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# Development of an integrated intraspecific map of chickpea (*Cicer arietinum* L.) using two recombinant inbred line populations

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**Abstract** A composite intraspecific linkage map of chickpea was developed by integrating individual maps developed from two $F_{8:9}$ RIL populations with one common parent. Different molecular markers viz. RAPD, ISSR, RGA, SSR and ASAP were analyzed along with three yield related traits: double podding, seeds per pod and seed weight. A total of 273 markers and 186 RILs were used to generate the map with eight linkage groups at a LOD score of ≥3.0 and maximum recombination fraction of 0.4. The map spanned 739.6 cM with 230 markers at an average distance of 3.2 cM between markers. The predominantly used SSR markers facilitated identification of homologous linkage groups from the previously published interspecific linkage map of chickpea and confirmed conservation of the SSR markers across the two maps as well as the variation in terms of marker distance and order. The double podding gene was tagged by the markers NCPGR33 and UBC249z at 2.0 and 1.1 cM, respectively. Whereas, seeds per pod, was tagged by the markers TA2x and UBC465 at 0.1 and 1.8 cM, respectively. Eight QTLs were identified that influence seed weight. The joint map approach allowed mapping a large number of markers with a moderate coverage of the chickpea genome and few linkage gaps.

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## Introduction

Chickpea (*Cicer arietinum* L.) is a self-fertilizing diploid ($2n = 2x = 16$) grain legume, grown in more than 30 countries of Central and South Asia, southern Europe, northern and eastern Africa, Australia, South America and North America. It is the second most important pulse crop worldwide in terms of area under cultivation (11.2 Mha) after dry beans, but ranks third in production (9.1 Mt) following dry beans and peas (FAOSTAT Database http://www.faostat.fao.org/, 2006). It is a traditional low-input and low yielding crop in the farming systems of Indian subcontinent and Near East, where it is an integral part of the daily diet of majority of the population. The low yield of chickpea is mostly due to its susceptibility to various biotic and abiotic stresses. Molecular marker based linkage maps have been useful in identifying and localizing important genes controlling both qualitatively and quantitatively inherited traits in a wide range of species (Tanksley et al. 1989). Marker assisted selection (MAS) of agronomically desirable traits such as yield, quality, biotic and abiotic stress resistance, etc. requires an intraspecific linkage map saturated with co-dominant and single-locus PCR based markers like SSRs. The SSRs also enable transfer of linkage information among maps developed from different populations and can be used as anchors to combine the maps to develop a highly saturated consensus map.

Mapping of the chickpea genome has been of interest to identify genomic locations of disease resistance genes (Winter et al. 2000) and other yield related traits (Cho et al. 2002; Rajesh et al. 2002a). However, due to very low polymorphism (Udupa et al. 1993; Labdi et al. 1996), progress in chickpea genomic research has been relatively slow, compared with legumes like soybean and *Medicago*. Nevertheless, a few genetic maps have been initially reported in chickpea based on morphological and isozyme markers (Gaur and Slinkard 1990; Kazan et al. 1993; Simon and Muehlbauer 1997) followed by DNA markers (Winter et al. 2000). Till date, five intraspecific linkage maps have been reported in chickpea, by using mainly SSRs (Cho et al. 2002, 2004; Flandez-Galvez et al. 2003; Udupa and Baum 2003) and RAPDs (Cobos et al. 2005). Even though there are common markers between these maps, integrating them has not been done and their usage has become limited. To further saturate the intraspecific linkage map of chickpea and to locate the yield related traits, we developed an integrated linkage map using SSRs as anchor markers from two RIL populations.

