Fine-Scale Analysis of Parasite Resistance Genes in the Red Flour Beetle, *Tribolium castaneum*

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ABSTRACT Parasite infection impacts population dynamics through effects on fitness and fecundity of the individual host. In addition to the known roles of environmental factors, host susceptibility to parasites has a genetic basis that has not been well characterized. We previously mapped quantitative trait loci (QTL) for susceptibility to rat tapeworm (*Hymenolepis diminuta*) infection in *Tribolium castaneum* using dominant AFLP markers; however, the resistance genes were not identified. Here, we refined the QTL locations and increased the marker density in the QTL regions using new microsatellite markers, sequence-tagged site markers, and single-strand conformational polymorphism markers. Resistance QTL in three linkage groups (*LG3*, *LG6*, and *LG8*) were each mapped to intervals <1.0 cM between two codominant markers. The effects of 21 genes in the three QTL regions were investigated by using quantitative RT-PCR analysis, and transcription profiles were obtained from the resistant *TIW1* and the susceptible *cSM* strains. Based on transcription data, eight genes were selected for RNA interference analysis to investigate their possible roles in *H. diminuta* resistance, including cytochrome P450 (LOC657454) and Toll-like receptor 13 (*TLR13*, LOC662131). The transcription of P450 and *TLR13* genes in the resistant *TIW1* strains was reduced more than ninefold relative to the control. Moreover, the effects of gene knockdown of P450 and *TLR13* caused resistant beetles to become susceptible to tapeworm infection, which strongly suggests an important role for each in *T. castaneum* resistance to *H. diminuta* infection.

PARASITES exert negative effects on host survivorship and reproductive success, and in turn hosts develop resistance to parasites by reducing susceptibility to infection. Despite the importance to medicine and agriculture of host resistance to parasitism, and the fact that host resistance is genetically determined, the genetics of parasite resistance in insect hosts is not well known. Infection of the red flour beetle, *Tribolium castaneum*, by the rat tapeworm, *Hymenolepis diminuta*, has been well characterized with regard to host–parasite interactions (Keymer and Anderson 1979; Zhong *et al.* 2003, 2005). The system also represents an excellent model to study the evolutionary genetics of resistance to parasite infection, as infection can be easily controlled and monitored in the laboratory. Serving as an intermediate host, infection of *Tribolium* by *H. diminuta* occurs upon ingestion of the parasite eggs in rat feces. The eggs then hatch and develop into cysticercoids that are capable of infecting the mammalian host and amplifying when ingested, but cannot be horizontally or vertically transmitted.

Previously, we used AFLP markers to identify three major quantitative trait loci (QTL) that affected *T. castaneum* beetle susceptibility to *H. diminuta* on linkage groups *LG3* [hds 4(*3*, *L1B1.69*)], *LG6* [hds(*6*, *L1A16.141*)], and *LG8* [hds(8, *L6B2.100*)]. The gene action at QTL hds(*3*, *L1B1.69*) and hds(*8*, *L6B2.100*) was overdominance in the resistant *TIW1* strain. In contrast, the gene action at QTL hds(*6*, *L1A16.141*) was overdominance in the resistant *TIW1* strain. In contrast, the gene action at QTL hds[*6*, *L1A16.141*] was underdominance or recessive in resistant *TIW1* strain (Zhong *et al.* 2003, 2005). Fine-mapping of QTL can be achieved by increasing marker density within the chromosomal region of interest, increasing the number of individuals for which phenotypic information can be obtained, or by increasing the accuracy of assigning QTL genotypes (Nezer *et al.* 2003). QTL should be defined by informative, codominant markers within a 1- to

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Raw genotype and phenotype data used for QTL analysis have been deposited in the Dryad Repository (http://dx.doi.org/10.5061/dryad.bk90m).
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2-cM range or less to facilitate candidate gene identification (Yu *et al.* 2006). However, the QTL that we identified were positioned within large marker intervals due to the dominant AFLP markers used in the linkage analysis (Adarichev *et al.* 2003). In addition, the genomic regions containing the QTL were too large for positional cloning. Thus, the resistance genes could not be mapped with a reasonable degree of certainty, nor could sufficient sequence information be obtained.

To decrease the size of the marker intervals obtained in our previous study and facilitate the identification of the *T. castaneum* resistance genes, we applied the advanced intercross lines (AIL) method for high-resolution fine-mapping of QTL (Darvasi and Soller 1995). An AIL is generated by random intercross breeding of two inbred strains for several generations. As such, AIL can accumulate many more recombination events than the conventional methods. AIL has been used to refine multiple proximally located QTL (Iraqi *et al.* 2000; Wang *et al.* 2003; Jagodic *et al.* 2004). Recent advances in *Tribolium* genome resources, including the development of a high-resolution bacterial artificial chromosome (BAC) fingerprint map that integrates genetic, physical, and comparative mapping information and genome sequence data (Tribolium Genome Sequencing Consortium 2008), provide a powerful means of identifying new high-density markers for QTL analysis. Utilizing this technology, we increased the codominant marker density in the QTL regions associated with resistance to *H. diminuta* and refined the map position of the parasite resistance QTL. We conducted high-resolution QTL analysis in the 15th generation (G_{15}) of an advanced intercross line, focusing specifically on the three previously identified QTL (Zhong *et al.* 2003). Based on the results of the QTL analysis, transcription profiles were obtained for selected candidate genes in resistant and susceptible populations, and RNA interference (RNAi) was used to investigate the function of putative resistance genes with regard to the resistant and susceptible phenotypes.

