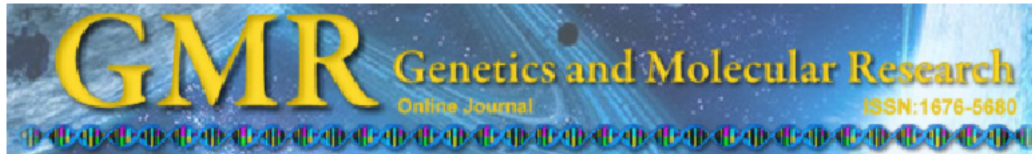

Genetic structure and diversity analysis of the primary gene pool of chickpea using SSR markers

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ABSTRACT. Members of the primary gene pool of the chickpea, including 38 accessions of *Cicer arietinum*, six of *C. reticulatum* and four of *C. echinospermum* grown in India were investigated using 100 SSR markers to analyze their genetic structure, diversity and relationships. We found considerable diversity, with a mean of 4.8 alleles per locus (ranging from 2 to 11); polymorphic information content ranged from 0.040 to 0.803, with a mean of 0.536. Most of the diversity was confined to the wild species, which had higher values of polymorphic information content, gene diversity and heterozygosity than the cultivated species, suggesting a narrow genetic base for cultivated chickpea. An unrooted neighbor-joining tree, principal coordinate analysis and population structure analysis revealed differentiation between the cultivated accessions and the wild species; three cultivated accessions were in an

intermediate position, demonstrating introgression within the cultivated group. Better understanding of the structure, diversity and relationships within and among the members of this primary gene pool will contribute to more efficient identification, conservation and utilization of chickpea germplasm for allele mining, association genetics, mapping and cloning gene(s) and applied breeding to widen the genetic base of this cultivated species, for the development of elite lines with superior yield and improved adaptation to diverse environments.

Key words: Chickpea; Genetic diversity; Molecular markers; Population structure; Principal coordinate analysis

INTRODUCTION

Chickpea (*Cicer arietinum* L.) with a genome size of 732 Mbp is a self-pollinated, diploid (2n = 2x = 16) cool season pulse crop grown in more than 44 countries representing all the continents under eight geographically diverse agro-climatic conditions (Croser et al., 2003). In addition to being a major source of dietary protein for humans in semiarid tropical regions, chickpea plays an important role in the maintenance of soil fertility particularly in dry rainfed areas. It ranks fourth in production (9.8 Mt) after soybean (222.3 Mt), dry bean (19.7 Mt) and dry pea (10.4 Mt) (FAO, 2009). Over 95% of the area, production and consumption of chickpea is in developing countries and the majority of the world's chickpea is grown in South Asia and the Mediterranean region with India being the largest producer.

Despite intensive breeding efforts over several decades, the average global chickpea yield, 0.9 t/ha (FAO, 2009) is far below its yield potential of 5 t/ha (Sudupak et al., 2002). A narrow genetic base and sexual incompatibility with other *Cicer* wild types, which carry the sources for resistance and tolerance to various biotic and abiotic stresses, contribute to the limited progress in the improvement of chickpea yield. In order to enhance the genetic potential one has to assess the extent and the pattern of real diversity available in the existing cultivated and wild accessions. World cultivated chickpea germplasm lacks the diversity that may include traits needed for effective improvement of the crop. It is in this context that the wild annual species, especially *C. reticulatum* and *C. echinospermum*, have drawn the attention of breeders, because they possess many agronomically desirable traits and are cross-compatible with *C. arietinum*.

Knowledge and management of the genetic diversity and relationship within and between the cultivated chickpea and its wild relatives are of paramount importance and may ensure the long-term success of chickpea improvement programs. Traditionally, a number of marker systems such as plant morphology, crossability data, karyotypes, seed storage protein analysis, and enzymes have been used to study the relationship between the *Cicer* species (Croser et al., 2003). Subsequently, DNA-based markers such as RAPD (Iruela et al., 2002; Sudupak et al., 2002), RFLP (Udupa et al., 1993), AFLP (Nguyen et al., 2004), and ISSR (Sudupak et al., 2004) were used to study genetic diversity and relationships in chickpea with most of these studies reporting abundant diversity in wild *Cicer* but narrow genetic variation in cultivated chickpea. However, recent reports on the generation of a range of genomic resources including identification of new SSR markers worldwide (Lichtenzveig et al., 2005;

Choudhary et al., 2006; Sethy et al., 2006a,b; Varshney et al., 2007; Nayak et al., 2010) have aided in different areas of genome analysis like genetic diversity (intra- and interspecific) and cultivar identification (Udupa et al., 1999; Chowdhury et al., 2002; Singh et al., 2008b; Upadhyaya et al., 2008; Sefera et al., 2011), construction of genetic linkage maps (Tekeoglu et al., 2002; Nayak et al., 2010), identification of QTLs (Cobos et al., 2005), and MAS (Gupta and Varshney, 2000).

Intensive efforts on the characterization of cultivated chickpea using SSR markers have been made worldwide (Chowdhury et al., 2002; Singh et al., 2008b; Sefera et al., 2011). However, studies related to molecular diversity and population structure of the members of the primary gene pool (*C. arietinum*, *C. reticulatum* and *C. echinospermum*) are relatively meager (Udupa et al., 1999; Upadhyaya et al., 2008). The present study was thus undertaken to analyze the nature of genetic structure and the level of genetic diversity and relationships within and between the popular chickpea cultivars and breeding lines and two of its closest wild relatives using a wide set of SSR markers. The study can supply information about putative domestication events, evolutionary relationships and the gene flow between the cultivated chickpea and its wild relatives and will therefore provide opportunities for breeders and molecular biologists to use diverse accessions for varied applications in chickpea genomics and breeding.

MATERIAL AND METHODS

Plant materials

Forty-eight chickpea accessions representing the members of the primary gene pool, including 38 accessions of *C. arietinum*, 6 of *C. reticulatum* and 4 of *C. echinospermum*, were analyzed in this study. Cultivated chickpea comprised of 26 desi and 12 kabuli accessions (Table 1). The material was obtained from the Pulse Research Laboratory, Indian Agricultural Research Institute, New Delhi, India.

