Advancing the STMS genomic resources for defining new locations on the intraspecific genetic linkage map of chickpea (Cicer arietinum L.)

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Abstract

Background: Chickpea (Cicer arietinum L.) is an economically important cool season grain legume crop that is valued for its nutritive seeds having high protein content. However, several biotic and abiotic stresses and the low genetic variability in the chickpea genome have continuously hindered the chickpea molecular breeding programs. STMS (Sequence Tagged Microsatellite Sites) markers which are preferred for the construction of saturated linkage maps in several crop species, have also emerged as the most efficient and reliable source for detecting allelic diversity in chickpea. However, the number of STMS markers reported in chickpea is still limited and moreover exhibit low rates of both inter and intraspecific polymorphism, thereby limiting the positions of the SSR markers especially on the intraspecific linkage maps of chickpea. Hence, this study was undertaken with the aim of developing additional STMS markers and utilizing them for advancing the genetic linkage map of chickpea which would have applications in QTL identification, MAS and for de novo assembly of high throughput whole genome sequence data.

Results: A microsatellite enriched library of chickpea (enriched for (GT/CA)n and (GA/CT)n repeats) was constructed from which 387 putative microsatellite containing clones were identified. From these, 254 STMS primers were designed of which 181 were developed as functional markers. An intraspecific mapping population of chickpea, [ICCV-2 (single podded) x JG-62 (double podded)] and comprising of 126 RILs, was genotyped for mapping. Of the 522 chickpea STMS markers (including the double-podding trait, screened for parental polymorphism, 226 (43.3%) were polymorphic in the parents and were used to genotype the RILs. At a LOD score of 3.5, eight linkage groups defining the position of 138 markers were obtained that spanned 630.9 cM with an average marker density of 4.57 cM. Further, based on the common loci present between the current map and the previously published chickpea intraspecific map, integration of maps was performed which revealed improvement of marker density and saturation of the region in the vicinity of sfl (double-podding) gene thereby bringing about an advancement of the current map.

Conclusion: An arsenal of 181 new chickpea STMS markers was reported. The developed intraspecific linkage map defined map positions of 138 markers which included 101 new locations. Map integration with a previously published map was carried out which revealed an advanced map with improved density. This study is a major contribution towards providing advanced genomic resources which will facilitate chickpea geneticists and molecular breeders in developing superior genotypes with improved traits.

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Background
Molecular genetic maps covering extensive parts of the genome are essential tools for genomics research, throwing light on genome organization, facilitating marker-assisted breeding of agriculturally important quantitative and qualitative traits and map-based cloning of important genes. Currently the co-dominant microsatellite based STMS markers remain a standard for the construction of highly saturated linkage maps in several economically important crop plants such as wheat [1], barley [2], maize [3], tobacco [4], sunflower [5], rose [6], apple [7], tomato [8] and legumes like soybean [9,10] and peanut [11].

Even though considerable progress has been achieved in many crops for studying the genetics of quantitative traits, in the 2nd (after bean, based on harvested area) most important grain legume crop i.e. chickpea (Cicer arietinum L.; 2n = 2x = 16) (FAOSTAT 2009; http://faostat.fao.org/site/567/default.aspx), genomics-assisted programs have moved at a slow pace. The crop has a genome size of 740 Mb and is primarily cultivated in arid and semi-arid areas of the world. Despite it being a protein-rich food, the current average yield of chickpea is only 798 Kg/ha which is far below the potential yield of 6.0 t/ha and is relatively low as compared to pea (1,468.7 Kg/ha) (FAOSTAT 2009). Susceptibility of the chickpea crop to various biotic and abiotic stresses and the low levels of genetic variability are the major constraints to its improvement [12,13]. Moreover, owing to the extremely low levels of genetic polymorphism [14,15], progress towards the development of a sufficient number of polymorphic markers has been limited. Therefore in order to reap the benefits of enabling biotechnologies for crop improvement, there is a pressing need to increase the availability of genomic resources which serve as tools to assist in plant breeding programs. Hence, the central goal of current chickpea researchers is to enrich genomic resources such as molecular markers, especially SSRs, and genetic linkage maps, comprising loci of both economic and scientific importance [13].

Among the vast repertoire of molecular markers currently available, STMS markers have emerged as the best tool to address the allelic diversity in chickpea [16-19]. Further, owing to their ability of interspecific transferability, STMS markers have been reported to be the most elite anchor markers for merging different genetic maps and for setting up a high genome coverage consensus map in chickpea [13,20]. Unfortunately, unlike other legumes like Medicago and soybean, till date in chickpea only about 800 STMS markers have been reported [16,18,21-26], and of these only 30-40% are expected to be polymorphic. Nevertheless, microsatellites which are known to be abundant and uniformly distributed in the chickpea genome have been used to develop a genotyping kit for chickpea [19], analyze genetic relationships among Cicer species [23,27] and assess levels of cross-transferability [28,29]. Further, these markers have been applied for the construction of intraspecific [30-36] and interspecific [21,26,37-39] genetic linkage maps and for mapping genes of agronomic importance such as disease resistance [37,39,40] and yield related traits [30,41,42], thereby demonstrating that SSRs are ideal tools for broad applications in basic and applied plant biology [43,44]. However, all these studies have repeatedly used only the limited set of available STMS markers and not more than 120 STMS markers have been mapped on the intraspecific linkage maps currently available [30,34,35]. Hence these maps have been of limited use as genomic regions harboring genes of important traits are not yet sufficiently saturated to apply MAS in plant breeding programs. Therefore, the immediate need to map new genomic locations and merge different genetic maps to saturate the intraspecific maps for uniform genome coverage was clearly evident.

Hence the present study was undertaken with the objective of developing a large number of STMS markers which could be utilized by the chickpea community for various applications in chickpea genomics. Next, these markers along with the other published STMS markers were used to advance the intraspecific genetic linkage map of chickpea by defining many new genomic locations. Finally, data of already published loci was integrated with our map to further saturate genomic regions.

Results
Characterization of microsatellites and development of STMS markers
Four thousand recombinant clones from the (GT/CA) and (GA/CT) microsatellite enriched library were screened which resulted in the identification of 387 clones that were sequenced. Assembly yielded a set of 22 contig and 314 singleton DNA sequences which summarized a total of 336 unique chickpea sequences. SSR mining revealed that 37 of these either contained an SSR sequence of <5 repeats or did not contain any microsatellites. Moreover, primers could not be designed against 45 of the sequences due to insufficient length of SSR-flanking sequences. Ultimately, 254 (75.5%) primer pairs were designed that flanked the microsatellite motifs. All these primer pairs were validated by PCR using genomic DNA from a set of four C. arietinum accessions. Of these, 48 (18.8%) primer pairs produced no PCR products under a number of annealing/elongation temperature combinations, 25 (9.8%) amplified anomalous fragments and 181 yielded fragments of expected sizes. The sequences of these 181 functionally validated primers and the respective microsatellite motifs are listed in Table 1.
Table 1 List of 181 novel chickpea STMS markers developed in this study; the locus name, type of repeat motif, primer sequences, annealing temperature (Tm), expected product size (bp), number of amplified alleles (Na), and GenBank accession numbers are mentioned

