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Determination of Genetic Relationship among Some Varieties of Chickpea (*Cicer arietinum* L) in Sulaimani by RAPD and ISSR Markers

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Abstract

The molecular evaluation of five chickpea (*Cicer arietinum* L.) varieties [Rania, Chamchamal, Sangaw, FLIP98-133c (screened for their very sensibility to *Ascochyta rabiei*) and FLIP83-48c (screened for their resistance to *Ascochyta rabiei*)] at the University of Sulaimani, College of Agriculture, in 2009-2010 was conducted to assess the genetic diversity and relationship of chickpea genotypes using RAPD and ISSR markers. Five primers of RAPD and ISSR were used of which all primers gave amplification products. On average, 5.8 bands per primer were observed by RAPD and 6.6 bands per primer by ISSR markers. In RAPD, the varieties shared 55.17% polymorphic bands, whereas they shared 63.63% polymorphic bands in ISSR analysis. Cluster analysis by RAPD and ISSR markers revealed clear distinct diversity between genotypes. Rania and Chamchamal showed more similarity than others varieties according to the RAPD data. FLIP83-48c showed the highest dissimilarity comparing with the other varieties. In ISSR analysis, Chamchamal and Sangaw showed more similarity than others varieties. Rania revealed the highest dissimilarity comparing with the others of varieties. Combination of RAPD and ISSR data shared that FLIP83-48c showed the highest dissimilarity comparing with the other varieties. The results showed that ISSR and RAPD analysis for diversity can provide practical information for the management of genetic resources in chickpea breeding program.

Keywords: Chickpea, RAPD, ISSR, Genetic diversity.

1. Introduction

Chickpea (Cicer arietinum L.), as the third most important cool season food legume in the world after dry beans and peas (FAO, 2006), is a diploid, with 2n = 2x = 16 (Arumuganathan *et al.,* 1991) and has a genome size of approximately 931 Mbp. Moreover, chickpea pod covers and seed coats can also be used as fodder. In grain legumes, proteins are an important seed component and are responsible for their relevant nutritional a socio-economic importance. The chickpea seed is a good source of carbohydrates and proteins, which together constitute 80% of the total dry seed weight (Talebi *et al.,* 2008). Two main types of chickpea cultivars are grown globally kabuli and desi, representing two diverse gene pools. The knowledge of genetic diversity is a useful tool in gene-bank management and breeding experiments like tagging of germplasm, identification and/or elimination of duplicates in the gene stock and establishment of core collections Genetic diversity among the parents is a prerequisite to improve the chances of selecting better segregates for various characters (Dwevedi *et al.,* 2009).

Differences between genotypes with regard to agronomic, morphological, biochemical (e.g. storage proteins, isozymes), and molecular characteristics are either indirect or direct representations of differences at the DNA level and are therefore expected to provide information about genetic relationships. The assessment of genetic diversity is important not only for crop improvement but also for efficient management and conservation of germplasm resources. For this purpose 5 varieties of chickpea were analyzed by using random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers.

Polymerase chain reaction (PCR) method, using arbitrary primers, has been widely utilized in the last 20 years. DNA markers have proved valuable in crop breeding, especially in studies on genetic diversity and gene mapping. The commonly used PCR-based DNA marker systems are RAPD, ISSR, amplified fragment length polymorphism (AFLP) and more recently simple sequence repeats (SSRs) or microsatellites (Gupta *et al.,* 2000). The major limitations of some of these methods are high cost of AFLP and the need to know the flanking

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sequences to develop species specific primers for SSR polymorphism.

The RAPD technique, based on the PCR, is one of the most commonly used molecular markers. RAPD markers are amplification products of anonymous DNA sequence using single, short and arbitrary oligonucleotide primer; thus, they do not require prior knowledge of DNA sequence. Low expense efficiency in developing a large number of DNA markers in a short time and requirement for less sophisticated equipment has made the RAPD technique valuable (Bardakci, 2001). RAPD identification techniques can be used at any stage of plant development and they are not affected by environment factors (Lisek *et al.*,2006). The reproducibility of the RAPD techniques can be influenced by variable factor, such as concentration of MgCl2, DNA template; DNA polymerase (Iqbal *et al*., 2002); number of primer; primer sequence; number of PCR cycles (Nkongolo *et al*., 2002) and annealing temperature (Schiliro *et al*., 2001).

