AFLP-Based Genetic Linkage Map for the Red Flour Beetle  
(*Tribolium castaneum*)

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**Abstract**

The red flour beetle (*Tribolium castaneum*) is a major pest of stored grain and grain products and a popular model species for a variety of ecological, evolutionary, and developmental biology studies. Development of a linkage map based on reproducible and highly polymorphic molecular markers would greatly facilitate research in these disciplines. We have developed a genetic linkage map using 269 amplified fragment length polymorphism (AFLP) markers. Ten previously known random amplified polymorphic DNA (RAPD) markers were used as anchor markers for linkage group assignment. The linkage map was constructed through genotyping two independent F2 segregating populations with 48 AFLP primer combinations. Each primer combination generated an average of 4.6 AFLP markers eligible for linkage mapping. The length of the integrated map is 573 cM, giving an average marker resolution of 2.0 cM and an average physical distance per genetic distance of 350 kb/cM. A cluster of loci on linkage group 3 exhibited significant segregation distortion. We have also identified six X-linked and two Y-linked markers. Five mapped AFLP fragments were sequenced and converted to sequence-tagged site (STS) markers.

Beetles represent the largest order of insects and have immense economic importance. The red flour beetle (*Tribolium castaneum*) is an important pest of stored grain and grain products. Its rapid life cycle and ease of maintaining laboratory cultures on a simple medium of wheat flour and yeast has made this species a popular choice as a model organism for studying ecology (Lewis and Austad 1994; Lewis and Jutkiewicz 1998; Wade 1984; Yan et al. 1998) and quantitative, population, and developmental genetics (Brown et al. 1994; Haas et al. 2001; Lorenzen et al. 2002; Shippy et al. 2000; Sulston and Anderson 1998; Wade and Beeman 1994). Its genome size is only 200 Mb and most repetitive sequences, as in *Drosophila*, occur in a pattern of long interspersions (Alvarez-Fuster et al. 1991; Brown et al. 1990). The karyotype reveals 10 chromosomes and a sex determination system in which females are XX and males XY (Beeman and Brown 1999; Smith 1952; Stuart and Mocelin 1995). The map of morphological variants includes approximately 80 loci comprising nine linkage groups (LGs) (Beeman and Brown 1999; Sokoloff 1977). Genetic linkage mapping has proven to be a powerful tool for gene localization, gene isolation, marker-assisted selection, and evolutionary studies. The first *T. castaneum* molecular linkage map was constructed using a backcross population and consisted of 123 random amplified polymorphic DNA (RAPD) markers, 6 identified genes, and 5 morphological markers (Beeman and Brown 1999). Using the linkage map, a major pyrethroid resistance gene, *PyR-1*, has been mapped in *T. castaneum* (Stuart et al. 1998).

Amplified fragment length polymorphism (AFLP) is a polymerase chain reaction (PCR)-based multilocus fingerprinting technique (Vos et al. 1995). It offers improved reproducibility compared to randomly amplified polymorphic DNA (RAPD) markers because of more stringent reaction conditions, but does not require prior information of the PCR primers for the study organism (Vos et al. 1995). Because of these features, AFLP has been widely employed for genetic mapping in plant and animal species, such as rice (Mackill et al. 1996; Price et al. 2000), wheat (Lotti et al. 2000), barley (Becker et al. 1995; Castiglioni et al. 1998; Powell et al. 1997; Qi et al. 1998), maize (Castiglioni et al. 1999; Vuylsteke et al. 1999), soybean (Keim et al. 1997; Liu et al. 2000), tomato (Haanstra et al. 1999; Saliba-Colombani et al. 2000), lettuce (Jeuken et al. 2001), melon (Oliver et al.
F1 intercrosses were used in the study. The parents, F1, and F2 populations following the chloroform/phenol method of Severson (1997). The parents and F1's were used to establish the segregation pattern of the molecular markers. All individuals were subjected to genotyping with AFLP markers according to Vos et al. (1995), with some modifications. Briefly, genomic DNA was double-digested with EcoRI and MseI. The DNA fragments were ligated with EcoRI and MseI adaptors, generating template DNA for PCR amplification. Two primers used for PCR amplification were designed, based on the adaptor sequences and restriction site sequences. Selective nucleotide sequences were added to the 3' end of each primer. PCR amplification was conducted in two steps: preselective amplification and selective amplification. Preselective amplification used EcoRI primer (5'-GACTGCGTACCAATTCC-3') and MseI primer (5'-GATGAGTCCTGAGTAA-3'). The EcoRI and MseI primers in the selective amplification used three additional nucleotides in the 3' end, therefore each primer combination amplified different subsets of all the fragments in the total digest. In order to detect the PCR products with the Li-Cor automated DNA analyzer (Li-Cor Inc., Lincoln, NE), the EcoRI primers were labeled with a fluorescent dye (infrared dye, IRD800). For selective amplification, a total of 300 primer combinations were screened. Forty-eight primer pairs that produced fragments with clear dominant inheritance patterns and reproducibility were used for linkage analysis (Table 1).

