RAPD-Based Genetic Linkage Maps of *Tribolium castaneum*

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ABSTRACT

A genetic map of the red flour beetle (*Tribolium castaneum*) integrating molecular with morphological markers was constructed using a backcross population of 147 siblings. The map defines 10 linkage groups (LGs), presumably corresponding to the 10 chromosomes, and consists of 122 randomly amplified polymorphic DNA (RAPD) markers, six molecular markers representing identified genes, and five morphological markers. The total map length is 570 cM, giving an average marker resolution of 4.3 cM. The average physical distance per genetic distance was estimated at 350 kb/cM. A cluster of loci showing distorted segregation was detected on LG9. The process of converting RAPD markers to sequence-tagged site markers was initiated: 18 RAPD markers were cloned and sequenced, and single-strand conformational polymorphisms were identified for 4 of the 18. The map positions of all 4 coincided with those of the parent RAPD markers.

THE red flour beetle, *Tribolium castaneum*, is a genetically pliant organism and is becoming increasingly popular as a genetic model (Beeman *et al.* 1992, 1996a; Brown *et al.* 1994). It has a small genome size of 200 Mb and the repetitive sequences occur in a pattern of long interspersion, as in Drosophila (Brown *et al.* 1990). The karyotype reveals 10 chromosomes and a sex determination system in which females are XX and males XY (Smith 1952; Stuart and Mocelin 1995). The map of morphological and pigmentation variants includes ~80 loci comprising nine linkage groups (Sokoloff 1977; our unpublished observations). *T. castaneum* is highly tolerant of inbreeding, and several near-homozygous lines are now available.

We have accumulated a collection of balancer chromosomes that covers a total of ~185 map units on six linkage groups. This corresponds to roughly half of the 360 total map units (nine linkage groups) known prior to the present work. We have used such balancer chromosomes to facilitate mutagenesis screens and complementation analyses and to preserve recessive lethal mutations in balanced, true-breeding stocks. However, until high-density, whole-genome recombination maps are available we will not know the extent of the genome for which balancers are still lacking.

In other genetic model species, high-resolution genetic mapping is an important means for identifying disease genes and revealing the genetic basis of complex traits controlled by quantitative loci. It also facilitates positional cloning of new genes, helps to identify genes affecting agronomic traits in plants, and, more generally, aids in the discovery and characterization of genes and gene pathways affecting important or useful traits (*e.g.*, Postlethwait *et al.* 1994; Hunt and Page 1995; Segre *et al.* 1995; Cai *et al.* 1997; Knapik *et al.* 1998). To provide a basis for future genomics research in *T. castaneum*, we created a whole-genome recombination map on the basis of randomly amplified polymorphic DNA (RAPD) loci (Williams *et al.* 1990). Our immediate purposes were (1) to facilitate development of improved balancer chromosomes, (2) to estimate the relationship between physical and recombination distances in *T. castaneum* for the purpose of assessing the feasibility of map-based cloning in this species, and (3) to develop anchor loci for future mapping.

MATERIALS AND METHODS

Beetle strains and genetic crosses: The map was based on a single-pair backcross family. The T strain from Uttar Pradesh, India (Thomson *et al.* 1995) was chosen as the source of the (dominant) RAPD markers. Southern blot analysis of genomic fragments of the midrepetitive retrotransposon *Woot* for a series of *T. castaneum* strains had suggested that the T strain was highly divergent when compared to a variety of other strains from diverse geographic locations, at least by the criterion of *Woot* structure and insertion site (Beeman *et al.* 1996b; Beeman and Stauth 1997). The multiple marker strain (mms) is of North American origin and is homozygous for the five recessive visible markers: *Abdominal-missing abdominal sternites (A^{mas}*, linkage group [Lg] 2); *aureate* (*au*, LG 3); *sooty* (*s*, LG 4); *ruby* (*rb*, LG 5); and *antennapedia* (*ap*, LG 8). Although both strains have been reared in the laboratory for at least 10 yr, and each has experienced several genetic bottlenecks, near-isogenic lines were not yet available at the time

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this study was initiated. Thus, each of the two mapping strains shows a considerable degree of within-strain polymorphism.