Materials and Methods

Mapping populations

Two *T. castaneum* strains, *cSM* and *TIW1*, were used to set up segregating populations for QTL high-resolution mapping. Both were standard laboratory strains that have been used in genetic linkage mapping and other ecological and evolutionary genetics studies. *TIW1* is less susceptible to tapeworm infection than is *cSM*, but there is considerable intrastrain variability in beetle susceptibility to parasites. The two strains have been reared in the laboratory for >15 years. The origin of the two strains and their F_1 and F_2 segregation populations have been published (Zhong *et al.* 2003, 2005). The first population was generated from pairwise mating between a *TIW1* male and a *cSM* female and an F_1 intercross (cross 1). The second was from pairwise mating between a *cSM* male and a *TIW1* female and an F_1 intercross (cross 2). Two AIL G_{15} (after 15 generations of inbreeding in cross 1 or cross 2) segregation populations were generated by random intercross breeding in this study. Beetles were raised in 8-dram shell vials (25 mm × 95 mm) containing ~5 g standard medium (95% by weight fine-sifted whole-wheat flour and 5% dried powdered brewer's yeast). Experimental vials were maintained in a dark incubator regulated at 29° and 70% relative humidity. All beetles used for QTL fine-mapping studies were raised under the same conditions.

Tapeworm infection and DNA extraction

Fresh rat feces mixed with *H. diminuta* eggs were obtained from Carolina Biological Supply Company (Burlington, NC), which has maintained rats infected with *H. diminuta* for the past 40 years. To identify the tapeworm parasite genotypes, the DNA of *H. diminuta* eggs was extracted, and the PCR fragments of the mitochondrial cytochrome c oxidase subunit I (COI) gene and ribosomal internal transcribed spacer 2 (ITS2) gene were sequenced using the PCR primers (COI gene: 5′-CGGGTATTGGCTGAACATTT-3′ and 5′-ACACTCGACGAGGTAAACCA-3′; ITS2 gene: 5′-GAACTGTATGCGGTGGATCA-3′ and 5′-AAGTTCAGCGGGTAATCACG-3′). Three haplotypes of the COI gene sequence were identified with one to two mutations for the 957-bp PCR fragment (GenBank accession nos. KC990401–KC990403), and seven unique ITS2 sequences were detected with three microsatellite polymorphic regions for the PCR fragment 756–769 bp (GenBank accession nos. KC990404–KC990410). Phylogenetic analysis of three COI gene sequences indicated that the tapeworm parasites are most closely related to the USA strain (GenBank accession no. AF314223). Therefore, the parasites used in the present study are mixed genotypes.

The parental *TIW1* and *cSM* populations, F_1 and G_{15} individuals from reciprocal crosses of *TIW1* and *cSM* strains, were evaluated for tapeworm susceptibility using the infection and dissection methods described previously (Pai and Yan 2003). A total of 300 female and 300 male beetles of each cross from the G_{15} segregating population were exposed to tapeworm eggs following the previously established infection protocol (Pai and Yan 2003). Large sample size is critical for placing the resistance genes in relation to closely linked molecular markers with high confidence. Two weeks post infection, 200 live beetles of each sex were selected and dissected in a cold DNA extraction buffer to determine infection intensity. The beetle carcasses were collected for DNA extraction and genotyping, and the parasite tissues were discarded (Zhong *et al.* 2003).

Screening and development of molecular markers

Three major QTL on *LG3* [hds(*3*, *L1B1.69*)], *LG6* [hds(*6*, *L1A16.141*)], and LG8 [hds(*8*, *L6B2.100*)] were selected for QTL high-resolution mapping. The primer sequences for microsatellite and sequence-tagged site (STS) markers previously developed in *T. castaneum* (Demuth *et al.* 2007; Lorenzen *et al*. 2005) were used to screen for polymorphism between *TIW1* and *cSM* parent strains surrounding the three QTL regions of ~10 megabases. To increase the marker

density, we developed new microsatellite and single-strand conformational polymorphism (SSCP) markers in the QTL region. The microsatellite markers were developed based on the *Tribolium* genome sequences using the online software WebSat (http://wsmartins.net/websat/). The SSCP markers were designed based on the sequences of the intron region of genes surrounding the QTL regions using the Primer3 software (http://frodo.wi.mit.edu/primer3/). The sequences of forward and reverse primers are listed in Supporting Information, Table S1. Marker polymorphism was screened using the method described previously (Zhong *et al.* 2004, 2006) and the Li-Cor model 4300 automated DNA analyzer (Li-Cor, Lincoln, NE). The Gene ImagIR 4.33 software (Li-Cor) program was used to quantify allele size based on the pattern of the height of signal peaks.

