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ORIGINAL PAPER

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Geographical patterns of genetic variation in the world collections of wild annual *Cicer* characterized by amplified fragment length polymorphisms

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Abstract *Cicer reticulatum*, *C. echinospermum*, *C. bijugum*, *C. judaicum*, *C. pinnatifidum*, *C. cuneatum* and *C. yamashitae* are wild annual *Cicer* species and potential donors of valuable traits to improve chickpea (*C. arietinum*). As part of a large project to characterize and evaluate wild annual *Cicer* collections held in the world gene banks, AFLP markers were used to study genetic variation in these species. The main aim of this study was to characterize geographical patterns of genetic variation in wild annual *Cicer* germplasm. Phylogenetic analysis of 146 wild annual *Cicer* accessions (including two accessions in the perennial *C. anatolicum* and six cultivars of chickpea) revealed four distinct groups corresponding well to primary, secondary and tertiary gene pools of chickpea. Some possible misidentified or mislabelled accessions were identified, and ILWC 242 is proposed as a hybrid between *C. reticulatum* and *C. echinospermum*. The extent of genetic diversity varied considerably and was unbalanced between species with greatest genetic diversity found in *C. judaicum*. For the first time geographic patterns of genetic variation in *C. reticulatum*, *C. echinospermum*, *C. bijugum*, *C. judaicum* and *C. pinnatifidum* were established using AFLP markers. Based on the current 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*. This information provides a solid basis for the design of future collections and in situ conservation programs for wild annual *Cicer*.

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Introduction

Understanding the extent and geographic patterns of genetic diversity within wild annual *Cicer* species is essential for effective future collection, the development of conservation strategies and efficient use of genetic resources for improvement of chickpea (*Cicer arietinum*) cultivars. Wild annual *Cicer* includes eight species: *C. bijugum*, *C. chorassanicum*, *C. cuneatum*, *C. echinospermum*, *C. judaicum*, *C. pinnatifidum*, *C. reticulatum* and *C. yamashitae*. They are found in the Mediterranean Basin, from western to central Asia, with some isolated accessions along the Red Sea coast of northeastern Africa (Berger et al. 2003). This area varies in habitat, topographic and climatic conditions according to the *Cicer* collection passport data. Long-term evolution and adaptation to these conditions makes these wild annual species rich in resistance genes for a range of biotic and abiotic stresses experienced in chickpea production (Muehlbauer et al. 1994; Singh et al. 1998). Accessions (124) of these species have been collected and are held in nine gene banks worldwide (Berger et al. 2003). Thereafter they have been exploited for application in various chickpea breeding programs (Singh and Ocampo 1997; Stamigna et al. 2000). The diversity available in different traits of the wild annual *Cicer* collections is very valuable, since the effectiveness of improvement in any crop depends upon the extent and nature of phenotypic and genotypic variation present in different traits of the broader population (Heslop-Harrison 2002). However, the present world collection probably represents only a small fraction of the diversity of wild annual *Cicer* (Abbo et al. 2003; Berger et al. 2003). Its further collection is imperative. Currently the geographical

