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Karyological studies in the genus *Lolium* in Iran

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Abstract

The genus *Lolium* is one of the most important categories of temperate forage grasses that is diploid (2n=2x=14), although some populations have been reported as tetraploid (2n=4x=28). Seeds were collected or provided from Genebanks. Karyotipic parameters like the length of long and short arm (arm length ratio), Total form percentage, and relative length of shortest chromosome (centromere index) were measured. Levels of ploidy and chromosome numbers were studied for 20 populations. Of these, 16 were diploid and 4 were tetraploid. Karyological data were recorded on at least five well-prepared cells and analyzed by SAS software. Analysis of variance showed that populations have significant differences in long arm, short arm and total length of chromosomes. Interspecific relationships between populations based on the karyological traits were discussed. Also, it was shown that *L. persicum* had the most symmetric chromosomes and *L. temulentum* and *L. rigidum* chromosomes were asymmetric.

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Introduction

Karyological studies are very important, because chromosomes include genes containing information about phenotype of the plants. Interpretation of processes resulting in genetic variation and evolution, are possible by chromosome studies (Hajimoniri, 1999). Gupta (1995) defined karyotype and explained that similarities and differences between the plant taxons may arise from phylogenetic relationships. Karyotypic characters (such as chromosome length, arm ratios, and secondary constrictions) can be useful for individual chromosomes identification and phylogenetic studies.

The genus *Lolium* L. family Poaceae (= Gramineae), subfamily festucoideae, belongs to the tribe festuceae Nees (Tezvelev, 1989; Zwierzykowski and Naganowski, 1996). The *Lolium* species are diploids with 2n=14; though some populations are polyploids (Loos, 1993; Jenkin, 1954). Several authors studied karyotypes of most *Lolium* species with use of classical (Essad, 1954; Malik and Thomas, 1966; Thomas, 1977, 1981) and modern cytogenetic methods (Thomas *et al.,* 1996, 1997; Harper *et al.,* 2004; Książczyk *et al.,* 2010). Essad (1954) studied karyotypes of five species of *Lolium* and suggested three classes for the genus. A detailed investigation of karyotype has been conducted by Malik and Thomas (1996). Klinga (1986) used seeds belonging to populations of two species of *L. multiflorum* and *L. perenne* for chromosome numbering. Total frequency of aneuploids in two populations of *L. multiflorum* was 6.52% and 7.50%, since it was 9.41% for *L. perenne*. Mirzaii-Nodushan and Nadarkhani (2001) studied karyotypes of nine populations of *L. multiflorum* and *L. rigidum*. They measured karyological traits such as arm length, chromosome number and symmetry on diploid and tetraploid populations. Findings of several authors suggested that in-breedings (*L. temulentum* and *L. persicum*) were different from the other species of the genus (Naylor, 1960; Hutchinson *et al.,* 1979; Thomas, 1981; Loos, 1993). Terrell (1968) had a comprehensive review on the genus *Lolium*. He recognized eight species in the genus and divided it to two sections based on breeding system. C-banding patterns of *L. temulentum* were completely distinct from the others; whereas they had longer chromosomes. Karyotypic studies of *L. temulentum*, also, indicated that the total length of the chromosome complement in the inbreeders is about 40% higher than outbreeders (Thomas, 1981). Loos (1993a) studied seven species of *Lolium* and showed that inbreeding species easily separated from outbreedings. The aim of this study was to explain the relationship between the genus *Lolium* based on karyotype characters in different species and morphological and other traits, especially on *L. persicum* that is endemic to Iran.

Materials and methods

20 seed populations (accessions) used for this study, but 12 accessions were taken from RIFR Genebank (www.RIFR.ac.ir). Three accessions from populations taken from ICRANS Genebank (www.Esfahan.areo.ir) and the rest ones were collected in Iran. Two populations were combinations of diploid and tetraploid (Table 1).

