ONLINE RESOURCES

Molecular diversity and phylogeny in geographical collection of chickpea (*Cicer* sp.) accessions

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Introduction

Chickpea (*Cicer arietinum* L.) is the third most important pulse crop in the world and India is the largest producer of this crop. Nevertheless, its yield in India is low (0.7 tone per hectare (t/ha)) as compared to Australia, Egypt, Israel and Italy (1 t/ha) (FAOSTAT 2008, http://faostat.fao.org/). There has been a significant change in the scenario of chickpea cultivation in India during the past three decades. The expansion of irrigated agriculture in northern India has led to displacement of chickpea with wheat in large area. As a result, the area under chickpea reduced from 3.2 million ha to 1.0 million ha in northern and northwestern India (Punjab, Haryana and Uttar Pradesh), while it increased from 2.6 million ha to 4.3 million ha in central and southern India (Madhya Pradesh, Maharashtra, Andhra Pradesh and Karnataka) from 1985 to 1990. Because of relatively warm environments in central and southern India, the crop is challenged by *Fusarium* wilt, a major yield reducing disease, while in northwestern India, due to cooler environments, the crop is exposed to a severe foliar disease *Ascochyta* blight.

The narrow genetic base among cultivated chickpea accessions is limiting genetic improvement of chickpea through breeding efforts. Understanding the extent of natural variation among cultivated chickpea and wild accessions at molecular level is essential to develop prebreeding and breeding strategies for chickpea. Until recently, the low intra-specific and inter-specific polymorphism in chickpea accessions detected by molecular markers and the scarcity of co-dominant DNA-based markers were serious constraints that hindered the preparation of dense molecular genetic maps or tagging of important traits in chickpea. However, recent studies using STMS markers reveal fairly high levels of variation at these loci and are therefore the marker system of choice for breeding applications in chickpea. Great progress has been made in the development of genetic maps (Sethy *et al.* 2006; Radhika *et al.* 2007; Choudhary *et al.* 2009; Bharadwaj *et al.* 2011; Gour *et al.* 2011) and transcription profiling techniques which help to identify genomic regions and genes underlying stress responses in chickpea.

The productivity potential of chickpea has stagnated around 638 kg/ha with most of the yield potentials of released chickpeas being around 25 quintals/ha (Singh 2010). An insight into diversity among chickpea accessions and their relatedness would provide valuable guidance to the breeders and germplasm people for initiating prebreeding efforts. Wild accessions of chickpea are valuable sources of genes for resistance to various abiotic and biotic stresses (Singh 2005) in planning future crossing programmes for goal oriented efforts towards increasing the genetic base of chickpea varieties being released. With a view to discern the genetic distance between the cultivated chickpea of Indian subcontinent and that of Western Asian Mediterranean. Asian region and wild chickpea accessions, molecular diversity study was undertaken with 50 accessions of chickpea obtained from ICARDA, Aleppo, Syria, ICRISAT, Hyderabad, India, NBPGR, New Delhi, India and IARI, New Delhi, India.

Material and methods

The plant material included 50 chickpea germplasm accessions comprising 12 from International Centre for Agricultural Research in the Dry Areas (ICARDA), 12 from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), three from National Bureau of Plant Genetic Resources (NBPGR), New Delhi and 15 from Division of Genetics, Chickpea Section, Indian Agricultural

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Table 1. List of germplasm accessions used along with their source, biological status, type, region and origin.

