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Phylogenetic relationships of *Elymus* L. and related genera (Poaceae) based on the nuclear ribosomal internal transcribed spacer sequences

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Abstract: To help in the clarification of the taxonomic status of *Elymus* L. and related genera *Agropyron* Gaertn., *Leymus* Hochst., *Hordelymus* (Jess.) Harz, and *Brachypodium* P.Beauv., the sequence diversity in the internal transcribed spacer (ITS) region of nuclear ribosomal DNA was studied. ITS data of 64 *Triticeae* accessions including 40 *Elymus*, 12 *Agropyron*, 7 *Leymus*, and 4 *Hordelymus*, and 1 *Brachypodium* as an out-group were analysed. The molecular diversity statistics indicated that the most diverse genus is *Elymus* among the studied genera. The constructed phylogenetic tree by the maximum parsimony method revealed that one specimen of *Elymus*, *E. pycnanthus* (Godr.) Melderis, clustered with species of *Agropyron*. Molecular diversity statistics also indicated that *E. pycnanthus* is distantly related to other species of *Elymus*, but is closer to *Agropyron* species. The finding of strong affinity of *Elymus* to the species of *Leymus* and *Agropyron* supports the view that the taxonomy of *Elymus* is further complicated by the role of hybridisation among different ancestral genera.

Key words: *Elymus*, molecular diversity, phylogeny, ITS, *Triticeae*

Elymus L. ve akraba cinslerin (Poaceae) nüklear ribozomal iç transkripsiyonu aralayıcı dizisi kullanılarak filogenetik akrabalıkları

Özet: *Elymus* L. ve bu cinse akraba *Agropyron* Gaertn., *Leymus* Hochst., *Hordelymus* (Jess.) Harz ve *Brachypodium* P.Beauv. cinslerinin taksonomik statülerini açıklamaya yardımcı olmak için nüklear ribozomal DNA bölgesinde bulunan iç transkripsiyonu aralayıcı (ITS) bölgesinin dizi çeşitliliği çalışılmıştır. 40 *Elymus*, 12 *Agropyron*, 7 *Leymus*, 4 *Hordelymus* ve dış-grup olarak kullanılan 1 *Brachypodium* olmak üzere 64 *Triticeae* örneğinin ITS bölgesinden alınan verileri analiz edilmiştir. Moleküler çeşitlilik verileri, çalışılan cinsler içerisinde en fazla çeşitliliğin *Elymus* cinsinde varolduğunu göstermiştir. Maximum parsimony metodu kullanılarak elde edilen filogenetik ağaç, *Elymus* cinsi içerisinde bulunan bir türün, *E. pycnanthus* (Godr.) Melderis, *Agropyron* cinsi ile beraber grup oluşturduğunu göstermiştir. Aynı zamanda moleküler çeşitlilik verileri, *E. pycnanthus* türünün *Elymus* cinsine ait türlere daha uzak, fakat *Agropyron* türlerine daha yakın olduğunu göstermiştir. *Elymus* cinsinin *Leymus* ve *Agropyron* cinsine ait olan türlerle yakın ilişkisi, *Elymus* taksonomisinin farklı atasal cinslerle aralarında gerçekleşen melezlenme ile dahada karmaşıklaştığı görüşünü desteklemektedir.

Anahtar sözcükler: *Elymus*, moleküler çeşitlilik, filogeni, ITS, *Triticeae*

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Introduction

The grass family (Poaceae) is an important plant family for agriculture that includes major cereals (the tribe *Triticeae*). It is also taxonomically challenging for botanists. Data from cytogenetic analyses were used to describe systematic relationships of the tribe, to mark limits of the genera and clarify the ancestry of many species in the past (Dewey, 1982, 1984; Löve, 1984). Among the various views on generic classification within *Triticeae*, the treatments by Stebbins (1956) and Löve (1984, 1986) represent opposite extremes. Stebbins (1956) argued for lumping all species into a single genus, and Löve (1986) split the *Triticeae* into 39 genera. These studies indicated that among the most challenging problems within the *Triticeae* is the delimitation of the genus *Elymus* and its relationships with related genera of the tribe.

