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Relationships among some regional species of the genus *Lolium* L. based on morphological and molecular markers

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Abstract

This study dealt with 89 accessions representing 40 populations of five species (*Lolium perenne*, *Lolium multiflorum*, *Lolium rigidum*, *Lolium temulentum* and *Lolium persicum*) distributed over 14 Mediterranean and Middle Eastern countries (Morocco, Algeria, Tunisia, Libya, Egypt, Palestine, Jordan, Iraq, Iran, Turkey, Cyprus, Romania, Greece and Albania) distributed over three continents (Africa, Asia and Europe). We depended on 80 qualitative and quantitative morphological characters with 172 character states. The genetic polymorphism and gene diversity was assessed using five polymorphic inter simple sequence repeat primers. Based on Morphological observations, the studied species were separated into two groups, the first combines *L. perenne*, *L. multiflorum* and *L. rigidum*, where, *L. perenne* is more associated to *L. multiflorum* than to *L. rigidum*; the second combines *L. temulentum* and *L. persicum*; to some extent geographical distribution influenced the relationships among the populations. High degree of gene diversity with polymorphism percentage of 95.62% was detected among the studied species. Genetically, high degrees of genetic distance are observed among the studied populations; the geographical distribution has a great influence on the population's relationships. The Molecular

data also confirmed the separation of two groups, the first combines *L. perenne*, *L. multiflorum* and *L. rigidum* (as resulted from morphological data), but here, *L. perenne* is more associated to *L. rigidum* than to *L. multiflorum*; and the second combines *L. temulentum* and *L. persicum*. No infraspecific taxa below the studied species were observed.

Keywords: *Lolium* L., Taxonomic relationships, Mediterranean species, Genetic Diversity, ISSR.

Introduction

The grass family (Poaceae) is one of the largest families that developed 60 million years ago. It includes some 10000 species with high economic importance, especially the essential cereal crops that are obligate in the daily diet (Kellogg, 2001). The family involves great morphological, physiological, ecological, and genetic diversity (Kellogg, 1998). Based on cytological and molecular studies, the genus *Lolium* L. belongs to the monophyletic tribe Poeae, which is one of 12 tribes belonging to subfamily Pooideae (Catalan *et al*., 1997; Kellogg, 1998; Gaut, 2001). *Lolium* consists of about eight recognized diploid species with a chromosome number of 2n=14 (Terrell, 1968).

Lolium is native to Europe, temperate Asia and North Africa, most of its species are distributed around the temperate regions of the world (Polok, 2007). The Mediterranean basin was supposed to be the origin of all ryegrasses (Charmet and Balfourier, 1994). It is believed that the coastal region of Syria may be the geographical origin of this genus (Dinelli *et al*., 2004).

According to Zohary (1946), three species of *Lolium* were recognized in Iraq. Cuénod *et al*., (1954) discussed four species from Tunisia including three varieties. Quezel and Santa (1962) described five species from Algeria including two subspecies, while Bor (1970) recorded six species from Iran. Mill (1985) discussed the morphological characters of the genus *Lolium* L. in the Turkish flora and provided six species including 11 varieties. Meikle (1985) recorded five species from Cyprus and Feinbrun (1986) depended on morphological characters and chromosome number to differentiate six species in Palestine including three subspecies, two varieties and two

formas, Sherif and Siddiqi (1988) recognized four species in Libya, Cope and Hosni (1991) recorded presence of four species in Egypt, Boulos (1995) considered four species in Egypt including three formas, Cope (2005) recorded presence of four species in Egypt, also, Boulos (2009) considered four species in Egypt.

Lolium (ryegrasses) straws can be fermented into liquid fuel at high temperatures (Lawford and Rousseau, 2003; Schell *et al*., 2003) and can be treated with cellulases during the fermentation process to improve the liberation of free sugars to convert plant biomass into biofuels, also, their cell wall composition can be altered through genetic modification to improve the nutritional value for animal consumption and convert the plant biomass into bioethanol (Baldwin *et al*., 2007).

Lolium perenne L. (perennial ryegrass) is used in temperate region due to its rapid establishment, adaptability, and nutrition values (Thorogood, 2003), where it is considered as the most appreciated temperate grass in Europe (Bothe *et al*., 2016). *Lolium temulentum* L. (Poison ryegrass) is very similar to wheat and in some regions it is referred to as true wheat (*Triticum* species), it is very difficult to separate the seeds of both from each other, in addition, its seeds are poisonous to people and livestock; it can be used for fodder and for erosion control (CABI: www.cabi.org/isc/ 2011). *L. temulentum* was proposed as a model species for genomic studies of cool-season forage and turf grasses because it is closely related to the important cultivated *Lolium* species and its short life cycle '2–3 months' (Baldwin and Dombrowski, 2006). *Lolium persicum* is resistant to herbicides and can produce seeds following herbicide applications; it is a competitive weed in dry land wheat and oat production (Bussan and Trainor, 2001).

Lolium multiflorum (Italian ryegrass) is an excellent model for increasing organic matter and improving soil structure, for providing erosion control, for quick growth and establishment, and very good for taking up and storing excess nitrogen, for animal grazing and for providing lasting residue (Evensen *et al*., 1998); it tolerates wet, moderate acidity and high levels of soil pH and poorly drained soils (Hannaway *et al*., 1999). *Lolium rigidum* (Rigid ryegrass) is planted in Australia as a forage crop (Niknam *et al*., 2002), it can hybridize with *L. perenne* and *L. multiflorum*, as well as

some species of *Festuca* (Terrell, op. cit.); it tolerates a wide range of pH and salt and osmotic stress (Chauhan *et al*., 2006).

The studies dealing with the phylogeny of *Lolium* agreed that it is a monophyletic genus, while the debate was about the common ancestor of the genus. The total nuclear DNA content in *L. perenne* present in lower amount (4.2 pg) than in the other species, which indicates that *L. perenne* may be the common ancestor of the genus (Thomas, 1981); also, protein studies suggested that the ancestral form of *Lolium* is most like the present *L. perenne* (Bulińska-Radomska and Lester, 1988). In contrast, other authors agreed that the common ancestor may be *L. rigidum* (Malik, 1967; Charmet and Balfourier, 1994), while Polok (2007) concluded that the subgenus *Schedonorus* may be the common ancestor of this genus.

The relationships among *Lolium* species was studied using different markers. Based on RAPD (Stammers *et al*., 1995; Ma *et al*., 2013) and AFLP data (Polok *et al*., 2006), it is found that *L. perenne, L. multiflorum* and *L. rigidum* are closely related to each other with *L. multiflorum* is closer to *L. perenne* than to *L. rigidum* and *L. temulentum* is closer to *L. persicum*. On other hand, *L. multiflorum* was observed to be closer to *L. rigidum* than to *L. perenne* according to the isozyme data (Charmet and Balfourier op. cit.; Bennett *et al*., 2002).

Polok (2007) speculated that *L. perenne* and *L. multiflorum* populations always mixed completely and there is no strong species boundary or reproductive barriers between them.

According to different marker analyzed, she proposed that the two species have nearly the same level of polymorphism and could be considered as one species (*L. perenne* subsp. *perenne* and *L. perenne* subsp. *multiflorum*); and *L. rigidum* is closely related to them, while *L. temulentum* and *L. persicum* are very close to each other. Also, Zielinski *et al*. (1997) proposed *L. perenne* and *L. multiflorum* as one species based on isoenzymes and RAPD.

Guan *et al*. (2017) suggested high genetic diversity of nine *Lolium* species based on SSR markers; they found that there were great differentiations between populations of

L. perenne and within populations of *L. multiflorum*. In dependence on some morphological characters, Gasior *et al*. (2016) found less phenotypic variation in accessions of *L. perenne* collected from across Europe.

Morphological traits are very important and significant in discrimination between taxa, but they have a number of limitations including low polymorphism, low heritability, late expression and susceptibility to environmental influences (Smith and Smith, 1992).

On the other hand, DNA molecular markers do not have such limitations. They can be used to detect variation at the DNA level and are effective tools to distinguish between closely related genotypes and the choice of the technique depends on the objective of the study, skills and facilities available (Beyene *et al*., 2005); in addition to their advantages: fast and useful for analyzing large collections of populations to determine the amount of genetic diversity within single populations and to identify genetic relationships among the populations (Peter-Schmid *et al*., 2008).