Table 1 Accessions of wild *Cicer* and chickpea cultivars used in AFLP analysis

Accession code[a]	Species[b]	Origin[c]	Latitude (°N)	Longitude (°E)	Accession code	Species	Origin	Latitude (°N)	Longitude (°E)
PI 599087	*Cana*	TUR	38.50	42.26	ILWC 41	*Cj*	SYR	34.98	35.95
PI 561078	*Cana*	TUR	38.60	42.25	ILWC 47	*Cj*	SYR	34.99	35.91
ILWC 277	*Cb*	SYR	32.71	36.78	ILWC 282	*Cj*	SYR	34.99	36.01
PI 599066	*Cb*	IRA	36.35	44.17	ILWC 279	*Cj*	SYR	34.99	36.04
ILWC 42(1)	*Cb*	SYR	36.46	37.00	ILWC 208	*Cj*	SYR	35.04	35.95
ILWC 42(2)	*Cb*	SYR	36.46	37.00	ILWC 211	*Cj*	SYR	35.18	35.92
ILWC 209	*Cb*	SYR	36.48	36.99	ILWC 48	*Cj*	SYR	35.25	35.97
ILWC 243(1)	*Cb*	TUR	37.05	38.40	ILWC 148	*Cj*	TUR	37.08	37.42
ILWC 243(2)	*Cb*	TUR	37.05	38.40	ILWC 33(1)	*Cj*	TUR	38.70	39.25
ILWC 32(1)	*Cb*	TUR	37.05	37.33	ILWC 33(2)	*Cj*	TUR	38.70	39.25
ILWC 32(2)	*Cb*	TUR	37.05	37.33	W6 12535	*Cj*	NA	–	–
ILWC 240(1)	*Cb*	TUR	37.23	39.27	ILWC 38	*Cj*	LBN	–	–
ILWC 240(2)	*Cb*	TUR	37.23	39.27	ICCW89	*Cj*	NA	–	–
ILWC 241	*Cb*	TUR	37.33	39.37	ILWC 96	*Cp*	PAL	31.78	35.22
PI 599051	*Cb*	TUR	37.55	42.43	ILWC 159	*Cp*	PAL	32.59	35.08
ILWC 7(1)	*Cb*	TUR	37.55	41.00	ILWC 261	*Cp*	SYR	33.42	35.97
ILWC 7(2)	*Cb*	TUR	37.55	41.00	ILWC 213	*Cp*	SYR	33.62	36.07
ILWC 284	*Cb*	TUR	37.55	41.00	ILWC 49	*Cp*	SYR	33.74	36.12
ILWC 285	*Cb*	TUR	37.55	41.02	ILWC 262	*Cp*	SYR	33.74	36.41
ILWC 286	*Cb*	TUR	37.55	41.05	ILWC 212	*Cp*	SYR	33.75	36.13
ILWC 217	*Cb*	TUR	37.55	41.00	ILWC 263	*Cp*	SYR	33.81	36.52
ILWC228(1)	*Cb*	TUR	37.92	40.13	ILWC 60	*Cp*	LBN	34.17	35.65
ILWC 228(2)	*Cb*	TUR	37.92	40.13	ILWC 224(1)	*Cp*	TUR	37.08	37.42
PI 599049	*Cb*	TUR	37.93	42.33	ILWC 224(2)	*Cp*	TUR	37.08	37.42
ILWC34(1)	*Cb*	TUR	38.00	40.33	ILWC 29	*Cp*	TUR	37.08	37.42
ILWC 34(2)	*Cb*	TUR	38.00	40.33	ILWC 236	*Cp*	TUR	37.30	38.77
ILWC 227	*Cb*	TUR	38.00	40.33	ICCW88	*Cp*	TUR	37.52	34.77
ILWC 8	*Cb*	TUR	38.12	40.00	ILWC 78	*Cp*	TUR	37.55	41.00
ILWC 220	*Cb*	TUR	38.12	40.00	PI 599045	*Cp*	TUR	37.55	40.93
ILWC 260(1)	*Cb*	TUR	–	–	ILWC 289	*Cp*	TUR	37.55	40.93
ILWC 260(2)	*Cb*	TUR	–	–	ILWC 226	*Cp*	TUR	37.77	36.82
W6 10150	*Cb*	TUR	–	–	ILWC 82	*Cp*	TUR	38.12	40.00
ILWC 37	*Ccun*	ETH	14.17	38.75	ILWC 248	*Cp*	TUR	38.12	39.98
ILWC 40	*Ccun*	ETH	14.17	38.75	ILWC 251	*Cp*	TUR	38.42	40.37
ILWC 238(1)	*Ce*	TUR	36.85	37.30	ILWC 9	*Cp*	TUR	38.70	39.25
ILWC 238(2)	*Ce*	TUR	36.85	37.30	ILWC 249	*Cp*	TUR	38.70	39.25
ILWC 235	*Ce*	TUR	36.90	37.37	ILWC 250(1)	*Cp*	TUR	38.75	38.80
ILWC 246(1)	*Ce*	TUR	37.67	39.17	ILWC 250(2)	*Cp*	TUR	38.75	38.80
ILWC 246(2)	*Ce*	TUR	37.67	39.17	ILWC 51	*Cp*	NA	–	–
ILWC 35(1)	*Ce*	TUR	37.72	39.50	ILWC 242(1)	*Cr*	TUR	37.28	38.77
ILWC 35(2)	*Ce*	TUR	37.72	39.50	ILWC 242(2)	*Cr*	TUR	37.28	38.77
ILWC 245(1)	*Ce*	TUR	37.72	39.50	ILWC 237(1)	*Cr*	TUR	37.30	38.77
ILWC 245(2)	*Ce*	TUR	37.72	39.50	ILWC 237(2)	*Cr*	TUR	37.30	38.77
ILWC 239(1)	*Ce*	TUR	37.72	39.63	ILWC 216(1)	*Cr*	TUR	37.37	40.80
ILWC 239(2)	*Ce*	TUR	37.72	39.63	ILWC 216(2)	*Cr*	TUR	37.37	40.80
ILWC 39	*Ce*	TUR	37.72	39.50	ILWC 257	*Cr*	TUR	37.48	43.63
ILWC 259	*Ce*	TUR	37.72	39.50	ILWC 290	*Cr*	TUR	37.55	40.97
PI 599040	*Ce*	TUR	37.72	38.58	ILWC 21(1)	*Cr*	TUR	37.55	40.98
ILWC 288	*Ce*	TUR	37.85	39.82	ILWC 21(2)	*Cr*	TUR	37.55	40.98
ILWC 20(1)	*Cj*	PAL	31.78	35.22	ILWC 218	*Cr*	TUR	37.55	41.00
ILWC 20(2)	*Cj*	PAL	31.78	35.22	ILWC 219	*Cr*	TUR	37.55	40.98
PI 458559	*Cj*	PAL	31.83	35.05	ILWC 36(1)	*Cr*	TUR	37.57	40.97
ILWC 256	*Cj*	JOR	32.52	35.78	ILWC 36(2)	*Cr*	TUR	37.57	40.97
ILWC 255	*Cj*	JOR	32.52	35.82	PI 599050	*Cr*	TUR	37.60	42.33
ILWC 30	*Cj*	PAL	32.59	35.08	PI 599042	*Cr*	TUR	37.60	40.50
ILWC 31	*Cj*	JOR	32.62	35.78	ILWC 258	*Cr*	TUR	37.75	38.28
ILWC 273	*Cj*	LBN	33.68	35.57	ILWC 247(1)	*Cr*	TUR	37.77	38.28
ILWC 283	*Cj*	SYR	33.72	36.11	ILWC 247(2)	*Cr*	TUR	37.77	38.28
ILWC 272	*Cj*	LBN	33.80	35.53	PI 599073	*Cr*	TUR	37.88	40.23
ILWC 4	*Cj*	LBN	34.17	35.65	ILWC 229(1)	*Cr*	TUR	37.92	40.13
ILWC 274	*Cj*	LBN	34.25	35.67	ILWC 229(2)	*Cr*	TUR	37.92	40.13
ILWC 276	*Cj*	LBN	34.27	35.92	ILWC 81(1)	*Cr*	TUR	38.12	40.00
ILWC 275	*Cj*	LBN	34.33	35.92	ILWC 81(2)	*Cr*	TUR	38.12	40.00
ILWC 43	*Cj*	SYR	34.81	36.01	PI 599092	*Cr*	TUR	38.33	39.73
ILWC 280	*Cj*	SYR	34.84	35.96	ILWC 214	*Cy*	AFG	34.58	69.33
ILWC 278	*Cj*	SYR	34.90	35.91	ILWC 215	*Cy*	AFG	34.60	69.38

Table 1 (Contd.)

Accession code[a]	Species[b]	Origin[c]	Latitude (°N)	Longitude (°E)	Accession code	Species	Origin	Latitude (°N)	Longitude (°E)
ILWC 46(1)	*Cj*	SYR	34.90	36.03	ILWC 3	*Cy*	AFG	34.67	69.72
ILWC 46(2)	*Cj*	SYR	34.90	36.03	Sona	*Cari*	AUS		
ILWC 44	*Cj*	SYR	34.92	35.93	Amethyst	*Cari*	AUS		
ILWC 45	*Cj*	SYR	34.93	36.00	Heera	*Cari*	AUS		
ILWC 210	*Cj*	SYR	34.97	35.92	Kaniva	*Cari*	AUS		
ILWC 207	*Cj*	SYR	34.97	35.92	Tyson	*Cari*	AUS		
ILWC 281	*Cj*	SYR	34.97	35.92	Bumper	*Cari*	AUS		

[a]ILWC accessions are from the International Centre for Agricultural Research in Dry Areas; PI and W6 from the US Department of Agriculture; and ICCW from the International Crops Research Institute for the Semi-Arid Tropics (1) from ICARDA gene bank, (2) from Australian gene bank

[b]*Cana C. anatolicum*; *Cb C. bijugum*; *Ccun C. cuneatum*; *Ce C. echinospermum*; *Cj C. judaicum*; *Cp C. pinnatifidum*; *Cr C. reticulatum*; *Cy C. yamashitae*; *Cari C. arietinum*

[c]*AFG* Afghanistan, *AUS* Australia, *ETH* Ethiopia, *IRA* Iraq, *LBN* Lebanon, *PAL* Palestine, *SYR* Syria, *TUR* Turkey, *NA* not available

patterns of genetic variation in the world collections of wild annual *Cicer* are unknown, and the locations of maximum genetic diversity for each wild annual *Cicer* species need to be assessed. This knowledge is necessary to develop strategies for future collections and in situ conservation (Sawkins et al. 2001).

