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ORIGINAL PAPER

EST-derived genic molecular markers: development and utilization for generating an advanced transcript map of chickpea

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Abstract Well-saturated linkage maps especially those based on expressed sequence tag (EST)-derived genic molecular markers (GMMs) are a pre-requisite for molecular breeding. This is especially true in important legumes such as chickpea where few simple sequence repeats (SSR) and even fewer GMM-based maps have been developed. Therefore, in this study, 2,496 ESTs were generated from chickpea seeds and utilized for the development of 487 novel EST-derived functional markers which included 125 EST-SSRs, 151 intron targeted primers (ITPs), 109 expressed sequence tag polymorphisms (ESTPs), and 102 single nucleotide polymorphisms (SNPs). Whereas EST-SSRs, ITPs, and ESTPs were developed by in silico analysis of the developed EST sequences, SNPs were identified by allele resequencing and their genotyping was performed using the Illumina GoldenGate Assay. Parental polymorphism was analyzed between *C. arietinum* ICC4958 and *C. reticulatum* PI489777, parents of the reference chickpea mapping population, using a total of 872 markers: 487 new gene-based markers developed in this study along with 385 previously published markers, of which 318 (36.5%) were found to be polymorphic and were used for genotyping. The genotypic data were integrated with the previously published data of 108 markers and an advanced linkage map was generated that contained 406 loci distributed on eight linkage groups that spanned 1,497.7 cM. The average marker density was 3.68 cM and the average number of markers per LG was 50.8. Among the mapped markers, 303 new genomic locations were defined that included 177 gene-based and 126 gSSRs (genomic SSRs) thereby producing the most advanced gene-rich map of chickpea solely based on co-dominant markers.

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Introduction

Chickpea (*Cicer arietinum* L.) is an autogamous cool-season diploid ($2n = 2x = 16$) legume having a genome size of 740 Mbp (Arumuganathan and Earle 1991), which is 1.5 times greater than that of the model legume *Medicago truncatula*. The crop is valued for its nutritive seeds that serve as an important source of plant-based dietary protein especially in developing countries. Constituting 15% of the world's pulse production, over 95% of the chickpea production area and consumption occurs in developing countries, with a major contribution (65%) from India (FAOSTAT 2009; http://faostat.fao.org/site/567/default.aspx#ancor). However, despite its agricultural value and continuous demand, no major breakthrough for yield enhancement has occurred mainly due to low genome variability and susceptibility of the crop to several biotic and abiotic stresses (Ryan 1997; Ahmad et al. 2005; Millan et al. 2006). Recently with the development of modern tools, chickpea genomics research has significantly

progressed as evidenced by the availability of genomic resources such as molecular markers and linkage maps (Nayak et al. 2010; Gaur et al. 2011; Gujaria et al. 2011), bacterial artificial chromosome (BAC) libraries (Rajesh et al. 2004; Lichtenzveig et al. 2005; Zhang et al. 2010), and cDNA libraries (Buhariwalla et al. 2005; Coram and Pang 2005; Varshney et al. 2009; Ashraf et al. 2009; Deokar et al. 2011) from which approx. 40,000 ESTs are available in the NCBI EST database. High-throughput transcriptome data of chickpea were recently reported based on sequencing of short reads using the Illumina Genome Analyzer II platform (Garg et al. 2011a) and long reads through Roche 454 pyrosequencing (Garg et al. 2011b; Hiremath et al. 2011). Even though these reported the development of molecular markers, their validation and utilization for map construction were not carried out. Additional marker coverage coupled with validation of markers in agronomically acceptable genetic backgrounds is required to broaden applications of genomics-assisted breeding in chickpea.

For various applications in plant genetics and breeding, PCR-compatible markers based on microsatellites, also known as simple sequence repeats (SSRs) are often considered the most efficient and reliable source for detecting genetic variation in several crop species (Varshney et al. 2007) including chickpea (Sharma et al. 1995; Hüttel et al. 1999; Sethy et al. 2003, 2006). Published linkage maps have been generated in both intra-specific (Radhika et al. 2007; Gaur et al. 2011) as well as inter-specific crosses (Winter et al. 2000; Nayak et al. 2010; Gujaria et al. 2011) using amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR) and sequence tagged microsatellite site (STMS) markers. However, levels of polymorphism observed in chickpea are usually low (Winter et al. 1999, 2000), therefore a very large repertoire of co-dominant markers are needed for mapping a sufficiently high number of loci on a dense genetic map of chickpea which could assist in tagging genes/QTLs for qualitative and quantitative agronomic traits and serve as a platform for de novo assembly of high-throughput whole genome sequence data.

With the recent escalating emphasis on functional genomics studies in several organisms, key focus has shifted on the generation of functional genic molecular markers (GMMs) which are derived from transcript sequences. In this regard, the expanding EST databases have provided an attractive resource for development of different types of cost-effective EST-based markers such as EST-SSRs, intron targeted primers (ITPs) and expressed sequence tag polymorphisms (ESTPs). More recently, single nucleotide polymorphisms (SNPs), considered the most abundant form of genetic variation, have been suggested to be ideally suitable for multiple applications especially for increasing marker density, QTL mapping and high-throughput marker-assisted selection. All these categories of EST-based markers are derived from genes of known function and provide an opportunity for mapping the gene-rich regions of the genome. Several studies have been conducted for linkage analysis in different crop species using EST-SSRs (Yi et al. 2006; Senthilvel et al. 2008; Shirasawa et al. 2010a, 2011; Sraphet et al. 2011), ITPs (Choi et al. 2004; Panjabi et al. 2008; Shirasawa et al. 2010a), ESTPs (Temesgen et al. 2001; Brown et al. 2001) and gene-based SNP loci (Choi et al. 2007; Hyten et al. 2008; Eckert et al. 2009; Muchero et al. 2009; Shirasawa et al. 2010b). High-density genetic maps of gene-based markers represent a powerful resource to enhance genome analysis, thus providing an important opportunity to directly tag genes related to agronomical traits. For example, in the study of Park et al. 2005 and Guo et al. 2007, the gene-rich linkage map of cotton was constructed on which a fiber quality trait was tagged successfully. However in chickpea, there are limited reports available on EST-based markers (Buhariwalla et al. 2005; Choudhary et al. 2009; Varshney et al. 2009; Nayak et al. 2010; Gujaria et al. 2011) and their defined map positions (Pfaff and Kahl 2003; Nayak et al. 2010; Gujaria et al. 2011). Therefore in chickpea, it is imperative to enrich the EST database and utilize this resource to generate EST-based functional markers to increase both marker resources and marker density at important genomic regions.

