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Development of EST derived microsatellite markers in chickpea and their validation in diversity analysis

Subhojit Datta*, Mayank Kaashyap[1] and Prasoonpal Gupta
Biotechnology Unit, ICAR-Indian Institute of Pulses Research, Kanpur 208 024, India

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Microsatellites are widely used as genetic markers because they are co-dominant, multi-allelic, easily scorable and highly polymorphic. In order to enhance availability of genomic resources, microsatellite loci were identified from chickpea (*Cicer arietinum* L.), the third most important grain legume in the world. A total of 20 SSR markers were developed from EST clones of wilt resistant cultivar (JG 315) of chickpea. Chickpea varieties (15) were analyzed for genetic diversity with these markers, which produced a total of 35 alleles with a mean of 1.5 alleles per primer. About 5 markers were polymorphic in the selected genotypes and observed heterozygosity ranged from 0.12 to 0.87 with an average of 0.32. These microsatellite markers will be useful in diversity analysis, mapping agronomically important traits and marker assisted breeding in chickpea.

Keywords: Chickpea, ESTs, heterozygosity, microsatellites, polymorphism, simple sequence repeat (SSR) markers

Introduction

Despite enormous agronomic importance, the average productivity of chickpea has been very low mainly because of yield losses caused by several biotic and abiotic stresses. Conventional breeding efforts did not make much progress in improving productivity mainly due to major effect of environmental factors and their interaction with genotypes, making empirical selection for yield and its components ineffective. Marker-assisted breeding holds the promise of potential crop improvement by increasing efficiency of breeding methods. Expressed sequence tags (ESTs) provide an effective approach for gene discovery and transcript pattern characterization and emerges as a cost effective, valuable source for molecular marker development[1]. The EST-SSRs are useful for many applications in plant genetics and breeding, such as, molecular mapping, genetic diversity analysis and cross-transferability across related species and genera[2].

In chickpea, microsatellite markers have been preferably used in germplasm classification, identification of genotypes[3] and genetic linkage map construction[4-6]. Considerable progress has been made in recent years in development and characterization of number of microsatellite markers of food and model legumes[1,7-9]. Genome-wide, polymorphic EST-SSR markers are the prerequisite of genetic mapping and marker assisted improvement in chickpea, as they directly provide link to a gene of interest being originated from the expressing part of genome. In the present study, we report isolation and characterization of 22 new SSR markers from chickpea EST library.

Materials and Methods

In order to isolate the microsatellite repeat motifs, a set of EST clones were constructed from wilt resistant chickpea cultivar JG 315. Total RNA was isolated from 3-wk-old leaves of chickpea and mRNA was isolated using RNeasy mini kit (QIAGEN GmbH, Germany). Double stranded cDNA was synthesized using SMART PCR cDNA library construction kit (Clontech, CA). Sticky ends of PCR amplicons were ligated to pTZ57/RT vector (Fermentas, USA) and transformed into XL1-blue MRF' *Escherichia coli* strain (Stratagene, La Jolla, USA). Recombinants were selected and plasmids isolated using QIAprep spin mini kit. From 200 recombinant clones, 35 were randomly selected for sequencing using Big Dye terminator technique in an ABI 3100 genetic analyzer apparatus (Applied Biosystems, USA) based on Sanger's method. A set of 35 EST sequences is submitted to NCBI databases (www.ncbi.nlm.nih.gov)

*Author for correspondence:
subhojit@email.com; subhojitdatta1@gmail.com
Present address: Division of Crop Improvement, ICAR-Central Research Institute for Jute and Allied Fibres, Barrackpore, Kolkata 700 120, India

under the accession numbers GE 213101 to GE 213135. Based on sequences of the regions flanking the microsatellite motifs, SSR primers were designed using Primer 3 software[10] to amplify the repeats containing loci.

The markers were validated for their ability to establish genetic relationship within a set of 15 chickpea cultivars including exotic, kabuli, biotic and abiotic stress tolerant varieties and one wild progenitor (*C. reticulatum*) of chickpea (Table 1). Genomic DNA was isolated by CTAB method and PCR reactions were performed in 20 µL volume containing 25 ng of template DNA, 20 pmoles each forward and reverse primers, 25 µM of each dNTPs (MBI Fermentas, USA) and 0.6 U of *Taq* DNA polymerase (Bangalore Genei, Bengaluru, India). The PCR program was run on PTC-200 (MJ Research, USA) thermocycler consisting of initial denaturation at 94°C for 3 min, followed by 39 cycles each consisting of denaturation at 94°C for 1 min, annealing at appropriate temperature (Table 2) for 1 min and elongation at 72°C for 2 min. Final extension step included 72°C incubation for 7 min. The amplified DNA fragments were resolved on ethidium bromide stained agarose gel (2%) in 1× TBE buffer. The gels were visualized on trans-UV and photographed in BioRad Gel Doc XR 2.0. The dendrogram was constructed using NTSYS-pc software *ver* 2.1[11].

