Genetic Diversity in Iranian Chickpea (*Cicer arietinum* L.) Landraces as Revealed by Microsatellite Markers

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Abstract: To estimate the genetic diversity of chickpea germplasm from Iran, a total of 307 landraces from 4 regions including: northern areas (29 from Ardebil, 3 from Qazvin and 5 from Mazanderan provinces), temperate (16 from Kermanshah, 2 from Semnan, 54 from Khorasan and 20 from Kerman provinces), semi-arid (28 from Ghom and 56 from Isfahan provinces) and cold areas (15 from West Azarbayjan, 52 from Tehran and 27 from East Azarbayjan provinces) were analysed using 16 microsatellite loci. The number of alleles per microsatellite locus ranged from 8 to 29, with an average of 19.31 per locus. A high level of genetic diversity in the northern area (*He* = 0.76*)*, even with a limited number of available landraces (37) compared with the other three regions (84–94), might confirm the northern Persia as part of the chickpea centre of origin. The neighbour-joining tree showed a low relationship between molecular divergence and the geographical grouping of chickpea. Moreover, cluster analyses based on molecular data showed that the northern area was separated clearly from the other three regions, indicating a physical barrier or geographical and environmental differences among these regions. A wide genetic diversity of Iranian chickpea landraces is a critical component for future selection and use of this germplasm for future breeding of chickpea.

Keywords: *Cicer arietinum*; chickpea; genetic structure; germplasm; microsatellite

Chickpea (*Cicer arietinum* L.) is a self-pollinated, diploid ($2n = 2x = 16$), and the third most important grain legume crop of the world. This grain legume was domesticated in association with other crops as part of the evolution of agriculture in the Fertile Crescent 12 000–10 000 years ago (Zohary & Hopf 2000). The crop most probably originates from Turkey and Syria (Harlan 1992). However, Zeven and De Wet (1982) suggested that chickpea has different secondary centres of diversity located in at least four regions; the Near East Region (comprising the Fertile Crescent), Hindustani Region (basically the current India and East Pakistan), Central Asian Region (with Afghanistan, Western Pakistan, Iran and the south of the former USSR) and the Mediterranean Region (including Lebanon and Palestine).

This species is an important food legume in several countries including Algeria, Ethiopia, India, Iran, Mexico, Morocco, Myanmar, Pakistan, Spain, Syria, Tanzania, Tunisia, and Turkey (Upadhyaya *et al.* 2002).

Iran is considered as a major centre of diversity for chickpea (Zeven & De Wet 1982). Moreover, based on the number of accessions, Iranian chickpea collection ranks the third in the world after ICRISAT and ICARDA collections (FAO 1998). However, little breeding work has been done in Iranian chickpea cultivars, and consequently, chickpea yield in Iran is limited by climatic factors, water availability and genotype (Soltani *et al.* 1999).

The knowledge of genetic diversity in cultivated and wild relatives has a significant impact on the

improvement of crop plants and this information has been successfully used for efficient germplasm management, fingerprinting and genotype selection. Genetic diversity can be estimated using phenotypic identification or molecular markers. However, morphological traits have a number of limitations including low polymorphism, low heritability, late expression, and may be controlled by epistatic and pleiotropic gene effects (Eivazi *et al.* 2008). Molecular markers are a useful complement to morphological characterization of accessions because they are plentiful, independent of plant tissue or environmental effects, and allow cultivar identification very early in plant development (Manifesto *et al.* 2001).

A number of different molecular assays have been used to determine genetic relationships between the chickpea accessions. But some of these molecular markers, such as biochemical (Ahmad & Slinkard 1992; Labdi *et al.* 1996) and DNA-based markers like RFLPs (Udupa *et al.* 1993) and RAPDs (Sant *et al.* 1999; Chowdhary *et al.* 2002; Iruela *et al.* 2002; Sudupak *et al.* 2002) were unable to address the genetic variation within chickpea accessions. However, it was previously shown that microsatellite marker systems could efficiently be used for detecting the genetic variation within chickpea cultivars (Huttel *et al.* 1999; Udupa *et al.* 1999). Simple sequence repeats (SSRs) are common and informative molecular markers used for genetic diversity studies because of their simplicity, high levels of polymorphism, high reproducibility, and co-dominant inheritance patterns (Powell *et al.* 1996). Although the rich germplasm of chickpea has been reported from Iran, however, little is known about the diversity of these accessions in Iran. Even though geographical distribution and climatic pattern of Iranian accessions of chickpea was evaluated in a previous study (Garoosi & Vojdani 1993), they could not differentiate the origin sites according to the evaluated traits. In the present study, we used microsatellite markers to investigate molecular variation in a collection of *C. arietinum* landraces sampled from four geographical areas in Iran.

