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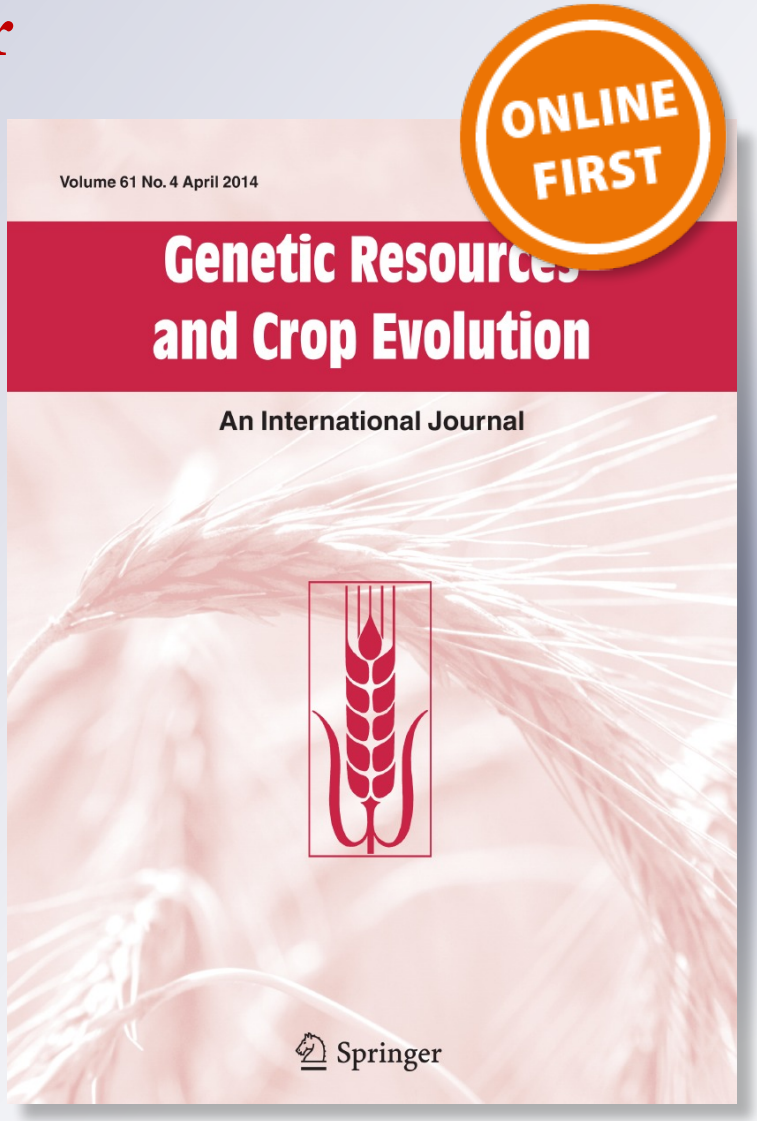

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RESEARCH ARTICLE

Target region amplification polymorphism (TRAP) for assessing genetic diversity and marker-trait associations in chickpea (*Cicer arietinum* L.) germplasm

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Abstract The effective utilization of crop diversity held in genebanks depends on our knowledge of useful traits and available markers associated with the target traits. Target region amplification polymorphism (TRAP) was used to evaluate the genetic diversity and underlying relationships among 263 accessions of chickpea landraces maintained by the USDA-ARS Western Regional Plant Introduction Station in Pullman, WA, USA. Two-hundred sixty-two TRAP markers were amplified by eight primer combinations. Altogether, 110 (42 %) markers were polymorphic, the other 152 (58 %) displayed no variation. These polymorphic markers revealed important differences among the accessions, with an estimated, mean pairwise genetic distance of 25.82 %, ranging from 2.8 to 50.0 %. Genetic distance analysis divided the accessions into two major groups, with 113 and 150 accessions each, and substantial association between molecular diversity and geographic origin was evident. Bayesian analysis of population structure revealed two groups ($K = 2$) with evidence for six sub-groups. Additionally, the population structure of a subset of 110 lines was determined ($K = 3$) for testing marker-trait associations (MTAs). Phenotypic traits included the concentrations of protein and nine mineral elements in the seeds. Two MTAs were significant ($p < 0.01$) for concentrations of Ca and K, and three MTAs were significant for Cu and Ni concentrations. The results indicate that this population is useful for genome-wide association studies on other economic traits given the level of genetic

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diversity uncovered and the marker-trait associations in seed minerals discovered.

