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Molecular Phylogenetic Analyses of Perennial Ryegrass (*Lolium perenne* L.) Populations Selected from the Flora of Türkiye

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ABSTRACT

Perennial ryegrass (*Lolium perenne* L.) is an important forage species widely used in grassland-meadow ecosystems and turfgrass management due to its high forage yield, feed quality, and strong adaptation ability. The effectiveness of breeding programs in this species depends on the accurate determination of genetic differences and phylogenetic relationships among the genetic materials to be used. This study was conducted to reveal, at the molecular level, the phylogenetic relationships among potential *L. perenne* samples collected from the natural distribution areas of Türkiye's Central Anatolia and Mediterranean regions. Phylogenetic analyses indicate that annual species (*L. rigidum* and *L. temulentum*) have made a significant contribution to the evolution of perennial ryegrasses. One of the most important findings of this study is that it confirms the origin of existing perennial ryegrass populations from annual ryegrasses, based on the sampled Anatolian ryegrasses, and provides important insights regarding local populations. In the phylogenetic tree constructed using chloroplast sequences, it is clearly observed from the haplotypes that, except for the Eskil populations, the sampled perennial ryegrasses possess an evolutionary history different from the *L. perenne* taxon and exhibit distinct maternal inheritance patterns. The fact that the sampled from Eskil (LP 16, 17, 18) share a common haplotype with both *L. multiflorum* and *L. perenne* in terms of maternal inheritance suggests a close relationship between biennial and perennial species at the maternal lineage level. Network analyses based on ITS sequences revealed a wide ribotype diversity, while those based on *rpl32* pointed to low haplotype variation and diversity. The presence of different ribotypes, in particular, indicates that perennial ryegrasses have arisen through a more complex natural evolutionary process than previously recognized and perhaps natural hybridisation could have been effective in occuring different lineages by natural crosses and gene flow among *Lolium* and its relatives in Poaceae. These results support the idea that interspecific gene flow plays an important role in the evolutionary history of *Lolium* species and that more taxa or hybrid populations with perennial growth habits are present within Türkiye's natural flora. Furthermore, the study highlights the importance of molecular analyses in determining genetic diversity and guiding parental selection in perennial ryegrass breeding programs.

Keywords: *Lolium perenne*, ITS, rDNA, *rpl32*, cpDNA, network analysis

Introduction

Perennial ryegrass (*Lolium perenne* L.) is a strategic species among cool-season forage crops, distinguished by its high forage quality, strong adaptive capacity, and intensive tillering ability. Thanks to its rapid germination and effective ground-covering characteristics, it is widely used in grassland-pasture establishment and forage production systems across extensive geographical regions such as Europe, North America, New Zealand, and Australia (Wilkins & Humphreys, 2003). Due to its vigorous vegetative growth, it is preferred in pasture improvement, erosion control, and turf establishment and is considered one of the most extensively bred forage species worldwide (Stewart & Hayes, 2011).

L. perenne is also notable for its high nutritional value, with crude protein content ranging between 14-22% and digestible organic matter between 65-80%. Owing to these properties, it plays a critical role in meeting the roughage requirements of dairy and beef production systems. Its ability to withstand intensive grazing, high regrowth capacity, and dense leaf structure makes it one of the fundamental species in sustainable pasture systems (O'Donovan et al., 2017; O'Donovan et al., 2021).

In Türkiye, *L. perenne* is widely used in pasture and meadow establishment as well as erosion control projects in regions such as Eastern Marmara, the Black Sea, the Mediterranean, and Central Anatolia due to its high adaptability, drought tolerance, and soil-binding capacity (Aygün & Olgun, 2013; Surmen et al., 2013). However, it has been reported that naturally occurring populations within Türkiye's flora have not been sufficiently investigated genetically, and local genetic resources may possess a largely unexplored variation potential (Erdoğdu et al., 2018; Özer, 2015).

The success of plant breeding programs depends on the diversity of the genetic material used and the accurate selection of parents. As in many cultivated species, variation among *L. perenne* cultivars is limited, raising concerns regarding its narrow genetic base (Ahmed et al., 2014; Guan et al., 2017; Karn & Jasieniuk, 2017). Furthermore, increasing environmental pressures such as drought, salinity, and heat stress under climate change, have increased the need for new populations with high adaptive capacity. Therefore, accurately determining genetic variation, identifying local genetic resources, and incorporating them into breeding programs constitute essential goals of current research efforts (Sampoux et al., 2011).

Morphology-based variation analyses may be insufficient for determining genetic relationships because they are easily influenced by environmental conditions. Consequently, molecular marker-based characterization studies have become widely used in the evaluation of plant genetic resources in recent years (Cruzan, 1998). DNA markers provide effective tools for analyzing genetic similarity and diversity, determining population structure, identifying phylogenetic relationships, and supporting parental selection in breeding programs (Jones et al., 2002; Dar et al., 2019).

