CSIRO PUBLISHING
www.publish.csiro.au/journals/cp *Crop & Pasture Science*, 2011, **62**, 177–187

Characterisation and genetic diversity analysis of selected chickpea cultivars of nine countries using simple sequence repeat (SSR) markers

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Abstract. The genomic DNA profiles of 48 chickpea cultivars released in nine countries and of historical significance to the chickpea breeding programs at ICRISAT and in Ethiopia were evaluated using 48 simple sequence repeat (SSR) markers. Across the cultivars, a total of 504 alleles representing the 48 SSR loci were detected with frequencies ranging from three to 22 (mean 10.5) alleles per locus. The polymorphism information content (PIC) for the SSR markers varied from 0.37 to 0.91 (mean 0.77). A subset of only three highly informative SSR markers (TA176, TA2, TA180) enabled complete discrimination among all 48 chickpea cultivars tested. Hierarchical neighbour-joining UPGMA cluster analysis based on simple matching dissimilarity matrix resolved the 48 cultivars into two major clusters representing *desi* and *kabuli* types. These cluster groupings of the cultivars were consistent with the pedigree information available for the cultivars as to the phenotypic classes of chickpea types. Analysis of the temporal patterns of the SSR diversity by classifying 48 chickpea cultivars into four periods of release revealed increasing tendencies in the overall genetic diversity from 0.42 for the earliest varieties developed in the 1970s to 0.62 for those released in the 1980s, and reached a maximum and equivalent level of 0.72 for the varieties developed in the 1990s and 2000s. Overall, the study ascertained that SSRs provide powerful marker tools in revealing genetic diversity and relationships in chickpeas, thereby proving useful for selection of parents in breeding programs and also for DNA fingerprint identification of cultivars.

Additional keywords: chickpea, molecular markers, genetic diversity, microsatellites, SSR.

Introduction

Chickpea (*Cicer arietinum* L.), a self-pollinated species with $2n = 2x = 16$ chromosomes and a genome size of ~740 Mbp (Arumuganathan and Earle 1991), is the third most important grain legume crop in the world after dry beans and peas in terms of gross production volume as well as acreage (FAO 2008). Chickpea is a staple food for people living in many of the most populated regions of the world and is grown in South Asia, West Asia, North Africa, East Africa, southern Europe, Australia, and North America (Singh 1997). It serves as an important source of protein in human diet and plays an important role in enriching soil fertility.

Based on 23 characters, cultivated chickpeas have been divided into two broad groups as microsperma (*desi*) and macrosperma (*kabuli*) types (Moreno and Cubero 1978). The *kabuli* types are generally grown in the Mediterranean region including Southern Europe, Western Asia, and Northern Africa; while the *desi* types are grown mainly in Ethiopia and the Indian subcontinent. *Desi* chickpeas are characterised by angular seed shape, dark seed coat, pink flowers, anthocyanin pigmentation of stems, rough seed surface, and either semi-erect or semi-spreading growth habit; *kabuli* types are generally characterised by owl-shaped and beige-coloured seeds, white flowers, smooth seed surface, lack of anthocyanin pigmentation, and semi-spreading growth habit (Pundir *et al*. 1985).

Molecular markers have, particularly in recent years, been widely used in genetic analyses, breeding studies, and investigations of genetic diversity and the relationships between and among cultivated crop species and their wild relatives (Gupta and Varshney 2004). This is because, compared to morphological markers, molecular markers offer the relative advantages of deciphering high polymorphism (genetic diversity) and independence from confounding effects of environments and physiological stages of plants. Besides, morphological markers are phenotypic traits, and it is often difficult to equate phenotype and genotype since similar

10.1071/CP10165 1836-0947/11/020177

phenotypes can have different genetic make-up (Qiu *et al*. 1995). In contrast, molecular markers can reflect changes at the DNA level; hence, they reflect the actual genetic variation and relationships among accessions and their common ancestry more accurately than phenotypic markers (Glaszmann *et al*. 2010).

Genetic diversity studies based on restriction fragment length polymorphisms (RFLPs; Udupa *et al*. 1993) and random amplified polymorphic DNA (RAPDs; Sonnante *et al*. 1997; Sant *et al*. 1999; Singh *et al*. 2003), however, show a low level of genetic variation among chickpea cultivars. The low polymorphism might presumably be attributed to narrow ancestry and the self-pollinated nature of the crop (Van Rheenen 1992). In another study, RFLP and RAPD analysis of 26 accessions could not differentiate between *desi* and *kabuli* accessions (Banerjee *et al*. 1999). On the other hand, higher levels of polymorphism were detected using microsatellite or simple sequence repeat (SSR) based probes or markers (Serret *et al*. 1997; Udupa *et al*. 1999; Chowdhury *et al*. 2002; Upadhyaya *et al*. 2008). Recently, several laboratories have developed a large number of SSR markers for chickpea (Varshney *et al*. 2007, 2009; Nayak *et al*. 2010). Among the most important features that render SSR markers suitable for DNA fingerprinting and analysis of genetic relatedness or diversity are co-dominance inheritance, multiple allelism, high informativeness and discriminatory power, PCR-based analytical methodology, and reproducibility (Thudi *et al*. 2010). It has been shown that SSR markers are three times more efficient than dominant markers for intra-specific analysis, and equally as efficient as other dominant markers in detecting inter-specific variability (Nybom 2004).

In the present study, therefore, the genomic DNA profiles of 48 chickpea genotypes, including 46 released varieties and two advanced lines from India and Ethiopia, were assayed using 48 SSR markers with the following objectives: (*i*) to assess the extent and pattern of genetic diversity and relationships among the chickpea genotypes; and (*ii*) to assess temporal trends of the effects of chickpea breeding programs on the level of diversity of modern chickpea varieties as to whether breeding has caused any erosion of the genetic resources base over time.

