Environmental and Experimental Biology (2014) 12: 161–166 *Original Paper*

Genetic diversity and population structure analysis of landrace and improved chickpea (*Cicer arietinum*) genotypes using morphological and microsatellite markers

Zohreh Hajibarat[1], Abbas Saidi[1]*, Zahra Hajibarat[1], Reza Talebi[2]

[1]Department of Biotechnology, College of New Technologies and Energy Engineering, Shahid Beheshti University, GC, Tehran, Iran
[2]Department of Agronomy and Plant Breeding, Sanandaj Branch, Islamic Azad University, Sanandaj, Iran

*Corresponding author, E-mail: abbas.saidi@gmail.com

Abstract

Genetic diversity, population structure and relationships of 48 chickpea genotypes comprising 19 Iranian landrace and 29 international lines and cultivars were studied using 38 SSR markers and seven morphological characters. High diversity and coefficients of variation were recorded for all morphological characters. We found considerable diversity, with a mean of three alleles per locus (ranging from 1 to 7); polymorphic information content ranged from 0 to 0.77, with a mean of 0.48. Based on unweighted neighbour joining clustering for morphological and molecular data, genotypes grouped into four and five distinct groups, respectively. Results showed that the introduction of genetic materials from exotic sources broadened the genetic base of the national chickpea breeding programme. Further implications of the findings of this study can be useful for selective breeding of specific traits and in enhancing the genetic base of breeding programmes.

Key words: chickpea, morphology, SSR markers, genetic diversity, population structure.
Abbreviations: AFLP, amplified fragment length polymorphism; ICARDA, International Centre for Agricultural Research in the Dry Areas; RAPD, random amplified polymorphic DNA; SSR, simple-sequenced repeats; STMS, sequenced tagged microsatellite sites.

Introduction

Chickpea (*Cicer arietinum* L.) is a cool season grain legume with high nutritive value and is the third most important pulse crop in the world after soybean and beans, covering an area of 11.5 million ha (FAOSTAT 2009, http://faostat.fao.org). 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 rain fed areas (Choudhary et al. 2012).

Iran, as one of the main origin centres of genetic diversity for chickpea (van der Maesen 1987; Talebi et al. 2008b), possesses a large number of chickpea germplasm collections from different geographical regions (Naghavi et al. 2005; Ghaffari et al. 2014). For effective utilization of these germplasm collections in breeding programmes, genetic characterization in terms of measure of the extent and pattern of genetic diversity within and between populations (Rubenstein et al. 2005) is essential (Carvalho 2004). This characterization is not only to unveil the magnitude of genetic diversity available in the germplasm for conservation purposes, but also to determine genes useful for possible progress in future breeding programmes. Screening and selection would more likely result in better and promising genotypes if germplasm sources were genetically diverse (Keneni et al. 2011).

Genetic characterization can be made by different methods, ranging from conventional methods like the use of descriptor lists of morphological characters, as well as biochemical and molecular methods (Carvalho 2004; de Vicente et al. 2005; Keneni et al. 2011). Morphological characters are the strongest determinants of the agronomic value and taxonomic classification of plants. Compared with other methods, morphological evaluations are direct, inexpensive and easy. However, errors can arise; furthermore, morphological estimations are more dependent on the environment (Jannatabadi et al. 2014). Additionally, some genetically related cultivars are morphologically very similar and it is difficult to distinguish between them by visual comparison. Also, genetically distant material can show very similar morphology due to cultivation selection/pressure.

DNA analysis could help to differentiate genotypes accurately and may be used in cultivar identification (Castro et al. 2011). For chickpea, various marker systems such as amplified fragment length polymorphism (AFLP; Talebi et al. 2008b), random amplified polymorphic DNA (RAPD; Talebi et al. 2008a) and microsatellite markers like simple-sequenced repeats (SSR) or sequenced tagged microsatellite sites (STMS; Saeed et al. 2011; Keneni et al. 2011; Ghaffari et al. 2014) have been used for diversity analysis. The present study was aimed to characterize Iranian landrace

and improved commercial cultivars of chickpea by the use of microsatellite and morphological markers, as well as to determine the potential utility of these markers for cultivar characterization.

