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Investigation of genetic variation of lentil lines using random amplified polymorphic DNA (RAPD) and intron-exon splice junctions (ISJ) analysis

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ABSTRACT: In order to investigation of genetic variation among lentil lines and genetic relationship these to Cistan local cultivar, DNA of 20 lentil lines and Cistan local cultivar were analysed using random amplified polymorphic DNA (RAPD) and intron-exon splice junctions (ISJ) markers. PIC, MI, percentage of polymorphic bands (P), Nei ' s gene diversity (H), Shannon' s information index (I) were calculated for both random, semi random and combined of random and semi random primers. For both matrices, genetic similarity (GS_{RAPD} and GS_{ISJ}) were calculated according to the Jaccard similarity coefficient. Correlation between two similarity matrices obtained with two markers types was estimated. The similarity matrix per marker was subjected to cluster analysis by the unweighted pair-group method (UPGMA). The $\overline{PIC}$ of RAPD was as $\overline{PIC}$ as ISJ, but MI index of ISJ (average 6.095) was much higher than RAPD (average 4.49). Mean of genetic similarity of RAPD marker was higher than ISJ marker, and combined of RAPD and ISJ markers. Thus in order to estimate of genetic diversity in lentil lines, ISJ marker was better than RAPD marker and combined of RAPD and ISJ markers was also better than RAPD marker. For $RAPD_s$ the similarity values ranged from 0.166 between TN2026 line and TN2464 line, to 0.697 between TN1086 line and TN1087 line. For ISJ, the values ranged from 0.052 between TN1084 line and TN2462 line, to 0.312 between TN2457 line and KC210031 line, and for RAPD +ISJ the values ranged from 0.147 between TN2420 line and TN2026 line, to 0.471 between TN1087 line and TN1086 line. In the ISJ markers the UPGMA cluster diagram have showed 3 major clusters: one cluster included the TN2026 line, the second cluster were contained TN2128, TN2022, TN2461, TN2464, TN1885, TN2463 and the third cluster were contained rest of lentil lines. In RAPD and combined of RAPD and ISJ markers, the UPGMA cluster diagram have showed 2 major clusters. Cistan local cultivar had higher genetic distance than TN2026 line in each three methods of grouping. Therefore in order to favourable genes transfer from TN2026 line to Cistan local cultivar should be considerable genetic distance.

Keywords: genetic diversity, lentil line, Cistan local cultivar, RAPD, ISJ

Introduction

Lentil is included in the cool season food legume group of pulses and is an important source of protein and fibre in human diet. Moreover, they are also valuable as feed and fodder for livestock, and play an important role in crop rotations because their nitrogen fixing capability (Durán and Vega, 2004). The assessment of genetic variation and genetic similarities is a major concern of plant breeders and population geneticists, because it facilitates the efficient sampling and utilisation of germ plasm resource (Maqbool and McNeil, 1996). Various methods have been employed to estimate genetic diversity. Morphological trait measurements are commonly used parameters since they provide a simple technique of quantifying genetic variation while simultaneously assessing genotype performance under relevant growing environments (Fufa *et*

al., 2005). However, assessing morphological traits is labor-intensive and the phenotypic plasticity of plants makes environmental variation a major problem. Molecular markers have a number of perceived advantages over the measurement of morphology for the assessment of genetic diversity. Marker assisted selection (MAS) is used to monitor breeding programs at very early stages of plant development and is independent of the growth conditions (XiaoPeng *et al.,* 2008). One such molecular system, Semi specific PCR and the use of primers with partial homology to sequences of intron-exon Junctions was developed by Weining and Langridge (1991) and RAPD system which developed by Williams *et al.,* (1990). The RAPD system is useful for many crops, but has low rate of polymorphism, along difficulties with the reproducibility of the results. ISJ seems to be an alternative to RAPD and other tedious and expensive methods such as RFLP and AFLP (Gawel and Iwona 2002). These allow the generation of great diversity of markers without any additional sequence information (Gawel and Iwona 2002, Weining and Langridge 1991). Hence, it has been widely used in assessments of genetic diversity (Vahabi *et al.,* 2008, Gawel and Iwona., 2002, Malorzata et al., 2002, Przetakiewize *et al.,* 2002) and chromosome identification (Allahdoo *et al.,* 2009). Sonnate and Pignone (2001) used RAPD and ISSR markers for assessment of genetic variation in a collection of lentil, they revealed the dendrogram generated from ISSR data was quite different from that generated from $RAPD_s$ ones. The aim of this work was to obtain estimates for genetic relationship of lentil lines and Cistan local cultivar that may be used in further programs. In the course of this work, we employed diversity assessment systems based on two polymerase chain reaction (PCR)-based methods (RAPD and ISJ).

Materials and methods

Plant material

A total of 20 lentil lines and Cistan local cultivar were used in this research, lentil lines were prepared by ICARDA institute (table 1).