Keywords *Cicer arietinum* · Population structure · Seed minerals · Seed protein

Introduction

Chickpea (*Cicer arietinum* L.) is an economically important crop for India, the Middle East, North Africa and Ethiopia. It is the third most important pulse crop in the world after common bean (*Phaseolus vulgaris* L.) and pea (*Pisum sativum* L.) (FAOSTAT 2013). The genus *Cicer* contains 42 wild species including nine annuals and 33 perennials. Almost all these species have a chromosome number $2n = 2x = 16$ (Labdi et al. 1996). The genome of the domestic chickpea is about 740 Mb in size (Arumuganathan and Earle 1991) and has been sequenced recently (Varshney et al. 2013). Chickpea is a self-pollinated crop, with natural cross pollination levels typically below 1 % (Singh 1987). Globally, >90 % of chickpea is produced and consumed in Asia, with >70 % produced in India. Chickpea provides a protein-rich supplement to cereal-based diets—its seeds contain from 12 to 30 % protein, 30 to 57 % starch, 3 to 9 % fat, 1 to 13 % crude fiber, 5 to 12 % soluble sugar and 2 to 4 % ash (Williams and Singh 1987; Wood and Grusak 2007). Chickpea crops restore and maintain soil fertility by biological nitrogen fixation and are an integral part of many cereal-based cropping systems (Kantar et al. 2007). Biotic and abiotic stresses can result in reduced productivity of chickpea (Singh et al. 1994). The number of pathogens affecting chickpea has increased threefold since 1996 (Nene et al. 1996). The primary fungal diseases of chickpea are Fusarium wilt, caused by *Fusarium oxysporum* Schlechtend.: Fr. f. sp. *ciceris* (Padwick) T. Matuo and K. Sato and Ascochyta blight, caused by *Ascochyta rabiei* (Pass.) Labr. [teleomorph *Didymella rabiei* (Kovatsch.) Arx] (Smithson et al. 1985).

Domestication has resulted in a genetic bottleneck reducing genetic diversity of many cultivated crops, including chickpea (Abbo et al. 2003). This reduction in genetic diversity may severely limit the potential of future breeding efforts to select for adaptive traits, such as resistance to biotic and abiotic stresses, thereby affecting the stability of crop yields (Labdi et al. 1996). Pedigree information of qualitative and quantitative traits, biochemical and isozyme markers, seed storage-protein analysis and DNA-based markers have all been exploited to examine patterns of genetic diversity in many legume crops (Upadhyaya et al. 2011). The main advantages of using molecular markers to assess genetic diversity include their ability to account for the effects of selection, the fact that they are neutral in regards to fitness (Holderegger et al. 2006), and they can be abundant in chickpea (Hiremath et al. 2012). Additionally, the advent of PCR technology and the ability to detect single-base pair differences have facilitated marker use in the analysis of phylogenetic relationships, cultivar identification, genetic diversity, parentage determination, and marker-assisted selection in many plant genera (Sharma et al. 1995), as well as leading to the powerful tool of association mapping (Zhu et al. 2008).

Earlier diversity studies have focused on taxonomic relationships and genetic diversity in *Cicer* species by using allozymes (Sudupak and Kence 2004; Tayyar and Waines 1996; Labdi et al. 1996; Ahmad et al. 1992; Kazan and Muehlbauer 1991), seed storage proteins (Ahmad and Slinkard 1992), RAPDs (Ahmad 1999; Iruela et al. 2002; Javadi and Yamaguchi 2004; Rao et al. 2007), AFLPs (Nguyen et al. 2008; Shan et al. 2005), microsatellite loci (Udupa et al. 1999; Choumane et al. 2000), and more recently ISSRs (Choudhary et al. 2013), along with gene sequences (Singh et al. 2008; Javadi et al. 2007). Within *C. arietinum*, the largest genetic diversity study to date is of a composite collection in chickpea (3,000 accessions), representing the entire spectrum of genetic diversity present in ICRISAT and ICARDA genebanks (Upadhyaya et al. 2006). The composite core was genotyped by using 48 SSR markers to study genetic diversity and for identifying a reference set of 300 diverse accessions (Upadhyaya et al. 2008). This composite collection showed rich allelic diversity (1,791 alleles, 36 alleles per locus, 994 rare and 797 common alleles, gene diversity from 0.534 to 0.964), and a number of unique alleles (kabuli's 117, desi's 310, wild *Cicer* 74; Mediterranean 122, west Asia 121, and south and south east Asia 123). The first high-throughput gene-based diversity study examined 58 accessions by using 2005 SNP assays with 1,341 polymorphic SNPs; it revealed two main clusters of the only two wild species examined and 56 cultivated accessions (Hiremath et al. 2012). Other chickpea genetic diversity studies include ISSRs of 38 mostly Indian accessions (Choudhary et al. 2013), 155

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Ethiopian accessions based on 33 SSRs (Keneni et al. 2012), 15 Italian accessions using SSRs (Zaccardelli et al. 2012), and RFLPs of 30 accessions (Serret et al. 1997).

Knowledge of levels of genetic diversity and phenetic relationships among conserved crop germplasm collections is essential for establishing, managing and ensuring the long-term success of crop improvement programs (Gebhardt et al. 2004). TRAP is a simple PCR-based marker technique, which uses available DNA sequence information to detect genetic variation at the DNA level (Hu and Vick 2003). TRAP uses a fixed primer designed against known DNA sequences and pairs it with arbitrary primers that target the intron or exon regions with an AT- or GC- rich core (Li and Quiros 2001) to amplify DNA fragments. Published studies applying TRAP to lettuce (Hu et al. 2005), sugarcane (Alwala et al. 2006), spinach (Hu et al. 2007), sunflower (Yue et al. 2009), faba bean (Kwon et al. 2010) and pea (Kwon et al. 2013) have suggested that TRAP is a useful marker system to determine germplasm diversity.

