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Phylogenetic analysis in the genus *Cicer* and cultivated chickpea using RAPD and ISSR markers

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Abstract Seventy five accessions belonging to 14 species of the genus *Cicer* were analysed with PCR-based molecular markers to determine their phylogenetic relationships. Eight of the species were annuals and included the Section Monocicer which contains cultivated chickpea (*Cicer arietinum* L.). The remaining six species were perennials (five from Section Polycicer and one from Section Acanthocicer). More than one accession per species was analysed in most of the wild species; within *C. arietinum,* 26 accessions including Kabuli and Desi types, were studied. RAPD analyses using 12 primers gave 234 polymorphic fragments. Variability within species was detected. A dendrogram based on the Jaccard similarity index showed that the distribution pattern of variability between species was related to both growth habit and geographical origin. An accession of *Cicer reticulatum* was closer to accessions of *Cicer echinospermum* than to the four remaining of *C. reticulatum,* suggesting the possibility of gene flow between species. Cluster analysis for cultivated chickpea differentiated Kabuli and Desi types but we did not detect a clear relationship between groups and the geographical origin of the accessions.

Keywords Chickpea · *Cicer sp.* · Genetic diversity · ISSR · RAPD · Phylogeny

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Introduction

The genus *Cicer* belongs to the family Fabaceae and the tribe Cicereae Alef. It includes 33 perennials, eight annual and one unspecified wild species as well as the cultivated chickpea, *Cicer arietinum* L. (Van der Maesen 1987). Chickpea is the third most important grain legume crop in the world. Two main types are widely accepted by chickpea breeders: "Kabuli" (white flower, large and cream coloured seeds) and "Desi" (purple flower, small angular and dark seeds). Kabuli types have been grown traditionally in the Mediterranean basin and central Asia, while Desi types have been mainly produced in the Indian subcontinent, East Africa, Central Asia, and to a limited extent in the Mediterranean basin.

It is commonly accepted that Kabuli chickpea originated from the Desi type in the Mediterranean basin (Moreno and Cubero 1978; Hawtin and Singh 1980; Salimath et al. 1984; Gil and Cubero 1993). The two main types differ in several important traits. In order to exploit the variation in these two types, Desi×Kabuli crosses have been carried out by breeders; however, the transfer of genes between the two major types has been slow (Bahl 1980; Hawting and Singh 1980; Maynez et al. 1993).

Resistance to biotic and abiotic stresses has been found in wild *Cicer* species (Singh 1990; Singh et al. 1994) but, to-date, only crosses between *C. arietinum* and *Cicer reticulatum* or *Cicer echinospermum* (Ladizinsky and Adler 1976a, b; Singh and Ocampo 1993, 1997) have been successful. There have been reports suggesting that interspecific hybrids of cultivated chickpea with *Cicer pinnatifidum*, *Cicer judaicum* and *Cicer bijugum* are possible (Singh et al. 1994; Badami et al. 1997). However, these results have not been confirmed. In order to make use of the valuable genetic variability found in wild species a major effort should be made towards introgression of genes from wild relatives into the cultigen (Singh 1990; Singh and Ocampo 1997).

Seed-protein electrophoresis (Ladizinsky and Adler 1975; Vairinhos and Murray 1983; Ahmad and Slinkard 1992) and isozyme analysis (Kazán and Muehlbauer 1991; Ahmad et al. 1992; Labdi et al. 1996; Tayyar and Wainess 1996) have been used to establish genetic relationships among the *Cicer* species. These studies revealed minimal intra-specifc polymorphism, particularly within *C.arietinum*. However, the availability of a large number of polymorphic markers is required for progress in any kind of genetic study such as diversity or linkage analysis. Recently, random amplified polymorphic DNA (RAPD) analysis have been applied to study genetic relationships among nine annual *Cicer* species (Ahmad 1999). It was shown that RAPD markers can be a useful tool for studies of phylogenetic relationships within *Cicer*. However, information about genetic variation within species could not be determined because only one accession per species was utilised. Other DNA markers such as ISSRs (Ratnaparke et al. 1998a, b), microsatellite-based RFLPs (Weising et al. 1992; Sharma et al. 1995; Serret et al. 1997) or STMSs (Sant et al. 1999; Udupa et al. 1999; Winter et al. 1999, 2000) have revealed a high level of polymorphism and have been used to estimate variability within *C.arietinum*; however, these types of markers have not been used to determine relationships between species. On the whole, genetic relationships within *Cicer* sp. based on molecular data agree with other types of analyses used to determine relationships between species. For example, expectations based on karyotype analysis (Ohri and Pal 1991; Ocampo et al. 1992) agree with results of crossability studies (Ladizinsky and Adler 1976 a, b; Singh and Ocampo 1993).