DNA extraction

Young-leaf tissue (0.5 g) from each lentil line was ground in liquid nitrogen to a fine powder and total genomic DNA was extracted using the Zidani *et al.,* (2005) method with minor changes. The quality and quantity of DNAs were determined in 1% agarose gel and Biophotometer (model Biorad.), respectively for PCR analysis (Zidani *et al.,* 2005).

PCR analysis

From a total of 25 random primers and 18 semi random primers, 9 random primers and 12 semi random primers, which had clear band patterns, were chosen for PCR amplification of the genomic DNA samples (Table 2 and 3). The RAPD reaction mixture (total volume = 25 μl) were contained 40 ng DNA, 1X PCR buffer, 0.8 μM primers, 200 μM of dNTPs, 3 mM $MgCl_2$, and 1 unit polymerase Taq DNA (Fermentas, USA). An initial, preheating step (2 min at 94^{o}C) was carried out in order to achieve a hot start. Subsequently, denaturation at 94^{o}C for 30 s and annealing at 36^{o}C at 60 s for random primers, 58 ^{o}C at 60 s for semi random 15-mer primers and 63^{o}C at 60 s for semi random 18-mer primers, and extension at 72^{o}C for 90 s. The subsequent 40 cycles each consisted of the steps: denaturation, annealing and extension. After the last cycle, an extension step of 72^{o}C for 8 min was performed.

Data analysis

In the RAPD and ISJ molecular analysis, the banding patterns were scored as present (1) or absent (0) for each primer pair. Only strong, reproducible and clearly distinguished bands were used in the analysis. The polymorphic information content (PIC) value was calculated for both molecular marker systems using formula $PIC = [\Sigma 2P_i(1-P_i)]$ (Mohammadi and Prasanna 2003). Where P_i is frequency of the i th band. MI was calculated as $MI = \overline{\overline{PIC}} \times n\beta$ (Tams *et al.,* 2005), where $\overline{\overline{PIC}}$ is the average PIC-value, n is the number of band detected and β is proportion of polymorphic bands. For both marker, and combined of both markers the number of bands, percentage of polymorphic bands (PPB), Nei's gene diversity (H), Shannon' s information index (I), the observed number of alleles (Na) and the effective number of alleles (Ne) (Lewontin, 1972; Nei, 1973) were calculated using the program POPGEN 1.32 (Yeh et al., 1997). For both

matrices, genetic similarity estimates (GS_{RAPD} and GS_{ISJ}) were calculated according to the Jaccard similarity coefficient. Correlation between two similarity matrices obtained with the two markers types was estimated. A Mantel Z-test reveals the correspondence of two matrices. The significance of Z was determined by comparing the observed Z-value with a critical Z-value after 1000 permutation. The similarity matrix per marker was subjected to cluster analysis by the unweighted pair-group method (UPGMA). Cophenetic correlation was calculated to test for goodness of fit between GS-values obtained from the cluster and the original GS matrix. Principle component analyses were calculated for each marker. Computations were performed with appropriate procedures of software NTSYS-pc version 2.11 (Rolhf, 2000).

Table 1. Lentil lines used.

N	line	no	line
1	TN1084	12	TN2026
2	KC210034	13	TN2437
3	TN2463	14	TN1960
4	TN1087	15	TN2128
5	TN1086	16	TN2022
6	TN1885	17	TN2457
7	TN2420	18	KC210031
8	TN2464	19	TN2458
9	TN2461	20	TN2439
10	TN2434	21	CISTAN
11	TN2462		

N:.....

Table 2. Semi- random primers sequence used for amplification of lentil lines DNA.

Semi random primer	sequence (5`-3`)	Annealing temperature
IT34	5` GCGGCATCAGGTAAG3`	58
IT31	5`GAAGCCGCAGGTAAG 3`	58
IT36	5`GTCGACCCAGGTAAG 3`	58
IT1	5` CCGGCAGGTAAGT 3`	63
IT2	5` GCAGAGGGCCAGGTAAGT 3`	63
IT32	5` GACTCGCCAGGTAAG 3`	58
IT33	5` GATGCCCCAGGTAAG 3`	58
ET32	5` ACTTACCTGGGCACG 3`	58
ET33	5` ACCTACCTGGCCGAT 3`	58
ET34	5` ACCTACCTGGGCGAG 3`	58
ET36	5` ACCTACCTGGGGCTC 3`	58
ET38	5` ACTTACCTGAGGCGCGAC 3`	63
ET31	5` ACTTACCTGGGCCAG 3`	58
ET42	5` ACTTACCTGCCTACGCGG 3`	63
IT3	5` CGTCGGCCACAGGTAAGT 3`	63
IT5	5`GGTGCGGGACAGGTAAGT 3`	63
IT4	5` CGCGGAGAGCAGGTAAGT 3`	63
IT6	5`CCTGGAGGCCAGGTAAGT 3`	63

Table 3. Random primers sequence used for amplification of lentil lines DNA.

