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DNA polymorphisms in chickpea accessions as revealed by PCR-based markers

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Abstract

Chickpea is a food legume which is alleged to be a preferred source of protein next only to milk. Germplasm of cultivated chickpea available is deficient in desired genetic variation. Genetic manipulations therefore, necessitate the genetic exploitation of its related annual and wild species. 42 RAPD and 41 ISSR markers were employed to ascertain polymorphism across 20 genotypes which were collected from 10 different geographical areas of the world. RAPD marker detected 51% genetic polymorphisms while ISSR marker detected 54 %. With an average of 6.5 each RAPD primer amplified 5-8 bands. Similarly with an average of 7.9 each ISSR primer amplified 4-12 bands. The cluster dendrogram demonstrated a similarity coefficient range from 0.80 to 0.92 due to RAPD markers, whereas with ISSR primers the cluster dendrogram showed similarity coefficient of 0.60 to 1.00. Accessions from same geographical area seem to be genetically similar than those from geographically distant and isolated ones. When however compared, interestingly the ISSR dendrogram showed more correlation with pedigree data than the RAPD dendrogram. The variability index worked out in the present study ranges from 0.79 to 0.96. Since the ultimate reason for such studies is selection of diverse genetic accessions for their recommendation to breeding programmers, the accessions like ICC6263, ICC6306 and ICC17160 can be recommended as parents. Further breeding programmes can therefore be planned to procure additional variation complexes in chickpea genetic stocks.

Key words: Chickpea, RAPD, ISSR, Polymorphism.

Introduction

Chickpea constitutes one of the major food legumes in countries across the world, both developed and developing (1). Crop improvement programmes are being vigorously pursued. However, traditional classic breeding approaches are very slow. Further possibilities to ascertain any useful traits from wild germplasm are limited due to strong crossability barriers. Plant Genetic Resources constitute an important components of the world agro-biodiversity. International Crop Research Institute for Semi-Arid Tropics (ICRISAT) Patancheru, (A.P.) India has been front runner in collecting and preserving more than 17,000 chickpea accessions from various parts of the world (2). Germplasm with broad genetic variability is a primary need to breed and develop disease free stable high yielding cultivars. Such diverse genetic lines provide an opportunity to create favorable gene combinations in cultivars with a probability of producing elite genotype. The genetic diversity thus increases in proportion to the number of genes by which the parents differ (3). In recent past, reference genome of the CDC frontier kabuli chickpea variety has been sequenced and by 2013 genome sequences of 90 cultivated and wild genotypes from 10 different countries were made available online. This draft revealed 28,269 genes in kabui chickpea providing clues on the sequences that can be useful for crop improvement. Recent breeding efforts over the past 60 years have been restricted to the limited introduction of diverse germplasm. More so, being a self-pollinated winter crop, available germplasm of cultivated chickpea shows a low genetic variation profile. This therefore, further necessitates the exploitation of germplasm from other related annual *Cicer* species vis-à-vis from both wild and cultivated forms for the desired genetic traits. Such molecular breeding approaches may further provide insights into both genome diversity and domestication (4, 5).

This study is therefore an attempt to access the polymorphism at molecular level within and amongst the various accessions of chickpea totaling 20 in all. The interesting facet of the work being the representation of those chickpea genotypes encompassing various geoniches of the world. There seems to be a genuine lack of information regarding comparative evaluation of molecular signatures in pulses. There are therefore issues which pertain to marker suitability, their number and combinations which could be employed as appropriate markers of utility for desired parameters. This therefore becomes a primary need in every crop species for better and effective management, characterization and utilization of germplasm pool to its large scale exploitation. The same therefore stands true for chickpea also. Advances in genomic research have provided new tools in crop improvement programmes and use of markers is one of those. Genotyping using DNA markers has now become an integral parameter for labelling genetic signatures in plant breeding and germplasm management. These now are seen to provide fresh inquisitive methods for observing genetic difference among species. Time taken is less with other larger advantages over conventional methods. Employing presently available molecular techniques, DNA markers of interest can be deve-

loped using PCR-based approaches for total or segmental plant genome analysis. Properties of molecular markers and their applications in research have already been reviewed (6).

