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Research Article

Genetic Diversity Analysis between Different Varieties of Chickpea (*Cicer arietinum L.*) Using SSR Markers

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Abstract

The paper aims at evaluating genetic diversity among genomes of chickpea comprising of 5 different varieties with the help of simple sequence repeats (SSR) molecular markers. Genomic DNA isolated from all varieties was checked with 15 different SSR markers specific for ENDPOINT PCR using PCR based techniques. Amplification bands with different markers enabled identification of the genomic regions responsible for Drought Tolerance in chickpea. All 15 SSR markers chosen gave monomorphic bands. A hierarchical tree was also constructed using UPGMA Dendogram for figuring out the exact genetic distance of cultivars using band amplification data. It depicted GUJ-1 and GUJ-2 are closest of all cultivars. GUJ-5 is at the center having GUJ-3 and UJJAVAL at an almost equal distance but GUJ-5 and GUJ-3 are more related. Physiological data also supported this genetic evidence.

Keywords: Chickpea; Genetic diversity; SSR; PCR; scoring; Dendogram; Field Analysis.

Abbreviations: MAS: Marker Assisted Selection; SSR: Simple Sequence Repeat; PCR: Polymerase Chain Reaction; AGE: Agarose Gel Electrophoresis; QTL: Quantitative Trait Loci; UPGMA: Unweighted Pair Group Method with Arithmetic Mean; WPGMA: Weighted Pair Group Method with Arithmetic Mean; DT: Drought Tolerance

Introduction

Chickpea (*Cicer arietenum L.)* is an important legume crop belonging to the genus *Cicer L.* with 43 species (Singh, 1997). It is the third most important food legume crop worldwide with major production areas being the Indian sub-continent, West Asia and North Africa (WANA). In terms of area under cultivation, chickpea ranks as the third most important legume after Soybeans and dry beans respectively, with a total of about 12.1 million tons produced in 2016. India is the largest producer contributing 64% of the global chickpea production, followed by Pakistan, Iran, Turkey and Australia *(FAOSTAT).*Locally known as 'Chana', the two main varieties cultivated in India

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are the 'DesiChana' with smaller, coarser and dark coloured seedsand the 'Kabuli Chana' having a larger, smoother and cream coloured seeds(sajja *et al.,*2017) Chickpea serves as a rich and cheap source of protein, amino acids, iron and folic acids particularly in the developing countries with semi-arid to arid climate that largely face nutritional deficiencies (Wallace *et al.,* 2016) The Chickpea plant forms a symbiotic association with Nitrogen-fixing bacteria in the soil and when rotated with cereal crops, helps in retaining the soil fertility. It is also an important animal fodder (Subramaniam *et al.,* 2017).

The State of Gujarat in the West of India has been an important producer and consumer of chickpea (Tailyor and Francis 2012). Owing to its largely semi-arid climate and a population that predominantly adheres to vegetarian diet, chickpea is vital in the diet to tackle pertaining issues including malnutrition and protein deficiency in the region.

Marker assisted selection (MAS) in crops has an enormous potential to overcome the shortcomings of conventional breeding programs and greatly increase the production and the quality of the crops (Amad *et al.,* 2014).All the different molecular markers currently available including restriction fragment length polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Microsatellites/SSR (Simple Sequence Repeats), Random Amplified Polymorphic DNA (RAPD) etc. have their own advantages and disadvantages. Of these, the microsatellites owing to their abundance, high polymorphism and co dominant nature serve as the best candidate for genetic diversity studies (Gajibhiya *et al*., 2012) Microsatellite markers have been developed and used for measuring genetic diversity in chickpea (Parida *et al.,*2015)

Materials and Methods

Plant Material and DNA Extraction

The genetic diversity of five cultivars was analyzed in this study. Seeds of five different varieties namely GUJ-1, GUJ-2, GUJ-3, GUJ-5 and UJJAVAL were procured from Gujarat State Seed Corporation Limited. Seeds of each variety were planted in pots in a net house on the campus of the Division of Biological and Life Sciences, Ahmedabad University on 21st November 2017. They were observed daily to check for poding and flowering and were grown for 3 and half months. Fresh young leaves were collected at time of 50% flowering from three replicates of each cultivar for DNA isolation using the CTAB method (Chandra and Tewari, 2017), (Doyle and Doyle 1990) with the help of 2 fixing solutions 1) Absolute Alcohol (99.99%), and 2) Alcohol: Chloroform (70:30) (Sharma *et al.,*2010). The DNA concentration was quantified using a multi-plate reader [Synergy HT BioTek]. Qualitative and quantitative analysis was conducted using UV-VIS spectroscopic readings and agarose gel electrophoresis images are shown in Table 1.

