in chloroplast sequences at

Table 3. Intrapopulational estimates of genetic variability in *B. hordeaceus* based on allozyme frequencies at 17 loci: mean number of alleles per locus (*A*), proportion of polymorphic loci (*P*), and mean genetic diversity (H_u). Genotype frequencies pooled for 6 polymorphic loci (ENP, ACP1, PGI1, PGI2, G6PDH1, ME1): double homozygotes (I) and intergenomic heterozygotes (II).

	A	*P*	H_u	I	II
Ksar	1.35	0.35	0.16	0.25	0.75
Bouzadjar	1.35	0.35	0.18	-	1.00
O-Sebkha	1.23	0.23	0.12	0.33	0.77
Larhat	1.35	0.35	0.16	0.16	0.84
Ouricia	1.41	0.35	0.18	0.03	0.97
Biban	1.35	0.35	0.18	-	1.00
Chelia	1.35	0.35	0.16	0.20	0.80
Djurdjura	1.35	0.35	0.18	0.03	0.97
Sahel	1.29	0.29	0.14	0.22	0.78
MS-Michel	1.41	0.35	0.19	0.12	0.88
Mont Dol	1.23	0.23	0.12	0.33	0.77
Rennes	1.41	0.35	0.17	0.25	0.75
Hirel	1.35	0.35	0.17	0.18	0.82
Loctudy	1.23	0.23	0.12	0.33	0.77
P-Raz	1.17	0.17	0.09	0.50	0.50
Mean	1.325	0.314	0.159	0.195	0.825
s.e.	0.019	0.016	0.007	0.144	0.129

the infrasectional level (AINOUCHE & BAYER, unpubl.), whereas nuclear ITS sequences revealed more variation (AINOUCHE & BAYER 1997).

Genomic DNA was extracted from fresh leaves for *B. hordeaceus* (3 accessions) and for all the diploid species listed in Tab. 2. DNA extraction, ITS amplification and sequencing protocols followed the procedure described in AINOUCHE & BAYER (1997). The ITS sequences (ITS1 and ITS2) were easily aligned visually, as only a few small (1bp) insertion-deletions were detected. Genome Sequence Data Base (GenBank) accession numbers are sequential from U83358 to U83368 and from U83380 to U83383 for the diploid species. Accession numbers of *B. hordeaceus* are U 83376 and U 83377 for ITS1 and ITS2 respectively. The 5.8S coding sequence separating the ITS1 and ITS2 regions is not considered in this study, as no variation was found among the species analyzed. We also introduced three diploid *Bromus* species representing other sections in this study: *Bromus sterilis* L. (sect. *Genea* DUMORT., GenBank accession numbers U 83354 and U 83355 for ITS1 and ITS2 respectively), *B. anomalus* RUPR. ex FOURN. (sect. *Pnigma* DUMORT., GenBank accession numbers U83352 and U 82353) and *B. catharticus* VAHL (sect. *Ceratochloa* P. BEAUV., GenBank accession number U82325 et U82326). Two diploid outgroup species were used, *Hordeum vulgare* L. (*Triticeae*) and *Avena longiglumis* DURIEU (*Aveneae*), as the *Triticeae* are known to be a sister group of the *Bromeae*, and the *Aveneae* belong to an adjacent clade of the *Bromeae-Triticeae* (HSIAO et al. 1995). We used published sequences with GenBank accession numbers Z11758 and Z11759 for *A. longiglumis* and *H. vulgare*, respectively (from HSIAO et al. 1994). Phylogenetic analysis was performed with PAUP 3.1.1 (SWOFFORD 1993). Branch and bound search (with uninformative characters excluded) was performed *via* stepwise addition of furthest sequences. The relative support of the various clades was determined by bootstrap analysis (FELSENSTEIN 1985) with 100 replicates, and by decay analyses (BREMER 1988, DONOGHUE et al. 1992) performed using a converse constraint (ENFORCE CONVERSE command) method of BAUM et al. (1994).

RESULTS

Cytogenetic analysis

All the species analyzed displayed symmetric karyotypes with mainly metacentric chromosomes (Fig. 1). The chromosome pair no. 1 of *B. hordeaceus* frequently presented a secondary constriction (satellite). Secondary constrictions (not shown) were also encountered

A plot of the 11 taxa onto the first three axes of the principal coordinate analysis is presented in Fig. 2. On the basis of the first two axes, the diploid *B. danthoniae* is clearly differentiated from the other taxa. The tetraploid *B. hordeaceus* which occupies an intermediate position in the group formed by the diploid *B. arvensis*, *B. alopecuros*, and *B. intermedius*, also appears close to *B. caroli-henrici* and *B. brachystachys*. Another group is composed of *B. briziformis*, *B. squarrosus*, *B. japonicus* and *B. scoparius*, where *B. squarrosus* displays a rather isolated position with regard to the third axis.