Genotyping of AIL mapping population

The polymorphic markers between parents were selected to genotype two G_{15} segregation populations. The detection of PCR products and PCR conditions were described previously (Zhong *et al.* 2004, 2006). A total of 800 individuals were genotyped in the two G_{15} populations using 54 polymorphic markers (Table S1). PCR products were resolved on denaturizing polyacrylamide gels and were detected by using the Li-Cor Model 4300 automated DNA analyzer (Li-Cor). Genotypes were evaluated manually and double-checked by the same individual..

Double-stranded RNA synthesis and RNAi analysis

Double-stranded RNA (dsRNA) was synthesized using the Ambion MEGAscript high-yield transcription kit as described previously (Tomoyasu *et al.* 2008). The sequences of gene-specific primers (marked with footnote *a* in Table 1) for dsRNA synthesizing and the size of the dsRNA are listed in Table 1. Total RNA was extracted from *cSM* and *TIW1* adult beetles. The dsRNA of genes on *LG3* and *LG8* were synthesized from the total RNA extract from the *TIW1* strain (dsRNA-T), while the dsRNA of genes on *LG6* were synthesized from the total RNA extract from the *cSM* strain (dsRNA-C). Approximately 300–500 ng of dsRNA was injected into each beetle pupa (dsRNA-T for the *TIW1* strain, dsRNA-C for *cSM* strain). For each RNAi experiment, 100 female pupae were injected with dsRNA and another 100 female pupae were injected with injection buffer as control. Injected pupae were then kept in a dark incubator at 29° and 70% relative humidity. Two weeks later, half the adult beetles were evaluated for tapeworm susceptibility using the infection and dissection methods previously described (Pai and Yan 2003). The other half of the beetles was used for gene expression analysis.

Quantitative RT-PCR analysis

To test the expression difference between the two parental strains (resistant *TIW1* beetles and susceptible *cSM* strains), we used quantitative real-time PCR (qRT-PCR) analysis and selected the genes surrounding the QTL regions. A total of 21 genes in the three QTL regions were selected (Table 1). Total RNA was extracted from a pool of 20 resistant *TIW1* beetles and 20 susceptible *cSM* beetles (2-week-old female adults) in the absence of parasite infection, using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA). The RNA was then treated with DNase I. Similarly, total RNA was extracted from 20 dsRNA-injected beetles and 20 control beetles at 14 days post infection. The poly(A^+) messenger RNA (mRNA) was isolated from the total RNA by the Oligotex mRNA kit (Qiagen) according to the Qiagen protocol. The purified mRNA samples were used for reverse transcription, using the iScriptTM cDNA synthesis kit (Bio-Rad, Hercules, CA). The qRT-PCR primers for each candidate gene were designed using Primer 3.0, based on the corresponding complementary DNA sequences obtained from GenBank. The qRT-PCR analysis was performed using the method described previously (Wang *et al.* 2010). The ribosomal protein RPS3 and RPS18 genes were used as the standard for expression normalization for each gene because of their stable expression in *T. castaneum* after exposure to bacteria (Lord *et al.* 2010). The qRT-PCR assays were conducted in triplicate, and the average value of the triplicate was used for analysis of gene expression differences.

Linkage analysis and statistics

Linkage analysis between molecular markers and resistance QTL was performed using GNU R 2.6.0 with the QTL package version 1.07–12 (R/qtl) (Broman *et al.* 2003). The physical map is derived from the National Center for Biotechnology Information (NCBI) *Tribolium* genome sequence at http://www.ncbi.nlm.nih.gov. Marker positions were obtained from the NCBI *Tribolium* genome Build 2.1 statistics. Data were analyzed by implementing a nonparametric model for quantitative traits. Confidence intervals (C.I.'s) for QTL were defined as the region within the maximum logarithm of odds (LOD). To further evaluate identified QTL, a multiple QTL model test was performed using R/qtl software. Analysis was performed for the two reciprocal crosses of G_{15} populations separately and also in a combined analysis to increase statistical power. Separate analyses were performed with the multiple imputation method (normal model) with 64 simulations (step = 2, ndraws = 64) in R/qtl. Because different populations were used in the combined cross analysis, marker regression with 64 simulations was used. Only physical positions for the markers were used in the combined analysis since they are constant between populations in contrast to genetic positions. The experiment-wise significance threshold levels were determined by the permutation method in R/qtl using 10,000 permutations (Broman *et al.* 2003).

