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Genetic Diversity Analysis in Chickpea Employing ISSR Markers

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Abstract

In the present study, inter simple sequence repeat (ISSR) markers were employed to estimate genetic diversity in 13 accessions of chickpea including cultivated and wild. Among all these anchored ISSR primers tested, pentanucleotide repeat primer UBC-879 produced better amplification patterns. A total of 150 bands were amplified in a molecular weight range of 100-2000 bps revealing an average of 21.4 bands per primers and 1.64 bands per primer per genotype. The repeats $(GA)_8C$, $(AG)_8YT$, $(GA)_8YC$, $(AG)_8C$, $(GTT)_6$ and $(GT)_8YC$ give least amplification. UPGMA dendrogram constructed between these accessions depicted three major clusters. Based on genetic origin and diversity index viz. ICC-14051, ICC-13441, ICC-15518, ICC-12537, and ICC-17121 recommended to be selected as a parent in future breeding programmes for chickpea.

Keywords: Chickpea; ISSR; Legumes; PCR

Introduction

Genetic diversity is the essence of crop improvement. Traditionally, diversity is examined by measuring variation in morphological parameters, biochemical parameters or using molecular markers. Environmental factors influence the expression of morphological and biochemical traits. The DNA based molecular markers are currently used for Marker Assisted Selection (MAS). ISSR or Simple Sequence Repeats (SSRs) are short tandem repetitive DNA sequences with a repeat length of few (1-6) base pairs [1]. These sequences are abundant, dispersed throughout the genome and are highly polymorphic in comparison with other molecular markers [2].

The applications of ISSR markers in plants are well known [3]. These are PCR based markers that permits detection of polymorphism in microsatellite and inter-microsatellite loci, without prior knowledge of DNA sequence [4]. In this technology, the SSR primers have either 5' or 3' extension of one or more bases including anchor sequences thereby making the profile easier for analysis [5].

Chickpea (*Cicer arietinum* L.) belongs to the family of leguminosae, is the third most important pulse crop of the world and India is the largest producer country [6]. Technically, it belongs to the tribe *Cicereae* Alef of the family *Leguminosae* with genus *Cicer* and species *arietinum*. Chickpea seeds are nutritious due to the presence of protein, carbohydrates, vitamins and minerals [7]. It contains well-balanced amino acids and low levels of anti-nutritional factors in comparison to other grain legumes [8]. Some of the biotic and abiotic factors reduce chickpea production worldwide and search for elite genotypes are being continuously carried out employing PCR-based markers [3]. The Indian Institute of Pulses Research, Kanpur, India maintains more than 3000 chickpea accessions as a primary gene pool stock (www.iipr.res.in). Assessment of genetic diversity from the available genetic stocks is a key factor aimed at improvement of crop performance [9]. In our earlier reports, ISSR-PCR analysis showed correlation with pedigree data amongst the 20 accessions of chickpea which included the species of *C. arietinum* and *C. reticulatium* [10]. Present investigation was undertaken to analyse genetic diversity between the 13 chickpea accession of *C. arietinum* and *C. reticulatum* available in the primary gene pool accessions.

Materials and Methods

Chemcials

Agarose, CTAB, Tris-HCl, EDTA, SDS, sodium chloride were purchased from Hi Media. dNTPs and Taq DNA polymerase was obtained from MBI, Fermentas, Richlands B.C. ISSR primers were obtained from Operon Technologies Ltd., (Alameda, California). All other chemicals and reagents used were of analytical grade.

Seed materials

A representative set of 13 accessions of *Cicer* that included cultivated and wild. Agronomic details of these accessions are given in Table 1. The collections encompassing eight geographic origins of world are shown in the Figure 1. All accessions were obtained from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) Patancheru, Hyderabad, India, under MTA understanding.

DNA isolation

DNA was isolated using the CTAB extraction method of Talebi et al. [11] with minor modifications. Hundred milligram seed material was ground in liquid nitrogen followed by homogenization with 1 ml freshly prepared extraction buffer. To this, 20% SDS was added and incubated at 60°C for 30 min. Then after, 92 µl of 5 M NaCl was added and subsequently, 75 µl of CTAB solution was added and re-incubated at 65°C for 15 min. To this cocktail, 300 µl of chloroform: Isoamyl alcohol mix (24:1) was added. This was followed by centrifugation at 12,000 g for 15 min at 4°C in a Sigma centrifuge 3-16 K. Chloroform: Isoamyl alcohol mix was readded to the supernatant in 1:1 volume and re-centrifuged at 12,000 g for 15 min at 4°C. Subsequently, precipitation