Elymus was the first described as a genus by Linnaeus (1753). Hitchcock and Green (1929) designated *E. sibiricus* as the type species of the genus. During the revision of *Triticeae* in *Flora of the USSR*, Nevski (1934) proposed considerable changes in the generic subdivision within *Triticeae*. The perennial taxa included in *Agropyron* were referred to 4 genera: *Agropyron* s. str., *Elytrigia* Desv., *Roegneria* C.Koch, and *Anthosachne* Steud. The species of *Elymus* were divided into 6 genera: *Elymus* s. str. (correct name *Leymus* Hochst.), *Aneurolepidium* Nevski, *Malacurus* Nevski, *Clinelymus* (Griseb.) Nevski (*Elymus* s. str.), *Terella* Nevski, and *Psathyrostachys* Nevski. (Assadi & Runemark, 1995).

Runemark and Heneen (1968) expanded the generic concept of *Elymus* to include the genera *Agropyron*, *Elytrigia*, *Roegneria*, *Aneurolepidium*, *Terella*, *Hystrix (Asperella)*, and *Sitanion*. This treatment was followed by Melderis (1985). However, Melderis proposed *Agropyron* s. str. as a separate genus for the crested wheatgrasses. Löve (1984, 1986) presented a new generic system for the *Triticeae*, based on genomic constituents. He used a narrow generic concept and the perennials referred by Bentham (1882) to *Agropyron* and *Elymus* were split into 14 genera.

The taxonomy of *Elymus* is extremely complex because of the enormous morphological variation within and between species, diversity of habitats where the genus originates, and hybridisation within *Elymus* as well as between *Elymus* and other genera of *Triticeae*. Some other problems are associated with the unreliability and discrepancies of morphological data that make taxonomists hold different opinions in delimitation studies in the genus *Elymus* (Hitchcock, 1951; Keng, 1959; Tzvelev, 1976).

The last revision of *Elymus* in Turkey was made by Melderis (1985) in Davis' *Flora of Turkey and the East Aegean Islands*, in which 19 species were recognised. Since the publication of the flora, an additional species, *Elymus hoffmanni*, has been described from Turkey (Jensen & Asay, 1996). After publication of the flora and its supplements, the new grass taxa were compiled in supplements for publication (Davis et al., 1988; Güner et al., 2000). In addition to these, taxonomical and palynological studies were performed on certain genera (Doğan, 1988, 1991, 1992, 1997, 1999; Cabi & Doğan, 2009; Cabi et al., 2009; Özler et al., 2009; Cabi et al., 2010a; Cabi et al., 2010b; Cabi & Doğan, 2010).

Helfgott and Mason-Gramer (2004) stated that 75% of the about 350 *Triticeae* species whose chromosome number is known have a polyploidy origin. *Elymus* has its origin through natural hybridisation of a few related genera in the tribe *Triticeae* (Dewey, 1984; Löve, 1984), and thus it has close genetic similarities with *Leymus*, *Agropyron*, and *Hordelymus*. Previous cytological studies suggest that *Elymus* involves 5 basic genomes, namely the St, Y, H, P, and W in various combinations (Lu, 1994). The Turkish *Elymus* species, which are the focus of this study, can be defined as those allopolyploid species with at least one set of *Pseudoroegneria* (Nevski) A.Löve (St) genomes. *Elymus* may be diploid, tetraploid, hexaploid, or octoploid, and may combine the St genome with H genome from *Hordeum* L., P from *Agropyron* Gaertner, and Y from *Roegneria*. Genome symbols and cytological structures of the studied specimen are given in Table 1 with their references.

Cytogenetic study of polyploidy species like those of *Elymus* is not only difficult, but also less informative for resolving phylogenetic problems of plant genera. However, phylogenetic relationships of polyploid species have been facilitated by the development of molecular techniques that assist to

Table 1. The genome designations and cytological status of the species of *Elymus* and its closely related genera used in the study.

