

# A Composite Genetic Map of the Parasitoid Wasp *Trichogramma brassicae* Based on RAPD Markers

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## ABSTRACT

Three linkage maps of the genome of the microhymenopteran *Trichogramma brassicae* were constructed from the analysis of segregation of random amplified polymorphic DNA markers in three F<sub>2</sub> populations. These populations were composed of the haploid male progeny of several virgin F<sub>1</sub> females, which resulted from the breeding of four parental lines that were nearly fixed for different random amplified polymorphic DNA markers and that were polymorphic for longevity and fecundity characters. As the order of markers common to the three mapping populations was found to be well conserved, a composite linkage map was constructed. Eighty-four markers were organized into five linkage groups and two pairs. The mean interval between two markers was 17.7 cM, and the map spanned 1330 cM.

**T**RICHOGRAMMA are minute parasitoid wasps, less than 1 mm in length, that parasitize the eggs of several lepidopteran species. Many studies focus on these hymenopteran species because of their importance in biological control of some lepidopteran pests in crops (Stinner 1977; Li 1994). Biological control using natural enemies (*e.g.*, parasitoids or predators) represents a successful pest control strategy that provides an alternative to the use of polluting pesticides and to the occurrence of resistance among pest species. However, in contrast to other animals that are economically important in agriculture, natural enemies have not yet been submitted to any improvement program. Genetic variation in traits involved in reproductive strategy has been demonstrated for *Trichogramma brassicae* Bezdenko (Hymenoptera; Trichogrammatidae; Wajnberg 1994). This provides the basis for improving the efficiency of this species to control the European corn borer, *Ostrinia nubilalis* Hubner (Lepidoptera; Pyralidae).

In this respect, identification of loci controlling some of the traits that are involved in biological control efficiency could help breeding programs by making the screening of insects easier. Indeed, it could be easier and faster to look at molecular markers that are linked to genes involved in parasitization performances, rather than to check for the variation in the corresponding biological traits (*e.g.*, fecundity, sex ratio, longevity, or emergence rate) in such a small insect. These traits are complex and are probably determined by several

quantitative trait loci (QTL). We aim to apply QTL mapping strategies (Lander and Botstein 1989) to localize loci that influence such biological features in *T. brassicae* and to construct a linkage map for this species. Although very few hymenopteran genomes have been mapped (Whiting 1961; Saul 1993), this strategy has proved to be efficient in the case of the honey bee. The honey bee genome has been extensively mapped with random amplified polymorphic DNA (RAPD) markers (Hunt and Page 1995), and the genetic map that was obtained has allowed the identification of chromosomal regions that affect foraging behavior (Hunt *et al.* 1995).

Because of the minute size of *Trichogramma* and therefore the limited amount of DNA that can be extracted from a single individual, methods based on polymerase chain reaction (PCR) were used to reveal DNA polymorphism in *T. brassicae*. RAPDs have been successfully used for detecting variability in this species (Vanlerberghe-Masutti 1994a,b); therefore, these markers were selected for genetic mapping. Recently, RAPD maps have been developed for a large number of plant species, such as *Arabidopsis* (Reiter *et al.* 1992), slash pine (Nelson *et al.* 1993), norway spruce (Binelli and Bucci 1994), eucalyptus (Grattapaglia and Sederoff 1995), and for several insect species, such as the honey bee (Hunt and Page 1995), the silkworm (Promboon *et al.* 1995), the parasitic wasp *Bracon hebetor*, and the mosquito *Aedes aegypti* (Antolin *et al.* 1996). While this technique is fast and convenient, the major drawback of RAPD markers is their dominant mode of inheritance. However, *Trichogramma* is especially amenable for genetic analysis using these markers because of haplodiploidy; females are diploid and males, which develop

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parthenogenetically from unfertilized eggs, are haploid. Thus, analysis of haploid males provides complete information about the heterozygous loci of their mother that is not confounded by the dominance effect. This strategy of genetic mapping of haploid phases with RAPD markers has been successfully applied to megagametophytes of conifers (Tulsieram *et al.* 1992; Nelson *et al.* 1993; Binelli and Bucci 1994) and to hymenopteran males (Hunt and Page 1995; Kazmer *et al.* 1995; Antolin *et al.* 1996). Dominant RAPD markers are then fully informative, and unambiguous assignment of the linkage phase, coupling or repulsion, in the progeny of parents of unknown phase is possible (Raeder and Broda 1986; Hulbert *et al.* 1988). Similar effects have been obtained using recombinant inbred lines (Reiter *et al.* 1992) or pseudo-testcross populations (Grattapaglia and Sederoff 1995).

