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Genetic Variability and Karyotype Analysis for 13 Accessions of *Lolium multiflorum*

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Abstract

Genetic variations were studied for 13 accessions of *Lolium multiflorum* using cytogenetical traits. Karyotype was prepared for 5 metaphases cells of each accession and the traits of total length (TL), long arm (LA), short arm (SA), arm ratio (AR) and centromer index (CI) were determined by micromeasure software. Six accessions were diploid and seven accessions were tetraploid, the basic chromosome number was x=7, and also three and six satellites were observed for diploid and tetraploid accessions, respectively. The accessions had an asymmetry karyotype, and variations were significant (at 1% level of probability) between accessions for karyotypic characters based on analysis of variance (ANOVA). Results of mean comparison showed that the diploid accessions had higher mean chromosome length than tetraploid accessions. The results of cluster analysis by Ward's method based on the values of intrachromosomal asymmetry index (A1) and interchromosomal asymmetry index (A2) showed that the studied accessions were fallen in three groups. The tetraploid accessions had higher intrachromosomal asymmetry than the diploid accessions. Principal components analysis based on karyotypic traits for accessions showed the first two components captured 84.37% of the total variance. Principal component analysis for grouping accessions based on Scatter plot identified four distinctive groups.

Keywords: Cytogenetic, Genetic variation, Karyotype, *Lolium multiflorum*.

INTRODUCTION

The genus *Lolium* L., family Poaceae (Geraminea), subfamily festucoideae, belongs to the tribe festuceae Nees (Zwierzykowski and Naganowski, 1996). This genus is one of the most important categories of temperate forage grasses that are diploid (2n = 2x = 14), although some accessions have been reported as tetraploid (2n = 4x = 28) (Mirjalili, 2014). The *Lolium* genus contains only eight species, including the agronomically important taxa annual or Italian ryegrass (*Lolium multiflorum* Lam.) and perennial ryegrass (*Lolium perenne* L.) (Lundqvist, 1962; Cornish *et al*., 1979), which are used for forage and turf purposes throughout the temperate regions of the world including North and South America, South Africa, Australia, and New Zealand (Lamp *et al.,* 2001).

Breeding programs are dependent on genetic variation for the development of improved cultivars. Therefore, the knowledge of genetic diversity is pertinent to improving overall plant characteristics which will allow for a systematic sampling of germplasm for breeding and conservation purposes (Che and Li, 2007). A rich and diverse germplasm collection is the backbone of every successful crop improvement programs. The genetic variability is the raw material to crop breeding industry in which selection acts to evolve superior genotypes. Morphological characteristics are the strongest determinants of the agronomic value and taxonomic classification of plants. Compared with other methods morphological evaluations are direct, inexpensive, and easy. However, errors can arise; furthermore, morphological estimations are more dependent on environment (Chowdhury *et al.,* 2002; Iruela *et al.*, 2002; Sudupak *et al*., 2002).

Interpretation of processes resulting in genetic variation and evolution are made 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. Karyological studies are very important, because chromosomes include genes containing information about phenotype of the plants. Mirzaei-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. The aim of this study was to explain the genetic diversity between accessions of *Lolium multiflorum* based on karyological characters.

MATERIALS AND METHODS

Plant materials

In order to evaluate the genetic variation, 13 accessions of *L. multiflorum* were prepared from gene bank of Research Institute of Forests and Rangelands, Tehran, Iran (Table 1).

Cytological studies

For karyotype study, freshly grown root tips were collected from the germinated seed, pretreated with α-Bromonaphtaline (4 h), and fixed in Levitsky solution (Solution A: chromium trioxide and solution B: Formaldehyde 37%) for 16 hours. Root tips were then hydrolyzed in 1%

Table 1. Gene bank code and origin of accessions of *L. multiflorum.*

Gene bank code	Origin	Number	Gene bank code	Origin	Number
usa	USA	G7	390	Italy	G1
374	Italy	G8	393	Italy	G2
1766	Netherlands	G9	374"	Italy	G3
1765	Netherlands	G10	1551	Russia	G4
1624	Netherlands	G11	1151	Unknown	G5
vi	Russia	G12	1268	France	G6
plc-Early	Unknown	G13			

