



**ISOENZYME DIVERSITY AND
PHYLOGENETIC AFFINITIES AMONG
THE EURASIAN ANNUAL BROMES
(*BROMUS* L., POACEAE)**

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To the memory of my mother

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LIST OF ORIGINAL PUBLICATIONS

This thesis is a summary of the following publications which are referred to by Roman numerals in the text.

- I Oja, T. & Jaaska, V. 1996. Isoenzyme data on the genetic divergence and allopolyploidy in the section *Genea* of the grass genus *Bromus* (Poaceae). *Hereditas* 125: 249–255.
- II Oja, T. 1998. Isoenzyme diversity and phylogenetic affinities in the section *Bromus* of the grass genus *Bromus* (Poaceae). *Biochemical Systematics and Ecology*, accepted.
- III Oja, T. & Jaaska, V. 1998. Allozyme diversity and phylogenetic relationships among the diploid annual bromes (*Bromus*, Poaceae). *Annales Botanici Fennici*, accepted.
- IV Oja, T. 1998. Genetic variation in two widespread diploid brome grass species, *Bromus tectorum* L. and *B. sterilis* L. (Poaceae), submitted to *New Phytologist*.

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ABSTRACT

Electrophoretic variation of isoenzyme characters of eleven enzymes (ADH, AAD, MDH, SKD, AAT, SOD, PRX, PGD, PGI, LAP, EST) has been studied with the use of PAGE in 21 annual brome species: six of the section *Genea*, fourteen of the section *Bromus* and one of the section *Boissiera* of the genus *Bromus*. Most species could be distinguished from each other by diagnostic isoenzyme phenotypes. Only two pairs of morphologically similar species, *B. diandrus* – *B. rigidus* of sect. *Genea* and *B. japonicus* – *B. squarrosus* of sect. *Bromus*, revealed identical zymograms. The other two morphologically related species pair of the type section, *B. hordeaceus* – *B. interruptus*, and *B. arvensis* – *B. intermedius*, showed partially overlapping isozyme phenotypes.

All diploid species lacked heterozygous allozyme phenotypes, indicating prevalent autogamy and self-fertilization. The two *Genea* diploids, *B. sterilis* and *B. tectorum*, proved clearly differentiated by alternate allozymes of seven isozymes. Populations of *B. tectorum* and *B. sterilis* from different sites of their Eurasian ranges had low genetic diversity but showed differentiation into eleven (*B. tectorum*) and six (*B. sterilis*) multilocus allozyme lineages. Geographically marginal European populations had only some of the multilocus allozyme lineages distributed in the Near East region, indicating weedy colonization of the two species by only a few successful genotypes.

Phylogenetic relationships and genetic differentiation among eleven diploid brome species were evaluated by cladistic and phenetic analysis of allozyme diversity. The placement of *B. pumilio* in its own section *Boissiera* was supported by its basal position in a separate clade. Morphologically uniform *B. intermedius* was the most polymorphic species, revealing six isoenzyme lineages. The diploids of the section *Genea* were distinguished in a separate cluster on both cladistic and phenetic allozyme trees.

All polyploids studied revealed different fixed heterozygosities of several heterozygotes with codominant homoeozymes, suggesting their allopolyploid nature. Morphologically similar polyploids *B. madritensis* and *B. rubens* differed in seven heterozygotes out of fourteen studied, supporting their specific recognition. One genome of the polyploid species of the section *Genea* corresponded closely to that of *B. sterilis*. Tetraploids *B. secalinus* and *B. commutatus* had very similar enzyme phenotypes, indicating their strong genetic affinity, but still differed in PRX-F homoeozymes of one composite genome. Diploid *B. alopecuroides* appears to be the donor of one genome in the tetraploid *B. lanceolatus*. *Bromus pseudosecalinus* could not be one of the diploid progenitors for *B. secalinus*. 6-PGD gene is duplicated and diverged in several diploids of the section *Bromus*, but species of the section *Genea* had no 6-PGD gene duplication.

1. INTRODUCTION

Bromus L. (Poaceae) is a taxonomically complex genus with about 130 species of annual and perennial, diploid and polyploid brome grasses of wide geographic distribution in Eurasia, North and South America, Africa and Australia. Up to now, there is no consensus on the taxonomic treatment of *Bromus*. The genus has been divided into seven sections: *Bromus*, *Genea*, *Pnigma*, *Ceratochloa*, *Nevskiella*, *Neobromus* and *Boissiera* (Smith, 1970, 1985), seven subgenera: *Neobromus*, *Ceratochloa*, *Festucaria*, *Stenobromus*, *Nevskiella*, *Bromus* and *Boissiera* (Stebbins, 1981) or even seven different genera *Anisantha*, *Bromus*, *Bromopsis*, *Ceratochloa*, *Boissiera*, *Nevskiella* and *Littledalea* (Tzvelev, 1976). The number and rank of the divisions depends on the characters used as a basis of their delimitation (serological, cytological and morphological, respectively), and phylogenetic relationships among them are still inconsistent. Sales (1993) doubted in the reality of the section *Genea* as an independent taxonomic unit. She noted that there is a continuous range of variation between section *Bromus* and section *Genea* via the *B. pectinatus* complex of section *Bromus*. This complex was suggested to link the *Genea* species with the section *Bromus* through the diploid *B. japonicus*. Recently, three papers have been published (Ainouche and Bayer, 1997; Pillay and Hilu, 1995; Pillay, 1996) reporting the use of advanced contemporary molecular methods with cpDNA and rDNA markers in the investigations of the genetic relationships in genus *Bromus*. Pillay and Hilu (1995), on the basis of the cpDNA data, suggested that *B. sterilis* and *B. tectorum* are recently derived species that share a common maternal ancestry. They also found that subgenera *Stenobromus* and *Bromus* are not distinct entities and probably originated from similar ancestors related to the subgenus *Festucaria*. Pillay (1996) concluded it unlikely that the subgenera vs. sections of *Bromus* had independent origins. However, Ainouche and Bayer (1997) showed sections *Pnigma*, *Genea* and *Bromus* in separate monophyletic clades on the rDNA ITS sequence cladogram. Thus, despite of this new molecular information, phylogenetic relationships among the subdivisions of *Bromus* are still far from complete clarification.

In addition to inconsistent intrageneric classification, there is a lot of problems about the species delimitation within sections, due to a great and frequently continuous morphological variability and unknown genetic relationships among them. The brome species often share the same habitats (dry places in general, roadsides, waste places, on calcareous, clay, sandy or rocky soils) and grow intermixed and sympatrically. The existence of intermediate plants frequently makes their exact identification difficult, although the differences between typical specimens of the species are clearly defined. The great morphological diversity within the group will give the impression that there is a continuous range of variation from one extreme of the section to the other

(Smith, 1970, 1972, 1980, 1981, 1986; Esnault, 1984; Esnault and Huon, 1985; Sales, 1991, 1993, 1994). The number of species included in the section *Bromus* remarkably varies (from 30 to 40), depending on the author of the classification and on the accepted synonymy (Smith, 1970; Tzvelev, 1976). Some of the species are considered together in aggregate species in some taxonomic works, e.g. *B. mollis* aggregate (Smith, 1968), some of them are considered conspecific in some treatments, e.g. *B. commutatus* and *B. racemosus* (Wilson, 1956).

Morphological characters have traditionally been the most important criteria in making taxonomic decisions. However, reliance only on shared morphological characters could contribute to taxonomic confusion due to widespread parallel, convergent and reticulate evolution in the grasses.

Polyploidy has played a significant role in the evolution of the genus *Bromus*. Cytogenetic studies of artificial interspecific hybrids in the sections *Bromus* and *Pnigma* have established allopolyploid nature of the some polyploids (Armstrong, 1977, 1981). However, no similar cytogenetic evidence is available with respect to the *Genea* species.

Isoenzyme analysis is a well-known general approach in taxonomy which makes it possible to assay many individuals for isoenzyme characters quite easily and quickly (Gottlieb, 1977; Crawford, 1989). Isoenzyme data provide the opportunity for tests of congruence between the isoenzyme patterns (genetic markers) and species delimitation on the basis of morphological characters. For example, Kahler, Krzakowa and Allard (1981) found that the *Genea* species could be distinguished by starch gel electrophoretic phenotypes of four enzymes: aspartate aminotransferase, phosphoglucosomerase, peroxidase and esterase, Krzakowa and Kraupe (1981) described inter- and intrapopulation variation of esterase and aspartate aminotransferase isozymes among *B. tectorum* populations from Poland and five other European countries. They suggested that other isoenzyme systems may uncover additional differences and may allow the determination of the extent of variation. Novak, Mack and Soltis (1991) studied allozyme variation within *B. tectorum* populations in North America. The late studies of Ainouche, Misset and Huon (1995, 1996) successfully used isoenzyme electrophoresis in population investigations of *Bromus* species.

Thus, isoenzymes enable relatively simply to analyze genotype — phenotype relationships among the species and populations.

2. OBJECTIVES

The main purpose of the present study was to assess genetic diversity and phylogenetic relationships among the Eurasian annual bromes with the use of electrophoretic isoenzyme characters. The particular objectives were:

- to find diagnostic isoenzymes for the identification of brome species and for the evaluation of genetic divergence;
- to analyze the differentiation among the bromegrasses between and within the sections by the electrophoretic allozyme characters;
- to analyze a correspondence of the isoenzyme differentiation with the morphological species delimitation;
- to establish the allo- or autoploid nature of the brome polyploids and which diploids are their suitable progenitors by isoenzymes;
- to assess phylogenetic relationships and genetic divergence among the diploid annual bromes by cladistic and phenetic analysis of allozyme characters;
- to characterize the allozyme diversity and its geographic distribution in the two *Genea* diploids, *B. tectorum* and *B. sterilis*.

3. MATERIAL AND METHODS

3.1. Plant material

The seed accessions from Turkey and Central Russia were collected by the author, accessions from Syria, Armenia, Kirghizia, Uzbekistan and Northern Ossetia were kindly provided by V. Jaaska. The accessions of European origin, collected in the wild from known locations, were received from botanical gardens. They were grown in the experimental field to have vouchers to verify identifications. Vouchers are deposited in TAA, Herbarium of the Institute of Zoology and Botany (Tartu, Estonia). The following *Bromus* L. species and accessions were available for this study:

Section *Genea*

1. *B. tectorum* L.: 61 accessions, originated from Turkey (17), Germany (14), Syria (13), Spain (5), France (3), Central Russia (2), Italy (1), Greece (1), Hungary (1), Slovakia (1), Czech republic (2) and Romania (1), including six Turkish accessions of *B. tectorum* L. ssp. *lucidus* Sales (= *B. sericeus* Drob.).
2. *B. sterilis* L.: 48 accessions, originated from Germany (19), France (11), Turkey (7), Syria (3), Italy (3), Greece (1), Portugal (1), Hungary (1), Romania (1) and Spain (1).
3. *B. rubens* L.: 1 accession, originating from Spain.
4. *B. madritensis* L.: 10 accessions, originating from Belgium (3), Italy (4), France (2), and Georgia (1).
5. The *B. diandrus* – *B. rigidus* complex: 18 accessions, originating from Belgium (4), France (5), Italy (5), Holland (2), Portugal (1), and Turkey (1).

Section *Bromus*

1. *B. danthoniae* Trin.: 2 Turkish accessions.
2. *B. alopecuroides* Poir.: 3 Turkish accessions.
3. *B. scoparius* L.: 3 accessions, originated from Kirghizia (1), Greece (1) and Central Asia (1).
4. *B. japonicus* Thunb.: 21 accessions, originated from Turkey (8), Central Russia (7), Armenia (1), Kirghizia (1), Germany (1), Hungary (2) and Czechia (1).
5. *B. squarrosus* L.: 16 accessions, originated from Turkey (10), Hungary (2), Switzerland (1), Greece (1) and Spain (2).
6. *B. intermedius* Guss.: 17 accessions, originated from Turkey.

7. *B. pseudosecalinus* P. Smith: 1 accession, originated from England.
8. *B. arvensis* L.: 6 accessions, originated from France (4) and Germany (2).
9. *B. secalinus* L.: 12 accessions, originated from Germany (6), France (4), Switzerland (1) and Poland (1).
10. *B. commutatus* Schrad.: 8 accessions, originated from Germany (3), France (3), Italy (1) and Romania (1).
11. *B. lanceolatus* Roth.: 4 accessions, originated from Turkey.
12. *B. pseudodanthoniae* Drob. (according to Tzvelev, 1976): 5 accessions, originated from Kirghizia (2), Uzbekistan (2) and Turkey (1).
13. *B. hordeaceus* L.: 44 accessions, originated from France (16), Germany (15), Italy (4), Northern Ossetia (2), Spain (1), Central Russia (1), Hungary (1), Austria (1), Italy (1), Switzerland (1) and Belgium (1).
14. *B. interruptus* (Hack.) Druce: 2 accessions, originated from England.

Section *Boissiera*

1. *B. pumilio* (Trin.) P. Smith: 1 accession, originated from Denmark.

For comparison, as an outgroup species in a cladistic study of phylogenetic relationships, were analysed two accessions of *Hordeum bogdanii* Wilensky, originated from Pakistan and China. Both were received from Dr. Roland von Bothmer of the Swedish Agricultural University, Svalöv, Sweden.

3.2. Isoenzyme analysis

The following enzymes were examined: aliphatic alcohol dehydrogenase (ADH, EC 1.1.1.1), aromatic alcohol dehydrogenase (AAD, EC 1.1.1.90 and 1.1.1.91), malate dehydrogenase (MDH, EC 1.1.1.37), shikimate dehydrogenase (SKD, EC 1.1.1.25), aspartate aminotransferase (AAT, EC 2.6.1.1), superoxide dismutase (SOD, EC 1.15.1.1), 6-phosphogluconate dehydrogenase (PGD, EC 1.1.1.44), phosphoglucoisomerase (PGI, EC 5.3.1.9), peroxidase (PRX, EC 1.11.1.7), leucine aminopeptidase (LAP, EC 1.11.1.7), and esterase (EST, EC 3.1.1.2).

Enzyme extracts were prepared from the shoots (the primary leaf with the coleoptile) of 4–10 days old, etiolated seedlings by grinding in 0.1 M Tris — 0.05 MEDTA buffer containing either 5 mM cysteine or 5 mM dithiothreitol. After adding 20–50 mg of a sucrose — Sephadex G — 200 mixture (4:1) to increase their viscosity, the extracts were subjected to electrophoresis in vertical polyacrylamide gel slabs (120 × 70 × 2 mm).

The following three gel-buffer systems and two catholytes were applied for different enzymes to achieve better band resolution:

Gel 1: 10% acrylamide, 0.15 % N,N'-bisacrylamide (Bis), 0.25 M Tris, and 0.1 M HCl; applied for PGI, SKD and PGD with the 2-alanine catholyte.

Gel 2: 10% acrylamide, 0.15 % Bis, 0.15 M Tris, and 0.1 M HCl; applied for ADH, AAD, MDH, AAT and PRX with the glycine catalyte.

Gel 3: 10% acrylamide, 0.15 % Bis, 0.25 M Tris, and 0.1 M Tris hydrochloride; applied for SOD and EST with the glycine catalyte.

N,N,N',N'-Tetramethylethylenediamine (0.05 ml%), riboflavine (0.5 mg%) and ammonium persulfate (1 mg%) were added to the gel mixtures to initiate and catalyse their photopolymerization between two day-light fluorescent bulbs during 1 h.

The two catholytes used consisted of 80 mM glycine or 2-alanine with 10 mM Tris. The lower anode buffer was always 0.1 M Tris — acetate with the initial pH about 8.9. It was used repeatedly as long as the pH remained over 7.

Electrophoresis in the anodal direction was carried out in an ice — refrigerated plexiglass apparatus by applying a pulsed current at 15 mA and 20–30 V/cm for about 2–2.5 h until the marker dye, bromphenol blue, reached the gel end.

After electrophoresis, the gels were stained for isoenzymes by applying standard histochemical methods (Weeden and Wendel, 1989) with modifications as described in Jaaska and Jaaska (1986, 1990).

Four individual seedlings of each accession were routinely analyzed for all isoenzymes. Up to ten individuals were analyzed for some enzymes and accessions, in order to check the constancy or polymorphism of the isoenzyme patterns. Isoenzyme phenotypes were interpreted on the basis of existing knowledge of isoenzyme structure and genetic control as summarized by Wendel and Weeden (1989).

3.3. Isoenzyme nomenclature and designation

The term “isozyme” has been proposed by Markert and Møller (1959) to describe all multiple molecular forms of the same enzyme irrespective of their nature, i.e. *sensu lato*. Later, when isozymes received a wide use in population genetic studies, many authors find it comfortable to use the term only for those molecular forms which are defined genetically and differ in their amino-acid structure. Soon it has become a custom to apply the term isozyme for enzyme forms encoded by different loci and to use a related term allozyme to specify enzyme variants encoded by alleles of the same locus. However, there are still other cases of genetically controlled isozymes, e.g. by duplicated genes in diploids and allopolyploids. Jaaska and Jaaska (1984) found it preferable and

more ethic to retain the priority *sensu lato* definition of Markert and Møller of isozymes and have proposed to use special terms for different kinds of isozymes. Thus, nomenclature distinguishing genetically heterologous, homoeologous and homologous isoenzymes (abbreviatedly heterozymes, homoeozymes and allozymes, respectively). Heterozymes (= isozymes *s. str. auct.*, non *sensu lato* of Markert and Møller, 1959), i.e. isoenzymes encoded by separate loci of a diploid genome are designated by capital letters followed by a number, reflecting allozymic variants in the order of decreasing electrophoretic mobility. Electrophoretically different isoenzymes encoded by homologous loci of composite genomes of polyploids, indicating their genetic homoeology, i.e. only partial homology of genomes, are named homoeozymes. Heterozygous phenotypes are designated by codominant isozymes separated by a diagonal line, i.e. as a fraction. Term “fixed heterozygosity” is used when heterozygous phenotypes of isoenzymes were observed for all analyzed individuals of polyploids without segregation into homozygous phenotypes.

3.4. Data analysis

Pee-Wee programm vers. 2.5.1 (Goloboff, 1993) which found trees with best total fit of characters on a tree and calculated Decay indexes (DI) of branch support (Bremer, 1994) was used for the cladistic analysis of the allozyme data. Phenetic analysis was performed with the TYTAN program provided by Dr. A. Batko (Warsaw University, Poland) by applying Manhattan distance combined with UPGMA method. Both analyses were conducted on the multilocus allozyme lineages that were identified among the species studied. The isoenzyme data matrix was made using allozymes as binary absence/presence characters. Rare morphs were excluded from the matrix. The autogamous diploid perennial *Hordeum bogdanii* of the sister tribe *Triticeae* Dum. was chosen as an outgroup for comparison in cladistic analysis.

4. RESULTS AND DISCUSSION

4.1. Isoenzyme diversity and phylogenetic affinities in the section *Genea*

In contrast to the intraspecific isoenzyme constancy, the two diploids, *B. sterilis* and *B. tectorum*, were differentiated from each other by the following eight isozymes, AAD-B, AAT-C, AAT-D, AAT-F, PRX-F, LAP-A, EST-A and EST-F (I, IV, Table 1). This indicates that the genetic divergence of the two diploids by the eight heterozymes has occurred at their speciation, while their subsequent weedy expansion was not accompanied by the appearance of significant allozymic variation, except rare variants of MDH-A and PRX-F.

All polyploids of the section *Genea*, *B. rubens*, *B. madritensis* and *B. diandrus* (incl. *B. rigidus*), revealed fixed heterozygosities at several isoenzyme loci, indicating that they may be allopolyploids. Fixed heterozygosity is taken as evidence in favour of an allopolyploid nature of polyploids (Soltis and Rieseberg, 1986; Soltis and Soltis, 1993). It should, however, be noted that fixed heterozygosity alone is not a firm proof of allopolyploidy, but it shows the hybrid origin of the polyploid. A polyploid showing fixed heterozygosity can arise from a hybrid between different genotypes of the same diploid, i.e. may be an autoploid with diploidized genomes. However, the numerous accessions of the diploids *B. sterilis* and *B. tectorum* analyzed, revealed no allozymes corresponding to the homoeozymes MDH-A1, SKD-A3, AAT-B2, AAT-B1, AAT-C2, SOD-A1, SOD-A3, PGD-A3, PGD-A2 which were observed in fixed heterozygous phenotypes of polyploids. Therefore, our results support the conclusion that the *Genea* polyploids are allopolyploids.

The appearance of several different fixed heterozygous isoenzymes in a polyploid is frequently taken as evidence for its recurrent origin from different genotypes of diploid progenitors (reviewed in Soltis and Soltis, 1993). The polyploids *B. madritensis* and *B. diandrus s.l.* also showed alternative fixed heterozygosities among different accessions which could be taken as an evidence in favour of their multiple origins. However, a possibility that the multiple heterozygous phenotypes have arisen subsequent to the initial polyploidization should also be considered.