In this study, we used four parental lines that exhibit different RAPD markers to construct three different  $F_2$  populations of haploid males, because specific genomic regions may lack segregating markers in one population and, consequently, mapping would not be possible in these regions. The three maps obtained have allowed us to confirm the reliability of RAPDs for genetic mapping of the *T. brassicae* genome. Moreover, as these maps have revealed a good colinearity, the three populations were pooled to construct a composite genetic map. In addition, the number of chromosomes of this species was determined so that the genome coverage of this map could be ascertained.

## MATERIALS AND METHODS

**Insects:** The *T. brassicae* wasps that were used in this study were taken from a laboratory culture of 80 isofemale lines that have been reared separately on the flour moth *Ephestia kuehniella* (Lepidoptera; Pyralidae) for several years in our laboratory (more than 100 generations at the time of this study). Each isofemale line was an inbred line that was initiated from the progeny of a single female mated with one of her sons, followed by brother-sister mating. Thus, the inbreeding coefficient should be close to 1, and it is highly probable that each line is nearly homozygous.

**Karyotypes:** Five virgin and five mated *T. brassicae* females were allowed to oviposit in eggs of *E. kuehniella* for 3 hr. Twenty-four hours later, five eggs per *T. brassicae* female were isolated from parasitized eggs of *E. kuehniella* and were dissected in 5% colchicine. After incubation for 2 hr 30 min at 20°, *T. brassicae* tissues were transferred onto a microscope slide in a drop of 2% lactic acid orcein and flattened with a cover slide to a monolayer of cells. After staining for 24 hr at 20°, chromosomes in metaphase plates were photographed using a Zeiss microscope ( $\times 1000$ ). Three to six metaphase plates were counted per slide.

**DNA extraction and RAPD assay:** DNA was extracted from single wasps using a chelating resin as described in Vanlerberghe-Masutti (1994a). For large-scale screening reasons, a single set of PCR conditions was chosen. Amplification reactions were performed in a 25  $\mu$ l volume, according to the protocol of Williams *et al.* (1990), containing 1  $\mu$ l of chelex supernatant, 100 ng of 10-nucleotide primer of arbitrary se-

quence (Operon Technology, Alameda, CA, or Eurogentec, Seraing, Belgium) and 0.4 unit of *Taq* DNA polymerase (Applied Biosystems). The cycling program consisted of 40 amplification cycles (94° for 30 sec, 36° for 30 sec, 70° for 80 sec), followed by a final incubation at 70° for 10 min. Amplifications were performed in 96-well microtiter plates in the PTC-100 thermocycler of MJ Research. The amplification products were electrophoresed on a 1.4% agarose gel at 7.2 V/cm for 2 hr and afterward were stained with ethidium bromide and photographed under UV light.

**Parental lines:** Thirty-two of the 80 isofemale inbred lines of *T. brassicae* were screened for DNA polymorphism using 10 random 10-base primers. Four lines (A, B, C, and D), which had different RAPD patterns and were polymorphic for longevity and fecundity, were selected to construct the  $F_2$  mapping populations. To limit residual heterozygosity within these four parental lines, four to six virgin females of each line were isolated, and eight sons of each were submitted to a polymorphism survey with the 10 RAPD primers. According to the results of this screening, the most homogeneous lineage was selected to initiate a new parental isofemale line.