The objective of this study was to enrich the resource of EST-based genic markers and develop a transcript map of chickpea. To maximize the potential of detecting polymorphisms, different types of EST-based markers, namely EST-SSRs, ITPs, ESTPs and SNPs were examined and these novel genic markers were integrated together with previously published STMS markers to generate an improved transcript map of chickpea.

Materials and methods

Plant material and DNA extraction

Cicer arietinum cv. ICCV2 (single-podded, Kabuli type) was used for the construction of the cDNA libraries. For linkage analysis and map construction, the inter-specific mapping population consisting of 129 RILs (recombinant inbred lines) derived from a cross between *C. arietinum* ICC4958 (a *fusarium* wilt resistant and drought tolerant) × *C. reticulatum* PI489777 (wild annual species, *fusarium* wilt susceptible) was used. The population is used internationally as a reference mapping population for chickpea mapping and was developed and provided by

Dr. Fred Muehlbauer, Washington State University, USA. Genomic DNA of all 129 RILs along with the parental genotypes was extracted from leaf samples according to the CTAB method (Doyle and Doyle 1987). The quality and quantity of genomic DNA were determined by agarose-gel electrophoresis using known concentrations of uncut λ DNA as standard.

EST generation and development of EST-based markers

Chickpea (cv. ICCV2) seeds from various stages of development ranging from 7 to 25 DAF (days after flower opening) were collected from field grown plants. Total RNA was isolated using the LiCl method as described earlier (Choudhary et al. 2009). cDNA libraries were constructed using the BD SMART cDNA synthesis kit (Clontech) according to the manufacturer's protocol. Double stranded cDNA fragments were cloned into the pCR2.1-TOPO vector as described by the manufacturer (Invitrogen). Random 5′ sequencing of recombinant clones was done using the BigDye Terminator technology (Applied Biosystems) in an ABI Prism 3700 automated DNA sequencer. After sequence trimming (removing low quality sequences, vector sequences and short reads <100 bp), the remaining high-quality chickpea ESTs were utilized for the development of different EST-based markers including EST-SSRs, ITPs, ESTPs and SNPs. ESTs were subjected to BLASTX analysis at a cut-off value of $1e-10$, in order to assign their putative function. All ESTs were submitted to GenBank for obtaining accession numbers.

EST-SSRs

For the development of EST-SSR markers, the program SSRIT (Simple Sequence Repeat Identification Tool; http://www.gramene.org/db/markers/ssrtool) was used to identify SSRs from chickpea transcripts. The minimum repeat unit was defined as five for dinucleotides and four for the higher order motifs including tri-, tetra-, penta-, and hexanucleotides. Duplicated SSR containing sequences were removed using the CAP3 program (http://pbil.univlyon1.fr/cap3.php; Huang and Madan 1999). STMS primer pairs were designed using the program Primer 3.0 (http://frodo.wi.mit.edu/primer3/; Rozen and Skaletsky 1997) according to the criteria mentioned in Choudhary et al. 2009 and the markers were designated as 'CESSR'.

Intron targeted polymorphism

For designing the ITP primers, the Potential Intron Polymorphism (PIP) program (Yang et al. 2007) was used. The program predicts the intron–exon junctions in the query species by comparing the query sequences (in this case, chickpea EST sequences) against the *Arabidopsis thaliana* genomic sequences and then designs intron-flanking exon–exon based primers in the query EST sequences. The designed ITP primers were designated as 'PIP'.

Expressed sequence tag polymorphism

For developing ESTP primers, the conventional approach was used in which a random EST region was targeted for designing PCR primer pairs that would generate length polymorphism. These were designated as 'CEST'.

Some of the chickpea EST-SSR and ESTP primers developed in this study amplified larger than expected sized products possibly due to the presence of intronic sequences. Some of these could not be resolved by gel electrophoresis and were difficult to score for polymorphism in the mapping population. Hence the higher sized amplified products were sequenced directly to allow new primers to be designed that could amplify smaller products. For this the genomic sequence was aligned with its EST sequence using the Splign program of NCBI and intronic sequences were identified. New primer pairs were then designed using the Primer 3.0 program (Rozen and Skaletsky 1997) to amplify smaller sized fragments that could be clearly resolved by gel electrophoresis.

Single nucleotide polymorphism

Many of the EST-based markers (across all categories) did not reveal length polymorphism and amplified same sized fragments in both the parents of the mapping population. For identification of SNPs, these similar sized PCR amplicons from the parental genotypes, *C. arietinum* ICC4958 and *C. reticulatum* PI489777, were amplified and resequenced. Prior to sequencing, the PCR products were cleaned by treatment with ExoSap-IT (USB) following the manufacturer's instructions. The resulting sequence chromatogram files were visually screened for presence of SNPs. Sequences were aligned using the Bioedit vs 7.0.5 program to identify SNPs. To design the Oligo Pool All (OPA) for performing the Illumina GoldenGate assay, only those SNPs were considered that had no other SNPs or indels in the 30 bp region immediately upstream and downstream of the depicted SNP. Sequences harboring the SNPs were then submitted to Illumina for processing by Illumina Array Design Tool (ADT) which generated scores for each SNP ranging from 0 to 1. SNPs with designability score >0.6 were selected for inclusion in the custom OPA for use in the GoldenGate assay. The SNP markers were designated as 'ESNP'.