Materials and methods

Plant materials

Forty-eight chickpea cultivars were used in this study. Table 1 summarises the cultivars, along with the respective passport pedigree and code at the time of breeding, given names, country and year of release, and type (*desi* or *kabuli*). These genotypes included 32 cultivars developed at ICRISAT and released in different countries (such as India, Bangladesh, Nepal, Myanmar, Sudan, Kenya, Australia, and USA); 14 cultivars released in Ethiopia through the national chickpea-breeding program during the last four decades; and two advanced breeding lines, one each from chickpea breeding programs of Ethiopia and ICRISAT.

DNA extraction

Seeds of each test cultivar were planted in 20-cm-diameter pots filled with soil and maintained in a glasshouse. About 80 mg of fresh leaf sample was collected from 15-day-old seedlings for genomic DNA extraction. A high-throughput mini-DNA isolation protocol (Mace *et al*. 2003) was adopted to isolate DNA from the leaf tissues in 96-well plate format. DNA quantification, quality check, and normalisation to 5 ng/μL concentration were done on agarose gel (0.8%) using a lambda DNA standard (MBI Fermentas, USA).

SSR markers

Forty-eight SSR markers (Hüttel *et al*. 1999; Winter *et al*. 1999; Sethy *et al*. 2003), including 31 mapped on the chickpea genome (Hüttel *et al*. 1999; Winter *et al*. 2000), were used (Table 2). Most of these markers had been screened for polymorphism and selected by Upadhyaya *et al*. (2008) for diversity analysis of the chickpea composite collection.

Polymerase chain reaction (PCR) and fragment analysis

PCR reactions were performed in 5 μL reaction volume, containing 5 ng genomic DNA, 2 pmol primers, 10 mM $MgCl_2$, 2 mM dNTPs, 0.1U *Taq* polymerase (Applied Biosystems, USA), 10× PCR buffer (Applied Biosystems, USA), and M13-forward primer with dye colour. PCR amplification was carried out using touchdown methodology involving initial denaturation for 3 min at 94°C, followed by 10 cycles of 94°C for 20 s, 65°C for 20 s, and 72°C for 30 s, then 40 cycles of 94°C for 30 s, 58°C annealing temperature for 50 s, and 72°C for 30 s and a final extension for 20 min at 72°C.

The PCR products were checked for amplification on 1.2% agarose gel containing 0.5 μL/10 mL ethidium bromide (10 mg/mL) with a 100-base pair DNA ladder by running it at a constant voltage of 90 V for 25 min. Amplification was visualised under UV illumination using the Uvi-Tech gel documentation system (DOL-008.XD, England). Subsequently, PCR products generated by four different fluorescence dye-labelled primers were pooled in equal volumes, and 1.0 μL each of FAM, VIC, NED, and PET labelled product (amplicon) were mixed with 7 μL of formamide (Applied Biosystems, USA), 0.05 μL of GeneScan™ 500 LIZ® Size Standard (Applied Biosystems, USA), and 2.95 μL of distilled water. DNA fragments (amplicons) were denatured and size fractioned using capillary electrophoresis on an ABI 3730 DNA Genetic Analyzer (Applied Biosystems, USA).

Data collection and analysis

Allelic data (peaks) observed on capillary electrophoresis were interpreted on GeneMapper 4 software (Applied Biosystems, USA) by using the internal LIZ-500 size standard. Allelic size data were subjected to the AlleloBin program developed at ICRISAT (www.icrisat.org/test1.asp?software=AlleloBin) for binning raw data and allele calling based on the repeat units of SSR motifs for the corresponding marker. Called allelic data were used to determine the accurate size of the allele tested against its standard deviation (Idury and Cardon 1997).

Polymorphism information content (PIC), as determined by the total number of detected alleles and the number of alleles per locus for the SSR markers, was estimated using PowerMarker V3.0 (Liu and Muse 2005; http://statgen.ncsu.edu/powermarker/). The PIC for each SSR marker, as

described by Botstein *et al*. (1980) and later modified for self-pollinated species by Anderson *et al*. (1993), was calculated as:

$$PIC = 1 - \sum_{j=1}^{n} P_{ij}^2$$

where P_{ij} is the frequency of the *j*th allele for the *i*th marker, and then summed over *n* alleles.

For understanding genetic variation (genetic diversity) as well as analysis of temporal diversity, the average PIC values were computed over all loci (Weir 1996). For the relationships among chickpea genotypes analysed, allelic data were used to develop dendrograms by using neighbour-joining unweighted pair-group method with arithmetic averages (UPGMA) clustering of simple matching dissimilarity indices with the help of the DARwin-5.0 program (Perrier *et al*. 2003). To measure the goodness-of-fit for the cluster analysis, a cophenetic correlation value between the original dissimilarity index and the cophenetic matrix given by the UPGMA clustering process was calculated. Factorial analysis of correspondence was also computed using DARwin 5.0 to determine the genetic relationships among individuals.

Results

Microsatellite allelic diversity

All of the 48 SSR markers used proved polymorphic across the 48 chickpea cultivars examined; as such, in aggregate they allowed detection of a total of 504 alleles (Table 2). Among the 48 SSR markers, the number of alleles detected per locus ranged from three for the markers NCPGR7, CaSTMS21, GAA40, and TS84 to 22 for the single marker TA176, with an average of 10.5 alleles per locus. The PIC, calculated as a relative measure of informativeness for each of the 48 SSR markers, ranged from 0.37 for GAA40 to 0.90 for three of the SSR markers (i.e. TA176, TS104, TA28, and TAA58), with an average value of 0.77 (Table 2). The majority of the SSR markers (except NCPGR7, CaSTMS21, GAA40, and TS84) showed a high level of polymorphism, as these markers, across 48 chickpea cultivars, displayed from three to 22 alleles per locus and PIC values from 0.55 to 0.90. Furthermore, the number of alleles per locus showed highly significant correlations with both the genetic diversity ($r = 0.86$, $P < 0.0001$) and the PIC ($r = 0.87$, $P < 0.0001$) of the SSR markers. Interestingly, three SSR markers (TA176, TA2, and TA180) with PIC values of 0.89–0.90 were found to be highly polymorphic and most informative; these three markers together enabled complete discrimination or distinction of the 48 chickpea cultivars analysed.