In *Lolium* species, various molecular marker systems such as RAPD, AFLP, SSR, and ISSR have been successfully applied (Warpeha et al., 1998; Nie et al., 2019). However, rDNA ITS and cpDNA-based markers are reported to be more reliable and widely used, especially for uncovering phylogenetic relationships (Hand et al., 2010). The ITS region is frequently preferred in phylogenetic analyses due to its high evolutionary rate and strong discriminatory power at the species level (White et al., 1990; Baldwin et al., 1995). The cpDNA *rpl32* intron, on the other hand, is accepted as a reliable marker for tracing hybridization and gene flow among species due to its maternal inheritance pattern (Shaw et al., 2007).

The genus *Lolium* is evolutionarily closely related to the genus *Festuca*, and together they form the *Lolium-Festuca* complex (Hand et al., 2010). Molecular studies have demonstrated substantial genomic homology between these two genera and the occurrence of frequent natural hybridization events (Jenkin, 1955; Inda et al., 2008). The integrated genome structures that result from such hybridizations often lead to polytomies in phylogenetic analyses, making taxonomic separation of species more difficult (Torrecilla & Catalán, 2002; Hand et al., 2010; Cheng et al., 2016).

Intense gene flow has been reported particularly between perennial *L. perenne* and annual species such as *L. rigidum*, *L. multiflorum*, and *L. temulentum* (Hu et al., 2011). Moreover, gene exchange between *Festuca arundinacea* and *L. perenne* has also been documented, suggesting that these two species may have evolved from a shared ancestor. This highlights the critical importance of cpDNA- and rDNA-based phylogenetic studies for understanding the evolutionary history of *Lolium* species (Balfourier et al., 2000; Tamura et al., 2011).

Türkiye is among the regions exhibiting high biodiversity and substantial variability and hosts rich genetic diversity within the Poaceae family. It is believed that *Lolium* species have historically been distributed across Anatolia and that local *L. perenne* populations possess high adaptive capacity and genetic differentiation potential. However, studies addressing the molecular characterization of natural *L. perenne* of Türkiye remain limited, and the existing genetic diversity has not yet been fully elucidated scientifically. Therefore, this study was conducted to determine phylogenetic relationships among perennial ryegrass (*Lolium perenne* L.) collected from the natural vegetation of the Central Anatolia and Mediterranean regions of Türkiye. For this purpose, the ITS region of ribosomal DNA (rDNA) and the *rpl32* region of chloroplast DNA (cpDNA) were amplified and sequenced. The obtained sequence data were used to perform phylogenetic analyses (Parsimony and Network), and evolutionary relationships among the wild populations of *Lolium'* were revealed. The results are expected to contribute to parental selection in perennial ryegrass breeding programs and shed light on the scientific evaluation of local *Lolium* genetic resources within Türkiye's flora.

Materials and Methods

Material

In this study, wild perennial ryegrass (*Lolium perenne* L.) collected from the natural flora of Türkiye were used as a source of plant material for DNA extractions and PCR sequences works. A total of 18 wild population belonging to naturally distributed *L. perenne* L. populations in the Central Anatolia and Mediterranean regions were included. The studied accessions are preserved at Selçuk University, Faculty of Agriculture, Department of Field Crops. In addition, nuclear and chloroplast sequences of *L. rigidum, L. multiflorum*, and *Festuca arundinacea* taxa obtained from the gene bank were incorporated into the data matrix. The provinces, collection number or codes, names, latitude, longitude, elevation data, and morphological characteristics of the sample used in the study are presented in Table 1.

Method

Total genomic DNA was isolated from fresh leaf tissues and silica gel-dried samples using the method of Doyle and Doyle (1987), with modifications by Soltis et al. (1991) and Cullings (1992). Approximately 0.01 g of leaf material was homogenized in CTAB extraction buffer and incubated at 65 °C for 4 hours. DNA was then purified through chloroform/isopropanol extractions, washed with 70% ethanol, and dissolved in 1× TAE buffer. DNA concentration was determined using a NanoDrop 2000 spectrophotometer.

DNA samples were loaded onto a 1.2% agarose gel with bromophenol blue and visualized under a UV transilluminator.

The internal transcribed spacer (ITS) region and the chloroplast *rpl32-trnL (UAG)* region were amplified separately by PCR. For the ITS region, ITS1 and ITS4 primers were used, and the PCR program was initiated at 94°C followed by 30 amplification cycles (White et al., 1990). The amplification of the *rpl32-trnL* (UAG) chloroplast gene region was carried out using the method of Shaw et al. (2007).

The samples used in the molecular analyses are shown in Table 2.

