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Diversity analysis of chickpea (*Cicer arietinum*) cultivars using STMS markers

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ABSTRACT

STMS primer pairs could clearly dissect the genetic diversity and relationship of chickpea (*Cicer arietinum* L.) varieties. Out of 55 STMS primer pairs, 35 generated polymorphism with an average 2.49 amplicons/primer pair. Polymorphic information content ranged from 0.48 to 1.0 and genetic similarity between cultivars ranged from 0.10 to 0.77. Dendrogram constructed based on UPGMA and bootstrapping of the data showed 2 distinct clusters with 2 sub-clusters. The grouping was on the basis of the ancestry. Association of sub-clustering appears to follow ancestry, seed type and flower colour which is supported by high boot strap values. It highlights the urgency for effective supplementation of pedigree data and other morphological data with the database generated by STMS marker to efficiently unearth the genetic inter-relationship among the genotypes as well as finger print the varieties for their protection. A high rate of polymorphism and occurrence of unique alleles or rare STMS alleles provides an immense opportunity for generation of comprehensive fingerprint database.

Key words: Chickpea, Clustering, Diversity, STMS markers

Chickpea (*Cicer arietinum* L.) is a self-pollinated, diploid (2n = 16), annual grain legume crop. It is the third most important grain legume crop of the dry areas of the Indian sub-continent, North Africa and West Asia constituting an important source of dietary protein. Chickpea also plays a major role in biological nitrogen fixation thereby contributing to crop rotation and sustaining soil productivity, nevertheless, its yield in India is low (0.7 tonne/ha) as compared to Australia, Egypt, Israel and Italy (1 tonne/ha). Indian Agricultural Research Institute (IARI), New Delhi is one of a premier institutes in India due to its contributions in chickpea breeding activities and released 28 varieties. An insight into genetic base of chickpea varieties released from IARI would provide valuable guidance to the breeders in planning future crossing programmes and directing the goal-oriented efforts towards increasing the genetic base of chickpea varieties being released. The challenge now is to use the recently developed molecular marker techniques to improve the efficiency of chickpea breeding.

Conventional breeding approaches have not greatly improved yield. Chickpea breeders throughout the world are focusing on increasing yield by pyramiding genes for resistance/tolerance into elite germplasm. Molecular markers have been shown to play a crucial role in crop improvement programmes. Such markers serve as efficient and powerful tools for marker-assisted selection of agronomically important traits. Molecular marker technologies help in improving the efficiency of breeding several-fold since selection is not directly on the trait of interest but on the molecular marker tightly linked to the trait, thereby accelerating the generation of new varieties, especially when the characters are difficult to score. In addition to these applications, cultivated chickpea has low level of genetic polymorphism. However, now availability of large number of microsatellite markers is offering immense scope in assessing the diversity and of utilizing the diverse lines in map construction. They also provide new insights into genome analysis, help in germplasm characterization, phylogenetic analysis and genetic diagnostics. It is important to characterize the genetic diversity in plant species since they serve as a resource base for as yet unidentified genetic information. Germplasm collections needs to be analyzed using for estimating the genetic variability.

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Considering the above a study was conducted to check for the efficiency of SSR markers in diversity analysis of some released chickpea varieties.

MATERIALS AND METHODS

The plant material consisted of 14 chickpea cultivars developed by Division of Genetics of the Institute and released by Central Variety Release Committee, Department of Agriculture and Co-operation, Government of India (Table 1). DNA from 100 mg of fresh young leaf tissue was collected in the winter (*rabi*) season of 2007–08, immediately frozen in liquid nitrogen and stored at –80ºC. Isolation of DNA was carried out in 2008–09 using modified CTAB method. Fifty STMS loci were screened in the varieties of which only 35 were polymorphic (Table 2). The STMS markers were synthesized from Bioneer, South Korea. Biorad MyCycler thermal cycler, USA was used to carry out amplifications in 10 µl volumes which had 20–25 ng plant genomic DNA, 10×Tris buffer (15 mM $MgCl_2$ and Gelatine) of Banglore Genei, India, 10 mM dNTP mix, 1.0 µl primer and 0.3 µl of 3U/µl Taq (Banglore Genei, India). PCR analysis was taken up by having preparation of 150 sec. at 90ºC, followed by 18 cycles of denaturation at 94ºC for 20 sec., annealing for 50 sec. at 50ºC (touch down of 0.5ºC for every repeat cycle) and 1 min. elongation at 72ºC for 50 sec. Further 20 cycles of denaturation at 94ºC for 20 sec., annealing for 50 sec. at 55ºC and 50 sec. elongation at 72ºC were given and finally extension at 72ºC for 7 min. were performed.