Genetic diversity and relationships among chickpea cultivars

The average genetic dissimilarity index among the 48 chickpea cultivars computed as the weighted mean for all of the pair-wise comparisons of the simple matching dissimilarity indices based on all of the 48 SSR markers ranged from 0.75 for cultivar CO 4 to 0.90 for cultivar DZ-10-11 (Table 1). High average genetic distance values were noted for cultivars DZ-10-11 (0.90), Dubie (0.87), and DZ-10-4 (0.87), indicating that these cultivars shared the lowest number of alleles with the rest of the test chickpea cultivars.

The actual genetic dissimilarity coefficients computed for all of the 1128 possible pair-wise comparisons of the 48 chickpea test cultivars using the 48 SSR markers (data not shown) varied from 0.17 (between the two *kabuli* chickpea cultivars Habru and Hawat/Chefe) to 1.00 (between *kabuli* cultivar Monino and *desi* advanced breeding line ICCV 92006), with a mean of 0.82. In addition, the frequency distribution analysis of all the pair comparisons showed a concentration of dissimilarity values in the classes 0.80–0.90, with the lowest class limit values of 0.10–0.20 indicating maximum similarity and the uppermost class range values of 0.90–1.00 indicating maximum divergence among the test chickpea cultivars (see Accessory Publication).

A high cophenetic correlation coefficient ($r = 0.92$) was observed between the original simple matching dissimilarity indices and the indices generated by the clustering process. The dendrogram from the neighbour-joining UPGMA (DARwin Program) cluster analysis of the pair-wise simple matching dissimilarity coefficients matrix taking into account all of the SSR loci profiles resulting from the 48 SSR markers resolved all 48 chickpea cultivars examined into two major clusters representing *desi* (Cl I) and *kabuli* types (Cl II), and a third small group (Cl III) consisting of three cultivars (DZ-10-4, DZ-10-11, and Dubie) released in Ethiopia from landrace collections (Fig. 1). Of these three cultivars, DZ-10-4 is an intermediate (pea-shape-type) chickpea, and DZ-10-11 and Dubie are both small-seeded *desi*-type old varieties released in the early 1970s. In general, the dendrogram showed clear separation between *desi*- and *kabuli*-type chickpea genotypes, with the exception of the unexpected grouping of the cultivars BG1053 (*kabuli*) and Himchanal (*desi*) in *desi* and *kabuli* type clusters, respectively.

In addition to the two higher level, major clusters (*kabuli* and *desi* chickpea types), further lower level hierarchies of the cluster analysis dendrogram depicted the segregation of both the *kabuli* and the *desi* primary clusters into three distinct sub-groups (Fig. 1). The three sub-clusters (SC) of the *kabuli*-type broad clusters are: one sub-group comprising clutivars released in India and shared by cultivars released in Nepal, Sudan and Ethiopia (SC 2.1); the second distinct sub-group of only clutivars released in Ethiopia (SC 2.2); and the third sub-group, cultivars released in Ethiopia, Sudan and India (SC 2.3). Likewise, the *desi*-type primary broad cluster of chickpea genotypes revealed sub-clustering of varieties released in Ethiopia and India into one distinct sub-group (SC 1.1), and the formation of other two different sub-groups, each non-distinctly containing a mix of varieties released in India, Ethiopia, Australia, Nepal, Kenya, Bangladesh, Myanmar, the Sudan, and USA (SC 1.2 and SC 1.3).

Principal coordinate analysis (PCA) of the molecular data showed the first coordinates were important; PCs 1, 2, and 3 accounted for 62.5% of the variation. The PCA plots of PC 1 *v.* PC 2 using factorial analysis of DARwin 5 showed wide dispersion of genotypes in the four quadrants (Fig. 2). Quadrants II and III have eight and 11 accessions, respectively, and all except one accession in each quadrant are *kabuli* genotypes; Myles and Dubie are *desi* accessions in Quadrants II and III, respectively. Except BG 10, all other accessions in Quadrant I are *desi* type. Similarly, Dz-10-4 an

Table 1. List of 48 chickpea test cultivars, along with their respective passport breeding pedigree and code, given names, country and year of release, type of chickpea group (*desi* or *kabuli*), and average genetic distance (GD) by cultivar