Genus name	Species name	Genome symbols	Cytology	References
Elymus	*tauri*	StP or St	2n=2x=14 or 4x=28	Wang et al., 1986; Yen et al., 2005; Zhang et al., 2009
	libanoticus	St	2n=14	Dewey, 1972
	sosnowskyi	Not designated	Unknown	
	repens subsp. *elongatiformis*	StStStHH	2n = 6x = 42	Assadi & Runemark, 1995
	repens subsp. *repens*	StStStHH	2n = 6x = 42	Assadi & Runemark, 1995
	clivorum	Not designated	Unknown	
	pycnanthus	EStP	2n = 6x = 42	Refoufi et al., 2001
	lazicus subsp. *divaricatus*	Not designated	Unknown	
	lazicus subsp. *lazicus*	Not designated	Unknown	
	caninus	StStHH	2n = 4x = 28	Dewey, 1970; Readinbaugh et al., 2000; Zhang et al., 2009
	transhyrcanus	StStStHH	2n = 6x = 42	Dewey, 1972
	elongatus subsp. *elongates*	E^e	2n=2x=14	Wang, 1985
	farctus subsp. *bessarabicus*	E^b	2n=2x=14	Wang, 1985
	hispidus subsp. *barbulatus*	SJJ, E^b E^b St or E^e E^b St	2n=6x=42	Liu &Wang, 1993; Chen et al., 1998
Agropyron	*cristatum* subsp. *pectinatum*	P	2n=4x=42	Assadi, 1995
	cristatum subsp. *incanum*	P	2n=6x=42	Assadi, 1995
Leymus	*cappadocicus*	Not designated	Unknown	
	racemosus subsp. *sabulosus*	NsXm	2n = 4x = 28	Anamthawat-Jonsson & Bödvarsdöttir, 2001
Hordelymus	*europeous*	XoXr		Bothmer et al., 1994
Brachypodium	*sylvaticum*	-	-	

determine genetic similarities in plant species (Wendel, 2000; Soltis et al., 2003). One of the most preferred techniques to determine genetic relationships of plants at lower taxonomic levels involves sequence diversity study of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (Baldwin et al., 1995; Hsiao et al., 1995; Wendel et al., 1995; Zhang et al., 2002; Hao et al., 2004). The ITS region plays a role in ribosomal maturation and processing of small and large-subunit rRNAs (Muster et al., 1990; Liang & Fournier, 1997; Joseph et al., 1999). The evolutionary origin of the ITS is considered to be an intron-like structure (Torres, 1990) flanked by highly conserved regions from which universal primers can be obtained (White et al., 1990). The small size of the ITS region makes this region easy to amplify, even from herbarium material that is dry. The other advantage in sequencing the ITS region is that it is non-coding and so includes a relatively high level of variability.

The objective of the present study was to shed light on the taxonomic problems of the genus *Elymus* by studying molecular diversity in the nuclear ribosomal ITS region and constructing a phylogenic tree of *Elymus* and its closely related genera such as *Leymus*, *Agropyron*, and *Hordelymus*.

Materials and methods

Plant material

A total of 64 *Triticeae* accessions were used in the current study including 40 *Elymus*, 12 *Agropyron*, 7 *Leymus*, and 4 *Hordelymus* accessions that represent 12, 1, 2, and 1 species, respectively. *Brachypodium sylvaticum* was used as an out-group based on phylogenetic studies of *Poaceae* (Hsiao et al., 1995; Gaut, 2002) since sequence information of the ITS region was available for only this genus. The leaf materials were obtained from a large collection which was part of a herbarium collection and a taxonomic

study dealing with *Elymus*, *Agropyron*, *Leymus* and *Hordelymus* species. For each species, leaf samples from genotypes originating from different locations within the natural range of the species in Turkey were used for DNA extraction.