PCR amplification

Validation of PCR-based markers (EST-SSRs, ITPs, and ESTPs) was done by amplification of the desired sized fragments from chickpea cv. ICCV2 genomic DNA. PCR was performed in a BIORAD thermal cycler (Icycler) using 25–30 ng of chickpea genomic DNA in a 10 µl reaction mix containing 0.5 mM dNTPs, 0.75 µM of each primer, 0.1 U of Taq DNA polymerase (Titanium^{TM} Taq, Clontech) and 1× PCR buffer (Titanium^{TM} Taq buffer, Clontech). A touchdown PCR protocol was used, which was described earlier (Choudhary et al. 2009). The amplified products were resolved on either 6% polyacrylamide gels or 3% Metaphor agarose gels stained with ethidium bromide.

Parental polymorphism and genotyping

For analysis of parental polymorphism, all the gene-based markers developed in this study along with 370 chickpea markers published earlier, but not mapped in the inter-specific cross, were screened for polymorphism between chickpea cultivar *C. arietinum* ICC4958 and its wild relative *C. reticulatum* PI489777, the parental lines of the inter-specific mapping population. The 370 previously published markers included 272 genomic SSR markers (gSSRs) (NCPGR series) reported by our group (Sethy et al. 2003, 2006; Gaur et al. 2011), 38 gSSRs (H-series) reported by Lichtenzveig et al. (2005) and 60 EST-SSR markers also reported earlier by our group (Choudhary et al. 2009). Additionally, 15 EST-SSRs from *Medicago truncatula* (designated MtEST) reported by Gutierrez et al. (2005) were also used for polymorphism analysis.

Genotyping was carried out with all the polymorphic markers by PCR amplification of genomic DNA from the 129 RILs and the parents as described above. The amplified products were electrophoresed on 6% PAGE stained with EtBr and analyzed using Typhoon 9210 imager (Amersham Biosciences). The banding patterns were scored as 'A' representing *C. arietinum* ICC4958 and 'B' representing *C. reticulatum* PI489777. In case of SNPs, genotyping was performed according to the standard protocol of the Illumina GoldenGate assay (Fan et al. 2003). For this 250 ng of genomic DNA (50 ng/µl) of each RIL was used along with the custom OPA (described above) and the genotyping was carried out by Sandor Proteomics Pvt. Ltd., India using the Illumina Bead Array Express Reader. The data analysis was carried out using the GenomeStudio software (Illumina, San Diego, USA).

Linkage analysis

Genotypic data with all the polymorphic gene-based and genomic markers were generated on 129 RILs of the inter-specific mapping population. Additionally, genotypic data of 105 STMS markers and 3 markers for resistance to *Fusarium* wilt races i.e. *Foc*0, *Foc*4 and *Foc*5, utilized earlier to generate the core chickpea map (Winter et al. 2000) were kindly provided by Dr. Fred Muehlbauer, Washington State University, USA and were integrated with our data for linkage analysis and map anchoring.

The Chi square test was performed for identification of markers with aberrant segregation using the locus genotyping frequencies of Joinmap ver. 4.0 (van Ooijen 2006). To identify linkage groups, grouping of markers was performed using the minimum independence LOD threshold of 3 and a maximum of 6 with a step of 0.5. At LOD 4, groups were converted to maps with the help of the regression algorithm with the following settings: used linkages with recombination frequency smaller than 0.49, LOD larger than 0.01, number of added loci after which to perform a ripple of 2 and Kosambi's mapping function (Kosambi 1994) was applied for calculation of map distances. Based on the positions and groupings of the STMS markers in the previously published maps of chickpea (Winter et al. 2000; Nayak et al. 2010; Millan et al. 2010), the LGs were numbered accordingly and designated with Arabic numerals.

Results

In the present study, a cDNA library was constructed from the developing seeds of *C. arietinum* with the aim to develop EST-based genic markers for chickpea. The library yielded 2,496 high-quality EST sequences which were systematically exploited for the development of different types of EST-based markers. In all, 487 novel, EST-derived functional markers were developed which included 125 EST-SSRs, 151 ITPs, 109 ESTPs and 102 SNPs.

Identification of microsatellites and development of EST-SSR markers

For the development of EST-SSR markers, 399 SSR containing EST sequences (16.0%) were identified and assembled into a total of 282 sequences harboring 323 SSR loci, of which 272 SSRs were found to be perfect repeats. Among the wide range of motifs identified, trinucleotide repeat motifs (170; 52.6%) were the most abundant followed by di-(91; 28.2%), penta-(32; 9.9%) and tetra-(30; 9.3%) nucleotide motifs (Fig. 1a). Among the trinucleotide motifs, the AAG motif was most frequent (57; 33.5%) followed by AAT (24; 14.1%), ACC (20; 11.7%) and AGC (17; 10.0%) (Fig. 1b). Among dinucleotides, the GA/CT motif (70; 76.9%) was the most frequent followed by AT/TA, AC/TG and a small fraction of GC/CG motifs (1; 1.09%) (Fig. 1c).