Preparations were made using fresh root tips that grown from seeds treated by fungicide in the petri dish at 25◦C. The seeds of unknown species and *L. perenne* seeds were stored at 4◦C for one week. Root tips were treated with α-Bromonaphtaline for 2.5 hours followed by fixation in a glacial acetic acid and absolute alcohol mixture (1:3) for 24 hours at room temperature. Root tips were then hydrolyzed in 1% HCl at 60◦C for five minutes, therefore, hematoxiline was used for chromosome staining. Karyological data were recorded on at least five well-prepared cells at metaphase stage for each population. Chromosome pairs were identified and arranged on the basis of Levan *et al.,* (1964). Pearson correlation coefficient was estimated for the total length of long arm (L), short arm (S), and L/S and S/L ratios of the corresponding chromosomes of the populations studied.

parameters such as total form percentage (TF %), differences in range of relative length (DRL), shortest chromosome relative length (S %), total length of the

chromosomes (TL), shortest chromosome length to longest chromosome length ratio (S/L) and total length average for each population were calculated. Total form percentage was estimated to assess karyotype symmetry. Analysis of variance was performed on the data recorded on the karyotypic traits using SAS software (SAS Institute, 2000). Cluster analysis was performed on the data for drawing tree diagram of populations using UPGMA in STATISTICA software (Statsoft, 1995).

Results

Chromosome numbering showed that 16 populations were diploid and 4 were tetraploid (Table 1). Two populations (No. 17 and 22) were combinations of diploid and tetraploid. Karyotype formula, total form percentage and other parameters studied, are presented in Table 2. Analysis of variance showed significant differences between populations, chromosomes and their interactions for most of karyological traits recommending further analysis of the traits (Tables 3, 4, 5, 6).

Table 1. Populations name, origin and number used for chromosome numbering and karyotype preparation.

No	Population name	Origin	Abbreviation	Ploidy	Species
1	G1	collected	Lte1	Diploid	*L. temulentum*
2	G2	collected	Lps21	Diploid	*L. persicum*
3	G3	collected	Lps59	Diploid	*L. persicum*
4	G4	RIFRI	Llo20	Diploid	*L. loliaceum*
5	G5	collected	Llo35	Diploid	*L. loliacum*
6	G6	RIFRI	Lmu4	Diploid	*L. multiflorum*
7	G7	collected	Lmu30	Diploid	*L. multiflorum*
9	G8	ICNRI	Lpr25	Diploid	*L. perenne*
12	G9	collected	LOL1	Diploid	*L. rigidum*
13	G10	collected	LR3	Diploid	*L. rigidum*
15	G11	RIFRI	LR2	Diploid	*L. rigidum*
16	G12	ICNRI	LR5	Diploid	*L. rigidum*
17	G13	RIFRI	LR4	Diploid	*L. rigidum*
18	G14	RIFRI	LI1	Diploid	*L. multiflorum*
19	G15	RIFRI	LI2	Diploid	*L. multiflorum*
20	G16	RIFRI	LI3	Diploid	*L. multiflorum*
22	G17	RIFRI	LM2	Diploid	*L. multiflorum*
8	G18	RIFRI	Lpr2	Tetraploid	*L. perenne*
14	G19	RIFRI	LR1	Tetraploid	*L. rigidum*
17	G20	RIFRI	LR4	Tetraploid	*L. rigidum*
21	G21	ICNRI	LM1	Tetraploid	*L. multiflorum*
22	G22	RIFRI	LM2	Tetraploid	*L. multiflorum*

Results indicated that not only the studied traits differ between populations and also between chromosomes within populations, but also the rate of change is not constant between different populations.

Discussion

Results showed that the populations could be categorized into six groups on the basis of (i) long arm, (ii) short arm and (iii) total length; although most of them have overlaps. According to dendrogram of cluster analysis, the populations can be divided into two main groups of diploids and tetraploids (Fig. 2). This division is clearly due to different levels of ploidy and morphological traits originated from them.

Studies on the compatibility and fertility of interspecific hybrids reported by Essad (1954) and Jenkin (1954) were among the reasons, why Terrell (1968) introduced two sections for the *Lolium*. This separation is confirmed by morphological and electrophoretic seed proteins (Bulinska-Radomska and Lester, 1985) and also by enzyme system electrophoresis (Emoto, 1985; Charmet and Balfourier, 1994) analyses.

