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ORIGINAL ARTICLE

iPBS-Retrotransposons-based genetic diversity and relationship among wild annual *Cicer* species

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Abstract Lack of requisite genetic variation in cultivated species has necessitated systematic collection, documentation and evaluation of wild *Cicer* species for use in chickpea variety improvement programs. *Cicer arietinum* has very narrow genetic variation, and the use of a wild relative in chickpea breeding could provide a good opportunity for increasing the available genetic variation of cultivated chickpea. Genetic diversity and the relationship of 71 accessions, from the core area of chickpea origin and domestication (Southeastern Turkey), belonging to five wild annual species and one cultivated species (*Cicer arietinum*) were analysed using iPBS-retrotransposon and ISSR markers. A total of 136 scorable bands were detected using 10 ISSR primers among 71 accessions belonging to 6 species, out of which 135 were polymorphic (99.3 %), with an average of 13.5 polymorphic fragments per primer, whereas iPBS detected 130 bands with 100 % polymorphism with an average of 13.0 bands per primer. *C. echinospermum* and *C. pinnatifidum* were the most diverse among species, whereas *C. arietinum* exhibited lower polymorphism. The average polymorphism information contents (PIC) value for both marker systems was 0.91. The clustering of the accessions and species within groups was almost similar, when iPBS and ISSR NeighborNet (NNet) planar graphs were compared. Further detailed studies are indispensable in order to collect *Cicer* germplasm, especially *C. reticulatum*, from southeastern Turkey particularly, from Karacadağ Mountain for preservation, management of this species, and to study their genetic diversity at molecular level. This study also demonstrates the utility and role of iPBS-retrotransposons, a dominant and ubiquitous part of eukaryotic genomes, for diversity studies in wild chickpea and in cultivated chickpea.

Keywords Wild *Cicer* · Genetic diversity · iPBS-Retrotransposons · ISSR · Genetic resources · Turkey

Abbreviations

iPBS	Inter primer binding site
ISSR	Inter simple sequence repeat
PCR	Polymerase chain reaction
CTAB	Cetyl trimethyl ammonium bromide
LTR	Long terminal repeats
PIC	Polymorphism information content

Introduction

Chickpea (*Cicer arietinum* L.) is one of the oldest grain legume and it is currently cultivated in tropical, subtropical, and temperate regions of about 45 countries (Ozer et al. 2010). The chickpea is a self-pollinated crop with chromosome number of 2n=16 and belongs to genus *Cicer*, comprising 33 perennials and 9 annual species. *Cicer arietinum* is the only cultivated species belonging to the genus *Cicer*. The other wild annual *Cicer* species includes; *C. reticulatum*, *C. echinospermum*, *C. pinnatifidum*, *C. judaicum*, *C. bijugum*, *C. cuneatum*, *C. chorassanicum* and *C.*

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yamashitae. The domesticated chickpea has been divided into two distinct chickpeas, namely microsperma or 'desi' and macrosperma or 'kabuli'. Wild *Cicer* species are found in Mediterranean region, Northern Africa, Central and Western Asia. Most of these species are widely distributed in Turkey, Syria, Afghanistan, Ethiopia, Sudan, Egypt and the Indian subcontinent. Wild *Cicer* species have been divided into three different groups based on their crossability with the cultivated chickpea. First group is the most important group among annual *Cicer* species having cultivated species *Cicer arietinum*, along with its close relative *C. reticulatum* and *C. echinospermum*, and these have been used in chickpea breeding programs in the last few years. The second group consists of *C. bijugum*, *C. judaicum* and *C. pinnatifidum* and the third group has only one annual species *C. cuneatum* originated in Ethipio (Singh et al. 2008). Interspecies hybridisation is possible within the group, but not between the groups (Singh et al. 2008).

Understanding the extent and nature of genetic variation in crop species has important implications in crop improvement and the conservation of plant genetic resources (Comertpay et al. 2012). For many crop plants continuous cycles of controlled breeding over thousands of years have led to narrow genetic backgrounds (Tanksley and McCouch 1997). The genetic diversity and relationship among wild *Cicer* species has been previously studied using morphological traits, seed storage protein (Ladizinsky and Adler 1975; Vairinhos and Murray 1983; Ahmad and Slinkard 1992), isozymes (Ahmad et al. 1992; Labdi et al. 1996; Tayyar and Waines 1996) and karyotype (Ocampo et al. 1992; Tayyar et al. 1994). However, these markers are limited, because they are few in number or lack adequate levels in chickpea. In the recent decade, PCR based molecular marker techniques (AFLP, RAPD, SSR, ISSR, etc.) have been used to study the DNA polymorphism for selection of desire parents for improvement of cultivars through breeding and also vastly used in the studying of the genetic relationship among crops of different species.

Until now, very few reports investigated the level of genetic variation between the accessions of wild *Cicer*. In the last decade, different researcher groups have studied the genetic diversity and relatedness among annual *Cicer* species by means of different DNA based molecular markers (Ahmad 1999; Iruela et al. 2002; Rajesh et al. 2002; Sudupak 2004). In earlier reports, RAPD markers were first employed to determine phylogenetic relationships in the genus *Cicer* (Ahmad 1999; Iruela et al. 2002; Sudupak et al. 2002). Sudupak et al. (2002) have studied the genetic relationships with RAPD markers using nearly the same set of *Cicer* accessions. The same group of researchers (Sudupak et al. 2004) again used AFLP markers to assess genetic relationships among 47 *Cicer* accessions, representing four perennial and six annual species, including chickpea with distribution in Turkey. Iruela et al. (2002) analysed genetic relationship and diversity among the 75 accessions belonging to 14 species of the genus *Cicer* and belonging to different parts using ISSR and RAPD markers. Rajesh et al. (2002) studied the genetic diversity among six annual and seven perennial wild species using ISSR markers and suggested that wild annual species of *cicer* were not monophyletic in nature. In another study, Shan et al. (2005) studied the phylogenetic analysis of 146 wild annual *Cicer* accessions and revealed four distinct groups, corresponding well to primary, secondary and tertiary gene pools of chickpea. Based on their collections, the maximum genetic diversity of *C. reticulatum*, *C. echinospermum*, *C. bijugum* and *C.pinnatifidum* was found in southeastern Turkey, while Palestine was the centre of maximum genetic variation for *C. judaicum*. They emphasised that locations with the highest genetic variation would attract more attention for future collection and conservation activities.

Inter simple sequence repeats (ISSR) have been widely employed to determine evolutionary relationships (Zhou et al. 2005; Chen et al. 2006) and levels of genetic variation among wild and cultivated plants (Qiu et al. 2003; Wu et al. 2006). ISSR amplify DNA segments between two microsatellite repeat regions (Zietkiewicz et al. 1994). This technique is simple, fast and efficient for genetic diversity analysis. ISSR analyses have high reproducibility and repeatability and can simultaneously identify multiple polymorphisms at various loci throughout the genome, depending upon the amount of variation between the cultivars being studied (Baloch et al. 2010).