Inter simple sequence repeat (ISSR) technique is a PCR based method, which involves amplification of DNA fragment present between two identical microsatellite repeats oriented in opposite direction. In this method, primers (16–25 bp) which may be di-nucleotide, tri-nucleotide, tetra-nucleotide or penta-nucleotide are used in a single primer PCR targeting multiple genomic loci to amplify inter- SSR sequences of different sizes (Reddy, 2002).

The present study was carried out to assess the taxonomic relationships among the studied species that represent the Mediterranean basin and Middle East region by using morphological markers and ISSR molecular marker, that provide successful numerical assessment of the species relationships.

Materials and Methods

Collection of plant material

The present work concerned with study of 89 accessions of five species of the genus *Lolium* L., these accessions represent 14 countries distributed over three continents (Table 1). These accessions include fresh plant material collected by the author during

periods of flowering and fruiting (March-April) of the years 2014 and 2015, plant specimens kept in the Egyptian herbaria of Cairo University Herbarium (CAI), Herbarium Flora of Phytotaxonomy Research, Agriculture Research Center (CAIM) as well as seeds obtained from the seed- bank of United states Department of Agriculture (USDA) and the seed- bank of Institut für Pflanzengenetik und Kulturpflanzenforschung 'Leibniz Institute of Plant Genetics and Crop Plant Research' (IPK).

For morphological description, specimens provided as seeds were planted until maturation then were examined by a binocular head zoom stereo microscope, model number MS003A in addition to examination of the herbarium specimens. Identification of the collected specimens was confirmed by revision with the specimens kept in CAI, CAIM; the abbreviations of "Index herbariorum" ed. 8 (Holmgren *et al*., 1990). The nomenclature was made according to Täckholm (1974), Cope and Hosni (op. cit.) and Cope (op. cit.).

The collected specimens were kept in the herbarium of Department of Botany, Faculty of Science, University of Fayoum (proposed abbreviation FAY).

Distribution of the studied species in the studied countries of the Mediterranean basin and Middle East region, is shown in Map 1.

Morphological Analysis

Morphological data of the studied species is based on 80 morphological characters and 172 character states. Both qualitative and quantitative features were selected that describes the whole plant, culms, leaves, inflorescences, spikelets, florets and caryopses (Table 2). The qualitative characters were scored as binary while the quantitative ones were scored as multi-state characters. The phenotypic characters of the accessions of the five studied species were used as an operational taxonomic unit (OUTs). The dendrogram was obtained by using average linkage agglomerative clustering method according to Schütze and Silverstein (1997) and two-dimensional plot was obtained by principal component analysis (PCA) correlation according to Pearson (1901). All the computations were made by using Community Analysis Package (CAP) program (Seaby and Henderson, 2007).

Molecular Assessment

DNA preparation

Seeds of 86 accessions (15 seeds/accession) were germinated in clean sterile petri dishes on double layered filter papers and 10 mm distilled water in incubator in dark then light at 23°C for ten days. DNA samples were extracted from 89 plants (fresh green juvenile leaves of the 86 previously germinated accessions, in addition to leaves of 3 herbarium specimens). DNA isolation was made according to the protocol attached with kit of ZR Plant/Seed DNA MiniPrep, Cat. No. D6020 (www.zymoresearch.com).

ISSR genotyping

The reaction mixture with a total volume of 25 µl contained 3 µl DNA, 0.1% 3 µl primer, 12.5 µl of master mix (GeneDirex, OnePCR^{TM}, Cat. MB203-0100) diluted up to 19 µl with sterile distilled water.

The amplification reactions was carried out by using PCR Biometra thermocycler as the following: preheating at 98°C, denaturation at 94°C for 5 min, followed by 42 cycles each consists of the following steps: denaturation at 92°C for 1 min, annealing at 44.6°and 49° for 1 min and 10 sec, extension at 72°C for 2 min; final extension at 72°C for 5 min and hold at 4°C.

Amplification product analysis

A modified protocol according to (Kirkpatrick, 1991) was followed: the amplified DNA for all samples (7 µl) were loaded on 2% agarose gel (2 gm agarose up to 100 ml 1X SB buffer) containing ethidium bromide (5.6 µl) and electrophoresed by using electrophoresis unit (WIDE mini-sub-cell GT BIO-RAD) at 100 constant volt for 1.5 h by using 1X SB running buffer and was determined with (Syngene Bio imaging) UV transilluminator. The amplified fragments were compared with 0.1µg/ µl 100 bp ladder (Fermentas, GeneRuler) and 56µg/ 500µl 50bp DNA ladder RTU (GeneDirex, Cat. No. DM012-R500).

Data analysis

The genotypic characters were scored as a binary matrix as present (1) or absent (0) for each fragment. Genic variation statistics for all loci of all populations were calculated by using PopGene version 1.32 of Yeh *et al*. (2000). These statistics include Mean and standard deviation of Observed number of alleles (na), Effective number of alleles (ne) according to Kimura and Crow (1964), Nei's gene diversity (h) according to Nei (1973) and Shannon's information index (I) according to Lewontin (1972).

Nei's analysis of gene diversity in subdivided populations according to Nei (1987), including: Estimate of gene flow (Nm), Number of polymorphic loci and percentage of polymorphic loci; Nei's unbiased measures of genetic distance according to Nei (1978). Dendrogram based on Nei's (1978) Genetic distance: Method = UPGMA modified from NEIGHBOR procedure of PHYLIP Version 3.5, was obtained also. Two-dimensional plot was obtained by principal component analysis (PCA) correlation.

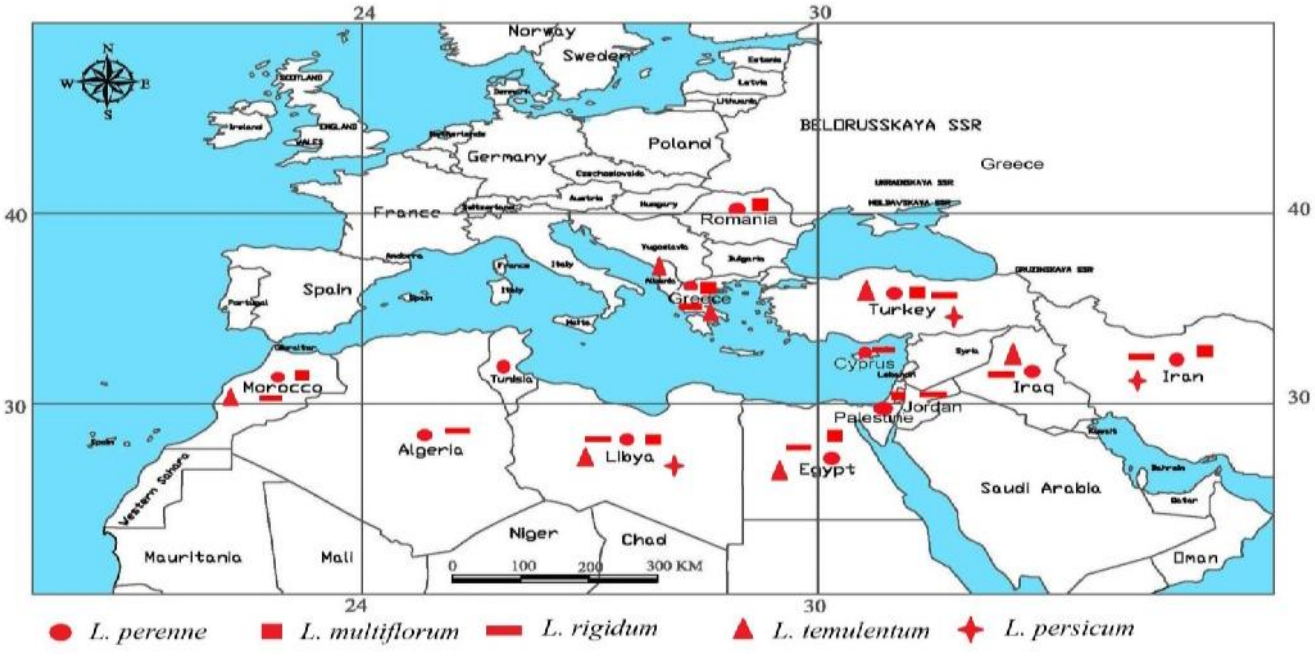
Table 1: Information about the accessions of *Lolium* that are included in both morphological and ISSR analyses.