**Construction of mapping populations:** According to the preliminary primer screening of the parental lines, the three most informative crosses ( $D \times A$ ,  $D \times C$ , and  $B \times C$ ) were selected for the construction of the  $F_2$  mapping populations. Once again, to limit the potential impact of residual heterozygosity in the four parental lines, those individuals presenting the highest number of null alleles (*i.e.*, absence of the band) for 20 markers were used as the female parent (diploid). Because the number of offspring that a Trichogramma female produces will rarely exceed 40 individuals, each  $F_2$  mapping population of  $\sim 200$  haploid males was obtained from several virgin  $F_1$  females, resulting from two or three repetitions of the cross (Table 1). For each mapping population,  $F_2$  progenies were divided into two equal parts to obtain two "sister" populations, each having 93 individuals. One population was used for mapping (Table 1), while the DNA of the sister population was stored at  $-20^\circ$  for further completion of the map.

**Primer screening:** Two individuals from each parental line were screened using 272 10-mer primers from several random primer kits (Operon Technology). Those primers that revealed polymorphic bands for the two parental lines were tested on six additional individuals per line. The RAPD fragments that were both polymorphic between two lines and homogeneous within lines were selected for genetic mapping of the three  $F_2$  populations. Polymorphic markers were denoted by the primer designation, and a letter was added when several markers were obtained from a single primer.

**Scoring of markers:** For each primer that was screened, reliability in marker assessment was ensured by including two positive controls corresponding to amplification of parental DNAs. Each amplification was repeated once to confirm repeatability or to solve amplification problems. Markers with unclear or poorly resolved amplification patterns were removed, while the others were scored for presence or absence in every individual of the mapping population. The scoring process was performed twice by two different people. The two readings were compared, and differences were either resolved or the data were considered to be ambiguous and were removed.

**Linkage analyses:** Segregation of markers in each  $F_2$  population was tested for goodness-of-fit to the expected Mendelian segregation ratio (*i.e.*, 1:1 for haploid inbred of a heterozygous female) using a chi-square test. Markers that showed highly significant segregation distortion ( $P < 0.001$ ) were excluded from linkage analysis. Markers that showed significant distortions ( $P < 0.01$ ) were included in linkage groups only if their

**TABLE 1**  
**Structure of the three  $F_2$  mapping populations**

Mapping populations	Repetitions per cross	$F_1$ females per repetition	$F_2$ progeny size of each $F_1$ female
$D \times A$	3	4	16, 10, 9, 9
		2	10, 10
		3	12, 10, 7
$D \times C$	2	4	22, 20, 17, 17
		2	14, 3
$B \times C$	2	4	19, 18, 18, 8
		2	18, 12

Number of repetitions per cross, number of  $F_1$  females per repetition, and progeny size of each  $F_1$  female are indicated.

presence did not disturb the order that was established without them.

The composite population was constructed by pooling the scores from individual populations. Missing values were assigned to individuals in populations with monomorphic bands.

Grouping of markers was performed with MAPMAKER (Lander *et al.* 1987) using a minimum LOD score of 4 and a maximal recombination fraction of 0.4 as linkage thresholds. CARTAGENE (Schiex and Gaspin 1997) was used for marker ordering, because the number of markers was either too large (essentially in pooled data) to find a good order with MAPMAKER or the level of missing data was too high to use the two-point procedure in the composite population. This software combines an optimized version of the expectation maximization (EM) algorithm (Dempster *et al.* 1977) for backcross data, with local search techniques originating from artificial intelligence and operations research. Like MAPMAKER, the CARTAGENE program also uses the EM algorithm to obtain maximum likelihood recombination fractions under a given order. The local search techniques were chosen for their well-known adequacy to solve the "traveling salesman problem," a problem closely related to marker ordering (Papadimitriou and Steiglitz 1982). CARTAGENE permits the ordering of a

large set of markers using the maximum likelihood criterion and has been found to provide significantly better results than the program JoinMap (Stam 1993), in terms of likelihood for pooled data. This difference could be due to the criterion used (multipoint likelihood for CARTAGENE vs. two points likelihood for JoinMap) or to the power of the local search algorithm of CARTAGENE (Schiex and Gaspin 1997). With small groups (less than six markers), results have been cross-checked with MAPMAKER and were always found to be in agreement. Recombination fractions were converted into map distances using the Haldane function (1919) because no information was available on the occurrence of chiasma interferences in *Trichogramma*.

