

Molecular Diversity Assessment in Chickpea through RAPD and ISSR Markers

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Abstract Chickpea (*Cicer arietinum* L.) is a cool season food legume in the family of *Fabaceae*. The exploration of genetically variable accessions is the key source of germplasm conservation for future selecting parents for breeding. PCR based analysis employing RAPD and ISSR marker are one of the established techniques of estimation of genetic diversity. Twelve chickpea accessions were analyzed by ten RAPD and ISSR primers of which all primers gave amplification products. The results showed that the level of DNA polymorphism in these accessions is low. RAPD analysis yielded 5.9 bands per primer while ISSR analysis produce 2.8 bands per primer. The average percentage of molecular polymorphism as produced by RAPD and ISSR primers was 67%. The cluster dendrogram demonstrated a similarity coefficient range of 0.10 to 0.66 due to RAPD markers, whereas ISSR markers showed 0.60 to 0.80. The variability index worked out in the present study ranges from 0.49 to 0.78. The present study determines DNA polymorphism in chickpea accessions as revealed by RAPD and ISSR markers. Overall the study ascertained that RAPD and ISSR marker provide powerful tools in revealing genetic diversity in chickpea.

Keywords: chickpea, RAPD, ISSR, polymorphism

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1. Introduction

Chickpea, is the second most important legume in the world after dry beans and peas (FAO, 2012). The chickpea seeds are the good source of carbohydrates and proteins, which together constitute 80% of the total dry seed weight [1]. Two main types of chickpea cultivars are grown globally i.e. kabuli and desi, representing two diverse gene pools. The knowledge of genetic diversity is useful in breeding experiments vis-à-vis 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 agronomic traits [2]. The differences at the DNA level are therefore expected to provide information about genetic relationships.

Polymerase chain reaction (PCR) is an *in-vitro* DNA amplification method, using arbitrary primers, has been widely employed in plant genotyping. The PCR based markers have been widely used because of their technical simplicity and ease of screening large number of samples in short period of time. The commonly used PCR-based DNA markers viz. RAPD and ISSR are mostly used to discriminate closely related genotypes [3]. The RAPD amplifications based on the PCR, is one of the most commonly used molecular markers. RAPD markers are the amplification products of anonymous DNA sequence using single, short and arbitrary oligonucleotide primers

thus they do not require prior knowledge of DNA sequence. RAPD identification techniques can be used at any stage of plant development and they are not affected by environment factors [4]. ISSR-PCR is a technique of choice mostly employed for testing of genetic diversity [5]. It is rapidly being used by the research community in various fields of crop improvement [6]. The technique is useful in examining genetic diversity, phylogenetic studies, gene tagging, genome mapping and evolutionary biology in a wide range of crop species [7].

The monophyletic origin of chickpea is responsible for its narrow genetic base, which inhibits its improvement via conventional breeding as reviewed [7]. There are however, opportunities to expand the genetic diversity of the crop by accessing the secondary gene pool present in its wild relatives. Marker assisted selection (MAS) provides an efficient means of selecting specific alleles and also a parent for developing the crosses between chickpea. To tap genetic diversity at DNA levels PCR based analysis provide a better means. The aim of this study is to evaluate the genetic diversity in chickpea accessions employing RAPD and ISSR markers.

2. Materials and Methods

2.1. Plant Material

A representative set of 12 accessions of *Cicer arietinum* L. that included chickpea genotypes used for analysis.

Agronomic details of all these accessions are given (Table 1). All accessions were obtained from Indian Institute of Pulses Research, Kanpur (U.P.) under MTA understanding. 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.