The objective of this study was to apply TRAP to evaluate the genetic diversity, relationships and population structure of 263 chickpea lines that constitute the USDA chickpea single plant collection (CSP) derived by selecting one representative plant from each of the 263 heterogeneous landraces and to investigate marker-trait associations with seed nutrient concentrations.

Materials and methods

Plant materials

The chickpea germplasm used in this study were 263 accessions that constitute a portion of the USDA-ARS USDA CSP developed for association mapping studies. Each line of CSP was derived from one representative plant selected from a heterogeneous landrace accession. Table S1 in supplementary material lists the PI numbers, names and geographic origins of the accessions from which CSP was formed.

Nutrient phenotyping

Each of the 110 accessions of the USDA chickpea core collection were grown in 5L black plastic pots (6 plants per pot, one pot per accession) filled with a growing medium composed of 2 parts Metro-Mix 360 (Scotts-Sierra Horticultural Products Co., Marysville, OH, USA) and 1 part vermiculite (Strong-Lite Medium Vermiculite, Sun Gro Horticulture Co, Seneca, IL, USA). Note that of the 263 accessions genotyped in this study (see below), only 110 accessions were phenotyped due to funding limitations. Plants were grown in a greenhouse, 22 ± 3 °C/day and 20 ± 3 °C/night, with a 15-h day, 9-h night cycle. A complete fertilizer mixture was provided to each pot twice daily, delivered with an automated dripline system. The mineral salts included: 1.0 mM KNO_3, 0.4 mM $Ca(NO_3)_2$, 0.1 mM $MgSO_4$, 0.15 mM KH_2PO_4 and 25 μM $CaCl_2$, 25 μM H_3BO_3, 2 μM $MnSO_4$, 2 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM H_2MoO_4, 0.1 μM $NiSO_4$, and 10 μM Fe(III)-*N*, *N*′-ethylenebis[2-(2-hydroxyphenyl)-glycine] (Sprint 138; Becker-Underwood, Inc., Ames, IA, USA). Seeds were harvested from all six plants of each accession at full maturity and dried at 65–70 °C before being ground as one bulk homogenate. Two sub-samples (0.25 g dry weight each) of each accession's bulk sample were digested independently, using a mixture of nitric and perchloric acids as previously described (Narayanan et al. 2007). Dried digestates were resuspended in nitric acid and analyzed for Ca, Mg, K, P, Fe, Zn, Mn, Cu, and Ni concentrations by using inductively coupled plasma atomic emission spectrometry (CIROS ICP Model FCE12; Spectro, Kleve, Germany). Tomato leaf standards (SRM 1573A; National Institute of Standards and Technology, Gaithersburg, MD, USA) were digested and analyzed along with the chickpea samples to ensure accurate instrument calibration. Mineral values from the two sub-samples were averaged for each accession. These averaged values were reported in the GRIN database (www.ars-grin.gov) as ppm (parts per million) on a dry weight basis.

Seed nitrogen concentrations were determined by using a LECO FP-528 Nitrogen/Protein Determinator (Leco Corp., St. Joseph, MI, USA). Two sub-samples (0.15 g each) of each accession's bulk sample were analyzed for nitrogen concentration. Each analytical sample was measured two times internally by the instrument. The two sub-sample values were then averaged to get a representative nitrogen concentration value for each accession. Protein concentrations were calculated by using a

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conversion factor of 5.44 [(seed nitrogen concentration) × 5.44 = (seed protein concentration)] (Mossé 1990).

Genomic DNA extraction

Leaf samples were taken from four plants of each accession, placed in liquid nitrogen and held at −80 °C. Genomic DNA was extracted from the frozen leaf tissue with DNeasy 96 Plant Kits (Qiagen, Valencia, CA, USA) following the manufacturer's instructions.

TRAP marker generation

Our TRAP protocol followed that of Hu and Vick (2003) and used three fixed and four arbitrary primers (Table 1). Four arbitrary primers and one fixed primer used in the current study are based on those described by Hu et al. (2005). The other two fixed primers (prefix, MIR) were designed against micro RNA sequences in *Arabidopsis thaliana* (L.) Heynh. (Kwon et al. 2010). Four sets of PCR with 8 primer combinations were run with all the DNA samples (Table 2). TRAP amplification was carried out according to Hu et al.'s (2007) protocol. A total volume of 10 µl was used for PCR amplification, which contained 1 µl of genomic DNA (10 ng/µl), 0.2 µl of the fixed primer (10 pmol/µl), 0.2 µl each of 700- and 800-IR dye labeled arbitrary primers (1 pmol/µl), 0.8 µl of dNTP (2.5 mM), 0.3 µl of $MgCl_2$ (50 mM), 1.0 µl of 10× PCR buffer, 0.2 µl of Taq polymerase (5 unit/µl; Bioline, Taunton, MA, USA), and 6.3 µl water. PCR was performed by initially denaturing template DNA at 94 °C (2 min) followed by 5 cycles at 94 °C (45 s), 40 °C (45 s) and 72 °C (60 s), then 35 cycles at 94 °C (45 s), 50 °C (45 s) and 72 °C (60 s) and final extension at 72 °C (7 min). PCR was carried out in GenAmp 9,700 thermal cyclers (Applied Biosystem, Foster City, CA, USA). Amplified products were separated on a 6.5 % polyacrylamide sequencing gel in a Li-Cor Global DNA Sequencer (Li-Cor Biosciences, Lincoln, NE, USA) by electrophoresis at 1,500 V for 3 h. The images were collected and scored manually.

