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Integration of sequence tagged microsatellite sites to the chickpea genetic map

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Abstract Fifty sequence-tagged microsatellite site (STMS) markers and a resistant gene-analog (RGA) locus were integrated into a chickpea (*Cicer arietinum* L., 2n = 2*x* = 16 chromosomes) genetic map that was previously constructed using 142 F_6-derived recombinant inbred lines (RILs) from a cross of *C. arietinum* × *Cicer reticulatum* Lad. The map covers 1,174.5 cM with an average distance of 7.0 cM between markers in nine linkage groups (LGs). Nine markers including the RGA showed distorted segregation ($P < 0.05$). The majority of the newly integrated markers were mapped to marker-dense regions of the LGs. Six co-dominant STMS markers were integrated into two previously reported major quantitative trait loci (QTLs) conferring resistance to Ascochyta blight caused by *Ascochyta rabiei* (Pass.) Labr. Using common STMS markers as anchors, three maps developed from different mapping populations were joined, and genes for resistance to Ascochyta blight, Fusarium wilt (caused by *Fusarium oxysporum* Schlechtend.: Fr. f. sp. *ciceris*), and for agronomically important traits were located on the combined linkage map. The integration of co-dominant STMS markers improves the map of chickpea and makes it possible to consider additional fine mapping of the genome and also map-based cloning of important disease resistance genes.

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Introduction

Genetic maps are powerful tools for studies of evolution, germplasm improvement, inheritance of important traits, marker-assisted selection (MAS) and positional cloning of agriculturally important genes. In an ideal map, different types of informative markers are spaced throughout the genome and newly integrated markers show linkage to existing markers (Paterson 1996). The localization of agronomically important genes along with those markers maximizes the potential for MAS using flanking markers. When they are closely linked to the important genes, polymerase chain reaction (PCR)-based markers can facilitate screening large populations in a breeding program for genes of interest. If the markers are co-dominant they can be more effectively used in early generation selection because they allow the determination of desirable alleles either in a homozygous or heterozygous state.

Several skeletal genetic linkage maps have been constructed for chickpea (*Cicer arietinum* L., 2n = 2*x* = 16 chromosomes) using morphological, isozyme (Gaur and Slinkard 1990; Kazan et al. 1993), restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) (Simon and Muehlbauer 1997), sequence tagged microsatellite sites (STMS), amplified fragment length polymorphism (AFLP) and DNA amplification fingerprint (DAF) (Winter et al. 1999). Winter et al. (2000) mapped the genes for resistance to Fusarium wilt races 4 and 5 on LG 2 using a RIL population derived from a cross between ICC-4958 (*Cicer arietinum*) and PI 498777 (*Cicer reticulatum* Lad.). Because of the almost complete lack of common markers between these maps, map integration has not been possible and information from these maps has had limited use.

A recent linkage map of chickpea was developed using RILs of an interspecific cross between FLIP 84-92C