ISSR-PCR is a technique overcomes most of these limitations (Zietkiewicz *et al*., 1994). It is rapidly being used by the research community in various fields of plant improvement (Godwin *et al.*, 1997). The technique is useful in areas of genetic diversity, phylogenetic studies, gene tagging, genome mapping and evolutionary biology in a wide range of crop species.

The aim of this study is to evaluate the genetic diversity of chickpea varieties by RAPD and ISSR markers.

2. Materials and Methods

2.1. Plant material

Five varieties of chickpea including Rania, Chamchamal, Sangaw, FLIP98-133c (screened for their very sensibility to Ascochyta rabiei) and FLIP83-48c (screened for their resistance to Ascochyta rabiei) were used in this study (Table 1). All varieties were obtained from Sulaimani Agricultural Research Center, Sulaimani, Iraq. Healthy seeds with identical dimensions were selected by visual observation.

2.2. Genomic DNA extraction and purification

Seeds were planted in a pot for three weeks at University of Sulaimani, College of Agriculture. Watering was done once a day and, after three weeks, healthy leaves were harvested. Total DNA was extracted from three weeks young chickpea leaves following the CTAB procedure (Cingilli *et al*., 2005).

2.3. RAPD analysis

Eight primers were used in this study, only five primers gave the products (Table 2). The reaction mixture (25 µl) contained 10× assay buffer, 2.5 mM MgCl2, 400 µM dNTP's (Fermantas), 5 pmoles of primer, 100 ng template DNA and 1 U of Taq DNA Polymerase (Fermantas). Amplification was carried out in a thermo-cycler (Master cycler) for 40 cycles, each consisting of a denaturation step at 94 °C for 1 min, annealing at 34 and 36 °C for 50 second and an extension step at 72 °C for 2 min. An initial denaturation step at 94 °C for 5 min, and a final synthesis step of 6 min at 72 °C were also included. Amplification products were separated on 1.5% agarose gel in 1X TAE (Tris base, acetic acid and EDTA) buffer.

2.4. ISSR analysis

PCR amplification was performed as described by Ratnaparkhe *et al.* (1998) with some modifications. Eight primers (UBC primers) were used. Only five primers gave the products (Table 3). Amplification was carried for 40 cycles, each consisting of a denaturation step at 94 °C for 1 min, annealing at 50 °C & 52 °C for 1 min. and an extension step at 72°C for 2 min. An initial denaturation step at 94 °C for 5 min, and a final synthesis step of 6 min at 72 °C were also included. Amplification products were separated on 1.5% agarose gel in 1X TAE buffer.

2.5. Data analysis

Following Lynch and Milligan (Lynch *et al.,* 1994) assumptions, each amplified product was treated as an independent locus and assigned numbers in order of decreasing molecular weight. DNA fragment profiles representing a consensus of two independent replicates were scored in a binary fission with '0' indicating the absence and '1' indicating presence of band. Using the binary data, a similarity matrix was constructed using the Jaccard coefficient (Jaccard, 1908), which was further subjected to clustering analysis and a dendrogram was generated. A cophenetic matrix was constructed using the matrix that was used to generate the clusters. A correlation between the cophenetic matrix and the similarity matrix was determined by using SPSS version 18 (Masumbuko *et al.,* 2003).

3. Results and Discussion

3.1. RAPD analysis

RAPD analysis revealed a good polymorphism among chickpea varieties (Figure 1). Five random primers of RAPD were used in this study. An average of 5.8 bands per primer was observed in a total of 29 bands. From RAPD data 44.83% of common bands and 55.17% (Table 4) of polymorphic bands were observed among chickpea varieties. The primer RAPD-2 and RAPD-4 gave rise to maximum bands (7) and RAPD-3 showed the least number of bands (4).

Cluster analysis was carried out depending on the results of RAPD analysis using the SPSS analysis to find the diversity among the given varieties of chickpea as shown in the dendrogram (Figure 2). At Jaccard dissimilarity of distance 1 Rania and Chamchamal showed more similarity than others varieties.