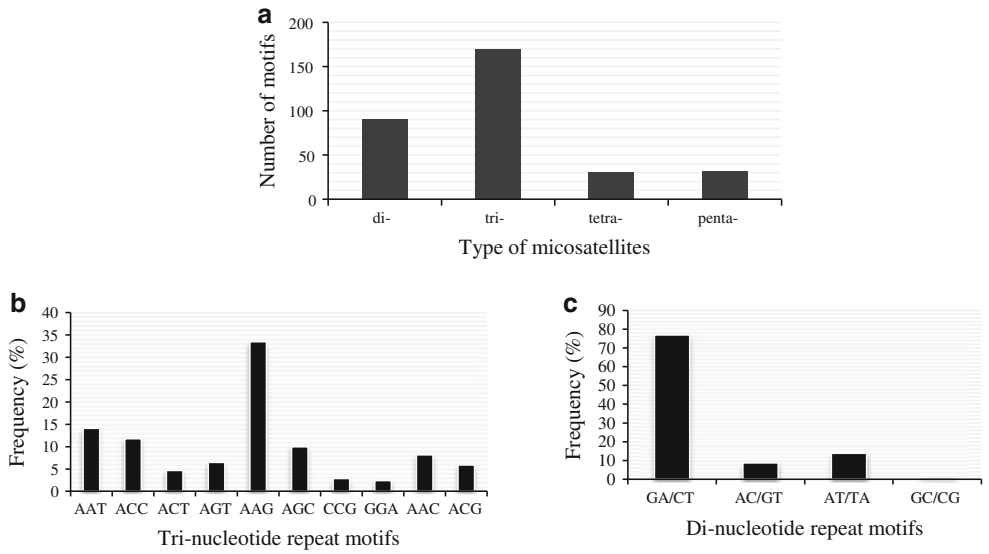

Fig. 1 **a** Frequency of occurrence of microsatellites based on type of repeat motifs. **b** Frequency distribution of tri-nucleotide and **c** di-nucleotide motifs in chickpea ESTs

Of the 282 unique sequences that contained one or more SSR motifs, 175 sequences were amenable to primer design in the region flanking the SSR motifs. For the remaining sequences, attrition was due to one of the following reasons: (a) small length of the microsatellite containing EST sequence, (b) repeat motifs were too close to the cloning sites of ESTs and (c) the flanking sequences were inappropriate for designing high-quality primer pairs (e.g. low GC content). Validation of these 175 primer pairs in *C. arietinum* cv. ICCV2 and Pusa 362 yielded 125 functional EST-SSR markers which were designated as the CESSR series and are listed in Supplementary Table 1 along with their sequences and GenBank accession numbers. The remaining 50 primer pairs either did not amplify or produced complex banding patterns. Several EST-SSR primer pairs produced fragments larger than the expected sizes, suggesting the presence of introns in the amplified genomic DNA. Some of the higher sized fragments were sequenced and new primer pairs (CESSR432, CESSR433, and CESSR434; Supplementary Table 1) were designed in order to amplify products that could be easily resolved by gel electrophoresis.

Development of EST-derived ITP and ESTP markers

For the development of ITPs in this study, the online program 'PIP' (Yang et al. 2007) was utilized. The program designed a total of 220 intron-flanking exon–exon based chickpea primers that were predicted to amplify approx. 100 bp fragments if without intron sequences. For validation, genomic DNA of chickpea cv. ICCV2 was amplified using the ITP primers which yielded 151 functional primers that produced alleles larger than 100 bp and predictably contained introns. These functional markers were designated as the PIP series and are listed in Supplementary Table 2. The remaining 69 primers could not be validated as 30 either did not amplify or produced very large fragments or complex banding patterns, while 39 primers amplified fragments of less than 100 bp that did not contain introns. The 151 ITP markers amplified products that ranged in size from 100 to 820 bp and thus were expected to contain introns. Several ITP markers also amplified more than one fragment (Supplementary Table 2).

For the development of ESTP primers, random regions within ESTs were targeted for primer design. 142 ESTP primers were designed from the generated chickpea EST sequences and validated in chickpea cultivar ICCV2. Amplification resulted in 109 primers producing expected sized bands indicating a high success rate of 76.8%. These were designated as the CEST series and are listed in Supplementary Table 3. The remaining 33 markers could not be validated as 19 primers amplified genomic regions measurably larger than those predicted from their corresponding ESTs and the remaining 14 primers did not amplify at all even under varying amplification conditions.

Identification of SNPs

Out of the repertoire of all the developed EST-based markers in this study, some were monomorphic in amplicon length between the parental lines (*C. arietinum* ICC4958 and *C. reticulatum* PI489777). Of these, 222 loci were resequenced from both the parental lines for the

identification of SNPs. Hence, a total of 47,184 bp from coding regions were analyzed for SNP discovery as calculated by adding the sizes of the expected fragments. A total of 121 single base changes and 18 indels were observed in the expressed sequences thereby revealing one SNP per 390 bp in the EST sequences analyzed. Nucleotide diversity (total no. of SNPs/total no. of aligned bases) was estimated to be 0.0025. Of the 121 SNP loci, 102 had highly conserved flanking regions and were processed for assigning the Illumina ADT designability scores. These SNPs were designated ESNP1-102 and are reported in Supplementary Table 4. Of these, 71 SNPs were found to have scores >0.6 and were used for developing the Illumina GoldenGate assay for genotyping.

Screening for parental polymorphism and genotyping with polymorphic markers

In this study, a large number of 872 co-dominant molecular markers that included both EST-based genic markers (developed in this study and some reported earlier) and genomic SSRs (gSSRs) (reported earlier but yet unmapped in the inter-specific mapping population) were used for analysis of parental polymorphism between *C. arietinum* ICC4958 and *C. reticulatum* PI489777. All the markers used in this study are listed in Table 1. The 460 EST-based markers utilized for polymorphism analysis included 185 EST-SSRs (125 developed in this study and 60 from Choudhary et al. 2009), 151 ITP and 109 ESTP markers along with 15 MtEST primers from Gutierrez et al. (2005). Additionally, 310 gSSRs were used which consisted of 272 NCPGR series by Sethy et al. (2003, 2006) and Gaur et al. (2011) and 38 H-series by Lichtenzveig et al. (2005) (Table 1). Among 770 markers in total, 260 markers (33.76%) produced clear and consistent polymorphic banding patterns between the parental lines. These 260 polymorphic markers which included 52 EST-SSRs, 51 ITPs, 25 ESTPs, 2 MtESTs, and 130 gSSRs (Table 1) were genotyped across the 129 individuals of the RIL population.

In addition to the 770 PCR-based markers mentioned above, 102 SNPs were also analyzed. Of these, 71 SNPs could be queried with the Illumina GoldenGate assay based on the ADT designability scores of >0.6. Genotyping using the assay revealed that 58 out of 71 (81.7%) represented true SNPs in the chickpea lines used and the data of 13 false SNPs were not considered for map generation. In total, parental polymorphism was analyzed using 872 markers (including 487 newly developed genic markers) and 318 markers (36.5%) were found to be polymorphic.