DNA isolation and genotyping

Genomic DNA was isolated from young leaf tissue using the CTAB procedure described by Saghai-Maroof et al. (1984) with minor modifications. DNA was quantified using a spectrophotometer and maintained at -20°C. One hundred polymorphic microsatellite loci of 210 tested were analyzed in this study (Lichtenzveig et al., 2005; Sethy et al., 2006b). Amplification was carried out in a 10-µL reaction volume containing 30 ng DNA, 1X buffer, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 µM each of the forward and reverse primer and 0.5 U *Taq* DNA polymerase (Bangalore Genei, Bangalore, India) in a thermocycler (Biometra, Gottingen, Germany) programmed for 35 cycles, consisting of denaturation at 94°C for 1 min, annealing at 50°-60°C (depending on the primer) for 1 min and extension at 72°C for 2 min. An initial denaturation at 94°C for 5 min and a final synthesis of 10 min at 72°C were also included. Amplified products were resolved on 4% super-fine resolution agarose gels (Amresco, Solon, USA) in 1X TAE buffer stained with ethidium bromide and photographed under UV light. The size of the amplified fragments was determined using a 100-bp ladder (Fermentas Life Science, USA).

Table 1. Detailed information of 48 chickpea (*Cicer*) accessions used in the study.

S. No.	Accession No.	Biological status	Species	Origin	Seed type
1	ICC162	Traditional cultivar	*C. arietinum*	India	Desi
2	ICC1932	Traditional cultivar	*C. arietinum*	India	Desi
3	ICC4918	Landrace	*C. arietinum*	India	Desi
4	ICC4951	Landrace	*C. arietinum*	India	Desi
5	ICC4958	Advanced cultivar	*C. arietinum*	India	Desi
6	ICC4993	Advanced cultivar	*C. arietinum*	North Africa	Desi
7	ICC8151	Landrace	*C. arietinum*	USA	Kabuli
8	ICC8159	Landrace	*C. arietinum*	India	Desi
9	ICC5003	Advanced cultivar	*C. arietinum*	India	Desi
10	ICC8933	Landrace	*C. arietinum*	India	Desi
11	ICC12968	Advanced cultivar	*C. arietinum*	India	Kabuli
12	ICCV10	Released variety	*C. arietinum*	India	Desi
13	ICCV88506	Released variety	*C. arietinum*	India	Desi
14	ICCV96029	Released variety	*C. arietinum*	India	Desi
15	ICCV96030	Released variety	*C. arietinum*	India	Desi
16	ICCV93954	Released variety	*C. arietinum*	India	Desi
17	ILC202	Genetic stock	*C. arietinum*	USSR	Kabuli
18	ILC3279	Genetic stock	*C. arietinum*	USSR	Kabuli
19	Flip87-8C	Breeding line	*C. arietinum*	Syria	Kabuli
20	IC118913	Released variety	*C. arietinum*	India	Desi
21	IC296131	Released variety	*C. arietinum*	India	Desi
22	IC296132	Released variety	*C. arietinum*	India	Desi
23	IC296133	Released variety	*C. arietinum*	India	Desi
24	IC244250	Released variety	*C. arietinum*	India	Desi
25	IC244160	Traditional cultivar	*C. arietinum*	India	Desi
26	IC244243	Released variety	*C. arietinum*	India	Kabuli
27	IC296376	Released variety	*C. arietinum*	India	Kabuli
28	IC411513	Released variety	*C. arietinum*	India	Desi
29	IC411514	Released variety	*C. arietinum*	India	Kabuli
30	IC449069	Released variety	*C. arietinum*	India	Kabuli
31	EC539009	Genetic stock	*C. arietinum*	Spain	Kabuli
32	BG315	Breeding line	*C. arietinum*	India	Kabuli
33	BG374	Breeding line	*C. arietinum*	India	Desi
34	BG1004	Breeding line	*C. arietinum*	India	Desi
35	SBD377	Breeding line	*C. arietinum*	India	Desi
36	IPC92-1	Breeding line	*C. arietinum*	India	Desi
37	PG95333	Breeding line	*C. arietinum*	India	Kabuli
38	Brachid Mutant	Genetic stock	*C. arietinum*	India	Desi
39	EC556270	Wild	*C. reticulatum*	Syria	-
40	ILWC104	Wild	*C. reticulatum*	Turkey	-
41	ICC17121	Wild	*C. reticulatum*	Turkey	-
42	ICC17123	Wild	*C. reticulatum*	Turkey	-
43	ICC17124	Wild	*C. reticulatum*	Turkey	-
44	ICC17160	Wild	*C. reticulatum*	Turkey	-
45	ILWC35	Wild	*C. echinospermum*	Turkey	-
46	ILWC181	Wild	*C. echinospermum*	Turkey	-
47	ILWC179	Wild	*C. echinospermum*	Turkey	-
48	ILWC180	Wild	*C. echinospermum*	Turkey	-

Data analysis

The alleles amplified by each SSR primer pair were scored across all the genotypes. The basic statistics of genetic diversity such as number of alleles per locus, observed heterozygosity (H_O), gene diversity and polymorphic information content (PIC) were calculated for the entire set of 48 accessions as well as for each of the two groups (wild and cultivated) separately using the POWERMARKER 3.25 software (Liu and Muse, 2005). H_O is calculated as number of heterozygous genotypes divided by the number of total genotypes observed at the locus. Gene diversity estimates the probability that two alleles at any locus are different from each other. The

PIC value measures the polymorphism observed in a group of genotypes at a specified locus. Pearson correlation coefficient was used to evaluate the relationships between the number of alleles per locus, PIC and gene diversity using the SAS software (SAS Institute, 1998).