$$\%TF = \frac{\sum_{i-1}^{n} SA_i}{\sum_{i-1}^{n} TL_i} \times 100$$

$$DRL = \%RL_{Max} - \%RL_{Min}$$

$$VRC = \frac{\sum_{i=1}^{n} TL_i}{n}$$

$$A_1 = 1 - \frac{\sum_{i=1}^{n} \frac{S_i}{L_i}}{n}$$

$$A_2 = \frac{S\,\overline{d}}{\overline{X}}$$

NaOH at 60°C for 8 minutes; therefore, hematoxiline was used for chromosome staining (4 h). Cytotype cells were recorded on at least five well-prepared cells at metaphase stage for each population by microscope Olympus BH2 monitoring systems (At magnification of 2775). The traits of total length of chromosomes (TL), short arm length (SA), long arm length (LA), arm ratio (AR), and centromer index (CI) were measured by MicroMeasure software. Then the parameter of short arm relative length percentage (S%), long arm relative length percentage (L%), relative length percentage (RL%), total form percentage (TF%), difference of range relative length (DRL), value of relative chromatin (VRC), intrachromosomal asymmetry index (A_1), and interchromosomal asymmetry index (A_2) were calculated. The types of chromosomes were identified according to Levan *et al.* (1964), and karyotype symmetry was determined according to Stebbins (1971).

Statistical analyses

Analysis of variance was performed on the data recorded on the karyotypic traits using SPSS software package. Mean comparison were performed by Duncan's test at 5% levels of probability for means of karyotypic traits for accessions. Principal components analysis was performed on the data using SPSS software.

RESULTS

Karyotype analysis

The results showed that six accessions (374, 1766, vi, 1624, plc and 1765) were diploid, seven accessions (393, 1551, 374", 1151, 390, 1268 and usa) were tetraploid (Fig. 1), and the basic chromosome number was x = 7. Three and six satellites were observed for diploid and tetraploid accessions, respectively (Table 2).

Karyotypic formula was varied for survived accessions, so that it was 5m + 3sm + 6st for accessions 374 and 1766, and 2m + 2sm + 3st for accessions 374" and 1151. Karyotypic formula for 393, 1268 and usa was 3m + 4sm. Also, the vi had 3m + 5sm + 6st chromosomes. Accessions 1624 had 8m + 6st chromosomes, karyotypic formula was 4m+4sm+6st for plc, and finally, it was 5m + 9sm, 3m + 3sm + 1st and 3m + 1sm + 3st for 1765, 1551 and 390, respectively. In terms of the Stebbins' system, the karyotype of accessions seizes 2A classes, which are considered as median asymmetrical karyotypic in this system. Based on the intrachromosomal asymmetry index (A1) and total form percentage (TF%), accessions 374, vi, plc and 390 had the highest value for intrachromosomal asymmetry (A_1) and the lowest value for TF%. Therefore, these accessions had the most karyotypic evolution. On the other hand, 1624, 1765, 393 and usa had the lowest karyotypic evolution based on these parameters. Accessions 1765 and 1268 had the lowest interchromosomal asymmetry index (A_2) and the lowest karyotypic evolution and accessions vi and usa had an asymmetric karyotype and had the most karyotypic evolution based on interchromosomal asymmetry index (A_2) parameters. The diagram of the accessions distribution based on the values of intra-

Table 2. Karyotypic characters for studied accessions.

Gene bank code	2n	DRL	TF%	A1	A2	SC	K.F
374	2n = 4x = 28	4.066	26.637	0.5237	0.1794	2A	5m + 3sm + 6st
1766	2n = 4x = 28	4.217	28.521	0.4977	0.1737	2A	5m + 3sm + 6st
vi	2n = 4x = 28	4.566	27.493	0.5117	0.1909	2A	3m + 5sm + 6st
1624	2n = 4x = 28	3.780	30.896	0.4270	0.1715	2A	8m + 6sm
plc	2n = 4x = 28	4.156	27.873	0.5070	0.1740	2A	4m + 4sm + 6st
1765	2n = 4x = 28	3.898	30.256	0.4451	0.1649	2A	5m + 9sm
393	2n = 2x = 14	6.921	30.115	0.4453	0.1753	2A	3m + 4sm
1551	2n = 2x = 14	6.690	29.204	0.4661	0.1745	2A	3m + 3sm + 1st
374"	2n = 2x = 14	7.344	28.615	0.4968	0.1757	2A	2m + 2sm + 3st
1151	2n = 2x = 14	6.690	29.204	0.4661	0.1745	2A	2m + 2sm + 3st
390	2n = 2x = 14	7.146	26.053	0.5294	0.1789	2A	3m + 1sm + 3st
1268	2n = 2x = 14	5.699	29.220	0.4772	0.1550	2A	3m + 4sm
usa	2n = 2x = 14	6.693	30.032	0.4457	0.1897	2A	3m + 4sm