Use of Random amplified polymorphic DNA (RAPD) is one such amplification based multi locus markers. It is a simplest and fastest detection technique, successfully employed for the determination of intraspecific genetic diversity in several legume seeds vis. *Vigna radiata* (7), *Lens culinaris* (8), *Vigna unguiculata* (9), *Phaseolus vulgaris* L (10), *Glycine max* (11), *Pisum sativum* (12) and *Cajanus cajan* (13). This analysis has also been used in the study of chickpea species cum lines for their genetic diversity (14), phylogeny (15), gene tagging (16) and evolutionary biology (17). Inter simple sequence repeats (ISSR) analysis is an another important molecular technique involving PCR amplification of targeted DNA regions between adjacent and inversely oriented microsatellites. It uses a single simple sequence repeat (SSR) motif. ISSR markers which are linked to certain traits of agronomic importance in crops have been sequenced and are being used as Sequence Tagged Sites (STS) in the marker aided selection of these sites (17). ISSR fingerprinting has successfully been applied to determine genetic diversity and relationships in a number of crop species (18, 19, 20).

The published data worldwide with regard to genetic diversity analysis of chickpea using molecular markers is diverse in itself. Such reports include chickpea genotypes of Indian origin (21) from those of north-west of Iran (22) Turkey (23). However, DNA marker based genetic variance studies among diverse genotypes collected from other world collections are scanty. Present study therefore entail at identifying polymorphy within accessions employing RAPD and ISSR markers. This data can then further be converted into discrete character data for assessing inter genetic relationships. The estimates of PCR based analysis can help to identify accessions with a potential for pulse improvement programmes. Also the data can be employed to document the markers in gene banks for plant genetic resources of chickpea posterity.

Figure 1. The country wise localization of *Cicer* accessions from different parts of world as used in the present study.

Materials and methods

Plant materials

The plant material procured as seeds comprised of twenty accessions of chickpea which also included the species of *Cicer arietinum* and *Cicer reticulatum.* These collections were encompass ten different geographic origin of world as shown in the fig.1. Of the 20 seed types, fifteen were cultivated and five wild type accessions. The passport details of these are given in the table 1. These accessions were collected from International Crop Research Institute for Semi-Arid Tropics (ICRISAT) Patancheru, (A.P.) India.

For DNA extraction, a single seed from each accession was germinated in sterile pot with sterile soil and allowed to grow in a growth chamber for 2 to 3 weeks. Fresh young leaves of the seedlings were harvested at 15 days.

DNA isolation

DNA was isolated from the leaves of each accession, using the CTAB extraction method of Talebi (24) with minor modification. 100mg leaf material was ground in the liquid nitrogen followed by homogenization with 1ml freshly prepared extraction buffer. To this, 20% was added and incubated at 60ºC for 30 minutes. Then

Table 1. List of *Cicer* accessions used in the study *vis-a-vis* their types and places of origin.