Using the data provided by 100 SSR markers, three types of analysis were performed to group the accessions and investigate the relationship and structure of the genetic diversity. From the data matrix that listed the alleles at each SSR marker locus, an unrooted neighbor-joining (NJ) tree was constructed with the POWERMARKER 3.25 software using Rogers' genetic distance (Rogers, 1972) and the tree was visualized with the TREEVIEW 1.6.6 software (Page, 1996). Using the data matrix for the presence or absence of each allele, a principal coordinate analysis (PCoA) was performed with the NTSYS-pc 2.1 software (Rohlf, 2000) and the two principal coordinates were used to visualize the dispersion of accessions. For the analysis of population structure, a Bayesian model-based analysis was performed using the STRUCTURE 2.1 software (Pritchard et al., 2000). This software assumes a model in which there are K populations (clusters), which contribute to the genotype of each individual and each is characterized by a set of allele frequencies at each marker locus. A Monte Carlo Markov chain method was used to estimate allele frequencies in each of the K populations and the degree of admixture for each individual plant. The number of clusters was inferred using 10 independent runs with 1,000,000 iterations and a burn-in period of 30,000 following the admixture ancestry model and correlated allele frequencies with the K value ranging from 1 to 7. A procedure described by Evanno et al. (2005) to determine ΔK was used to strengthen the results (in terms of real number of clusters). The results generated by this software were visualized in a graphical bar plot of membership coefficients for each K value.

RESULTS

SSR analysis

Forty-eight chickpea accessions were screened using 210 SSR markers, which produced a total of 581 alleles. Of these, 100 SSR markers, which produced 480 alleles, were found to be polymorphic and used for further analysis. The number of alleles per locus ranged from 2 (H3A052, H4E08, H1I08, H3G031, NCPGR27, H5G12, NCPGR44, and NCPGR76) to 11 (NCPGR74) with an average of 4.8 alleles (Table 2). PIC ranged from 0.040 (H4E08) to 0.803 (H2I20) with an average of 0.536. As PIC is a function of allele number and frequency, markers (NCPGR74, NCPGR42, H3D05, H3G06 and H2I20) with a higher number of alleles generally had higher PIC values and vice versa. Gene diversity ranged from 0.041 (H4E08) to 0.825 (H2I20) with a mean of 0.577 in 48 accessions. Average heterozygosity detected was 1.2%, which ranged from 0.0 to 10.0%, with excess of heterozygotes at 4 loci, H3F09, NCPGR33, NCPGR74 and H4D02 (Table 2). The number of alleles per locus showed a significant and positive relationship with both PIC ($r = 0.812$, $P < 0.0001$) and gene diversity ($r = 0.774$, $P < 0.0001$).

To explore genetic diversity among the accessions within each of the groups of wild and cultivated chickpea, four parameters of genetic diversity were also calculated separately for each group (Table 3). A total of 328 alleles were observed in the wild group, ranging from 1 to 6, with an average of 3.28 alleles per locus and 366 alleles were observed in the cultivated group, which ranged from 1 to 9, with an average of 3.66. The mean values of PIC, gene diversity and H_O for wild accessions were 0.487, 0.543 and 3.4, respectively, which were higher than the group of cultivated germplasm (0.449, 0.496 and 0.5, respectively).

Table 2. Allele number, polymorphic information content (PIC), gene diversity, and heterozygosity (H_O) obtained after screening 48 chickpea accessions at 100 SSR loci analyzed in this study.

No.	Marker	Allele No.	PIC	Gene diversity	H_O (%)	No.	Marker	Allele No.	PIC	Gene diversity	H_O (%)
1	H2I20	8	0.803	0.825	2.13	51	H1F21	4	0.596	0.650	0.00
2	H3DO5	9	0.793	0.813	2.13	52	H1B09	3	0.530	0.598	0.00
3	H3GO6	8	0.750	0.775	4.17	53	H5A08	4	0.558	0.621	0.00
4	H5E11	6	0.756	0.787	0.00	54	H2J09	3	0.424	0.486	0.00
5	H2J20	7	0.794	0.818	2.13	55	H4H06	4	0.607	0.666	0.00
6	H5H06	5	0.460	0.492	0.00	56	H3A09	4	0.407	0.447	0.00
7	H4G11	5	0.716	0.756	0.00	57	H1E06	6	0.559	0.590	0.00
8	H5B04	7	0.783	0.810	2.33	58	H3G031	2	0.169	0.187	0.00
9	H1H13	4	0.564	0.636	0.00	59	H1H14	6	0.636	0.666	6.38
10	H6D11	7	0.686	0.721	0.00	60	H1P17	5	0.586	0.632	0.00
11	H3E04	5	0.693	0.739	0.00	61	NCPGR50	4	0.675	0.727	4.17
12	H4A04	5	0.654	0.698	0.00	62	NCPGR27	2	0.195	0.219	0.00
13	H3EO52	7	0.636	0.675	0.00	63	NCPGR28	3	0.371	0.406	0.00
14	H1I16	7	0.577	0.621	4.44	64	NCPGR33	5	0.701	0.746	8.33
15	H1A18	6	0.725	0.760	0.00	65	NCPGR37	7	0.651	0.697	0.00
16	H1F05	6	0.701	0.742	0.00	66	NCPGR48	5	0.705	0.749	4.17
17	H4H01	6	0.740	0.775	0.00	67	NCPGR21	6	0.797	0.822	4.17
18	H4H08	3	0.370	0.458	0.00	68	NCPGR42	9	0.790	0.813	0.00
19	H3A052	2	0.110	0.117	0.00	69	NCPGR89	5	0.634	0.688	0.00
20	H1H20	6	0.623	0.672	0.00	70	NCPGR93	5	0.682	0.727	0.00
21	H4D08	6	0.702	0.743	2.13	71	NCPGR74	11	0.761	0.787	8.33
22	H4G10	3	0.310	0.362	0.00	72	NCPGR57	7	0.730	0.767	2.08
23	H4E08	2	0.040	0.041	0.00	73	NCPGR81	7	0.715	0.747	0.00
24	H5F021	4	0.556	0.614	0.00	74	NCPGR99	3	0.293	0.338	0.00
25	H5G032	6	0.711	0.753	2.13	75	NCPGR72	4	0.328	0.353	0.00
26	H4G01	5	0.607	0.671	0.00	76	NCPGR53	3	0.132	0.137	6.25
27	H6E09	6	0.642	0.680	0.00	77	H5G12	2	0.218	0.249	0.00
28	H1L161	4	0.450	0.532	0.00	78	H4D02	8	0.727	0.764	10.00
29	H1D221	6	0.688	0.725	0.00	79	NCPGR43	3	0.324	0.352	0.00
30	H6C07	6	0.700	0.743	2.27	80	NCPGR44	2	0.110	0.117	0.00
31	H3F08	7	0.712	0.741	0.00	81	NCPGR51	3	0.244	0.260	0.00
32	H3A10	4	0.680	0.728	0.00	82	NCPGR41	3	0.359	0.401	6.25
33	H4B09	6	0.589	0.622	0.00	83	NCPGR34	5	0.399	0.420	2.08
34	H1B02	5	0.540	0.600	0.00	84	NCPGR39	4	0.540	0.617	0.00
35	H4H02	3	0.498	0.564	0.00	85	NCPGR40	4	0.497	0.576	0.00
36	NCPGR69	7	0.720	0.753	4.26	86	NCPGR46	5	0.443	0.471	2.08
37	NCPGR90	5	0.741	0.777	6.25	87	TA80	7	0.687	0.714	0.00
38	H3C06	6	0.709	0.739	2.08	88	NCPGR52	5	0.509	0.576	2.17
39	H3H04	3	0.543	0.622	0.00	89	NCPGR54	3	0.264	0.288	0.00
40	H3F09	4	0.552	0.616	8.33	90	NCPGR55	3	0.178	0.190	0.00
41	H4F03	7	0.747	0.778	0.00	91	NCPGR75	3	0.178	0.190	0.00
42	H3H07	4	0.583	0.650	0.00	92	NCPGR76	2	0.077	0.080	0.00
43	H6C11	4	0.673	0.721	0.00	93	NCPGR77	4	0.577	0.628	0.00
44	H1F14	6	0.709	0.748	0.00	94	NCPGR59	5	0.444	0.471	2.08
45	H1G11	3	0.231	0.254	0.00	95	NCPGR62	3	0.307	0.344	0.00
46	H1H15	5	0.594	0.650	4.17	96	NCPGR63	3	0.432	0.538	0.00
47	H1I08	2	0.331	0.418	0.00	97	NCPGR65	4	0.443	0.484	0.00
48	H1G16	5	0.628	0.675	0.00	98	NCPGR68	5	0.507	0.546	0.00
49	H4E09	4	0.626	0.683	0.00	99	NCPGR86	3	0.188	0.205	2.08
50	H5H032	4	0.483	0.573	0.00	100	NCPGR91	3	0.441	0.530	0.00