	Accession number	Accession identifier	Source	Biological status	Biological type	Geographical region	Origin
1	ICC 4993	RABAT	ICRISAT, India	Genetic stock	*Cicer arietinum*	Morocco	Africa
2	IC 449069	Pusa 1108	IARI, New Delhi	Released variety	*C. arietinum*	Northern India	India
3	ILWC 104	IG 72933	ICARDA, Syria	Wild species	*C. reticulatum*	Mardin	Turkey
4	IC 550411	Pusa 5028	IARI, New Delhi	Released variety	*C. arietinum*	Northern India	India
5	ICC 12968	ICCV 2	ICRISAT, India	Released variety	*C. arietinum*	Southern India	India
6	ILC 3279	NEC 141	ICARDA, Syria	Genetic stock	*C. arietinum*	Syria	Syria
7	ICC 1932	P-1559	ICRISAT, India	Genetic stock	*C. arietinum*	Northern India	India
8	ICC 4951	JG 62	ICRISAT, India	Landrace	*C. arietinum*	Central India	India
9	ICC 17124	ICC 17124	ICRISAT, India	Wild species	*C. reticulatum*	Mardin	Turkey
10	ICC 4958	ICC 4958	ICRISAT, India	Genetic stock	*C. arietinum*	Central India	India
11	ICC 5003	K 850	ICRISAT, India	Released variety	*C. arietinum*	India	India
12	IC 244160	BG 390	IARI, New Delhi	Released variety	*C. arietinum*	Northern India	India
13	EC-539009	EC-539009	NBPGR, New Delhi	Genetic stock	*C. arietinum*	Spain	Spain
14	ICC 8933	WR 315	ICRISAT, India	Genetic stock	*C. arietinum*	Northern India	India
15	ILC 202	Vyr 32	ICARDA, Syria	Genetic stock	*C. arietinum*	Syria	Syria
16	IC 244217	Pusa 1003	IARI, New Delhi	Released variety	*C. arietinum*	Northeast India	India
17	Flip 90-166	Flip 90-166	ICARDA, Syria	Breeding line	*C. arietinum*	Syria	Syria
18	ICCV 96029	ICCV-96029	ICRISAT, India	Released variety	*C. arietinum*	India	India
19	ILWC235	IG 73064	ICARDA, Syria	Wild species	*C. echinospermum*	Gaziantep	Turkey
20	IC 244243	Pusa 1053	IARI, New Delhi	Released variety	*C. arietinum*	India	India
21	ICCV 96030	ICCV-96030	ICRISAT, India	Released variety	*C. arietinum*	India	India
22	SBD 377	SBD 377	IARI, New Delhi	Genetic stock	*C. arietinum*	India	India
23	ICC 3935	ICC 3935	ICRISAT, India	Genetic stock	*C. arietinum*	Iran	Iran
24	ICCV 88503	ICCV 88503	ICRISAT, India	Released variety	*C. arietinum*	Iran	Iran
25	IC 244250	Pusa Dharwar Pragati 72	IARI, New Delhi	Released variety	*C. arietinum*	Central India	India
26	ILWC31	IG 69970	ICARDA, Syria	Wild species	*C. judaicum*	Irbid	Jordan
27	ICC 8159	NEC 2306	ICRISAT, India	Genetic stock	*C. arietinum*	India	India
28	EC 556270	EC 556270	NBPGR, New Delhi	Wild species	*C. reticulatum*	Syria	Syria
29	IC 296132	Pusa 372	IARI, New Delhi	Released variety	*C. arietinum*	Northern India	India
30	EC 556288	EC 556288	NBPGR, New Delhi	Wild species	*C. judaicum*	Syria	Syria
31	ICC 162	P 136-1	ICRISAT, India	Genetic stock	*C. arietinum*	Northern India	India
32	ICCV 88506	ICCV-88506	ICRISAT, India	Released variety	*C. arietinum*	India	India
33	IC 296131	Pusa 362	IARI, New Delhi	Released variety	*C. arietinum*	Northern India	India
34	ICCV 10	ICCV 10	ICRISAT, India	Released variety	*C. arietinum*	India	India
35	ICC 1882	ICC 1882	ICRISAT, India	Genetic stock	*C. arietinum*	India	India
36	IC 296133	Pusa 256	IARI, New Delhi	Released variety	*C. arietinum*	India	India
37	ILWC43	IG 69982	ICARDA, Syria	Wild species	*C. judaicum*	Tartous	Syria
38	ICC 17160	ICC 17160	ICRISAT, India	Wild species	*C. reticulatum*	Turkey	Turkey
39	ILWC 148	IG 72977	ICARDA, Syria	Wild species	*C. judaicum*	Gaziantep	Turkey
40	IC 296376	Pusa 1088	IARI, New Delhi	Released variety	*C. arietinum*	Northern India	India
41	ILWC179	IG 73008	ICARDA, Syria	Wild species	*C. echinospermum*	Urfa	Turkey
42	IC 411513	Pusa 1103	IARI, New Delhi	Released variety	*C. arietinum*	Northern India	India
43	ILWC230	IG 73059	ICARDA, Syria	Wild species	*C. echinospermum*	Urfa	Turkey
44	IC 411514	Pusa 1105	IARI, New Delhi	Released variety	*C. arietinum*	Northern India	India
45	ILWC238	IG 73067	ICARDA, Syria	Wild species	*C. echinospermum*	Gaziantep	Turkey
46	Flip 87-8C	Flip 87-8C	ICARDA, Syria	Breeding line	*C. arietinum*	Syria	Syria
47	ICC 17121	ICC 17121	ICRISAT, India	Wild species	*C. reticulatum*	Mardin	Turkey
48	BG 2024	Pusa 2024	IARI, New Delhi	Released variety	*C. arietinum*	Northern India	India
49	ICC 17123	ICC 17123	ICRISAT, India	Wild species	*C. reticulatum*	Mardin	Turkey
50	BGD 112	BGD 112	IARI, New Delhi	Genetic stock	*C. arietinum*	Northern India	India