Random primer	Sequence (5`-3`)	Annealing temperature
OPF03	5`CCTGATCACC 3`	36
OPN06	5` GAGACGCACA 3`	36
OPM06	5` CTGGGCAACT 3`	36
OPC11	5` AAAGCTGCGG 3`	36
NO CODE4	5` GACCGACCCA 3`	36
NOCODE2	5` TGCCGAGCTG 3`	36
OPN05	5` ACTGAACGCC 3`	36
OPN14	5` TCGTGCGGGT 3`	36
OPF01	5` ACGGATCCTG 3`	36
OPN15	5` CAGCGACTGT 3`	36
OPM04	5` GGCGGTTGTC 3`	36
OPC05	5` GATGACCGCC 3`	36
OPN16	5` AAGCGACCTG 3`	36
OPN02	5`ACAACGCCTC 3`	36
OPW18	5` TTCAGGGCAC 3`	36
OPH19	5` CTGACCAGCC 3`	36
OPG09	5` TGAGCCTCAC 3`	36
NOCODE1	5`CAGGCCCTTC 3`	36
OPI10	5`ACAACGCGAG 3`	36
OPJ19	5` GGACACCACT 3`	36
OPJ12	5` GTCCCGTGGT 3`	36

Results

Polymorphism content of random and semi random primers

Among the pre-screened primers, 12 semi random and 9 random primers amplified polymorphic, repetitive DNA bands (table 1and 2). Random primers produced a total of 187 bands, 133 bands (61.4%) which were polymorphic bands, used for band scoring (figure 1). The bands were characterized based on size and ranged from approximately 200-5000 bp. The number of amplified bands varied from 6 (OPN-06) to 36 (OPN-14), with an average of 20.77 bands per primer (table 3).

Semi random primers produced a total of 308 bands, 293 bands (88.3%) were polymorphic bands. The bands were characterized based on size and ranged from approximately 200-4800 bp. Number of amplified bands varied from 12 (IT33) to 43 (ET36), with an average of 25.66 bands per primer (table 3).

For semi random primers average PIC-values ranged from 0.138 to 0.316 with mean of 0.269, the MI ranged from 2.45 to 13.11 and averaged 6.095. for random primers, average PIC- values ranged from 0.192 to 0.354, with mean of 0.272, the MI ranged from 1.29 to 7.21 and averaged 4.49 (table 3).

Estimation of similarity and diversity indices using RAPD, ISJ and combined RAPD and ISJ

For all pair wise comparisons of GS estimates, GS_{RAPD} ranged from 0.166 to 0.697 with an average of 0.468 , GS_{ISJ} ranged from 0.052 to 0.312 with an average of 0.211. $GS_{RAPD+ISJ}$ ranged from 0.147 to 0.471 with an average of 0.329. The cophenetic correlation was 0.814 for GS_{RAPD} and 0.607 for GS_{ISJ}. The correlation between GS_{ISJ} and GS_{RAPD} was 0.173, Mantel Z-test revealed a low and non-significant correlation of GS_{ISJ} and GS_{RAPD} matrices.

For comparing and calculating genetic data in the RAPD marker, the mean of observed alleles number (na) was 1.96, the mean of effective number of alleles (Kimura and Crow 1964) was 1.22, the mean Nei's gene diversity (h) was 0.198 and the mean of Shannon' s Information index (I) was 0.306. For the ISJ marker, the observed average number of alleles (na) was 1.7, the mean of effective number of alles was 1.33, the mean of Nei ' s gene diversity (h) was 0.1628 and mean of Shannon's Information index (I) was 0.2837. Therefore, content of h and I indices in ISJ marker was higher than RAPD marker (table 7).

Clustering pattern based on random and semi random primers and combined random and semi random primers

Both kinds of markers and combined of RAPD and ISJ, were used to estimate the similarity between lentil lines by the Jaccard similarity index. For $RAPD_s$ the similarity values ranged from 0.166 between TN2026 line and TN2464 line, to 0.697 between TN1086 line and TN1087 line (table 5), for ISJ_s the values ranged from 0.052 between TN1084 line and TN2462 line, to 0.312 between TN2457 line and KC210031 line (table 6), and for RAPD+ISJ the values ranged from 0.147 between TN2420 line and TN2026 line, to 0.471 between TN1087 line and TN1086 line (table 7).

The dendrogram obtained from UPGMA cluster analysis based on random primers, semi random primers and combined of random and semi random primers are present in figure 2, 3 and 4. on ISJ markers the UPGMA cluster diagram have showed 3 major clusters: one cluster included the TN2026 line, the second cluster contained TN2128, TN2022, TN2461, TN2464, TN1885, TN2463 and the third cluster were contained rest of lentil lines (Fig 2). TN2026 line had the highest genetic distance to other of lentil lines. In the RAPD and combined of the RAPD and ISJ markers, the UPGMA cluster diagram have showed 2 major clusters: one cluster included TN2026 line and the second cluster were contained rest of lentil lines (Fig 1 and 3). In each 3 methods of clustering TN2026 line had the highest genetic distance to other of lines. Cistan local cultivar using RAPD, ISJ and RAPD+ISJ has lower genetic similarity than TN2026 line, but using RAPD marker had higher genetic similarity than TN2439 line (table 6), using ISJ marker had higher genetic similarity than KC210034 line and TN2437 line (table 5), and using the of RAPD and ISJ markers, had higher genetic similarity than TN2439 line (table 7).