No.	Breeding code	Breeding pedigree	Name	Country of release	Type	Year of release	Av. GD[A]
1	ICCL 82106	(P 99 × NEC 108) × Radhey	Akaki	Ethiopia	Desi	1995	0.81
2	FLIP 89-84C	X87TH186/ICC 14198 × FLIP 82-150C	Arerti	Ethiopia	Kabuli	1999	0.83
3	ICCV 92318	(ICCV 2 × Surutato 77) × ICC 7344	Hawat/Chefe	Sudan/Ethiopia	Kabuli	1999/04	0.8
4	Dubie	Collection	Dubie	Ethiopia	Desi	1978	0.87
5	DZ-10-11	Collection	DZ-10-11	Ethiopia	Desi	1974	0.9
6	DZ-10-4	Collection	Dz-10-4	Ethiopia	Interm	1974	0.87
7	FLIP 97-263C	X94TH71/FLIP 87-59 C × UC 15	Ejere	Ethiopia	Kabuli	2005	0.83
8	FLIP 88-42C	X85TH230/ILC 3395 × FLIP 83-13C	Habru	Ethiopia	Kabuli	2004	0.81
9	ICCC 37	P 481 × (JG 62 × P 1630)	Kranthi	India	Desi	1989	0.85
10	ICCL 81248	P 481 × (JG 62 × P 1630)	Nabin	Bangladesh	Desi	1987	0.86
11	ICCL 82108	(JG 62 × WR 315) × (P 1363-1 × PRR 1)	Kalika	Nepal	Desi	1990	0.82
12	ICCL 83105	(K 850 × T 3) × (JG 62 × BEG 482)	Barichola 3	Bangladesh	Desi	1993	0.82
13	ICCL 83110	(K 850 × T 3) × (JG 62 × BEG 482)	ICCL 83110	Kenya	Desi	1986	0.81
14	ICCL 87207	K 850 × ICCL 80074	Vishal	India	Desi	1995	0.79
15	ICCV 1	H 208 × T 3	ICCC 4/Sita	India/Nepal	Desi	1983/87	0.86
16	ICCV 10	P 1231 × P 1265	Bharati/Barichola 2	India/Bangladesh	Desi	1992/93	0.78
17	ICCV 2	{(K 850 × GW 5/7) × P 458} × (L 550 × Guamuchil)	Swetha/WadHamid/Yezin 3	India/Sudan/Myanmar	Kabuli	1989/99	0.8
18	ICCV 3	{(K 850 × GW 5/7) × P 458} × (L 550 × Guamuchil)	BG 1053	India	Kabuli	2000	0.79
29	ICCV 6	L 550 × L 2	Koselee	Nepal	Kabuli	1990	0.76
20	ICCV 88202	PRR 1 × ICCC 1	Sona/Yezin 4	Australia/Myanmar	Desi	1998/00	0.81
21	ICCV 89314	ICCL 80074 × ICCC 30	Dilaji	India	Desi	2000	0.83
22	ICCV 89509	(L 550 × Radhey) × (K 850 × H 208)	Atmor	Sudan	Kabuli	1999	0.78
23	ICCV 90201	GL 769 × P 919	Himchana 2	India	Desi	2003	0.84
24	ICCV 91302	ICCV 32 × (K 4 × Chaffa)	Burgeig	Sudan	Kabuli	1999	0.79
25	ICCV 92006	(GW 517 × ICCC 37) × ICC 12271	Line/ICCV 92006	Ethiopia	Desi	–	0.79
26	ICCV 92311	(ICCV 2 × Surutato 77) × ICC 7344	KAK 2	India	Kabuli	2000	0.8
27	ICCV 92337	(ICCV 2 × Surutato 77) × ICC 7344	JGK 1	India	Kabuli	2002	0.79
28	ICCV 92809	(BDN 9-3 × K 1184) × ICP 87440	Myles	USA	Desi	1994	0.83

29	ICCV 92944	(GW 517 × P 326) × ICCL 83149	Yezin 6/JG 14	India/Myanmar/Sudan	Desi	2004/08	0.82
30	ICCV 93952	(ICCC 37 × GW 517) × ICCV 17	JAKI 9218	India	Desi	2006	0.83
31	ICCV 93954	(Phule G-5 × Narsingpur Bold) × ICCC 37	JG 11	India	Desi	1999	0.8
32	ICCV 93958	ICCC 42 × ICC 12237	CO 4	India	Desi	1999	0.75
33	ICCV 94954	ICCC 42 × BG 256	JG 130	India	Desi	2000	0.77
34	ICCV 95311	(ICCC 32 × ICCL 80004) × [(ICCC 49 × FLIP 82-8C) × ICCV 3]	Vihar	India	Kabuli	2002	0.79
35	ICCV 95332	(ICCC 32 × L 144) × (ICCC 49 × FLIP 82-1C) × ICCV 3	JGK 2	India	Kabuli	2006	0.76
36	ICCV 95418	(ICC 7676 × ICC 32) × [(ICC 49 × FLIP 82-1C) × ICCV 3)]	Virat	India	Kabuli	2001	0.76
37	ICCV 96329	(ICCL 8001 × ICCC 32) × (ICCC 49 × FLIP 82-1C) × ICCV 3	LBeG 7	India	Kabuli	2006	0.78
38	ICCV 96836	(BDN 9-3 × K 1184) × ICP 87440	Genesis 836	Australia	Desi	2005	0.82
49	ICCV 96970	(ICCC 42 × ICCV 88506) × (KPG 59 × JK 74)	JG 16	India	Desi	2001	0.81
40	ICCV-97105	ICCV 10 × GL 769	Line/ICCV 97105	ICRISAT	Desi	–	0.8
41	ICCX-810800	GL 629 × ILC 202	Himchana 1	India	Desi	1999	0.81
42	ICCX-820065	JG 1258 × BDN 9-3	GG 2	India	Desi	1998	0.79
43	Mariye	K 850 × F 378 (Sel from ICCX 730089)	Mariye	Ethiopia	Desi	1985	0.79
44	Monino	Not known	Monino	Ethiopia	Kabuli	Not known	0.87
45	ICCX-910112-6	(ICCV 88102 × ICCV 10) × ICC 4958	Natoli	Ethiopia	Desi	2005	0.83
46	ICCV-93512	ICCC 33 × (L 144 × E 100 Y(M))	Shasho	Ethiopia	Kabuli	2000	0.76
47	FLIP 97-266C	X94TH75/FLIP87-58C × UC 15	Teji	Ethiopia	Kabuli	2005	0.83
48	ICCL 82104	(Annigeri × Chaffa) × (Rabat × F 378)	Worku	Ethiopia	Desi	1993	0.8

[A]Based on pair-wise comparisons of each cultivar with the other 47 cultivars analysed.