| S. No. | Locus | Repeat motif | Primer sequence (5’→3’) | Tm (°C) | Size (bp) | Na | GenBank Acc. No. |
|-------|-------|--------------|--------------------------|--------|----------|----|-----------------|
| 1     | NCPGR101 | (CT)_{18} | TCTGCTTTTGTCAGAAGAT/TGAATACGCGTACCTGGTGG | 59.3   | 291      | 1   | EU877268        |
| 2     | NCPGR102 | (CA)_{2}N_{10}(CA)_{13} | GCGTGATCACTCATCAATA/TTAACATGGGCACT | 55.4   | 240      | 1   | EU877269        |
| 3     | NCPGR103 | (CT)_{2}tc(CT)_{21} | ACAACCATATCTTTGGCC/GTCTATGAGAAAACCGGAGAA | 55.0   | 213      | 1   | EU877270        |
| 4     | NCPGR104 | (GA)_{21} | GCTAAGAGATGATATGGA/GGCACTCTGGAGATGTCAT | 54.3   | 221      | 1   | EU877271        |
| 5     | NCPGR105 | (CT)_{2}at(CT)_{2}at(CT)_{2}at(CT)_{2}at(CT)_{2}at(CT)_{18} | TTTTTTTGTCAGAAGAT/TGAATACGCGTACCTGGTGG | 54.5   | 261      | 1   | EU877272        |
| 6     | NCPGR106 | (GA)_{19} | ATTTGGCTAACATGTGTT/GTCAATGATGTTTGTTTCAAGA | 54.5   | 229      | 1   | EU877273        |
| 7     | NCPGR107 | (CT)_{22} | AAAACATATATGCTCCTCA/CCCTACCGAGATGTCGT | 54.0   | 244      | 1   | EU877274        |
| 8     | NCPGR108 | (CT)_{20}(GT)_{6} | AGTTCAAGCTCTTTGATGT/CCGGGAAACAGGAGGAAGGA | 54.5   | 278      | 1   | EU877275        |
| 9     | NCPGR109 | (CT)_{2}cccc(CT)_{10} | TAGCTCAAGAGATATGC/AAAACATATATGCTCCTCA | 55.1   | 285      | 1   | EU877276        |
| 10    | NCPGR110 | (AT)_{6}(GT)_{2}gc(GT)_{2}at(GT)_{2}ct(GT)_{10} | CAAAGGATTAGTGGAA/GGAAAGAACATGTTGTGTTG | 55.2   | 217      | 2   | EU877277        |
| 11    | NCPGR111 | (CT)_{22} | AATACAACCGCTATTGGA/CCCTACCGAGATGTCGT | 54.5   | 247      | 1   | EU877278        |
| 12    | NCPGR112 | (CA)_{2}cg(CA)_{2}cg(CA)_{2}cg(CA)_{12} | TTTTTTTTCCCATCATC/CTTACACCGAGATGTCGT | 54.5   | 290      | 3   | EU877279        |
| 13    | NCPGR113 | (CT)_{1}ca(CT)_{2}(CT)_{1}ca(CT)_{6} | ATCTCCTCCTCTCCTCTCTCGT/CCCTACCACTCATACACCA | 58.0   | 299      | 1   | EU877280        |
| 14    | NCPGR114 | (GA)_{2}gg(GA)_{9} | TAAAGGGGGACATCATTG/CGGTGTTACCTGATACAC | 55.0   | 279      | 1   | EU877281        |
| 15    | NCPGR115 | (CT)_{18} | TGGAGCCCAAATGATGCT/CTTACCTCGAGGAGATTG | 60.2   | 213      | 1   | EU877282        |
| 16    | NCPGR116 | (GA)_{21} | ATATTCTTCTTCCGGGAC/AGGGGAAAAGATATCTACAC | 55.4   | 295      | 1   | EU877283        |
| 17    | NCPGR117 | (CT)_{23} | GAATCTTCTCTCTCAGG/CTTGACACGAGAAAGATTG | 54.5   | 199      | 1   | EU877284        |
| 18    | NCPGR118 | (GT)_{2}2(GA)_{18} | GAGTTGATTTGTTGATT/ACGGGAAATATCTCAGG | 55.5   | 224      | 1   | EU877285        |
| 19    | NCPGR119 | (CT)_{1}N_{10}(CT)_{10} | GTTGGCTCTCTCTCTCTCTCTCT/TCACACCGGACCGACAA | 60.1   | 234      | 1   | EU877286        |
| 20    | NCPGR120 | (GT)_{20} | GCCGCAAGGTGGATATTAG/TATGGTCTTCTTCCTACAC | 54.7   | 300      | 4   | EU877287        |
| 21    | NCPGR121 | (GT)_{1}N_{8}(GA)_{15} | TGGTTGTTGAAAAGAACAA/TGGTTGTTGAAATCTCGGACTG | 58.9   | 215      | 1   | EU877288        |
| 22    | NCPGR122 | (GA)_{1}3g(GA)_{2}(GA)_{8}a(GA)_{9} | TGGTTGTTGGAATCTTCTG/TTGGTAGGATAGAGACACCC | 55.0   | 289      | 2   | EU877289        |
| 23    | NCPGR123 | (CT)_{25} | CTTCTGGGACTCAGGGAAT/GCTCTGTCAGGAAACGCAAAAA | 55.0   | 273      | 1   | EU877290        |
| 24    | NCPGR124 | (CT)_{20} | TGGTTGTTGGAATCTTCTG/ACTGAACTTTTGGAAAGG | 54.3   | 140      | 1   | EU877291        |
| 25    | NCPGR125 | (CT)_{25} | CTTCTGGGACTCAGGGAAT/GCTCTGTCAGGAAACGCAAAAA | 55.5   | 239      | 1   | EU877292        |
| 26    | NCPGR126 | (CT)_{1}N_{2}(CT)_{2}t(CT)_{3} | AGAAGTGGGACAAACACCTT/TTGGTGGGCACTATGATACCTTCCTG | 59.1   | 324      | 1   | EU877293        |
| 27    | NCPGR127 | (GA)_{18} | CACGAAAGCCGAGAATTAG/CTTCTTCTTCATGATGCTGCGG | 55.5   | 279      | 1   | EU877294        |
| 28    | NCPGR128 | (CA)_{2}cg(CA)_{2}CGCA_{4} (CA)_{2}N_{10}(CG)_{4} (CA)_{10} | GCAATGAGCAACATTCTT/CGTCGAGCACTTTCCGCT | 56.2   | 290      | 1   | EU877295        |
Table 1 List of 181 novel chickpea STMS markers developed in this study; the locus name, type of repeat motif, primer sequences, annealing temperature (Tm), expected product size (bp), number of amplified alleles (Na), and GenBank accession numbers are mentioned (Continued)