At distance 20, there are 3 groups: group 1: Rania and Chamchamal, group 2: Sangaw and FLIP98-133c, group 3: FLIP83-48c. At distance 25, Rania, Chamchamal, Sangaw and FLIP98-133c are grouped into one cluster while FLIP83-48c varieties in another cluster. FLIP83-48c showed more dissimilarity distance with the rest of the varieties. The similarity matrix varied from 0.08 to 0.88 in chickpea varieties. The highest value of similarity matrix was registered by Chamchamal and Rania while the lowest value of similarity matrix was recorded by Chamchamal and FLIP83-48c (Table 5). In this investigation, RAPD markers showed a high level of polymorphism and a high number of clearly amplified bands.

The RAPD data reported in this study is in agreement with that obtained by other researchers. Extensive DNA

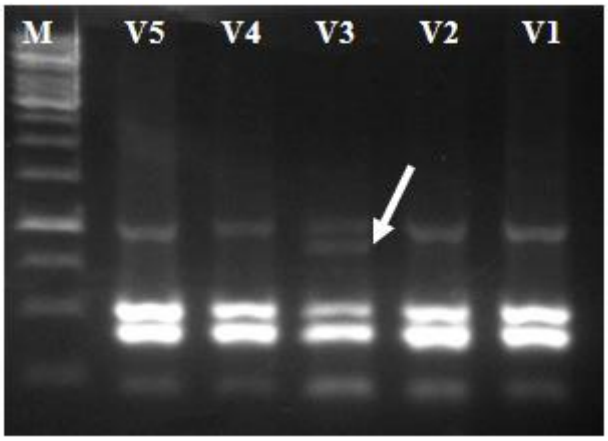

Figure 1. Agarose gel (1.5%) showing the amplified product using RAPD-3 primer. Lane (M): 1 kb DNA ladder, lane (V1): Rania, lane (V2): Chamchamal, lane (V3): Sangaw, lane (V4): FLIP98-133c and lane (V5): FLIP83-48c.

