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M.A. Sudupak · M.S. Akkaya · A. Kence

Analysis of genetic relationships among perennial and annual *Cicer* species growing in Turkey using RAPD markers

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Abstract Random amplified polymorphic DNA (RAPD) fragments were used to assess genetic relationships among *Cicer* spp. growing in Turkey. Seven 10-mer primers selected from a 50 random oligonucleotide primer set, depending on their ability to amplify genomic DNA in all species, were used to detect RAPD variation in 43 wild and cultivated accessions representing ten species. These primers yielded 95 reproducible amplification products, 92 of which were polymorphic. Pairwise genetic distances of accessions estimated according to Nei and Li (1979) were used to produce a dendrogram using UPGMA. The dendrogram contained two main clusters, one of which comprised accessions of the four perennial species (*Cicer montbretii, Cicer isauricum, Cicer anatolicum* and *Cicer incisum*) together with the accessions of the three annual species (*Cicer pinnatifidum, Cicer judaicum* and *Cicer bijugum*), and the other cluster included the remaining three annual species (*Cicer echinospermum, Cicer reticulatum* and *Cicer arietinum*). Analysis of RAPD variation showed that *C. incisum* is the most similar perennial species to annuals, and *C. reticulatum* is the closest annual species to chickpea. These results generally agree with our allozyme study which was carried out using same *Cicer* collection and previous studies of relationships among annual species. The results also show that RAPD markers can be used to distinguish *Cicer* species and to survey genetic variation and relationships among taxonomic units in this genus.

Keywords *Cicer* · RAPD · Species relationship · Chickpea · DNA variation

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M.A. Sudupak (✉)
Department of Biology, College of Arts and Sciences,
Erciyes University, Kayseri 38060, Turkey
e-mail: sudupak@erciyes.edu.tr
Tel.: 90-(352)-437-4901-33059, Fax: 90-(352)-437-4933

M.S. Akkaya
Department of Chemistry, College of Arts and Sciences,
Middle East Technical University, Ankara, Turkey

M.A. Sudupak · A. Kence
Department of Biology, College of Arts and Sciences,
Middle East Technical University, Ankara, Turkey

Introduction

The genus *Cicer* (Fabaceae) contains 43 species, nine of which are annual including chickpea (*Cicer arietinum* L.), and the rest are perennial. *Cicer* species are predominantly self-pollinating, and the chromosome number of annual species is 2n = 16 (van der Maesen 1987, Ahmad 2000). Chickpea is one of the most important pulse crops in the world. It is grown in South Asia, West Asia, North Africa, East Africa, southern Europe, Australia, South and North America (Singh 1997). Chickpea is also extensively cultivated throughout Turkey (630,000 hectares with an annual production of 600,000 metric tons in 1999), one of the largest chickpea exporter providing 31% of the World's exported chickpea (FAO 2000).

Understanding and the management of the genetic diversity present within cultivated species and its wild relatives are critical for crop improvement studies. Use of wild species had significant implications in improving several characteristics of major cereals and tomato (Xiao et al. 1996; Hoisington et al. 1999). As an important grain legume, investigation of relatedness and genetic diversity within and among species of the genus *Cicer* and its effective evaluations are an obvious necessity to identify a new source of germplasms bearing valuable genes, which may be used in the improvement of the chickpea. It is generally accepted that the average yield potential of chickpea is below its presumed potential, and efforts to improve the productivity of this crop by conventional breeding-means were not very effective (Singh et al. 1994). One of the reasons for this drawback was speculated to be the lack of traits in the world chickpea germplasm collection needed for effective improvement (Robertson et al. 1997). However, a number of wild

Cicer accessions appear to harbor genes for resistance to a number of biotic and abiotic stresses (Muehlbauer et al. 1994; Singh et al. 1994). These accessions are utilized in interspecific hybridization experiments to improve the chickpea (Singh and Ocampo 1997).

Several groups have studied the genetic diversity and relatedness among annual *Cicer* species by means of hybridization, electrophoresis of seed storage proteins and isozymes (Ladizinsky and Adler 1976; van der Maesen 1987; Ahmad and Slinkard 1992; Ahmad et al. 1992; Kazan and Muehlbauer 1993; Labadi et al. 1996; Tayyar and Waines 1996). Based on these studies, annual species were classified into four groups. The first group included the chickpea (*C. arietinum* L.) and its closest relatives (*Cicer reticulatum* Ladiz. and *Cicer echinospermum* P.H. Davis). The second group included *Cicer pinnatifidum* Jaub & spp., *Cicer judaicum* Boiss. and *Cicer bijugum* K.H. Rech. The remaining two annual species (*Cicer cuneatum* and *Cicer chorassanicum*) formed the last two groups. Recently, analysis of allozyme variation at 12 loci among six annual and four perennial *Cicer* species (the same species used in this study) also confirmed the same grouping for the six of these annual species, and revealed the relationships between four perennial species with these annuals and among themselves (Sudupak and Kence, unpublished results).