The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, USA) and sequenced. After the obtained sequences were edited in the Chromas Lite 2.1 program. The sequences were aligned using MEGA 6 and BioEdit software, and a data matrix was generated by comparing base pairs for phylogenetic analyses (Swofford, 1990). Phylogenetic networks were analyzed using Network4613 and the beta version of PAUP 4.0 (Swofford, 2003).

Results and Discussion

Molecular Findings

DNA Isolation

Genomic DNA isolated from *L. perenne* accessions collected from their natural distribution areas was determined to be approximately 10-15 kb in size. The DNA purity ratios (A260/A280) ranged between 2.01 and 2.07, indicating low protein contamination and sufficient quality for downstream molecular analyses (Sambrook & Russell, 2001). Nucleic acid concentrations varied between 984 and 2467 ng/µl. Similar levels of DNA purity and concentration obtained from plant tissues have previously been reported as adequate for PCR-based molecular analyses (Doyle & Doyle, 1987; Porebski et al., 1997).

ITS Results

The internal transcribed spacer (ITS) region of the nuclear DNA was amplified at a length of 500-750 bp. Owing to the clarity and distinctness of the amplified bands, the products were purified and sequenced. ITS sequences obtained from 23 samples were aligned using the BioEdit software, and a data matrix was constructed. The final alignment comprised 579 bp, of which 555 characters were constant, 9 were variable, and 15 were parsimony-informative. Parsimony analyses yielded a Consistency Index (CI) of 0.727, a Retention Index (RI) of 0.625, and a Homoplasy Index (HI) of 0.273. These values are consistent with the moderate levels of homoplasy commonly reported in ITS-based phylogenetic studies of grasses (Gaut et al., 2000; Torrecilla & Catalán, 2002).

Phylogenetic analyses indicated that the majority of the collected samples clustered within the same polytomic clade as foreign *L. perenne* taxa (Figure 1; BS 66%; PP 0.91). This finding is in agreement with previous studies reporting close genetic relationships among perennial ryegrass populations across broad geographic regions (Catalán et al., 2004; Cheng et al., 2016). In contrast, sample LP18 was evaluated as a taxon of possible hybrid origin involving *L. perenne* × *L. multiflorum* or *L. rigidum*. Similar ITS-based evidence of hybrid origin within the genus *Lolium* has been reported previously (Gaut et al., 2000; Cheng et al., 2016).

Bayesian analyses further separated the Central Anatolian and Mediterranean populations into two distinct subclades, while certain samples (LP4, LP9, and LP14) exhibited close genetic relationships (Figure 2). Such geographic structuring has frequently been observed in ITS-based phylogenetic analyses of *Lolium* populations (Torrecilla & Catalán, 2002). Network analyses suggested that LP18 may have originated from foreign perennial ryegrass populations (Figure 3), supporting the view that network approaches are more informative than strictly bifurcating trees for

revealing complex evolutionary processes such as gene flow and hybridization (Posada & Crandall, 2001).

***rpl32* Analysis Results**

The chloroplast DNA *rpl32* gene region was amplified at a length of 900-1000 bp (Figure 4). The resulting sequences were aligned using BioEdit, yielding a data matrix with a total length of 866 bp, including 812 constant and 37 variable characters. Parsimony analyses resulted in a Consistency Index (CI) of 0.873, a Retention Index (RI) of 0.867, and a Homoplasy Index (HI) of 0.127, indicating that chloroplast DNA regions provide reliable phylogenetic signals (Shaw et al., 2007).

The *rpl32* phylogenetic trees exhibited lower resolution compared to the ITS results. However, network analyses improved phylogenetic resolution, particularly among closely related taxa (Posada & Crandall, 2001). Haplotype analyses based on the *rpl32* region revealed that a substantial proportion of naturally occurring perennial grass populations in Türkiye's are more closely related to *Festuca arundinacea* in terms of maternal inheritance. Given the predominantly maternal inheritance of chloroplast DNA, this finding is important for understanding hybridization and gene flow processes (McGrath et al., 2006; Diekmann et al., 2012).

In contrast, the Eskil populations (LP16-18) shared the same haplotype with *L. perenne* and *L. multiflorum,* suggesting a common maternal origin. The separation of LP16, LP17, and LP18 from other natural ryegrass populations and their close relationship with the annual species *L. temulentum* var. *arvense* and *L. rigidum* indicate that these populations may have arisen through gene flow between annual and perennial taxa (Figure 4). Network analyses further supported the possible hybrid origin of these populations (Figure 5), a pattern previously reported in *Lolium* species (Catalán et al., 2004; Cheng et al., 2016).

When ITS and *rpl32* analyses were evaluated together, most of the collected natural ryegrass accessions showed a moderate genetic relationship with *L. perenne*. Nevertheless, some populations, particularly LP18, appeared to be of hybrid origin and may have experienced gene flow with different *Lolium* species. The combined use of nuclear and chloroplast DNA data provided robust insights into the phylogenetic structure and evolutionary relationships of the studied populations (Gaut et al., 2000; Torrecilla & Catalán, 2002).