Polymorphism screening of AFLP products was conducted on the Li-Cor model 4200 automated DNA analyzer using 6.5% polyacrylamide gel. Gel electrophoresis was maintained under a constant temperature of 45°C. Allele sizes were determined using GENE IMAGIR computer software provided by the manufacturer (Li-Cor Inc.).

**Materials and Methods**

**Beetle Strains and Genetic Crosses**

Two F2 segregating families from reciprocal pairwise mating between T. castaneum strains cSM and TIW1, and F1 intercrosses were used in the study. The cSM strain was kindly provided by Dr. Micheal Wade of Indiana University and TIW1 by Dr. Richard Beeman of the Grain Marketing & Production Research Center, U.S. Department of Agriculture. The cSM strain has been used extensively in various ecological and genetic studies (Stevens 1989; Wade 1977; Yan 1997; Yan and Stevens 1995), and TIW1 was used for construction of an RAPD-based linkage map (Beeman and Brown 1999). The two strains have been reared in the laboratory for more than 10 years. We pooled DNA samples from 10 individuals from each strain for marker polymorphism screening. The first segregating population (R13) included 123 F2 individuals (68 females and 55 males) from pairwise mating between TIW1 males and cSM females and 4 F1 brother-sister matings. The second population (S13) comprised 120 F2 s (59 females and 61 males) and was produced from pairwise mating between TIW1 females and cSM males and 5 F1 brother-sister matings. All beetles were raised in 8-dram shell vials containing approximately 5 g standard medium (95% by weight fine sifted whole wheat flour and 5% dried powdered brewer's yeast) in a dark incubator regulated at 29°C and 70% relative humidity.

**DNA Extraction and AFLP Analysis**

Genomic DNA was extracted individually from all the parents, F1, and F2 populations following the chloroform/phenol method of Severson (1997). The parents and
Table 1. AFLP primer sequences and combinations used for *T. castaneum* genome mapping

<table>
<thead>
<tr>
<th>EcoRI primers (5'–3')</th>
<th>Primer combinations used for genome mapping</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1 = GACTGCAGGTACCAATTCAAC</td>
<td>L1/1/B1, L1/A1, L1/A2, L1/A3, L1/A4, L1/A9, L1/A16, L1/A18</td>
</tr>
<tr>
<td>L2 = GACTGCGTACCACATTCAAC</td>
<td>L2/B1, L2/A1, L2/A2, L2/A3, L2/A8, L2/A16, L2/A18, L2/A28</td>
</tr>
<tr>
<td>L5 = GACTGCGTACCAATTCCAG</td>
<td>L5/A16</td>
</tr>
<tr>
<td>L7 = GACTGCGTACCAATTCCAGG</td>
<td>L8/A1, L8/A2, L8/A4, L8/A16, L8/A18</td>
</tr>
</tbody>
</table>

The L1/A16 primer combination produced the most qualified polymorphic fragments in both crosses (12 for R13 and 9 for S13), whereas the L3/A8 primer combination had the least (1 for both crosses).