Item	Species name	Origin	Locality (collection site)	Plant ID or Accession number	Last date of update/ Collection date	Seed bank/ Herb.
1	*L. perenne*	Morocco	Near Meknes/Boufekrane, 37 km south of Meknes on road S331, Mrirt-Meknes.	PI 598864	2000/ 1994	USDA
2	*L. perenne*	Morocco	3 km from center of Ifrane toward El-Hajeb on road S309.	PI 598873	1998/ 1994	USDA
3	*L. perenne*	Morocco	2 km west of Azerzou on P33, Zeida to K. Tadla.	PI 598892	1998/ 1994	USDA
4	*L. perenne*	Algeria	____	PI 231583	2003	USDA
5	*L. perenne*	Algeria	____	PI 231585	2003	USDA
6	*L. perenne*	Algeria	____	PI 231616	2003	USDA
7	*L. perenne*	Tunisia	Near Teboursouk, 6 k west of Teboursouk on C75.	PI 610924	2001/ 1994	USDA
8	*L. perenne*	Tunisia	Near Bizerte, 16 k west of Bizerte on C51.	PI 610926	2001/ 1994	USDA

9	*L. perenne*	Tunisia	Near Nefza, 16 k south of Nefza on C52.	PI 610958	2001/ 1994	USDA
10	*L. perenne*	Libya	Cyrenaica.	PI 231565	2003	USDA
11	*L. perenne*	Libya	Cyrenaica.	PI 231566	2003	USDA
12	*L. perenne*	Libya	Cyrenaica.	PI 231568	2003	USDA
13	*L. perenne*	Egypt	Ras-el-Hekma.	PI 239730	2003	USDA
14	*L. perenne*	Palestine	Goret El- Lout, Khan Yunis.	———	1955 (collection date)	CAI
15	*L. perenne*	Iraq	97 km. north of Mosul.	PI 254898	2004	USDA
16	*L. perenne*	Iran	Market, Tehran.	PI 222527	1997	USDA
17	*L. perenne*	Iran	From Livestock Station, Moghan Steppes, Zaerbaijan.	PI 223385	2003	USDA
18	*L. perenne*	Iran	From cultivated fields near Ahwaz.	PI 227020	2003	USDA
19	*L. perenne*	Turkey	2km S Kizilhisar, Denizli province.	PI 545665	2004/ 1984	USDA
20	*L. perenne*	Turkey	Weedy area, Ankara.	PI 598510	1998/ 1992	USDA
21	*L. perenne*	Turkey	City border, Bolu, Ankara.	PI 598520	1998	USDA
22	*L. perenne*	Cyprus	———	PI 204086	2003	USDA
23	*L. perenne*	Cyprus	———	PI 206376	2003	USDA
24	*L. perenne*	Cyprus	———	PI 206377	2003	USDA
25	*L. perenne*	Romania	Faragau, Jud. Mures.	GR 8834	2003	IPK
26	*L. perenne*	Romania	Dipsa (Jud. Bistrita - Nasaud).	GR 8838	2003	IPK
27	*L. perenne*	Romania	Sanger, Cimpia Transi Praniei, Mures District.	GR 9508	2003	IPK
28	*L. perenne*	Greece	———	PI 199252	2003	USDA
29	*L. perenne*	Greece	———	PI 231599	2003	USDA
30	*L. perenne*	Greece	———	PI 231600	2003	USDA
31	*L. multiflorum*	Morocco	———	PI 202509	2003	USDA
32	*L. multiflorum*	Libya	El- Ryayna between Rumia and Zintan.	———	1970 (Collection date)	CAI
33	*L. multiflorum*	Egypt	———	PI 343155	1997	USDA
34	*L. multiflorum*	Egypt	———	PI 343156	1997	USDA
35	*L. multiflorum*	Palestine	———	PI 200344	2003	USDA
36	*L. multiflorum*	Iran	Market, Tehran.	PI 222526	2003	USDA
37	*L. multiflorum*	Turkey	Seed store, Istanbul, Istanbul.	PI 170519	2003/ 1948	USDA
38	*L. multiflorum*	Turkey	3km E Kirsehir, Kirsehir province.	PI 545668	1992/ 1984	USDA
39	*L. multiflorum*	Turkey	15km SE Van, Van province.	PI 545671	1992/ 1984	USDA
40	*L. multiflorum*	Romania	———	GR 2608	1983	IPK
41	*L. multiflorum*	Romania	———	GR 9070	2003	IPK
42	*L. multiflorum*	Romania	———	GR 9075	2003	IPK
43	*L. multiflorum*	Greece	———	PI 199251	2003	USDA

44	*L. rigidum*	Morocco	____	PI 239779	2003	USDA
45	*L. rigidum*	Morocco	____	PI 239781	2003	USDA
46	*L. rigidum*	Morocco	Flat grassland, sandy loam soil over sandstone, 7km from Ben-Slimane, Bouznika, Ben Slimane	PI 516608	2004/ 1983	USDA
47	*L. rigidum*	Algeria	____	PI 239750	2003	USDA
48	*L. rigidum*	Algeria	____	PI 239755	2003	USDA
49	*L. rigidum*	Algeria	____	PI 239761	2003	USDA
50	*L. rigidum*	Libya	____	PI 239735	2003	USDA
51	*L. rigidum*	Libya	____	PI 239737	2003	USDA
52	*L. rigidum*	Libya	____	PI 239738	2003	USDA
53	*L. rigidum*	Egypt	Fouka.	PI 239731	2003	USDA
54	*L. rigidum*	Egypt	Shepparaton, Victoria.	PI 250804	2004	USDA
55	*L. rigidum*	Egypt	Shepparaton, Victoria.	PI 250806	2004	USDA
56	*L. rigidum*	Jordan	____	PI 202676	2003	USDA
57	*L. rigidum*	Iraq	70 km. east of Kirkuk.	PI 254899	2004	USDA
58	*L. rigidum*	Iraq	Hilla.	GR 11896	1987/ 1986	IPK
59	*L. rigidum*	Iran	Mosj.-Gol.	PI 239795	2003	USDA
60	*L. rigidum*	Iran	Susa.	PI 239796	2003	USDA
61	*L. rigidum*	Iran	Ahwaz.	PI 239798	2003	USDA
62	*L. rigidum*	Turkey	Roadside between Mardin and Nusaybin.	PI 298416	2004	USDA
63	*L. rigidum*	Turkey	14km N Gaziantep toward Yavuzeli, Gaziantep province.	PI 545606	1992/ 1984	USDA
64	*L. rigidum*	Turkey	Hwy 380 at Jct. Diyarbakir-Bismil Rd., Diyarbakir province.	PI 545612	1992/ 1984	USDA
65	*L. rigidum*	Cyprus	____	PI 204081	1997	USDA
66	*L. rigidum*	Cyprus	____	PI 204083	1997	USDA
67	*L. rigidum*	Cyprus	Morphou.	PI 239733	2003	USDA
68	*L. rigidum*	Greece	Levadia.	PI 239792	2003	USDA
69	*L. rigidum*	Greece	Igoumenitsa.	PI 239793	2003	USDA
70	*L. temulentum*	Morocco	____	PI 391427	2004	USDA
71	*L. temulentum*	Morocco	13km west of Tetouan, roadside.	PI 422589	2004/ 1975	USDA
72	*L. temulentum*	Libya	Fezzan / Al Awaynat.	GR 5453	1983/ 1983	IPK
73	*L. temulentum*	Egypt	Beheira Province, El-Dilingat.	____	1987 (Collection date)	CAI
74	*L. temulentum*	Iraq	____	GR 12759	1979	IPK
75	*L. temulentum*	Iraq	____	GR 12760	1979	IPK
76	*L. temulentum*	Turkey	Zigana Pass, between Torul and Macka.	PI 206691	2003	USDA
77	*L. temulentum*	Turkey	13km N Buldan Jct., Denizli province.	PI 545641	1992/ 1984	USDA
78	*L. temulentum*	Turkey	9km SE Ayvacik, Canakkale province.	PI 545644	1992/ 1984	USDA

79	*L. temulentum*	Albania	Fushë-Dukat, Prov. Vlorë.	GR 11902	1995/ 1994	IPK
80	*L. temulentum*	Albania	Katundishtë, Prov. Këlcyr-Përmet.	GR 11903	1995/ 1994	IPK
81	*L. temulentum*	Albania	Surrel, 20 km NO von Tirana, Hausgarten mit zahlreichem Material.	GR 11907	1995/ 1993	IPK
82	*L. temulentum*	Greece	Karyai, Athos.	PI 249725	1997	USDA
83	*L. persicum*	Libya	Fezzan / Aqar.	GR 5452	1983/ 1983	IPK
84	*L. persicum*	Iran	Livestock Station, Sarab, Azerbaijan.	PI 222807	2004/ 1954	USDA
85	*L. persicum*	Iran	12 miles east of Sanandaj, Kurdistan.	PI 229764	2004	USDA
86	*L. persicum*	Iran	Faridan.	PI 230110	2004	USDA
87	*L. persicum*	Turkey	31km SW Golbasi, Adiyaman province.	PI 545637	1997/ 1984	USDA
88	*L. persicum*	Turkey	12km NE Ankara city limit sign, Ankara province.	PI 545661	1992/ 1984	USDA
89	*L. persicum*	Turkey	53km E. Gole, Kars province.	PI 545680	1992/ 1984	USDA



Map 1: Distribution of the studied species over the studied countries of the Mediterranean and Middle East.