Construction of the linkage map

With the intention of constructing a dense linkage map of chickpea using the (*C. arietinum* ICC4958 × *C. reticulatum* PI489777) reference population, which is being used by the chickpea community worldwide, attempts were made to assign new genomic locations to the large number of co-dominant markers generated in this study as well as to some markers previously developed but not used for map generation. Genotypic data were generated with 318 polymorphic markers and were integrated with data derived from 108 previously mapped genomic STMS loci including 3 loci for resistance to *Fusarium* wilt races i.e. *Foc*0, *Foc*4 and *Foc*5 (Winter et al. 2000). The positions of the 108 previously mapped genomic STMS loci served as a framework for the construction of the linkage map and for assigning names to the LGs. The number of markers utilized and mapped in this study is summarized in Table 1.

Linkage analysis revealed that 406 loci mapped onto 8 linkage groups (corresponding to the haploid chromosome number of chickpea), which were assigned names LG1-8 according to Winter et al. 2000, based on marker similarity and concurrence between corresponding LGs (Fig. 2). The map spanned 1,497.7 cM with an average marker

Table 1 Summary of the markers utilized in the present study for the construction of the inter-specific linkage map of chickpea (*C. arietinum* ICC4958 × *C. reticulatum* PI489777)

	Markers analyzed	Markers polymorphic (%)	Markers mapped
EST-SSR (125 from this study + 60 from Choudhary et al. 2009)	185	52 (28.1)	47
ITP (from this study)	151	51 (33.8)	49
ESTP (from this study)	109	25 (22.9)	22
SNP (from this study)	102	58 (56.9)	57
MtEST (Gutierrez et al. 2005)	15	02 (13.3)	02
gSSRs [272 NCPGR + 38 H-series] (Gaur et al. 2011; Sethy et al. 2003, 2006; Lichtenzveig et al. 2005)	310	130 (41.9)	126
STMS markers (Winter et al. 1999; Hüttel et al. 1999)	108	108	103
Total	980	426	406

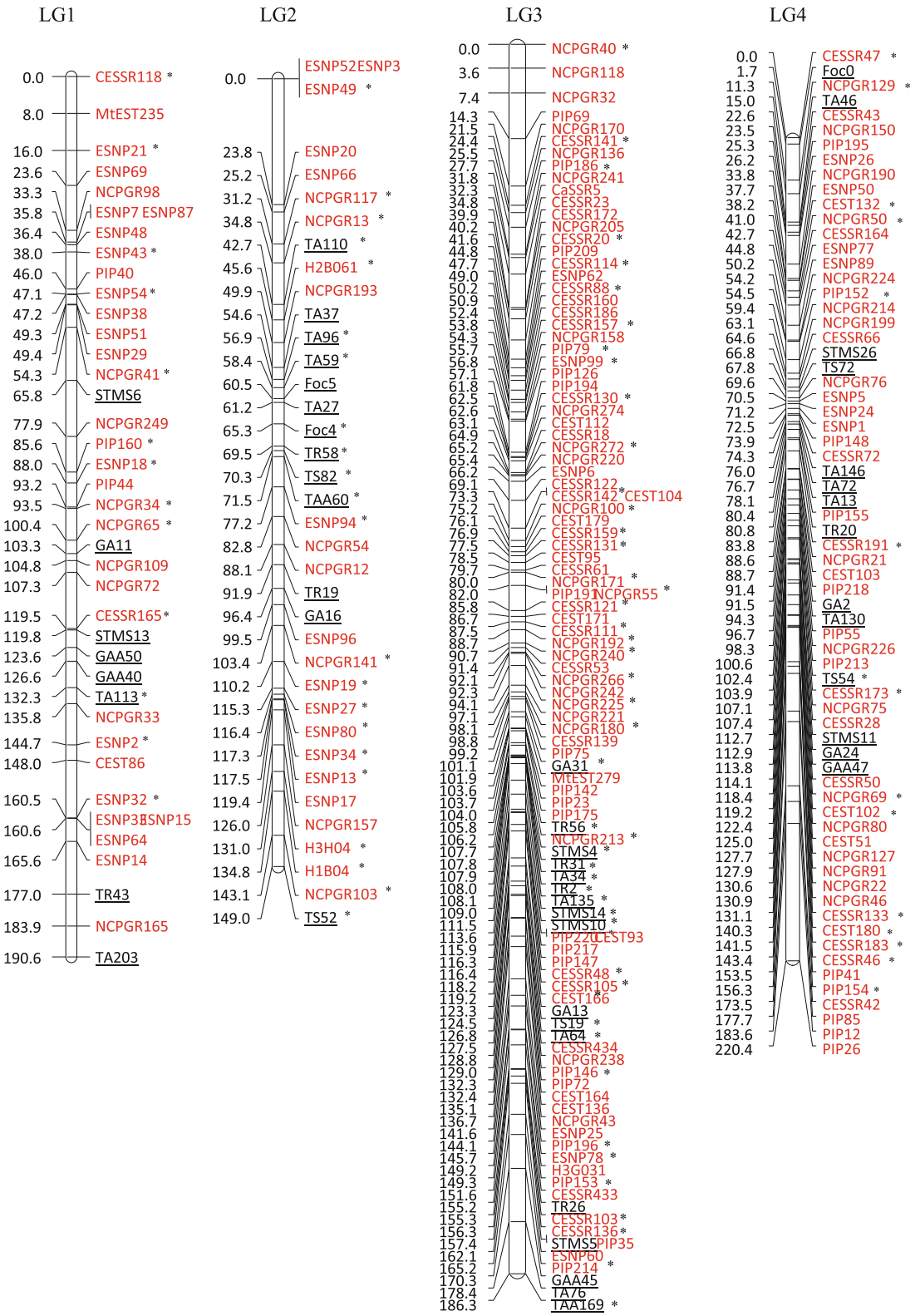

Fig. 2 The inter-specific linkage map of chickpea [*C. arietinum* (ICC 4958) × *C. reticulatum* (PI 489777)] was generated using JoinMap ver. 4.0. The map consists of eight linkage groups (LGs) spanning 1,497.7 cM. LGs were designated in Arabic numerals corresponding to the map of Winter et al. (2000) and are mentioned at the top of each LG. Distances of the loci (cM) are shown to the *left* and the name of loci are shown to the *right* side of the linkage groups. STMS markers which were used as the anchor markers (Winter et al. 2000; Hüttel et al. 1999) are *underlined*. *Markers showing distortion