Research Institute (IARI), New Delhi (table 1). The accessions had 13 wildtypes and 37 annual cultivated lines. Leaf DNA of about 100 mg of fresh young leaf tissue was collected during December 2009–2010, immediately frozen in liquid nitrogen and stored at −80°C. Isolation of DNA was carried out in 2010 using modifications to the CTAB method of Doyle and Doyle (1987). A total of 100 sequence tagged microsatellite site (STMS) loci were screened in the accessions of which only 71 were polymorphic (table 2). The STMS markers were synthesized as per the sequences of Winter *et al.* (2000) from Bioneer, Daejeon, South Korea. BioRad MyCycler thermal cycler, Richmond, USA was used to carry out amplifications in 10 μL volume reaction mixture. This mixture contained 1 μL of 20 ng plant genomic DNA, 1.6 μL of 10× Tris buffer (15 mM $MgCl_2$ and gelatine), 1 μL of 10 mM dNTP mix, 1.0 μL each of forward and reverse primer and 0.3 μL of 3 U μL^{-1} *Taq* polymerase. PCR was performed with following conditions 150 s at 90°C followed by 18 cycles of denaturation at 94°C for 20 s, annealing for 50 s at 50°C (touch down of 0.5°C for every repeat cycle) and 1 min elongation at 72°C for 50 s. Further 20 cycles of denaturation at 94°C for 20 s, annealing for 50 s at 55°C and 50 s elongation at 72°C were given and final extension at 72°C for 7 min were performed. The resolution of PCR products was done on six per cent polyacrylamide gels. 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. Reconfirmation of null allele was done and the bands which were appearing as artifact or bands which were either diffused or highly faint and null alleles even after reconfirmation were

Table 2. STMS primers used for polymorphism and genetic diversity studies.

Primer name	Total no. of alleles produced	Linkage group	PIC value	Primer name	Total no. of alleles produced	Linkage group	PIC value
TA 179	3	5[a]	0.558	CaSTMS21	2	–	0.460
TA 47	3	2[b]	0.563	GA 16	5	2[a]	0.765
TA71	4	5[a]	0.650	TA 110	5	2[a]	0.775
TA103	3	2[b]	0.409	TA 194	4	2[a]	0.650
TA 117	3	–	0.562	TR 58	3	2[a]	0.359
TA 113	3	1[a]	0.613	TA 144	6	–	0.726
TR 24	2	6[b]	0.463	TA 186	5	–	0.653
TA140	2	5[a]	0.461	GA 20	3	–	0.646
TA180	2	7[a]	0.537	TA 80	3	6[a]	0.577
TA 200	5	–	0.770	TA 114	3	–	0.458
TA 3	4	9[a]	0.642	TA 203	4	1[a]	0.582
TR 31	4	3[a]	0.541	TR 56	4	3[a]	0.509
TS 43	3	5[a]	0.644	TA 176	4	6[a]	0.439
GAA 40	2	1[a]	0.409	TA 53	4	2[b]	0.481
GAA 50	2	–	0.446	TS 46	5	–	0.689
CaSTMS 4	2	6[d]	0.695	TA 2	5	4[a]	0.702
CaSTMS 10	1	3[a]	0.588	TA 8	5	1[a]	0.726
CaSTMS 14	2	3[a]	0.713	TA 13	2	4[a]	0.771
CaSTMS 15	3	6[a]	0.852	TA 18	3	7[a]	0.487
CaSTMS16	4	–	0.858	TA 21	6	7[a]	0.573
CaSTMS 19	4	5[a]	0.714	TA 27	2	2[a]	0.564
CaSTMS 22	5	5[a]	0.698	TA 28	2	6[d]	0.498
CaSTMS 25	4	15[a]	0.661	TA 34	3	3[a]	0.661
CaSTMS 28	3	3[a]	0.652	TA 37	4	2[a]	0.660
TA 45	5	7[d]	0.715	TA 43	3	8[d]	0.698
TA 59	3	3[d]	0.708	TA 125	5	5[c]	0.493
TA 64	5	3[a]	0.689	TR 7	1	6[a]	0.841
TA 72	2	4[c]	0.634	TR 20	2	4[a]	0.762
TA 93	2	–	0.733	TR 26	2	3[a]	0.723
TA 96	3	8[d]	0.711	TR 29	2	5[c]	0.691
TA 106	2	6[a]	0.687	TR 59	2	5[a]	0.812
TA 110	1	3[d]	0.581	TS 12	3	7[d]	0.641
TS 53	3	5[c]	0.531	TS 29	2	–	0.722
TS 72	2	4[c]	0.651	TS 45	3	8[a]	0.689
TAA 58	4	7[a]	0.810	TAA 137	2	–	0.573
				CS 27	4	2[a]	0.812