As in many other taxonomic groups, Turkey has a considerable diversity in the genus *Cicer*. A quarter of the known wild *Cicer* species shows a distribution in Turkey (Davis 1973; van der Maesen 1987). Turkey resides on one of the centers of origin proposed for chickpea by Vavilov. A number of other investigators have recognized southeastern Turkey to be the center of origin for chickpea (Ladizinsky and Adler 1976; van der Maesen 1987; Singh 1997). Therefore, the assessment and efficient conservation of genetic resources in Turkey exhibit a prime importance for chickpea improvement. Although relationships among annual species were studied to a certain extent, only in two studies were perennial species examined with annuals, one of which included only *Cicer anatolicum* and the other one studied two perennial species *C. anatolicum* with *Cicer songoricum* revealing conflicting results in grouping *C. anatolicum* with annual species (Kazan et al. 1991; Tayyar and Waines 1996).

DNA sequence polymorphisms are used extensively to probe genetic variation in plant populations and determine relationships. A number of molecular markers including restriction fragment length polymorphisms (RFLPs, Botstein et al. 1980) and random amplified polymorphic DNAs (RAPDs, Williams et al. 1990) have been developed and widely used to study plant genomes. Advantages of these marker systems include the detection of a potentially unlimited number of loci covering nearly the entire genome, independent of various factors (Whitkus et al. 1994; Rafalski et al. 1996; Kumar 1999). RAPD analysis requires use of the polymerase chain reaction (PCR) with 10-mer primers to generate random amplified fragments of DNA. The RAPD technique has several advantages such as the ease and rapidity of analysis, a relatively low cost, the availability of large numbers of primers and the requirement of a very small amount of DNA for analysis (Williams et al. 1990; Whitkus et al. 1994; Weising et al. 1995). However, a number of disadvantages are also associated with RAPDs, such as limited reproducibility and dominant inheritance, which have to be taken into consideration when using RAPDs as genetic markers (Devos and Gale 1992; Whitkus et al. 1994). Advantages associated with RAPDs have made them a favorite marker technique in mapping, the determination of phylogenetic relationships and genetic diversity, and the identification of cultivars and parents in a number of plant species (Skroch et al. 1993; Abo-elwafa et al. 1995; Sharma et al. 1995a; Weising et al. 1995; Simon and Muehlbauer 1997; Butos et al. 1998; Wolff and Morgan Richards 1998).

Although a number of isozyme studies have provided considerable insight into genetic diversity and relationships among annual species, only a few DNA-based approaches have been utilized in *Cicer*. Weising et al. (1992) and Sharma et al. (1995b) have assayed simple sequence repeat polymorphisms in chickpea with hybridization-based oligonucleotide fingerprinting and found it very informative. Udupa et al. (1999) have recently utilized PCR-based simple sequence repeat polymorphism in chickpea, and two closely related wild species (*C. reticulatum* and *C. echinospermum*) found high levels of polymorphism (Weising et al. 1992; Sharma et al. 1995b; Udupa et al. 1999). Recently, Ahmad (1999) has used RAPD markers to asses the genetic relationships among annual species. The aim of the present study was to use RAPD markers to asses the intra- and inter-specific genetic relationships among accessions of perennial and annual species in Turkey. Some of the specific aims were the determination of species relationships among annual and perennials growing in Turkey, the determination of the closest perennial relative(s) of the annual species and the determination of the closest wild species to the cultivated species.

Materials and methods

Plant material and DNA extraction

The plant material used in this study included 43 wild and cultivated *Cicer* accessions representing five annual and four perennial *Cicer* spp. with distribution in Turkey. The origin and the source of each accession for each *Cicer* species are given in Table 1. Accessions of perennial species and some of the accessions of annual species were collected from their natural habitats, and others were obtained from the International Center for Agricultural Research in Dry Areas (ICARDA), Aleppo, Syria, the Aegean Agricultural Research Institute (AARI), Menmen, Izmir, and the Anatolian Agricultural Research Institute (AARI*), Eskisehir. To obtain leaf material for DNA extraction, seeds of accessions for annual species and for two perennial species (*C. anatolicum* and *Cicer incisum*) were germinated and grown in a growth chamber. After 3–4 weeks of growth, the youngest leaves of the seedlings were used to extract DNA. Attempts to germinate seeds of the accessions for the remaining perennial species were unsuccessful. The plant material for them was the youngest dried leaflets, which were ground in liquid nitrogen and used to extract DNA (Weising et al. 1995). No significant degradation of DNA and differences in RAPD pat-