Table 1. Agronomic details of *Cicer* accessions used in the study

S.N.	Accession	Agronomic details
1	ILWC 292	Nematode resistant
2	IPC-11-64	Tall heighted and large seeded
3	IPC-12-277	Kabuli type and tolerant against pod borer
4	EC 556270	Wild type and wilt resistant
5	IPC-12-01	Desi type and wilt resistant
6	ICC-17148	Wild type and wilt resistant
7	IPC-12-03	Wilt resistant
8	IPC-12-257	Small seeded and wilt resistant
9	ILWC-142	Wild and heat tolerant
10	IPC 12-88	Resistance to wilt
11	IPC 12-11	Small seeded and wilt resistant
12	IPC 11-13	Tall heighted and bold seeded

2.2. DNA Isolation

DNA was isolated from the leaves of each accession, using the CTAB extraction method of Talebi et al., [1] with minor modification. 100mg leaf material was ground in the liquid nitrogen followed by homogenization with 1ml freshly prepared extraction buffer. To this, 20% SDS was added and incubated at 60°C for 30 minutes. Then after, 92µl of 5M NaCl was added and subsequently, 75µl of CTAB solution was added and reincubated at 65°C for 15 minutes. To this cocktail, 300µl of chloroform: isoamyl alcohol mix (24:1) was added. This was followed by centrifugation at 12000g for 15 minutes at 4°C 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 4°C. Subsequently precipitation was done by adding chilled isopropanol 40% v/v as final concentration. The precipitated DNA was then centrifuged as a 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 -20°C.

2.3. RAPD and ISSR Primers

A set of ten RAPD primers (Operon Tech. Inc. Alameda, USA) and ten ISSR primers representing di, tri, tetra and pentamer repeats (procured from the Biotechnology Laboratory, University of British Columbia, Canada) were used in the study. Details of both the primers are represented in the Table 2 and 3 respectively.

2.3.1. RAPD-PCR

The PCR procedure for DNA amplification according to the method of Williams et al., [8] 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 MgCl₂; 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 was made at 72°C for 7 min.

2.3.2. ISSR-PCR

The PCR procedure for DNA amplification according to the method of Welsh et al., and Bhagyawant et al., [9,10] was followed with minor modifications. The reaction cocktail contained 25µl reaction volumes containing 10mM Tris-HCl pH 9.0; 50 mM KCl; 0.1% Triton-x-100; 1.5mM MgCl₂; 0.1mM dNTP; 2mM primer; 0.5 unit of TaqDNA 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 1 minute, followed by polymerization at 72°C for 2 minutes. Final extension step was carried out at 72°C for 7 minutes.

2.4. Agarose Gel Electrophoresis and Dendrogram Construction

The PCR products were separated on 1.5 % agarose gel in 1X TAE by electrophoresis at 100 V for 3h and bands were detected by ethidium bromide staining. 3kb standard molecular weight of *MBI, Fermentas, Richlands B.C* was used as a marker. Clearly resolved and unambiguous bands were scored visually for their presence or absence with each RAPD and ISSR markers. The DNA fragment profiles representing a consensus of two independent replicates were scored in the form of a matrix with '1' and '0', which indicate the presence and absence of bands in each accessions respectively. The individual RAPD and ISSR amplified profiles were analysed for genetic diversity. Jaccard's similarity coefficient [11] was estimated from these binary data using Past [12] software.

Table 2. RAPD primers used in the present study

S.N.	Primer	Sequence motif	T _m	% GC
01.	OPA-18	5'-AGGTGACCGT-3'	32°C	60%
02.	OPG-11	5'-TGCCCCGTCTG-3'	34°C	70%
03.	OPG-04	5'-AGCGTGTCTG-3'	32°C	60%
04.	OPA-12	5'-TCGGCGATAG-3'	32°C	60%
05.	OPA-17	5'-GACCGCTTGT-3'	32°C	60%
06.	OPA-11	5'-CAATCGCCGT-3'	32°C	56%
07.	OPA-08	5'-GTGACGTAGG-3'	32°C	80%
08.	OPAC-06	5'-GGCTTCGCAA-3'	32°C	60%
09.	OPZ-06	5'-GTGCCGTTCA-3'	32°C	60%
10.	OPZ-10	5'-CCGACAACC-3'	30°C	62.2%