Table 1—Description of chickpea genotypes used for diversity analysis

No.	Name of the genotype	Special features
1	BG 1005	Kabuli, donor for wilt tolerance
2	KAK 2	Kabuli, semi-spreading plants, bold, white seeded
3	JG 11	Donor for wilt tolerance
4	TYSON	Australian genotype
5	ICCV 10	Exotic, donor for wilt and dry root rot tolerance
6	BGD 72	Semi-erect plants, medium bold seeds, tolerant to wilt
7	CSG 8962	Donor for mild salinity and wilt tolerance
8	BG 1003	Tolerant to wilt
9	K 850	Kabuli, erect plants with dark green foliage, reddish
10	KANIVA	Australian, kabuli and donor for drought tolerance
11	HC 3	Donor for wilt tolerance
12	JG 315	Donor for wilt tolerance
13	E 100 Ym	Donor for short internode trait
14	RSG 888	Donor for drought and dry root rot tolerance
15	*C. reticulatum* (ICC 17196)	Crossable (primary gene pool) and resistant to pod borer

Results and Discussion

A set of 35 EST sequences were submitted to NCBI databases (www.ncbi.nlm.nih.gov) under the accession numbers GE 213101 to GE 213135. Sequence analysis of SSR motifs from a set of ESTs revealed that 19 were perfect repeats including dimers, trimers, tetramers and pentamers repeated more than twice, while 3 were compound repeats (Table 2). The copy number of dinucleotide repeats varied from 3 to 39, trinucleotide repeats varied from 2 to 14 and tetranucleotoide repeats from 2 to 6. The most frequently appearing dinucleotide motifs were GA, followed by TA; whereas among the trinucleotides, ATT was predominant followed by AAG.

The markers were validated for their ability to establish genetic relationship within a set of 15 chickpea cultivars including exotic, kabuli, biotic and abiotic stress tolerant varieties and one wild progenitor (*C. reticulatum*) of chickpea (Table 1). All the 22 primer pairs used amplified fragments of expected length across all the 15 chickpea cultivars. A total of 35 alleles were amplified with an average of 1.5 alleles per primer. Primer pairs IPCM 8, IPCM 11, IPCM 13 and IPCM 14 amplified a maximum number of three alleles in 15 chickpea genotypes. Five primer pairs, namely IPCM 8, IPCM 11, IPCM 14, IPCM 20 and IPCM 31 produced highly polymorphic amplicons. The observed heterozygosity values ranged from 0.12 (IPCM 3, IPCM 4, IPCM 10, IPCM 12) to 0.87 (IPCM 20) with an average of 0.32. Clustering analysis indicated the levels of intraspecific genetic polymorphism and efficiency of these SSR markers in identification of genotypes on the basis of special features they share (Fig. 1). Both Kabuli chickpea BG 1005 and KAK 2 cluster close together (Cluster IA) and similar is the case with JG11 and ICCV10, which share common trait of wilt tolerance (Cluster IC). Cluster IB depicts unambiguous clustering of donors for disease resistance, namely, HC3, RSG888, JG315 and *C. reticulatum* (ICC 17196).

In conclusion, the study demonstrates that the microsatellite markers characterized here will prove useful in assessing both the intra and interspecific genetic polymorphism within genus *Cicer* and also

Table 2—Description of chickpea microsatellite markers developed including number of alleles, motifs, product size and heterozygosity

