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Genetic variation of *Bromus hordeaceus s. lato* (Poaceae) over its core distribution range

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Using isozyme analysis, we studied the genetic diversity among 316 *Bromus hordeaceus* (Poaceae) accessions of different geographic origin and belonging to five subspecies. Eight putative isozyme loci revealed remarkable allozyme variation. The isozyme variation occurred as different fixed homozygous and heterozygous phenotypes without segregation among the seed progeny. We detected no subspecies-specific allozymes. Two isozymes tend to differentiate among subspecies, indicating that subspecies of *B. hordeaceus* are still undergoing contemporary process of divergence. The differentiation into 65 multilocus isozyme lineages (MLILs) was not related to the geographic origin of *B. hordeaceus* accessions. *Bromus hordeaceus* did not lose genetic variation towards its range margins: we found 7.7% out of total MLILs in the northernmost part of a core distribution range and 7.7%–40% in the southern part. We recorded silencing of one homeoallelic isozyme gene in the allotetraploid genome of *B. hordeaceus* for accessions of different geographic origins.

Key words: *Bromus hordeaceus*, genetic variation, geographic distribution, multilocus isozyme lineages, range margins

Introduction

Taxonomically and evolutionarily the genus *Bromus* is a complicated unit (Smith 1970, Stebbins 1981), in which Smith (1983) nominated sect. *Bromus* as a "taxonomic nightmare". The section *Bromus* incorporates several taxonomically controversial species groups, one of them being the *B. hordeaceus s. lato* complex (Smith 1968). *Bromus hordeaceus s. lato* is a predominantly self-pollinated, allotetraploid invasive weed (2n = 28) with a wide distribution pattern. It occurs throughout Europe, Mediterranean countries, and SW Asia. Stebbins (1981) recognized SW Asia as a center of diversity for many brome species, including *B. hordeaceus s. lato*. Smith (1986), on the basis of the Webb's (1985) criteria for determining native or introduced status, claimed that the native distribution of *B. hordeaceus* is primarily outside of SW Asia in the eastern Mediterranean. According to several studies (Jain *et al.* 1970, Brown 1984, Jain 1978), outcrossing rate in *B. hordeaceus* ranged from 1% to 18% in different geographical regions.

The *B. hordeaceus* species complex has been the subject of many taxonomic papers

(Stebbins 1981, Smith 1981, 1986, Ainouche & Bayer 1996, Ainouche *et al*. 1995, 1996, 1999, Oja 1998) and still retains many unanswered questions. This complex is well known for its remarkable morphological variation that led to recognition of five subspecies. Three of them are "ecotype subspecies" and related to particular habitat conditions: *B. hordeaceus* subsp. *ferronii* to oceanic cliffs; *B. hordeaceus* subsp. *thominii* to maritime dunes and *B. hordeaceus* subsp. *molliformis* to coastal sand and rocks. *Bromus hordeaceus* ssp. *hordeaceus* (syn. *B. mollis*) is a weed that occurs in different ecological and geographical conditions, e.g., in ruderal sites. The distribution of *Bromus hordeaceus* ssp. *hordeaceus* greatly expanded during last century, being now worldwide.

Recently, Spalton (2001) described *B. hordeaceus* subsp. *longipedicellatus* that resembles *B. commutatus* in general appearance. He suggested that the former is most closely related to *B. hordeaceus* subsp. *hordeaceus* and can be distinguished from it by having longer culms and panicles with longer pedicels and branches. Spalton (2001) hypothesized that *B. hordeaceus* subsp. *longipedicellatus* may be a result from a gene transfer from *B. hordeaceus* into *B. racemosus* or *B. arvensis*, with *hordeaceus* genes becoming dominant. Acedo and Llamas (1999) described a new annual brome-grass from the Iberian Peninsula, *B. cabrerensis* as belonging to *Bromus* sect. *Bromus*. *Bromus cabrerensis* is a tetraploid and closely related to *B. hordeaceus*, from which it clearly differs by having large panicles with longer panicle branches and numerous spikelets. The general description of *B. cabrerensis* resembles the description of *B. hordeaceus* subsp. *longipedicellatus* (Spalton 2001).

Jain *et al*. (1970) found significant differences in the levels of genetic variation among populations of *B. mollis* (*B. hordeaceus* subsp. *hordeaceus*) in California and showed that it is predominantly a self-pollinated species with average outcrossing of 4.8%. They also found little evidence of geographical differentiation and concluded that heterozygous advantage plays only a minor role in the maintenance of polymorphisms in the species. On the basis of two isozyme loci, Lönn (1993) found a very high degree of selfing in *B. hordeaceus* but substantial spatial genetic structure associated with microhabitat factors on the Baltic island of Öland.

On the other hand, Ainouche *et al*. (1995) reported consistent gene flow and a low level of interpopulation differentiation among Mediterranean populations of *B. hordeaceus*; this species exhibits strong invasive behavior there. Ainouche *et al*. (1996) studied the allozyme variation in the *B. hordeaceus* populations from Algeria and France and found a low variability within the region, but some populations were geographically correlated. However, Ainouche *et al*. (1999) found no divergence in the nrDNA ITS sequences among 15 Atlantic and Mediterranean populations of *B. hordeaceus* that previously revealed differentiation at enzyme loci. In that study, all populations contained the same homozygous and heterozygous loci, suggesting high rates of selfing. They also found no genetic differentiation among the four subspecies. Similarly, Oja (1998) observed that intraspecific allozyme variation in 44 accessions of *B. hordeaceus* from the European individuals was independent of the morphological differentiation into three ecotype-subspecies. Thus, all previous attempts to unravel the complex genetic relationships in this species on the basis of different molecular markers were confined to a small part of the species distribution and were based on a small number of populations. The relationships and diversity in this species complex are still not clear.