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S. No.	Chickpea accessions	Type	Origin	Agronomic details	Geolocation (Latitude, Longitude)
1	ICC-14051	*Cicer arietinum* (L.)	Ethiopia	Drought tolerant	8.97°N, 42.22°E
2	ICC-7554	*Cicer arietinum* (L.)	Iran	Resistant to fungal disease	32.42°N, 53.68°E
3	ICC -2307	*Cicer arietinum* (L.)	Myanmar	*Helicoverpa* resistant	20.78°N, 97.02°E
4	ICC-13441	*Cicer arietinum* (L.)	Iran	*Helicoverpa* resistant	32.42°N, 53.68°E
5	ICC-15518	*Cicer arietinum* (L.)	Morocco	Drought tolerant	34.34°N, 5.32°W
6	ICC-1422	*Cicer arietinum* (L.)	India	Water use efficiency	17.38°N, 78.48°E
7	ICC-11627	*Cicer arietinum* (L.)	India	*Ascochyta blight* resistant	31.53°N, 75.91°E
8	ICC-6263	*Cicer arietinum* (L.)	Russia	Salinity stress tolerant	61.52°N, 105.31°E
9	ICC-12537	*Cicer arietinum* (L.)	Ethiopia	*Helicoverpa* resistant	8.85°N, 38.77°E
10	ICC-11944	*Cicer arietinum* (L.)	Nepal	*Ascochytablight* resistant	28.12°N, 82.30°E
11	ICC-306	*Cicer arietinum* (L.)	Russia	Resistant to pod borer	61.52°N, 105.31°E
12	ICC-4958	*Cicer arietinum* (L.)	India	Drought tolerant	17.38°N, 78.48°E
13	ICC-17121	*Cicer reticulatum*	Turkey	Higher branches	37.53°N, 40.88°E

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

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

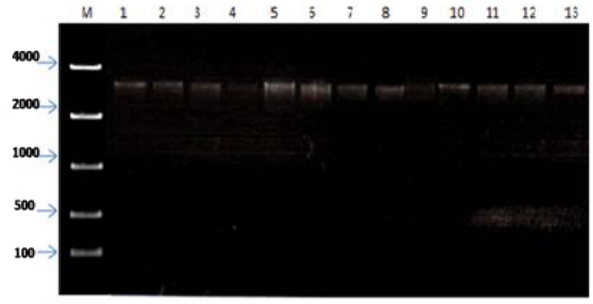

Figure 2: DNA quality of 13 chickpea accessions on 1.2% agarose gel.

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 and dissolved in 100 µl of Tris-EDTA buffer (19 mM Tris-HCl pH 8.0; 1 mM EDTA pH 8.0). The quality of extracted DNA was checked on 1.2% agarose gel (Figure 2) and further stored at -20°C until use.

ISSR-PCR amplifications

The PCR procedure for DNA amplification was employed according to the method of Welsh and Mcclelland [4]. The reaction cocktail contained 25 µl reaction volumes containing 10 mM Tris-HCl pH 9.0; 50 mM KCl; 0.1% Triton-x-100; 1.5 mM $MgCl_2$; 0.1 mM dNTP; 2 mM primer; 0.5 unit of Taq DNA polymerase 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 min, followed by denaturation at 94°C for 1 min. The annealing was performed at 56°C for 1 min, followed by polymerization at 72°C for 2 min. Final extension was carried out at 72°C for 7 min. Details of ISSR primers are represented in the Table 2.

Agarose gel electrophoresis

The PCR products were separated on 1.5% agarose gels in 1X TAE by electrophoresis at 100 V for 3 h and bands were detected by ethidium bromide staining. 4 kb standard DNA molecular weight was used as a marker (Figure 2). Clearly resolved bands were scored visually for their presence or absence. Jaccard's similarity coefficient [12] was estimated from these binary data using Past [13] software.

Results and Discussion

The chickpea accessions possessing important agronomic characters like higher yield potential with early maturity and wilt resistance were analyzed for estimating genetic diversity using 10 ISSR primers. The primers tested were two pentanucleotide viz. $(CTTCA)_3$ and $(GGAGA)_3$, three trinucleotide $(ATG)_6$, $(GTT)_6$ $(TAT)_6$ and five dinucleotide anchor repeat primers. Among all these anchored ISSR primers tested, pentanucleotide repeat primer UBC-879 produced better amplification patterns. The 3 primers did not amplify DNA of any chickpea genotypes tested. Such non-amplifying primers were also been reported earlier [14]. A total of 150 bands were amplified across the 13 genotypes with these primers in a molecular weight range of 100-2000 bps revealing an average of 21.4 bands per primers and 1.64 bands per primer per genotype. The total number of bands amplified by 3' anchored primers varied from 20-50. The primer $(CTTCA)_3$ has amplified maximum number of 80 bands across the these accessions analyzed. Whereas the primer sequence $(AG)_8C$ has amplified least number of bands (40). The repeats $(GA)_8C$, $(AG)_8YT$, $(GA)_8YC$, $(AG)_8C$, $(GTT)_6$ and $(GT)_8YC$ give least amplification in the present study. This may be due to the region that chickpea genomic sequences may not be in the range of amplification by Taq DNA polymerase.