Table 3. ISSR primers used in the present study

S.N.	Primer	Sequence motif	T _m	% GC
1	UBC 808	AGAGAGAGAGAGAGAGC	52°C	52.9%
2	UBC 811	GAGAGAGAGAGAGAGAC	52°C	52.9%
3	UBC 823	TCTCTCTCTCTCTCC	52°C	52.9%
4	UBC 835	AGAGAGAGAGAGAGAGYC	52°C	52.9%
5	UBC 836	AGAGAGAGAGAGAGAGYA	52°C	52.9%
6	UBC 847	CACACACACACACARC	52°C	52.9%
7	UBC 848	CACACACACACACARG	52°C	52.9%
8	UBC 855	ACACACACACACACYT	52°C	52.9%
9	UBC 864	ATGATGATGATGATGATG	48°C	33.3%
10	UBC 866	CTCCTCCTCCTCCTCTC	60°C	66.7%

2.5. Results

2.5.1. RAPD Amplification Profiles

RAPD analysis revealed DNA polymorphism among the chickpea accessions that are tested. Each primer yielded distinct and easily detectable bands of variable intensities and thus used for scoring of polymorphic fragments. Out of ten RAPD primers, one was found to be non-amplifying (OPAC-06). Such kind of non-amplifying primers were also reported earlier in chickpea (Bhagyawant *et al.*, 2008). Rest of the RAPD primers yielded a total of 59 bands in a molecular weight range of 500-2000 bps. The average number of bands per primer per accession accounted to 5.9. Maximum number of 9 bands was scored by OPA-18 while least number of bands were shown by OPG-11 and OPZ-10. The total polymorphic bands as generated by these RAPD primers are 16. Highest no. of polymorphic bands were obtained with primer OPG-04 and lowest polymorphic bands were obtained in primer OPZ-06 and OPA-11. The representative RAPD-PCR amplification patterns as generated by primer OPA-18 and OPA-11 are shown in Figure 1 and Figure 2 respectively.

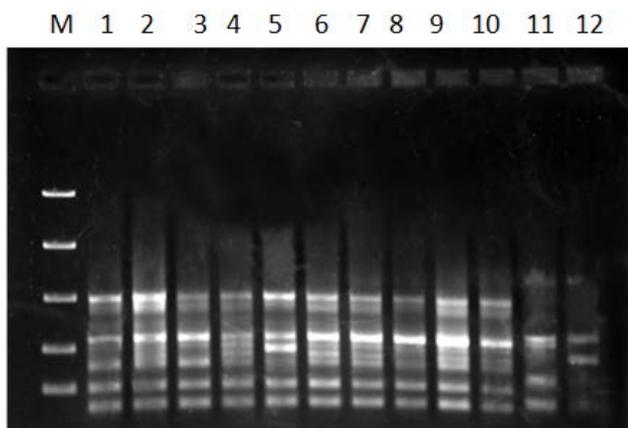


Figure 1. Amplification of 12 chickpea accessions with primer OPA-18
M: 3Kb markers B: Blank; Lane 1 -12: ILWC292, IPC-11-64, IPC-12-277, EC 556270, IPC-12-01, ICC-17148, IPC-12-03, IPC-12-257, ILWC-142, IPC 12-88, IPC 12-11, IPC 11-13

Based on this RAPD-PCR amplification data, a consensus was scored in a binary matrix with negative (0) indicating the absence and positive (1) indicating presence of the bands using software PAST. Similarity index values arrived from the polymorphic data gave the extent of

genetic relatedness among accessions. The accessions ILWC-292, IPC-11-13, IPC-12-277 and IPC-12-11 showed lowest similarity index of 0.10 and 0.14 respectively. Accessions ICC-17148 and IPC-12-03 showed highest similarity index of 0.66. A similarity matrix was constructed employing the Jaccard coefficient using binary data. For dendrogram construction this data was further subjected to UPGMA cluster analysis in the Figure 5. The present study has shown that % of DNA polymorphism of chickpea accessions varied from 0-66 % with mean of 27.4% with maximum no. of polymorphism percent in primer OPZ-10. By and large a measurable genetic diversity existed among the chickpea accessions that are tested.