To create the mapping family, a virgin T female was mated with an mms male. A single, virgin F_1 female was then backcrossed to her mms father. Approximately 400 backcross progeny were harvested and scored individually for segregation of each of the five recessive visible mutant markers derived from the mms father. Although meiotic recombination occurs with approximately equal frequency in both sexes of *T. castaneum*, we based the map on recombination analysis in an F_1 female only, to avoid the uncertain parentage that would have resulted from backcross of an F_1 male to his (nonvirgin) mother.

DNA extraction: After scoring the backcross progeny for visible mutant phenotypes, genomic DNA was prepared individually from each of the 400 progeny as well as the 3 parental beetles involved in the cross and backcross. For most DNA preparations we used the single-insect DNA extraction method of Livak (1984), as modified by Black and DuTeau (1997). We further modified the procedure with the addition of a chloroform/phenol extraction step prior to ethanol precipitation. After removal of ethanol, each DNA preparation was dissolved in 500 μl of TE. In a few cases we used the Wizard genomic DNA isolation kit (Promega, Madison, WI). Aliquots of each DNA were held at 4° for analysis or at −80° for long-term storage. These DNAs were used as templates in a PCR-based search for segregating RAPD markers.

Primer screening: Approximately 1000 decamer oligonucleotide primers of arbitrary sequence (Operon Technologies, Alameda, CA) were screened by PCR using three single-insect DNAs each from the mms and T strains as templates. We retained only those primers that amplified one or more fragments from all three T DNAs but not from any of the mms DNAs. This prescreening procedure was designed to identify markers that would be homogeneous within strains and thus reproducible in subsequent mapping crosses. Primers that met this criterion were retested using segregant DNAs from the backcross.

Polymerase chain reactions: PCR reactions were run in PTC-100 thermocyclers (MJ Research, Watertown, MA) and contained 50 mm KCl, 10 mm Tris-Cl, 0.1% Triton X-100, 0.2 mm dNTPs, 2 mm $MgCl_2$, 1 μl of DNA extract, 25 pmol of a single decamer primer, and 1.25 units of Promega Taq polymerase in a total volume of 25 μl at pH 9. The temperature program for RAPD PCR was 45 cycles of 94° 1′, 35° 1′, and 72° 2′, followed by a 7′ final extension at 72°. The temperature program for specific PCR (see below) was 94° 2′, followed by 36 cycles of 92° 30″, 55° 30″, and 72° 30″, with a 5′ final extension at 72°.

Conversion of RAPD to sequence-tagged site (STS) markers: To convert RAPD markers to specific-sequence, or STS markers, gel regions containing desired DNA fragments were excised from agarose gels and the DNA leached into water overnight at 4°. Target fragments were then reamplified using the original decamer primer. After gel purification and excision, the DNAs were isolated using the Promega Wizard PCR kit and cloned using a TA-cloning kit (Invitrogen, San Diego, CA) in conjunction with the pCRII-TOPO vector. DNA sequences of inserts were determined using a thermosequenase kit (Amersham, Piscataway, NJ) with ^{33}P-labeled dideoxynucleotide terminators. We then made specific primer pairs for each and searched for useful polymorphisms using single-strand conformational polymorphism (SSCP) analysis.