Table 1 Target region amplification polymorphism (TRAP) primer names and sequences used in the current study

Primer name	Sequences (5′–3′)
Fixed primers	
B14G14B[a]	AAT CTC AAG GAC AAA AGG
MIR 156A[b]	GAT CTC TTT GGC CTG TC
MIR 159A[b]	GAT CCT TGG TTC TTT GG
Arbitrary primers	
Sa4-700[a]	TTA CCT TGG TCA TAC AAC ATT
Sa12-700[a]	TTC TAG GTA ATC CAA CAA CA
Ga3-800[a]	TCA TCT CAA ACC ATC TAC AC
Ga5-800[a]	GGA ACC AAA CAC ATG AAG A

[a] Hu et al. (2005)

[b] Kwon et al. (2010)

Data analysis

Each amplified fragment was treated as a unit character and scored as a binary code 1 and 0 for presence and absence, respectively. To ensure reliability, only prominent bands were scored. Hierarchical cluster analysis was conducted by using the unweighted pair group method with arithmetic mean method based on the allele-sharing distance (UPGMA) (Sneath and Sokal 1973) by PowerMarker version 3.25 (Liu and Muse 2005), and the resulting tree was displayed with Mega4 (Tamura et al. 2007). The accessions analyzed are landraces subjected to multiple, arbitrary selection cycles, so evolutionary distances should not be relevant as they would be for wild, proximal populations separated by natural geologic events and thus subjected to natural selection from an original panmictic population. UPGMA clustering is widely used because it produces less distortion from the resemblance matrix to the tree (Romesburg 2004) and produces trees with a high cophenetic correlation coefficient (Farris 1969). Additionally, UPGMA similarities are factual, no speculative phylogenetic inferences are implied, and allow for comparison with published chickpea studies (Sokal and Rohlf 1962; Pejic et al. 1998; Hiremath et al. 2012; Choudhary et al. 2013). GenAlEx version 6.1 was used to measure the Shannon's information index and percentage of polymorphism (Peakall and Smouse 2006).

The population genetic structure of 263 chickpea accessions and 110 accessions of the seed nutrient concentration study was determined by using STRUCTURE 2.3.3 (Pritchard et al. 2000; Falush et al. 2003, 2007; Hubisz et al. 2009). STRUCTURE is a Bayesian clustering program which uses the genotype data to

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Table 2 Distribution of TRAP markers amplified by the eight primer combinations among 263 chickpea germplasm accessions

PCR	Primer combination	Monomorphic fragments	Polymorphic fragments	Total fragments	% monomorphic	% polymorphic	Shannon's information index
F01	B14G14B + Sa12-700	22	23	45	48.9	51.1	0.295
	B14G14B + Ga5-800	16	12	28	57.1	42.9	0.336
		38	35	73	52.0	48.0	0.316
F02	MIR156A + Sa12-700	15	27	42	37.7	64.3	0.462
	MIR156A + Ga5-800	10	11	21	47.6	52.4	0.268
		25	38	63	39.7	60.3	0.365
F03	MIR159A + Sa4-700	29	14	43	67.4	32.6	0.324
	MIR159A + Ga3-800	21	5	26	80.7	19.2	0.613
		50	19	69	72.5	27.5	0.469
F04	MIR159A + Sa12-700	21	11	32	65.6	34.4	0.423
	MIR159A + Ga5-800	18	7	25	72.0	28.0	0.593
		39	18	57	68.4	31.6	0.508
	Total	152	110	262	58.0	42.0	
Mean	Per PCR	38	27.5	65.5			0.414
	Per primer combination	19	13.75	32.8			0.414

describe and visualize population structure based on allele frequencies of the data. The model assumes that there are K putative subpopulations in which each locus is characterized by a set of allele frequencies. The value of K is set by user when analyzing the dataset and the program will calculate the probability for each K value. The K value resulting in the highest probability (an average of ten simulations) represents the number of subpopulations in the samples. The default model of the program was utilized with the admixture option selected and correlated allele frequencies included between the populations. In addition to the estimated log probability, the following *ad hoc* statistics suggested by Evanno et al. (2005) were performed: the rate of change of the log probability of data with respect to the number of clusters $[L'(K) = \Pr(X|K) - \Pr(X|K-1)]$; the absolute value of the rate of change of the likelihood distribution $[|L''K| = |L'(K+1) - L'(K)|]$; and the absolute value of the rate of change divided by standard deviation of the 20 original simulations $[\Delta K = \mu|L''K|/s\ [L(K)]$ with Structure Harvester (Earl and von Holdt 2012). Replicate STRUCTURE runs (3) and cluster matching between populations and subpopulations was achieved with CLUMPP software (Jakobsson and Rosenberg 2007) run in Structure Harvester.

Marker-trait associations were determined with TASSEL 3.0.1 (Bradbury et al. 2007; Yu et al. 2005). Three tests for significance were performed. First, a single factor analysis (SFA) of variance was run using each marker as the independent variable and comparing the mean performance of each allelic class. Second, Q GLM (general linear model) was used on the chosen Q-matrix derived from STRUCTURE with 10,000 permutations to test the marker significance and to determine the experiment-wise P value for each marker's significance. Third, the Q + K MLM (mixed linear model) method was used to determine a kinship matrix and the population structure Q matrix. SPAGeDi (Hardy and Vekemans 2002) was used to calculate kinship (K) coefficients.