The present study was conducted to determine the genetic variability between and within *Cicer* species using RAPD markers. Our aims were to: (1) provide a better understanding of the phylogenetic relationships between annual and perennial *Cicer* species, and (2) determine the degree to which PCR based markers such us as RAPDs or ISSRs are able to assess the variation and genetic relationship between Desi and Kabuli types of the cultivated chickpea.

Material and methods

Plant material

A total of 75 accessions representing eight annual and six perennial *Cicer* species were used in this study (Table 1). The number of accessions included for each of the annual species were: 26 for *C. arietinum* L., five for *C. reticulatum* Ladiz., five for *C. echinospermum* P.H.Davis., five for *C. pinnatifidum* Jaub. & Sp., five for *C. judaicum* Boiss, five for *C. bijugum* K.H. Rech, four for *Cicer yamashitae* Kitamura, and five for *Cicer cuneatum* Hochst. ex Rich. The number of accessions analysed for each perennial species were: four for *Cicer anatolicum* Alef., two for *Cicer macracanthum* M. Pop., five for *Cicer microphyllum* Benth., one for each *Cicer multijugum* van der Maesen and *Cicer canariense* Santos Gerra & Lewisand, and two for *Cicer oxyodon* Boiss & Hoh. The 26 cultivated chickpea accessions represented Desi and Kabuli types and a large portion of its global growing area (Table 1). Bulked DNA of five individuals per accession was prepared from glasshouse-grown plants to analyse variability both between accessions and species.

DNA extraction

About 100 mg of young leaf tissue was excised and immediately frozen in liquid nitrogen and stored at –80°C. DNA was isolated using the CTAB method of Lassner et al. (1989) with the modifications described by Torres et al. (1993). The DNA final concentration was determined by agarose-gel electrophoresis using known concentrations of uncut λ DNA as standard.

PCR amplification and electrophoresis

Primers were screened for consistency and their ability to produce at least one polymorphism in *C. arietinum* (Hajj Moussa et al. 1996). Twelve 10-mer oligonucleotides (Operon Technologies, Alameda Calif.) were used for inter-specific analysis (Table 2). Variability within *C. arietinum* was evaluated using ten RAPD and eight ISSR primers (Table 2).

Optimal reaction conditions for RAPD analysis was established according to Welsh and McClelland (1990) and Williams et al. (1990). Amplification was carried out in 25-µl reactions containing: 20 to 40 ng of plant genomic DNA, buffer (50 mM KCl, 10 mM Tris-HCL, 0.1% Triton X-100), 1.5 mM of $MgCL_2$, 100 mM of each dNTP, 2–4 mM of primer and 0.6 units of *Taq* DNA polymerase (Promega). Amplification was achieved in a Perkin Elmer Cetus DNA Thermal Cycler programmed for 40 cycles with the following temperature profile: 1 min at 94°C, 2 min at 35°C, 2 min at 72°C. Cycling was concluded with a final extension at 72°C for 8 min.

ISSR analysis was performed following the Ratnaparkhe et al. (1998b) protocol. In 25-µl reactions the volume contained: 30 ng of genomic DNA in 10 mM Tris-HCl, 50 mM of KCl, 0.1% Triton X-100, 2.5 mM of $MgCl_2$, 0.2 mM of dNTP, 0.24 µM of primer and 1 unit of *Taq* polymerase (Promega). PCR amplification was done using a Perkin Elmer Cetus programmed for 35 cycles with the following temperature profile: 94°C for 30 s, 50°C for 30 s and 72°C for 2 min followed by a final extension at 72°C for 10 min. Amplification products were electrophoresed in a mixed 1% agarose, 1% Nu-Sieve agarose, 1×TBE gel, visualized by ethidium staining and photographed under UV light.

Data analysis

Electrophoretic data were scored for the presence (1) or absence (0) of PCR fragments using *Bioimage Whole Band Analyser* software (BIO IMAGE, Michigan). The match to pattern option using reference templates gave a binary data matrix for each primer for the 75 accessions of *Cicer* sp. The combination of the whole set of data generated an extensive matrix from which Jaccard's coefficient of similarity was calculated using NTSYS software (Exeter Software, New York**).** Starting from these data, a new similarity matrix was obtained by the average of all possible pair-wise combinations between species. This similarity matrix was converted into a dissimilarity matrix (disimilarity=1-similarity) and a dendrogram was constructed by the Neighbor-joining method (Saitou and Nei 1987) using PHYLIP ver. 3.5 software (Joseph Felsenstein, University of Washington, Seattle, Washington).

