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Evaluation of microsatellite markers to discriminate induced mutation lines, hybrid lines and cultigens in chickpea (*Cicer arietinum* L)

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Abstract

A total of 47 chickpea (*Cicer arietinum* L) accessions including 21 induced mutation lines, 17 hybrid lines, 5 local cultigens, and 4 non-nodulating lines were examined for their growth characteristics in the field. Genotypes of these accessions were also examined using 10 SSR (microsatellite) markers. Eight of the 10 markers were polymorphic and the number of alleles ranged from 2 to 16, with the average of 7.4 per locus. PIC values ranged from 0.227 to 0.876, with an average of 0.636. The accessions comprised 29 Desi and 18 Kabuli phenotypes. The average PIC was 0.582 in Desi and 0.577 in Kabuli phenotypes. Significant genetic differentiation was revealed between Desi and Kabuli phenotypes by AMOVA under stepwise mutation assumption ($R_{ST} = 0.239$, $P \leq 0.001$). A considerable amount of genetic variation was observed in the mutation accumulation lines tested. PIC for the accessions derived from the Desi parental line C-44 was 0.443 and that of Pb-91 was 0.422, which accounts for about 66 to 70% of the total variation within our collection, respectively. UPGMA and ME trees classified the accessions into 6 groups and all but 6 accessions could be clearly separated. Grouping was mostly the same in the two phylogenetic trees, but the branching order differed greatly. Recent introgression among the parental lines is suggested for this reason. Our study indicates that SSR markers are useful tools for discriminating induced mutation lines as well as hybrid lines.

Keywords: Desi, genetic variation, growth characters, Kubuli, nodulation, SSR

Abbreviations: AMOVA_analysis of molecular variance; AFLP_amplified fragment length polymorphism; ICARDA_International Center for Agricultural Research in the Dry Areas, Syria; ICRISAT_International Crops Research Institute for the Semi-Arid Tropics, India; ME_minimum-evolution method; NIFA_Nuclear Institute for Food and Agriculture, Pakistan; PCA_principal component analysis; PIC_polymorphic information content; RAPD_random amplified polymorphic DNA; RFLP_restriction fragment length polymorphism; SSR_simple sequence repeat; UPGMA_unweighted pair-group method using arithmetic averages.

Introduction

Chickpea (*Cicer arietinum* L.) is one of the world's most important crops but less-studied leguminous food crops with nearly 10×10^6 ha grown across North and Central America, the Mediterranean Basin, East Africa, the Middle East, Asia and Oceania (FAOSTAT, 2004). While in developed countries it represents a major crop for export, in developing countries it provides a protein-rich supplement to cereal-based diets. The oldest report concerning chickpeas dates from 5,450 BC (Helbaek, 1959) and there is evidence that it has been cultivated for at least 7,000 years (van der Maesen, 1972). Chickpeas are generally grouped into 2 types: the Desi type with small, angular, dark-colored seeds, and the Kabuli type with large, smooth-coated, beige seeds. It is commonly accepted that Kabuli chickpea originated from the Desi type in the Mediterranean Basin (Moreno and Cubero, 1978; Hawtin and Singh, 1981). The Kabuli cultivars are cultivated principally in the Mediterranean Basin, the Near East and America where the entire seeds are used for human consumption after soaking and boiling. The large, smooth-coated and rapid-cooking Kabuli chickpea seed is usually preferred for human consumption. The Desi type are cultivated in the Indian subcontinent and East Africa and usually dehulled and split before cooking. Although most chickpeas are produced for human consumption, they also provide the livestock industry with an alternative protein and energy feedstuff (Christodoulou et al., 2005). Chickpea seed contains 29% protein, 59% carbohydrate, 3% fiber, 5% oil and 4% ash. Chickpea protein is rich in lysine and arginine but is deficient in the sulfur-containing amino acids methionine and cysteine (Iqbal et al., 2006). Chickpea is also a good source of absorbable Ca, P, Mg, Fe and K (Christodoulou et al., 2005).