Table 2: Qualitative and quantitative features used as morphological markers.

Characters	Character states	Code	Characters	Character states	Code
1. Longevity	1. Annual.	1	43. Surface of glumes	89. Glabrous.	1
	2. Annual or biennial.	2			
	3. Perennial.	3			
2. Life form	4. Tufted.	1			
	5. Spreading.	2			
3. Branching	6. Unbranched.	1			
	7. Branched at base.	2	44. Apex of glumes	90. Acute.	1
4. Erection of culms	8. Erect.	1		91. Acuminate.	2
	9. Decumbent.	2		92. Obtuse.	3
	10. Prostrate.	3		93. Sub-obtuse.	4
	11. Subprostrate.	4	45. Curvature of glumes	94. Rounded on the back.	1
5. Surface of culms	12. Glabrous.	1		95. Broad on the back.	2
	13. Scaberulous below spike.	2	46. Number of nerves of glumes	96. ≤6.	1
6. Length of culms/ cm	14. <105.	1		97. >6.	2
	15. >105.	2	47. Central nerve of glumes	98. Pointed.	1
7. Number of nodes	16. ≤3.	1		99. Not.	2
	17. >3.	2	48. Length of glumes/ mm	100. <13.	1
8. Surface of leaf sheath	18. Glabrous.	1		101. >13.	2
	19. Scaberulous.	2	49. Width of glumes/ mm	102. ≤2.	1
9. Colour of leaf sheath at node	20. Green.	1		103. >2.	2
	21. Purplish.	2	50. Presence of lower glume	104. Only on terminal spikelets.	1
10. Attachment of sheath to culm	22. Loose.	1	51. Texure of lower glumes	105. Firm.	1
	23. Tightly clasping and loose below.	2		106. Very firm.	2
11. Shape of ligules	24. Truncate.	1	52. Texure of upper glumes	107. Firm.	1
	25. Obtuse.	2		108. Muticous.	2
12. Texure of ligules	26. Membranous.	1	53. Upper glumes according to presence of awns	109. Awned.	1
13. Length of ligules/ mm	27. ≤2.5.	1		110. Awnless.	2
	28. >2.5.	2	54. Length of awns of upper glumes/ mm	111. 8.5.	1
14. Presence of auricles	29. Present.	1		112. Absent.	2
	30. Absent.	2	55. Number of florets	113. <8.	1
15. Overlapping of auricles (if present)	31. Extremely overlapped.	1		114. >8.	2
	32. Not.	2	56. Overlapping of lemmas	115. Overlapped.	1
16. Length of	33. <12.	1		116. Not.	2

leaf sheath/ cm	34. >12.	2	57. Shape of lemmas	117. Ovate.	1
17. Apex of leaf blades	35. Acute.	1	58. Surface of lemmas	118. Oblong to lanceolate.	1
	36. Acuminate.	2		119. Lanceolate.	2
18. Texure of leaf blades	37. Firm on upper surface and glossy on lower one.	1		120. Elliptic to ovate.	3
	38. Firm on both surfaces.	2		121. Oblong.	4
19. Surface of leaf blades	39. Glabrous.	1		122. Glabrous.	5
	40. Scabrid.	2		123. Glabrous but rough on margins.	6
20. Behaviour of leaf blades when young	41. Folded.	1	59. Apex of lemmas	124. Acute.	1
21. Length of leaf blades/ cm	42. <30.	1		125. Obtuse.	2
	43. >30.	2		126. Two toothed.	3
22. Width of leaf blades/ mm	44. ≤7.	1	60. Texure of apex of lemmas	127. Firm.	1
	45. >7.	2		128. Hyaline.	2
23. Erection of spikes	46. Erect.	1	61. Curvature of lemmas	129. Rounded on the back.	1
	47. Curved.	2		130. Broad on the back.	2
24. Stifness of spikes	48. Stiff.	1	62. Turgidity of lemmas at maturity	131. Very turgid.	1
	49. Not.	2		132. Not.	2
25. Denesity of spikes	50. Dense.	1	63. Number of nerves of lemmas	133. ≤4.	1
	51. Loose.	2		134. >4.	2
26. Length of spikes/ cm	52. ≤30.	1	64. Length of lemmas/ mm	135. ≤6.5.	1
	53. >30.	2		136. >6.5.	2
27. Spike central axis	54. Cylinderical.	1	65. Width of lemmas/ mm	137. <6.5.	1
	55. Flattened.	2		138. >6.5.	2
28. Surface of spike central axis	56. Glabrous.	1	66. Lemmas according to presence of awns	139. Awned.	1
	57. Scabridulous.	2		140. Awnless.	2
	58. Scabrous.	3	67. Straightness of awns	141. Straight.	1
29. Shape of spikelets	59. Oblong to elliptic.	1		142. Absent.	2
	60. Oblong to lanceolate.	2	68. Thickness of awns	143. Very fine.	1
	61. Oblong.	3		144. Absent.	2
	62. Ovate.	4	69. Origin of awns	145. Below apex of lemma.	1
	63. Elliptic.	5		146. Absent.	2
30. Compression of spikelets	64. Compressed.	1	70. Length of awns of lemmas/ mm	147. <14.5.	1
	65. Swollen.	2		148. >14.5.	2
31. Direction of	66. Ascending.	1		149. Absent.	3

spikelets					
32. Erection of spikelets	67. Erect.	1	71. Shape of palea	150. Keeled.	1
33. Stalk of spikelets	68. Sessile.	1	72. Surface of palea	151. Glabrous.	1
34. Arrangement of spikelets on the axis	69. Alternate on both sides.	1		152. Scabrid.	2
35. Number of spikelets	70. <14.	1		153. Finely scabrid.	3
	71. >14.	2	73. Length of palea/ mm	154. ≤6.5.	1
36. Length of spikelets (excluding awns)/ cm	72. ≤2.5.	1		155. >6.5.	2
	73. >2.5.	2	74. Width of palea/ mm	156. <1.5.	1
				157. >1.5.	2
			75. Length of anthers/ mm	158. <2.	1
37. Width of spikelets/ mm	74. ≤4.5.	1		159. >2.	2
	75. >4.5.	2	76. Shape of caryopsis	160. Ovate.	1
38. Concavities of rachillae	76. Present.	1		161. Oblong to lanceolate.	2
	77. Absent.	2		162. Lanceolate.	3
39. Length of glumes in comparison with spikelets	78. G shorter than S.	1		163. Elliptic to oblong.	4
	79. G longer than S.	2		164. Oblong.	5
	80. G longer than or as long as S.	3	77. Compression of caryopsis	165. Swollen.	1
	81. G as long as S.	4		166. Compressed.	2
40. Texure of margin of glumes	82. Firm.	1	78. Length of caryopsis/ mm	167. <7.5.	1
	83. Hyaline.	2		168. >7.5.	2
41. Shape of margin of glumes	84. Wing like.	1	79. Width of caryopsis/ mm	169. ≤1.5.	1
	85. Not.	2		170. >1.5.	2
42. Shape of glumes	86. Oblong to lanceolate.	1	80. Colour of caryopsis	171. Light brown.	1
	87. Oblong.	2		172. Dark brown.	2
	88. Lanceolate.	3			

Results

1. Morphological treatment

The dendrogram based on morphological data matrix (Figure 1), it was branched into two clusters, the first cluster combined accessions of *L. persicum* and *L. temulentum*;

while, the second one combined accessions of *L. rigidum*, *L. multiflorum* and *L. perenne*. The second cluster was sub-branched into two sub-clusters, the first sub-cluster included accessions of *L. rigidum* while the second one combined accessions of *L. multiflorum* and *L. perenne*.

The two-dimensional plot based on principal component analysis (PCA) correlation of morphological data illustrated the separation of the studied accessions into five groups which represented the five studied species of *Lolium*; morphologically, each group was sub-grouped according to the degree of dissimilarities among them (Figure 2).