Table 1 *Cicer* species and their accessions used in RAPD analysis

Cicer species	No[a]	Accession	Source[b]	Origin[a]
C. montbretii Jaub.	1	Kaz dagi	This study	Çanakkale
C. isauricum Dav.	2	Kurucaova	This study	Konya
C. anatolicum Alef.	3	Kazanci	This study	Konya
	4	Erciyes	This study	Kayseri
C. incisum (wild) K. Mally	5	Yahyali	This study	Kayseri
C. pinnatifidum Jaub & Spp.	6	Besni	This study	Adiyaman
	7	Eregli	This study	Konya
	8	Avcilar	This study	K. Maras
	9	Erkenek	This study	K. Maras
	10	ILWC 33	ICARDA	Adana
	11	ILWC 236	ICARDA	Urfa
	12	ILWC 9	ICARDA	Elazig
	13	ILWC 250	ICARDA	Elazig
	14	ILWC 29	ICARDA	Gaziantep
	15	ILWC 78	ICARDA	Mardin
	16	ILWC 248	ICARDA	Diyarbakir
	17	ILWC 251	ICARDA	Diyarbakir
	18	Kir obasi	This study	Içel, Mut
C. echinospermum P.H. Davis	19	ILWC 239	ICARDA	Urfa
	20	ILWC 245	ICARDA	Urfa
	21	ILWC 246	ICARDA	Urfa
	22	TR 39241	AARI	Bingöl
	23	TR 54962	AARI	Diyarbakir
	24	ILWC 235	ICARDA	Gaziantep
	25	ILWC 238	ICARDA	Gaziantep
C. judaicum Boiss.	26	ILWC 148	ICARDA	Gaziantep
C. bijugum K.H. Rech.	27	ILWC 240	ICARDA	Urfa
	28	ILWC 241	ICARDA	Urfa
	29	ILWC 243	ICARDA	Urfa
	30	ILWC 8	ICARDA	Diyarbakir
	31	ILWC 177	ICARDA	Diyarbakir
	32	ILWC 62	ICARDA	Mardin
C. reticulatum Ladz.	33	ILWC 36	ICARDA	Mardin
	34	ILWC 182	ICARDA	Mardin
	35	ILWC 216	ICARDA	Mardin
	36	ILWC 218	ICARDA	Mardin
	37	ILWC 81	ICARDA	Diyarbakir
	38	ILWC 247	ICARDA	Adiyaman
	39	TR 54961	AARI	Siirt
C. arietinum L.	40	Canitez 89	AARI*	Anatolian
	41	Eser 87	AARI*	Agricultural
	42	Diyar 95	AARI*	Research
	43	Aydin 92	AARI*	Institute

[a] ICARDA: International Center for Agricultural Research in Dry Areas (ICARDA); Aleppo, Syria. Anatolian Agricultural Research Institute, Eskisehir; AARI: Aegean Agricultural Research Institute, Menmen, Izmir

[b] None of the accessions has same locality, and detailed collection addresses are not given here. They can be requested from the author

[c] The number coding each accession corresponds to the numbers in figures and graphs

terns were detected between fresh frozen and dried material when compared in *C. anatolicum* and *C. incisum*, for which both types of material were available.

The CTAB DNA extraction procedure, as described in Hulbert and Bennetzen (1991), was employed to isolate DNA from *Cicer* samples. The procedure was scaled down to use a 1.5-ml Eppendorf tubes. Briefly, the extraction procedure was as follows; leaf material was ground in liquid nitrogen until it becomes a fine powder using a pre-cooled pointed screwdriver in a 1.5-ml Eppendorf tube. The powder was suspended in 600–700 µl of 2 × CTAB extraction buffer (1.4 M of NaCl, 2% hexadecyltrimethylamonium bromide (Sigma), 1% β-mercapto ethanol, 100 mM of Tris–HCl, pH 8) and incubated in a water bath at 65 °C for 30–45 min. An equal volume of chloroform/isoamyl alcohol (24:1) was added to the mixture, inverted a few times and shaken slowly for 30 min at room temperature. The emulsion was centrifuged at 10,000 g for 10 min and the supernatant was transferred into a new centrifuge tube. Chloroform extraction was repeated twice and, before the last extraction, RNA was digested by adding 0.5 µl of RNAse A and incubating at 37 °C for 1 h. Supernatant was transferred into a fresh tube, and DNA was precipitated by adding 0.8 vol of isopropyl alcohol and inverting it several times. The DNA pellet was precipitated with a brief spin followed twice with 70% ethanol washes. The DNA pellet was air dried and dissolved in an appropriate volume of sterile ddH_2O. Concentrations of DNA samples were determined by running on 1% agarose gel with 1 µg of *Hin*dIII-digested λ DNA.