Over recent decades, molecular marker technology has developed into a valuable tool for plant breeding. A number of techniques (e.g. RFLP, RAPD, AFLP, SSR) can be used as DNA markers linked to traits of interest, directing selection towards these markers instead of selecting for a phenotype (Edwards and Mogg, 2001). The small genome size (740 Mb), short life cycle (3 to 6 months) and high economic importance as a food crop legume make chickpea an important species for genomics research. Molecular markers and linkage maps are the prerequisites for undertaking molecular breeding activities. However, the progress towards development of a reasonable number of molecular markers has been very slow in cultivated varieties of chickpea. One of the main reasons for this may be attributed to the low level of genetic diversity present in the cultivated gene pools of this species, at least with the detection tools that are currently available (Sharma et al., 1995, Rajesh et al, 2002). Although several genetic linkage maps using various markers and genomic tools have become available, sequencing

efforts and use of available resources have been limited in chickpea genomic research. Among various molecular markers currently available, SSR or microsatellite markers are often chosen as the preferred markers for a variety of applications in breeding because of their multi-allelic nature, co-dominant inheritance, relative abundance and extensive genome coverage (Gupta and Varshney, 2000). As a result, several hundred SSR markers have been developed and are available in chickpea (Hüttle et al., 1999; Winter et al., 1999). By examining the vast collection of chickpeas covering a broad geographic range, a sufficient amount of genetic variation has been reported (Serret et al., 1997; Udupa et al., 1999; Imtiaz et al., 2008; Upadhyaya et al., 2008). Together with availability of high density linkage map (Winter et al., 2000), highly polymorphic marker system like SSR would be of great value in QTL mapping and marker assisted selection for various important traits (Singh et al., 2008). Genetic variation not only invests crop varieties with the capacity to adapt to various environments, and resistant pests and diseases, but is also a necessary resource for improving yield and quality required for food. In order to enhance the genetic diversity of cultivars, it is necessary to utilize exotic and diverse germplasm. Induction of mutation by irradiation and hybrid formation are other methods of enhancing genetic variation in cultivated crops. Thus far no study has been done to evaluate the genetic variation harbored in such experimental lines and to identify each other. The materials used in this study include radiation-induced mutation lines from single parental lines as well as lines derived from hybrids of two or three parental lines, which have been developed and maintained in NIFA, ICARDA and ICRISAT. We also included several local cultigens grown at the Ahmedwala Research Station in Karak, Pakistan, and spontaneously occurring non-nodulating lines. The purpose of this study was to explore the extent of genetic diversity of such experimental laboratory stock and to test the performance of SSR markers to discriminate closely related lines such as induced-mutation lines and whether SSRs can be used as genetic markers for future breeding programs for crop improvement.

Material and methods

Sample Collection

A total of 47 accessions of chickpea were used for this study (Table 1). They included 29 Desi and 18 Kabuli accessions. Among Desi accessions, 20 accessions (nos. 2 to 18, and nos. 21 to 23) were procured from NIFA, which were produced through radiation-induced mutation of Desi lines C-44, Pb-91 and 6153. The accession no. 1 was a hybrid of C-44 (Desi) and ILC-195 (Kabuli) from NIFA. Two Desi accessions (no.19 and 20) were sent from ICARDA and derived from a hybrid of JG74 (Desi) and ICC12071 (Kabuli). Those lines that originated from hybrids between Desi and Kabuli lines (nos. 1, 19 and 20) were classified as Desi by their phenotype. Among 18 Kabuli germplasm, 15 (nos. 26 to 39) were given by ICARDA and originated from crosses between two or three Kabuli parental lines. One (no. 40) was induced mutation lines from ILC-195 and was procured from NIFA. Five accessions (4 Desi, nos. 41, 42, 43, and 44; and 1 Kabuli, no. 45) were local collections (cultigens) of Ahmedwala Research Station. Four accessions, 2 Kabuli (nos. 46 and 47) and 2 Desi (nos. 48 and 49) were spontaneous non-nodulating mutant lines sent from ICRISAT. All genotypes of hybrid origin were in advanced generations (more than 7 generations), and further segregation of these genotypes was not expected.

Field Experiment and statistical analysis

The field experiment was carried out in the experimental farm of the Faculty of Agriculture, Ehime University, Matsuyama, Japan. The 47 accessions of chickpea were grown in randomized complete block design with 3 replications. No fertilizer was applied to the field. Data was recorded for leaf area, number of leaflets per leaf and number of nodules per plant before harvest while seed weight was measured after harvest. For data collection 5 plants were selected randomly in each plot, and then data was averaged for plots and for replications for each accession. Difference in the average phenotypic values between Desi and Kabuli accessions were tested by analysis of variance (ANOVA). Association between morphological traits was revealed by principal component analysis (PCA) based on correlation coefficients. All statistical analyses were performed using JMP for Windows version 5.0 (SAS Institute Inc., NC, USA).

DNA Extraction

Seeds of chickpea were sown in 27 cm diameter pots containing 7.5 Kg of sandy soil with 5 seeds per pot in a green house. DNA was extracted separately from fresh leaves of 5 2-week-old plants for each accession using modified CTAB method described by Doyle and Doyle (1990).