2. Genotypic data information

A total of 137 fragments were resulted from the five primers, 131 (95.62%) fragments were polymorphic ranged in size from 150 to 1750 bp, polymorphism percentage of the used primers ranged from 92 to 100% (Table 3).

Generally, the greatest average value of Nei's gene diversity and Shannon's index (measure of gene diversity) in all the 40 studied populations were observed in *L. multiflorum* from Turkey. At the species level, the highest average level of gene diversity within populations of *L. perenne* was observed in Cyprus, while the population of *L. multiflorum* from Turkey showed the highest value. Considering populations of *L. rigidum*, the highest values of gene diversity were observed in that representing Libya, *L. temulentum* from Iraq, and in *L. persicum* from Turkey (Table 4). However, the remaining populations of the five studied species showed nearly similar gene diversity measures (Table 4).

The highest number and percentage of polymorphic loci all over the studied populations were observed in the populations of *L. perenne* from Cyprus and Greece and the population of *L. multiflorum* from Turkey. On the other hand, the populations of *L. rigidum* from Libya and Egypt; *L. temulentum* from Iraq and *L. persicum* from Turkey showed the highest number and percentage of polymorphic loci (Table 4).

The greatest average value of observed number of alleles (na) was estimated in the populations of *L. perenne* from Cyprus and Greece, *L. multiflorum* from Turkey, *L. rigidum* from Libya and Egypt, *L. temulentum* from Iraq and *L. persicum* from

Turkey. While the greatest average value of effective number of alleles (ne) was estimated in the populations of *L. perenne* from Cyprus, *L. multiflorum* from Turkey, *L. rigidum* from Libya, *L. temulentum* from Iraq and *L. persicum* from Turkey (Table 4).

Nei's analysis of gene diversity in the studied populations showed high level of the total gene diversity with an average of 0.2038, low level of gene flow among the populations with an average of 0.0417; it was found that the mean of gene diversity within population was lower than proportion differentiation among the populations (Table 5).

In general, for all studied species, high levels were noticed in the mean values of observed and effective number of alleles (1.9562 and 1.2826) respectively, Nei's gene diversity (0.1966) and Shannon's information index (0.3283) as shown in Table 4.

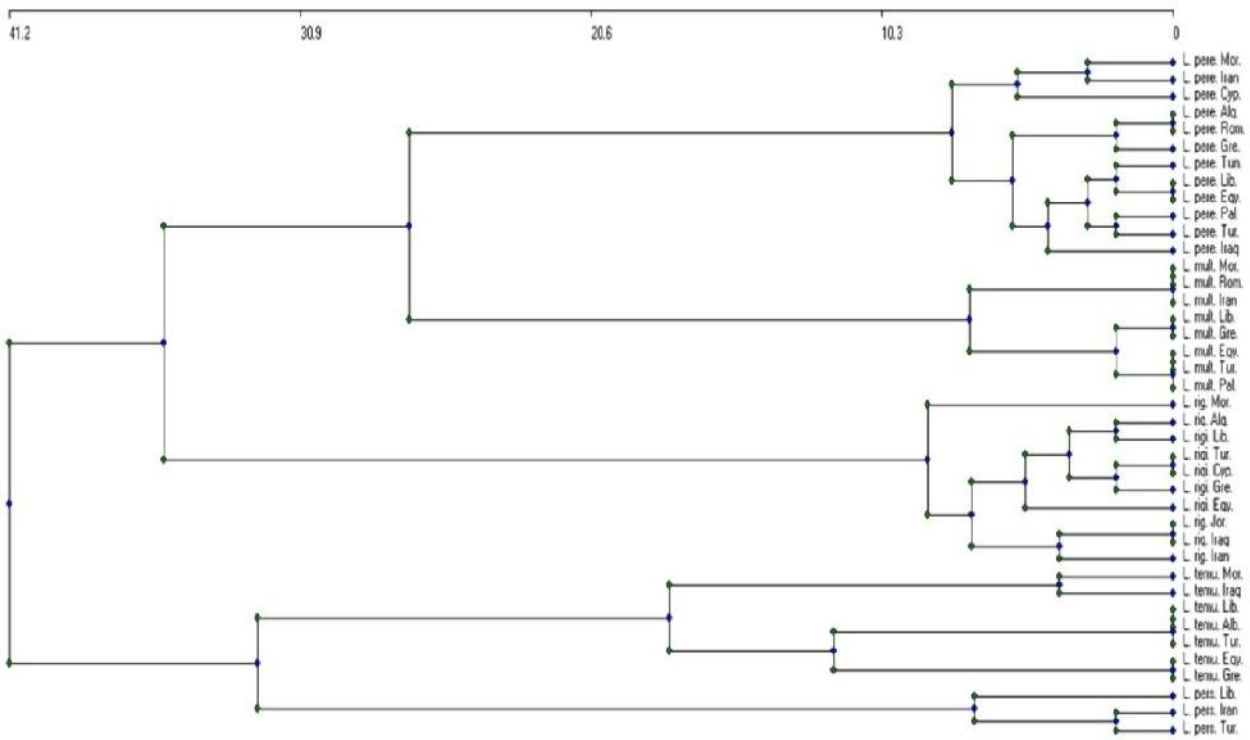


Figure 1: Dendrogram based on morphological data**.T**

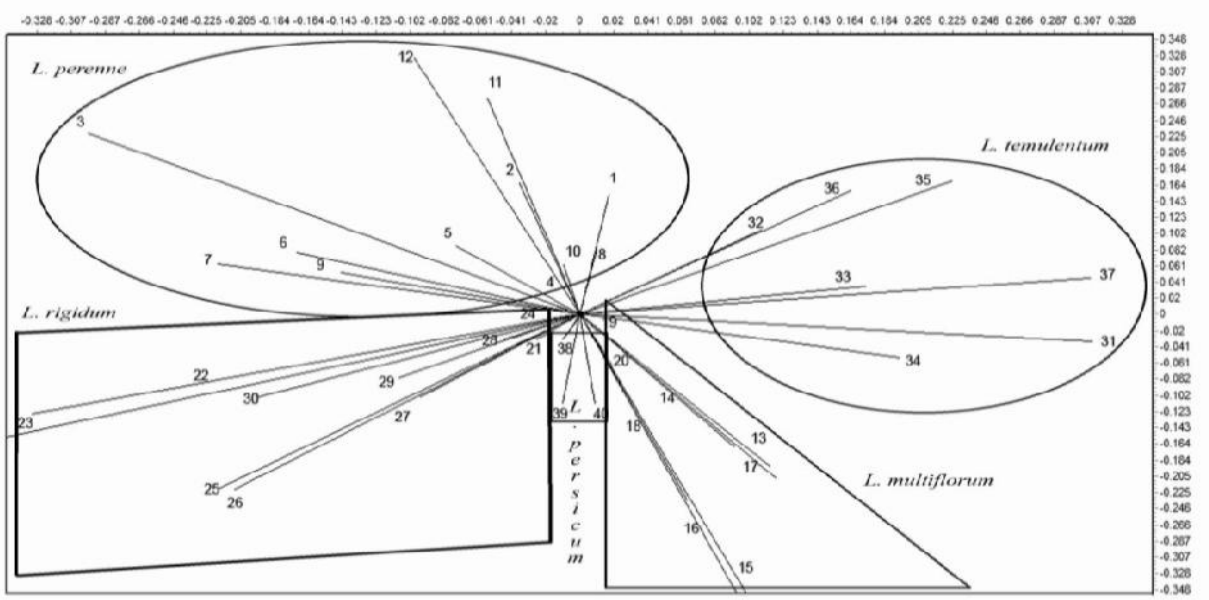


Figure 2: Two-dimensional plot obtained by principal component analysis (PCA) correlation based on morphological data.

The dendrogram based on genetic distance was classified into two clusters, the first combined the populations of *L. persicum* and *L. temulentum* and the second cluster combined populations of *L. multiflorum*, *L. rigidum* and *L. perenne*; in the second cluster, *L. perenne* more associated to *L. rigidum* than to *L. multiflorum* (Figure 3).Two-dimensional plot based on principal component analysis (PCA) correlation was obtained by using CAP program, which supported separation of the five studied species of *Lolium*, each group also was sub-grouped according to the degree of dissimilarities among them (Figure 4).