**RAPD Primer Screening**

Among the 50 RAPD primers screened, 24 produced one or more *TIW1*- or *cM*-unique fragments and they were further screened for inheritance pattern using parents, F1, and F2 individuals. Twelve primers yielded consistently amplified and dominant-segregating fragments. A total of 17 and 14 RAPD fragments were scored for linkage analysis for crosses R13 and S13, respectively. Of these, 11 (64.7%) dominant alleles were descended from the *TIW1* strain in cross R13, while in cross S13, 11 (78.6%) fragments were derived from the *TIW1* strain. All markers with dominant alleles descended from the *TIW1* strain are underlined in Figure 2. Eleven fragments were common to the two crosses.

**Construction of an Independent Map**

The linkage map derived from the R13 cross contained 208 AFLP and 15 RAPD markers that were assigned to 10
LGs at the LOD threshold of 4.5 (*T. castaneum* has 10 chromosomes). Twenty-one AFLP and two RAPD markers could not be assigned to any LG. The total map distance was 534 cM. A total of 30 markers (14.5%) showed significant segregation distortion from the Mendelian segregation ratio, and most of these markers are on one arm of LG3 (Figure 2).

For the S13 cross, 197 AFLP and 12 RAPD markers were mapped to 10 LGs, with a total map length of 486 cM. Fourteen AFLP and two RAPD markers could not be assigned to any LG. Twenty-eight markers (13.4%) exhibited significant segregation distortion from the Mendelian segregation ratio, mostly on the upper arm of LG3 (Figure 2).

### Construction of an Integrated Map

The linkage maps generated from the two independent segregating populations were very similar with respect to marker order and map distance on each LG. Alignment of the two maps revealed that the markers common to the two maps fell in the same LGs. Consequently an integrated map, comprising markers from both populations, was constructed using a LOD threshold of 4.0.

The new integrated linkage map consists of 269 AFLP markers and 18 RAPD markers (Figure 2). Of the 18 RAPD markers, 10 were previously mapped by Beeman and Brown (1999). LG2–LG10 contain 23–41 markers, but LG1 has only 6 markers. The total recombination distance over the 10 LGs was 573 cM after correction for double crossovers using the Kosambi function, similar to the length of the RAPD map (Beeman and Brown 1999). Given that the estimated genome size of *T. castaneum* is 200 Mb, the average physical distance per recombination distance is about 350 kb/cM. LG2–LG10 of our AFLP-based linkage map (Figure 2) likely correspond to the same nine LGs of the RAPD-based map of Beeman and Brown (1999). This notion was supported by the fact that the molecular sizes of our RAPD markers were the same, and that the two linked RAPD markers, AP13.650 and G9.600, were mapped to the same chromosome location on linkage group 10 as Beeman and Brown’s (1999) map. The AFLP markers apparently did not show significant clustering near centromeres or the distal region of chromosomes, suggesting that they provide good coverage of the genome (Figure 2).
Sex-Linked Markers

LG1 represents the X chromosome because the markers on LG1 segregate only in the males, not in the females, of the F2 population derived from the F1 intercross. Two AFLP markers (L3A9.129 and L3A9.161) showed a Y-linked pattern of inheritance because the AFLP fragments were expressed in the parental male, F1 males, and all F2 male progeny, but were absent in the female parent, F1, and F2 females.

Conversion of AFLP Markers to STS Markers

Among the nine AFLP fragments that we excised for sequencing, three fragments (L6A18.294, L6A18.298, L4A2.224) did not yield good-quality sequences. STS primers were designed for six fragments with high-quality sequences, of which five primer pairs yielded successful amplification. We further screened the STS markers for polymorphism with the TIIW1 and cM strains and found that four STS markers converted from AFLP fragments L2A8.377, L3B1.155, L4A2.290, and L6A18.150 were polymorphic between the two strains and segregated as codominant markers (Figure 3). The STS primer pair for AFLP fragment L3B1.187 yielded monomorphism in the two beetle strains, and the L2A8.228 primers did not produce successful PCR amplification. The successful STS primer sequences are shown in Table 2.