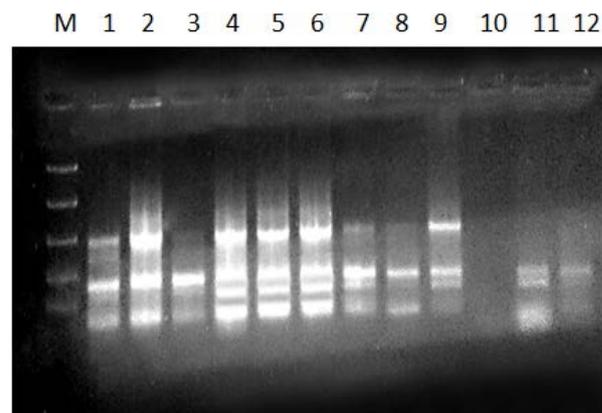


Figure 2. Amplification of 12 chickpea accessions with primer OPA-11
Lane identification as per Figure 1

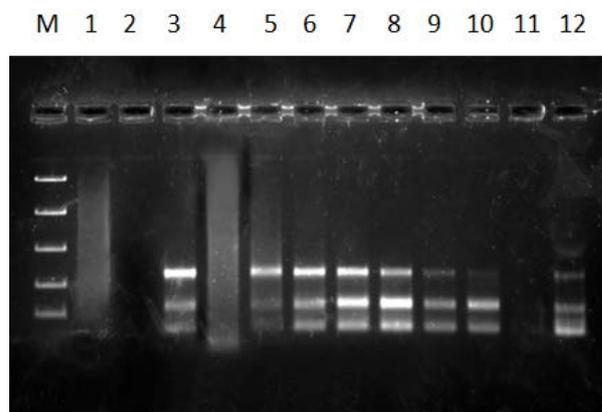


Figure 3. Amplification of 12 chickpea accessions with primer UBC-835
Lane identification as per Figure 1

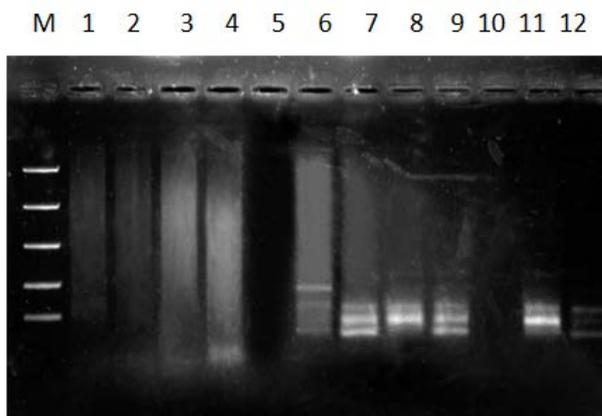


Figure 4. Amplification of 12 chickpea accessions with primer UBC-836
Lane identification as per Figure 1