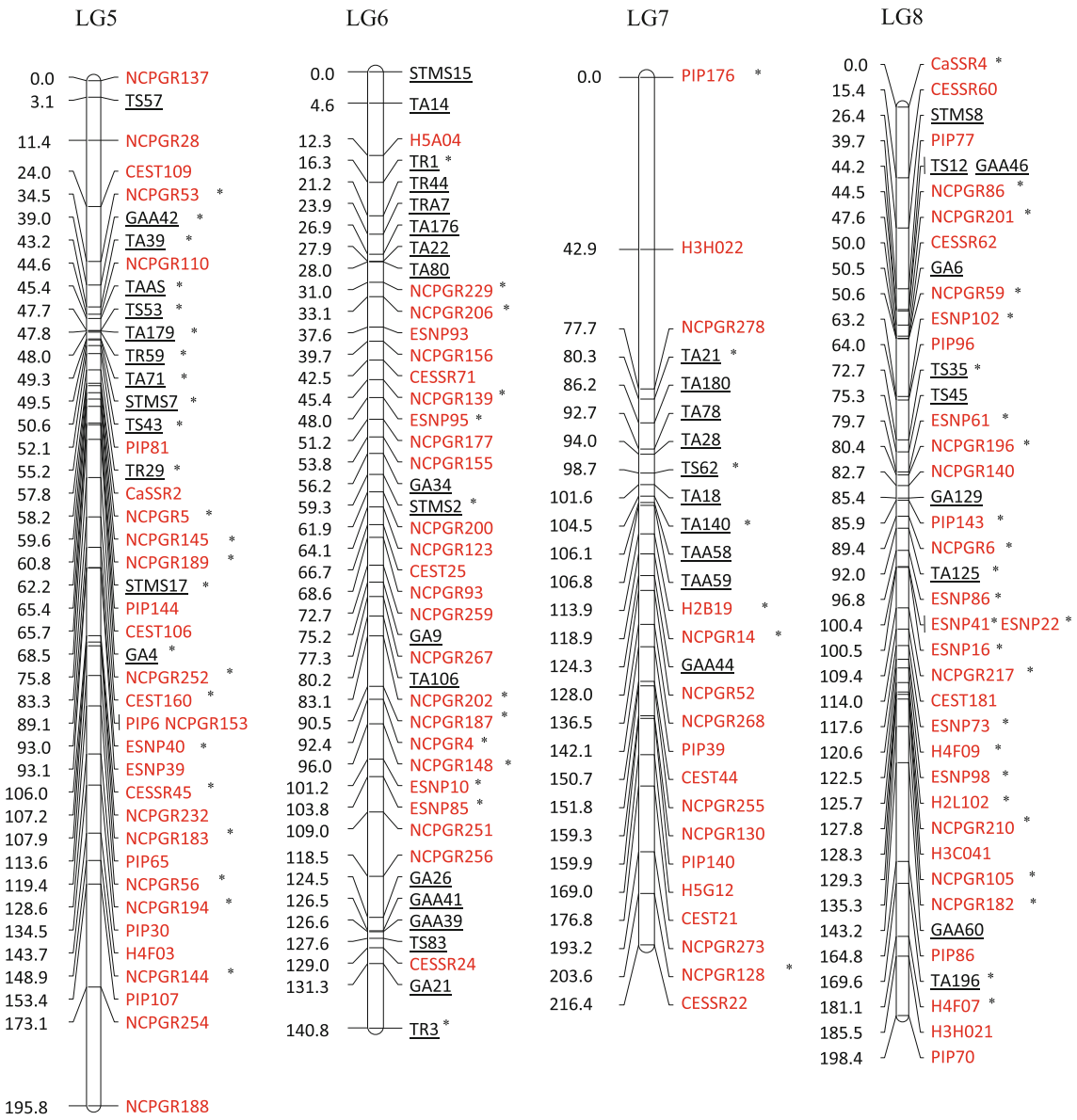

Fig. 2 continued

density of 3.68 cM (Table 2) where the total number of markers in individual LGs varied from 27 to 105, and the marker density varied from 1.77 cM in LG3 to 8.01 cM in LG7 (Table 2). On average, one LG covered 187.2 cM and contained 50.8 markers. Considering the 740 Mbp estimated size of the chickpea genome (Arumuganathan and Earle 1991), 1 cM distance in the present map approximately equaled 0.5 Mbp. The 406 markers in the present map included 303 new markers and 103 previously mapped genomic STMS markers (Winter et al. 2000). Of the new markers, 177 were gene based (47 EST-SSRs, 49 ITPs, 22 ESTPs, 57 SNPs, and 2 MtEST) and 126 were gSSRs.

Segregation distortion was observed for 176 (41.3%) loci as determined by χ^2 test ($P < 0.05$) and out of these 167 were mapped (Fig. 2). Interestingly, the majority of the distorted markers (124) skewed toward the wild annual parent *C. reticulatum* (female) whereas only 52 markers skewed toward the cultigen *C. arietinum*. Thus, on a whole genome basis, the frequency of distorted female markers appeared to be two times more (70.0%) as compared to the distorted male markers (29.3%).