| Marker   | Repeat Motif | Primer Sequences | Tm  | Na | Accession Number |
|----------|--------------|------------------|-----|----|-----------------|
| NCPGR129 | (GT)_{21}    | AGGAGAATTTAATACCGGA/ GAGATTGAGTTGACGGTT | 54.5 | 293 | EU877296        |
| NCPGR130 | (CT)_{48}tt(CT)_{2} | GATACTGTTGAAAAATGGAA/ CAACTGCTTCTGAAATTTGCG | 55.5 | 245 | EU877297        |
| NCPGR131 | (GA)_{14}at(GA)_{4}aa(GA)_{3} | CTATGCGAGGATTCTCAT/ ATACCTGGCACATCCTGTTT | 54.3 | 290 | EU877298        |
| NCPGR132 | (GT)_{16}(GA)_{2} | GAAGATCTGGGAGGATTGTTA/ CGGGGACTAAACAAAGTGTATG | 55.5 | 242 | EU877299        |
| NCPGR133 | (CT)_{19}   | TGAGTTGAAGGTTTGAAGAAG/ AAGTTCCACTTACACAATTGCAA | 55.5 | 265 | EU877300        |
| NCPGR134 | (GT)_{24}(GA)_{22} | CATCCATGAGGATCCTCTT/ TTGTGCTTCCACACTCCCTCTCTC | 57.6 | 250 | EU877301        |
| NCPGR135 | (CA)_{4}cg(CA)_{5}(CG)_{2}(CA)_{5} (TA)_{3} | GAGGAACATTCGAGATT/ TATCTGATGATGAATGCGG | 55.5 | 234 | EU877302        |
| NCPGR136 | (GT)_{6}gc(GT)_{3}ac (GT)gc(GT)_{4}gg(GT)_{10} | GGAGCTGAGTGGAGTGGCTT/ GTATCCTCCGTTCTCCATTC | 54.0 | 132 | EU877303        |
| NCPGR137 | (GT)_{3}ct(GT)_{2}ct(GT)_{3}gg(GT)_{5} | GTGATGCGACCATGTGAAAA/ CGGAGGACTAAACAAAGTGAAG | 58.0 | 287 | EU877304        |
| NCPGR138 | (CT)_{2}cc(CT)_{2}ccc(CT)_{4} | ATTCCTATGCAAGTTTGTTG/ TGGGATTTCAATGGTGAATG | 54.3 | 213 | EU877305        |
| NCPGR139 | (GA)_{40}   | TGTTGCTTGGTTGGTTGGA/ CATGACCTTAGGATGAAACCA | 56.5 | 245 | EU877306        |
| NCPGR140 | (GT)_{14}gc(GT)gc(GT)gc (GT)_{10} | ATIGGGTTGAAGGTTCTGAGG/ TTTATATTTCTACCCACAG | 55.0 | 264 | EU877307        |
| NCPGR141 | (GA)_{4a}(GA)_{13}aa(GA)_{9} | ACTCAAAAGGACAGAACAAGCA/ AGGTTTACGACTCATGC | 55.5 | 211 | EU877308        |
| NCPGR142 | (CT)_{24}   | TAACTCCATTTGGTTGGAGA/ TAAACCTATATGTTGACGGG | 54.5 | 263 | EU877309        |
| NCPGR143 | (GT)_{4}(GA)_{22} | TACTTCCATCTCCTGAGAA/ GAATGAAAAATGTTGAAAACG | 54.5 | 220 | EU877310        |
| NCPGR144 | (GT)_{2}gt(GT)_{3}(GA)_{2} | TCTGAAACAGGTTTCTCAG/ TTCATGAGTCTCAGACACCT | 55.5 | 252 | EU877311        |
| NCPGR145 | (CT)_{1}(CACT)_{2}(CT)_{13}ca(CA)_{4}(CT)_{4}gtca (CT)_{11} | CCCATGAGATAATGCTGGCA/ ATCTAGGCGCAAGGATGAGTC | 56.3 | 316 | EU877312        |
| NCPGR146 | (CT)_{10}(CA)_{12} | AAAGTGGAATTCGCCAACA/ GAGTTGATTTGTTGTTTAGG | 55.4 | 225 | EU877313        |
| NCPGR147 | (CT)_{22}(CA)_{15} | TGATGAAATACACCTTGACCTATT/ CGATGATATTTCTGAGCAGAC | 55.5 | 219 | EU877314        |
| NCPGR148 | (GA)_{12}n_{2}(GA)_{9} | ACACAAGGCTATGGATAGA/ GCTTTGATTTATGCTTTGG | 55.9 | 285 | EU877315        |
| NCPGR149 | (GA)_{27} | TTATATAAGAGGGGCTCTCA/ AACTCAGATCTCCTGAGC | 60.0 | 202 | EU877316        |
| NCPGR150 | (AT)_{4}(GT)_{16} | GGAGCCGCAACAGAATCAA/ GGTTAAGGATGCGCCATAG | 54.5 | 287 | EU877317        |
| NCPGR151 | (CA)_{4}(TA)_{9} | AAATCTGTTGTTGCGACCT/ GGAATAAATGCTGTTGTTGGG | 54.5 | 284 | EU877318        |
| NCPGR152 | (GA)_{16} | AAGCGAGCTTCTCTCCATCA/ CCGTGGAGACTAACCCTTGT | 60.4 | 221 | EU877319        |
| NCPGR153 | (CT)_{16} | TGCCCTCAAATCTACTTCACT/ AGTGGGACGCTAGGAAATACC | 55.6 | 281 | EU877320        |
| NCPGR154 | (CT)_{13}CA_{13}(CT)_{12}N_{2}(CT)_{2}N_{4}(CT)_{2}n_{2}(CT)_{3} | CGCACTCCTAAGGCGCTT/ GTGCGAAACGCAAACTTGGG | 58.9 | 271 | EU877321        |
| NCPGR155 | (GA)_{18} | GGAAAAATAATGAGGAGGAGA/ TGCTCACATTTTCTCCTCT | 55.0 | 281 | EU877322        |
| NCPGR156 | (CA)_{4}(TA)_{5} | CGATTATGGTTCATCCTCCCTTT/ ATTCACAGGCCTCAACATC | 55.5 | 261 | EU877323        |
| Locus | Repeat Motif | Primer Sequences | Annealing (Tm) | Expected Size (bp) | Number of Amplified Alleles (Na) | GenBank Accession Numbers |
|-------|--------------|------------------|---------------|------------------|-------------------------------|-------------------------|
| NCPGR157 | (CA),d(TA) | TCCGTGACTGTGAACAA/ TGGGATTACACTGGATAAGG | 55.1 | 203 | 1 | EU877324 |
| NCPGR158 | (CT),tc(TT) | TAAAGCTGGAAACTCGAAAG/ TAACTCTTCTTCTTCTCCTC | 55.1 | 179 | 1 | EU877325 |
| NCPGR159 | (GT),GCj,GTj,ggcc(GT)j,GCj,Na(GC)j,GTj,GCj | TGTAATTTTCTCGCTGCTTGT/ GGCAATGAGCAACTTTTCCT | 59.3 | 285 | 1 | EU877326 |
| NCPGR160 | (GT),j,GAj | TTGGACGACAAATACCTGGA/ CGGCGACTAATAATCTCAAG | 59.9 | 241 | 1 | EU877327 |
| NCPGR161 | (CT) | ACCATCGCAATTCTGTGT/ CCCCCTCTACAAAGCCATGAA | 60.5 | 238 | 1 | EU877328 |
| NCPGR162 | (CT) | GCCGTGACTATTCCTCTCA/ TAAGCGAGGACTAGCTCGTA | 57.8 | 139 | 1 | EU877329 |
| NCPGR163 | (GA) | CAAAATCGCCTCGAACAACA/ TCCCCCCTCTCTCTCTCCT | 60.0 | 164 | 1 | EU877330 |
| NCPGR164 | (CT),j,ct(CT) | CCATAACATCACTCTCTTC/ TCCTCTCTTGATGTCGGG | 54.0 | 211 | 1 | EU877331 |
| NCPGR165 | (GA) | TCAGAAAGAACACAGAAGACG/ CAGCAACATATTGGACAC | 55.5 | 233 | 1 | EU877332 |
| NCPGR166 | (CT),j,ca(CT) | TGGATTGTGTATCCAAAAGG/ CAGCATCATCAAAGGTGCAT | 59.6 | 197 | 1 | EU877333 |
| NCPGR167 | (AT),j,GT | AGATGTGACCAAAGTAAGGGTG/ ATGTGACCAAAGTAAGGGTG | 55.5 | 266 | 1 | EU877334 |
| NCPGR168 | (GA) | TCCATACCCGAGGCTCA/ CCGGCGACTAATAATCTCAAG | 60.4 | 243 | 1 | EU877335 |
| NCPGR169 | (CT),j,(CA),j,CT,GCj,GAj | CCCCCCTCTCTCTCTCTCT | 54.6 | 256 | 2 | EU877336 |
| NCPGR170 | (CT),j,GAj | AGCTGAATTACCTCATAAACAG/ GAGGTGACCTTCGATTTGAAT | 55.9 | 224 | 1 | EU877337 |
| NCPGR171 | (GA) | AAAAGAGAAAAGCAAGGAGG/ AAAACCCCCTAAATTCAGAA | 55.0 | 205 | 1 | EU877338 |
| NCPGR172 | (AC) | TCCGTGACTGTGAACAA/ TGGGATTACACTGGATAAGG | 54.5 | 181 | 1 | EU877339 |
| NCPGR173 | (AT),j,GT | AATCCCGAGGAGGAGGAGGAGG/ ATGTGACCAAAGTAAGGGTG | 54.5 | 266 | 1 | EU877340 |
| NCPGR174 | (CA),j,TAj | TGGGCGGTGTTTGATGAAAT / GTGTGACCAAAGTAAGGGTG | 54.5 | 170 | 1 | EU877341 |
| NCPGR175 | (CA),j,CT,GAj | AAAACCGGAGTGTTTAAAG/ CGCATTAAAGAACCAGA | 56.0 | 232 | 1 | EU877342 |
| NCPGR176 | (AT),j,GT | TGGAAAGGATGTGGAAAC/ GGCAGTAGGAAGAAGA | 56.3 | 234 | 1 | EU877343 |
| NCPGR177 | (GA) | GGCCCCGAAAATGAGGAGG/ GGCAGTAGGAAGAAGA | 56.1 | 253 | 1 | EU877344 |
| NCPGR178 | (CA),j,aa(CA) | CCCCCCTCTCTCTCTCTC | 54.5 | 181 | 1 | EU877345 |
| NCPGR179 | (CT) | TACCAAAAAGCTCGCCTCA/ GGAAGGGTGAAGGAGA | 62.0 | 335 | 1 | EU877346 |
| NCPGR180 | (CA),j,TAj | TCCGTTGAAGAAGGAGA/ GGTGACCAAAGTAAGGGTG | 55.5 | 283 | 1 | EU877347 |
| NCPGR181 | (TA),j,GTj,TGj | GAAATGGAAGAATGGAAGA/ GGTGACCAAAGTAAGGGTG | 54.5 | 264 | 2 | EU877348 |
| NCPGR182 | (CA),j,TAj | CCCCCCGGAAAAGGAAAC/ GCATTAAAGAACCAGA | 54.5 | 190 | 1 | EU877349 |
| NCPGR183 | (GA),j,ggata(GA) | AAAACCGGGGAGGCTGATGGA/ GGTGACCAAAGTAAGGGTG | 60.5 | 236 | 1 | EU877350 |
| NCPGR184 | (AT),j,GT | CTCTGTGAGGAAATAGGAGAAT/ CACGTGATGAAAGAGAGGAGA | 55.5 | 252 | 1 | EU877351 |
| NCPGR185 | (CT),j,gcg(CT) | TCCGTGACTGTGAACAA/ TGGGATTACACTGGATAAGG | 59.4 | 242 | 1 | EU877352 |
| No. | Locus Name | Repeat Motif | Primer Sequences | Annealing Temp. (°C) | Product Size (bp) | Amplified Alleles | GenBank Accession Numbers |
|-----|------------|--------------|------------------|---------------------|------------------|-------------------|--------------------------|
| 86  | NCPGR186   | (CA)_4(TA)_5 | GTGCATCCATGTGAAAGATTT/ AACCAGAGTGAACGGAATA | 55.0               | 228              | 2                | EU877353                |
| 87  | NCPGR187   | (CT)_14(tcc) | CTCCTACGCTGGTTATG/ TAAACAACGCTGCAATG | 54.5               | 152              | 1                | EU877354                |
| 88  | NCPGR188   | (TA)_2(tg) | GTTATATTGCGGCCAAG/ TGCACTTCTCCACACCTTC | 56.0               | 181              | 1                | EU877355                |
| 89  | NCPGR189   | (CT)_10(CT)_5(CACT)_2(CT)_10 | TGCCGACATGGTAGATG/ ATGGCAAGAAGGTAGTCTATA | 54.5               | 297              | 1                | EU877356                |
| 90  | NCPGR190   | (AT)_4(GT)_5 | CTCCTGCTGATGCGAC/ GACGCACTTATGAGCCA | 54.5               | 289              | 1                | EU877357                |
| 91  | NCPGR191   | (TA)_4(TG)_3(TG)_2(TG)_3 | TTACGGTCTATG/ ATGGCAAGAAGGTAGTCTATA | 55.1               | 269              | 1                | EU877358                |
| 92  | NCPGR192   | (TA)_3(TG)_1 | TGACGGTACATG/ GACGCACTTATGAGCCA | 55.1               | 203              | 1                | EU877359                |
| 93  | NCPGR193   | (AT)_3(tgt) | CGCTGAAACATGAAACAG/ AAACGGGTTTCAAGAAG | 58.3               | 232              | 1                | EU877360                |
| 94  | NCPGR194   | (TG)_3| ATGGCAAGAAGGTAGTCTATA | 54.5               | 190              | 1                | EU877361                |
| 95  | NCPGR195   | (CA)_12| TGGCACAATGTATGTATTGAA/ ATGGCAAGAAGGTAGTCTATA | 54.0               | 211              | 1                | EU877362                |
| 96  | NCPGR196   | (CT)_17 | TCTATGTACTTCTATTG/ ATGGCAAGAAGGTAGTCTATA | 55.5               | 226              | 1                | EU877363                |
| 97  | NCPGR197   | (CT)_17 | TCTGGATATATGCACTTATCGAAGAATG/ CAACTGAAACGGAATGAG | 54.0               | 241              | 1                | EU877364                |
| 98  | NCPGR198   | (GA)_18 | TTCCAAATATCTCGAGGACAT/ GACGCACTTATGAGCCA | 54.5               | 188              | 1                | EU877365                |
| 99  | NCPGR199   | (GA)_27 | GCCAAGAAGGTAGTCTATA | 55.5               | 196              | 1                | EU877366                |
| 100 | NCPGR200   | (GA)_24 | GACGCACTTATGAGCCA | 55.5               | 250              | 1                | EU877367                |
| 101 | NCPGR201   | (CT)_13(CA)_12 | TCTATGTCGATG/ GACGCACTTATGAGCCA | 55.5               | 269              | 1                | EU877368                |
| 102 | NCPGR202   | (CT)_25 | ATGGCAAGAAGGTAGTCTATA | 56.5               | 259              | 1                | EU877369                |
| 103 | NCPGR203   | (GA)_21 | GACGCACTTATGAGCCA | 55.5               | 157              | 1                | EU877370                |
| 104 | NCPGR204   | (CT)_17 | TTCATGGTTATGCACTTATCGAAGAATG/ CAACTGAAACGGAATGAG | 59.2               | 181              | 1                | EU877371                |
| 105 | NCPGR205   | (CA)_12(TA)_5 | GACGCACTTATGAGCCA | 56.5               | 267              | 1                | EU877372                |
| 106 | NCPGR206   | (GA)_2(aa)(GA)_2 | ACAAACGAACGGAACGGA | 54.3               | 252              | 1                | EU877373                |
| 107 | NCPGR207   | (CA)_10(CT)_8 | GCAACGAGAAGAATCTGTTG/ GCAATGGATTGAAATGAGAAGG | 57.5               | 281              | 1                | EU877374                |
| 108 | NCPGR208   | (CT)_14 | GACGCACTTATGAGCCA | 54.6               | 178              | 1                | EU877375                |
| 109 | NCPGR209   | (GT)_14(GT)_14(GT)_14(GT)_14(GT)_14 | ATGGCACTTATGAGCCA | 55.5               | 161              | 1                | EU877376                |
| 110 | NCPGR210   | (GA)_13 | AAGTGTAACAGGTAACGCTG/ CCTCATGATGGAAGATAGGGG | 55.5               | 224              | 1                | EU877377                |
| 111 | NCPGR211   | (CT)_14 | ATCCACTGATGATCCGAG/ CGCTCAGCTACCCCACAAAATC | 60.0               | 213              | 1                | EU877378                |
| 112 | NCPGR212   | (GA)_6(GT)_12 | CAGCTCATAAACCAAGGACTGC/ TCAATCCCAAATAATTCTTATT | 55.0               | 190              | 1                | EU877379                |
| 113 | NCPGR213   | (CT)_12(CA)_12 | TTGATGATGAGATTCCTCCC/ CCCGACTATTTCCTCCATAA | 54.5               | 220              | 1                | EU877380                |
Table 1 List of 181 novel chickpea STMS markers developed in this study; the locus name, type of repeat motif, primer sequences, annealing temperature (T_m), expected product size (bp), number of amplified alleles (N_a), and GenBank accession numbers are mentioned (Continued)