Since autogamous species display most of their variation among populations, and within-population diversity is relatively low (Hamrick & Godt 1990), we decided to study accessions collected from different geographical regions throughout the species' range in order to describe the intraspecific allozyme differentiation. In this paper, we investigated the distribution of genetic variation among natural populations throughout the core centre of distribution range of the species. This study employed isozymes to assess the genetic diversity within and among populations of *B. hordeaceus* because our previous studies (Oja *et al*. 2003, Oja 2005, Oja & Paal 2007) showed that allozyme analysis provided valuable data for characterising the genetic diversity and breeding system of brome species, and a recent

comparative study (Conte *et al*. 2008) supported the reliability of allozyme markers to estimate a population's genetic structure. The goal of our work is to evaluate the distribution of isozyme variation among the different subspecies of *B. hordeaceus s. lato* and among accessions of different geographic origin.

Material and methods

Plant material

We collected mature panicles from a few (1–3) neighboring plants from natural populations from different parts of the distribution area. Lawrence Spalton kindly provided seed accessions of *B. hordeaceus* subsp. *longipedicellatus* from UK. We received some seed accessions of wild origin from known locations from the U.S.A. National Plant Germplasm System Collection, and from botanical gardens. We grew vouchers for checking identifications from selected seed accessions in a garden and preserved them in the herbarium of the Institute of Ecology and Earth Sciences of the University of Tartu. In total, we analyzed 316 accessions from the following countries: Bulgaria (89), France (68), Germany (43), Spain (35), Italy (13), England (17), Ukraine (14), Estonia (13), Switzerland (5), Greece (3), Portugal (3), Uzbekistan (2), Russia (2), Lithuania (2), Serbia (1), Sweden (1), Latvia (1), Iraq (1), Iran (1), USA (2), including five of *B. hordeaceus* ssp. *molliformis*, six of *B. hordeaceus* ssp. *longipedicellatus*; seven of *B. hordeaceus* ssp. *ferronii* and four accessions of *B. hordeaceus* ssp. *thominii* (*see* Fig. 1). A detailed list of the accessions is available upon request.

Isozyme analysis and nomenclature

We examined isozymes of six enzymes: shikimate dehydrogenase (SKD, EC 1.1.1.25), 6-phosphogluconate dehydrogenase (PGD, EC 1.1.1.44), aspartate aminotransferase (AAT, EC 2.6.1.1), superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (PRX, EC 1.11.1.7) and phosphoglucoisomerase (PGI, EC 5.3.1.9). We performed vertical PAGE (Jaaska & Jaaska 1990, Oja 1999) and analyzed 4–32 individual seedlings of each accession for all isozymes. We interpreted isozyme phenotypes on the basis of existing knowledge of isozyme structure and genetic control (Wendel & Weeden 1989). The isozyme nomenclature follows the description we give in our previous studies (Oja 1998). We designated isozymes encoded by separate loci of a diploid genome by capital letters followed by a number, reflecting allozyme variants in the order of decreasing mobility. We designated heterozygous phenotypes as a fraction of codominant allozymes separated by a diagonal line. We describe the results at the level of isozyme phenotypes.

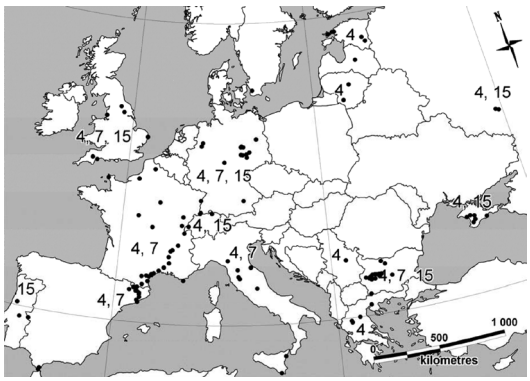

Fig. 1. Geographic distribution of the study populations of *Bromus hordeaceus*. Populations from USA, Iraq, Iran and Uzbekistan are not shown. Numbers designate MLILs.

Data analysis

We performed phenetic analyses of the isozyme data with the PAUP* program (Swofford 2000) using neighbour-joining and UPGMA clustering methods, with a mean Manhattan differences as a distance measure. We coded allozymes as binary (presence/absence) characters. We did not perform a traditional population genetic analysis, because in case of *B. hordeaceus* we found only fixed homo- or heterozygosities without segregation. Therefore, the genetic diversity of the species consists of different genetic lineages and it is irrelevant to calculate different indices.

Results

Isozyme variation of *Bromus hordeaceus*

Six enzymes with eight isozymes encoded by respective putative loci revealed remarkable genetic variation. All isozymes were polymorphic, with at least two different allozymes. PRX-F and PGI-A were most variable with six and seven allozymes, respectively, whereas SOD-B was almost monomorphic for B2, except rare SOD-B3 found in three accessions of *B. hordeaceus*. All isozyme variation occurred as different fixed homozygous or fixed heterozygous phenotypes without segregation among the seed progeny of accessions. We detected no subspecies-specific allozymes. The electrophoretic variants of eight isozymes among 316 accessions of *B. hordeaceus s. lato* are listed in Table 1.