Distances between the 26 accessions of *C. arietinum* were determined from a binary matrix including only the loci polymorphic within this species. Two morphological characters, flower colour and seed coat thickness, were included in the analysis. As indicated previously, a disimilarity matrix (1-similarity) was obtained from Jaccard's coefficient and the Neighbor-joining method was employed to generate a dendrogram.

Table 1 Accessions, source, origin and botanical Section of the annual and perennial species used in this study

Species		Accessions	Source[1]	Origin	Botanical section
Annual					
C. arietinum	(desi type)	CA1276, CA1351	CIFA	Mexico	Monocicer
		CA1592	CIFA	Hungary	
		CA1713, CA1744	CIFA	Iran	
		CA1713, CA1744	CIFA	Iran	
		CA1776	CIFA	Morocco	
		CA1540, CA1796, CA2077, CA2138	CIFA	Spain	
		JG62, WR315	ICRISAT	India	
		CA1853	ICRISAT	India	
	(kabuli type)	CA2016, CA2065, CA2133, CA2139, CA2156	CIFA	Spain	Monocicer
		ICCL1001	ICARDA	India	
		ILC72, ILC200, ILC3279	ICARDA	Old USSR (Caucaso)	
		ILC2912	ICARDA	Afghanistan	
		ILC484	ICARDA	Turkey	
		CA1990	CIFA	Mexico	
		P2245	ICRISAT	unknown	
C. reticulatum		ILWC242, ILCW36, ILCW114, ICCW45, W62074	ICARDA, USDA	Turkey	Monocicer
C. echinospermum		W610151	USDA	Syria	Monocicer
		ILWC181, ILWC246, PI489776, W61984	ICARDA, USDA	Turkey	
C. pinnatifidum		IG73054, PI458556, PI510654	ICARDA, USSA	Turkey	Monocicer
		W66158, W610160	USDA	Syria	
C. judaicum		IG6997	ICARDA	Lebanon	Monocicer
		PI458556, PI510659	USA	Israel	
		PI572536, W66154	USDA	Syria	
C. bijugum		ILCW70, ILCW34, W610150	ICARDA, USDA	Turkey	Monocicer
		W610148, W610149	USDA	Syria, Iraq	
C. yamashitae		ILWC55, ILWC215, PI504550, PI510657	USDA	Afghanistan	Monocicer
C. cuneatum		IG69976, ILWC37, IG73016, ILWC187, PI458554	ICARDA, USDA	Ethiopia	Monocicer
Perennial					
C. anatolicum		PI561078, PI383626, W614183, PI383626	USDA	Turkey	Polycicer
C. multijugum		W611189	USDA	Uzbekistan	Polycicer
C. macracanthum		W611180, W611179	USDA	Pakistan	Acanthocicer
C. microphyllum		W69395, W611181, W614187	USDA	India	Polycicer
		W611186, W611188	USDA	Pakistan	
C. canariense		PI557453	USDA	Spain	Polycicer
C. oxyodon		PI561084, PI561103	USDA	Turkey	Polycicer

[1] CIFA, Centro de Investigación y Formación Agraria, Córdoba, Spain; ICRISAT, International Crops Research Institute for the Semiarid-Tropics, Hyderabad, India; ICARDA Center for Agricultural Research in Dry Areas, Aleppo, Syria; USDA, United States Department of Agriculture, USA

Results

Inter-specific variation

Amplification of genomic DNA of the 75 accessions using 12 primers for RAPD analysis yielded 234 fragments that could be scored. Representative banding patterns are shown in Fig. 1. All the chosen primers amplified fragments across the 14 species studied, with a number of amplified fragments per reaction ranking from 24 to 12 and which varied in size from 218 to 2,048 bp (see Table 2). Most fragments were shared by at least two accessions. Twenty six fragments (11.1%) of the total scored were found to be specific and monomorphic to a particular species. *C. bijugum* and *C. cuneatum* were the species with the highest number of specific and monomorphic bands (5), while *C. reticulatum, C. echinospermum, C. pinnatifidum and C. anatolicum* did not show this kind of species-distinctive fragment.