The original collections have been duplicated frequently and housed in different gene banks. As a result 572 wild annual *Cicer* entries exist mainly in nine world gene banks, although only 124 original accessions (among them eight accessions have lost viability) have been recorded among the eight wild annual *Cicer* species (Abbo et al. 2003; Berger et al. 2003). Materials are often duplicated and sent to chickpea researchers. During exchange, transfer and multiplication of seeds, misidentification and mislabelling may occur, which can affect chickpea improvement programs. However, no attempt has been made to examine the similarity of the same accessions held in different gene banks.

Reliable knowledge of phylogenetic relationships within the genus *Cicer* is a crucial aspect of practical breeding (Croser et al. 2003; Zeid et al. 2003). A number of marker systems have been used to study relationships within *Cicer* and are reviewed by Croser et al. (2003) and reported in recent papers (Nguyen et al. 2004; Sudupak 2004; Sudupak et al. 2004). They include DNA molecular markers, plant morphology, crossability data, karyotypes, enzymes and seed storage protein analyses. In addition to different marker systems, they also used different accessions or selections in their studies. This makes comparison of results published to date difficult. Most studies conclude that *C. reticulatum* and *C. echinospermum* are closely related to *C. arietinum* and form a phylogenetic group. The second group includes *C. bijugum* and *C. judaicum*. There is a disagreement as to whether *C. pinnatifidum* should be placed in the first group with *C. reticulatum*, *C. echinospermum* and *C. arietinum* (Iruela et al. 2002), or in the second group with *C. bijugum* and *C. judaicum* (Nguyen et al. 2004; Sudupak 2004; Sudupak et al. 2004). Other wild annual species, *C. chorassanicum*, *C. yamashitae* and *C. cuneatum*, and perennial species are more distantly related to *C. arietinum*, although again different grouping of these species occurs. For example, Iruela et al. (2002) reported *C. cuneatum* has a closer relationship with *C. arietinum* than *C. yamashitae* and perennial *C. anatolicum*, while Nguyen et al. (2004) indicated that *C. cuneatum* is more distantly related than *C. yamashitae* and *C. anatolicum*. It is essential that all of the available wild annual *Cicer* accessions in the world collections should be used to estimate genetic variation and to clarify systematic classification in wild annual *Cicer*.

DNA molecular markers have more advantages than phenotypic markers, since they are free of environmental influences when determining genetic variability (Virk et al. 1995; Serret et al. 1997). Amongst DNA-based markers, AFLP is one of the most reliable tools, which produces much polymorphism and enjoys high repeatability and resolution (Capo-chichi et al. 2001). AFLP analysis has been used for the study of geographical genetic variation and discovery of misidentifications in two species of *Stylosanthes* Sw. (Sawkins et al. 2001) and examination of phylogenetic relationships in many other legume crops, such as in velvetbean (*Mucuna* sp., Capo-chichi et al. 2001) and faba bean (Zeid et al. 2003).

In this study we expected to characterise the geographical patterns of genetic variation in world collections of wild annual *Cicer*, which would provide recommendations for future collection, characterisation, utilisation and conservation.

Materials and methods

Plant materials

A total of 146 accessions were used in this study (Table 1). They were obtained from the International Centre for Agricultural Research in Dry Areas (ICARDA, Aleppo, Syria), Tamworth Centre for Crop Improvement (New South Wales Agriculture, Australia), International Crops Research Institute for the Semi-Arid Tropics (ICRISAT, Patancheru, India) and the US Department of Agriculture (USDA). They included 104 original wild annual *Cicer* accessions in *C. reticulatum*,

C. echinospermum, *C. bijugum*, *C. judaicum*, *C. pinnatifidum*, *C. cuneatum* and *C. yamashitae* and eight accessions isolated from a mixture. Among these, 26 accessions had two samples from both ICARDA and Australian collections. Six Australian chickpea cultivars (Sona, Amethyst, Heera and Tyson as representatives of desi type; Kaniva and Bumper as representatives of kabuli type) were included in the study. Two accessions (PI 599087 and PI 561078) in the perennial species, *C. anatolicum*, were also included as an outgroup.

All seeds were sterilized in 2.5% sodium hypochlorite solution for 1 min and then rinsed with tap water. Disinfected seeds were placed on moist filter paper (Whatman No. 3) in petri dishes at room temperature for germination. In general seeds germinated after 2–3 days; otherwise, hard seed coats were nicked to allow germination. Germinated seeds were transferred into pots and placed in a temperature controlled glasshouse (22/18 °C) with natural daylight at the University of Western Australia, Perth.

AFLP analysis

DNA was extracted from young leaves bulked from three to five individual plants from an accession, using a Nucleon Phytopure Extraction Kit (Amersham Biosciences), following the protocol provided. AFLP markers were generated using the protocol described by Shan et al. (2004). Sequences of adaptors were:

*Eco*RI adaptor: 5′	CTC	GTA	GAC	TGC	GTA	CC			
			CTG	ACG	CAT	GGT	TAA		5′
*Mse*I adaptor: 5′	GAC	GAT	GAG	TCC	TGA	G			
		A	CTC	AGG	ACT	CAT			5′

Preselective amplification primers were *Eco*RI + 3′ A and *Mse*I + 3′ C. Six primer combinations for selective amplification of target DNA sequences were used. They were *Eco*RI-AAC/*Mse*I + CAA, *Eco*RI -AAG/*Mse*I + CAA, *Eco*RI−ACC/*Mse*I + CAA, *Eco*RI−ACG/*Mse*I + CAC, *Eco*RI−ACT/*Mse*I + CAC and *Eco*RI−AGC/*Mse*I + CAC. Gels were analysed with the GeneScan software (version 3.0, PerkinElmer). AFLP fragments, mainly from 100 to 400 base pairs, were scored as binary code using the software package Genographer (Benham 2001), with the presence of a fragment as 1 and the absence of a fragment as 0. Only unambiguous fragments were scored and used for analysis.

Statistical analysis of genetic variation

The similarity coefficients and distances were calculated as follows:

- Nei–Li genetic similarity coefficient (S_{ij}) (Nei and Li 1979): $S_{ij} = 2a/(b + c)$.
- Nei–Li genetic distance (D_{ij}): $D_{ij} = 1 - S_{ij}$.
- Jaccard's coefficient (S_{ij}) (Shi 1993): $S_{ij} = a/(a + b + c)$ where S_{ij} is the similarity between samples i and j; a is the number of fragments common to both samples (positive matches); b the number of fragments restricted to i; c the number of fragments restricted to j.
- Mean character distance was calculated using the software package PAUP (Swofford 2002).
- Shan's difference index (DI) was developed to show genetic variation between accessions within a species. DI of an accession was an average value of D_{ij} between this accession and all other accessions within a species. The value of DI was from 0 to 1. The larger the DI, the more different this accession was from all the other accessions.
- Gene diversity (H): H is an average value of gene diversity of a locus (h) over all loci as a measure of genetic variation within a selfing population. Gene diversity of a locus (h) was calculated as follows (Nei 1987):

$$h = 1 - \sum_{i=1}^{m} x_i^2 \quad \text{when } n \geq 50,$$

$$h = \frac{n}{n-1}\left(1 - \sum_{i=1}^{m} x_i^2\right) \quad \text{when } n < 50$$

where x_i is the population frequency of the ith allele at a locus, m is the number of alleles and n is the number of accessions analysed.

