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ORIGINAL PAPER

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Identification of microsatellite markers from *Cicer reticulatum*: molecular variation and phylogenetic analysis

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Abstract Microsatellite sequences were cloned and sequenced from *Cicer reticulatum*, the wild annual progenitor of chickpea (*C. arietinum* L.). Based on the flanking sequences of the microsatellite motifs, 11 sequence-tagged microsatellite site (STMS) markers were developed. These markers were used for phylogenetic analysis of 29 accessions representing all the nine annual *Cicer* species. The 11 primer pairs amplified distinct fragments in all the annual species demonstrating high levels of sequence conservation at these loci. Efficient marker transferability (97%) of the *C. reticulatum* STMS markers across other species of the genus was observed as compared to microsatellite markers from the cultivated species. Variability in the size and number of alleles was obtained with an average of 5.8 alleles per locus. Sequence analysis at three homologous microsatellite loci revealed that the microsatellite allele variation was mainly due to differences in the copy number of the tandem repeats. However, other factors such as (1) point mutations, (2) insertion/deletion events in the flanking region, (3) expansion of closely spaced microsatellites and (4) repeat conversion in the amplified microsatellite loci were also responsible for allelic variation. An unweighted pairgroup method with arithmetic averages (UPGMA)-based dendrogram was obtained, which clearly distinguished all the accessions (except two *C. judaicum* accessions) from one another and revealed intra- as well as inter-species variability in the genus. An annual *Cicer* phylogeny was depicted which established the higher similarity between *C. arietinum* and *C. reticulatum*. The placement of *C. pinnatifidum* in the second crossability group and its closeness to *C. bijugum* was supported. Two species, *C. yamashitae* and *C. chorassanicum*, were grouped distinctly and seemed to be genetically diverse from members of the first crossability group. Our data support the distinct placement of *C. cuneatum* as well as a revised classification regarding its placement.

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Introduction

The genus *Cicer* (family Fabaceae, tribe *Cicereae* Alef.) consists of 43 species of which 9 are annual including cultivated chickpea, 33 are perennial and one is unclassified. The annual species have been classified into two sections, *Monocicer* and *Chamaecicer*, based on morphological characteristics, life cycle and geographical distribution (van der Maesen 1987). Eight of the annual species (*Cicer arietinum*, *C. reticulatum*, *C. echinospermum*, *C. bijugum*, *C. judaicum*, *C. pinnatifidum*, *C. yamashitae* and *C. cuneatum*) belong to the section *Monocicer*, while section *Chamaecicer* contains the last known annual species, *C. chorassanicum*.

The importance of the wild annuals is due to chickpea, the third most important grain legume of the world. Chickpea is an excellent human and animal feed because of high protein content and plays a major role in agricultural practices by fixing atmospheric nitrogen. Improving the historic low yield of chickpea and increasing tolerance to biotic and abiotic stresses are major breeding aims. It is in this context that the wild annual species of chickpea have drawn the attention of breeders since they possess many agronomically desirable traits (Muehlbauer et al. 1994; Singh and Ocampo 1997; Upadhyaya 2003). Moreover, they share annual growth habits and chromosome number ($2n = 16$) with chickpea, which makes them amenable to hybridization (Singh and Ocampo 1997; Croser et al. 2003; Berger et al. 2003). No successful hybridization between the wild perennial and cultivated germplasm has yet been reported (Croser et al. 2003). However, various attempts made to cross the annual species with *C. arietinum* have

yielded fully fertile hybrids only with *C. reticulatum*, whereas with *C. echinospermum* partially sterile hybrids have been obtained. Hybridization of *C. arietinum* with *C. bijugum*, *C. judaicum* and *C. pinnatifidum* produces hybrids with low fertility and poor seed set. Sterile hybrids were produced whenever *C. arietinum* was crossed with *C. cuneatum* or *C. yamashitae* whereas *C. chorassanicum* has never been crossed (Croser et al. 2003). Hence, in order to successfully utilize the wild annual germplasm for widening the genetic base of cultivated chickpea, genome analysis of the wild annuals and their species inter-relationships need to be investigated further.

Efforts have been made to study the phylogeny of the wild annuals using morphological traits and biochemical markers like seed storage proteins, allozymes, isozymes and karyotyping (review by Croser et al. 2003). DNA-based molecular marker techniques like RAPD (Ahmad 1999; Sudupak et al. 2002; Iruela et al. 2002), microsatellites (Choumane et al. 2000), ISSR (Rajesh et al. 2002; Sudupak 2004), AFLP (Sudupak et al. 2004; Nguyen et al. 2004) and chloroplast sequence analysis (Javadi and Yamaguchi 2004) were utilized for phylogenetic analysis. There is a general consensus about the members of the first crossability group which contains *C. arietinum* along with *C. reticulatum* (Ahmad 1999; Iruela et al. 2002; Rajesh et al. 2002; Sudupak et al. 2002, 2004; Javadi and Yamaguchi 2004; Nguyen et al. 2004), suggested to be the annual progenitor of chickpea (Ladizinsky and Adler 1975), and *C. echinospermum*, suggested to have played a significant role in the evolution of cultivated chickpea (Tayyar and Waines 1996). The second crossability group contains *C. bijugum*, *C. judaicum* and *C. pinnatifidum* (Ahmad 1999; Sudupak et al. 2002, 2004; Sudupak 2004; Nguyen et al. 2004). The last three species, *C. yamashitae*, *C. chorassanicum* and *C. cuneatum*, were either excluded from many studies or were differentially positioned with respect to the cultivated germplasm. The RAPD data of Ahmad (1999) placed *C. yamashitae* and *C. chorassanicum* next to the first crossability group and the ISSR-based study of Rajesh et al. (2002) placed *C. yamashitae* along with *C. bijugum* and *C. judaicum*. Hence the interspecific relationships between all the nine annual *Cicer* species need to be addressed better for use in future breeding programmes.