Molecular phylogeny based on ITS sequences

A description of the ITS region in bromes has been previously detailed in AINOUCHE & BAYER (1997). In this study, analysis of ITS1 and ITS2 sequences provided 156 variable nucleotide sites among which 82 were phylogenetically informative.

A branch and bound search yielded one most parsimonious tree (Fig. 3) of 147 steps. Section *Bromus* is monophyletic, with all diploid and the tetraploid species appearing as a sister group of *B. sterilis*, *B. anomalus* and *B. catharticus*, which all belong to other sections. *Bromus caroli-henrici* is basal in the section, with 14 autapomorphies. The tetraploid *B. hordeaceus* also displays many autapomorphies (13) and appears as a sister group of the other remaining diploid species. The latter forms a low, and probably recently-diverged well-supported group of species including three short-branched clades (1–5 changes): the first one is composed of *B. alopecuros* and *B. scoparius*, the second one of *B. pseudobrachystachys*, *B. japonicus*, *B. squarrosus*, *B. arvensis* and *B. intermedius*, and the third one of *B. danthoniae* and *B. briziformis*.

Within *B. hordeaceus*, the two subspecies *hordeaceus* and *molliformis* have the same ITS sequence, as do Mediterranean and Atlantic populations of subsp. *hordeaceus*. These same populations, however, have diverged at enzyme loci (AINOUCHE et al. 1996). Sequence heterogeneity was not encountered in any of the tetraploid individuals, except for one (autapomorphic) polymorphic G/C site in the ITS2 region.

DISCUSSION

Genetic diversity in *B. hordeaceus*

The genetic data on *B. hordeaceus* presented in this study allow us to summarize our knowledge on this invasive weed. Different colonizing strategies are encountered among plants (BROWN & MARSHALL 1981, DAEHLER 1998), and it is interesting to note that *Bromus hordeaceus* displays several features often shared by successful invaders (WARWICK 1990, MEERTS et al. 1998): a short life cycle as an annual species, associated with a predominantly autogamous breeding system which allows uniparental reproduction after long distance dispersal (BAKER 1974). Intragenomic homozygosity resulting from autogamy is counter-balanced by gene duplication and fixed intergenomic heterozygosity resulting from polyploidy. The resulting diversity then provides weedy populations with an enhanced capacity to respond to new selection pressures upon colonization (BARRETT & SHORE 1989). Moreover, in polyploids, genetic diversity is enhanced by recurrent (i.e. multiple), rather than unique polyploidization events, during polyploid species formation (SOLTIS & SOLTIS 1993). This is consistent with the different multilocus genotypes encountered in the *B. hordeaceus* populations examined (AINOUCHE et al. 1996).