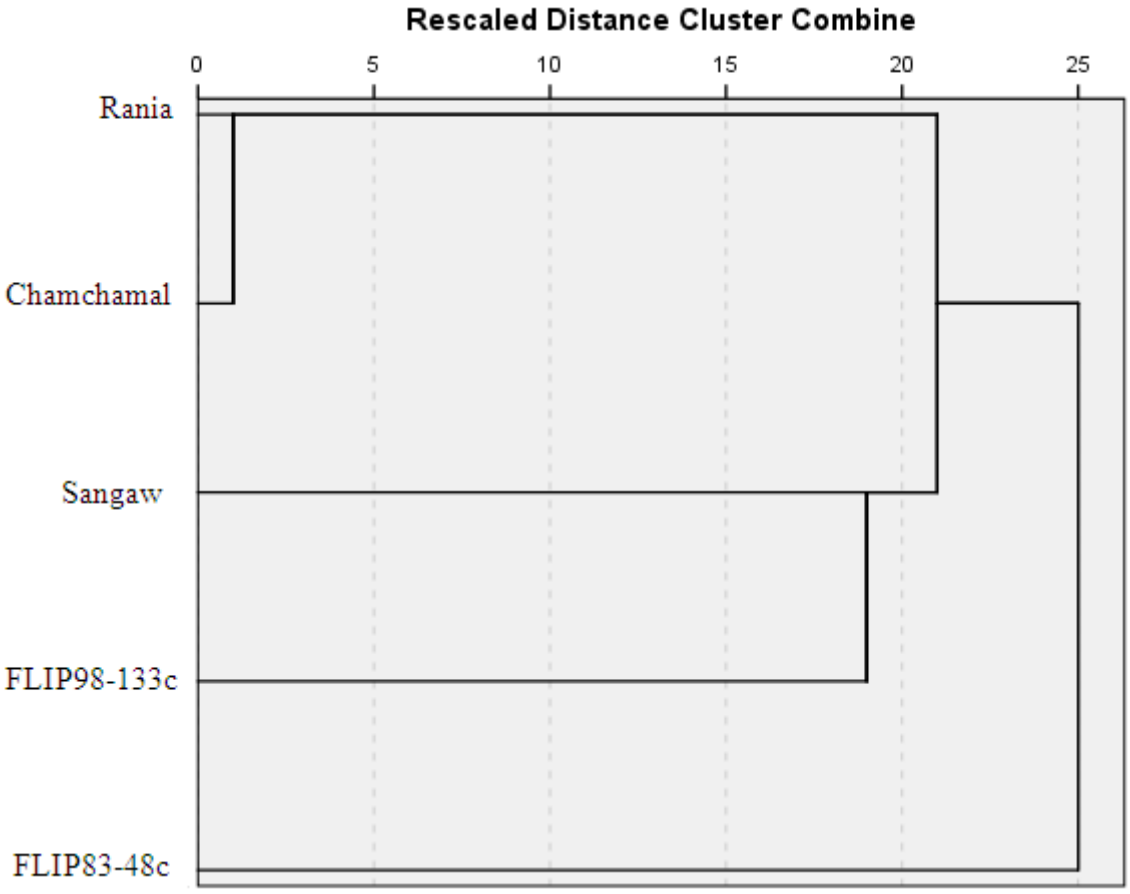

Figure 2. Dendrogram of chickpea varieties showing the genetic similarity based on RAPD data by using cluster analysis.

Table 1. Some agronomic data of chickpea varieties.

Varieties	*Ascochyta rabiei* reaction	Days to maturity
Rania	Susceptible	105
Chamchamal	Susceptible	110
Sangaw	Susceptible	120
FLIP98-133c	Very susceptible	115
FLIP83-48c	Resistant	130

Table 2. RAPD primer with their sequences, percentage of GC and annealing temperature.

Primers	Sequence	GC%	Annealing temperature (^{o}C)
RAPD-1	5'GCGAGTGTG '3	60	36
RAPD-2	5'TCGCTGGTGT '3	60	36
RAPD-3	5'ACAACGCCTC '3	60	36
RAPD-4	5'GGGAACGTGT '3	60	34
RAPD-5	5'GTGATCGCAG '3	60	34

Table 3. ISSR primer with their sequences, percentage of GC and annealing temperature.

Primers	Sequence	GC%	Annealing temperature (^{o}C)
ISSR-1	5' GAAGAAGAAGAAGAAGAA 3'	33	50
ISSR-2	5' ACACACACACACACAC GG 3'	55	50
ISSR-3	5' TGTGTGTGTGTGTGTGAA 3'	44	52
ISSR-4	5' ACACACACACACACACTT 3'	44	52
ISSR-5	5' TGTGTGTGTGTGTGTGGA 3'	50	52

Table 4. Number of amplified fragment, polymorphic fragments, polymorphism percentage, monomorphic fragments and monomorphism percentage based on RAPD data.

Primers	Amplified fragments	Polymorphic fragments	Polymorphism %	Monomorphic fragments	Monomorphism %
RAPD-1	5	3	60	2	40
RAPD-2	7	3	42.85	4	57.14
RAPD-3	4	1	25	3	75
RAPD-4	7	3	42.85	4	57.14
RAPD-5	6	6	100	0	0
Total	**29**	**16**	**55.17**	**13**	**44.83**
Average	**5.8**	**3.2**	**55.17**	**2.6**	**44.83**

Table 5. Jaccard similarity matrix showing the relationship among chickpea varieties based on RAPD data.

	Similarity matrix: Jaccard				
Varieties	**Rania**	**Chamchamal**	**Sangaw**	**FLIP98-133c**	**FLIP83-48c**
Rania	1.00				
Chamchamal	0.88	1.00			
Sangaw	0.31	0.33	1.00		
FLIP98-133c	0.25	0.27	0.33	1.00	
FLIP83-48c	0.15	0.08	0.14	0.27	1.00