The wild annual progenitor of chickpea, *C. reticulatum*, is of particular interest to breeders since it makes fertile crosses with cultivated chickpea. Moreover it is particularly promising as it has been identified as a source of resistance/tolerance to Fusarium wilt (Haware et al. 1992; Singh et al. 1994, 1998), Ascochyta blight (Haware et al. 1992; Singh et al. 1998; Collard et al. 2001), cyst nematodes (Singh et al. 1996), leaf miner, bruchid and cold (Singh et al. 1994, 1998). Genome analysis and molecular marker development from this particular species will help in trait introgression programmes into chickpea and their subsequent monitoring. Moreover, being a wild species as well as the progenitor of cultivated chickpea, it was expected that the markers developed for *C. reticulatum* would be more uniformly and efficiently transferred to both wild and cultivated species as compared to the cultivated chickpea-derived markers leading to the depiction of a clear phylogeny of the genus. Additionally, the *C. reticulatum* markers will also greatly assist in chickpea mapping programmes, since extensive linkage maps of chickpea have been constructed using the interspecific crosses between *C. arietinum* × *C. reticulatum* (Winter et al. 1999, 2000; Tekeoglu et al. 2002).

Microsatellite markers [simple sequence repeats (SSRs) or sequence-tagged microsatellite site (STMS)] have been used extensively in genetic diversity analysis and DNA typing in recent years (Choumane et al. 2000; Abe et al. 2003; He et al. 2003). Microsatellite markers have also been developed from *C. arietinum* (Huttel et al. 1999; Winter et al. 1999; Sethy et al. 2003; Lichtenzveig et al. 2005). They consist of 1–6 bp tandem repeat regions that are ubiquitous with uniform distribution in the genome. Important advantages like co-dominant inheritance, multiple alleles in a single locus, high informativeness, discriminatory power and reproducibility make them suitable for fingerprinting and analyses of genetic relatedness. It has recently been shown that STMS markers are three times more efficient as compared to dominant markers for intraspecific analysis and are equally efficient as other dominant markers in detecting interspecific variability (Nybom 2004). Hence, the present study was undertaken with the aim of (1) isolating microsatellite markers from *C. reticulatum*, (2) investigating the cross-species transferability of the *C. reticulatum* markers, (3) elucidating the molecular basis of allelic variation at homologous loci and (4) establishing the phylogenetic relationships among the annual species of the genus *Cicer*. To our knowledge this is the first report of the isolation of microsatellite markers from a wild species of this genus, namely *C. reticulatum*, and their use in phylogenetic analysis.

Materials and methods

Plant material and DNA extraction

A total of 29 accessions representing all nine annual species of genus *Cicer* were analysed in this study (Table 1). These include: nine accessions of *C. arietinum* L., four of *C. reticulatum* Ladiz, one of *C. echinospermum* P.H. Davis, four of *C. pinnatifidum* Jaub. and Sp., four of *C. judaicum* Boiss, three of *C. bijugum* K.H. Rech, two of *C. yamashitae* Kitamura and one accession each of *C. cuneatum* Hochst. Ex Rich and *C. chorassanicum*. All the plant materials were obtained from ICRISAT (International Crops Research Institute for the Semi-Arid Tropics), India, except Pusa362 which was obtained from Indian Agricultural Research Institute (IARI), India. The nine chickpea accessions include

Table 1 List of *Cicer* accessions analysed in this study along with species names and source country

Sl no.	Accession no./name	Species	Source country
1.	Pusa362	*C. arietinum*[a]	India
2.	ICCV2	*C. arietinum*	India
3.	JG62	*C. arietinum*[a]	India
4.	ICC6263	*C. arietinum*	Russia and CIS
5.	ICC15802	*C. arietinum*	Syria
6.	ICC15518	*C. arietinum*	Morocco
7.	ICC13077	*C. arietinum*	India
8.	ICC15606	*C. arietinum*[a]	India
9.	ICC16487	*C. arietinum*[a]	Pakistan
10.	ICC17121	*C. reticulatum*	Turkey
11.	ICC17123	*C. reticulatum*	Turkey
12.	ICC17163	*C. reticulatum*	Turkey
13.	ICC17164	*C. reticulatum*	Turkey
14.	ICC17159	*C. echinospermum*	Israel
15.	ICC17122	*C. bijugum*	Turkey
16.	ICC17125	*C. bijugum*	Turkey
17.	ICC17187	*C. bijugum*	Syria
18.	ICC17126	*C. pinnatifidum*	Turkey
19.	ICC17155	*C. pinnatifidum*	Turkey
20.	ICC17200	*C. pinnatifidum*	Syria
21.	ICC17209	*C. pinnatifidum*	Syria
22.	ICC17148	*C. judaicum*	Lebanon
23.	ICC17150	*C. judaicum*	Israel
24.	ICC17188	*C. judaicum*	Syria
25.	ICC17204	*C. judaicum*	India
26.	ICC17116	*C. yamashitae*	Afghanistan
27.	ICC17117	*C. yamashitae*	Afghanistan
28.	ICC17141	*C. chorassanicum*	Afghanistan
29.	ICC17162	*C. cuneatum*	Ethiopia

[a]The desi chickpea (*C. arietinum*) accessions

four desi and five kabuli accessions (Table 1). All the accessions were grown at the field site of NCPGR. DNA was isolated from fresh and young leaf tissue using GenElute plant genomic DNA kit (Sigma). The final concentration was determined by agarose gel electrophoresis by comparison with known concentrations of λ-DNA.

Isolation of microsatellite sequences

Microsatellite sequences from *C. reticulatum* (ICC17123) were isolated using the method of Fisher et al. (1996). Briefly, 50 ng of genomic DNA was amplified in 20 µl reaction volume containing 4 µM degenerate primer KKVRVRV$(CT)_6$, buffer (20 mM Tris–HCl, 50 mM KCl), 2.5 mM $MgCl_2$, 100 mM each dNTP and 1 U of Taq DNA Polymerase (Life Technologies). The amplification profile used was as follows: initial denaturation of 2 min at 94°C followed by 40 cycles: denaturation for 10 s at 94°C, annealing for 10 s at 57°C and extension for 10 s at 72°C with a final extension for 20 min at 72°C. The amplified products were electrophoresed on 1.2% agarose gels and cloned in pGEM-T vector (Promega, USA). After transformation in DH5α cells (Invitrogen, USA), recombinants were selected on Luria Agar plates containing ampicillin, IPTG and X-gal.