Principle component analysis was carried out based on Jaccard genetic similarity using random, semi random and combined of random and semi random primers. The first component accounted for 10.84 of the total variation, the second component accounted for 6.87 of the total variation and the third component, accounted for 8.63 of the total variation using the RAPD markers. For the ISJ marker, the first component three was explained 21.03 of the total variation and for the combined of RAPD and ISJ 21.38 of the total variation (table 8).

Table 3: degree of polymorphism, mean of PIC and MI for random and semi random primers applied to 21 lentil lines

primer	Total band	N.P.B	$\overline{PIC}$	MI
Semi random				
ET32	37	31	0.316	5.10
ET36	43	43	0.305	13.11
ET33	32	26	0.315	6.18
ET38	26	26	0.277	7.2
ET34	32	31	0.253	7.61
IT2	26	26	0.138	3.58
IT33	12	12	0.238	2.85
IT31	13	13	0.189	2.45
IT34	19	17	0.306	5.19
IT1	22	22	0.235	5.17
IT32	19	19	0.217	4.12
IT36	27	27	0.264	7.12
mean	25.66	24.41	0.269	6.095
random				
NO COD2	10	9	0.192	1.72
OPF01	14	9	0.354	3.1
OPN 05	27	27	0.272	7.34
NO COD 4	21	13	0.318	4.19
OPF03	22	11	0.329	3.61
OPN06	6	4	0.324	1.29
OPC 11	24	19	0.336	6.91
OPM 06	27	19	0.324	5.10
OPN 14	36	22	0.328	7.21
mean	20.77	14.77	0.272	4.49

Table 4- Genetic similarity based on Jaccard similarity coefficient, between 20 lentil lines and Cistan local cultivar using semi random primers.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	1																				
2	0.176	1																			
3	0.151	0.1	1																		
4	0.244	0.197	0.123	1																	
5	0.226	0.185	0.236	0.259	1																
6	0.14	0.123	0.227	0.206	0.147	1															
7	0.11	0.116	0.19	0.106	0.144	0.195	1														
8	0.129	0.168	0.276	0.206	0.135	0.304	0.139	1													
9	0.137	0.166	0.166	0.137	0.11	0.225	0.15	0.31	1												
10	0.16	0.154	0.123	0.207	0.193	0.163	0.135	0.252	0.2	1											
11	0.052	0.212	0.101	0.15	0.185	0.164	0.149	0.125	0.162	0.207	1										
12	0.151	0.126	0.106	0.142	0.18	0.144	0.105	0.154	0.13	0.163	0.139	1									
13	0.172	0.265	0.135	0.164	0.181	0.15	0.133	0.163	0.188	0.202	0.194	0.163	1								
14	0.227	0.2	0.164	0.175	0.214	0.22	0.219	0.15	0.171	0.208	0.231	0.162	0.297	1							
15	0.17	0.069	0.183	0.053	0.109	0.16	0.18	0.105	0.189	0.133	0.177	0.122	0.125	0.17	1						
16	0.161	0.142	0.136	0.151	0.107	0.218	0.12	0.191	0.204	0.163	0.133	0.164	0.148	0.173	0.126	1					
17	0.131	0.186	0.16	0.187	0.138	0.264	0.275	0.208	0.223	0.223	0.202	0.135	0.197	0.272	0.2	0.17	1				
18	0.277	0.207	0.13	0.21	0.254	0.172	0.187	0.142	0.15	0.181	0.197	0.191	0.182	0.243	0.134	0.161	0.312	1			
19	0.161	0.13	0.238	0.096	0.144	0.152	0.166	0.177	0.204	0.219	0.133	0.107	0.223	0.255	0.212	0.135	0.23	0.207	1		
20	0.141	0.089	0.185	0.142	0.135	0.175	0.181	0.16	0.157	0.173	0.188	0.16	0.174	0.219	0.15	0.177	0.262	0.157	0.237	1	
21	0.09	0.206	0.183	0.179	0.181	0.163	0.164	0.175	0.188	0.202	0.194	0.075	0.205	0.172	0.094	0.081	0.168	0.16	0.134	0.104	1

