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The study of genetic variation for *Lolium perenne* using ISSR molecular markers

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Abstract

Genetic variation for 12 accessions of *Lolium perenne* were surveyed using the number of 12 ISSR primers, that the number of 10 primers can be scored. The ISSR primers can be produced the number of 62 bands, which the polymorphism was showed for the number of 47 bands. The average of bands was 6.2 for each primer. The primer of IS_9 showed the highest number of band (11 bands) and IS_{15} showed the lowest number of band (3 bands). A desirable polymorphism between genotype was observed based on ISSR markers, which the primers of IS_9, IS_{10}, IS_{13} and IS_{16} were determined for genetic variation study in *Lolium perenne* as desirable primers. Average PIC in the used primers was 0.33 that the highest amount of PIC related to primer IS_{12} that amount of PIC was 0.45 in this primers and primers IS_3 and IS_{14} with the lowest value of PIC don't have ability in the separation of accessions. Yatsyn (G11) had the most genetic distance with Grenisle (G7). Cluster analysis and Scatter plot based on first and second axis from principal coordinate analysis for genotypes, showed that genetic variation did not agreement with the geographical distribution. Therefore the genotypes which had the most genetic distance based on used ISSR marker can be considered as suitable material in breeding programs for using hetrosis.

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Introduction

Perennial ryegrass (*Lolium perenne* L.) is the most important grass species in temperate climates of the world. Perennial ryegrass is a diploid species (2n=2x=14) with a two-locus self incompatibility system, which ensures a high degree of genetic variation in populations (Bolaric *et al.,* 2005a). *Lolium perenne* (*Poaceae*) was chosen as the model species and small populations of 15 different cultivars of this species were established in each experimental community. *Lolium perenne* is one of most important grass species in central Europe and worldwide used for the sowing and regeneration of temperate agricultural grassland. Its productivity is stimulated by nitrogen fertilization and it has a high fodder quality and grazing tolerance. Beside its importance as a fodder crop, perennial ryegrass is frequently used as a turf species in lawns (Beddows, 1967).

Knowledge of genetic variation is a useful tool in genebank management, helping in the establishment of core collections, facilitating efficient sampling and utilization of germplasm (identifying and/or eliminating duplicates in the gene stock), and selecting of desirable genotypes to be used in breeding programs (Elham *et al.,* 2010; Vishwanath *et al.,* 2011). On the other hands, understanding genetic diversity of certain species is not only useful in addressing questions about evolutionary process and the development of conservation strategies, but also a prerequisite for efficient use of genetic resources in breeding programs. Interest in the genetic structure of natural populations of grass species has been increased in the last few years due to the necessity of broadening the knowledge of genetic variations in cultivated species (Che and Li, 2007). Assessment of genetic diversity with molecular markers is a promising alternative. Molecular characterization can also be re-applied after years of maintenance and new accessions can be related to existing collections. Moreover, molecular characterization of genetic diversity provides base information, which can be used to select a promising range of accessions for different breeding programs (Roldan-Ruiz *et al.,* 2001). 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). Inter Simple Sequence Repeat (ISSR) is a dominant molecular marker revealed in mass. ISSR has recently been developed as an anonymous, RAPD–like approach that accesses variation in the numerous microsatellite regions dispersed throughout the various genomes and circumvents the challenge of characterizing individual loci that other molecular approaches require. They are characterized by mono-, di- or multi - nucleotide repeats that have 4-10 repeat units' side-by-side. Extremely high variability combined with greater robustness in repeatability experiments and less prone to changing band patterns with changes in constituent or DNA concentration template make them superior to other readily available marker systems in investigations of genetic variation (Fang and Roose, 1997). Genetic diversity based on Molecular markers for within different species of *Lolium* was reported by many researchers (Elazreg *et al.,* 2011; Vieira *et al.,* 2004; Majidi and Mirlohi, 2010).

Thus, the present study aimed to determine and assess the genetic variability of the *Lolium* collection and better understand the genetic diversity structure of the available accessions using ISSR molecular markers.