Table 2. Allelic variation of the microsatellite markers employed for genotyping of 48 chickpea cultivars
PIC, Polymorphism information content. For linkage group, N indicates that the marker is not mapped

No.	Marker	Linkage group	Variation in product size (bp)	Predominating allele (bp) and its frequency	No. of alleles	PIC
1	CaSTMS21	LG1	190–194	190 (0.55)	3	0.40
2	GA16	LG2	253–351	257 (0.33)	11	0.80
3	GA26	LG6	213–237	233 (0.31)	8	0.77
4	GAA40	LG1	231–243	240 (0.67)	3	0.37
5	NCPGR4	N	179–197	193 (0.45)	4	0.63
6	NCPGR7	N	217–223	223 (0.55)	3	0.40
7	TA103II	N	188–212	203 (0.42)	9	0.74
8	TA11	N	242–275	254 (0.26)	9	0.82
9	TA110	LG2	213–243	228 (0.23)	9	0.83
10	TA113	LG1	207–231	222 (0.41)	9	0.75
11	TA116	N	235–283	253 (0.33)	11	0.78
12	TA118	N	202–274	208 (0.19)	14	0.88
13	TA130	LG4	191–242	221 (0.50)	9	0.66
14	TA132	N	178–232	199 (0.24)	13	0.86
15	TA135	LG3	175–205	193 (0.55)	7	0.60
16	TA14	LG6	273–306	291 (0.25)	9	0.83
17	TA142	N	143–156	146 (0.34)	5	0.69
18	TA144	LG8	219–294	219 (0.32)	14	0.83
19	TA176	LG6	208–328	220 (0.23)	22	0.90
20	TA180	LG7	194–236	206 (0.17)	13	0.89
21	TA194	LG2	119–164	149 (0.31)	11	0.79
22	TA2	LG4	149–203	164 (0.18)	16	0.89
23	TA203	LG1	213–285	243 (0.20)	16	0.88
24	TA206	N	317–407	389 (0.24)	11	0.84
25	TA22	N	220–310	232 (0.21)	16	0.89
26	TA28	N	304–393	340 (0.16)	17	0.90
27	TA3	LG8	302–314	311 (0.53)	4	0.55
28	TA37	LG2	285–327	300 (0.31)	10	0.78
29	TA42	N	179–221	206 (0.21)	13	0.88
30	TA64	LG3	235–283	253 (0.33)	11	0.78
31	TA71	LG5	201–249	219 (0.29)	13	0.85
32	TA76s	N	222–240	228 (0.42)	8	0.70
33	TA80	LG6	219–258	219 (0.38)	11	0.78
34	TA96	LG2	272–313	297 (0.36)	12	0.76
35	TAA104	N	188–212	191 (0.27)	8	0.81
36	TAA58	LG7	279–357	324 (0.15)	16	0.90
37	TR1	LG6	201–243	231 (0.23)	12	0.85
38	TR20	LG4	154–175	172 (0.42)	7	0.73
39	TR29	LG5	197–251	236 (0.26)	13	0.83
40	TR43	N	299–377	299 (0.28)	13	0.84
41	TR59	LG5	165–189	171 (0.36)	9	0.79
42	TR7	LG6	179–227	203 (0.35)	11	0.77
43	TS104	N	156–213	207 (0.19)	19	0.90
44	TS17	N	233–266	236 (0.23), 242 (0.23)	8	0.81
45	TS17x	N	258–291	261 (0.23), 267 (0.23)	8	0.81
46	TS45	LG8	242–269	263 (0.45)	8	0.66
47	TS72	LG11	241–313	253 (0.21)	15	0.86
48	TS84	N	246–252	246 (0.54)	3	0.47
Total					504	
Mean					10.5	0.77

intermediate type released in Ethiopia, all other accessions are *desi* type in Quadrant IV.

Temporal genetic diversity of chickpea cultivars

With an objective to assess the temporal patterns of genetic diversity of the chickpea, and thereby to assess whether breeding has resulted in dwindling of the genetic diversity of the varieties, all 48 chickpea genotypes were classified into four groups according to their period of release: (*1*) old cultivars released before 1980 (1970–1980 or 1970s), (*2*) varieties released 1981–1990 (or 1980s), (*3*) those released 1991–2000 (or 1990s), and (*4*) modern varieties released post 2000. Number of alleles, and average PIC values, were computed on the basis of