Conclusions

In this study, molecular characterization of perennial *Lolium* species collected from natural flora of Türkiye revealed important findings about the evolutionary history, gene flow, and speciation dynamics of the genus. Phylogenetic analyses showed that the majority of the studied populations had different ribotypes resulting from natural gene flow and hybridization. In particular, the fact that the LP17 genotype exhibits an intermediate position between annual and perennial groups supports the idea that natural hybridization is an effective mechanism in speciation. In addition, chloroplast region analyses indicate a significant gene flow between annual and perennial *Lolium* populations and suggest that the maternal origin is largely based on annual species. These results reveal that natural hybridization and backcrossing are fundamental processes shaping the genetic structure of perennial ryegrass populations.

The obtained phylogenetic data show that the genus *Lolium* exhibits a monophyletic structure and that its common ancestor is most likely related to the diploid *Festuca pratensis*. Annual taxa were found to have made independent contributions to the evolutionary history of perennial *Lolium* populations in Türkiye. This suggests that *Lolium* populations exhibiting a perennial appearance in nature may not be limited to *L. perenne* alone, and that more perennial grass types exist in natural conditions. In general, it appears that different perennial grasses arose as a result of natural hybridization between annual *Lolium* species and closely related *Festuca* taxa. In this context, mimicking the natural hybridization processes described in this study could significantly contribute to the development of new and superior grass varieties through biotechnological approaches.

Acknowledgements

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Table 1. The codes and names of the *Lolium perenne* L. samples used in the study, along with their locality, latitude, longitude, elevation information, and morphological characteristics.

Sample No	Sample Code	Genotype Name	Location Name	Latitude (North)	Longitude (East)	Elevation (m)	Plant Height (cm)	Spike Length (cm)	Plant Height / Spike Length Ratio	Last Internode Length (cm)	Leaf Length (cm)	Leaf Width (cm)	Leaf Length / Leaf Width Ratio	Leaf Texture (1 = narrow-fine, 9 = broad-coarse)	Density (1 = very sparse, 9 = maximum density)	Growth Habit (1 = erect, 9 = prostrate)	Seasonal Color Change (1 = straw yellow/ brown, 9 = dark green)	Spike Formation Tendency (1 = none or very weak, 9 = very strong)
1	**LP 1**	A -24	Cihanbeyli-Konya	38.42	32.44	989	34.4	15.2	2.26	5.1	6.5	3.7	1.76	2.6	4.8	6.5	6.8	7.0
2	**LP 2**	A -43	Between Ankara and Kulu-ANKARA	39.26	32.51	109	33.7	13.6	2.48	8.3	7.7	4.1	1.88	5.2	4.4	1.6	7.3	8.0
3	**LP 3**	A -134	Akşehir-KONYA	38.27	31.19	981	31.2	12.6	2.48	6.7	9.3	4.1	2.27	5.0	5.6	6.8	5.8	5.0
4	**LP 4**	A -149	Akşehir-KONYA	38.29	31.21	983	30.8	13.4	2.30	4.6	8.2	3.8	2.16	3.1	4.7	4.5	6.3	5.4
5	**LP 5**	A-155	Akşehir-KONYA	38.30	31.22	984	31.0	12.5	2.48	4.7	9.3	4.5	2.07	4.6	5.6	6.8	6.6	5.2
6	**LP 6**	B-1	Akören-KONYA	37.28	32.14	118	32.4	13.4	2.42	4.5	7.4	3.5	2.11	5.2	3.0	6.4	6.6	5.9
7	**LP 7**	B-35	Kırkpınar Village, Çifteler-ESKİŞEHİR	39.12	31.07	914	33.1	13.0	2.55	5.3	8.1	3.4	2.38	4.1	5.1	6.0	6.9	7.7
8	**LP 8**	B-59	Between Karaman and Mut -MERSİN	36.56	33.16	150	30.2	11.8	2.56	5.3	6.0	3.7	1.62	4.9	4.2	6.3	6.7	6.9
9	**LP 9**	B-110	Seydişehir-Taraşçı-KONYA	37.27	31.40	121	36.4	14.2	2.56	7.2	8.3	3.8	2.18	4.3	4.1	3.9	5.4	8.0
10	**LP 10**	B-117	Between Şarkikaraağaç and Beyşehir	37.45	31.24	113	33.3	11.2	2.97	6.0	6.9	4.0	1.73	5.2	4.3	3.4	6.9	7.1
11	**LP 11**	G-500	Yarpuz-ANTALYA	37.08	31.50	125	32.7	11.9	2.75	7.2	5.1	2.4	2.13	3.2	6.2	3.2	6.1	8.3
12	**LP 12**	G-501	Yarpuz-ANTALYA	37.09	31.51	124	32.8	12.5	2.62	7.1	6.1	3.1	1.97	4.3	6.1	3.2	5.5	8.1
13	**LP 13**	G-504	Akbelenli-ANTALYA	37.35	30.52	966	29.9	11.0	2.72	4.9	5.0	2.3	2.17	5.0	6.2	5.7	5.7	6.8
14	**LP 14**	G-506	Eğrigöl-ANTALYA	36.55	32.12	206	34.8	14.0	2.49	6.3	5.6	2.5	2.24	3.2	5.7	2.6	5.4	8.6
15	**LP 15**	234	Eskil (Sıcak Plateau)-AKSARAY	38.12	33.23	101	*	*	*	*	*	*	*	*	*	*	*	*
16	**LP 16**	601	Eskil (Sıcak Plateau)-AKSARAY	38.10	33.24	101	*	*	*	*	*	*	*	*	*	*	*	*
17	**LP 17**	602	Eskil (Sıcak Plateau)-AKSARAY	38.11	33.24	101	*	*	*	*	*	*	*	*	*	*	*	*
18	**LP 18**	603	Eskil (Sıcak Plateau)-AKSARAY	38.11	33.23	100	*	*	*	*	*	*	*	*	*	*	*	*

