Ann. Bot. Fennici 35: 123–130
Helsinki 30 June 1998
ISSN 0003-3847

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

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Received 14 October 1997, accepted 11 March 1998

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 independent 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 alopecuros* 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 perfomed 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 1998).

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). No. = 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 A	MDH A	SKD A	AAT B	AAT C	SOD A	SOD B	PRX F	PGD A|B	PGI A
Hordeum bogdanii	2	3	**2f**	3	**6**	**2**	2	**1**	**9**	**3**	5
Section *Boissiera*											
B. pumilio Eu	1	3	2	2	**6**	3	2	2	**1**	2|3	**7**
Section *Bromus*											
B. danthoniae T	2	3	3	2	5	3	2	2	6	2|3	5
B. alopecuros T1	2	3	2	2	5	3	4	2	**2s**	2	4
B. alopecuros T2	1	3	2	2	5	3	4	**1**	**2**	2	3
B. scoparius CA1	2	3	3	**3s**	4	3	**6**	2	4	2	4
B. scoparius CA2	1	**7**	3	2	4	3	**6**	2	4	2	4
B. japonicus CR	8	3	3	2	5	3	4	2	6	2|3	4
B. jap-sq T1	9	3	3	2	4	3	4	2	6	2|3	4
B. jap-sq T2	2	3	3	2	4	3	4	2	6	2|3	5
B. squarrosus T1	2	3	3	2	5	3	4	2	**6f**	2|3	4
B. squarrosus T2	5	3	3	2	4	3	4	2	6	**1|3**	4
B. squarrosus CR	1	3	3	2	4	3	4	2	5	2|3	4
B. intermedius T1	1	3	2	2	5	3	4	2	5	2|3	4
B. intermedius T2	1	3	2	3	4	3	4	2	5	2|3	4
B. intermedius T3	2	3	2	3	5	3	4	2	5,**3**	2|3	4
B. intermedius T4	4	3	2	3	4	3	4	2	5	2|3	3
B. intermedius T5	7	3	2	3	5	3	4	2	5	2|3	3
B. intermedius T6	1	3	2	3	5	3	4	2	4	2|3	3
B. arvensis T1	1	3	2	2	5	3	4	2	5	2|3	3
B. arvensis T2	1	3	2	3	5	3	4	2	4	2|3	5
B. arvensis Eu	12	3	2	2	4	3	4	2	4	2|3	3
B. pseudosecalinus Eu	1	3	3	3	**3**	3	4	2	5	**2|4**	4
Section *Genea*											
B. sterilis T1	4	4	3	2	5	3	4	2	8	2	7
B. sterilis T2	2	4	3	2	5	3	4	2	8	2	6
B. sterilis T3	1	4	3	2	5	3	4	2	8	2	5
B. sterilis Eu	20	4	3	2	5	3	4	2	8,6r	2	6
B. tectorum T	17	4	3	2	5	**1**	4	2	5	2	6
B. tectorum 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 heterozyme 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 heterozymes (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. tectorum* 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 heterozymes. 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 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 *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 heterozymes 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. Heterozymes differed remarkably in the extent of genetic variability, AAT-C and SOD-B were the most conservative heterozymes in this section, with one morph in common to most species. PRX-F, PGI-A and PGD-A were the most variable heterozymes 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 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 (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 (1985b) 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 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. 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).

