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Genetic Relationships among Chickpea (*Cicer arietinum*) Elite Lines Based on RAPD and Agronomic Markers

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ABSTRACT

Utilization of diverse germplasm is needed to enhance the genetic diversity of cultivars. Genetically diverse lines provide ample opportunity to create favourable gene combinations and the probability of producing a unique genotypes increases in proportion to the number of gene by which the parents differ. The objective of this study was to evaluate the genetic relationships of 36 chickpea germplasm accessions using morphological traits and RAPD markers. Out of 33 primers, nine primers generated 44 polymorphic markers. The average polymorphic information content (PIC) was 0.43, ranging from 0.68 to 0.12. The lowest and the highest PIC value were recorded for primer OPB10 and OPJ-20, respectively. The average genetic distance (GD), based on F_{st} values among the 36 accessions was 0.397, ranging from 0.59 to 0.12. Cluster analysis based on morphological traits separated the accessions into three groups and based on RAPD data accessions formed in four distinct groups. The RAPD analysis clearly indicated that even with nine polymorphic primers, reliable estimation of genetic diversity could be obtained. The markers generated by RAPD assays can provide practical information for the management of genetic diversity.

Key Words: Chickpea; Genetic diversity; RAPD; Morphological traits

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the second most important cool season pulse crop in the world and is grown in at least 33 countries including central and west Asia, south Europe, Ethiopia, North Africa, north and South America and Australia (Ladizinsky & Alder, 1976; Singh & Ocampo, 1997). Chickpea is important because it provides food for humans as well as for livestock. Furthermore, 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 important. The chickpea seed is a good source of carbohydrates and proteins, which together constitute 80% of the total dry seed weight. West Asia, including Iran is known to be a genetic diversity centre and rich in both landrace and wild relatives of chickpea (Singh & Ocampo, 1997). Modern plant breeding and agricultural systems have narrowed the genetic base of cultivated chickpea (Robertson *et al.*, 1997). This has promoted the search for new sources of variation that might be used in plant breeding programs (Brown *et al.*, 1990).

Assessment of the extent of genetic variability within chickpea is fundamental for chickpea breeding and the conservation of genetic resources and is particularly useful as a general guide in the choice of parents for breeding hybrids. Criteria for the estimation of the genetic diversity can be different, which include morphological traits (Upadhaya *et al.*, 2007) or molecular markers (Sharma *et al.*, 1995). Molecular markers have proved to be valuable tools in the characterization and evaluation of genetic diversity within and between species and populations. A number of DNA based markers are now available for the effective quantification of genetic variation in plant species. Restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) have been applied successfully and have provided considerable genetic information in a number of plant species (Vos *et al.*, 1995; Xu *et al.*, 2000). These techniques are slow and expensive and are not amenable for assessment of genetic variation in large scale population genetic studies. More recently, PCR-based RAPD and simple sequence repeat (SSR) markers requiring small amounts of DNA have also been developed (Williams *et al*., 1990). SSR markers have proved to be polymorphic but require nucleotide information for primer design (Sun *et al*., 1998). RAPD methodology overcomes this limitation; considerable polymorphic markers can be obtained with relative ease from minute amounts of genomic DNA without prior knowledge of sequence information.

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RAPD technique are highly polymorphic and useful in studies on chickpea for genetic diversity (Ratnaparkhe *et al.*, 1998), phylogeny (Iruela *et al.*, 2002), gene tagging (Rajesh *et al.*, 2002) and evolutionary biology (Reddy *at al.*, 2002). Abundance and polymorphism of 38 different microsatellite have been studied in four chickpea accessions (Sharma *et al*., 1995). To our knowledge, no comprehensive effort has been made to investigate Iranian elite chickpea genotypes using DNA markers. The aims of this research were to study genetic diversity in the Iranian elite chickpea germplasm using RAPD markers, morphological traits and to provide a better understanding of the genetic diversity in chickpea collection for development of superior chickpea genotypes.