Materials and methods

Plant material and field evaluation

Forty eight chickpea (*Cicer arietinum* L.) accessions comprised 19 landrace accessions from different geographical locations of Iran and 29 improved genotypes provided by the Iranian Seed and Plant Improvement Institute and International Centre for Agricultural Research in the Dry Areas (ICARDA) were considered for the study of genetic variation using morphological and SSR markers (Table 1). Field experiments were laid out in randomized complete block design with three replications in 2011 – 2012. Seeds were hand drilled and each plot consisted of a three rows of 3 m with spacing of 0.3 m between rows and 10 cm between plants within a row. Six plants were randomly chosen from each plot to measure the number of seeds per plant, number of pods per plant, plant height, 100-seed weight, plant biomass and seed yield and harvest index determined as (seed yield / plant biomass) × 100.

DNA extraction and SSR analysis

Total genomic DNA was extracted from 2 g fresh leaves of each genotype following a CTAB extraction protocol (Lassner et al. 1989). A total of 60 SSR markers initially were screened in the genotypes, of which 38 were polymorphic. SSR markers used in this study were developed by Winter et al. (2000) and distributed through the all linkage groups of the chickpea genetic linkage map. PCR was performed in a total reaction volume of 20 µL containing 1U Taq DNA polymerase (Cinnagen, Iran), 10 mMTris–HCl pH8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 mM of each dNTPs (Cinnagen, Iran), 10 pmol of each primer and 20 ng of template DNA, using a Eppendorf ThermoCycler (Germany). Amplifications were programmed for an initial step at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at the required Tm for 1 min and elongation at 72 °C for 2 min, followed by a final elongation step at 72 °C for 7 min.

PCR products were analyzed using 3% Methaphor agarose electrophoresis gels stained with ethidium bromide. Frequencies of incidence of all polymorphic alleles for each SSR markers were calculated and used for determining statistical parameters. Alleles were numbered as 'a1', 'a2' etc., sequentially from the largest to the smallest band. No distinction was made between amplified products of varied intensity, when the amplified products were within the expected size range. Number of alleles, effective number of alleles, gene diversity and polymorphism information content were calculated by GENALEX 6.1 software (Peakal, Smouse 2006). Tree construction following an unrooted NJ tree using a similarity matrix was carried through DARwin

Table 1. Details of 48 chickpea genotypes tested, including pedigree and origin/source

No.	Name	Pedigree (Origin)
1	L7	Landrace (Iran)
2	CCV2	Short term check (ICARDA)
3	ILC482	Drought tolerance check (Iran)
4	Flip2005.5c	X99TH154/ILC5901XILC3397 (ICARDA)
5	LMR144	X99TH154/ILC5901XILC3397 (ICARDA)
6	8EL93IM2446	Not traced (ICARDA)
7	L28	Landrace (Iran)
8	L69	Landrace (Iran)
9	L38	Landrace (Iran)
10	Azad	Not traced (Iran)
11	L26	Landrace (Iran)
12	L18	Landrace (Iran)
13	Hashem	Not traced Iran
14	Flip03-45c	×99TH 6/FLIP91-14C ×FLIP90-19C
15	L13	Landrace (Iran)
16	L21	Landrace (Iran)
17	L60	Landrace (Iran)
18	ILC533	Not traced (ICARDA)
19	Flipo3.27c	X98TH86/[(ILC267XFLIP89-4C)XHB-1]) XS95345 (ICARDA)
20	L37	Landrace (Iran)
21	ILC263	Susceptible Ascochyta blight check (ICARDA)
22	L50	Landrace (Iran)
23	L68	Landrace (Iran)
24	LMR159	X99TH154/ILC5901XILC3397 (ICARDA)
25	L45	Landrace (Iran)
26	L33	Landrace (Iran)
27	LMR29	x99TH151/ILC3805xILC5901 (ICARDA)
28	ILC1929	Not traced (ICARDA)
29	Arman	Not traced Iran
30	ILC3279	Landrace/Long term check (ICARDA)
31	Flip2005.1c	x99TH151/ILC3805xILC3397
32	LMR153	x99TH154/ILC5901xILC3397
33	ILC588	Short term check (ICARDA)
34	Flip2005.3c	x99TH154/ILC5901xILC3397
35	LMR81	x99TH153/ILC3805xILC5309
36	Piruz	Not traced (Iran)
37	L32	Landrace (Iran)
38	L5	Landrace (Iran)
39	L58	Landrace (Iran)
40	Flip04-20c	×00 TH35/FLIP 98-25C × S99442 (ICARDA)
41	Flip01-48c	X98TH30/FLIP 93-55C x S 96231 (ICARDA)
42	Kaka	Not traced (Iran)
43	Jam	Not traced (Iran)
44	ILC3397	Not traced (ICARDA)
45	Flip5187-3C	Drought tolerance check (ICARDA)
46	LMR165	x99TH155/ILC5901xILC5309 (ICARDA)
47	L17	Landrace (Iran)
48	LMR134	x99TH154/ILC5901xILC3397 (ICARDA)