The three *Genea* polyploids had different isozyme phenotypes and fixed heterozygosities at several heterozyme loci, indicating their different genome composition and independent allopolyploid origin from different diploid progenitors. The PRX isoenzyme data indicated that *B. sterilis* could be a genome donor for the polyploid species *B. madritensis* and for the *B. diandrus - rigidus* complex, and *B. tectorum* — for *B. rubens* (Fig. 1).

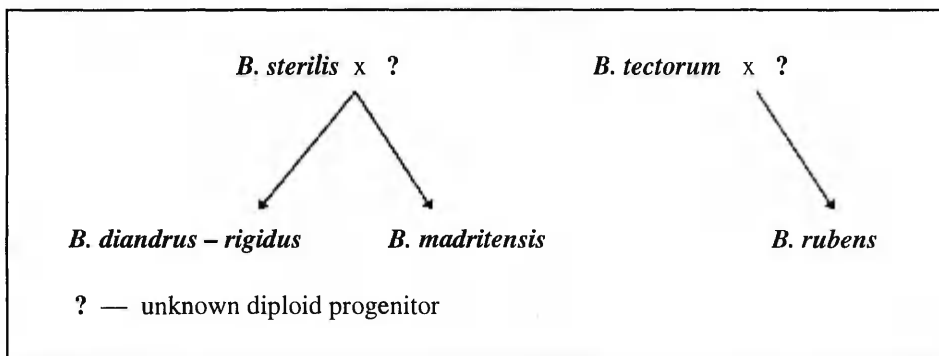


Figure 1. Probable diploid progenitors for the polyploids in section *Genea*.

However, *B. tectorum* was monomorphic for a unique allozyme B3 of AAD-B, which argues against this diploid as the donor of a full genome to any of the *Genea* polyploid species. Unfortunately, *Bromus fasciculatus* which is another possible candidate as a diploid genome donor was not available for the present study. Our isoenzyme results suggest that *B. diandrus* and *B. rigidus* are closely related not only morphologically, but also genetically, and do not deserve the rank of separate species. Out of 14 heterozyms, 10 were monomorphic and shared in the two taxa. Variations observed in four heterozyms (AAT-C, SOD-A, PGI-B, and LAP-A) were not related to differentiation by morphological characters (I, Table 1). This supports the conclusion of a recent multidisciplinary investigation of Sales (1993) that *B. diandrus* and *B. rigidus* are not distinct enough (on the basis of morphological, geographical and ecological evidences) to be given a taxonomic rank higher than a variety, *B. diandrus* Roth var. *diandrus* and *B. diandrus* Roth var. *rigidus* (Roth) Sales, respectively. Hence, they may be considered as members of a polyploid complex with tetra-, hexa-, and octoploid cytotypes (Esnault and Huon, 1987).

Bromus madritensis and *B. rubens*, which were recorded as subspecies by Sales (1993) on the basis of their resemblance in morphological characters, proved distinctly different by at least eight isoenzymes (ADH-A, MDH-A, SKD-A, AAT-B, AAT-C, PRX-F, PGI-B, LAP-A). These results show a degree genetic differentiation between these polyploids, which merit recognition as separate species.

4.2. Isoenzyme diversity and phylogenetic affinities in the section *Bromus*

Most species of the section could be distinguished from each other by diagnostic isoenzyme phenotypes (Table 1 and II, Fig. 1). *Bromus japonicus* and *B. squarrosus*, however, revealed identical zymograms, providing new evidence

about their very close genetic relationships. While studying the material of these species from Central Russia and Turkey, a very common problem for all taxonomists appeared. Using available keys (e.g. Smith, 1980, 1985) it is almost impossible to resolve the variability of accessions encountered in nature. Some individuals were typical *B. japonicus*, some others were typical *B. squarrosus*, but most accessions could be identified only as belonging to the *B. japonicus-squarrosus* complex because they combined morphological characters of both taxa. None of the morphological characters helped to distinguish the two species, neither "good, conservative characters, like lemma size and shape, nor rather less useful, more plastic panicle characters" (Smith and Sales, 1993). There seems to be a continuous range of variation from *B. japonicus* to *B. squarrosus*. Isoenzyme variation was independent from their differentiation by morphology; typical *B. japonicus*, typical *B. squarrosus* and intermediates revealed identical isoenzyme phenotypes. Serological findings (Smith, 1972) also support that the diploid pair *B. squarrosus* and *B. japonicus* are closely related. Our isoenzyme results suggest that *B. japonicus* and *B. squarrosus* are closely related not only morphologically, but also genetically, and their ranking into separate species remains questionable, needing more detailed investigations.

Bromus scoparius and *B. intermedius* which are morphologically easily distinguishable are also clearly distinguished from each other and from the previously mentioned *B. japonicus - squarrosus* complex on the basis of the isoenzyme evidence, in agreement with the serological studies of Smith (1972).

In addition to the interspecific variation, intraspecific allozyme differentiation between local populations (accessions) was observed in five of eight diploid species. *Bromus alopecuros*, *B. scoparius*, *B. japonicus*, *B. squarrosus* and *B. intermedius* revealed intraspecific variation with two of the allozymes ADH-A, SKD-A, PGD-A, AAT-B, SOD-A, PRX-F, PGI-A (Tab. 1).

In our study, no heterozygous phenotypes of any heterozyme, except PGD-A, were observed in the diploid taxa. This indicates prevalent selfing in all diploids examined, since seed accessions collected from plants in nature were analyzed.

Diploid *B. pseudosecalinus* which is morphologically very similar to the tetraploid *B. secalinus* was found to be serologically very different from latter and Smith (1972) supposed that "morphological resemblance is fortuitous, and does not imply similar ancestry". Our results showed that *B. pseudosecalinus* differ from *B. secalinus* in morphs of MDH-A and AAT-B, suggesting it is unlikely to be the diploid ancestor of this tetraploid, in agreement with the serological evidence.

Pillay and Hilu (1995) suggested close relations between *B. secalinus* and *B. alopecuros* on the basis of the cpDNA. Our results, showing different MDH-A, AAT-B and PRX-F allozymes, suggest that *B. alopecuros* does not suite to be a diploid parent for *B. secalinus*. Smith (1972) concluded that *B. danthoniae* is

likely to be one of the diploid ancestors of *B. lanceolatus* because of the serological similarity. Our data, however, does not support this due to a difference in allozymes of SOD-A. Instead, our allozyme data indicate that *B. alopecuroides* could be one of the genome donors for the tetraploid *B. lanceolatus*.

Table 1. Electrophoretic variants of alcohol (ADH), aromatic alcohol (AAD), malate (MDH), shikimate (SKD), aspartate aminotransferase (AAT), superoxide dismutase (SOD), peroxidase (PRX), 6-phosphogluconate dehydrogenase (PGD) and phosphoglucoisomerase (PGI) heterozygotes in *Bromus* species of the section *Bromus*: n — the number of accessions analyzed, s designates a slightly slower and f a slightly faster variant of the numbered morph; species-specific variants are in bold

taxa	N	ADH	AAD	MDH	SKD	AAT	AAT	SOD	SOD	PRX	PGD	PGI
		A	C	A	A	B	C	A	B	F	A	A
diploids												
danthoniae	2	3	3	3	2	5	3	2	2	6	2 3	5
alopeuroides	3	3	3	2	2	5	3	4	2, 1	2, 2s	2	4, 3
scoparius	3	3,7	3	3	2,3s	4	3	6	2	4	2	4
japonicus	21	3	3	3	2	4, 5	3	4	2	5, 6, 6f	2 3, 1 3	4, 5
squarrosus	16	3	3	3	2	4, 5	3	4	2	5, 6, 6f	2 3, 1 3	4, 5
arvensis	12	3	3	2	2	4	3	4	2	4	2 3	3
intermedius	17	3	3	2	2, 3	4, 5	3	4	2	5, 4, 3	2 3	3, 4
pseudo-secalinus	1	3	3	3	3	5	3	4	2	5	2 4	4
tetraploids												
secalinus	12	3	3	3s	2 3	4 5f	3	4	2	5, 5 6	2 3 4	4,4 5
commutatus	8	3	3	3s	2 3	4 5f	3	4	2	3 5, 4 5	2 3 4	4,4 5
lanceolatus	4	3	3	2 3	2	4 5	3	4	2	2 6, 2s 6	2 3	3 5,4
pseudodanthoniae	5	3	3	3	2	4 5f	3	2 4	2	4 6, 4 7	2 3	4 6
hordeaceus	44	3,3 5	3	3,3s, 0 3	2	4 5	3, 2 3	2 4	2	4 6, 4 7	2 3	4 5
interruptus	2	3	1 3	3	2	4 5	3	2 4	2	4 6	2 3	4 5

Polyploids *B. secalinus* and *B. commutatus* had very similar isoenzyme phenotypes. Out of 11 heterozygotes, 10 were monomorphic and shared in the two

taxa, supporting their close genetic affinity. Especially indicative is a unique, shared double heterozygosity of PGD-A2/3/4, suggesting the second duplication with divergence of the PGD-A locus. The isoenzyme data on the close phylogenetic affinity of the two species are in accordance with the morphological similarity of these species, their serological homogeneity (Smith, 1972) and hybridization data suggesting that they possibly have had common progenitors (Wilson, 1956). The isoenzyme data, however, revealed a clear difference between them in PRX-F, in support of their recognition as separate species. The two tetraploids may have one common genome indicated by shared PRX-F homoeozymes F5, whereas the second genome is differentiated by homoeozymes F6 in *B. secalinus* versus F3 and F4 in *B. commutatus*.

Tetraploids *B. hordeaceus* and *B. interruptus*, in addition to their close morphological resemblance, also shared identical fixed heterozygosities at AAD-C, AAT-B, SOD-A, PRX-F, PGD-A and PGI-A and the same homozygous phenotypes of ADH-A, MDH-A, SKD-A, AAT-C and SOD-B. The only difference was that *B. hordeaceus* was more polymorphic, perhaps due to much higher number of accessions analysed. Our isoenzyme results support the suggestion of Smith (1981) that *B. interruptus* may be interpreted as an ecotype subspecies (crop contaminant) of *B. hordeaceus*. Intraspecific allozyme variation observed in *B. hordeaceus* was independent from the morphological differentiation which recognized three ecotypes-subspecies in *B. hordeaceus*. Similar findings were reported by Ainouche and Bayer (1996) in their recent paper.

Stebbins (1981) concluded on the basis of the cytogenetic data that *B. hordeaceus* and *B. lanceolatus* are allotetraploids. Our data about fixed heterozygosities at several isoenzyme loci in all studied tetraploids, including the two above mentioned species, suggest their hybrid origin and support the hypothesis that they all are allopolyploids. In particular, all tetraploids showed fixed heterozygosity of AAT-B, PRX-F, PGD-A and PGI-A. Intraspecific polymorphism of PRX-F and PGI-A in *B. secalinus* and *B. commutatus* and of AAT-C in *B. hordeaceus* may reflect inactivation of one homoeoallele of a composite genome among accessions of these tetraploids. The diploid ancestor of one genome of polyploids, however, remains unknown, since homoeozymes ADH-A5, AAD-C1, AAT-C2, PRX-F7 and PGI-A6 of polyploids were not detected among the diploids studied.

4.3. Phylogenetic relationships and genetic differentiation among diploid annual bromes

A characteristic feature of the diploid annual bromes examined, in contrast to polyploids, was a total absence of heterozygous allozyme phenotypes of most heterozygotes except PGD, despite of a considerable number of individuals analyzed and the existence of intraspecific allozyme polymorphism of several heterozygotes (III, Table 1). This result is in accord with the data about autogamy and self-fertilization of annual bromes (Smith, 1972) and shows prevalent inbreeding in the natural populations of all diploids examined. No case of heterozygous isozyme phenotype was recorded in the accessions *B. intermedius* and *B. squarrosus* studied, in contrast to a recent report of Ainouche *et al.* (1995).

PGD was exceptional among the enzymes analyzed by showing fixed heterozygosity with permanent three-banded phenotypes in several diploids. Out of ten species studied, only *B. alopecuroides* and *B. scoparius* from the type section and *B. sterilis* and *B. tectorum* from the section *Genea*, had homozygous variant A2. The most plausible explanation for the permanent occurrence of a symmetrical three-banded, heterozygous phenotype of dimeric 6-PGD in the autogamous diploids is that it is encoded by a duplicated locus with divergent alleles. It is likely that the permanent three-banded phenotype reflects heterozygosity due to gene duplication with concurrent modification and the appearance of a modified, paralogous locus encoding heterozygote PGD-B.

The important consequence of prevalent self-fertilizing breeding system in the diploid bromes examined is their intraspecific differentiation into distinct multilocus allozyme genotypes maintained as multilocus allozyme (isozyme) lineages. Thus, isoenzyme analyses revealed two isozyme lineages in *B. alopecuroides* with different allozymes of 3 heterozygotes (SOD-B, PRX-F and PGI-A) and in *B. scoparius* with differences in two allozymes of ADH-A and SKD-A. *Bromus arvensis* from Turkey also showed two isozyme phenotypes (differentiated by SKD-A and PGI-A) which both differed from the European accessions in AAT-B allozymes (III, Table 1).

Accessions of *B. sterilis*, from Turkey, displayed three isozyme lineages, which differed only in PGI-A allozymes. The European accessions were identical with the *B. sterilis* lineage T2 from Turkey, except some having PRX-F6 instead of common PRX-F8. All accessions of *B. tectorum* from Turkey were monomorphic, whereas some accessions from Europe had only one additional allozyme MDH-A2. Six Turkish accessions which could be attributed to *B. tectorum* ssp. *lucidus* by general morphology (synaptospermous, but still with lower glume 1-veined) had absolutely identical isozyme phenotype to *B. tectorum* subsp. *tectorum*. This evidence is consistent with the conclusion of Sales (1991) who reduced *B. sericeus* to *B. tectorum* subsp. *lucidus* on the basis

of the morphological and reproductive characters, favouring even its lower taxonomic rank as a mere morphological variety.

The accessions attributed to the *B. japonicus-squarrosus* complex by morphology displayed homologous variation with shared morphs at several heterozygotes. In general, taxonomists have recognized that *B. japonicus* is a very variable taxon with at least two subspecies: typical subsp. *japonicus* and subsp. *anatolicus*, the latter almost exactly fitting the description of *B. squarrosus* (Smith, 1980, 1985a). Our experience with growing voucher reproductions shows the extremely great influence of the environmental conditions on the bromegrass phenotype. For example, an original accession of *B. japonicus* collected in Turkey had a lower lemma 10×6 mm and uppermost spikelet 20 mm, while its reproduction in Estonia had a lower lemma 13×8 mm and uppermost spikelet 35 mm, thus resembling more *B. squarrosus*. There seems to be a continuous range of variation from *B. japonicus* to *B. squarrosus*. A set of eleven Turkish accessions were morphologically intermediate between typical *B. japonicus* and *B. squarrosus* (designed as jap-sq in Table 1 of III). They were differentiated into two isozyme lineages. The data show that genetic differentiation in the complex by allozymes is independent of differentiation by morphology.

The allozyme data, thus, suggest that *B. japonicus* and *B. squarrosus* are closely related not only morphologically, but also genetically. The complex needs more detailed investigations to decide about a correct taxonomic rank of *B. japonicus* and *B. squarrosus*, but our allozyme data favour their intraspecific status.

Isoenzyme evidence about three unique allozymes in its electrophoretic phenotype clearly shows that *B. pumilio* stands apart from the other diploids of the section *Bromus*, where it has been put in some treatments (Smith, 1970). This is in accord with its divergence by morphology and dispersal mechanism (Smith, 1985b). The suggestion to put *B. pumilio* in its own section *Boissiera* (Smith, 1985b) is thus supported by our allozyme data. Contrary to a report about the tetraploid chromosome number for *B. pumilio* (Smith, 1972), our isoenzyme results showed that *B. pumilio* should be a diploid species. This was confirmed by showing that our accession of *B. pumilio* has $2n = 14$ (Laarmann, 1997, unpublished). The diploid chromosome number was also given by Avdulov (1931).

Bromus intermedius was the most polymorphic species among the diploids studied, revealing six isozyme lineages. Despite of remarkable allozyme variability, all accessions of *B. intermedius* examined were morphologically quite uniform with slender, tangled panicle branches and hairy spikelets with recurved awns, as described by Smith (1985a). Remarkable is that reproductions of Turkish accessions of *B. intermedius* in Estonia didn't show such a strong effect of environmental growing conditions on a plant phenotype as it was observed in the case of *B. japonicus-squarrosus*.

The ten heterozymes of 8 enzymes examined revealed 36 shared and 14 unique morphs (putative allozymes) of different occurrence frequency and pattern within and among the species. Heterozymes differed remarkably in the extent of genetic variability, AAT-C and SOD-B were the most conservative heterozymes, with one morph in common to most species. PRX-F, PGI-A and PGD-A were the most variable heterozymes with 9, 5 and 4 morphs, respectively.

Cladistic analysis of the allozymic variation pattern among the species and their isozyme lineages was performed on the basis of the presence/absence (0/1) data matrix of 36 phylogenetically informative, shared allozyme characters, compiled from the data in Table 1. Analysis of the data matrix with Pee-Wee gave 18 trees of best fit, having a length of 87 steps and a total fit of 178.9. The consensus tree of the 18 fittest trees (III, Fig. 1) shows that *B. pumilio* is linked to *Hordeum bogdanii* as the outgroup species and is basally paraphyletic with respect to all other diploids studied. This indicates its outstanding position in the genus and supports its recognition in a separate section by Smith (1985b). Another distinctly differentiated species among annual diploids is *B. danthoniae*, which is placed basally paraphyletic to the other species of the section *Bromus*. Decay indexes of the *B. danthoniae* clade from the sister clades of the *B. japonicus-squarrosus* complex and of *B. pumilio* are 3.4 and 4.3, respectively. Smith (1985b) also supposed that *B. pumilio* is most closely related to *B. danthoniae* and *B. alopecuroides* in the genus. The clear allozyme differentiation of *B. danthoniae* is congruent with its divergence by a unique morphological character (this species has three-awned lemmas) in the section *Bromus*.

The *Bromus japonicus-squarrosus* complex has a central position on the allozyme cladogram, but was not clearly resolved into two different species. Morphologically intermediate allozyme lineage *japonicus-squarrosus* T2 together with the typical *B. japonicus* from the Central Russia are basal in the consensus cladogram and are only weakly (DI only 0.4) differentiated from the intermediate lineage T1 and the typical *B. squarrosus* lineages T1 and T2.

The six morphologically cryptic allozyme lineages within *B. intermedius* are less differentiated from the *B. japonicus-squarrosus* complex (DI = 0.8), being divided into two closely related groups and mixed with *B. arvensis*.

Ainouche and Bayer (1997) also found that the species couples *B. japonicus* – *B. squarrosus* and *B. intermedius* – *B. arvensis* composed the group with not well resolved position on the ITS — based phylogenetic tree.

Bromus alopecuroides and *B. scoparius* of the section *Bromus* are linked on the allozyme cladogram as sister species and formed one subclade with *B. sterilis* and *B. tectorum* belonging to the section *Genea*. *Bromus alopecuroides* seems to be less diverged from the *B. japonicus-squarrosus* complex, than *B. scoparius*, with the Bremer's supports (DI values) 1.0 and 3.8, respectively.

Section *Genea* is recognised on the cladogram as a monophyletic group which is well diverged from central *B. japonicus-squarrosus* complex (DI = 4.1) and is linked to the *B. alopecuros* – *B. scoparius* clade. The cladogram shows *B. tectorum* and *B. sterilis* T2 lineage as sister species, supporting their close phylogenetic affinity. The cpDNA data also suggest that *B. sterilis* and *B. tectorum* are recently derived species that share a common maternal ancestry (Pillay and Hilu, 1995). Sales (1993) suggested that section *Genea* is linked with the type section via *B. pectinatus* which was not available for our study.

Bromus pumilio and *B. danthoniae* are two basally ancestral brome species on the phylogram that revealed the duplication of the PGD locus with PGD-AB2/3, whereas the outgroup species, perennial *Hordeum bogdanii* showed no duplication. The same duplication was found in most of the diploids studied. This indicates that the duplication with the emergence of paralogous PGD-A2 has occurred in *B. pumilio* and is maintained in most diploid bromes. Only *B. alopecuros*, *B. scoparius* and diploids of the section *Genea* (*B. sterilis* and *B. tectorum*) which fell within the same, derived subclade on the cladogram again lacked the duplication and had only PGD-A2. This suggests inactivation of the duplicated PGD-B3 in this derived subclade. Further divergence of duplicated PGD-AB with the appearance of PGD-A1 and PGD-B4 was detected for *B. squarrosus* T2 and *B. pseudosecalinus*, respectively.

