The use of microsatellite markers in the annual and perennial Cicer species growing in Turkey

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Abstract: Several types of molecular markers have been used in plant breeding and genetic diversity for a wide range of applications. Generally, single-locus co-dominant markers are preferred, because they make it possible to tag and map the same loci in many different populations and even species. Probably the best markers in this respect are microsatellites. The analysis based on microsatellites in our study has revealed the usefulness of these markers in the identification of polymorphism in chickpea genome, which was earlier thought to be less polymorphic. Such markers will be highly efficient in identifying specific markers linked to the trait of interest. The plant material used in this study included 43 wild and 2 cultivated *Cicer* accessions representing annual and perennial *Cicer* with distribution in Turkey. Ten STMS primers were selected from *Cicer* species depending on their ability to amplify genomic DNA in all species. Separation of the PCR products on agarose gels revealed single bands of the expected size with 10 of the primer pairs. DNA from *C. arietinum*, *C. reticulatum*, *C. echinospermum*, *C. pinnatifidum*, *C. bijugum*, and *C. anatolicum* amplified with the primers GA2, GA24, TA13, GAA47, TA46, TA130, TA72, TA146, TS54, TS72 respectively, were sequenced and compared with the chickpea sequence. The DNA of one accession for each species has been amplified with 45 chickpea-derived STMS primer pairs. Amplification resulted either in the presence or absence of products. For other STMS loci, only three STMS/species combinations were successful which could be used as specific markers (GAA47, TS54 and TA72). We examined whether and to which extent STMS primers designed for the cultigen could also be applied to genome analysis of wild *Cicer* species

Key words: chickpea, DNA isolation, molecular markers, STMS.

Abbreviations: PCR – polymerase chain reaction, RAPD – random amplified polymorphic DNA, SSR – simple sequence repeat, STMS – sequence tagged microsatellite site.

Introduction

The genus *Cicer* belongs to the family *Fabaceae* and the tribe *Cicereae* Alef. It includes 33 perennials, eight annuals, and one unspecified wild species as well as cultivated chickpea. *Cicer* species are predominantly self-pollinating, and the chromosome number of annual species is diploid $2n = 16$ (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 and East Africa, Southern Europe, Australia, South and North America (Singh, 1997). Chickpea most probably originated from an area of present-day south-eastern Turkey and adjoining areas of Syria. Turkey is one of the largest chickpea exporter providing 31% of the world's export (FAO 2000). 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 & Cubero, 1978; Gil & Cubero, 1993). The two main types differ in several important traits. In order to exploit the variation in these two types, Desi × Kabuli crosses were carried out by breeders; however, the transfer of genes between the two major types was slow (Maynez et al., 1993). Resistance to biotic and abiotic stresses was found in wild *Cicer* species (Singh, 1990; Singh et al., 1994). Chickpea breeding aims at high yield combined with resistance to biotic stresses and tolerance to drought and cold. Despite its agronomical importance and the international efforts in conventional breeding, productivity of the crop has not yet been significantly

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improved. In recent years, use of molecular markers has facilitated breeding of crop plants, including resistance breeding (Winter & Kahl, 1995). Molecular marker technology has made it possible to generate a genetic map of chickpea that is promising for use in marker-assisted selection and positional cloning of agronomically important genes (Udupa et al., 1993; Labdi et al., 1996). Several groups have studied the genetic diversity and relatedness among annual *Cicer* species by means of hybridization, electrophoresis of seed proteins and isozymes (Ladizinsky & Adler, 1976; Kazan & Muehlbauer, 1991; Ahmad et al., 1992; Labdi et al., 1996; Tayyar & Wainess, 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 (*C. reticulatum*) and (*C. echinospermum*). The second group included *C. pinnatifidum* Jaub., *C. judaicum* Boiss., and *C. bijugum.* The remaining two annual species (*C. cuneatum* and *C. chorassanicum*) formed the last two groups (Sudupak et al., 2002). *C. soongaricum*, *C. anatolicum* and *C. yamashitae* (Singh, 1997). Recently random amplified polymorphic DNA (RAPD) analysis has 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 the genus *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 inter simple sequence repeat (Ratnaparkhe et al., 1998), sequence-tagged microsatellite sites (Sant et al., 1999; Udupa et al., 1999; Winter et al, 1999) have revealed a high level of polymorphism and have been used to estimate variability within cultivated genotypes. Microsatellites are short stretches of tandem repeats of simple DNA sequence units, each 1–5 bp long. Such microsatellites are dispersed in all eukaryote nuclear genomes (Goodfellow, 1992) as well as in some prokaryote genomes (Field & Wills, 1996). All evidence suggests that these sequences exhibit exceptional levels of polymorphism, due to variability in the number of tandem repeats at a given locus (Schlötterer et al., 1997). In recent years microsatellites have become the marker of choice for many applications. Their abundance, high level of repeat-number polymorphism, manifested as the occurrence of a large number of alleles per locus, and co-dominant inheritance has facilitated their extensive use in genome mapping (Dib et al., 1996). Recently, Hüttel et al. (1997) and Winter et al. (1997) developed locus-specific (sequence tagged) microsatellite markers in chickpea. STMS markers are used for the subject, microsatellites are abundant and highly polymorphic, and therefore chosen for studies of both basic and applied genetics in chickpea (Anonymous, 1996).