## RESULTS

**Karyotypes:** The number of chromosomes counted in metaphase plates from fertilized eggs (female) laid by mated females was twice the number of chromosomes counted from unfertilized eggs (male) laid by virgin females. Therefore, karyotype experiments confirmed the haplo-diploidy of the species *T. brassicae* and showed that the haploid chromosome number was five (Figure 1), as in most chalcidoids (Crozier 1977). The female karyotypes that were studied were organized into four

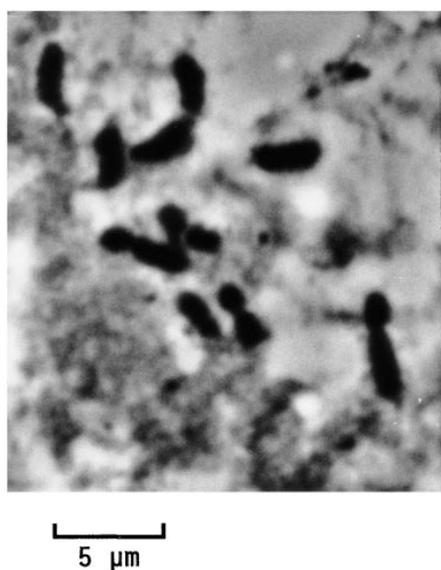


Figure 1.—Chromosomes in metaphase plate from a *T. brassicae* female. Five pairs of chromosomes are identified.

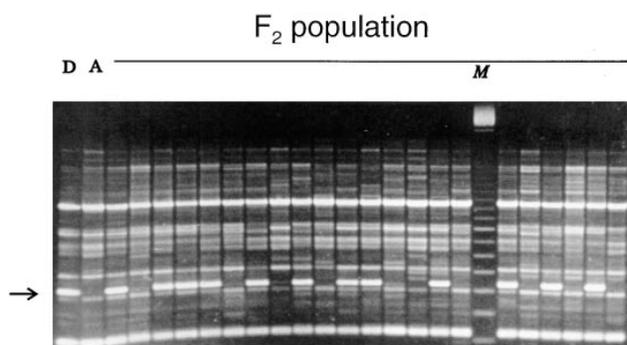


Figure 2.—Example of RAPD marker segregation in  $F_2$  haploid males from population  $D \times A$ . Dominant RAPD marker N4 indicated by an arrow is present in the mother of the  $F_1$  virgin female (from line D in the first lane on the left) and absent in the father (from line A, second lane). Lane M corresponds to a 100-bp ladder.

**TABLE 2**  
**Distribution of the informative markers in the three mapping populations**

	D × A	D × C	B × C
D × A	60	26	12
D × C		55	17
B × C			43

The number of informative markers between each pair of populations is indicated.

pairs of small chromosomes, with a size 2.4–3.2  $\mu\text{m}$ , and one pair of larger chromosomes of 4.8  $\mu\text{m}$ .

**Polymorphism:** From the 272 random primers that were screened, 15 (5.5%) did not yield amplification products, and 164 (60.3%) did not detect any informative polymorphism between the parents, irrespective of whether they were monomorphic or variable within lines. The remaining 93 primers (34.2 %) revealed 143 polymorphic bands (*i.e.*, markers). Amplifications of the three  $F_2$  mapping populations were performed with these primers. Twenty primers, generating 37 markers, were discarded from further analysis due to amplification problems or nonreproducibility. The remaining 73 primers that revealed 106 markers were scored in at least one of the three mapping populations (Figure 2), yielding an average of 1.45 markers per selected primer. Of the 106 markers selected, 60 were informative on the cross D × A, 55 on D × C, and 43 on B × C. Forty-eight markers doubly segregated in two mapping populations, and three segregated in the three populations (Table 2). Although the female parents were initially chosen on the basis of 20 markers in the preliminary screening to present an excess of null alleles, as compared to the male parents, the amount of scoreable markers gave an equivalent number of recessive markers in both parents of D × A (31/29) and D × C (26/28) populations.