Discussion

In this study we developed an AFLP-based linkage map for the red flour beetle (*T. castaneum*). *T. castaneum* has been used as a model organism for studying ecology and quantitative, population, developmental, and insecticide resistance genetics. The availability of molecular linkage maps is very valuable to enhance the research in these disciplines. Our map consists of 269 AFLP and 18 RAPD markers and has integrated 10 RAPD markers previously studied by Beeman and Brown (1999). The current map has a total length of 573 cM and an average marker resolution of 2.0 cM.

The AFLP linkage map was generated through in-
tegration of two linkage maps from two independent segregating populations. Although the two maps differ in the total map distance by about 10% (534 cM for cross R13 and 486 cM for cross S13, respectively), the order of the markers and marker assignment to individual LGs were remarkably similar. The difference in map length between the two maps can be explained by two reasons. First, cross R13 contained 14 more markers than cross S13. The map distance could be inflated when the average marker distance becomes smaller (Lincoln and Lander 1992). Second, more markers in cross R13 appeared to be mapped to the distal portion of the LG than cross S13. Markers on the telomere region tend to have a higher recombination distance than those near the centromere (Harushima et al. 1998; Saal and Wricke 2002; Young et al. 1999). However, good correspondence between the two maps in marker order makes integration of the two maps possible. The integrated map consists of 287 markers, covering all 10 LGs with a total map distance of 573 cM, similar to the length of the RAPD linkage map (Beeman and Brown 1999). However, the average marker resolution is only 2.0 cM between adjacent markers, about 50% higher than the RAPD linkage map. Therefore this study demonstrated that AFLP is a reproducible and relatively efficient marker for linkage map construction.

Figure 3. Segregation patterns of STS markers converted from mapped AFLP fragments. PCR products were electrophoresed on 5.5% denaturing polyacrylamide gels. P1 and P2 represent parent T7W1 and cSM strains, respectively.

Our linkage map used polymorphic AFLP fragments with a clear dominant inheritance pattern; that is, the eligible fragments must show complete dominance expression in one parent strain and complete recessive expression in the other strain, and all F1 individuals must be heterozygous. Several studies have demonstrated that some AFLP fragments segregate in codominant manner (Castiglioni et al. 1999; Piepho and Koch 2000; Yin et al. 2002); however, it is extremely difficult to identify codominantly segregating fragments from the polyacrylamide gel with several hundred AFLP fragments. Therefore we did not use the codominant AFLP markers. From the 48 pairs of AFLP primer combinations used in this study, we identified an average of 4.8 eligible fragments per primer combination for linkage mapping. This success rate was comparable to an AFLP linkage map study in teff that produced an average of 4.5 eligible fragments per primer combination (Bai et al. 1999), but was significantly lower than barley (Castiglioni et al. 1998) and silkworm (Tan et al. 2001).

We observed a small portion of AFLP markers that did not follow the Mendelian segregation ratio (14.5% and 13.4% for crosses R13 and S13, respectively). Most of those markers are on the upper arm of LG3 for both segregating
Both STS forward (F) and reverse (R) primers are shown.