Gene expression data obtained from qRT-PCRs for 21 loci in both *TIW1* and *cSM* strains were analyzed as a one-way ANOVA. To assess differences in tapeworm infection intensity with or without dsRNA injection (RNAi), the gene expression ratio data obtained from qRT-PCRs in eight selected loci in the *TIW1* or *cSM* strain were also explored using one-way ANOVA. We used pairwise Student's *t*-tests to detect

Table 1 Genes selected for RNAi assay

No.	Locus[a]	*LG*	Location (cM)	Gene name	Forward primer	Reverse primer	Size (bp)
1	LOC100142401	3	13.7	Similar to discoidin domain receptor CG33531-PA	ATCTATGGGTGTCGCTGGAC	TGTTCTTACGCGTGTCGTTC	244
2	LOC657454[a]	3	14.1	Similar to probable cytochrome P450	ACTCGCTGAAAGACCGAAAA	AAGTGTTCTTCCGGGTGTTG	277
3	LOC100141631[a]	3	14.2	Similar to AGAP006113-PA	CCTGGAGGACACCTCGAATA	CGTCACCATATTGACGCAAC	232
4	LOC657788	3	15.2	Hypothetical protein LOC657788	TCTCCACTGCAGACACCAAG	AACCCAATTTTTCCGATTCC	387
5	LOC659770	3	15.4	Similar to CG5642-PA	TGGATTCCATCCCTGATGAT	AGCTCTGAGCCACTCTCCAG	310
6	LOC660033	3	15.5	Similar to CG1221-PA, isoform A	AGTGAACGAGGAGCCAAGAA	GTTGGGCTTCAGTTTGTCGT	243
7	LOC654972	3	16.7	Similar to CG5547-PD, isoform D	GCGCTTTTGATCTCTTCCAC	TCAAACTCCAGCCTGTTCCT	406
8	LOC663292	6	7.8	Similar to CG4225-PA	CTTGGTTCAGTCCCGTTTGT	CAGAAGGGAAAAGGGGTAGG	172
9	LOC663441[a]	6	7.9	Similar to CG1512-PB, isoform B	CAGAAACTGTCCGATGCTGA	AAGAATTGTGCCCCTGACTG	216
10	LOC663587[a]	6	8.1	Similar to CG2052-PB, isoform B	CAGCATCTCAGAAACCACGA	ATTGGGGTCCTTGTGTGTGT	234
11	LOC663602[a]	6	8.7	Similar to CG7332-PA	CCGAAACTAAAACCGACCAA	GCACCAATGAAAACACATCG	249
12	LOC655450	6	9.6	Similar to leucine-rich repeat containing 47	CCCTCCGGAAATTAACCAAT	CCTTTTGTTCGCTCTTTTGC	239
13	LOC656106	6	9.7	Similar to ubiquitin-specific protease 2	TAACCGAAGACGGCATAACC	AGTTGGCCGGTGAGTATTTG	228
14	LOC661773	8	10.5	Similar to potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 1	TGGTGGCAATGAGCAAAATA	CATAGCGGAACACCCTGTTT	231
15	LOC661984	8	10.9	Similar to CG10889-PA	AGTGCACATACGGCAACAAA	ACACCACGACATGTTGCACT	195
16	LOC662131[a]	8	11.0	Similar to Toll-like receptor 13	ACGGTTTGGACAACTTGGAG	CCCTCAGGTGCGTATTTGTT	228
17	LOC662235[a]	8	11.2	Similar to CG17839-PB, isoform B	CTGTTGTTTTGGCCCTTGTT	GTTCTTCCAGCCTTGCACTC	245
18	LOC662348[a]	8	11.4	Similar to CG8503-PA	AGAAGCAGTTCTTGCCCAAA	TCGTGTCCATTGACCTGAAA	208
19	LOC662521	8	11.8	Similar to myeloid leukemia factor (myelodysplasia-myeloid leukemia factor)	ACCGCATGAGCAACTCTCTT	TAATACCCCCAGGAGCTGTG	198
20	LOC663023	8	12.0	Similar to glyceraldehyde-3-phosphate dehydrogenase II	CAAAGTTATCCCGGCTTTGA	AAATCGACGAGTGCGTATCC	230
21	LOC663134	8	12.1	Similar to endothelin-converting enzyme 2	TTTTTACCGCTCCTGCCTTA	AAAATCAAATTCCCCGGTTC	237

Locus name, location, product size, and primer sequence of qRT-PCR of the candidate genes in the QTL regions in *T. castaneum*.

[a] The dsRNA of genes on *LG3* and *LG8* were synthesized from the total RNA extract from the *TIW1* strain (dsRNA-T), while the dsRNA of genes on *LG6* were synthesized from the total RNA extract from the *cSM* strain (dsRNA-C).

statistically significant differences in the absolute average infection intensity of tapeworm between dsRNA and buffer injections. JMP software package (SAS Institute Inc., Cary, NC) was used to perform the statistical analysis.