Phenetic analysis was performed on the basis of the presence/absence data matrix of the 36 shared plus 14 unique allozymes compiled from Table 1 of III, by applying Manhattan distance with the UPGMA method of clustering.

The allozyme phenogram (III, Fig. 2) is rather similar to the cladogram in the general topology by recognizing similar clusters of related species; but supplementing it by evaluating the extent of genetic divergence between the species and allozyme lineages. Thus, *B. pumilio* is distinguished in a separate phenetic branch, so as it also appeared in a separate clade on the cladogram.

The phenogram shows that *B. japonicus* CR and *B. squarrosus* T1 are the least divergent couple which is linked with other allozyme lineages of the *B. japonicus-squarrosus* complex in the same cluster. *Bromus danthoniae* is linked with the *B. japonicus-squarrosus* cluster at a higher divergence distance. Like on the cladogram, the *B. intermedius*-*B. arvensis* couple is joined in a separate clade which is linked with the *B. japonicus-squarrosus* cluster at a higher divergence distance. Diploids *B. sterilis* and *B. tectorum*, belonging to the section *Genea* appear on both cladistic and phenetic trees in a separate cluster which is linked to the section *Bromus* through the *B. alopecuros*.

The most significant difference between the phenogram and cladogram is in the position of *B. scoparius* which is, according to the phenogram, the most divergent species among the annual diploids studied, supported by unique allozymes ADH-A7, SKD-A3 and SOD-A6, followed by *B. pumilio* with unique allozymes AAT-B6, PRX-F1 and PGI-A7 (III, Table 1).

4.4. Geographic distribution of the allozyme variation in *Bromus tectorum* and *B. sterilis*

Bromus sterilis and *B. tectorum* may be considered as successful weedy colonizers which have occupied large territories to both east- and westward from their presumable Near-East centre of origin. The isoenzyme data in Table 1 of IV characterize the genetic heterogeneity among the populations of different geographic origin within the *B. tectorum* and *B. sterilis*. The accessions from Turkey and Syria within their native East-Mediterranean area displayed more interpopulational variability than accessions from other regions. For example, *B. sterilis* and *B. tectorum* from Turkey revealed four multilocus isozyme lineages, whereas numerous accessions from Germany had only two lineages. The number of accessions analysed from these regions was almost equal. On the basis of the polymorphic EST-E and EST-F data, *B. tectorum* from Syria is divided into four multilocus allozyme lineages, whereas *B. tectorum* from France has only two lineages. Levels of genetic variation are, however, geographically heterogeneous.

Many of the populations within the species appear to belong to identical multilocus lineages in different geographic regions, having different ecological conditions. Thus, the most common *B. tectorum* allozyme genotype from Turkey was also found in Syria, France, Germany, Spain, Hungary, Greece and Slovakia. Another common *B. tectorum* genotype was found in Turkey, Germany, Syria and Central Russia (Fig. 3). A common genotype of *B. sterilis* was found everywhere throughout its Eurasian distribution (Fig. 2).

Two contrasting explanations of the weedy adaptation have been proposed (Baker, 1965, 1974; Bradshaw, 1965; Barrett and Richardson, 1986). According to one, the effective colonization relies on the extensive genetic polymorphism needed for the successful colonization throughout large territories with the development of locally adapted "specialist" genotypes. Another colonization strategy relies on the development of a limited number of "general purpose" genotypes which are adaptable to a wide range of environmental conditions due to their significant phenotypic plasticity.

Only little intrapopulational allozymic variability was observed in the brome diploids throughout their huge distribution ranges. Thus, only eight populations out of total 109 *B. tectorum* and *B. sterilis* examined revealed genetic variability with rare additional ADH-A, MDH-A, AAT-B, AAT-C and SOD-B allozymes. Similarly, Novak, Mack & Soltis (1991) also detected only low allozyme variation within *B. tectorum* populations in North America. Eurasian populations of *B. tectorum*, however, exhibited greater genetic allozyme differentiation than those in North America, and most differentiated were populations from SW Asia, the presumable native range of this species (Novak & Mack, 1993).

The population genetic structure established for many self-pollinating, weed species clearly indicates that a high level of genetic variation is not a prelimi-

nary condition for successful colonization (Barrett and Shore, 1989). The low level of genetic variation in *B. tectorum* and *B. sterilis* is similar to that reported for other autogamous plant species. For example, autogamous weedy grasses of *Setaria* spp. showed low genetic diversity (Wang *et al.*, 1995 a, b) in six different countries from both the Old and New World. Selfing allotetraploid *Elymus canadensis* revealed low allozyme variation without relationship to climatic differences between the regions of origin (Sanders *et al.*, 1979). Very low levels of allozyme polymorphism were found in five colonizing North American weed species which sharply contrasted with their substantial inter- and intrapopulational variation in morphology and phenology (Warwick, 1988).



Figure 2. Geographic distribution of allozyme lineages of *Bromus sterilis*. Lineage D is common everywhere, lineages E and F from France and Germany, respectively, are new lineages not found in Near East — the presumable centre of origin of the species.

On the contrary, Nevo *et al.* (1979, 1986 a, b) revealed rich allozyme variation in *Hordeum spontaneum* from Turkey, Iran and Israel and showed that genetic variation in wild barley populations is correlated with the local environment and is partly adaptive. Investigations of *Avena barbata* from Israel (Kahler *et*

al., 1980) and California (Clegg and Allard, 1972; Hamrick and Allard, 1972) also have shown that allozyme diversity is greater in SW Asian populations and is correlated with the environment.

Our results also indicate the presence of greater allozyme variability in the central part of the distribution area (Turkey, Syria). Marginal populations (West Europe, Central Russia) were less variable. The same tendency was also marked for *Hordeum jubatum* (Shumaker and Babble, 1980) and for *H. secalinum* (Cronberg *et al.* 1996). The allozyme variation patterns for *B. tectorum* and *B. sterilis* showed that migration from their native Near East region and adaptation to the different ecological conditions were not accompanied by the appearance of considerable genetic divergence. Thus, the presence of a common allozyme genotype of *B. sterilis* throughout its Eurasian distribution and two most common genotypes of *B. tectorum* suggests that the “general purpose” genotype paradigm of weedy colonization could be applicable in case of the two taxa studied.

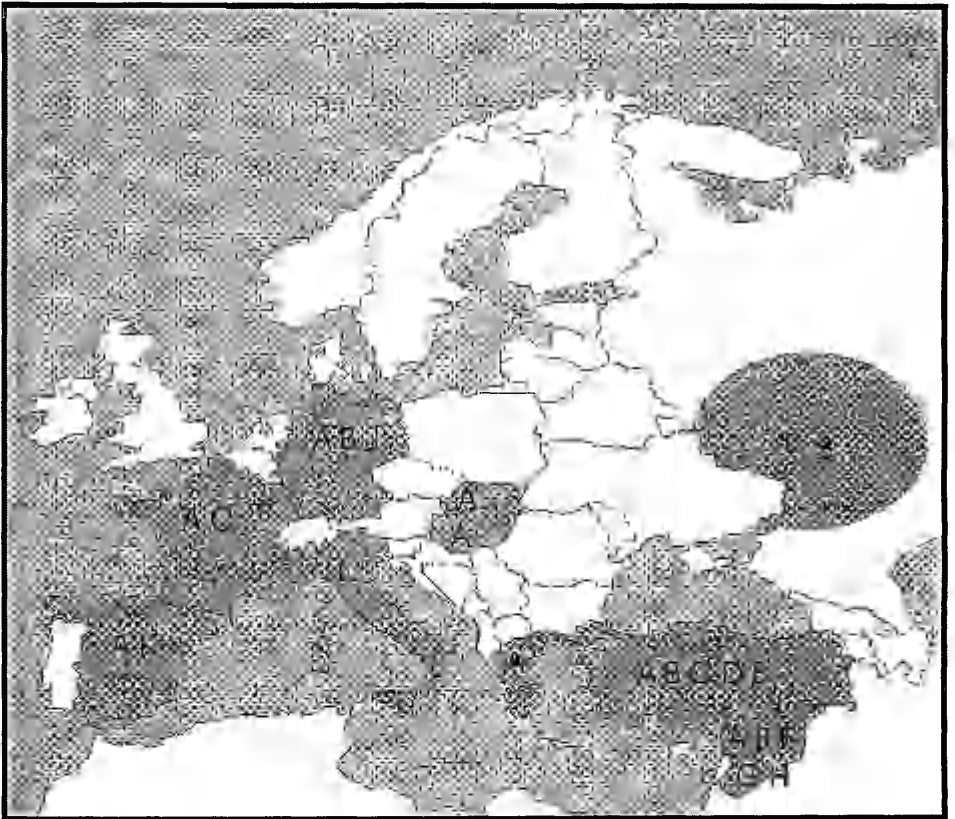


Figure 3. Geographic distribution of the multilocus allozyme lineages of *Bromus tectorum*. Lineages A and B are two lineages more common everywhere, lineages J, K and L are new lineages from Germany, Spain and Italy, respectively, not found in Near East — the presumable centre of origin of the species.

The data presented here demonstrate the usefulness of isozyme gene markers for biogeographic investigations, but more extensive sampling both within and among accessions from different parts of the ranges is needed for more sound conclusions.

5. CONCLUSIONS

Isoenzyme characters of eleven enzymes were analysed for 21 annual brome species, belonging to sections *Bromus*, *Genea* and *Boissiera* of the genus *Bromus*.

The results obtained demonstrate that isoenzymes are good molecular markers for genetic differentiation of brome grass species. Thus, 13 species out of 21 studied could be unambiguously distinguished by allozymic phenotypes.

Only two morphologically very similar species pairs: *B. diandrus* – *B. rigidus* (section *Genea*) and *B. japonicus* – *B. squarrosus* (section *Bromus*) revealed identical zymograms, in agreement with their close relationships by morphology, and indicating that their ranking into separate species remains questionable. The *B. japonicus* – *squarrosus* complex was also not clearly resolved into separate clades on the allozyme cladogram. The other two species pairs of the type section, *B. hordeaceus* – *B. interruptus* and *B. arvensis* – *B. intermedius*, showed partially overlapping isozyme phenotypes.

All ten diploids studied revealed a total absence of heterozygous allozyme phenotypes of most heterozymes except PGD, despite of a considerable number of individuals analyzed and the existence of intraspecific allozyme polymorphism of several heterozymes. This results show the prevalence of self-fertilizing breeding system in the natural populations of diploid bromes.

PGD was exceptional among the enzymes analyzed by showing fixed heterozygosity in several diploids. Only *B. alopecuros* and *B. scoparius* from the type section and *B. sterilis* – *B. tectorum* from the section *Genea*, had a homozygous phenotype. The most plausible explanation for the fixed heterozygosity of dimeric 6-PGD in the autogamous diploids is that it is encoded by two duplicated paralogous loci with divergent alleles.

The important consequence of prevalent self-fertilizing breeding system in the diploid bromes examined is their intraspecific differentiation into distinct multilocus allozyme genotypes maintained as multilocus allozyme (isozyme) lineages.

Section *Genea* is recognised on the cladogram as a monophyletic group which is well diverged from central *B. japonicus* – *squarrosus* complex.

Unique allozyme phenotype of *B. pumilio* and its basally paraphyletic position on the cladogram supports its outstanding position in the section *Bromus*, and its putting in a separate section *Boissiera*.

Another distinctly differentiated species among annual diploids is *B. danthoniae* which is placed basally paraphyletic to the other species of the section *Bromus*. This is congruent with its divergence by a unique morphological character (this species has three-awned lemmas) in the section *Bromus*.

Bromus intermedius was the most polymorphic species among the diploids studied, revealing six isozyme lineages. Despite of remarkable allozyme vari-

ability, all accessions of *B. intermedius* examined were morphologically quite uniform.

Bromus pseudosecalinus differed from *B. secalinus* in having different morphs of MDH-A and AAT-B, suggesting it is unlikely to be the diploid ancestor of this tetraploid.

Our data revealed fixed heterozygosity at several isoenzyme loci in all studied tetraploids, suggesting a hybrid origin and supporting the hypothesis that they all are allopolyploids. In particular, *B. alopecuros* could be one of the genome donors for the tetraploid *B. lanceolatus*.

The PRX isoenzyme data indicated that *B. sterilis* could be a genome donor for the polyploid species *B. madritensis* and the *B. diandrus* – *rigidus* complex, and *B. tectorum* for *B. rubens*. *Bromus tectorum* was monomorphic for a unique allozyme B3 of AAD-B, which argues against this diploid as the donor of a full genome to any of the *Genea* polyploid species.

The morphologically similar polyploids *B. madritensis* and *B. rubens* (section *Genea*) differed in seven heterozymes out of fourteen studied, supporting their specific recognition.

Tetraploids *B. secalinus* and *B. commutatus* (section *Bromus*) had very similar enzyme phenotypes, indicating their strong genetic affinity, but still differed in PRX-F homoeozymes of one composite genome.

Intraspecific allozyme variation observed in *B. hordeaceus* was independent from the morphological differentiation which recognized three ecotypes-subspecies in *B. hordeaceus*.

The isoenzyme results support the suggestion that *B. interruptus* may be interpreted as an ecotype subspecies (crop contaminant) of *B. hordeaceus*.

The accessions of *B. tectorum* and *B. sterilis* from Turkey and Syria within their native East-Mediterranean area displayed more interpopulational variability than accessions in marginal regions. Many of the populations within this two species appear to belong to identical multilocus lineages in different geographic regions, with different ecological conditions. Thus, the most common *B. tectorum* allozyme genotype from Turkey was also found in Syria, France, Germany, Spain, Hungary, Greece and Slovakia. Another common *B. tectorum* genotype was found in Turkey, Germany, Syria and Central Russia. A common genotype of *B. sterilis* was found everywhere throughout its Eurasian distribution.

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ISOENSÜÜMTUNNUSTE VARIEERUVUS JA FÜLOGENEETILISED SEOSSED EURAASIA ÜHEAASTASTEL LUSTETEL (*BROMUS* L., POACEAE)

Kokkuvõte

Kõrreliste perekond luste (*Bromus* L.) on taksonoomiliselt keeruline taimerühm, kus on rohkesti nomenklatuurseid ja fülogeneetilisi probleeme. Perekonda kuulub umbes 130 liiki, ühe- ja mitmeaastaseid, diploidseid ja polüloidseid, mis on levinud laialt Euraasias, Põhja- ja Lõuna-Ameerikas ning Austraalias. Paljud liigid on edukad kolonisaatorid ja umbrohud. Siamaani pole suudetud luua perekonna üldtunnustatud klassifikatsiooni. Eri uurijad on jaotanud perekonna vastavalt kasutatud tunnuste loomule kas seitsmeks sektsiooniks, alamperekonnaks või isegi seitsmeks perekonnaks. Paljud kahtlevad perekonna taksonite monofüleetilises päritolus.

Suure morfoloogilise plastilisuse ja liikide vähe uuritud geneetiliste seoste tõttu on sama palju probleeme liikide identifitseerimisega sektsioonides. Paljud liigid on morfoloogiliselt väga sarnased, mõnesid vaadeldakse kompleksidena või on pakutud neid liita. Osal liikidel on hulk eri ajal erinevate autorite pakutud sünonüüme, samas liigikirjeldused on kattuvad. See kõik teeb perekonna keeruliseks, kuid samas väga huvitavaks uurimisobjektiks.

Oma uuringutes kasutasime morfoloogia täienduseks isoensüümtunnuseid, sest elektroforeetiline isoensüümanalüüs on meetod, mis võimaldab võrdlemisi lihtsalt ja ruttu analüüsida paljude indiviidide geneetiliselt vahetult määratud valgulisi fenotüüpe. Seega lubavad fenotüüpsed isoensüümtunnused selgitada liikide ja populatsioonide genotüüpsid seosid.

Uuriti elektroforeetiliste isoensüümtunnuste liigisisese ja liikidevahelise varieeruvuse seaduspärasusi lustete perekonna *Bromus* L. Euraasia üheaastaste liikide erinevat geograafilist päritolu proovidest. Selgitati uuritavate liikide geneetilist diferentseerumist seoses nende mikroevolutsiooni ja geograafilise levikuga. Leiti, et isoensüümtunnused sobivad hästi lustete fülogeneetiliseks uurimiseks. Uuritud 11 ensüümi (ADH, AAD, MDH, SKD, AAT, SOD, PRX, PGD, PGI, LAP, EST) lubavad teha järgnevad järeldusi.

Selgitati liigispetsiifilised allosüümid ja isoensüümfenotüübid, mis võimaldavad eristada 13 liiki 21 uuritust. Ainult kaks morfoloogiliselt omavahel väga sarnast liigipaari, *B. diandrus* – *B. rigidus* sektsioonist *Genea* ja *B. japonicus* – *B. squarrosus* sektsioonist *Bromus*, osutusid eristamatuks, mis pakub uut tõestust nende geneetilisest lähedusest, kuid samas teeb küsitavaks nende liigilise staatuse.

Kõigil uuritud diploididel puudusid isoensüümfenotüüpides täielikult heterostügooidid, mis viitab domineerivale autogaamiale ja iseviljastumisele ning liigisisesele divergentsile multilookuselisteks genotüüpideks, mida saab tuvastada isoensüümfenotüüpide kaudu.

Bromus pumilio unikaalne isoensüümfenotüüp ja basaalne eristumine kladogrammil on täielikult kooskõlas selle liigi morfoloogiliste iseärasustega ja toetab tema eraldamist omaette seksiooni *Boissiera*.

Tüüpseksiooni liikidest osutus *B. danthoniae* teistest selgesti erinevaks ja basaalselt parafüleetiliseks kladogrammil, mis on kooskõlas tema eristumisega unikaalse morfoloogilise tunnuse — kolmeohtelise sõkla järgi.

Seksiooni *Genea* kuuluvad diploidid *B. tectorum* ja *B. sterilis* moodustasid eraldi klasteri nii kladogrammil kui fenogrammil, tunnistades seksiooni *Genea* monofüleetilist päritolu. Nende liikide populatsioonid erinevatest Euraasia paikadest näitasid diferentseerumist allosüümseteks liinideks (*B. tectorum* 11-ks ja *B. sterilis* 6-ks). Geograafiliselt perifeersetele Euroopa populatsioonidele olid iseloomulikud ainult mõned allosüümised liinid paljudest, mis on levinud Lähis-Idas, nende liikide oletatavas päritolukeskuses. Seega võib oletada, et liikide *B. tectorum* ja *B. sterilis* puhul on umbrohtudena laiemas levikus edukaks osutunud vaid üksikud valitud genotüübid.

Kõik uuritud polüploidid näitasid mitmeid erinevaid fikseeritud heterostügootseid isoensüümfenotüüpe, toetades sellega hüpoteesi nende sõltumatust allopolüploidsest päritolust eri diploidide vahelistest hübriididest. Morfoloogiliselt väga sarnased polüploidid *B. madritensis* ja *B. rubens* (seksioon *Genea*), mida on pakutud ühendada üheks liigiks, erinevad isoensüümtunnuste alusel oluliselt, mis põhjendab nende tunnustamist erinevate liikidena. Samuti väga sarnased tüüpseksiooni tetraploidid *B. commutatus* ja *B. secalinus* näitasid peaaegu identseid ensüümfenotüüpe siiski selge erinevusega ühes heterostüümis.

Diploid *B. alopecuros* sobib tetraploidi *B. lanceolatus* ühe genoomi doonoriks. Vastupidi, *B. pseudosecalinus*, mis morfoloogilise sarnasuse poolest võiks olla tetraploidi *B. secalinus* diploidseks eellasteks, isoensüümtunnuste järgi selleks ei sobi.

Tuvastati 6-PGD geeni duplitseerumine ja divergents mitmetel tüüpseksiooni diploididel, seksiooni *Genea* kuuluvatel liikidel seda ei leitud.

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Isoenzyme data on the genetic divergence and allopolyploidy in the section *Genea* of the grass genus *Bromus* (Poaceae)

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Electrophoretic variation of isoenzyme characters of ten enzymes (ADH, AAD, MDH, SKD, AAT, SOD, PRX, PGD, PGI, LAP) has been studied in five brome species, comprising the section *Genea* of the genus *Bromus*. The three polyploids, *B. madritensis*, *B. rubens* and *B. diandrus* (incl. *B. rigidus*), exhibited different fixed heterozygosities of several heterozymes, suggesting their allopolyploid nature. The morphologically similar polyploids *B. madritensis* and *B. rubens* differed in seven heterozymes out of fourteen studied, supporting their specific recognition. The two diploids, *B. sterilis* and *B. tectorum*, differed in four heterozymes. One genome of the polyploid species corresponded closely to that of *B. sterilis*. All species of the section could be distinguished from each other by diagnostic isoenzyme phenotypes. *Bromus diandrus* and *B. rigidus* revealed identical zymograms, providing new support for their conspecific recognition.

