General and Applied Plant Physiology – 2009, Volume 35 (1–2), pp. 03–12

Available online at www.bio21.bas.bg/ipp/

PHYLOGENETIC DIVERSITY AND RELATIONSHIP AMONG ANNUAL *CICER* SPECIES USING RANDOM AMPLIFIED POLYMORPHIC DNA MARKERS

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Received: *07 March 2008* Accepted: *02 December 2008*

Summary. Genetic variation among cultivated chickpea (*Cicer arietinum* L.) and six other related annual wild species of *Cicer* was evaluated with random amplified polymorphic DNA (RAPD). Among the 42 random 10-mer primers tested, only 9 amplified genomic DNA across all species. Three main species groups were identified by UPGMA clustering using Nei's pair-wise distance calculations. Group I included the cultivated species *C. arietinum*, *C. reticulatum* and *C. echinospermum*. Within this group, *C. reticulatum* accessions were clustered closest to the *C. C. arietinum*. *C. yamashitae*. The second cluster was separated from the other clusters. Group III (the annual tertiary group) included *C. judaicum, C. pinnatifidum* and *C. cuneatum*. These results were in accordance with the common hypothesis that *C. reticulatum* was the progenitor species of cultivated chickpea. Our results also show that RAPD markers can be used for studying *Cicer* species and largely confirm the known relationships among taxonomic units in this genus.

Keywords: chickpea, annual *Cicer* species, genetic diversity, RAPD, DNA fingerprinting.

INTRODUCTION

The genus *Cicer* belongs to the family L*eguminosae*, subfamily P*apilionoideae*, tribe *Cicereae* Alef and comprises 43 species, nine of which being annual including chickpea (*Cicer arietinum* L.), while the rest are perennial (Vander Maesen, 1987). Chickpea is currently cultivated in over 40 countries worldwide and grown on 11 million hectares producing around 9 millions Mt in 2005 growing season (FAO, 2006). Two main types of chickpea are useful for plant breeding

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purposes, "Kabuli" (white flower, large and cream colored seeds) and "Desi" (purple flowers, small angular and dark seeds). World germplasm collections of cultivated chickpea are lacking in diversity that may include traits needed for effective improvement of the crop (Robertson et al., 1997). It has been recognized that interspecific hybridization will increase the variation and can be useful for the plant breeding purpose in a recalcitrant crop like chickpea (Singh et al., 1994; Van Rheenen et al., 1993). This is further exemplified by the utilization of two related wild species, *C. reticulatum* and *C. echinospermum*, in plant breeding programs (Singh and Ocampo, 1993, 1997; Singh et al., 1994). The better understanding of the genetic diversity present within cultivated species and its wild relatives is critical for crop improvement studies. The use of wild species had a significant implication in improving several characteristics of major cereals and tomato (Xiao et al., 1996; Hoisington et al., 1999). As an important grain legume, investigations of relatedness and genetic diversity within and among species of genus *Cicer* and its effective evaluation are an obvious necessity to identify a new source of germplasm bearing valuable genes, which may be used in the improvement of the chickpea cultivars. It is generally accepted that the average yield potential of chickpea is low 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 experiment to improve chickpea (Singh and Ocampo, 1997). Seed-protein electrophoresis (Ladizinsky and Adler, 1975; Vairinhos and Murray, 1983; Ahmad and Slinkard, 1992) and isozyme analysis (Kazan and Muehlbauer, 1991; Ahmad et al., 1992; Labdi et al., 1996; Tayyar and Waines, 1996) have been used to establish genetic relationships among the *Cicer* species. These studies revealed minimal intra-specific polymorphisms, particularly within *C. arietinum*. However, the availability of a large number of polymorphic markers is required for progress in any kind of genetic studies, such as diversity or linkage analyses. Random amplified polymorphic DNA (RAPD) analysis has been applied to study genetic relationship among nine annual *Cicer* species (Ahmad, 1999; Iruela et al., 2002; Sudapak et al., 2002). It has been shown that RAPD markers can be a useful tool for studies of phylogenetic relationships within *Cicer* spp. 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 the results of cross ability studies (Ladizinsky and Alder, 1976a, b; Singh and Ocampo, 1993). The aim of the present study was to use RAPD markers to asses the intra- and inter-specific genetic relationships among accessions of annual *Cicer* species and the determination of the wild species closest to the cultivated species.