The aim of the present study was to use STMS markers determination of the wild species closest to the cultivated *Cicer* (perennial and annual species) growing in Turkey. It is the first report on the use of microsatellite markers for DNA fingerprinting of chickpea genotypes. Furthermore, the primer sets designed for chickpea in this work turned out to be useful markers for breeding program purposes and identification of wild and cultivated *Cicer* accessions what was the aim of this work.

Table 1. Accessions and source of the annual and perennial chickpea genotypes used in this study originated from Turkey.

Species	Accessions	Source
Annual		
C. arietinum	ILC502	ICARDA
	ILC484	ICARDA
	Canıtez89	AARI
	Eser87	AARI
	Aydın92	AARI
	Damla	AARI
	FLIP84-92C(3)	WSU
	ILC3560	ICARDA
C. reticulatum	ILWC242	ICARDA
	ILCW36	ICARDA
	ILCW114	ICARDA
	ICCW45	ICARDA
	W62074	ICARDA
	ILWC231	ICARDA
	ILWC36	ICARDA
	ILWC182	ICARDA
	ILWC216	ICARDA
	ILWC218	ICARDA
	ILWC81	ICARDA
	TR54961	CIFC
	PI599072	WSU
C. echinospermum	TR39241	CIFC
	ILWC246	ICARDA
	ILWC235	ICARDA
	PI489776	WSU
	W61984	CIFC
C. pinnatifidum	IG73054	ICARDA
	PI458556	WSU
	PI510654	WSU
	ILWC33	ICARDA
	ILWC236	ICARDA
	ILWC250	ICARDA
	ILWC248	ICARDA
	ILWC251	ICARDA
C. bijugum	ILCW70	ICARDA
	ILCW34	ICARDA
	W610150	ICARDA
	ILWC240	ICARDA
	ILWC241	ICARDA
	ILWC243	ICARDA
	ILWC8	ICARDA
Perennial		
C. anatolicum	PI561078	WSU
	PI383626	WSU
	Kazancı	ICARDA
	W614183	WSU

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Table 2. Sequences of microsatellite primers used to amplify *Cicer* genomic DNA.

Marker	Linkage Group	Molecular weight (bp)	Sequence
GA 2	2	144	TGCATTGGAAATACAGCATGA/ AATTTTGGTTCGCCACAAAC
GA 24	2	203	TTGCCAAAACCAATAACTCTG/ TCCCTTTTACACAAGGCCAG
TA 13	2	243	TAAGTTAAGGGACCAACGAA/CAAGTT GGAGTCAAACCAAT
GAA 47	2	169	CACTCCTCATGCCAACTCCT/ AAAATGGAATAGTCGTATGGGG
TA 46	2	152	TTTATTGCAATAAAACTCATTTCTTATC/ TTCTTTTTGTGTGAAAAAAAAATATAGTGA
TA 130	2	219	TCTTTCTTTGCTTCCAATGT/ GTAAATCCCACGAGAAATCAA
TA 72	2	256	GAAAGATTTAAAAGATTTTCCACGTTA/ TTAGAAGCATATTGTTGGGATAAGAGT
TA 146	2	161	CTAAGTTTAATATGTTAGTCCTTAAATTAT/ ACGAACGCAACATTAATTTTATATT
TS 54	2	209	TACAAGTTAAAAATGAATAAATATTAATA/ GAAATTTAGAGAGTCAAGCTTTAC
TS 72	2	264	CAAACAATCACTAAAAGTATTTGCTCT/

STMS primers are described in WINTER et al. (1999).

