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Application of ISSR molecular markers in genetic diversity of three *Trifolium* species

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Abstract

Genetic variation for of 14 accessions of three *Trifolium* species of evaluated using 10 ISSR primers. In total 75 bands (loci) were polymorph in accessions studied. Primer IS_{12} with 11 bands had the highest and primer IS_{15} with 4 bands had the lowest number of bands. The primers of IS_{12}, IS_{13} and IS_{10} considering polymorphism information content (PIC) were useful for polymorphism study in *Trifolium* species genus in the future researches. AMOVA revealed that 29% of the total variance was due to differences between species and 71% was due to differences within species. *T.prateuse* specie with the Shannon's information index (I) and Nei's gene diversity (He) had the highest variety between reviewed **species**, and *T.hybridum* specie had the lowest variety. In addition the species *T.prateuse* had the highest similarity with T.*fragiferum*. These results were confirmed by cluster analysis and principal coordinate analysis of species. Cluster analysis for reviewing accession, classified them in three groups. Cluster analysis and Scatter plot based on first and second axis from principal coordinate analysis for accessions, showed that the primers ISSR could clearly separate species and accessions of each species were placed to each other.

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Introduction

The clover genus, *Trifolium* L., belongs to one of the largest groups in the family Fabaceae. It contains about 255 species (Zohary and Heller, 1984; Gillet and Taylor, 2001), and of those the highly cultured ones are especially agronomical important (Coombe, 1972; Zohary and Heller, 1984). It is particularly common in the northern hemisphere. Major centers are found in North America (60–65 species), Africa (25–30 species), and Eurasia (150–160 species) (Zohary and Heller, 1984). The methods currently used for plant diversity studies include not only analyses of agro-biological traits but also biochemical-molecular techniques. Special emphasis is attached to the research into nucleic acids, which are direct agents of genetic information transfer (Ghariani *et al.,* 2003). 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 (Che and Li, 2007). In recent years, many new alternative and promising marker techniques have been developed in line with the rapid growth of genomic research (Gupta and Rustgi, 2004). 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). 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 variations based on Molecular markers for between and within different species of *Trifolium* were reported by many researchers (Gustine *et al.,* 2001; Kolliker *et al.,* 2002; Dabkeviciene *et al.,* 2011). In this study we have used the ISSR marker to determine genetic variation between and within species of Trifolium.

Materials and methods

Plant materials

In order to evaluate the genetic variation, 14 accessions of three species *Trifolium* 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. PCR amplification was performed in 20 µl reaction containing 1× PCR buffer, 30 ng sample DNA, 2.5 µM primers, 200 µM of each dNTP, 1.5–2.5 mM MgCl2 and 1.5 unit of Taq DNA polymerase (Cinnagene, Iran).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 for 5 min at 72°C was performed 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). Data were analyzed using Gen ALEX 6.2 and Nei's gene diversity (He) (Nei and Li, 1973), Shannon's information index (I) (Shannon, 1948) were calculated for each of species. Cluster analysis, similarity matrix and principal coordinate analysis axis were carried out for 14 accession using Darwin and Gen ALEX 6.2.

Results

ISSR Polymorphism

Primers sequences, code, number of bands scored, percent of polymorphic bands (PPB) and polymorphism information content (PIC) were showed for ISSR primers in Table 2.

Table 1. List of 14 accessions from 3 species *Trifolium*.

Gen bank cod	Origin	species	Number	Gen bank cod	Origin	species	Number
20258	UNKNOW	*T. fragiferum*	8	2580	AUSTRALIA	*T. fragiferum*	1
1250	ZANJAN	*T.hybridum*	9	2324	FAO	*T.hybridum*	2
134	UNKNOW	*T. fragiferum*	10	618	UNKNOW	*T.pratense*	3
720	TABRIZ	*T. fragiferum*	11	2056	USA	*T.hybridum*	4
716	TABRIZ	*T.pratense*	12	1753	URMIA	*T.pratense*	5
324	KARJ	*T.pratense*	13	2056	UNKNOW	*T.pratense*	6
2139	AUST RALIA	*T. fragiferum*	14	1451	HAMEDAN	*T.pratense*	7

For all primers, the number of 75 bands was scored that percent of polymorphism was 100% for all primers. IS_{12} primer with 11 bands had the highest and primer IS_{15} with 4 bands had the lowest number of bands. Band pattern of accessions for IS_9 showed in Figure 1.

Table 2. ISSR primers used in this study and some summary results.