## Materials and methods

### Plant material and DNA extraction

Two $F_{8:9}$ recombinant inbred line (RIL) populations advanced by single seed descent method were used in the present study. The populations were derived from two intraspecific crosses, JG62 × Vijay (JV population) and Vijay × ICC4958 (VI population), and comprised 197 and 108 lines, respectively. Of the three *Desi* genotypes, JG62 is double podded and susceptible to fusarium wilt; Vijay is wilt resistant, drought tolerant variety having high pod number and wider adaptability, whereas ICC4958 is late wilting, drought tolerant and bold seeded cultivar. The two RIL populations were grown in the field at Pulses Research Station, Mahatma Phule Krishi Vidyapeeth (MPKV), Rahuri, India in 2002–2003. Ninety-three RILs from each population were randomly chosen and used for marker analysis. DNA was extracted from individual samples according to the method described by Simon and Muehlbauer (1997).

### PCR analysis

The primers used in the present study included 800 RAPD (UBC1-800), 100 ISSR (UBC801-900), 24 RGA, 1 ASAP (CS27) and 510 chickpea SSR primers. Optimal PCR conditions were established for each primer type and all the marker loci were scored at least twice to minimize interpretation errors.

### RAPD and ISSR analyses

The RAPD and ISSR primers were obtained from the University of British Columbia (UBC), Canada, and analyzed as described by Winter et al. (2000) and Ratnaparkhe et al. (1998), respectively. The amplification products were electrophoretically separated on 2% agarose gels and the banding patterns were visualized on a UV-transilluminator after staining the gels with ethidium bromide. Only clear and reproducible polymorphic bands were scored as loci.

### RGA analysis

Twenty-four resistance gene analog (RGA) primers described by Chen et al. (1998) were used. PCR amplifications were performed according to Rajesh et al. (2002b) in 15 $\mu$L reaction volumes with minor modifications. The amplification products were separated on either 6% denaturing polyacrylamide gels that were silver stained or 3% MetaPhor agarose gels followed by staining with ethidium bromide.

### SSR analysis

The SSR analysis included 28 primers reported by Huttel et al. (1999), 174 primers by Winter et al. (1999), 95 primers by Sethy et al. (2003, 2006a, b), 200 primers by Lichtenzveig et al. (2005) and 13 primers by Choudhary et al. (2006). Fifteen *Medicago truncatula* SSRs (Eujayl et al. 2004) were also used to check cross-species utility of the primers. PCR amplifications were performed in 15 $\mu$L reaction volumes as described by Winter et al. (1999) with minor modifications. Majority of the PCR products were resolved as stated in RGA analysis. However, the bands with narrow allelic size variation were separated on 6% denaturing polyacrylamide gels using $\alpha$-$^{32}$P or $\gamma$-$^{33}$P labeled dATPs (BRIT, India).

### Scoring of yield related traits

Data for three yield traits, seeds per pod (VI population), double podding and seed weight (JV population) were collected from two replications. For collecting data on seeds per pod, one plant from each RIL was randomly selected and five pods were randomly chosen. The lines were similarly scored for double poddedness or single poddedness after pod setting. The data on seed weight were recorded from the JV population by taking the average weight of 100 seeds of each RIL for three consecutive years (2003–2005).

### Linkage analysis

The $\chi^2$ test was used to assess goodness-of-fit to the expected 1:1 segregation ratio for each marker. All mark-

ers including those with distorted segregation were used for linkage analysis performed using JoinMap Ver. 3.0 (van Ooijen and Voorrips 2001). The markers were classified into linkage groups (LGs) using the minimum LOD threshold of 3.0 and maximum recombination fraction of 0.4. Kosambi mapping function was used to estimate the map distances (Kosambi 1944). Two separate maps were initially developed for each population, which were integrated later using the common markers. The heterogeneity of recombination rate between these common markers in the two maps was tested using the $\chi^2$ test as implemented in JoinMap Ver. 3.0. Comparison of the present map with the interspecific map developed by Winter et al. (2000) was performed using MapChart Ver. 2.2 (Voorrips 2002). In the comparison, the LGs of the present map were designated with Arabic numerals, whereas the LGs of the map of Winter et al. (2000) were designated with Roman numerals.