Materials and methods

Plant materials

In order to evaluate the genetic variation, 12 accessions of *Lolium perenne* were prepared from gene bank of Research Institute of Forests and Rangelands, Tehran, Iran (Table 1).

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.

Template DNA was initially denatured at 92°C for 5 min, followed by 35 cycles of PCR amplification under the following parameters: denaturation for 30 seconds at 95°C, primer annealing for 30 seconds at the temperature based on primer temperature (Temperatures of annealing in this study was 50, 55 and 60 °C) and primer extension for 1 min at 72°C. A final incubation was performed for 5 min at 72°C 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. Principal coordinate analysis performed to better interpret the genetic variation between accessions and finally the molecular variance analysis performed for the three groups from cluster analysis. Statistical analyses were done by Darwin 5 and Gen ALEX 6.2.

Results

ISSR polymorphism

Genetic variation for 12 accessions of *Lolium perenne* were surveyed using the number of 12 ISSR primers, that the number of 10 primers can be scored. For all primers, the number of 62 bands was scored that polymorphism was observed for 47 of them. The average of bands was 6.2 for each primer. IS_9 primer with 11 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 5.1 for 12 genotypes that genotype Spelga (G12) had the most and genotype Fontoon (G10) had the lowest band. Band pattern of accessions for IS_9 showed in Fig. 1.

Table 1. Gen bank cod and Origin of accessions of *Lolium perenne.*

Gene bank code	Name genotype	Origin	Number	Gen bank cod	Name genotype	Origin	Number
1303	Grenisle	Southern *Ireland*	G7	1309	Aubisque	Netherlands	G1
1312	Tyrone	Northern *Ireland*	G8	1307	Green Gold	Southern *Ireland*	G2
1311	Napoleon	*Denmark*	G9	1308	Magician	Southern *Ireland*	G3
1330	Fontoon	Southern *Ireland*	G10	1305	Carat	Netherlands	G4
1302	Yatsyn	New Zealand	G11	1313	Gilford	Ireland	G5
1306	Spelga	Northern *Ireland*	G12	1301	Moy	Northern *Ireland*	G6

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

Average of polymorphism percent was 80.24%. The lowest percent of polymorphism belonged to IS_{15} (50%) and the highest percent of polymorphism was 100% for primers IS_3, IS_9, IS_{10}, IS_{13} and IS_{16}. Average of PIC for all primers was 0.33 that the highest value of PIC related to IS_{12} and the lowest belonged to IS_3 and IS_{14} (Table 3).

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.

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

ISSR code	Primer sequence	NSB	NPB	PPB	PIC
IS_3	5' GAGAGAGAGAGAGAGAYC 3'	4	4	100%	0.257
IS_5	5' AG AG AG AG AG AG AG AGC 3'	11	8	72.73%	0.304
IS_9	5' CTCTCTCTCTCTCTCTG 3'	4	4	100%	0.368
IS_{10}	5' GAGAGAGAGAGAGAGARC 3'	8	8	100%	0.339
IS_{11}	5' ACACACACACACACACC 3'	7	4	57.14%	0.344
IS_{12}	5' TGTGTGTGTGTGTGTGG 3'	5	3	60%	0.458
IS_{13}	5' AGAGAGAGAGAGAGAGYT 3'	3	3	100%	0.324
IS_{14}	5' GACAGACAGACAGACA 3'	8	5	62.50%	0.280
IS_{15}	5' GGATGGATGGATGGAT 3'	8	4	50%	0.336
IS_{16}	5'DBDACACACACACACACA3'	4	4	100%	0.340
	Average	6.2	4.7	80.24	0.335

Similarity Matrix

Similarity matrix based on Dice's coefficient for accessions showed that (Table 4) the average of Similarity between accessions was 0.79 and the range of similarity was 0.70 [Between the accessions of Yatsyn (G11) and Grenisle (G7)] to 0.87 [Between the accessions of Aubisque (G1) with Grenisle (G7)].