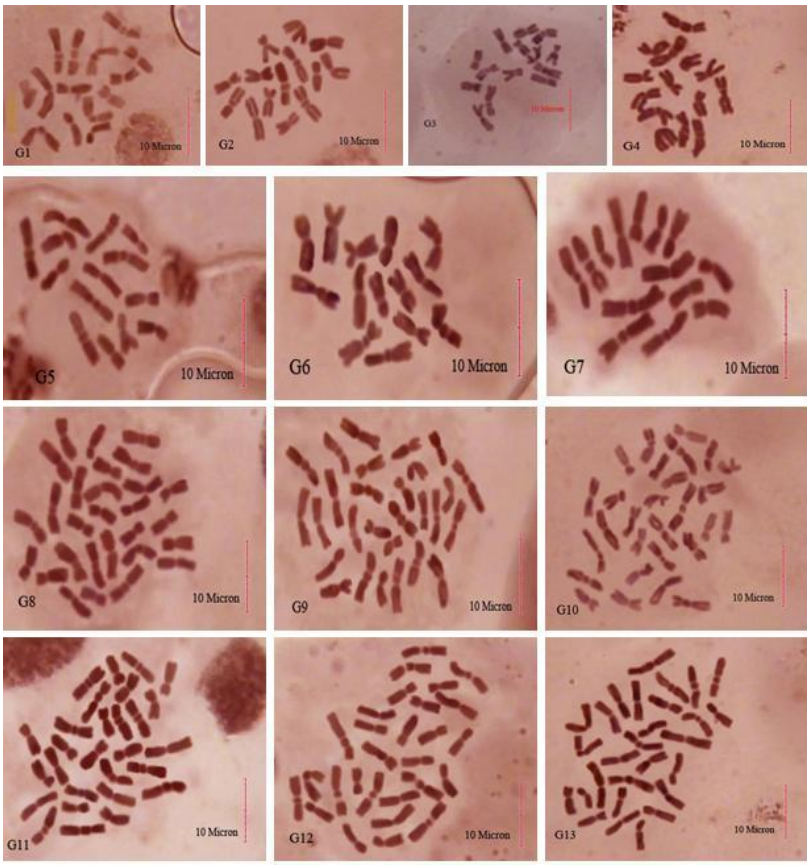

Fig. 1. Cytotypes of studied accessions.

chromosomal asymmetry index (A_1) and interchromosomal asymmetry index (A_2) and accessions grouped using cluster analysis of Ward's method based on the values of intrachromosomal asymmetry index (A_1) and interchromosomal asymmetry index (A_2) showed that the studied accessions fell into three groups (Fig. 2).

Analysis of variance and means comparison

The results of variance analysis showed a significant variation (at the 1% level of probability) between the accessions for chromosomal traits. So, the tested germplasm varied for karyotypic characteristics (Table 3). Also, the results of mean comparison by Duncan's test (Table 4) revealed that the diploid accessions 393, 1551, 390 and usa and the tetraploid accession plc had the highest length of total chromosome, and long and short arms. The tetraploid accessions 1624

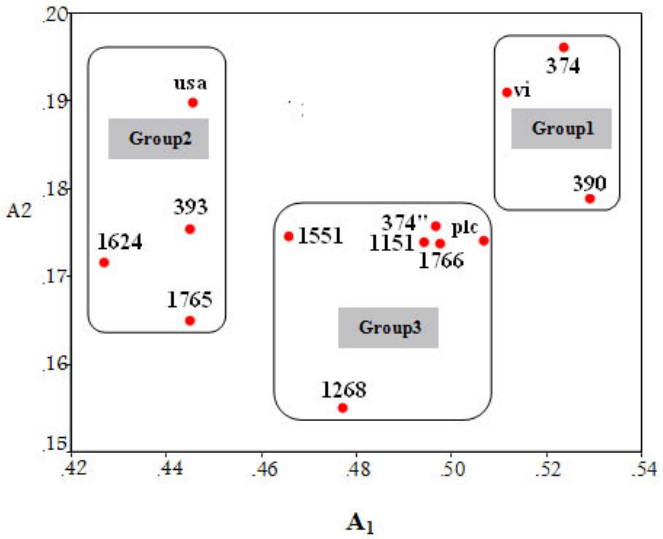

Fig. 2. Scatter plot for accessions based on A1 and A2.

Table 3. Analysis of variance for karyotypic traits of the studied accessions.