PCR and Marker Analysis

Fifteen polymorphic SSR (Simple Sequence Repeats) markers were selected for the study: ICCM0249, TAA170, STMS11, CaGM00495, CaGM00515, ICCeM0050, CAM1577, NCPGR223, NCPGR138, ICCM0105, ICCM0228, ICCM0301, CaM0040, CaM0489, CaM0803. Markers were selected from the published data set of (Nayak *et al.,* 2010*)* and *(*Winter *et al.,* 2000). The sequence and temperature of each is mentioned in Table 1.

The PCR reactions were performed with a total volume of 20µl containing: (a) 0.2µl around 400-500ng genomic DNA (b) 0.2µl 10mM dNTPs (c) 0.5µl forward and reverse primers (d) 2µl 1mM $MgCl_2$(e) 0.5µl 1U Taq polymerase (Dream Tag, Fermentas) (f) 2µl 10X Assay PCR buffer (Dream Taq buffer, Fermentas) (g) 14.0µl of molecular biology grade water. The reactions were performed under the following conditions: Initial denaturation at 95°C for 4 minutes; followed by 40 cycles of denaturation at 94°C for 1 minute each; variable annealing temperatures for 1 minute 20 seconds and an extension at 72°C for 1 minute followed by a final extension at 72°C for 10 minutes. The DNA amplifications were determined by gel electrophoresis using 1% Agarose gel containing Ethidium Bromide. The sample was then prepared by adding 4µl of Bromophenol blue dye to 20µl of DNA sample, followed by loading of 15µl solution in the prepared wells. The results were then visualized using a Gel Doc [Image quant LAS 500]. The PCR amplification of the markers was performed on Eppendorf master Cycler (40) using GeneAmp® software.

Statistical Analysis

The following field data was collected from three replicates of each cultivar: (i) Time taken for 50% flowering (ii) Root length (iii) Shoot length (iv)Number of leaves/plant (leaflets) (v) Number of pods/plant (vi) Seed yield per pod/plant (vii) Seed weight. The observed values have been shown in Table 1.4.

To understand the phenotypic variation and co-relation between the cultivars, these parameters were statistically analyzed using OPSTAT online statistical package (Hisar Agricultural University) to obtain the standard deviation and the Pearson correlation matrix.

Binary scoring of all the cultivars based on their amplification profile on the agarose gel was performed. The UPGMA cluster analysis with Dendro-UPGMA freely available software was performed and dendogram was obtained to check exact genetic distance between cultivars.

Results and Discussions

DNA was isolated from young juvenile leaves and quality of DNA was tested using quantitative and qualitative analysis. Good intact bands on AGE gel certified its quality and the Ratio of the 260/280 in spectrophotometric measurements from 1.8 to 2.0 indicates presence of a pure DNA and determining its quality. RNAse (2µl) treatment

was given to sample with subsequent incubation in the water bath for 45 minutes at 80°C, to avoid any RNA contamination.

15 SSR markers where chosen from well-known literature of (Nayak *et al*., 2010) and (Winter *et al*., 2000) and there electrophorograms were checked to assure there banding patterns. Table 1 shows the list of SSR markers chosen for testing and their primer sequence and product length.

Table 1 represents data of the selected 15 micro-satellite primers after isolating the genomic DNA from the gene pool of 5 cultivars and were screened to carry out genomic analysis. These SSR markers were amplified using polymerase chain reaction to find genetic distance between the cultivar varieties chosen. AGE was performed on the PCR products using a 0.8-1.0% agarose gel. NCPGR223 and ICCM0105 amplification was observed in 0.8% gel (AGE) because of their high molecular weight and the photographs of obtained bands were taken in the Gel Doc as shown from Figure 1 to 5. Different markers showed bands at different position with different varieties.