PIC	Percentage of polymorphic bands(PPB)	No. of bands scored	Primer sequence	ISSR code
0.399	7	7	5' GAGAGAGAGAGAGAGAYC 3'	IS3
0.389	10	10	5' AG AG AG AG AG AG AG AG AGC 3'	IS5
0.392	10	10	5' CTCTCTCTCTCTCTCTG 3'	IS9
0.426	6	6	5' GAGAGAGAGAGAGAGARC 3'	IS10
0.381	5	5	5' ACACACACACACACACC 3'	IS11
0.435	11	11	5' TGTGTGTGTGTGTGTGG 3'	IS12
0.421	7	7	5' AGAGAGAGAGAGAGAGYT 3'	IS13
0.427	10	10	5' GACAGACAGACAGACA 3'	IS14
0.401	4	4	5' GGATGGATGGATGGAT 3'	IS15
0.420	5	5	5'DBDACACACACACACACA3'	IS16
0.409	7.5	7.5		Average

Average of PIC in the used Primers was 0.409, that the highest number of PIC related to Primers IS_{12}, IS_{13} and IS_{10} that amount of PIC were 0.43 and 0.42 in these Primers. Primers $IS_{11}$and IS_5 with the lowest amount of PIC had not ability in the separation of accession.

Molecular variance analysis

Analysis of molecular variance was performed for ISSR bands to determine of significant difference between populations of accessions based on three species. Indicated that as there is considerable genetic diversity between species, between accessions of each of species, there is genetic diversity that diversity between species was %29 and within species was 71% that indicating, there is difference between studying species accordance to germplasm (Table 3).

Table 3. Molecular variance analysis.

S.O.V	Df	SS	MS	Est. Var.	Var%	Stat	Value	P
Between species	2	77.11	36.56	5.24	29%	PhiPT	0. 288*	0.010
Within species	11	142.53	12.96	12.96	71%			
Total	13	215.64		18.20	100%			

Table 4. Statistical analysis of genetic diversity of 3 species.

Percentage of polymorphic bands (PPB)	Nei's gene diversity (He)	Shannon's information index(I)	Number of simple	species
60	0.22(0.024)	0.32(0.034)	5	*T.fragiferum*
58.67	0.21(0.022)	0.32(0.32)	3	*T.hybridum*
77.32	0.28(0.021)	0.41(0.30)	6	*T.prateuse*

Genetic variation

Shannon's information index (I), Nei's gene diversity (He) and percentage of polymorphic bands were calculated for each species (Table 4). The genetic diversity of *T.prateuse* was relatively high, and the PPB, He and I of *T.prateuse* were 77.32%, 0.28 and 0.41, respectively. Species of *T.hybridum* had the lowest genetic diversity among the species, that is, *PPB* = 58.67%, He = 0.21 and I = 0.32. Considering to this parameter, there is the highest genetic diversity between accession of *T.prateuse* and the lowest related to *T.hybridum*.

Table 5. Similarity matrix for studying 14 accessions based on Dice's coefficient.

accession	T.fr 2580	T.hy 2324	T.pr 618	T.hy 2056	T.pr 1753	T.pr 2086	T.pr 1451	T.fr 20258	T.hy 1250	T.fr 134	T.fr 720	T.pr 716	T.pr 324
T.hy 2324	0.286												
T.pr 618	0.433	0.486											
T.hy 2056	0.464	0.526	0.441										
T.pr 1753	0.500	0.426	0.679	0.441									
T.pr 2086	0.580	0.429	0.677	0.618	0.528								
T.pr 1451	0.521	0.426	0.536	0.541	0.639	0.634							
T.fr 20258	0.394	0.481	0.473	0.597	0.393	0.507	0.546						
T.hy 1250	0.769	0.320	0.415	0.469	0.464	0.525	0.590	0.567					
T.fr 134	0.646	0.340	0.351	0.455	0.455	0.579	0.642	0.389	0.735				
T.fr 720	0.712	0.328	0.452	0.514	0.551	0.608	0.621	0.487	0.800	0.775			
T.pr 716	0.657	0.400	0.508	0.500	0.688	0.595	0.684	0.371	0.647	0.583	0.667		
T.pr 324	0.585	0.491	0.561	0.455	0.563	0.649	0.734	0.457	0.620	0.611	0.641	0.714	
T.fr 2139	0.727	0.296	0.393	0.478	0.571	0.603	0.625	0.451	0.778	0.658	0.810	0.676	0.676

Similarity matrix

Similarity matrix based on Dice's coefficient for all accessions (Table 5) showed that accession T.hy 1250 with accession T.fr 720 had the highest value of similarity. On the other hand, accession T.fr 2580 and accession T.hy 2324 had the lowest similarity.