SSCP analysis: SSCP analysis can reveal (codominant) dimorphisms between allelic PCR fragments of identical length but nonidentical sequence (Orita *et al.* 1989). For SSCP mapping we designed pairs of specific primers 18–24 nucleotides (nt) in length that would amplify unique fragments of known sequence. These included both RAPD-derived STS markers and genes that had been previously characterized. To improve chances for detection of SSCPs, we targeted fragments that were <250 nt in length. After specific PCR amplification (see above), 7 μl of each PCR product was added to 4 μl of sequencing stop buffer (Amersham thermosequenase kit). The samples were then denatured at 85° for 3 min, placed in ice water, and 5–7 μl aliquots were loaded immediately onto precast 4–20% gradient PAGE (1× TBE) gels (Novex, Encinitas, CA) and run at a constant 250 V at 6° for 1–3 hr, depending on fragment size. Bands of ssDNA were visualized with ethidium bromide. If no segregating dimorphism was apparent in the mapping family, PAGE was repeated at 12° or 18°, or a second pair of specific primers was designed, targeting an adjacent fragment.

Linkage analysis: Preliminary mapping was accomplished with JoinMap software (Stam 1993, version 1.4 for the MacIntosh). The entire analysis was done using data from a single family. Only 150 of the 400 backcross progeny were used in the mapping analysis, because that number proved sufficient for statistical confidence. Not all progeny were scored for all markers, and many genotypes were scored as unknown. The segregation type was coded as backcross (of a heterozygote to a homozygous recessive, Aa × aa). Markers segregating as in an F_1 × self were excluded from the analysis, as were all paternally derived dominant markers. Each marker was checked for significant deviation from the expected 1:1 segregation by chi square analysis. All segregating markers were derived from the female parent (including the wild-type alleles of the recessive visible mutant markers) and thus all linked markers were in coupling (*cis*) configuration. The vast majority of markers were dominant, but, because phase relationships were unambiguous and markers segregating as F_1 × self were omitted, these dominant markers were equivalent to codominant markers in information content. The critical value for linkage detection was set at LOD = 3.0. Additional mapping analysis was done using Map Manager XP version 5 for Windows, created by Kenneth F. Manly and available at the Web site http://mcbio.med.buffalo.edu/mapmgr.html/. Observed recombination frequencies were converted to map distances using the Kosambi mapping function. The "hide locus" function of Map Manager was used to determine the effect on map expansion of inclusion of each locus. In addition, the "find double crossovers" function of Map Manager was used to detect possible genotyping errors.

RESULTS

Primer screening: Of ~1000 decamer primers screened using single-insect DNAs from the mms and T strains, ~200 amplified one or more T-specific fragments and were rescreened using segregant DNAs from the backcross. Of these, 79 amplified one or more reliable and properly segregating RAPD markers. Although markers were preselected to be representative of the parent strains rather than specific to the particular individuals used in the mapping cross, many new segregating markers were discovered while the backcross DNAs were screened with preselected RAPD primers. Of these (individual-specific) markers, those shown to be derived from the T parent were also included in the map. All RAPD markers and SSCP fragments used in mapping were confirmed to have been derived from the female (T) parent. Thus, all segregating markers on each linkage group were *in cis* configuration. We discarded ~10

additional, widely scattered RAPD markers that appeared to be of the backcross type but deviated significantly from the expected 1:1 segregation ratio (chi square test, $P < 0.05$). The LG9 clusters AO121, AC193, A12, and AC61 segregated about 2:1, deviating significantly from the expected 1:1 (*e.g.*, for AC193, the frequency of the material allele = 0.68, $0.05 > P > 0.01$). These markers were retained, because independent tests have indicated segregation distortion in this region (our unpublished observations).