Since 2006, as a part of a revisional study of the tribe *Triticeae* Dumort. in Turkey, the authors have carried out extensive field studies and collected a large number of specimens of the genus *Elymus*. The specimens were sampled following the methodology outlined in Davis and Heywood (1973). The specimens were cross-checked with the keys provided by Melderis (1985). The specimens were also compared with the specimens cited in the *Flora of Turkey* that are kept at E, K, BM, ANK, EDTU, EGE, GAZI, HUB, ISTE, and ISTF herbaria (herbarium codes from Holmgren et al., 1990). Geographic and topographic information on all collected accessions and the ones taken from National Center for Biotechnology Information (NCBI) are provided in Table 2.

DNA extraction, ITS amplification and sequencing

Total DNA was extracted from fresh young leaf tissues of each accession with a slightly modified CTAB (cetyltrimethylammonium bromide) protocol (Doyle & Doyle, 1987). The entire ITS region (ITS1-5.8-ITS2) was amplified by polymerase chain reaction (PCR) using the primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS-L (5'TCGTAACAAGGTTTCCGTAGGTG-3') (Hsiao et al., 1995). The 18S-26S nuclear ribosomal RNA (nrDNA) gene family has proven to be a valuable tool for phylogeny reconstruction in plants (Hillis & Dixon, 1991; Hamby & Zimmer, 1992), especially at the family and higher taxonomic levels by DNA sequencing (Hamby & Zimmer, 1992) as well as among closely related genera or species (Kim & Mabry, 1991). Amplification of the ITS1, 5.8S, and ITS2 region was conducted in a volume of 50 µL, while the reaction mixture contained about 30 µL of sterile water, 5 µL of 10× reaction buffer, 1 µL of a mix of each 10 mM dNTP, 4 µL $MgCl_2$ (25 mM), using 0.15 µL (5 U/µL) of the Taq polymerase (Promega, Madison, USA), 2 µL of each primer (0.1 µg), and 3 µL of template. The thermal cycling for PCR comprised incubation at 94 °C for 3 min, and 35 cycles, each with 1 min denaturation at 94 °C, 1 min annealing at 50 °C, and an extension of 2.5 min at 72 °C with 2 s added to each subsequent polymerisation step in a Eppendorf Mastercycler PCR, USA (Daniel et al., 1994). The PCR products were electrophoresed on 1.0% agarose gels and band products were sent with forward and reverse primers for sequencing to REFGEN Gene Research and Biotechnology Company (METU-Teknopark, Ankara, Turkey).

Data analysis

Chromatograms were manually checked and edited using Chromas 2.01 (Chromas version 2.01; www.technelysium.com.au.chromas.html) and sequences were aligned with CLUSTAL X (Thompson et al., 1999) and refined manually. The boundaries of ITS1, 5.8S, and ITS2 regions in each sample were determined through comparison with sequences of the corresponding segments in *Elymus repens* (accession number: DQ859051) (Mahelka et al., 2007), *Elymus caninus* (accession number: AY740898) (Liu et al., 2006), *Elymus hispidus* (accession number: DQ859054) (Mahelka et al., 2007), *Agropyron cristatum* (accession number: AY740892) (Liu et al., 2006), *Leymus racemosus* (accession number: EF602021) (Sha et al., 2008), and *Brachypodium sylvaticum* (accession number: AJ608155) (Blattner, 2004). These ITS sequences were obtained directly from the GenBank database (Table 2) (www.ncbi.nlm.nih.gov). Since some of the sequences obtained from the same taxon were identical, only one sequence was included in the dataset. Alignment of the sequences was performed visually, as gaps were few and easily interpreted. Insertion/deletion gaps were treated as missing data. The basic sequence statistics, including nucleotide frequencies, and variability in different regions of the sequences were computed by MEGA 4 (Tamura et al., 2007). In addition to these analyses, all sequences were also used to estimate genetic diversity, which is useful in molecular population genetics studies. Nei's (1973) genetic diversity parameters, total genetic diversity, within genus-genetic diversity, and genetic distance among genera, were analyzed with MEGA 4 software (Tamura et al., 2007).

Phylogenetic analyses of the sequence data were performed using maximum parsimony (MP) in PAUP version 4.0b10 (Swofford, 2003). For MP analysis, all

Table 2. Geographic regions, topographic information and GenBank accession numbers of all studied specimens.