Source: Obtained from TÜBİTAK projects No. 106O159 and 110O312. ***:** *Accessions 234, 601, 602, and 603 used in this study were included later; since data for these Accessions were not available, they were not included in the table.*

Table 2. Numerical and molecular samples used in the study.

Collection Code	Samples	ITS	*rpl32*
LP1_A24	*Lolium perenne*	+	+
LP2_A43	*Lolium perenne*		+
LP3_A134	*Lolium perenne*	+	+
LP4_A149	*Lolium perenne*	+	+
LP5_A155	*Lolium perenne*	+	+
LP6_B1	*Lolium perenne*	+	+
LP7_B35	*Lolium perenne*	+	+
LP8_B59	*Lolium perenne*	+	+
LP9_B110	*Lolium perenne*	+	+
LP10_B117	*Lolium perenne*	+	+
LP11_G500	*Lolium perenne*	+	+
LP12_G501	*Lolium perenne*	+	+
LP13_G504	*Lolium perenne*	+	+
LP14_G506	*Lolium perenne*	+	+
LP15_234	*Lolium perenne*	+	+
LP16_601	*Lolium perenne*	+	+
LP17_602	*Lolium perenne*	+	+
LP18_603	*Lolium perenne*	+	+
KJ599446_*rpl32* KJ598998_ITS	*Lolium multiflorum*	+	+
KJ599447_*rpl*32 KJ598999_ITS	*Lolium perenne*	+	+
KJ599448_*rpl32* KJ599000_ITS	*Lolium rigidum*	+	+
KJ599411_*rpl32* KJ598964_ITS	*Lolium temulentum* var. *arvense*	+	+
KJ599444_*rpl32* AF171180_ITS	*Festuca pratensis*	+	+
EF379060_ITS	*Festuca gigantea*	+	
KJ599440_*rpl32*	*Festuca arundinacea*		+