Retrotransposons are mobile genetic elements, which transpose explicatively through RNA intermediates, which contribute to the physical size of the host genome. They are found in all major eukaryote divisions and comprise major fractions of the genomes of plant (SanMiguel et al. 1996; Pearce et al. 1996). Retrotransposons integrate to new positions in the genome via an RNA intermediate, and insert new cDNA copies back into the genome. They can be mobilised via a replicative mechanism by which many daughter copies are generated and inserted into the genome, thereby increasing genome size (SanMiguel et al. 1998). Retrotransposons can be separated into two groups: the LTR (long terminal repeat) and the non-LTR retrotransposons. LTR retrotransposons are present dominantly in the plant genome. Retrotransposons (RTNs) consist of long terminal repeats (LTRs) with a highly conserved terminus that has been exploited for primer design in the development of retrotransposons-based markers, such as inter-retrotransposon amplified polymorphism (IRAP) and retrotransposon microsatellite-amplified polymorphism (REMAP; Kalendar et al. 1999; Kalendar and Schulman 2006). Kalendar et al. (2010) describe a novel PCR-based method, useful both as a marker system in its own right and for the rapid isolation of retrotransposon termini and full-length elements, making it ideal for "orphan crops" and other species with underdeveloped marker systems. The method, *iPBS amplification*, is based on the virtually universal presence of a tRNA complement as a reverse transcriptase primer binding site (PBS) in LTR retrotransposons. Moreover, iPBS has proved to a

powerful DNA fingerprinting technique without previous knowledge of a sequence (Kalendar et al. 2010).

Botanical, genetic, and archaeological evidence points to chickpea originating from wild species *C. reticulatum*, within the Fertile Crescent, in the area that comprises modern-day Southeastern Turkey and adjoining parts of Syria (Toker 2009). Therefore, this area contains diverse chickpea germplasm with having several wild *Cicer* species. Most of the known wild species, particularly, *C. reticulatum* shows only distribution in Turkey. Therefore, knowing the extent, amount and the distribution of available genetic diversity conserved in germplasm from Turkey is crucially important for unlocking that diversity and for their potential utilisation in a chickpea improvement program. Few studies have been conducted to study the chickpea germplasm collected from Turkey. For example, Sudupak et al. (2002, 2004) reported the genetic diversity and relatedness of perennial and annual *Cicer* germplasm from Turkey. The indications from these studies were that the Turkish *Cicer* genetic resources harboured a substantial amount of genetic diversity. However, the germplasm evaluated so far represent only a small subset of available resources. Moreover, iPBS-retrotransposon genetic markers have not been employed in the genetic diversity studies of *Cicer*. Therefore, present investigation was undertaken with the objective to characterise the seven widely distributed species of *Cicer* from Southeastern Turkey using iPBS and ISSR markers.

Materials and methods

Plant material and DNA extraction

A total of 71 accessions consisting of: 18 *C. bijugum,* 12 *C. pinnatifidum,* 8 *C. echinospermum,* 18 *C. reticulatum*, 5 *C. judaicum* and 10 *C. arietinum* were used. Wild *Cicer* species were kindly obtained from ICARDA (International Center of Agricultural Research in Dry Areas) gene bank. The identification number and collection site of the accessions of *Cicer* species has been given in Table 1. Bulk DNA of 8 individuals per accession was prepared from young leaves of 2 weeks old plants grown under greenhouse conditions. DNA was extracted according to the method described by Doyle and Doyle (1990), with minor modifications (Ozkan et al. 2005).

iPBS marker analysis

Initially, 83 iPBS primers were used to screen four chickpea species, in order to determine which primer produces a sharp and clear banding profile. Primer names, their annealing temperature and banding pattern are illustrated in Supplementary Table 1. Ten of these primers, which produced good-excellent PCR products were selected for genotyping the whole set of *Cicer* accessions. The PCR mix (25 µl) for iPBS analysis consisted of: 10 ng template DNA, 3X Dream *Taq* Green Buffer (Fermentas), 5 mM dNTP, 10 µM primer and 1.75 U/µl Dream *Taq* DNA polymerase (Fermentas). The PCR thermal cycling profile was as follows: initial denaturation of 5 min at 95 °C; 30 cycles of 95 °C for 15 s, 50–65 °C for 1 min, 68 °C for 1 min and final extension at 72 °C for 5 min (Kalendar et al. 2010). The PCR products were separated 1.7 % agarose gel and stained with ethidium bromide. Gel images were captured by UV transilluminator (Fig. 1).

ISSR marker analysis

Inter simple sequence repeat (ISSR) amplification was carried out according to Zietkiewicz et al. (1994) with minor modifications (Kafkas et al. 2006). A total 32 ISSR primers were tested for ISSR amplification on four chickpea species: 10 of these which produced sharp and clear banding profile were used for genotyping of all chickpea accessions and cultivars. The PCR volume consisted of 25 µl, containing 10 ng template DNA, 2.5X Dream *Taq* Green Buffer, 2.5 mM dNTP, 5 µM primer and 1 U/µl Dream *Taq* DNA polymerase. The PCR reaction program consisted of: 1 cycle at 94 °C for 2 min; 40 cycles of 95 °C for 1 min, 50–52 °C for 1 min, 72 °C for 2 min and a final extension step of 72 °C for 5 min. The ISSR amplification products were separated 1.7 % agarose gel and stained with ethidium bromide. Gel images were captured under UV light.

Data analysis

Autoradiographs were scored for the absence (0) or presence (1) of iPBS and ISSR marker bands, manually and independently, by at least two persons. Only those fragments that could be clearly scored were used. Genetic diversity among chickpea species and within chickpea species were calculated by using computer program Popgene ver. 1.32 (Yeh et al. 2000). Genetic similarity and genetic distance among chickpea species was estimated according to Nei (1972) for pairwise comparison based on the proportion of shared bands produced by each primer. For ISSR and iPBS primers, the mean polymorphism information content (PIC) was calculated following the formula described by De Riek et al. (2001). Average Shannon information index (I) and gene diversity (h) were measured by using Popgene statistical software ver. 1.32 (Yeh et al. 2000). NeighborNet (NNet) planar graphs of iPBS and ISSR Nei distances (1987) between individuals were constructed using SplitsTree v4.11 (Huson and Bryant 2006) software.