MATERIALS AND METHODS

Plant material

The plant material used in this study was obtained from the International Center for Agricultural Research in Dry Area (ICARDA), Alepo, Syria and included 19 wild *Cicer* accessions representing seven annual *Cicer* spp. The origin and the source of each accession are given in Table l.

Genomic DNA extraction

Three seeds of each accession were sown in pots and after 10 days total genomic DNA was extracted from 2 g of young leaves collected from one of the plants of each accession using the CTAB method according to Lassner et al. (1989) with the modification described by Torres et al. (1993). The DNA final concentration was determined by agarose-gel electrophoresis using a known concentration of uncut λ DNA as a standard.

RAPD analyses

RAPD analyses were performed using 42 random 10-mer oligonucleotide primers from Roche Technologies (GRE). The amplification reaction was carried out in 25 µl volume containing 15 ng of genomic DNA, 10 mM Tris-HCL (pH 9.0), 50 mM KCl, 0.1 % Triton X-100, 1.5 mM $MgCl_2$, 0.1 mM each of dATP, dCTP, dGTP and dTTP, 2 mM primer, 1 unit of Taq DNA

Table 1. Accession, source and origin/collection site of the annual *Cicer* species used for RAPD analysis.

Species	Accession	Source[a]	Origin
C. echinospermum	ILWC35	ICARDA	Turkey
	ILWC181	ICARDA	Turkey
	ILWC288	ICARDA	Turkey
C. reticulatum	ILWC36	ICARDA	Turkey
	ILWC231	ICARDA	Turkey
	ILWC114	ICARDA	Turkey
C. pinnatifidum	ILWC49	ICARDA	Syrian
	ILWC225	ICARDA	Turkey
	ILWC212	ICARDA	Syrian
C. judacium	ILWC38	ICARDA	Lebanon
	ILWC46	ICARDA	Syrian
C. cuneatum	ILWC37	ICARDA	Ethiopia
C. yamashitae	ILWC55	ICARDA	Afghanistan
	ILWC215	ICARDA	Afghanistan
	ILWC214	ICARDA	Afghanistan
C. arietinum	Jam	ICARDA	Iran
	Kaka	ICARDA	Iran
	Pirooz	ICARDA	Iran
	FLIP97-111C	ICARDA	Syrian

[a]ICARDA, International Center for Agricultural Research in Dry Areas

polymerase (Sinagene). Amplifications were carried out in a thermo-cycler (Eppendorf) programmed for 35 cycles with an initial strand separation at 94°C, 1 min at 37°C and 2 min 72°C. After 35 cycles, there was a final extension step of 8 min at 72°C. Amplification products were separated in 2 % agarose gel and detected by staining with ethidium bromide. Standard molecular weight markers were used in each electrophoresis run.

Data Analysis

The RAPD bands were visually scored as either present (1) or absent (0) for each accession and each primer. The binary matrix was then used to measure the pair-wise genetic distance using Nei's (1978) unbiased genetic distance within POPGENE version 1.32 (Yeh et al., 1997). A dendrogram showing the genetic relationships among 19 accessions was constructed using the pair-wise genetic distance values based on the unweighed pair-group method with NTSYS-pc software (Ver. 1.7, Rohlf 1992).

RESULTS AND DISCUSSION

Out of 42 RAPD primers, 9 produced 81 reproducible polymorphic fragments (Table 2). The RAPD primer OPB10 generated the highest number of polymorphic bands (13 fragments) (Fig. 1) and the lowest (3 fragments) were generated by OPQ05 primer (Table 2). Estimates of pair-wise genetic distances among studied species ranged from 0.232 to 0.689 with an average of 0.392. (Table 3). The lowest genetic distance (0.232) was observed between *C. judacium* and *C. pinnatifidum*, whereas the highest genetic distance (0.689) was observed between *C. cuneatum* and *C. echinospermum*. The dendrogram constructed by the UPGMA method demonstrated that three main sub groupings existed in the collection (Fig. 2). Group I (the primary and secondary cross ability groups) documented by Ladizinsky and Adler (1976) clustered *C. arietinum* between *C. echinospermum* and *C. reticulatum*. *C. yamashitae*, the second cluster, was separated from the