MATERIAL AND METHODS

Plant materials. A total of 307 germplasm accessions of kabuli-type chickpea, collected from 12 provinces of Iran, were used in this study. To investigate the geographical distribution of genetic diversity, chickpea growing areas were divided into four regions (Figure 1): Northern areas (29 from Ardebil, 3 from Qazvin and 5 from Mazanderan provinces), temperate (16 from Kermanshah, 2 from Semnan, 54 from Khorasan and 20 from Kerman provinces), semi-arid (28 from Ghom and 56 from Isfahan provinces) and cold areas (15 from West Azarbayjan, 52 from Tehran and 27 from East Azarbayjan provinces) (Table 1, Figure 1). All these landraces were previously studied for morphological variations (Naghavi

Table 1. The geographical origins of the 307 accessions of chickpea used in this study; NOR, SEA, COL and TEM represent Northern area, semi-arid, cold and temperate regions, respectively

Population	Code	Sample size	Province	Accession No.
Northern area	NOR	37	Ardebil	1–29
			Qazvin	30–32
			Mazanderan	33–37
Temperate	TEM	92	Kermanshah	38–53
			Semnan	54–55
			Khorasan	56–109
			Kerman	110–129
Semi arid	SEA	84	Ghom	130–157
			Isfahan	158–213
Cold	COL	94	Azarbayjan West	214–228
			Tehran	229–280
			Azarbayjan East	281–307

Figure 1. The geographical origins of 307 landraces of chickpea used in this study; NOR, SEA, COL and TEM represent Northern area, semi-arid, cold and temperate regions, respectively

& Jahansouz 2005). These accessions were procured from the Gene Bank at the University of Tehran, Karaj, Iran.

DNA extraction and microsatellite analyses. Total genomic DNA of one plant per landrace was isolated from leaf material using a CTAB method (Saghai-Maroof *et al.* 1984) with minor modifications. A set of 16 SSR primer pairs (Huttel *et al.* 1999) was used to amplify the genomic DNA of all 307 landraces. PCR was performed in 5 µl reaction volume with final concentrations of 5 ng DNA, 2mM $MgCl_2$, 0.12mM of dNTPs, 1X PCR buffer, 1 pmole of forward primer labelled with a fluorescent dye (either 6-Fam or Vic or Ned or Pet from Applied Biosystems, Carlsbad, USA), 1 pmole of reverse primer and 0.1U of *Taq* DNA polymerase (Bioline, London, UK) in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, Carlsbad, USA). PCR amplification was carried out using a touch-down methodology with 15 min initial denaturing (to activate *Taq* DNA polymerase), followed by 10 cycles of denaturing at 94°C for 15 s, annealing at 60°C for 20 s (temperature reduced by 1°C for each cycle) and extension at 72°C for 30 s. This was followed by 40 cycles of denaturation at 94°C for 10 s, annealing at 54°C for 20 s and extension at 72°C for 30 s with the final extension of 20 min at 72°C. PCR products of different dyes were pooled together along with internal size standard (GeneScan™ 500 LIZ® from Applied Biosystems, Carlsbad, USA) and capillary electrophoresis was carried out using an ABI 3700 Genetic Analyser (Applied Biosystems, Carlsbad, USA). Sizing of PCR amplified fragments was done based on the relative migration of internal size standard using GeneScan 3.7 software and allele calling was done by Genotyper 3.7 software (Applied Biosystems, Carlsbad, USA).

Data analysis. The results of amplification reactions from all landraces were scored and the following statistics of genetic variation within different chickpea regions were computed as averages over loci using the GENAlEX 6.1 software (Peakal & Smouse 2006): number of observed alleles (*Na*), polymorphism information content (PIC), number of private alleles, effective number of alleles (*Ne*), Shannon's information index (*I*), analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) and gene diversity (*He*) computed according to Nei (1978). The relationships of the landraces were estimated from the SSR data by employing the Neighbour-Joining (NJ) clustering method on simple matching allele frequency-based distance matrix using DARwin5 5.0.146 computer software (Perrier *et al.* 2003).