Jaccard's coefficient of similarity between all accessions of the 14 species ranged from 0.30 to 0.98. Cultivated chickpea, with the highest number of accessions (n=26), showed one of the highest mean values for the similarity index within species (0.86), with a minimum

Table 2 Sequences of oligonucleotide primers used for inter-specific and *C. arietinum* intra-specific variability analyses

Primer	Sequence (5´ to 3´)	No. of bands analysed	Band molecular weight (bp)
OPAB-17	TCG CAT CCA G	15	1,700–424
OPAC-09	AGA GCG TAC C	23	1,995–380
OPAF-16	TCC CGG TGA G	17	1,920–544
OPC-05	GAT GAC CGC C	21	1,790–513
OPI-13	CTG GGG CTG A	23	1,675–431
OPJ-20	AAG CGG CCT C	24	1,632–545
OPM-05	GGG AAC GTG T	22	2,048–299
OPS-01	CTA CTG CGC T	12	1,906–520
OPT-13	AGG ACT GCC A	20	1,503–375
OPX-14	ACA GGT GCT G	17	1,501–255
OPY-02	CAT CGC CGC A	23	2,185–218
OPZ-10	CCG ACA AAC C	19	1,709–419
OPAE-09[a]	TGC CAC GAG G	1	1,402
OPAE-20[a]	TTG ACC CCA G	1	1,100
OPAF-08[a]	CTC TGC CTG A	1	1,125
OPF-14[a]	TGC TGC AGG T	1	638
OPI-02[a]	GGA GGA GAG G	1	1,300
OPI-12[a]	AGA GGG CAC A	2	1,740–1,528
MER5[a]	CTG TGC TGT G	1	1,641
OPQ-05[a]	CCG CGT CTT G	1	538
OPX-04[a]	CCG CTA CCG A	1	693
OPY-20[a]	AGC CGT GGA A	1	1,624
UBC-807[b]	AGA GAG AGA GAG AGA GT	1	260
UBC-809[b]	AGA GAG AGA GAG AGA GG	1	972
UBC-811[b]	GAG AGA GAG AGA GAG AC	1	992
UBC-856[b]	ACA CAC ACA CAC ACA CYA	1	529
UBC-858[b]	TGT GTG TGT GTG TGT GRT	1	438
UBC-880[b]	GGA GAG GAG AGG AGA	1	247
UBC-884[b]	HBH AGA GAG AGA GAG AG	1	532
UBC-885[b]	BHB GAG AGA GAG AGA GA	3	1,246–441

[a] and [b]: used only in *C. arietinum* analyses
[b] primers for ISSR markers, four of them have mixed base positions: *B* indicates C, G or T; *H* indicates A, C or T; *R* purine, *Y* pyrimidine

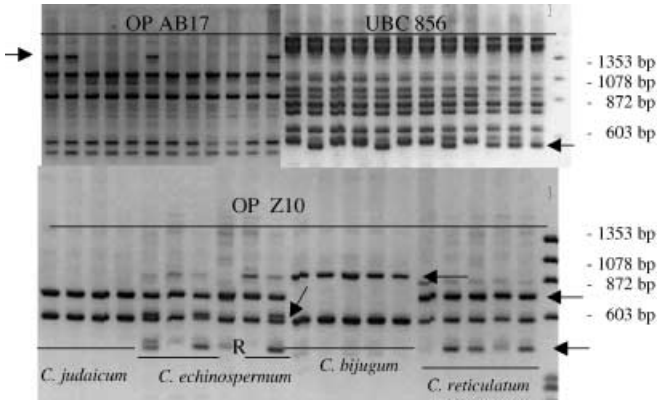


Fig. 1 Amplification patterns from RAPD and ISSR primers (AB-17, OPZ-10 and UBC-856) used for analyses of intra-specific variability in *C. arietinum* (upper gel) and inter-specific variability among four *Cicer* species (lower gel). Discriminatory bands are marked by *arrows*. *Lane R* reference sample of *C. arietinum*. Molecular-weight reference fragments (*lane M*) were derived from an ØX174 OP/*Hae*III digest

value of 0.67 and a maximum of 0.98 (Table 3). In this species, only 31 fragments were polymorphic (13.2%). *C. macracanthum* showed also a coefficient of similarity of 0.86 but with only two accessions. The rest of the species with more than one accession showed average values of similarity between 0.45 and 0.78, with *C. bijugum* and *C. cuneatum* having the lowest genetic diversity (0.78 and 0.74 respectively). These results confirm the low level of genetic diversity within *C. arietinum* compared to the wild species.