Linkage analysis: A total of 122 markers were used for mapping analysis, or an average of 1.5 RAPD markers for each informative primer. In addition to the 122 RAPD markers and 5 visible mutant markers, we also positioned six genes on the map using SSCP analysis. These were *hunchback* (*hb*), *hairy* (*h*), *engrailed* (*en*), *even-skipped* (*eve*), *distalless* (*dll*), and *cytochrome P450* (*cyp4*). Data for all loci that caused a map expansion of >3 units and for all individuals involved in suspicious double recombination events were reexamined for accuracy and uncertain genotype assignments verified or discarded. Map expansions of >4 map units associated with nine retained loci are indicated. Six of these are located on LG2. All pairs of adjacent loci were linked at LOD > 3.0 with a single exception (indicated on the map) on LG6. JoinMap creates linkage groups and orders loci by highest LOD score rather than by closest linkage. Consequently, it worked well for identifying linkage groups and gave roughly correct gene orders, but sometimes incorrectly assigned nonzero recombination distances between tightly linked markers on the "best" map. Map Manager creates linkage groups and orders loci by closest linkage rather than by LOD score and therefore does not work well for datasets that contain too many unknowns. This is because it does not assign lower importance to linkages that are based on fewer progeny typings. Because our dataset included many "unknown" genotypes, Map Manager was less successful than JoinMap in assigning correct linkage groups. However, we preferred to use Map Manager for manual readjustment of marker orders, identification of suspect datapoints, and statistical analysis.

The 133 markers were distributed over 10 linkage groups, presumably corresponding to the 10 chromosomes of *T. castaneum* (Figure 1). This correspondence, as well as the absence of orphan loci, suggests that the map gives good coverage of the genome. The total recombination distance over the 10 linkage groups is 570 cM, after correction for double crossovers using the Kosambi function, giving an average marker separation of 4.3 cM. The haploid genome size of *T. castaneum* has been measured at 0.21 pg = 200 Mb (Brown *et al.* 1990) using reassociation kinetics. Alvarez-Fuster *et al.* (1991) independently measured the haploid genome size at 0.20 pg by spectrophotometric analysis of Feulgen-stained spermatids. Thus, the average physical distance per recombination distance is calculated to be 200 Mb/570 cM, or 350 kb/cM. Several tightly linked clusters of 3–4 markers each were observed at scattered locations. Some of these could represent centromeric regions, which are known to be recombinationally suppressed in Drosophila (Stephan and Mitchell 1992). For example, there is a tightly linked cluster of 4 RAPDs at position 51 in the center of LG3, previously shown to represent a metacentric chromosome (Beeman and Stuart 1990).

Conversion of RAPD to STS markers: Eighteen RAPD markers were cloned and converted to targeted PCR, or STS markers. They are AB31, AC191, AF181, AI202, AJ191, AP131, AR61, G121, L41, L71, L72, S102, U11, W11, X162, X63, Y61, and Y62. After specific PCR, useful SSCP dimorphisms were detected in 4 of the 18 STS markers, namely AC191, AJ191, X63, and U11 (Table 1). The correct map positions for all 4 were confirmed by SSCP mapping. Sequences of primers used to amplify fragments of the six known genes are shown in Table 2.

Sex chromosomes and linkage group correlations: After constructing the RAPD linkage maps, we attempted to correlate all nine currently identified visible mutant linkage groups with the corresponding RAPD linkage groups. RAPD autosomal linkage groups 2, 3, 4, 5, and 8 were correlated with the corresponding mutant linkage groups by the inclusion of one visible marker corresponding to each of these LGs, as described above. Autosomal linkage group 9 was identified by incorporation of the cytochrome P450 locus, cyp4, previously mapped onto visible LG9 (J. J. Stuart and R. W. Beeman, unpublished results).

In *T. castaneum* and other Tenebrionid beetles, females and males are karyotypically XX and XY, respectively. The X linkage group could not be identified from the RAPD mapping data, because in backcrosses of heterozygous females to homozygous or hemizygous null males, dominant, maternally derived X-linked markers segregate in a manner indistinguishable from that of autosomal markers. RAPD LG7 was shown to be autosomal through the use of a codominant SSCP at the *even-skipped* (*eve*) locus. We monitored segregation of the SSCP in the progeny of a single pair (mms × T) in which the two parents were each homozygous for alternative SSCP alleles. All progeny of either sex inherited both alleles, demonstrating that LG7 is autosomal. LG10 was confirmed to be autosomal through the use of specific primers derived from the DNA sequence of the cloned U11 RAPD fragment. Like the original 10-mer-primed U11 fragment, the specifically primed U11 fragment was amplifiable only from the T strain. Monitoring U11 segregation in the F_1 of a single pair cross of T male × mms female revealed an autosomal mode of inheritance. RAPD LG1 was shown to be X-linked through the use of the RAPD markers AB31 and AG83, each found only in the T strain. Both markers were tightly linked to sex (*i.e.*, they were faithfully transmitted