5.0.128 (Perrier et al. 2003) analysis. Bootstrap analysis using 1000 bootstrap values was performed for the node construction. For the analysis of population structure, a Bayesian model-based analysis was performed using STRUCTURE 2.1 software (Pritchard et al. 2000). This software assumes a model in which there are K populations (clusters) that 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 simultaneous runs with 1000 replications using the admixture model and correlated allele frequencies with the K value ranging from 1 to 10.

Results

Diversity of morphological characteristics

The results of variance analysis of seven morphological traits showed significant differences among the examined genotypes, indicating the presence of variability that can be exploited through selection (Table 2). For each of the traits evaluated, descriptive statistics, including the extreme genotype mean values along with the corresponding genotypes, the mean, median, range, variance with their coefficient of variation are summarized in Table 3. Among traits, grain yield (g per plant) ranged from 4.12 to 22 with a mean value of 10.11 g per plant. High differences between the maximum and minimum mean values were found for all other traits. A dendrogram was constructed from the standardized values of morphological traits and genotypes grouped in four distinct clusters (Fig. 1). The first cluster contained nine genotypes of which two of them were Iranian landrace and the remaining were genotypes originated from ICARDA. Thirty five genotypes were grouped in the second cluster and ILC263 with Pirouz (Iranian drought tolerance check) were grouped in third cluster. Two genotypes (L60 and ILC533) were grouped in cluster IV (Fig. 1).

SSR allelic polymorphism, genetic diversity and population structure

In total, 38 SSR loci covering various bin locations on different linkage groups were used for genetic diversity analysis in 48 chickpea genotypes (Table 4). The 38 SSR loci analyzed produced 117 alleles with an average of three alleles per marker. The number of alleles ranged from 1 to 7, whereas the maximum was observed in TAA170. PIC ranged from 0 (TR1) to 0.77 (TAA170) with an average of 0.48. Gene diversity ranged from 0 to 0.78 with a mean of 0.5 in 48 accessions. The number of alleles per locus showed a significant and positive relationship with both PIC (r= 0.64, $P < 0.01$) and gene diversity ($r = 0.68$, $P < 0.01$). Cluster analysis using the un-weighted neighbor joining clustering algorithm clearly delineated the genotypes in five major clusters (Fig. 2). Cluster I contained ten genotypes of which eight of them were Iranian landrace genotypes. Cluster II included seven genotypes of which two Iranian improved cultivars ('Hashem' and 'Azad') grouped with Iranian landrace accessions. In cluster III, three Iranian chickpea cultivars ('Kaka', 'Jam' and 'Pirouz') were grouped with genotypes that originated from ICARDA. Cluster IV and V contained six and five genotypes, respectively, with most of them originating from ICARDA (Fig. 2). Genetic structure of the germplasm was further explored using the Bayesian clustering model implemented in the STRUCTURE software. 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, landrace and cultivated (Fig. 3) with both clusters showing uniform structure (Fig. 3). All the accessions of the landrace and cultivated cluster have relatively high (>80%) membership in their clusters.

Discussion

The present study aimed at characterizing the genetic diversity of landrace and advanced chickpea germplasm using SSR and morphological attributes and determining the potential utility of these markers. Studies on genetic diversity and relationships among landraces and improved varieties are not only useful for germplasm conservation, but also facilitate use of the genetic resources in crop improvement programmes (Imtiaz et al. 2008; Saeed et al. 2011; Choudhary et al. 2012).

In this study, 48 chickpea genotypes (19 landrace accessions and 29 improved cultivars and lines) were characterized using seven morphological characteristics

Table 2. Analysis of variance for seven morphological traits in 48 chickpea genotypes. HI, harvest index; TSW, 100-seed weight; SP, number of seeds/plant; BIO, plant biomass; NPP, number of pods per plant; PH, plant height. *, ** significant at P = 0.05 and 0.01, respectively

S.O.V	d.f	Mean square						
		HI (%)	TSW	Yield (g plant^{-1})	SP	BIO (g plant^{-1})	NPP	PH (cm)
Replication	2	88.58**	61.77*	19.12*	119.4*	136.14**	0.92	0.89
Genotype	47	192.23**	150.14**	57.41**	415.24**	359.13**	3.71**	64.77**
Error	92	9.14	10.77	2.78	27.14	15.72	0.37	6.18