Discussion

The ever expanding EST databases strengthened by both structural and functional genomics projects have led to the development of contemporary genetic marker systems that

Table 2 Distribution of markers in the eight linkage groups of an inter-specific linkage map of chickpea (*C. arietinum* ICC4958 × *C. reticulatum* PI489777)

LGs	Markers mapped	Map length (cM)	Average marker density (cM)	Skewed markers (%)
LG1	41	190.6	4.65	13 (31.7)
LG2	37	149.0	4.03	22 (59.5)
LG3	105	186.3	1.77	48 (45.7)
LG4	68	220.4	3.24	15 (22.0)
LG5	43	195.8	4.55	24 (55.8)
LG6	43	140.8	3.27	13 (30.2)
LG7	27	216.4	8.01	7 (25.9)
LG8	42	198.4	4.72	25 (59.5)
8	406	1497.7	3.68	167 (41.1)

have been exploited for enhancement of crop characteristics. Recently there have been several examples of plant species where EST-derived genic markers were successfully developed and utilized for the generation of linkage maps, including cassava (Sraphet et al. 2011), *Raphanus sativus* (Shirasawa et al. 2011), tomato (Shirasawa 2010a, b), pearl millet (Senthilvel et al. 2008) and soybean (Choi et al. 2007). In comparison, EST-derived GMMs in chickpea that have been successfully validated and utilized for mapping have been limited to 281 GMMs validated and 126 mapped (Gujaria et al. 2011). Therefore, to enhance the repertoire of chickpea GMMs, various kinds of EST-based markers totaling to 487 were developed in this study and utilized to construct a transcript map of chickpea that defines 303 new genomic locations of which 177 were GMMs.

Mining of microsatellites from the 2,496 chickpea EST sequences yielded 399 (16%) SSR containing ESTs which were comparable to previous results (Choudhary et al. 2009) and those obtained in other dicot species (Kumpatla and Mukhopadhyay 2005). Further, the higher proportion of trimeric SSRs followed by dimeric SSRs was in close agreement with observations in monocot and dicot plants (Kantety et al. 2002; Tian et al. 2004; Yi et al. 2006), even though several studies have shown exceptions as well, and this may sometimes depend on the criteria used for SSR mining (Varshney et al. 2005). In terms of converting identified SSRs into potential SSR markers, 175 primer pairs could be designed of which 125 (71.42%) proved to be functional. Generally, it has been reported that SSR amplification rates usually range from 60 to 90% in plants (Varshney et al. 2005).

With the need to identify additional efficient tools for genetic analysis, attempts were made in this study to generate EST-based markers other than SSRs, namely ITPs, ESTPs and SNPs. Intronic regions, which are reported to be more variable than exonic regions due to less evolutionary constraints provide a good source of potentially neutral genetic markers for use in linkage mapping, phylogeny, evolutionary and comparative genomic studies. Initially this was restricted only to model plants, where fully characterized genes/whole genome sequences are available (Holland et al. 2001; Wang et al. 2005). However recently, comparative genomics has facilitated applying this strategy in non-model crops where only EST sequences are available. Using ESTs of the target species and genomic sequences of *Arabidopsis* homologs, Choi et al. (2004), Wei et al. (2005) and Panjabi et al. (2008) successfully designed ITPs and demonstrated their utility for linkage mapping. Moreover, the ITP markers were also found to be highly suitable for cross-transferability studies and thus useful for identification of genetic determinants of a trait even in less-studied taxa (Choi et al. 2004; Wang et al. 2005). With the development of web-based programs like PIP (Yang et al. 2007) and GeMProspector (Fredslund et al. 2006), the designing of ITPs accelerated more rapidly even in orphan crops. Similarly in this study of chickpea, a high success rate of validation of ITP markers was observed (151/220; 68.6%) which was comparable with an earlier study in chickpea which reported 71.9% (Gujaria et al. 2011). The capability of the ITP markers to amplify intronic regions pointed to its efficiency in primer template alignment which could be attributed to accuracy and reliability of the program. This result further indicates that intron positions are highly conserved in plants and therefore using model plants to predict intron positions in non-model plants is feasible (Yang et al. 2007).

With the establishment of high-throughput SNP genotyping systems, it has now become feasible to map the entire functional gene-space rather than a small set of candidate genes. The Illumina GoldenGate SNP genotyping assay has been widely used for the genetic analysis of several crop species including the complex and the polyploid genomes (Eckert et al. 2009; Akhunov et al. 2009; Shirasawa et al. 2010b). The present study also led to the identification of 121 SNPs after resequencing of 222 chickpea loci. Our estimated SNP frequency of 1 in 390 bp in coding sequences in chickpea is higher than that estimated for wheat (1 in 1,000 bp; Bryan et al. 1999), human (1 in 1,000 bp; Sachidanandam et al. 2001) and *Arabidopsis* (1 in 3.3 kb; Jander et al. 2002). However, SNP frequency in chickpea is comparable to another legume, soybean, which has 1 SNP per 425 bp (Hyten et al. 2006). For SNP genotyping, the proportion of SNPs that can be converted into working assays is a very important factor that influences the cost and efficiency of genotyping. In the present study, the Illumina Golden Gate assay was used for the first time for genotyping in chickpea and proved to be

highly efficient. Of the 121 SNPs identified, 102 were selected for obtaining the Illumina ADT scores, based on which 71 (69.6%) were converted into working assays and were genotyped in the mapping population. This conversion rate depends upon the presence of neighboring polymorphisms and repetitive elements around a query SNP as well as on Illumina's proprietary bioinformatics screening procedures implemented in ADT (Fan et al. 2003). Of the 71 genotyped SNPs, 58 SNPs (81.7%) were identified to be true SNPs. Similarly, such high success rates of genotyping by the GoldenGate assay have been obtained previously in pine (Eckert et al. 2009), spruce (Pavy et al. 2008) and barley (Rostoks et al. 2006). In comparison, when genotyping is done by other methods such as the conversion of SNPs to CAPS, then the efficiency of genotyping is much lower as demonstrated in chickpea itself (37.06%; Gujaria et al. 2011). Therefore, SNP genotyping by the Illumina GoldenGate Assay may be recommended as the method of choice for high-throughput genotyping and mapping efforts in chickpea and other plants.