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Section *Genea* Dum. of the genus *Bromus* L. comprises weedy, annual brome grasses widely distributed in Mediterranean countries, SW Asia, and northern Europe. It includes the following species, which display considerable variation in karyotypes and ploidy levels, including intraspecific ploidy cytotypes: *B. sterilis* L. ($2n = 14$), *B. fasciculatus* Presl ($2n = 14$), *B. tectorum* L. ($2n = 14$), *B. rubens* L. ($2n = 28$), *B. madritensis* L. ($2n = 28, 42$), *B. diandrus* Roth ($2n = 28, 42, 56$), *B. rigidus* Roth ($2n = 28, 42, 56, 70$) (CUGNAC 1934, 1937; KNOWLES 1944; OVADIAHU-YAVIN 1969; TZVELEV 1976; STEBBINS 1981). Of the diploids, *B. sterilis* is basically a Euro-Siberian species, whereas *B. tectorum* is of Euro-Siberian and Irano-Turanian distribution, *B. fasciculatus* is confined to the E Mediterranean and western parts of SW Asia. The polyploids are mainly or exclusively Mediterranean taxa. They often share the same habitat and grow intermixed and sympatrically. The existence of intermediate types makes identification difficult, and morphological characters may not alone permit resolution of these species. The great morphological diversity within the group will give the impression that there is a continuous range of variation from one extreme of the section to the other (SMITH 1970, 1972, 1980, 1981, 1985, 1986; ESNAULT 1984; ESNAULT and HÜON 1985; SALES 1991, 1993, 1994).

Polyploidy has played a significant role in the evolution of the section *Genea*, as well as in other

sections of the genus *Bromus*. Cytogenetic studies of artificial interspecific hybrids in the sections *Bromus* and *Pnigma* have established allopolyploid nature of the polyploids (ARMSTRONG 1977, 1981). However, no similar cytogenetic evidence is available with respect to the *Genea* species.

Isoenzymes have successfully been employed to establish allo- versus autoploid nature of plant polyploids, and to identify their putative diploid progenitors (CRAWFORD 1985; SOLTIS and SOLTIS 1993). Fixed heterozygosity of isoenzymes in allopolyploids is used as a criterion to distinguish them from autoploids. Fixed enzyme heterozygosity in allopolyploids is maintained by diploidization of their composite genomes with a resultant disomic inheritance, whereas autoploids show polysomic inheritance of isoenzymes (SOLTIS and RIESEBERG 1986).

KAHLER et al. (1981) found that the *Genea* species could be unambiguously distinguished by starch gel electrophoretic phenotypes of four enzymes: aspartate aminotransferase, phosphoglucosomerase, peroxidase and esterase. They suggested that other isoenzyme systems may uncover additional differences and may allow the determination of the extent of variation.

The purpose of the present study was to find additional diagnostic isoenzymes for the identification of *Genea* species and for the evaluation of genetic divergence. Isoenzymes are used as genetic

markers to determine the allo- or autoploid nature of the *Genea* polyploids and the likelihood that *B. sterilis* and *B. tectorum* are their diploid progenitors.

Materials and methods

Plant material

Most of the seed accessions, collected in the wild from known locations, were received from botanical gardens. Some accessions were collected in Azerbaijan, Georgia, Kirghizia, Northern Ossetia, Syria, and Spain by Vello Jaaska and personal acquaintances of the authors.

1. *B. tectorum*: 24 accessions, originating from Germany (7), Italy (1), Greece (1), Slovakia (1), Czechia (1), Spain (2), Syria (2), Northern Ossetia (1), Kirghizia (4), Hungary (1), Switzerland (1).

2. *B. sterilis*: 21 accessions, originating from France (7), Belgium (4), Azerbaijan (1), Germany (7), Greece (1), and Spain (1).

3. *B. rubens*: 1 accession, originating from Spain.

4. *B. madritensis*: 10 accessions, originating from Belgium (3), Italy (4), France (2), and Georgia (1).

5. The *B. diandrus* — *B. rigidus* complex: 18 accessions, originating from Belgium (4), France (5), Italy (5), Holland (2), Portugal (1), and Turkey (1).

Seed accessions of *B. fasciculatus* appeared to be not available for this study.

Most seed accessions were grown in order to verify their identity. Vouchers are deposited at TAA, Herbarium of the Institute of Zoology and Botany (Tartu, Estonia).

Isoenzyme analysis and nomenclature

Enzyme extracts were prepared from the shoots (the primary leaf with the coleoptile) of 5–10 days old, etiolated seedlings, subjected to electrophoresis in vertical polyacrylamide gel slabs and stained as described in JAASKA and JAASKA (1986, 1990). Four individual seedlings of each accession were routinely analyzed for all isoenzymes. Up to eight individuals were analyzed for some enzymes and accessions, including the sole accession of *B. rubens*, to check reproducibility. The nomenclature distinguishing genetically heterologous, homoeologous, and homologous isoenzymes, abbreviately

heterozymes, homoeozymes, and allozymes, respectively, is followed (JAASKA and JAASKA (1984). Heterozymes, i.e., isoenzymes encoded by separate loci of a diploid genome, are designated by capital letters followed by a number, reflecting allozymic variants in the order of decreasing mobility. Electrophoretically different isoenzymes encoded by homologous loci of composite genomes of polyploids, evidencing their genetic homoeology, i.e., only partial homology of genomes, are named homoeozymes. Heterozygous phenotypes are designated by codominant isozymes separated by a diagonal line, i.e., as a fraction. Term "fixed heterozygosity" is used when heterozygous phenotypes of isoenzymes were observed for all analyzed individuals of polyploids without segregation into homozygous phenotypes.

Results

The data on the isoenzyme variation among the six brome grass species are summarized in Table 1. The zymograms of the taxa studied are presented schematically in Fig. 1 (a–j). Their genetic interpretation is based on the available information about subunit structure of the enzymes studied and follows the principles described by WENDEL and WEEDEN (1989). Heterozygous phenotypes of dimeric enzymes could be distinguished on the zymograms as symmetrical triplets with codominant isoenzyme variants as flanking bands, whereas monomeric enzymes showed two-banded heterozygous phenotypes.

Aliphatic alcohol dehydrogenase

ADH, EC 1.1.1.1, zymograms (Fig. 1a) showed three phenotypes of the constitutive heterozyme ADH-A. The diploids *B. sterilis* and *B. tectorum* had the same homozygous isoenzyme A4. The polyploids, except *B. madritensis*, showed a fixation of heterozygosity for A3/4. *B. madritensis* revealed the homozygous band A3, not recorded among the diploids. Sometimes, additional, faster, and fainter bands of anaerobically inductive ADH isoenzymes were observed, but they were not recorded because of their inconsistent appearance.

Aromatic alcohol dehydrogenase

AAD, EC 1.1.1.90 and 1.1.1.91, zymograms (Fig. 1b) revealed three heterozymes designated A, B, C

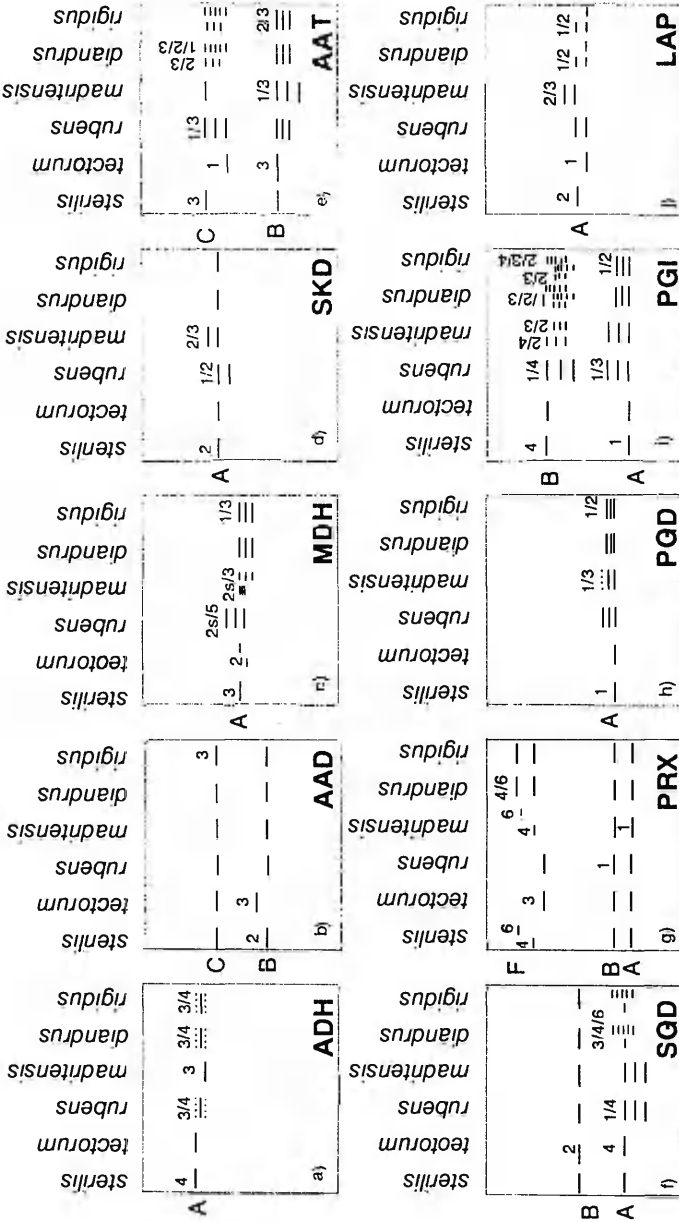


Fig. 1. Schematic phenotypes of ADH (a), AAD (b), MDH (c), SKD (d), AAT (e), SOD (f), PRX (g), PGD (h), PGI (i) and LAP (j) for six *Bromus* species. The origin at the top, the anode at the bottom (2s designates a slightly slower morph than 2).

Table 1. Electrophoretic variants of alcohol dehydrogenase (ADH), aromatic alcohol dehydrogenase (AAD), malate dehydrogenase (MDH), shikimate dehydrogenase (SKD), aspartate aminotransferase (AAT), superoxide dismutase (SOD), peroxidase (PRX), 6-phosphogluconate dehydrogenase (PGD), phosphoglucoisomerase (PGI), and leucine aminopeptidase (LAP) heterozygotes in *Bromus* species of the section *Genea*: n = the number of accessions analyzed, 2s designates a slightly slower morph than 2

Taxa	n	ADH A	AAD C	AAD B	MDH A	SKD A	AAT B	AAT C	SOD A	SOD B	PRX F	PGD A	PGI A	PGI B	LAP A
<i>sterilis</i>	21	4	3	2	3	2	3	3	4	2	6, 4	1	1	4	2
<i>tectorum</i>	24	4	3	3	3, 2	2	3	1	4	2	3	1	1	4	1
<i>rubens</i>	1	3/4	3	2	2s/5	1/2	2/3	1/3	1/4	2	3	1/3	1/3	1/4	1/2
<i>madritensis</i>	10	3	3	2	2s/3, 1/3	2/3	1/3	3	1/4	2	6	1/3	1/3	2/4, 2/3	2/4
<i>diandrus</i> & <i>rigidus</i>	18	3/4	3	2	1/3	2	2/3	2/3, 1/2/3	4 3/4/6	2	4/6	1/2	1/2	2.3, 2/3/4 1/2/3	1

in order of their decreasing mobility. They differed in their coenzyme specificity: AAD-A and AAD-B required NADP for the activity, while AAD-C was NAD-specific. The fastest band, A, was frequently too faint for an exact recording and, is not shown on Fig. 1b. *Bromus tectorum* was monomorphic for a unique AAD-B3, whereas all other species had an allozyme B2. Accordingly, AAD-B distinguishes the two diploids, and indicates that *B. tectorum* is unlikely to be the donor for polyploids. AAD-C was invariant for C3 in all taxa.

Malate dehydrogenase

MDH, EC 1.1.1.37 and 1.1.1.40, zymograms (Fig. 1c) showed the homozygous variant A3 in both diploids, *B. tectorum* and *B. sterilis*. The tetraploid *B. madritensis* revealed intraspecific variation among the accessions with heterozygous triplets A1/3 or A2s/3, whereas the related tetraploid *B. rubens* had a different heterozygous phenotype, A2s/5 (2s designates a slightly slower morph than A2). The *B. diandrus-rigidus* complex was monomorphic for a heterozygous triplet A1/3. Homoeozymes A1 and A5 of polyploids were not detected among the accessions of diploids analysed.

Shikimate dehydrogenase

SKD, EC 1.1.1.25, zymograms (Fig. 1d) revealed a two-banded heterozygous phenotype A2/3 as monomorphic in *B. madritensis*, but a different two-banded phenotype A1/2 for *B. rubens*. Both diploids and the *B. diandrus-rigidus* polyploids had the same homozygous variant A2, whereas ho-

moeozymes A3 of *B. madritensis* and A1 of *B. rubens* were not observed among the accessions of either diploid.

Aspartate aminotransferase

AAT, EC 2.6.1.1, Fig. 1e revealed two major zones of independent variable patterns, designated as heterozygote AAT-B and AAT-C, supplemented by fainter bands of several minor heterozygotes which could not always be recorded correctly. The faster major heterozygote AAT-B was homozygous and monomorphic for B3 in diploids and showed fixed heterozygosity with triplet B1/3 in *B. madritensis* and with B2/3 in other polyploids. The homoeozymes B1 and B2 of polyploids were not found in diploids. The slower isozyme AAT-C was homozygous, but different in the diploids: C3 in *B. sterilis* and C1 in *B. tectorum*. *Bromus madritensis* was homozygous and monomorphic for the same C3 allozyme, like *B. sterilis*. The other polyploids revealed fixed heterozygous triplet phenotypes: *B. rubens* — C1/3, *B. diandrus* and *B. rigidus* — C2/3. Some of the accessions of *B. rigidus* and *B. diandrus* revealed fixed double heterozygosity for five-banded C1/2/3, giving evidence for the allohexaploid nature of these accessions. The C2 of polyploids was not recorded among diploids. Thus, AAT isoenzyme phenotypes made it possible to distinguish between all taxa, except *B. diandrus* and *B. rigidus*. The *B. diandrus-rigidus* complex showed variation in AAT-C, but this was independent of their differentiation by morphology. The typical *diandrus*-like plants had broad, lax panicles, long branches, scar of rachilla segment round, whereas typical *rigidus*-like individuals were with the

narrow erect panicles, short branches, scar of rachilla segment elliptic. However, the difference between *diandrus* and *rigidus* was often so subtle that many specimens could be identified as a mere *B. diandrus* s.l.

Superoxide dismutase

SOD, EC 1.15.1.1, zymograms (Fig. 1f) showed zones of two heterozygotes: SOD-A and SOD-B. The slower SOD-B was monomorphic for B2 in all taxa. The faster SOD-A was monomorphic for the homozygous allozyme A4 in diploids and heterozygous for A1/4 in *B. madritensis* and *B. rubens*. The *B. diandrus-rigidus* complex showed variation with two phenotypes, homozygous A4 and double heterozygous A3/4/6, but, as in the case with AAT-C, this variation was not related to their morphology. Four accessions of the *B. diandrus-rigidus* complex (two *diandrus*-like and two *rigidus*-like) showed fixed double heterozygosity of SOD-A with a six-banded phenotype A3/4/6, which indicates at least their hexaploid ploidy level. Variants A1, A3, and A6, characteristic of polyploids, were not detected among the diploids.

Peroxidase

PRX, EC 1.11.1.7, zymograms (Fig. 1g) revealed several bands of different intensity and mobility. The most remarkable interspecific variation was found for the slow heterozygote PRX-F. *B. sterilis* and *B. madritensis* revealed homologous intraspecific variation with shared homozygous F6 in most accessions of both species and with F4 in some accessions of these species. *B. tectorum* and *B. rubens* shared F3. The accessions of the *B. diandrus-B. rigidus* complex all revealed heterozygous F4/6 doublet. PRX-F makes it possible to distinguish both diploids and the three polyploid species, and identifies *B. sterilis* as a genome donor for *B. madritensis*, *B. diandrus*, and *B. rigidus*, but *B. tectorum* only for *B. rubens*. The two fast isoenzymes, PRX-A and PRX-B, were invariant for the same homozygous morphs A1 and B1 common to all species.

6-Phosphogluconate dehydrogenase

PGD, EC 1.1.1.44, zymograms (Fig. 1h) showed homozygous monomorphic phenotype A1 in diploids *B. sterilis* and *B. tectorum*, heterozygous triplet phenotype A1/3 in *B. madritensis* and *B.*

rubens and a different heterozygous triplet A1/2 in the *B. diandrus-rigidus* complex.

Phosphoglucoisomerase

PGI, EC 5.3.1.9, like AAT and SOD, revealed two major band zones of independent interspecific variation pattern, designated heterozygotes PGI-A and PGI-B (Fig. 1i). The diploid species *B. sterilis* and *B. tectorum* were monomorphic for the same variants of both isozymes A1 and B4. The polyploid *B. madritensis* showed intraspecific variation of PGI-B among the accessions with two heterozygous triplets—B2/3 and B2/4, whereas *B. rubens* revealed a different fixed heterozygosity with the triplet B1/4. The *B. diandrus-rigidus* complex showed heterozygous B2/3 and double heterozygous B1/2/3 and B2/3/4 phenotypes, the latter two suggesting the hexaploid nature of some accessions. PGI-A revealed the same heterozygous triplet A1/3 for *B. madritensis* and *B. rubens*, but a different heterozygous triplet A1/2 for the *B. diandrus-rigidus* complex. The variants A2, A3 and B1, B2, B3 of polyploids were not detected among the diploids studied.

Leucine aminopeptidase

LAP, EC 3.4.11.1, revealed four electrophoretic phenotypes of heterozygote A (Fig. 1j). The diploids, *B. tectorum* and *B. sterilis*, were monomorphic for the two different variants, A1 and A2, respectively. The polyploids, *B. madritensis* and *B. rubens*, had different two-banded heterozygous phenotypes, A2/3 and A1/2, respectively. The *B. diandrus-rigidus* complex showed variation with two phenotypes, homozygous A1 and heterozygous A1/2, which was not correlated with their morphology. *Diandrus*-like and *rigidus*-like plants shared the same A1 and A1/2 phenotypes. Variant A3, characteristic for *B. madritensis* was not detected among diploids.

Discussion

The results obtained demonstrate that isoenzymes are good molecular markers for genetic differentiation of brome grass species in section *Genea* of the genus *Bromus*, confirming and extending the results of KAHLER et al. (1981). Of the thirteen putative heterozygotes specified, nine revealed interspecific differences and four (AAD-C, SOD-B, PRX-A, and PRX-B) proved invariant.

Only little interpopulational allozymic variability was observed in the diploids throughout their huge distribution ranges. Only MDH-A and PRX-F showed intraspecific variation (between accessions of different geographic origin) with two allozymes in one of the diploids, whereas all other heterozygotes were monomorphic (Table 1). Similarly, NOVAK et al. (1991) detected a low variation with only 1–2 rare allozymes in addition to the prevalent allozyme for 7 of 25 isoenzymes studied and no heterozygote individual in the North American populations of *B. tectorum*. KRZAKOWA and KRAUPE (1981), however, described both inter- and intra-populational variation with nine esterase and two aspartate aminotransferase phenotypes for natural populations of *B. tectorum* in Poland and five other European countries. This shows that isoenzymes may considerably differ in the extent and pattern of intraspecific variation in the autogamous species.

In contrast to the intraspecific isoenzyme constancy, the two diploids, *B. sterilis* and *B. tectorum*, were differentiated from each other by the following four isozymes, AAD-B, AAT-C, PRX-F, and LAP-A (Table 1). This indicates that the genetic divergence of the two diploids by the four heterozygotes has occurred at their speciation, while their subsequent weedy expansion was not accompanied by the appearance of significant allozymic variation, except rare variants of MDH-A and PRX-F.

Our isoenzyme results suggest that *B. diandrus* and *B. rigidus* are closely related not only morphologically, but also genetically, and do not deserve the rank of separate species. Out of 14 heterozygotes, 10 were monomorphic and shared in the two taxa. Variations observed in four heterozygotes (AAT-C, SOD-A, PGI-B, and LAP-A) were not related to differentiation by morphological characters. This supports the conclusion of a recent multidisciplinary investigation of SALES (1993) that *B. diandrus* and *B. rigidus* are not distinct enough (on the basis of morphological, geographical and ecological evidences) to be given a taxonomic rank higher than a variety: *B. diandrus* Roth var. *dian-drus* and *B. diandrus* Roth var. *rigidus* (Roth) Sales, respectively. Hence, they may be considered as members of a polyploid complex with tetra-, hexa-, and octoploid cytotypes (ESNAULT and HUON 1987).

Bromus madritensis and *B. rubens*, which were recorded as subspecies by SALES (1993) on the basis of their resemblance in morphological char-

acters, proved distinctly different by at least eight isoenzymes (ADH-A, MDH-A, SKD-A, AAT-B, AAT-C, PRX-F, PGI-B, LAP-A). These results show a degree of genetic differentiation between these polyploids, which merits them to be recognized as separate species.