polymorphism has been reported using RAPD markers in several other crops (Iruela *et al.,* 2002; Hou *et al.,* 2005). The RAPD-based dendrogram of chickpea genotypes displayed the genetic relationships between these accessions, which accorded with previous studies on chickpea (Ahmad *et al.,* 1992; Tayyar *et al.,* 1996 and Iruela *et al.,* 2002). Although the *Cicer* species are predominantly self-pollinating, more variation was observed among them. The reason for this genetic variation could be that the specific accessions were heterozygous at some marker loci. Similar observations were reported in pea, lentil (Simon *et al.,* 1997), and chickpea (Moussa *et al*., 1996; Sant *et al*., 1999). Iruela *et al.,* 2002) showed that RAPD markers successfully identified genetic variation in *Cicer*. The variation identified was greater than that revealed by the isozymes or seed storage proteins used in previous studies of genetic relationships among annual *Cicer* species (Ahmad *et al.,* 1992; Labdi *et al.,* 1996; Tayyar *et al.,* 1996). Further, large amount of genetic variation which exists between chickpea genotypes can be used efficiently for gene tagging and genome mapping of crosses to introgression the favorable traits such as high yield potential, disease and insect resistance into the cultivated genotypes. Thus, RAPD markers were good indicators of morphological divergence.

3.2. ISSR analysis

The importance and need of chickpea varieties at global level requires evaluation of germplasm to assist the future breeding programs. Hence, it is essential to characterize chickpea germplasm using markers like PCR-based marker such as RFLPs, RAPDs and microsatellites. Five primers (ISSR-1, ISSR-2, ISSR-3, ISSR-4, and ISSR-5) were found to be polymorphic (Figure 3). On an average, 6.6 bands per primer were observed in a total of 33 bands (Table 6). The varieties shared 36.37% common bands and 63.63% polymorphic bands with ISSR markers. Out of five polymorphic ISSR primers, ISSR-2 given the maximum bands (11) and ISSR-3 showed least number of bands (2).

Dendrogram cluster analysis, resulted from ISSR using the SPSS analysis, showed diversity among the given varieties on the bases of similarity matrix of Jaccard. The similarity matrix varied from 0.16 to 1.00 in chickpea varieties (Table 7). The highest value of similarity matrix was registered by Chamchamal and Sangaw while the lowest value of similarity matrix was recorded by Rania and FLIP83-48c. The diagram (Figure 4) revealed four main groups: group 1 includes Chamchamal and Sangaw, group 2 contains FLIP98-133c, group 3 contains FLIP83-48c and group 4 includes Rania. At Jaccard dissimilarity of distance 1, the varieties: Chamchamal and Sangaw showed more similarity than others varieties. At distance 17, there are 3 groups: group 1includes Chamchamal, Sangaw and FLIP98-133c, group 2 contains FLIP83-48c and group 3 contains Rania. The varieties Rania and FLIP83-48c showed more dissimilarity distance with the rest of the varieties.

When compared to the RAPD dendrogram, the ISSR dendrogram showed more correlation with the pedigree data, which shows that the ISSR markers are the most efficient marker system, because of their capacity to reveal several informative bands from single amplification. Similar observations were reported by Bornet and Branchard (2001), Fernandez *et al.* (2002) in barley and Qian *et al.* (2001) in rice. Since ISSR markers are dominant, the similarity at the sequence level of monomorphic bands can be questioned. But numerous studies verified that most co-migrating fragments are identical by descent, at least at the intraspecific level (Wu *et al.,* 2000; Sales *et al.,* 2001). Ratnaparkhe *et al.* (1998) studied the inheritance of Inter-simple sequence repeat polymorphisms and linkage analysis with *Fusarium* resistance gene in chickpea. They demonstrated that a simple sequence repeat (AC) 8YT was linked to the gene for resistance to Fusarium wilt race 4. Rao *et al.* (2007) reported the ISSR fingerprinting in cultivated chickpea and its wild progenitor to correlate the relationship measures based on pedigree data and morphological traits for the selection of good parental material in chickpea breeding programs. Rajesh *et al.* (2003) reported that genetic relationship analysis based on ISSRs supports the morphological and crossability data, ISSRs prove to be an efficient marker system. The diversity thus observed with microsatellites in the chickpea germplasm is probably due to the use of landraces throughout most of the Indian subcontinent (Malhotra *et al.,* 1987; Sant *et al.,* 1999), and even today these landraces are being used for the development of elite cultivars. However, the genetic diversity between the various landraces still remains to be studied and molecular markers will be greatly useful in quantifying this diversity.