| Locus   | Locus Type | Primer Sequences | T_m  | N_a | Accession |
|---------|------------|------------------|------|-----|-----------|
| 114     | NCPGR214   | (CA)_n(TA)_m    | 54.5 | 225 | EU877381  |
| 115     | NCPGR215   | (CA)_n(CA)_m(stt) | 54.5 | 195 | EU877382  |
| 116     | NCPGR216   | (CA)_n(ttt)TA_m | 55.4 | 286 | EU877383  |
| 117     | NCPGR217   | (TG)_n         | 55.1 | 171 | EU877384  |
| 118     | NCPGR218   | (AT)_n(GT)_m   | 54.5 | 275 | EU877385  |
| 119     | NCPGR219   | (CA)_n         | 54.5 | 237 | EU877386  |
| 120     | NCPGR220   | (GT)_n(GA)_m   | 54.5 | 255 | EU877387  |
| 121     | NCPGR221   | (CA)_n(cga(CA)_m(cg(CA)_n(TA)_m | 55.0 | 260 | EU877388  |
| 122     | NCPGR222   | (CT)_n         | 56.5 | 165 | EU877389  |
| 123     | NCPGR223   | (CA)_n         | 56.5 | 267 | EU877390  |
| 124     | NCPGR224   | (AT)_n(GT)_m   | 54.7 | 225 | EU877391  |
| 125     | NCPGR225   | (CA)_n         | 55.2 | 203 | EU877392  |
| 126     | NCPGR226   | (CT)_n         | 55.3 | 205 | EU877393  |
| 127     | NCPGR227   | (CA)_n         | 59.9 | 207 | EU877394  |
| 128     | NCPGR228   | (CT)_n         | 57.0 | 236 | EU877395  |
| 129     | NCPGR229   | (GA)_n         | 57.9 | 158 | EU877396  |
| 130     | NCPGR230   | (GA)_n         | 56.7 | 242 | EU877397  |
| 131     | NCPGR231   | (GA)_n         | 59.4 | 226 | EU877398  |
| 132     | NCPGR232   | (GA)_n         | 56.5 | 265 | EU877399  |
| 133     | NCPGR233   | (CA)_n         | 59.5 | 243 | EU877400  |
| 134     | NCPGR234   | (GA)_n         | 59.5 | 188 | EU877401  |
| 135     | NCPGR235   | (CA)_n         | 59.7 | 182 | EU877402  |
| 136     | NCPGR236   | (GT)_n(GA)_m   | 56.5 | 200 | EU877403  |
| 137     | NCPGR237   | (GA)_n         | 59.9 | 314 | EU877404  |
| 138     | NCPGR238   | (GA)_n         | 56.5 | 273 | EU877405  |
| 139     | NCPGR239   | (CA)_n         | 56.5 | 137 | EU877406  |
| 140     | NCPGR240   | (GA)_n         | 55.0 | 238 | EU877407  |
| 141     | NCPGR241   | (TA)_n         | 58.7 | 250 | EU877408  |
| 142     | NCPGR242   | (CT)_n         | 58.5 | 145 | EU877409  |
Table 1 List of 181 novel chickpea STMS markers developed in this study; the locus name, type of repeat motif, primer sequences, annealing temperature (T_m), expected product size (bp), number of amplified alleles (N_a), and GenBank accession numbers are mentioned (Continued)