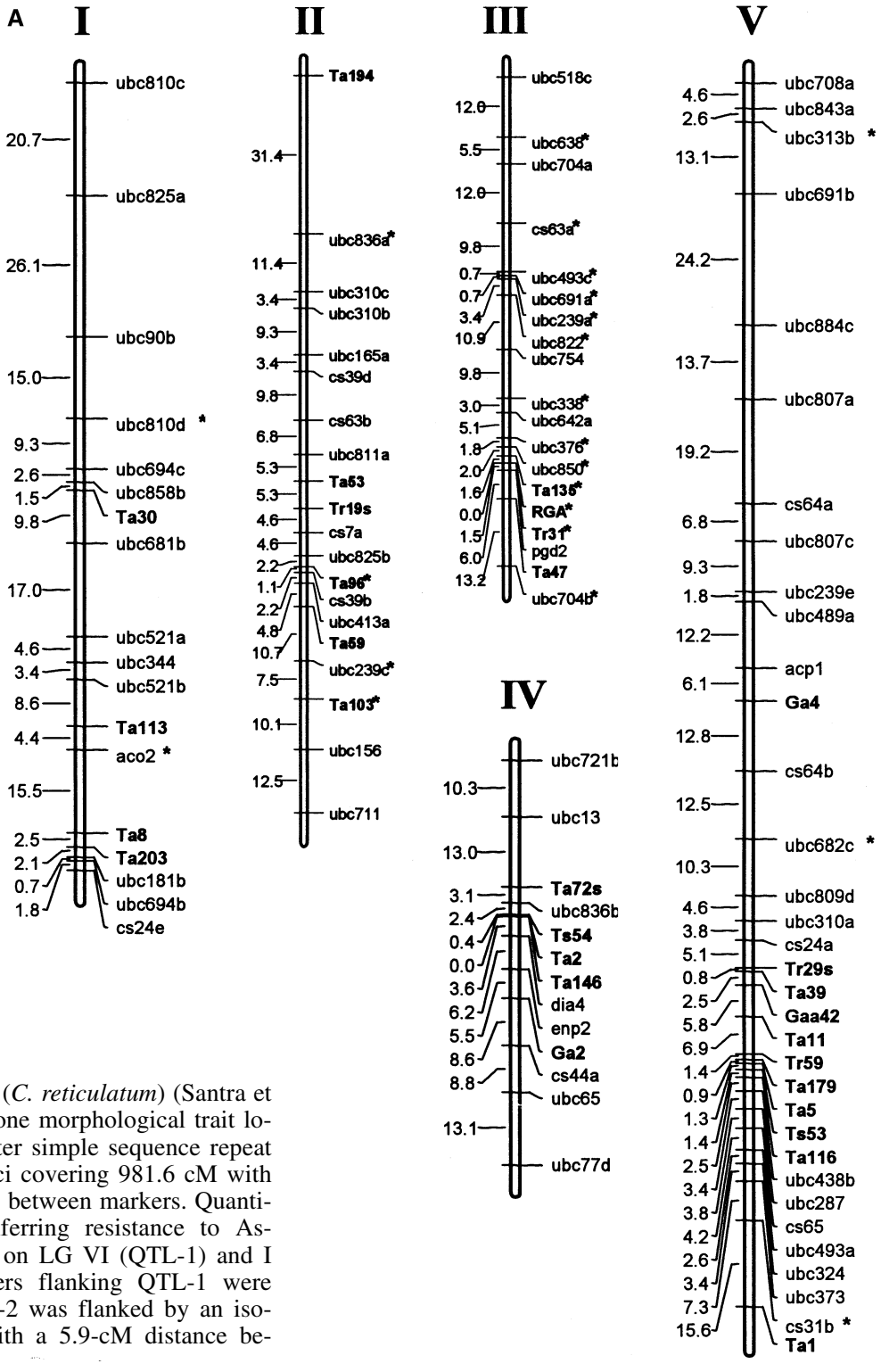


Fig. 1A, B Linkage map of the chickpea genome. STMS markers and the RGA are in *bold*. Markers showing distorted segregation are indicated by *($P < 0.05$)

(*C. arietinum*) and PI 599072 (*C. reticulatum*) (Santra et al. 2000). The map included one morphological trait locus, nine isozyme loci, 17 inter simple sequence repeat (ISSR) loci and 90 RAPD loci covering 981.6 cM with an average distance of 8.4 cM between markers. Quantitative trait loci (QTLs) conferring resistance to Ascochyta blight were detected on LG VI (QTL-1) and I (QTL-2). Two RAPD markers flanking QTL-1 were 10.7-cM apart, whereas QTL-2 was flanked by an isozyme and a ISSR marker with a 5.9-cM distance between markers.

Many of the markers on the map of Santra et al. (2000) are not polymorphic between cultivated chickpea lines (Tekeoglu and Santra, unpublished), and their use for MAS is therefore limited. Markers that are polymorphic within cultivated chickpea are needed for MAS. In addition, the number of markers in the QTL regions for Ascochyta blight resistance needs to be increased, which should also reduce the distance between flanking markers and improve the resolution of the QTLs. The objectives of this study were: (1) to increase the marker density of the existing chickpea map by integrating STMS markers; (2) to identify STMS markers located in the vicinity of QTLs conferring Ascochyta blight resistance; and (3) to identify common LGs between this map and previously published genetic maps of chickpea.

