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Genetic diversity of Lolium Multiflorum accessions using ISSR molecular markers

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ABSTRACT: Genetic diversity based on different markers, plays a key role in breeding programs and one of the most important criteria for selection of Parents. In this study the genetic diversity among 13 accessions of *Lolium Multiflorum* was investigated using 9 ISSR primers. These primers could identify 66 bands. The average of bands was 7.36 for each primer. Average PIC in the used primers was 0.404 that the highest amount of PIC related to primers IS_{11}, IS_{14} and IS_{16} and primers IS_5, IS_{11} and IS_{13} with the lowest value of PIC don't have ability in the separation of accessions. A desirable polymorphism between genotype was observed based on ISSR markers, which the primers of IS_9, IS_{10}, IS_{11}, IS_{14} and IS_{16} were determined for genetic variation study in *Lolium Multiflorum* as desirable primers. Also, cluster analysis and principal coordinate analysis using Dice similarity coefficients were calculated and dendrogram was drawn using the UPGMA for 13 accessions. Grouping of accessions indicated that genetic variations do not agreement with the geographical distribution of accessions. The coordinate analysis was performed using of the similarity matrix and confirmed the results of cluster analysis.

***Keywords*:** Lolium Multiflorum, genetic diversity, accessions, ISSR molecular markers

INTRODUCTION

Lolium multiflorum is a pasture species utilized largely for its ability to provide winter grazing for sheep in semi-intensive and intensive livestock production systems (Eckard, 1986). Among the temperate climate forage species, annual ryegrass (Lolium multiflorum) is the most widely used annual forage grass (Maia, 1995) because of its high productivity and palatability, excellent grow and easy plan stability (Carambula, 1971). The origins of annual ryegrass are still undefined, but we do know that this species was among the natural vegetation in the fields of northern Italy, where it probably originated (Spedding and Diekmahns, 1972). 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 of crop breeding industry on 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). Molecular markers provide a robust estimate of genetic similarity that often was not obtained using morphological data alone (Surendhar et al., 2009). Often, the initial objective of DNA profiling of populations is to determine diversity among populations in order to develop genetically distinct subsets of populations in a breeding

program or to check for duplicates in a gene bank. In these cases, it may be possible to determine diversity among populations by profiling bulked DNA of the individuals (Rouf et al., 2002). One of the markers that using to study genetic diversity is Inter Simple Sequence Repeats or ISSR that is based on PCR method and it is also semi-random marker. This marker is used to amplification DNA fragment between two micro satellites which is placed inversely in genotype of a species. This marker is much more efficient than RAPD (Sicard et al., 2005). The purpose of this study was to evaluate the genetic diversity of 13 accessions of *Lolium Multiflorum* using ISSR molecular markers.

MATERIALS AND METHODS

***Plant Materials*:**

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

Table 1. Gen bank cod and origin of accessions of Lolium Multiflorum

Gen bank cod	Origin	Number	Gen bank cod	Origin	Number
1765	Netherlands	G7	Vi	Russia	G1
1766	Netherlands	G8	1551	Russia	G2
usa	USA	G9	374	Italia	G3
1268	France	G10	390	Italia	G4
Plc-Early	Unknown	G11	393	Italia	G5
1151	Unknown	G12	1624	Netherlands	G6
"374	Italia	G13			

***DNA Extraction and ISSR Method*:**

Total genomic DNA was extracted for young leaves of greenhouse-grown plants using a modified CTAB (Murry and Tompson, 1980) with modification described by(De la Rosa et al., 2002). Quality and quantity of extracted DNA were examined using 0.8% agarose gel. The compounds of polymerase chain reaction were carried out according to Table 2.

Table 2. Compounds of optimized ISSR reaction

To provide 20 µl	Compounds of a sample
12.6 µl	Water distillated twice
2 µl	Buffer PCR (X10)
1.5 µl	Colored manyazium (50 mmol)
0.4 µl	Nucleotides mixture (10 mmol)
1.2 µl	Primer (10 µmol)
0.3 µl	Tag polymerase
2 µl	DNA (10 ng)
20 µl	Total

Template DNA was initially denatured at 92oC for 5 min, followed by 35 cycles of PCR amplification under the following parameters: denaturation for 30 seconds at 95oC, primer annealing for 30 seconds at the temperature based on primer temperature (Temperatures of annealing in this study was 50, 55 and 60 oC) and primer extension for 1 min at 72oC. A final incubation was performed for 5 min at 72oC to ensure that the primer extension reaction proceeded to completion. The PCR amplified products were separated by electrophoresis on a 1.5% agarose gels using TBE buffer. The gels were put in the Ethidium bromide for 30-45 min and visualized by gel document.