Legend: 1: Population 1 (accessions of *L. perenne* from Morocco). **2:** Population 2 (accessions of *L. perenne* from Algeria). **3:** Population 3 (accessions of *L. perenne* from Tunisia). **4:** Population 4 (accessions of *L. perenne* from Libya). **5:** Population 5 (accession of *L. perenne* from Egypt). **6:** Population 6 (accession of *L. perenne* from Palestine). **7:** Population 7 (accession of *L. perenne* from Iraq). **8:** Population 8 (accessions of *L. perenne* from Iran). **9:** Population 9 (accessions of *L. perenne* from Turkey). **10:** Population 10 (accessions of *L. perenne* from Cyprus). **11:** Population 11 (accessions of *L. perenne* from Romania). **12:** Population 12 (accessions of *L. perenne* from Greece). **13:** Population 13 (accession of *L. multiflorum* from

Morocco). **14:** Population 14 (accession of *L. multiflorum* from Libya). **15:** Population 15 (accessions of *L. multiflorum* from Egypt). **16:** Population 16 (accession of *L. multiflorum* from Palestine). **17:** Population 17 (accession of *L. multiflorum* from Iran). **18:** Population 18 (accessions of *L. multiflorum* from Turkey). **19:** Population 19 (accessions of *L. multiflorum* from Romania). **20:** Population 20 (accession of *L. multiflorum* from Greece). **21:** Population 21 (accessions of *L. rigidum* from Morocco). **22:** Population 22 (accessions of *L. rigidum* from Algeria). **23:** Population 23 (accessions of *L. rigidum* from Libya). **24:** Population 24 (accessions of *L. rigidum* from Egypt). **25:** Population 25 (accession of *L. rigidum* from Jordan). **26:** Population 26 (accessions of *L. rigidum* from Iraq). **27:** Populatio n 27 (accessions of *L. rigidum* from Iran). **28:** Population 28 (accessions of *L. rigidum* from Turkey). **29:** Population 29 (accessions of *L. rigidum* from Cyprus). **30:** Population 30 (accessions of *L. rigidum* from Greece). **31:** Population 31 (accessions of *L. temulentum* from Morocco). **32:** Population 32 (accession of *L. temulentum* from Libya). **33:** Population 33 (accession of *L. temulentum* from Egypt). **34:** Population 34 (accessions of *L. temulentum* from Iraq). **35:** Population 35 (accessions of *L. temulentum* from Turkey). **36:** Population 36 (accessions of *L. temulentum* from Albania). **37:** Population 37 (accession of *L. temulentum* from Greece). **38:** Population 38 (accession of *L. persicum* from Libya). **39:** Population 39 (accessions of *L. persicum* from Iran). **40:** Population 40 (accessions of *L. persicum* from Turkey).

Table 3: Characteristics of ISSR primers used for detection of polymorphism in the studied genotypes.

Primer name	Sequence	Annealing Temp. (° C)	Total loci	Poly-morphic loci	Poly-morphism (%)	Range of fragments size (bp)
ISSR 809	GAGGAGAGAGAGAGAGG	49	25	23	92	200-1750
ISSR 810	GAGAGAGAGAGAGAGAT	44.6	32	31	96.87	150-1750
ISSR 812	GAGAGAGAGAGAGAGAA	44.6	28	27	96.42	>150-1750
ISSR 813	CTCTCTCTCTCTCTCTT	44.6	26	24	92.30	200-1750
ISSR 834	AGAGAGAGAGAGAGAGCTT	49	26	26	100	>150-1750

Table 4: Genic variation statistics for all loci of all populations according to Nei (1987).

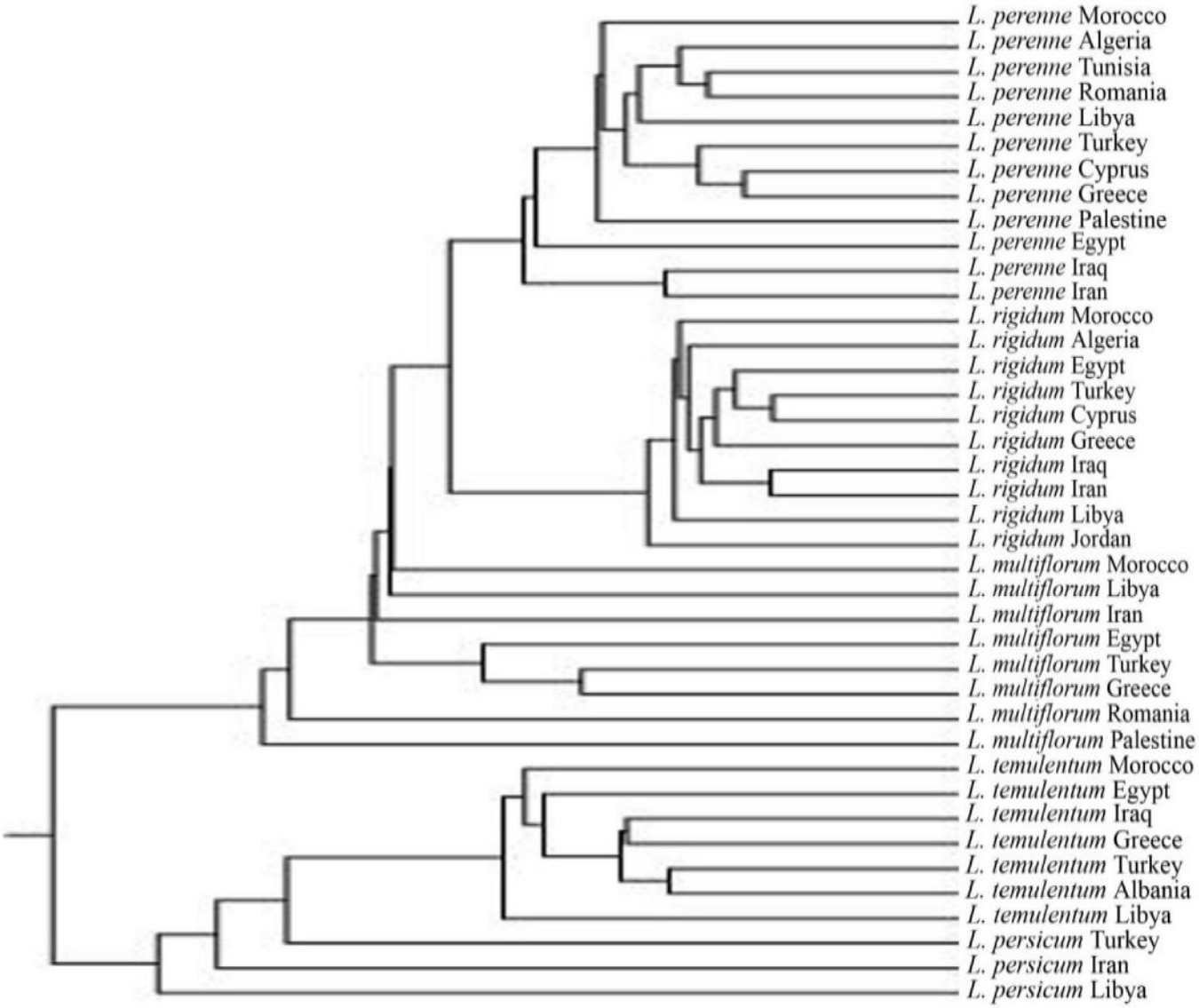
Populations		Summary	Observed number of alleles (na)	Effective number of alleles (ne)	Nei's gene diversity (h)	Shannon's information index (I)	No. of polymor-phic loci	% of polymor-phic loci
Pop. Name	Sample size							
Pop. 1	3	Mean	1.0219	1.0209	0.0107	0.0149	3	2.19 %
		St. dev.	0.1469	0.1400	0.0717	0.1000		
Pop. 2	3	Mean	1.0219	1.0094	0.0066	0.0104	3	2.19 %
		St. dev.	0.1469	0.0628	0.0440	0.0700		
Pop. 3	3	Mean	1.0657	1.0396	0.0238	0.0358	9	6.57 %
		St. dev.	0.2487	0.1629	0.0930	0.1377		
Pop. 4	3	Mean	1.0876	1.0490	0.0304	0.0462	12	8.76 %
		St. dev.	0.2837	0.1725	0.1013	0.1520		
Pop. 5	1	Mean	1.0000	1.0000	0.0000	0.0000	0	0.00 %
		St. dev.	0.0000	0.0000	0.0000	0.0000		
Pop. 6	1	Mean	1.0000	1.0000	0.0000	0.0000	0	0.00 %
		St. dev.	0.0000	0.0000	0.0000	0.0000		
Pop. 7	1	Mean	1.0000	1.0000	0.0000	0.0000	0	0.00 %
		St. dev.	0.0000	0.0000	0.0000	0.0000		
Pop. 8	3	Mean	1.0584	1.0365	0.0216	0.0323	8	5.84 %
		St. dev.	0.2353	0.1595	0.0899	0.1324		
Pop. 9	3	Mean	1.0949	1.0521	0.0326	0.0497	13	9.49 %
		St. dev.	0.2941	0.1754	0.1039	0.1564		
Pop. 10	3	Mean	1.1095	1.0737	0.0424	0.0626	15	10.95 %
		St. dev.	0.3134	0.2282	0.1254	0.1825		
Pop. 11	3	Mean	1.0511	1.0449	0.0236	0.0333	7	5.11 %
		St. dev.	0.2210	0.1985	0.1030	0.1450		
Pop. 12	3	Mean	1.1095	1.0660	0.0397	0.0597	15	10.95 %
		St. dev.	0.3134	0.2061	0.1174	0.1737		
Pop. 13	1	Mean	1.0000	1.0000	0.0000	0.0000	0	0.00 %
		St. dev.	0.0000	0.0000	0.0000	0.0000		
Pop. 14	1	Mean	1.0000	1.0000	0.0000	0.0000	0	0.00 %
		St. dev.	0.0000	0.0000	0.0000	0.0000		
Pop. 15	2	Mean	1.0584	1.0413	0.0242	0.0353	8	5.84 %
		St. dev.	0.2353	0.1664	0.0975	0.1423		
Pop. 16	1	Mean	1.0000	1.0000	0.0000	0.0000	0	0.00 %
		St. dev.	0.0000	0.0000	0.0000	0.0000		
Pop. 17	1	Mean	1.0000	1.0000	0.0000	0.0000	0	0.00 %
		St. dev.	0.0000	0.0000	0.0000	0.0000		
Pop. 18	3	Mean	1.1241	1.0838	0.0482	0.0711	17	12.41 %
		St. dev.	0.3309	0.2419	0.1327	0.1930		
Pop. 19	3	Mean	1.0438	1.0226	0.0145	0.0224	6	4.38 %
		St. dev.	0.2054	0.1136	0.0696	0.1061		
Pop.	1	Mean	1.0000	1.0000	0.0000	0.0000	0	0.00 %