Table 1 Passport data of the Turkish chickpea species and cultivars

Cicer species/Accession	Province	Geographical location
C. bijugum		
ILWC 69946	Mardin	35 km to Midyat; 14 km from Savur
ILWC 70002	Mardin	35 km to Midyat; 14 km from Savur
ILWC 70007	Mardin	35 km to Midyat; 14 km from Savur
ILWC 70008	Mardin	35 km to Midyat; 14 km from Savur
ILWC 70010	Mardin	35 km to Midyat; 14 km from Savur
ILWC 109207	Mardin	5.8 km E of Dereici from Savur to Midyat. Edge of wild wheat field.
ILWC 69947	Diyarbakır	33 km from Diyarbakir to Ergani
ILWC 70018	Diyarbakir	33 km from Diyarbakir to Ergani
ILWC 70022	Diyarbakir	33 km from Diyarbakir to Ergani
ILWC 73006	Diyarbakir	28 km E of Diyarbakir
ILWC 73007	Diyarbakir	28 km E of Diyarbakir
ILWC 73049	Diyarbakir	33 km from Diyarbakir to Ergani
ILWC 73056	Diyarbakir	28 km E of Diyarbakir
ILWC 73057	Diyarbakir	13 km NW Diyarbakir on road to Maden, in cultivated field
ILWC 69971	Gaziantep	Near Gaziantep; field edge
ILWC 73069	Urfa	Payamli; 62 km E of Urfa on Viransehir road
ILWC 73070	Urfa	Mecrihan; 54 km E of Urfa on Viransehir road
ILWC 109209	Siirt	63.1 km E of Siirt rd. to Pervari or 7.6 km E of rd. to Doganca.
C. pinnatifidum		
ILWC 73050	Elazig	Harput; road to FatihAhmet rubble only; not in smooth soil
ILWC 69948	Elazig	Harput; road to FatihAhmet rubble only; not in smooth soil
ILWC 70025	Elazig	Harput; road to FatihAhmet rubble only; not in smooth soil
ILWC 73000	Elazig	Harput; road to FatihAhmet rubble only; not in smooth soil
ILWC 73078	Elazig	Near Harput on road from Elazig across road from Cemetary;
ILWC 73079	Elazig	11 km after Keban dam towards Arapkir
ILWC 69972	Adana	- - - - - - – - - - - - - – - - - - - – - - - - - - -
ILWC 72985	Gaziantep	5 km E of Gaziantep to Marash. Clay soil mixed with stones, calcareous bedrock
ILWC 73065	Urfa	Pirhalli; 2 kmN of Pirhalli; 4 kmS of Borzova
ILWC 109212	Mardin	3.5 km E of Savur on Savur-Midyat road.
ILWC 70021	Diyarbakir	33 km from Diyarbakir to Ergani
ILWC 73080	Diyarbakir	On Malatya-Kayseri road 4 kmW of Doganlar village and 320 km E of Kayseri
C. echinospermum		
ILWC 73075	Urfa	42 kmW of Diyarbakir on Diyarbakir to Siverek road
ILWC 73068	Urfa	22 km ESE Siverek on Karacadag road
ILWC 73009	Urfa	20 km E Siverek
ILWC 73010	Urfa	20 km E Siverek
ILWC 73088	Urfa	26 km E of Siverek on south side of Diyarbakir to Urfa road

Table 1 (continued)

Cicer species/Accession	Province	Geographical location
ILWC 73064	Gaziantep	7 kmS of Zeytinili on Gaziantep to Kilis road
ILWC 73067	Gaziantep	300 mW Toreli on road to Capali
ILWC 109211	Diyarbakir	1 kmS of road, protected, ungrazed, 20 km E Karacadag to Siverek
C. reticulatum		
ILWC 69960	Mardin	12 km from Savur to Midyat
ILWC 69975	Mardin	8 km E of Savur on road to Midyat. Edges of stone pile, calcareous bedrock
ILWC 73011	Mardin	8 km E of Savur on road to Midyat. Edges of stone pile, calcareous bedrock
ILWC 73012	Mardin	8 km E of Savur on road to Midyat. Edges of stone pile, calcareous bedrock
ILWC 73013	Mardin	8 km E of Savur on road to Midyat. Edges of stone pile, calcareous bedrock
ILWC 73045	Mardin	10 km to Savur from Mardin; Pinazdare stone rubble, vineyard
ILWC 109213	Mardin	1 km E of Dereci. N facing, edge of vineyard.
ILWC 73082	Mardin	10 km to Savur from Mardin; Pinazdare stone rubble, vineyard
ILWC 73083	Mardin	35 km to Midyat; 14 km from Savur
ILWC 73060	Mardin	8 km E of Savur on road to Midyat. Edges of stone pile, calcareous bedrock
ILWC 70020	Diyarbakir	33 km from Diyarbakir to Ergani
ILWC 73058	Diyarbakir	13 km NW Diyarbakir on road to Maden, in cultivated field
ILWC 73086	Hakkari	1.5 km SW of Gimenli on Hakkari-Cukurca road. Above Zap River.
ILWC 73087	Malatya	20 km E of Golbasho on road to Adiyaman, limestone slope
ILWC 73066	Urfa	Pirhalli; 2 kmN of Pirhalli; 4 kmS of Borzova
ILWC 73076	Adiyaman	- -
C. judaicum		
ILWC 72997	Elazig	Harput; road to FatihAhmet rubble only; not in smooth soil
ILWC 73002	Elazig	Harput; road to FatihAhmet rubble only; not in smooth soil
ILWC 73003	Elazig	Harput; road to FatihAhmet rubble only; not in smooth soil
ILWC 73005	Elazig	Harput; road to FatihAhmet rubble only; not in smooth soil
ILWC 72981	Gaziantep	5 km E of Gaziantep to Marash. Clay soil mixed with stones
C. arietinum	Registration	Maintainer
DİYAR	20.04.1995	Gap International Agricultural Research and Training Center/Diyarbakır
G. SARISI	01.05.1991	Gap International Agricultural Research and Training Center/Diyarbakır
CEVDET BEY	15.05.1998	Aegean Agricultural Research Institute
63-C	- - - - - - -	- -
GÜLÜMSER	27.04.2001	Blacksea Agricultural Research Institute
ÇAĞATAY	27.04.2001	Blacksea Agricultural Research Institute
AKSU	06.04.2009	Agricultural Research Sitation of the Eastern Mediterranean Crossing Region/Kahramanmaraş
CANITEZ	- - - - - - -	- -
AYDIN	11.05.1992	Aegean Agricultural Research Institute
AZKAN	06.04.2009	Transitional Zone Agricultural Research Institute/Eskisehir

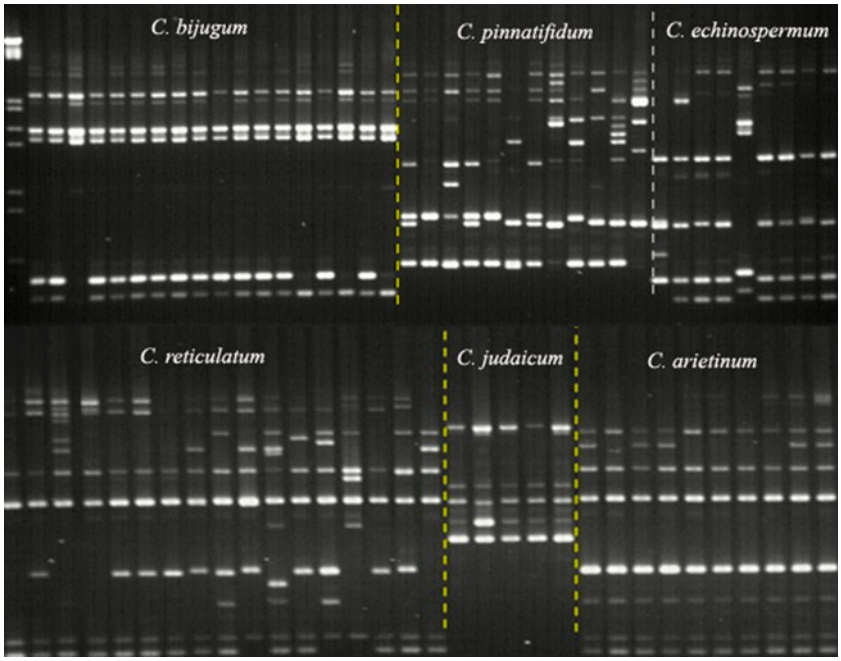

Fig. 1 DNA profiles of six *Cicer* species; *C. bijugum*, *C. pinnatifidum*, *C. echinospermum*, *C. reticulatum, C. judaicum, C. arietinum* based on iPBS-retrotransposons marker (iPBS2074)

Results

iPBS analysis

Eighty-three iPBS primers were tested on four *Cicer* species for initial screening. 55 iPBS primers did not give any PCR product, whereas 8 primers gave poor banding profiles, 13 primers gave good banding profiles, and 5 primers gave excellent PCR fragments (Supplementary Table 1). Ten iPBS primers yielding strong and reproducible polymorphic bands, were selected for further analysis. The ten iPBS primers generated a total 130 scorable bands and all of them were found polymorphic (%100), with an average of 13.0 polymorphic fragment per primer (Table 3). The number of bands per primer ranged from 8 (iPBS2392) to 17 (iPBS2074), with an average of 13 bands per primer.