| No. | Locus Name | Type of Repeat Motif | Primer Sequences | Annealing Temperature (T_m) | Expected Product Size (bp) | Number of Amplified Alleles (N_a) | GenBank Accession Numbers |
|-----|------------|----------------------|------------------|----------------------------|---------------------------|----------------------------------|--------------------------|
| 143 | NCPGR243   | (CA)_13              | TGGCTTGCGGAGAATGTA/GGCCGGCTTTGACTTTGCA | 58.7                       | 206                        | 1                                 | EU877410                 |
| 144 | NCPGR244   | (CT)_{c(CA)}_{13}    | TGGACTACTGAACTTTTGTGGA/TTGGACTTTCTGACG  | 59.2                       | 200                        | 1                                 | EU877411                 |
| 145 | NCPGR245   | (CA)_13              | GGAGCTGGACTAAATGGGCA/AAAGGATGAGCAGTGGAAAAGA | 54.5                       | 148                        | 1                                 | EU877412                 |
| 146 | NCPGR246   | (CA)_13              | GGTGACTACCAACCATAGGA/ACCATTACCAGAAAACCAGTA | 54.5                       | 154                        | 1                                 | EU877413                 |
| 147 | NCPGR247   | (GT)_12              | CAACTGATTGTCTCTCTCTGA/GGTTGACTAAATATGGCG  | 54.5                       | 105                        | 1                                 | EU877414                 |
| 148 | NCPGR248   | (GT)_12              | GCCATTGTTGAAAGGAGGA/GCCGGTGGAACCTACATG   | 59.8                       | 230                        | 1                                 | EU877415                 |
| 149 | NCPGR249   | (CA)_{d(CG)}_{3(CA)}_{10} | CTTCTTGATTGCGGTA/GTTTCGACCTAAATTTCACG    | 55.5                       | 231                        | 1                                 | EU877416                 |
| 150 | NCPGR250   | (CA)_10              | GGCGGTGGACTACCTGTGA/TGGCTAAGCCTTCCATT    | 57.9                       | 243                        | 1                                 | EU877417                 |
| 151 | NCPGR251   | (CA)_13              | AATGGGTTAATTTGACTTGGAT/TTATGGGCAACCAATACCTTTT | 54.0                       | 282                        | 1                                 | EU877418                 |
| 152 | NCPGR252   | (CA)_12              | GTGCTCTGAGGAAATACATGAT/GGTTGTTGAGGCCAGCATACTG | 54.3                       | 187                        | 1                                 | EU877419                 |
| 153 | NCPGR253   | (GT)_{12(N21(GT)21)} | ACATTGTTGGAGAACCTCATT/GGTTGACTAAATATGGCG  | 60.0                       | 236                        | 1                                 | EU877420                 |
| 154 | NCPGR254   | (AT)_{12(GT)}_{11}   | GCCCTTTCTCTCTTCTCA/CCCAAGAAGACAAACAAACAC | 54.5                       | 298                        | 1                                 | EU877421                 |
| 155 | NCPGR255   | (GT)_12              | TCATGGGTTGCAAGACTCTG/GCCCTCTCAAAAAGTGAACCT | 54.0                       | 258                        | 2                                 | EU877422                 |
| 156 | NCPGR256   | (CA)_12              | AATGGGTTAATTTGACTTGGCT/TATGGGCAAACTAAAATCATT | 54.2                       | 280                        | 1                                 | EU877423                 |
| 157 | NCPGR257   | (GT)_{2(gc(GT))4}    | CCCAAAGGCTTGGCAGAAATGC/GCCGGTGACTCTCTTCCATT | 58.2                       | 182                        | 1                                 | EU877424                 |
| 158 | NCPGR258   | (CT)_{atca(CT)4}     | TTATTTGCATTTGATGACTTTG/TTGTTGATGAAATCTGGAAGAG | 56.5                       | 250                        | 1                                 | EU877425                 |
| 159 | NCPGR259   | (GT)_12              | TATAGCCTAAGGCGAACATG/TGGTTGTAAGGGAAATAG  | 55.6                       | 185                        | 1                                 | EU877426                 |
| 160 | NCPGR260   | (GT)_12              | CGGGTCTTTCTTCTCTCTCATT/ATTAGGTTGGTAAAGCGCAA | 56.5                       | 247                        | 1                                 | EU877427                 |
| 161 | NCPGR261   | (CA)_{3(CA)}_{10}    | GATTGTTGACCTTTAACTCAT/GCTCTGAGTTGGTCTCTGGA | 58.9                       | 300                        | 1                                 | EU877428                 |
| 162 | NCPGR262   | (GT)_13              | GATAAGGCGATACCTTGG/GGCTTGGAATACATATCAT  | 55.0                       | 185                        | 1                                 | EU877429                 |
| 163 | NCPGR263   | (GT)_10              | CAAAGATGAGTTGTGGAATGATG/GTATGTACCTCCTGGTTTCCC | 55.5                       | 111                        | 2                                 | EU877430                 |
| 164 | NCPGR264   | (GT)_{gg(GT)2(23)gg(GT)2(3)} | TGGGAAATTCGTTGTTCTTCT/GTAAAGGAGATGGAAAGAAGC | 57.1                       | 221                        | 2                                 | EU877431                 |
| 165 | NCPGR265   | (GT)_{11(CT)}_{2}    | GTGGTTGTCCTGTCCTGCR/CAATCCACACACATACACAGT | 54.5                       | 195                        | 1                                 | EU877432                 |
| 166 | NCPGR266   | (CA)_{12}            | GTGGTAAATCGATGAGGAA/GTTGTTGTTGTTGTTGCTTG  | 54.5                       | 195                        | 1                                 | EU877433                 |
| 167 | NCPGR267   | (TA)_{2(CA)}_{13}    | ATTAACTGTGCTGAGGAAA/TATAGCCTAAGGCGCAAACAT | 54.5                       | 279                        | 1                                 | EU877434                 |
| 168 | NCPGR268   | (GT)_{11}            | TCAACTAAGGATTGCTCG/GAAAGCTGAGAGATGGAAACAA | 54.5                       | 296                        | 1                                 | EU877435                 |
| 169 | NCPGR269   | (GT)_{2}             | CTTGGAAATCTGAAATGGAAGT/ATAAGGGAAAAGGACCA | 60.5                       | 221                        | 2                                 | EU877436                 |
| 170 | NCPGR270   | (GTATGTAT)_{2(GT)10} | GGGTTGAAATGCTGAGAAGATGG/GCTGAGATAATACCCACATG | 60.0                       | 236                        | 1                                 | EU877437                 |
As expected, these 181 SSR containing sequences were rich in (GT/CA)_n and (GA/CT)_n motifs and based on the structural organization, the repeat motifs were classified as perfect (72, 39.7%), imperfect (26, 14.3%), compound (45, 24.8%) and interrupted (38, 20.9%). However, the predominance of CA repeats was observed (78 clones; 43.0%) compared to CT repeats (68 clones; 37.5%) while CA and CT compound motifs were found in the remaining clones (19.0%). High variability in the numbers of microsatellite motifs were found at these loci with the maximum number of uninterrupted GA and CA units being 47 (NCPGR163) and 40 (NCPGR235) respectively. However, many long repeat motifs were also present like (GA)_40 at NCPGR139, and (GT)_20 at NCPGR120. The longest stretch of compound microsatellite motif was found in NCPGR236 with repeat motif (GT)_12(GA)_25. But the majority of the repeat motifs comprised of 12-30 repeat units. 160 primer pairs (83.39%) amplified single alleles whereas, 21 primers (11.6%) produced 2-4 alleles (Table 1). Moreover, with 44 out of the 181 primer pairs, intraspecific variability was clearly detectable among four chickpea accessions even by resolution on simple agarose gel (data not shown).