Results

TRAP amplification profiles

TRAP amplification of 263 chickpea accessions was carried out with eight primer combinations which successfully amplified a total of 262 recordable fragments. Of the 262 amplicons scored, 152 (58 %) were monomorphic and 110 (42 %) were polymorphic

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among the accessions. The number of polymorphic fragments amplified by each primer combination ranged from 11 (MIR 156A + Ga5_800) to 23 (B14G14B + Sa12_700) and the sizes of the amplified fragments ranged from 100 to 850 base pairs (Fig. S1 in supplementary material). For each primer combination, an average of 32.75 total fragments were scored and an average of 13.75 were polymorphic (Table 2). The highest level of polymorphism (64.3 %) was obtained with primer combination MIR 156A + Sa12_700 (Fig. S1 in supplementary material), and the lowest level (19.2 %) was obtained with primer combination MIR 159A + Ga3_800.

Genetic distance among entries

Differences among accessions at the DNA level were determined by comparing the genetic distance coefficients for 34,453 pair-wise comparisons. When estimated from the 110 informative markers, the average pair-wise genetic distance was found to be 25.82, with a range from 2.8 to 50.0. The most closely related four pairs of accessions, which showed less genetic distance (2.8) among them, were PI 360660/PI 360383, PI 360660/PI 360493, PI 360660/PI 451410, and PI 360660/PI 454346. Germplasm accession PI 360660 originated from Israel while accessions PI 360383, PI 360493, PI 451410 and PI 451346 originated from Iran. Similarly, the least related pairs of accessions showed dramatic differences, with genetic distances $\geq$ 47.3, including PI 451085/PI 502994, PI 360641/PI 451084 and PI 451085/PI 509156. Accessions PI 502994 and PI 509156 originated from Syria and Turkey, respectively. The other accessions (PI 451085, PI 360641, and PI 451084) originated from Iran.

Population structure

Population analysis was performed with a predefined number of subpopulations (K) ranging from 1 to 10. A Bayesian clustering method was used to assign accessions to two groups ($K = 2$) selected based on the Delta$K$ method of Evanno et al. (2005) (Figs. 1, 2). On that basis, 150 and 113 accessions were included in population group I (red bars) and group II (blue bars), respectively (Fig. 1). This representation provides an estimate of the degree of admixture of the accessions (Fig. 1).

Fig. 1 Dendogram and model-based population structure of 263 accessions based on TRAP marker polymorphisms where the populations presented are $K = 2$ and $K = 6$. Each accession is represented by a *horizontal bar* partitioned by color, with each color representing the estimated membership fraction

The result indicated that when two subpopulations were assumed ($K = 2$), 69 of 263 (26.2 %) accessions had the estimated ancestry membership (q) of $\leq$0.95 with one of the two groups, and 62 (23.6 %) had q values <0.75. On the other hand, 132 (50.2 %) accessions had q values >0.95. Within population groups, 111 (74.0 %) of the 150 population group I accessions showed q values >0.75 (red bars) as did 88 (77.9 %) of the 113 accessions in population group II (blue bars).

The Delta$K$ provides evidence for six sub-groups ($K = 6$) within the 263 accessions (Figs. 1, 2). There was general alignment of the dendrogram clustering with the population-structural analysis (Fig. 1). When K was increased from two to six (assuming that six subpopulations exist), significant changes in membership appeared for accessions in both groups. The group I accessions were separated into four major groups, whereas group II was divided into three major groups. One subgroup, designated by orange bars, was prominently split in both population groups of the dendrogram (Fig. 1). In spite of increasing K, most of the accessions still had high q values at $K = 2$. High q values (proportion of the genome of an individual originating from each inferred population) are greater than $\leq$0.95 membership in a group (K). Thirty-two accessions had q values >0.95, and 33 accessions had q values between 0.75 and 0.95 in $K = 6$. In the case of group II, 21 accessions had q values >0.95, and 26 accessions had q values between 0.75 and 0.95 in $K = 6$. This means that 65 of 111 accessions in group I and 45 of 88 accessions in group II possessed the same membership group from $K = 2$ to $K = 6$.

The population structure of the subset of 110 lines was also determined as ($K = 3$) with the highest probability and ($K = 6$) with the second highest probability, both with a similar probability value, and, the DeltaK value of Evanno et al. (2005) also provides support for those clusters (Figs. S2, S3 in supplementary material).