S. N.	*Cicer species*	Accessions	Source	Seed types	Origin
01	*C. arietinum*	ICC1180	ICRISAT	Desi	India
02	*C. arietinum*	ICC14051	ICRISAT	Desi	Ethopia
03	*C. arietinum*	ICC13441	ICRISAT	Kabuli	Iran
04	*C. arietinum*	ICC15518	ICRISAT	Kabuli	Nepal
05	*C. arietinum*	ICC11944	ICRISAT	Desi	Morocco
06	*C. arietinum*	ICC12328	ICRISAT	Desi	Cyprus
07	*C. arietinum*	ICC6306	ICRISAT	Desi	Russia &CIS
08	*C. arietinum*	ICC16269	ICRISAT	Desi	Malawi
09	*C. arietinum*	ICC637	ICRISAT	Desi	India
10	*C. arietinum*	ICC5434	ICRISAT	Desi	India
11	*C. arietinum*	ICC7554	ICRISAT	Desi	Iran
12	*C. arietinum*	ICC12537	ICRISAT	Desi	Ethiopia
13	*C. arietinum*	ICC6537	ICRISAT	Desi	Iran
14	*C. arietinum*	ICC 12028	ICRISAT	Desi	Mexico
15	*C. arietinum*	ICC6263	ICRISAT	Desi	Russia & CIS
16	*C. reticulatum*	ICC17164	ICRISAT	Desi	Turkey
17	*C. reticulatum*	ICC17163	ICRISAT	Desi	Turkey
18	*C. reticulatum*	ICC17123	ICRISAT	Desi	Turkey
19	*C. reticulatum*	ICC17121	ICRISAT	Desi	Turkey
20	*C. reticulatum*	ICC17160	ICRISAT	Desi	Turkey

after, 92µl of 5M NaCl was added and subsequently, 75µl of CTAB solution was further added and reincubated done at 65ºC for 15 minutes in a water bath. To this cocktail, 300µl of chloroform: isoamyl alcohol mix (24:1) was added. This was followed by centrifugation at 12000g for 15 minutes at 4ºC in a Sigma centrifuge 3-16 K. Chloroform: isoamyl alcohol mix was readded to the supernatant in 1:1 volume and recentrifuged at 12000g for 15 minutes at 4ºC. Subsequently precipitation was done by adding chilled isopropanol 40% v/v as final concentration. The precipitated DNA was then centrifuged as pellet and cleared with 70% ethanol. The ethanol washed DNA was air dried overnight and dissolved in 100µl of Tris-EDTA buffer (19mM Tris-HCl pH 8.0; 1mM EDTA pH 8.0). Extracted DNA was stored at -20ºC.

RAPD and ISSR primers

42 RAPD primers (Operon Tech. Inc. Almeda, USA) were used for analysis. A set of 41 ISSR primers representing di, tri, tetra and pentamer repeats were procured from the Biotechnology Laboratory, University of British Columbia, Canada. Out of these, 15 RAPD and 19 ISSR primers that formed clear and reproducible polymorphic bands. Only these were therefore chosen and used for further genotyping of chickpea accessions. Details of both the primers i.e. RAPD and ISSR are represented in the table 2 and 3 respectively.

RAPD-PCR

The PCR procedure of Williams and Bhagyawant (25, 18) was followed with some minor modifications. Reaction was carried out in 25µl reaction volumes containing 10mM Tris-HCl pH 9.0; 50mM KCl; 0.1% Triton-x-100; 1.5mM $MgCl_2$; 0.1mM dNTP; 2mM primer; 0.5 unit of Taq DNA polymerase (*MBI, Fermentas, Richlands B. C., Qld*) and 25 ng template DNA. Amplifications were carried out in a thermo-cycler (Bio-Rad 3.03 version) programmed for 35 cycle with an initial melting at 94°C for 4 min followed by denaturation at 94°C for 1 min. The annealing was performed at 37°C for 1 min, then followed by polymerization at 72°C for 2 min. Final extension step was through at 72°C for 7 min.

ISSR-PCR

The PCR procedure of Welsh (26) was followed with minor modification as follows. The reaction cocktail contained 25µl reaction volumes containing 10mM Tris-HCl pH 9.0; 50mM KCl; 0.1% Triton-x-100; 1.5mM $MgCl_2$; 0.1mM dNTP; 2mM primer; 0.5 unit of *Taq* DNA polymerase (*MBI, Fermentas, Richlands B. C.*) and 25 ng template DNA. Amplifications were carried out in a Bio-Rad 3.03 *version* thermo-cycler. Programme was set for 35 cycles with an initial melting at 94°C for 4 minutes, followed by denaturation at 94°C for 1 minute. The annealing was performed at 56°C for

Table 2. Representative RAPD primers and their sequences along with some amplification characteristics.