Plasmid isolation, sequencing and primer design

Plasmids were isolated from 5 ml of overnight grown cultures according to Sambrook et al. (1989). Plasmid DNAs were sequenced on ABI 3700 Prism automated DNA sequencer using Big Dye Terminator reaction kit (Applied Biosystems). Sequences were analysed and the microsatellite motifs identified using the TROLL software (Castelo et al. 2002). STMS primers were designed from regions flanking the microsatellite motifs using Primer3 software (Rozen and Skaletsky 1997). All the primers were synthesized from BioBasic Inc., Canada.

Amplification of SSR loci and electrophoresis

The designed *C. reticulatum* primer pairs (designated CrtSSRs; Table 2) were first used for amplifying DNA from the *C. reticulatum* (Table 1) in order to validate the correct size of the amplified fragment. Next the CrtSSR primer pairs were utilized to analyse the 29 annual *Cicer* accessions (Table 1). Amplifications were carried out in 15 µl of reaction mixture containing 40–50 ng of genomic DNA, PCR buffer (20 mM Tris–HCl, 50 mM KCl), 1.5 mM $MgCl_2$, 100 mM of each dNTP, 1 mM of each primer and 0.6 U of Taq DNA Polymerase (Invitrogen). Amplifications were performed in a BIORAD thermal cycler (iCycler model) using a touchdown amplification profile. The amplification cycles were: initial denaturation of 2 min at 94°C followed by 18 cycles of denaturation for 20 s at 94°C, touchdown from 64°C to 56°C with 0.5°C decrease in each cycle followed by extension at 72°C for 50 s. The next 20 cycles were denaturation for 20 s at 94°C, annealing at 55°C for 50 s and extension at 72°C for 50 s followed by final extension of 7 min at 72°C. Following successful amplification the products were electrophoresed on 6% polyacrylamide gels or high-resolution agarose gels (Hormaza 2002). The gels were stained with ethidium bromide and photographed in a gel documentation system (Alpha Innotech Corp.).

Cloning and sequencing of microsatellite alleles

Genomic DNAs from *C. arietinum* (Pusa362, ICC13077 and ICC15518), *C. reticulatum* (ICC17163), *C. judaicum* (ICC17188) and *C. cuneatum* (ICC17162) were amplified with primer pairs CrtSSR2, CrtSSR51 and CrtSSR74 using the amplification reactions mentioned above. Bands representing allelic size variants were eluted from 6% PAGE gels, cloned into pGEM-T vector (Promega) and transformed into DH5α cells. Following blue-white selection, plasmids were isolated manually and sequenced as mentioned above. For each individual fragment 4–6 random clones were sequenced. The multiple sequence alignments along with the originally cloned allele from *C. reticulatum* were performed using CLUSTALW (1.83).

Table 2 Sequences of *C. reticulatum* STMS primer pairs along with the repeat motifs, expected product length, number and size of alleles obtained and percentage of cross-species transferability

Primer name	Sequence	Motif	Product length (bp)	No. of alleles	Size range of alleles (bp)	Transfer ability (%)
CrtSSR2	F: GAGGTTTGGTGAAGGTATGA R: GTGCTGGTTCTTCTTCGTTA	$(TTCTC)_6$	262	9	191–354	100
CrtSSR19	F: TCATAGCCATACAGTAGCA R: TTTTCATATGGCTCAACAAA	$(CTG)_4$	155	9	147–175	100
CrtSSR31	F: AGCGGCTAAGGTAAGAAGAG R: AGATAATCAGAGAGATATTTTCACAA	$(ATGTG)_3(GGTT)_3$	207	5	195–213	100
CrtSSR35	F: GAGGAAGAAGATCGATGAAA R: TTGGTGGACAATAAAATTAAGA	$(GA)_8...(GA)_5$	270	2	264–270	100
CrtSSR46	F: AGTATAGTGGGGGAAGACCT R: GTGGCAGAGGAACTGATAGA	$(GTT)_2...(GA)_8$	244	1	244	100
CrtSSR47	F: TGAGGCCTAAGAGTACCAAA R: TCTCATCAGGAACAACAACA	$(GTT)_4$	128	7	124–175	100
CrtSSR51	F: TTGGAATTCCTTTTACGAGA R: ACACACACCACAAAGAACAA	$(CT)_{10}$	257	9	188–300	100
CrtSSR69	F: GAGAGAGAGACAGAAAGGCA R: TTGGAATTCCTTTTACGAGA	$(CA)_5...(GA)_9$	279	7	276–346	100
CrtSSR74	F: TAACTGCCATCATGAACTGA R: AAAACGTCCATAACAACGTC	$(TTA)_5$	233	7	224–274	100
CrtSSR75	F: GTTTCATTTGCCTTTATTGC R: ACACGTTAATGTTGTGACGA	$(TAA)_4C(TAA)$	240	4	238–435	100
CrtSSR79	F: TCATTGCCGAATCTTATTTT R: TTGTATCATAAAAACTGATGGACT	$(A)_{11}(AATA)_3(A)_{20}$	283	4	283–350	66

Data analysis

The gels were scored both manually and with the help of the gel documentation system. The allelic bands for all 29 accessions were scored in a binary matrix where 1 represents presence and 0 represents the absence of the band. Jaccard's similarity coefficient was calculated for the data matrix. The similarity ($D=1-S$) matrix was then used for cluster analysis using the unweighted pairgroup method with arithmetic averages (UPGMA) and a dendrogram was constructed using NTSYS-pc (Version 2.1, Rohlf 1994). The reliability of the dendrogram was evaluated with 1,000 bootstraps using WinBoot software (Yap and Nelson 1996).