In addition, relationships of the four chickpea growing regions were estimated from the SSR data using the UPGMA clustering method on the basis of Nei's unbiased genetic distance (1978). The UPGMA tree was constructed using NTsys program ver. 2.0 (Rohlf 1998).

RESULTS

Genetic diversity

A total of 309 alleles were identified by 16 SSR markers across all landraces. The numbers of alleles per primer varied from 8 for TA46 to 29 for TA28, with an average of 19.31 alleles per locus in the total collection. The highest and the lowest PIC values were 0.92 (TA28) and 0.36 (TA46), respectively, with an average of 0.75 (Table 2). Moreover, the highest and the lowest gene diversity was observed for TA64 (0.903) and TA46 (0.359), respectively.

In addition, a total of 106 unique (private) alleles were identified using 16 SSR primers, 26 such alleles appeared in Northern area, 18 in temperate, 18 in semi-arid and 44 in cold regions (Table 3).

Table 2. Number of alleles and polymorphism information content (PIC) in the 307 landraces of chickpea for each microsatellite locus

Locus	No. of alleles	PIC	*Ho*	*He*
CaSTMS15	15	0.77	0.003	0.768
CASTMS2	23	0.88	0.003	0.865
CaSTMS21	14	0.43	0.000	0.432
TA113	14	0.74	0.006	0.731
TA116	24	0.81	0.000	0.802
TA117	23	0.88	0.009	0.865
TA14	20	0.81	0.003	0.800
TA21	24	0.89	0.000	0.864
TA22	27	0.85	0.007	0.845
TA27	9	0.69	0.007	0.689
TA46	8	0.36	0.000	0.359
TA64	23	0.91	0.003	0.903
TA71	18	0.79	0.000	0.778
TA72	23	0.72	0.000	0.716
TA76s	15	0.64	0.000	0.639
TA28	29	0.92	0.003	0.900
Mean	19.31	0.75	0.003	0.747
SE	6.22	–	0.001	0.020

Ho – observed heterozygosity; *He* – Nei's (1973) gene diversity; SE – standard error

Genetic structure of the origin sites

In general, the growing regions included in this study showed a relatively high level of genetic diversity with *Na* = 12.4, *Ne* = 5.38, *He* = 0.74 and *I* = 1.86 (Table 3). It can also be seen that the landraces from Northern area, with the least numbers of landraces (37), showed a high level of Nei's gene diversity and Shannon's information index (0.76 and 1.88, respectively).

Table 3. Genetic diversity estimates for four growing regions of chickpea based on 16 microsatellite loci

Region	Code	Sample size	*Na*	*Ne*	*I*	*Ho*	*He*	Unique alleles
Northern area	NOR	37	10.65 (0.84)	5.50 (0.630)	1.88 (0.122)	0.003 (0.002)	0.76 (0.037)	26
Temperate	TEM	92	11.67 (1.21)	5.51 (0.81)	1.83 (0.144)	0.002 (0.001)	0.74 (0.03)	18
Semi arid	SEA	84	12.00 (1.04)	5.22 (0.608)	1.85 (0.135)	0.002 (0.001)	0.74 (0.041)	18
Cold	COL	94	14.25 (1.19)	5.30 (0.768)	1.88 (0.145)	0.003 (0.002)	0.73 (0.045)	44
Mean			12.140	5.38	1.86	0.003	0.74	26.5

Na – observed number of alleles; *Ne* – effective number of alleles; *Ho* – observed heterozygosity; *He* – Nei's (1973) gene diversity; *I* – Shannon's information index; standard error is given in the brackets; NOR, SEA, COL and TEM represent Northern area, semi-arid, cold and temperate regions, respectively

Table 4. Analysis of molecular variance (AMOVA) for 307 *Cicer arietinum* individuals among and within regions

Source of variation	df	Sum of squares	Variance components	Percentage of variation
Among regions	3	107	0.149	1
Within regions	303	7445.4	24.573	99
Total	306	7552.4	24.772	

df – degree of freedom

Partitioning the variation within and between origin sites using an analysis of molecular variance (AMOVA) showed that 1% of the total genetic variation existed among growing regions (Table 4).