S.N.	Primer	Sequence	Number of bands	Number of polymorphic bands	% Polymorphism
01	B10	CTGCTGGGAC	145	34	23.4
02	OPA04	AATCGGGCTG	131	65	49.6
03	OPA07	GAAACGGGTG	168	123	73.2
04	OPA08	GTGACGTAGG	179	128	71.50
05	OPA11	CAATCGCCGT	71	43	60.5
06	OPA12	TCGGCGATAG	167	142	85.02
07	OPA15	TTCCGAACCC	130	92	70.76
08	OPA16	AGCCAGCGAA	87	45	51.72
09	OPA17	GACCGCTTGT	118	67	56.77
10	OPA18	AGGTGACCGT	101	87	86.13
11	OPA20	CAAACGTCGG	94	39	41.48
12	OPE10	GTTGCGATCC	138	55	39.85
13	OPZ06	GTGCCGTTCA	147	53	36.05
14	OPZ10	CCGACAACC	131	65	49.61
15	OPZ16	TCCCCATCAC	81	06	7.40

Table 3. Representative ISSR primers and their sequences along with some amplification characteristics.

S.N.	Primer	Sequence	Number of bands	Number of polymorphic bands	(%) Polymorphism
01	IS-01	$(TAA)_6$	113	48	42.47
02	IS-05	$(CAG)_6$	129	54	41.86
03	IS-07	$(CCT)_6$	152	12	7.89
04	IS-09	$(TCC)_6$	136	136	100
05	IS-12	$(ACT)_6$	65	65	100
06	IS-23	$(TG)_8G$	160	105	65.62
07	IS-27	$(AC)_8YA$	154	54	35.06
08	IS-32	$(GGC)_6$	102	39	38.23

1 minute, followed by polymerization at 72°C for 2 minutes. Final extension step was carried out at 72°C for 7 minutes.

Agarose gel electrophoresis and dendrogram construction

Amplification products were separated by electrophoresis on 1.2 % agarose gel run in 1X TAE. Bands were detected by ethidium bromide staining. 3kb standard molecular weight of *MBI, Fermentas, Richlands B.C* were used as a marker. The electrophoretic run of the PCR product was visualized under UV-trans illuminator and further documented using Applied Bio system gel doc system (27). A similarity matrix was constructed employing the Jaccard coefficient using binary data. For dendrogram construction this data was further subjected to UPGMA cluster analysis. Software package InforBio (*version 5.26*) was employed for analysis (28).

Results

DNA fingerprint database has been generated using two different PCR- based molecular markers like RAPD and ISSR for 20 chickpea accessions. Results indicate that primers used in the present study were able to distinguish each of the 20 accessions. Salient features of fingerprint database obtained using different markers are as follows;

RAPD amplification profiles

Representative maker figures as produced by four RAPD primers viz OPA-07, OPA-08, OPA-12 and OPA-18 revealed a computable polymorphisms among chickpea genotypes. 27 primers gave either sub-optimal or non-distinct amplification of PCR-products, hence discarded. Only remaining 15 primers that generated reproducible RAPD patterns were subsequently used for analysis. RAPD primers yielded 1888 bands ranging between 300-3000bp. Each genotype produced 5 to 8 bands with an average of 6.25 bands. 15 primers produced 974 polymorphic bands. The reproducible polymorphic bands therefore average 64.93 per primer amounting to 51.53 percent polymorphism. Maximum of 11 bands and a minimum of single band were exhibited by primers OPA-07 and OPA-11 respectively. The representative PCR-RAPD results are discussed as under;

Primer OPA-08

OPA-08 RAPD primer generated 179 bands out of which lanes 03, 12, 14 and 15 depicts light bands. Bands ranged between 500-2000bp. Accession ICC12328 showed maximum bands whereas accession ICC17160 shows least number of 03 bands. The average number of bands per accessions works out to be 8.95. Therefore polymorphism calculated due to OPA-08 primer is 71.50%. Presence and clarity of accession specific bands in accessions ICC13441 and ICC12028 reveals that the Primer OPA-08uniquely identifies accessions in a molecular weight range of 450 and 1200bp (Fig. 2).