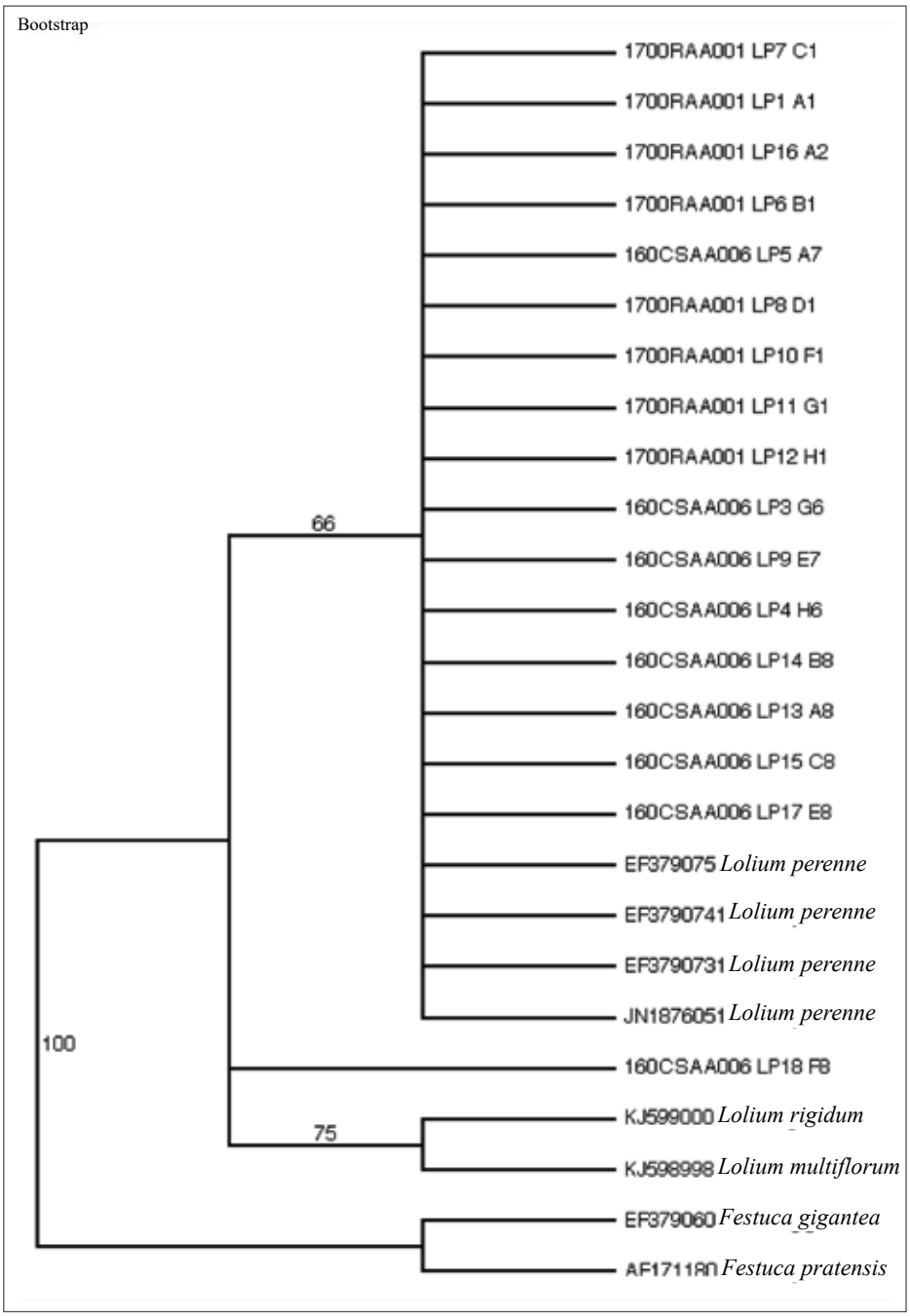

Figure 1. Phylogenetic tree showing the evolutionary relationships of *Lolium* taxa and accessions generated by parsimony analyses of ITS sequences.