Results

Phenotypic variability in susceptibility to tapeworm parasites in AIL

Infection intensity of the *cSM* and *TIW1* parental and the resultant F_1 progeny was reported previously, with the *cSM* and F_1 populations being significantly more susceptible to tapeworm parasite infection than the *TIW1* (Zhong *et al.* 2003, 2005). In the present study, the individuals of two G_{15} AIL populations were exposed to feces obtained from *H. diminuta*-infected rats. A total of 400 G_{15} AIL beetles in each cross were infected with tapeworm parasites. The average infection intensity of cross 1 AIL populations was 5.3 $\pm$ 0.7 (range 0–28; $n = 400$) and 3.85 $\pm$ 0.5 (range 0–22; $n = 400$) for cross 2. There was no difference in the parasite infection intensity between male and female beetles for cross 1 and cross 2, which suggests that sex is not a factor for susceptibility to tapeworm infection.

High-resolution mapping of the QTL [hds*(3, L1B1.69)*] region on linkage group *LG3*

A 14.17-cM (~5.0-Mb) region of the QTL *LG3* [*hds*(*3*, *L1B1.69*)] was selected for high-resolution mapping (map position: 6.48–20.65 cM). Previously described markers (Zhong *et al.* 2004; Demuth *et al.* 2007) were used to screen for polymorphism between *cSM* and *TIW1* parent strains. Of the 42 markers previously reported on *LG3*, 9 were polymorphic between the two parental strains. Ninety-two new primers were subsequently designed based on *Tribolium* genome sequences in the QTL region on *LG3*; 10 of these were polymorphic between the two parental strains. Thus, a total of 19 markers were used to genotype the two AIL populations (Table S1). Linkage analysis was performed in two AIL populations separately and confirmed the locus with a high-LOD score (>8) in the two AILs. The combined population linkage analysis indicated an LOD score of 9.2 at an interval of the two markers Tca3.4924 and Tca3.4970 for the QTL (Figure 1, Figure S1A, and Table S1). The QTL region was narrowed to a genomic region of ~0.13 cM (46 kb) within a 95% C.I. Based on the *Tribolium* genomic database, this region contains four predicted genes, including a candidate gene, cytochrome P450 (GenBank accession no. XM_963914) (Figure S1A), an important enzyme system involved in insecticide metabolism.

High-resolution mapping of the QTL [hds(6, L1A16.141)] region on linkage group LG6

A 14.31-cM (~5.0-Mb) region of the QTL *LG6* [hds(*6*, *L1A16.141*)] was selected for high-resolution mapping by the same method used for the analysis on *LG3*. Of 17 markers previously reported in the region (Demuth *et al.* 2007), 9 markers identified polymorphisms in the parental strains and 11 new markers were then developed, which identified additional polymorphisms in this region. A total of 20 markers were used to genotype the two AIL populations (Table S1). Linkage analysis was performed in two AIL populations separately, and the locus was confirmed with a high-LOD score of >10 in the two AILs. The combined population linkage analysis indicated an LOD score of 13.1 at an interval of the two markers Tca6.2726 and Tca6.2926 for the QTL (Figure 1, Figure S1B, and Table S1). The QTL region was narrowed to a genomic region of ~0.55 cM (192 kb) within a 95% C.I. Based on the *Tribolium* genomic database, this region contains 11 predicted genes of unknown function (Figure S1B).

High-resolution mapping of the QTL [hds*(8, L6B2.100)*] region on linkage group *LG8*

A 9.93-cM (~3.48-Mb) region of the QTL *LG8* [hds(*8*, *L6B2.100*)] was selected for high-resolution mapping. Of 20 markers previously reported in the region (Demuth *et al.* 2007), seven markers identified polymorphisms in the parental strains, and eight newly developed markers identified additional polymorphisms (Table S1), resulting in a total of 15 markers that were used to genotype the two AIL populations.

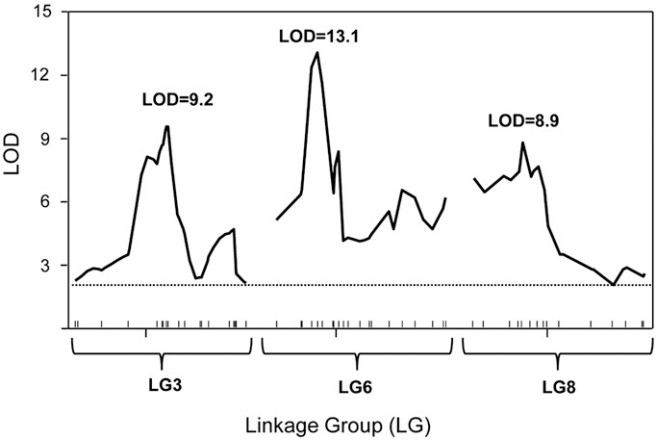

Figure 1 Fine-mapping QTL-associated beetle susceptibility to tapeworm parasite in AIL (G_{15}). LOD scores are presented on the *y*-axis and physical positions (in centimorgans) of the linkage group are given on the *x*-axis. LOD score values were determined using R/qtl software (Broman *et al.* 2003). The physical position of the markers was retrieved from the *T. castaneum* genome database at http://www.ncbi.nlm.nih.gov/genome/guide/beetle/index.html.