Similarity search using the BLASTN program at NCBI revealed that the chickpea microsatellite containing sequences had homology with a variety of sequences including repetitive DNA, ribosomal DNA as well as coding sequences of genes and unknown proteins from diverse plant genomes. Forty eight of the sequences were found to be similar to the M. truncatula BAC clones whereas 5 sequences showed similarity to known proteins or predicted genes of the same plant. Of the 14 sequences found to be similar to the chickpea genome, only two sequences (NCPGR160, NCPGR164) were similar to the chickpea polypyrimidine track-binding protein (ptb) (AJ549383) and beta-galactosidase genes (AJ012687) respectively, while the remaining sequences were similar to retrotransposons and ribosomal DNA.

**Identification of polymorphic markers and genotyping for linkage analysis**

In the present study, a total of 522 chickpea STMS markers (Table 2) including 265 NCPGR series markers developed by us, 150 H-series markers developed by Lichtenzveig et al. 2005 [24] and 107 markers developed and mapped by Hüttel et al. 1999; Winter et al. 1999 [16,21] were used to identify polymorphic primers between ICCV-2 and JG-62, the parental lines of the mapping population. Of the 522 STMS primer pairs, only 226 (43.3%) primer pairs (109 (48.2%) NCPGR series, 69 (30.5%) H-series [24] and 48 (21.2%) of Hüttel et al. 1999; Winter et al. 1999 [16,21]) produced clear and consistent polymorphic banding patterns between the parental lines (Table 2). These 226 polymorphic primers were further used to genotype all the 126 individuals of the RIL population. Genotyping data was obtained for all 226 chickpea STMS markers along with 1 morphological marker (double-podding) and used for linkage analysis.

### Table 1 List of 181 novel chickpea STMS markers developed in this study; the locus name, type of repeat motif, primer sequences, annealing temperature (T_m), expected product size (bp), number of amplified alleles (N_a), and Gen-Bank accession numbers are mentioned (Continued)

| Locus  | Repeat Motif | Primer Sequence            | T_m  | N_a  | Accession Number |
|-------|--------------|----------------------------|------|------|-----------------|
| NCPGR271 | (CA)_13     | TGGAAATTTGCTGTGAGACATGA/CGGGAGGTGAAGACGAGT | 59.1 | 355  | EU877438        |
| NCPGR272 | (AT)_(GT)_13 | TGGAAATTTGCTGTGAGACATGA/CGGGAGGTGAAGACGAGT | 54.5 | 233  | EU877439        |
| NCPGR273 | (CA)_11     | CCACTTTTACAAAAGTGAACTCT/TCAGCAGATGAGACATCG | 54.6 | 273  | EU877440        |
| NCPGR274 | (GT)_12     | GTGTGTTGTCTGTTTCTGAA/TTTGAAGACAACTCAATCC  | 55.9 | 268  | EU877441        |
| NCPGR275 | (CA)_(TA)_3 | CGAGGAACCTGCATTCTCT/TCCTGAGCCTGATTAAA   | 58.0 | 355  | EU877442        |
| NCPGR276 | (CA)_(CA)   | CGAGGAACCTGCATTCTCT/TCCTGAGCCTGATTAAA   | 56.9 | 257  | EU877443        |
| NCPGR277 | (CT)_17     | CACGCTCTCCATTATTTGGATTTC/ACATGAACTCGTCCAAAAC  | 56.5 | 278  | EU877445        |
| NCPGR278 | (GT)_(GT)_(GT)_(GT)   | TGAGATCCATCGACTATTGGAAAA/CACTTCCTCAAAAGTGAAACC  | 56.0 | 250  | EU877445        |
| NCPGR279 | (CT)_(CT)_(CT)_(CT)   | TTTTGGGTCTTACTCTTCAGCC/ATTAAAAGCTGAGGAGGAAGAAA  | 54.5 | 248  | EU877445        |
| NCPGR280 | (GT)_(GT)   | GCAATGATTGGTTCTCTTCAGCT/TTTGGGTCTTACTCTTCAGCT  | 56.5 | 207  | EU877445        |
| NCPGR281 | (GT)_9      | GCAATGATTGGTTCTCTTCAGCT/TTTGGGTCTTACTCTTCAGCT  | 56.5 | 114  | EU877448        |
Table 2 Summary of the STMS markers used in the present study for the construction of the intraspecific linkage map of chickpea (Cicer arietinum ICCV-2 X JG-62)

| Markers analyzed | Markers polymorphic in parents | Markers mapped No. (%) | Markers distorted |
|------------------|--------------------------------|------------------------|------------------|
| NCPGR            | 265                            | 109                    | 66 (60.55%)      | 38               |
| Lichtenzveig et al. 2005 [24] | 150                            | 69                     | 35 (50.72%)      | 23               |
| Winter et al. 1999 [21]       | 107                            | 48                     | 36 (75.00%)      | 9                |
| Hüttel et al. 1999 [16]       |                                |                        |                  |                  |
| Total             | 522                            | 226                    | 137 (60.61%)     | 70               |

Development of an intraspecific linkage map

JoinMap ver. 4.0 [45] was used to develop the intraspecific genetic linkage map using 227 markers of which 137 STMS and 1 morphological trait (sfl) were mapped at a LOD score of 3.5 (Figure 1). The 137 STMS mapped markers included 66 of NCPGR series, 35 of H series [24], and 36 markers of Hüttel et al. 1999 and Winter et al. 1999 [16,21] (Table 2). The current linkage map covered 630.9 cM spanning 8 linkage groups with an average marker density of 4.57 cM (Figure 1). There was a large variation in the lengths of individual linkage groups that varied from a maximum of 205.4 cM to a minimum of 29.8 cM and genome coverage varying from 96.0% (LG6) to 33.0% (LG3). Relative to the estimated physical size of the chickpea genome (750 Mbp) [46], 1 cM distance in the present map approximately equals to 1.18 Mbp.

In order to facilitate comparisons with the previously published studies, the maps of Winter et al. 2000 [37] and Millan et al. 2010 [20] were considered as reference maps and the LGs in our map were named (LGI-VIII) to conform to these maps [20,37] based on the common set of 30 markers present in the LGs (Figure 1). The current map (Figure 1) revealed that the markers were not distributed evenly throughout the genome as some of the linkage groups were densely populated with markers while other LGs were sparsely packed (Figure 1). LGVI was the largest linkage group both in terms of size (205.4 cM) and number of mapped markers (61). It defined new positions of 34 NCPGR series and 12 H-series markers with an average marker density (DAv) of 3.36 cM. The double-podding gene (sfl) also mapped to this linkage group and was flanked by TA80 and NCPGR128 at 3.7 cM and 3.0 cM respectively. This linkage group shared 8 markers (TA14, TA22, TA176, TA80, TR44, TS24, Tr35 and STMS2) with the corresponding LGVI of the interspecific map [37]. LGV spanned 65.6 cM, harbouring 11 markers and shared 4 common markers (TR59, TS43, TA5, and TA42) with LGV [37]. LGIV was composed of 26 loci containing 14 NCPGR series and 4 H-series markers spanning 101.3 cM with average marker density of 3.89 cM and contained 5 common STMS loci namely TR20, TA2, TA72, TA130 and TA146 with LGIV of Winter et al. 2000 [37]. LGVIII was one of the smallest linkage group, having marker density of 3.76 cM and defined positions of 9 NCPGR series markers. LGI spanned 64.6 cM with 12 markers mapped at an average marker density of 5.38 cM and corresponds to LGI [37] as they shared 3 loci namely TA8, TR43 and TA203. LGII had 10 markers and shared 2 common markers (TA59 and TA96) with LGII [37]. LGVII spanned 52.9 cM and had an average marker density of 17.6 cM, but did not possess any common markers from Winter et al. 2000 [37]. LGIII was the smallest linkage group spanning 29.8 cM that housed only 2 markers, one of which (TA64) was common with LGIII of Winter et al. 2000 [37]. The wide range of marker density (3.36 in LGVI to 17.6 in LGVII) indicated differing degrees of saturation of linkage groups with the new set of markers.

Of the 226 STMS markers analyzed, 70 (31.0%) markers did not segregate according to the expected Mendelian ratio. Out of these 70, the majority of markers (43; 61.4%) showed slight deviation from the ratio while 27 loci (38.5%) exhibited significantly high segregation distortion. Further, analysis revealed that the frequency of distorted female markers appeared to be double (43 markers; 61.4%) as compared to distorted male markers (27 markers; 38.6%). Of 70 loci, 23 (32.8%) markers were mapped and most of them resided on LGVI and LGVII and were indicated by arrows on the linkage groups (Figure 1).