CaGM00515 and CaM0040 showed monomorphic bands at 240 Kb, ICCM0301 at 180Kb, CAM0489 at 160kb, CaM0803 at 150Kb, CaM1577 at 230Kb, NCPGR223 at 260Kb, ICCeM0050 at 210Kb and ICCM0105 at 280Kb. CaGM00495 showed monomorphic bands at 320Kbforall cultivars except 2nd variety with band at345Kb, ICCM0228 at 220Kbfor V1 and 270Kb for rest varieties.

All the electrophorograms shown above depicts the product length of all primers with different varieties and most of them match with Table 1. Based on these bands binary scoring was created and with that scoring data was used to generate dendogram.

Table 1: list of selected markers.

Marker	Forward primer (5' to 3')	Reverse Primer (5' to 3')	Product length
ICCM0249	TTTCTTCGCATGGGCTTAAC	GGAGATTTGTTGGGTAGGCTC	193
TAA170	TATAGAGTGAGAAGAAGCAAAGAGGAG	TATTTGCATCAATGTTCTGTAGTGTTT	259
STMS11	ATATCATAAACCCCCAC	GTATCTACTTGTAATATTCTCTTCTCT	232
CaGM00495	CCACCACATTTCATCACTCG	TTAGGGTCTCCGTCGTATGG	345
CaGM00515	ATCGATTTGGGGGAAATAGG	AGACAAAGCCATAACCGTGG	234
ICCeM0050	GCGAATCAATGTTCACAAGC	GAGGGAACACCAACTCCAAA	198
CAM1577	TCCTTTGTTTTTCCTTTCTTCCT	AATGCGTTACGGGTGAAATG	237
NCPGR223	TGGGTTTCTTTTCTTGAAGC	AGTGGGTTGAGAAATTACGG	267
NCPGR138	ATTCCAAATTGCTGTTGTTTG	TGTGGATTTTAGTTGCAATG	213
ICCM0105	TGCTTCCTTTTCAATCACCA	TGACAAAGGACAAATAAGTGTTTTA	280
ICCM0228	TGGACGTAGGTTGTTGTGGA	GGACCGGGAGTCCCTTATTA	274
ICCM0301	ATGGCCAAAATGAACTCCAG	AAAAGAGAAGGTTCCATCGG	173
CaM0040	TTGGTTTATTTGGGTTGGGA	GCTTGATTTACATGAACTGAACTGA	237
CaM0489	GGAGGGGGAGCAATAATAGG	TGATTTGTCTCATGCCGCTA	150
CaM0803	CAGAATCCTCATCGTCGGAT	TCGCAACATTTAGCAGCATC	132

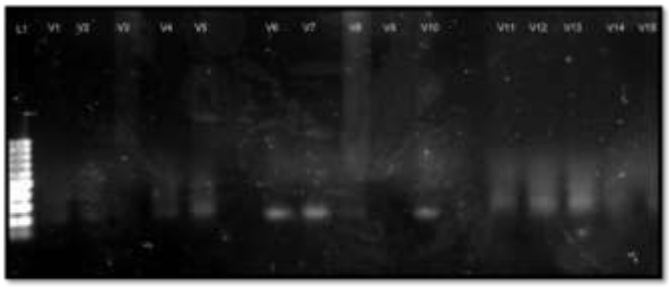

Fig. 1: L1 is a 100bp ladder; the wells V1-V5 shows amplification profile of 5 varieties for the primer CAM1577 V6-V10 represents bands with the primer ICCM0301 and the wells V11-V15 with the primer CAM0040.

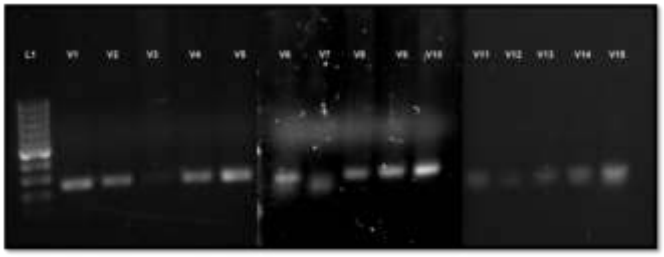

Fig. 2: V1-V5 wells show bands of PCR product of all 5 varieties resulted from primer CaGM00515 V6-V10 shows from primer ICCeM0050, and wells V11-V15 shows bands of 5 varieties by primer CaM0803.