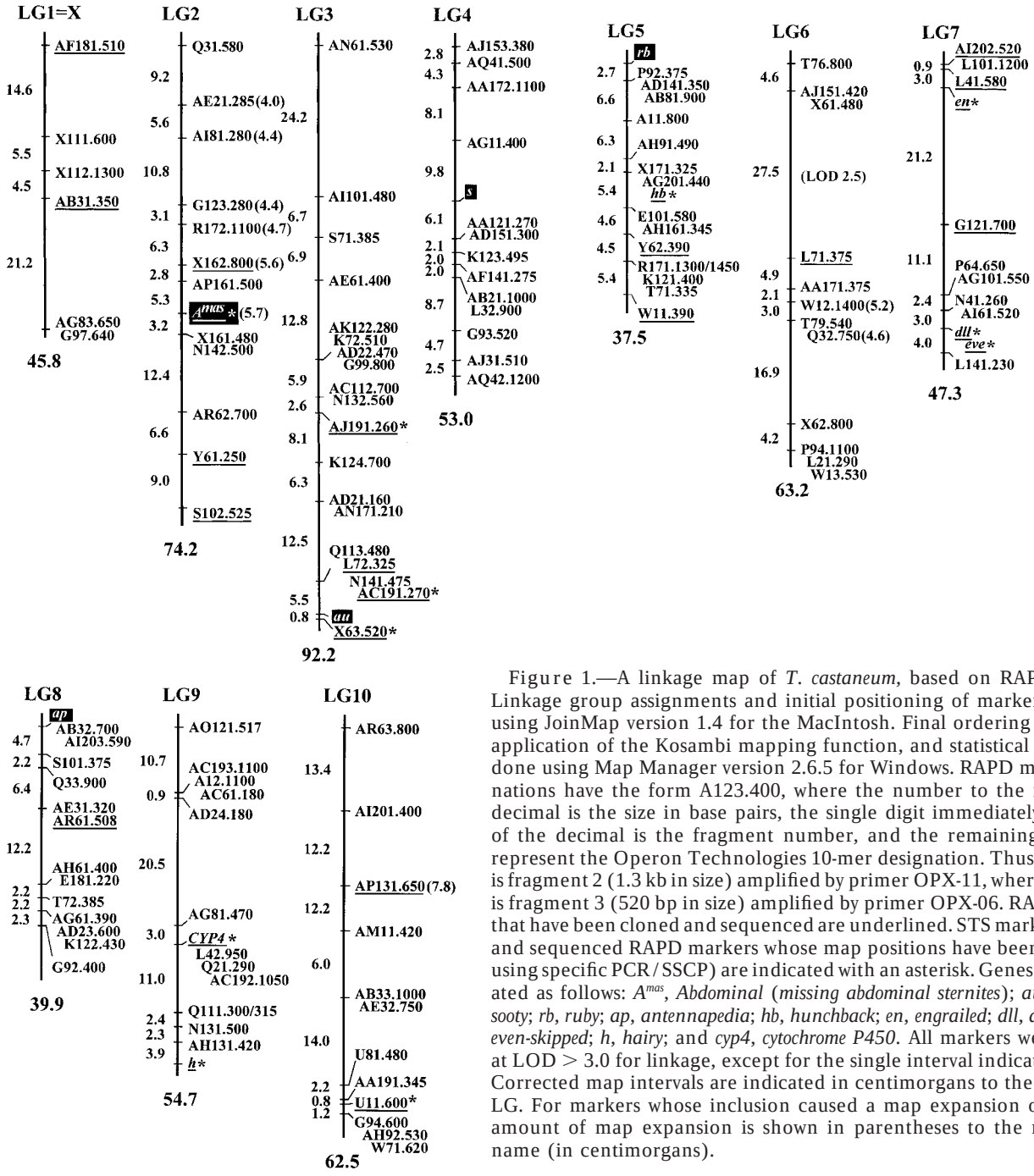

Figure 1.—A linkage map of *T. castaneum*, based on RAPD markers. Linkage group assignments and initial positioning of markers was done using JoinMap version 1.4 for the MacIntosh. Final ordering of markers, application of the Kosambi mapping function, and statistical analysis was done using Map Manager version 2.6.5 for Windows. RAPD marker designations have the form A123.400, where the number to the right of the decimal is the size in base pairs, the single digit immediately to the left of the decimal is the fragment number, and the remaining characters represent the Operon Technologies 10-mer designation. Thus, X112.1300 is fragment 2 (1.3 kb in size) amplified by primer OPX-11, whereas X63.520 is fragment 3 (520 bp in size) amplified by primer OPX-06. RAPD markers that have been cloned and sequenced are underlined. STS markers (cloned and sequenced RAPD markers whose map positions have been confirmed using specific PCR/SSCP) are indicated with an asterisk. Genes are abbreviated as follows: A^{mas}, *Abdominal* (*missing abdominal sternites*); *au*, *aureate*; *s*, *sooty*; *rb*, *ruby*; *ap*, *antennapedia*; *hb*, *hunchback*; *en*, *engrailed*; *dll*, *distalless*; *eve*, *even-skipped*; *h*, *hairy*; and *cyp4*, *cytochrome P450*. All markers were mapped at LOD > 3.0 for linkage, except for the single interval indicated on LG6. Corrected map intervals are indicated in centimorgans to the left of each LG. For markers whose inclusion caused a map expansion of >4.0, the amount of map expansion is shown in parentheses to the right of the name (in centimorgans).