Table 5- Genetic similarity based on Jaccard similarity coefficient, between 20 lentil lines and Cistan local cultivar using random primers.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	1																				
2	0.45	1																			
3	0.506	0.542	1																		
4	0.524	0.538	0.626	1																	
5	0.538	0.5	0.539	0.697	1																
6	0.605	0.539	0.565	0.723	0.657	1															
7	0.434	0.373	0.476	0.521	0.56	0.567	1														
8	0.328	0.333	0.451	0.457	0.447	0.523	0.617	1													
9	0.447	0.404	0.528	0.586	0.54	0.589	0.476	0.426	1												
10	0.407	0.34	0.405	0.506	0.541	0.547	0.428	0.448	0.553	1											
11	0.41	0.372	0.369	0.454	0.486	0.453	0.387	0.269	0.469	0.446	1										
12	0.28	0.277	0.261	0.255	0.229	0.263	0.197	0.166	0.241	0.206	0.188	1									
13	0.466	0.436	0.445	0.487	0.407	0.526	0.309	0.298	0.424	0.364	0.447	0.227	1								
14	0.525	0.41	0.546	0.58	0.5	0.602	0.416	0.432	0.467	0.447	0.413	0.206	0.591	1							
15	0.364	0.397	0.42	0.506	0.458	0.507	0.333	0.258	0.397	0.415	0.491	0.237	0.532	0.424	1						
16	0.467	0.406	0.486	0.626	0.519	0.608	0.391	0.428	0.55	0.529	0.47	0.261	0.486	0.546	0.606	1					
17	0.323	0.363	0.474	0.434	0.424	0.432	0.403	0.395	0.377	0.246	0.375	0.173	0.377	0.409	0.442	0.403	1				
18	0.474	0.511	0.577	0.476	0.545	0.512	0.462	0.437	0.415	0.376	0.416	0.313	0.415	0.421	0.408	0.473	0.508	1			
19	0.32	0.404	0.351	0.365	0.371	0.415	0.365	0.311	0.385	0.287	0.323	0.311	0.347	0.308	0.396	0.351	0.48	0.478	1		
20	0.594	0.47	0.486	0.525	0.583	0.633	0.476	0.426	0.552	0.485	0.469	0.241	0.444	0.506	0.417	0.528	0.4	0.513	0.426	1	
21	0.564	0.522	0.451	0.488	0.536	0.6	0.4	0.375	0.506	0.486	0.493	0.318	0.468	0.524	0.407	0.545	0.352	0.571	0.397	0.752	1

Table 6- Genetic similarity based on Jaccard similarity coefficient, between 20 lentil lines and Cistan local cultivar using combined of RAPD and ISJ primers.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	1																				
2	0.307	1																			
3	0.316	0.319	1																		
4	0.38	0.372	0.378	1																	
5	0.365	0.336	0.388	0.471	1																
6	0.333	0.314	0.393	0.447	0.365	1															
7	0.251	0.242	0.335	0.308	0.328	0.362	1														
8	0.207	0.241	0.335	0.318	0.258	0.394	0.308	1													
9	0.269	0.281	0.337	0.345	0.293	0.385	0.293	0.358	1												
10	0.267	0.244	0.258	0.352	0.345	0.327	0.263	0.328	0.348	1											
11	0.207	0.295	0.239	0.306	0.326	0.3	0.263	0.185	0.301	0.316	1										
12	0.208	0.198	0.181	0.198	0.203	0.199	0.147	0.159	0.18	0.183	0.162	1									
13	0.303	0.352	0.29	0.327	0.289	0.316	0.219	0.22	0.297	0.278	0.316	0.193	1								
14	0.357	0.302	0.343	0.366	0.344	0.387	0.311	0.26	0.301	0.313	0.318	0.182	0.432	1							
15	0.262	0.23	0.31	0.277	0.272	0.322	0.258	0.17	0.288	0.25	0.33	0.176	0.313	0.29	1						
16	0.3	0.274	0.312	0.283	0.294	0.397	0.25	0.289	0.361	0.324	0.293	0.21	0.307	0.341	0.348	1					
17	0.21	0.269	0.3	0.304	0.257	0.339	0.333	0.275	0.289	0.234	0.279	0.152	0.276	0.333	0.305	0.272	1				
18	0.36	0.34	0.315	0.331	0.377	0.311	0.3	0.245	0.257	0.262	0.291	0.242	0.281	0.319	0.25	0.292	0.389	1			
19	0.233	0.261	0.297	0.23	0.25	0.272	0.259	0.231	0.287	0.251	0.223	0.195	0.283	0.281	0.302	0.238	0.333	0.317	1		
20	0.353	0.288	0.357	0.354	0.356	0.4	0.338	0.279	0.35	0.328	0.304	0.202	0.318	0.364	0.3	0.363	0.333	0.316	0.338	1	
21	0.297	0.367	0.326	0.343	0.352	0.366	0.283	0.263	0.339	0.337	0.344	0.187	0.337	0.337	0.253	0.3	0.254	0.333	0.262	0.428	1

Table 7- Diversity indices in lentil lines using random (RAPD), semi random (ISJ) primers and combined of random and semi random primers in the lentil lines.

Index/primer	Random primers (RAPD)	Semi random primers (ISJ)	Combined random and semi random primers
Mean of na*	1.96	1.7	1.86
Mean of ne*	1.22	1.33	1.26
Mean of h*	0.198	0.1628	0.177
Mean of I*	0.306	0.283	0.292
P.P.B*	96.38	70.88	86.24

*na= Observed number of alleles, * ne = Effective number of alleles [Kimura and Crow (1964)], * h = Nei's (1973) gene diversity, * I = Shannon's Information index [Lewontin (1972)] and PPB The percentage of polymorphic bands.