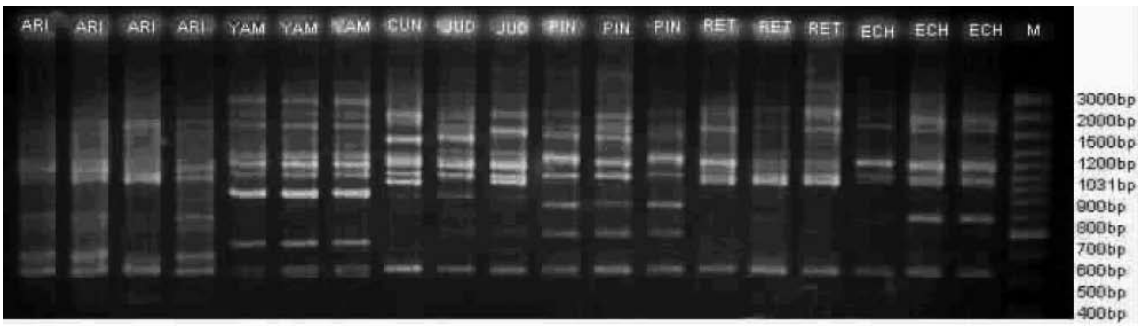

Fig. 1. Genomic DNA amplification pattern in seven annual C*icer* species with 10-mer random primer OPB-10 (**M** molecular-weight marker, **ECH** *C. echinospermum*, **RET** *C. reticulatum*, **PIN** *C. pinnatifidum*, **JUD** *C. judacium*, **CUN** *C. cuneatum*, **YAM** *C. yamashitae*. **ARI** *C. arietinum*).

Table 2. Sequence of random primers used to amplify *Cicer* genomic DNA and deduce genetic relationship.

Primer	Primer DNA sequence (5'-3')	Percentage G+C content	Approximate size of fragments [kb]	No. of polymorphic bands
PC05	GATGACCGCC	80	370-2600	9
OPZ-10	CCGACAAACC	60	350-1750	9
OPM-05	GGGAACGTGT	60	280-1800	10
OPJ-20	AAGCGGCCTC	70	300-1650	10
OPB-10	CTGCTGGGAC	70	330-2400	13
OPAC-09	AGAGCGTACC	60	290-2100	9
Bio-27	TGACGCGCTC	70	240-1800	10
OPI-13	CTGGGGCTGA	70	420-1700	8
OPQ-05	CCGCGTCTTG	70	320-1480	3

other clusters. Group III (the annual tertiary group) included *C. judaicum, C. pinnatifidum* and *C. cuneatum*.

Intra- and inter- annual *Cicer* specific genetic relationships

Many chickpea breeding programmes are focused on improving the genetic potential both to increase yield and to provide protection against abiotic and biotic stresses. In order to enhance genetic potential, there must be a comprehensive understanding of the amount and pattern of genetic variation that exists within and between the available cultivated and wild accessions. To date there have been very few reports investigating the level of genetic variation between the accessions of *Cicer* and wild *Cicer* for the generation of intraspecific or interspecific populations of chickpea. Although *Cicer* species are predominantly self-pollinating, more variation was observed among them. Considerable variation was observed between wild accessions and cultivated chickpea in RAPD analysis. Iruela et al. (2002) showed that RAPD markers successfully identified genetic variation in *Cicer*. The variation identified was greater than that observed by the isozymes or seed storage proteins used in previous studies of genetic relationships among annual *Cicer* species (Ahmed et al., 1992; Labdi at al., 1996).

Wild *Cicer* species closest to the cultivated species

Our results further proved a close genetic relationship among *C. arietinum, C. reticulatum* and *C. echinospermum* at the DNA level and confirmed the common hypothesis that *C. reticulatum* is the progenitor species of cultivated chickpea, however, previous studies using crossability, karyotype, isozyme and seed protein analyses reported the close genetic relationship among *C. arietinum*, *C. reticulatum* and *C. echinospermum* (Ladizinsky and Alder, 1976; Ohri and Pal, 1991; Ahmad et al., 1992; Tayyar

Table 3. Pair-wise genetic distance between *Cicer* species based on RAPD analysis.