Band patterns for each of the microsatellites markers were recorded for each genotype by assigning a letter to each band. Alleles were numbered as 'A1', 'A2' etc. sequentially from the largest to the smallest sized band. No distinction was made between amplified products of varied intensity, when the amplified products were within the expected size range. Any band thought to be an artifact or bands which were either diffused or highly faint or those that were difficult to score due to multiple bands were considered as 'missing data' and was not considered while analyzing the genetic similarities. The polymorphic bands were scored in a spread sheet format with '0' representing absence of band and '1' representing the presence of band. 'Null allele' for any specific marker in any genotype was again considered as absence of band (designated as '0'). The null alleles were reconfirmed. Monomorphic data were excluded from the studies except in cases where at least one genotype showed a null allele, clearly indicating absence of STMS primer binding site. Any marker with more than or equal to 30% missing data across various genotype was excluded from the analysis. The data was analyzed in NTSYS-PC software (version 2.21b). Bootstraps were done using Free Tree and Tree view software. For Clustering, UPGMA was used based on the similarity matrix generated on combined data. Polymorphic information content for each STMS primer pair was calculated.

RESULTS AND DISCUSSIONS

Fifty STMS primer pairs could amplify 1–4 loci/primer pair. Out of these 35 STMS primer pairs were polymorphic while 15 were monomorphic. They could generate 87 amplicons giving on an average 2.49 amplicons/primer pair (Table 2). A high degree of molecular polymorphism was exhibited by all the markers studied. The polymorphic international content ranged from 0.48 to 1.0 and genetic similarity between cultivars ranged from 0.10 to 0.77. Further 87 out of 102 amplicons were polymorphic (85.2%) indicating considerable variability in the material under study. The Jacards similarity matrix dendrogram constructed using the UPGMA method showed the genetic similarity between cultivars ranged from 0.10 to 0.77. Bootstrap values of each cluster were high at the node of major clusters which indicates that the cluster phenogram so obtained (Fig 1) is reliable

Table 1. List of 14 chickpea varieties with their parentage used

Name	Parentage	Type
'Pusa 1103'	('Pusa 256' × *Cicer reticulatum*) × 'Pusa 362'	*Desi*
'Pusa 5023'	'FLIP 90-166' × 'BG 1072' × 'BG 1082' × 'BG 1073'	*Kabuli*
'Pusa 1003'	'ICCV 32' × 'Rabat'	*Kabuli*
'Pusa 391'	'ICCV 3935' × 'Pusa 256'	*Desi*
'Pusa 1053'	'ICCV 3' × 'FLIP 88-120'	*Kabuli*
'Pusa 362'	('BG 203 × P179') × ('BG 303')	*Desi*
'Pusa 256'	('JG 62 × 850-3/27') × ('L 550 × H 208')	*Desi*
'Pusa 372'	'P 1231' × 'P1265'	*Desi*
'Pusa 1088'	('Pusa 256' × 'ICCV 32') × ('ICCV 32')	*Kabuli*
'Pusa 5028'	('SBD 377' × 'Pusa 362') × ('SBD 377' × 'BGD 72')	*Desi*
'Pusa 1105'	('C 104' × 'BG 1003') × ('ICC 88503' × 'BG 1048')	*Kabuli*
'Pusa Dharwar Pragati' (BGD 72)	'Pusa 256' × 'E100 YM' × 'Pusa 256'	*Desi*
'Pusa 1108'	('BG 315' × 'ILC 72') × ('ICCV 13' × 'FLIP 85-11') × ('ICCV 32' × 'SURUTOTO 77')	*Kabuli*
'Pusa 2024'	('BG 261' × 'ICC 88503') × ('GL 920' × 'BG 1003')	*Kabuli*