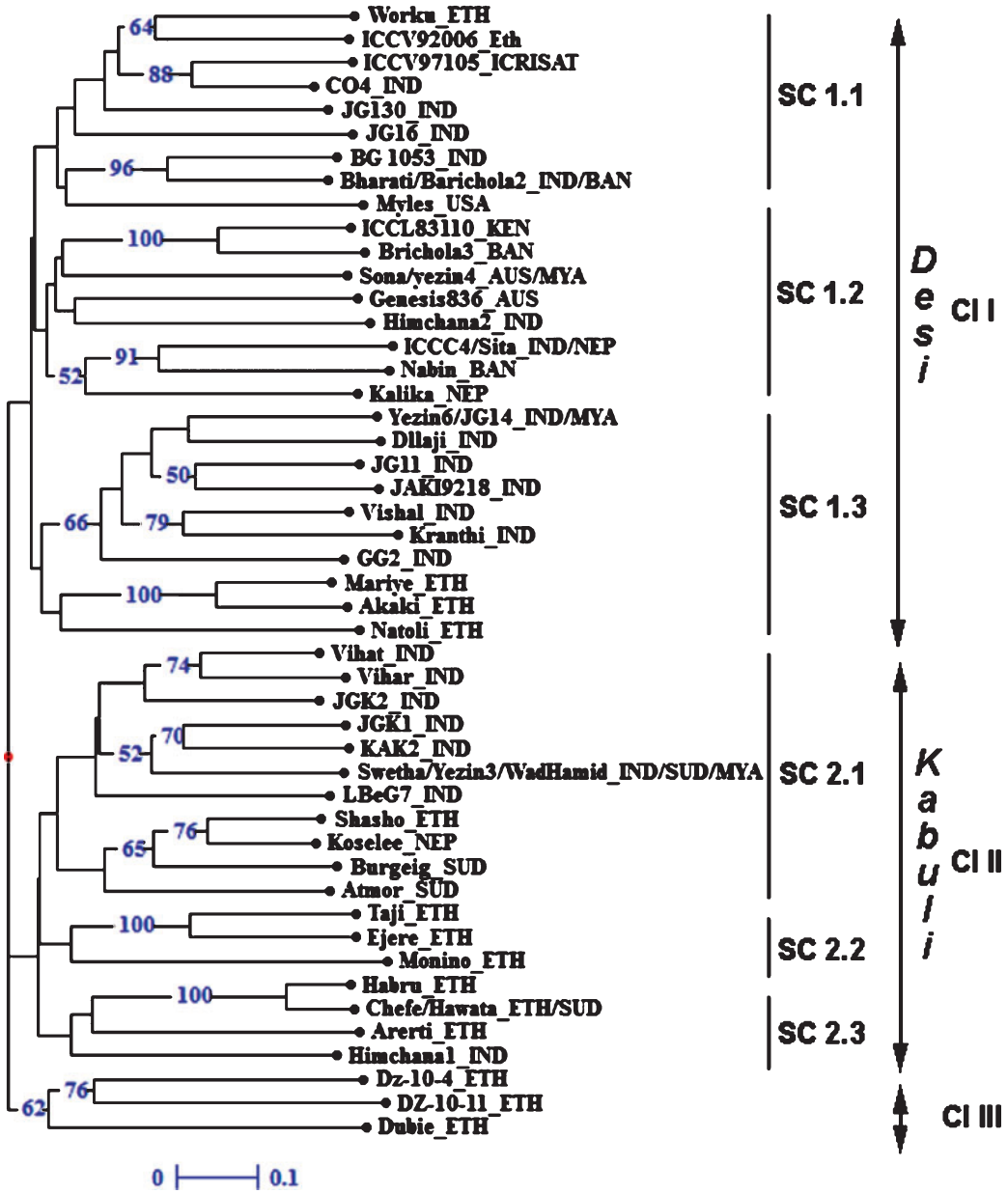

Fig. 1. Dendrogram for 48 chickpea cultivars derived from UPGMA cluster analysis using simple matching dissimilarity index based on 48 SSR markers. The numbers are bootstrap values based on 1000 iterations. Cultivars names were given according to country of release (AUS, Australia; BAG, Bangladesh; ETH, Ethiopia; IND, India; KEN, Kenya; MYA, Myanmar; NEP, Nepal; SUD, Sudan).

the periods of release for the cultivars. Differences in the average PIC values between the four periods of release were evaluated by analysis of variance (SAS Institute 1998). PIC values were calculated for the cultivars grouped within each period of release at each locus. SSR marker loci were used as blocks to separate the variation among loci from the error term and increase the sensitivity of the statistical analysis.

The PIC values computed based on the SSR allelic marker profiles of the various cultivars as mentioned above exhibited significant effects ($P < 0.05$) of breeding (variety) release periods on the overall genetic diversity of the chickpea cultivars released. Pair-wise comparisons revealed that the PIC values were lowest (43%) for the old varieties of the 1970s, and then the average genetic diversity increased progressively and substantially ($P < 0.05$) to ~66% for the cultivars of the 1980s, and reached an equivalent peak of 72% for the cultivars released 1991–2000 (1990s) and post 2000 (Table 3).

Discussion

Assessment of genetic variation and understanding genetic relationships in germplasm collections are indispensable for

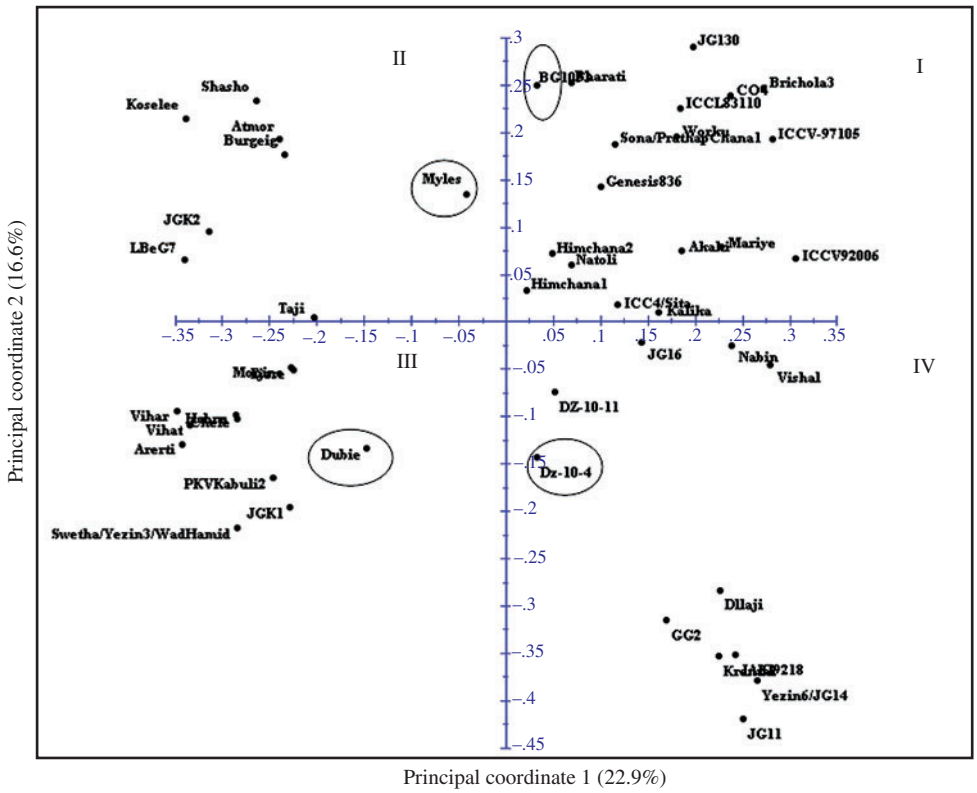

Fig. 2. Principal coordinate analysis of 48 chickpea cultivars based on 48 SSR markers. The diagram shows the position of each cultivar in the space spanned by the first two coordinates of a relatively simple matching dissimilarity matrix. The genotypes circled are *desi*, *kabuli*, and intermediate types that are exceptions in each quadrant.