RAPD assay

Fifty 10-mer random oligonucleotide primers [10-mer Set 50/1(1)], obtained from Dr. John Hobbs (Biotechnology Laboratory, University of British Columbia, Vancouver, B.C., Canada), were used to amplify *Cicer* genomic DNA. Following an initial screening, seven primers that amplified clear and reproducible bands in all *Cicer* species were used to study RAPD variation in the accessions (see Table 2). The amplification reaction volume was 25 µl, each containing 10 mM of Tris–HCl (pH 9.0), 50 mM of KCl, 0.1% Triton X-100, 1.5 mM of $MgCl_2$, 100 µM of each dATP, dCTP, dGTP and dTTP, 2 µM of primer, 0.8 units of *Taq* DNA polymerase (Promega) and 30 ng of genomic DNA. PCR reactions were performed in a thermocycler with a heated lid (Techne Inc.) programmed for one cycle, consisting of an initial denaturation at 94 °C for 3 min, annealing at 36 °C for 1 min and an extension step at 72 °C for 2 min, followed by 39 cycles of a dena-

Table 2 Sequences of random primers used to amplify *Cicer* genomic DNA and deduce genetic relationships

Primer	Sequence 5′ → 3′	Number of RAPD bands	Size (bp)	
			Min.	Max.
UBC # 5	CCT GGG TTC C	14	250	1500
UBC # 6	CCT GGG CCT A	13	300	2000
UBC # 23	CCC GCC TTC A	12	450	1300
UBC # 30	CCG GCC TTA G	18	200	1500
UBC # 34	CCG GCC CCA A	16	150	1500
UBC # 38	CCG GGG AAA A	14	200	2500
UBC # 44	TTA CCC CGG C	8	300	850
Total		95		

turation step at 94 °C for 30 s, an annealing step at 36 °C for 1 min and an extension step at 72 °C for 2 min. Amplification was terminated by a final extension step of 5 min at 72 °C. PCR products were electrophoresed in 1.5% agarose gels and visualized by staining with ethidium bromide (0.4 μg/ml) and UV illumination. Molecular-weight markers were used to estimate the sizes of amplification products and to compare duplicate reactions.

Data analysis

RAPD assays were repeated twice for each primer and only the reproducible bands were scored, with specific attention to the repeatability of polymorphic bands. The amplification products at each band were treated as a separate character and scored as 1 (present)/0 (absent), and a rectangular binary data matrix of 43 × 95 was prepared. Statistical analyses of the data matrix were carried out using the NTSYS-pc (Ver. 1.7, Rohlf 1992). A similarity matrix was obtained using Dice's coefficient as in Nei and Li (1979), and it was converted into distances using the formula $GD_{ab} = -\ln(S_{ab})$, where S_{ab} is the measure of genetic similarity between accessions a and b and is defined as $S_{ab} = 2N_{ab}/N_a + N_b$, where N_{ab} is the number of common bands present in accessions a and b, and N_a and N_b are the sum of the scored bands in accessions a and b respectively. The distance matrix was then used for cluster analysis of *Cicer* accessions using the unweighted pair-group method with the arithmetic mean (UPGMA) procedure. Principle co-ordinate analysis (PCO) of pairwise genetic distances was carried out using SYN-TAX-pc (Ver. 5, Podani 1993) to determine the ability of 95 RAPD bands to display the inter-relationships among *Cicer* accessions and species.

Results

In the present study, genomic DNA isolated from accessions of different *Cicer* species was successfully amplified following optimization of the amount of template DNA, *Taq* DNA polymerase and $MgCl_2$ concentrations, and also the thermal profile of the amplification reactions. Fifty 10-mer primers were screened on individuals representing each species. Seven primers showing consistently reproducible and simple amplification products were selected to screen *Cicer* accessions (Table 2). Assaying RAPD variation in accessions of *Cicer* species with these primers yielded 95 bands (ranging 0.15–2.5 kb in size), 92 of which were polymorphic. Two of these RAPD patterns obtained using primers UBC # 1-30 and UBC # 1-44 are given in Fig. 1. The sequences of primers, the number of RAPD fragments they amplified, and their approximate size are given in Table 2. The number of bands varied from 8 to 18 with the primer employed. The primer UBC # 1-44 has amplified the lowest number of RAPD bands (Fig. 1B; Table 2), and primer UBC # 1-30 amplified the highest number of RAPD markers (Fig. 1A; Table 2). As is shown in Fig. 1A, a shift in RAPD patterns of three annual species (*C. echinospermum, C. reticulatum* and *C. arietinum*) were observed with three of the primers that we used.

Table 3 Genetic variability at 95 RAPD loci in five *Cicer* species, which were represented by more than one accessions

Cicer species	A[a]	P[b]	Mean gene diversity
C. pinnatifidum	1.284	28.42	0.0847 ± 0.150[c]
C. bijugum	1.073	7.37	0.0220 ± 0.006
C. echinospermum	1.136	13.68	0.0455 ± 0.118
C. reticulatum	1.168	16.84	0.0610 ± 0.143
C. arietinum	1.01	1.05	0.0039 ± 0.038
For 43 accessions	1.94	96.84	0.2412 ± 0.030

[a] A: Mean number of observed alleles
[b] P: Percent of polymorphic loci
[c] Standard errors

Genetic variation in species represented by more than one accession was measured in terms of the percent of polymorphic loci, the mean number alleles per loci and the mean gene diversity. RAPD variation was relatively high among accessions of different *Cicer* species, but variation within species was low. *C. pinnatifidum* represented by 13 accessions was the most polymorphic species followed by *C. reticulatum* and *C. echinospermum* (Table 3). The observed level of variation was comparable to the previous isozyme reports.