Amplification of DNA

Ten SSR (microsatellite) markers including 5 dinucleotide and 5 trinucleotide repeats were used for genotyping. Eight markers were screened from *C. arietinum* BAC libraries (Lichtenzveig et al., 2005) and 2 were *Cicer arietinum* sequence-tagged microsatellite site (CaSTMS) markers screened from a genomic library of the same species (Hüttle et al., 1999). Primers were labeled with FAM, HEX, and NED fluorescent dyes (Applied Biosystems, Foster City, USA). Sequences of the primer pairs for each marker are listed in Table 2. The reaction mixture for amplification of markers contained 8.4 µl of distilled water, 1.25 µl of 10× PCR buffer, 1.25 µl of 2 mM deoxynucleotide mix, 0.5 µM forward and reverse primers, 0.1 U of Taq DNA polymerase (Blend Taq, Toyobo, Osaka, Japan) and 5 ng of genomic DNA in a total volume of 12.5 µl. Microsatellite amplification was carried out using the following cycling parameters: preheating for 3 min at 95°C followed by 25 cycles of denaturing at 94°C for 30 sec, annealing at primer specific temperatures of 48–60°C for 30 sec (see Table 2), and extension for 1 min at 72°C. Reactions were completed by incubation at 72°C for 1 min and holding at 4°C. The PCR products were denatured for 2 min at 95°C and separated by capillary electrophoresis on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). GeneMapper software (Applied Biosystems) was used for sizing and genotyping microsatellite alleles.

Microsatellite Data Analysis

Genetic diversity parameters were computed using GenAlEx 6 software (Peakall and Smouse, 2006). The following statistics were estimated: average number of alleles (N_a), and effective number of alleles (N_e). Polymorphic information content (PIC) of each microsatellite locus was determined as described by Weir (1996): $PIC = 1 - \sum p_i^2$, where p_i is the frequency of the *i*th allele in the examined test lines. The estimation of genetic differentiation was performed using AMOVA implimented in

Table 1. Description of chickpea accessions used in this study

No.	Entry	Phenotype	Genotype	Parentage[A]	Origin	H[B]
1	NDC-122	Desi	Nod^+	C-44 × ILC-195	NIFA	0
2	NDC-727	Desi	Nod^+	C-44/M	NIFA	0.25
3	NDC-728-5	Desi	Nod^+	C-44/M	NIFA	0.375
4	NDC-730-2	Desi	Nod^+	C-44/M	NIFA	0.125
5	NDC-15-1	Desi	Nod^+	Pb-91/M	NIFA	0
6	NDC-15-2	Desi	Nod^+	Pb-91/M	NIFA	0
7	NDC-15-3	Desi	Nod^+	Pb-91/M	NIFA	0
8	NDC-15-4	Desi	Nod^+	Pb-91/M	NIFA	0
9	NDC-4-15-1	Desi	Nod^+	C-44/M	NIFA	0
10	NDC-4-15-2	Desi	Nod^+	C-44/M	NIFA	0
11	NDC-4-15-3	Desi	Nod^+	C-44/M	NIFA	0
12	NDC-4-20-1	Desi	Nod^+	C-44/M	NIFA	0
13	NDC-4-20-2	Desi	Nod^+	C-44/M	NIFA	0.375
14	NDC-4-20-3	Desi	Nod^+	C-44/M	NIFA	0
15	NDC-4-20-4	Desi	Nod^+	C-44/M	NIFA	0.5
16	NDC-4-20-5	Desi	Nod^+	C-44/M	NIFA	0.125
17	NDC-4-20-6	Desi	Nod^+	C-44/M	NIFA	0
18	NDC-4-20-7	Desi	Nod^+	C-44/M	NIFA	0.125
19	NDC-5-S10	Desi	Nod^+	JG74 × ICC12071	ICARDA	0
20	NDC-5-S11	Desi	Nod^+	JG74 × ICC12071	ICARDA	0.375
21	NIFA-88	Desi	Nod^+	6153	NIFA	0
22	NIFA-95	Desi	Nod^+	6153/M	NIFA	0.125
23	NIFA-2005	Desi	Nod^+	Pb-91/M	NIFA	0
26	NKC-10-99	Kabuli	Nod^+	FlIP98-138C × SEL99TH15039	ICARDA	0
27	NKC-5-S12	Kabuli	Nod^+	BAHODIR × SEL99TER85530	ICARDA	0
28	NKC-5-S13	Kabuli	Nod^+	SEL99TH15039 × S98008	ICARDA	0.25
29	NKC-5-S14	Kabuli	Nod^+	SEL99TH15039 × S98008	ICARDA	0.125
30	NKC-5-S15	Kabuli	Nod^+	FLIP98-15C × S98033	ICARDA	0.375
31	NKC-5-S16	Kabuli	Nod^+	S99456 × SEL99TER85314	ICARDA	0.625
32	NKC-5-S17	Kabuli	Nod^+	S99456 × SEL99TER85314	ICARDA	0
33	NKC-5-S18	Kabuli	Nod^+	(ILC4291 × FLIP98-129C) × S98008	ICARDA	0.375
34	NKC-5-S19	Kabuli	Nod^+	(ILC4291 × FLIP98-129C) × S98008	ICARDA	0
35	NKC-5-S20	Kabuli	Nod^+	FLIP98-138C × SEL99TH15039	ICARDA	0
36	NKC-5-S21	Kabuli	Nod^+	GLK95069 × SEL99TER85530	ICARDA	0
37	NKC-5-S22	Kabuli	Nod^+	CA9783007 × SEL99TER85534	ICARDA	0
38	NKC-5-S23	Kabuli	Nod^+	CA9783007 × SEL99TER85534	ICARDA	0
39	NKC-5-S24	Kabuli	Nod^+	CA9783007 × SEL99TER85534	ICARDA	0
40	HASSAN-2K	Kabuli	Nod^+	ILC-195/M	NIFA	0
41	Karak 1	Desi	Nod^+	Local selection	Karak	0
42	Karak 2	Desi	Nod^+	Local selection	Karak	0
43	Karak 3	Desi	Nod^+	Local selection	Karak	0
44	Sheenghar	Desi	Nod^+	Local selection	Karak	0
45	Lawaaghar	Kabuli	Nod^+	Local selection	Karak	0
46	ICC 4993	Kabuli	Nod^-	Rabat	Morocco	0
47	ICC 19183	Kabuli	Nod^-	ICC 4993NN	ICRISAT	0