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

accessions	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11
G2	0.822										
G3	0.795	0.787									
G4	0.714	0.825	0.782								
G5	0.873	0.805	0.818	0.785							
G6	0.778	0.732	0.809	0.775	0.84						
G7	0.836	0.8	0.771	0.767	0.8	0.747					
G8	0.776	0.831	0.738	0.8	0.831	0.753	0.817				
G9	0.827	0.814	0.839	0.786	0.86	0.814	0.795	0.815			
G10	0.787	0.789	0.747	0.764	0.829	0.743	0.857	0.862	0.865		
G11	0.747	0.736	0.766	0.800	0.767	0.782	0.709	0.756	0.791	0.784	
G12	0.744	0.8	0.804	0.75	0.778	0.778	0.786	0.8	0.809	0.736	0.812

Cluster Analysis

UPGMA hierarchical clustering for grouping accessions based on Dice's coefficient (Fig. 2) were identified the three distinctive groups. The first group consisted of accessions Yatsyn (G11), Spelga (G12), Magician (G3) and Moy (6G), which the average similarity was 0.79 for this group. The second group included accessions of Carat (G4), Green Gold (G2) and Tyrone (G8), which the average similarity coefficient was 0.81 for this group. The third group consisted of Aubisque (G1), Gilford (G5), Grenisle (G7), Napoleon (G9) and Fontoon (G10). The average of Dice's coefficient was 0.83 for this group.

Cluster analysis of the similarity matrix for grouping showed, the most similar among Third group and Second group, and the most distance for accession in groups Second and first. (Table5).

Table 5. Similar groups from the cluster analysis.

Groups	C_1	C_2	C_3
C_1	1		
C_2	0.767	1	
C_3	0.778	0.795	1

Molecular Variance Analysis

Analysis of molecular variance was performed for ISSR bands to determine of significant difference between groupings of accessions based on cluster analysis (Table 6). The results showed a significant ($P<0.01$) difference between groups and the portion of variance percent for between group and within group were 13% and 87%, respectively.

Principal Coordinate Analysis

Scatter plot for accessions based on first (33.25) and second (17.26) axis from principal coordinate analysis (Fig. 3) showed that Genetic variation did not matching with the geographical distribution of accessions. These results confirmed by cluster analysis and similarity matrix.

Table 6. Molecular variance analysis.

S.O.V	Df	SS	MS	Est. Var.	Var%	PhiPT
Between group	2	27.63	13.82	1.323	13%	0.133*
Within group	9	77.70	8.633	8.633	87%	
Total	11	105.33		9.957	100%	

Est. Var; Calculated variance for Within and between group %Var; Percent of variance of each source to total variance.

Discussion

The results revealed that ISSR markers are suitable tools for detecting the genetic variation in *Lolium* accessions. According to this study a Significant variation was observed among accessions. We believe that there needs a molecular markers studies as a complementary studies for the morphological traits in the field. It will reduce the amount of materials for study as well as the costs of experiments. This technique has been used to study a variety of plants. The results of this study Ghariani *et al* (2003) were consistent. The Average percentage of polymorphism was 80.24% that showed a good polymorphism between genotypes. Between 10 used primers, the primers IS_3, IS_9, IS_{10}, IS_{13} and IS_{16} showed 100% polymorphism that indicate high ability for survey molecular variation and high variation among genotypes. PIC values estimate the discriminatory power of a marker. The mean PIC values for markers used in present study were 0.33. Marker with high PIC values such as IS_{12} could be effectively used in genetic diversity studies in *Lolium*. Efficiency of ISSR primers were reported by other researchers to determine of genetic diversity between and within different *Lolium* species (Pivoriene *et al.,* 2008; Hu *et al.,* 2011; Posselt *et al.,* 2006; Bolaric *et al.,* 2005b). The similarity between accessions based on Dice's coefficient was high, therefore can be stated that there was a low genetic variation among accessions. It was noticed that the majority of the similarity coefficients between accessions was close to 0.78. This indicated the close relationships between the evaluated accessions, though they are collected from different origin. Grouping of accessions based on cluster analysis and principal coordinate analysis indicated that genetic variations do not in agreement with the geographical distribution of accessions.