Our data about fixed heterozygosity in the section *Genea* at several isoenzyme loci substantiate the hypothesis that all polyploids in the section *Genea*, *B. rubens*, *B. madritensis* and *B. diandrus* (incl. *B. rigidus*), are allopolyploids. Fixed heterozygosity is taken as evidence in favour of an allopolyploid nature of polyploids (SOLTIS and RIESEBERG 1986; SOLTIS and SOLTIS 1993). It should be noted that fixed heterozygosity alone is not a firm proof of allopolyploidy, but it shows the hybrid origin of the polyploid. A polyploid showing fixed heterozygosity can arise from a hybrid between different genotypes of the same diploid, i.e., is actually an autopolyploid with diploidized genomes. However, the numerous accessions of the diploids *B. sterilis* and *B. tectorum* analyzed, revealed no allozymes corresponding to the homoeozymes MDH-A1, SKD-A3, AAT-B2, AAT-B1, AAT-C2, SOD-A1, SOD-A3, PGD-A3, PGD-A2 which were observed in fixed heterozygous phenotypes of polyploids. Our results support the conclusion that the *Genea* polyploids are allopolyploids.

The appearance of several different fixed heterozygous isoenzymes in a polyploid is frequently taken as evidence for its recurrent origin from different genotypes of diploid progenitors (reviewed in SOLTIS and SOLTIS 1993). The polyploids *B. madritensis* and *B. diandrus* s.l. also showed alternative fixed heterozygosities among different accessions which could be taken as an evidence in favour of their multiple origins. However, a possibility that the multiple heterozygous phenotypes have arisen subsequent to the initial polyploidization, should also be seriously considered.

The three *Genea* polyploids had different phenotypes and fixed heterozygosities at several heterozygote loci, indicating their different genome composition and independent allopolyploid origin from different diploid progenitors. The PRX isoenzyme data indicated that *B. sterilis* could be a genome donor for the polyploid species *B. madritensis* and the *B. diandrus-rigidus* complex, and *B. tectorum* for *B. rubens*. *Bromus tectorum* was monomorphic for a unique allozyme B3 of AAD-B, which argues against this diploid as the donor of a full genome to any of the *Genea* polyploid species. Unfortu-

nately, *Bromus fasciculatus*, which is another possible candidate as diploid genome donor, was not available for the present study.

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Isoenzyme diversity and phylogenetic affinities in the section *Bromus* of the grass genus *Bromus* (Poaceae)

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Abstract

Allozyme variation of eleven heterozymes of nine enzymes was analyzed for fourteen annual brome species in the type section of the genus *Bromus* with the use of PAGE. Diploids *B. japonicus* and *B. squarrosus* had identical zymograms, providing new evidence about their very close genetic relationships. Tetraploids *B. secalinus* and *B. commutatus* had very similar enzyme phenotypes, indicating their strong genetic affinity, but still differed in PRX-F homoeozymes of one composite genome. Diploid *B. alopecuros* appears to be the donor of one genome in the tetraploid *B. lanceolatus*. *Bromus pseudosecalinus* could not be one of the diploid progenitors for *B. secalinus*. 6-PGD gene is duplicated and diverged in several diploids of the section *Bromus*. All tetraploids studied exhibited different fixed heterozygosities of several heterozymes, suggesting their allopolyploid nature. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: *Bromus*; Poaceae; Isoenzymes; Allozyme diversity; Taxonomy; Phylogenetic relationships

1. Introduction

Section *Bromus* of the genus *Bromus* comprises annual or biennial species which are characterized by loose or dense panicles of lanceolate or ovate-lanceolate, more or less terete or slightly compressed spikelets. The lower glumes are 3–5-nerved, and the upper glumes are 5–7-nerved. Lemmas are rounded on the back, rounded, angled, or rarely toothed on the margins, and have one terminal notch. Below the notch arise the awns (1–3), straight and erect or divaricate and recurved, rarely absent. The grains are linear, or elliptical, sometimes being longitudinally rolled with the groove on the

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adaxial side (Smith, 1970, 1972). The section is widespread in Eurasia and a number of species have been introduced into the New World, Africa and Australia. The number of species included in the section remarkably varies (from 30 to 40), depending on the author of the classification and on the accepted synonymy (Smith, 1970; Tzvelev, 1976). Some of the species are considered together in aggregate species in some taxonomic works, e.g. *B. mollis* aggregate (Smith, 1968), some of them are considered conspecific in some treatments, e.g. *B. commutatus* and *B. racemosus* (Wilson, 1956). They are weedy grasses, seldom found in closed communities. All species are predominantly self-fertilizing, occasional crossing maintains a high level of variation in many of the taxa (Knowles, 1944). Polyploidy is common in this section but is probably restricted to the tetraploid level (Stebbins, 1981).

This section has been regarded as a taxonomically difficult group for a long time (Smith, 1970, 1972). Many of the species are hard to identify by morphology alone, because their diagnostic features are often quantitative and subtly different or even overlapping. Their high degree of phenotypic plasticity in morphology depending on the edaphic conditions, complicate the use of morphometric characters for identification. Genetic relationships and general variation of many of the species are poorly known.

Hybridization experiments between species of the section *Bromus* and with species of other sections of the genus have been performed by Cugnac (1935), Knowles (1944) and Jahn (1959). Usually, hybridization was found impossible. It seems that genetic barriers have quite effectively isolated section *Bromus* from other sections. In addition to the best-known serological study by Smith (1970, 1972) and cytogenetic study by Stebbins (1981) some recent papers about the whole genus *Bromus* (Pillay and Hilu, 1995; Pillay, 1996) and about population genetics among the species, belonging to section *Bromus* (Ainouche *et al.*, 1995, 1996; Ainouche and Bayer, 1996) have been published. The latter study successfully used isoenzyme electrophoresis. Our previous paper about section *Genea* (Oja and Jaaska, 1996) showed that isoenzymes suits to be a good molecular markers for genetic studies among the annual brome grasses.

The goal of the present study was to analyze the differentiation among the brome grasses of this section on the basis of the isoenzyme characters and to compare allozyme diversity in their native Mediterranean area and peripheral regions (Western Europe and Central European Russia).

A correspondence of the isoenzyme differentiation with the morphological species delimitation was also analyzed. Intraspecific allozyme variability among accessions from taxa from different geographic origin were compared.

2. Materials and methods

2.1. Plant material

The seed accessions from Turkey and Central Russia were collected by the author, accessions from Armenia, Kirghizia, Uzbekistan and Northern Ossetia were kindly provided by V. Jaaska. The accessions of European origin, collected in the wild from known locations, were received from botanical gardens. They were grown in the

experimental field to have vouchers to verify identifications. Vouchers are deposited in TAA, Herbarium of the Institute of Zoology and Botany (Tartu, Estonia). The following accessions were available for this study:

B. danthoniae Trin.: Turkey (2); *B. alopecuros* Poir.: Turkey (3); *B. scoparius* L.: Kirghizia (1), Greece (1) and Central Asia (1); *B. japonicus* Thunb.: Turkey (8), Central Russia (8), Armenia (1), Kirghizia (1), Germany (1), Hungary (2) and Czech Republic (1); *B. squarrosus* L.: Turkey (10), Hungary (2), Switzerland (1), Greece (1), Central Russia (1) and Spain (2); *B. arvensis* L.: France (4) and Germany (2); *B. intermedius* Guss.: Turkey (17); *B. pseudosecalinus* P. Smith: England (1); *B. secalinus* L.: Germany (6), France (4), Switzerland (1) and Poland (1); *B. commutatus* Schrad.: Germany (3), France (3), Italy (1) and Romania (1); *B. lanceolatus* Roth.: Turkey (4); *B. pseudodanthoniae* Drob. (according to Tzvelev, 1976): Kirghizia (2), Uzbekistan (2) and Turkey (1); *B. hordeaceus* L.: France (16), Germany (15), Italy (4), Northern Ossetia (2), Spain (1), Central Russia (1), Hungary (1), Austria (1), Italy (1), Switzerland (1) and Belgium (1); *B. interruptus* (Hack.) Druce: England (2).

2.2. Isoenzyme analysis and nomenclature

Enzyme extracts were prepared from the shoots (the primary leaf with the coleoptile) of 5–10 days old etiolated seedlings, subjected to electrophoresis in vertical polyacrylamide gel slabs and stained as described in Jaaska and Jaaska (1986, 1990). Four individual seedlings of each accession were routinely analyzed for all isoenzymes. From eight to twelve individuals were analyzed for some enzymes and accessions, in order to check the constancy or polymorphism of the isoenzyme patterns. The nomenclature distinguishing genetically heterologous, homoeologous and homologous isoenzymes, is abbreviated to heterozymes, homoeozymes and allozymes, respectively, is followed (Jaaska and Jaaska, 1984). Heterozymes (= isozymes *s. str. auct.*, non *sensu lato* of Markert and Moller, 1959), i.e. isoenzymes encoded by separate loci of a diploid genome which are designated by capital letters followed by a number, reflecting allozymic variants in the order of decreasing electrophoretic mobility. Electrophoretically different isoenzymes encoded by homologous loci of composite genomes of polyploids, indicating their genetic homoeology, i.e. only partial homology of genomes, are named homoeozymes. Heterozygous phenotypes are designated by codominant isozymes separated by a diagonal line, i.e. as a fraction. Term “fixed heterozygosity” is used when heterozygous phenotypes of isoenzymes were observed for all analyzed individuals of polyploids without segregation into homozygous phenotypes.

3. Results

The zymograms of the fourteen brome grass species studied and the data on the isoenzyme variation are presented schematically in Fig. 1a–i. Genetic interpretation of

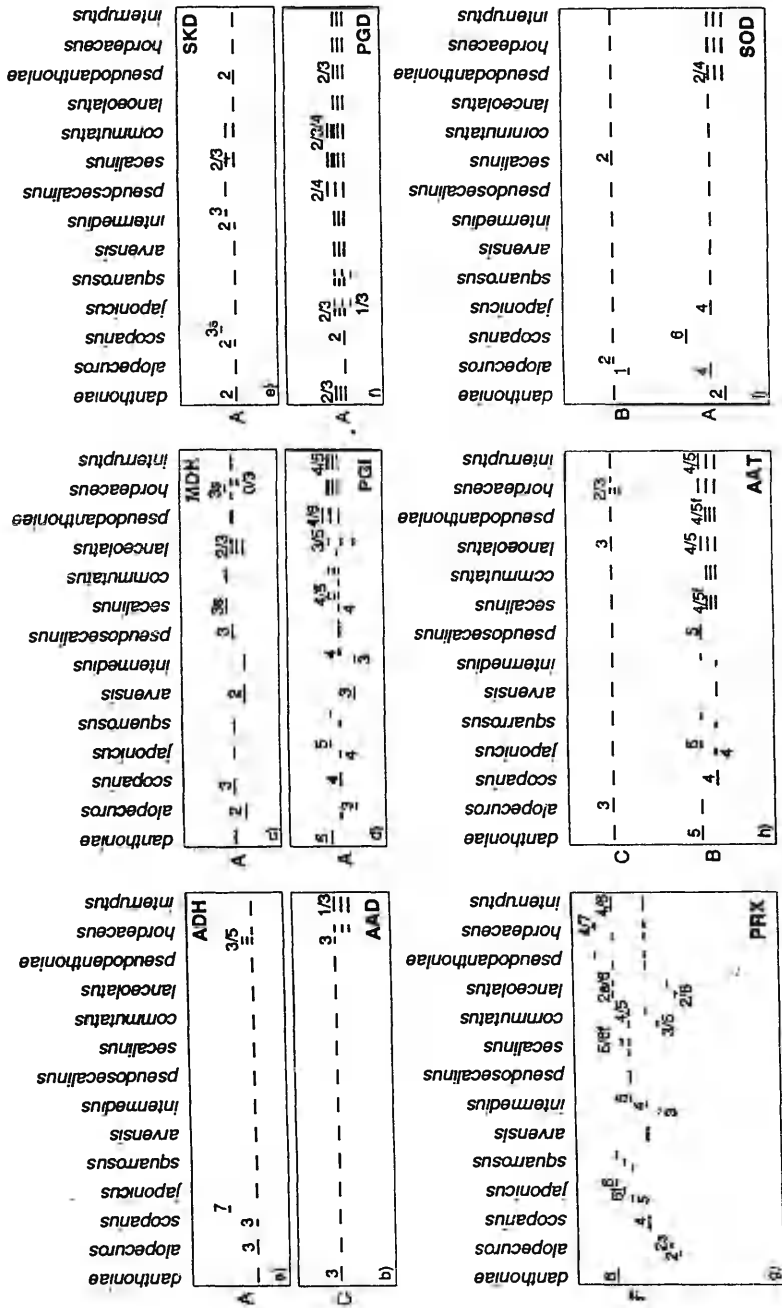


Fig. 1. Schematic phenotypes of AAD (a), AAD (b), MDH (c), PGI (d), SKD (e), PGD (f), PRX (g), AAT (h) and SOD (i) for 14 sections *Brumus* species. The origin at the top, the anode - at the bottom (* denotes a slightly slower and / a slightly faster variant of the numbered morph).

zymograms is based on the available information about subunit structure of the enzymes studied and following the principles described by Weeden and Wendel (1989) and by Wendel and Weeden (1989). Heterozygous phenotypes of dimeric enzymes could easily be distinguished on the zymograms as symmetrical triplets with codominant isoenzyme morphs as flanking bands, whereas monomeric enzymes showed two-banded heterozygous phenotypes.

3.1. Variability of isoenzyme phenotypes

Aliphatic alcohol dehydrogenase (ADH, EC 1.1.1.1) zymograms (Fig. 1a) showed homozygous A3 monomorphic in all diploids except *B. scoparius* which revealed intraspecific variation among the accessions with allozymes A3 (two accessions) and unique A7 (one accession). All tetraploids shared the same A3, with only *B. hordeaceus* displaying intraspecific variation with A3 and heterozygous A3/5. Variant A5 was not recorded among the diploids studied.

Aromatic alcohol dehydrogenase (AAD, EC 1.1.1.90 and 1.1.1.91) zymograms (Fig. 1b) stained in the presence of both NAD and NADP coenzymes showed three zones of bands, designated as heterozymes A, B, C in order of their decreasing mobility. Fast-moving NADP-specific AAD-A and AAD-B were too faint for the exact recording and are not shown in Fig. 1b. NAD-specific AAD-C was quite uniform in this section. All diploids revealed homozygous AAD-C3. Tetraploids were also monomorphic with C3, except *B. hordeaceus*, which showed C1/3 in most accessions, and *B. interruptus* which showed only heterozygous C1/3 phenotype. Allozyme C1 was not detected among the diploids analyzed. Thus, ADH and AAD were the least variable enzymes for all taxa and therefore less informative in the section *Bromus*.

Malate dehydrogenase (MDH, EC 1.1.1.37 and 1.1.1.40) zymograms (Fig. 1c) showed divergence among the species with three homozygous phenotypes A2, A3 and A3s (slightly slower, than A3 morph): diploids *B. alopecuros*, *B. intermedius* and *B. arvensis* shared an A2, diploids *B. japonicus*, *B. squarrosus*, *B. danthoniae*, *B. scoparius*, *B. pseudosecalinus* and tetraploids *B. pseudodanthoniae* and *B. interruptus* shared A3, whereas *B. secalinus* and *B. commutatus* had A3s. The tetraploid *B. lanceolatus* had fixed heterozygosity with three-banded A2/3, and the tetraploid *B. hordeaceus* displayed intraspecific polymorphism with A3, A3s, and two-banded, heterozygous A0/3, with 0 designating the absent flanking band in a triplet and reflecting putative inactivated homoeozyme.

Phosphoglucosomerase (PGI, EC 5.3.1.9) (Fig. 1d) zymograms of diploids revealed three one-banded, homozygous phenotypes of PGI-A: *B. danthoniae* had a homozygous A5, *B. pseudosecalinus* and *B. scoparius* A4, and *B. arvensis* A3. *Bromus alopecuros* and *B. intermedius* showed homologous intraspecific variation with shared A3 and A4. *Bromus japonicus* and *B. squarrosus* Turkish accessions share variants A4 and A5, whereas accessions of these taxa from other regions displayed only A4. Tetraploids *B. secalinus* and *B. commutatus* had a similar intraspecific variation with A4 and A4/5, whereas *hordeaceus* and *B. interruptus* shared heterozygous triplet A4/5. *Bromus lanceolatus* and *B. pseudodanthoniae* revealed different fixed heterozygosities with unique triplet A3/5 and A 4/6, respectively.

Shikimate dehydrogenase (SKD, EC 1.1.1.25) zymograms (Fig. 1e) revealed three homozygous variants A2, A3 and A3s. Diploids *B. danthoniae*, *B. alopecuros*, *B. japonicus*, *B. squarrosus* and *B. arvensis* were monomorphic for the same allozyme A2, *B. pseudosecalinus* had an allozyme A3, and *B. intermedius* showed intraspecific variation with both allozymes as homozygous phenotypes. *Bromus scoparius* displayed intraspecific polymorphism with morphs A2 and unique A3s. Tetraploids *B. secalinus* and *B. commutatus* shared heterozygous A2/3, all other polyploids were monomorphic for homozygous A2.

6-Phosphoglyconate dehydrogenase (PGD, EC 1.1.1.44) zymograms (Fig. 1f) showed four electrophoretic phenotypes among diploid taxa. *Bromus alopecuros* and *B. scoparius* were monomorphic for homozygous A2, while other diploids revealed three variants of different fixed heterozygosity. *Bromus pseudosecalinus* showed unique heterozygous triplet A2/4, *B. danthoniae*, *B. arvensis* and *B. intermedius* shared triplet A2/3. Turkish accessions of *B. japonicus* and *B. squarrosus* showed homologous intraspecific variation with two heterozygous triplets, A1/3 and A2/3, while all samples from other regions were monomorphic for A2/3. Since all other enzymes without exception showed homozygous morphs for these diploid taxa, the discovery of fixed heterozygosity suggests divergence of a PGD gene duplication in some diploid species in the section *Bromus*. Tetraploids *B. secalinus* and *B. commutatus* revealed double heterozygosity with five-banded A2/3/4, while remaining tetraploids shared three-banded A2/3.

Peroxidase (PRX, EC 1.11.1.7) zymograms (Fig. 1g) revealed several bands of different intensity and mobility. The most remarkable and distinct interspecific variation which could be recorded was found for the slow heterozygote PRX-F. *B. danthoniae* revealed F6, *B. pseudosecalinus*-F5 as homozygous variants, *B. arvensis* and *B. scoparius* shared-F4. Other diploids showed intraspecific variation: *B. alopecuros* with allozymes F2 and F2s (2s designates a slightly slower morph than 2), *B. intermedius* with morphs F5, F4 and rare F3, *B. japonicus* and *B. squarrosus* were polymorphic for the same alleles, F5, F6f and F6. Almost all tetraploids showed intraspecific polymorphism and different fixed heterozygosities: *B. commutatus* with F3/5 and F4/5, *B. secalinus* with F5 and F5/6f, and *B. lanceolatus* with morphs F2/6 and F2s/6. *Bromus pseudodanthoniae* and *B. hordeaceus* revealed homologous intraspecific variation with heterozygous F4/6 and F4/7 morphs. Only *B. interruptus* was monomorphic for F4/6. Thus, PRX-F proved to be a good informative heterozygote for distinguishing the species in the section *Bromus*.

Aspartate aminotransferase (AAT, EC 2.6.1.1.) (Fig. 1h) revealed two major zones of independent variation pattern, designated as heterozygotes AAT-B and AAT-C. The slower heterozygote AAT-C was homozygous and monomorphic for C3 in all diploids and tetraploids, except *B. hordeaceus* which displayed intraspecific variation with heterozygous C2/3 and C3. The faster isozyme AAT-B was homozygous, but variable among the diploids: *B. danthoniae*, *B. alopecuros* and *B. pseudosecalinus* shared B5, whereas *B. scoparius* and *B. arvensis* had B4. Intraspecific variation with homozygous B4 and B5 was detected in *B. japonicus*, *B. squarrosus* and *B. intermedius*. Most accessions of *B. japonicus* and *B. squarrosus* from Turkey revealed B4 (only 3 accessions out of 17 had B5), whereas most samples from other regions (13 out of 21) had

B5. All tetraploids studied displayed fixed heterozygosity of AAT-B: B4/5 for *B. lanceolatus*, *B. hordeaceus* and *B. interruptus* and the slightly different B4/5f (5f designed slightly faster morph, than 5) for *B. secalinus*, *B. commutatus* and *B. pseudodanthoniae*.