Cluster analysis

A genetic distance-based analysis was performed by calculating the Rogers' distance, the scaled-Euclidean distance frequencies among all the accessions and constructing an NJ dendrogram. The average distance based on all the markers among all the accessions was 0.573 and ranged from 0.060 to 0.844, indicating that there was a high amount of genetic

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Table 3. Allele number, gene diversity, heterozygoty (H_O), and polymorphic information content (PIC) obtained per SSR locus in the wild (W) and cultivated (C) populations.

No.	Marker	Allele No.		Gene diversity		H_O (%)		PIC	
		W	C	W	C	W	C	W	C
1	H2I20	6	6	0.785	0.812	10.00	0.00	0.756	0.786
2	H3DO5	2	9	0.346	0.844	0.00	2.60	0.286	0.825
3	H3GO6	5	5	0.640	0.673	20.00	0.00	0.603	0.631
4	H5E11	4	5	0.667	0.739	0.00	0.00	0.607	0.696
5	H2J20	3	7	0.494	0.824	0.00	2.60	0.438	0.800
6	H5H06	3	4	0.494	0.472	0.00	0.00	0.438	0.428
7	H4G11	3	5	0.593	0.738	0.00	0.00	0.527	0.692
8	H5B04	3	6	0.611	0.783	0.00	2.70	0.536	0.749
9	H1H13	2	4	0.180	0.619	0.00	0.00	0.164	0.564
10	H6D11	4	5	0.691	0.621	0.00	0.00	0.640	0.573
11	H3E04	3	3	0.370	0.655	0.00	0.00	0.340	0.580
12	H4A04	3	5	0.656	0.659	0.00	0.00	0.582	0.609
13	H3EO52	4	4	0.580	0.661	0.00	0.00	0.535	0.604
14	H1I16	6	2	0.780	0.408	20.00	0.00	0.749	0.325
15	H1A18	4	6	0.667	0.766	0.00	0.00	0.620	0.731
16	H1F05	4	5	0.694	0.697	0.00	0.00	0.641	0.647
17	H4H01	3	4	0.560	0.699	0.00	0.00	0.499	0.643
18	H4H08	2	3	0.420	0.467	0.00	0.00	0.332	0.380
19	H3A052	1	2	0.000	0.145	0.00	0.00	0.000	0.135
20	H1H20	3	5	0.460	0.573	0.00	0.00	0.410	0.498
21	H4D08	5	6	0.716	0.699	0.00	2.60	0.677	0.646
22	H4G10	2	2	0.180	0.388	0.00	0.00	0.164	0.313
23	H4E08	2	1	0.180	0.000	0.00	0.00	0.164	0.000
24	H5F021	4	4	0.720	0.533	0.00	0.00	0.672	0.478
25	H5G032	4	5	0.595	0.750	10.00	0.00	0.531	0.707
26	H4G01	4	4	0.700	0.655	0.00	0.00	0.645	0.588
27	H6E09	4	5	0.700	0.600	0.00	0.00	0.645	0.554
28	H1L161	2	3	0.480	0.454	0.00	0.00	0.365	0.373
29	H1D221	3	4	0.460	0.626	0.00	0.00	0.410	0.568
30	H6C07	5	5	0.735	0.689	11.10	0.00	0.693	0.633
31	H3F08	5	5	0.780	0.634	0.00	0.00	0.745	0.595
32	H3A10	3	4	0.620	0.683	0.00	0.00	0.548	0.636
33	H4B09	5	5	0.760	0.545	0.00	0.00	0.720	0.515
34	H1B02	4	3	0.640	0.541	0.00	0.00	0.581	0.482
35	H4H02	2	2	0.219	0.432	0.00	0.00	0.195	0.339
36	NCPGR69	5	4	0.645	0.639	10.00	2.70	0.611	0.579
37	NCPGR90	3	4	0.580	0.701	0.00	7.90	0.492	0.642
38	H3C06	4	5	0.615	0.639	10.00	0.00	0.562	0.602
39	H3H04	3	3	0.620	0.589	0.00	0.00	0.548	0.510
40	H3F09	3	3	0.660	0.481	0.00	10.50	0.586	0.405
41	H4F03	4	4	0.700	0.697	0.00	0.00	0.645	0.644
42	H3H07	3	4	0.460	0.633	0.00	0.00	0.410	0.563
43	H6C11	4	4	0.700	0.705	0.00	0.00	0.645	0.655
44	H1F14	4	4	0.580	0.661	0.00	0.00	0.535	0.604
45	H1G11	3	2	0.580	0.051	0.00	0.00	0.492	0.050
46	H1H15	2	4	0.180	0.500	0.00	5.30	0.164	0.418
47	H1I08	2	2	0.320	0.438	0.00	0.00	0.269	0.342
48	H1G16	4	3	0.700	0.539	0.00	0.00	0.645	0.467