**Segregation of RAPD markers:** Mendelian segregation distortions were found in the three mapping populations, although to a lesser extent in population D × C. Two markers from population D × A and three from

B × C deviated at  $P < 0.001$  and were discarded. Although four markers from D × A, three from D × C, and three from B × C deviated at  $P < 0.05$ , only one marker from B × C was excluded from linkage analysis because its presence disturbed the order that was obtained without it. In general, distortion ratios did not appear to be preferentially skewed toward one parent or one allele. Moreover, within a population, distortions were shared with all repetitions.

**Map construction:** During map construction, discrepancies in colinearity between the three maps and the high number of unlinked markers have drawn our attention to several markers. All of them were checked for errors in scoring. Few errors were detected, but a large number of markers were judged *a posteriori* to be too difficult to score and were then removed. These corrections have allowed the linkage of the previously unlinked marker E7a to group IV of map D × A and have allowed a slightly better adequacy of the three individual maps. Maps are presented in Table 3 and Figure 3. Individual maps were comprised of up to 43 markers, organized into four to six linkage groups that contained more than two markers. Each group contained between 3 and 14 markers and covered up to 358.8 cM. The percentage of markers from each population that remained unlinked ranged from 11.4 to 14%. Three unlinked markers from D × A, two from D × C, and one from B × C were distorted. The composite map contained 84 markers organized into five linkage groups and two pairs. It spanned 1330 cM, with an average distance between two adjacent markers of 17.7 cM.

Several maps that had a maximum likelihood similar to that of the best map were found for the three populations and the composite one. For groups II, IV, and V, the best order was the same for the three individual maps and the composite map. A similar result was obtained for the D × C map and the composite map for group I and for B × C map and the composite map for group III. For the other comparisons, the log-likelihood of the most likely gene order in each population was compared with the log-likelihood of the most likely gene order from the composite population. As differences in LOD for group I (−0.47 for D × A and −0.85 for B × C) and group III (−0.9 for D × A and −1.7 for D ×

**TABLE 3**  
**Characteristics of the three individual maps and the composite one**

Population	No. of linked markers	No. of groups + pairs	Unlinked markers (%)	Map size (cM)	Mean interval between two markers (cM)
D × A	43	5 + 2	14	873.7	24.3
D × C	39	4	13.3	644.6	18.4
B × C	31	6 + 1	11.4	598.7	24.9
Composite	84	5 + 2	8.7	1330	17.7

The percentage of unlinked markers was only calculated for markers showing Mendelian segregation.