Superoxide dismutase (SOD, EC 1.15.1.1) zymograms (Fig. 1i) showed zones of two heterozymes: SOD-A and SOD-B. The slower SOD-B was monomorphic for B2 in all accessions except one of the three studied of *B. alopecuros* which showed the unique B1. SOD-A displayed variation with three morphs: *B. danthoniae* and *B. scoparius* had unique allozymes A2 and A6, respectively, specific to these species, while *B. alopecuros*, *B. arvensis*, *B. pseudosecalinus*, *B. intermedius*, *B. japonicus* and *B. squarrosus* were monomorphic for A4. Tetraploids *B. secalinus*, *B. commutatus* and *B. lanceolatus* shared homozygous A4, whereas *B. pseudodanthoniae*, *B. hordeaceus* and *B. interruptus* had fixed heterozygosity with a three-banded phenotype A2/4.

3.2. Allozyme diversity

The putative 11 heterozymes of nine enzymes examined revealed 38 morphs (putative allozymes) of different occurrence within and among the species. Heterozymes differed remarkably in the extent of genetic variability, ADH-A, AAD-A, AAT-C and SOD-B were the most conservative heterozymes, with one morph common to the most species. PRX-F, MDH-A and PGI-A were the most variable heterozymes with 8, 5 and 4 morphs, respectively. PRX-F and PGI-A also revealed considerable intraspecific variability. Some pairs of species (*B. japonicus*, *B. squarrosus*, and *B. secalinus* / *B. commutatus*, and *B. hordeaceus*, *B. interruptus*) displayed homologous variation with shared morphs at several heterozymes. In accordance with the assumption that the greatest genetic diversity occurs in the centre of origin of a species (SW Asia-E Mediterranean area is the presumable centre of their origin), accessions from Turkey demonstrated greater interpopulational variability than the accessions from other parts of the range. In *B. japonicus* and *B. squarrosus* accessions from Turkey, three heterozymes (SKD-A, PGD-A and PGI-A) showed two allozymes and PRX-F showed three allozymes. Accessions from Europe and Central Russia, however, were monomorphic for the SKD-A, PGD-A and PGI-A and PRX-F revealed only two allozymes.

4. Discussion

The results obtained demonstrate that isoenzymes are good molecular markers for the study of genetic differentiation among species in the section *Bromus* of the genus *Bromus*. Eleven putative heterozymes revealed interspecific differences. Most species of the section could be distinguished from each other by diagnostic isoenzyme phenotypes. *Bromus japonicus* and *B. squarrosus*, however, revealed identical zymograms, providing new evidence about their very close genetic relationships. While studying the material of these species from Central Russia and Turkey, a very common problem for all taxonomists appeared. Using available keys (e.g. Smith, 1980,

1985) it is almost impossible to resolve the variability of accessions encountered in nature. Some individuals were typical *B. japonicus*, some typical *B. squarrosus*, but most accessions could be identified only as *B. japonicus-squarrosus* complex as they had combined morphological characters of both taxa. None of the morphological characters helped to distinguish the two species, neither “good, conservative characters, like lemma size and shape, nor rather less useful, more plastic panicle characters” (Smith and Sales, 1993). There seems to be a continuous range of variation from *B. japonicus* to *B. squarrosus*. Isoenzyme variation was independent from their differentiation by morphology; typical *B. japonicus*, typical *B. squarrosus* and intermediates revealed identical isoenzyme phenotypes. Serological findings (Smith, 1972) also support that the diploid pair *B. squarrosus* and *B. japonicus* is closely related. Our isoenzyme results suggest that *B. japonicus* and *B. squarrosus* are closely related not only morphologically, but also genetically, and their ranking into separate species remains questionable, needing more detailed investigations.

Bromus scoparius and *B. intermedius* which are morphologically easily distinguishable are also clearly distinguished from each other and from the previously mentioned *B. japonicus-squarrosus* complex on the basis of the isoenzyme evidence, in agreement with the serological studies of Smith (1972).

In addition to the interspecific variation, intraspecific allozyme differentiation between local populations (accessions) was observed in five of eight diploid species. *Bromus alopecuroides*, *B. scoparius*, *B. japonicus*, *B. squarrosus* and *B. intermedius* revealed intraspecific variation with two of the allozymes ADH-A, SKD-A, PGD-A, AAT-B, SOD-A, PRX-F, PGI-A.

In the recent paper Ainouche *et al.* (1995) suggested that self-fertilizing diploid species *B. intermedius* and *B. squarrosus* may have substantial amounts of allogamy owing to the high levels of heterozygosity at several loci. In our study, no heterozygous phenotypes of any heterozygote, except PGD-A, were observed in the diploid taxa. Our results indicate high rates of selfing in all diploids examined, since seed accessions collected from plants in nature were analyzed.

The appearance of fixed heterozygous phenotypes of PGD in six of the eight diploid taxa supports the hypothesis of a 6-PGD gene duplication and divergence among the diploid taxa of the section *Bromus*. Out of eight, only two taxa, *B. alopecuroides* and *B. scoparius* had homozygous variant A2 with no evidence of the 6-PGD gene duplication. The most likely explanation for the permanent appearance of a symmetrical three-banded, heterozygous phenotype of dimeric 6-PGD in these autogamous diploids is that it is encoded by a duplicated locus with divergent alleles. This hypothesis assumes the duplication of the primary 6-PGD locus with subsequent modification of the duplicated locus producing an isoenzyme of slower mobility and lower specific activity, as indicated by a relatively lower intensity of the slower band. Our previous study showed that taxa, belonging to section *Genea* had no 6-PGD gene duplication (Oja and Jaaska, 1996). Similar 6-PGD gene duplication has been reported for the grass genera *Eleusine* (Werth *et al.*, 1993) but in genus *Bromus* it has not been recorded up till now. Other enzymes duplications have been found in *Dactylis* (Lumaret, 1986) and *Secale* (Rebordinos and de la Vega, 1988). Although gene duplications are rare events, there may have been three such events giving

rise to independent lineages of *Bromus* species each exhibiting two allozymes of PGD.

The diploid *B. pseudosecalinus* morphologically very similar to the tetraploid *B. secalinus*, was found to be serologically very different from latter and Smith (1972) supposed that "morphological resemblance is fortuitous, and does not imply similar ancestry". Our results also showed that *B. pseudosecalinus* differed from *B. secalinus* in having different morphs of MDH-A and AAT-B, suggesting it is unlikely to be the diploid ancestor of this tetraploid.

Smith (1972) placed *B. secalinus* and *B. alopecuroides* in separate groups on the basis of morphological and serological differences. On the contrary, Pillay and Hilu (1995) suggested close relations between them (on the basis of the cpDNA). Our results, showing different MDH-A, AAT-B and PRX-F alleles, suggest that *B. alopecuroides* does not suit as a diploid parent for *B. secalinus*. Smith (1972) concluded that *B. danthonia* is likely to be one of the diploid ancestors of *B. lanceolatus* because of the serological similarity. Our data, however, does not support this due to difference in allozymes of SOD-A. Instead, our allozyme data indicate that *B. alopecuroides* could be one of the genome donors for the tetraploid *B. lanceolatus*.

Polyploids *B. secalinus* and *B. commutatus* had very similar isoenzyme phenotypes. Out of 11 heterozygotes, 10 were monomorphic and shared in the two taxa, supporting their close genetic affinity. Especially indicative is a unique, shared double heterozygosity of PGD-A2/3/4, suggesting the second duplication with divergence of the PGD-A locus. The isoenzyme data on the close phylogenetic affinity of the two species are in accordance with the morphological similarity of these species, their serological homogeneity (Smith, 1972) and hybridization data suggesting that they possibly have had common progenitors (Wilson, 1956). The isoenzyme data, however, revealed a clear difference between them in PRX-F, in support of their recognition as separate species. The two tetraploids may have one common genome indicated by shared homoeozym F5, whereas the second genome is differentiated by homoeozymes F6 in *B. secalinus* versus F3 and F4 in *B. commutatus*.

Tetraploids *B. hordeaceus* and *B. interruptus* in addition to the close morphological resemblance, also shared identical fixed heterozygosities at AAD-C, AAT-B, SOD-A, PRX-F, PGD-A and PGI-A and the same homozygous phenotypes of ADH-A, MDH-A, SKD-A, AAT-C and SOD-B. The only difference was that *B. hordeaceus* was more polymorphic, perhaps due to much higher number of accessions analysed. Our isoenzyme results support the suggestion of Smith (1981) that *B. interruptus* may be interpreted as an ecotype subspecies (crop contaminant) of *B. hordeaceus*.

Intraspecific allozyme variation observed in *B. hordeaceus* was independent from the morphological differentiation which recognized 3 ecotype-subspecies in *B. hordeaceus*. Similar findings were reported by Ainouche *et al.* in their recent paper.

Stebbins (1981) concluded on the basis of the cytogenetic data that *B. hordeaceus* and *B. lanceolatus* are tetraploids. Our data revealed fixed heterozygosity at several isoenzyme loci in all studied tetraploids, including the two above-mentioned species, suggesting a hybrid origin and supporting the hypothesis that they all are allopolyploids. In particular, all tetraploids showed fixed heterozygosity of AAT-B, PRX-F,

PGD-A and PGI-A. Intraspecific polymorphism of PRX-F and PGI-A in *B. secalinus* and *B. commutatus* and of AAT-C in *B. hordeaceus* may reflect inactivation of one homoeoallele of a composite genome among accessions of these tetraploids. The diploid ancestor of one genome of polyploids, however, remains unknown, since homoeozymes ADH-A5, AAD-C1, AAT-C2, PRX-F7 and PGI-A6 of polyploids were not detected among the diploids studied.

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Allozyme diversity and phylogenetic relationships
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Allozyme diversity and phylogenetic relationships among diploid annual bromes (*Bromus*, Poaceae)

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Phylogenetic relationships and genetic differentiation among eleven diploid annual brome species were evaluated by cladistic and phenetic analysis of the allozyme diversity of eight enzymes detected by PAGE. All species lacked heterozygous allozyme phenotypes, indicating prevalent autogamy and self-fertilization. *Bromus japonicus* Thunb. and *B. squarrosus* L. had the same allozymes, supporting their close genetic affinity. The placement of *B. pumilio* (Trin.) P. M. Smith in its own section *Boissiera* (Hochst. ex. Steudel) P. M. Smith was supported by its basal position in a separate clade. Morphologically uniform *B. intermedius* Guss. was the most polymorphic species, revealing six isoenzyme lineages. The diploids of the section *Genea* Dum. were distinguished in a separate cluster on both cladistic and phenetic allozyme trees.

Key words: *Bromus*, isoenzymes, phylogenetic relationships, Poaceae, taxonomy

INTRODUCTION

Bromus L. (Poaceae) is a taxonomically complex genus with about 130 species of annual and perennial, diploid and polyploid brome grasses of wide geographic distribution in Eurasia, North and South America, Africa and Australia. There is still no consensus on the taxonomic treatment of *Bromus*. The genus has been divided into six sections (Smith 1972), seven subgenera (Stebbins 1981) or even five different genera (Tsvelev 1976). The number and rank of the divisions depends on the characters used as a basis for their delimitation (serological, cytological and morphological, respectively), and phylogenetic relationships among them are still inconsistent. Sales (1993) doubted the reality of the section *Genea* Dum. as an in-

dependent taxonomic unit. She noted that there is a continuous range of variation between section *Bromus* and section *Genea* via the *B. pectinatus* Thunb. complex of section *Bromus*. This complex was suggested in order to link the *Genea* species with the section *Bromus* through the diploid *B. japonicus*. Recently, Pillay and Hilu 1995 and Pillay 1996 reported the use of advanced contemporary molecular methods with cpDNA and rDNA markers in the investigations of the genetic relationships in the genus *Bromus*. Pillay and Hilu (1995), on the basis of the cpDNA data, suggested that *B. sterilis* L. and *B. tectorum* L. are recently derived species that share a common maternal ancestry. They also found that subgenera *Stenobromus* (Griseb.) Hackel and *Bromus* are not distinct entities and probably originated from simi-

lar ancestors related to the subgenus *Festucaria* (Coss. & Dur.) Hackel. Pillay (1996) concluded it unlikely that the subgenera vs. sections of *Bromus* had independent origins. Despite this new molecular information, phylogenetic relationships among the subdivisions of *Bromus* are still far from clear.

In addition to inconsistent intrageneric classification, there is a lot of confusion about species delimitation within the sections, due to their great and frequently continuous morphological variability, and less well known genetic relationships among them. Among the diploids, some species are easily distinguished by unique morphological characters, like *Bromus pumilio* (Trin.) P. M. Smith and *B. danthoniae* Trin., but other diploids of the type section are less clearly differentiated and difficult to identify since their diagnostic characters are often overlapping and difficult to describe exactly (Smith 1973). Smith and Sales (1993) tried to clarify the taxonomy of some difficult taxa of the section *Bromus* and recognized four affinity groups among them. Ainouche and Bayer (1996) confirmed the weak divergence of the diploid species within the section *Bromus* according to the ITS sequences of the nrDNA.

The goal of the present study was to analyze the differentiation among diploid brome grasses between and within sections on the basis of electrophoretic allozyme characters and to compare allozyme diversity between taxa in their native East Mediterranean and other regions (Europe and Central Russia). Cladistic and phenetic analysis of the allozyme characters was used to understand the phylogenetic relationships and genetic affinities among diploid species as ancestral to polyploids. This was necessary in order to clarify the relationships within the whole genus. We also analyzed how divergence by isoenzyme phenotypes was correlated with the morphological species delimitation.

MATERIAL AND METHODS

Plant material

Most of the seeds were collected in Turkey and Central Russia by the first author. The accessions of other geographical origins, also collected in the wild from known locations, were received from different botanical gardens.

1. *Bromus pumilio* (Trin.) P. Smith: 1 accession, from Denmark.
2. *Bromus danthoniae* Trin.: 2 Turkish accessions.
3. *Bromus alopecuroides* Poir.: 3 Turkish accessions.
4. *Bromus scoparius* L.: 3 accessions, from Kirghizia (1), Greece (1) and Central Asia (1).
5. *Bromus japonicus* Thunb.: 21 accessions, from Turkey (8), Central Russia (7), Armenia (1), Kirghizia (1), Germany (1), Hungary (2) and the Czech Republic (1).
6. *Bromus squarrosus* L.: 16 accessions, from Turkey (10), Hungary (2), Switzerland (1), Greece (1) and Spain (2).
7. *Bromus intermedius* Guss.: 17 accessions, from Turkey.
8. *Bromus pseudosecalinus* P. Smith: 1 accession, from England.
9. *Bromus sterilis* L.: 27 accessions, from Turkey (7) and from central and southern Europe (20).
10. *Bromus tectorum* L.: 34 accessions, from central and southern Europe (17) and from Turkey (17), including six Turkish accessions of *B. tectorum* L. ssp. *lucidus* Sales (= *B. sericeus* Drob.)
11. *Hordeum bogdanii* Wilensky: 2 accessions, from Pakistan and China.

Vouchers are deposited in TAA, Herbarium of the Institute of Zoology and Botany (Tartu, Estonia). Some of them were grown from the seed accessions in order to check their botanical identifications.

Isoenzyme analysis and nomenclature

Enzyme extracts were prepared from the shoots (the primary leaf with the coleoptile) of 5–10-day-old, etiolated seedlings subjected to electrophoresis in vertical polyacrylamide gel slabs and stained as described in Jaaska and Jaaska (1986, 1990). Four individual seedlings of each accession were routinely analyzed for all isoenzymes. For some enzymes and accessions, up to ten individuals were analyzed in order to check the constancy or polymorphism of the isoenzyme patterns. Isoenzymes encoded by separate loci of a diploid genome are designated by capital letters followed by a number denoting the allozymic variants (in short, morphs) in the order of decreasing mobility. Heterozygous phenotypes are designated by codominant allozymes separated by a diagonal line, i.e. as a fraction. The term "fixed heterozygosity" is used when heterozygous phenotypes of isoenzymes were observed for all analyzed individuals without segregation into homozygous phenotypes.

Data analysis

Pee-Wee program vers. 2.5.1 (Goloboff 1993) which finds trees with the best total fit of characters and calculates decay indexes (DI) of branch support (Bremer 1994) was used for the cladistic analysis of the allozyme data. Phenetic analysis was performed with the TYTAN program provided by Dr. A. Batko (Warsaw University, Poland) by applying the

Manhattan distance method combined with the UPGMA method. Both analyses were conducted on the multilocus allozyme lineages identified in the species studied. The isoenzyme data matrix was constructed using allozymes as binary absence/presence characters. Rare morphs were excluded from the matrix. The autogamous diploid perennial *Hordeum bogdanii* Wilensky of the sister tribe *Triticeae* Dum. was chosen as an outgroup for comparison in cladistic analysis.

RESULTS AND DISCUSSION

Data on the isoenzyme variation among the ten brome-grass species is summarized in Table 1.

The genetic interpretation of zymograms followed the results of our previous comparative studies of isoenzymes in diploid and polyploid bromes (Oja & Jaaska 1996, Oja 1997).

A characteristic feature of the diploid annual bromes examined was the total absence of heterozygous allozyme phenotypes of most heterozymes except PGD, despite the existence of intraspecific allozyme polymorphism of several heterozymes. This result is in accordance with the data on the autogamy and self-fertilization of annual bromes (Smith 1972) and shows prevalent inbreeding in the natural populations of all diploids examined.

Table 1. Electrophoretic variants of alcohol (ADH), malate (MDH) and shikimate (SKD) dehydrogenase, aspartate aminotransferase (AAT), superoxide dismutase (SOD), peroxidase (PRX), 6-phosphogluconate dehydrogenase (PGD) and phosphoglucoisomerase (PGI) heterozymes in *Bromus* species: Allozyme lineages within the species are numbered, with their geographic origin designed with letter T (Turkey), CR (Central Russia), Eu (Europe) and CA (Central Asia). N = the number of accessions analyzed, s = a slightly slower variant of the numbered morph, and f = a slightly faster variant of the numbered morph, r = a rare morph. The unique (autapomorphic) allozymes are in bold.

Species and allozyme lineages	No.	ADH	MDH	SKD	AAT		SOD		PRX	PGD	PGI
		A	A	A	B	C	A	B	F	AIB	A
<i>Hordeum bogdanii</i>	2	3	2f	3	6	2	2	1	9	3	5
Section Boissiera											
<i>B. pumilio</i> Eu	1	3	2	2	6	3	2	2	1	2 3	7
Section Bromus											
<i>B. danthoniae</i> T	2	3	3	2	5	3	2	2	6	2 3	5
<i>B. alopecuroides</i> T1	2	3	2	2	5	3	4	2	2s	2	4
<i>B. alopecuroides</i> T2	1	3	2	2	5	3	4	1	2	2	3
<i>B. scoparius</i> CA1	2	3	3	3s	4	3	6	2	4	2	4
<i>B. scoparius</i> CA2	1	7	3	2	4	3	6	2	4	2	4
<i>B. japonicus</i> CR	8	3	3	2	5	3	4	2	6	2 3	4
<i>B. jap-sq</i> T1	9	3	3	2	4	3	4	2	6	2 3	4
<i>B. jap-sq</i> T2	2	3	3	2	4	3	4	2	6	2 3	5
<i>B. squarrosus</i> T1	2	3	3	2	5	3	4	2	6f	2 3	4
<i>B. squarrosus</i> T2	5	3	3	2	4	3	4	2	6	1 3	4
<i>B. squarrosus</i> CR	1	3	3	2	4	3	4	2	5	2 3	4
<i>B. intermedius</i> T1	1	3	2	2	5	3	4	2	5	2 3	4
<i>B. intermedius</i> T2	1	3	2	3	4	3	4	2	5	2 3	4
<i>B. intermedius</i> T3	2	3	2	3	5	3	4	2	5,3	2 3	4
<i>B. intermedius</i> T4	4	3	2	3	4	3	4	2	5	2 3	3
<i>B. intermedius</i> T5	7	3	2	3	5	3	4	2	5	2 3	3
<i>B. intermedius</i> T6	1	3	2	3	5	3	4	2	4	2 3	3
<i>B. arvensis</i> T1	1	3	2	2	5	3	4	2	5	2 3	3
<i>B. arvensis</i> T2	1	3	2	3	5	3	4	2	4	2 3	5
<i>B. arvensis</i> Eu	12	3	2	2	4	3	4	2	4	2 3	3
<i>B. pseudosecalinus</i> Eu	1	3	3	3	3	3	4	2	5	2 4	4
Section Genea											
<i>B. sterilis</i> T1	4	4	3	2	5	3	4	2	8	2	7
<i>B. sterilis</i> T2	2	4	3	2	5	3	4	2	8	2	6
<i>B. sterilis</i> T3	1	4	3	2	5	3	4	2	8	2	5
<i>B. sterilis</i> Eu	20	4	3	2	5	3	4	2	8,6r	2	6
<i>B. tectorum</i> T	17	4	3	2	5	1	4	2	5	2	6
<i>B. tectorum</i> Eu	24	4	3,2r	2	5	1	4	2	5	2	6

No case of heterozygous isozyme phenotype was recorded in the accessions *Bromus intermedius* and *B. squarrosus*, in contrast to the recent findings of Ainouche *et al.* (1995).