effective management and use of genetic resources in crop breeding, as well as providing insurance against unforeseen threats (e.g. climate change) to agricultural production. Past efforts on assessment of genetic diversity in chickpea suggested that low molecular variation exists in cultivated chickpea (Udupa *et al*. 1993; Sonnante *et al*. 1997; Sant *et al*. 1999; Singh *et al*. 2003). However, with the discovery of large numbers of genomic SSR markers (Varshney *et al*. 2007; Nayak *et al*. 2010), it is now possible to conduct extensive molecular diversity studies for identifying genetically diverse germplasm with beneficial traits for use in chickpea improvement programs. In our study, all of the SSR markers used showed polymorphism and detected a total of 504 alleles with an average of 10.5 alleles per locus, and average PIC value of 0.77.

Results of the PCA were in agreement with those of the neighbour-joining dendrogram, with two major groups detected; one clearly represents the *desi* cultivars and the other, the *kabuli* cultivars (Figs 1 and 2). Division of the accessions into two major groups showed that there was correspondence between the grouping of cultivars released in Ethiopian and India. Ten of the 48 loci examined detected unique alleles in the case of Dubie, a *desi*-type released in Ethiopia, and this genotype interestingly shared a greater proportion of alleles with *kabuli* cultivars (at the other 38 loci examined). Hence, this genotype was uniquely placed along with the *kabuli* germplasm in Quadrant II (Fig. 2). Pedigree-based grouping is seen in the case of cultivars released in India; for instance, Dilaji, GG2, JAKI 9218, Yezin6/JG 14, JG 11, Vishal, and Kranthi were clustered together (Figs 1 and 2). Interestingly, three *desi* cultivars, Genesis 836, Myles and GG2, possess BDN 9-3 as one of the common parents in their pedigrees (Table 1); however, they are grouped in three different quadrants (Quadrants I, II, and IV, respectively) (Fig. 2). The PCA analysis also suggests that the cultivars released in different counties are diverse, which is evident from dispersion of chickpea cultivars in all four quadrants.

Other genetic diversity studies based on SSR markers have been conducted. For instance, based on the use of 22 SSR markers in four genotypes, Hüttel *et al*. (1999) identified two to four alleles, while Singh *et al*. (2008*b*) obtained two to five alleles and an average PIC value of 0.78 among 21 chickpea cultivars using 18 STMS markers. In a study conducted on a large germplasm collection (2915 genotypes) with 48 SSR markers, Upadhyaya *et al*. (2008) reported an average 35 alleles and 0.85 PIC value. Higher allele numbers and PIC values detected in the present study compared with the studies of Hüttel *et al*. (1999) and Singh *et al*. (2008*b*) can be attributed to the use of selected highly polymorphic markers from our earlier study (Upadhyaya *et al*. 2008). On the other hand, a low level of diversity relative to Upadhyaya *et al*. (2008) can be attributed to the use of only 2% of genotypes in the present study compared with Upadhyaya *et al*. (2008). Nevertheless, the observation of a highly significant ($P < 0.0001$) and positive correlation between number of

Table 3. Comparison of chickpea cultivars used in this study according to their period of release

Means followed by the same letter are not significantly different ($P > 0.05$, Tukey's test)

Period of release	No. of varieties	Total no. of alleles	Av. no. of alleles/marker	Av. genetic diversity (PIC)
All cultivars	48	504	10.5	0.77a
2000s	17	321	6.69	0.72a
1990s	20	346	7.21	0.72a
1980s	8	223	4.65	0.66b
1970s	3	115	2.40	0.43c

alleles and PIC in the present study agreed with results of Upadhyaya *et al*. (2008).

As three SSR markers, namely TA176, TA2, and TA180, were found to be highly polymorphic and most informative based on their ability to distinguish all 48 chickpea cultivars examined, this underlies the utility of these marker loci to generate specific genetic fingerprints for each genotype, useful for variety identification and protection, genetic purity analysis, and other studies. In other words, these results suggest the reliability of SSR markers for DNA fingerprinting and genetic diversity analysis of chickpea cultivars.

On the basis of morphology, cultivated chickpeas are normally classified into two major distinct forms known as *desi* and *kabuli* types, and a third, rare group designated as pea-shaped types, characterised by medium–small seed size, and cream-coloured seeds (Moreno and Cubero 1978). Similarly, the neighbour-joining UPGMA analysis clustered the 48 chickpea test cultivars in the current study into the two major groups, *kabuli* and *desi* types, and a third, other small group comprised three the chickpea varieties released in Ethiopia: the single pea-shaped variety in the study (Dz-10-4) and the *desi* varieties Dubie and DZ-10-11. Further down in the hierarchy, the two broad (*kabuli*- and *desi*-type) clusters were subdivided, each into three sub-groups in accordance with their breeding background and origin. In fact, some marker-based genetic diversity studies conducted earlier in chickpea reported differentiation of genotypes either according to distinct forms and/or as per passport information such as pedigree information and origin (Iruela *et al*. 2002; Upadhyaya *et al*. 2008). However, some studies, such as conducted by Lin *et al*. (2008) based on microsatellite-anchored fragment length polymorphism analysis of 24 chickpea cultivars released in Australia, failed to reveal not only differentiation between the two major chickpea types, but also the general pedigree relationships among the chickpea cultivars.