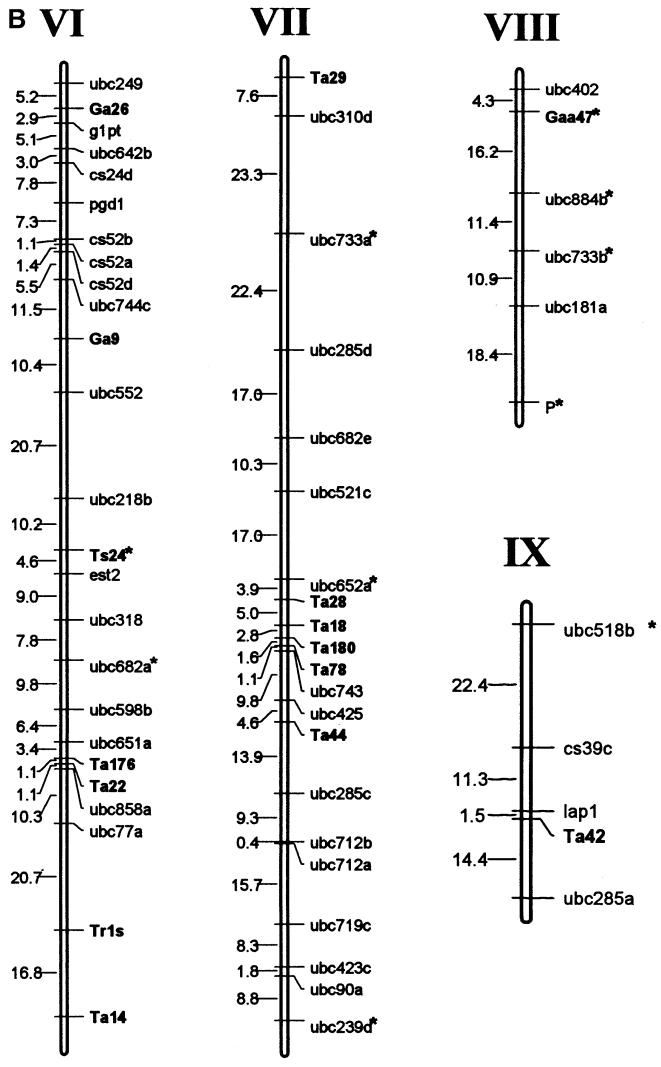


Fig. 1A, B (continued)

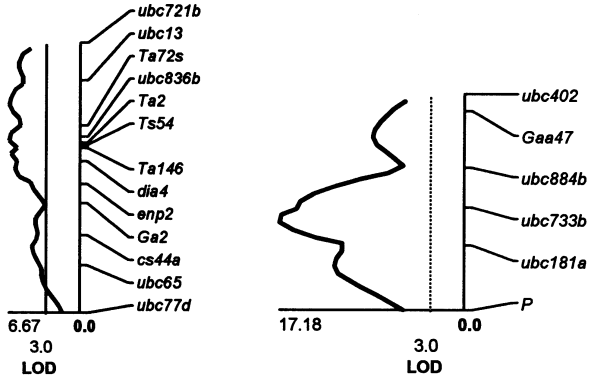


Fig. 2 QTLs for Ascochyta blight resistance located on LGs IV and VIII of the *Cicer* genome

Materials and methods

A population of 217 recombinant inbred lines (RILs) were derived by single-seed descent from a single F_1 plant of FLIP 84-92C × PI 599072 (Tekeoglu et al. 2000). One hundred and forty two RILs were randomly chosen from this population by Santra et al. (2000) and used to construct the existing chickpea genetic map that contains QTLs for Ascochyta blight resistance. The same 142 RILs were used in this study to integrate one resistant gene analog (RGA) and 50 STMS markers into the map. STMS primers were obtained from Gibco BRL Life Tech., Genosys Biotech. Inc. and Integrated DNA Tech., USA, and the RGA was obtained from Gibco BRL Life Tech., USA. DNA was extracted from F_7-derived F_8 plants of each RIL according to the method described in Simon and Muehlbauer (1997). Sixty five primer pairs of STMS markers were chosen to represent all LGs in previously reported STMS maps of chickpea (Winter et al. 1999, 2000). The PCR procedures and the primer names and sequences for STMS markers have been described in Huttel et al. (1999) and Winter et al. (1999). Ten RGA primer pairs were used in the initial parental screening to detect polymorphism between parents. The PCR procedure and the names and sequences for RGA primers were described by Chen et al. (1998).

All amplifications were conducted using Perkin Elmer 9600 and 9700 thermocyclers. PCR products were electrophoresed on either 2% agarose gels or on 6% denaturing polyacrylamide gels. Agarose gels were stained with ethidium bromide and polyacrylamide gels were stained using a commercially available silver-stain protocol (Promega #Q4132). Since STMS markers are locus specific, a single band from each primer pair was visualized and scored for each parent and RIL on the gels. RILs showing both parental bands were considered heterozygous for that locus and, for data analysis purposes, were treated as missing data.