Gene diversity varied from 0.05 to 0.50, with a mean value of 0.27 among 71 genotypes. Gene diversity per iPBS primer ranged from 0.15 (iPBS2375) to 0.33 (iPBS2374). Shannon's information index per iPBS primer varied from 0.27 (iPBS2375) to 0.50 (iPBS2374). The mean PIC value ranged from 0.86 (iPBS2392) to 0.95 (iPBS2276, iPBS2277 and iPBS2232) (Table 3). *iPBS analysis results* revealed that the highest genetic variation was found in *C. echinospermum,* in which 54 polymorphic loci were detected among 8 accessions with a mean gene diversity of 0.121 (Table 2) The lowest genetic variation was detected within *C. arietinum*. Highest genetic similarities (0.97 and 0.90) of *C.*

Table 2 Genetic variation among *Cicer* species collected from southeastern Turkey; core area of diversity

Species	iPBS						ISSR				
	NA	TB	NPL	PAL%	PWS%	GD	TB	NPL	PAL%	PWS%	GD
C. bijugum	18	35	9	6.92	25.71	0.017	58	14	10.29	24.13	0.029
C. pinnatifidum	12	43	34	26.15	79.07	0.100	49	39	28.68	79.59	0.104
C. echinospermum	8	60	54	41.54	90.00	0.121	44	31	22.79	70.45	0.075
C. reticulatum	18	38	22	16.92	57.89	0.046	38	19	13.97	50.00	0.045
C. judaicum	5	37	14	10.77	37.83	0.035	42	2	1.47	4.76	0.005
C. arietinum	10	28	3	2.31	10.71	0.011	28	1	0.74	3.57	0.002
for all accessions	71	130*	130**	100	–	0.266	136*	135**	99.26	–	0.293

NA number of accession, *TB* total bands within each species, *NPL* number of polymorphic bands with each species, PAL% polymorphic locus percentage with other species, PWS% polymorphism percentage within species, *GD* gene diversity, * total scorable bands for all species, ** polymorphic bands for all species

arietinum were observed with *C. reticulatum* and *C. echinospermum*, respectively. Lowest genetic similarity (0.55 and 0.59) was found between *C. judaicum* and *C. bijugum*, and *C. arietinum* and *C. judaicum,* respectively (Table 4). The NeighborNet (NNet) planar graph clearly split the 71 accessions of *Cicer* species into four different clusters, each cluster having accessions from the same species (Fig. 2).

A comparison of genetic similarity values obtained from ISSR and iPBS, performed by the Mantel test, detected a statistically significant correlation coefficient (r=0.89).

ISSR analysis

A total of 136 scorable bands were detected using 10 ISSR primers, among 71 accessions consisting of 61 Turkish wild *Cicer* accessions and 10 Turkish commercial chickpea cultivars (Table 3). Out of 136 bands, 135 were polymorphic (%99.3). The number of bands per primer ranged from 12 (UBC884 and UBC885) to 18 (UBC890), with an average of 13.5 bands per primer. Gene diversity (h) ranged from 0.00 to 0.50, with a mean value of 0.29 among 71 *Cicer* genotypes. Gene diversity per ISSR primer ranged from 0.23 (UBC826) to 0.35 (UBC888). Shannon's information index per ISSR primer ranged from 0.38 (UBC826) to 0.52 (UBC888). The highest PIC value was 0.95 for UBC885 and UBC826 primers, whereas the lowest PIC value was 0.87 for UBC891 (Table 3).

Genetic diversity was calculated within each of the *Cicer* species (Table 2). ISSR analysis results showed that the highest genetic variation was found in *C. pinnatifidum,* in which 39 polymorphic loci were detected among 12 accessions, with a mean gene diversity of 0.104. The lowest genetic variation was detected within *C. arietinum*. Highest genetic similarity of *C. arietinum* (0.958 and 0.894) was observed with *C. reticulatum* and *C. echinospermum* respectively. Lowest genetic similarity was found between *C. bijugum and C. judaicum* (Table 4). In the NeighborNet (NNet) planar graph, the 71 accessions of *Cicer* species and cultivars into four different groups according to their species level (Fig. 3).

iPBS/ISSR/combine analysis

To obtain a more precise picture, two data sets (iPBS and ISSR) were combined and genetic similarities were calculated among 71 accessions consisting of 61 Turkish wild *Cicer* accessions and 10 Turkish commercial chickpea