Genetic Similarity for three species based on Dice's coefficient showed that the highest of distance between T.hybridum and T.prateuse species (0.65) and the highest of Similarity relates to T.fragiferum and T.prateuse (0.75).

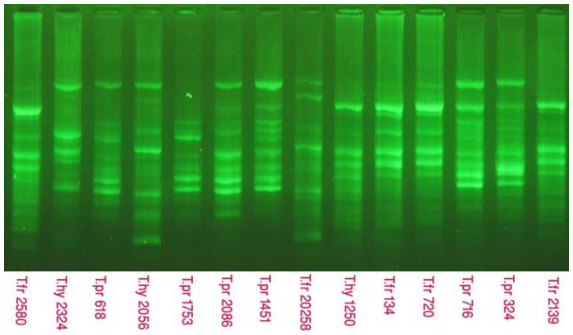

Fig. 1. The band pattern for accessions using IS_9 primer.

Cluster analysis

UPGMA hierarchical clustering for grouping accessions based on Dice's coefficient (Fig. 2) were identified the three distinctive groups. The Results showed that the primers ISSR could clearly separate species and accessions of each species were placed to each other.

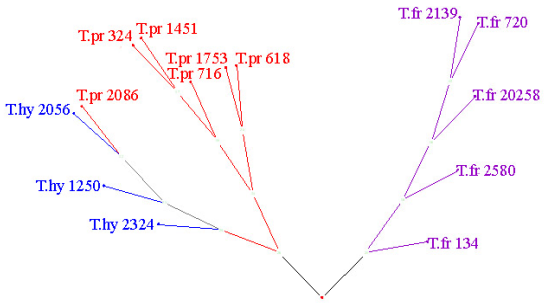

Fig. 2. Dendrogram of cluster analysis for accessions based Dice's coefficient by UPGMA.

Cluster Analysis was performed for studied species, were identified the two distinctive groups. That its results were corresponding to achieved results of similarity matrix of species indicating that species *T.hybridum* had the highest interval that the other species (Fig.3).

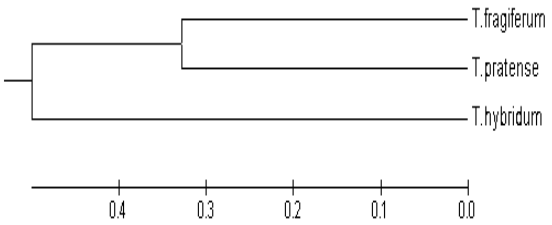

Fig. 3. Dendrogram of cluster analysis for species by UPGMA method.

Principal coordinate analysis

Scatter plot for accessions based on first (31.36) and second (21.02) axis from principal coordinate analysis (Fig. 4) showed that accessions of each species were placed together. These results confirmed by Similarity matrix and cluster analysis.

Discussion

Polymorphism

The current study confirmed the importance of molecular studies beside the morphological data in detecting genetic variation among genotypes in selecting diverse parents to carry out a new crossing program successfully. 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. The results revealed that ISSR markers are suitable tools for detecting of genetic variation in *Trifolium* species. The average PPB in the present investigation indicated a high polymorphism among *Trifolium* accessions based on ISSR primers. In the current study, a total number of 75 alleles were detected using 10 ISSR selective markers, with an average of 7.5 allele per locus, suggested the presence of a considerable polymorphism at studied ISSR loci and revealed a high level of genetic diversity in the existing *Trifolium* germplasm which is close to that obtained by Jahufer *et al.,* (2003) and Gustine *et al.,* (2002). PIC values estimate the discriminatory power of a marker. The mean PIC values for markers used in present study were 0.40. Markers with high PIC

values such as IS_{12}, IS_{13} and IS_{10} could be effectively used in genetic diversity studies in *Trifolium*. Genetic diversity in species *Trifolium* was studied by Dabkeviciene *et al.,* (2011) based on RAPD and ISSR marker and they reported a different PIC for used primers.

Genetic variation

Analysis of molecular variance was performed for ISSR bands to determine of significant difference between populations of accessions based on species, Indicating that only 29% of the multiplied sited had influenced in the examiner of the diversity between species. Results indicated that there was a well variation between studying species considering to Shannon's information index (I) and Nei's gene diversity (He) which, it was revealed that *T.prateuse* species had highest variation between its accession and the lowest one belongs to *T.hybridum* species.

Genetic Similarity for three species based on Dice's coefficient showed that the highest of Similarity relates to T.fragiferum and T.prateuse, Indicates that gene flow between these species can be done.