Shikimate dehydrogenase (SKD) zymograms revealed three different morphs, a common homozygous morph A2, and fixed heterozygous phenotypes A1/2 and A2/3. We found SKD A1/2 in accessions of *B. hordeaceus* subsp. *hordeaceus* from France, Spain and Bulgaria. From the five accessions of *B. hordeaceus* subsp. *molliformis* we studied, four show SKD A2/3. In all accessions of *B. hordeaceus s. lato* we studied, 6-phosphoglyconate dehydrogenase (PGD) zymograms showed different fixed heterozygous or double heterozygous variants with no homozygous phenotypes. PGD A2/3 was the most common variant in different regions. We detected some rare region-specific heterozygous phenotypes: A1/3 in one Bulgarian accession and A2/4 in one France accession. Our previous studies showed that several species of the section *Bromus* exhibited PGD gene duplication (Oja 1998, Oja 2002), so that diploids were fixed for different heterozygous triplets and tetraploids for different double heterozygosities. As evident from the occurrence of double-heterozygous PGD A2/3/6, A1/2/3 and A2/3/4, tetraploid *B. hordeaceus* also should possess putative PGD gene duplication in some populations (Table 2). Aspartate aminotransferase (AAT) zymograms revealed two major zones of bands that we attribute to heterozymes AAT-B and AAT-C. The faster heterozyme AAT-B showed variation with four different heterozygous and two different homozygous phenotypes. All accessions we studied displayed the fixation of AAT-B for either homozygous or heterozygous phenotypes, but never segregation between individuals within accessions. The most common fixed heterozygous triplet among accessions of different geographic origin was B4/5. Variant B3/5 was fixed for three accessions from Bulgaria and one accession from Germany. We observed AAT-B4/6 in four accessions from France. The slower heterozyme AAT-C was more monomorphic and revealed prevalence of accessions with homozygous C3. Some accessions displayed fixed heterozygous triplet C2/3. Only one accession of *B. hordeaceus* from Spain revealed another fixed heterozygous vari-

Table 1. Electrophoretic variants of SKD, AAT, SOD, PRX, PGI and 6PGD among *Bromus hordeaceus s. lato* 316 accessions. A, B, C designate heterozymes, numbers designate allozymes; f = a slightly faster and s = a slightly slower variant of the numbered morph.

SKD A	6PgD A	AAT B	AAT C	SOD A	SOD B	PRX F	PGJ A
2	2/3	3/5	3	2/4	2	4	4/5
1/2	2/3/6	4/5	2/3	2/6	3	4/6	2/4
2/3	1/2/3	4	3/4	4		4/5	3/4
	2/3/4	5	1/3			6	2/5f
	1/3	4/5s				2/5	2/5
	2/4	4/6				4/7	4/6
						5	3/5
						3/5	5
						3/6	4
						5/6	5s
						3/4	

ant B3/4, and one accession from France had C1/3. The AAT-B and AAT-C isozyme phenotypes do not distinguish between the subspecies of *B. hordeaceus* but show some region-specific allozymes in heterozygous phenotypes. Superoxide dismutase (SOD) zymograms also showed two zones of bands attributed to two putative heterozymes, SOD-A and SOD-B. The slower SOD-B was almost totally monomorphic for homozygous B2, except three accessions of *B. hordeaceus* subsp. *hordeaceus* with B3, originated from Estonia, Uzbekistan and Greece. The fastest SOD-A displayed heterozygous A2/4 as the prevalent variant among accessions of various geographic origins, whereas *B. hordeaceus* subsp. *molliformis* showed mainly homozygous A4. Only one accession from Italy shows heterozygous variant A2/6. Superoxide dismutase was the least variable enzyme. Peroxidase (PRX) was the most polymorphic isozyme with six different allozymes. Eleven different phenotypes, three homozygous and eight heterozygous were recorded among accessions of *B. hordeaceus*. The most common fixed heterozygous phenotype was two-banded F4/6. We detected some rare region-specific heterozygous phenotypes: F4/7 for four German accessions, F3/6 for two Ukraine accessions, F2/5 in two France and one Spain accessions, F5/6 only in one Italy accessions, and F3/4 as unique for one France accession. Despite the remarkable intraspecific variation in PRX-F, we did not detect the subspecies-specific allozymes. Phosphoglucoisomerase (PGI) zymograms revealed one band zone attributed to heterozyme PGI-A. PGI-A was also quite variable with seven putative allozymes and ten different phenotypes, three homozygous and seven heterozygous. Among seven fixed heterozygotes the most common was A4/5. We found PGI-A4/6 and PGI-A2/5f (5f designates a slightly faster morph than A5) each for only one accession from Spain and Bulgaria, respectively. Our results revealed three homozygous phenotypes, A4, A5 and A5s (5s designates a slightly slower morph than A5). We found unique homozygous phenotype A5s for one accession from Germany. However, extensive intraspecific variation of PGI-A among the accessions of *B. hordeaceus* did not allow to distinguish subspecies.

Mating mode of *B. hordeaceus*

Isozyme variation we observed in *B. hordeaceus* revealed significant allozyme variation, but without segregation into homo- and heterozygous phenotypes among the seed progeny within accessions. We revealed either different fixed homozygotes or different fixed heterozygotes in accessions. These results reaffirm the alloploid nature of *B. hordeaceus* and shows intrinsic self-fertilization in all the populations we studied. Only 13 accessions from France, Spain, Germany and Bulgaria out of 316 were polymorphic, displaying some rare homozygotes in addition to a prevalent heterozygous phenotype. We chose nine populations from differ-

Table 2. Electrophoretic variants of SKD, AAT, SOD, PRX, PGI and 6PGD in nine populations of *Bromus hordeaceus s. lato*. A, B, C designate heterozymes, numbers designate allozymes and homeozymes; *n* = number of individuals analyzed.