Phylogenetic analysis

Nei–Li genetic similarity, Jaccard's coefficient and mean character distance were used to construct phylogenetic trees by the unweighted pair group method using arithmetic averages (UPGMA) and nearest neighbour (NN) approach. PAUP (Swofford 2002) was used to calculate bootstrap values for 1,000 replicates to evaluate branch support for the phylogenetic trees.

Determination of geographic patterns of genetic diversity in wild annual *Cicer* species

Values for Shan's DI were plotted for each accession on a three-dimensional graph against the geographic coordinates for its site of collection. If an accession had two samples (coming from different gene banks), an average value of DI was plotted on the graph. A location for a cluster of accessions was then arbitrarily defined according to the natural cluster of collections based on their geographical proximity. The definition of these locations was not rigid but acknowledged after consulting with curator and germplasm collectors. The gene diversity for a species and a natural cluster were calculated. Accessions without geographic

coordinates and possible misidentifications were not included in this analysis. The location with maximum genetic variation for each species was identified. This analysis was not carried out in *C. cuneatum* and *C. yamashitae* due to limited accessions available in these species.

Examination of similarity in some wild annual *Cicer* accessions

Twenty-six accessions from both ICARDA and Australian gene banks were randomly selected without any background knowledge for the examination of the similarity of the wild annual *Cicer* collections housed in different gene banks. When similarity between the two samples of the same accession from different sources was outside the species similarity range, the one that clustered in a different species was taken as a possible misidentification or mislabelling. Crosschecks were also made based on plant morphology, pod and seed features. These accessions were not included in further analyses.

Results

Phylogenetic relationships based on AFLP markers

Analysis of the 146 *Cicer* accessions with six selective amplification primer pairs identified a total of 455 AFLP markers, of which 447 (98%) were polymorphic in the whole collection. Phylogenetic trees constructed by UPGMA, NN based on Nei–Li, Jaccard's similarity indexes or mean character distance produced the same tree topologies. A dendrogram constructed by UPGMA based on Nei–Li similarity index is presented (Fig. 1). High-bootstrap indices indicated the robustness of clusters. Based on this study annual *Cicer* germplasm

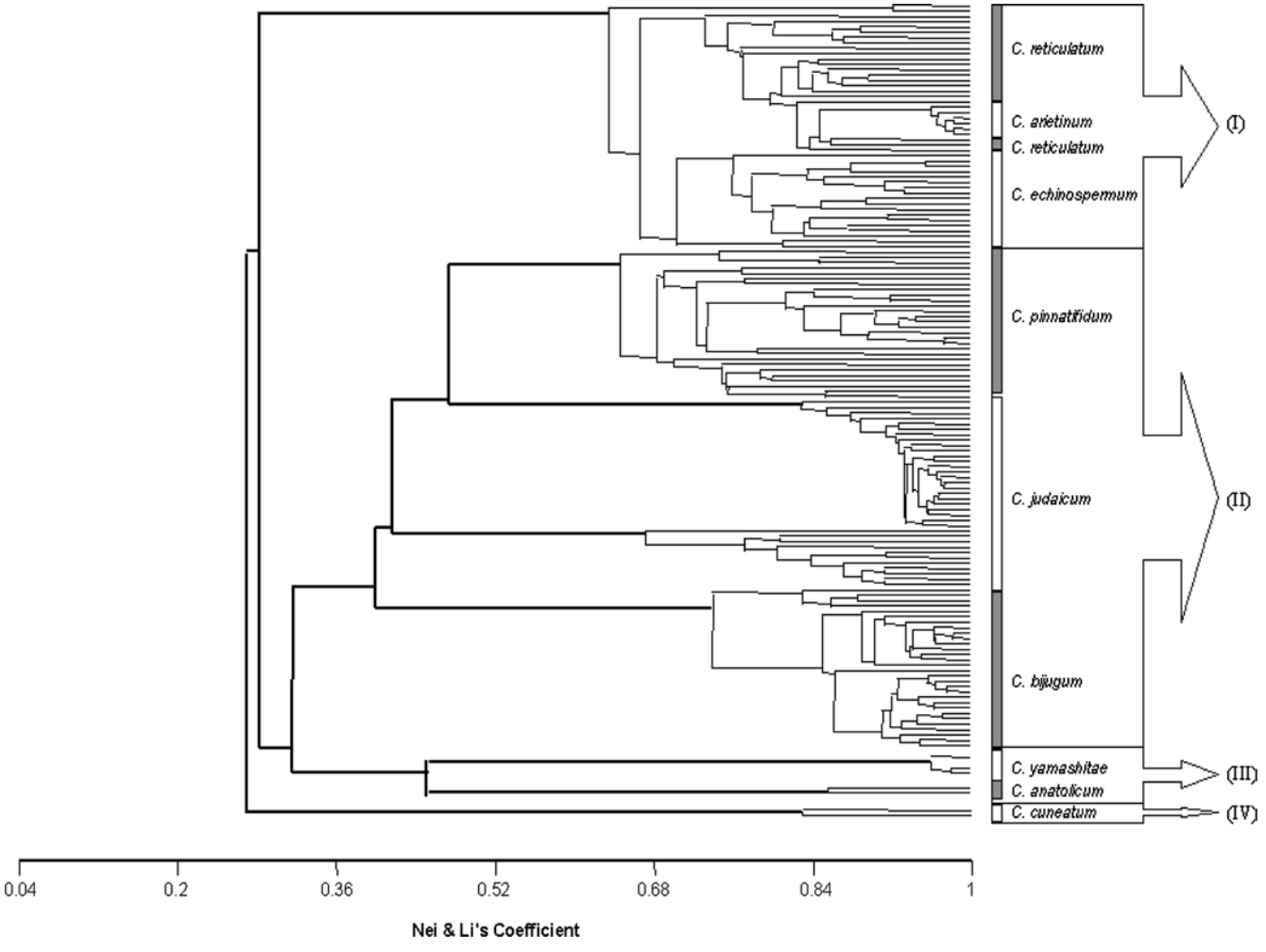

Fig. 1 A dendrogram constructed for 140 accessions of the wild annual *Cicer*, two accessions of a perennial species *C. anatolicum* and six chickpea cultivars, based on Nei–Li similarity index from AFLP data using unweighted pair group method using arithmetic averages (*UPGMA*). Four groups were identified. Group I included *C. reticulatum*, *C. echinospermum* and *C. arietinum*. Group II had *C. bijugum*, *C. judaicum* and *C. pinnatifidum*. Group III was *C. yamashitae* and *C. anatolicum*. Group IV was *C. cuneatum*