Clustering of populations

The neighbour-joining tree showed two major cluster groups (group I and II). Most landraces from cold area were in group I, whereas group II mainly consisted of accessions from temperate and semi-arid regions. However, there were some exceptions in each group. Moreover, accessions of Northern areas belonged to both groups (Figure 2).

The largest genetic distance (0.092) among the four regions was found between the Northern and cold areas. Meanwhile, the smallest genetic distance (0.044) was observed between cold and semi-arid regions. According to the cluster analysis (UPGMA)

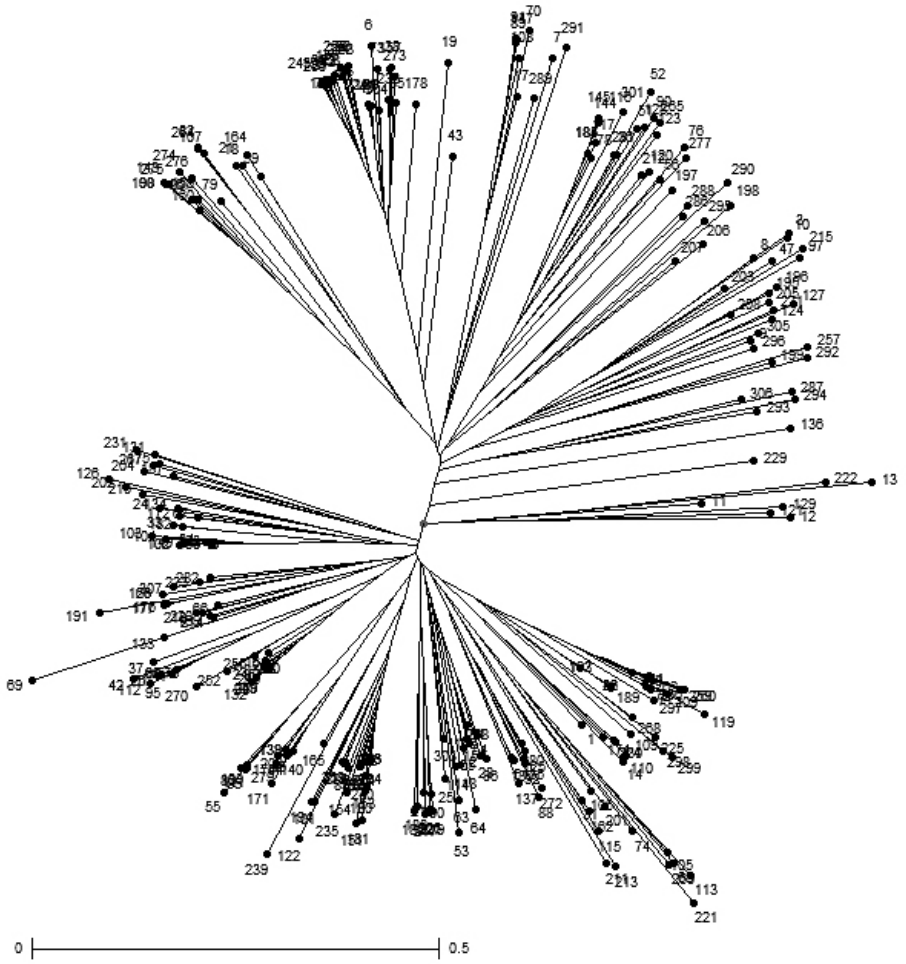

Figure 2. Unweighted neighbour-joining tree based on the simple matching dissimilarity matrix of 16 SSR markers diagram genotyped across the 307 landraces of chickpea; for accession number see Table 1

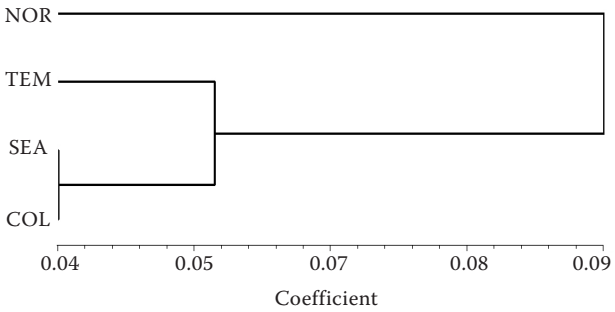

Figure 3. Dendrogram of genetic relationships between four growing regions of chickpea as reconstructed by the UPGMA method based on the genetic distance obtained from Nei's genetic distances (1978) of SSR data; NOR, SEA, COL and TEM represent Northern area, semi-arid, cold and temperate regions, respectively

the two semi-arid and cold regions showed a closer genetic relationship. While, high genetic distances were found between the landraces from Northern area and the other regions (Figure 3).