20		St. dev.	0.0000	0.0000	0.0000	0.0000		
Pop. 21	3	Mean	1.0730	1.0427	0.0260	0.0393	10	7.30 %
		St. dev.	0.2611	0.1662	0.0959	0.1427		
Pop. 22	3	Mean	1.0803	1.0497	0.0296	0.0442	11	8.03 %
		St. dev.	0.2727	0.1835	0.1037	0.1529		
Pop. 23	3	Mean	1.1022	1.0629	0.0375	0.0562	14	10.22 %
		St. dev.	0.3040	0.2038	0.1152	0.1700		
Pop. 24	3	Mean	1.1022	1.0476	0.0320	0.0502	14	10.22 %
		St. dev.	0.3040	0.1480	0.0965	0.1503		
Pop. 25	1	Mean	1.0000	1.0000	0.0000	0.0000	0	0.00 %
		St. dev.	0.0000	0.0000	0.0000	0.0000		
Pop. 26	2	Mean	1.0584	1.0413	0.0242	0.0353	8	5.84 %
		St. dev.	0.2353	0.1664	0.0975	0.1423		
Pop. 27	3	Mean	1.0876	1.0567	0.0331	0.0492	12	8.76 %
		St. dev.	0.2837	0.1990	0.1108	0.1622		
Pop. 28	3	Mean	1.0730	1.0427	0.0260	0.0393	10	7.30 %
		St. dev.	0.2611	0.1662	0.0959	0.1427		
Pop. 29	3	Mean	1.0803	1.0459	0.0282	0.0427	11	8.03 %
		St. dev.	0.2727	0.1694	0.0987	0.1475		
Pop. 30	2	Mean	1.0438	1.0310	0.0181	0.0265	6	4.38 %
		St. dev.	0.2054	0.1452	0.0851	0.1242		
Pop. 31	2	Mean	1.0000	1.0000	0.0000	0.0000	0	0.00 %
		St. dev.	0.0000	0.0000	0.0000	0.0000		
Pop. 32	1	Mean	1.0000	1.0000	0.0000	0.0000	0	0.00 %
		St. dev.	0.0000	0.0000	0.0000	0.0000		
Pop. 33	1	Mean	1.0000	1.0000	0.0000	0.0000	0	0.00 %
		St. dev.	0.0000	0.0000	0.0000	0.0000		
Pop. 34	2	Mean	1.0365	1.0258	0.0151	0.0221	5	3.65 %
		St. dev.	0.1882	0.1331	0.0780	0.1138		
Pop. 35	3	Mean	1.0219	1.0132	0.0079	0.0119	3	2.19 %
		St. dev.	0.1469	0.0959	0.0548	0.0813		
Pop. 36	3	Mean	1.0000	1.0000	0.0000	0.0000	0	0.00 %
		St. dev.	0.0000	0.0000	0.0000	0.0000		
Pop. 37	1	Mean	1.0000	1.0000	0.0000	0.0000	0	0.00 %
		St. dev.	0.0000	0.0000	0.0000	0.0000		
Pop. 38	1	Mean	1.0000	1.0000	0.0000	0.0000	0	0.00 %
		St. dev.	0.0000	0.0000	0.0000	0.0000		
Pop. 39	3	Mean	1.0365	1.0271	0.0151	0.0219	5	3.65 %
		St. dev.	0.1882	0.1483	0.0797	0.1144		
Pop. 40	3	Mean	1.0438	1.0302	0.0172	0.0254	6	4.38 %
		St. dev.	0.2054	0.1522	0.0833	0.1208		
All pops.	89	Mean	1.9562	1.2826	0.1966	0.3283		
		St. dev.	0.2054	0.2392	0.1305	0.1803		

Table 5: Nei's analysis of gene diversity in subdivided populations according to Nei (1987).

Populations		Summary	Total gene diversity (Ht)	Mean gene diversity within population (Hs)	Proportion differentiation among populations (Gst)	Estimate of gene flow (Nm)	No. of poly-morphic loci	(%) poly-morphic loci
Pop. name	Sample size							
All pops.	89	Mean	0.2038	0.0157	0.9229	0.0417	131	95.62%
		St. dev.	0.0169	0.0009				

Figure 3 : Dendrogram based on genetic distance according to Nei (1978):



Method=UPGMA modified from NEIGHBOR procedure of PHYLIP Version 35

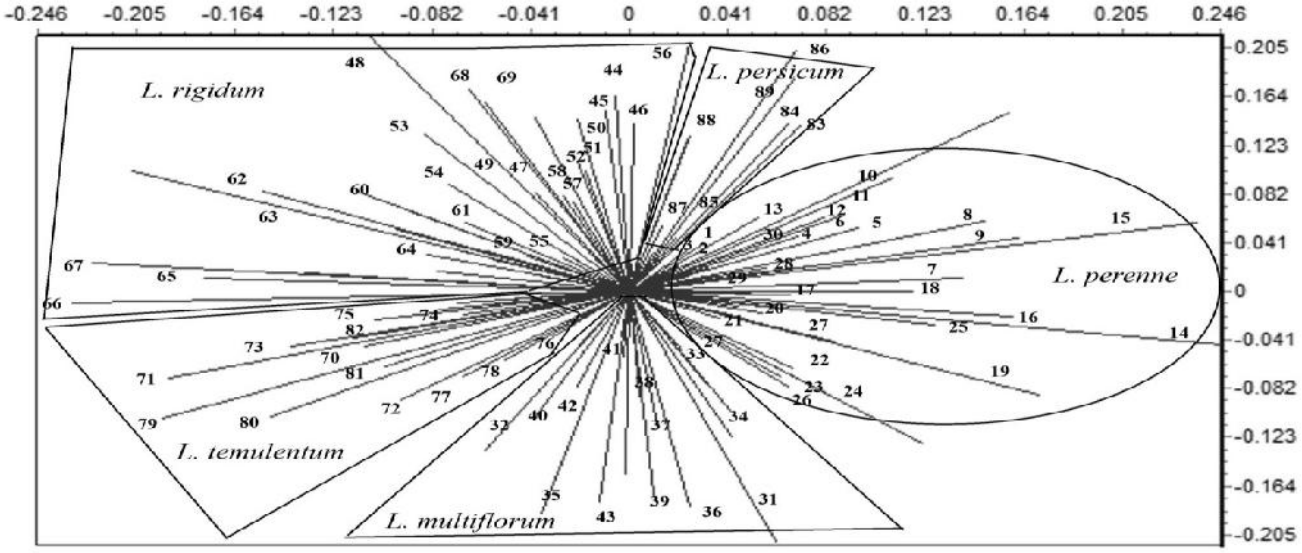


Figure 4: Two-dimensional plot obtained by principal component analysis (PCA) correlation based on molecular data**.**