Primer name	Sequence (5′- 3′)	Tm (°C)	Expected product size	Obtained product size	Motif	Alleles	Expected heterozygosity
IIPR 1	F: CCGTTCCGACAATTTGAAGT	58.3	184	300	(GAA)2(TTTA)2(TGT)4	1	0.24
	R: ATTGGGAGGATCCCATTGA	58.0					
IIPR 2	F: GACAAAACAACCTCCCAAGAAA	58.0	258	250	(ATT)6	1	0.36
	R: GACGACAACAACAACAACAACA	58.0					
IIPR 3	F: CTCTTCCTTGCAACCGAAAC	60.4	209	200	(TTC)4	1	0.12
	R: TCTCTGCAGCATTGAGGAAA	58.3					
IIPR 4	F: TTTTGAATCGATCCACCTGA	56.3	244	235	(TATTT)2 (TTAT)2	1	0.12
	R: GCAAATCGCATGGCATATTA	56.3					
IIPR 5	F: GAGCCCTGAAATGAAGAAAGAA	58.0	387	400	(AAAT)5	1	0.78
	R: CACCTTTGAGCCCTAGTCTGTT	62.0					
IIPR 6	F: GATTCGCCCTTTCGAGCG	62.0	156	373	(CT)20	1	0.25
	R: TGGTGAGAGAAGCAAGACCCAT	58.0					
IIPR 7	F: TGAATTTTGTTTACCACCCCTC	58.0	157	250	(AG)20	1	0.36
	R: TTTGGCTTATTCTGTTCTTCCC	58.0					
IIPR 8	F: GACAAAACAACCTCCCAGAAA	58.0	279	219, 260, 500	(TTG)6	3	0.81
	R: AACAACGACAACAACAACAACG	58.0					
IIPR 9	F: AAACACCTCTCTTCACCTTCCA	60.8	221	204, 176	(ATA)3	2	0.68
	R: CCAACACTTTAATTAGCAAAACA	57.3					
IIPR 10	F: TCACACTTCCACAACAAAATGA	57.0	203	188	(AAG)3	1	0.12
	R: GGAGCTCAAAAGTGGCAAAG	60.4					
IIPR 11	F: GGTCATGTTGATTTCTCACCAA	58.0	337	250, 195, 181	(AAAT)6	3	0.79
	R: GAACTTTCCGCACACGTTATG	59.0					
IIPR 12	F: GCTTTGAGGGAGTTTGCATC	60.4	186	162	(ACACA)2	1	0.12
	R: CAATCGCATGTCATTCTTTCA	56.7					
IIPR 13	F: ATACGACGACGATTCTGGATTT	58.0	170	247, 155, 110	(GA)36	3	0.66
	R:TTCTCACATCTCTCTCTCTCTCTCTC	58.0					
IIPR 14	F:ACCTCCGTCCACACATTTCTAC	62.0	224	312, 200, 162	(GA)39	3	0.83
	R:GTCGAAGCCATTGTTTTGTTG	57.0					
IIPR 16	F: AAATTGGACAACCGCCTCTA	58.3	212	200	(TA)4(TA)3 (TA)3	1	0.24
	R: CCCCAGGGAATAAAGGAAAA	58.3					
IIPR 17	F: TGTCAGTGGATCACCAATTAGC	60.0	354	356	(TAT)7	1	0.25
	R: CAATCCCCATAAGATGAACTCC	60.0					
IIPR 20	F: GTTACAAGTCGCCATTCCAAA	57.0	398	326, 300	(ATA)14	2	0.87
	R: CATTTGTCTCGTTCACATACCG	60.0					
IIPR 21	F: GGGCCATACATCAAACACAAT	57.0	249	210	(GA)20	1	0.36
	R: CCACATTCTTTAGCACATGGAA	57.0					
IIPR 25	F: GGCACATGGTTCCTCTTAAACT	60.0	300	327	(AG)20	1	0.36
	R: CCATCACACCTTATTGCTTTCA	58.0					
IIPR 26	F: TGCTCATGCTTACTTCTTCCTG	60.0	220	400	(CAA)5	1	0.25
	R: GTCGGCTTGGCTCATGTAAT	58.0					
IIPR 27	F: CATGCTCCCCTAATTGACATA	57.0	355	197, 232	(AT)32	2	0.49
	R: AGGCATAAATCCATCTGCATAC	58.0					
IIPR 31	F: TCCGATGGAACCTTCTCTTTTA	58.0	400	396	(TTA)7	1	0.00
	R: CTCTTCGGGGTCGTATTGATT	59.0					

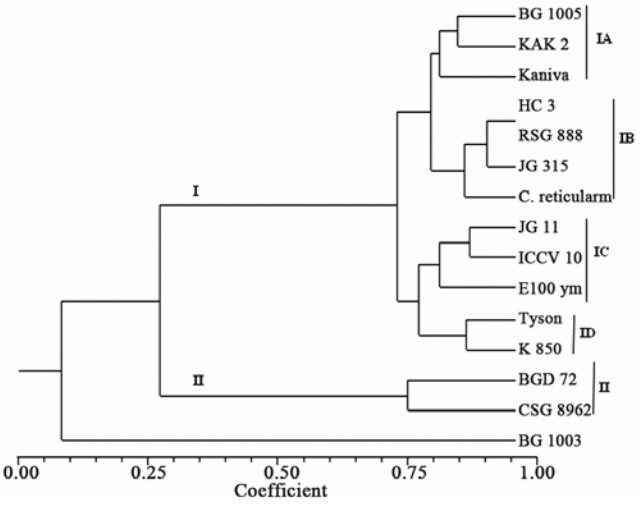

Fig. 1—Clustering of chickpea genotypes using Jaccards similarity co-efficient based on SSR data.

aid in construction of linkage maps and comparative mapping in chickpea.

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