Map compilation and integration

Comparison of our map with the recently published intraspecific map of chickpea [34] was carried out. Since the LGs in Radhika et al. 2007 [34] were not named according to Winter et al. 2000 [37], hence 47 common markers between our map (Figure 1) and that of Radhika et al. 2007 [34] were identified which were distributed across five LGs. Hence five of our linkage groups namely LGII, LGIV, LGV, LGVI and LGVIII were integrated with the corresponding LG3, LG2, LG1, LG4, and LG6 respectively of Radhika et al. 2007 [34] using the program BioMercator ver. 2.1 [47]. The map of the 5 compiled LGs (designated LGs A-E; Figure 2) illustrated that even though the overall map lengths of the projected LGs remained almost same but the marker density improved dramatically. For example, after
Figure 1 The intraspecific linkage map of chickpea. The intraspecific linkage map of chickpea based on RILs of *C. arietinum* (ICCV-2) × *C. arietinum* (JG-62) was generated with STMS markers using JoinMap version 4.0. The name of the linkage groups, the number of mapped markers and the Average Marker Density (DAv) is mentioned at the top of each LG. Newly mapped markers (NCPGR-series and H-series) are shown in blue colour and the morphological marker (double-podding, sfl) is shown in a shaded box. Arrows represent the markers showing distortion.
Figure 2 Map of projected linkage groups. Markers from the LGs of 2 maps namely ours (from Figure 1) and Radhika et al. 2007 [34] (marked by *) were combined to obtain the 5 projected LGs (designated A-E). The software BioMercator ver. 2.1 [47] was used for the integration of the individual LGs. Markers shown in black colour are from the map of Radhika et al. 2007 [34] whereas markers from our map (Figure 1) are in blue. Total number of markers and the Average Marker Density (DAv) is mentioned above each LG.
combining our LGVI (61 markers) with LG4 [34] (26 markers) the inter-marker distance improved to 1.88 cM from 3.36 cM (LG A; Figure 2). This combined LG A clearly helped in fine mapping of sfl region such that flanking markers TA80, NCPGR78, H5B08, and NCPGR 128 which have been shown to be closely associated with the sfl gene in the previous maps [34] and in our map (Figure 1), now position more closely at a distance of 2.5 cM, 1.9 cM, 1.9 cM, and 2.1 cM respectively from the sfl region. Remarkable improvement was also obtained when our LGIV was combined with LG2 of Radhika et al. 2007 [34] (72 markers) to accommodate 94 positions with marker density of 1.51 (LG B; Figure 2). Similarly, projections of our LGII, LGV and LGVIII on LG3, LG1 and LG6 of Radhika et al. 2007 [34] respectively, substantially improved the marker densities of each of the LGs (LGs C, D, E; Figure 2).

**Discussion**

Availability of the chickpea genomic resources is still in its infancy. Most imperative among these are the SSR markers, ESTs and a saturated linkage map. A critical mass of polymorphic SSR markers is still limited in chickpea as only about 800 have been reported till date [16,18,21-26] of which only about 30% are expected to be polymorphic. Hence, keeping in mind the limited number of available SSR markers coupled with the low levels of polymorphism in chickpea, it was necessary to generate several additional SSR markers which could be used to construct high-density genetic linkage maps of chickpea. Although several intraspecific linkage maps are available for chickpea with various mapping populations [20,30,33-36], all these maps have been constructed employing only the STMS markers reported in earlier [16,21] as well as later studies [18,24]. Therefore, as expected, all these maps have exhibited similar genomic locations and similar marker order, and are therefore of limited use. Thus, the primary goal of the present study was to generate new STMS markers and use them to construct an intraspecific genetic linkage map of chickpea to decipher new unmapped regions of the genome. Moreover the integration of this genomic information with a recently available intraspecific specific map was done to substantially increase the marker density, thereby facilitating the saturation of the linkage map.

The important contribution of the present study was the development of a major genomic resource comprising of 181 genomic STMS markers developed from the microsatellite enriched library of chickpea. Use of this enrichment method [48] significantly increased the efficiency of SSR marker development since about 10% of the recombinants contained SSR motifs in agreement with earlier reports [48,49]. Moreover a very stringent criterion was used to select the SSR motifs against which STMS primers were developed. Most of the SSRs selected belong to the class I type [50] which include SSRs greater than 20 bp in length and are therefore more polymorphic and more useful as genetic markers. This was clearly evident from the data of polymorphism analysis (Table 2) which showed that 41.1% of our markers (NCPGR series) were polymorphic. Hence the developed STMS markers provide a resource which in future may be utilized for the analysis of genetic diversity, map integration and QTL analysis.

Another achievement of this study was the advancement of the linkage map. Not only were the newly developed 181 STMS markers used for map generation, but 341 additional STMS markers, reported earlier but mostly unmapped, were also used (Table 2). Hence, a total of 522 microsatellite markers were used to screen for polymorphism between ICCV-2 and JG-62, the parental lines of the intraspecific RIL mapping population, and this revealed 226 (43.3%) polymorphic markers. This level of polymorphism was fairly high for a crop like chickpea which has a narrow genetic base and was comparable with earlier studies in chickpea which reported 30-40% polymorphism between the parental lines of the various intraspecific mapping populations [31,32,34,35].

The present linkage map defined 138 map positions which were distributed non-randomly and unevenly over 8 linkage groups. The map spanned 630.9 cM which was comparable with the previous map (739.6 cM) [34]. The map length was larger than the other intraspecific maps (426.99 cM) [20], (534.4 cM) [33], (419 cM) [32], (318.2 cM) [31], (419.7 cM) [36] but smaller than the map (1285 cM) reported by Taran et al. 2007 [35]. Several factors, including population size and the nature and number of markers used in the analysis, may contribute to the difference in map coverage on different populations. Moreover, differences in linkage intensities among different crosses might be responsible for differences in the map coverage [51]. A remarkable feature of this map was the 101 new genomic locations that were defined in this study (which included 66 NCPGR series and 35 H-series markers) in the backdrop of the previously mapped STMS markers [37]. These new locations would be beneficial to chickpea breeders to tag important genes and QTLs. Even though the number of linkage groups defined in this study were the same as expected for chickpea haploid number (n = 8) the density of the markers indicated the need to add more markers to the small groups which would then coalesce and be integrated to construct the detailed genetic linkage map.

About 31.0% of markers used for linkage analysis did not follow the expected Mendelian ratios. This could be compared with the studies [34,37] in chickpea and with other plant species such as *Arabidopsis* [52], rice [53]
and Medicago [54-56]. From the genetic mapping projects, it is clear that variations from expected Mendelian ratios are common within both interspecific and intraspecific crosses [54], however generally higher percentage of allelic distortion was observed in the former case. Hence, the mapping of new STMS markers on the intraspecific genetic linkage map was preferred as it would serve chickpea breeders more accurately than interspecific maps by alleviating problems like marker distortion [30,33]. In tomato, Paran et al. 1995 [57] reported a significant increase in the number of loci that differed, possibly due to the intraspecific nature of the parents during propagation of the RILs. A similar level of segregation distortion was also reported for mungbean from F3 to F7 population [58,59]. Interestingly, the distorted markers in the present map were majorly concentrated on linkage groups VI and VII suggesting that some structural reasons might be responsible for this distortion. Moreover, most of the distorted loci (61.4%) were skewed in favour of the maternal alleles i.e. JG-62. This might be due to accumulation of distorted alleles in the population with progressive cycles of selfing undergone in the development of the RILs [33].

In the current map non-random distribution and clustering of markers was observed for most linkage groups leading to large variations in the marker density. This might be attributed to the fact that microsatellite sequences in the chickpea genome may cluster around centromeres [60]. Similar clustering of microsatellites around the centromere has been observed in various plant species like sugarbeet [61], barley [62,63], tomato [64,65] and several other Triticeae species [63]. Several factors are responsible for this clustering of genomic SSRs on genetic linkage maps, major being their non-random physical distribution in plant genomes [66,67], reduced recombination in centromeric regions [68,69] and the genomic origin of DNA sequences used for SSR development [70].

Currently, the primary goal in chickpea research programs worldwide is to generate the consensus linkage map and to increase the marker density i.e. to place as many markers as possible into a single map. Comparison of the present intraspecific map of chickpea (Figure 1) with the interspecific map developed by Winter et al. 2000 [37] and the consensus map of Millan et al. 2010 [20] revealed high linkage conservation in at least 6 linkage groups and hence we were able to designate our LGs in accordance with these maps. However, the map distances and marker orders of the common SSR markers differed, possibly due to the intraspecific nature of our mapping population. Nevertheless, by developing separate intraspecific maps for C. arietinum and C. reticulatum using common STMS markers and comparing the map positions might provide the molecular insight into the chromosomal rearrangement events and evolution of chickpea from its wild progenitor C. reticulatum. In this context, it was felt that map comparisons and integration with existing intraspecific maps would be more significant. Therefore an effort has been made in the present study to integrate the available information from the intraspecific maps in order to construct a more dense and saturated linkage map of chickpea. The program BioMercator [47] allows merging different individual genetic maps even without the availability of raw genotyping data. Considering the common loci as bridges between maps, this program provides the possibility of building the compiled map by iterative projection. Since common markers were identified on 5 LGs of our map and the recently reported map [34], it was possible to combine these data using the program BioMercator ver. 2.1 [47] (Figure 2). Five highly resolved LGs (LG A-E; Figure 2) were generated with substantially improved marker densities. Such marker densities are highly desirable as they make application of MAS and map-based cloning possible. Also, highly dense maps are now proving useful for de novo sequence assembly of next generation whole genome sequence data by facilitating the anchoring and orienting of the scaffolds [71].