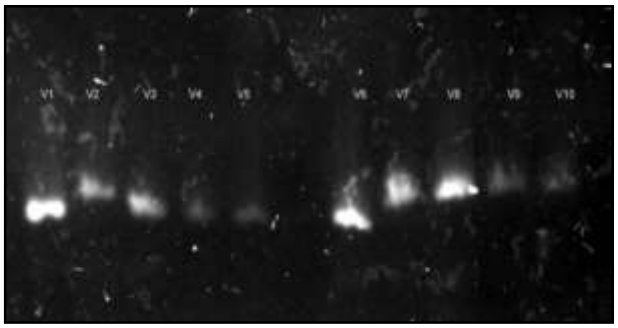

Fig. 3: V1-V5 is 5 varieties with CaGM00495 and ICCM0228 is from V6-V10.

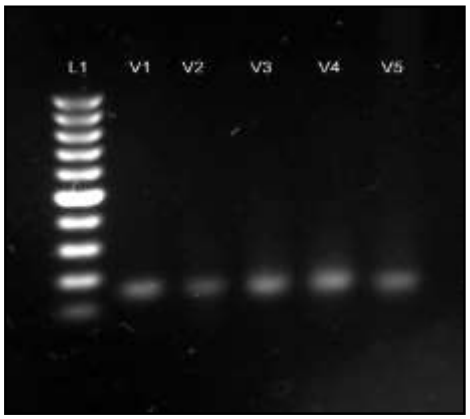

Fig. 4: L1 is a 100bp ladder, V1-V5 is PCR product with CaM0489.

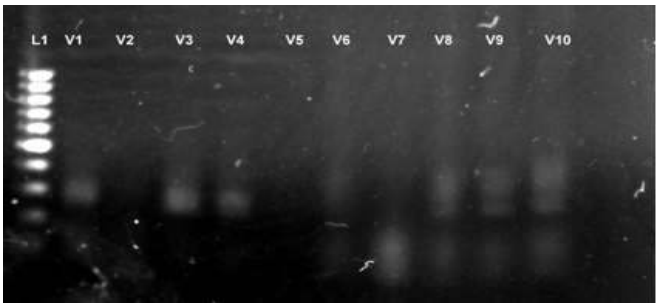

Fig. 5: L1 is a 100bp ladder; the wells**,** V1-V5 is 5 varieties with NCPGR223 and ICCM0105 is from V6-V10.

Dendro-UPGMA is a web server that allows free construction of dendrograms, using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) or WPGMA (Weighted Pair Group Method with Arithmetic Mean) algorithm. A dendrogram is a diagram frequently used to illustrate the arrangement of different clusters produced by hierarchical clustering between similar sets of data. They are frequently used in biology to show clustering between genes or samples. It consists of U-shaped lines that connect data points in a hierarchical tree. The length of lines represents the distance between two data points that are connected (Wilkinson *et.al* 2009) (cold spring harbor protocol)

According to the result we can interpret data as GUJ-1 and GUJ-2 are closest among all the cultivars chosen.GUJ-5 and GUJ-3 are closely related to each other, followed by UJJAVAL.GUJ- 5 is nearest to center node with UJJAVAL on its one side and rest all on others.GUJ-3 is in center of GUJ- 5 and GUJ-2 showing equal distance to both. This proves genetic diversity of cultivars with no one so closely related to come in same cluster but genetically related.