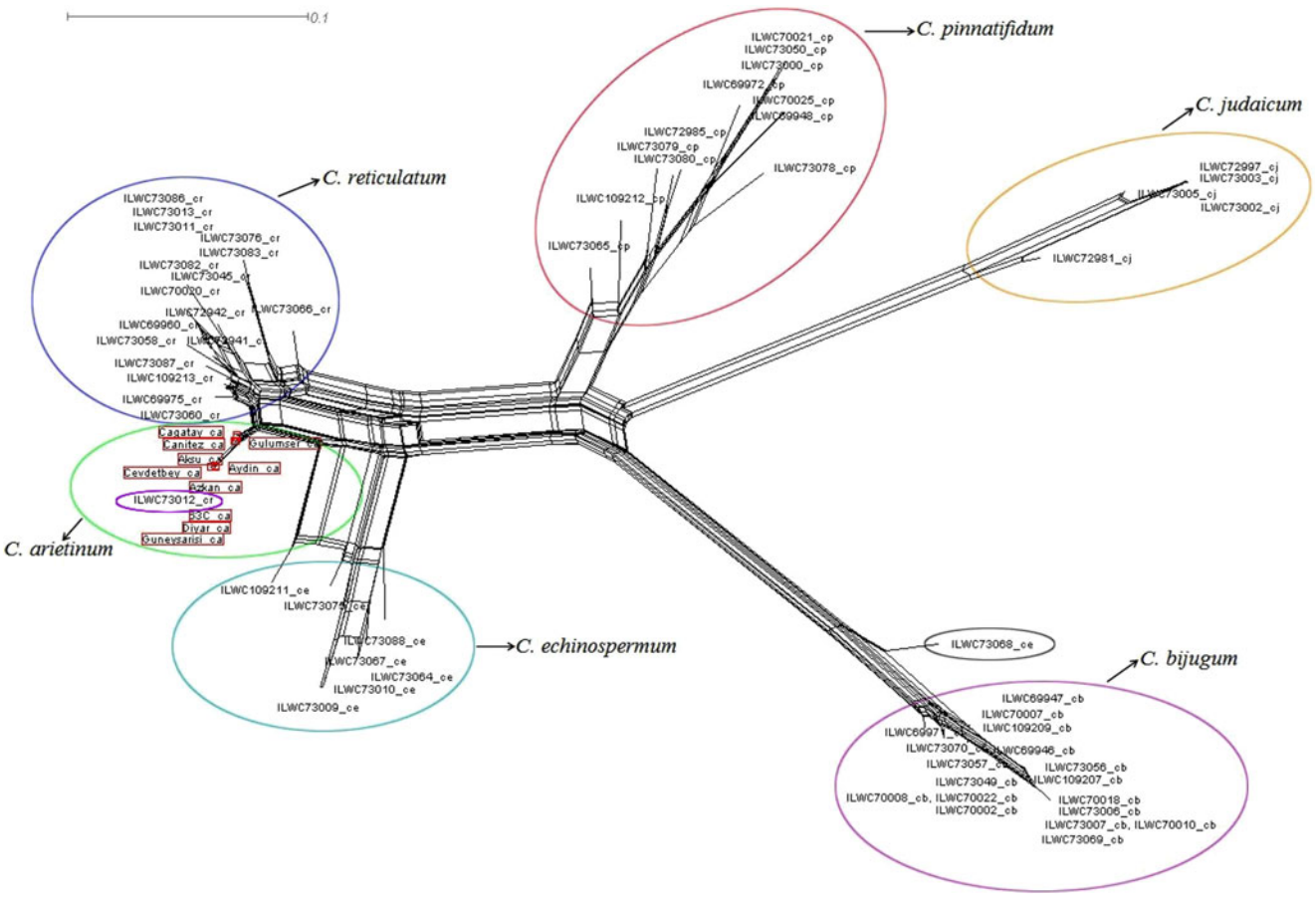

Fig. 2 Neighbor Net (NNet) planargraph of six *Cicer* species consisting of 71 accessions and cultivars based on iPBS-retrotransposons markers

Table 3 Information about the iPBS and ISSR primers used in this study, including their sequence, diversity index, PIC value, number of total and polymorphic bands

Markers	Sequence	Number of bands		*Diversity* (Mean)		
iPBS/ISSR		Total	Polymorphic	*h*	I	PIC
iPBS2074	GCTCTGATACCA	17	17	0.28	0.44	0.93
iPBS2228	CATTGGCTCTTGATACCA	14	14	0.29	0.45	0.89
iPBS2232	AGAGAGGCTCGGATACCA	15	15	0.27	0.42	0.95
iPBS2249	AACCGACCTCTGATACCA	15	15	0.31	0.47	0.89
iPBS2276	ACCTCTGATACCA	10	10	0.26	0.42	0.95
iPBS2277	GGCGATGATACCA	16	16	0.27	0.43	0.95
iPBS2373	GAACTTGCTCCGATGCCA	14	14	0.25	0.40	0.89
iPBS2374	CCCAGCAAACCA	11	11	0.33	0.50	0.89
iPBS2375	TCGCATCAACCA	10	10	0.15	0.27	0.87
iPBS2392	TAGATGGTGCCA	8	8	0.18	0.31	0.86
Total	–	130	130	–	–	–
Average	–	13.0	13.0	0.27	0.41	0.91
UBC808	AGAGAGAGAGAGAGAGC	12	11	0.30	0.45	0.89
UBC810	GAGAGAGAGAGAGAGAT	14	14	0.28	0.44	0.88
UBC823	TCTCTCTCTCTCTCTCC	14	14	0.30	0.46	0.91
UBC826	ACACACACACACACACC	13	13	0.23	0.38	0.95
UBC884	HBHAGAGAGAGAGAGAG	12	12	0.29	0.46	0.93
UBC885	BHBGAGAGAGAGAGAGA	12	12	0.30	0.46	0.95
UBC886	VDVCTCTCTCTCTCTCT	14	14	0.31	0.47	0.94
UBC888	BDBCACACACACACACA	13	13	0.35	0.52	0.90
UBC890	VHVGTGTGTGTGTGTGT	18	18	0.32	0.48	0.91
UBC891	HVH TGTGTGTGTGTGTG	14	14	0.25	0.40	0.87
Total	–	136	135	–	–	–
Average	–	13.6	13.5	0.29	0.45	0.91

*(h)*gene diversity (Nei 1972), *(I)* Shannon information index, *PIC* polymorphism information content

cultivars. The NeighborNet (NNet) planar graph combining the information from iPBS and ISSR (Fig. 4) had the main characteristics of both independent tree (Figs. 2 and 3). The analysis was clearly split among 71 accessions, consisting of 61 Turkish wild *Cicer* accessions and 10 Turkish commercial chickpea cultivars, into five groups. All accessions

Table 4 Genetic similarity and genetic distance coefficient Nei (1972) among six *Cicer* species

Species	iPBSMarkers					
	Cb	Cp	Ce	Cr	Cj	Ca
C. bijugum	****	0.6762	0.6982	0.6175	0.5547	0.6293
C. pinnatifidum	0.3913	****	0.7898	0.7722	0.6760	0.7532
C. echinospermum	0.3592	0.2360	****	0.8855	0.6131	0.8904
C. reticulatum	0.4821	0.2585	0.1216	****	0.6114	0.9656
C. judaicum	0.5893	0.3915	0.4892	0.4920	****	0.5915
C. arietinum	0.4632	0.2834	0.1161	0.0350	0.5250	****
Species	ISSR Markers					
	Cb	Cp	Ce	Cr	Cj	Ca
C. bijugum	****	0.7044	0.5763	0.5408	0.4247	0.5524
C. pinnatifidum	0.3504	****	0.7436	0.7047	0.6013	0.7020
C. echinospermum	0.5512	0.2962	****	0.9344	0.6152	0.8941
C. reticulatum	0.6146	0.3500	0.0678	****	0.5995	0.9581
C. judaicum	0.8564	0.5086	0.4858	0.5116	****	0.5933
C. arietinum	0.5935	0.3538	0.1119	0.0428	0.5221	****