Table 2 Genetic variation in the world collection of annual wild *Cicer* species and *C. arietinum*

Species	Number of original accessions	Gene diversity, *H*	Nei–Li genetic similarity coefficient, S_{ij}	
			Range	Mean ± SD
C. bijugum	20	0.070	0.65–0.98	0.85 ± 0.08
C. chorassanicum	2	–	–	–
C. cuneatum	1	0.062	–	–
C. echinospermum	10	0.092	0.56–0.96	0.79 ± 0.08
C. judaicum	34	0.116	0.38–0.97	0.71 ± 0.23
C. pinnatifidum	28	0.092	0.52–0.97	0.72 ± 0.08
C. reticulatum	18	0.087	0.55–0.92	0.76 ± 0.07
C. yamashitae	3	0.015	0.95–0.98	0.97 ± 0.02
C. arietinum	–	0.012	0.95–0.98	0.97 ± 0.01

SD Standard deviation

could be divided into four groups. The first group included *C. echinospermum*, *C. reticulatum* and *C. arietinum*, with bootstrap of 100%. The second group consisted of *C. bijugum*, *C. judaicum* and *C. pinnatifidum*, with bootstrap of 88%. The third group was *C. yamashitae* and *C. anatolicum* (perennial). *C. cuneatum* formed a unique cluster.

Genetic variation within species

The genetic variation in wild annual *Cicer* collections was 0.234. Genetic diversity within annual *Cicer* species was in the order *C. judaicum* > *C. echinospermum* > *C. pinnatifidum* > *C. reticulatum* > *C. bijugum* > *C. cuneatum* > *C. yamashitae* > *C. arietinum* (Table 2). *C. arietinum* and *C. yamashitae* had the least genetic variation. The genetic diversity in *C. echinospermum* and *C. reticulatum* was more than seven times that found in *C. arietinum*. *C. judaicum* had the largest genetic variation. Genetic diversity of *C. bijugum* and *C. cuneatum* was roughly half of that in *C. judaicum*, but still five times that found in *C. arietinum*.

Geographic pattern of genetic variation

C. reticulatum and C. echinospermum

C. reticulatum and *C. echinospermum* belong to group I (Fig. 1). Both species were collected from a very limited area (Fig. 2). In *C. echinospermum* all of the accessions could be divided into two natural collection clusters (Fig. 2a). Most accessions came from the location at latitude 37.67–37.85°N with longitude 38.58–39.63°E belonging to cluster I, with genetic diversity of 0.081, but cluster II had higher genetic diversity of 0.123. Accessions in cluster II were collected from the area at latitude 36.85–36.90°N with longitude 37.30–37.37°E. This area was identified as the location with maximum genetic diversity for the species *C. echinospermum*.

In *C. reticulatum* three natural collection clusters were recognized (Fig. 2b). Cluster II enjoyed highest genetic diversity, which was higher than the species value (Table 2). This cluster was located at latitude 37.55–37.92°N with longitude 40.13–41.00°E. All other clusters had lower average genetic diversity and were lower than the species value.

C. bijugum, C. judaicum and C. pinnatifidum

These three species were recognized as members of group II (Fig. 1). In *C. bijugum* three geographic collection clusters were identified (Fig. 3a). Cluster II had most accessions, which came from the area confined by latitude 37.23–38.12°N and longitude 39.27–41.05°E. This cluster possessed the highest genetic diversity, greater than the genetic diversity value of this species (Table 2). The other two clusters had lower genetic diversity than the genetic diversity value of the species.

In *C. judaicum* four geographic collection clusters were identified (Fig. 3b). Cluster IV enjoyed highest genetic diversity. Although cluster I had most accessions, it had lower genetic diversity than other groups. The genetic diversity was reduced from cluster IV northwards to cluster III, cluster II and cluster I. The location at latitude 31.78–32.59°N with longitude 35.05–35.22°E, where cluster IV was identified, was taken as the location with maximum genetic variation for the species *C. judaicum*.

In *C. pinnatifidum* four geographic collection clusters were identified (Fig. 3c). All four clusters contained accessions very different from each other with high *DI*. However, cluster I enjoyed the highest genetic diversity, which was greater than the genetic diversity of this species (Table 2). All other clusters had lower genetic diversity than the value of the species. The location at latitude 37.3–38.75°N with longitude 38.77–40.93°E, where cluster I was identified was taken as the location with maximum genetic diversity for the species *C. pinnatifidum*.

Geographical locations of maximum genetic variation

Locations with maximum genetic variation for each species were plotted on a map (Fig. 4). These locations were found in southeastern Turkey for *C. reticulatum*, *C. echinospermum*, *C. pinnatifidum* and *C. bijugum*. The area of maximum genetic variation for *C. judaicum* was in the Palestine region.

Similarity between some wild annual *Cicer* accessions

Similarity between two samples of 26 accessions from both ICARDA and Australian collections (the latter

originally obtained from those in ICARDA or ICRISAT) were examined. Pairs of samples from the two sources were similar for most accessions with Nei–Li similarity of 0.639–0.901. These fell into a similarity range of the species to which the accession belonged (Table 2), and clustered in their own species. However, there were exceptions. Four pairs of accessions from two sources had low similarities and one sample of a pair clustered in another species. This occurred in *C. bijugum* ILWC 260(2), *C. echinospermum* ILWC 238(1), *C. judaicum* ILWC 46(1) and *C. reticulatum* ILWC 36(2) (Table 3). The similarity between these accessions and their own species (Table 3) was out of the similarity range of that species (Table 2). Another two pairs of accessions, *C. judaicum* ILWC 33(1) versus ILWC 33(2) and *C. reticulatum* ILWC 242(1) versus ILWC 242(2), were very similar with similarity within their species similarity range respectively. However, ILWC 33 clustered in *C. pinnatifidum* and ILWC 242 clustered in *C. echinospermum*. Interestingly it was hard to allocate ILWC 33 into *C. pinnatifidum* and define ILWC 242 as an accession in *C. echinospermum* according to the similarity comparison (Tables 2, 3).