According to Figure 1, results obtained from karyotypic parameters showed that the species can be divided into two groups, in-breeding species at the right side (G1, G2, G3) and out-breedings at the left.

Our findings were consistent with those of Charmet and Balfourier (1994), who used enzyme variation of *Lolium* species and showed that in-breeding species (*L. persicum* and *L. temulentum*) are grouped together and distinct from out-breedings (*L. rigidum*, *L. perenne* and *L. multiflorum*). As shown in Figures 1 and 2, *L. temulentum* is separated from the rest. This separation can be explained by the differences of *L. temulentum* from the others, although it has similarities with inbreedings. Essad (1954) based on size and symmetry of chromosomes suggested that the genus *Lolium* divided into three classes: 1) *L. temulentum* – *L. remotum*, 2) *L. perenne* – *L. multiflorum*, and 3) *L. rigidum*; the latest resembles the second group more than the first. Malik and Thomas (1966) found that the basic karyotype was similar in the two groups. This study confirmed results of Malik and Thomas and showed that *L. temulentum*, in the absence of *L. remotum*, made a distinct group; although another in-breeding species (*L. persicum*) was replaced.

Table 2. Karyotypic symmetry parameters used for studied populations.

Population	TF%	D.R.L	S%	TL	S/L	Total Meam	Karyotype formula
G1	39.408	12.033	8.348	39.89	0.409	5.698	5M+2SM
G2	42.559	7.49	10.387	30.71	0.581	4.387	7M
G3	43.950	8.511	10.261	33.72	0.546	4.817	7M
G4	42.900	10.725	9.363	29.37	0.466	4.196	7M
G5	41.341	9.755	9.056	31.47	0.481	4.495	6M+1SM
G6	40.477	9.997	9.365	34.81	0.483	4.973	7M
G7	41.985	9.013	10.011	36.06	0.526	5.151	6M+1SM
G8	40.575	8.309	10.087	30.93	0.548	4.418	5M+2SM
G9	41.680	12.013	08.177	31.55	0.405	4.507	6M+1SM
G10	42.505	8.008	10.502	32.09	0.567	4.584	7M
G11	39.187	8.584	9.805	34.85	0.533	3.511	6M+1SM
G12	39.962	9.220	9.660	31.87	0.511	4.540	5M+2SM
G13	39.920	9.179	9.379	29.96	0.505	4.280	5M+2SM
G14	41.480	9.542	9.153	30.81	0.489	4.401	7M
G15	42.077	7.412	10.607	31.30	0.588	4.471	6M+1SM
G16	41.137	7.985	10.103	38.70	0.558	5.528	6M+1SM
G17	42.876	8.439	9.120	30.81	0.519	4.401	6M+1SM
G18	40.838	5.168	4.559	78.75	0.468	5.625	11M+3SM
G19	41.346	4.724	4.550	62.86	0.490	4.490	11M+3SM
G20	41.797	5.686	3.737	61.87	0.396	4.419	13M+1SM
G21	39.620	4.616	5.151	57.85	0.527	4.132	12M+2SM
G22	41.608	5.850	4.114	70.25	0.412	5.018	12M+2SM

Table 3. Analysis of variance of the data recorded on the karyotypic traits on diploid populations.

Source of variation	Degree of freedom	Mean Square (MS)				
		Long arm	Short arm	Total length	L/S	S/L
Genotype	16	3.16**	1.72**	8.97**	0.24ns	0.04ns
Chromosome	6	21.68**	21.89**	86.63**	1.90**	0.304**
Interaction	96	0.12ns	0.11ns	0.24ns	0.15ns	0.020ns
Error	376	0.20	0.15	0.39	0.22	0.30

** = significant at 1% level. *= significant at 5%level. ns= non-significant.

Terrell (1968) pointed out that two species of inbreeding group - *L. persicum* and *L. temulentum*, together with *L. remotum*, originated from common ancestral form in the southwest of Asia. *L. persicum*, was, also, restricted to the southwest of Asia, and originated from the same common ancestral of two other inbreeding species, or derived from the same prototype that the other two originated (Charmet and Balfourier, 1994). There are many morphological similarities between *L. persicum* and *L. temulentum* (Loos, 1993). These similarities might be interpreted as grouping near them.