Segregation of marker loci was tested for a fit to the expected Mendelian ratio of 1:1 using the χ^2 test ($P > 0.05$). Linkage analysis was performed using Mapmaker/Exp 3.0 (Lander et al. 1987). LGs were established at a constant LOD score of 4.0 and a recombination value of 0.25 by two-point analysis using the "group" command. Once STMS markers were assigned to previously reported LGs, the markers were then integrated into that group by applying the multipoint analysis "try" function. The most-likely order of loci within a group was determined using the multipoint "compare" command and these orders were verified using the "ripple" command. The Kosambi mapping function was used to determine cM distances between markers (Kosambi 1994). Double-crossovers were checked by the "double-crossover" command in Map Manager QTb (version 2.8) (Manly 1998).

Results

Integration of markers into the chickpea genetic map

With the integration of STMS markers, the current chickpea genetic map comprises 167 markers covering 1,174.5 cM with an average distance of 7.0 cM between markers. Forty three STMS markers and a RGA locus were incorporated into nine LGs of the previously constructed chickpea genetic map, but six STMS markers remained unlinked (Fig. 1). Most of the markers were located on previously larger LGs. For example, only one STMS marker was mapped to each of LGs VIII and IX to bring the number of markers in those groups to 6 and 5, respectively (Table 1). On the other hand, 11 STMS markers were mapped to LG V along with 23 previously mapped markers.

Of the 65 STMS primer pairs we used, nine gave no amplification and six were monomorphic. Segregation was distorted ($P < 0.05$) for seven STMS markers; six in favor of *C. reticulatum* and one in favor of the cultivated