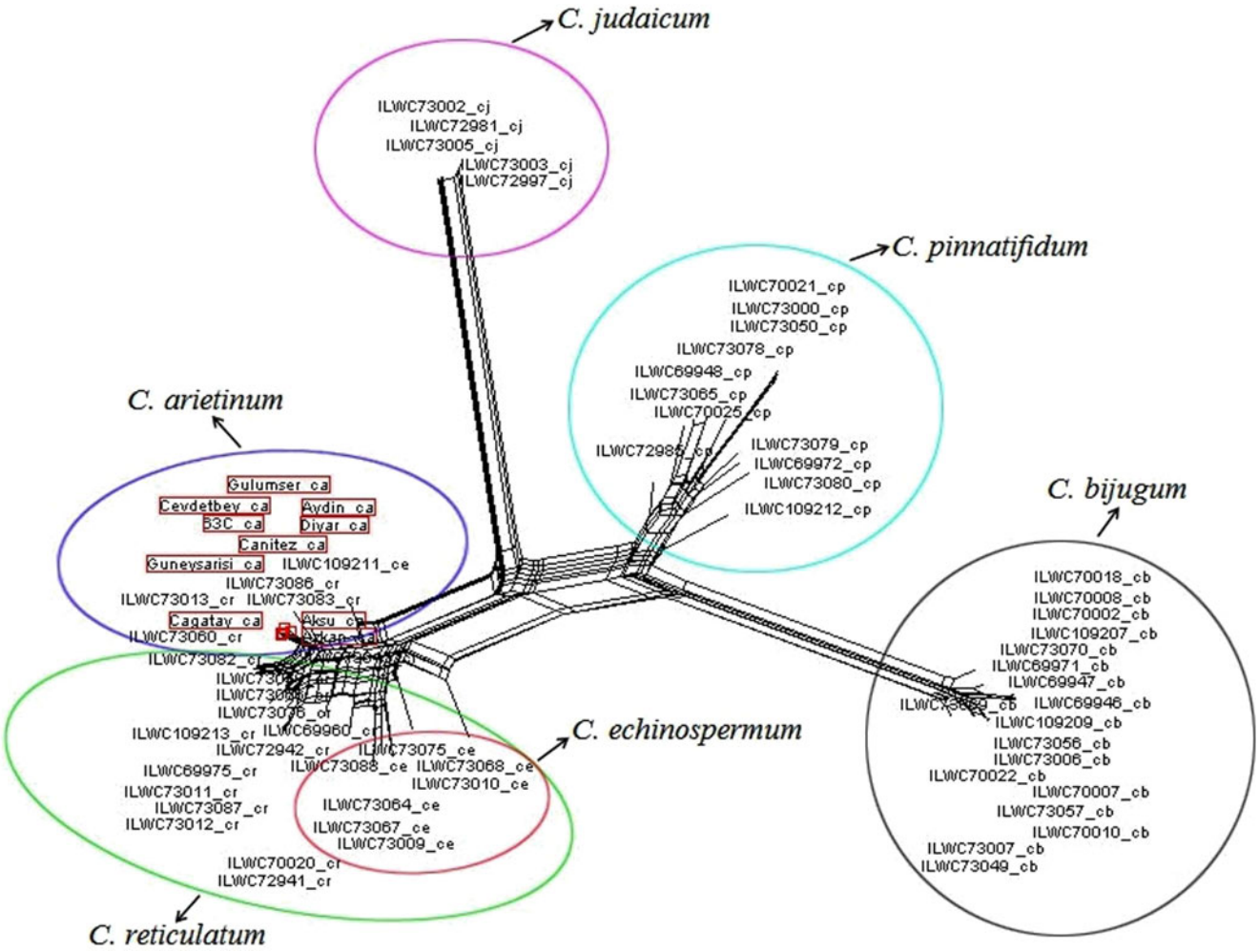

Fig. 3 Neighbor Net (NNet) planar graph of six *Cicer* species consisting of 71 accessions and cultivars based on ISSR markers

belonging to the same species were clustered together, except for one accessions ILWC73068 (*C. echinospermum*), that was placed under different group.

Discussion

In apparent contradiction with the lack of genetic diversity in chickpea indicated by molecular markers studies, chickpea genome shows environmentally induced but, heritable genomic changes. The activation of transposons elements could be proposed as the phenomena behind this genomic plasticity. Transposable elements, particularly the retrotransposons, comprise much to most of plant genomes; their replication generates genomic diversity and makes them an excellent source of molecular markers (Schulman et al. 2004). Retrotransposons markers, due to their general applicability, simplicity of their implementation and genotype resolution systems, have been widely applied in numerous evolutionary and genetic studies, such as genetic diversity, in different crop plants, such as rice (Branco et al. 2007), grapevine species and cultivars identification (Onofrio et al. 2010), and *Citrus* genus and its relative (Biswas et al. 2010), for the determination of phylogenetic relationships in *Crocus* (Alavi-Kia et al. 2008) and *Aegilops tauschii* (Saeidi et al. 2008); for gene mapping in barley (Manninen et al. 2000); for uncovering the polyploidisation-induced genetic restructuring in triticale (Bento et al. 2008). The limiting factors in the development retrotransposons markers for a new species rely on product size variation, and require cloning and sequence information for designing of primers matching the flanking genomic DNA at each specific site. Recently, Kalendar et al. (2010) described a novel PCR based method that overcomes these difficulties and can both isolate LTR retrotransposons in virtually any organism, as well as serve as a universal marker system in its own right. The iPBS (inter primer binding site) method is based on the amplification between the reverse transcriptase primer binding site in LTR retrotransposons. Unlike methods for retrotransposons isolation that rely conserved protein coding domains, iPBS primers also directly visualised polymorphism for retrotransposons loci in the genome. (Kalendar

et al. 2010). The iPBS is only retrotransposons-based marker system that has shown visualised polymorphism throughout the plant kingdom and for animals as well.

In both monocot and dicot plants, retrotransposons comprise highly heterogeneous populations, whose members frequently span different genera. Several retrotransposons have been shown to be highly polymorphic for insert location within plant species (Queen et al. 2004). For example, The BARE-1 element is a good example of this; BARE-1 was originally isolated from barley, and has since been shown to have close homologues in wheat, oat and rye (Pearce et al. 1997; Gribbon et al. 1999). However, most of transposons marker systems working in one species may not be useful in other species. However, iPBS markers developed by Kalendar et al. (2010) are referred to as "*Universal retrotrasposons markers*", and could be utilised in all plant and animal species.

Using recently developed and introduced protocol of iPBS retrotransposons markers (Kalendar et al. 2010), we have studied the genetic diversity of the *Cicer* species, as well as few cultivars of cultivated chickpea. Kalendar et al. (2010) described these iPBS retrotransposons markers as universal markers and, additionally, iPBS retrotransposons had not been used for genetic diversity studies in any crop species including chickpea. We used different statistical aspect to compare the performance of iPBS-retrotrasposons markers with ISSR markers, including a measure of genetic polymorphism, the efficiency of polymorphism detection, a measure of marker efficiency in detecting polymorphisms and the capacity of different techniques to deduce genetic relationships between accessions. In the present study, iPBS and ISSR banding pattern of the *Cicer* genotypes exhibited high level of polymorphism. We observed that a number of polymorphic loci and polymorphism percentage obtained through iPBS markers were higher, compared with ISSR markers in *C. echinospermum*, *C. reticulatum*, *C. judaicum* and *C. arietinum*. However, it was lower in *C. pinnatifidum*, and *C. bijugum*. The iPBS markers generated 54 polymorphic bands out of a total of 60 bands in *C. echinospermum*, whereas ISSR markers detected a total of 44 fragments, out of which 31