PGD was exceptional among the enzymes analyzed by showing fixed heterozygosity with permanent three-banded phenotypes in several diploids. Of the ten species studied, only *Bromus alopecuros* and *B. scoparius* from the type section and *B. sterilis*, *B. tectorum* from the *Genea* section, had the homozygous variant A2. The most plausible explanation for the permanent occurrence of a symmetrical three-banded, heterozygous phenotype of dimeric 6-PGD in the autogamous diploids is that it is encoded by a duplicated locus with divergent alleles. It is likely that the permanent three-banded phenotype reflects heterozygosity due to gene duplication with concurrent modification and the appearance of a modified, paralogous locus encoding heterozygote PGD-B.

Intraspecific variation with two allozymes of ADH-A, SKD-A, PGD-A, AAT-B, SOD-B, PRX-F and PGI-A was observed in all seven diploids for which sufficient numbers of accessions were analysed: *Bromus alopecuros*, *B. scoparius*, *B. japonicus*, *B. squarrosus*, *B. intermedius*, *B. arvensis*, and *B. sterilis*. An important consequence of the prevalent self-fertilizing breeding system in the diploid bromes examined is their intraspecific differentiation into distinct multilocus allozyme genotypes maintained as multilocus allozyme (isozyme) lineages. Thus, isoenzyme analyses revealed two isozyme lineages in *B. alopecuros* with different allozymes of 3 heterozygotes (SOD-B, PRX-F and PGI-A) and in *B. scoparius* with differences in two allozymes of ADH-A and SKD-A. *Bromus arvensis* from Turkey also showed two isozyme phenotypes (differentiated by SKD-A and PGI-A) which both differed from the European accessions in AAT-B allozymes.

Accessions of *Bromus sterilis*, from Turkey, displayed three isozyme lineages, which differed only in PGI-A allozymes. The European accessions were identical with the *B. sterilis* lineage T2 from Turkey, except some having PRX-F6 instead of common PRX-F8. All accessions of *B. tectorum* from Turkey were monomorphic, whereas some accessions from Europe had only one additional allozyme MDH-A2. Six Turkish accessions which could be attributed to *B. tecto-*

rum ssp. lucidus by general morphology (synaptospermous, but still with lower glume 1-veined) had an isozyme phenotype absolutely identical to that of *B. tectorum* subsp. *tectorum*. This evidence is consistent with the conclusion of Sales (1991) who reduced *B. sericeus* to *B. tectorum* subsp. *lucidus* on the basis of the morphological and reproductive characters, and favouring even the lower taxonomic rank of mere morphological variety.

The accessions attributed to the *Bromus japonicus-squarrosus* complex by morphology displayed homologous variation with shared morphs at several heterozygotes. The accessions from Turkey demonstrated relatively greater interpopulational variability than the accessions from other parts of the species range. The Turkish accessions of *B. japonicus-squarrosus* showed polymorphism of SKD-A, PGD-A and PGI-A with two allozymes and of PRX-F with three allozymes. The accessions from Europe and Central Russia attributed by morphology to typical *B. japonicus* and *B. squarrosus*, respectively, were differentiated from each other by alternative allozymes of AAT-B and PRX-F. In general, taxonomists have recognized that *B. japonicus* is a highly variable taxon with at least two subspecies: typical subsp. *japonicus* and subsp. *anatolicus*, the latter almost exactly fitting the description of *B. squarrosus* (Smith 1980, 1985a). Our experience with growing voucher reproductions has shown the great influence of environmental conditions on brome-grass phenotype. For example, an original accession of *B. japonicus* collected in Turkey had a lower lemma 10 × 6 mm and uppermost spikelet 20 mm, while its reproduction in Estonia had a lower lemma 13 × 8 mm and uppermost spikelet 35 mm, thus resembling *B. squarrosus* more. There seems to be a continuous range of variation from *B. japonicus* to *B. squarrosus*. A set of eleven Turkish accessions was morphologically intermediate between typical *B. japonicus* and *B. squarrosus* (designed as *jap-sq* in Table 1). They were differentiated into two isozyme lineages. The data in Table 1 show that genetic differentiation in the complex by allozymes is independent of differentiation by morphology.

The allozyme data suggests that *Bromus japonicus* and *B. squarrosus* are closely related not only morphologically, but also genetically. The complex needs more detailed investigations in

order to determine the correct taxonomic rank of *B. japonicus* and *B. squarrosus*, but our allozyme data favours their intraspecific status.

Isoenzyme evidence on three unique allozymes in its electrophoretic phenotype clearly shows that *Bromus pumilio* stands apart from the other diploids of the section *Bromus*, where it has been placed in some treatments (Smith 1970). This is in accordance with its divergence by morphological and dispersal mechanism characteristics (Smith 1985b). The suggestion to put *B. pumilio* in its own section *Boissiera* (Smith 1985b) is thus supported by our allozyme data. Contrary to a report about the tetraploid chromosome number for *B. pumilio* (Smith 1972), our isoenzyme results showed that *B. pumilio* should be a diploid species. This was confirmed by showing that our accession of *B. pumilio* had $2n = 14$ (H. Laarmann unpubl.). The diploid chromosome number was also given by Avdulov (1931).

Bromus intermedius was the most polymorphic species among the diploids studied, revealing six isozyme lineages. Despite remarkable allozyme variability, all the examined accessions of *B. intermedius* were morphologically quite uniform with slender, tangled panicle branches and hairy spikelets with recurved awns, as described by Smith (1985a). It is remarkable that the phenotype of specimens of Turkish accessions of *B. intermedius* reproduced in Estonia was less affected by environmental growing conditions than in the case of *B. japonicus-squarrosus*.

The ten heterozygotes of the eight enzymes examined revealed 36 shared and 14 unique morphs (putative allozymes) occurring with different frequencies and patterns within and among the species. Heterozygotes differed remarkably in the extent of genetic variability, AAT-C and SOD-B were the most conservative heterozygotes in this section, with one morph in common to most species. PRX-F, PGI-A and PGD-A were the most variable heterozygotes with nine, five and four morphs, respectively.

Cladistic analysis of the allozymic variation pattern among the species and their isozyme lineages was performed on the basis of the presence/absence (0/1) data matrix of 36 phylogenetically informative shared allozyme characters compiled from the data in Table 1. Analysis of the data matrix with Péc-Wee gave 18 trees of best fit, having

a length of 87 steps and a total fit of 178.9. The consensus tree of the 18 fittest trees (Fig. 1) shows that *Bromus pumilio* is linked to *Hordeum bogdanii* as the outgroup species and is basally paraphyletic with respect to all other diploids studied. This indicates its outstanding position in the genus and supports its recognition in a separate section by Smith (1985b). Another distinctly differentiated species among annual diploids seems to be *B. danthoniae* which is basally paraphyletic to the other species of the section *Bromus*. Decay indexes of the *B. danthoniae* clade from the sister clades of the *B. japonicus-squarrosus* complex and of *B. pumilio* are 3.4 and 4.3 respectively. Smith (1985ab) also supposed that *B. pumilio* is most closely related to *B. danthoniae* and *B. alopecuros* in the genus. The clear allozyme differentiation of *B. danthoniae* is congruent with its divergence by a unique morphological character (this species has a three-awned lemma) in the section *Bromus*.

The *Bromus japonicus-squarrosus* complex has a central position on the allozyme cladogram, but was not clearly resolved into two different species. The morphologically intermediate allozyme lineage *japonicus-squarrosus* T2 together with the typical *B. japonicus* from Central Russia are basal in the consensus cladogram and are only weakly (DI only 0.4) differentiated from the intermediate lineage T1 and the typical *B. squarrosus* lineages T1 and T2.

Bromus alopecuros and *B. scoparius* of the section *Bromus* are linked on the cladogram as sister species and formed one subclade with species belonging to the section *Genea*. *Bromus alopecuros* seems to be less diverged from the *B. japonicus-squarrosus* complex, than *B. scoparius*, with the Bremer's supports (DI values) 1.0 and 3.8 respectively.

Section *Genea* is recognised on the cladogram as a monophyletic group which is well diverged from the central *Bromus japonicus-squarrosus* complex (DI = 4.1) and is linked to the *B. alopecuros-B. scoparius* clade. The cladogram shows the *B. tectorum* and *B. sterilis* T2 lineage as sister species, supporting their close phylogenetic affinity. The cpDNA data also suggests that *B. sterilis* and *B. tectorum* are recently derived species that share a common maternal ancestry (Pillay & Hilu 1995).

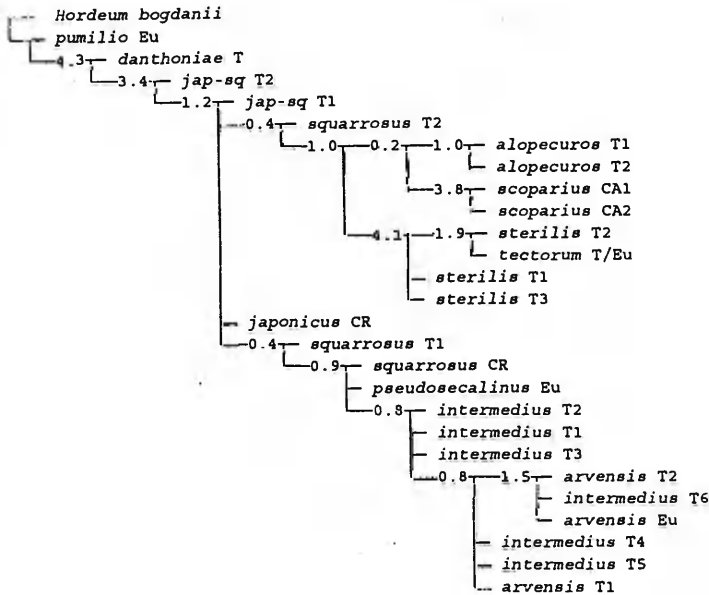


Fig. 1. The consensus tree of the 18 fittest trees for the diploid annual *Bromus* species from the Pee-Wee analyses of the data matrix of 36 shared allozymes of 10 heterozygotes. Decay indexes are given at branches.

The six morphologically cryptic allozyme lineages within *Bromus intermedius* are less differentiated from the *B. japonicus-squarrosus* complex (DI = 0.8), being divided into two closely related groups and mixed with *B. arvensis*. The recent study by Ainouche and Bayer (1997) on the basis of rDNA ITS sequences also showed that the diploid bromes *Bromus japonicus*, *B. squarrosus*, *B. arvensis* and *B. intermedius* belong to a weakly differentiated clade within the section *Bromus*, which agrees well with our results. Both the ITS and allozyme cladograms reveal the same sister species couples, e.g. *B. japonicus-B. squarrosus* and *B. intermedius-B. arvensis*.

The only remarkable discrepancy between the ITS and allozyme data is in the phylogenetic position of the section *Genea*. Thus, the ITS cladogram placed *Bromus tectorum* of the section *Genea* as a sister species to *B. anomalus* Rupr. ex Fourm. of the section *Pnigma* Dum. (Ainouche & Bayer 1997), whereas our allozyme cladogram linked the *Genea* diploids to the *B. alopecuros-B. scoparius* subclade of the type section. The possible reason for this disagreement is that no diploid *Pnigma* species was available in our study, and therefore the cladistic program found the most closely related species of those studied.

Bromus pumilio and *B. danthoniae* are two basally ancestral brome species on the phylogram that revealed the duplication of the PGD locus with PGD-AB2/3, whereas the outgroup species, perennial *Hordeum bogdanii*, showed no duplication. The same duplication was found in most of the diploids studied. This indicates that duplication with the emergence of paralogous PGD-A2 has occurred in *B. pumilio* and is maintained in most diploid bromes. Only *B. alopecuros*, *B. scoparius* and diploids of the section *Genea* (*B. sterilis* and *B. tectorum*), which fell within the same derived subclade on the cladogram again, lacked the duplication and had only PGD-A2. This suggests inactivation of the duplicated PGD-B3 in this derived subclade. Further divergence of duplicated PGD-AB with the appearance of PGD-A1 and PGD-B4 was detected for *B. squarrosus* T2 and *B. pseudosecalinus*, respectively.

Phenetic analysis was performed on the basis of the presence/absence data matrix of the 36 shared, plus 14 unique, allozymes compiled from Table 1, by applying Manhattan distance with the UPGMA method of clustering.

The allozyme phenogram (Fig. 2) is rather similar to the cladogram in the general topology in recognizing similar clusters of related species, but

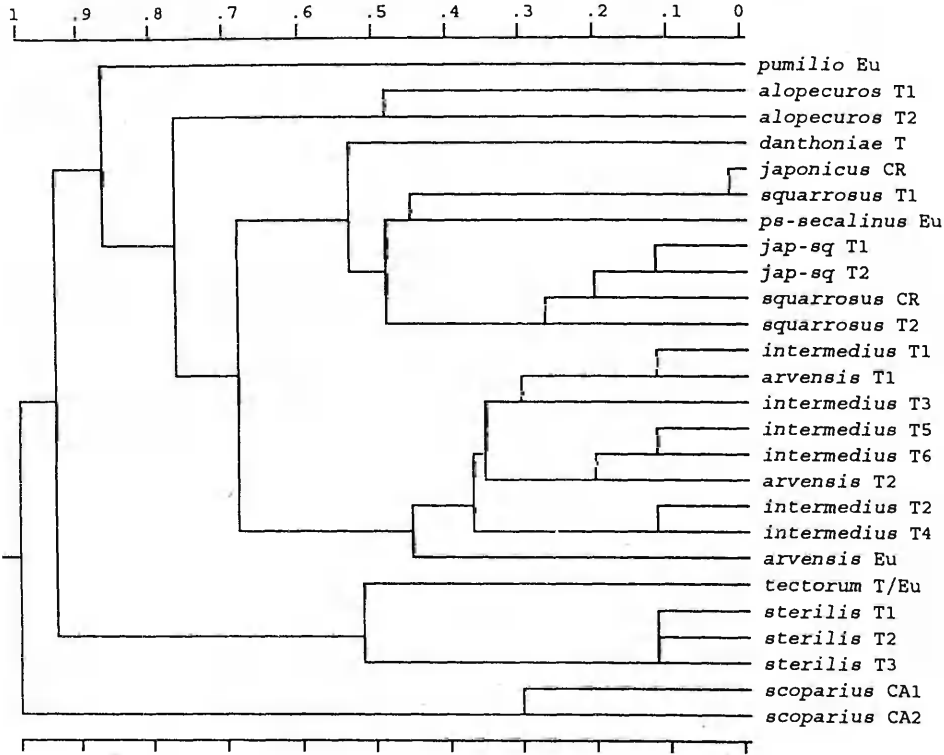


Fig. 2. UPGMA phenogram of Manhattan distances for the diploid annual bromes, based on a presence/absence data matrix of 36 shared and 14 unique allozymes of 10 heterozygotes compiled from Table 1. Scale relative, 0 = minimal distance.

supplementing it by evaluating the extent of genetic divergence between the species and allozyme lineages.

Thus, *Bromus pumilio* is distinguished in a separate phenetic branch, having also appeared in a separate clade on the cladogram.

The phenogram shows that *Bromus japonicus* CR and *B. squarrosus* T1 are the least divergent couple linked with other allozyme lineages of the *B. japonicus-squarrosus* complex in the same cluster. *Bromus danthoniae* is linked with the *B. japonicus-squarrosus* cluster at a higher divergence distance. As in the cladogram, the *B. intermedius*–*B. arvensis* couple is joined in a separate clade which is linked with the *B. japonicus-squarrosus* cluster at a higher divergence distance. Diploids *B. sterilis* and *B. tectorum*, belonging to the section *Genea*, appear on both cladistic and phe-

netic trees in a separate cluster which is linked to *B. alopecurus* of the section *Bromus*.

The most significant difference between the phenogram and cladogram is in the position of *Bromus scoparius* which, according to the phenogram, is the most divergent species among the annual diploids studied, supported by unique allozymes ADH-A7, SKD-A3 and SOD-A6, followed by *B. pumilio* with unique allozymes AAT-B6, PRX-F1 and PGI-A7 (Table 1).

The results presented above should be considered only as a preliminary attempt to understand phylogenetic relationships in the genus *Bromus* by applying phenetic and cladistic analysis of the variability pattern of molecular allozyme characters among the ancestral diploid species. Future studies involving perennial diploids of the section *Pnigma*, which were not available for the

present study, as well as more accessions and species of annual diploids with the use of a larger number of molecular markers will hopefully give a more substantiated picture of phylogenetic relationships in the brome genus at the ancestral diploid level.

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Table 1. Electrophoretic variants of alcohol (ADH), malate (MDH) and shikimate (SKD) dehydrogenase, aspartate aminotransferase (AAT), superoxide dismutase (SOD), peroxidase (PRX), 6-phosphogluconate dehydrogenase (PGD) and phosphoglucoisomerase (PGI) heterozymes in *Bromus* species : Allozyme lineages within the species are numbered, with their geographic origin designed with letter T (Turkey), CR (Central Russia), Eu (Europe) and CA (Central Asia). N - the number of accessions analyzed, s designates a slightly slower, and f - a slightly faster variant of the numbered morph, r - a rare morph. The unique (autapomorphic) allozymes are in bold.

Species and allozyme lineages	N	ADH A	MDH A	SKD A	AAT B	AAT C	SOD A	SOD B	PRX F	PGD A B	PGI A
<i>Hordeum bogdanii</i>	2	3	2f	3	6	2	2	1	9	3	5
Section Boissiera											
<i>B. pumilio</i> Eu	1	3	2	2	6	3	2	2	1	2 3	7
Section Bromus											
<i>B. danthoniae</i> T	2	3	3	2	5	3	2	2	6	2 3	5
<i>B. alopecuros</i> T1	2	3	2	2	5	3	4	2	2s	2	4
<i>B. alopecuros</i> T2	1	3	2	2	5	3	4	1	2	2	3
<i>B. scoparius</i> CA1	2	3	3	3s	4	3	6	2	4	2	4
<i>B. scoparius</i> CA2	1	7	3	2	4	3	6	2	4	2	4
<i>B. japonicus</i> CR	8	3	3	2	5	3	4	2	6	2 3	4
<i>B. jap-sq</i> T1	9	3	3	2	4	3	4	2	6	2 3	4
<i>B. jap-sq</i> T2	2	3	3	2	4	3	4	2	6	2 3	5
<i>B. squarrosus</i> T1	2	3	3	2	5	3	4	2	6f	2 3	4
<i>B. squarrosus</i> T2	5	3	3	2	4	3	4	2	6	1 3	4
<i>B. squarrosus</i> CR	1	3	3	2	4	3	4	2	5	2 3	4
<i>B. intermedius</i> T1	1	3	2	2	5	3	4	2	5	2 3	4
<i>B. intermedius</i> T2	1	3	2	3	4	3	4	2	5	2 3	4
<i>B. intermedius</i> T3	2	3	2	3	5	3	4	2	5, 3r	2 3	4
<i>B. intermedius</i> T4	4	3	2	3	4	3	4	2	5	2 3	3
<i>B. intermedius</i> T5	7	3	2	3	5	3	4	2	5	2 3	3
<i>B. intermedius</i> T6	1	3	2	3	5	3	4	2	4	2 3	3
<i>B. arvensis</i> T1	1	3	2	2	5	3	4	2	5	2 3	3
<i>B. arvensis</i> T2	1	3	2	3	5	3	4	2	4	2 3	5
<i>B. arvensis</i> Eu	12	3	2	2	4	3	4	2	4	2 3	3
<i>B. pseudosecal.</i> Eu	1	3	3	3	3	3	4	2	5	2 4	4
Section Genea											
<i>B. sterilis</i> T1	4	4	3	2	5	3	4	2	8	2	7
<i>B. sterilis</i> T2	2	4	3	2	5	3	4	2	8	2	6
<i>B. sterilis</i> T3	1	4	3	2	5	3	4	2	8	2	5
<i>B. sterilis</i> Eu	20	4	3	2	5	3	4	2	8,6r	2	6
<i>B. tectorum</i> T	17	4	3	2	5	1	4	2	5	2	6
<i>B. tectorum</i> Eu	24	4	3,2r	2	5	1	4	2	5	2	6

IV

Oja, T. 1998.
Genetic variation in two widespread diploid brome grass species,
Bromus tectorum L. and *B. sterilis* L. (Poaceae),
submitted to New Phytologist.

**GENETIC VARIATION IN TWO WIDESPREAD DIPLOID
BROMEGRASS SPECIES,
Bromus tectorum L. AND *B. sterilis* L. (Poaceae).**

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SUMMARY

Genetic diversity among 61 accessions of *Bromus tectorum* and 48 of *B. sterilis* from different sites of their Eurasian ranges has been studied by electrophoretic analysis of ten enzymes encoded by 18 loci. The two species proved clearly differentiated by alternate allozymes of seven isozymes. Populations of both taxa had low genetic diversity but showed differentiation into eleven (*B. tectorum*) and six (*B. sterilis*) multilocus allozyme lineages. Geographically marginal European populations had only some of the multilocus allozyme lineages distributed in the Near East region, indicating weedy colonization of the two species by only a few successful genotypes.