DISCUSSION

In this study, 307 landraces of chickpea were genotyped at 16 SSR loci in order to detect genetic diversity among the four chickpea growing regions of Iran. The number of alleles per marker is considered to be a good indicator of genetic variability (Nevo 1978). Compared with previously published data on chickpea using SSR markers, results of this study revealed from 8 to 29 alleles with an average of 19.31 alleles per locus (Table 2), which is more than the value found by Udupa *et al.* (1999) and Sethy *et al.* (2006), while being less than what was reported by Upadhyaya *et al.* (2008). These differences might be partially explained by the utilization of chickpea landraces with different population size and also from different geographical origins, as well as by the use of different SSR markers.

In addition, our microsatellite survey of the four growing regions of *C. arietinum* indicates a high level of genetic variation. In previous studies a narrow genetic base of chickpea was reported, as isozymes (Kazan & Muehlbauer 1991; Ahmad & Slinkard 1992; Labdi *et al.* 1996), RFLPs (Udupa *et al.* 1993) and RAPDs (Sant *et al.* 1999) revealed very low polymorphism in this species. However, a high level of genetic variation was reported using SSR markers (Udupa *et al.* 1999). This discrepancy could be attributed to different marker systems. As pointed out by many studies, microsatellites possessed higher resolving power among various genetic markers (Powell *et al.* 1996). However, in addition to the above-mentioned advantages, microsatellites have also disadvantages, namely the risk of homoplasy and higher mutation rates (Lowe *et al.* 2004).

The chickpea from four Iranian regions showed a number of private (unique) alleles (Table 3). The maximum of specific alleles (44) was seen in the cold area while both temperate and semi-arid areas showed the minimum of specific alleles (18). The landraces from both cold and Northern areas revealed (nearly 2 and 1.5 times, respectively) more unique alleles compared with the landraces from temperate and semi-arid areas. This may be explained by the fact that the landraces were gathered from a wide range of agroecosystems and different climatic conditions in cold and Northern areas. In addition, a high level of genetic diversity in Northern area (*He* = 0.74), even with a limited number of available accessions (37), compared with the other three growing regions (84–94), might confirm the Northern Persia as part of the origin centre of chickpea (De Candolle 1883).

Results of the Neighbour-Joining tree showed two major cluster groups. Although studied landraces are not randomly distributed in both groups, there is no strong relationship between molecular divergence and geographical distribution of landraces (Figure 2). It seems that this material has been exchanged among farmers of different regions. Aghaei *et al.* (2000) also found a high level of diversity for most morphological characters in wild accessions of chickpea from Iran. However, the evaluated landraces had not been separated according to geographical distribution. In agreement with this result, Garoosi and Vojdani (1993) also found no relationship between genetic diversity and geographical distribution in Iranian chickpea landraces. However, cluster analyses of the chickpea growing regions using molecular data showed that the Northern area separated clearly from the other three regions (Figure 3), a reflection of the geographical and evolutionary differences in genetic diversities among growing regions. We would expect that a greater sampling and increasing the number of landraces in each region would be valuable to conclude more precisely the relationship between diversity and geographical origin.

In the present study, microsatellite data indicate that genetic variability mainly existed among *C. arietinum* landraces rather than among the growing regions. This is a clear indication that the extent of inbreeding within populations is high, but the extent of genetic differentiation among populations is only low or moderate as the narrowness of the genetic base is one of the principal causes of the slow progress of chickpea breeding. A wide genetic diversity of Iranian native chickpea found in this and other studies (Aghaei *et al.* 2000; Naghavi & Jahansouz 2005) is a critical component for future selection and use of this germplasm for future breeding of chickpea.

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