Mean similarity values within species were higher than between species indicating that accessions belonging to the same species were accurately grouped (Table 3). One exception was a single accession of *C. reticulatum* (ILWC242) that was closer to *C. echinospermum* (similarity index mean=0.55) than to the rest of the *C. reticulatum* accessions (similarity index mean=0.46). This accession was matched with the *C. echinospermum* group in a last step with a low average similarity index (0.55). Besides, this accession shows a seed morphology intermediate between *C. reticulatum* and *C. echinospermum*; even under the binocular microscope it was possible to detect short hairs in some areas on the surface of the seed (*C. reticulatum* seed is glabrous; *C. echinospermum* one shows long and dense hairs).

Cluster analysis based on disimilarity values between species generated a dendrogram that represent the phylogenetic relationships among the 14 species under study (Fig. 2). A high co-phenetic correlation coefficient (0.96) between the disimilarity matrix and the Neighbor-joining clustering method was obtained.

According to our results, the species under study are distributed in four groups. Group I included the perennial species of Asian origin (from East Turkey to Himalaya). Group II is only formed by *C. yamashitae,* an annual and endemic species of a region in Afghanistan close to

Table 3 Jaccard's coefficient of similarity values for eight annual and six perennial species of the genus *Cicer*. The range of values for accession pairs compared between species are shown in parentheses

Species	ari.[a]	ret.	ech.	pin.	jud.	bij.	ana.	mul.	mac.	mic.	oxy.	yam.	can.	cun.
ari.	**0.86** (0.67–0.98)													
ret.	0.45 (0.33–0.55)	**0.54** (0.40–0.77)												
ech.	0.47 (0.37–0.55)	0.43 (0.28–0.61)	**0.66** (0.57–0.81)											
pin.	0.25 (0.16–0.31	0.23 (0.13–0.36)	0.31 (0.20–0.45)	**0.48 (0.31–0.67)**										
jud.	0.25 (0.18–0.36)	0.17 (0.09–0.29)	0.22 (0.15–0.32)	0.23 (0.15–0.29)	**0.65 (0.32–0.92)**									
bij.	0.15 (0.12–0.16)	0.13 (0.08–0.17)	0.16 (0.11–0.20)	0.23 (0.13–0.31)	0.24 (0.20–0.28)	**0.78 (0.69–0.91)**								
ana.	0.21 (0.15–0.26)	0.16 (0.12–0.20)	0.19 (0.15–0.22)	0.17 (0.13–0.22)	0.19 (0.14–0.24)	0.14 (0.10–0.18)	**0.41 (0.30–0.53)**							
mul.	0.18 (0.15–0.21)	0.18 (0.14–0.23)	0.23 (0.21–0.15)	0.18 (0.15–0.23)	0.18 (0.14–0.23)	0.14 (0.12–0.15)	0.26 (0.20–0.29)	**1** –						
mac.	0.14 (0.12–0.17)	0.12 (0.08–0.17)	0.15 (0.10–0.17)	0.16 (0.13–0.20)	0.16 (0.14–0.19)	0.14 (0.11–0.15)	0.21 (0.16–0.24)	0.18 (0.16–0.20)	**0.86** –					
mic.	0.16 (0.11–0.21	0.14 (0.10–0.20)	0.18 (0.12–0.23)	0.18 (0.14–0.23)	0.15 (0.10–0.19)	0.12 (0.09–0.16)	0.25 (0.19–0.28)	0.30 (0.21–0.33)	0.31 (0.24–0.40)	**0.62 (0.54–0.71)**				
oxy.	0.20 (0.17–0.23)	0.19 (0.15–0.23)	0.19 (0.15–0.24)	0.16 (0.13–0.17)	0.12 (0.07–0.18)	0.11 (0.05–0.16)	0.19 (0.14–0.22)	0.16 (0.13–0.19)	0.19 (0.15–0.23)	0.16 (0.12–0.20)	**0.47** –			
yam.	0.16 (0.11–0.22)	0.16 (0.11–0.21)	0.20 (0.14–0.27)	0.21 (0.13–0.32)	0.16 (0.10–0.23)	0.14 (0.11–0.19)	0.17 (0.10–0.23)	0.18 (0.13–0.22)	0.13 (0.11–0.15)	0.17 (0.13–0.21)	0.17 (0.12–0.21)	**0.61 (0.45–0.72)**		
can.	0.10 (0.08–0.12)	0.09 0.04–0.13)	0.11 (0.08–0.12)	0.14 (0.08–0.18)	0.09 (0.08–0.10)	0.10 (0.09–0.12)	0.11 (0.06–0.18	0.12 (–)	0.14 (0.13–0.14)	0.07 (0.04–0.08)	0.07 (0.06–0.07)	0.09 (0.07–0.11)	**1** –	
cun.	0.14 (0.11–0.22)	0.11 (0.06–0.19)	0.13 (0.08–0.23)	0.14 (0.10–0.20)	0.12 (0.09–0.18)	0.11 (0.09–0.13)	0.10 (0.05–0.17)	0.11 (0.10–0.14)	0.10 (0.08–0.13)	0.10 (0.08–0.13)	0.12 (0.09–0.15)	0.12 (0.07–0.19)	0.23 (0.08–0.26)	**0.74 (0.54–0.91)**