The RAPD data matrix was used to compute pairwise genetic distances of accessions according to Nei and Li's (1979) coefficient. These coefficients were employed to generate a dendrogram of *Cicer* accessions using UPGMA (Fig. 2). In the dendrogram, *Cicer* accessions formed two main clusters, one of which contained accessions of both perennial and annual species, and the other group had only accessions of three genetically close annual species. Within these groups, except *C. reticulatum* and *C. arietinum*, accessions of *Cicer bijugum, Cicer pinnatifidum, C. echinospermum* and *C. anatolicum* formed distinct species clusters. Accessions of *C. arietinum* and *C. reticulatum* did not form separate clusters in-

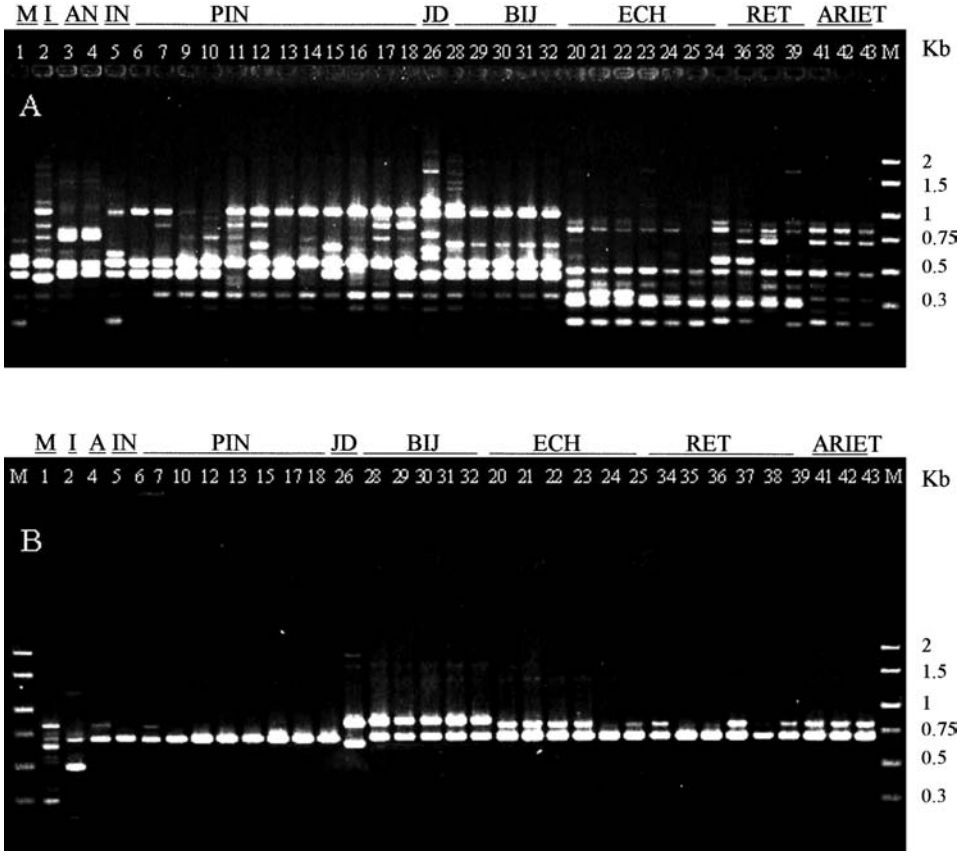

Fig. 1 Amplification patterns of cultivated and wild *Cicer* accessions generated by using arbitrary primers UBC # 1-30 (A) and UBC # 1-44 (B). *M* indicates the size marker, and the number given above each lane corresponds to the accession code listed in Table 1. Abbreviations for species are as follows: *M C. montbretii*; *I C. isauricum*; *A/AN C. anatolicum*; *IN C. incisum*; *PIN C. pinnatifidum*; *JD C. judaicum*; *BIJ C. bijugum*; *ECH C. echinospermum*; *RET C. reticulatum*; *ARIET C. arietinum*