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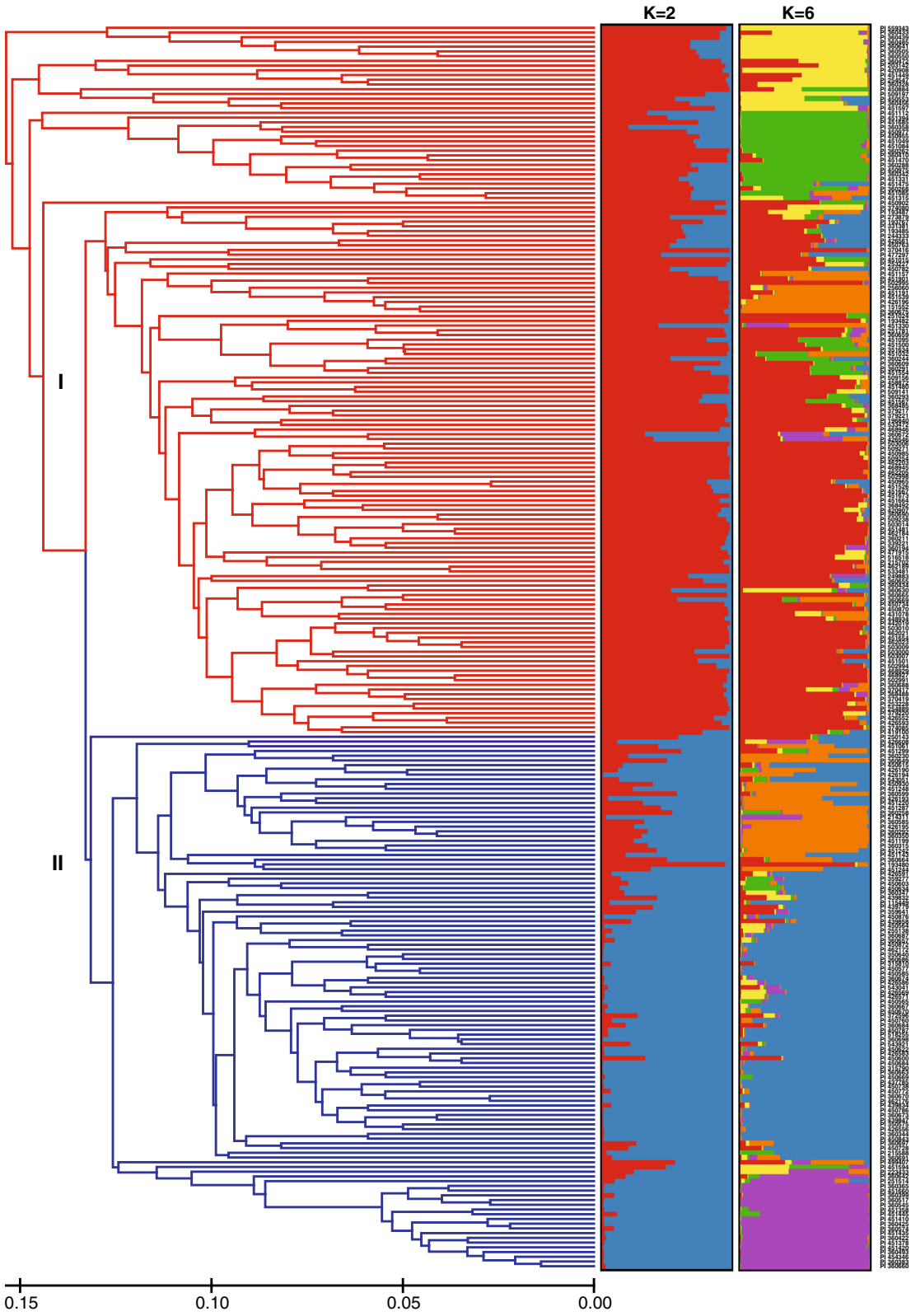

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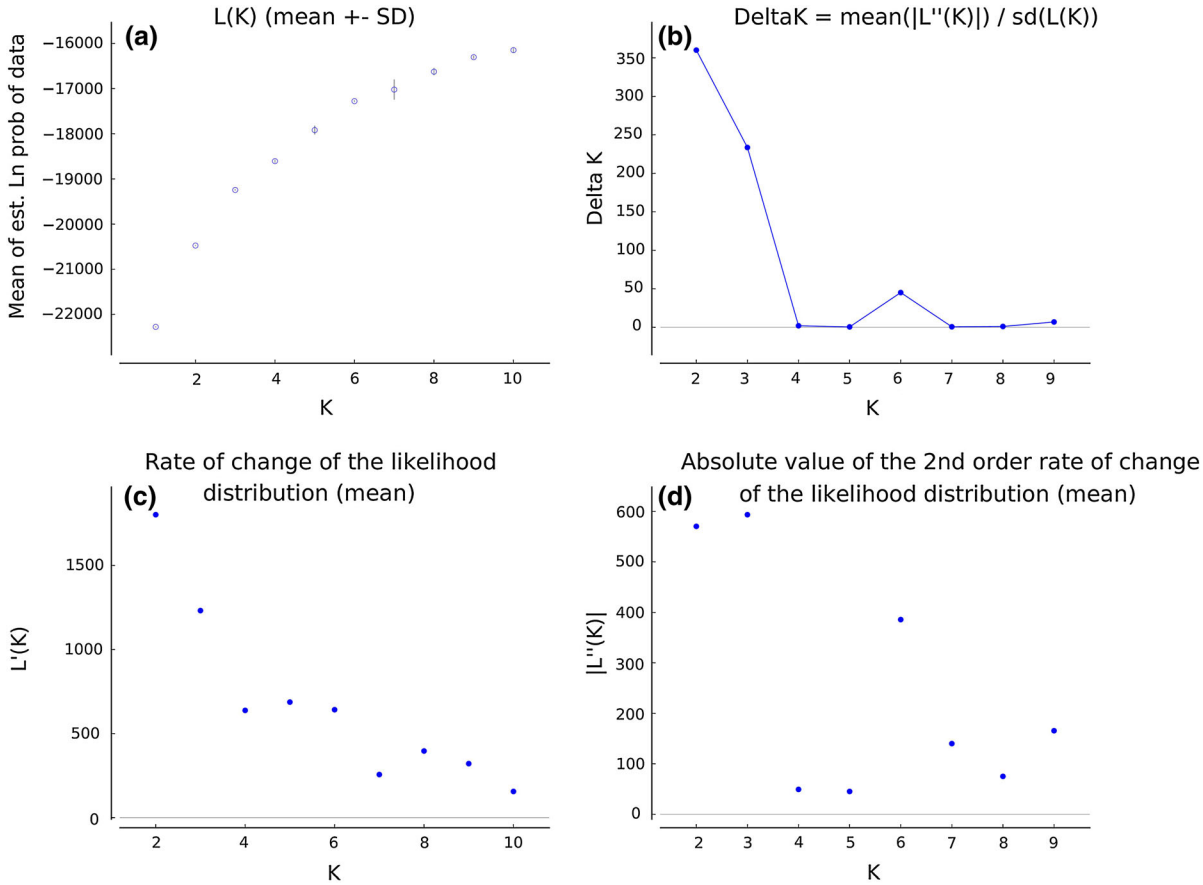