– Unknown; [a]Winter *et al.* (2000); [b]Tekeoglu *et al.* (2002); [c]Nayak *et al.* (2010); [d]Bharadwaj *et al.* (2011).

not considered for analysis. Tree construction following unrooted NJ tree using similarity matrix was carried through DARwin 5.0.128 (Perrier *et al.* 2003) analysis. Bootstrap analysis using 1000 bootstrap values was performed for the node construction.

Results and discussions

In the present study, a total of 100 STMS loci were analysed, covering various bin locations on different linkage group. Seventy-one STMS loci, in the genetic material under study were found to be highly polymorphic (table 2). Only data from these polymorphic STMS loci were utilized for further statistical analysis. The 71 STMS loci analysed produced 224 alleles with an average of 3.210 alleles per marker. The number of alleles ranged from 1 to 6. Based on the allele frequencies, the PIC (polymorphism information content) values were estimated for different STMS loci analysed. The PIC values ranged from 0.409 to 0.858 with an average PIC of 0.633. A high PIC value in chickpea microsatellite analysis was also reported by Udupa *et al.* (1999) and Upadhyaya *et al.* (2008), who attributed this to polymorphism of TAA motif in chickpea. Diversity index (DI) values ranged from 0.321 to 0.908 with TA 110 showing maximum DI and PIC values. The range of DI was very high in cluster 2 comprising the chickpea collections from central Asian region and ranged from 0.721 to 0.908 while it was narrow in cluster I-D with the values ranging from 0.321 to 0.442. Wild species showed a DI range of 0.801 to 0.908. Jomová *et al.* (2009) also reported high DI and PIC values while analysing 49 chickpea germplasm accessions. The high DI values indicate the lack of close genetic relatedness in the accessions analysed and the greater PIC values indicate the suitability of the microsatellites used for diversity analysis (figure 2). Results from the present study support the observations of several workers on the potential utility of molecular markers in characterization (Satyavathi *et al.* 2006; Sethy *et al.* 2006; Bharadwaj *et al.* 2010). There was reasonably a high rate of polymorphism which points towards the scope for further