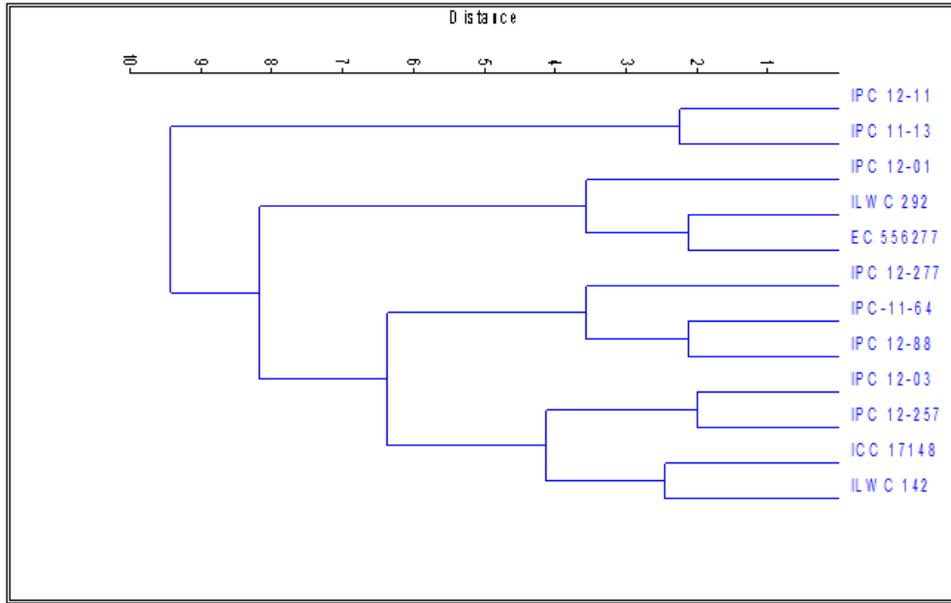


Figure 5. Dendrogram of 12 chickpea accessions based on RAPD-PCR amplification

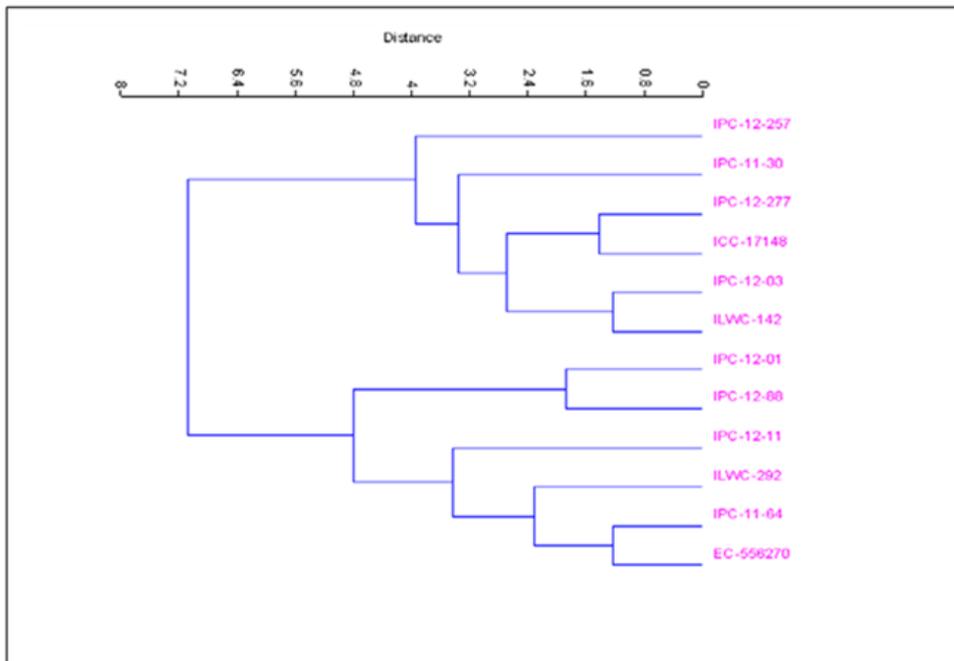


Figure 6. Dendrogram of 12 chickpea accessions based on ISSR-PCR amplification

2.5.2. ISSR Amplification Profiles

The ISSR primers tested were eight dinucleotide [(AG)₈C, (GA)₈C, (TC)₈C, (AG)₈YC, (AG)₈YA, (CA)₈RC,(CA)₈RG,(AC)₈YT] and two trinucleotide [(ATG)₆, (CTC)₆] 3' anchored repeat primers. Among the dinucleotide repeats, (CA)₈RG and (AC)₈YT produced better amplification patterns. A total of 28 bands were amplified across 12 accessions with 10 ISSR primers, revealing an average of 2.8 bands per primer and 0.23 bands per primer per genotype. Assaying ISSR variation in *Cicer* accessions yielded a total of 28 bands ranging molecular weight between 500-2000 bps. Highest polymorphism was obtained with primer (AC)₈YT and no polymorphism was obtained in primer (AG)₈C, (AG)₈YC, (CA)₈RC, (ATG)₆ and (CTC)₆. Ten ISSR primers were screened against twelve selected chickpea DNA samples