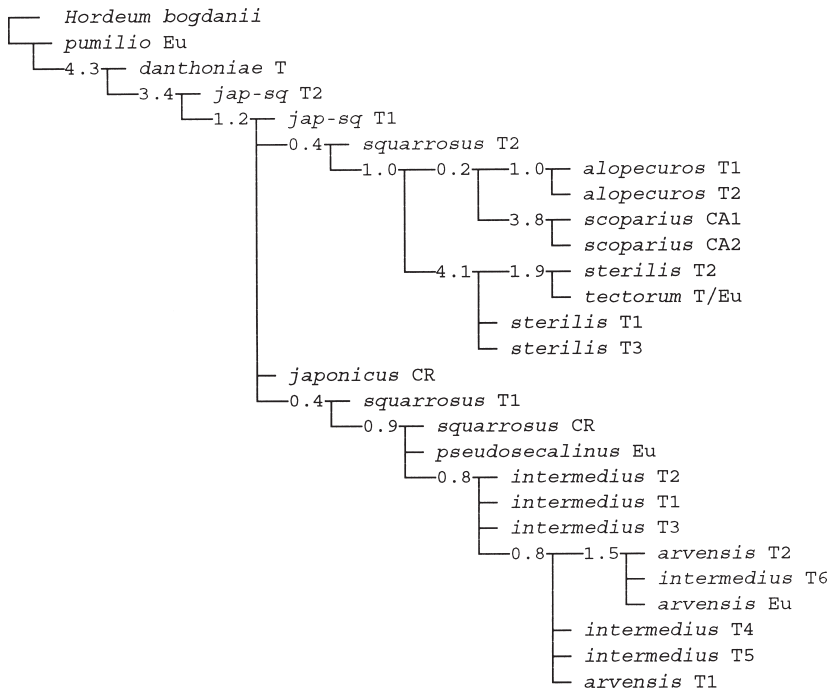

Fig. 1. The consensus tree of the 18 fittest trees for the diploid annual *Bromus* species from the Pee-Wee analyses of the data matrix of 36 shared allozymes of 10 heterozymes. Decay indexes are given at branches.

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 Fourn. 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

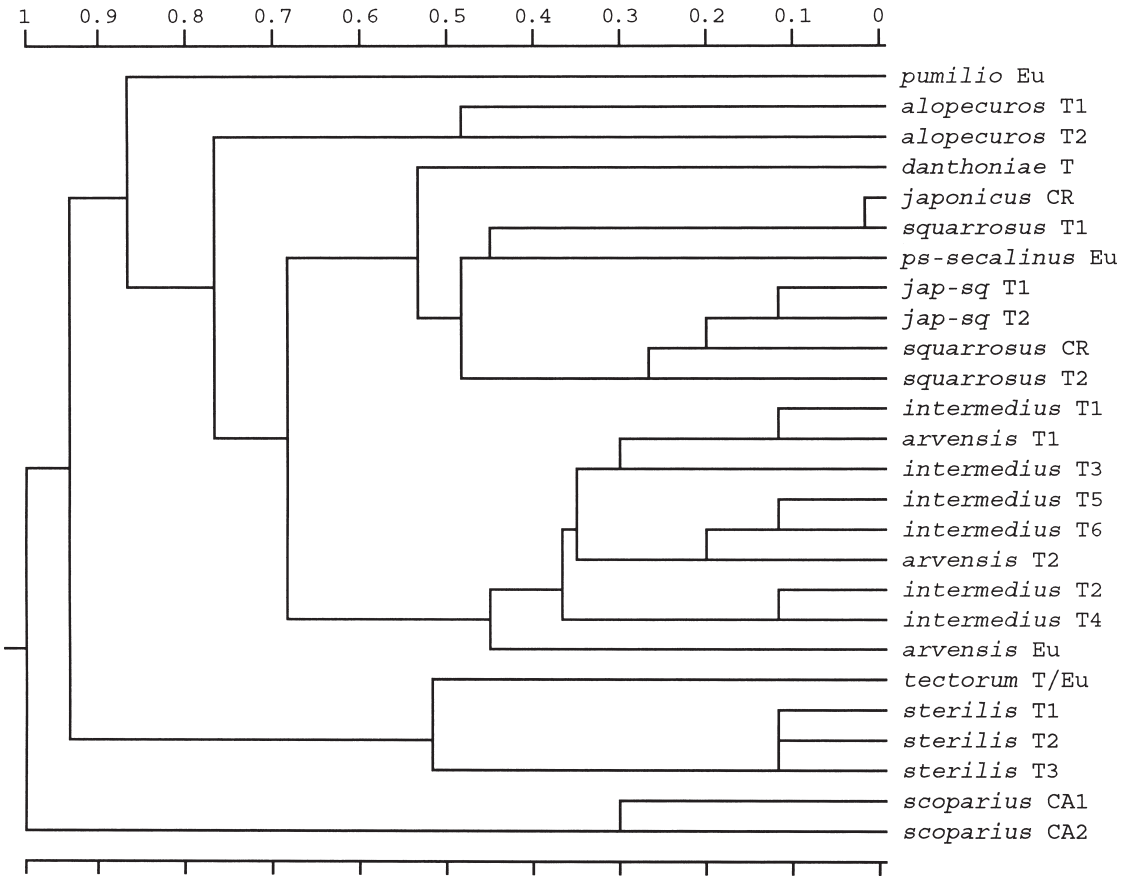

Fig. 2. UPGMA phenogram of Manhattan distances for the diploid annual bromes, based on a presence/absence data matrix of 36 shared and 14 unique allozymes of 10 heterozymes compiled from Table 1. Scale relative, 0 = minimal distance.

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 phenetic trees in a separate cluster which is linked to *B. alopecuros* 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.

Acknowledgements: The research was supported partly by the grant Nr. 2010 from the Estonian Science Foundation to Dr. V. Jaaska. We thank Prof. Erast Parmasto (Department of Mycology, Institute of Zoology and Botany, University of Tartu) for his assistance with the computer programs.

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