paternally to daughters but never to sons) in a single pair cross of a T male × mms female (data not shown).

Y-linked RAPD markers: In the course of this work we observed several dominant RAPD markers that were segregating in the backcross male, but were absent in both the T female parent and the F_1 daughter. Three of these, namely AI21, AC21, and AB121 (fragment sizes 290, 290, and 1100 nt, respectively) showed a Y-linked pattern of inheritance, *i.e.*, they appeared in the parental male and in all backcross male progeny but in none of the parental or backcross females.

DISCUSSION

Variability of RAPD marker alleles: Although we chose unrelated parental strains from North America and India in an attempt to maximize the frequency of useful dimorphisms, the degree of heterozygosity of the hybrid female was somewhat disappointing. In the honeybee (*Apis mellifera*), 13% of 1000 random 10-mers were ultimately used for mapping (Hunt and Page 1995), comparable to our 8% of 1000 tested. However, we found an average of only 1.5 useful markers per primer, in comparison to 2.8 informative bands per primer in

TABLE 1

STS primer sequences used for mapping

RAPD locus	Map position[a]	STS primer sequences (5′–3′)	GenBank accession no.	dbSTS no.
AC191	3 (86)	F CCGCCTGTATCAGACTTACTG R CCCCTTTTCCAACGATAAC	G41772	61996
AJ191	3 (59)	F ACATTCAAATTCAGTCAGCAGCAC R CGGCTCGACCCCATAAAACC	G41773	61997
X63	3 (92)	F GCCAGAGGGATGTAAATGCATAG R GGCGGCTTTGTACGAATTTTTC	G41774	61998
U11	10 (61)	F GGTCCTGAGAATAGTGCTTTCGTTG R ACGTCACCACCATCATCATCATC	G41775	61999

Primer pairs forward (F) and reverse (R) amplify specific fragments that identify a strain dimorphism between mms and T, either on agarose gels or by SSCP.