Table 3. Descriptive statistics for seven morphological traits evaluated on 30 landrace chickpea genotypes. . HI, harvest index; TSW, 100-seed weight; SP, number of seeds/plant; BIO, plant biomass; NPP, number of pods per plant; PH, plant height; CV, coefficient of variation

Variable	Min	Max	Mean	Median	Variance	CV%
HI	9.12	48.47	23.18	23.11	69.77	31.78
TSW	7.74	38.65	22.57	21.77	39.14	22.87
Yield (g plant^{-1})	4.12	22	10.11	9.78	20.67	34.17
SP	14	121	49.88	45.41	680.25	33.48
BIO	24.78	79	41.55	39.16	128.37	19.47
NPP	15	124	7.99	5	0.98	15.14
PH	21	49	29.77	40.12	25.17	8.65

and 38 SSR markers located among eight LG of the chickpea map. The Iranian landrace chickpea showed a high level of morphological diversity for most of the traits observed, which may be useful for future breeding endeavors. Diversity analysis using 38 SSR markers produced 117 alleles, with an average of three alleles per marker. This suggested the presence of considerable polymorphism at the studied microsatellite loci and revealed a moderate level of genetic diversity in the existing chickpea germplasm, which is similar to the results obtained by Khan et al. (2010) and Ghaffari et al (2014).

Narrow genetic variation had been reported in chickpea germplasm by various researchers (Singh et al. 2003; Upadhaya et al. 2012), but it was now possible to conduct an extensive molecular diversity study in chickpea using large number of SSR markers to identify genetically diverse germplasm with potentially beneficial traits for chickpea improvement programmes. In the current study, heterozygosity was detected in genotypes that ranged from 0 (TR1) to 0.78 (TAA170) with mean of 0.50, which

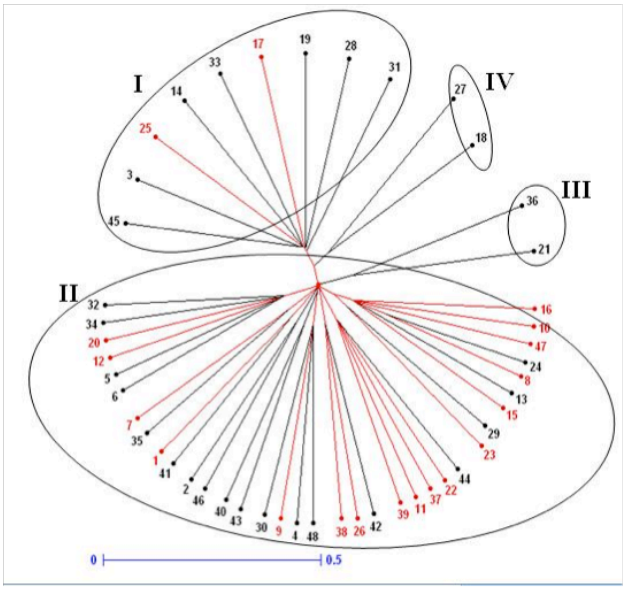

Fig. 1. Unweighted pair grouping method of arithmetic averages dendrogram of Iranian landrace (red color) and improved chickpea genotypes based on genetic distances computed from morphological traits.

Table 4. Number of allel (Na), number of effecticve allel (Ne), gene diversity (He) and polymorphism information content (PIC) observed in 48 chickpea genotypes with 38 microsatellite markers

Marker	Na	Ne	He	PIC
Cstms4	2	1.88	0.47	0.47
Cstms5	2	1.09	0.08	0.08
TA110	3	1.28	0.22	0.22
TS 43	3	2.00	0.51	0.52
GA20	3	2.68	0.63	0.62
GA24	2	1.23	0.19	0.19
GA34	4	2.79	0.65	0.64
TA53	4	1.98	0.50	0.50
TA96	3	1.90	0.48	0.49
TR1	1	1.00	0.00	0.00
TA159	4	2.92	0.66	0.66
TA5	3	1.81	0.45	0.45
TA113	3	2.13	0.54	0.53
TA76	2	1.88	0.47	0.47
TAA27	2	1.70	0.42	0.14
TR58	3	1.83	0.46	0.45
TA28	4	2.93	0.67	0.66
TA59	2	1.98	0.50	0.49
TA118	2	1.95	0.49	0.49
TS35	4	1.97	0.50	0.51
TR59	2	2.00	0.50	0.50
TR20	3	2.44	0.60	0.59
TR19	3	2.42	0.59	0.59
TA22	3	2.36	0.58	0.58
TA130	2	1.23	0.19	0.19
TA78	4	3.15	0.69	0.68
TA176	4	3.23	0.70	0.69
TA25	4	3.01	0.68	0.67
TS72	4	3.24	0.70	0.70
TA47	3	2.57	0.62	0.61
TA37	3	2.46	0.60	0.60
TAA170	7	4.35	0.78	0.77
TS 12	4	2.28	0.57	0.56
TS 45	3	1.57	0.37	0.05
TA146	3	2.52	0.61	0.60
TA72	3	1.21	0.17	0.17
TA3	2	1.97	0.50	0.49
TA39	4	2.89	0.66	0.64