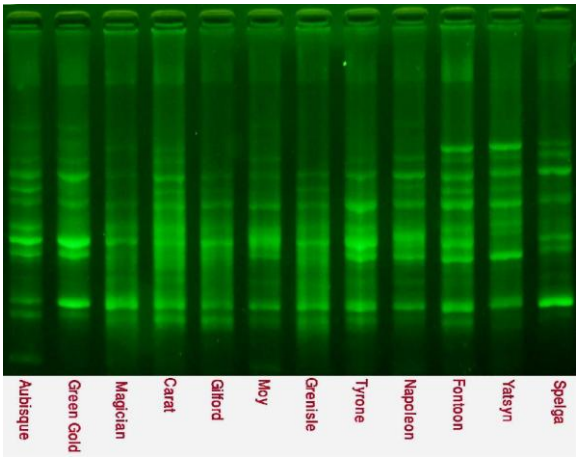

Fig. 1. The band pattern for accessions using IS_9 primer.

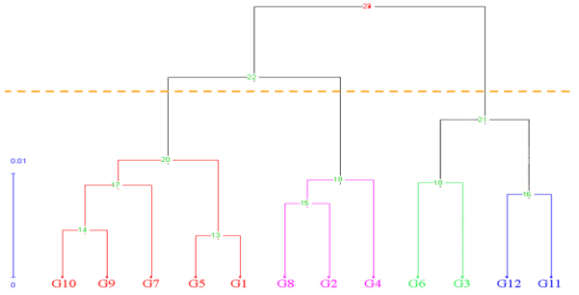

Fig. 2. Dendrogram of cluster analysis for accessions based Dice's coefficient by UPGMA.

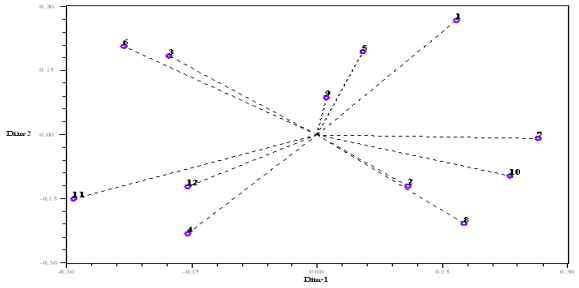

Fig. 3. Scatter plot for accessions based on two first axes from principal coordinate analysis.

There are several possible explanations for such results: some of them connected with nature and structure of different molecular markers that designed from various regions of genome. Another problem was the possibility of overestimating genetic similarity because fragments with the same size could have different origins. However, results of cluster analysis confirmed by molecular variance analysis, on the other hands the results of estimated variance showed that the genetic variation within group was more than between group and this result was due to high genetic variation between accessions. Information about current genetic diversity permits the classification of our available germplasm into various/heterotic groups, which is particularly important to hybrid/cross-breeding programs in *Lolium*. Even though the genetic mechanisms that explain heterosis are not fully understood, it is well documented that crosses between unrelated and genetically distant parents, show greater hybrid vigor than crosses between closely related parents. The magnitude and pattern of genetic variation detected in this study can be useful for more systematic germplasm management and utilization in breeding programs.

References

Anderson JA, Church JE, Autrique SD, Thanksley S, Sorrells ME. 1993. Optimizing parental selection for genetic linkage map. Journal of Genome **36,** 181-188.

Beddows AR. 1967. Biological flora of the British Isles. Lolium perenne L. Journal of Ecology **55,** 567–587.

Bolaric S, Barth S, Melchinger AE, Posselt U K. 2005a. Genetic diversity in European perennial ryegrass cultivars investigated with RAPD markers. Plant breeding **124(2),** 161-166. http://dx.doi.org/10.1111/j.1439-0523.2004.01032.x

Bolaric S, Barth S, Melchinger AE, Posselt U K. 2005b. Molecular characterization of genetic diversity in European germplasm of perennial ryegrass. Euphytica **146(1-2),** 39-44. http://dx.doi.org/10.1007/s10681-005-4439-1

Casler MD, Stendal CA, Kapich L, Vogel KP. 2007. Genetic diversity, plant adaptation regions, and gene pools for switchgrass. Crop Science **47,** 2261–2273.