MATERIALS AND METHODS

Plant materials and morphological traits. Thirty six chickpea (*Cicer arietinum* L.) genotypes were obtained from the Agricultural Research Station of Kordestan, Sanandaj, Iran. (Table I) and evaluated at the Kurdistan Agricultural Research Station, 30 km west of Sanandaj, Iran, in 2005. The trials were carried out in a randomized complete block design with three replicates: each plot consisting of one row (2 m length) with 10 plants sown by hand. Measurements and observations of examined characters were done on five plants which had been randomly chosen in the mid-row of each plat. The following measurements and observation were made: day to heading and maturity, plant height, number of pods per 10 plants, number of seeds per 10 pods, 100-seed weight, number of primary and secondary branches and yield per plant.

RAPD analysis. Healthy leaves harvested from the plants were used for DNA extraction. Total genomic DNA was isolated using a CTAB method (Saghai-Maroof *et al.*, 1988) with minor modifications. DNA quantity and quality were measured with a UV-spectrophotometer. RAPD assay were performed using 33 random 10-mer oligonucleotide primers from Sina-Gene technologies (Iran). After screening 33 primers, RAPD analysis was conducted using 9 most polymorphic primers (Table II). PCR reaction were carried out in a 25 µL reaction mix containing approximately 15 ng template DNA, 2.5 pmol of each dNTP (Sina-Gene), 5 pmol of a single 10-mer primer, 1 unit of Taq DNA polymerase (Sina-Gene). The buffer [10 mm Tris-HCl, PH 8.8 at 25°C, 1.5 mm $MgCl_2$, 50 mm KCl, 0.1% Triton X-100] used as per instructions of the manufacturer. The PCR reaction were run in a Ependorf thermal cycler (Ependorf, Germany) programmed for an initial denaturation step of 3 min at 94°C followed by 35 cycle of 30 s at 94°C, 30 s at 34°C and 1.5 min at 72°C. A final elongation step of 5 min at 72°C was included. The PCR products were separated on 1.5% agarose gel run in 1 X TBE and stained with ethidium bromide. The gels were visualized with a UV trans-illuminator. All PCR reactions were run in triplicate and only reproducible and clear bands were scored.

Data analysis. The RAPD markers were scored for presence (1), absence (0) or as missing observation (9) and each band regarded as locus. The genetic similarities (GS) were calculated according to Nei and Li (1979). Based on similarity matrix, a dendrogram showing the genetic relationships between genotypes was constructed using the un-weighted pair group method with arithmetic average (UPGMA) through the software NTSYS-pc program (Rohlf, 1992). Polymorphic information content (PIC) values were calculated for each RAPD primers according to the formula: $PIC= 1- \Sigma(P_{ij})^2$, where P_{ij} is the frequency of the i^{th} pattern revealed by the j^{th} primer summed across all patterns revealed by the primers (Botstein *et al*., 1980). The average genetic distance (GD) between any two cultivars is represented by its F_{st} value and refers to as inter-cultivar distance. F_{st} values were used as input data for two-dimensional principal coordinate analysis (2D PcoA) (Huff, 1997). Data for morphological traits were standardized as described by Rodan-Ruiz *et al*. (2001) and used to calculate a dendrogram showing the genetic relationship between genotypes. The 2D PcoA was made on the basis of a distance matrix.

Table I. List of 36 chickpea accessions used for genetic diversity studies

Entry No.	Name	Entry No.	Name
1	FLIP97-111C	19	FLIP01-39C
2	FLIP98-91C	20	FLIP01-40C
3	FLIP98-117C	21	FLIP01-43C
4	FLIP99-26C	22	FLIP01-49C
5	FLIP99-45C	23	FLIP01-50C
6	FLIP99-46C	24	FLIP01-52C
7	FLIP00-1C	25	FLIP01-54C
8	FLIP00-18C	26	FLIP01-57C
9	FLIP00-20C	27	FLIP02-06C
10	FLIP00-24C	28	FLIP02-15C
11	FLIP01-1C	29	FLIP02-21C
12	FLIP01-5C	30	FLIP02-47C
13	FLIP01-6C	31	FLIP02-84C
14	FLIP01-24C	32	ILC482
15	FLIP01-30C	33	FLIP82-150C
16	FLIP01-32C	34	FLIP88-85C
17	FLIP01-33C	35	FLIP93-93C
18	FLIP01-38C	36	JAM