Results

Isolation and characterization of *C. reticulatum* microsatellite sequences

The method of Fisher et al. (1996) was very effective in identifying microsatellite sequences from the *C. reticulatum* genome. More than 1,000 recombinants were obtained on selection plates of which 110 clones were randomly sequenced. Of these 18 were found to be redundant. The average insert size was 410 bp. All the sequenced clones contained a $(GA)_n$ motif at the 5′ end and a $(CT)_n$ motif at the 3′ end. However, in 14 of the 92 sequences, additional internal microsatellite motifs were identified between the two flanking repeat motifs at both the ends. The composition of the internal microsatellite motifs varied, consisting of mononucleotide to pentanucleotide repeats. Of the 14 internal microsatellites, 12 were considered for designing STMS primers (designated as CrtSSR) since the remaining two motifs were too close to the cloning site to be converted into STMS markers. The 12 CrtSSRs were validated by amplification in four accessions of *C. reticulatum*. Eleven primer pairs successfully amplified fragments of the expected size, whereas one primer pair failed to amplify the correct-sized product and was excluded. The primer pair CrtSSR35 amplified products in three out of the four *C. reticulatum* accessions indicating the presence of null alleles (Fig. 1). The sequences of the 11 CrtSSR primer pairs utilized in this study are listed in Table 2. All the 11 microsatellite-containing sequences were submitted to the GenBank and have the accession numbers AY760077 to AY760087. Homology search for all these sequences in the GenBank database revealed that two sequences (CrtSSR19 and CrtSSR75) were homologous to specific proteins from *A. thaliana* (At1g49600, BX826355; e values $6e^{-05}$ and $1e^{-22}$, respectively), whereas another sequence (CrtSSR46) had homology with repeated regions from *Vigna radiata* (AF320012; e value $7e^{-44}$).

Transferability of CrtSSR markers across species of the genus *Cicer*

The 11 CrtSSR primer pairs were used to amplify genomic DNA from the 29 accessions (Table 1) representing all 9 annual *Cicer* species. Variable number and size of bands were obtained with these primers across the

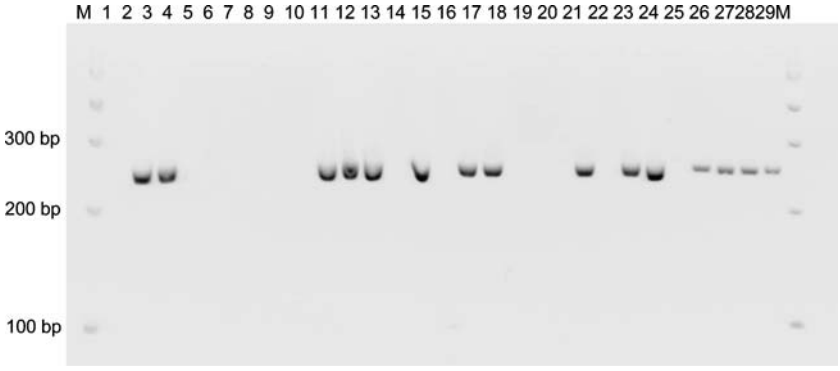

Fig. 1 Amplification profile of *Cicer* accessions obtained using primer pair CrtSSR35. PCR-amplified products were resolved on 6% PAGE gel. *Lanes* represent 1–9 *C. arietinum*, 10–13 *C. reticulatum*, 14 *C. echinospermum*, 15–18 *C. pinnatifidum*, 19–22 *C. judaicum*, 23–25 *C. bijugum*, 26 and 27 *C. yamashitae*, 28 *C. chorassanicum* and 29 *C. cuneatum*. *M* indicates 100 bp ladder

species. Of the 11 primer pairs tested, 9 primers amplified polymorphic alleles across all the species, 1 primer pair (CrtSSR46) produced monomorphic amplification profile in all the 29 accessions and the primer pair CrtSSR79 amplified 6 of the 9 annual species, i.e. *C. arietinum*, *C. reticulatum*, *C. echinospermum*, *C. pinnatifidum*, *C. judaicum* and *C. yamashitae*. Primer pair CrtSSR35 amplified only few accessions from each species (three accessions of *C. reticulatum*, two accessions each of *C. arietinum*, *C. bijugum*, *C. pinnatifidum*, *C. yamashitae* and one accession each of *C. echinospermum*, *C. judaicum*, *C. chorassanicum and C. cuneatum*) indicating the existence of null alleles (Fig. 1). Eight primer pairs CrtSSR2, CrtSSR19, CrtSSR47, CrtSSR51, CrtSSR69, CrtSSR74, CrtSSR75 and CrtSSR79 were polymorphic between *C. reticulatum* and *C. arietinum* while CrtSSR2, CrtSSR47, CrtSSR51, CrtSSR69 and CrtSSR79 were polymorphic within chickpea. The overall CrtSSR marker analysis with all the primer pairs revealed 64 alleles at the 11 loci. A maximum of 9 alleles with primer pairs, CrtSSR2, CrtSSR19 and CrtSSR51, were obtained, while a minimum of 1 allele with CrtSSR46 was obtained with an average of 5.8 alleles per locus. The size range of the alleles varied from 124 to 435 bp. The alleles amplified in *C. cuneatum* and *C. chorassanicum* were most distinct and showed maximum length variation. The characteristics of the cross-species amplification profiles obtained with the CrtSSR primers are summarized in Table 2.

The CrtSSR primers also revealed intraspecific polymorphism within accessions of *Cicer* species. More than one accession of *C. arietinum*, *C. reticulatum*, *C. bijugum*, *C. pinnatifidum*, *C. judaicum* and *C. yamashitae* was included in this study (Table 1). Intraspecific polymorphism was due to two reasons: (1) difference in the sizes of amplified bands and (2) difference in the number of amplified bands. However, variation in the number of amplified alleles was the major source of intraspecific polymorphism.

Sequence analysis of homologous alleles

To understand the molecular basis of variation in the size and number of amplification products within and between the species, size variant alleles at the homologous loci (CrtSSR2, CrtSSR51 and CRtSSR74) were cloned, sequenced and compared with the originally cloned allele from *C. reticulatum*. Sequence analysis of the length variant alleles amplified with the CrtSSR2 primer pair (Fig. 2a) revealed that the 251 bp allele of *C. reticulatum* ICC17163 had only three copies of the basic repeat motif (TTCTC) as compared to six copies in the 262 bp originally cloned allele from *C. reticulatum* ICC17123. In case of *C. arietinum* cv. Pusa362, the repeat region had a compound motif $(TTCTC)_5(TTTTC)_3$ unlike the perfect motif in the *C. reticulatum* accessions. Moreover, expansion of the 4 bp (CTCA) motif (nucleotide position 176) was observed only in *C. reticulatum* ICC17163 making the repeat structure $(CTCA)_3$. In addition to these, a single base insertion "A" was observed in both the *C. reticulatum* accessions at nucleotide position 58.