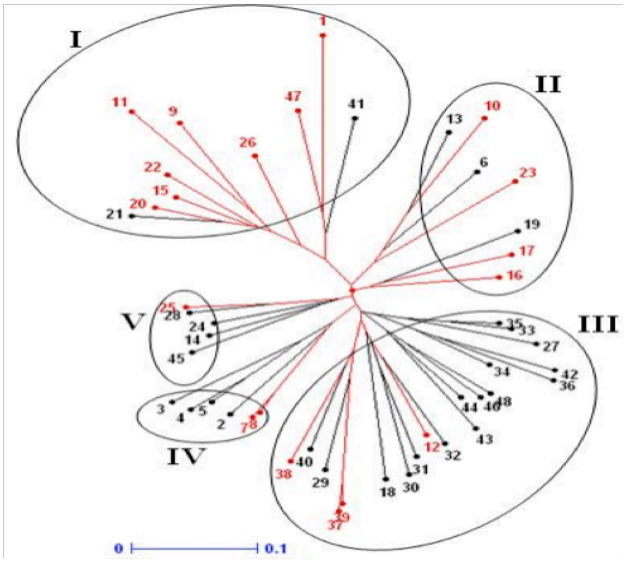

Fig. 2. Unweighted pair grouping method of arithmetic averages dendrogram of Iranian landrace (red color) and improved chickpea genotypes based on genetic distances computed from 38 SSR molecular markers.

is similar or relatively higher than values reported by Upadhaya et al. (2008) and Saeed et al. (2011), respectively. Cluster analysis using morphological and SSR markers separated all chickpea genotypes into four and five distinct groups, respectively. Most Iranian landraces accessions studied in present research were grouped relatively close together and this close relation between molecular genetic variability may be reflected to close geographic sources of these accessions.

The existing genetic diversity observed in advanced breeding lines developed at ICARDA indicated the efforts underway to widen the genetic base of chickpea for various traits. The selection of genotypes for this study was primarily based on different geographical origins/ or morphological characteristics. Therefore, we believe that research on additional molecular markers for morphological traits in the field are needed as complementary studies. This will reduce the amount of materials for study as well as the costs of experiments. The relationship observed using molecular markers may provide information on the history and

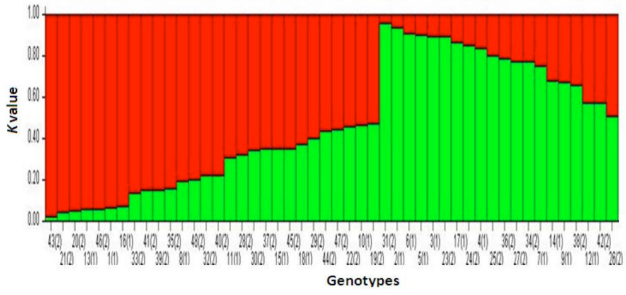

Fig. 3. Genetic relatedness of 48 chickpea accessions based on 38 SSR markers and analyzed by the structure programme.

biology of genotypes, but it does not necessarily reflect what may be observed with respect to agronomic traits (Metais et al. 2000). In conclusion, results of the present study indicate that the extent of genetic variability in the Iranian landraces and improved lines developed at ICARDA seem to have remained quite constant. Information about the current genetic diversity permits the classification of our available germplasm into various/ heterotic groups, which is particularly important to hybrid/cross-breeding programmes for chickpea.

Acknowledgements

This study was supported by the Scientific and Technological Research Council of Shahid Beheshti University, Tehran, Iran and Islamic Azad Univerity, Sanandaj Branch, Iran.

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Received 30 July 2014; received in revised form 14 September 2014; accepted 19 November 2014