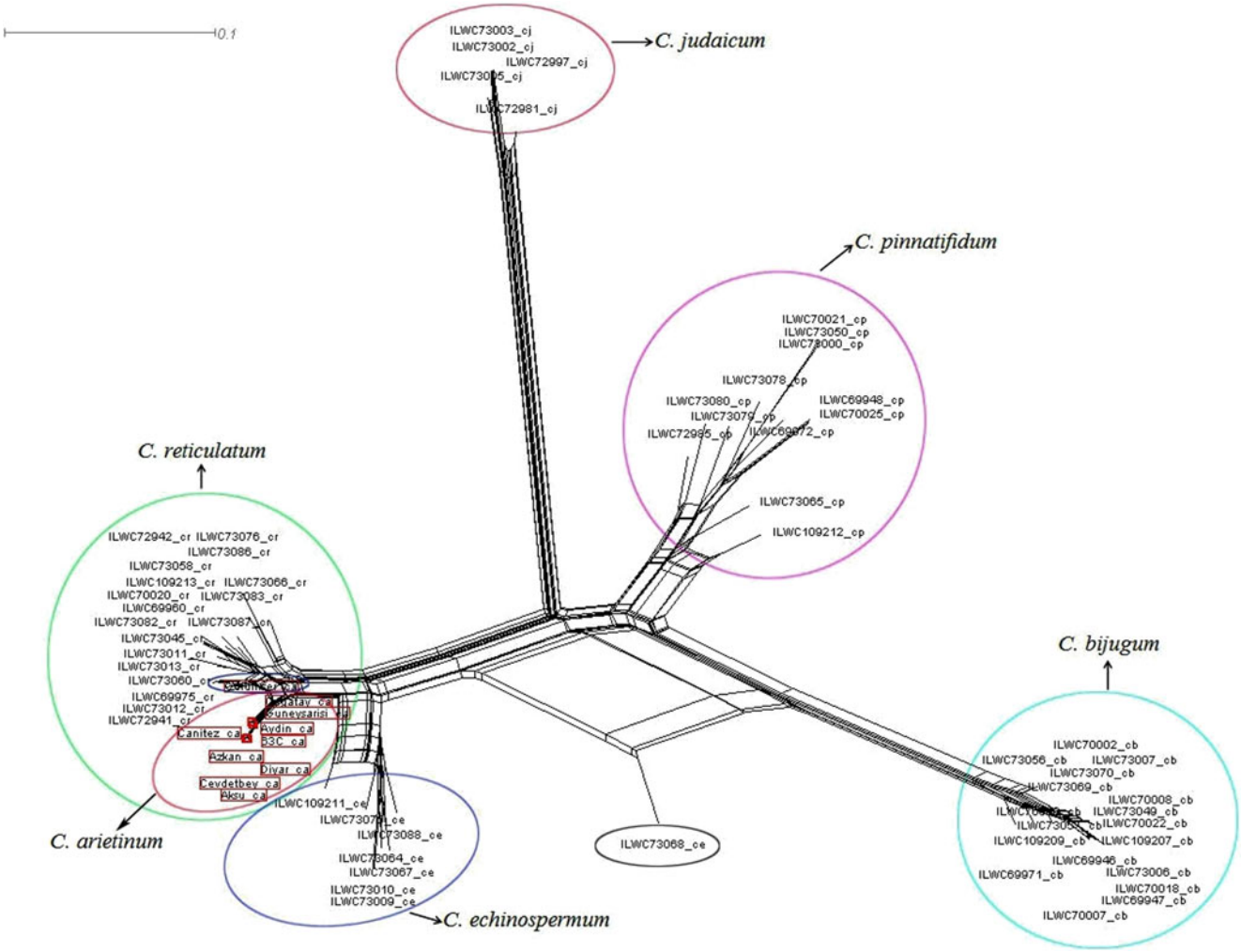

Fig. 4 Neighbor Net (NNet) planar graph of six *Cicer* species consisting of 71 accessions and cultivars based on combined analysis of iPBS + ISSR markers

fragments were polymorphic. In the case of *C. reticulatum*, both markers were discovered to have a total of 38 PCR fragments, but number of polymorphic bands through iPBS marker (22) were higher than ISSR marker. In total 130 bands were generated through 10 iPBS primers with 100 % polymorphism. In fact, iPBS have equal or even higher capacity to reveal polymorphism and offer great potential to determine intra and interspecific diversity. In case of *C. arietinum*, iPBS markers generated total 28 bands, out of which 3 bands were polymorphic, whereas in the case of ISSR, only one out of 28 bands was polymorphic. The results are comparable with other marker systems used for characterisation of *Cicer* species, such as ISSR (Iruela et al. 2002; Rajesh et al. 2002; Rao et al. 2007), RAPD (Sudupak et al. 2002; Talebi et al. 2009), and AFLP (Sudupak et al. 2004; Nguyen et al. 2004; Shan et al. 2005). Our results also revealed that *C. arietinum* has least genetic diversity, which is consistent with the earlier previous studies describing the narrow genetic base of cultivated chickpea (Abbo et al. 2003; Nguyen et al. 2004; Shan et al. 2005). This reinforces the necessity of broadening the genetic basis of cultivated chickpea through utilization of wild resources inbreeding program for introducing favourable alleles into commercial varieties. Among its wild relatives, *C. echinospermum* enjoyed highest genetic diversity, compared to other species, according to iPBS marker. This is in contrast to the findings of Shan et al. (2005), who demonstrated that *C. echinospermum,* have lower genetic variability than *C. judaicum*. *C. pinnatifidum* was the second most diverse species, according to iPBS, and most diverse according to ISSR analysis. *C. reticulatum* was also the third most diverse species. *C. echinospermum* and *C. reticulatum* are most important species for chickpea improvement as they are crossable with the cultivated chickpea. These results are most important in revealing the significant higher diversity of *C. echinospermum* and *C. reticulatum*, compared with previous studies.

Various molecular markers show a different efficiency for evaluating DNA polymorphism in genus *Cicer*. For instance, Sudupak et al. (2004) obtained 306 scorable bands using three AFLP marker combinations for 47 *Cicer* accessions representing ten *Cicer* species. They reported that *C. pinnatifidum* was the most polymorphic, followed by *C. reticulatum* and *C. arietinum* had the least genetic variation among the *Cicer* species. Nguyen et al. (2004) also found that the most variable species was *C. pinnatifidum*, for which 102 polymorphic loci were detected among 17 accessions with an overall gene diversity (h) of 0.126 using five AFLP primer combinations. Rajesh et al. (2002), using fifteen ISSR primers in 13 *Cicer* species, observed 6.6 polymorphic bands per primer, while Sudupak (2004), using six ISSR primers in 6 *Cicer* species, observed 25 polymorphic bands per primer. Rao et al. (2007) detected the 176 and 66 bands using 29 RAPD and 6 ISSR primers among 19 chickpea cultivars and five accessions of its wild progenitor *Cicer reticulatum* L.. AFLP, RAPD and ISSR markers have been extensively used as molecular markers systems for detecting polymorphism and genetic relationship among *Cicer* species in the last years. According to the literature, RAPD is a polymorphic marker in *Cicer*, but has many disadvantages, such as a problem with reproducibility, while AFLP markers, instead of producing much polymorphism, high repeatability and resolution, have many drawbacks, such as high cost, difficulty of performance and a time-consuming technique. Here, we reported the use of iPBS markers for detecting polymorphism among 71 accessions belonging to 6 species, including cultivated one, obtained 130 bands with 100 % polymorphism, with an average of 13.0 bands per primer. Therefore, we compared iPBS markers with ISSR markers as ISSR markers are dominant, reliable, simple and easy to perform. Using 10 ISSR markers, we observed 135 out of 136 bands were polymorphic (99.3 %), with an average of 13.5 polymorphic fragments per primer.