The linkage analysis was performed separately, and the locus was confirmed with a high-LOD score of >8 in the two AILs. The combined population linkage analysis indicated an LOD score of 8.9 at an interval of the two markers Tca8.3781 and Tca8.4007 for the QTL (Figure 1, Figure S1C, and Table S1). The QTL region was narrowed to a genomic region of ~0.70 cM (245 kb) with a 95% C.I. This region contains 12 predicted genes, including a candidate gene, Toll-like receptor 13 (*TLR13*; GenBank accession no. XM_968248) (Figure S1C), which has been implicated in innate immunity and endotoxin susceptibility in mice (Roach *et al.* 2005; Mishra *et al.* 2008; Shi *et al.* 2009).

Gene expression differences between resistant and susceptible strains

The transcription profiles of genes associated with the loci identified in the resistant *TIW1* and susceptible *cSM* strains (2-week-old female adults) were examined using qRT-PCR. A total of 21 genes surrounding the three QTL regions were selected for qRT-PCR analysis: seven genes on *LG3*, six genes on *LG6*, and 8 genes on *LG8* (Table 1, Figure 2). The range of expression level between the resistant and susceptible strain was a 1.3- to 8.3-fold difference for *LG3*, 1.6- to 5.9-fold for *LG6*, and 1.2- to 4.3-fold for *LG8* (Figure 2). Nineteen genes of the 21 selected genes showed significant differences in gene expression ($P < 0.05$) between resistant and susceptible strains. The resistant *TIW1* strain had a higher expression level for the genes on *LG3* and *LG8* whereas a lower expression level was found for the genes on *LG6* (Figure 2). These results are consistent with the overdominance of QTL hds[*3*, *L1B1.69*] on *LG3* and hds[*8*, *L6B2.100*] on *LG8* and underdominance or recessive of QTL hds[*6*, *L1A16.141*] on *LG6* in the resistant *TIW1* strain (Zhong *et al.* 2003, 2005).

RNAi effect on parasite infection intensity and gene expression level changes

A total of eight genes were selected for RNAi analysis (Table 2). RNAi was performed by injecting the beetle pupae and by subsequently examining the parasite infection intensity and comparing the mRNA levels with those of buffer-injected controls. Significant differences in parasite infection densities were observed for genes on *LG3* and *LG8*, but not on *LG6* (Table 2). The dsRNA injected beetles had significantly more parasites compared to control beetles (buffer-injected) in the resistant *TIW1* strain, but not in the susceptible *cSM* strain. Transcription levels for the selected genes were significantly altered after RNAi treatment for both the resistant and the susceptible strains (Table 3). Both genes (LOC657454 and LOC100141631) on *LG3* had significantly reduced transcription (10- to 13-fold changes, $P < 0.001$). Similar to the two genes on *LG3*, the three genes used for RNAi analysis on *LG8* had a significant gene knockdown effect (3- to 9-fold changes, $P < 0.01$). The *TLR13* gene (LOC662131) had the highest gene knockdown effect (9.49-fold change, $P < 0.001$) in the resistant *TIW1* strain (Table 3). While no significant difference in parasite infection intensity was observed for genes on *LG6* as a result of RNAi treatment, the transcription levels of the three selected *LG6* genes were significantly altered.

Discussion

To refine the QTL associated with *T. castaneum* susceptibility to *H. diminuta* infection, we generated two AIL populations from resistant *TIW1* and susceptible *cSM* strains. Using 29 newly developed microsatellite and STS markers, together with 25 previously published microsatellite markers, we were able to identify three tapeworm-resistant QTL in genome regions of ~0.13 cM on *LG3*, 0.55 cM on *LG6*, and 0.70 cM on *LG8*. The QTL on *LG3* contain sequences homologous to cytochrome P450 (*CPYIXF2*), a family of enzymes involved in insecticide activation and detoxification. The QTL on *LG8* contain gene *TLR13*, a novel member of the Toll-like receptor family (Shi *et al.* 2009), which plays a key role in the innate immune system. Because these QTL regions may include genes that play a role in inhibiting parasite development in the beetle host, we designated these genes as candidate genes for resistance to *H. diminuta* infection.

A biological basis for the involvement of the candidate genes in resistance to tapeworm infection may exist. Cytochrome P450s constitute the largest gene superfamily found in nature, displaying a wide variety of functions. However, only a small subset of these cytochrome P450 genes is involved in insecticide metabolism and the innate immune response. Insect cytochrome P450s are known to play an important role in detoxifying insecticides, such as pyrethroids (Feyereisen 2005; Karunker *et al.* 2009). The most important character of insect cytochrome P450s is that they are constitutively overexpressed in the insecticide-resistant