48	ICC4918 NN	Desi	Nod$^-$	Annigeri	India	0
49	ICC19181NN	Desi	Nod$^-$	ICC 435NN	ICRISAT	0

[A]The lines indicated by /M is mutation induction lines. [B]Heterozygosity.

Table 2. Primers used for amplifying chickpea microsatellite regions

Locus	Primer	Sequence (5'-3')	Annealing temp.	Motif	Repeat
H1116	F	GACATGAAATTCGGTGCATT	52°C	GA	20
	R	AACGCCCTAAACCTCTTGGT			
H1F17	F	GGGGAGGAAGAAGATGGAA	48°C	TA	27
	R	GCGTTATGGGTGGAAATGGTA			
H3C06	F	AATTTCGTGAATCATTAAAAATAGAGG	55°C	TAA	23
	R	CACATGACTATCTAGACATTTTATTTATC			
H3A10	F	TTTAAGGCTTCAGGTATTGATTTCT	55°C	TTA	24
	R	TCACACATGCCAACTTAAAATAAAA			
H3A07	F	GCGACACCTATTCCTCTTTTCTA	58°C	TTA	20
	R	TCATTTTTGGAATATTTTAGTGACAA			
H2J09	F	AACGAAAAACAAGGGAGAAAAA	52°C	GA	18
	R	TATTTCTTTGACTCCCCCTAACTT			
H1B11	F	GCAGCTGTTGACATCTAATTTTG	60°C	TAA	20
	R	ACCGAAAACACTTGTGATTGTTA			
H6G07	F	TCTATCAGAGATATTAAGTTGAACG	60°C	TAA	23
	R	CGTGACAGAATTAGCCTCTTGT			
CaSTMS19	F	TGAAGCTGGGGGTTCCTTG	50°C	AT	15
	R	TCAATTGAGTCGCGACGAGAG			
CaSTMS25	F	TACACTACTGCTATTGATATGTGGT	50°C	CT	19
	R	GACAATGCCTTTTTCCTT			

GenAlEx 6. Random permutation test with 999 shuffles was performed under the assumption of stepwise mutation model (Slatkin, 1995). Phylogenetic analysis was completed using UPGMA and ME method using MEGA4 software (Tamura et al., 2007) based on the genetic distance matrix constructed by the method of Smouse and Peakall (1999).