[a] ari.=*C. arietinum*; ret.=*C. reticulatum*; ech.=*C. echinospermum*; pin.=*C. pinnatifidum*; jud.=*C. judaicum*; bij.=*C. bijugum*; ana.=*C. anatolicum*; mul.=*C. multijugum*; mac.=*C. macracanthum*; mic.=*C. microphyllum*; oxy.=*C. oxyodon*; yam.=*C. yamashitae;* can.=*C. canariense* and cun.=*C. cuneatum*

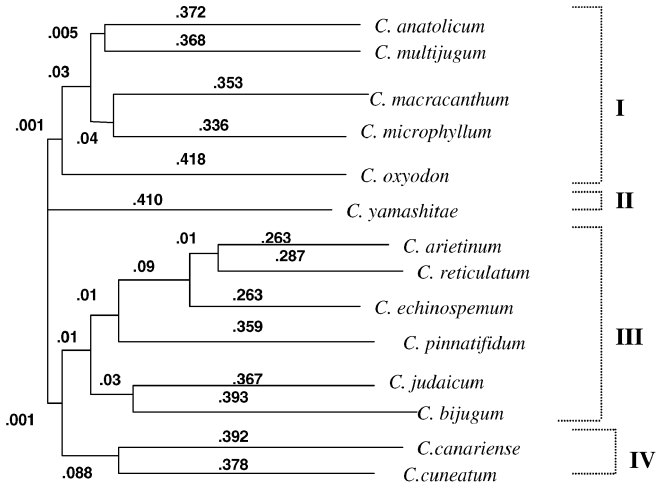


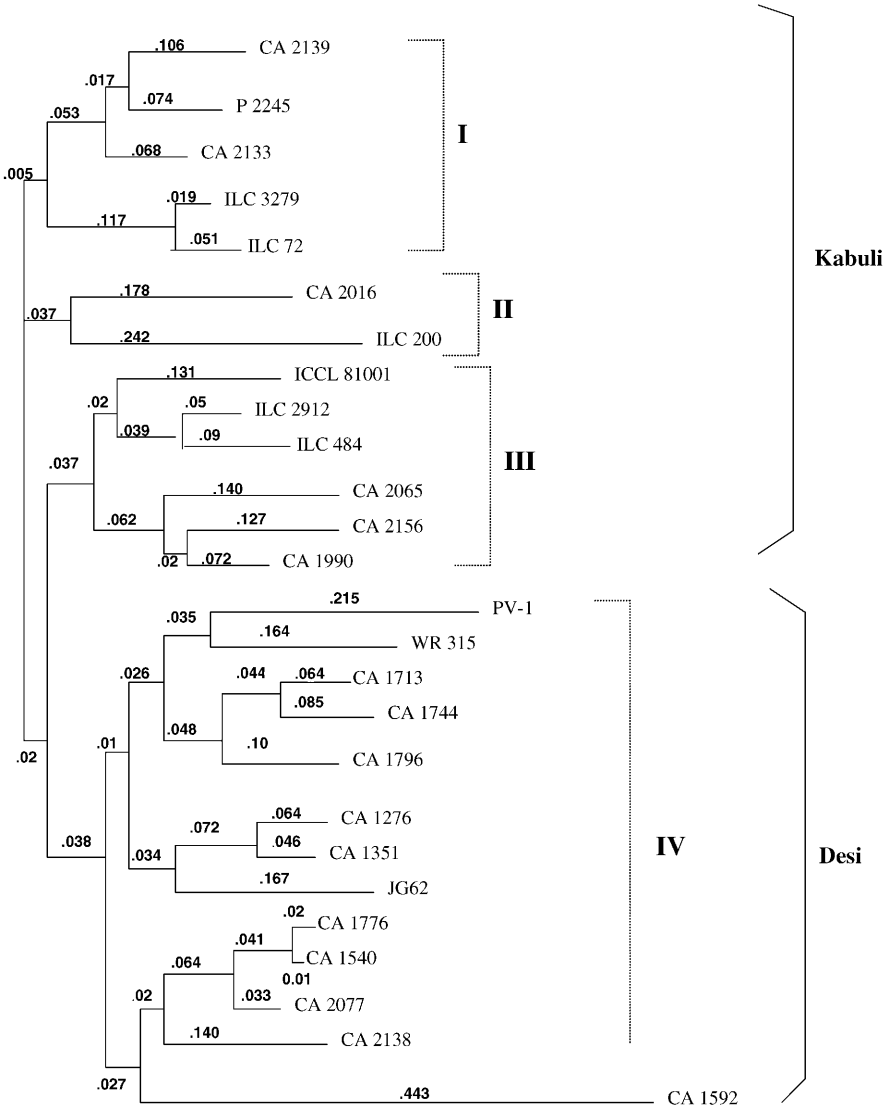
Fig. 2 Dendrogram of 14 annual and perennial species of the genus *Cicer* derived from analysis using RAPD markers and based on distances obtained from the disimilarity matrix (1-Jaccard index) and neighbor-joining clustering method. Numbers refer to distances