Seeds procured from Gujarat State Seed Corporation limited were sown in November 2017. On monitoring them religiously it was observed that GUJ-3 and GUJ-5 exhibited flowering at 40th day. GUJ-1 showed 50% flowering, GUJ-3 and GUJ-5 started showing poding at 51th day. On 58th day all pots had poding except UJJAVAL. Flowering was observed in UJJAVAL on 63rddaybut turning tables around it resulted as a highest yielding plant. The mature plants were harvested and field data was collected in replicates whose photographs are shown in Fig. 1 to 5

Fig. 6 represents a dendogram depicting genetical distance between 5 chosen cultivars. Here it's clearly observed that cluster 2 is nearest to center with cluster 3 closest to it and cluster 1 little far serving almost same distance. Cluster 4 and 5 are closest to each other from all other clusters, but away from node. Cluster 3 is almost equally away from cluster 2 and 4. Hence, cluster 4 and 5 are genetically closest to each other, followed by cluster 2 and 3, then comes cluster 3 and 4 and lastly cluster 1 and 2. None of the cluster present shows sub clustering proving none of the variety chosen are close relatives.

Table 3 shows Pearson correlation between different cultivars and their characteristics are shown in above table. Nearer the value towards 1 more significant would be data. Mean of all the three replicates was taken for all characters. This data of Table 3 Opstat was used for correlation analysis in Haryana Agriculture University (off campus user option) and correlation as shown in above table was obtained. Root dry weight, shoot dry weight, shoot length and number of pods all when compared with root length data in column 1 it shows which means that as root length increases its shoot length, root and shoot dry weight and number/weight of pod is also increasing significantly. The rest of the data has been interpreted in a similar manner. This high significance proves data correct.

Fig. 6: A dendogram depicting genetical distance between 5 chosen cultivars.

Table 2: Physiological data

Parameters	**GUJ 1**	**GUJ 2**	**GUJ 3**	**GUJ 5**	**UJJAVAL**
RL	5.10 ± 036	6.27 ± 025	5.93 ± 031	6.23 ± 0.25	6.93 ± 012
RDW	0.052 ± 0.01	0.063 ± 0	0.053 ± 0.01	0.049 ± 0	0.096 ± 0
SL	33.00 ± 2	31.00 ± 6.56	33.00 ± 2	31.33 ± 1.53	46.67 ± 4.51
SDW	0.271 ± 0	0.337 ± 0.04	0.551 ± 0.04	0.246 ± 0.03	1.066 ± 0.15
PODS	3.33 ± 0.58	2.33 ± 0.58	4.00 ± 1.73	1.67 ± 0.58	7.00 ± 1.73
BRANCHES	2.00 ± 0	1.33 ± 0.58	2.00 ± 0	1.00 ± 0	3.67 ± 0.58
SEED WT.	0.221 ± 0.01	0.200 ± 0.06	0.267 ± 0.05	0.187 ± 0.05	0.433 ± 0.11

Columns depicts the variety chosen and rows shows the mean values of the triplicates data of various parameters chosen where RL= root length, RDW= root dry weight, SL= shoot length, SDW= shoot dry weight, PODS= number of pods obtained from each plant, BRANCHES= number of branches starting from 1 root system and SEED WT. = weight of total seeds obtained from all pods.

Table 3: Pearson Correlation Matrix

	RL	RDW	SL	SDW	PODS
RL	1				
RDW	1.000**	1			
SL	0.995**	0.996**	1		
SDW	1.000**	0.999**	0.995**	1	
PODS	0.995**	0.996**	1.000**	0.994**	1

NS = negative significant co-relation, * means 0.01% significant and ** means 0.05% significant co-relation.