Material and methods

Plant material

The plant material used in this study included 43 wild and 2 cultivated *Cicer* accessions representing annual and perennial *Cicer* species with distribution in Turkey. The origin and the source of each accession for each *Cicer* species are given in (Tab. 1).

Molecular materials

STMS primers were selected from Linkage Group 2 in this study (Tab. 2). All microsatellite primers are described in WINTER et al. (1999). STMS primers were synthesized by Invitrogen, (Invitrogen, PO. BOX: 2312 Groningen, England).

DNA Extraction

Plants were grown in the greenhouse, and DNA was isolated from young leaflets using a modified cetyl-trimethylammoniumbromide (CTAB) protocol (WEISING et al., 1995). The DNA final concentration was determined by agarose gel electrophoresis using known concentrations of uncut λ DNA Hind III as standard.

Microsatellite Analysis

Primers were 22–32 nucleotides long with annealing temperatures around 55 ℃. Thirty-five cycles of PCR were performed on 50 ng DNA in 20 μL of ethidium-bromide reaction buffer containing 2 μM primers, 1.5 μM $MgCl_2$, 250 μM dNTP and 0.4 unit of Taq DNA polymerase. Initial denaturation of DNA at 96 ℃ for 2 min was followed by 35 cycles of denaturation at 96 ℃ for 20 s and annealing of primers at 55 ℃ for 50 s and elongation at 60 ℃ for 50 s, and a final extension at 60 ℃ for 5 min. The final elongation segment was held for 8 min. Polymerase chain reactions were carried out in a PTC-100 thermocyler (MJ Research, USA). The PCR products were separated electrophoretically in 2% (w/v) agarose gel using 1xTBE buffer (SAMBROOK et al., 1989). Amplification products were separated on 2% agarose gels in 1xTBE buffer. Banding patterns were visualized on a UV – transilluminator after staining the gels with ethidium-bromide. To score the loci, STMS gels were photographed on Polaroid 667 black and white films. All STMS reactions were repeated at least two times.

Result and discussion

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 thermal profile of the amplification reactions. Ten primers (GAA47, TS54, and TA72) showing consistently reproducible and simple amplification products were selected to screen *Cicer* accessions (Tab. 3) and (Fig. 1). Various approaches ranging from morphology to molecular techniques were used to infer patterns of diversity and relationships among plant species. STMS analysis is one of these approaches, which was used in a number of plant DNA variations and genetic relationships (SKROCH et al., 1993; BUTOS et al., 1998). In the present study, STMS markers were used to deduce intra- and inter-species relationships and to examine STMS variation more comprehensively in 45 wild and cultivated accessions representing ten *Cicer* species. Two of our main goals were to develop molecular markers for their use in breeding programmes, and to isolate genes for their use in transgenic approaches to chickpea improvement. Phenotypic differences between the individual accessions have provided the basis for successful plant breeding. However, phenotypic appearance is not always a good indicator of genetic potential. Recently, the ability to detect polymorphism at the DNA level has profoundly changed plant genetic analysis and is poised to impact on chickpea breeding and plant biotechnology.