Results

Genetic Variation

All the primer pairs generated reproducible and easily readable microsatellite patterns. Eight of the 10 primer pairs generated more than 2 alleles (polymorphic), whilst 2 SSR primer pairs (H1B11 and H6G07) amplified only one allele (monomorphic). These 2 monomorphic loci were excluded from the analysis. A total of 58 alleles were detected in the 47 accessions of chickpea (Table 3). The number of alleles per locus ranged from 2 (CaSTMS19) to 16 (H3A10), with the average of 7.4 per locus. The effective number of alleles (N_e) ranged from 1.29 (CaSTMS25) to 8.06 (H3A10) with an average of 3.66. Between Desi and Kabuli morphs, 46 alleles were found in Desi, and 42 alleles were found in the Kabuli, whilst 30 alleles were shared by both. A total of 28 private alleles (16 in Desi and 12 in Kabuli) were detected. The average numbers of allele per locus in Desi and Kabuli morphs were 5.87 and 5.25, respectively, while their mean effective numbers were 2.78 and 3.40, respectively (Table 3). Across all the polymorphic loci, 6 alleles (0.3%) of the 58 alleles were classified as rare (present at a frequency of <1%), whereas 36 alleles (70.1%) were common alleles (1–20%) and 16 alleles (27.6%) were frequent alleles (> 20%). The PIC values ranged from 0.227 (CaSTMS25) to 0.876 (H3A10), with an average of 0.636 (Table 3). Both CaSTMS markers showed relatively low PIC values. The average PIC was 0.582 in Desi and 0.577 in Kabuli, and these values were very similar. AMOVA was used to partition the genetic variation between morphs, between individual accessions within each morph and within individuals (Table 4). Significant differentiation (R_{ST} = 0.239, $P \leq 0.001$) was obtained between the two morphs. Segregating loci were detected in 14 of our composite chickpea accessions, ranging from 1 to 5 loci per accession with an average heterozygosity from 0.125 to 0.625 (Table 1). This resulted in an overall mean heterozygosity of 0.088.

Genetic Relationship among Accessions

The UPGMA tree based on the genetic distance (Fig. 1a) shows that all the accessions were divided into 6 monophyletic groups (A, B, C, D, E and F) and 2 outliers (nos. 47 and 49). Group A consists of 3 Desi (nos. 1, 43 and 44) and 1 Kabuli (no. 40) accession. Accessions 43 and 44 were the local collections of Karak, Pakistan. Groups B and C both consist of only Desi lines. Among Group B accessions, nos. 2, 9, 11, 14 and 15 were derived from the same induced mutation line (C-44), while nos. 7 and 8 were from another induced mutation line (Pb-91). Nine accessions (nos. 3, 4, 5, 6, 10, 12, 13, 16, 17, and 23) in Group C had the same parentage of C-44, with accession 23 located in the basal position. Group D includes 12 accessions containing both Desi (nos. 18, 19, 20, 21 and 22) and Kabuli (nos. 26, 27, 28, 30, 31, 32, 33 and 34) accessions. Lines 30 and 31 had the same parental origin. Group E is composed of only Kabuli lines (accessions 29, 35, 36, 37, 38, 39 and 46) with accession 46 (local collection from Morocco) in the basal position. All the accessions included in the Group F are the local collections from Karak, Pakistan, and this group consists of 2 Desi (nos. 41 and 42) and one Kabuli (no. 45) accession. The accessions 47 and 49 are basal in the tree. The UPGMA tree locates the root at the middle point of the branch connecting accession 47; however, the branching associations among the groups are ambiguous because they are connected with very short branches. Bootstrap values for all these branches are very low (less than 50%). An ME tree was constructed and is shown in Fig. 1b. In this tree, 6 monophyletic groups (A', B', C', D', E' and F') were recognized.