Population	Geographic location	*n*	SKD A	6PGD A	AAT B	AAT C	SOD A	SOD B	PRX F	PGJ A
221/99	USA, Colorado	26	2	2/3	4/5	3	2/4	2	4/6	4
444/01	Sweitz, La Sarraz	32	2	1/2/3	4/5	3	2/4	2	4/6	4/5
231/01	France, Nimes	23	2	2/3	4/5;5	3	2/4	2	4/6	4/5
436/01	Germany, Moosburg	21	2	2/3	4/5;4	3	2/4	2	4/6	4/5
482/01	Spain, Tarifa	21	2	2/3	4/5	3,3/4	2/4	2	4/5	4/5
211/99	Estonia, Vormsi	21	2	2/3	4/5	3	2/4	2	4/6	4/5
239/96	Central Russia, Voronezh	21	2	2/3	4/5	3	2/4	2	4/6	4/5
218/06	Ukraine, Crimea, Pervomaiskoye	24	2	2/3/4	4/5	3	2/4	2	4/6	4/5
376/00	Bulgaria, Sofia	21		2/3	4/5	3	2/4	2	4/6	4/5

ent regions, including three accessions (231/01, 436/01 and 482/01) from 13 polymorphic populations (Table 2) for the quantitative characterization of mating mode in *B. hordeaceus*. We analyzed 21–32 individuals from these populations for obtaining reliable results. The extensive sampling showed no deviation from disomic inheritance in nine populations. As a result, all individuals from these nine populations behaved like selfed progenies and exhibited either fixed heterozygous or fixed homozygous phenotypes, without segregation into homozygotes in heterozygous populations. Only AAT-B and AAT-C loci in three polymorphic populations (231/01, 436/01 and 482/01) showed some rare individuals with homozygous allozymes of one consisting allele of prevalent fixed heterozygous phenotypes, but never segregation into both different homozygous allozymes. These findings indicate putative inactivation of one homeologous locus in the allotetraploid genome, not outcrossing events. Our results showed that nearly complete self-fertilization is characteristic of the *B. hordeaceus* populations of different geographical location (Table 2).

The geographic and subspecific distribution of isozyme variation

Given the virtually complete autogamous breeding mode, the genetic diversity of *B. hordeaceus* is divided into different genetic lineages or, in this study, multilocus isozyme lineages (MLILs) (for the summarized data on the differentiation of the isozyme variation into MLILs *see* Table 3). In total, we determined 65 MLILs among the 316 accessions originating from different regions

Table 3. Multilocus isozyme lineages (MLILs) among 316 seed accessions of the *Bromus hordeaceus* species complex: h = ssp. *hordeaceus*; m = ssp. *molliformis*; l = ssp. *longipedicellatus*; th = ssp. *thominii*; f = ssp. *ferronii*. Abbreviations of geographic regions: Fr = France, Bg = Bulgaria, It = Italy, Pt = Portugal, Es = Spain, GB = England, US = United States of America, Uz = Uzbekistan; Gr = Greece, Ch = Switzerland, De = Germany, Ru = Russia, Ir = Iran; Iq = Iraq, Yu = Serbia, Sw = Sweden, Ee = Estonia, Lv = Latvia, Lt = Lithuania, Ukr = Ukraine. The number of seed samples from a particular region is given in brackets.

MLIL	Sub-species	SKD A	6PGD A	AAT B	AAT C	SOD A	SOD B	PRX F	PGI A	Geographical region
1	h	2	2/3/4	4/5	3	2/4	2	4	4/5	Fr(1); Bg(1);
2	h	1/2	2/3	4/5	3	2/4	2	4/6	4/5	Fr(3); Es(1); Bg(3);
3	h	2	2/3	4/5	3/4	2/4	2	4/6	4/5	Es(1);
4	h; m; l; th; f	2	2/3	4/5	3	2/4	2	4/6	4/5	Fr(21); Es(14); Ch(4); De(18); Bg(36); GB(6); Ru(1); Ukr(7); It(6); Ee(8); Uz(2); Gr(1); Lv (1); Lt(2); Sw(1); Yu(1)
5	h	2	2/3	4/5	2/3	2/4	2	4/6	4/5	Fr(4); Es(6); Bg(1);
6	h	2	2/3	4/5	3	2/4	2	6	4/5	Es(1); Bg(1);
7	h; m	2	2/3	4/5	3	2/4	2	4/6	2/4	Fr(9); Es(1); De(1); Bg(6); GB(4); It(1), Gr(1); UZ(1)
8	h	2	2/3	4/6	3	2/4	2	4	4/5	Es(1);
9	h	1/2	2/3	4/5	3	2/4	2	4/6	4/5	Fr(2); Es(2); Bg(2);
10	h	1/2	2/3	4/5	3	2/4	2	4	4/5	Fr(1)
11	h	2	2/3	4/5	3	2/4	2	2/5	4/5	Fr(2); Es(1);
12	h	2	2/3	4/6	3	2/4	2	2/5	4/5	Fr(2);
13	h	2	2/3/6	4/5	3	2/4	2	4/6	4/5	Fr(1); Es(1);
14	h	2	2/3/6	4/5	2/3	2/4	2	4/6	4/5	Fr(1); Bg(2);
15	h; l	2	1/2/3	4/5	3	2/4	2	4/6	4/5	Ch(1); De(10); Bg(9); Pt(3); GB(1); Ru(1); Ukr (1)
16	h	2	1/2/3	4/5	3	2/4	2	4/7	4/5	De(1);
17	h	2	2/3	4/5	3	2/4	2	4/6	3/4	Fr(2);
18	h	2	2/3	4/5	3	2/6	2	4/6	4/5	It(1)
19	h	2	2/3	4/5	2/3	2/4	2	4/6	4/5	FRr2); Bg(4);
20	h	2	2/3/4	4/5	2/3	2/4	2	4/6	4/5	De(2);

continued

throughout their core geographic distribution. The most common lineage MLIL-4 includes 139 accessions from 16 different geographic locations and from all five subspecies. We found two other common lineages, MLIL-7 and MLIL-15, in 24 and 26 accessions, respectively. Among the remaining 62 MLILs, 31 are rare, local MLILs — only one accession from a single location representing each. Most of the rare MLILs reflects the putative differentiation at the tetraploid level by mutational silencing of one locus or rise of new allele. The data in Table 3 show that three