S.o.V	df	TL	LA	SA	CI	AR
Accessions	12	0.794**	0.183**	0.128**	0.001**	0.299**
Error	52	0.154	0.057	0.019	0.0002	0.036
CV (%)		5.95	6.59	7.25	4.5	8.54
Average		6.58	3.63	1.890	0.30	2.23

**: significant at the 1% level of probability.

and 1765 and diploid accessions 393, 1268 and usa had the highest centromer index and the lowest intrachromosomal asymmetry. The tetraploid accessions 374 and plc and diploid accession 390 had the highest arm ratio (AR) and intrachromosomal asymmetry index (A_1).

Principal component analysis

The results of principal components analysis based on karyotypic traits for accessions showed that the first two components captured 84.37% of the total variance (Table 5). The most important traits for the first component included the length of short arm (SA), centromer

Table 4. The means comparison by Duncan's test for studied accessions at the 5% levels.

Accessions	TL(µ)	LA(µ)	SA(µ)	CI	AR
374	6.31 cd	3.48 cd	1.640 e	0.274 f	2.47 a
1766	6.03 d	3.39 d	1.720 de	0.290 cdef	2.40 ab
vi	6.55 bcd	3.68 abcd	1.802 cde	0.285 def	2.40 ab
1624	6.40 bcd	3.43 d	1.976 abc	0.318 a	1.88 c
plc	6.84 abcd	3.85 ab	1.907 bcd	0.286 def	2.51 a
1765	6.52 bcd	3.55 bcd	1.972 abc	0.312 ab	1.97 c
393	7.14 a	3.82 abc	2.149 a	0.309 abc	1.96 c
1551	7.11 a	3.80 abc	2.076 ab	0.298 bcde	2.15 bc
374"	6.36 cd	3.55 bcd	1.819 cde	0.291 cdef	2.37 ab
1151	6.35 cd	3.56 bcd	1.825 cde	0.292 cdef	2.35 ab
390	6.93 abc	3.92 a	1.805 cde	0.279 ef	2.56 a
1268	5.98 d	3.36 d	1.748 de	0.302 abcd	2.05 c
usa	7.08 ab	3.78 abc	2.126 a	0.308 abc	1.96 c

*In each column, means with the similar letters were not significantly different at the 5% level of probability using Duncan's test.

Table 5. Eigen value, percentage of variance, cumulative variance for the first and second principal components.

Traits	PRIN1	PRIN2
TL	0.495	0.858
LA	0.202	0.945
SA	0.922	0.367
CI	0.917	-0.384
AR	-0.897	0.347
VRC	0.495	0.858
DRL	0.199	0.504
TF%	0.894	-0.398
A1	-0.943	0.293
A2	-0.333	0.554
Eigen value	4.865	3.573
Eigen vector	48.647	35.726
Percentage of cumulative variance	48.647	84.373

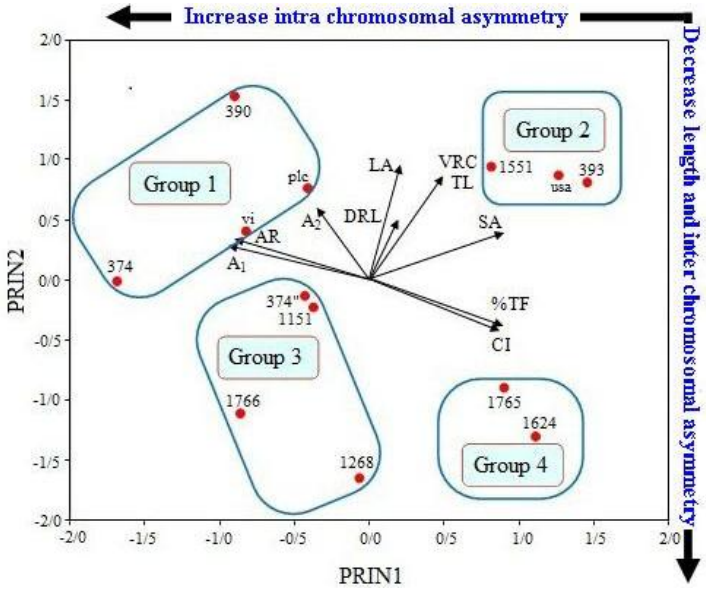

Fig. 3. Biplot of karyotypic characters in the studied accessions based on two first components and grouping of accessions.

index (CI), and total form percentage of chromosome (TF%) with the highest positive coefficients of eigen vectors and arm ratio (AR) and intrachromosomal asymmetry index (A_1) with the highest negative coefficients of Eigen vectors. For the second component, the total length (TL) of chromosome, long arm (LA), value of relative chromatin (VRC), difference of range relative length (DRL) and interchromosomal asymmetry index (A_2) played the most important role for total variation. According to the biplot, the accessions were divided into four groups (Fig. 3).