Table 2 STMS primer sequence, linkage group and PIC value

Primer	Repeat motifs		Primer sequence (5′–3′)	Total no. of alleles produced	Linkage group	PIC value
TA 36	$(TAA)_{41}$	F	TTTAATATTTTACCTTATTAGGAATTGAGA	2	*	0.512
		R	TTCAACTTAAGACATGAAATTTGTTTTTT			
TA 179	$(TAA)_{20}$	F	CAGAAGACGCAGTTTGAATAACTT	2	LG 5[@]	0.761
	$(TAAA)_8$	R	CGAGAGAGAGAAAGGAAGAAGAG			
TA 47	$(TAA)_{21}$	F	TTTTTATAGGTGTCTTTTTGTTGTCTTT	3	LG 2[$]	0.894
		R	TCTGAATAGGAAATAAGAAAGGTAGGTT			
TA71	$(AAT)_{32}$	F	CGATTTAACACAAAACACAAA	2	LG 5[@]	0.671
		R	CCTATCCATTGTCATCTCGT			
TA103	$(AAT)_{31}$	F	TGAAATATCTAATGTTGCAATTAGGAC	2	LG 2[$]	0.480
		R	TATGGATCACATCAAAGAAATAAAAT			
TA 117	$(ATT)_{52}$	F	GAAAATCCCAAATTTTTCTTCTTCT	2	*	
		R	AACCTTATTTAAGAATATGAGAAACACA			0.852
TA 113	$(TAA)_{26}$	F	TGCAAAAACTATTACGTTAATACCA	2	LG 1[@]	0.571
		R	TTGTGTGTAATGGATTGAGTATCTCTT			
TR 60	$(TAA)_{26}$	F	TGAGTCAAAACAAAGAACTTG	2	*	0.649
		R	CTACCGGAAATTTCATTGAC			
TS 24	$(TAA)_3$ TAC	F	GTAGAAAGAAAACTGACATGGTTGAG	4	LG 1[@]	1.000
	$(TAA)_{48}$	R	GCCTAACCCAATAATACCTTCTTTT			
TR 24	$(TTA)_{29}$	F	AACAACTTCCTCTTATTTTCCA	3	LG 6[$]	0.926
		R	CAGTAAAAATCAGCCCAAAC			
TA146	$(TTA)_{29}$	F	CTAAGTTTAATATGTTAGTCCTTAAATTAT	3	LG 4[@]	0.896
		R	ACGAACGCAACATTAATTTTATATT			
TA140	$(TAA)_5TT(A)_3$	F	TTTTGGCATGTTGTAGTAATCATATTT	2	LG 5[@]	0.577
	$(TAA)_{18}$	R	TGAAATGAAAAAGAAAAGGAAAAAGTA			
TA180	$(TAA)_{24}$	F	CATCGTGAATATTGAAGGGT	2	LG 7[@]	0.602
		R	CGGTAAATAAGTTTCCCTCC			
TA 200	$(TAA)_{37}$	F	TTTCTCCTCTACTATTATGATCACCAG	2	*	0.538
		R	TTGAGAGGGTTAGAACTCATTATGTTT			
TA 3	$(TAA)_{11}$	F	AATCTCAAAATTCCCCAAAT	2	LG 9[@]	0.726
		R	ATCGAGGAGAGAAGAACCAT			
TR 31	$(TAA)_{20}$ $T(A)_5$	F	CTTAATCGCACATTTACTCTAAAATCA	2	LG 3[@]	0.788
	$(TAA)_9$	R	ATCCATTAAAACACGGTTACCTATAAT			
TS 43	$(ATT)_{33}$	F	AAGTTTGGTCATAACACACATTCAATA	4	LG 5[@]	0.996
		R	TAAATTCACAAACTCAATTTATTGGC			
GA 102	$(CT)_{13}$	F	CAGAGAACCACATGTTTAGTTGAA	2	*	0.842
		R	AGTTTTGATGCGTGCCATTT			
GAA 40	$(CTT)_9$	F	TTGACGCAGAGAACTCTCAA	2	LG 1[@]	0.631
		R	ATTGGTGTGATGGGTGGATT			
GAA 50	$(CTT)_9$	F	TTCGTTCCCATCAACATTCA	2	*	0.524
		R	CCCTCCCGTATTCATACCAA			
CaSTMS 21	$(CT)_9$ ATCT-	F	CTACAGTCTTTTGTTCTTCTAGCTT	2	*	0.612
	$(CTTT)_2$ $(CT)_4$	R	ATATTTTTTAAGAGGCCTTTTGGTAG			