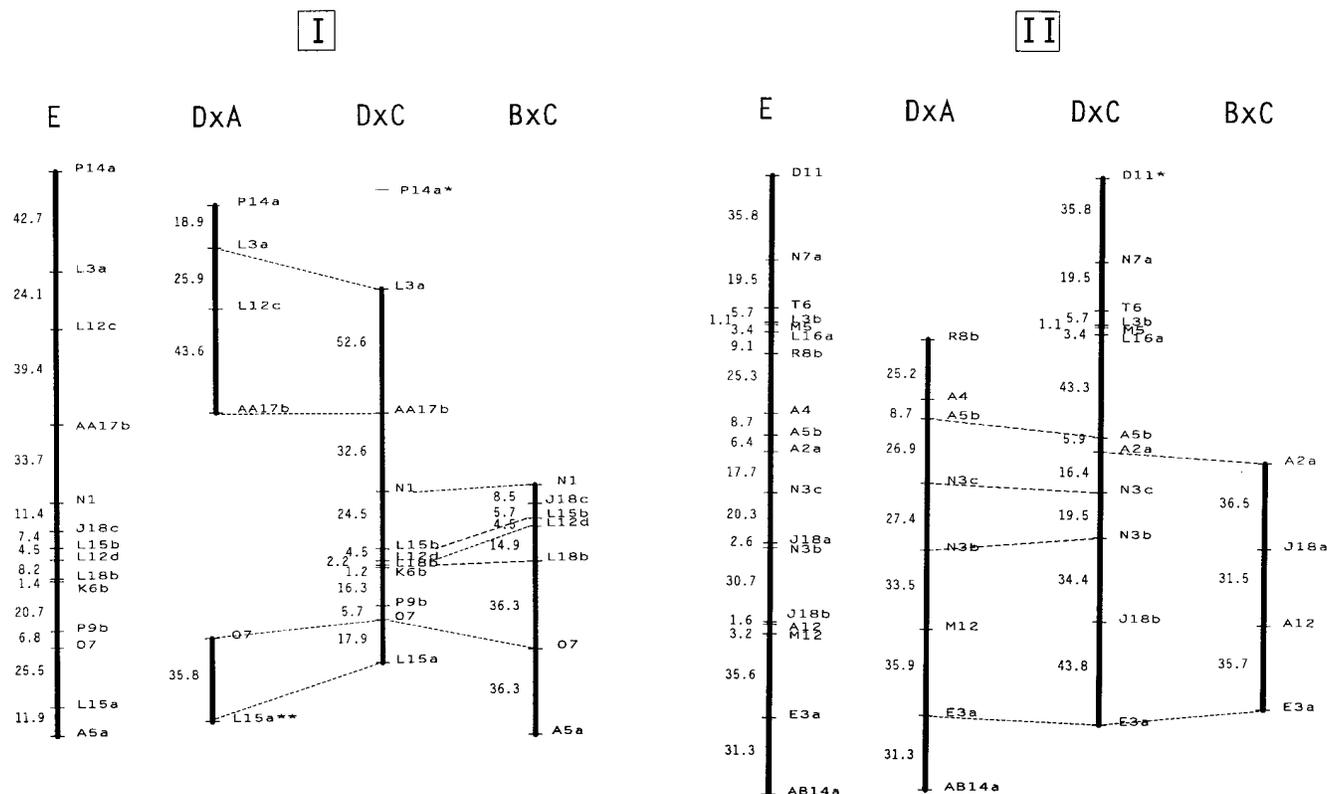


Figure 3.—Composite linkage map (E) and individual linkage maps  $D \times A$ ,  $D \times C$ , and  $B \times C$ . Markers are organized according to the five groups of the composite map. These five groups are named arbitrarily from I to V. The relative position of markers common to two individual maps are indicated. One additional pair of markers (R8a-D15a : 35.8 cM) is obtained on population  $D \times A$  and another one (E16a-E16b : 26.8 cM) on  $B \times C$ . These two pairs remain isolated in the composite map. Markers presenting segregation distortions significant at  $P < 0.01^{**}$  and  $P < 0.05^*$  are indicated.

C) were not significant (*i.e.*,  $<3$ ), the corresponding genetic order of the composite map was retained.

#### DISCUSSION

In this study, three genetic maps of  $F_2$  male populations of *T. brassicae* were constructed with 106 RAPD markers. Although the number of markers common to the three maps was low, a composite map of five linkage groups and two pairs was constructed. Considering the high level of missing scores in the composite map, from zero for a marker informative in the three populations to at least 67% for a marker informative in only one population, the good correspondence between the three individual maps and the composite one confirms the reliability of RAPD markers for mapping in haplo-diploid species. Moreover, the fact that population  $D \times C$  is sharing the female parent with population  $D \times A$  and the male parent with population  $B \times C$  validates the construction of the composite map.

The proportions of unlinked markers obtained in this study (Table 3) are similar to those reported in other studies that have presented an unsaturated map (Gebhardt *et al.* 1989; Faure *et al.* 1993; Lefebvre *et al.* 1995). The high level of markers that are unlinked and

the two pairs tend to suggest that we are far from completion of the map. Thus, the five groups obtained may not correspond to the five chromosomes of the species. The same type of result was reported by Promboon *et al.* (1995) in *Bombyx mori* ( $n = 28$ ) for which 169 RAPD markers were used to construct a genetic map composed of 29 linkage groups covering  $\sim 900$  cM (32 cM per chromosome) and 10 unlinked loci. In the parasitic wasp *Bracon hebetor* ( $n = 10$ ), 79 RAPD markers were mapped into 4 large linkage groups and 9 small, covering 1156 cM, with an average distance of 17 cM between 2 markers (Antolin *et al.* 1996). The composite map of *T. brassicae* already spans 1330 cM, yielding an average of 266 cM per chromosome, and the remaining 11 unlinked markers should extend this size. The average chromosomal length found for *Apis mellifera* was 215.6 cM (3450 cM obtained for 16 chromosomes, with an average spacing of 1 marker per 9.1 cM; Hunt and Page 1995). Although these authors used the Kosambi function for their map distances, which reduces the size of the linkage groups compared to the Haldane function that we used, we consider that the size of the linkage groups in *T. brassicae* should be as large as in *Apis mellifera*. Therefore, as for the honey bee, we suspect a high recombination rate for *T. brassicae*. The number