Primer OPA-12

The Primer OPA-12 generated 167 bands. Majority of these bands were observed in a molecular weight

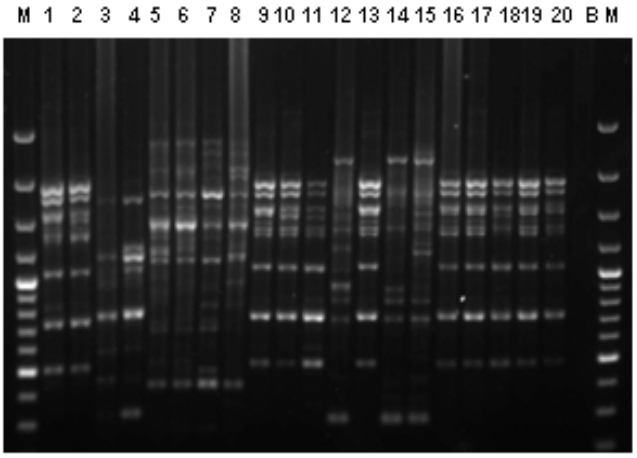

Figure 2. Amplification of 20 accessions of Cicer with primer OPA-08 M: 3Kb markers B: Blank; Lane1-5: 17121, 17123, 17160, 17163, 17164 Lane 6-20: 5434, 13441, 11944, 15518, 12537, 12328, 6306, 6263, 637, 12028, 7554, 14051, 6537, 16269, 1180.

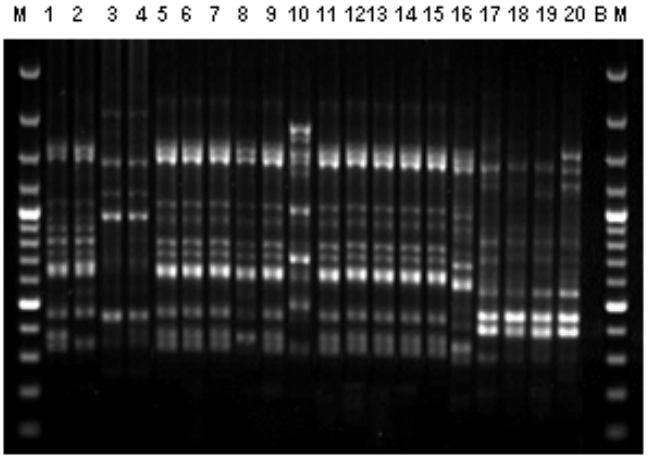

Figure 3. Amplification of 20 accessions of Cicer with primer OPA-12 Lane identification as per fig. 2.

range between 300 to 2200bp. The band of molecular weight of 1600bp is however, present in all the accessions. Out of 167 bands, 142 bands are polymorphic. Number of bands per accession averages 8.35, hence showed polymorphism percentage of 85.02 due to primer OPA-12. The accessions ICC17160 and ICC17163 produced least amplification of DNA bands show a string-out amplification patterns. This pattern is absent in other accessions analysed. Once again due to presence and clarity of strain specific bands in accessions ICC12537 and ICC7554 the primer OPA-12 uniquely identifies varieties in a molecular weight range of 500bp, 600bp and 1900bp respectively (fig. 3).