Contd...

Table 1 Concluded

Primer	Repeat motifs		Primer sequence (5′–3′)	Total no. of alleles produced	Linkage group	PIC value
GA 16	$(GA)_{22}$	F R	CACCTCGTACCATGGTTTCTG TAAATTTCATCCTCTCCGGC	2	LG 2[@]	0.774
TA 110	$(TTA)_{22}$	F R	ACACTATAGGTATAGGCATTTAGGCAA TTCTTTATAAATATCAGACCGGAAAGA	3	LG 2[@]	0.886
TA 194	$(TTA)_{21}$	F R	TTTTTGGCTTATTAGACTGACTT TTGCCATAAAATACAAAATCC	3	LG 2[@]	0.858
TR 58	$(TAA)_{21}$	F R	CTCTATATTTGTTTGTTTTTCGTTTTG TAAAATGTGTAGGGTGCAGAATAAATA	2	LG 2[@]	0.498
TA 144	$(TAA)_{27}$	F R	TATTTTAATCCGGTGAATATTACCTTT GTGGAGTCACTATCAACAATCATACAT	2	*	0.854
TA 186	$(TTA)_{20}$	F R	ACAAAATTCTAAAAGTTCCTTCTACCA GTTGTTAGTCGAATAATTGAGAAAAAGA	3	*	0.680
TA 53	$(TTA)_{57}$	F R	GGAGAAAAATGGTAGTTTAAAGAGTACTAA AAAAATATGAAGACTAACTTTGCATTTA	3	LG 2[$]	0.842
GA 20	$(CT)_{23}$	F R	TATGCACCACACCTCGTACC TGACGGAATTCGTGATGTGT	2	*	0.721
TA 80	$(TTA)_{23}$	F R	CGAATTTTTACATCCGTAATG AATCAATCCATTTTGCATTC	3	LG 6[@]	0.872
TA 114	$(AAT)_{52}$	F R	TCCATNTAGAGTAGGATNTTNTTGGA TGATACATGAGTTATTCAAGACCCTAA	4	*	0.941
TA 203	$(TAA)_{43}$	F R	ATAAAGGTTTGATCCCCATT TGTGCATTCAGATACATGCT	3	LG 1[@]	0.758
TR 56	$(TAA)_{21}$	F R	TTGATTCTCTCACGTGTAATTC ATTTTTGATTACCGTTGTGGT	3	LG 3[@]	0.824
TA 176	$(TAA)_{20}(GAA)_9$	F R	ATTTGGCTTAAACCCTCTTC TTTATGCTTCCTCTTCTTCG	3	LG 6[@]	0.861
TS 46	$(TAA)_{46}(CAA)_2$ $(TAA)_3$	F R	GTTGATATTTTTGTGTGTGCGTAG TAATTACTTGCAAAAATAAATGGACAC	2	*	0.513

*- Unknown; @ - Winter *et al.* 2000; $ - Tekeoglu *et al.* 2002

indicator of molecular diversity and the grouping so obtained would be stable even in addition of newer markers and there is less chance of a change in this grouping pattern. This study revealed that all the 14 varieties grouped into 2 major clusters with two sub-groups each and 2 ungrouped genotypes each. A critical examination of these 2 clusters clearly indicate that the grouping was primarily based on seed type, viz *desi* or *kabuli* and within a group the molecular diversity by descent appears to play a major role as the varieties with same ancestors tended to go together.