RESULTS

Morphological analyses. In variance analysis the genotype variance was significant for six of the nine observed traits (plant high, 100 seed weight, day to maturity, number of pods per 10 plants, number of seeds per 10 pods & yield) (Table III). This indicated that differences existed between the accessions for these traits. Thirty six genotypes were grouped into three clusters (Fig. 1). Cluster-I contained maximum number (28) of genotypes. These genotypes were of medium flowering duration, medium pod number and medium grain yield per plant. Cluster-II comprised three genotypes, which were of earliest flowering duration, less number of pods and low grain yield. Five genotypes falling in cluster-III were of later flowering duration, high number of pods and high grain yield. The principal coordinate analysis (PcoA) was used to provide a reduced dimension

Table II. Nucleotide sequences of 9 polymorphic primers and PIC values in RAPD analysis

Primer	Primer Sequence 5′ to 3′	Percentage G+C content	Approximate size of fragment (kb)	PIC Value
OPA10	GTGATCGCAG	60	320-2100	0.53
OPJ06	TCGTTCCGCA	60	300-1650	0.49
OPM05	GGGAACGTGT	60	250-1950	0.47
OPJ20	AAGCGGCCTC	70	260-1950	0.68
OPB10	CTGCTGGGAC	70	400-1500	0.12
OPAC09	AGAGCGTACC	60	270-2200	0.46
OPF09	CCAAGCTTCC	60	300-1750	0.55
OPI-13	CTGGGGCTGA	70	220-1600	0.33
OPQ05	CCGCGTCTTG	70	270-1480	0.26

Table III. Estimation of components of variance due to genotypes for 9 agronomic traits in chickpea germplasm evaluated at Kurdistan Agricultural Research Station, Sanandaj, Iran

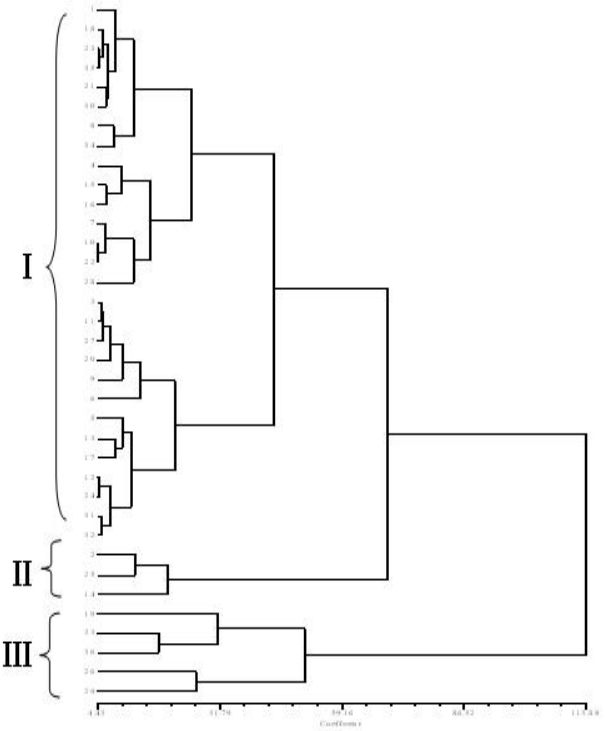
S.O.V	df	Plant hight	100 seed weight	Primary branches (no.)	Secondary branch (n.)	Day to maturity	Day to heading	Pods per plants (no.)	10 Seeds per 10 pod (no.)	Yield (kg ha^{-1})
Replication®	2	10.56	39.3*	0.75	51.17	419.6*	250.34	18.34**	40.5**	427282.4**
Variety	35	20.52*	38.8*	0.50	3.99	781.08**	397.2	18.64**	22.81*	83380.92**
Error	70	9.86	7.6	0.34	5.59	292.6	220.45	6.16	11.04	42661.7
C.V.	-	18.2	13.56	21.2	22.1	8.7	19.7	6.33	9.1	10.27

* Significant at P= 0.05; ** Significant at P= 0.01

model that would indicate measured differences among 36 diverse accessions identified in this study (Fig. 3).