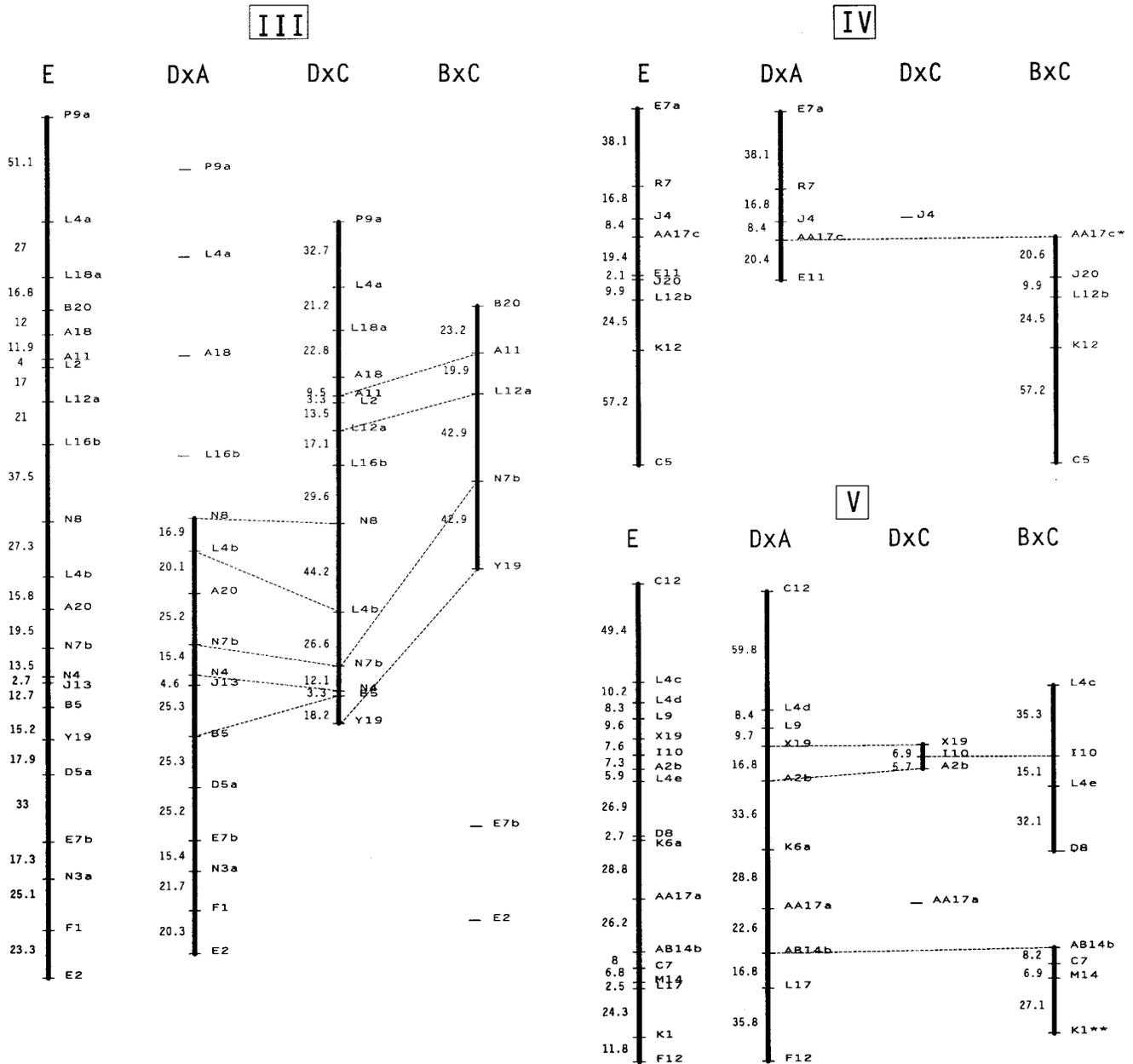


Figure 3.—Continued.