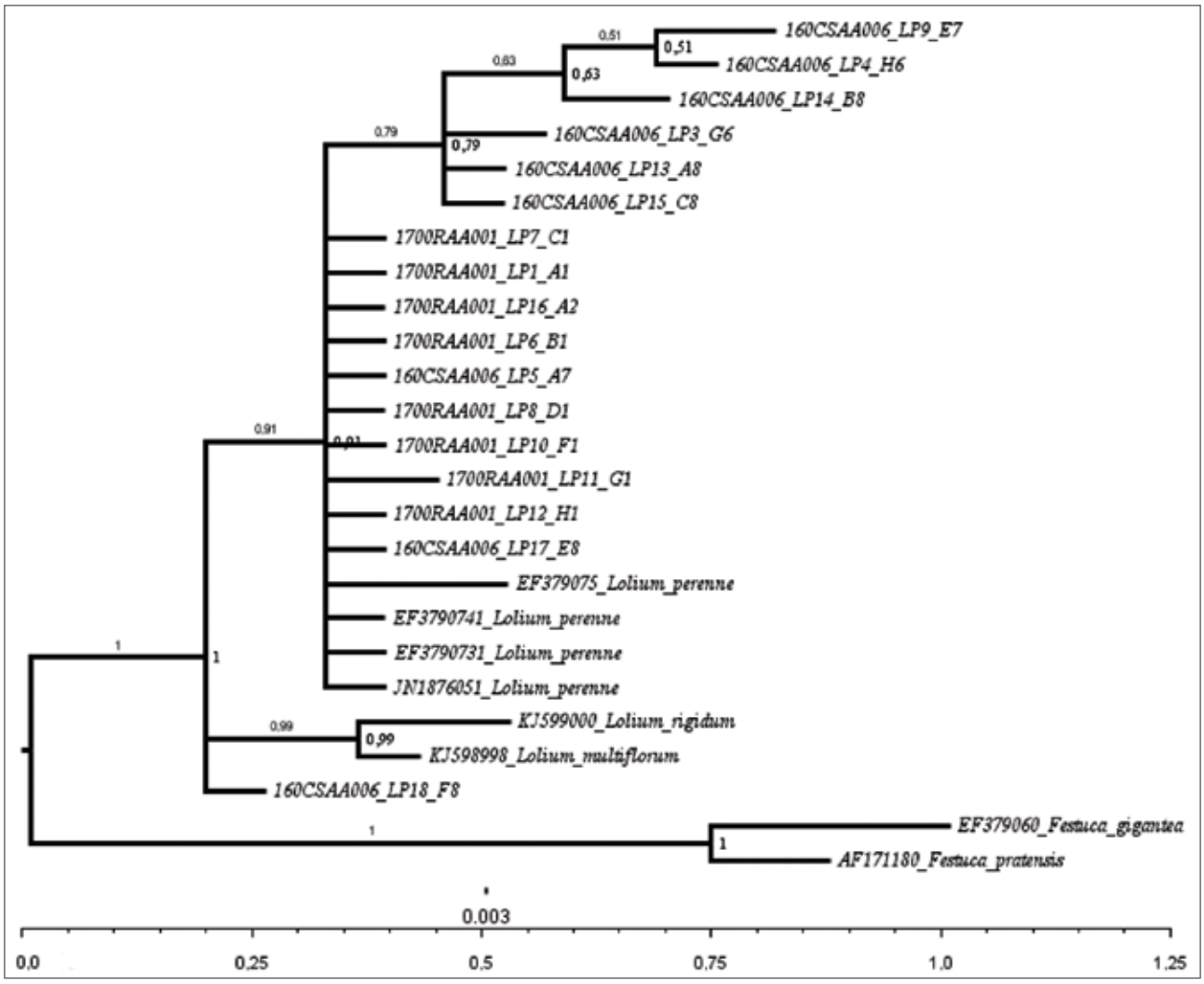

Figure 2. Phylogenetical tree showing the phylogenetic relationships of *Lolium* taxa and populations, constructed by Bayesian analysis of ITS sequences.

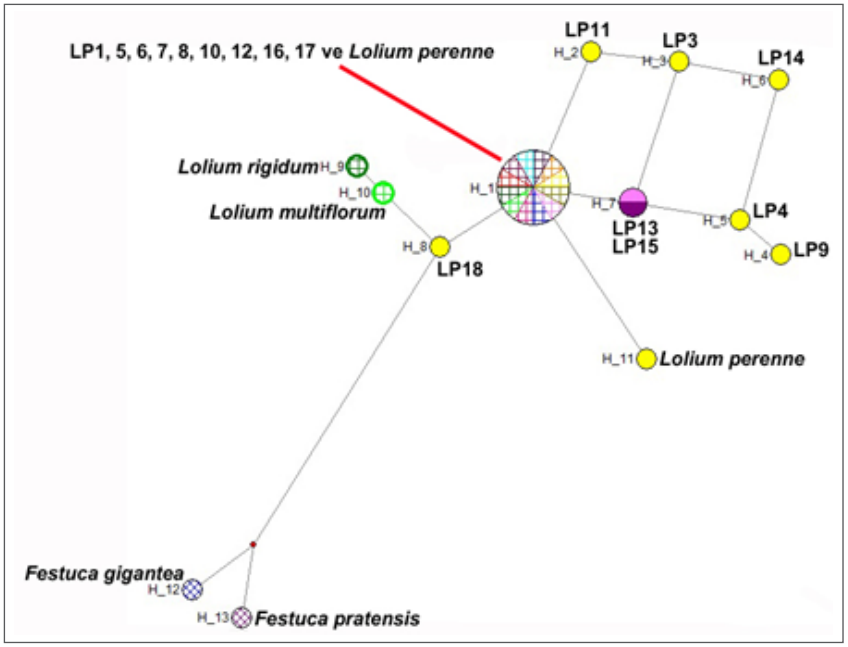

Figure 3. Phylogenetic network of *Lolium perenne* and closely related taxa generated from the aligned ITS gene region sequences.