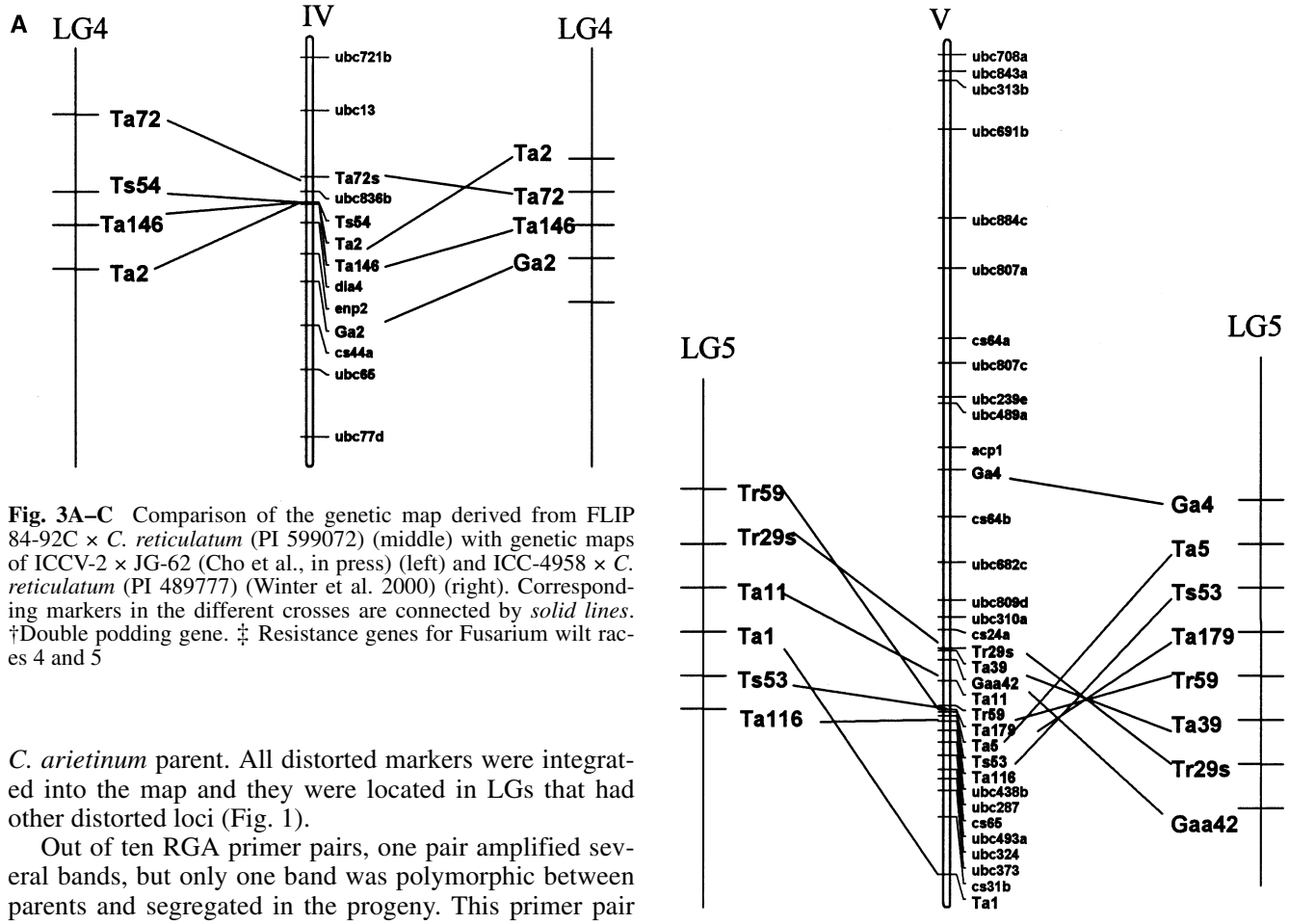


Fig. 3A–C Comparison of the genetic map derived from FLIP 84-92C × *C. reticulatum* (PI 599072) (middle) with genetic maps of ICCV-2 × JG-62 (Cho et al., in press) (left) and ICC-4958 × *C. reticulatum* (PI 489777) (Winter et al. 2000) (right). Corresponding markers in the different crosses are connected by *solid lines*. †Double podding gene. ‡ Resistance genes for Fusarium wilt races 4 and 5

C. arietinum parent. All distorted markers were integrated into the map and they were located in LGs that had other distorted loci (Fig. 1).

Out of ten RGA primer pairs, one pair amplified several bands, but only one band was polymorphic between parents and segregated in the progeny. This primer pair was designed based on the leucine-rich repeat region of the *Xa21* resistance gene in rice (Chen et al. 1998). The RGA was mapped to LG III and showed distorted segregation in favor of the wild parent. This LG had the largest number of distorted loci (13 out of 19) throughout the map. LG VIII had six loci, four of which had distorted segregation ratios.

The amount of distortion for STMS markers was 14%; however, 19% of all loci on the map were distorted. The distorted loci appeared to be clustered in certain LGs rather than certain regions of each LG. Also, clusters of marker-rich regions were obvious on each LG except VII and IX.

Although only one STMS marker was linked to QTL-1 for blight resistance on LG VII, five STMS markers were mapped within QTL-2 located on LG IV (Fig. 2). Based on the addition of STMS markers, the QTLs for Ascochyta blight resistance could be defined more fully, providing co-dominant markers in those critical regions.

Table 1 Distribution of markers and the estimated lengths of the nine linkage groups that comprise the chickpea genetic map

Linkage group	cM	Markers					
		Isozyme	RAPD	ISSR	STMS	Other[a]	Total
1	145.6	1	9	4	4		18
2	146.4		11	3	6		20
3	99.0	1	12	2	3	1	19
4	75.0	2	5	1	5		13
5	226.5	1	17	5	11		34
6	183.1	3	14	1	7		25
7	188.1		15		6		21
8	61.2		3	1	1	1	6
9	49.6	1	3		1		5
Unlinked		2	22	4	6		34
Total	1,174.5	11	111	21	50	2	195