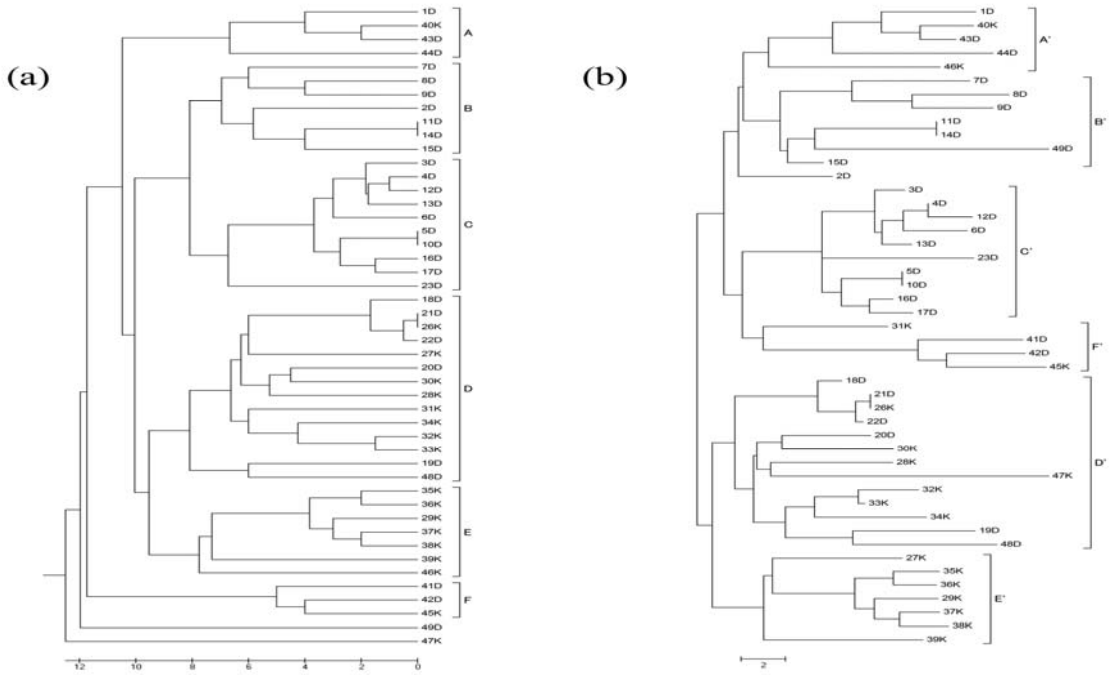

Fig 1. (a) UPGMA tree showing phylogenetic relationship between 47 chickpea accessions. (b) ME tree showing phylogenetic relationship between 47 chickpea accessions. In both trees branch length is proportional to the genetic distance. Scales are shown below.

Table 3. Genetic diversity statistics of 47 chickpea accessions

		Locus								
	Parameters[A]	H1116	H1F17	H3C06	H3A10	H3A07	H2J09	CaSTMS19	CaSTMS25	Mean
All Genotypes										
	N	47	47	47	47	47	47	47	47	47
	N_a	5	7	12	16	6	7	2	3	7.37
	N_e	3.261	3.648	5.381	8.062	3.344	2.543	1.641	1.294	3.66
	PIC	0.693	0.726	0.814	0.876	0.701	0.607	0.39	0.227	0.636
Kabuli vs Desi										
Desi	N	29	29	29	29	29	29	29	29	29
	N_a	4	7	10	10	5	6	2	2	5.87
	N_e	2.346	3.228	3.526	5.021	2.339	2.507	1.859	1.312	2.78
	PIC	0.574	0.69	0.716	0.801	0.573	0.601	0.462	0.238	0.592
Kabuli	N	18	18	18	18	18	18	18	18	18
	N_a	3	5	8	12	5	4	2	3	5.25
	N_e	2.418	2.582	2.838	7.714	3.682	2.445	1.246	1.256	3.40
	PIC	0.586	0.613	0.829	0.87	0.728	0.591	0.198	0.204	0.577

[A]N, no. accessions examined; N_a, actual number of alleles; N_e, effective number of alleles; PIC, polymorphic information content.

Accession 2 was a sister to the cluster of Groups A' and B'. These groupings are mostly the same as the grouping of the UPGMA tree (Group A in UPGMA corresponds to A' in ME, and so on) with some minor differences. Accession 46 in Group E of the UPGMA tree was displaced to Group A' in the ME tree. Accession 49 was included in Group B' in the ME tree. The composition of Group C' in the ME tree is the same as for Group C in the UPGMA tree. Accession 31, which was in Group D of the UPGMA tree, was included in Group F' in the ME tree. Accession 27, which was in Group E' of the ME tree was in Group D in the UPGMA. Branching associations among the groups in the ME tree were very different from that of the UPGMA tree, reflecting ambiguous associations among the groups. Non-nodulating lines (accessions 46, 47, 48, and 49) were not closely related in either of the trees.

Grouping of Chickpea Accessions Based on Morphological Traits

Means of the 4 morphological traits in Desi and Kabuli accessions are shown in Table 5 with their standard errors. ANOVA showed that there were significant difference in the number of leaflets and nodules per plant. Both were larger in Desi accessions. Correlations among the 4 morphological traits (number of nodules per plant, seed weight, number of leaflets per leaf, and leaf area) are summarized in Table 6. Nodulation was significantly positively correlated with the leaf area and the leaflet number ($P \leq 0.01$ for both). The correlation between leaf area and leaflet number was also highly significant ($P \leq 0.01$). The seed weight did not show any significant correlation with any of the other traits. PCA was performed based on the covari-

Table 4. Summary of AMOVA of the microsatellite data of 47 chickpea accessions

Source[A]	d.f.	SS[B]	MS[C]	Est. Var.[D]	%
Among morphs	1	7087.7	7087.7	140.7***	24%
Among accessions	45	37647.6	836.6	389.3***	66%
Within accessions	47	2725.5	58.0	58.0***	10%
Total	93	47460.9		588.0	100%

*** Significant at $P \leq 0.001$. [A]Morphs are Desi and Kubuli. [B]Sum of square. [C]Expected mean square. [D]Estimated variance.