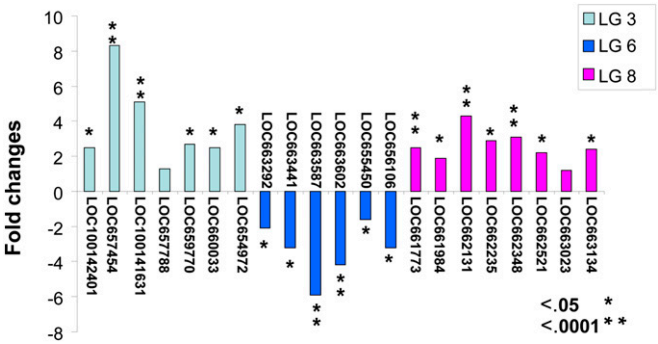

Figure 2 Gene expression difference (in fold) between the resistant *TIW1* strain and the susceptible *cSM* strain in the absence of parasite infection. A positive value indicates that the resistant strain had a higher mRNA level, while a negative value indicates that the susceptible strain had higher mRNA level.

phenotype, causing enhanced metabolic detoxification of insecticides (Feyereisen 2005; Zhu *et al.* 2008). In *Tribolium*, >200 sequences of P450 genes have been registered in the GenBank database, but the physiological functions of P450s remain largely unknown. Zhu *et al.* (2010) identified a P450 gene, *CYP6BQ9* responsible for the majority of deltamethrin resistance that showed a >200-fold increase in expression in the deltamethrin-resistant QTC279 strain when compared with a deltamethrin-susceptible Lab-S strain.

In our present study, we found that the resistant *TIW1* strain had significantly higher transcription of *CPYIXF2* (LOC657454, probable cytochrome P450) compared to the susceptible *cSM* strain in the absence of parasite infection. Our analysis showed that injection of dsRNA corresponding to *CYPIXF2* sequences in the resistant *TIW1* strain (LOC657454) resulted in a significant increase in infection intensity that was accompanied by a significant decrease in the transcription of *CYPIXF2*. These data strongly suggest that this P450 gene is involved in resistance to tapeworm infection.

Toll-like receptors (TLRs) are an important family of pattern-recognition receptors that play a key role in the innate immune system (Roach *et al.* 2005). The Toll receptor was initially identified in *Drosophila melanogaster* for its role in embryonic development and was recognized as a key regulator of immune response (Leulier and Lemaitre 2008). TLRs have been identified in many animal species, including pigs (Shinkai *et al.* 2006), chickens (Fukui *et al.* 2001), fish (Tsujita *et al.* 2004), and insects (Tauszig *et al.* 2000; Imamura and Yamakawa 2002; Aronstein and Saldivar 2005). It has been estimated that most mammalian species have between 10 and 15 types of Toll-like receptors (Roach *et al.* 2005). Insect TLRs and mammalian TLRs are evolutionarily conserved and share characteristic domain organization (Gangloff *et al.* 2003; Roach *et al.* 2005). It has been demonstrated that experimental infection of humans with the malaria parasite *Plasmodium falciparum* can enhance TLR-mediated responses (Franklin *et al.* 2009). In *T. castaneum*, Zou *et al.* (2007) reported nine genes (TLRs 1–4, TLRs 6–10) that encode Toll and Toll homologs. Zhang *et al.* (2004) demonstrated that *TLR11* recognizes

Table 2 RNAi effect on parasite infection intensity

Strain/treatment (injection)	*LG*	*n*	Infection intensity (mean ± SE)	Significance[a]
TIW1 (dsRNA-T)				
LOC657454 (dsRNA)	3	44	5.30 ± 0.93	A
LOC662131 (dsRNA)	8	44	4.48 ± 0.60	A
LOC100141631 (dsRNA)	3	38	2.42 ± 0.40	B
LOC662348 (dsRNA)	8	40	1.85 ± 0.41	BC
LOC662235 (dsRNA)	8	45	1.58 ± 0.32	BC
Control (injection buffer)		48	0.60 ± 0.15	C
cSM (dsRNA-C)				
LOC663441 (dsRNA)	6	43	7.74 ± 1.03	A
LOC663587 (dsRNA)	6	42	8.81 ± 1.08	A
LOC663602 (dsRNA)	6	44	7.91 ± 1.10	A
Control (injection buffer)		47	8.60 ± 1.07	A

Student's *t*-test was performed to compare the difference. *n*, number of beetles.
[a] RNAi effect on parasite infection. Means with the same letter are not significantly different.

urinary pathogenic *Escherichia coli*. *TLR13* expression was evident in brain cells of mice, both infected and uninfected with the *Mesocestoides corti* parasite, and parasite infection caused a several-fold increase in mRNA and protein levels of *TLR13* (Mishra *et al.* 2008). In our study, the *TLR13* gene of the resistant *TIW1* strain exhibited a significantly higher *TLR13* mRNA level than the susceptible *cSM* strain in the absence of parasite infection. RNAi analysis showed that injection of dsRNA corresponding to the *TLR13* gene sequences (LOC662131) resulted in a significant gene knockdown effect in the resistant *TIW1* strain, as evidenced by the presence of a significantly higher number of parasites than in the buffer-injected controls. Therefore, these data strongly suggest the role of *TLR13* in *T. castaneum* resistance to *H. diminuta* infection.