Table 3. Continued

MLIL	Sub-species	SKD A	6PGD A	AAT B	AAT C	SOD A	SOD B	PRX F	PGI A	Geographical region
21	h	2	2/3	4/5	3	2/4	2	4/6	2/5f	Bg(1);
22	h	2	2/3	3/5	3	2/4	2	4/6	2/5	Bg(3);
23	h	2/3	2/3	4/5	2/3	2/4	2	4/6	4/5	Es(1); Bg(1);
24	h	1/2	2/3	4/5	2/3	2/4	2	4/6	4/5	Fr(1);
25	h	2	1/3	4/5	3	2/4	2	4/6	4/5	Bg(1)
26	h	2	1/2/3	4/5	2/3	2/4	2	4/6	5	Bg(1);
27	h	2	2/3	5	3	2/4	2	4/6	4/5	Fr(2); Ee(1); Ir(1); Iq(1)
28	h	1/2	2/3	5	3	2/4	2	4/6	4/5	Fr(1);
29	h; l	2	2/3	4/5	3	2/4	2	4/6	5	Bg(3); GB(1); Ee(1)
30	l	2	1/2/3	4/5	3	2/4	2	5	4	GB(1);
31	h	2/3	2/3	4/5	3	2/4	2	4	4/5	Es(1);
32	h; m	2/3	2/3/6	4/5	3	4	2	3/5	4	Bg(1); It(1)
33	h; m	2/3	2/3/6	4/5	3	4	2	5	4	Bg(2); It(2)
34	h	2	2/3	4	3	4	2	5	4	De(1); Bg(2);
35	h	2	2/3	4/5	3	2/4	2	4/6	3/5	Fr(1); De(1);
36	h	2	2/3	4/5	1/3	2/4	2	4/6	4/5	Fr(1);
37	h	2	2/4	4/5	1/3	2/4	2	4/6	4/5	Fr(1);
38	h	2	2/3	4/5/s	3/4	2/4	2	4/6	4/5	Es(1);
39	h	2	2/3	4/5	3	2/4	2	4/7	4/5	De(1);
40	h	2	2/3	4/5	3	2/4	2	4	4/5	Fr(1); Es(1);
41	h	2	2/3	4/6	3	2/4	2	4/6	4/5	Fr(4);
42	h	2	2/3/6	4/5	3	2/4	2	4/7	4/5	De(2);
43	h	2	2/3	4/5	3	2/4	3	4/6	4/5	Ee(1); Fr(1); Gr(1); De(2); It(1)
44	h	2	2/3	4/5	3	2/4	2	4/6	5s	De(1);
45	h	2	2/3	4/5	2/3	2/4	2	4/6	5	Bg(1);
46	h	2	2/3	4/5	3	2/4	2	4/6	4/6	Bg(1);
47	h	2	2/3	4/5	2	2/4	2	4/6	2/4	Es(1); De(1);
48	h	2	2/3	4/5	3	2/4	2	4/6	2/5	Bg(1)
49	h	1/2	2/3	4/5	3	2/4	2	4/6	5	Bg(1);
50	h	2	2/3	4/5	3	2/4	2	4/6	4	Bg(1); US(2)
51	h	1/2	2/3	4/5	3	2/4	2	4/6	2/4	Fr(1); Es(1); Bg(1);
52	m	2/3	2/3/6	4/5	3	4	2	5/6	4	It(1)
53	h	2	1/2/3	3/5	3	2/4	2	4/6	4/5	De(1);
54	h	2	2/3	4/5	3	2/4	2	3/4	4/6	Fr(1);
55	h	2	2/3/4	4/5	3	2/4	2	4/6	2/4	Fr(1);
56	h	2	2/3	4/5	2/3	2/4	2	6	4/5	Es(1)
57	h	2	2/3	4/5	3	2/4	2	4/5	4/6	Es(1);
58	h	2	2/3	4/5	2/3	2/4	2	4/5	4/5	Fr(1); Bg(2); Ee(1)
59	h	2	2/3	4/5	3	2/4	2	5	3/5	De(1); Bg(1);
60	h	2	1/2/3	4/5	3	2/4	2	4/6	2/4	GB(1)
61	h	2/3	2/3	4/5	3	2/4	2	4/6	2/4	GB(1)
62	h	2/3	2/3	4/5	3	2/4	2	4/6	4/5	GB(3)
63	h	2	2/3/4	4/5	3	2/4	2	4/6	4/5	Ukr (4)
64	h	2	2/3/4	4/5	3	2/4	2	3/6	4/5	Ukr (1)
65	h	2	2/3	4/5	3	2/4	2	3/6	4/5	Ukr(1)

widespread MLILs: 4, 7, and 15 had either the same or even lower values of fixed heterozygosities than rare MLILs. For example, the two most common MLILs 4 and 7 have five heterozygous loci, whereas rare MLILs 23 and 24 have seven heterozygous loci; evidence against the adaptive importance of greater number of heterozygosities in colonizing species. The three more frequent MLILs consisted of accessions belonging to different subspecies of *B. hordeaceus*. Thus, the differentiation into MLILs is not consistent with the subspecific morphological delimitation.