Apart from the Ethiopian-released cultivars , which emanated from three sources (ICRISAT, ICARDA, and selection from landrace collections), all the other chickpea cultivars analysed in the present study were crossbreeds from the ICRISAT's breeding program and released in different countries, with the same cultivar(s) sometimes released in more than one country at different times. Moreover, since most of the cultivars originating from ICRISAT's breeding program were developed from multiple crosses of more than two parents, and for some of them also from crosses involving *desi*- and *kabuli*-type parents (Gaur *et al*. 2007), inheritance of common alleles from their respective parental progenitors would not be unexpected. Despite this, however, the SSR markers in the present study allowed efficient and complete discrimination between major chickpea types and the origin of the cultivars in accordance with their initial passport and pedigree information.

The average genetic similarity values of the cultivars showed that DZ-11-10, Dubie, DZ-11-10, and Monino shared the lowest number of alleles with all the other cultivars; hence, these four cultivars are the most genetically distinct types. This finding is in agreement with the pedigree and ancestral relationships of the cultivars, as DZ-11-10, Dubie, and Dz-11-10 are cultivars released in Ethiopia through selection from landraces, while Monino is a new introduction to Ethiopia from Mexico. Further, PCA results also indicate that Dubie and Dz-11-10 are distinct from the *desi* cultivars (Fig. 2) and share alleles of *kabuli*-type cultivars. Similarly, Upadhyaya *et al*. (2008) reported that accessions from the East Africa region are highly polymorphic and genetically diverse with beneficial traits; Anbessa and Bejiga (2002) also asserted the consideration of Ethiopia as the secondary centre of diversity for chickpeas. The difference in genetic diversity between the old varieties developed through collection from landraces (released in 1970s) and those developed by crossing (after 1980s) were clearly distinguishable. Our study showing increasing temporal trends of overall genetic diversity of chickpea cultivars released over the different periods demonstrates successful efforts of chickpea-breeding programs with novel approaches towards broadening the genetic variability base, particularly with respect to combating biotic and abiotic stresses. However, the equivalent mean PIC values (72%) for the 1990s and for the period after 2000 suggests that an apparent peak might have been reached and, therefore, requires careful consideration in chickpea-breeding programs. To that end, Van Rheenen *et al*. (1993) earlier asserted that chickpea is 'a recalcitrant crop species,' as it has not been very amenable to genetic improvement despite extensive breeding efforts for the last three decades.

Our findings do not support the general belief that the practice of modern intense breeding inevitably leads to a decline in the genetic variation available for crop improvement. The concept of 'genetic erosion' was first assumed more than 30 years ago (Harlan 1972) and refers to a dramatic shift in population structure or allele frequencies within a species as a result of natural processes or breeding activity. This issue led to concern over the ability of plant breeders to adequately respond to the dynamics of pathogens, climate, and agricultural practices by developing new genotypes rather than relying upon a narrowing germplasm base. However, comprehensive studies on crop diversity have shown no declining trends of genetic variation (Manifesto *et al*. 2001; Landjeva *et al*. 2006). There has probably been a certain narrowing due to the utilisation of a few 'key' varieties for hybridisation. However, the consistent utilisation of diverse materials from different gene pools, including *desi* × *kabuli* and inter-specific crosses with wild relatives, coupled with induced mutagenesis to incorporate other genes conferring resistance/tolerance to biotic and abiotic stresses (Gaur *et al*. 2007) has, indeed, enhanced the broadening rather than narrowing of the genetic diversity resources base.

A recent review by Singh *et al*. (2008*a*) indicated that the valuable genetic resources present in the primary gene pool were

successfully utilised in plant-breeding programs for genetic enhancement in chickpea. However, the results of the current study showed a tendency of achievement of an apparent plateau of genetic diversity, particularly over the last two decades. In addition, the world germplasm collections of cultivated chickpea are said to be lacking in diversity that may include traits needed for effective improvement of the crop (Robertson *et al*. 1997; Collard *et al*. 2003) despite the prevalence of several biotic and abiotic stresses constraining the production of the crop. This challenge may be overcome by looking to the wild relatives to widen the genetic base available for breeding programs through inter-specific hybridisation (Singh and Ocampo 1997; Glaszmann *et al*. 2010). Similarly, Nguyen *et al*. (2004) observed, by employing AFLP analysis, that perennial tertiary species may be as valuable as annual tertiary species for increasing variation to incorporate novel germplasm into cultigens. However, the review of Singh *et al*. (2008*a*) pointed out that hybridisation of the secondary and tertiary gene pool with the cultivated species is often limited by reproductive barriers. Thus, an appropriate cross-bridge might be needed to overcome the reproductive barriers to use the potential merits of these gene pools.

In conclusion, most of the studies on polymorphism of molecular markers in chickpea indicate the presence of limited genetic variability in the cultivated species. However, our study shows that a few selected polymorphic SSR markers were enough to discriminate among chickpea cultivars studied. Therefore, SSR markers can provide a robust and highly discriminatory marker system that can greatly facilitate cultivar tagging and identification as well as genetic diversity studies such as sequence-based diagnostics, and promote integration and comparison of datasets from laboratories throughout the chickpea genetics and breeding research community. The present results will also help chickpea breeders in the selection of parent material in breeding programs.

Acknowledgments

The authors are thankful to Chickpea Breeding programs of ICRISAT and Debre Ziet Agricultural Research Center (DZARC) of EIAR for providing the seeds of cultivars used in this study. Thanks are due to Tropical Legumes-I (TL-I) and Tropical Legume-II (TL-II) projects of Generation Challenge Program (www.generationcp.org) and Bill and Melinda Gates Foundation (BMGF) for supporting this study.

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Manuscript received 11 May 2010, accepted 20 January 2011