RAPD analyses. The average polymorphic information content (PIC) was 0.43, ranging from 0.68 to 0.12. The lowest and the highest PIC value were recorded for primer OPB10 and OPJ-20, respectively. The average GD, based on F_{st} values among the 36 accessions was 0.397, ranged from 0.59 to 0.12. Of the 33 primers screened, three primers did not amplify or gave poor amplification products, while seven primers showed complex or non-repeatable banding patterns. Twenty three primers produced reproducible bands and of these, only 9 primers generated 44 polymorphic markers (Table II). In order to determine the ability of RAPD analysis to display genetic relationships among accessions, principle co-ordinate analysis (PCo) was carried out and accessions were plotted in the coordinate system for the first two coordinates which accounted for 61.30 and 9.96% of the variation respectively. PCo provided a better graphical illustration and a clear separation of species (Fig. 4). From the UPGMA dendrogram (Fig. 2), it was discernible that the material taken for the analysis can be divided in to four major group. Cluster-I and II contained maximum number of genotypes. These genotypes, as result obtained from morphological-based dendrogram, were of medium flowering duration and medium grain yield per plant. Other two clusters completely accorded with morphological-based dendrogram.

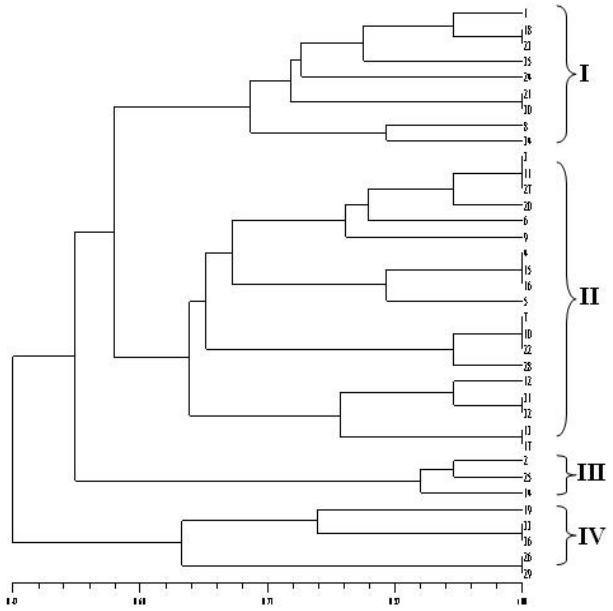
Fig. 1. Dendrogram of 36 chickpea accessions based on morphological data using UPGMA



DISCUSSION

In this investigation, RAPD markers showed a high level of polymorphism and a high number of clearly amplified bands (Fig. 5). Extensive DNA polymorphism has been reported using RAPD markers in several other crops plants (Hilu & Stalker, 1995; 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 & Waines, 1996; Iruela *et al.*, 2002). It was evident from the results that the dendrogram based on RAPD-markers was accord with the dendrogram based on morphological traits, as reported for other crops (Loarce *et al.*, 1996; Fernandez *et al.*, 2002).

Fig. 2. Dendrogram of 36 chickpea accessions based on RAPD data using UPGMA



Pairs of cultivars numbers 12 and 24, 34 and 4, 5 and 13 were closely related in the morphological based analysis but much less related in RAPD analysis. Weak correlation between RAPD data and agronomical records in research for other crops were reported by several authors (Barret *et al.*, 1998; Maric *et al.*, 2004). There are several possible explanations for such results: some of them connected with RAPD technique. Sensitivity of the working conditions and equipment used can influence the result of RAPD markers (Davos & Gale, 1992).