Based on the computed pairwise genetic similarity coefficients, a NeighborNet planner graph for all accessions was constructed separately for iPBS, ISSR (Figs. 2 and 3) and combined for both (Fig. 4). The dendrogram obtained from the iPBS similarity matrix (Fig. 2) is, apparently, the most representative of effective relationship between all *Cicer* species. The iPBS-based grouping among six *Cicer* species was fully consistent with grouping obtained using ISSR markers. All accessions were also grouped into four main groups. *C. echinospermum*, *C. reticulatum* and *C. arietinum* were clustered under the same group; In ISSR dendrogram, *C. echinospermum* accessions were not clearly distinct, instead, they were mixed with *C. reticulatum* and *C. arietinum*, however, iPBS markers were more sophisticated and efficient and showed that *C. reticulatum* is the closest species with cultivated chickpea, and *C. echinospermum* was the second closest species to *C. arietinum*. Here, again observing the NeighborNet graph, it is clear that one accession of *C. echinospermum* (ILWC-73012) was placed under the same group with cultivated chickpea varieties. One accession of *C. echinospermum* (ILWC-73068) was clustered closely to the accessions of *C. bijugum.* Besides, one accession of *C. judaicum* (ILWC-72981; Fig. 2) was separated from other *C. judaicum* species. This accession was only collected from the nearby region of Gaziantep, whereas, all other accessions, belonging to *C. bijugum*, were collected from Elazığ province.

In the case of ISSR, the graph distributed all 71 accessions into four different groups. Group A consisted of three species, *C. echinospermum*, *C. reticulatum* and *C. arietinum*, which is in agreement to the hypothesis that *C. echinospermum* and *C. reticulatum* are the closest species and progenitor of *C. arietinum*. In ISSR NeighborNet planner graph, all accessions were positioned into species clusters

with one exception: one accession of *C. echinospermum* (ILWC-109211) clustered closely with one accession of *C. reticulatum* (ILWC- 73086) and two accessions of cultivated species (Güneysarısı and Canıtez). This accession (ILWC-109211) was collected from Diyarbakir, whereas, other accessions were collected from Gaziantep and Urfa. Three accession of *C. reticulatum* (ILWC-73086, ILWC-73013, and ILWC-73083) are the closest species to *C. arietinum*, explaining the possible gene flow between these species. Cultivar "Gülümser" was the distant cultivar from the rest of the chickpea cultivars (Fig. 3).

The clustering of the accessions and species within groups was almost similar, when iPBS and ISSR dendrograms were compared. The NeighborNet planner graph obtained from the iPBS and ISSR (Figs. 2 and 3) separately, as well as combined similarity matrix for both markers (Fig. 4) is, apparently the most representative of the effective relationship between all *Cicer* species and *C. arietinum* cultivars. In agreement with the previous results of iPBS and ISSR separately as well as combined analysis also clustered all accessions belonging to the same species under the same group. Combined analysis divided all accessions in five groups. Group A consisted of *C. echinospermum*, *C. reticulatum* and *C. arietinum*. Among commercial varieties, cultivar "Aksu" was most different from the rest of the varieties. One accession of *C. echinospermum* (ILWC-73068) was clustered alone in a separate group between *C. echinospermum* and *C. bijugum*, as observed in iPBS graph (Fig. 2). This accession harbour a unique DNA profile and, therefore, is likely to have the greatest number of novel alleles, which were absent in the other accessions and, ultimately, resulted in the high polymorphism within *C. echinospermum*. This accession was collected from the Karacadağ road near Karacadağ Mountain. This region is located at the junction of the East Mediterranean and Anatolian regions, and is thought to be the place where "einkorn wheat" and various legume species such as lentil, chickpea, field pea, and faba bean were first domesticated (Ozkan et al. 2010). Extensive collection should be made in Southeastern Turkey, particularly Karacadağ region for *C. echinospermum* and *C. reticulatum*. It could be hypothesised that accession "ILWC-73068" might be a hybrid between *C. echinospermum* and *C. bijugum*. Such a natural hybrid could be useful for "bridging" crosses to introduce gene from incompatible species in cultivated chickpea. Further collection and study in Southeastern Turkey and surrounding areas is required for the conservation of *Cicer* genetic resources with maximum genetic diversity present.

iPBS-retrotransposon and ISSR marker systems differ in the nature of the evolutionary mechanism underlying their variation and their distribution in plant genome. The results obtained from both iPBS and ISSR marker system are strongly supported by the fact that they showed high correlation (r=0.89). Moreover, the number of polymorphic bands, PIC, polymorphism percentage is almost similar in both of these molecular marker systems, which was in accordance with similar studies on amaranth (Xu and Sun 2001) and radish (Muminovic et al. 2005).

Genetic improvement of chickpea remains slow, because of narrow genetic base or limited genetic variation among cultivated chickpea. Lack of requisite genetic variation in the cultivated species has necessitated systematic collection, documentation and evaluation of wild *Cicer* species for use in chickpea variety improvement programs. The wild relative of chickpea provides a good opportunity for increasing the genetic variation of cultivated chickpea, since the variability for resistance to biotic and abiotic stresses in the domesticated chickpeas is insufficient. Many improved cultivars of crops are vulnerable to insect pests as they lack the defense mechanisms of their wild progenitors. Singh et al. (2008) reported that the valuable genetic resources present in the primary gene pool were successfully utilised in plant-breeding programs for genetic enhancement in chickpea. However, the review of Singh et al. (2008) pointed out that hybridisation of the secondary and tertiary gene pool with the cultivated species is often limited by reproductive barriers. Thus, an appropriate cross-bridge might be needed to overcome the reproductive barriers to use the potential merits of these gene pools.

One of the major limitations in the characterisation of the chickpea germplasm is the lack of suitable marker system. The iPBS markers have been proven useful for assessing genetic differences among cultivars, populations and species with high reproducibility. Many features of retrotransposons make them appealing as the basis of molecular marker systems. They are ubiquitous, abundant and dispersed components of eukaryotic genomes. Their activity, simultaneously, leads to genome diversification and provides a means of its detection. Our results revealed that both iPBS markers and ISSR markers sufficiently determined genetic variation in *Cicer*, although iPBS markers were better in differentiating all accessions in comparison with ISSR markers in general. This study demonstrates the utility of retrotransposons, a dominant and ubiquitous part of eukaryotic genomes, for diversity studies. The present study suggested that iPBS retrotransposon based fingerprinting methods are useful tool for rapid characterisation of cicer germplasm. This approach could be efficiently employed for conservation and management of cicer genetic resources.

Lev-Yadun et al. (2000) proposed a "*core area*" for the origins of agriculture within the Fertile Crescent. This was based on the proposition that wild einkorn and wild emmer from this area are genetically more closely related to the domesticated crop plants than elsewhere; and that chickpea,

one of the founder crops and its wild relative *C. reticulatum* is restricted only to this region of southeast Turkey, which includes the Karacadağ mountain range (Ozkan et al. 2010). In the last few decades, due to heavy industrialisation, urbanisation and tourism activities, there is a serious threat of the disappearance of such natural wild resources. To stem this loss of genetic variation, conservation and reconnaissance of existing biodiversity are fundamental. Therefore, more detailed study is indispensable in order to collect *Cicer* germplasm, especially *C. reticulatum*, from southeastern Turkey, particularly, from Karacadağ mountain. This would promote preservation, management of this species, and study of their genetic diversity at molecular level, as well as screening germplasm for useful agronomic and quality traits, resistance against different biotic and abiotic stress. Furthermore, it would promote their utilisation in future breeding in order to enhance genetic diversity of modern chickpea varieties through hybridisation. A large amount of genetic diversity which existed in wild accessions, particularly in *C. reticulatum*, could be used efficiently by crossing wild and cultivar types in developing mapping population for gene/QTL tagging for insect, pest and disease resistance and introgression in cultivated varieties.

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