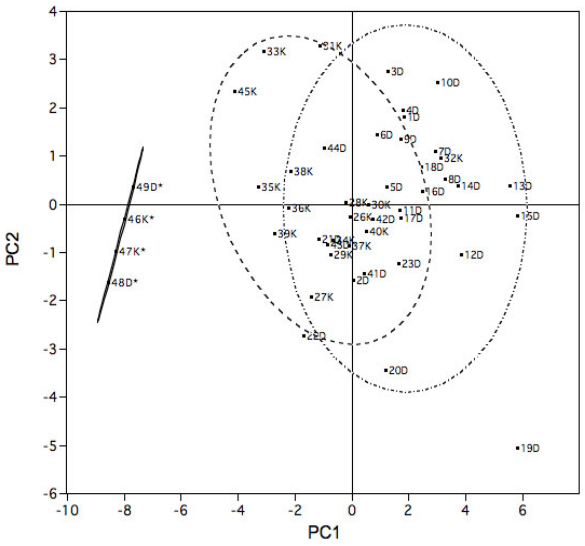

Fig 2. Scatter plot of principal component analysis based on morphological data. PC1: principal component 1; PC2: principal component 2. Dotted line and dashed line circles show the 90% density ellipse of Nod^+-Desi and Nod^+-Kabuli accessions, respectively. The accessions with asterisk show Nod^- accessions with 90% density ellipse of solid line.

ance matrix. PCA based on the 4 morphological traits allocated 72.1% and 16.4% of the variance to principal component 1 (PC1) and PC2, respectively, accounting for 88.5% of the total variance (Table 7). Nodule number showed the largest eigenvector for PC1. Similarly, leaf area and leaflet number were the first and second largest eigenvectors for PC2, respectively. Using PC1 and PC2 as X and Y axes, respectively, chickpea accessions were clustered graphically as shown in Fig. 2. This clearly differentiates the Nod^- accessions as an isolated group from the Nod^+ accessions. Among the Nod^+ accessions, Desi and Kabuli accessions formed overlapping groups, but their center was clearly distinguished.

Discussion

A collection of chickpea experimental lines for breeding programs was evaluated for their genetic variation, especially between Desi and Kabuli accessions. All the SSR markers examined were selected randomly from previously published data. In this study, 8 out of the 10 selected SSR markers showed polymorphism in our 47 chickpea accessions. The allele number per locus ranged from 2 to 16, averaging 7.32, and with PIC values of 0.227–0.876, averaging 0.636. These values are lower than those observed by Udupa et al. (1999), who reported an average number of alleles of 14.1 per locus and an average PIC of 0.86 for 12 SSR loci in 78 accessions of chickpea including 72 landraces, 4 cultivars and 2 wild species of the primary gene pool (i.e. *C. reticulatum* and *C. echinospermum*). Similarly, Imtiaz et al. (2008), who evaluated 48 accessions of chickpea comprising cultigens, landraces, and wild relatives using 21 SSR loci, detected an average of 16.9 alleles per locus and an average PIC value of 0.82. In a more comprehensive study, Upadhyaya et al. (2008) examined 2915 accessions from a vast collection of chickpea germplasm maintained in two gene banks in ICRISAT and ICARDA using 48 SSR markers. They reported that the number alleles per locus ranged from 14 to 67, with an average of 35, and PIC values from 0.467 to 0.974, averaging 0.854. In comparable soybean studies, Narvel et al. (2000) reported an average of 10.2 alleles per locus among 79 genotypes including 39 elite accessions (Elites) and 40 plant introductions (PIs) using 74 SSR loci. Average marker diversity among the PIs was 0.56 and that of the Elites was 0.50. Likewise Wang et al. (2006) calculated an average of 12.2 alleles per locus for 129 Chinese soybean accessions using 60 SSR loci and the PIC among accessions varied from 0.5 to 0.92 with a mean of 0.78. Although allele number is very much dependent on sample size, the possible explanation for the lower observed PIC value in our study could be that most of the accessions were advanced-generation laboratory stocks derived from a limited number of parental lines or hybrids, whereas many of the studies cited above used a larger number of accessions from geographically diverse areas including landraces and wild relatives. However, the materials used here still reveal a considerable amount of genetic variation. PIC values for the induced mutation lines derived from C-44 was 0.443 and from Pb-91 was 0.422. These contain about 50% of the variation that resides in the world collections of chickpea (Upadhyaya et al., 2008), and the SSR markers could effectively discriminate the lines derived from a same parental line. Thus the microsatellite technique proved to be a useful system for managing our experimental lines. Our result also showed the average heterozygosity of 0.088, which is rather high compared with reported range between 0 and 1% in chickpea natural cross-pollination (Singh, 1987). In this study, extensive care was taken to avoid inadvertent seed mixture and a single plant from each accession was selected and used for DNA extraction and analysis. Probably in some lines, especially in the lines of hybrid origin, genetic materials are still segregating. Desi and Kabuli accessions showed clear morphological difference especially in the leaflet number and nodule number (Table 5, and Fig. 2). The average PIC values for Desi and Kabuli morphs were 0.582 ± 0.061 and 0.577 ± 0.090, respectively, which are not significantly different from each other. Although significant differentiation in their allele frequency constitution was shown by AMOVA (Table 4), Desi and Kabuli groups were not clearly divided in UPGMA and ME phylogenetic trees (Figs. 1a and 1b). Some accessions formed monophyletic groups in phylogenetic tree such as in Group B and Group C for Desi accessions and Group E for Kabuli accessions in UPGMA tree, but others are paraphyletic (Fig. 1a). Apparently in our study, some accessions were derived from hybrids between Desi and Kabuli lines (such as accessions 1, 19,