Cicer species	*C. reticulatum*	*C. echinospermum*	*C. yamashitae*	*C. judacium*	*C. pinnatifidum*	*C. cunnetum*	*C. arietinum*
C. reticulatum	-						
C. echinospermum	0.243	-					
C. yamashitae	0.355	0.525	-				
C. judacium	0.331	0.476	0.390	-			
C. pinnatifidum	0.349	0.561	0.463	0.232	-		
C. cunnetum	0.435	0.689	0.480	0.458	0.251	-	
C. arietinum	0.237	0.355	0.304	0.304	0.318	0.494	-

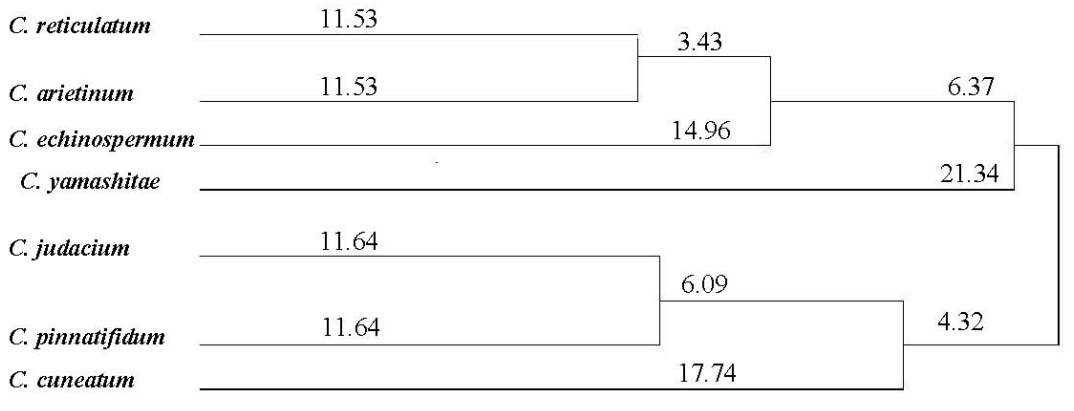

Fig. 2. Dendrogram of genetic relationships between seven annual *Cicer* species.

and Waines, 1996; Iruela et al., 2002; Rajesh et al., 2002; Sudupak at al., 2002; Nguyen et al., 2004). The implication of this information is that perhaps it might be relatively less experimental to utilize these two species for cultivated chickpea improvement. Closely related species commonly lose the ability to interbreed and become genetically isolated due to chromosomal structural mutations (Tayyar and Waines, 1996). Hybridization between *C. arietinum* and *C. reticulatum* showed normal chromosomal pairing and complete fertility (Ladizinsky and Alder, 1976; Singh and Ocampo, 1993), whereas those with *C. echinospermum* showed a reciprocal translocation difference and partial to complete sterility (Ladizinsky and Alder, 1976). Clearly, being the closest relative to the cultivated species, *C. reticulatum* is the prime candidate for the progenitor species.

C. cuneatum has been placed quite distinct from all other studied species. In our study, this spices had a very distinct RAPD amplification pattern as reported by Ahmad (1999), isozyme profile (Kazan and Muehlbauer, 1991; Ahmad et al., 1992), seed protein profile (Ahmad and Slinkard, 1992) and peculiar morphological features (Robertson et al., 1997). Furthermore, Robertson et al. (1997) reported that *C. cuneatum* was the only species that had a climbing growth habit, leaves that end in branched tendril, elliptical to pods and rounds seeds that lack the characteristic beak. An important goal in chickpea research remains the construction of detailed genetic linkage maps. However, in order to detect significant polymorphism, previous researchers using isozyme, RFLPs and morphological markers have focused on interspecific crosses of cultivated chickpea with the wild species *C. echinospermum* and *C. reticulatum* (Gaur and Slinkard, 1991; Kazan et al., 1993; Simon and Muehlbauer, 1997). In these cases of interspecific-drived mapping population and the segregation of markers sometimes deviated significantly from expected ratios (Gaur and Slinkard, 1991; Kazan et al., 1993).

Conclusion remarks

Chickpea has been characterized as a species with poor genetic variability (Ahmad et al., 1992; Van Rheenen, 1992; Simon and Muehlbauer, 1997), but it has recently been shown that ample genetic diversity exists for a short sequence tandem repeat (Weising et al., 1992; Sharma et al., 1995b). Thus, it is expected

that the utilization of a large number of accessions and the increased resolution associated with the large number of potential arbitrary primers available to the RAPD approach may provide sufficient markers to construct a genetic linkage map between carefully chosen cultivated chickpea accessions. Skewed segregation could thus be avoided and linkage data of more immediate relevance to chickpea breeding be provided.

Acknowledgements: We thank Mr. Seyed Mostafa Pirseyedi for providing software and for his helpful comments to cluster analyses.

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