3.3. RAPD and ISSR dendrogram

To decrease the inaccuracies of the independent techniques, a dendrogram was developed by pooling the data of both RAPD and ISSR. Two major clusters were observed in this dendrogram (Figure 5). Chamchmal and Sangaw grouped together into one major cluster, whereas

Rania, FLIP98-133c and FLIP83-48c formed the second, third and fourth cluster respectively. The similarity matrix

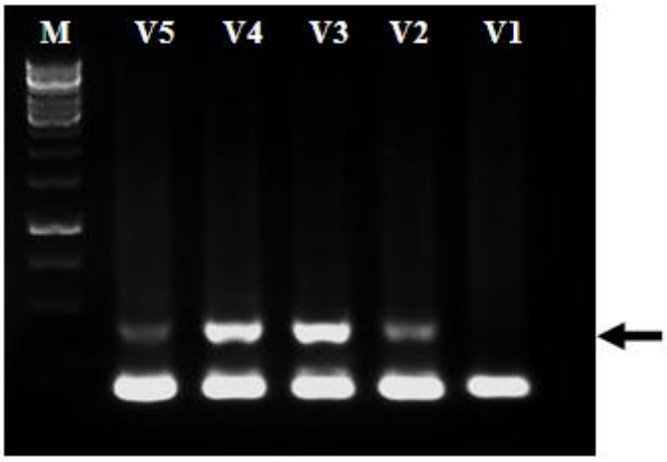

Figure 3. Agarose gel (1.5%) showing the amplified product using ISSR-3 primer. Lane (M): 1 kb DNA ladder, lane (V1): Rania, lane (V2): Chamchamal, lane (V3): Sangaw, lane (V4): FLIP98-133c, and lane (V5): FLIP83-48c.