Legend: 1, 2 and 3: Population 1 (accessions of *L. perenne* from Morocco). **4, 5 and 6:** Population 2 (accessions of *L. perenne* from Algeria). **7, 8 and 9:** Population 3 (accessions of *L. perenne* from Tunisia). **10, 11 and 12:** Population 4 (accessions of *L. perenne* from Libya). **13:** Population 5 (accession of *L. perenne* from Egypt). **14:** Population 6 (accession of *L. perenne* from Palestine). **15:** Population 7 (accession of *L. perenne* from Iraq). **16, 17 and 18:** Population 8 (accessions of *L.perenne* from Iran). **19, 20 and 21:** Population 9 (accessions of *L. perenne* from Turkey). **22, 23 and 24:** Population 10 (accessions of *L. perenne* from Cyprus). **25, 26 and 27:** Population 11 (accessions of *L. perenne* from Romania). **28, 29 and 30:** Population 12 (accessions of *L. perenne* from Greece). **31:** Population 13 (accession of *L. multiflorum* from Morocco). **32:** Population 14 (accession of *L. multiflorum* from Libya). **33 and 34:** Population 15 (accessions of *L. multiflorum* from Egypt). **35:** Population 16 (accession of *L. multiflorum* from Palestine). **36:** Population 17 (accession of *L. multiflorum* from Iran). **37, 38 and 39:** Population 18 (accessions of *L. multiflorum* from Turkey). **40, 41 and 42:** Population 19 (accessions of *L. multiflorum* from Romania). **43:** Population 20 (accession of *L. multiflorum* from Greece). **44, 45 and 46:** Population 21 (accessions of *L. rigidum* from Morocco). **47, 48 and 49:** Population 22 (accessions of *L. rigidum* from Algeria). **50, 51 and 52:** Population 23 (accessions of *L. rigidum* from Libya). **53, 54 and 55:** Population 24 (accessions of *L. rigidum* from Egypt). **56:** Population 25 (accession of *L. rigidum*

from Jordan). **57 and 58:** Population 26 (accessions of *L. rigidum* from Iraq). **59, 60 and 61:** Population 27 (accessions of *L. rigidum* from Iran). **62, 63 and 64:** Population 28 (accessions of *L. rigidum* from Turkey). **65, 66 and 67:** Population 29 (accessions of *L. rigidum* from Cyprus). **68 and 69:** Population 30 (accessions of *L. rigidum* from Greece). **70 and 71:** Population 31 (accessions of *L. temulentum* from Morocco). **72:** Population 32 (accession of *L. temulentum* from Libya). **73:** Population 33 (accession of *L. temulentum* from Egypt). **74 and 75:** Population 34 (accessions of *L. temulentum* from Iraq). **76, 77 and 78:** Population 35 (accessions of *L. temulentum* from Turkey). **79, 80 and 81:** Population 36 (accessions of *L. temulentum* from Albania). **82:** Population 37 (accession of *L. temulentum* from Greece). **83:** Population 38 (accession of *L. persicum* from Libya). **84, 85 and 86:** Population 39 (accessions of *L. persicum* from Iran). **87, 88 and 89:** Population 40 (accessions of *L. persicum* from Turkey).

Discussion

Diversity is controlled by immigration, drift, selection or competition, heterogeneity, connectivity and fragmentation of regional landscapes. Identity, abundance and diversity of habitats influence the abundance of species live in certain areas (Noss, 1990). In the present study, the morphological diversity was investigated by scoring 80 characters and 172 character states including: habit, culms, leaves, inflorescences, inflorescences axes, spikelets, florets and caryopses to determine the relationships among these species by applying numerical analyses.

The agglomerative clustering based on morphological data revealed the separation of five groups that represent the five studied species., *L. rigidum*, *L. multiflorum* and *L. perenne* share morphological characters as small size of flowers and fruits and firm texture of leaf blade on the upper surface while glossy on the lower one; *L. perenne* is more associated with *L. multiflorum* than with *L. rigidum* as they have oblong to elliptic spikelets, overlapped and oblong lemmas and their glumes shorter than the spikelets; also, *L. persicum* and *L. temulentum* are closely associated with each other, where they have large size of flowers and fruits, firm texture of leaf blade on the both surfaces and their glumes as long as the spikelets. In this work, the resulted cluster as concluded by Stammers *et al*., 1995; Polok *et al*., 2006; Ma *et al*., 2013. Within each

species, populations were sub-grouped geographically according to degree of morphological dissimilarities. High level of morphological similarity was observed between majorities of the populations within each species from different origins, these similarities may be due to highly similar ecological and topographical features of the studied countries over short distances.

The present study showed polymorphism percentage as high as 95.62% for all populations, where we obtained 131 polymorphic fragments from a total number of 137 fragments. The relationships among these species reflected a high degree of divergence among the studied populations according to the geographical distribution of the accessions.

The highest degrees of gene diversity for all populations were observed among the accessions of *L. perenne* population from Cyprus, *L. rigidum* from Libya, *L. temulentum* from Iraq and *L. persicum* from Turkey. *L. multiflorum* from Turkey not only showed the highest degree of gene diversity, but also the highest degrees of Shannon's information index, observed number of alleles and effective number of alleles. From these results we can predict that Turkey may be the center of origin of the studied *Lolium* species. As the result of Blackmore *et al*. (2015) there is a great correlation between geographic distribution and genetic structure within and between populations of *L. perenne*.

Nei's gene diversity (h) has similar or the same values in some populations within each species (Table 4) this indicates that the gene diversity do not differ significantly among these populations.

In most of the studied populations it was observed that the average values of (na) was greater than that of (ne) at all loci which indicates that the allele frequencies are not equal for all alleles, while in remaining populations the average values of (na) equals that of (ne) which indicates extremely low level of gene diversity or absence of gene diversity in these populations (Table 4).

Nei's analysis of gene diversity of the studied populations (Nei, 1987) showed low level of gene flow among all the populations with an average of 0.0417 and showed high level of the total gene diversity (Ht) with an average of 0.2038 that reflects high

degree of complication and independence of the evolutionary line of these species. The level of Proportion differentiation among populations (Gst) is higher than Mean gene diversity within population (Hs), which refers to that the genetic variation within each species is due to differences among the populations not due to differences within each population.

It was found that the accession of *L. persicum* (GR 5452) from Libya (Fezzan / Aqar) collected in 1983 that was provided by IPK, is associated with the other accessions of *L. persicum* (from Iran and Turkey) morphologically and molecularly; but the origin of this accession is doubtful because it was not recorded in the Libyan flora 'Flora of Libya' (Sherif and Siddiqi, 1988). So we recommend revision of its collection in IPK to ensure its origin for future studies and to enable its study for other researchers.

Both two-dimensional plot based on principal component analysis (PCA) and the dendrogram based on Nei's (1978) genetic distance supported the separation of the studied accessions into five species. In the meantime, it supported the conclusion that *L. rigidum*, *L. multiflorum* and *L. perenne* are closely related to each other, while *L. perenne* is more related to *L. rigidum* than to *L. multiflorum* and *L. persicum* and *L. temulentum* are closely related to each other. Based on molecular data, within each species, the populations also were sub-grouped according to their geographical origin.

Geographical distribution of these species was made in the past due to climatic changes during glacial and interglacial periods since about 18000-25000 years ago, where species migrated northwards during the interglacial periods (Roberts, 1998), this may affect the genetic structure of the populations. Genetic differentiation among the European populations might be increased due to colonization of *Lolium* species from the Middle East during glaciation periods and their extinction during the interglacial periods or because the species came into Europe with the first farmers as weeds of cereal crops (Polok, 2007).

Finally, the difference between morphological results and molecular results (in relationships among *L. perenne*, *L. multiflorum* and *L. rigidum*) may be due to the evolutionary line of *Lolium* species and the genetic history of them, which impacted the genetic patterns of these species. This conclusion may affect the opinion that supports the point of view of that *L. perenne* and *L. multiflorum* are very associated

with each other (molecularly), another point of view was arose from this study and need to be confirmed by using further significant molecular tools in other future works.

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