[a] Other includes a resistant gene analog and morphological marker

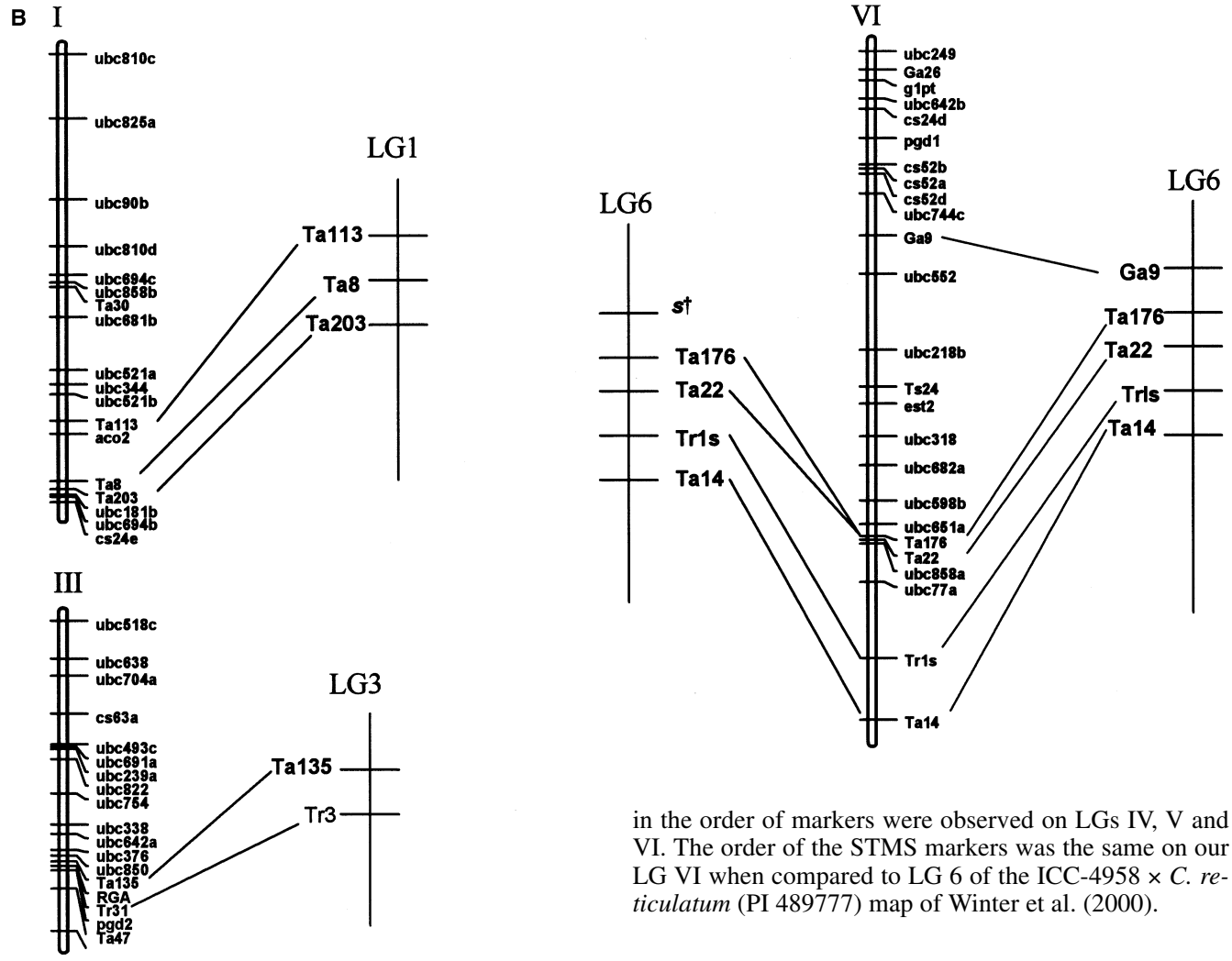


Fig. 3A–C (continued)

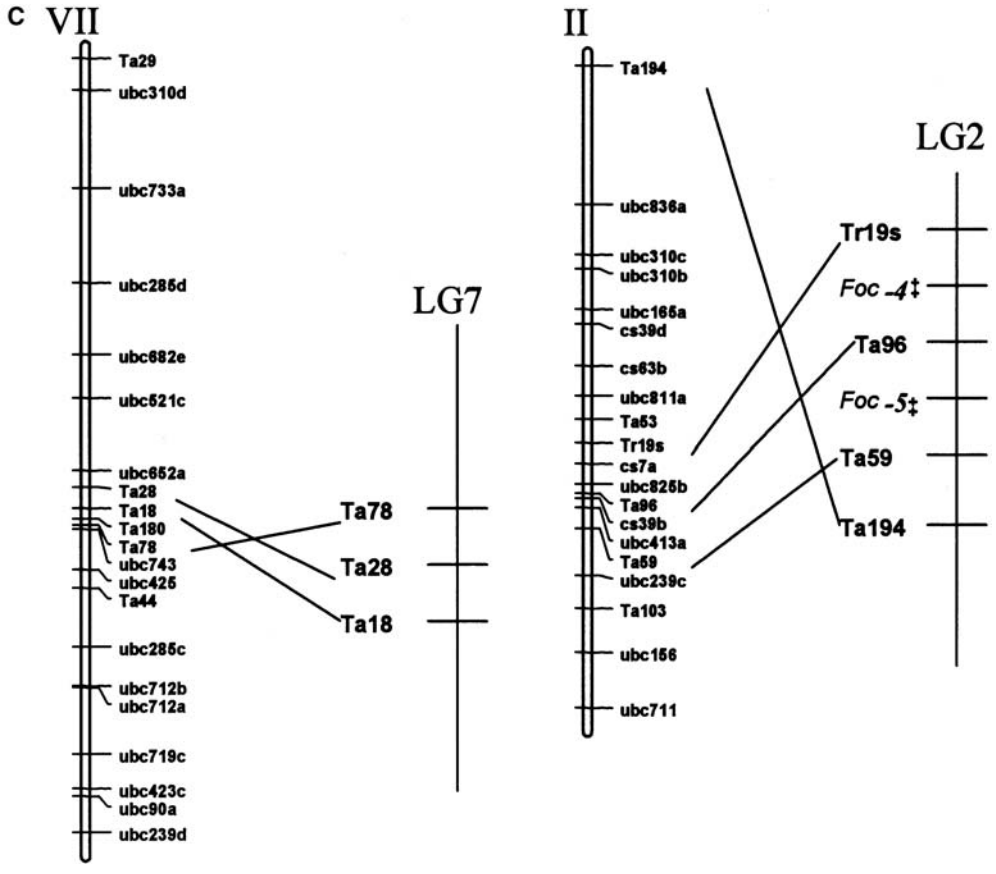
Comparison of the genetic maps of chickpea