Discussion

Genetic diversity between species was unbalanced and varied considerably in the world collections of wild annual *Cicer* germplasm. Our results also showed that cultivated *C. arietinum* had the least genetic variation. The result is consistent with the conclusion that chickpea has a very narrow genetic base (Abbo et al. 2003; Nguyen et al. 2004). Some difference in values for estimated genetic diversity between studies may be explained by the different number of accessions and

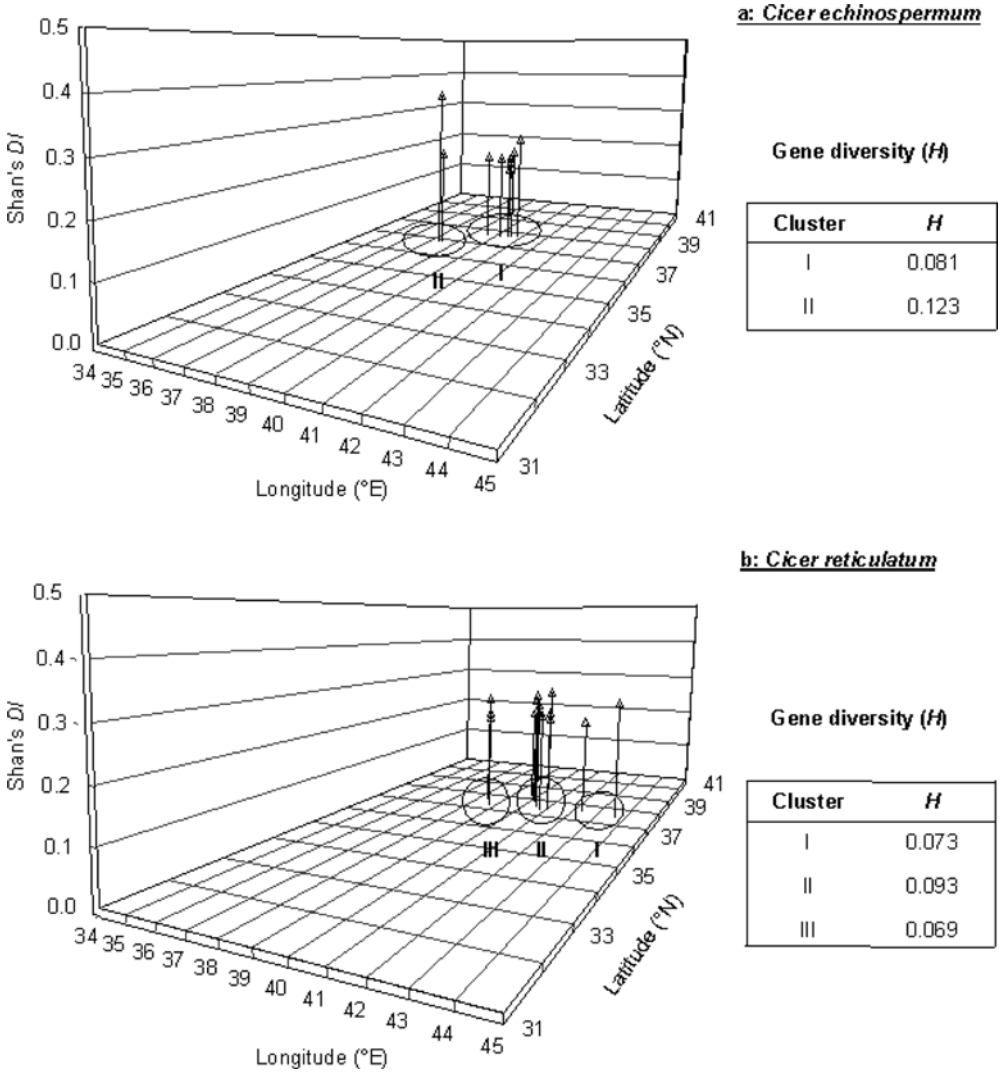

Fig. 2 Geographical pattern of genetic variation in **a** *C. echinospermum* and **b** *C. reticulatum*. Gene diversity (H) was calculated following Nei (1987). Shan's difference index (DI) of an accession was an average value of D_{ij} (Nei–Li genetic distance) between this accession and all other accessions within a species

polymorphism used in each study, but overall it is agreed that *C. arietinum* is far less variable than its wild annual relatives. This may explain why yield improvement of chickpea on a global basis has been slow when compared to cereal crops. This reinforces the necessity to introduce valuable genes from its wild relatives (Singh et al. 1994; Croser et al. 2003). Among its wild annual relatives *C. judaicum* enjoyed high genetic variation. It was collected from a wide range of habitats (Berger et al. 2003). This suggests a potential of broadening genetic diversity for other *Cicer* species. *C. reticulatum* and *C. echinospermum* are the most important species for chickpea improvement, since they are crossable with chickpea. However, they had lower genetic variability than *C. judaicum*. Both *C. reticulatum* and *C. echinospermum* were collected from a narrow strip within Turkey at latitude 36.85–38.33°N, confined within longitude 38.28–43.63°E for *C. reticulatum*, and 37.30–39.82°E for *C. echinospermum*. We suggest that immediate attention should be given to expand genetic diversity of these two species by targeting future collections.