to identify potential primers producing more number of polymorphic and repeatable fragments. ISSR analysis revealed a better polymorphism among these chickpea accessions. The representative ISSR amplification patterns as generated by primer UBC-835 and UBC-836 are shown in Figure 3 and Figure 4 respectively. The cluster analysis using PAST program and resulting dendrogram is shown in the Figure 6.

2.6. Discussion

The study presented here includes analysis of genetic similarity among 12 accessions of chickpea using RAPD and ISSR markers. The comparative marker analysis of RAPD and ISSR can provide practical information for DNA polymorphisms. Based on the RAPD dendrogram, accessions have been grouped into two major clusters i.e.

cluster I and II. Cluster I consisted of IPC 12-11 and IPC 12-13 which came as an out group. The cluster II comprises all the other 10 accessions viz ILWC 292, IPC-11-64, IPC-12-277, EC 556270, IPC-12-01, ICC-17148, IPC-12-03, IPC-12-257, ILWC-142 and IPC 12-88 were grouped separately. Similarly on the ISSR dendrogram, accessions have been grouped into two major clusters i.e. cluster I and II. Cluster I consisted of IPC 12-207, IPC 11-30, IPC-12-277, ICC-17148, IPC-12-03, ILWC-142 as an out group. The major cluster II comprises all the another accessions ILWC 292, IPC-11-64, EC 556270, IPC-12-01, IPC-12-11, IPC and IPC 12-88 were grouped separately which came as an out group.

This data generated out of such analysis needs to be explored for identifying diverse genetic stocks. Such comparative studies in legumes involving RAPD and ISSR markers have been successfully carried out previously in crops like Black gram [13], *Vigna* species [14] and Rice [15]. In present study out of ten RAPD primers one was found to be non-amplifying (OPAC-06), such kind of non-amplifying primers were also reported earlier [10]. 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. Equally use of wild species and landrace cultivars can provide a broad genetic variability base to such crop species [16].

The ISSR markers detect variation at DNA level in chickpea accession IPC-12-1. Such genetic variation obtained from ISSR markers may be useful in discriminating chickpea accessions which needs to be complemented with the morphological traits data. Further, the genetic variation that exists between these chickpea accessions can efficiently be used as directives in planning future chickpea breeding programmes. Such genetic diversity data can provide practical information for selection of parental material and thus may assist in forecasting chickpea breeding strategies. ISSR markers are highly polymorphic and are useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology. In addition, these markers have been successfully used to study diversity and phylogenetic relationships in chickpea for the last decade [10, 17-19]. Therefore, in order to have comprehensive and substantial information about the extent of genetic variability to tap the polymorphism, in a *Cicer* species, a combined approach involving morphological and PCR- based analysis needs to be undertaken.

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. 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. Wild relatives of chickpea could be one of the sources through which molecular breeding programmes for new genes can be planned and favorable re-combinations

created. The studies of Ahmad et al., [21] selected wild resistant genotypes using SSR marker TA194, that provided an opportunity to marker assisted breeding for yield improvement of the crops. RAPD and ISSR markers in combination suggested that ISSR markers are promising in detection of DNA polymorphisms in chickpea. Therefore, PCR generated data between distant populations needs to be correlated to morphological data for complementing each other. This will reduce time as well as the costs of field experimentations. Looking at the huge chickpea germplasm available in the gene bank and being a self-pollinated crop, genotype screening using DNA-based markers must be performed and documented for executing future breeding programmes.

Conflicts of Interest Statement

Authors declare that the work carried out by them is original and have no conflict of interests.

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