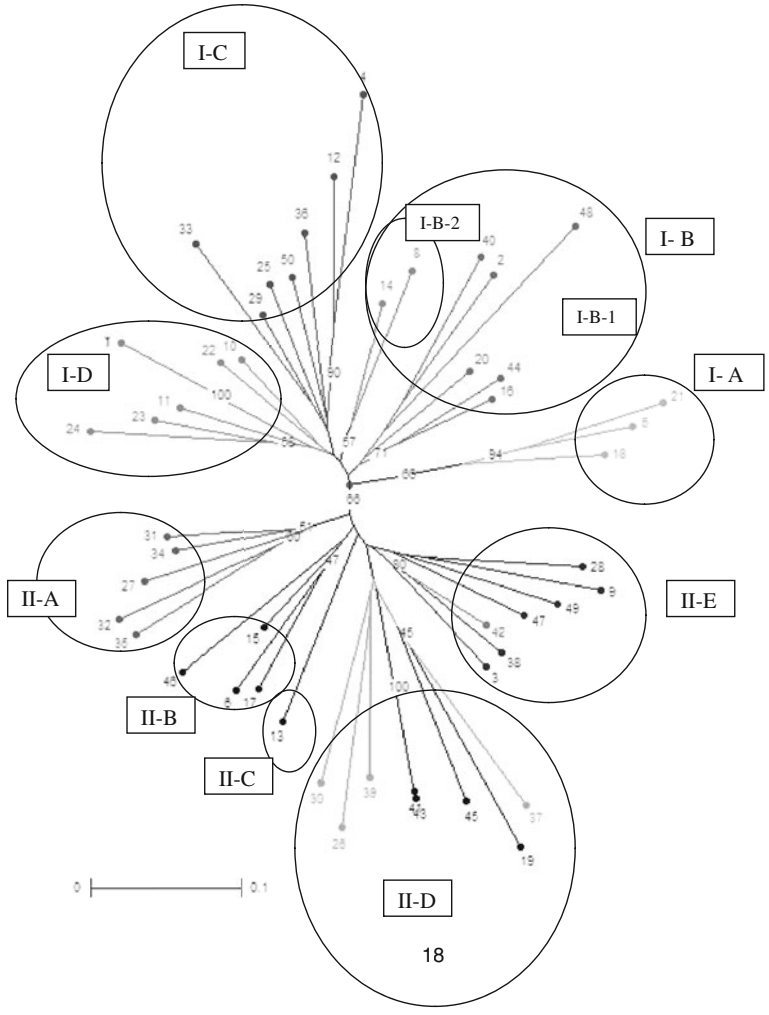

Figure 1. NJ phylogenetic tree using STMS markers in 50 chickpea accessions. Bootstrap values are indicated at the node of each cluster.

Table 3. Clustering based on Darwin's grouping of 50 chickpea accessions as revealed through SSR analysis.

Major cluster	Minor cluster	Number of accessions	Diversity index (DI range)	Accession name
I	I-A	3	0.341 to 0.486	ICCV-96029, ICC 12968, ICCV-96030
	I-B-1	6	0.452 to 0.789	IC 449069, IC 244217, IC 296376, IC 411514, BG-2024, IC 244243
	I-B-2	2	0.567 to 0.664	ICC 4951, ICC 8933
	I-C	7	0.642 to 0.869	IC 550411, IC 244160, IC 296133, BGD-112, ICC 244250, IC 296132, IC 296131
	I-D	7	0.321 to 0.442	ICC 4958, SBD-377, ICC 4993, ICC 5003, ICC 3935, ICCV 88503, ICC 1932
II	II-A	5	0.721 to 0.798	ICC 1882, ICCV 10, ICC 8159, ICC 162, ICCV 88506
	II-B	4	0.741 to 0.784	Flip 90-166, ILC 3279, ILC 202, Flip 87-8C
	II-C	1	1.0	EC 539009
	II-D	8	0.801 to 0.908	ILWC 43, ILWC 235 ILWC 238, ILWC 230, ILWC 179, ILWC 148, ILWC 31, EC 556288
	II-E	7	0.776 to 0.887	EC 556270, ICC 17124, ICC 17123, ICC 17121, IC 411513, ICC 17160, ILWC 104

utilization of these markers for chickpea improvement. The occurrence of unique alleles or rare STMS alleles provides an immense opportunity for generation of comprehensive fingerprint database.

The resources of many unique STMS alleles may be an indication of addition or deletion of small number of repeats (Goldstein and Pollock 1997) and most rational explanation for high mutation rate is polymerase slippage (Levinson and Gutman 1987). The sequence analysis at two microsatellite loci by Sethy *et al.* (2006), revealed copy number changes at repeats motifs as the major reason for microsatellite variability in chickpea.

The genetic similarity matrix was further analysed using UPGMA clustering algorithm by software programme DARwin 5.0.128 (figure 1). The radial branching clearly delineated the genotypes into two major clusters (table 3) with five sub groups and 25 genotypes each. The primary grouping appears to follow geographic distribution from where these germplasm lines were obtained i.e., source or more precisely the origin of cultivars. All the lines which were from Western Asia-Mediterranean grouped as one major cluster (II) including the wild progenitors while those from Indian subcontinent grouped as one major subcluster (I). Both arms in the radial tree between these two subclusters are quite diverse indicating large variability at molecular levels between the Syrian group and the Indian group. Within cluster I, the accessions of ICRISAT (IA, ID) and IARI (IC, IB2) were grouping into distinct subclusters, though at closer levels when compared to the Syrian group of cluster II. Within this cluster, all the kabuli and desi genotypes collected from IARI, New Delhi which are released varieties, grouped distinctively into two subclusters in cluster I. In cluster II also, the grouping followed a definite pattern with all the cultivated types of *C. arietinum* L. falling into distinct group while the wild *C. reticulatum* grouped into distinct subcluster II E and II D of cluster II with the genotype Pusa 1103 (IC 411513) falling into the subcluster II E. The parentage of Pusa 1103 included a wild relative *C. reticulatum*, Pusa 256 and Pusa 362. The study also conclusively proves Pusa 1103's wild lineage and its diverse breeding base. The accessions belonging to *C. judaicum* grouped as a separate subcluster close to the *C. echinospermum* group in cluster II