49	H4E09	3	4	0.580	0.654	0.00	0.00	0.492	0.584
50	H5H032	4	2	0.720	0.499	0.00	0.00	0.672	0.374
51	H1F21	3	3	0.580	0.553	0.00	0.00	0.492	0.491
52	H1B09	3	3	0.531	0.605	0.00	0.00	0.468	0.534
53	H5A08	3	3	0.620	0.605	0.00	0.00	0.548	0.534
54	H2J09	3	3	0.660	0.400	0.00	0.00	0.586	0.339
55	H4H06	4	3	0.640	0.594	0.00	0.00	0.581	0.528
56	H3A09	3	3	0.568	0.380	0.00	0.00	0.489	0.325
57	H1E06	4	5	0.688	0.436	0.00	0.00	0.630	0.414
58	H3G031	2	2	0.180	0.188	0.00	0.00	0.164	0.171
59	H1H14	3	4	0.515	0.564	30.00	0.00	0.460	0.524
60	H1P17	4	5	0.660	0.558	0.00	0.00	0.610	0.523
61	NC50	3	3	0.560	0.663	20.00	0.00	0.499	0.589
62	NCPGR27	2	2	0.180	0.229	0.00	0.00	0.164	0.202
63	NCPGR28	3	3	0.640	0.194	0.00	0.00	0.563	0.185
64	NCPGR33	4	5	0.580	0.713	20.00	5.30	0.535	0.662
65	NCPGR37	2	6	0.320	0.706	0.00	0.00	0.269	0.658
66	NCPGR48	2	5	0.500	0.745	20.00	0.00	0.375	0.699
67	NCPGR21	4	5	0.690	0.796	20.00	0.00	0.640	0.764
68	NCPGR42	4	6	0.640	0.748	0.00	0.00	0.581	0.710
69	NCPGR89	4	3	0.685	0.572	0.00	0.00	0.632	0.483
70	NCPGR93	3	5	0.620	0.670	0.00	0.00	0.548	0.610
71	NCPGR74	5	9	0.585	0.753	10.00	7.90	0.544	0.728
72	NCPGR57	4	5	0.715	0.681	10.00	0.00	0.665	0.620
73	NCPGR81	4	5	0.640	0.644	0.00	0.00	0.581	0.598
74	NCPGR99	2	3	0.420	0.309	0.00	0.00	0.332	0.275
75	NCPGR72	4	2	0.580	0.100	0.00	0.00	0.535	0.095
76	NCPGR53	2	2	0.375	0.051	30.00	0.00	0.305	0.050
77	H5G12	1	2	0.000	0.301	0.00	0.00	0.000	0.255
78	H4D02	6	4	0.816	0.699	42.90	3.00	0.790	0.639
79	NCPGR43	3	2	0.620	0.145	0.00	0.00	0.548	0.135
80	NCPGR44	1	2	0.000	0.145	0.00	0.00	0.000	0.135
81	NCPGR51	3	3	0.620	0.101	0.00	0.00	0.548	0.099
82	NCPGR41	2	2	0.455	0.010	30.00	0.00	0.352	0.095
83	NCPGR34	5	2	0.695	0.145	10.0	0.00	0.643	0.135
84	NCPGR39	2	3	0.180	0.608	0.00	0.00	0.164	0.531
85	NCPGR40	3	2	0.620	0.495	0.00	0.00	0.548	0.372
86	NCPGR46	5	3	0.725	0.277	10.00	0.00	0.681	0.257
87	TA80	3	7	0.560	0.605	0.00	0.00	0.499	0.584
88	NCPGR52	5	3	0.675	0.508	10.00	0.00	0.634	0.404
89	NCPGR54	3	1	0.560	0.000	0.00	0.00	0.499	0.000
90	NCPGR55	3	1	0.580	0.000	0.00	0.00	0.492	0.000
91	NCPGR75	2	2	0.480	0.051	0.00	0.00	0.365	0.050
92	NCPGR76	2	1	0.320	0.000	0.00	0.00	0.269	0.000
93	NCPGR77	2	4	0.444	0.632	0.00	0.00	0.346	0.578
94	NCPGR59	4	2	0.735	0.188	10.00	0.00	0.687	0.171
95	NCPGR62	2	1	0.320	0.000	0.00	0.00	0.269	0.000
96	NCPGR63	3	2	0.642	0.495	0.00	0.00	0.568	0.372
97	NCPGR65	3	2	0.460	0.301	0.00	0.00	0.410	0.255
98	NCPGR68	3	3	0.620	0.324	0.00	0.00	0.548	0.300
99	NCPGR86	3	1	0.545	0.000	10.00	0.00	0.442	0.000
100	NCPGR91	2	3	0.420	0.492	0.00	0.00	0.332	0.426

variation. The two cultivated chickpea accessions IC244250 and IC296133 with the lowest distance (0.060) were most closely related, whereas a wild accession ICC17121 and a cultivated accession ICCV96030 with the highest distance (0.844) were most distantly related. The distances were low among the pairs within the cultivated group (mean = 0.492, range = 0.060-0.704) as compared to the wild group (mean = 0.536, range = 0.271-0.712).