Fig. 2 The results of the population genetic sub-structure analysis generated from eight pairs of TRAP primers and 263 accessions of the USDA single-plant core collection based on the Evanno method (Evanno et al. 2005). **a** The average estimated log probability of the data for $K = 1$ to 10. **b** DeltaK, the rate of change of the log probability of the data with respect to the number of clusters. **c** The absolute values of the rate of change of the likelihood distribution. **d** The absolute value of the rate of change divided by the standard deviation of the original 20 simulations

Marker- trait associations

Marker-trait associations (MTAs) between the 110 markers in USDA CSP and ten seed nutrient concentrations in the chickpea core collection were explored by applying three statistical methods. Twenty-four significant MTAs ($p < 0.05$) were discovered for concentrations for seven seed components, including Ca, Cu, Fe, K. Ni, Zn and protein (Table 3). Five MTAs were significant at $p < 0.01$ when using the overall average of the three methods, with three MTAs highly significant at $p < 0.001$ for concentrations of Cu, K, and Ni.

Discussion

TRAP offers low cost genotyping for moderate throughput germplasm fingerprinting. In the experiment, eight primer combinations amplified a total of 262 fragments. With the arbitrary primers labeled with two different fluorescent dyes, two primer combinations were run in one PCR reaction. Each PCR amplified more than 60 fragments, comparable with that obtained with AFLP, which requires much lengthy and more complicated laboratory procedures. The polymorphic fragments discriminated all 263 genotypes from each other and the data analysis

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Table 3 Significance of tests for association analysis between 110 TRAP markers with seed concentrations for protein and several minerals, using 110 chickpea accessions and three statistical approaches

Trait	Marker	SFA	R^2	Q GLM	R^2	Q + K MLM	R^2	Overall average
Ca(mg/g)	**MIR159A_SA4_GA3_800_3**	***	**0.285**	**	**0.079**	**	**0.065**	**
	B14G14_SA12_GA5_700_6	***	0.109	**	0.054	*	0.035	*
	MIR159A_SA4_GA3_800_4	***	0.211	**	0.052	ns	0.035	*
Cu(μg/g)	**MIR159A_SA12_GA5_700_2**	***	**0.132**	***	**0.101**	***	**0.114**	***
	B14G14_SA12_GA5_700_23	**	**0.096**	**	**0.074**	**	**0.074**	**
	MIR159A_SA4_GA3_700_2	*	0.041	**	0.074	*	0.059	*
	B14G14_SA12_GA5_700_10	**	0.075	*	0.047	*	0.042	*
	MIR159A_SA4_GA3_700_13	**	0.082	ns	0.032	*	0.042	*
	B14G14_SA12_GA5_700_7	**	0.069	*	0.046	*	0.045	*
Fe(μg/g)	MIR159A_SA4_GA3_700_7	**	0.086	**	0.067	*	0.056	**
	MIR156A_SA12_GA5_700_23	**	0.091	**	0.057	ns	0.035	*
	MIR159A_SA4_GA3_700_2	***	0.123	**	0.065	*	0.050	*
	MIR156A_SA12_GA5_700_24	**	0.095	*	0.048	ns	0.033	*
K(mg/g)	**B14G14_SA12_GA5_700_14**	***	**0.139**	***	**0.110**	**	**0.100**	***
	MIR159A_SA4_GA3_700_2	*	0.056	**	0.108	**	0.083	**
	MIR159A_SA12_GA5_700_1	***	0.147	***	0.128	ns	0.030	*
	MIR159A_SA12_GA5_700_4	***	0.147	***	0.128	ns	0.030	*
	MIR156A_SA12_GA5_700_15	***	0.118	**	0.092	ns	0.035	*
	MIR159A_SA4_GA3_700_7	**	0.105	**	0.090	ns	0.029	*
	B14G14_SA12_GA5_700_9	**	0.075	*	0.054	ns	0.026	*
Ni(μg/g)	**B14G14_SA12_GA5_700_7**	***	**0.162**	***	**0.122**	**	**0.101**	***
Zn(μg/g)	MIR156A_SA12_GA5_700_23	*	0.043	**	0.074	*	0.037	*
Protein	MIR159A_SA12_GA5_800_1	**	0.091	**	0.076	*	0.061	**
	MIR159A_SA4_GA3_700_8	**	0.071	**	0.072	*	0.056	*