### QTL analysis

Windows QTL Cartographer Ver. 2.5 (Wang et al. 2006) was used for QTL analysis. Model 6 was adapted, and the control marker number and window size was 5 and 10 cM, respectively. The walk speed was 1 cM and the forward regression method was selected. LOD score peaks >2.0 indicated the existence of QTLs.

## Results

### Linkage maps for the two RIL populations

Marker analysis for both the populations was performed separately. Of the 1,226 primers (800 RAPD, 100 ISSR, 310 chickpea SSRs, 15 *Medicago truncatula* SSRs and 1 ASAP) screened between the parents of JV (JG62 × Vijay) population, only 116 (9.5%) primers revealed clear and consistent polymorphism generating 121 reproducible and segregating markers for linkage analysis. The 15 *Medicago* SSRs although produced amplifications, were not polymorphic with the parents. The linkage analysis revealed seven linkage groups with 106 markers (95 SSRs, 9 RAPDs, 1 ASAP and double podding) (results not shown). This map covered 509.3 cM at an average marker density of 4.8 cM. Sixteen markers containing four RAPDs, one ISSR and eleven SSRs were unlinked.

The parents of the VI population (Vijay and ICC4958) were screened with 1,434 primers (800 RAPDs, 100 ISSRs, 510 SSRs and 24 RGAs), which produced 167 segregating markers containing 113 SSRs, 35 RAPDs, 16 ISSR, two RGAs and one yield related trait (seeds per pod, *Spp*) in seven LGs (results not shown). Of the 24 RGAs screened, only one primer (RGAPtokin1) was polymorphic and produced two loci, which were mapped on LG-2. The map spanned 623.9 cM placing the markers at an average of 3.7 cM interval. Eleven SSR, two RAPD and one ISSR markers remained unlinked among the 181 markers analyzed.

### Integrated map

JoinMap Ver. 3.0 (van Ooijen and Voorrips 2001) was used for integrating the two individual maps using 43 common markers on five LGs. The joint segregation analysis with a total of 247 markers and 186 RILs produced an integrated map with 230 markers (containing 44 RAPDs, 16 ISSRs, 165 SSRs, 2 RGAs, 1 ASAP and two yield related traits, double podding gene (*Sfl* and *Spp*) in eight linkage groups (Fig. 1). The integrated intraspecific map, which covered 739.6 cM, had an average marker density of 3.2 cM. Relative to the estimated physical size of the chickpea genome (750 Mbp; Arumuganathan and Earle 1991), 1 cM distance in the present map approximately equals 1 Mbp.

LG-1 was the longest linkage group with 63 markers and spanned 132.4 cM at an average marker density of 2.1 cM. It shared 17 markers from three LGs (III, V and XIII) of the interspecific map of Winter et al. (2000) (Fig. 2). LG-2 was the densest linkage group with the average marker density of 1.8 cM and had 72 markers spanning 127.4 cM. The *Spp* trait and two RGA markers, RGA6x (234 bp) and RGA6y (272 bp), were mapped on this LG. This group corresponded to LGs I and IV of the interspecific map. The LG-3 corresponded mainly to LG-II but shared a single marker (TS12) from LG-VIII. Similar observation was also made by Flandez-Galvez et al. (2003). The LG-4 had 26 markers spanning 97.3 cM and shared four markers from LG-VI. The *Sfl* was also mapped on this LG and was flanked by NCPGR33 and UBC249z markers. LG-5 spanned 65.9 cM with 25 markers and corresponded to LG-VII of the interspecific map. However, it also shared the marker STMS25 from LG-XV. LG-6 had only seven markers and may correspond to LG-VIII of the map of Winter et al. (2000) and chromosome H of Vlacilova et al. (2002) as it contains the marker TS45. The LGs 7 and 8 were individual LGs from VI and JV populations, respectively, and comprised only RAPD markers. These LGs lacked common markers and could not be combined or compared with the LGs of Winter et al. (2000) map. Inversions were observed with respect to marker orders in all linkage groups between the present and the interspecific map of Winter et al. (2000).