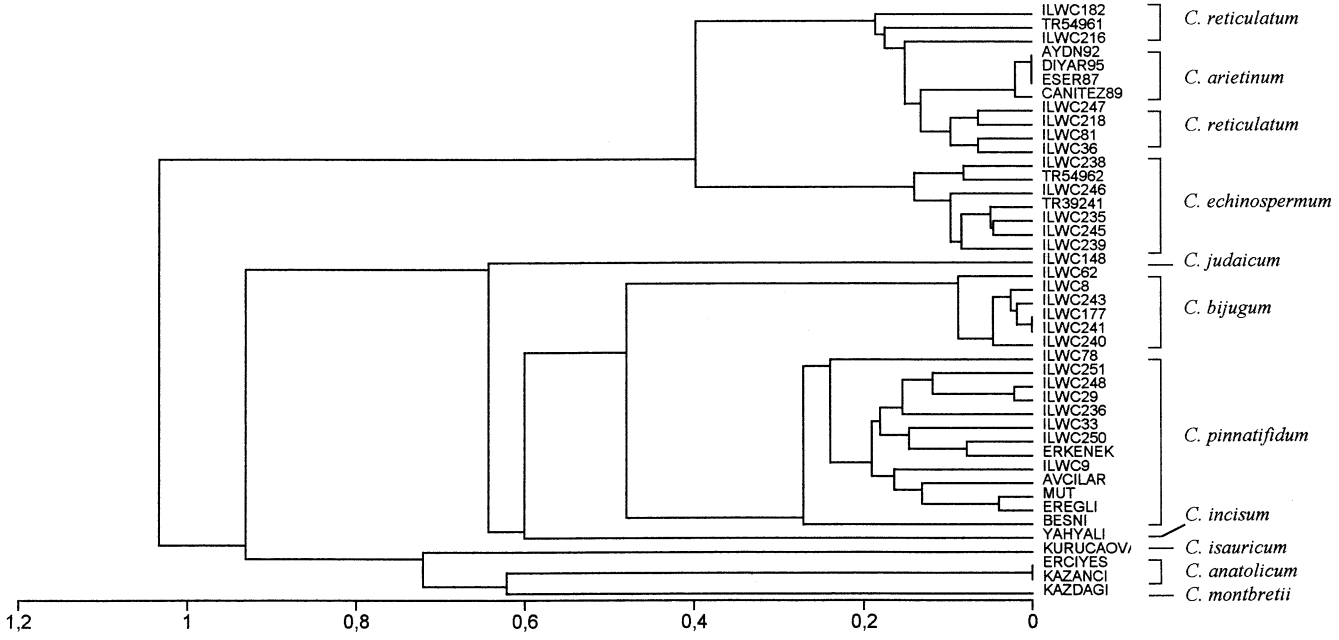

Fig. 2 Dendrogram showing the genetic relationships among 43 wild and cultivated *Cicer* accessions

dicating the close similarity among them. *Cicer judaicum*, which had distinct RAPD profiles with almost every primer, grouped outside the cluster containing the *C. pinnatifidum*, *C. bijugum* and the perennial species *C. incisum*. In addition, these species were found to be more closely related to perennial species than the group containing *C. echinospermum, C. reticulatum* and *C. arietinum*, which had very similar RAPD profiles and formed a separate cluster in the dendrogram (Fig. 2).

Mean genetic distances between *Cicer* species were computed by averaging the genetic distances for the re-

Table 4 Estimates of mean genetic distances between *Cicer* species computed by averaging the genetic distances between respective accessions

Cicer species	*C. mo.*	*C. isa*	*C. an*	*C. in.*	*C. pin.*	*C. ju.*	*C. bi.*	*C. ec.*	*C. re.*	*C. ar.*
C. montbretii	0.000									
C. isauricum	0.734	0.000								
C. anatolicum	0.622	0.715	0.000							
C. incisum	0.738	0.821	0.942	0.000						
C. pinnatifidum	0.895	0.715	0.986	0.598	0.142[a]					
C. judaicum	0.936	1.011	1.191	0.753	0.641	0.000				
C. bijugum	0.806	0.883	1.145	0.606	0.612	0.629	0.030			
C. echinospermum	0.979	1.155	0.964	0.945	0.928	1.124	0.990	0.066		
C. reticulatum	1.161	1.237	1.148	1.139	1.102	1.243	1.012	0.393	0.113	
C. arietinum	1.095	1.173	1.116	1.006	1.049	1.104	0.960	0.405	0.144	0.006

[a] Mean genetic distance within species

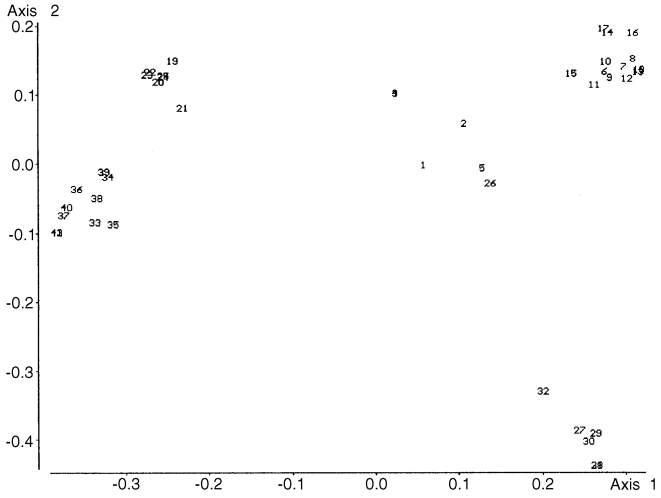

Fig. 3 Associations among 43 wild and cultivated *Cicer* accessions as revealed by principle co-ordinate analysis (PCO) based on genetic distance estimates, calculated for 95 RAPD bands. Axis 1 and Axis 2 correspond to first and second principle coordinates which account for 34.7 and 12.9% of the variation respectively. *Numbers* given in the plot represent the accessions listed in Table 1

spective accessions (Table 4). Among perennial species, *C. incisum* had the lowest mean genetic distance value to the annual species. Three species (*C. echinospermum, C. reticulatum* and *C. arietinum*) placed in the first branch of the dendrogram had considerably lower mean genetic distances ranging from 0.144 to 0.405 (Table 4, Fig. 2). *C. reticulatum* was the closest species to chickpea (0.144). Mean genetic distances within species with more than one accessions were also calculated by taking the averages of the distances for accessions.