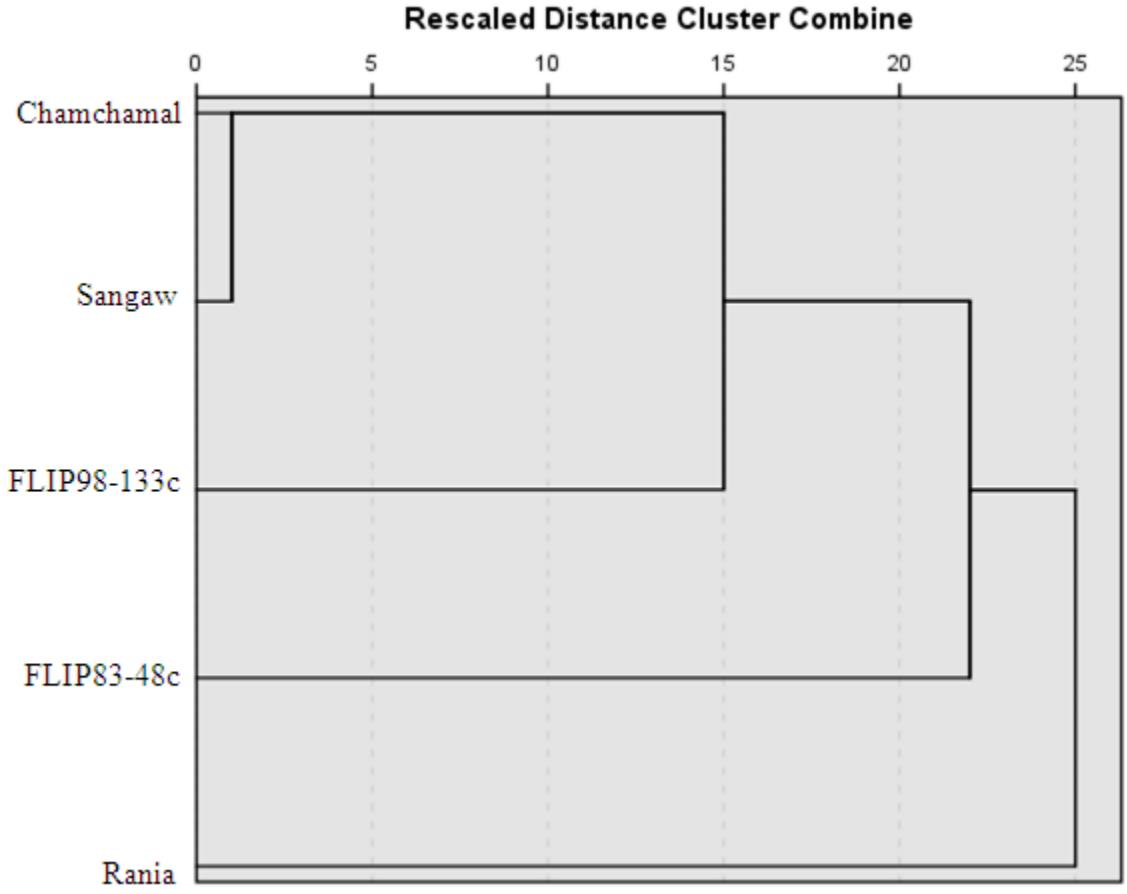

Figure 4. Dendrogram of chickpea varieties showing the genetic similarity based on ISSR data by using cluster analysis.