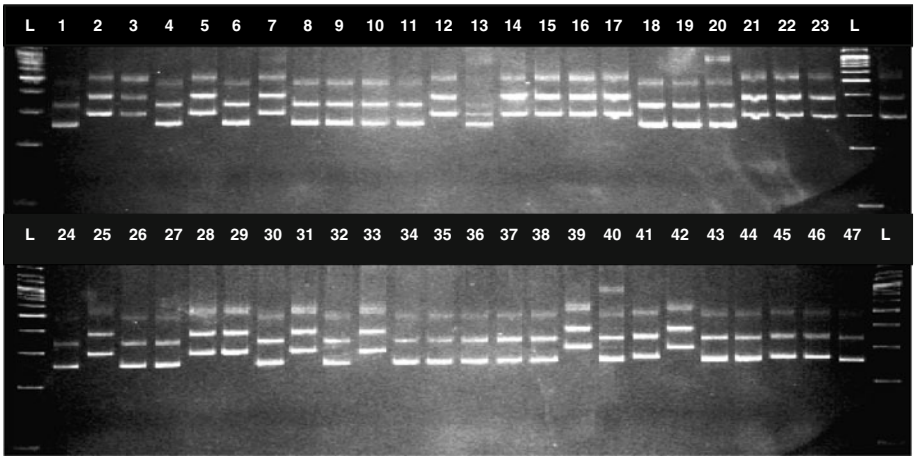

Figure 2. Polyacrylamide gel electrophoresis pictures of the chickpea genotypes with STMS primer TA 2.

(II D) indicating their relatedness in this group and these fall into secondary gene pool as per Singh (2005). They are farther away from the primary cultivated chickpea gene pool species of *C. arietinum* (II E). Comparing a geographically diverse chickpea collection of 36 lines using 25 SSR markers, Sethy *et al.* (2006), could discern the genetic distinctness among these collections. However, they could not establish the genetic relatedness as they reported noncongruence of genetic diversity with geographic diversity. Though this is true in many crops where cultivated or advanced breeding lines are used, when we use land races and genetic stocks as done in this study along with a large number of wild species which have not been subjected to breeding procedures of selection, then genetic diversity is in concomitance to geographic diversity as found in this investigation. This grouping could also clearly establish the distinctness of western Asia which happens to be the centre of origin of chickpea to those that have been developed from Indian subcontinent in concomitance with the present findings.

All the ICARDA lines of Syrian, Turkey and those from Spain origin were falling in near vicinity in subcluster IIB. This clearly brings out the distinctiveness of the Mediterranean group of lines from Syria and vicinity to be distinct from the Indian subcontinent lines. It is obvious as such because the ICRISAT germplasm has more than 60% accessions from Indian subcontinent and use of these accessions in developing advanced breeding lines and varieties by the breeders of Indian subcontinent repeatedly in their breeding programmes would have narrowed the genetic base of the varieties released at this centre. Further, adaptive selection from these lines by breeders in India while developing varieties suitable for Indian subcontinent would have led to development of Indian chickpea breeding materials with relatively narrower genetic base. The grouping of lines from Syria and wild species into a separate cluster indicates that they are diverse to the Indian subcontinent type and can serve as good sources for genetic base broadening in chickpea.

The occurrence of distinct groups of chickpea lines revealed through STMS analysis could possibly draw the attention of the chickpea breeders for effective prebreeding for breaking yield barriers. The initial gains obtained through use of germplasm from ICRISAT though has paid off, wider gains and introgression of alleles for more useful traits can occur if the prebreeding involves ICARDA germplasm or wild relatives. Importance of wild relatives of chickpea as a source of genes having resistance to biotic and abiotic traits has been documented by many chickpea workers (Singh 2005; Kaur *et al.* 2010).

Breeders can broaden the chickpea genetic base by resorting to prebreeding using wild relatives like *C. reticulatum* which is easily crossable with *C. arietinum*. The divergence of *C. arietinum* lines from Mediterranean region from those of the Indian subcontinent also indicates that greater genetic gains can be obtained by using these lines in breeding programmes. Further this study also conclusively proves the ability of STMS markers in discerning diversity.

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