Genus name	Species name	Location (Province)	Latitude	Longitude	Altitude (m)	GenBank Acc. No.
Elymus	*tauri*-1	Mersin	36°59.721′	34°16.672′	1415 m	GQ365144
	tauri-2	Karaman	36°37.151′	32°55.333′	1279 m	GQ365146
	tauri-3	Karaman	36°38.199′	32°55.510′	1491 m	GQ365147
	libanoticus-1	Niğde	37°47.601′	35°03.792′	1613 m	GQ365148
	libanoticus-2	Niğde	37°47.601′	35°03.792′	1613 m	GQ365149
	sosnowskyi-1	Erzurum	40°29.286′	41°55.906′	1536 m	GQ365150
	sosnowskyi-2	Erzurum	40°29.286′	41°55.906′	1536 m	GQ365151
	repens subsp. *elongatiformis*-1	Kayseri	38°12.678′	35°52.015′	1390 m	GQ365145
	repens subsp. *elongatiformis*-2	Malatya	38°22.529′	37°52.986′	1337 m	GQ373266
	repens subsp. *repens*-1	Ankara	39°53.778′	32°46.785′	902m	GQ373267
	repens subsp. *repens*-2	Kastamonu	41°15.953′	33°31.890′	1047 m	GQ373268
	clivorum-1	Muş	39°07.601′	41°28.027′	1463 m	GQ373269
	clivorum-2	Muş	39°07.601′	41°28.027′	1463 m	GQ373270
	clivorum-3	Muş	39°07.601′	41°28.027′	1463 m	GQ373271
	pycnanthus-1	Çanakkale	-	-	Sea level	GQ373272
	pycnanthus-2	İstanbul	41°02.827′	28°03.180′	Sea level	GQ373273
	pycnanthus-3	Sinop	42°01.576′	35°04.853′	1 m	GQ373274
	lazicus subsp. *divaricatus*-1	Sivas	39°00.012′	37°17.171′	1720 m	GQ373275
	lazicus subsp. *divaricatus*-2	Kayseri	38°21.082′	36°30.905′	2180 m	GQ373276
	lazicus subsp. *divaricatus*-3	Kayseri	38°56.350′	35°49.483′	1320 m	GQ373277
	lazicus subsp. *lazicus*-1	Trabzon	40°37.818′	39°23.699′	1766 m	GQ373278
	caninus-1	Ankara	40°26.769′	32°36.499′	1265 m	GQ373279
	caninus-2	Erzurum	40°00.778′	40°32.571′	2197 m	GQ373280
	caninus-3	Erzurum	40°00.778′	40°32.571′	2197 m	GQ373281
	caninus-4	Mersin	36°56.411′	34°30.123′	1257 m	GQ373282
	caninus-5	Mersin	36°56.411′	34°30.123′	1257 m	GQ373283
	caninus-6	Kastamonu	41°04.013′	33°45.023′	1854 m	GQ373284
	transhyrcanus-1	Erzincan	39°52.431′	39°33.350′	2277 m	GQ373285
	transhyrcanus-2	Erzurum	40°26.173′	41°36.171′	2089 m	GQ373286
	transhyrcanus-3	Erzurum	40°09.422′	41°01.606′	2024 m	GQ373287
	elongatus subsp. *elongatus*-1	Sivas	39°26.048′	37°02.321′	1380 m	GQ373288
	elongatus subsp. *elongatus*-2	Sivas	39°26.048′	37°02.321′	1380 m	GQ373289
	elongatus subsp. *elongatus*-3	Sivas	39°23.850′	37°04.938′	1426 m	GQ373290
	elongatus subsp. *elongatus*-4	Ankara	39°54.125′	32°46.986′	899 m	GQ373291
	farctus subsp. *bessarabicus*-1	Sinop	42°01.576′	35°04.853′	1 m	GQ373292
	farctus-1	İstanbul	41°03.377′	28°05.516′	23 m	GQ373293
	farctus-2	İstanbul	41°04.128′	28°08.468′	0 m	GQ373294
	hispidus subsp. *barbulatus*-1	Kırşehir	39°17.567′	33°56.859′	1430 m	GQ373295
	hispidus subsp. *barbulatus*-2	Ankara	39°37.683′	32°42.086′	1073 m	GQ373296
	hispidus subsp. *barbulatus*-3	Kırıkkale	39°58.591′	34°04.506′	1050 m	GQ373297

Table 2. Continued.