Table 4. Grouping of diploid populations based on mean square of karyotypic traits.

Population	Long arm	Short arm	Total length	L/S	S/L
1	3.455a	2.249a	5.701a	1.626ab	0.662b
2	2.518de	1.873cd	4.389d	1.430ab	0.754ab
3	2.704d	2.118ab	4.821bc	1.351b	0.783a
4	2.396e	1.806cd	4.201d	1.383ab	0.751ab
5	2.639de	1.862cd	4.499cd	1.544ab	0.711ab
6	2.959bc	2.018bc	4.975b	1.573ab	0.690ab
7	2.989b	2.168ab	5.156b	1.472ab	0.728ab
9	2.628de	1.796cd	4.423d	1.561ab	0.697ab
12	2.627de	1.883cd	4.509cd	1.473ab	0.723ab
13	2.636de	1.955bc	4.589cd	1.463ab	0.744ab
15	2.137f	1.378e	3.515e	1.654a	0.652b
16	2.728cd	1.819cd	4.546cd	1.595ab	0.686ab
17	2.572de	1.711d	4.282d	1.561ab	0.670b
18	2.575de	1.831cd	4.405d	1.502ab	0.710ab
19	2.590de	1.886cd	4.475cd	1.473ab	0.726ab
20	3.251a	2.280a	5.530a	1.538ab	0.699ab
22	2.514de	1.889dc	4.402d	1.470ab	0.745ab

Table 5. Analysis of variance of the data recorded on the karyotypic traits on tetraploid populations.

Source of variation	Degree of freedom	Mean Square (MS)				
		Long arm	Short arm	Total length	L/S	S/L
Genotype	4	4.214**	2.385**	12.65**	0.075**	0.011ns
Chromosome	13	4.453**	5.413**	19.24**	0.780**	0.145**
Interaction	52	0.31ns	0.110ns	0.142ns	0.137ns	0.019ns
Error	196	0.200	0.114	0.366	0.150	0.023

** = significant at 1% level. *= significant at 5%level. ns= non-significant.

Bulinska-Radomska and Lester (1985) on the basis of protein patterns similarity found that *L. multiflorum* and *L. rigidum* are the most similar among all outbreeding species. Also the karyomorphology of these three species are very close. However, their DNA amount is different. C-banding of *L. multiflorum* and *L. rigidum* are the same (Thomas, 1981). Hybridization between these three outbreeding species has been performed successfully (Terrell, 1966; Hutchinson *et al.,* 1979).

Table 6. grouping of diploid populations based on mean square of karyotypic traits.

Population	Long arm	Short arm	Total length	L/S	S/L
8	3.332a	2.299a	5.628a	1.563a	0.684a
14	2.636c	1.860c	4.495c	1.509a	0.700a
17	2.572c	1.850c	4.422c	1.475a	0.720a
21	2.497c	1.639d	4.135d	1.576a	0.685a
22	2.932b	2.091b	5.022b	1.504a	0.712a

L. rigidum and *L. loliaceum* have shown close relationships. According to Terrell (1968), these two species are considered as varieties of one species *L. rigidum*. He classified *L. loliaceum* as *L. rigidum* var. *rottbollioides*. Several authors have confirmed this idea in the recent years (Bennett, 2000; Mirjalili *et al.,* 2006, 2008).