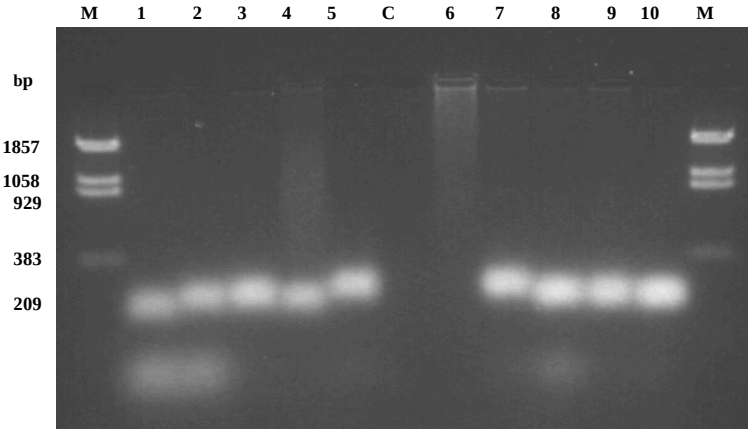

Fig. 1. Amplification patterns of the annual and perennial chickpea genotypes generated by using TS54 primer. Amplification products were electrophoresed in 2% agarose gels and stained with ethidium bromide. Lane 1 and 13 – DNA marker, Lane 2 – PI599072 (*C. reticulatum*), Lane 3 – FLIP84-92C(3) (*C. arietinum*), Lane 4 – Eser87 (*C. arietinum*), Lane 5 – TR54961 (*C. reticulatum*), Lane 6 – PI489776 (*C. echinospermum*), Lane 7 – C (control, none genomic DNA), Lane 8 – ILWC236 (*C. pinnatifidum*, no amplification), Lane 9 – PI561078 (*C. anatolicum*), Lane 10 – ILWC240 (*C. bijugum*), Lane 11 – ILWC241 (*C. bijugum*), Lane 12 – ILWC243 (*C. bijugum*). Molecular weights are given in bp.

Table 3. Conservation of microsatellite-flanking loci between different annual and perennial wild *Cicer* species.

Loci	C. ret (PI599072)	C. ari (FLIP84-92C)	C. ari (Eser87)	C. ret (TR54961)	C. ech (PI489776)	*C. pin* (ILWC236)	*C. ana* (PI561078)	*C. bij* (ILWC240)	*C. bij* (ILWC241)	*C. bij* (ILWC243)
GAA47	1	1	1	1	1	1	1	1	1	0
TS54	1	1	1	1	1	–	1	1	1	1
TA72	1	1	1	1	1	1	1	1	1	1

C. ana: C. anatolicum C. ari: C. arietinum, C. bij: C. bijugum, C. ech: C. echinospermum, C. pin: C. pinnatifidum, C. ret: C. reticulatum.
1 = indicates the presence of amplification products
0 = indicates the zero allele.
(–) = For TS54 microsatellite primers, *C . pinnatifidum* (ILWC236), no amplification
**Cicer* loci: represent the 10 primer pairs that give amplification products in all investigated species. (GA2, GA24, TA13, GAA47, TA46, TA130, TA72, TA146, TS54, TS72). Note that all markers are described in WINTER et al. (1999).

An important element of this development is the technology for detecting DNA sequence variation. Microsatellites, or simple sequence repeats (SSRs), provide an important intermediate technology for chickpea breeders or consumer, and a direct relevance to breeders since SSRs mean that diagnostic markers will have a high probability of detecting polymorphism in germplasm. Furthermore, the allelic differences between the *Cicer* accessions can be resolved on agarose gels providing an ideal co-dominant, diagnostic marker for use in chickpea breeding programmes. In addition to utilizing microsatellite size polymorphisms for cultivar identification, there are many potential applications for microsatellites in genetic analysis, such as linkage studies, evolutionary studies, physical mapping, and population studies (BECKER & HEUN, 1995). Since microsatellites combine extensive variation in total motif lengths with somatic stability and a co-dominant inheritance pattern, they are considered to be the ideal genetic markers (MORGANTE & OLIVIERI, 1993). Over the last two decades a number of DNA markers have been developed and put into use for different purposes in both animal and plant genetic research. Microsatellites are tandem repeats of short sequence motifs that occur ubiquitously in eukaryotic genomes. One of the main feature of this class of repetitive DNA is high level of variation among taxa, mainly expressed as a variable copy number of tandem repeats. Length variation of individual microsatellite loci is analyzed by PCR with a pair of locus-specific flanking primers. The DNA sequences flanking microsatellites are generally conserved within individuals of the same species, allowing the selection of PCR primers that will amplify the intervening SSR in all chickpea genotypes. Unlike the other PCR-based marker techniques SSRs are inherited in a co-dominant fashion. This allows one to discriminate between homoheterozygous states, and increases the efficiency of genetic mapping and population genetic studies (JONES et al., 1997). In future, the success of marker-assisted selection may depend on the possibility of tagging the favorable alleles themselves.

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