The dendrogram constructed clearly separated the members of wild *Cicer* and cultivated chickpea into two clusters (Figure 1). Cluster I corresponded to all the wild *Cicer* accessions grouping *C*. *echinospermum* accessions into subcluster IA and *C*. *reticulatum* accessions into subcluster IB. Cluster II, made up of cultivated chickpea accessions, was further divided into 4 subclusters (IIA-IID) of which subcluster IID was composed of 12 desi accessions (ICC8933, IC118913, IC296132, ICC4951, IC411513, IC244160, ICC5003, ICC4958, ICCV10, IPC92-1, IC296133, and IC244250) clearly distinguishing all the accessions except IC244250 and IC296133. Subcluster IIC revealed subgrouping of 5 desi accessions (ICCV88506, ICCV96030, ICCV96029, ICC162, and ICC1932) into one distinct subgroup and another distinct subgroup containing desi (IC296131, BG374, BG1004, and Brachid Mutant) and kabuli (Flip87-8C, EC539009, ILC3279, and PG95333) accessions non-distinctively in equal proportion. In subcluster IIB, again 3 desi accessions (ICC4918, SBD377 and ICCV93954) and six kabuli accessions (BG315, ICC12968, IC411514, IC449069, IC296376, and IC244243) form two distinct groups. ILC202 branched out in this subcluster showing less similarity with other accessions. Subcluster IIA constituted two desi (ICC4993 and ICC8159) and one kabuli (ICC8151) accession.

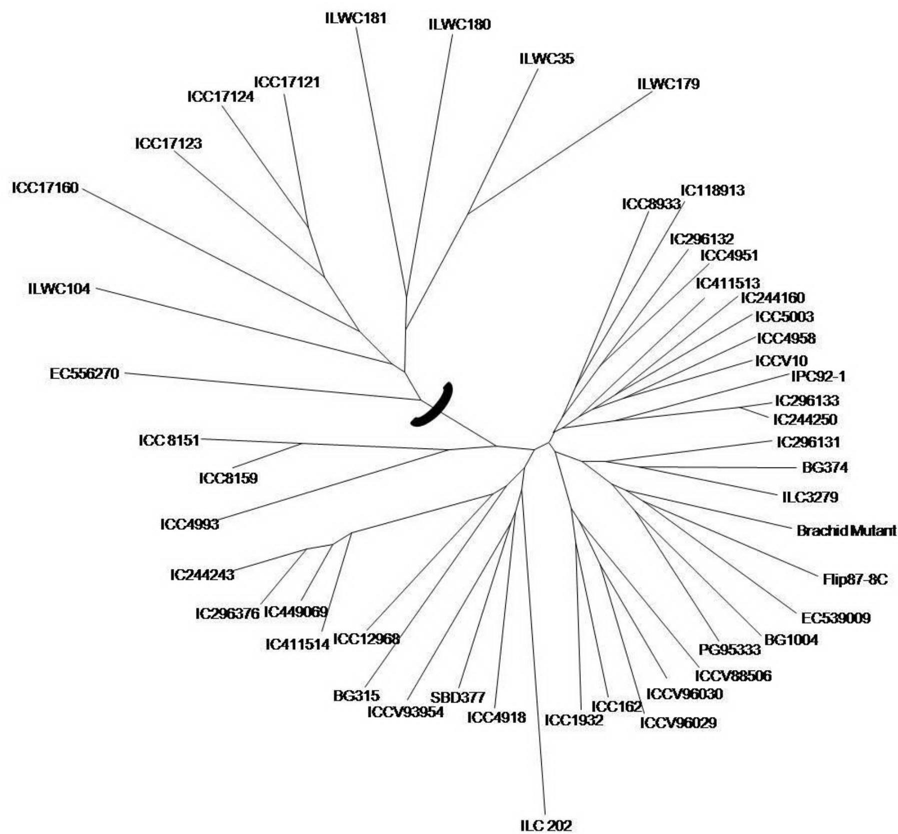

Figure 1. Unrooted neighbor-joining tree of 48 chickpea accessions based on Rogers' genetic distance calculated from 100 SSR markers.

Correlation of the dendrogram with the pedigree data revealed that the accessions with similar pedigree or common parentage generally clustered together. For instance, genotype pairs ICCV96029/ICCV96030 {pedigree: P458 [(K850 × GW-GW5/7) × (L550 × Gaumuchil916) × (ICC1069 × TCPS50467)]} and IC296132/ICCV10 [pedigree: (P1231 x P1265)] derived from the same cross were present in the same subclusters IIC and IID, respectively. ICC244250 [pedigree: (Pusa256 × E100/YM) × (Pusa256)], IC411513 [pedigree: (Pusa256 × *C. reticulatum*) × (Pusa362)] and IC244160 [pedigree: (Pusa256 × GG588)] having a common parent (Pusa256) were present in the same subcluster IID although IC296376 with the same common parent was present at a higher genetic distance from the other three accessions in the separate subcluster IIB. Likewise IC449069 {pedigree: [F_1 (BG315 × ILC72) × F_1 (ICCV13 × Flip85-11)] × F_1 (ICCV32 × SURUTOTO77)} and IC296376 [pedigree: F_1 (Pusa256 × ICCV32) × ICCV32] are present in the same subcluster IIB as they have a common parent (ICCV32). Some genotype pairs like BG315/IC449069 and ICC4951/IC296133 were closely related and present in the same subcluster IIB and IID, respectively, as the former genotype of each couple was one of the parents of the latter. It was clearly depicted that chickpea accessions did not strictly group as per geographical origin.

PCoA analysis

The PCoA further validated the results of the dendrogram. The two dimensional PCoA plot (Figure 2) separated all the accessions into two major clusters. All the wild accessions appeared at the right as a separate major cluster whereas the majority of the cultivated accessions appeared at the left. Four cultivated chickpea accessions (IC449069, IC244243, IC411514, and IC296376), all kabuli, were present at the top left as a separate small subcluster of the second major cluster as is also evident in the dendrogram. The cultivated accessions ICC4993, ICC8151 and ICC8159 had an intermediate position between wild and the cultivated chickpea.