At the CrtSSR51 locus (Fig. 2b), alleles differing in length were obtained in the two *C. reticulatum* accessions whereas in the chickpea accessions, both allelic length and number varied, i.e. in ICC15518, two fragments of sizes 248 bp (allele A) and 266 bp (allele B) were obtained. Sequence comparison of all the alleles showed that they differed in the copy number of the originally cloned repeat motifs which varied from $(CT)_{10}$ to $(CT)_{16}$ at position 219. Between the two *C. reticulatum* accessions, a 5 bp deletion was observed for ICC17163 from position 78 to 82. Comparing the chickpea alleles it was observed that the 250 bp Pusa362 allele and the 248 bp allele A of ICC15518 differed only in the number of repeat motifs, whereas between the A and B alleles of *C. arietinum* accn ICC15518, there was not only a repeat number variation [$(CT)_{13}$ in A and $(CT)_{14}$ in B], but also an expansion of a (TC) motif at nucleotide positions 105–120 (Fig. 2b). This expansion leading to the presence of $(TC)_2AT(TC)_5$ in ICC15518 B resulted in 16 bp length difference between the two alleles at this position.

The CrtSSR74 locus was amplified and sequenced from one member of each crossability group, i.e. *C. arietinum*, *C. judaicum* and *C. cuneatum*, and compared with the homologous allele from *C. reticulatum*. Variation in length as well as number of amplified products was observed (Fig. 2c). At this locus the

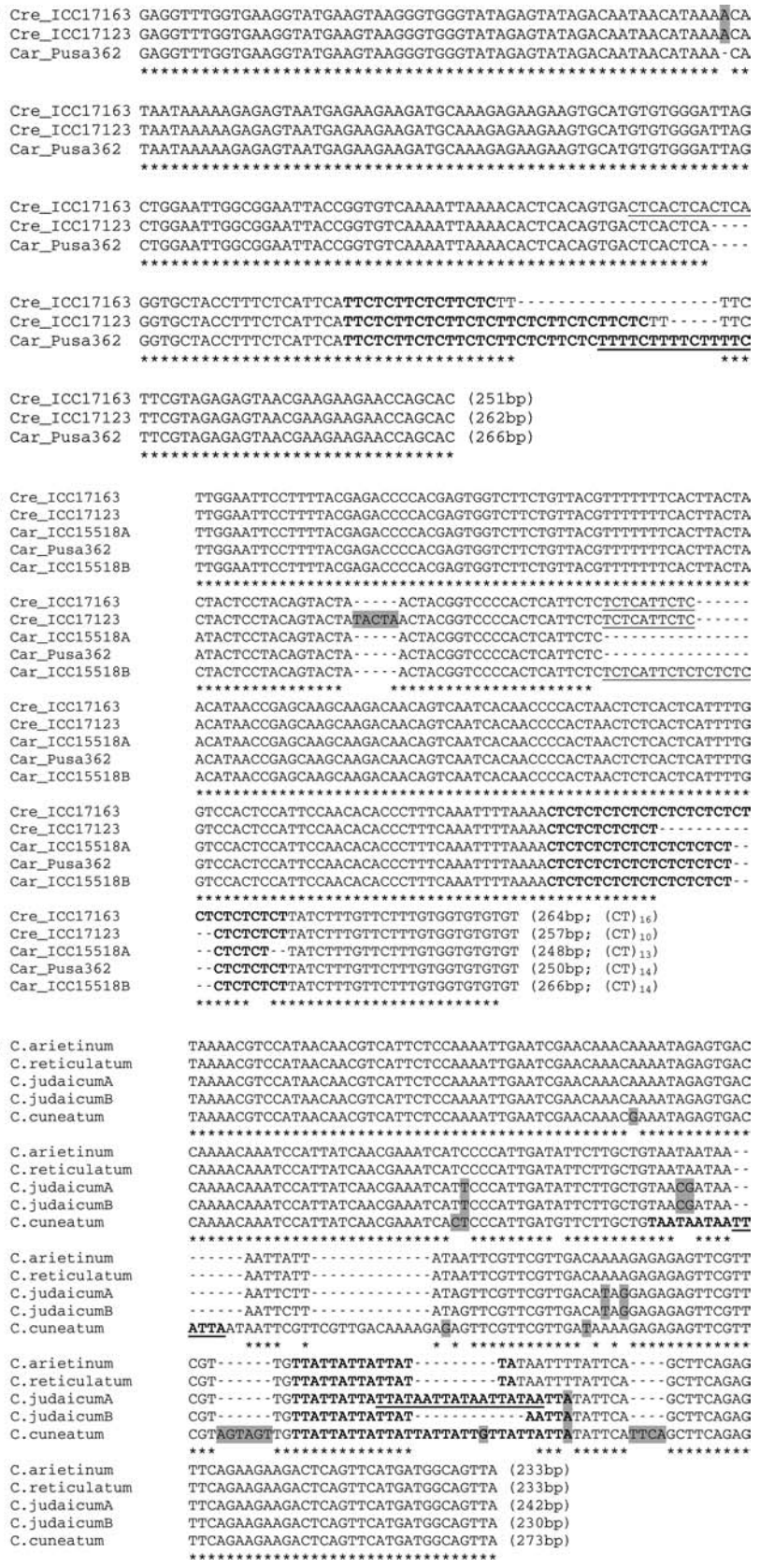

Fig. 2 Sequence comparison between homologous alleles amplified using STMS primer pairs CrtSSR2, CrtSSR51 and CrtSSR74. The repeat motifs are represented as *bold letters*. * indicates similar sequences and – indicates alignment gaps. *Shaded box* indicates base substitutions/insertions and *underlined microsatellite sequences* indicate repeat conversion/expansion. **a** Sequence alignment of alleles amplified using CrtSSR2 from *C. arietinum* (Pusa362), *C. reticulatum* (ICC17123) and C. *reticulatum* (ICC17163). **b** Sequence alignment of alleles amplified using CrtSSR51 from *C. arietinum* (Pusa362), *C. arietinum* (ICC15518), *C. reticulatum* (ICC17123) and *C. reticulatum* (ICC17163). ICC15518A and ICC15518B represent two alleles from the same accession. The numbers of repeat motifs are mentioned at the *end* of each sequence **c** Sequence alignment of alleles amplified using CrtSSR74 from *C. arietinum* (Pusa362), *C. reticulatum* (ICC17123), *C. judaicum* (ICC17188) and *C. cuneatum* (ICC17162). ICC17188A and ICC17188B indicate two alleles of the same *C. judaicum* accession