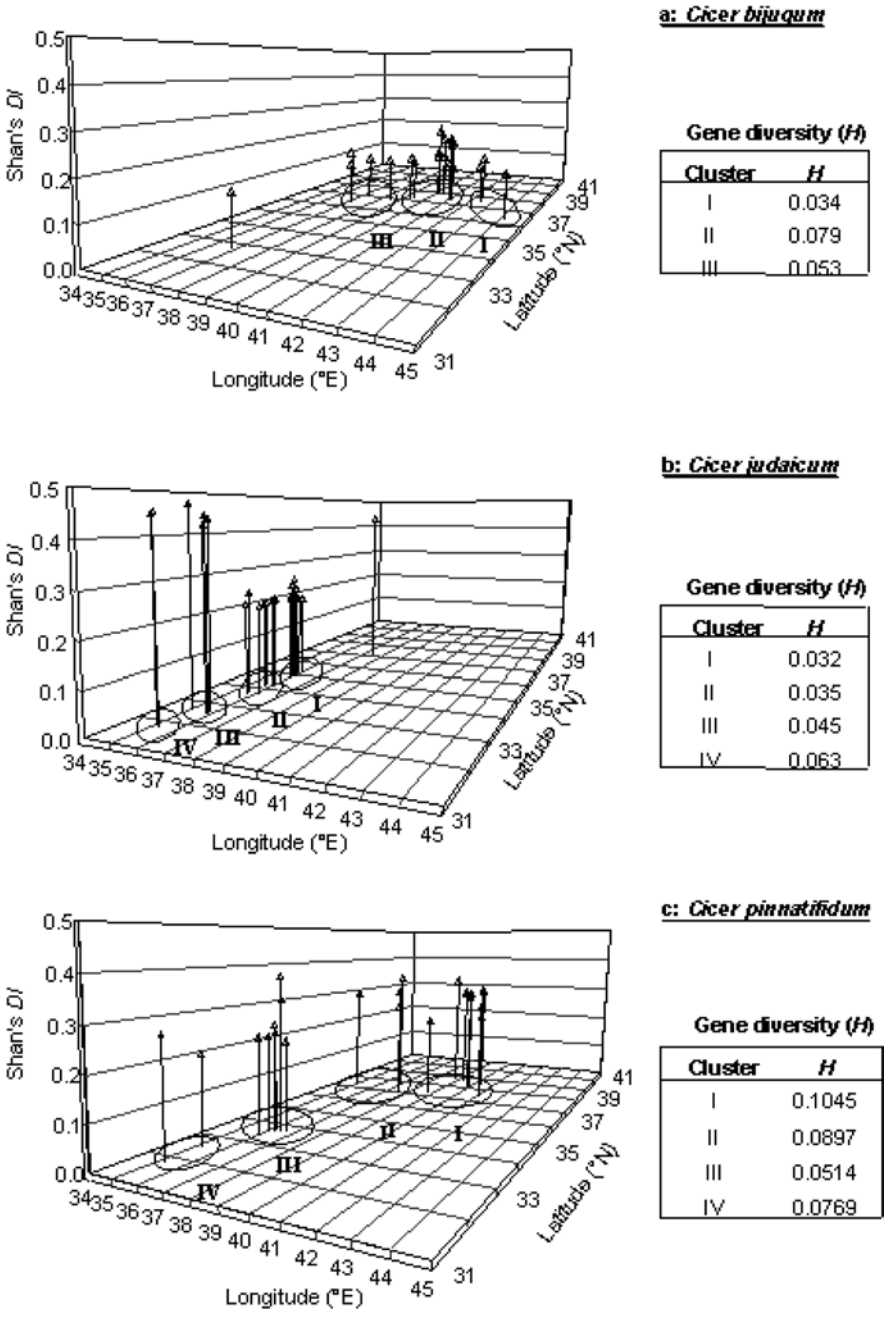

Fig. 3 Geographical pattern of genetic variation in **a** *C. bijugum*, **b** *C. judaicum* and **c** *C. pinnatifidum*. Gene diversity (*H*) was calculated following Nei (1987). Shan's DI of an accession was an average value of D_{ij} (Nei–Li genetic distance) between this accession and all other accessions within a species

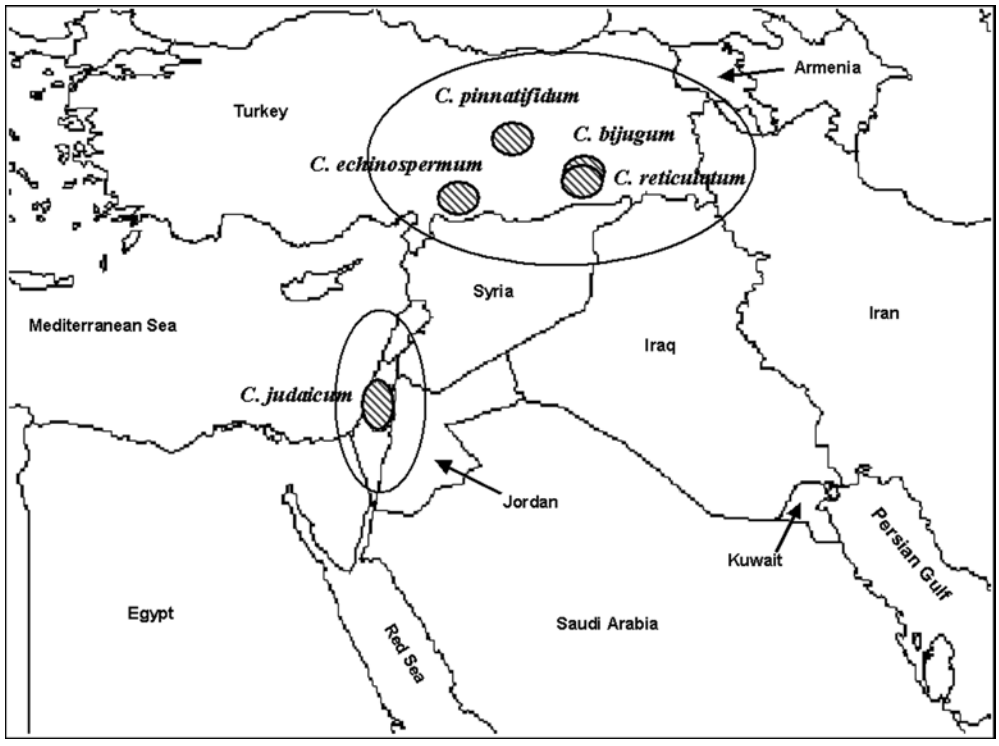

Fig. 4 Geographical locations of maximum genetic variation of the wild annual *Cicer* species. The locations are plotted on the map represented by *shaded ellipses* with a species name next to it. The locations with maximum genetic diversity were found in southeastern Turkey for species *C. bijugum*, *C. echinospermum*, *C. pinnatifidum* and *C. reticulatum*, and in Palestine for *C. judaicum*. The *large circles* surrounding the locations suggest an area with the best potential for future collections

If expansion of genetic variation is possible by more targeted collections, then geographic patterns of the *Cicer* genetic resources provide critical information to design such missions. It was clear that the locations having maximum genetic variation were found in southeastern Turkey for *C. reticulatum*, *C. echinospermum*, *C. pinnatifidum* and *C. bijugum*, and in the Palestine region for *C. judaicum*. Further collection and study in these locations and the surrounding areas is required for the conservation of *Cicer* genetic resources with the maximum genetic variation present. In the first instance extensive collection should be made in southeastern Turkey for *C. reticulatum*, *C. echinospermum*, *C. pinnatifidum* and *C. bijugum* and the surrounding areas including northern Syria, northern Iraq, northwestern Iran and southwestern Armenia. More collections should be considered in Palestine for *C. judaicum* and the surrounding areas including Israel, Syria, Lebanon, Jordan and northeastern Egypt. Secondly, further collection should be made in areas far from the centre of

Table 3 Genetic similarities for accessions (with same code) from International Centre for Agricultural Research in Dry Areas (ICARDA) and Australian gene banks