The comparison of genetic relationship pattern obtained by STMS matrix data with that of pedigree information available for 14 varieties showed a clearcut pattern of clustering that emerged based on pedigree lineage. In the sub cluster of cluster-I where 'Pusa Dharwar Pragati' grouped with 'Pusa 256', it can be seen that 'Pusa 256' appears thrice in the ancestry of 'Pusa Dharwar Pragati', both of which are bold-seeded *desi* types. Thus 7 genotypes were grouped in cluster 1, 5 in cluster 2 and the genotypes 'Pusa 1108' and 'Pusa 5028' were ungrouped. Association of clustering appears to follow ancestry, seed type and flower colour which is supported by high boot strap values. Rakesh Singh *et al*. (2008) reported similar clustering pattern based on seed type and flower colour while studying diversity using molecular markers. While carrying out STMS profiling, due consideration was given to stratified sampling of polymorphic STMS loci covering bin location on various chromosomes which has been indicated by the linkage group location in Table 2 as per the linkage groups reported by Winter *et al.* (2000) and Tekeoglu *et al.* (2002). The STMS polymorphism were assayed using a DNA pooling strategy, although it is not supposed to do as all the genotypes under study are pure

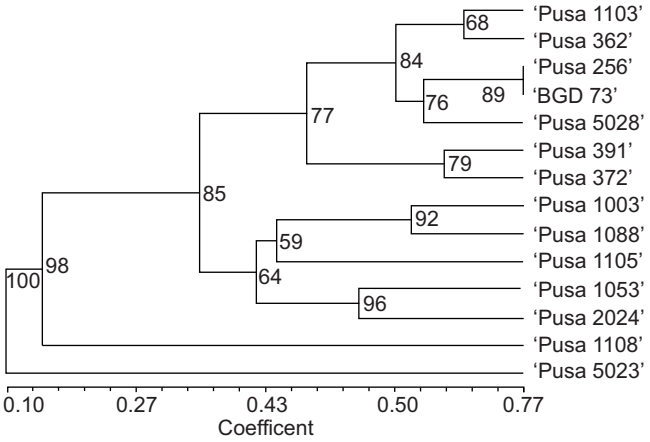

Fig 1 Dendrogram obtained through UPGMA analysis using STMS markers in 14 chickpea varieties. Bootstrap values are indicated at the node of each cluster

lines. The power and potential of SSR markers for a wide range of applications in genetic and breeding of chickpea has been well demonstrated by Flandez-Galvez *et al.* (2003), but still substantial numbers of chickpea microsatellites are not available in public domain. Microsatellite genotypic data from a number of loci have potential to provide unique allelic profiles or DNA fingerprints for establishing genotypes identity as well as in development of molecular linkage map of chickpea. Microsatellite or STMS is a group of repetitive DNA sequences that represents significant portion of eukaryotic genomes. The uniqueness and value of STMS arises from their multiallelic nature, co-dominant transmission, relative abundance and extensive genome coverage. Molecular markers being easily reproducible they have become favourite tools with breeders and biotechnologists to discern the traits as well as to study diversity among cultivars (Tara Satyavathi *et al.* 2005). In this study fourteen cultivars could easily be studied for their diversity using the 35 informative STMS primers. The 35 primers generated 2–4 alleles with 2.48 alleles/primer pair. However, no correlation could be derived from PIC and allele numbers in this study.

There was reasonably high rate of polymorphism for 14 out of 35 STMS markers loci in the present study. Among these TA 114, TS 43 and TS 24 exhibited higher polymorphism pointing towards the scope for further utilization of these markers for chickpea germplasm characterization. The occurrence of unique alleles or rare STMS alleles provides an immense opportunity for generation of comprehensive fingerprint database. The present analysis also gives an insight of the interrelationship among the genotypes and highlights the urgency for effective supplementation of pedigree data and other morphological data with the database generated by STMS marker to efficiently unearth the genetic inter-relationship among the genotypes as well as finger print the varieties for their protection.

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