According to many authors (e.g. Brown 1984, Arnaud-Haond *et al*. 2006, Besold *et al*. 2008) the peripheral populations located at the edges of the distribution range have lower genetic diversity than populations from the geographical center of the range. We found five MLILs (7.7% out of total MLILs number) among the 13 accessions from Estonia, representing the northernmost part of the core distribution range studied. The 17 accessions from England revealed seven different MLILs. The southern regions showed a considerably higher number of MLILs, e.g. 17 MLILs (26.1%) among the 35 accessions from Spain, 25 MLILs (38.5%) from the 68 accessions from France, and 26 MLILs (40%) among the 89 accessions from Bulgaria. However, we discovered only six different MLILs (9.2%) among the 13 accessions from Italy and only five MLILs among the 14 accessions from Ukraine. Thus, our results did not support the central-margin hypothesis about a loss of genetic variation towards range margins (Eckert *et al*. 2008).

We performed phenetic analysis with two methods to compare the genetic divergence between the three subspecies and MLILs in the whole species complex. The UPGMA phenogram of Manhattan distances (Fig. 2) and the neighbour-joining tree (Fig. 3) of the same data matrix showed that MLILs of *B. hordeaceus* resolved into many clusters of different constitution and position. Isozyme lineages of different subspecies within *B. hordeaceus s. lato* were intermingled into the same sub-clusters. This indicates that morphologically distinguishable subspecies in *B. hordeaceus* do not differentiate by isozymes. Two of the three common MLILs, four and 15, are in one cluster on both UPGMA and NJ trees, whereas the third common MLIL 7 is in a separate cluster. The main clusters and sub-clusters in both trees consisted of MLILs of different geographic regions. Both dendrograms indicate that the differentiation into MLILs is not associated with the geographic origin of *B. hordeaceus* accessions.

Discussion

Our results revealed differentiation into monomorphic homozygous and fixed heterozygous isozyme phenotypes indicating complete selfing in *B. hordeaceus*, as expected for an allotetraploid species. Similarly, Jain *et al*. (1970) reported high selfing rates in Californian populations of *B. hordeaceus*. Lönn (1993) also observed extremely high levels of selfing in *B. hordeaceus* from Öland. In our study, isozymes PGD-A, AAT-B, SOD-A, PRX-F and PGI-A revealed fixed heterozygosity in most accessions of different geographic origin and fixed homozygous phenotypes in only a limited number of accessions in *B. hordeaceus*. A possible reason for the appearance of homozygous genotypes may be silencing mutations at one homeolocus at the tetraploid level that the autogamous breeding system maintains as different lineages (MLILs). Various mechanisms of gene silencing, like inactivation of the expression of one homeolocus, or of its homeozyme, while the enzyme product of the second homoeolocus compensates for this loss, can cause the homozygous phenotypes at some isozyme loci in allopolyploids (Leitch & Bennett 1997, Soltis & Soltis 2000, Adams *et al*. 2003).

Different authors (Wilson *et al*. 1983, Gastony 1991, Allen & Eccleston 1998, Jaaska 1998, Oja 2002) describe the intraspecific loss of duplicate gene expression with differentiation into fixed hetero- *versus* homozygosities at different isozyme loci in many other allopolyploid species as an important component of the polyploid evolution. However, most of the accessions we included in the study revealed homozygous phenotypes of SKD-A, AAT-C and SOD-B, indicating that both parental diploids of the allotetraploid *B. hordeaceus* contributed identical alleles of the three isozymes to the two homeoloci of the tetraploid. Therefore, hetero-