[a] Map position is given as LG (cM), where cM refers to distance from "top" end of LGs as shown in Figure 1.

the honeybee, even though no attempt was made to cross unrelated strains in the latter case. Our decision to use only those markers that were *in cis* phase and backcross type limited the number markers we could incorporate into the map. In addition, because of the haplo-diploid system in Apis, all segregating markers are, in principle, scoreable in progeny drones. In our system, segregating markers derived from a homozygous male could not be used, because RAPD markers are usually dominant, and the backcross was to the male parent.

The efficiency of RAPD mapping might also be affected by the repetitive DNA content of the genome. Williams *et al.* (1990) reported that single decamer primers tend to amplify segments of repetitive DNA, because palindromic sequences are more highly represented in such regions. Thus, RAPD markers should be more frequently encountered in genomes with a higher content of repetitive DNA. This prediction is not borne out by our work, because Tribolium has a rather high content of repetitive DNA but gave a rather low yield of RAPD markers. According to Brown *et al.* (1990) highly repetitive sequences compose 23% of the genome of *T. castaneum*, but only 12% of the genome of *Drosophila melanogaster*, and only 4% of the genomes of *Anopheles quadrimaculatus* and *Sarcophago bullata*. If single decamer primers tend to amplify segments of repetitive DNA, then RAPD markers should tend to cluster (in repeat regions), rather than show random distribution on genetic linkage maps, particularly in species (such as *T. castaneum*) that show a long period interspersion (clumped) pattern of distribution of repetitive DNA. Because we found an average of less than two RAPD markers per primer used, there was little opportunity

TABLE 2

Gene-specific primer sequences used for mapping

Locus[a]	Map position[b]	Gene-specific primer sequences (5′–3′)	GenBank accession no.[c]
hb	5 (18)	F TCCAAGAAACGGCATAGAAAG R GCACGCGAAAAGAGGCAGTT	X91618
h	9 (55)	F TGTATGTCCTACCATGG R AGGAGATTCCTCAGCTC	—
en	7 (4)	F AAATCCCCGTCTGGCAAAATCAAT R GAGCATAATCCGCCTTCTTCAT	—
eve	7 (43)	F CAACTCAATCTCCCAGAAAG R GTGTTTCACCAGTAGGAGTC	U77974
dll	7 (43)	F AAGGCCAAAACATGATGGCAG R CCAATTGATTGTTGCCTCAAGTG	—
cyp4	9 (35)	F CCTAGTCCTTTGTGTAACCC R GGAGGCGGTCCTTGATGAG	—

Primer pairs forward (F) and reverse (R) amplify specific fragments that identify a strain dimorphism between mms and T by SSCP.

[a] *hb, hunchback; h, hairy; en, engrailed; eve, even-skipped; dll, distalless; cyp4, cytochrome P450*.

[b] Map position is given as LG (cM), where cM refers to distance from "top" end of LGs as shown in Figure 1.

[c] Sequences for *h, en, dll,* and *cyp4* have not yet been deposited in GenBank. Primers for these genes were based on unpublished sequences obtained from D. Tautz, S. Brown, A. Beermann and J. Stuart, respectively.

for clustering to occur in our map. However, Kesseli *et al.* (1994), Antolin *et al.* (1996), and others have observed nonrandom clustering of RAPD loci amplified by the same primer.

Tribolium as a genetic model organism: The average physical distance per recombination unit in *T. castaneum*, estimated at 350 kb/cM in the current work, in combination with the ease of high-resolution recombinational mapping, suggests that map-based cloning could be feasible in this species. Because of its superior genetic attributes, the availability of balancer chromosomes, the recent development of near-isogenic lines, and the importance of beetles as agronomic pests, further development of *T. castaneum* as a genetic model is warranted. We are producing new mapping families based on highly inbred lines, so that segregating molecular markers can be used in subsequent independent backcrosses. Thus, the map will be of generalized usefulness for any laboratory, and the list of useful markers will be cumulative. We are also in the process of developing gene transfer vectors and selectable transformation markers for this species.

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