Kabul (Van der Maesen 1987). Group III contains only annual species including *C. arietinum*, all from Near East. Group IV included two species at a relatively large distance from the rest, both of them being of African origin: *C. cuneatum* from East Africa and *C. canariense* from North-West Africa (Canary Islands), the former being an annual and the latter a perennial (Fig 2). Although groups IV and III clustered in the dendrogram, the distances from group IV to groups I, II and III were somewhat larger (>0.88) than the distances among these three groups (values from 0.83 to 0.84).

Variation within C. arietinum

A total of 52 markers were scored to study the variation within *C. arietinum*. Jaccard's similarity index obtained from these data varied between 0.19 and 0.97. The dendrogram obtained by the neighbor-joining method showed four main clusters (Fig. 3). Three groups (I, II

Fig. 3 Dendrogram derived from the analysis of 26 accessions of *C. arietinum* using RAPD and ISSR markers and based on distances obtained from the disimilarity matrix (1-Jaccard index) and neighbor-joining clustering method. *Numbers* refer to distances



and III) included accessions of the cultivated chickpea Kabuli type; the last group (IV) contained Desi-type chickpea accessions. A Desi accession CA1952, from Hungary, included in group IV is an extreme variant with low similarity values with respect to the other 25 accessions (values between 0.19 and 0.42). The cophenetic correlation between the dissimilarity matrix and the dendrogram obtained by the Neighbor-joining clustering method was 0.88.

There was not a clear grouping according to geographical origin; for example, two Kabuli accessions from old USSR, Caucaso (ILC72 and ILC3279) clustered together, but another sample from the same region (ILC200) was grouped apart (Fig. 3). Similar results were obtained for Kabuli accessions from Spain, only two of them were in the same group joined with a Mexican accession, CA1990, that could be considered of Spanish origin. Within the Desi type, accessions from the West Mediterranean countries (Spain and Morocco) clustered together except for the Spanish accession CA1796 that was grouped in another cluster with accessions from Iran and India. Two accessions from Mexico (CA1276 and CA1351) were clustered close to JG62 from India. This result can be easily explained as Desi accessions from ICRISAT (India) have been employed in the Mexican chickpea breeding programs (Andrade-Arias 1980).

Discussion

Our results show that RAPD markers successfully identified genetic variation in *Cicer*. The variation identified was greater than that revealed by the isozymes or seed-storage proteins used in previous studies of genetic relationships among annual Cicer species (Tuwafe et al. 1988; Kazan and Muehlbauer 1991; Ahmad and Slinkard 1992; Ahmad et al. 1992; Labdi et al. 1996; Tayyar and Wainess 1996). Perennial *Cicer* species have been studied for the first time using RAPD markers, whereas only *C. anatolicum* and *Cicer songoricum* were compared with annual species in previous reports using isozymes (Kazan and Muehlbauer 1991; Tayyar and Waines 1996).

The phylogenetic relationships obtained are in good agreement with the geographical distribution of *Cicer* sp. Group I included perennial species with a broader geographical distribution area and higher-altitude growing conditions than the annual species. Kazan and Muehlbauer (1991) reported a close relationship among *C. anatolicum* (a perennial*), C. reticulatum, C. echinospermum* and *C. arietinum*, while Tayyar and Waines (1996) did not find this close association; they grouped *C. anatolicum* with *Cicer songoricum* (perennial), *C. yamashitae* and *Cicer chorassanicum* (annuals). Our results suggest that *C. anatolicum* is more closely related to other perennials species included in this study (*C. multijugum, C. macracanthum, C. microphylum* and *C. oxyodon*).