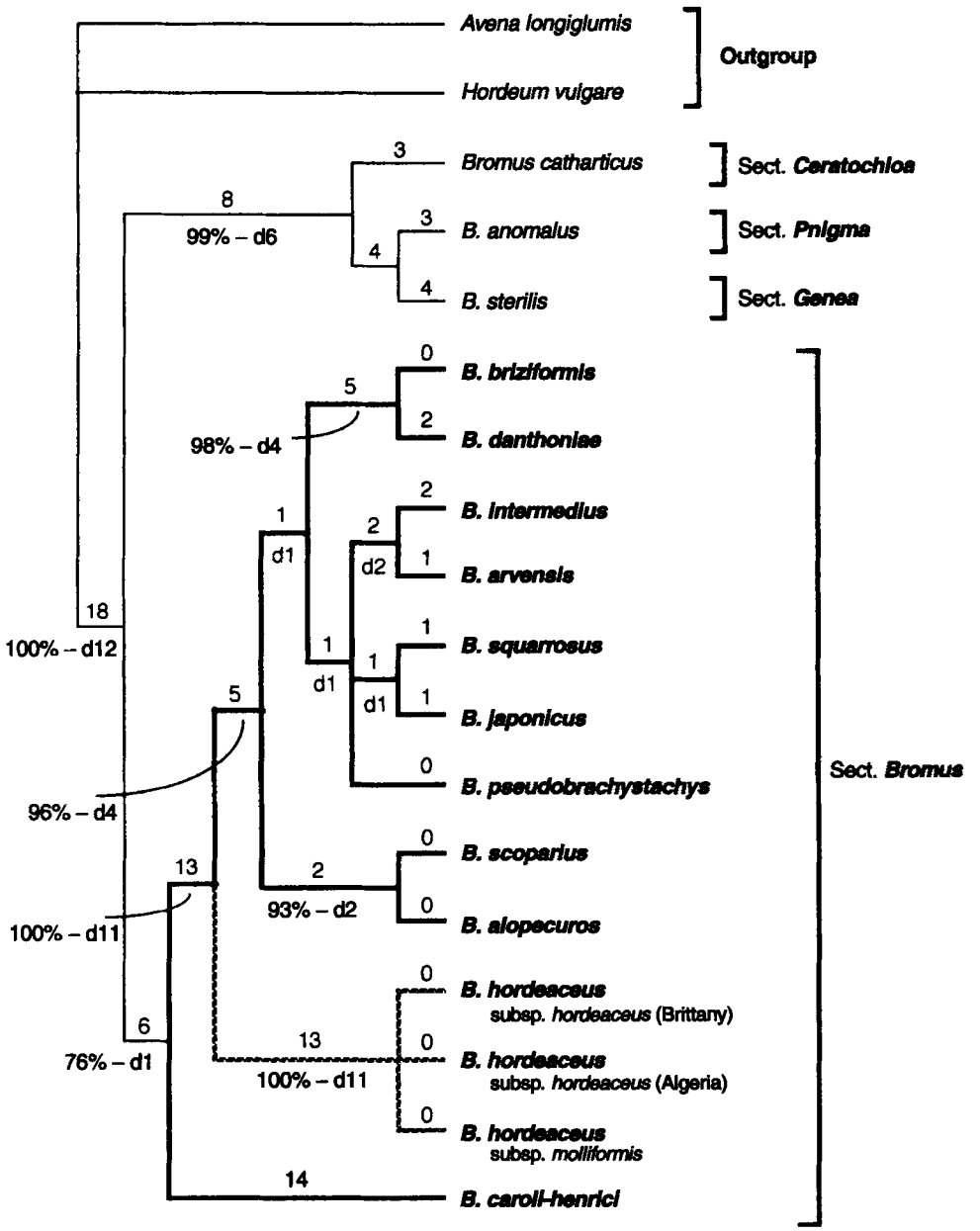

Fig. 3. Phylogenetic analysis (Branch and bound search, PAUP) based on ITS sequences. Bootstrap values (given as percentages) and decay index values are given below the branches, and the number of base pair changes is indicated above the branches.

We have found that within section *Bromus*, the diploid species present a more restricted distribution in natural, less-disturbed habitats, than the tetraploids in North Africa (AINOUCHE 1993; AINOUCHE et al. 1995). Invasiveness is not always correlated with polyploidy in annual bromes, however, as in section *Genea*. For example, the Mediterranean diploid and phenotypically plastic *B. tectorum* is an aggressive ruderal, which successfully colonized the New World (NOVACK & MACK 1993). Phenotypic plasticity, which is a feature frequently involved in the success of weedy species (MEERTS 1995), has also been reported in *B. mollis* (syn. *B. hordeaceus* subsp. *hordeaceus*) (JAIN 1978). A morphological analysis of North African populations revealed consistent plasticity for both vegetative and floral quantitative traits (AINOUCHE 1993). Although sampled over a larger range of ecological and geographical conditions, the North-African populations appeared less genetically differentiated than populations from Brittany in this study, suggesting the existence of higher levels of gene flow among the first ones (Nm = 3.756 and 1.066 respectively). In Australian *B. hordeaceus* populations, GOVINDAJARU (1989) reported higher values (Nm = 6.55). All these estimations represent rather consistent values for selfing species (GOVINDAJARU 1989). In selfing annual bromes, gene flow occurs essentially through seed dispersal by animals, or by humans (SMITH 1986). On the other hand, it has been shown in *B. hordeaceus*, that local differences in microhabitat influence the distribution of allozymes in Swedish populations (LÖNN 1993).