In order to determine the ability of RAPD analysis to display genetic relationships among accessions and species with a minimum distortion, principle co-ordinate analysis (PCO) was carried out, and accessions were plotted in the coordinate system for the first two coordinates which accounted for 34.7 and 12.9% of the variation respectively. PCO provided a better graphical illustration and a clear separation of species (Fig. 3). Except for *C. arietinum* and *C. reticulatum*, the accessions of the remaining species formed distinct groups and separated from each other. In addition, similarity matrices obtained using Nei and Li's formula (1979) and Jaccard's coefficient was compared to assess the equivalence of two similarity calculation methods using the matrix comparison procedure of SYN-TAX-pc, and nearly a total correlation was found ($r = 0.993$).

Discussion

Various approaches ranging from morphology to molecular techniques have been used to infer patterns of diversity and relationships among plant species. RAPD analysis is one of these approaches which was used in a number of plant taxa to detect inter- and intra-species DNA variation and genetic relationships (Skroch et al. 1993; Abo-elwafa et al. 1995; Link et al.1995; Sharma et al.1995a; Butos et al. 1998). In *Cicer*, Ahmad (1999) studied relationships among annual species with RAPD markers using bulked DNA samples from a single accession for each species. In the present study, RAPD markers were used to deduce intra- and inter-species relationships and examined RAPD variation more comprehensively in 43 wild and cultivated accessions representing ten *Cicer* species (annuals and perennials). This is the first study covering annual and perennial species for assessing inter-species DNA variation (in some, intra-species variation) and genetic relationships in the genus. Of the 50, 10-mer primers screened for their ability to amplify *Cicer* genomic DNA and amplification, products were obtained in about 15 of them. Among these primers, a few failed to amplify DNA in some species, and others did not have clear RAPD profiles, and were excluded from the analysis. Apparently, some of them required different amplification conditions than we used in this study. Seven primers with clear and scorable patterns were used to screen *Cicer* accessions (Table 2). However, if optimized, individually the other primers could also be used.

There are some concerns regarding the use of RAPDs in the analysis of genetic relationships. First, their reproducibility has been questioned because of the sensitivity

of DNA amplification with arbitrary primers to reaction conditions. Maintenance of consistent reaction conditions is critical for reproducible RAPD profiles. Second, the dominant inheritance of RAPD markers leads to the loss of information when compared to co-dominant markers. Last, the uncertainty of homology among the co-migrating amplification products was observed among different individuals or species. The homology of co-migrating amplified fragments can be determined either by Southern analysis using one of the bands as a probe (Thormann et al. 1994; Weising et al. 1995) or cleaving a shared RAPD band with 4-bp- or 6-bp-cutter restriction enzymes to see that the same cleavage patterns for the same band could be obtained among different genotypes (Butos et al. 1998). These studies indicate that nearly 20% of the co-migrating bands appear not to be homologous. In our PCR experiments, following the optimization of several parameters, *Cicer* genomic DNA was amplified reproducibly and clear RAPD profiles were obtained (Fig. 1A, B). These aspects of RAPD analysis were taken into account during data collection and analysis, and all shared bands were assumed to be homologous to each other.