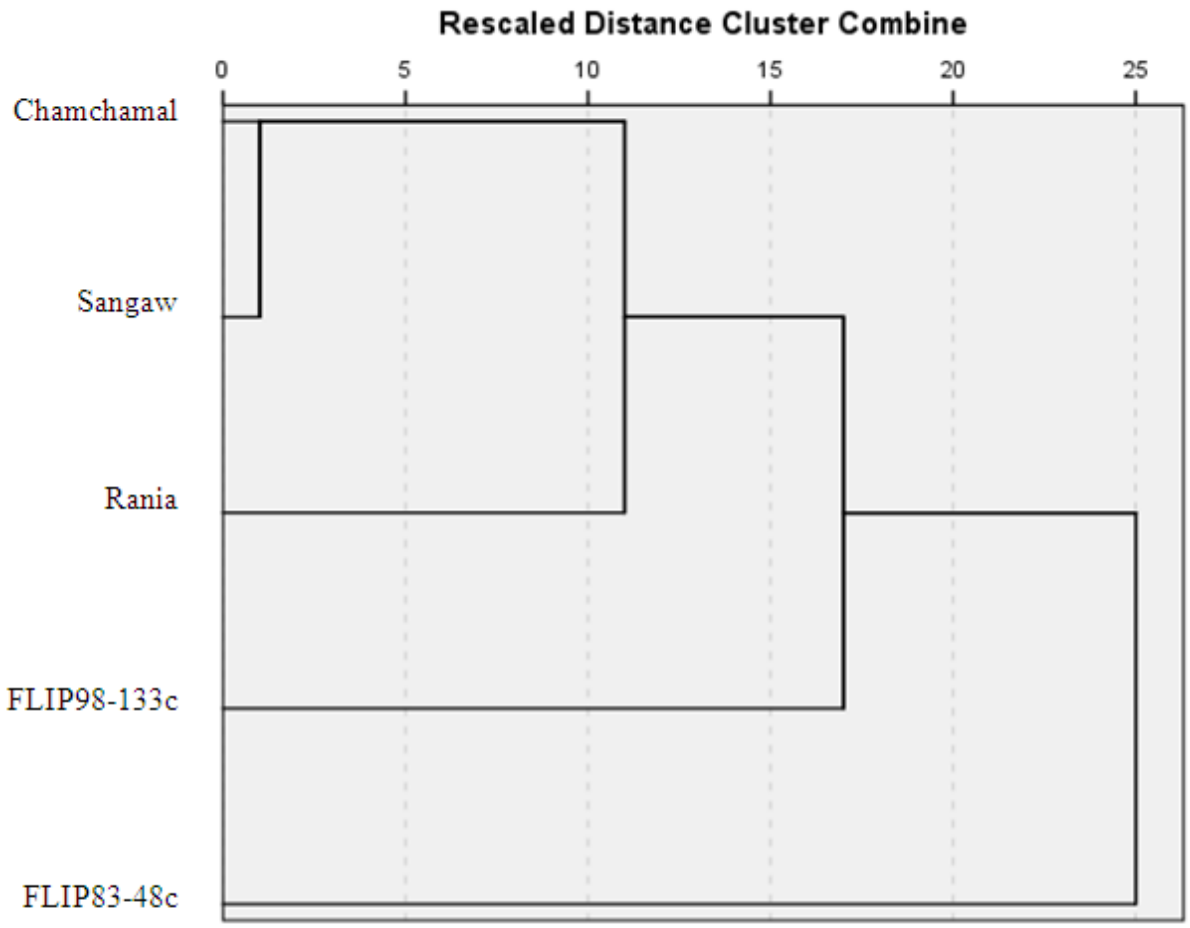

Figure 5. Dendrogram of chickpea varieties showing the genetic similarity based on RAPD and ISSR data by using cluster analysis.

Table 6. Number of amplified fragment, polymorphic fragments, polymorphism percentage, monomorphic fragments and monomorphism percentage based on ISSR data.

Primers	Amplified fragments	Polymorphic fragments	Polymorphism %	Monomorphic fragments	Monomorphism %
ISSR-1	8	5	62.5	3	37.5
ISSR-2	11	7	63.64	4	36.36
ISSR-3	2	1	50	1	50
ISSR-4	6	4	66.67	2	33.33
ISSR-5	6	4	66.67	2	33.33
Total	**33**	**21**	**63.63**	**12**	**36.37**
Average	**6.6**	**4.2**	**63.63**	**2.4**	**36.37**

Table 7. Jaccard Similarity matrix showing the relationship among chickpea varieties based on ISSR data.

	Similarity matrix: Jaccard				
Varieties	**Rania**	**Chamchamal**	**Sangaw**	**FLIP98-133c**	**FLIP83-48c**
Rania	1.00				
Chamchamal	0.44	1.00			
Sangaw	0.44	1.00	1.00		
FLIP98-133c	0.22	0.62	0.62	1.00	
FLIP83-48c	0.16	0.50	0.50	0.25	1.00

Table 8. Jaccard Similarity matrix showing the relationship among chickpea varieties based on RAPD and ISSR data.

	Similarity matrix Jaccard				
Varieties	**Rania**	**Chamchamal**	**Sangaw**	**FLIP98-133c**	**FLIP83-48c**
Rania	1.00				
Chamchamal	0.58	1.00			
Sangaw	0.38	0.65	1.00		
FLIP98-133c	0.23	0.46	0.48	1.00	
FLIP83-48c	0.16	0.30	0.32	0.26	1.00

for the chickpea varieties varies from 0.16 to 0.65 (Table 8), whereas the maximum value of similarity shared by Chamachamal and Sangaw whereas Rania and FLIP83-48c revealed the minimum values. FLIP83-48c showed the highest dissimilarity comparing with the others of varieties.

This observation was consistent with the study of Simon and Muehlbauer (1997), who detected variation within single *C. reticulatum* accession (PI 489777), used to generate an interspecific mapping population. Our results are in accordance with Iruela *et al.* (2002). Iruela reported the genetic diversity among *C. arietinum* varieties using RAPD and ISSR. Shan *et al*. (2005) showed that a natural hybrid could be useful for bridging crosses to introduce genes to chickpea from incompatible species given that *C. reticulatum* was the wild progenitor of chickpea.

ISSR analysis is more economical and reliable than that of RAPD. Earlier studies also reported that ISSR technique generates large number of polymorphisms in chickpea (Collard *et al*., 2003a). The phylogenetic relationship between *Cicer* species from this study was overall consistent with most previous studies (Croser *et al.*, 2003; Nguyen *et al.*, 2004; Sudupak, 2004).

4. Conclusions

The present investigation demonstrates the potential of RAPD and ISSR fingerprinting in detecting polymorphism among chickpea varieties. Varieties FLIP83-48c showed the highest dissimilarity comparing to others varieties. Genetic information obtained from RAPD and ISSR markers can be used in discriminating chickpea varieties and can complement the genetic information generated from the morphological traits. Further, the genetic variation which exists between chickpea varieties can be used efficiently in plant breeding.

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