Key words: *Bromus*, Poaceae, isozymes, allozyme diversity, geographic distribution.

INTRODUCTION

Bromus tectorum L. and *B. sterilis* L. are annual diploids which comprise the section *Genea* Dum. together with some polyploids (Smith, 1970). They are predominantly cleistogamous, colonizing weeds which are widely distributed in SW Asia and Mediterranean countries, extending to central and West Europe. *Bromus tectorum* has also been introduced in North America, Australia, and New Zealand, whereas *B. sterilis* is a Euro-Siberian species. They often share the same habitats and grow intermixed and sympatrically, but are easily distinguished by morphology (Smith, 1972; Sales, 1993, 1994) and are clearly differentiated by karyotypes (Kozuharov, Petrova & Ehrendorfer, 1981), indicating their reproductive isolation and specific distinctness. Our previous study (Oja & Jaaska, 1996) showed that the two species also differ genetically in alternate allozymes of four isozymes. At the same time, both species revealed astonishingly little interpopulational allozymic variability; only two isozymes out of 13 studied displayed variant allozymes in some accessions of different geographic origin (Oja & Jaaska, 1996). Similarly, Novak, Mack & Soltis (1991) also detected only low allozyme variation within *B. tectorum* populations in North America. Eurasian populations of *B. tectorum*, however, exhibited greater genetic allozyme differentiation than those in North America, and most differentiated were populations from SW Asia, the presumable native range of this species (Novak & Mack, 1993). Krzakowa & Kraupe (1981) described inter- and intrapopulational variation of esterase and aspartate aminotransferase isozymes among *B. tectorum* populations from Poland and five other European

countries. These studies showed that isoenzymes may considerably differ in the extent and geographic pattern of intraspecific variation in autogamous species.

This study presents more extensive data on the allozyme divergence and diversity in *B. tectorum* and *B. sterilis*, in supplement to our previous paper (Oja & Jaaska, 1996). Special objectives were to assess geographic distribution of the allozyme variation in the two diploids throughout their Eurasian range and to compare allozyme variability among presumable native Near East and marginal European accessions.

MATERIALS AND METHODS

Plant material

Seed accessions from Turkey and central Russia were collected by the author, accessions from Syria were collected and provided by Vello Jaaska (Institute of Zoology & Botany, Tartu). The accessions of other geographical origin, also collected in the wild from known locations, were received from different botanical gardens.

1. *Bromus tectorum* L.: 61 accessions, originated from Turkey (17), Syria (13), Germany (14), Spain (5), France (3), Central Russia (2), Italy (1), Greece (1), Hungary (1), Slovakia (1), Czech Republic (2) and Romania (1).

2. *Bromus sterilis* L.: 48 accessions, originated from Turkey (7), Syria (3), Germany (19), France (11), Italy (3), Greece (1), Portugal (1), Hungary (1), Romania (1) and Spain (1).

Vouchers are deposited in TAA, Herbarium of the Institute of Zoology and Botany (Tartu, Estonia). Some of them were grown from the seed accessions in order to check their botanical identifications.

Isoenzyme analysis and nomenclature

The following enzymes were examined: aliphatic alcohol dehydrogenase (ADH, EC 1.1.1.1), aromatic alcohol dehydrogenase (AAD, EC 1.1.1.90 and 1.1.1.91), malate dehydrogenase (MDH, EC 1.1.1.37), shikimate dehydrogenase (SKD, EC 1.1.1.25), aspartate aminotransferase (AAT, EC 2.6.1.1), superoxide dismutase (SOD, EC 1.15.1.1), 6-phosphogluconate dehydrogenase (PGD, EC 1.1.1.44), phosphoglucoisomerase (PGI, EC 5.3.1.9), peroxidase (PRX, EC 1.11.1.7) and esterase (EST, EC 3.1.1.2). Enzyme extracts were prepared from the shoots (the primary leaf with the coleoptile) of 4–10 days old, etiolated seedlings by grinding in 0.1 M Tris — 0.05 MEDTA buffer containing either 5 mM cysteine or 5 mM dithiothreitol. After adding 20–50 mg of a sucrose — Sephadex G — 200 mixture (4:1) to increase their viscosity, the extracts were subjected to electrophoresis in vertical polyacrylamide gel slabs (120 × 70 × 2 mm). The following three gel-buffer systems and two catholytes were applied for different enzymes to achieve better band resolution:

Gel 1: 10% acrylamide, 0.15 % N,N'-bisacrylamide (Bis), 0.25 M Tris, and 0.1 M HCl; applied for PGI, SKD and PGD with the 2-alanine catholyte.

Gel 2: 10% acrylamide, 0.15 % Bis, 0.15 M Tris, and 0.1 M HCl; applied for ADH, AAD, MDH, AAT and PRX with the glycine catalyte.

Gel 3: 10% acrylamide, 0.15 % Bis, 0.25 M Tris, and 0.1 M Tris hydrochloride; applied for SOD and EST with the glycine catalyze.

N,N,N',N'-Tetramethylethylenediamine (0.05 ml%), riboflavine (0.5 mg%) and ammonium persulfate (1 mg%) were added to the gel mixtures to initiate and catalyze their photopolymerization between two day-light fluorescent bulbs during 1 h.

The two catholytes used consisted of 80 mM glycine or 2-alanine with 10 mM Tris. The lower anode buffer was always 0.1 M Tris — acetate with the initial pH about 8.9, and it was used repeatedly as long as the pH remained over 7.

Electrophoresis in the anodal direction was carried out in an ice — refrigerated plexiglass apparatus by applying a pulsed current at 15 mA and 20–30 V/cm for about 2–2.5 h until the marker dye, bromphenol blue, reached the gel end.

After electrophoresis, the gels were stained for isoenzymes by applying standard histochemical methods (Weeden & Wendel 1989) with modifications as described in Jaaska & Jaaska (1986, 1990).

Four individual seedlings of each accession were routinely analyzed for all isoenzymes. Up to ten individuals were analyzed for some enzymes and accessions, in order to check the constancy or polymorphism of the isoenzyme patterns. Isoenzyme phenotypes were interpreted on the basis of existing knowledge of isoenzyme structure and genetic control as summarized by Wendel and Weeden (1989). Genetically heterologous isoenzymes encoded by separate loci of a diploid genome, named shortly heterozymes according to Jaaska & Jaaska (1984) and corresponding to isozymes *auct.*, *non* Markert & Müller (1959), are designated by capital letters followed by a number, reflecting allozymic variants (in short-morphs) in the order of decreasing mobility.

RESULTS

The data on the isoenzyme variation among the accessions of the two bromegrass species are summarized in Table 1. The data for the six invariant heterozymes which were monomorphic for a shared allozyme in both species, namely ADH-A4, AAD-C3, AAT-A1, SKD-A2, SOD-A4 and PGD-A2, were excluded for shortage from Table 1. The three other heterozymes, MDH-A, AAT-B and SOD-B, were also virtually monomorphic for the same morph in both species, except the appearance of a rare MDH-A2r in a single German accession of *B. tectorum*, of a rare AAT-B6 in a single Spanish accession of *B. tectorum*, and of a rare AAT-B7r and SOD-B1r in some individuals of a single Turkish accession of *B. sterilis* and *B. tectorum*, respectively (Table 1). Thus, in total, of the 18 heterozymes specified, nine proved virtually invariant for the same allozyme shared by the both species.

The data in Table 1 confirm the divergence of the two diploids by allozymes of AAD-B, AAT-C and PRX-F reported previously (Oja & Jaaska, 1996) and additionally show their divergence by allozymes of AAT-D, AAT-E, EST-A and EST-F. In addition to divergence by the major AAT-C, we noticed that *B. sterilis* and *B. tectorum* could also be distinguished by minor bands located on the zymograms between AAT-A and AAT-B. These bands, designed as AAT-D and AAT-E, could be indicated only after a more prolonged staining of gels than was needed to develop the major bands of AAT-B and AAT-C, and they were not scored in our previous paper (Oja & Jaaska, 1996). Both heterozymes, AAT-D and AAT-E, displayed interspecific divergence. All accessions of

B. tectorum lacked AAT-D (designed as AAT-D0), whereas *B. sterilis* had AAT-D1. AAT-E revealed allozymes E1 and E2 in *B. tectorum* and *B. sterilis*, respectively.

In total, *B. sterilis* and *B. tectorum* showed divergence by allozymes of seven heterozygotes, AAD-B, AAT-C, AAT-D, AAT-E, PRX-F, EST-A and EST-F (Table 1). Only a few additional variants were encountered in single individuals at these species-specific isozyme loci. This result indicates that the genetic differentiation with the appearance of species-specific allozymes was confined to the speciation event and time, which was followed by a genetic stasis throughout the whole following colonization history of both species, at least at the allozyme loci examined.

The seedling esterase zymograms revealed a complex banding pattern with at least six scorable heterozygotes, designed from A to F. The characteristic feature of the esterase isozyme system was that its pattern showed considerable changes in both the band number and intensity, depending on the seedling age and the tissue (the coleoptile, primary leaf or root). Some heterozygotes could also be distinguished by different staining colour, showing different specificity to the two substrates, 1- and 2-naphthyl acetates, in the same staining medium, as described in Jaaska (1980, 1992).

The fastest isozyme EST-A was consistently scorable for not more than 4-day old shoots and revealed a clear interspecific divergence with A2 in *B. sterilis* and A3 in *B. tectorum*. No intraspecific variation of EST-A was detected within both species. A fast group of three bands, designated as putative heterozygotes EST-B, EST-C and EST-D was difficult to homologize due to their partially overlapping mobilities and great dependence on the tissue and its age. These isoesterases, as less suitable genetic markers for population studies will therefore not be considered here.

EST-E was consistently scorable as a band doublet for 6-day old shoots with E2 shared by both species. However, it displayed some intraspecific variation with three variants in *B. tectorum* (prevalent E2, rare E3 and E0) and two variants in *B. sterilis* (prevalent E2 and rare E0). E0 specifies absence of the band, i.e. a presumable null-allele.

The slowest heterozygote EST-F was less dependent on the age of seedlings and was polymorphic in both species. Almost all accessions of *B. sterilis* showed F3, and only rare accessions F4 or F1. *B. tectorum* displayed interpopulational variation with two prevalent allozymes, F1 and F4, and three rare morphs, F5, F3 and F2.

EST-E and EST-F were the two most variable heterozygotes in *B. tectorum*, whereas most variable in *B. sterilis* were PRX-F and PGI-A with three allozymes in each.

A characteristic feature of the isozyme variability in the two bromes was total absence of two- or three-banded heterozygous allozyme phenotypes of polymorphic heterozygotes despite of a considerable number of individuals analyzed. All the variation was observed as single-banded electrophoretic variants attributed to allozymes. This result shows virtually complete inbreeding in natural populations of both diploids, in accordance with the data about autogamy and self-fertilization of annual bromes (Smith, 1972). Similarly, Novak *et al.* (1991) detected no heterozygous individuals in the North American populations of *B. tectorum*. An important consequence of the autogamous breeding in the two species is that their evolution occurs by divergence into multilocus genetic lineages, in contrast to reticulation in outcrossing species.

The isoenzyme data in Table 1 characterize the genetic heterogeneity among the populations of different geographic origin within the two species studied. The accessions from Turkey and Syria within their native East-Mediterranean area displayed more interpopulational variability than accessions from other regions. For example,

B. sterilis and *B. tectorum* from Turkey revealed four and five multilocus isozyme lineages, respectively, whereas numerous accessions from Germany had only three lineages in each species. The number of accessions analysed from these regions was almost equal. By the polymorphic EST-E and EST-F, *B. tectorum* from Syria is divided into five multilocus allozyme lineages, including three unique lineages, whereas *B. tectorum* from France has only two lineages, one of which corresponds to a Syrian lineage. Levels of genetic variation are, however, geographically heterogeneous.

Many of the populations within the species appear to belong to identical multilocus lineages in different geographic regions with different ecological conditions. Thus, the most common *B. tectorum* allozyme genotype Turkey 1 was also found in Syria, France, Germany, Spain, Hungary, Greece and Slovakia. Another common *B. tectorum* genotype Turkey 2 was also found in Syria, Germany, and Central Russia. In Germany we found both common lineages from Near East (Syria 3, 4), but in Central Russia — only one of them (Syria 3). The two French allozyme lineages of *B. tectorum*, France 1 and 2, correspond to respective Turkish lineages 3 and 1. A common genotype of *B. sterilis* (Turkey 4, Syria 1) was found everywhere throughout its Eurasian distribution. Only five new allozyme lineages could be found among geographically marginal European accessions of the two species: one French and one German lineage of *B. sterilis* with the appearance of unique PRX-F6 and PRX-F9, respectively, and three lineages of *B. tectorum* — one in Germany with unique MDH-A2, one in Spain with unique AAT-B6 and one in Italy with the appearance of unique EST-F3. Of the eight allozyme lineages of *B. tectorum* detected in the Near East region, five were confined to this region and only three were distributed to Europe.

DISCUSSION

Two contrasting explanations of the weedy adaptation and colonization have been proposed (Baker, 1965, 1974; Bradshaw, 1965; Barrett & Richardson, 1986). One strategy of effective colonization relies on extensive genetic polymorphism needed for successful colonization throughout large territories with the development of locally adapted “specialist” genotypes. Another strategy relies on development of a limited number of “general purpose” genotypes adaptable to a wide range of environmental conditions due to their significant phenotypic plasticity.

The two annual diploid brome studied, *B. sterilis* and *B. tectorum* may be considered as successful weedy colonizers which have occupied large territories to both east- and westward from their presumable Near-East centre of origin. Only little intrapopulation allozymic variability was observed in the diploids throughout their huge distribution ranges. Thus, only eight populations from total 109. *B. tectorum* and *B. sterilis* examined revealed intrapopulation polymorphism with rare additional AAT-B, SOD-B, PRX-F, PGI-A and EST-F allozymes in single individuals. This finding is in accordance with previously reported data (Novak *et al.*, 1991; Novak & Mack, 1993). The population genetic structure established for many self-pollinating weed species clearly indicates that high level of genetic variation is not a preliminary condition for successful colonization (Barrett & Shore, 1989). The low level of genetic variation in *B. tectorum* and *B. sterilis* is similar to that reported for several other autogamous plant species. For example, autogamous weedy grasses of *Setaria* spp. showed low genetic diversity

(Wang, Wendel & Dekker, 1995 a,b) in six different countries from both the Old and New World. Selfing allotetraploid *Elymus canadensis* revealed low allozyme variation without relationship to climatic differences between the regions of origin (Sanders, Hamrick & Holden, 1979). Very low levels of allozyme polymorphism were found in five colonizing North American weed species, in a sharp contrast with their substantial inter- and intrapopulational variation in morphology and phenology (Warwick, 1988). The colonizing ability of these autogamous species, including *B. sterilis* and *B. tectorum*, apparently lies on the limited number of widely adapted multilocus lineages.

On the contrary, Nevo *et al.* (1979, 1986 a, b) revealed rich allozyme variation in *Hordeum spontaneum* from Turkey, Iran and Israel and supposed that genetic variation in wild barley populations is correlated with the environment and is at least partly adaptive. Investigations of *Avena barbata* from Israel (Kahler *et al.*, 1980) and California (Clegg, Allard, 1972; Hamrick, Allard, 1972) also have shown that allozyme diversity is greater in SW Asian populations and correlated with environment. The weedy colonization of these species apparently lies on the use of an alternative evolutionary strategy based on the development of multiple locally adapted genotypes.

Our results also indicate the presence of greater allozyme variability in plants from the ancestral part of the distribution area (Turkey, Syria). Marginal populations (Europe, Central Russia) were less variable. The same tendency was marked for *Hordeum jubatum* (Shumaker & Babble, 1980) and for *H. secalinum* (Cronberg, Widen, Bothmer, 1996). The present data about allozyme variation patterns for *B. tectorum* and *B. sterilis* showed that long distance migration from their native area and adaptation to different ecological conditions were not accompanied by considerable genetic divergence. The colonizing expansion of both autogamous annuals from its presumably ancestral Near East centre of origin and divergence to Europe has led to development of only three new multilocus allozyme genotypes in *B. tectorum* (Germany 3, Spain 2 and Italy 1) and only two in *B. sterilis* (France 2 and Germany 3). Thus, the presence of a common allozyme genotype for *B. sterilis* throughout its Eurasian distribution and two most common allozyme genotypes for *B. tectorum* suggest that "general purpose" genotype paradigm could be applicable to the two species to explain their colonizing success.

Data presented here demonstrate the usefulness of isozyme gene markers for biogeographic investigations of autogamous annual bromes, but more extensive sampling both within and among accessions from different parts of the ranges is needed for more substantiated phylo- and ecogeographic conclusions.

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Table 1. Electrophoretic variants of aromatic alcohol dehydrogenase (AAD), malate dehydrogenase (MDH), aspartate aminotransferase (AAT), superoxide dismutase (SOD), peroxidase (PRX), phosphoglucosomerase (PGI) and esterase (EST) heterozymes in *Bromus* species. N — the number of accessions analyzed, s designates a slightly slower, and f — a slightly faster variant of the numbered morph, r — a rare morph. Rare and unique allozymes are in bold.

Species and geographic lineages	N	AAD	MDH	AAT	AAT	AAT	AAT	SOD	PRX	PGI	EST	EST	EST
		B	A	B	C	D	E	B	F	A	A	E	F
<i>Bromus sterilis</i>													
Turkey 1	4	2	3	5	3	1	2	2	8	7	2	2	3
Turkey 2	1	2	3	5	3	1	2	2	8	6	2	0	3
Turkey 3	1	2	3	5	3	1	2	2	8	5	2	2	3
Turkey 4	1	2	3	5,7r	3	1	2	2	8	6	2	2	3
Syria 1	3	2	3	5	3	1	2	2	8	6	2	2	3
France 1	10	2	3	5	3	1	2	2	8	6	2	2	3
France 2	1	2	3	5	3	1	2	2	6	6	2	2	3
Germany 1	16	2	3	5	3	1	2	2	8	6	2	2	3
Germany 2	1	2	3	5	3	1	2	2	8	6	2	0	3
Germany 3	2	2	3	5	3	1	2	2	9	6	2	2	3
Spain 1	1	2	3	5	3	1	2	2	8	6	2	2	3
Hungary 1	1	2	3	5	3	1	2	2	8	6	2	2	3,4r
Italy 1	3	2	3	5	3	1	2	2	8	6	2	2	3
<i>Bromus tectorum</i>													
Turkey 1	8	3	3	5	1	0	1	2	5,5sr	6,5r	3	2	1
Turkey 2	4	3	3	5	1	0	1	2,1r	5	6,5r	3	2	4
Turkey 3	2	3	3	5	1	0	1	2	5	6	3	3	1
Turkey 4	1	3	3	5	1	0	1	2	5	6	3	0	1
Turkey 5	2	3	3	5	1	0	1	2	5f	6	3	2	4
Syria 1	4	3	3	5	1	0	1	2	5	6	3	0	4
Syria 2	4	3	3	5	1	0	1	2	5	6	3	2	0
Syria 3	2	3	3	5	1	0	1	2	5	6	3	2	4
Syria 4	2	3	3	5	1	0	1	2	5	6	3	2	1
Syria 5	1	3	3	5	2	0	1	2	5	6	3	0	4
France 1	1	3	3	5	1	0	1	2	5	6	3	3	1
France 2	2	3	3	5	1	0	1	2	5	6	3	2	1
Germany 1	10	3	3	5	1	0	1	2	5	6	3	2	1
Germany 2	2	3	3	5	1	0	1	2	5	6	3	2	4
Germany 3	2	3	2	5	1	0	1	2	5	6	3	2	1
Spain 1	4	3	3	5	1	0	1	2	5	6	3	2	1
Spain 2	1	3	3	6	1	0	1	2	5	6	3	2	1
Hungary 1	1	3	3	5	1	0	1	2	5	6	3	2	1
Italy 1	1	3	3	5	1	0	1	2	5	6	3	2	3
Greece 1	1	3	3	5	1	0	1	2	5	6	3	2	1,5r
Slovakia 1	1	3	3	5	1	0	1	2	5	6	3	2	1
Central Russia 1	2	3	3	5	1	0	1	2	5	6	3	2	4

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Teadustegevus

Uurimisvaldkonnad: kõrreliste (perekond *Bromus*) fülogeneetiline süstemaatika, isoensüüm-tunnuste mitmekesisus ja geneetiline varieerumine. Populatsioonigeneetika. Olen osalenud "Taimede välimääraja" koostamisel.

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