Table 8. Principle component analysis using random, semi random and combined of random and semi random primers.

primer	eigenvalue	percent	cumulative
Random			
	1.28	10.84	10.84
	1.1	8.63	20.71
	0.96	6.87	29.35
Semi random			
	1.25	7.55	7.55
	1.13	6.86	14.41
	1.09	6.62	21.03
Random+semi random			
	1.046	7.45	7.45
	1.003	7.14	14.59
	0.954	6.79	21.38

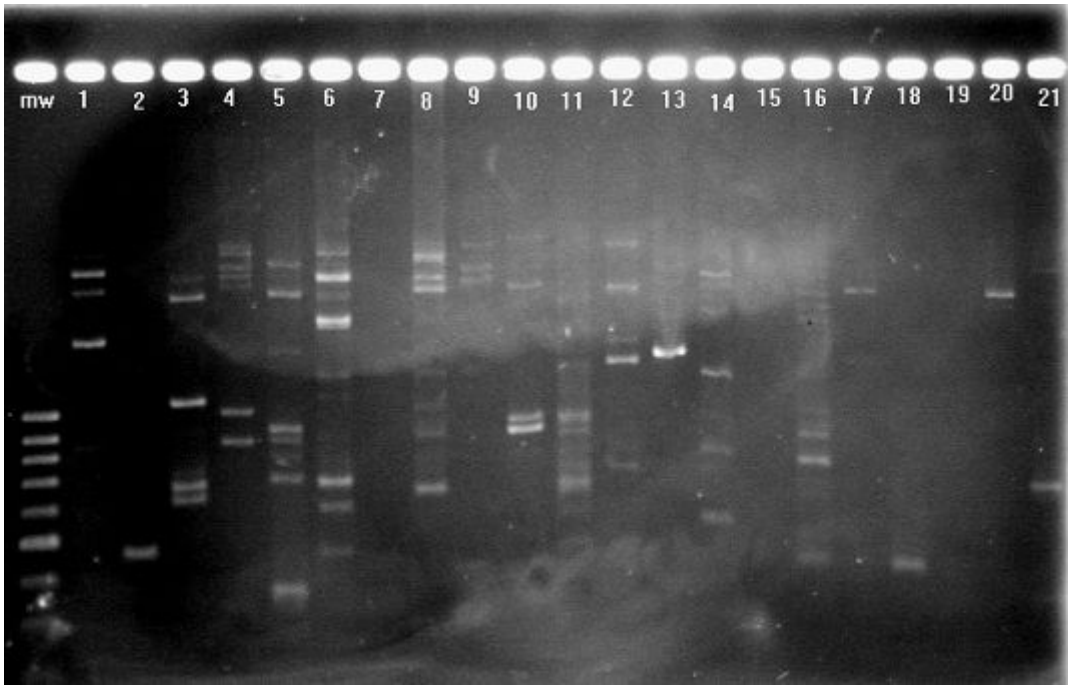

Figure 1. Amplified DNA using IT36 primer.

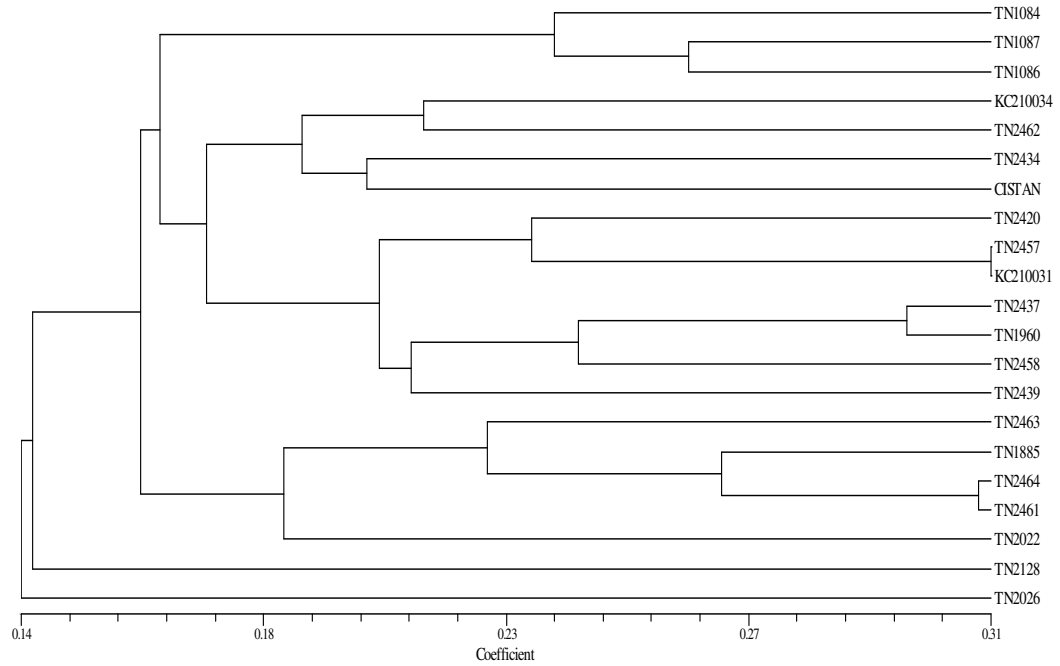

Figure 2. Dendrogram based on UPGMA using semi random primers in the lentil lines based in which coefficient? It must be mentioned in the title! The word “coefficient” in the down of figures must be removed or specify which coefficient is it! What the numbers at the right of dendrogram refer to?