The correlation between number of markers on each LG and length of the respective LG gave an indication of distribution of markers over the linkage groups. These correlation coefficients were 0.58, 0.72 and 0.70 ($P < 0.001$) for the maps of JV, VI and integrated map, respectively (data

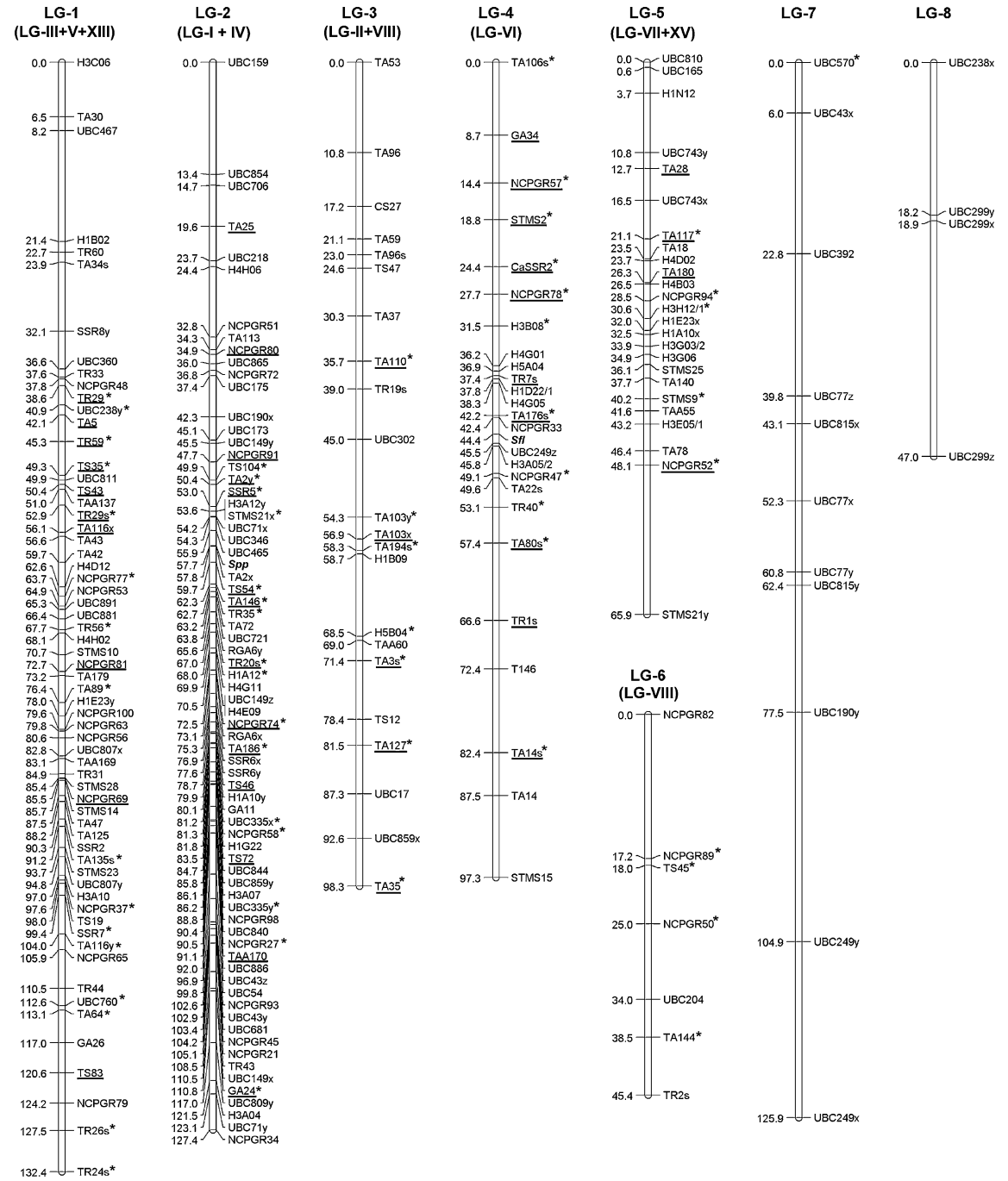


**Fig. 1** Composite map of the chickpea genome obtained by integrating the linkage maps of JV (JG62 × Vijay) and VI (Vijay × ICC4958) RIL populations. The markers common between the two maps have been *underlined* and those showing distorted segregation are indicated by *asterisks*. Corresponding LGs of Winter et al. (2000) reference map have been indicated in *Roman numerals* in parentheses

not shown), which indicates more random distribution of markers among the LGs in VI and integrated maps than the JV map. Of the 106 and 167 markers mapped in the JV and