Results obtained from karyotype symmetry, showed that the populations of species represented different patterns. Most of the populations had meta- and submetacentric (SM) chromosomes. On the basis of two parameters - TF% and S%, populations of *L. persicum* had the most symmetric chromosomes. Diploid populations of *L. rigidum*, *L. temulentum* and one tetraploid population of *L. multiflorum* had less

symmetry throughout the populations. Essad (1954) explained that karyotypes of *L. perenne* and *L. multiflorum* are very similar. According to Essad (1962) *L. perenne* was the original species of *Lolium*, which through progressive evolution gave rise to such form as *L. rigidum* (annual allogamous) and to an autogamous group of species representing maximum evolution of the group. Malik (1967) suggested that self-pollinating and cross-pollinating species of the genus *Lolium* originated from a common ancestral form. This original form was a cross-pollinating and annual with the basic chromosome number x=7. In addition, its karyotype consisted of chromosomes of nearly equal size with median and submedian position of centromers. However, Malik (1967) suggested that *L. rigidum* could be ancestral form. The results of Charmet and Lester (1994 suggested that the species *L. rigidum* was the common ancestral form of the genus and other species that had the most diversity. According to some authors, Mediterranean area was the origin of the genus and a hypothetical, common ancestral form should be the most related to *L. rigidum* (Malik, 1967; Borrill, 1976; Charmet and Balfourier, 1994).

Table 7. First three principal components: eigenvalues, percentage of total variance and the characters with the highest loadings on the first three principal components at diploid and tetraploid levels.

Principal component	1	2	3
Eigenvalues	3.058	1.927	1.264
% Total variation	38.225	24.096	15.806
Cumulative Eigenvalues	3.058	4.985	6.250
Cumulative %	38.225	62.322	78.128
Factor Loadings	TL: -0.956 S%: 0.874 L: 0.591	Tot: 0.780 S: 0.639 DRL: 0.601	S/L: 0.703 TF: 0.612 DRL: -0.526

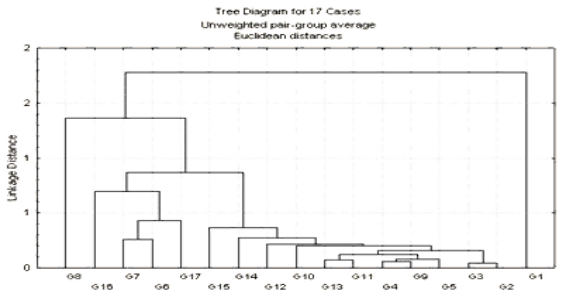

Fig. 1. Dendrogram produced by cluster analysis (UPGMA method) based on all karyological data in diploid populations.

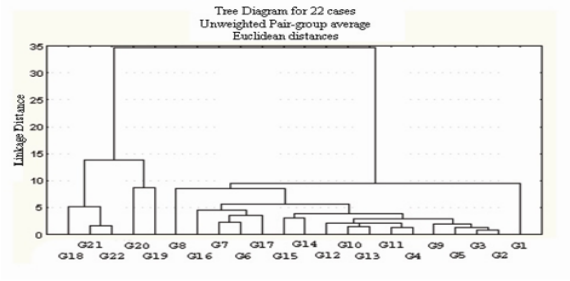

Fig. 2. Dendrogram produced by cluster analysis (UPGMA) for diploid and tetraploid populations on the all karyological parameters.

Despite of publishing of reports about phylogeny of the genus especially on the species *L. persicum*, some findings showed that this species could be ancient. On the basis of karyotype symmetry, *L. persicum* was the most symmetric and some populations of *L. rigidum* and *L. temulentum* were asymmetric. In addition, symmetric karyotype is older and the asymmetric ones are younger. Therefore, *L. persicum*, that is endemic to Iran, could be the oldest and two other species are younger. These results are in contrast with the previous findings, analyzed and explained in the absence of *L. persicum*.

Conclusions

All species of the genus *Lolium* are diploid, but due to breeding activities tetraploid varieties are found, especially in outbreeding species. karyological traits and morphological might be helpful in studying of taxonomic relationships between the species in the genus. According to previous reliable studies, *L. loliaceum* and *L. rigidum* are not two distinct species and they are two varieties of *L. rigidum*; two inbreeding species, containing *L. persicum* and *L. temulentum*, that morphological analyses showed their close relationships. Interpretation of phylogeny

of the genus *Lolium* using karyological traits is relatively difficult.

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