AFLP locus | Map position | STS primer sequences (5’–3’ | Size (bp) | GenBank accession no. | dbSTS no.
--- | --- | --- | --- | --- | ---
L2A8.377 | 3 (78) | F: GAATATTCCGCTCTCCCGCTGA R: TGGTATTGGCAAACCTCGAAG | 202 | BV079583 | 210460
L3B1.155 | 3 (15) | F: TCGAAAGTACAGGCAAAATACAG R: TCGACATCTGCGGTCTGTAATAA | 126 | BV079584 | 210461
L4A2.290 | 4 (33) | F: CCGATCATACAGGAGGAACC R: TTGGAAGCTATGGCAAGGT | 165 | BV079585 | 210462
L6A18.150 | 8 (48) | F: AAATTGCTAATTGAGGTCACC R: TTCACCTGAAAGGCTGTTGCG | 124 | BV079586 | 210463
L3B1.187 | 2 (55) | F: CATGTGGATATCCTGCAAAGG R: GCGTACCAATCTGCGAAG | 100 | BV079587 | 210464

Table 2. Primer sequences of STS markers converted from T. castaneum AFLP markers

* Map position is given as linkage group (genetic distance, in Kosambi centiMorgans, from the top end of the linkage group as shown in Figure 2).

* Both STS forward (F) and reverse (R) primers are shown.

populations, favoring alleles from the TIW1 strain over the cSM strain. Beeman and Brown (1999) found a cluster of RAPD loci on LG9 showing distorted segregation. The reason why alleles on one arm of LG3 are associated with reduced fitness in the cSM strain is unknown, but the extent of segregation skewness was similar or lower than other reported studies using AFLP markers (e.g., 20% in potato [Gebhardt et al. 1989], 44% in barley [Graner et al. 1991], 54% in silkworm [Tan et al. 2001], 65% in clubroot [Voorrips et al. 1997]). Distorted segregation of molecular markers may result from competition among gametes for preferential fertilization (Lytte 1992), sampling in finite mapping populations, and severe breakdown of long-chain DNA during DNA extraction from tissue samples (Faris et al. 1998). Although markers amplified near the centromere regions of the chromosomes also tend to have distorted segregation ratios (Faris et al. 1998), the markers that exhibited distorted segregation ratios in our study are within the region of 0–40 cM from the tip of LG3.

Several publications reported that AFLP markers generated from EcoRI/MseI restriction tend to cluster around centromere regions (Haanstra et al. 1999; Qi et al. 1998; Vuylstke et al. 1999; Young et al. 1999). We did not observe severe clustering of AFLP markers in the present study. It is worth noting that some AFLP markers generated by the same primer combinations were mapped to similar positions (e.g., LG2 [L2A16.154 and L2A16.155], LG3 [L4A28.126 and L4A28.136], LG5 [L6B1.58 and L6B1.129], LG7 [L4B2.81, L4B2.161, and L4B2.157], LG9 [L4B2.66, L4B2.102, and L4B2.103], and LG10 [L3A18.82, L3A18.120, and L3A18.121]). Perhaps these AFLP primer combinations have amplified gene clusters or repeated sequences that are physically linked.

The conversion of mapped AFLP fragments to STS markers will greatly improve the utility of AFLP markers for genetic studies. We have taken the first step to convert several AFLP markers to STS markers. During our first trial, six of nine AFLP fragments were sequenced, five STS primers yielded successful amplification, and four STS markers showed polymorphism with the two parental strains used to generate the linkage map when tested on denatured polyacrylamide gels. We anticipate a higher success rate of converting dominant AFLP markers to polymorphic, codominant STS markers in T. castaneum when some of our protocols are improved in later trials. Some low molecular weight AFLP fragments may be difficult to convert to useful STS markers because a minimum of 80–100 bp are required for reliable determination of polymorphism on polyacrylamide gels. However, the majority of AFLP fragments are above the minimum molecular weight. Thus the present study provided a useful foundation for developing a high-density STS map in T. castaneum.

In summary, we used AFLP markers to construct a linkage map for T. castaneum. The map consists of 269 AFLP and 18 RAPD markers, with an average marker resolution of 2 cM. Because AFLP amplification is highly reproducible, the development of an AFLP linkage map provides a valuable tool for studying beetle ecology and genetics, such as identification of strain-specific markers for tracking allele frequency changes and quantitative trait loci (QTL) analysis for agriculturally important or evolutionarily interesting traits.

References


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