The double-podding gene (sfl) which mapped on LGVI in our present map (Figure 1) was flanked by Ta80 and NCPGR128 at 3.7 cM and 3.0 cM respectively (Figure 1) and is known to have a positive yield stabilizing effect and it is independent of seed size [72]. Map compilation helped in saturating this region (LG A; Figure 2). Ta80 which had been earlier shown to be 4.84 cM from sfl [41] and 3.7 cM in our map (LG I, Figure 1), now in the projected LG A (Figure 2) was only 2.5 cM apart. Moreover the marker NCPGR78 was embedded between sfl and Ta80. In LGI (Figure 1) sfl was flanked by NCPGR128 at 3.0 cM which in LG A (Figure 2) reduced to 2.1 cM and accommodated 1 marker (H3B08) between them. Therefore it was clear that the compiled map would serve as a highly useful resource for future mapping projects.

Conclusions
In the present study, we enhanced the marker repertoire in chickpea by developing a set of 181 novel STMS markers from a microsatellite-enriched library, thereby providing researchers with advanced genomic resources for genomics-assisted breeding programs. To apply the developed resource in breeding, an advanced intraspecific genetic linkage map of chickpea was constructed. New genomic locations were mapped by utilization of new as well as the previously developed but unmapped STMS markers. Marker density was
substantially improved by merging the map data generated in this study with the available intraspecific map. Therefore this study will be directly useful in promoting future mapping projects, for dissection of complex agronomic traits and for anchoring and orienting the scaffolds required for assembly of next generation whole genome sequence data.

**Methods**

**Plant material and DNA isolation**

The intraspecific mapping population of chickpea was generated at ICRISAT, Patancheru, India by Dr Jagdish Kumar. Briefly, *C. arietinum* cv. ICCV-2 (donor parent, large seeds and single pods) a kabuli variety was crossed with *C. arietinum* cv. JG-62 (recipient parent, small seeds and double podded) a desi chickpea variety. The F₁ plant was self-pollinated to obtain the F₂ offspring that were further self-pollinated and advanced by single seed descent for next 10 generations to obtain recombinant inbred lines (RILs). A population of randomly selected 126 individuals was used for linkage analysis and map construction. All the plants were grown at the NIPGR field site. Genomic DNA from fresh leaf tissue of all the 126 RILs of intraspecific population along with the parental lines ICCV-2 and JG-62 was isolated using CTAB method [73]. The quality and quantity of all DNA samples were checked on agarose gels by comparison with known amounts of uncut λ DNA.

**Cloning and characterization of microsatellite rich regions**

Nuclear DNA of chickpea cv. Pusa 362 was isolated by using the protocol of Malmberg et al. 1985 [74]. The microsatellite enriched library was constructed [48] for the identification of (GT/CA)n, and (GA/CT)n repeats. Approximately 2.5 ng of microsatellite enriched eluted DNA was cloned into 10 ng of a modified pUC19 vector (pJVI) [48]. After transformation and blue-white selection on IXA (IPTG, X-gal and ampicillin) plates, the white colonies were transferred to Hybond N membrane (Amersham Biosciences, USA) and screened using γ[^32]P-ATP labelled (CA)10 and (CT)10 oligonucleotide probes. Plasmid DNA from the recombinant clones producing intense signal after autoradiography were isolated using the alkaline lysis method [75], purified by PEG precipitation and sequenced on ABI3700 Prism automated sequencer (Applied Biosystems, USA). To reduce the redundancy, DNA sequences were assembled using the CAP3 program (http://pbil.univ-lyon1.fr/cap3.php) [76]. Microsatellite detection was done using the TROLL program [77] where ≥5 dinucleotide and ≥4 trinucleotide motifs were selected. The microsatellite containing sequences were submitted to the GenBank for obtaining the accession numbers (EU877268-EU877448) and also subjected to BLASTN analysis at threshold value of 1E-05 for homology searches.

**STMS marker development and polymorphism analysis**

100-150 bp regions flanking the microsatellite motifs were identified for designing STMS primers. Primers were designed using the software Primer 3.0 (http://frodo.wi.mit.edu/primer3/) [78] and the criteria for primer design was as mentioned in Choudhary et al. 2009 [79]. The primer pairs were validated by amplification of the expected sized products from chickpea cv. Pusa362 genomic DNA and designated as NCPGR 101-281 (Table 1). The 181 STMS primers developed in this study (Table 1) along with 84 primers developed earlier in our laboratory (NCPGR 1-100) [18,22], 150 primers of H-series [24] and 107 primers reported in earlier studies in chickpea [16,21] were used for analysis of parental polymorphism (Table 2). All the primers were screened for polymorphism between chickpea accessions ICCV-2 and JG-62, the parental lines of the mapping population. Those that exhibited polymorphism were further used for genetic analysis of all the 126 individual RILs of the mapping population.

**Genotyping, linkage analysis and map construction**

Since only microsatellite based markers were used, SSR genotyping was done by PCR amplification of genomic DNA from the 126 RILs and the parents followed by gel electrophoresis. PCR reactions were carried in a 15 μl reaction volume containing 40-50 ng of genomic DNA, Titanium Taq PCR buffer (20 mM KOH, 10.6 mM KCl, 2.3 mM MgCl₂, 2.5 μg/ml BSA), 0.75 μM of each primer, 0.125 mM of each dNTP, and 0.5 U of Titanium Taq DNA polymerase (Takara, Clontech). The following touchdown amplification profile was used: (i) initial denaturation 94°C 3 min, (ii) 18 cycles of 94°C 50 s, 65°C 50 s [decreasing annealing temperature 0.5°C/ cycle], 72°C 50 s, (iii) 20 cycles of 94°C 50 s, 55°C 50 s, 72°C 50 s, and (iv) final extension 72°C 7 min. The amplified products were electrophoresed on 6% polyacrylamide gels or 3% Metaphor agarose gels depending upon the size range, stained with ethidium bromide and analyzed using the gel documentation system. The amplified banding patterns were scored as ‘A’ for ICCV-2 type banding pattern, ‘B’ for JG-62 type banding pattern and ‘H’ for heterozygous loci. Additionally, the RILs were also phenotyped for one morphological trait i.e. double-podding (sfl) which is reported to be a monogenic recessive trait [41]. The pod number per peduncle was scored for each of the RILs for three consecutive years (in the chickpea growing season of 2006, 2007 and 2008) at the institute field site.

Each segregating marker was tested for goodness of fit to the expected 1: 1 ratio by χ² test (p <0.05). All markers
including those with distorted distribution were used for linkage analysis and map calculations performed using JoinMap ver. 4.0 [45]. The markers were classified into linkage groups (LGs) using the minimum LOD threshold of 3.5 and maximum of 5.0 with recombination fraction of 0.4. Kosambi mapping function was used to estimate the map distances [80]. The LGs of the present map were designated with Roman numerals from I to VIII. Genome coverage was calculated according to Chakravarti et al. 1991 [81] i.e. Genome coverage = Map length/(Map length x [No. of loci +1]/No. of loci-1])

Map Projection
To build the consensus intraspecific linkage map of chickpea, the program BioMercator ver. 2.1 [47] was used. The program facilitates automatic compilations of several genetic maps by iterative projections of genes, loci and QTLs. Common loci between homologous LGs were compiled to compute specific distance ratios for each interval between two common loci. Using this criteria, LGs of our map were projected on LGs of reported map [34] through this program. Further, to saturate the regions harboring the double-podding (sfl) gene, further integration was carried out.

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Authors’ contributions
RG, SC, NKS and VG conducted the experimental work. RG, SC and SB compiled and analyzed all data and provided inputs for interpretation of results. RG, SC, NKS and BS wrote the manuscript in consultation with other co-authors. SB conceived, planned coordinated and supervised the overall study and finalized the manuscript. All authors read and approved the final manuscript.

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