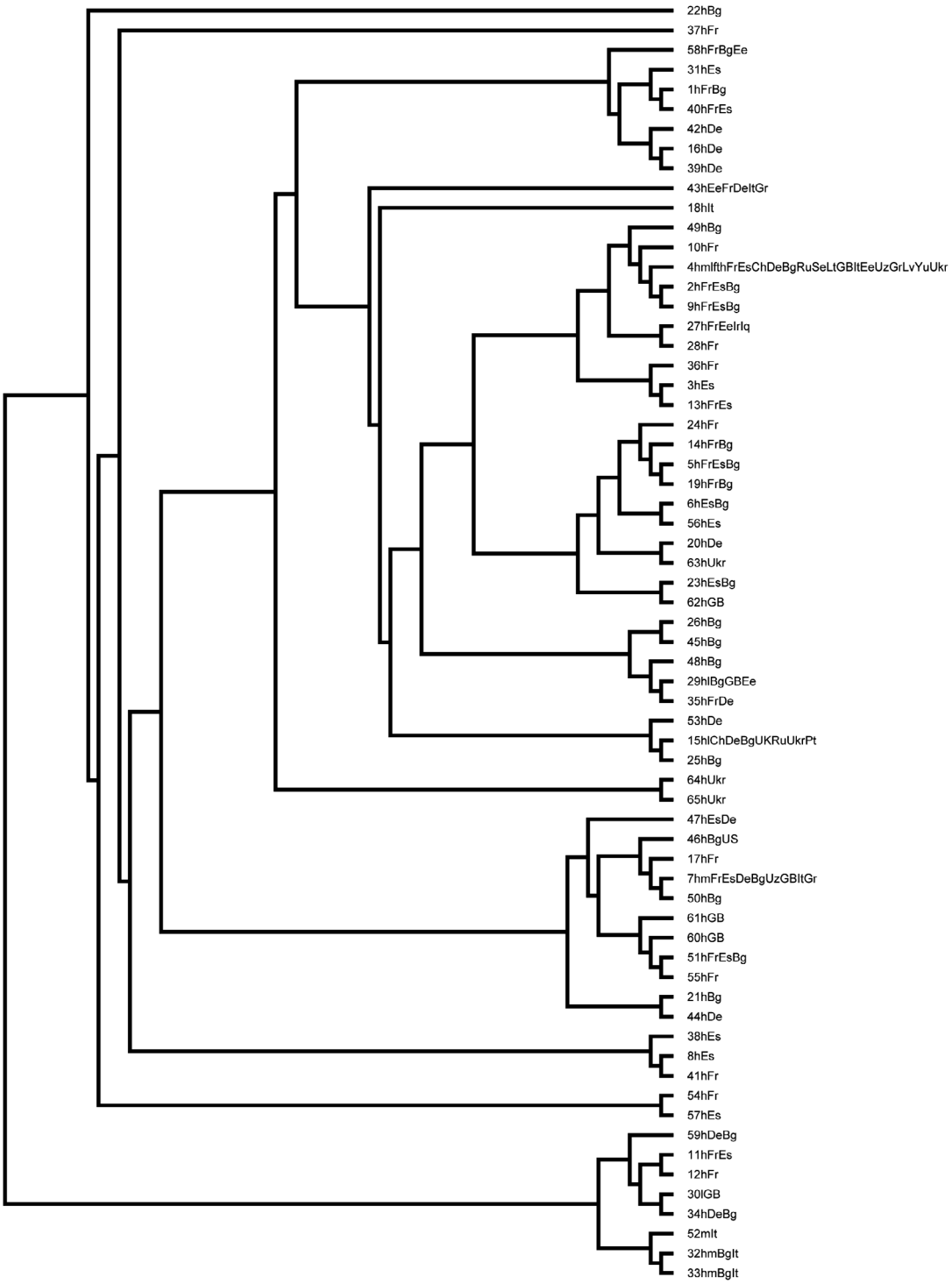

Fig. 2. UPGMA fenogram for *Bromus hordeaceus* MLILs geographic distribution. Numbering and geographic origins are given in Table 3.

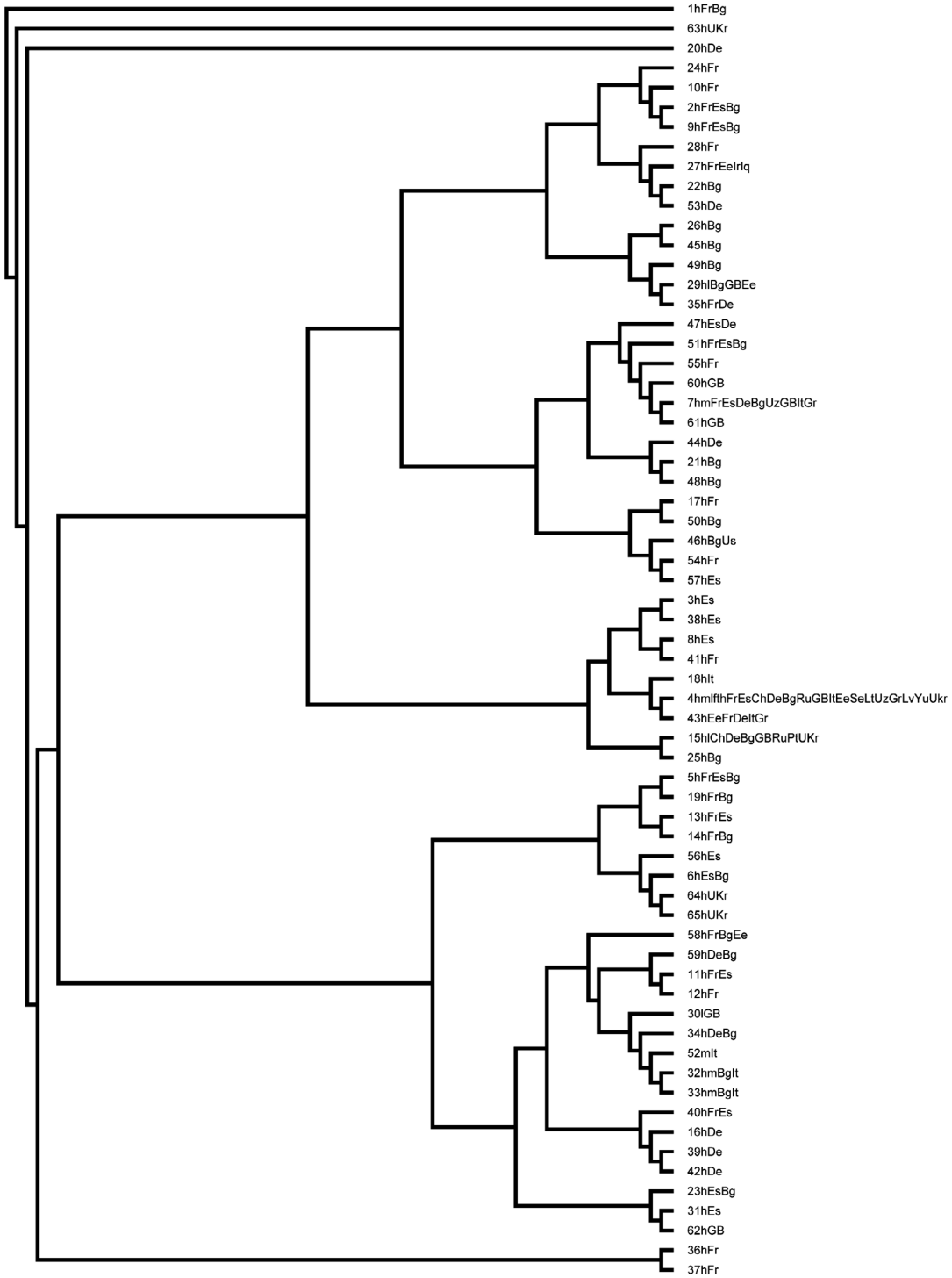

Fig. 3. NJ fenogram for *Bromus hordeaceus* MLILs geographic distribution. Numbering and geographic origins are given in Table 3.

zygosities we observed for these three isozymes probably reflect the rise of new alleles at the tetraploid level and subsequent dispersal to different geographic regions. Oja (2002) described the occurrence of MLILs with both homozygous and fixed heterozygous isozyme phenotypes for allotetraploid *B. madritensis*.