DISCUSSION

No variation in basic chromosome number was observed in the studied accessions, but there were two groups for ploidy level. The diploid accessions' average chromosome length was higher than that of tetraploid accessions. The species *L. multiflorum* has been reported to be diploid in most research (2n = 2x = 14) (Hovin and Hill, 1966; Evans and Mecefield, 1974), but some studies have reported the tetraploid species (Wu and Chen, 1991). On the other hand, the tetraploid accessions had higher intrachromosomal asymmetry than diploid accessions. Essad (1954) based on size and symmetry of chromosomes

suggested the genus *Lolium* to be 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.

The satellites were observed for all accessions, and variations were observed in the number, length and location of satellites on chromosomes. Mirzaei-Nodushan and Nadarkhani (2001) reported the number of one or two satellites for two species of *Lolium*. The accessions varied in karyotypic formula and all accessions were composed of metacentric and subtelocentric chromosomes. Essad (1954) studied karyotypes of five species of *Lolium* and suggested three classes for the genus. A detailed investigation of karyotype was conducted by Malik and Thomas (1966).

In terms of the Stebbins' system, the karyotype of accessions are considered as median asymmetrical karyotypic in this system and this index cannot show the diversity between accessions in this species. The accessions had an asymmetry karyotype and variation was significant between accessions in karyotypic characters. Karyotypic evolution for *L. multiflorum* was more than intrachromosomal asymmetry, given the fact that the studied accessions and intrachromosomal asymmetry were increased by the development of ploidy level from diploidy to tetraploidy. Terrell (1968) had a comprehensive review on the genus *Lolium*. He recognized eight species in the genus and divided it into two sections based on breeding system. The accessions 374, 390 and vi displayed the highest karyotypic asymmetry based on intra and interchromosomal asymmetry and had the highest karyotypic evolution. The accessions 1765, 1624, 393 and usa had the moderate of karyotipic evolution and the accessions 1268, 1551, 374", 1151, 1766 and plc had the lowest karyotypic evolution based on intrachromosomal asymmetry index (A1) and interchromosomal asymmetry index (A_2). Essad (1954) studied karyotypes of five species of *Lolium* and suggested three classes for the genus. In addition, the accessions 390, 374, vi and plc had the highest karyotypic evolution and the accessions 1624 and 1765 had the lowest one.

The results of variance analysis showed a significant variation (1% level) between the accessions in chromosomal traits. Cytogenetic divergence and extensive variations in karyotypic traits in *Lolium* germplasem were reported by other researchers (Mirzaei-Nodushan and Nadarkhani, 2001; Mirjalili, 2014). The results of principal components analysis based on karyotypic traits for accessions showed that the first two components accounted for 84.37% of the total variance. Multivariate methods of analysis, such as the PCA and cluster analyses used in the present study, revealed the germplasm groupings in many genetic resources such as *Lolium* (Mirjalili, 2014). According to the biplot, accessions 390, 374, vi and plc (Group 1) had the highest value based on the second component and the lowest values for the first component. So, these accessions had the highest chromosome length, intrachromosomal asymmetry index (A_1), interchromosomal asymmetry Index (A_2), and evolutionary accession based on karyotype characteristics. The accessions 1551, 393 and usa (Group 2) based on the first and second components showed the highest values implying that they had the highest chromosome length and asymmetric karyotype based on interchromosomal asymmetry and the lowest asymmetric karyotype based on intrachromosomal asymmetry. In addition, the accessions of Group 2 had the moderate of karyotipic evolution. The accessions 1151, 1268, 1766 and 374" (Group 3) had the lowest values for first and second components; therefore, they had the shortest chromosomes and the lowest symmetrical karyotypes based on interchromosomal asymmetry. The accessions of group 3 had the lowest karyotypic evolution based on intrachromosomal asymmetry. The accessions 1765 and 1624 (Group 4) had the lowest values for second component and the highest values for first component, so they had the shortest chromosomes and the lowest symmetrical karyotype. According to ploidy levels for accessions, we observed that the tetraploid accessions had the highest evolutionary of karyotypes. Mirjalili (2014) classified genotypes using karyotypic traits.

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