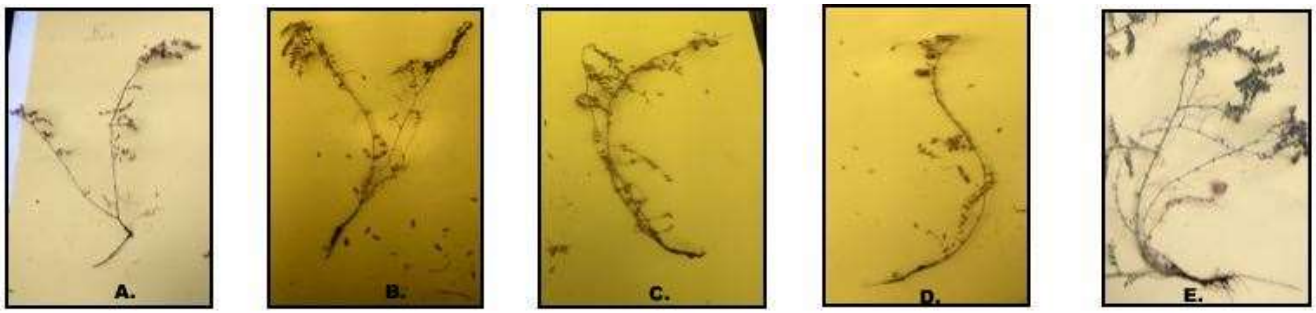

Fig. 7: The figures show one plant each, from entire pot of cultivars at the time of harvest. A.= GUJ-1, B.= GUJ-2, C. =GUJ-3, D.= GUJ-5 and E.= UJJAVAL.

Discussion

The analysis and selection of cultivars to be best suited for the semi-arid conditions was done on the backdrop of various parameters. The protocol followed for the isolation of the plant genomic DNA using the CTAB method without utilization of liquid nitrogen where different fixative agents were used of which absolute alcohol gave best result and DNA's quality was comparable to extraction with liquid nitrogen and this method is used in many research papers. Good quality positive yields were obtained, analyzed using electrophoresis and quantified using spectrophotometric analysis. The Genomic study on the five different cultivars was carried out using a set of known Chickpea SSR markers selected from the studies of markers identified by Nayak. The varieties selected in our studies were found to be coherence with the previous studies through amplification of the products using the Polymerase chain reaction and the fragments analyzed using electrophoresis method and visualized using the Image quant LAS 500. In the included literature, bands were observed at the following base pairs in the respective variety as shown in Table 1 CaGM00495 = 345bp, CaGM00515 = 234bp, ICCeM0050 = 198bp, CAM1577 = 237bp, NCPGR223 = 267bp, ICCM0105 = 280bp, ICCM0228 = 274bp, ICCM0301 = 173bp, CaM0040 = 237bp, CaM0489 = 150bp, and CaM0803 = 132bp, which corresponds to the results we obtained (Fig. 1-5). All markers chosen gave monomorphic bands according to literature and our results also showed all monomorphic bands adding proof to authenticity of our data.

The phylogenetic tree created using UPGMA method using online available software's reveal that GUJ-1 and GUJ-2 are closely related to each other, GUJ-5 and GUJ-3follows next, GUJ-5 is nearest to center node showing almost equal distance to GUJ – 3 and UJJAVAL. Dendogram proves that all variety and genetically distinct from each other with no sub-clustering proving no close relatives present. GUJ-5 and GUJ-3 AND GUJ-1 and GUJ -2 are 2 pairs showing relatedness with highest similarity in GUJ-1 and GUJ-2 in their genetic origin.

Significant differences were observed between the five cultivars selected for the study for all the parameters. Not only genetically, physiologically also the varieties had very different characteristics. The varieties GUJ-3 and GUJ-5 showed minimum days to flowering at 40 days followed by flowering in GUJ-1 and GUJ-2 on 51th day. The varieties of GUJ-3 and GUJ-5 displayed minimum days to maturity at 51 days while the varieties GUJ-1 and GUJ-5 yielded pods by the 58th day. We can clearly observe the relatedness of GUJ-1 and GUJ-2; and GUJ-3 and GUJ-5 in their physiological data, which proves our data of genetic relativeness which in turn affects their physiology showing physiological similarity. The variety UJJWAL displayed maximum time for flowering as well as time to maturity, but then also highest yielding variety with a good biomass and high number of branches. Comparatively root and shoot length of almost all variety was same yielding almost same number of pods with little heavier seeds in UJJAVAL and almost same in rest all. As height and number of branches were almost same there dry weight also was significantly co-related giving highly significant results in Opstat

analysis. Grain yield and the number of pods long with was selected as the standard to determine the varieties better suited for the semi-arid conditions of Gujarat. The variety UJJWAL produced plants with best physiological characteristics including the root length, shoot length, the root and shoot dry weight. The variety was also found to be the best suited for the harsh semi-arid climate of the state of Gujarat. UJJWAL was also found to produce the highest number of pods and seeds. Hence, the highest yield was observed in the variety UJJWAL that also had the best physiological dimensions and was found to be best suited for cultivation in Gujarat.

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