Agropyron	*cristatum*-1	Erzincan	39°49.133′	39°31.095′	2099 m	GQ373298
	cristatum-2	Erzincan	39°49.133′	39°31.095′	2099 m	GQ373299
	cristatum subsp. *pectinatum*-1	Sivas	39°24.428′	37°04.838′	1405 m	GQ373300
	cristatum subsp. *pectinatum*-2	Sivas	39°24.428′	37°04.838′	1405 m	GQ373301
	cristatum subsp. *pectinatum*-3	Kars	40°43.681′	43°25.498′	1642 m	GQ373302
	cristatum subsp. *pectinatum*-4	Kars	40°43.681′	43°25.498′	1642 m	GQ373303
	cristatum subsp. *pectinatum*-5	Kırıkkale	39°34.367′	33°26.303′	772 m	GQ373304
	cristatum subsp. *pectinatum*-6	Erzurum	39°42.061′	42°17.363′	1715 m	GQ373305
	cristatum subsp. *pectinatum*-7	Nevşehir	38°36.555′	34°44.513′	1318 m	GQ373306
	cristatum subsp. *incanum*-1	Erzurum	40°01.633′	40°31.340′	2401 m	GQ373307
	cristatum subsp. *incanum*-2	Erzurum	40°01.633′	40°31.340′	2401 m	GQ373308
	cristatum-3	Sivas	39°23.786′	37°04.988′	1434 m	GQ373309
Leymus	*cappadocicus*-1	Ankara	39°33.843′	31°48.627′	870 m	GQ373310
	cappadocicus-2	Aksaray	38°21.573′	33°25.085′	946 m	GQ373311
	cappadocicus-3	Ankara	39°34.610′	32°51.317′	1066 m	GQ373312
	racemosus-1	Sinop	42°01.576′	35°04.853′	1 m	GQ373313
	racemosus-2	Sinop	42°01.576′	35°04.853′	1 m	GQ373314
	racemosus-3	Sinop	42°01.576′	35°04.853′	1 m	GQ373315
	racemosus-4	İstanbul	41°04.256′	28°09.431′	1 m	GQ373316
Hordelymus	*europeous*-1	Kastamonu	41°04.013′	33°45.023′	1854 m	GQ373317
	europeous-2	Kastamonu	41°04.013′	33°45.023′	1854 m	GQ373318
	europeous-3	Kastamonu	41°04.013′	33°45.023′	1854 m	GQ373319
	europeous-4	Kastamonu	41°04.013′	33°45.023′	1854 m	GQ373320
Brachypodium	*sylvaticum*-1	Trabzon	40°37.818′	39°23.699′	1766 m	GQ373321
Elymus	*repens*	-	-	-	-	DQ859051*
	caninus	-	-	-	-	AY740898*
	hispidus	-	-	-	-	DQ859054*
Agropyron	*cristatum*	-	-	-	-	AY740892*
Leymus	*racemosus*	-	-	-	-	EF602021*
Brachypodium	*sylvaticum*	-	-	-	-	AJ608155*

• GenBank accession numbers for the ITS sequences of the genera *Elymus, Agropyron, Leymus*, and *Brachypodium* that were previously published

characters were equally weighted, gaps were treated as missing, and character states were treated as unordered. A heuristic search was implemented with 100 random additional sequence replicates, tree-bisection-reconnection (TBR) branch swapping, MULPARS option, and ACCTRAN optimisation.