C. arietinum and *C. reticulatum* sequences shared 100% homology. However, the 242 bp (allele A) and 230 bp (allele B) from *C. judaicum* differ from each other by 12 bp, but allele B possessed the same repeat structure as of chickpea and *C. reticulatum* with one (TTA to TAA) interruption, while allele A had a compound repeat

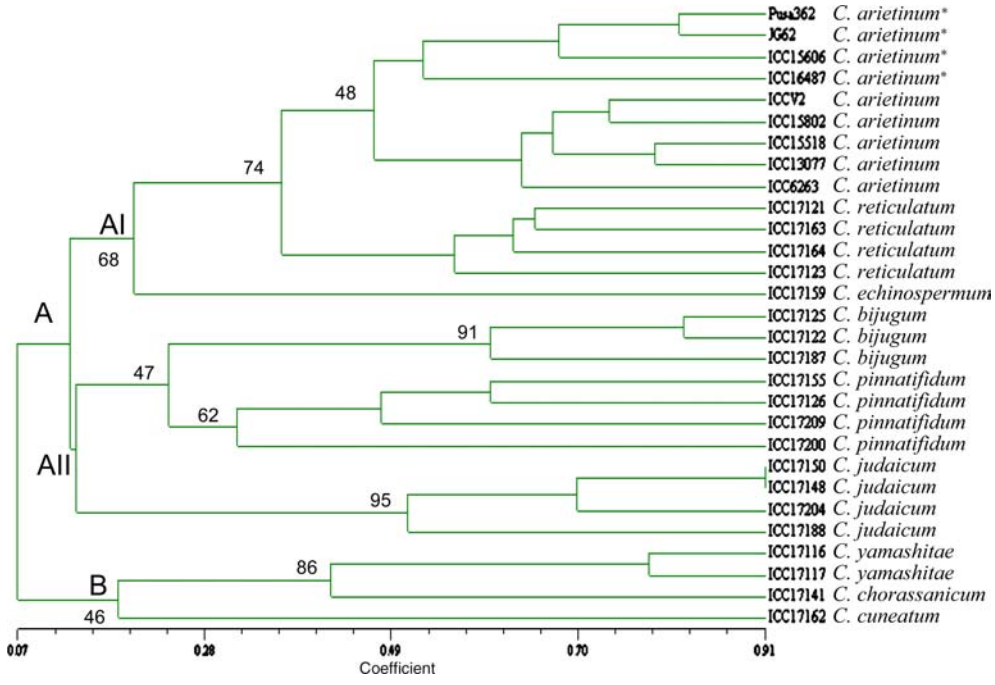

Fig. 3 The UPGMA-based dendrogram obtained with 11 *C. reticulatum* STMS (CrtSSR) markers. Species names and accession numbers/names are indicated along with each accession. Bootstrap values are indicated at the major nodes. * indicates the desi *C. arietinum* germplasm

structure $(TTA)_3(TTATAA)_3$ (Fig. 2c). Length variation as long as 40 bp was observed between *C. cuneatum* (273 bp) and *C. reticulatum* (233 bp) which could be due to (1) variation in the $(TTA)_n$ microsatellite repeat number with repeat interruption (TTA to TTG at seventh repeat motif); (2) two insertions, 13 bp insertion at position 134, 6 bp insertion at position 184; (3) one repeat conversion and expansion at positions 119–126 upstream of the originally cloned $(TTA)_n$ motif (Fig. 2c). In addition to these, different point mutations were detected in *C. judaicum* and *C. cuneatum* (Fig. 2c). However, at position 221 both the species *C. judaicum* and *C. cuneatum* shared a common point mutation (T to A) as compared to chickpea and *C. reticulatum*.

Phylogenetic analysis

The phylogenetic relationships among the annual species of the genus *Cicer* were evaluated by construction of the STMS-based dendrogram (Fig. 3). The dendrogram could distinguish all the accessions from one another except the two *C. judaicum* accessions. The 29 individual accessions were distributed into two distinct clusters, A and B. Cluster A was further divided into two subclusters AI and AII. The cluster AI contained the cultivated chickpea accessions along with the *C. reticulatum* and *C. echinospermum* accessions with 68% bootstrap support. The *C. echinospermum* accession (ICC17159) branched out in this cluster showing less similarity with chickpea than the *C. reticulatum* accessions (Fig. 3). The cultivated chickpea accessions in subcluster AI were further subdivided into two distinct groups, one containing the four desi accessions and the other having the five kabuli accessions. The second subcluster AII contains all the 11 accessions of *C. bijugum*, *C. judaicum* and *C. pinnatifidum*. In this subcluster *C. bijugum* and *C. pinnatifidum* accessions were placed closer to each other than *C. judaicum*, which formed a distinct subgroup (95% bootstrap support) within this cluster. Two *C. judaicum* accessions, ICC17148 and ICC17150, clustered at the same position. The other major cluster B contained all the five accessions of the three species, *C. yamashitae*, *C. chorassanicum* and *C. cuneatum*, showing that these species were distinct from the other six annual species in cluster A. Within this cluster, *C. yamashitae* and *C. chorassanicum* were closer to each other (87% bootstrap support) while the *C. cuneatum* accession ICC17162 was placed distinctly (Fig. 3).