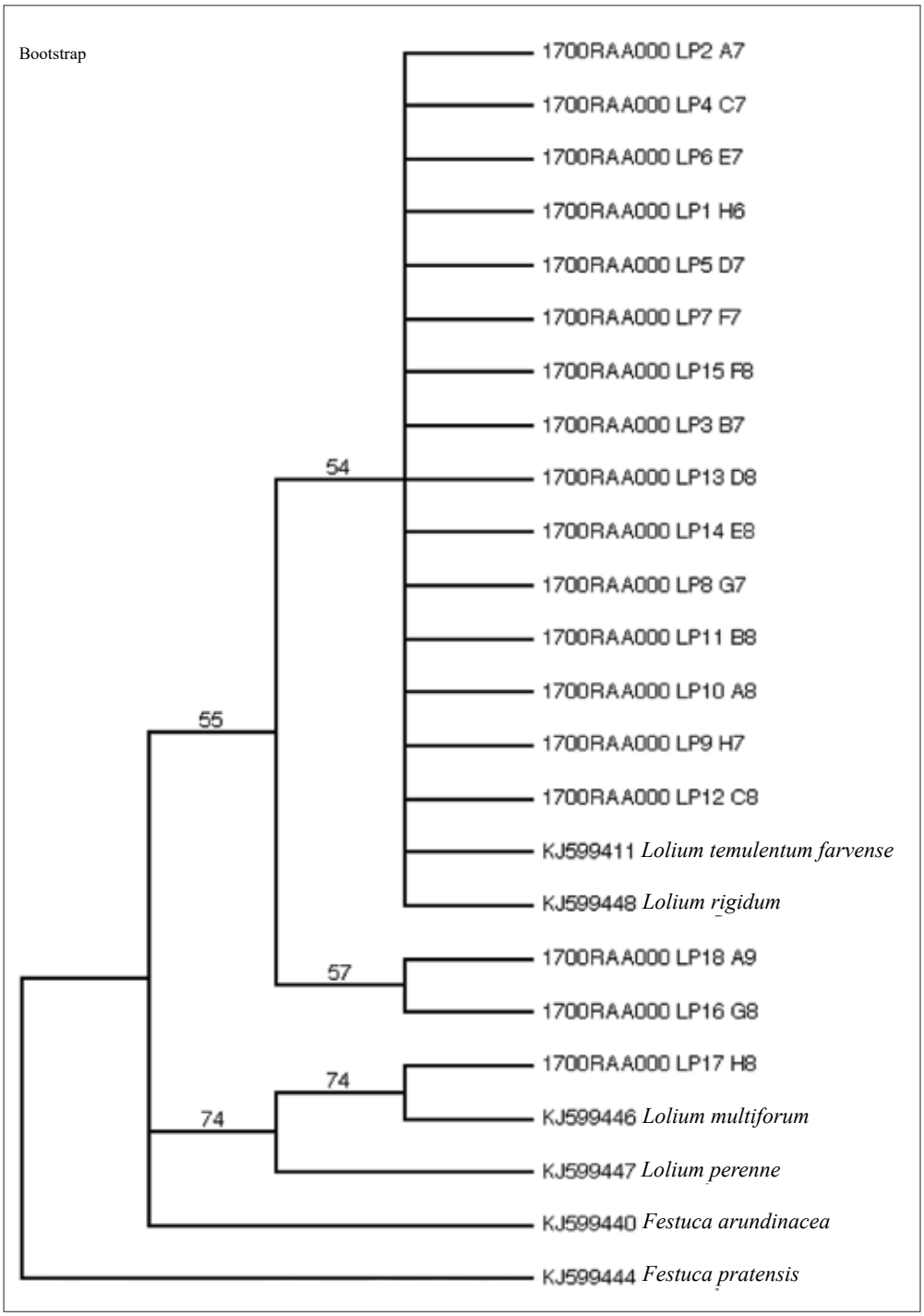

Figure 4. Phylogenetic tree showing the evolutionary relationships of *Lolium perenne* generated by parsimony analyses of *rpl32* sequences.

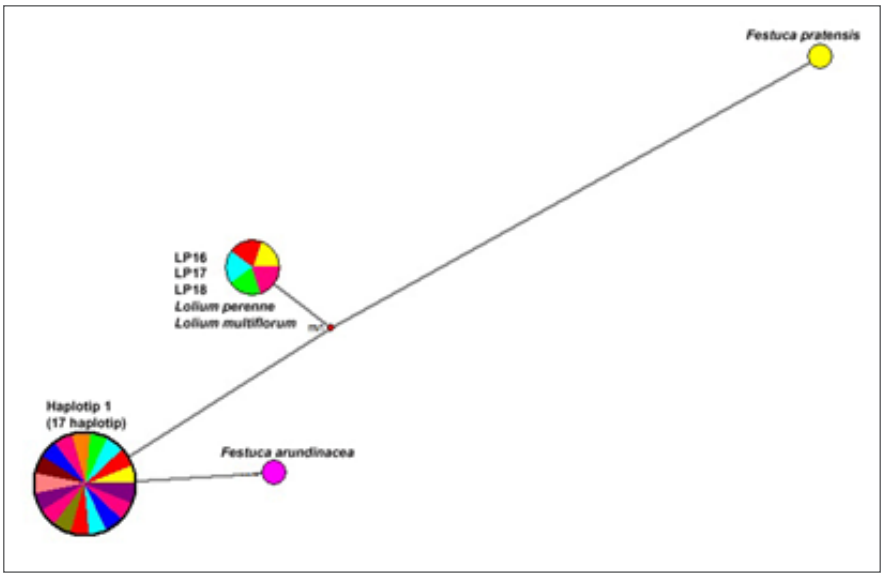

Figure 5. Phylogenetic network of *Lolium* taxa and populations generated by network analyses of *rpl32* gene sequences.

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