Several studies have compared the level of polymorphism obtained with microsatellites isolated from genomic and EST libraries in different systems and generally observed lower polymorphism in the latter case owing to the conserved nature of the genic regions (Cho et al. 2000; Varshney et al. 2005). Similar results were obtained in this study of chickpea where gSSRs demonstrated higher level of polymorphism (41.9%) compared to EST-SSRs (28.1%) between the parental lines of the chickpea inter-specific cross. The 41.9% polymorphism demonstrated by gSSRs was comparable with earlier studies carried out at inter/intra level in chickpea that had revealed rates of polymorphism varying from 30 to 50% (Hüttel et al. 1999; Winter et al. 1999, 2000; Udupa and Baum 2003; Cho et al. 2004; Radhika et al. 2007; Taran et al. 2007; Gaur et al. 2011). Further, the polymorphism rate of 28.1% of chickpea EST-SSRs was comparable to that reported with pepper EST-SSRs (29.2%; Yu et al. 2006) and higher than cotton (19.8%; Park et al. 2005) but lower than that reported earlier in chickpea (37%; Gujaria et al. 2011). Further, comparison amongst the three kinds of chickpea EST-based markers employed for mapping purpose revealed that the highest polymorphism was obtained with ITPs (33.8%) followed by EST-SSRs (28.1%) and lastly with exon-based ESTP (22.9%) markers. These results suggest that both introns and SSRs may serve as efficient sources of hypervariable markers in chickpea for analysis of genetic diversity, map saturation and comparative mapping studies.

In the present genetic map of chickpea, 403 markers were positioned along with three *Foc* loci, spanning 1,497.7 cM with an average marker density of 3.68 cM, which is a higher density than that reported by Nayak et al. (2010) (4.99 cM) in the same mapping population. Gujaria et al. (2011) recently reported a map with inter-marker density of 2.55 cM, although this map only covered 766.56 cM. The linkage map we developed in this study includes 303 new map locations in comparison to the earlier maps of Nayak et al. (2010) (175 new loci) and Gujaria et al. (2011) (62 new locations). Another advantage of the map described in this report is that it was constructed using only co-dominant markers in comparison to earlier maps that included larger proportions of dominant markers, which can be difficult to apply and reproduce. An additional distinguishing feature of our map was that it was a gene-rich map that contained 177 GMMs (47 EST-SSRs, 49 ITPs, 22 ESTPs, 57 SNPs, and 2 MtESTs). These anchored functional loci may prove useful not only for comparative mapping studies but also directly for MAS to improve related traits.

The markers on this map were distributed relatively evenly across linkage groups. However, gaps of >40 cM between markers were found at the ends of LG4 and LG7 and >20 cM gaps between markers were found at the ends of LG2 and LG5, demonstrating a lack of polymorphic markers in these regions. Generally, it has been noted that genomic SSRs tend to form clusters in heterochromatic regions (Ramsay et al. 2000; Areshchenkova and Ganal 2002; Shirasawa et al. 2010a), however in this study, since EST-derived markers were also included, the overall marker distribution was more or less even throughout all the LGs. The relative order of the anchored markers determined on previous maps (Winter et al. 2000; Nayak et al. 2010) was concurrent with the present map and for the first time the number of genetic markers were significantly improved on LGs 7 and 8 (Fig. 2). Moreover, mapping of the EST-derived markers considerably increased the marker density of LGs 1, 3 and 4 (Fig. 2), where the number of mapped EST-derived markers exceeded the gSSR markers. The EST-derived markers accounted for >69.3% of the loci in these regions, whereas they contributed only 43.9% to the map as a whole. Based on the genome coverage method from linkage data (Chakravarti et al. 1991), almost 98.85% of the chickpea genome was covered indicating that the map was fairly saturated.

The map generated in this study was a gene-rich map which accommodated 177 EST-derived loci. Of these, 73.4% showed significant similarity to known sequences while 26.6% to hypothetical/unknown proteins. Most gene products were predicted to function as enzymes involved in primary or secondary metabolism, in regulation or signal transduction pathways, and in biotic or abiotic stress responses. Such gene-derived loci are particularly useful for non-model organisms, since they provide better chances of identifying genes controlling complex traits in the absence of genome sequence data (Namroud et al. 2008).

The segregation distortion obtained with the current set of chickpea co-dominant markers (41.3%) was comparable to the marker distortion of 38% reported by Winter et al. (2000) on the same mapping population. This distortion may have been due to recombination suppression at meiosis or translocations and inversions that are common in inter-specific crosses. Other factors also responsible for segregation distortion include the types of mapping population (RILs/F2/BC) and the nature of cross etc. Generally, a higher percentage of allelic distortion is observed in inter-specific crosses as compared to the intra-specific crosses (Flandez-Galvez et al. 2003 found only 20.4% distortion in a chickpea F2 intra-specific population), and also increases with generational advancement from F2 through F7, as has been demonstrated in tomato (Paran et al. 1995) and mung bean (Lambrides et al. 2004). In our study, of the 176 distorted markers, the majority (70.4%) skewed in favor of the female parent (*C. reticulatum*). This was explained by the chromosomal rearrangements in chickpea relative to *C. reticulatum* in karyotyping studies (Galasso et al. 1996). The apparent clustering of distorted loci observed in our study (Fig. 2) was consistent with results obtained earlier in chickpea (Winter et al. 1999; Tekeoglu et al. 2002) and in other crops (Kidwell et al. 1993; Riaz et al. 2004) and generally resulted in less overall genome coverage (Tanksley et al. 1992).

In the present study, the repertoire of co-dominant molecular markers available for chickpea, especially the gene-derived markers (GMMs) spanning various categories, was greatly enhanced as 487 new, validated, functional markers were developed. Using a chickpea reference mapping population, an inter-specific linkage map was constructed that contained EST-derived genic markers anchored in the backdrop of genomic SSRs. This improved gene-rich linkage map will facilitate the identification of markers associated with a range of agronomic traits that can be examined in the inter-specific RIL population.

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