Statistical Analysis:

ISSR bands were treated as binary characters and coded accordingly (presence =1, absence = 0). The Number of scored bands (NSB), number of polymorphic bands (NPB), percentage of polymorphism bands (PPB) and polymorphism information content (PIC) calculated for each primer (Anderson et al., 1993). Similarity matrix computed based on Dice's coefficient and cluster analysis performed for grouping accessions based on Dice's coefficient by UPGMA methods. Cluster analysis, similarity matrix and principal coordinate analysis axis were carried out for 13 accessions using NTSYS and Darwin5.

RESULTS AND DISCUSSION

ISSR Polymorphism:

Band pattern of accessions for IS_{14} showed in Figure 1. Primers sequences, code, number of bands scored, number of polymorphic bands, percent of polymorphic bands (PPB) and polymorphism information content (PIC) were showed for ISSR primers in Table 3.

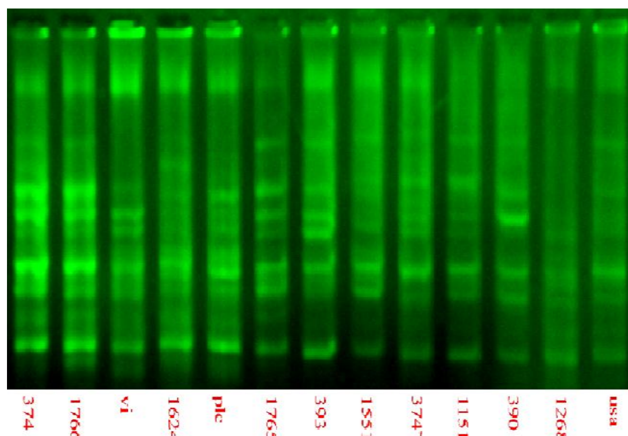

Figure 1. The band pattern for accessions using IS_{14} primer

In this study the genetic diversity among 13 accessions of *Lolium multiflorum* was investigated using 9 ISSR primers. For all primers, the number of 66 bands was scored that polymorphism was observed for 56 of them. The average of bands was 7.33 for each primer. IS_9 and IS_{12} primer with 10 bands had the highest and primer IS_{15} with 3 bands had the lowest number of bands. The average of bands for each primer was 5.07 for accessions that genotype 1151 (Unknown) had the most and genotype USA (USA) had the lowest band. Average of polymorphism percent was 83.46%. The lowest percent of polymorphism belonged to IS_{15} and IS_{11} (66.67%) and the highest percent of polymorphism was 100% for primers IS_9, IS_{10} and IS_{13}. Average of PIC for all primers was 0.404 that the highest value of PIC related to IS_{11}, IS_{14} and IS_{16} and the lowest belonged to IS_5, IS_{11} and IS_{13} (Table 3).

Table 3. ISSR primers used in this study and some summary results

ISSR code	Primer sequence	No. of bands scored	No. of polymorphic bands	Percentage of polymorphic bands	PIC
IS_5	5'- AG AG AG AG AG AG AG AGC-3'	8	6	75%	0.314
IS_9	5'- CT CT CT CT CT CT CT CTG-3'	10	10	100%	0.436
IS_{10}	5'- GA GA GA GA GA GA GA GA Rc-3'	5	5	100%	0.421
IS_{11}	5'-ACACACACACACACACC-3'	6	4	66.67%	0.450
IS_{12}	5'-TGTGTGTGTGTGTGTGG-3'	10	9	90%	0.400
IS_{13}	5'- AG AG AG AG AG AG AG AGYT-3'	7	7	100%	0.365
IS_{14}	5'- GACA GACA GACA GACA-3'	8	6	75%	0.477
IS_{15}	5'- GGATGGATGGATGGAT-3'	3	2	66.67%	0.323
IS_{16}	5'-DBDACACACACACACACA-3'	9	7	77.78%	0.450
Average		7.33	6.22	88.46%	0.404

Similarity Matrix:

Similarity matrix based on Dice's coefficient for accessions showed that (Table 4) the average of Similarity between accessions was 0.618 and the range of similarity was 0.507 [Between the accessions of 1151 (Unknown) and vi (Russia)] to 0.754 [Between the accessions of 374" (Italia) with 393 (Italia)].

Table 4. Similarity matrix for studying accessions based on Dice's coefficient

accessions	374	1766	vi	1624	plc	1765	393	1551	374"	1151	390	1268
1766	0.70											
vi	0.62	0.58										
1624	0.68	0.53	0.65									
plc	0.61	0.57	0.56	0.63								
1765	0.73	0.74	0.65	0.54	0.60							
393	0.68	0.57	0.64	0.62	0.67	0.68						
1551	0.50	0.58	0.53	0.65	0.55	0.61	0.60					
374"	0.59	0.56	0.31	0.62	0.70	0.71	0.75	0.60				
1151	0.62	0.65	0.50	0.57	0.60	0.68	0.60	0.67	0.63			
390	0.61	0.55	0.58	0.54	0.62	0.62	0.57	0.70	0.59	0.65		
1268	0.59	0.59	0.64	0.54	0.67	0.62	0.60	0.63	0.57	0.54	0.71	
usa	0.58	0.54	0.52	0.57	0.58	0.67	0.64	0.65	0.59	0.56	0.63	0.66