Our Group II contains only *C. yamashitae*. Several authors (Kazan and Muehlbauer 1991; Ahmad and Slinkard 1992; Labdi et al. 1996; Tayyar and Waines 1996) placed it far from the species comprising the two crossability groups proposed by Ladizinsky and Adler (1976a), in agrement with our results. The first crossability group is formed by *C. arietinum, C. echinospermum* and *C. reticulatum* and the second one by *C. bijugum, C. judaicum* and *C. pinnatifidum*. Recently, *C. yamashitae* was included in a cluster very close to the group containing cultivated chickpea by using RAPD markers (Ahmad 1999), but only one accession was used in his study. *C. yamashitae* is endemic in Afghanistan (Van der Maesen 1987), thus geographically very far from the area of Group III which contains *C. arietinum*.

Group III shows a more-reduced geographical distribution than Group I. This group contains the two first crossability groups proposed by Ladizinsky and Adler (1976a). Our results agree with *C. reticulatum* being the closest species to cultivated chickpea, as shown by many other studies involving seed storage-protein profiles (Ladizinsky and Adler 1975; Ahmad and Slinkard 1992), pollen and seed morphobiometric data (De Leonardis et al. 1996), phylogenetic studies using molecular markers (Ahmad et al. 1992; Labdi et al. 1996; Tayyar and Waines 1996; Ahmad 1999) and crossability (Ladinzinsky and Adler 1976a, b).

Some totally fertile recombinant inbred lines are possible in crosses between *C. arietinum* and *C. echinospermum* (Singh and Ocampo 1997). The fact that one of our accessions of *C. reticulatum* (ILWC 242) appeared to be close to *C. echinospermum*, and with a seed morphology intermediate between these two species, could be due to possible natural interspecific crossing. We have detected interspecific hybridisation between *C. reticulatum* and *C. arietinum* in a population of *C. reticulatum* coming from ICARDA. Thus, a certain gene flow among *C. arietinum, C. reticulatum* and *C. echinospermum* may exist under natural conditions. We agree with Tayyar and Waines (1996) that *C. echinospermum* may have played a role in the evolution of cultivated species. With regard to *C. judaicum, C. bijugum* and *C. pinnatifidum* our results are coincidental with Ahmad (1999) who concluded, using the RAPD technique, that *C. judaicum* and *C. bijugum* were the closest species in this group.

Species included in Group IV, *C. canariense* (a perennial) and *C. cuneatum* (an annual), are the only species with a climbing growth habit, elongate pods, globular seed and non-conspicuous beak (Van der Maesen 1987). Only one of these species (*C. cuneatum*) has been included in previous studies and was placed far away from all other species (Kazan and Muehlbauer 1991; Ahmad and Slinkard 1992; Ahmad et al. 1992; Tayyar and Wainess 1996; Ahmad 1999). In the present study, *C. canariense* clustered with *C. cuneatum*, both of them very distant from the rest of the species. Our results suggest that annual African species could have originated from a perennial stock in the same way that Asian perennials may have been the origin of two annuals (following Tayyar and Waines 1996).

Cultivated chickpea has minimal polymorphism for molecular markers as also revealed by seed protein (Ladizinsky and Adler 1975), isozyme (Tuwafe et al. 1988; Gaur and Slinkard 1990; Kazán and Muehlbauer 1991; Labdi et al. 1996; Tayyar and Wainess 1996) and RFLP (Udupa et al. 1993) analysis. In the present study, all primers were selected from previous reports (Hajj-Moussa et al. 1996; Ratnaparke et al. 1998a, b; Hajj Moussa et al. 2000) for providing polymorphic bands. Our results agree with other studies that show Kabuli and Desi chickpea are two distinct groups with different genetic backgrounds (Moreno and Cubero 1978; Hawtin and Singh 1980; Salimath et al. 1984; Gil et al. 1996).

The absence of a relationship between geographic origin and the clustering of the different accessions suggests that chickpea could during different historical periods have been spread from its centre of origin in the Near East due to human action. The study of a larger collection should provide a better knowledge about genetic diversity and its relationship with geographical origin. This information could be very valuable in the management of genetic resources in this species. The accession CA1592, a Desi type of Hungarian origin, is in fact isolated from the rest of the cultivated chickpea accessions, both Kabuli and Desi. It could be of interest for mapping purposes. This accession has been included in our crossing programs in order to broaden the genetic base of chickpea.

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