Table 5. Summary of ANOVA for the 4 morphological traits of 47 chickpea accessions[A]

	Mean[B]			
	Leaf area (cm^2)	Leaflet/plant	Seed weight (g)	Nodule/plant
Desi	7.14 ± 0.159	14.3 ± 0.098	0.719 ± 0.0165	8.89 ± 0.414
Kabuli	7.06 ± 0.202	12.9 ± 0.125	0.747 ± 0.0209	6.04 ± 0.538
F-ratio[C]	0.0878 (1, 45)	74.14 (1, 45)***	1.116 (1, 45)	17.60 (1, 41)***

*** $P \leq 0.001$. [A]Non-nodulated lines are excluded from the analysis of nodulation.
[B]The values are mean ± standard error.
[C]F-ratio testing the difference of Desi and Kabuli accessions. The numbers in parenthesis are the degree of freedom.

Table 6. Correlation among four morphological traits of 47 chickpea accessions

	Leaflet	Seed weight	Nodulation
Leaf area	0.3971**	0.0532	0.4365**
Leaflet		0.0024	0.4307**
Seed weight			–0.0699

Table 7. Principal component analysis on four morphological traits of 47 chickpea accessions

	PC1	PC2	PC3	PC4
Eigenvalue	11.5216	2.6242	1.7774	0.0614
Percent	72.0793	16.4170	11.1196	0.3841
Cum. Percent	72.0793	88.4963	99.6159	100.0000
Eigenvector				
Leaf	0.29749	0.68135	–0.66861	0.01471
Leaflet	0.28387	0.60556	0.74345	–0.00211
Seed weight	–0.00377	0.01524	–0.00814	0.99984
Nodulation	0.91154	–0.41088	–0.01335	0.00959

and 20), so that they made a group of mixture with both types. In the study of Upadhyaya et al. (2008) with 2915 accessions including 1668 Desi and 1167 Kabuli groups, Desi and Kubuli accessions are largely separated but include some paraphyletic members. This probably shows that these two groups have not been completely isolated and occasional hybridization between the two morphs might have occurred. AMOVA also showed significant differentiation between Nod^+ and Nod^- accessions (R_{ST} = 0.270, $P \leq 0.01$); however, Nod^- accessions are not monophyletic in either the UPGMA or ME trees (Figs. 1a and 1b). Probably mutations causing non-nodulation had occurred independently in different genetic backgrounds. Rupela (1992) reported that the frequency of Nod^- plants in 4 Nod^+ accessions ranged from 120 to 490 per million. He also reported that Nod^- selections were indistinguishable from their respective parents for plant growth except for nodulation, and yielded similarly to their Nod^+ accessions when supplied with 50 to 100 Kg N/ha, but on a low-N field, the Nod^- plants were light green and grew poorly. Singh et al. (1992) reported that a new Nod^- mutation that occurred in 1 accession (ICC435) was inherited in Mendelian recessive manner, but was non-allelic to the formerly reported mutants, and suggested that there are at least 6 loci controlling nodulation. This verifies the high mutation rate observed for this character. The high level of variability observed in microsatellite markers makes them suitable for application in identification of germplasms of local varieties, cultigens and cultivars (Udupa et al., 1999). Here, we have shown that all, but 3 pairs (accessions 5 and 10, accessions 11 and 14, and accessions 21 and 26, Figs. 1a and 1b), could be readily distinguished with these microsatellite markers. Since the number of markers we used was very limited, using a larger number of markers in even more closely related lines could certainly improve resolution. Thus, this method is extremely useful for breeding programs that utilize induced mutation lines and lines of hybrid origin.

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