VI populations, respectively, 44 and 17 markers did not segregate according to the expected Mendelian ratio ($P < 0.001$). However, only two markers (TA127 and TR29s) were distorted in both the populations. Different marker types exhibited different levels of skewness, however, SSRs were the most distorted. The skewed markers are indicated by asterisks on the integrated map (Fig. 1).

## Location of yield related traits

Three yield related traits, double podding (*Sfl*), seeds per pod (*Spp*) and seed weight (*Sw*) were mapped. The double podding locus (*Sfl*) on LG-4 was flanked by NCPGR33 and

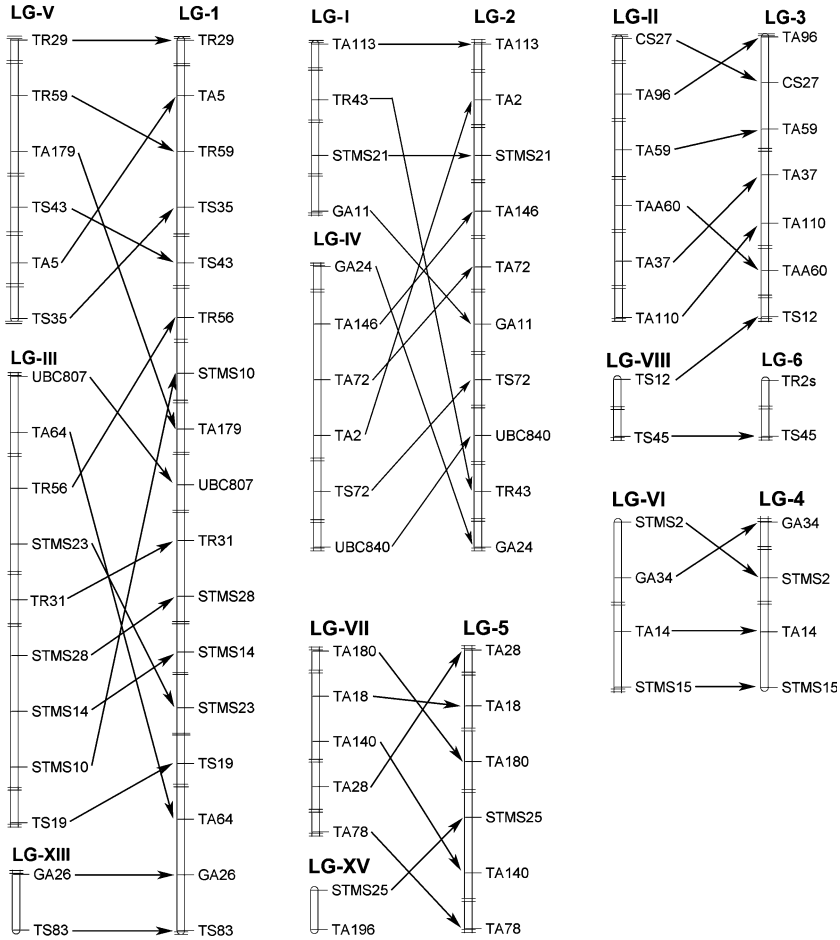


**Fig. 2** Comparison between the interspecific map of Winter et al. (2000) and the present composite map. The LGs of Winter et al. (2000) map are designated with *Roman numerals*, whereas those of the present map are designated with *Arabic numerals*