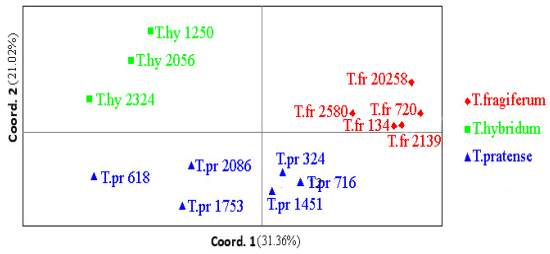

Fig. 4. Scatter plot for accessions based on two first axes from principal coordinate analysis.

Grouping

Studied genotypes were clustered in three groups based on UPGMA clustering method. Cluster analysis and Scatter plot based on first and second axis from principal coordinate analysis for accessions, showed that the primers ISSR could clearly separate species and accessions of each species were placed to each other. Overall, the results of the cluster analysis and principal coordinate analysis showed that the rate of correlation of genetic variation among species in high performance primers ISSR have *Trifolium*. The existing genetic diversity observed in advanced breeding lines developed at gen bank indicated the efforts underway to widen the genetic base of *Trifolium* for various traits. 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 *Trifolium*. 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.

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.

Che YH, Li LH. 2007. Genetic diversity of prolamines in Agropyron mongolicum Keng indigenous to northern China. Genet. Resour. Crop. Evol **54,** 1145–1151. http://dx.doi.org/10.1007/s10722-006-9006-7

Coombe DE. 1972. Trifolium L., In: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, Webb DA, editors. Flora Europaea, vol 2. Cambridge, U.K: Cambridge University Press, 157–172 p.

Dabkeviciene G, paplauskiene and vileinskas E. 2011. Assessment of genetic diversity in Trifolium SPP using ISSR AND RAPD markers. Journal of food agriculture 8Environment Vol. **9(1),** 210-214.

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. Mol. Ecol. Notes. **2**, 265-267.

Fang DQ, Roose ML. 1997. Identification of closely related citrus cultivars with inter-simple sequence repeats markers. Theor. Appl.Genet **95,** 408-417.
http://dx.doi.org/10.1007/s001220050577

Ghariani S, Trifi-Farah N, Chakroun M, Marghali S, Marrakchi M. 2003.Genetic diversity in Nissan perennial ryegrass revealed by ISSR markers // Genetic Resources and Crop Evolution. vol. 50, No. **8,** 809–815 p.

Gillet JMX, Taylor NL. 2001. The World of Clovers. Iowa, Ames, USA: Iowa State University Press.

Gupta PK, Rustgi S. 2004. Molecular markers from the transcribed/expressed region of the genome in higher plants. Funk Integr Genomics **4,** 139–16.
http://dx.doi.org/10.1007/s10142-004-0107-0

Gustine DL, Voigt PW, Brummer EC, Papadopoulos YA. 2002. Genetic variation of RAPD markers for North American white clover collections and cultivars. Crop Science **42(2),** 343-347 p.

Jahufer MZ, Barrett BA, Grifiths AG, w wood field DR. 2003**.** DNA fingerprinting and genetic relationships among white clover cultivars. Proceedings of the new Zealand Grassland Association **65,** 1690-163.

Kolliker R, jones ES, jahufer MZZ, forster J W. 2001. Bulked AFLP analysis for the assessment of genetic diversity in white clover (Trifolium repens L) E .uphitica **121,** 305-315.
http://dx.doi.org/10.1007/s00122-003-1248-6

Murry MG, Tompson WF. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acid Research **8,** 4321-4325.

Nei NM, Li W. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci **76,** 5269–5273, PMCID: PMC413122.

Paplauskiene V, Dabkeviciene G. 2012. A study of genetic diversity in Trifolium hybridum Varietied using of morphological characters and ISSR markers. ZEMDirbyste, Agriculture, Vol. No. 313-318 P.

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

Shannon CE. 1948. A mathematical theory of communication. Bell System Technical Journal **27**, 379-423.

Surendhar RCh, Prasad BA, Mallikarjuna S BP, Kaladhar K, Sarla N. 2009. ISSR markers based on GA and AG repeats reveal genetic relationship among rice varieties tolerant to drought, flood, or salinity. Journal of Zhejiang Univsity Science B **10(2),** 133–141.
http://dx.doi.org/10.1631/jzus.B0820183

Zohary M, Heller D. 1984. The genus Trifolium. Jerusalem, Israel: Israel Academy of Sciences and Humanities.