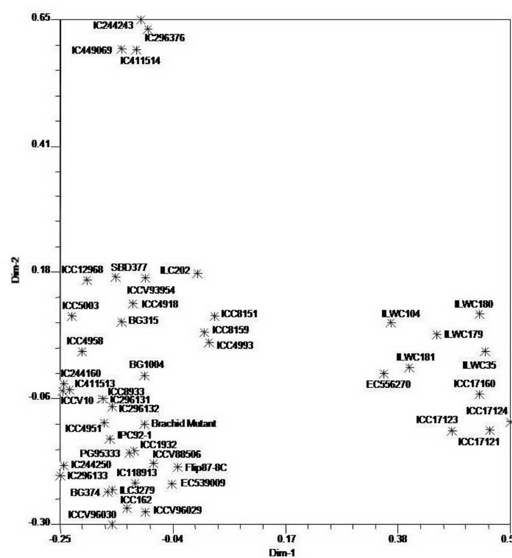

Figure 2. Two-dimensional plot obtained from principal coordinate analysis of 48 chickpea accessions using 100 SSR markers.

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Structure analysis

Genetic structure of the germplasm was further explored using the Bayesian clustering model implemented in the STRUCTURE software. The natural logarithm of the probability of the data, proportional to the posterior probability of K, depicted no clear peak for K from 1 to 7 and hence the determination of the true number of populations (K) was rather difficult. The rate of change of Napierian logarithm probability relative to the standard deviation (ΔK) as described by Evanno et al. (2005) was estimated. The results showed the highest peak at K = 2 indicating the presence of two major clusters: wild and cultivated (Figure 3) with both the clusters showing uniform structure. Structure is considered to be uniform when more than 80% of the accessions in one group have more than 80% of membership in this group.

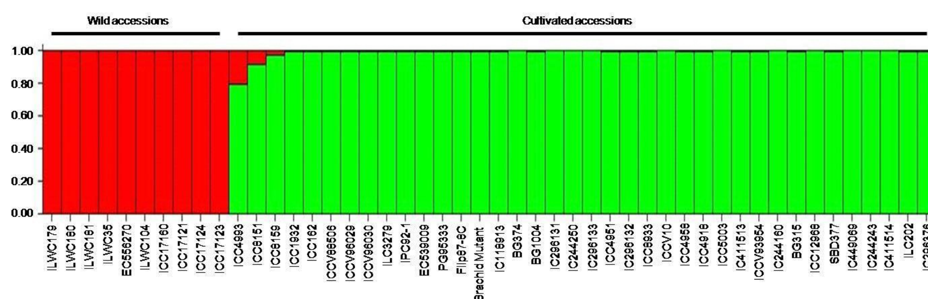

Figure 3. Genetic relatedness of 48 chickpea accessions based on 100 SSR markers and analyzed by the structure program.

The results indicate that all the accessions of the wild cluster have 100% membership in their cluster. In contrast, only 10 of 38 cultivated accessions showed 100% membership in their own cluster, 25 accessions showed very high membership (>99%) in their cluster while 3 accessions showed substantial membership in their cluster and a low level membership in the wild cluster indicating that these accessions are either wild-cultivated hybrids, introgressants or the evolutionary intermediate forms.

DISCUSSION

Evaluation of genetic diversity and an understanding of the genetic relationships in the germplasm collection are needed to be able to adopt effective conservation and management strategies and also facilitate the thorough utility of these genetic resources in crop improvement programs. World chickpea germplam has a narrow genetic base (Nguyen et al., 2004) and lacks the desirable traits needed for ready utilization in varietal improvement programs. Use of wild species provides a wider genetic base and is also a potential source of resistance genes for various biotic and abiotic stresses (Singh et al., 2008a). Thus, the investigation of the nature and structure of genetic diversity and relatedness within and among the cultivated chickpea and its wild relatives is an obvious necessity to identify new sources of germplasm bearing valuable genes for improving yield, grain quality and enhancing resistance to various biotic and abiotic stresses.

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Narrow genetic variation has been reported in cultivated chickpea in various studies although this conclusion is based on a limited number of germplasm and specific marker systems (Udupa et al., 1993; Singh et al., 2003). However, in chickpea, research efforts worldwide have led to the identification and characterization of a large number of SSR markers, which are now utilized extensively to study genetic diversity and relationships to identify genetically diverse germplasm with beneficial traits for use in chickpea improvement programs. SSR markers are three times as efficient as dominant markers for intraspecific analysis and are as efficient as other dominant markers in detecting interspecific variability (Nybom, 2004).

In this study, we evaluated 100 SSR markers in 48 chickpea accessions representing popular cultivated chickpea cultivars and breeding lines and its wild relatives, *C. reticulatum* and *C. echinospermum*. The SSR analysis showed considerable genetic diversity, detecting a total of 480 alleles with an average of 4.8 alleles per locus and average PIC of 0.536, which ranged from 0.040 to 0.803. The ability of SSRs to detect intra- as well as interspecific variation in chickpea has been demonstrated previously. For instance, Hüttel et al. (1999) detected 2 to 4 alleles at the intraspecific level in four genotypes using 22 SSR markers while Singh et al. (2003) used 12 SSR markers to analyze genetic diversity within 13 chickpea cultivars and obtained 1 to 4 alleles with an average of 2.58 alleles. Singh et al. (2008b) obtained 2 to 5 alleles and an average PIC of 0.78 among 21 chickpea cultivars using 18 SSR markers. Upadhyaya et al. (2008) conducted a large-scale study using a collection of 2915 genotypes and reported an average of 35 alleles per locus and PIC of 0.85 using 48 SSR markers, whereas Sefera et al. (2011) used 48 chickpea cultivars and detected an average of 10.5 alleles per locus and PIC of 0.77 using 48 SSR markers. The higher allele number detected in the present study compared to the studies of Hüttel et al. (1999), Singh et al. (2003) and Singh et al. (2008b) can be attributed to the use of a larger set of microsatellite markers. On the other hand, lower values of alleles and PIC relative to Upadhyaya et al. (2008) are due to the use of a much smaller germplasm set (2%). Lower values of the two parameters as compared to the study of Sefera et al. (2011) may be due to the use of the highly polymorphic markers that the authors selected from an earlier study (Upadhyaya et al., 2008). In this study, the number of alleles per locus showed a significant and positive correlation with both PIC and gene diversity and is in agreement with the results of Upadhyaya et al. (2008). Another aspect that was notable about the accessions analyzed is the presence of a very low proportion (1.2%) of heterozygous alleles. The heterozygosity in self-pollinating species such as chickpea mainly results from the low level (0 to 1.58%) of outcrossing as has been reported earlier (Gowda, 1981). Additionally, due to other possibilities like inbreeding depression at the loci in question or a higher mutation rate, the presence of heterozygotes cannot be completely ruled out in an otherwise self-pollinated crop.