UBC249z markers at 2.0 and 1.1 cM, respectively. *Spp* segregated in 1:1 ratio ($P < 0.05$) and was mapped on LG-2. It was flanked by TA2x and UBC465 at 0.1 and 1.8 cM, respectively. For seed weight, two significant QTLs (LOD > 3.0) and six putative QTLs (2.0 < LOD < 3.0) were identified on three LGs in the JV map (Table 1). Two QTLs (*Qncl.Sw1* on LG-1 and *Qncl.Sw7* on LG-4) were consistent across two environments, whereas other QTLs expressed in only one environment. *Qncl.Sw7* was also associated with *Sfl* (data not shown) and is presumably the same QTL identified by Abbo et al. (2005). These QTLs individually explained between 6 and 13% of the total phenotypic variance.

## Discussion

The main objective of this study was to construct a saturated intraspecific linkage map of chickpea using the consensus map approach. Such approach allows locating markers or genes for important traits, which might not segregate in one mapping population, but in the other. This is particularly important for crops like chickpea, where very low levels of polymorphism have initially been reported (Udupa et al. 1993; Labdi et al. 1996). We used the 43 common markers from the two separate maps as anchors to combine other markers from the maps into a composite

**Table 1** QTLs for seed weight detected in the JG62 × Vijay population

| QTL name | LG | Indicative marker | LOD score | Additive value | $R^2$ (×100) |
|---|---|---|---|---|---|
| *Qncl.Sw1** | 1 | TR56 | 2.56 | 1.05 | 7.4 |
| *Qncl.Sw2* | 1 | UBC238y | 3.19 | 1.27 | 11.4 |
| *Qncl.Sw3* | 1 | TA116x | 2.00 | 0.93 | 6.0 |
| *Qncl.Sw4* | 1 | STMS13 | 2.07 | −1.12 | 8.2 |
| *Qncl.Sw5* | 3 | UBC17 | 2.87 | −0.73 | 9.3 |
| *Qncl.Sw6* | 3 | TA53 | 2.65 | −1.33 | 12.4 |
| *Qncl.Sw7** | 4 | TR7s | 3.35 | 1.77 | 13.0 |
| *Qncl.Sw8* | 4 | STMS2 | 2.87 | −1.31 | 8.8 |

* Indicates QTLs stable across two environments

map. Such maps have been developed in pea, tomato, barley, etc. (Ellis et al. 1992; Tanksley et al. 1992; Kleinhofs et al. 1993). Availability of a saturated linkage map facilitates marker-assisted breeding as well as mapping of quantitative trait loci (QTLs). In *Vicia faba*, previously reported maps were used for identification of QTLs associated with seed weight and broomrape/ascochyta resistance (Vaz Patto et al. 1999; Roman et al. 2003, 2004).

The composite map developed in this study consisted of 230 markers distributed over eight LGs and covered 739.6 cM. The parental genotypes used for developing the mapping populations were diverse with respect to resistance to fusarium wilt, yield related traits (*Sfl*, *Spp*, pod number, seed weight) and drought tolerance. Integration of the individual maps developed from the two mapping populations allowed us to map more markers to obtain improved coverage of the chickpea genome and to fill linkage gaps in the individual maps. By integrating the two maps, the length of the composite map increased by 18.5%, from 623.9 cM (VI map) to 739.6 cM, while the number of mapped markers increased by 37.7%.

Marker distortion was evident in both the individual maps. However, it was more pronounced in JV than in VI population. The segregation distortion observed in JV population (41.5%) was comparable to that reported by Reiter et al. (1992) in *Arabidopsis* and Xu et al. (1997) in rice. While in the VI population, 10.2% of the loci deviated from the expected Mendelian segregation. Most of the distorted loci in the JV population were skewed in favor of the parent Vijay. This might be due to accumulation of distorted alleles in the population with progressive cycles of selfing undergone in the development of the RILs (Flandez-Galvez et al. 2003). In tomato, Paran et al. (1995) reported a significant increase in the number of loci that deviated from the expected Mendelian inheritance from $F_2$ to $F_7$. They accounted this increase to the cumulative effect of selection against the alleles of one of the parents during propagation of the RILs.