The RAPD-based dendrogram of *Cicer* accessions displayed the genetic relationships between annual and perennial species among the members of each of these groups, as well as the accessions for each species (Fig. 2). Relationships among six annual species obtained by RAPD analysis were generally in agreement with the previous studies of relationships which were carried out using various approaches, ranging from crossability to isozyme analysis (Ladizinsky and Adler 1976; Kazan and Muehlbauer 1993; Ahmad and Slinkard 1992; Ahmad et al. 1992; Tayyar and Waines 1996). However, different groupings were observed in two perennials when compared with previous studies (Kazan and Muehlbauer 1993; Tayyar and Waines 1996). Recently, using the same accession collection we have studied the allozyme variation in ten *Cicer* species and, despite some differences overall, grouping of the species in this study is in agreement with the phenetic tree based on our allozyme data (Sudupak and Kence, unpublished results). One of these differences was the grouping of three annual species (*C. bijugum, C. pinnatifidum* and *C. judaicum*) with perennials, but they formed a separate cluster on the branch bearing the perennials. When considering that species/accessions on the same cluster are closer to each other than the species/accessions on different clusters, these annuals are closer to perennials than the remaining annual species, based on RAPD analysis. This similarity was also apparent in the patterns generated by four of the seven primers. Within this cluster, the grouping of *C. judaicum* and *C. incisum* was another significant difference in the dendrogram. Previous allozyme and seed storage protein-variation studies, including our analysis, have indicated that *C. judaicum* is closer to *C. pinnatifidum*; but both Ahmad (1999) and the grouping we observed in this study surprisingly placed this species further away from the accessions of *C. pinnatifidum*, even outside the perennial species *C. incisum* (Fig. 2). Other differences were in the grouping of two perennial species *C. incisum* and *Cicer isauricum*. Similar to the allozyme analysis, *Cicer incisum* was the closest perennial to the annual species, but it closely clustered with three annual species in the first branch of the dendrogram, and as a group they were closer to the cluster containing perennial species than the branch containing the remaining annuals (Fig. 2). *C. isauricum* and two other perennial species (*C. anatolicum* and *Cicer montbretii*) formed a separate cluster, consistent with our previous analysis (Sudupak and Kence, unpublished results). In contrast to the allozyme study, RAPD analysis revealed that *C. anatolicum* is closer to *C. montbretii* than *C. isauricum*. Ahmad (1999) observed striking differences between grouping based on RAPD analysis and previous analysis of relationships among annual species. Although some differences were apparent as discussed above, generally, our grouping was compatible with our previous analysis of relationships among both annual and perennial *Cicer* species. Only the six annual species are common to both studies, and we do not know the placements of the annual species that were not studied here with respect to the annuals and perennials included in this study. In addition to the cluster analysis, principle co-ordinate analysis (PCO) was carried out to determine whether associations among accessions of *Cicer* species could be resolved further. PCO analysis grouped the accessions according to their respective species with the exception of the grouping observed among accessions of *C. arietinum* and *C. reticulatum*, similar to the cluster analysis (Fig. 3). Overall, PCO analysis using the first two principle coordinates provided a good grouping of accessions and species in the co-ordinate system, confirming the relationships in the dendrogram.

Differences observed between the RAPD-based tree and allozyme-based tree could be due to several reasons. The number of loci sampled in the two studies, the principle differences in the two techniques in sampling variation at different levels and loci from different portions of the genome, and the error associated with each technique and the method of estimating genetic distances, could cause these differences. Allozymes are products of structural genes and represent a proportion of the variation in coding regions. They may be under some selective processes. However, the RAPD technique scans anonymous DNA sequences and presumably covers the entire genome, and represents variation at the DNA level (Williams et al. 1990). In this respect, RAPD analysis appears to be less restrictive in surveying polymorphism in the entire genome. Hence, it is an efficient and quick method of screening DNA sequence polymorphisms at a very large number of loci. Despite the fact that the error associated with RAPD analysis could be largely due to the characteristics of RAPDs, the number of markers used to deduce relationships and the features of RAPDs mentioned above suggest that the RAPD-based tree could be more reliable.

RAPD analysis also enabled us to measure the genetic diversity among accessions of different species and among accessions within species, which were represented by more than one accession. The percent of polymorphic loci and the mean heterozygosity are the two parameters commonly used to measure genetic diversity. In general, estimated values of these parameters among accessions of different species were considerably high. However, the genetic diversity within species was limited. *C. arietinum* had the lowest, while *C. pinnatifidum* had the highest, genetic variability when compared to three other annual species (Table 3). The distribution of RAPD variation somewhat reflects the presence of limited intra-species variation. Previous studies also suggest that there is a correlation between the level of intra-specific RAPD variation and the reproductive system. Autogamous species are generally characterized by large inter-population RAPD variation (Kazan et al. 1991). This aspect of RAPD variation parallels the level of allozyme variation, and the magnitude of it appears to be close to the values reported for wild and cultivated lentil species (Sharma et al. 1995a).

When the information obtained in this study and in our previous analysis of allozyme variation among annual and perennial species are considered together, one could speculate on the evolution of the annual habit from the perennial state in the genus. In both studies, among perennial species, *C. incisum* is the genetically closest species to the annuals. Classification of this species with one annual species (*Cicer chorassanicum*) in the *Chamocicer* section based on morphology and life cycle also supports this grouping. Close clustering of *C. reticulatum* accessions with *C. arietinum* lines in both studies is consistent with the presumed progenitor hypothesis. Thus, both allozyme and RAPD variation reveal the same information regarding the evolution of life cycle in the genus *Cicer* and the closest wild species of *C. arietinum*. However, the observed relationships were estimated using accessions collected from Turkey, and it only includes approximately a quarter of the known wild species in the genus. The species spectrum of this study is insufficient to generalize the observed relationships to the entire genus. In addition, only the four *C. arietinum* lines were included to assess the grouping of this species with respect to annuals and perennials. Observed RAPD variation was not sufficient to say that RAPD analysis can be used to differentiate chickpea accessions. Among four varieties, only one could be differentiated from the other three, and the observed low level of variation somewhat reflects the limited genetic variation in this species.

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