Accession code[a]	Species[b]	Nest in specific group[b]	Nei–Li S_{ij} between accessions from two gene banks	Nei–Li S_{ij} with accessions in its own species group		Nei–Li *Sij* with accessions in its nesting species	
				Range	Mean ± SD	Range	Mean ± SD
ILWC 260(1)	*Cb*	*Cb*	0.28	0.75–0.97	0.89 ± 0.06	0.20–0.31	0.27 ± 0.03
ILWC 260(2)	*Cb*	*Ce*	0.28	0.22–0.35	0.30 ± 0.03	0.68–0.70	0.78 ± 0.05
ILWC 238(1)	*Ce*	*Cb*	0.28	0.25–0.36	0.31 ± 0.04	0.63–0.95	0.84 ± 0.07
ILWC 238(2)	*Ce*	*Ce*	0.28	0.56–0.70	0.64 ± 0.05	0.19–0.28	0.23 ± 0.03
ILWC 46(1)	*Cj*	*Ce*	0.30	0.23–0.33	0.30 ± 0.02	0.60–0.80	0.72 ± 0.06
ILWC 46(2)	*Cj*	*Cj*	0.30	0.40–0.89	0.73 ± 0.19	0.25–0.40	0.33 ± 0.04
ILWC 33(1)	*Cj*	*Cp*	0.66	0.42–0.54	0.49 ± 0.04	0.56–0.85	0.69 ± 0.06
ILWC 33(2)	*Cj*	*Cp*	0.66	0.33–0.53	0.47 ± 0.06	0.63–0.74	0.68 ± 0.05
ILWC 242(1)	*Cr*	*Ce*	0.89	0.55–0.72	0.67 ± 0.04	0.62–0.87	0.79 ± 0.06
ILWC 242(2)	*Cr*	*Ce*	0.89	0.60–0.74	0.66 ± 0.04	0.65–0.83	0.78 ± 0.05
ILWC 36(1)	*Cr*	*Cr*	0.22	0.57–0.81	0.71 ± 0.07	0.23–0.34	0.28 ± 0.03
ILWC 36(2)	*Cr*	*Cj*	0.22	0.19–0.33	0.28 ± 0.04	0.40–0.82	0.52 ± 0.15

[a] (1) From ICARDA gene bank, (2) from Australian gene bank
[b] See Table 1 footnote for abbreviations of species

maximum genetic variation, but with contrasting eco-geographic environments. These areas may have lower in-group genetic variation, but are likely to possess characteristic genotypes due to long-term evolution under habitats different from the most diverse centres.

This study revealed the strength of AFLP technique in not only detecting genetic variations within species and populations, but also identifying accessions. AFLP analysis identified eight entries from six accessions that were very different from all the other accessions within their own species. This showed that some misidentifications/mislabelling might have occurred during either collection or curation. Another possibility is that the original collections for these accessions were from a mixture. Some accessions have been obtained from separation from a mixture (Berger et al. 2003). Different species growing together and seed shattering are main causes for such errors in resource collection by seeds. Among these accessions ILWC 242 might have a hybrid origin. It was taken as an accession in *C. reticulatum*, but it clustered in *C. echinospermum*. This has also been observed in phylogenetic analyses using random amplified polymorphic DNA (RAPD) and ISSR markers (Iruela et al. 2002) and AFLPs (Sudupak et al. 2004). ILWC 242 was collected in Turkey where both *C. echinospermum* and *C. reticulatum* are found (Abbo et al. 2003). Natural hybridisation may have occurred since these two species are crossable (Singh and Ocampo 1993). Seed, pod and plant morphology of this accession appeared to be intermediate between *C. reticulatum* and *C. echinospermum* in our study. The genetic similarities of ILWC 242 with accessions from both *C. reticulatum* and *C. echinospermum* were high and the similarity ranges were within, or very close to, both species. Therefore, we suggest that ILWC 242 is a hybrid between *C. reticulatum* and *C. echinospermum*. Such a natural hybrid could be useful for 'bridging' crosses to introduce genes to chickpea from incompatible species, given that *C. reticulatum* was the wild progenitor of chickpea.

The phylogenetic relationship between *Cicer* species from this study was overall consistent with most previous studies (review by Croser et al. 2003; Nguyen et al. 2004; Sudupak 2004; Sudupak et al. 2004). We believe that the annual *Cicer* germplasm should be divided into three gene pools based on AFLP analysis following the model of wild annual *Cicer* proposed by Croser et al. (2003), which is based on the original definition of gene pools and crossability, molecular diversity, and karyotyping studies. Group I included *C. arietinum*, *C. reticulatum* and *C. echinospermum*, which were in the primary gene pool. Group II was composed of *C. bijugum*, *C. pinnatifidum* and *C. judaicum*, which were in the secondary gene pool. Group III and group IV included species in the tertiary gene pool. This model supports the phylogenetic relationships established from AFLP markers in this study. Therefore, the most recent evidence, despite the application of different methods, different accessions, different numbers of species and different statistical approaches to data analysis, is that *C. pinnatifidum* is grouped with *C. bijugum* and *C. judaicum* with a high bootstrap indices of 88% and not in the group with *C. reticulatum*, *C. echinospermum* and *C. arietinum* as suggested by Iruela et al. (2002).

Further research is needed to determine the possible perennial ancestor of annual *Cicer*. *C. anatolicum* is thought to be the most closely related perennial to annual *Cicer* species, except *C. cuneatum*, based on sequence tagged microsatellite site markers (Choumane et al. 2000) and allozyme analysis (Kazan and Muehlbauer 1991). AFLP analysis in this study and by Nguyen et al. (2004) agree that *C. cuneatum* is more distantly related to all other wild annual *Cicer* species than *C. anatolicum* suggesting that *C. cuneatum* branched off very early in the evolution of the annual *Cicer*. We cannot yet conclude whether *C. anatolicum* is the closest perennial species to the annual species, since only one perennial species was included in this analysis. Recent studies indicate that other perennial species, such as *C. incisum*, have a closer relationship with annual *Cicer* species than *C. anatolicum* (Sudupak 2004; Sudupak et al. 2004). There are 33 perennial species in the genus *Cicer* (van der Maesen 1987), but only limited germplasm is available in the world collections. More perennial species and greater numbers of accessions from each species need to be included in phylogenetic examinations for firm conclusions to be made regarding the perennial ancestor of annual *Cicer*.

In conclusion, for the first time geographic patterns of genetic variation in *C. reticulatum*, *C. echinospermum*, *C. bijugum*, *C. judaicum* and *C. pinnatifidum* were revealed using AFLP analysis. This study provided the most detailed picture of genetic variation of these species across their geographical collection range to date. The maximum genetic diversity of *C. reticulatum*, *C. echinospermum*, *C. bijugum* and *C. pinnatifidum* was found in southeastern Turkey based on the current available world collections, while Palestine was the centre of maximum genetic variation for *C. judaicum*. The location with the highest genetic variation would attract more attention for future collection and conservation activities if undisturbed, since more novel genetic variation is expected than in any other location. *C. arietinum* had the narrowest genetic variation while its wild annual relatives had much greater genetic variation, which could be used in chickpea improvement. However, the genetic diversity was unbalanced and varied considerably between species in the world collections of wild annual *Cicer* germplasm. The number of accessions and genetic diversity in the wild annual *Cicer* germplasm was very limited overall compared to other major collections, such as wheat, barley and rice (Virk et al. 1995; Abbo et al. 2003). However, the expansion of genetic diversity held for ex situ wild annual *Cicer* is possible, and this can be achieved by more targeted collections in those identified areas with the maximum genetic diversity and nearby sites.

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