ISSR amplification profiles

Of the 41 ISSR primers used for PCR studies, only 25 primers amplified chickpea DNA. Among the trinucleotide repeats tested, $(TAA)_6$, $(TCC)_6$, $(CCT)_6$, $(ACT)_7$, $(GGC)_6$, $(CAG)_6$ produced better amplification patterns. Eight of these ISSR primers on an average produced 126 bands. 64 bands shows average 54 % polymorphic accounting. The number of bands varied from four to twelve in IS-01 and IS-27 respectively. The primer sequences $(ACT)_6$ and $(GGC)_6$ amplified least number of bands viz. 65 and 102 respectively. $(TG)_8G$, amplified a maximum number of 160 bands. An average polymorphism of 54 % across all genotype is calcula-

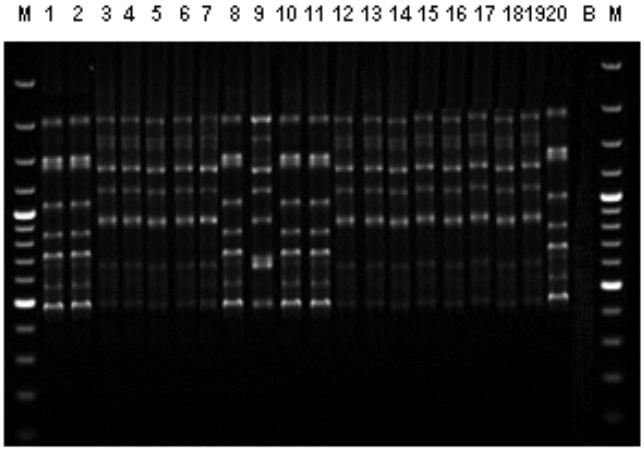

Figure 4. Amplification 20 accessions of Cicer with primer IS-23 $(TG)_8G$ Lane identification as per fig. 2.

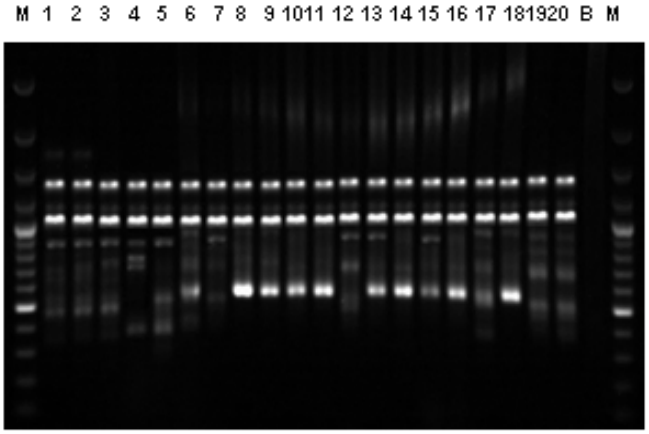

Figure 5. Amplification 20 accessions of Cicer with primer IS-32 $(GGC)_6$ Lane identification as per fig. 2.

ted. Figure 4 and 5 are the representative amplification patterns as generated by ISSR markers.

Cluster analysis

As per RAPD analysis, all wild accessions and ones from Turkey share one cluster. The accession ICC6306 which is cultivated in Russia and CIS countries is presented as an out group. Accessions ICC5434 and ICC637 cultivated in India are grouped together. ICC12537, ICC14051, ICC13441, ICC7554 accessions from Ethiopia and Iran constitute one cluster group (fig. 6) ISSR analysis clustered these accessions into two distinct groups (fig. 7). Except for ICC17164 and ICC17121, all wild accessions grouped together in the first cluster, whereas chickpea cultivars formed a second major cluster group. Second genotype group is divided into two sub groups. One of this subgroup is a monocultivar having ICC17164 accession alone in it. The other genotypes constitute a separate sub group. The similarity coefficient values for the cultivated chickpea lines in this UPGMA dendrogram range between 0.60 and 1.00. The values of wild accessions only range between 0.63 and 0.73 and those of *arietinum*, the similarity coefficient value range between 0.61 and 0.82. It was found that all the chickpea genotypes present in a cluster have similar pedigree or share a common parent. Compared to the RAPD, the ISSR dendrogram shows higher correlations with the pedigree data.