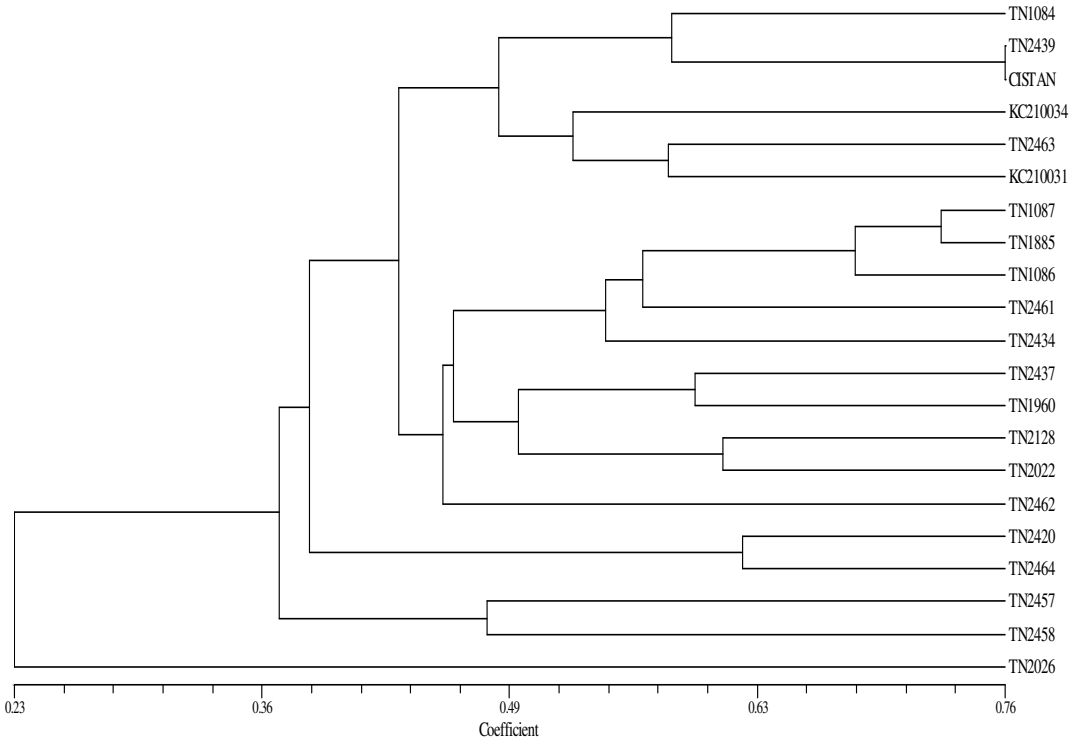

Figure 3. Dendrogram based on UPGMA, using random primers in the lentil lines

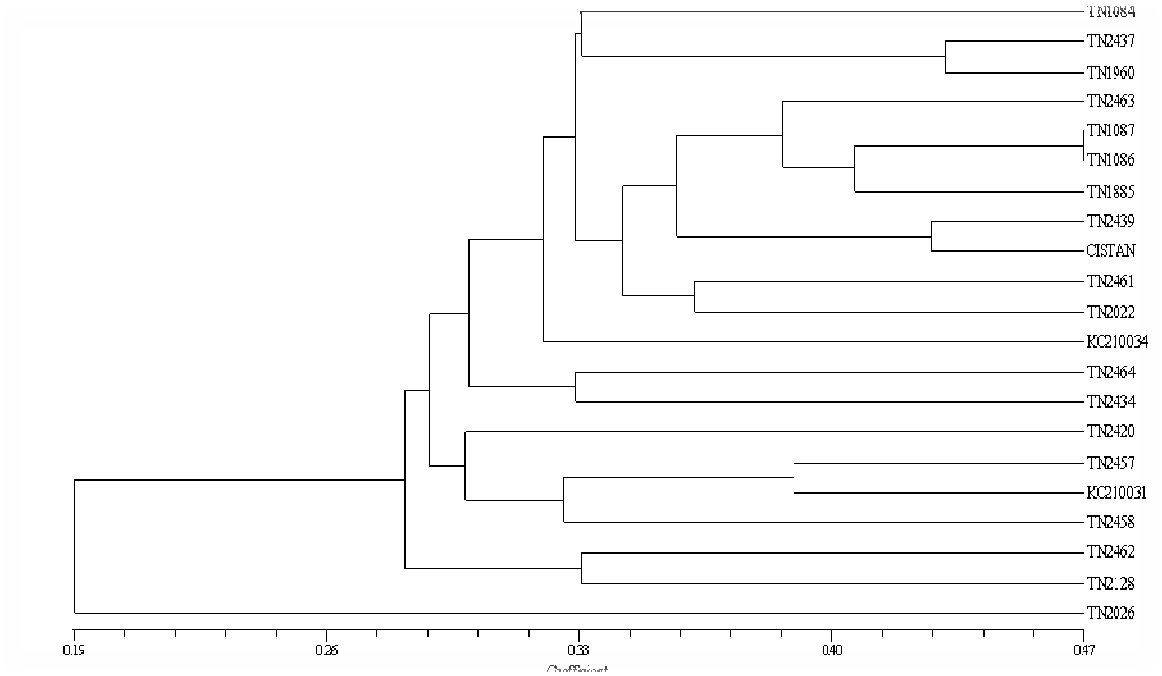

Figure 4. Dendrogram based on UPGMA using combined of semi random and random primers in the lentil lines.

Discussion

Comparison of RAPD and ISJ markers

The ISJ primers are valuable in detecting and mapping polymorphisms between wheat, barley, Faba triticale and tritipyrum (Weining and Langridge 1991, Vahabi *et al.,* 2007, Iqbal *et al*.,2002, Allahdoo *et al.,*2009). It is important that understand they also detect polymorphism between lines and cultivars of lentil. Figure 1 shows an example of the products obtained when the primer is used to amplify bands from 20 lines of lentil and one Cistan local cultivar. RAPD and ISJ markers have advantages and disadvantages for assessing genetic diversity, and many recent papers have demonstrated the high potential of two markers for population and species level studies and chromosome identification (Weining and Langridge 1991, Gawel and Iwona 2002 , Allahdoo *et al.,* 2009). In this work, the $\overline{PIC}$ of RAPD was as $\overline{PIC}$ as ISJ, but MI index of ISJ (average =6.095) was much higher than RAPD (average= 4.49). Mean of genetic similarity of the RAPD marker was higher than ISJ marker, and was higher than combined of RAPD and ISJ markers. Therefore in order to estimate of genetic diversity in lentil lines, ISJ marker was better than RAPD marker and combined of the RAPD and ISJ markers was also better than RAPD marker. This result supports the previous reports of suitability of the ISJ in the assessment of plant genetic variation (Weining and Langridge 1991, Gawel and Iwona 2002, Allahdoo *et al.,* 2009, Vahabi *et al.,* 2007). In the ISJ analysis, bands with an intermediate intensity in some DNA samples could occur because of heterozygosity or heterogeneity of the seed lot in the bulked sample of the lentil lines. Mantel test also revealed a low and non-significant correlation (r=0.17) for the matrices of GS_{RAPD} and GS_{ISJ}. These differences could be explained by: (1) different DNA segments targeted by two methods; (2) genomic regions sampled by the RAPD and ISJ markers maintain a different evolutionary process under selection. Therefore low and non-significant correlation GS_{RAPD} and GS_{ISJ} could be because of preferring ISJ marker to RAPD marker. Wu *et al.,*(2004) revealed a poor fit data analysis among population of *Oryza granulate* from Yunnan of Chinna for the matrices of GS_{RAPD} and GS_{ISSR} that agrees with our results using RAPD and ISJ markers in lentil lines. Tams *et al.,*(2005) revealed the moderate