No DNA sequence variation (this study), nor allozyme differentiation (AINOUCHE et al. 1996, and this study) have been encountered among the traditionally recognized subspecies of *B. hordeaceus*. We have found that apart from the fixed, diagnostic (SMITH 1980, SCHOLZ 1998) morphological characters (panicle shape, spikelet hairiness, awn width and curvature), a large range of overlapping morphological variation occurs among populations belonging to these subspecies (AINOUCHE 1993, AINOUCHE, unpubl.). No divergence of ITS sequences from the nrDNA was found between Atlantic and Mediterranean populations that differ at enzyme loci (AINOUCHE et al. 1996). This would indicate that the ITS sequences are not evolving fast enough to reveal recent intraspecific divergence in *B. hordeaceus*.

Origin of the tetraploid *B. hordeaceus*

The strong karyotype symmetry encountered in both the tetraploid *B. hordeaceus* and in the related diploid species, together with the weak genome size differentiation among the diploid species analyzed, make it difficult to distinguish the two genomes present in the tetraploid on the basis of chromosome morphology, even using differential chromosome staining procedures such as Giemsa C-banding (AINOUCHE, unpubl.). Prevailing bivalent pairing in meiotic configurations, and fixed non-segregating heterozygotic phenotypes (i.e. intergenomic heterozygotes) at enzyme loci indicate a disomic mode of inheritance which usually characterizes allopolyploids (polyphyletic origin) containing two independently--evolved genomes (DA SILVA & SOBRAL 1996). Anthropogenic disturbance is likely to encourage interspecific hybridization and the appearance of new allopolyploid species by breaking down ecological isolating barriers (reviewed in RAMSEY & SCHEMSKE 1998). Section *Bromus* is believed to have originated during the Pleistocene, and most of the extant species have evolved together with grazing and agriculture (STEBBINS 1981, SMITH 1986). Hybridization and polyploidy are known to have played a major role in the evolution of this group (STEBBINS 1981). On the basis of morphology and serology, SMITH (1972) suggested the diploid *B. scoparius* and *B. arvensis* as possible ancestors of *B. hordeaceus*. Moderate allozyme divergence was found among species of section *Bromus* occurring in North Africa

(AINOUCHE et al. 1995). Although more affinity was encountered between *B. hordeaceus* and the diploid *B. intermedius*, no diagnostic allele could be used to infer diploid-tetraploid species relationships and to confirm nor to discount *B. scoparius* and *B. arvensis* as progenitors of *B. hordeaceus* (AINOUCHE, unpubl.). Those intergenic chloroplast DNA spacers investigated to date are not evolving fast enough within section *Bromus* to provide enough phylogenetically informative data (AINOUCHE & BAYER, unpubl.). RAPD analysis revealed that *B. hordeaceus* expresses global molecular similarity with the group *B. arvensis*, *B. pseudobrachystachys*, *B. caroli-henrici*, *B. alopecuros* and *B. intermedius*. The molecular affinities encountered among the diploid species (e.g. *B. briziformis*, with *B. japonicus* and *B. squarrosus*, or *B. caroli-henrici* with *B. alopecuros)* are rather consistent with morphology (SMITH 1972, 1980). However, the molecular phylogeny obtained with ITS of nuclear ribosomal genes shows unambiguously that *B. hordeaceus* diverged earlier than all diploid species of section *Bromus* analyzed, except *B. caroli-henrici*. This suggests that at least one of the diploid ancestor of *B. hordeaceus* might have been an extinct or undiscovered species, perhaps related to *B. caroli-henrici*. This relatively distant origin of *B. hordeaceus* within the section is supported by the numerous (13) autapomorphies accumulated during its evolution, and also by the absence of intra-individual sequence polymorphism which could reflect interlocus concerted evolution (WENDEL et al. 1995). Sequence heterogeneity has been reported in species of hybrid origin (SANG et al. 1995), and it is more likely to be encountered in recent hybrid (and allopolyploid) species, as in the tetraploid *Bromus lanceolatus* and *Bromus secalinus* belonging to recently diverged clades of section *Bromus* (AINOUCHE & BAYER 1997), or in the young allopolyploid *Spartina anglica* (AINOUCHE et al., in prep.). Reconstructing the history of allopolyploids is an exciting, but not easy venture, and we must keep in mind the recent findings, which revealed that important genome rearrangements can take place very rapidly after the formation of the polyploid (SONG et al 1995, FELDMAN et al. 1997, LIU et al. 1998). This, together with the subsequent long-term genome evolution of both the polyploid and the parental species, may obscure reconstruction of the phylogeny (WENDEL & DOYLE 1998), and reinforce the need of combining different markers and approaches to elucidate the history of polyploids and to learn more about plant evolution.

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