Discussion

In the present study, microsatellite sequences from the wild annual progenitor of cultivated chickpea, namely *C. reticulatum*, were cloned, characterized and utilized for depicting the annual *Cicer* phylogeny and elucidating the basis of sequence variation at homologous microsatellite loci. Though microsatellite markers provide an attractive choice for mapping and estimating genetic diversity, the cost involved in their generation limit their use. To overcome this, many different methods for the identification of microsatellites have been developed and used in different species (Zane et al. 2002). The

microsatellite enrichment method utilized in this study (Fisher et al. 1996) proved to be very efficient. In this technique, degenerate primers anchored at the 5′ ends of microsatellites were used to amplify and clone two close and inverted SSRs along with the region between them. As expected, all (100%) the sequenced recombinants had microsatellite motifs at the 5′ and 3′ends. This turned out to be a limitation of the method as only one specific STMS primer could be designed. The use of one internal specific primer and one degenerate primer caused the amplification of non-specific bands in genomic DNA of some samples. In order to overcome this limitation, we identified at least 15% of the clones (14 out of 92) that contained an internal microsatellite motif in addition to the ones at the extreme ends. The 11 STMS primers reported in this study were therefore designed based on the regions flanking the internal microsatellite motifs. The internal microsatellites identified varied in size and sequence composition of the repeat motifs ranging from mono- to pentanucleotide repeats (Table 2). In most of the other conventionally used microsatellite enrichment methods, only motifs of one kind with a defined repeat unit and structure are identified depending upon the probe used for screening (Zane et al. 2002). In chickpea, $(GA)_n$, $(GAA)_n$ and $(TAA)_n$ microsatellite motifs have been identified from conventional genomic library (Winter et al. 1999) whereas Lichtenzveig et al. (2005) have constructed and screened a BAC library with $(GA)_n$, $(GAA)_n$, $(AT)_n$, $(TAA)_n$, $(TGA)_n$, $(CA)_n$, $(CAA)_n$ and $(CCA)_n$ motifs to identify microsatellites from the chickpea genome. Moreover, the microsatellite sequences isolated in the present study represented the coding and non-coding regions of *C. reticulatum* since they were found to have sequence homology with coding sequences of *A. thaliana* and repeated regions of other genomes. A potential disadvantage of this method was the redundancy of the clones. There were 18 redundant clones (16%) out of the 110 sequenced clones.

The STMS markers of *C. reticulatum* were successful in revealing polymorphism both across and within species as demonstrated by the high value of 5.8 alleles per locus (Table 2), thereby proving their utility in genetic diversity analysis and fingerprinting. The eight *C. reticulatum*-derived markers (see Results) will be useful in the interspecific mapping projects arising from crosses between *C. arietinum* × *C. reticulatum*. The overall banding patterns suggested that the variation in length as well as number of amplified alleles contributed equally to the intra- as well as interspecific polymorphism, which have been reported earlier by Choumane et al. (2000). Hence sequence comparison of microsatellite regions at three loci from different *Cicer* species was done in order to understand the basis of variation between alleles from (1) the same accession, (2) different accessions of the same species and (3) different species. Overall it was observed that there was large sequence conservation between the homologous alleles. However, the major reason for the length variability within microsatellite alleles was due to the difference in the copy number of repeat units. Additionally other factors responsible included (1) point mutations, (2) insertion/deletion events, (3) expansion of closely spaced microsatellites in the flanking region and (4) repeat conversion at microsatellite loci. Sequence comparison of the length variant alleles from the two *C. reticulatum* accessions demonstrated that the source of length polymorphism was the variation in the copy number of the repeat motifs (CrtSSR2 and CrtSSR51) as well as an insertion in ICC17123 at CrtSSR51 (Fig. 2). Sequence comparison between the two alleles from the chickpea accession ICC15518 at the CrtSSR51 locus revealed an expansion of the repeat motif in the upstream region as the major factor giving rise to length variant alleles in the same accession (Fig. 2b). When considering the allele variation between chickpea and *C. reticulatum*, it was observed that along with repeat number changes and expansion of repeats in the flanking regions, repeat conversion (perfect to compound microsatellite in CrtSSR2 locus) was an additional source of variation (Fig. 2a). Sequences of the *C. judaicum* and *C. cuneatum* alleles at the CrtSSR74 locus revealed the presence of species-specific point mutations (Fig. 2c). Moreover, repeat conversion in *C. judaicum* (A allele), repeat interruption (B allele) and longer repeat motif with interruption were detected in *C. cuneatum* (Fig. 2c). Polymorphism resulting due to variable number of tandem repeats and repeat interruption by base substitutions are now well accepted (Eisen 1999) and has been demonstrated in species such as *Glycine* (Peakall et al. 1998), *Zea* (Matsuoka et al. 2002) and *Pine* (Karhu et al. 2000; Gonzalez-Martinez et al. 2004). Choumane et al. (2000) compared the sequences of microsatellite loci from chickpea and the wild annual *Cicer* species and reported extensive repeat length variation and insertion/deletion events in the microsatellite flanking regions. However, other factors such as those mentioned at (3) and (4) above, though also responsible for length variation as demonstrated in the present study, were not observed by Choumane et al. (2000). Generation of diverse microsatellite alleles by single-base substitutions within the repeats or flanking sequences along with indels of non-repeated sequences has been well demonstrated in plants (Peakall et al. 1998; Matsuoka et al. 2002). But events like compound motif formation and repeat expansions in the microsatellite flanking regions are less documented for plant microsatellites and have been reported only in *Oryza* (Akagi et al. 1998) and *Vitis* (Di Gaspero et al. 2000). Hence, the *C. reticulatum* derived microsatellite loci were useful for understanding the factors underlying microsatellite evolution.

Transferability of markers across related species is desirable as it leads to the enrichment of molecular markers necessary for construction of high-density genetic maps as has been demonstrated in wheat (Korzun et al. 1999; Guyomarc'h et al. 2002), *Oryza* (Brondani et al. 2001), *Eucalyptus* (Marques et al. 2002) and *Olea* (Rallo et al. 2003). Transferability of the chickpea microsatellite markers across related genera of

the *Leguminosae* family has been investigated earlier (Pandian et al. 2000; Choumane et al. 2004). In the present study, cross-species amplification profiles showed that all the markers (except CrtSSR79) transferred efficiently to the other annual species depicting a high transferability of 97% as compared to the *C. arietinum* STMS markers which have been shown to have only 68% transferability across other annual species (Choumane et al. 2000). The marker CrtSSR79 finds only 66% transferability within the wild annuals while all the other markers were 100% transferable. Moreover, the sequence comparisons of the microsatellite regions amplified using the *C. reticulatum* derived primers clearly indicated a high degree of sequence conservation at these loci from annual *Cicer* species, thereby proving them to be homologous alleles which may be used as reliable syntenic markers for map generation and comparative mapping in the genus.