and significant correlation between GS_{AFLP} and GS_{SSR} (r=0.7) in winter wheat, that disagree with our results using RAPD and ISJ markers in lentil lines.
Variation indices including Nei' s gene diversity (h), Shannon Information index (I), in the ISJ marker was higher than RAPD marker that was demonstrated efficient of ISJ marker to RAPD marker.

Genetic diversity among lentil lines
The estimation of the degree of differentiation between materials included in a crossing program is useful, since it can help in selecting the different parents. Cistan local cultivar has high yield stability and adaptation to environmental unusable conditions, but has low yield. Molecular study of genetic resources collection has prepared identification and selection of favourable parents for crossing programs. Cistan local cultivar has higher genetic distance than line TN2026 in each three methods of grouping (table 4, 5, 6). Therefore in order to favourable genes transfer from TN2026 line to Cistan local cultivar should be considerable genetic distance. Using ISJ markers, this study showed that there is a high level of genetic diversity among lentil lines (ranged from 0.052 to 0.312 with an average of 0.211) and using RAPD marker lower level of genetic diversity among lentil lines. This result agrees with previous studies by Vahabi *et al.,* (2008) in pea landress and Duran and Vega (2004) in Lens accessions using RAPD and ISSR markers. In both types of markers, TN2026 line had higher genetic diversity than the other lines and was placed in segregation cluster. Principle component analyses were almost explained 21-22% of the total variation in the ISJ markers and the combined of RAPD and ISJ markers, but 29.35 % of the total variation were explained using the RAPD markers. For morphological characteristics first component three were explained 70-80 % of the total variation and genotypes grouping based on first component three according to the cluster analysis grouping, but for molecular data first component three were explained 10-20 % of the total variation, which it is not favourable for genotypes grouping, but it indicated favourable sampling of primers from the genome regions (Mohammadi *et al.,* 2003). Therefore semi random primers had favourable sampling from the lentil genome to random primers.

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