Seven LGs were consistent between our map and the linkage maps reported by Winter et al. (2000) and Cho et al. (in press). The map of Winter et al. (2000) was developed using a population of RILs derived from an interspecific cross and comprised eight large and eight small LGs. Two race-specific resistance gene loci for Fusarium wilt, *foc-4* and *foc-5*, were located on LG 2, one of the eight large LGs, and an STMS marker, Ta96, was mapped between these two resistance genes. Ta96 and three other STMS markers on LG 2 of this map were located on LG II of our map with a similar locus order (Fig. 3). Cho et al. (in press) reported an STMS marker map using a RIL population derived from an intraspecific cross of 'ICCV-2' × 'JG-62'. A morphological trait locus (*s*), the gene for double podding, segregated in the intraspecific cross and was located 7.3 cM from Ta176 on LG 6. Ta176 and three other common STMS markers were located on LG VI of our map and the order was the same as that of Cho et al. (in press) (Fig. 3). Differences in the order of markers were observed on LGs IV, V and VI. The order of the STMS markers was the same on our LG VI when compared to LG 6 of the ICC-4958 × *C. reticulatum* (PI 489777) map of Winter et al. (2000).

Discussion

The coverage and marker density of the chickpea genetic map was increased with the integration of STMS markers. Since the nuclear DNA content of chickpea is 738 Mbp/1C (Arumuganathan and Earle 1991), 1 cM of the genetic map would correspond to about 630 kbp. However, this value would change depending on which region of the map is being considered because markers are not equally distributed across the LGs. The present map has nine LGs, which is one more than the expected eight based on the number of chromosomes. The apparent additional LG may be the result of insufficient map density or the parameters used in the mapping procedures. To be useful for map-based cloning of disease resistance genes, additional markers need to be found that are closer to the genes of interest. Increased marker density around the genes of interest will provide a solid starting point for chromosome walking, which is one of the basic elements of map-based cloning (Tanksley et al. 1992). The use of artificial chromosome libraries, especially bacterial artificial chromosomes (BAC), for chromosome walking and also for physical mapping of the chickpea genome would become more feasible with a

Fig. 3A–C (continued)



linkage map of higher marker density (Woo et al. 1994). Increasing marker density, and the addition of genes for other agronomically important traits to a map, can be accomplished by using different mapping populations that segregate for important agronomic traits and markers that are common across the populations (Hayes et al. 1996; Weeden et al. 2000). Examples of these consensus maps have been reported for several crops (Ellis et al. 1992; Tanksley et al. 1992; Kleinhofs et al. 1993). Markers that were common across mapping populations are used as anchors to locate important genes to a particular LG. This approach allows locating important genes in a map where those genes may not segregate in the mapping population. Our results represent the first attempt to compose homologous LGs of published chickpea genetic maps that were developed using different mapping populations.