The cluster matrix method UPGMA was used and a dendrogram was constructed from a similarity matrix based on Jaccard's similarity coefficient values. The cophenetic correlation between ultrametric similarities of the tree and similarity matrix was high, indicating that the cluster analysis strongly represents the similarity data. The cluster analysis revealed the genetic relationships between the chickpea genotypes with similarity index values ranging from 0.11 to 0.19 with an average value of 0.15. Three broad clusters of chickpea genotypes were obtained from dendrogram as shown in Figure 3. Cluster one

S. No.	Primer	Anchor sequence	Tm (°C)	MW (Dalton)
1	UBC-809	5'-AGAGAGAGAGAGAGAGC-3'	47°C	5359
2	UBC-811	5'-GAGAGAGAGAGAGAGAC-3'	52°C	5359
3	UBC-834	5'-AGAGAGAGAGAGAGAGYT-3'	45°C	5374
4	UBC-841	5'-GAGAGAGAGAGAGAGAYC-3'	47°C	5359
5	UBC-850	5'-GTGTGTGTGTGTGTGTYC-3'	47°C	5287
6	UBC-864	5'-ATGATGATGATGATGATG-3'	48°C	5613
7	UBC-869	5'-GTTGTTGTTGTTGTTGTT-3'	41°C	5559
8	UBC-871	5'-TATTATTATTATTATTAT-3'	28°C	5469
9	UBC-879	5'-CTTCACTTCACTTCA-3'	36 °C	4439
10	UBC-880	5'-GGAGAGGAGAGGAGA-3'	45°C	4772

Table 2: ISSR primers used in the present study.

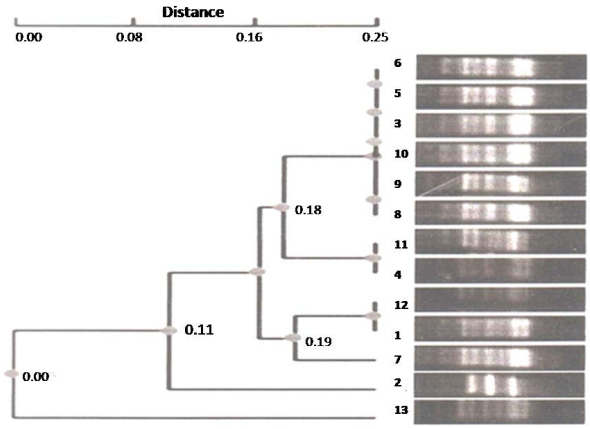

Figure 3: Dendrogram of chickpea accessions based on ISSR-PCR amplifications.

included chickpea accessions of ICC-12307, ICC-15518, ICC-1422, ICC-6263, ICC-12537, ICC-11944 and subclad of this cluster include accession ICC-6306 and ICC-13441. Cluster two includes accession ICC-14051 and ICC-4958 and subclade of this cluster include ICC-11627. Cluster three includes ICC-17121 as out group while genotype ICC-7554 has given polymorphy. The well-defined clustering was not observed between desi and kabuli genotype.

Applications of ISSR markers in genetic diversity analysis are becoming more popular for marker-assisted selection. Earlier studies of Ratnaparkhe et al. [15] revealed the inheritance of inter-simple sequence repeat polymorphisms and linkage analysis with *Fusarium* resistance gene in chickpea. The results shown that the ISSR loci they studied expressed virtually in complete agreement with the exception of mendalian segregation.

Literature perusal suggests that PCR can successfully forecast the growth habitats and geographic origin. Studies of Iruela et al. [16] in the genus *Cicer* and cultivated chickpea using combination of RAPD and ISSR markers of 26 accessions including kabuli and desi types were employed. Rao et al. [17], developed RAPD and ISSR fingerprinting in cultivated chickpea and its wild progenitor *Cicer reticulatum* L. They concluded ISSR analysis as a reliable attributes for estimation of genetic diversity than of RAPD. Fatemeh et al. [18], employed ISSR markers to fingerprint genetic diversity and conservation of landrace chickpea from north-west of Iran. ISSR markers are effectively scored for genetic diversity analysis of various crop plants. Amirul et al. [19], estimated genetic diversity among collected purslane accessions. Based on their assessment they recommended few of the accession that can be used as parents in future breeding program. In the present investigation, the polymorphic band as produced by primer UBC-879 may disseminate genetic diversity. The bands specific for accession no ICC-14051, ICC-13441, ICC-15518, ICC-12537, and ICC-17121 could be further developed into a SCAR marker. The present analysis also reflects the known phylogenetic relationships in chickpea. Understanding genetic diversity in the primary gene pool collection is crucial for any crop improvement program [20]. Chickpea being a self-pollinated crop has a narrow genetic base. Looking at huge chickpea germplasm available and to reduce costs of field experimentation, genotype screening using PCR based markers is a prerequisite for breeding programme. Based on genetic origin and diversity index viz. ICC-14051, ICC-13441, ICC-15518, ICC-12537 and ICC-17121 recommended to be selected as a parent in future breeding programmes for chickpea. More efforts are needed to screen chickpea accessions which can estimate genetic diversity within the *Cicer* species to identify elite genetic stocks.

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