Results

The alignment of sequences of the ITS region from *Elymus* and related genera varied from 600 to 605 nucleotides in length, and had 128 variable positions, of which 119 were parsimony-informative. The 5.8 rDNA was the most conserved region, with only 3 variable sites, while the ITS1 and ITS2 sequences had 55 and 70 polymorphic sites, respectively. Nucleotide deletions, insertions, or substitutions consisted of the main variable positions. On average, ITS1 is 220 bp and ITS2 is 215 bp in length. The average G + C content of the entire ITS sequence was 62.8%. The total genetic diversity calculated using the maximum composite likelihood method is 0.026. The genus *Elymus* was the most diverse (0.017) among the genera studied (Table 3). Mean genetic distance among the genera ranged from 0.014 between *Leymus* and *Agropyron* to 0.154 between *Elymus* and *Brachypodium* (Table 4).

Table 3. Mean genetic diversity of *Elymus*, *Agropyron*, *Leymus*, *Hordelymus*, and *Brachypodium*.

Genus	Genetic diversity
Elymus	0.017
Agropyron	0.001
Leymus	0.002
Hordelymus	0.001
Brachypodium	0.000

Heuristic searches performed on the ITS matrix resulted in 500 most parsimonious (MP) trees with a consistency index (CI) of 0.8649, and a retention index (RI) of 0.9575. In the strict consensus tree (Figure), there were 4 major clades supporting high bootstrap values. The first clade included all sampled species of *Elymus* except for *E. pycnanthus*, which was clustered in another group along with the genus *Agropyron*. In general, specimens of each species found in the first clade formed different subgroups with quite high bootstrap values. However, *E. lazicus-divaricatus* and *E. lazicus-lazicus* samples formed clusters with low bootstrap values.

The second and fourth clades only consisted of samples taken from *Leymus* and *Hordelymus*, respectively. In the third clade, *E. pycnanthus* was located with species of *Agropyron* with high bootstrap value (70%).

Discussion

Although the numbers of samples studied in *Agropyron* (12 accessions), *Leymus* (7 accessions), and *Hordelymus* (4 accessions) were not as large as in *Elymus* (40 accessions), ITS sequence data presented here provided a clear understanding of the phylogenetic relationships between *Elymus* and related genera. As expected, *Elymus* was the most diverse among the genera studied. Genetically, *Elymus* is closer to *Leymus* and *Agropyron* than *Hordelymus*. Liu et al. (2006) suggested that polyploid *Elymus* species are derived from polyploidisation through hybridisation between different ancestral genera, as indicated by cytological analyses (Dewey, 1984; Lu, 1994). For example, St-H genome *Elymus* species are derived from the hybridisation between *Pseudoroegneria* (St) and *Hordeum* (H). The homogenisation of ITS sequences in the allopolyploid *Elymus* is not completed, and sufficient time is needed to allow ITS sequences to be homogenised (Ainouche & Bayer, 1997). These multiple origins of the genome in *Elymus* are a very important evolutionary process in speciation that each polyploid species is formed frequently from different parental genotypes generating a diverse array of polyploid genotypes (Soltis & Soltis, 1999).

In the current study, 4 major clades were formed. The first clade consisted of all *Elymus* species including different genomic constitutions except *E. pycnanthus*. The second clade included only the Ns genome of *Leymus* species. The third clade was composed of *Agropyron* (P) and *E. pycnanthus* and in the last clade *Hordelymus* accessions formed a group.

In the first clade, subgroups were produced by different species found in *Elymus* with low and high bootstrap values. Within this clade, there were subgroups with strong bootstrap values including such as *E. elongatus* (100%), *E. farctus* (99%), and *E. repens* (92%). However, between these species

Table 4. Genetic distance between *Elymus* and related genera.