Among the two groups included in this study, higher genetic variability was found in the group of wild *Cicer* accessions with higher values for the diversity parameters (allele number, PIC and gene diversity) than cultivated chickpea. These results are in accordance with other studies, which concluded that a very low level of genetic variation exists in cultivated chickpea (Iruela et al., 2002; Nguyen et al., 2004; Rao et al., 2006). Differences in values for estimated genetic diversity parameters between studies may be explained by the different number of accessions, different number of loci examined and perhaps the nature of markers used in each study, but overall it is agreed that *C. arietinum* is far less variable than its wild annual relatives.

Rogers' (1972) genetic distance is a modified Euclidian distance and is best suited for

the estimation of genetic distances when the information on the evolutionary forces influencing the genotypes under consideration is not available and no specific mutation model can be attributed to the allelic variation observed at the SSR loci (Reif et al., 2005). Highest genetic similarity with the lowest distance of 0.060 was between two cultivated chickpea accessions (IC244250 and IC296133) and least genetic similarity with highest distance of 0.844 was between a wild accession ICC17121 and a cultivated accession ICCV96030.

Cluster analysis separated all chickpea accessions into two major clusters, wild and cultivated (Figure 1). Results generated from PCoA were also in agreement with those of the dendrogram. Cultivated chickpea was found to be more closely related to *C. reticulatum* than *C. echinospermum*. This result is supported by earlier studies using molecular markers such as RAPD (Iruela et al., 2002; Sudupak et al., 2002), AFLP (Nguyen et al., 2004; Sudupak et al., 2004) and SSR (Croser et al., 2003; Rao et al., 2006). Although a high degree of relatedness was measured between the cultivar pairs, all were clearly distinguished except two, IC244250 and IC296133. Microsatellite analysis divided cultivated chickpea into four subclusters (IIA-IID) in accordance with the seed morphology indicating that breeding lines had a tendency for clustering within kabuli and desi types to a great extent with the exception of grouping of some desi accessions in the kabuli type cluster and vice versa. Hence, our study agrees to a great extent with other studies, which indicate that cultivated chickpea comprising two gene pools, desi and kabuli, can easily be distinguished (Iruela et al., 2002; Sudupak et al., 2004; Upadhyaya et al., 2008; Sefera et al., 2011). Genetic relationships obtained are in good agreement with the known pedigree information. For instance, genotype pairs derived from the same cross or having a common parent were present in the same subclusters. The overall clustering pattern did not strictly follow the grouping of accessions according to their geographic origins. This may be due to the extensive germplasm exchange among farms from different geographical regions. In contrast, some accessions from the same geographical origins remained distinct and subclustered among themselves.

The NJ tree, PCoA and population structure analysis clearly differentiated the cultivated accessions from wild. In the PCoA plot (Figure 2) four kabuli accessions (IC449069, IC244243, IC411514, and IC296376) clustered together and diverged from the other cultivated accessions revealing distinct genetic nature. This distinct identity could be a consequence of deliberate selection criteria followed by the breeders in the development of these varieties to specifically suit the northwestern plain of India. Three cultivated accessions (ICC4993, ICC8151 and ICC8159) showed intermediate positions between cultivated and wild accessions in PCoA. The analysis of population structure (Figure 3) also revealed similar results showing varying degrees of introgression of wild germplasm in these three accessions in cultivated cluster, whereas the accessions included in the wild cluster showed uniform structure with 100% membership in their cluster indicating no gene flow or introgression. Introgression in the cultivated accessions of chickpea may be due to natural interspecific hybridization between wild and cultivated chickpea. The genetic distance-based approach also agreed with the results of the model-based approach and PCoA. The inferred genetic structure showed both uniform and introgressed populations. Introgressed genotypes may harbor interesting combinations of traits, such as high adaptability to environmental stresses, diseases or insects and may have other benefits such as higher nutritional quality and hence could be of interest for mapping purposes and can be included in crossing programs to broaden the genetic base of chickpea.

The low genetic diversity in *C. arietinum* reported in this study compared to its wild relatives supports the conclusion that chickpea has a narrow genetic base (Nguyen et al., 2004). These results indicate that despite extensive breeding efforts, the varieties under current cultivation are closely related among themselves. This is probably due to the use of few key varieties for hybridization. This explains why yield improvement and increased tolerance to various biotic and abiotic stresses have been slow in chickpea. Hence, it is imperative to broaden the genetic base of the cultivated chickpea to increase the yield and reduce its vulnerability to diseases and insect pests by introducing traits from across the wild members of the primary gene pool. Wild species of chickpea represent a potential source of new alleles for improving yield, quality and stress resistance in cultivated chickpea (Nguyen et al., 2004; Singh et al., 2008a). Unfortunately, the restricted distribution of cross-compatible wild relatives, their extremely poor representation in the world germplasm collection and the difficulty of interspecific hybridization limit the potential of this approach in chickpea. Hence, it is essential to increase the number of accessions in the primary gene pool to maximize the genetic diversity available for introgression into *C. arietinum*.

In conclusion, the present study revealed significant diversity and relationships and provided information on population structure among the members of the primary gene pool. The genetic diversity evaluated can provide the basis for future chickpea crop variety identification, conservation and management. The promising accessions identified through this investigation could also be potentially utilized by molecular biologists and plant breeders for allele mining, gene tagging, genome mapping, association genetics, and in applied breeding for developing elite lines/cultivars with higher yield and enhanced adaptation to environmental stresses leading to the broadening of the genetic base of breeding populations.

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