STMS markers were highly polymorphic in our mapping population (78%). High polymorphism of microsatellite markers in chickpea and other crops has been well documented (Saghai-Maroof et al. 1994; Akkaya et al. 1995; Ratnaparkhe et al. 1998; Weising et al. 1998). In addition to their abundance, STMS markers used in this study are locus specific and co-dominant (Weising et al. 1998). Co-dominant-type markers are important in map construction because they allow for differentiation between heterozygous and homozygous genotypes during scoring of alleles in the mapping population. Another important feature of co-dominant markers is their effectiveness for identifying desirable genotypes as homozygous at very early stages of selection. Probably the most important feature of co-dominant markers is their use in backcrossing desirable genes from usually less-adaptive germplasm to adapted cultivars. In these cases, co-dominant markers can be used to enhance the effectiveness of marker-assisted backcrossing by helping to eliminate linkage drag in a relatively short period of time when compared to the use of dominant markers for that purpose.

Identification and scoring of parental alleles of STMS markers and their segregation in the RILs was simple and unambiguous. Each primer pair amplified a single fragment from each parental line and the polymorphism was detected by size differences of the fragments that ranged from 4 bp up to 300 bp. Considerable size differences in STMS amplification products were reported for several *Cicer* species (Choumane et al. 2000). Distorted segregation observed in STMS markers (14%) was not as high as reported in Winter et al. (1999, 2000). This could be due to the smaller number of loci used in this study or bias from our choosing markers that were representative of defined LGs in their map. We may have inadvertently chosen only two or three markers from regions that were reported as having clusters of distorted markers in their map.

Clustering of loci with distorted segregation was also apparent in our study (Fig. 1). A high number of distorted loci from interspecific crosses has been reported for

other crops and this situation was more dramatic when RILs were used as the mapping population in comparison to F_2 populations, and is likely due to the accumulation of favorable alleles that provide a selection advantage for one of the parental types (Paran et al. 1995). Clustering of distorted markers, as apparent on LGs III and VIII of our map, is consistent with results from other crops (Kidwell et al. 1993; Kleinhofs et al. 1993). Segregation distortion may be the result of abnormal meiosis, which is a common phenomenon in interspecific crosses due to divergent chromosome homology between parents. Translocations and inversions are common causes of meiotic abnormalities in interspecific crosses. Karyotypic analysis of parental lines would provide valuable information in this regard.

Clustering of markers, either distorted or segregating normally, may be the result of suppressed recombination in those regions of the genome. It has been shown in yeast that the centromere has a negative effect on crossing-over called the "spindle fiber effect" (Lambie and Roeder 1986). Another reason for reduced recombination may be heterochromatin, due to its more condensed structure, when compared to euchromatin at meiosis (Tanksley et al. 1992). Ahmad and Hymowitz (1993) studied the chromosome structure of chickpea in pachytene and identified heterochromatin in regions that were proximal to the centromeres on each of the eight chromosomes. They also found that the entire short arm of chromosome 3 was heterochromatic and chromosome 6 had two small heterochromatic regions. Clusters of markers on all LGs except VIII and IX might indicate that these markers are located near the centromeres or are in heterochromatin-rich regions of the chickpea chromosomes.

The comparison of the genetic maps of chickpea constructed from interspecific and intraspecific crosses indicated only minor differences in the marker order overall. Order differences between tightly linked (about 1 cM) markers may have been due to the more than one likely order suggested for those markers by Mapmaker. The STMS marker order on LG V of our map was similar to that found by Cho et al. (in press) but differed somewhat from that of Winter et al. (2000); however, the differing order involved markers that were closely linked. Since there has been no report of this type of mapping study in chickpea, our results need further confirmation using other types of markers and possibly the development of a physical map of the *Cicer* genome. We did not observe any indication of translocations that would have resulted in grouping of some markers outside of their expected LGs. A lack of translocations was expected because *C. reticulatum* is the presumed progenitor of cultivated chickpea (van der Maesen 1987), and the progenies from crosses with *C. arietinum* have been fully fertile.

The relationship between maps based on different chickpea crosses provides a better understanding of genome organization in cultivated chickpea as well as related wild species. STMS markers are valuable for comparing cultivated *C. arietinum* with closely related wild species. Choumane et al. (2000) found that microsatellite-flanking sequences were highly conserved across eight annual and one perennial species of *Cicer*. Conservation of STMS markers across *Cicer* species would provide needed polymorphism for mapping the chickpea genome, and would overcome the current lack of polymorphism that has limited progress. In addition, STMS markers will aid the incorporation of genes for valuable traits into an improved chickpea map.

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