The tree obtained with the CrtSSR primers presented a clear phylogeny of the genus *Cicer* which was consistent with the interspecific relationships depicted in some of the previous reports (Kazan and Muehlbauer 1991; Ahmad and Slinkard 1992; Javadi and Yamaguchi 2004; Sudupak et al. 2004; Nguyen et al. 2004) but was in disagreement with others (Ahmad 1999; Iruela et al. 2002). The dendrogram revealed that members of subcluster AI (*C. arietinum*, *C. reticulatum* and *C. echinospermum*) were closely related and correspond to the primary genepool or the first crossability group as proposed by earlier studies based on seed protein, allozyme, AFLP and chloroplast sequences (Kazan and Muehlbauer 1991; Ahmad and Slinkard 1992; Sudupak et al. 2004; Nguyen et al. 2004; Javadi and Yamaguchi 2004). In this study, *C. reticulatum*, which has been proposed to be the progenitor of cultivated chickpea, was found to be more closely related to chickpea than to *C. echinospermum*. This finding was supported by the results obtained earlier with molecular markers such as RAPD (Ahmad 1999; Sudupak et al. 2002; Iruela et al. 2002) and AFLP (Sudupak et al. 2004; Nguyen et al. 2004) but was in conflict with the findings of the seed protein and allozyme-based studies (Kazan and Muehlbauer 1991; Ahmad and Slinkard 1992; Labdi et al. 1996; Tayyar and Waines 1996), which have found close similarities between *C. reticulatum* and *C. echinospermum*. The *C. reticulatum* microsatellite markers were also able to distinguish the two major groups (desi and kabuli) of cultivated chickpea which have been characterized earlier based on the seed morphology and genetic make-up (Moreno and Cubero 1978; Gil et al. 1996). Earlier studies with DNA-based markers (Iruela et al. 2002; Sudupak et al. 2004) have also indicated that the cultivated accessions comprise two genepools which can be easily distinguished. But, studying a diverse chickpea collection with the help of $(TAA)_n$ microsatellite markers, Udupa et al. (1999) could not find any clustering based on either geographic location or seed morphology. Of the CrtSSRs, 45% resulted in polymorphic amplification profiles within chickpea which is slightly higher than the chickpea-derived microsatellite markers (Lichtenzveig et al. 2005). Our data, supported by high-sequence conservation and reliable bootstrap values, lead us to speculate that *C. reticulatum* microsatellite markers were more powerful in depicting a clear *Cicer* phylogeny which could delineate the two genepools within chickpea.

The subcluster AII represents the secondary genepool (or second crossability group) and contains the accessions of *C. bijugum*, *C. judaicum* and *C. pinnatifidum*. These two accessions of *C. judaicum* (ICC17148, ICC17150) might represent the same original accession that has been duplicated in the seedbank as two independent accessions (Berger et al. 2003). This cluster provides evidence that *C. bijugum* and *C. pinnatifidum* accessions are closer to each other than accessions of *C. judaicum* in agreement with Sudupak et al. (2002) and Nguyen et al. (2004). However, closer association between *C. bijugum* and *C. judaicum* has been reported earlier (Iruela et al. 2002; Sudupak et al. 2004; Javadi and Yamaguchi 2004) while all the protein-based studies and ISSR-based study (Sudupak 2004) have shown closer association between *C. pinnatifidum* and *C. judaicum*. These three species in the second crossability group together represent the largest genetic variability as well as the highest number of seedbank entries amongst the wild annuals (Berger et al. 2003). Therefore, in order to resolve the conflicting interrelationships between these three species, an analysis of a larger germplasm collection of these species should be undertaken.

The second major cluster B contains the three species *C. yamashitae*, *C. chorassanicum* and *C. cuneatum*. Based on the higher genetic similarity between the accessions of *C. yamashitae* and *C. chorassanicum*, they could be classified as members of the third crossability group (tertiary genepool) which was in agreement with previous reports (Kazan and Muehlbauer 1991; Croser et al. 2003; Javadi and Yamaguchi 2004). The clustering together of these two species, even though they have different morphological characteristics and belong to different sections (*Monocicer* and *Chamaecicer*), was attributed to the similar range of geographic distribution of these two species (van der Maesen 1987; Kazan and Muehlbauer 1991). The third species in this cluster, *C. cuneatum*, was placed separately from all other species of the genus. The distinct placement of *C. cuneatum* is a common feature reported in all the earlier protein and DNA-based studies (Croser et al. 2003). When perennial species were included in the studies it always clustered along with one of them. This is well justified considering its morphological characteristics (globular seeds and climbing habit) which is in contrast with other annuals of the section *Monocicer* characterized by firm, erect or horizontal stems branched from the middle or base (Croser et al. 2003). Therefore, based on our results and the earlier evidences, it is likely that *C. cuneatum* either evolved from a different ancestor or may have diverged very early during the course of evolution of the

annual *Cicer* species. It may also be suggested that *C. cuneatum* may not be classified in the section *Monocicer* but may have a separate section of its own. Hence we suggest, as has also been proposed in earlier studies (Choumane et al. 2000; Javadi and Yamaguchi 2004), a revised classification of the genus especially regarding the placement of *C. cuneatum*.

In conclusion, it was demonstrated in *Cicer* that microsatellite sequences from *C. reticulatum*, the wild progenitor of chickpea, had higher cross-species transferability rates than microsatellite markers from cultivated species and had high sequence conservation at homologous loci within cultivated and wild annual species. Therefore they were more suitable and reliable for phylogenetic analysis and comparative mapping in the genus. These markers were powerful tools for studying microsatellite evolutionary events in the genus as well as suitable for inter- and intraspecific mapping projects. The molecular phylogeny depicted in this study contributed to a better understanding of the species relationships in the genus *Cicer* supporting the placement of *C. pinnatifidum* in the second crossability group as well as suggesting the need of a revised classification with respect to *C. cuneatum*.

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