of crossovers was counted in linkage groups composed of more than 8 markers for each haploid male of the three individual populations, and there was an average of 1.8 crossovers per linkage group (data not shown). This value is close to the two crossovers that was found in *A. mellifera* by Hunt and Page (1995). Considering that haploid male genomes proceed from only one of the two sister chromatids, this value should be doubled to get the number of crossovers per bivalent. As reviewed by Hunt and Page (1995) for 17 species of plants, animals, or fungi, most species have a number of two to three crossovers per bivalent, regardless of the physical size of the chromosome. They demonstrated that species with small chromosomes have more crossovers

per unit of physical distance, and this seems to be associated with the duration of the haploid stage in the life cycle. The few haplo-diploid insects, for which estimations of physical chromosome size are available, are characterized by small chromosomes. The wasp *Nasonia vitripennis* is a chalcidoid, as is *Trichogramma*, but it has large chromosomes (Rasch *et al.* 1977). This could represent an exception; however, the preliminary genetic map indicates a low recombination rate (Saul 1993). Thus considering that in hymenopterans crossover rates seem to be inversely correlated to chromosome size (Hunt and Page 1995), the high crossover rate found for *T. brassicae* would indicate that this species has small chromosomes. Initial results obtained

from the karyotyping of *T. brassicae* effectively indicates that this species has small chromosomes. Indeed, chromosome sizes found in this study range from 2.4 to 4.8  $\mu\text{m}$ , which is similar to the size found in other chalcidoids (Goodpasture 1975a) and hymenopterans (Goodpasture 1975b).

For individual maps, it was found that some maps had a maximum likelihood that was not significantly different from the best map (*i.e.*, LOD < 3). This suggests that in the absence of any additional information orders other than the one retained could have been chosen. Among these maps, it was decided that for each population the gene order that was common to the three populations would be retained. This rule was applied only four times among the 15 linkage groups constructed. The same strategy was used by Beavis and Grant (1991) during the construction of a composite map in maize, whereas Hunt and Page (1995) chose to increase the mapping population from 94 drones to 142 to confirm linkage for some loci.

In the present study, the linear orders that were retained showed perfect concordance between the three individual maps, although the distances between common markers revealed some differences. However, in most cases these distances were not significantly different. Regions of unequal recombination between populations have been found for most linkage groups during construction of composite maps (Beavis and Grant 1991; Kianan and Quiros 1992). If unequal recombination among populations exists, it raises the question of whether the data should be pooled to construct a composite map. But to span the greatest part of the genome, Beckmann and Soller (1983), Helentjaris *et al.* (1986), Burr *et al.* (1988), Beavis and Grant (1991), and Ellis *et al.* (1992) have agreed that a map should be derived from several crosses. A composite map may be particularly useful for experiences that require genetic markers uniformly dispersed throughout the genome and to compare QTL identified in different genetic backgrounds.

As there is no more DNA available from the  $F_2$  mapped populations, further completion of the *T. brassicae* map will be achieved on the sister population stored at 20° for population D  $\times$  C. The other two populations are informative for two complex traits involved in the reproductive strategy of Trichogramma, *i.e.*, fecundity and longevity, and therefore completion will be performed on the QTL mapping populations. These populations are at present under construction, using the same pattern as those previously mapped. As both genotyping and phenotyping cannot be made on a single Trichogramma, because of its minute size and its short life span, a strategy of QTL identification based on progeny testing will be used.  $F_2$  males will be individually crossed to two females (B and C for cross D  $\times$  A, and D and A for cross B  $\times$  C) before being scored for markers. Fecundity and longevity of these  $F_2$  males will

be evaluated from their  $F_3$  female progeny. Such a strategy is commonly used in cattle to evaluate milk performance of sires for example (Haley 1995). Completion of the maps for these new  $F_2$  populations will involve the scoring of a high number of previously mapped markers to anchor the two maps, and by increasing the mapping population size, the marker ordering in *T. brassicae* genetic maps may be improved.

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