	Elymus	*Agropyron*	*Leymus*	*Hordelymus*	*Brachypodium*
Elymus					
Agropyron	0.021				
Leymus	0.017	0.014			
Hordelymus	0.037	0.033	0.027		
Brachypodium	0.154	0.144	0.142	0.141	

Figure. The strict consensus tree of two most parsimonious (MP) trees inferred from the nrDNA ITS sequences of 12 taxa from *Elymus* and its related genera, CI = 0.8649, RI = 0.9575. Numbers at the branches indicate bootstrap supports.

involved subgroups, the bootstrap values were low. Cytological studies suggest that 5 basic genomes, namely the St, Y, H, P, and W, in various combinations constitute *Elymus* species (Lu, 1994). These low bootstrap values between *Elymus* species may have appeared because of the origin of *Elymus* through an allopolyploid process (Dewey, 1984; Lu, 1994). Hybridisation between different ancestral genera may cause different types of ITS sequences in *Elymus* (Liu et al., 2006) and homogenisation of ITS sequences in the allopolyploid *Elymus* requires a long time (Ainouche & Bayer, 1997); this process may explain the low bootstrap supports observed between the subgroups in clade I.

E. transhyrcanus (Nevski) Tzvelev (StStH) and *E. caninus* formed a tight subcluster under the first clade. Dewey's (1972) study of the amphiploid hybrid between *E. caninus* (L.) L. with the genomic constitution StH and *E. libanoticus* = *P. libanotica* (St) indicated that the hybrid is biologically equivalent to the hexaploid *E. transhyrcanus*. Assadi and Runemark (1995) proposed that *E. transhyrcanus* and *E. repens* (=*Elytrigia repens*) both have variants of the same genomic combination, StStH.

Morphologically *E. clivorum* (*Elytrigia clivorum* (Melderis) Valdés & H.Scholz.) and *E. repens* (StStH) are very close species. They differ from each other mainly by the indumentum and venation characters of the lower leaves. The former has lower leaves with prominent marginal veins bearing sparse spinulose cilia, while the latter does not. Remarkably, they are placed in different subgroups of the first clade in the constructed phylogenetic tree.

Elymus sosnowskyi is very similar to *E. libanoticus*, from which it may be distinguished by its narrowly lanceolate, acuminate-subulate, 3-veined glumes. Again, the samples from this species with strong bootstrap values were placed in a different subgroup in the first clade.

E. hispidus (*Thinopyrum intermedium*) and *E. elongatus* (*T. elongatum*) were treated in the genus *Thinopyrum* in some studies. In the current study, samples from these species were clearly grouped in different subgroups. Similarly, *E. farctus* populations formed a different sub-cluster, which was supported by very strong bootstrap values.

Refoufi et al. (2001) indicated that *E. pycnanthus* (=*Elytrigia pycnantha* or *Thinopyrum pycnanthum*) contains S, E, and P genomes using genomic in situ hybridisation (GISH) techniques. They also proposed that the P genome is closely related to that of *A. cristatum*. In the present study, *E. pycnanthus* samples showed strong linkage with specimens from the related genus *Agropyron* (70% bootstrap), suggesting the same maternal donor as *Agropyron cristatum*. This result may be produced by natural hybridisation of this species with the genus *Agropyron*. The abundant genetic diversity within *Elymus* species (0.017) could be explained by the occurrence of hybridisation since recurrent hybridisation promotes rapid adaptation of the *Elymus* species to different ecological habitats, resulting in the formation of many endemic genotypes and species as in the case of Turkey (Liu et al., 2006).

Genome analysis is a powerful and reliable tool in understanding phylogenetic relationships of *Triticeae* species. Classification based on genomic constitution in the *Triticeae* has been widely accepted by taxonomists (e.g. Wang, 1992; Lu, 1994; Yen et al., 2005). This study evaluates phylogenetic relationships between *Elymus* and related genera at a molecular level and also is the first time that many species of *Elymus* are analysed together. However, more comprehensive molecular analyses combining more species of *Elymus* and related genera are needed to clarify their phylogenetic relationships.

Despite considerable progress in our understanding of the taxonomy and phylogeny of the tribe, still further research is needed to understand the role of polyploidy and natural hybridisation in speciation. Furthermore, the taxonomic position of many members of the Triticeae remains uncertain, and the genomic constitution of many species and even genera remains unknown. Future investigations on the taxonomy of the genus *Elymus* L. in Turkey should focus on determining chromosome numbers and meiotic configurations for the species not studied previously.

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