Bradshaw (1965), Baker (1974) and Barrett and Richardson (1986) pointed out that weeds may display two contrasting modes of adaptation to variable environments either through harboring substantial genetic polymorphism, with the development of a large number of locally adapted "specialist" genotypes, or by phenotypic plasticity with a limited number of "general purpose" genotypes. Many isozyme studies revealed diminished genetic diversity both within and among populations of weed species, reflecting that only a limited number of widely adapted multilocus lineages are involved into the colonizing success of these autogamous species (reviewed in Barrett & Husband 1990, Novak *et al*. 1991, Novak & Mack 1993, Oja 1999, Dlugosch & Parker 2007). At the same time, some invasive species possess significant genetic diversity (Warwick *et al*. 1987, Green *et al*. 2001, Menchari *et al*. 2007).

Successful colonists are frequently generalists and carry significantly more variation than specialists (Barrett & Shore 1989). The presence of the most common MLIL-4 and of two widespread MLIL-7 and MLIL-15, throughout the species with Eurasian distribution range suggests that *B. hordeaceus* has "general purpose" genotypes that may contribute to its colonizing success (Fig. 1). Similarly, the colonizing weed *Arabidopsis thaliana* revealed identical multilocus haplotypes in 28 populations out of 37 over its distribution range (Bakker *et al*. 2006). Jain *et al*. (1970) found substantial phenotypic plasticity in *B. hordeaceus* ssp. *hordeaceus* (= *B. mollis*). Greenhouse progenies did not inherit the morphological differences of *B. mollis* the authors collected from different environments (Jain *et al*. 1970). Thus, autogamous breeding mode and great phenotypic plasticity with few "general purpose" genotypes seems to give *B. hordeaceus* a remarkable colonizing ability. Populations belonging to the most common MLIL-4 are widely scattered across the distribution range of the species, from Estonia to Uzbekistan, indicating the absence of a geographic pattern of isozyme differentiation in the *B. hordeaceus* populations. Similarly, several other studies (Innan *et al*. 1997, Breyne *et al*. 1999, Jorgensen & Mauricio 2004) based on different molecular markers showed that *A. thaliana*, a selfing invasive weed lacks any geographical correlation of genetic variation at a global scale. However, Beck *et al*. in their recent paper (2008), based on genome-wide variation at 10 loci from 167 globally distributed populations of *A. thaliana*, found an east-west genetic structure reflecting postglacial routes of recolonization. Unfortunately, we had only very limited accessions from the eastern part of the distribution range of *B. hordeaceus*, whereas accessions from Iran and Iraq revealed the same MLIL-27 as some accessions from France and Estonia. Accessions from Uzbekistan belong to the two most common MLIL-4 and MLIL-7.

Our data shows that northernmost populations from Estonia have the same MLILs, such as MLIL-4 throughout the Mediterranean region, MLIL-27 in Near East, MLIL-43 in central Asia, MLIL-4 in Iberian and Italian Peninsulas, and MLILs 4, 29, 43 and 58 in Balkan Peninsula. Multiple postglacial introduction events from different glacial refugia might be a possible explanation for this finding. In our previous study (Kull & Oja 2007) we found a similar pattern for *Carex loliacea* that possibly migrated to Estonia from two different locations: from the south (Poland) and from the east (Russia). Repeated migration and multiple introduction events during species range expansion would also explain the lack of geographic structure in allozyme variation of *B. hordeaceus*. Brochmann *et al*. (1992) found similar patterns for the Nordic *Draba* and Kucera *et al*. (2006) found the same pattern for *Cardamine impatiens*. Putative homogenizing factor might be dispersal throughout the habitats suitable for the species' survival. Rapid range expansion outside the Mediterranean suggests that *B. hordeaceus* is highly dispersive, and high dispersal rates have raised the level of geographical gene flow (Barret & Husband 1990). The dispersal ability and colonizing success of *B. hordeaceus* is obviously partially man-made. The geographical pat-

tern of genetic diversity of *B. hordeaceus* might be completely fuzzy due to effective spreading and intermingling among different colonization routes. Brown and Marshall (1981) found no differences in the average levels of genetic diversity between native English and introduced Australian populations of *B. mollis*.

Large numbers of different fixed heterozygosities are an indicator of high genetic diversity needed for adaptations of weeds during range expansion (Barrett & Husband 1990, Sakai *et al*. 2001). In our previous studies concerning the allotetraploid *B. madritensis* (Oja 2002), we indicated that most widespread MLILs do not have higher levels of fixed heterozygosity than the rare MLILs. Therefore, the fixed heterozygosities in itself are not related to geographical and ecological success of *B. hordeaceus*.

One of the common modes of plant speciation is an adaptational divergence in response to ecological factors. In case of ecotypes or subspecies of *B. hordeaceus* the morphologic differentiation is quite obvious. At the same time, in contrast to a considerable intraspecific allozymic diversity detected, we failed to find subspecies-specific allozymes again, consistent with our previous study based on 44 European accessions (Oja 1998, Ainouche *et al*. 1996). Subspecies of *B. hordeaceus* showed no clear allozymic divergence. However, two isozymes, SOD-A and SKD-A, indicate a slight tendency to differentiate among subspecies, given the idea that subspecies of *B. hordeaceus* might be of relatively recent origin by adapting to different habitats and these isozymes are a result of adaptive divergence.

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