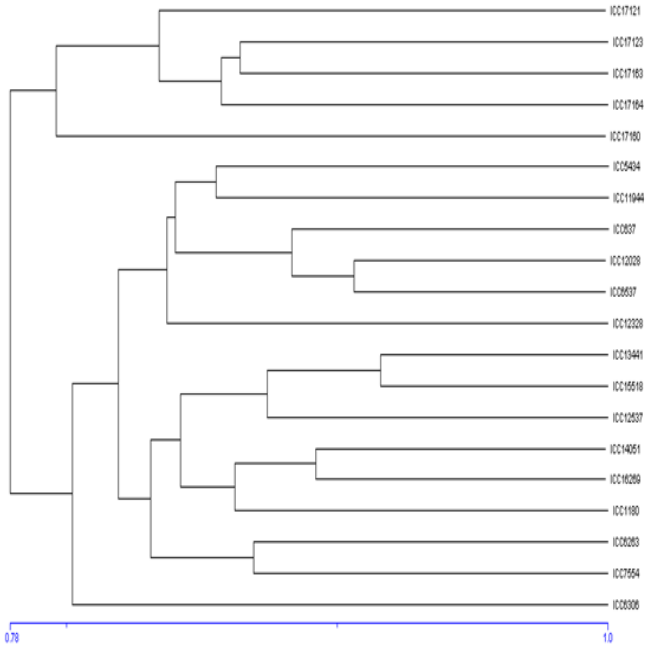

Figure 6. Dendrogram of 20 chickpea accessions based on RAPD polymorphic data using UPGMA.

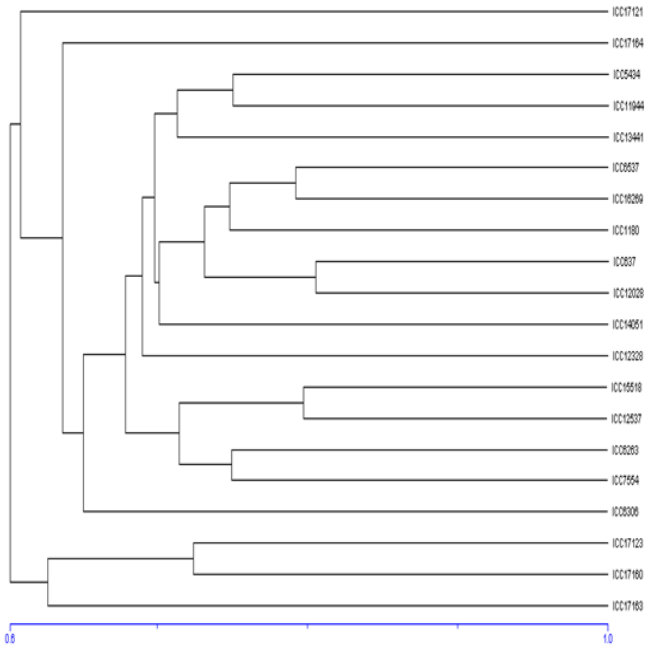

Figure 7. Dendrogram of 20 chickpea accessions based on ISSR polymorphic data using UPGMA.

Discussion

International Crop Research Institute for Semi-Arid Tropics (ICRISAT) Patancheru, (A.P.) India maintains over 17,000 germplasm accessions assembled and collected from various parts of the world. (www.icrisat.org.). World chickpea germplasm could readily be exploited and utilized in crop improvement programmes. Use of wild species and landrace cultivars provides a broad genetic varability base to crop species (22). It seems now feasible to conduct an extensive molecular diversity studies in this important legume crop. To indentify genetically diverse germplasm with beneficial traits for subsequent use in crop improvement programme is a posterity. Genetic diversity assessment is important for crop improvement, its efficient manage-