Another problem was the possibility of overestimating genetic similarity because fragments with the same size could have different origins. In addition to the cluster analysis, 2D principal component analysis (PcoA) was carried out to determine whether accessions of chickpea could be resolved further. PcoA analysis using the first two principal coordinates provided a good grouping of accessions in the co-ordinate system, confirming the relationships in the dendrogram. Differences observed between the RAPD-based PcoA and morphological trait-based PcoA could be due to several reasons. The number of loci sampled in the two studies, the error associated with each technique and the method of genetic distances, could cause the differences. On the other hand, the relationship observed using molecular markers may provide information on the history and biology of cultivars, but it does not necessarily reflected what may be observed with respect to agronomic traits (Metais *et al.*, 2000). Previous studies have shown that the measurements of genetic divergence obtained from morpho-agronomical traits are not completely similar to RAPD-based results. A possible explanation is because we do not know what linkage associations exist between markers and genes that control

Fig. 3. Two-dimensional principal coordinate analysis of cultivars examined based on morphological traits

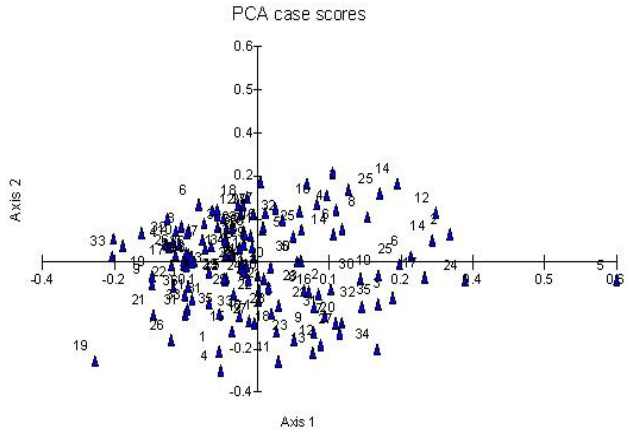
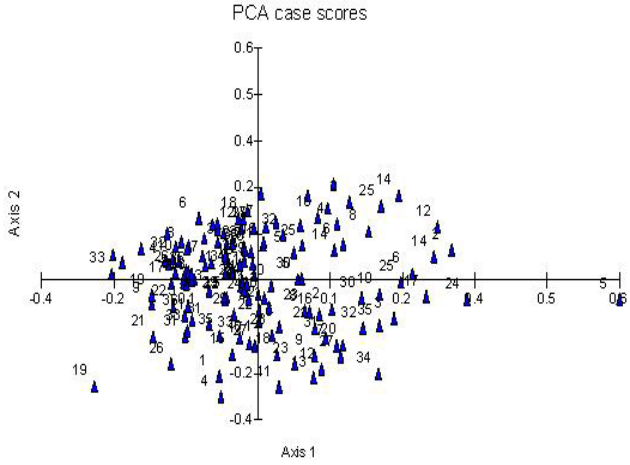


Fig. 4. Two-dimensional principal coordinate analysis of cultivars examined based on random amplified polymorphic DNA (RAPD) data



morpho-agronomical traits. For a trait like yield, it is likely that there were many controlling genes spread throughout the genome.

In present study, the correlation between the genetic distances obtained with RAPD and morphological traits, indicating that there is a strong multi-locus association between molecular and morphological traits in these cultivars. Thus, RAPD markers were good indicators of morphological divergence. Limited evaluation of a chickpea lines in this study led to the identification of genetic variation, which exists in the accessions. It is expected that when such diverse lines are involved in breeding programs, as a result of reshuffling of the alleles due to recombination, there are better chances for the appearance of transgressive segregation with beneficial traits that can be selected to extract high yielding lines with desirable trait combination. Further, large amount of genetic variation which exists between chickpea genotypes can be used efficiently for gene tagging and genome mapping of crosses to introgress the

favorable traits such as high yield potential, disease and insect resistance into the cultivated genotypes.

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