Che YH, Li LH. 2007. Genetic diversity of prolamines in Agropyron mongolicum Keng indigenous to northern China. Genetic Resources and Crop Evolution **54(5),** 1145–1151.

http://dx.doi.org/10.1007/s10722-006-9006-7

De La Rosa R, James C, Tobutt KR. 2002. Isolation and characterization of polymorphic microsatellite in olive (Olea europaea L.) and their transferability to other genera in Oleaceae. Molecular Ecology Notes **2,** 265-267.

Dice LR. 1945. Measures of the amount of ecologic association between species. Ecology **26(3),** 297-302.

Elazreg H, Chtourou-Ghorbel N, Ghariani S, Chakroun M, Trifi-Farah N. 2011. Studying genetic diversity of the Tunisian Lolium perenne and Festuca arundinacea with AFLP markers. Journal of Food, Agriculture & Environment **9(1),** 409-415.

Elham AA, El-Hady A, Atef AA, Haiba, Nagwa R, El-Hamid A, Aida A. 2010. Phylogenetic diversity and relationships of some tomato varieties by electrophoretic protein and RAPD analysis. Journal of American Science **6(11),** 434-441.

Fang DQ, Roose ML. 1997. Identification of closely related citrus cultivars with inter-simple sequence repeats markers. Theoretical and Applied Genetics. **95(3),** 408-417.
http://dx.doi.org/10.1007/s001220050577

Ghariani S, Trifi-Farah N, Chakroun M, Marghali S, Marrakchi M. 2003. Genetic diversity in Tunisian Perennial ryegrass revealed by ISSR markers. Genetic Resources and Crop Evolution **50(8),** 809-815.
http://dx.doi.org/10.1023/A:1025971730574

Hu T, Li H, Li D, Sun J, Fu J. 2011. Assessing genetic diversity of perennial ryegrass (Lolium perenne L.) from four continents by intersimple sequence repeat (ISSR) markers. African Journal of Biotechnology **10(83),** 19365-19374.

Majidi MM, Mirlohi A. 2010. Genetic similarities among Iranian populations of Festuca, Lolium, Bromus and Agropyron using amplified fragments length polymorphism (AFLP) markers. Iranian Journal of Biotechnology **8(1),** 16-23.

Murry MG, Tompson WF. 1980. Rapid isolation of high molecular weight plant DNA. Nucl Acids Res **8,** 4321-4325.

Pivoriene O, Pasakinskiene I. 2008. Genetic diversity assessment in perennial ryegrass and Festulolium by ISSR fingerprinting. Agriculture **95(2),** 125–133.

Posselt UK, Barre P, Brazauskas G, Turner L B. 2006. Comparative Analysis of Genetic Similarity between Perennial Ryegrass Genotypes Investigated With AFLPs, ISSRs, RAPDs and SSRs. Czech Journal of Genetics and Plant Breeding **42(3),** 87–94.

Roldan-Ruiz I, van Eeuwijk FA, Gilliland TJ, Dubreuil P, Dillmann C, Lallemand J, Loose M De, Baril CP. 2001. A comparative study of molecular and morphological methods of describing relationships between perennial ryegrass (Lolium perenne L.) varieties. Theor Appl Genet **103,** 1138–1150.
http://dx.doi.org/10.1007/s001220100571

Rouf Mian MA, Andrew AH, John CZ. 2002. Determination of Genetic Diversity in Tall Fescue with AFLP Markers. Crop Science **42,** 944-950.

Vieira EA, Castro CM, Oliveira ACd, Irajá F, Carvalho Fd, Zimmer PD, Martins LF. 2004. Genetic structure of annual ryegrass (Lolium multiflorum) populations estimated by RAPD. Scientia Agricola **61(4),** 407-413.
http://dx.doi.org/10.1590/S010390162004000400009

Vishwanath K, Prasanna KPR, Pallvi HM, Prasad S, Ramegowda DPJ. 2011. Identification of tomato (Lycopersicon esculentum) varieties through total soluble seed proteins. Research Journal of Agricultural Sciences **2(1),** 8-12.