ment and further conservation of germplasm (29). The genetic level manipulation for desired traits in chickpea is low; being a self-pollinated crop, inherits a very limited genetic variation in the germplasm. Chickpea is therefore known to be one of the recalcitrant crops. The chickpea cultivars exhibit meagre genetic variations due to low heterozygous loci. The 20 accessions subjected to RAPD analysis in the present investigation however shows a measurable amount of genetic diversity.Differences between genotypes vis a vis their agronomic, morphological, biochemical and molecular characteristics are either a direct or an indirect representation of differences at their DNA level (30). These therefore, provide acumulative information about their genetic descendance. Genetic fingerprinting in chickpea, for a long time was hampered by low variability in the chickpea genome. To have more compared data-sets by using combination of more competent markers has become a priority. Agrawal and Srivastava, (2010) analysed genetic diversity on chickpea cultivars of India using RAPD markers and found a very narrow range of 0.68 to 0.92 in the similarity index of sixty eight genotypes. This therefore indicated low variability amongst the chickpea cultivars of India. Inour results, the variability index range from 0.79 to 0.91 thus in conformity too (31).

Of late use of two marker systems like RAPD and ISSR comparative assessment of their efficacy in examining genetic diversity is followed by many workers in many other crops. Previously too, studies in the present lab are credited with using two marker system test to genetic diversity among chickpea cultivars grown in the central India (19, 23).The present investigation too therefore, demonstrates the efficacy of RAPD in distinguishing the seed accessions. The RAPD markers used here can also be employed in the selection of variation in parental materials in breeding programs. Data based on these PCR-RAPD analysis can also be tailored for tagging the gene of interest. This however, requires to convert such dominant RAPD markers into more informative co-dominant markers. This can be percieved by polymorphic bands and subsequently developing these into SCAR markers. Using such SCAR marker, RAPD data be used for genome mapping studies with additional authenticity and subsequently gene transfer strategies into susceptible cultivar can be developed (22). Whatever extent of the genetic diversity, it projects an imbalanced considerable variant presentation amongst the species of *Cicer* germplasm. It is however, imperative that this variation tagged by various markers at gene level can be used in the introduction of desirable traits in the wanted genotypes. (32, 33).

The prime difference into earlier work in our lab and in the present study has been the accession origin. Earlier chickpea germplasm represented small geographic centres whereas in this study global collections are analysed (34). The results in the present investigation infer that the ISSR primers constitute as efficient informative markers and can be further examined so as to correlate banding patterns with agronomic characteristics. This certainly necessitates therefore an effective collaboration between plant breeders, molecular biologists and agronomists in the exchange of germplasm leading to tagging of commercial genes of importance. For example here, the unique band as produced by the ISSR primer $(TAA)_6$ in the ICC6263 and ICC6306 accessions may serve as unique identifier of a phenotype having Russian origin. Need for more investigation using more number of primers however is not contested. Relationship measures based on pedigree data and morphological traits can further be correlated in minimizing any individual inconsistencies in chickpea. Further, now available large pool of genetic stocks at national and international levels can be effectively used to screen out variations employing PCR-based markers in a crop as important as chickpea.

Of genetic diversity in any crop for producing heterotic genotypes over the existing ones is fundamental. Adequate molecular characterization for agronomic and morphological traits is necessary to facilitate effective utilization of germplasm by plant breeders. Germplasm screening using PCR- based makers allow the extension of traditional breeding methods to tag the polymorphism in different seed accessions in a more precise and controlled manner. Genetic improvement within the species is usually expressed in terms of genetic allels that occur at each of the given set of observable loci. The present study in our lab is different from earlier in that here the selection of global germplasm and then subjecting to RAPD and ISSR markers in combination suggested that ISSR markers are promising in detection of polymorphisms in *Cicer* species. Therefore, meeting between distant populations with molecular studies is needed to complement each other. This will reduce time as well as costs of field experimentation. Looking at huge chickpea germplasm available and being a self-pollinated crop, genotype screening using DNA- based markers must be performed. Considering all these criteria and results, accessions that are far from each other by virtue of genetic origin and diversity index viz.ICC6263, ICC6306 and ICC17160 may be recommended to be selected as a parent and be used in future breeding programs for chickpea.

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