Cluster Analysis:

UPGMA hierarchical clustering for grouping accessions based on Dice's coefficient (Figure 2) were identified the three distinctive groups. The first group consisted of accessions 1766 (Netherlands), 1268 (France), 374" (Italia), Plc-Early (Unknown) and 1151 (Unknown), which the average similarity was 0.612 for this group. The second group included accessions of Vi (Russia), 1551 (Russia) and 1624 (Netherlands), which the average similarity coefficient was 0.616 for this group. The third group consisted of 390 (Italia), 393 (Italia), 374 (Italia), 1765 (Netherlands) and USA (USA). The average of Dice's coefficient was 0.646 for this group. Grouping of accessions indicated that genetic variations do not agreement with the geographical distribution of accessions. Therefore considering of genetic distance between these groups, using of the first and third group accessions can be useful in breeding programs, to utilization of heterosis.

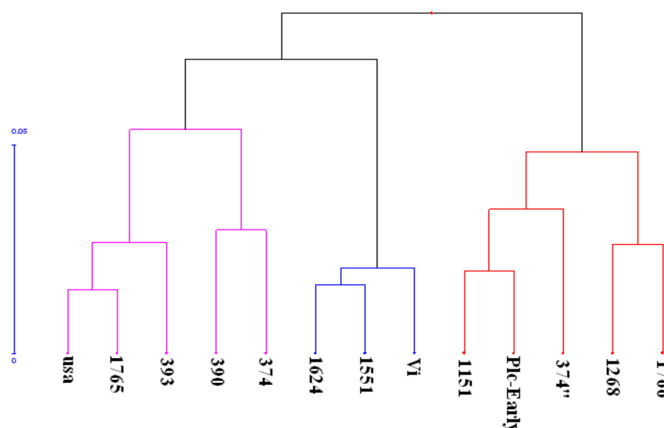

Figure 2. Dendrogram of cluster analysis for accessions based Dice's coefficient by UPGMA

***Principal Coordinate Analysis**:*

Scatter plot for accessions based on first and second axis (62.37%) from principal coordinate analysis (Figure 3) showed that Genetic variation did not matching with the geographical distribution of accessions. These results confirmed by cluster analysis and similarity matrix.

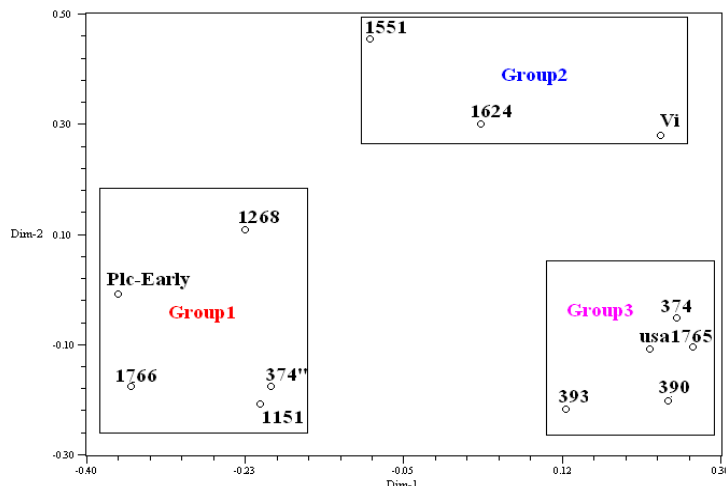

Figure 3. Scatter plot for accessions based on two first axes from principal coordinate analysis

According to this study a Significant variation was observed among accessions. This technique has been used to study a variety of plants. The results of this study (Vieira et al., 2004 and Bolaric et al., 2005) were consistent. PIC values estimate the discriminatory power of a marker. The Average PIC values for markers used in present study were 0.404. Marker with high PIC values such as IS_9, IS_{10}, IS_{11}, IS_{14} and IS_{16} could be effectively used in genetic diversity studies in Lolium. The similarity between accessions based on Dice's coefficient was high, therefore can be stated that there was a low genetic variation among accessions. 1151 (Unknown) had the most genetic distance with Vi (Russia). UPGMA hierarchical clustering for grouping accessions based on Dice's coefficient were identified the three distinctive groups. Grouping of accessions indicated that genetic variations do not agreement with the geographical distribution of accessions. Therefore considering of genetic distance between these groups, using of the first and third group accessions can be useful in breeding programs, to utilization of heterosis. Scatter plot for accessions based on first and second axis (62.37%) from principal coordinate analysis showed that Genetic variation did not matching with the geographical distribution of accessions. These results confirmed by cluster analysis and similarity matrix. Efficiency of ISSR primers were reported by other researchers to determine of genetic diversity between and within different Lolium species (Posselt et al., 2006; Hu et al., 2011; Pivoriene and Pasakinskiene, 2007; Pivoriene et al., 2008).

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