The highly significant correlation (0.70, $P < 0.001$) observed between the lengths of the LGs and the number of markers in the respective LGs, indicated random distribution of the markers in the map. However, non-uniform distribution of markers was observed in some linkage groups (Fig. 1). This might be due to non-random sampling of the genome by the primers used, by uneven distribution of recombination along the length of the LGs (Tanksley et al. 1992), or by clustering of some markers due to their preferential targeting of particular genomic regions (Castiglioni et al. 1999).

The *Sfl* gene was first tagged by Rajesh et al. (2002a) with the marker TA80 at 4.84 cM. In the present study, the gene has been tagged with two new markers NCPGR33 and UBC249z at 2.0 and 1.1 cM, respectively. The *Sfl* gene has a positive yield stabilizing effect and it is independent of seed size (Rubio et al. 2004). It reportedly increases seed yield by 10–18% under moisture-limiting conditions (Sheldrake et al. 1979; Kumar et al. 2000). The *Spp* trait was tagged by two flanking markers TA2x and UBC465 at 0.1 and 1.8 cM respectively. The seed size is determined by seed weight and is an important component of yield in chickpea (Upadhyaya et al. 2006). We identified two significant and six putative QTLs for seed weight in the JV population (Table 1). Upadhyaya et al. (2006) have reported seed weight to be controlled by at least two major genes and the two significant QTLs identified in the present study might correspond to these genes. Alternatively, the second significant QTL (*Qncl.Sw7*) might be the same QTL as identified by Abbo et al. (2005), which indicates the stability of this QTL across populations as well as across environments. The *Qncl.Sw7* was also associated with the *Sfl* gene, which would facilitate simultaneous selection of these two traits to improve yield. The markers associated with these traits would be useful to improve chickpea yields using marker-assisted selection (MAS) approach. The RGA markers have been used in different crops to identify disease resistance genes (Kanazin et al. 1996; Hays and Maroof 2000). As fusarium wilt is a major disease in chickpea, an effort was made to map the wilt resistance gene(s) using the RGA markers. However, only one of the 24 primers analyzed was polymorphic and produced two loci, none of which was linked to fusarium wilt resistance.

Comparison of the present intraspecific map of chickpea with the interspecific map developed by Winter et al. (2000) revealed high linkage conservation in at least five linkage groups. However, the map distances and marker orders of the common SSR markers differed, possibly due to the intraspecific nature of our mapping populations. The merging of more than one LGs of the interspecific map with single LGs of the intraspecific map was observed. This might be due to homology and subsequent resolution of the sequences that joined the linkage groups in the intraspecific mapping populations (Flandez-Galvez et al. 2003). By developing separate intraspecific maps for *C. arietinum* and *C. reticulatum* using common SSR markers and comparing them might provide the molecular insight of the likely chromosomal rearrangements that led to the evolution of *C. arietinum* from *C. reticulatum*.

The composite intraspecific linkage map developed in this study was an effort towards developing a saturated map of chickpea. As no single population would segregate for all the economic traits of interest, genes for those traits need to be mapped on linkage maps developed from different segregating populations. As the map becomes saturated with more markers, complex traits could be dissected and utilized efficiently in breeding programs. Further, establish-

ment of gene-specific markers on the map could be useful for marker-assisted selection and positional cloning of agronomically important genes. More SSRs need to be developed and mapped for developing a highly saturated linkage map of chickpea. The present intraspecific map will be helpful for mapping and tagging of the genes or QTLs governing traits such as biotic and abiotic stress resistance, agronomic characters and quality in chickpea breeding programs.

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