Traits/markers in bold are significant at $p < 0.01$ for all three tests

SFA single factor analysis of variance, *Q GLM* general linear model using the Q population structure matrix, *Q + K MLM* mixed linear model using the Q population structure matrix and the K kinship matrix, Overall, average p value of three methods

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; *ns* non significance

produced an informative dendogram and differentiated population structure in the collection. These results demonstrate that TRAP markers are useful for chickpea germplasm fingerprinting, and thus improving germplasm management of and promoting germplasm utilization. Although 42.0 % of the fragments amplified were found to be polymorphic, it is possible to improve the efficiency by adding a preliminary screening for primer combinations that amplify more polymorphic fragments from a stratified subset.

Based on our sample, two groups clearly could be distinguished in an unweighted neighbor-joining tree (Fig. 1). In our structural analysis, when comparing groups I and II ($K = 2$) to the $K = 6$ clusters, striking features are present (Fig. 1). For group I, 58 % of those accessions stayed in one cluster. The $K = 6$ cluster accessions were from 18 different countries of the 22 countries in the TRAP marker population, which supported no particular pattern of geographic clustering. For group II, 51 % of the accessions remained in one new cluster with $K = 6$, with indications of some degree of geographic clustering. For example, 53 % of the Indian accessions (24 of 45 total) stayed in one cluster under the $K = 6$ structure result. Accessions of Iranian origin are well represented (99 accessions), however they were members of all six clusters.

Geographic clustering of chickpea accessions based on SSRs was reported by Upadhyaya et al. (2008) after analysis of a large number (3,000) of

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accessions. In our study, the clustering pattern based on genetic distance showed a distinctive grouping of genotypes into separate clusters based on their geographical origin (Fig. 1, Table S1 in supplementary material). It should be pointed out that in the passport data of an accession, "the country of origin" is recorded as the country from which the accession was collected. Due to the human and other factors involved in moving germplasm from one place to another, it is difficult to determine the real origin of some accessions which could be originated from a region different from where they were collected. Therefore, genetic distance based on DNA marker genotypes is more accurate in interpreting the relationship between or among accessions than those geographic points of collection. Further clustering comparisons are hindered as unfortunately Upadhyaya et al. (2008) did not report the population structure analyses using similar Bayesian approaches presented here on the USDA collection. In a chickpea diversity study of 2005 SNPs with *C. arietinum* (56) and *C. reticulatum* (2) unsurprisingly two clusters were found by using UPGMA, one cluster of all the *C. reticulatum* and the second cluster of all the *C. arietinum* from 11 countries (Hiremath et al. 2012). Bayesian structure analysis was not presented, so again a direct comparison with the population structure of this USDA population is not possible. Overall, these studies point to limited genetic diversity within the cultivated chickpea in agreement with all previously published genetic studies of *C. arietinum* (Abbo et al. 2003).

Positive results for marker-trait associations for seed mineral nutrient traits indicate that the USDA CSP population may be useful for further genome-wide association studies using the large SNP sets available for chickpea (Hiremath et al. 2012). While all the minerals studied, as well as protein, are important for human nutrition and health, the identification of markers associated with seed Ca concentration are particularly noteworthy. Chickpea can be an important source of dietary Ca in Middle Eastern countries, where the consumption of dairy products (good sources of Ca) is generally limited (Abbo et al. 2000). Markers for seed Fe and Zn concentrations are also of interest due to the prevalence of Fe and Zn deficiencies in human populations throughout parts of the developing world where chickpea consumption is common (Pfeiffer and McClafferty 2007).

High-throughput genotyping approaches, restriction-site associated DNA (RAD) SNPs (Baird et al. 2008) and two-enzyme genotyping by sequencing (GBS) SNPs (Poland et al. 2012), are now available for chickpea and are approaching very affordable costs (Varshney et al. 2009). Understanding genetic processes in chickpea is greatly enhanced with the recent development of such genomic resources, including the publication of two draft genome sequences of chickpea (Jain et al. 2013; Varshney et al. 2013). Added to this wealth of sequence data are transcriptomes (Hiremath et al. 2011; Garg et al. 2011a, b; Molina et al. 2008; Mantri et al. 2007; Bhushan et al. 2007), BAC libraries (Rajesh et al. 2004; Lichtenzveig et al. 2005), SSRs from BAC end sequences (Thudi et al. 2011), the already mentioned high-throughput SNP assays of Hiremath et al. (2012), and the demonstration of whole-of-genome or candidate gene analysis for functional purposes (Coram and Pang 2005; Gremigni et al. 2005; Jayashree et al. 2005). These resources combined with the world's chickpea genetic resources will greatly enhance efforts in chickpea nutritional and productivity improvement.

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