Only a few invertebrate parasite-susceptibility QTL-mapping studies have been completed to date, and all have reported multiple regions involving parasite susceptibility, such as QTL affecting malaria-parasite and filarial-parasite infection in *Aedes aegypti* (Beerntsen *et al.* 1995; Severson *et al.* 1995, 1999; Morlais *et al.* 2003; Aronstein and Saldivar 2005) and in *Anopheles gambiae* (Menge *et al.* 2006). The polygenic, quantitative genetic patterns that we have observed in these experiments are supported by earlier studies that approached parasite resistance evolution as a quantitative character rather than a single genetic factor (or gene). Characterization of the *Tribolium* beetle in response to tapeworm parasite infection will provide valuable insights into the molecular basis of host resistance to parasites and coevolution between beetle host and tapeworm parasites. This will have important implications for the development of novel strategies for *Tribolium* pest control. The knowledge and tools developed in such studies are useful in analyzing natural host–parasite systems.

Gene expression analysis was performed on female beetles only; the effects of host sex and age on their immune response and resistance to parasite infection were not examined. Freitak *et al.* (2012) found that 54% of microRNAs exhibited gender-specific expression patterns upon exposure to environmental stress in *T. castaneum*. When we examined the between-sex variation in the expression of 29 immune-related genes in *T. castaneum*, we found significant among-strain variations in the response of the immune-related genes (Zhong *et al.* 2013). Furthermore, despite the fact that the tapeworm parasites used in the study have been maintained in the Carolina Supply Company for 40 years, we identified three genotypes by COI gene, suggesting that tapeworm parasites were not genetically homogenous. This is consistent with natural situations in which parasite populations are genetically heterogeneous. More studies are needed to determine the effects of interactions between parasite and host genotypes on host infectivity.

In summary, the tapeworm parasite resistance QTL of *T. castaneum* beetles were finely mapped to genomic regions in *T. castaneum* using AILs and high-density molecular markers. Two genes were identified as candidates for resistance genes to *H. diminuta* infection (*CPYIXF2* and *TLR13*) using transcription analysis and RNAi. This work not only lays a foundation for identification and cloning of tapeworm parasite resistance genes in the *Tribolium* host, but also improves the recognition of function in resistance genes to the indirectly transmitted macroparasites.

Table 3 RNAi effect on gene expression

Strain/locus name	*LG*	Expression changes (RNAi to control)	ANOVA (*P*-value)
TIW1			
LOC657454	3	−13.21	0.0002
LOC662131	8	−9.49	0.0008
LOC100141631	3	−10.31	<0.0001
LOC662348	8	−3.63	0.0015
LOC662235	8	−5.00	0.0074
cSM			
LOC663441	6	−6.56	0.0006
LOC663587	6	−15.00	<0.0001
LOC663602	6	−5.43	0.0046

The gene expression between RNAi and control groups is compared. ANOVA analysis for significance is listed.

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Fine-Scale Analysis of Parasite Resistance Genes in the Red Flour Beetle, *Tribolium castaneum*

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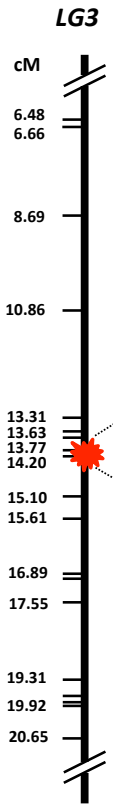
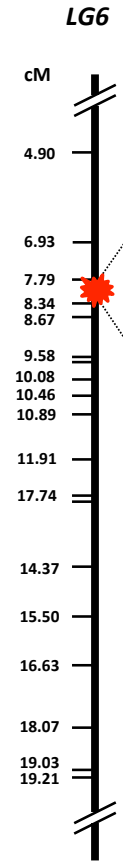


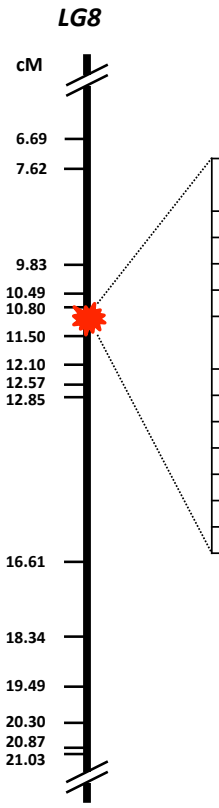
A

Position (cM)	Start	Symbol	Size (bp)	Function	Description
14.13	4946133	LOC657535	17156	mRNA	similar to CG2812-PA
14.13	4947077	LOC657454	3294	protein	similar to Probable cytochrome P450 9f2 (CYPIXF2)
14.19	4965706	LOC100141845	3565	ab initio	hypothetical protein LOC100141845
14.21	4972080	LOC100141631	6522	protein	similar to AGAP006113-PA



B

Position (cM)	Start	Symbol	Size (bp)	Function	Description
7.79	2726021	LOC663271	890	protein	similar to CG13618-PA
7.79	2726918	LOC663292	11247	mRNA	similar to CG4225-PA
7.85	2746389	LOC663334	8904	mRNA	similar to CG31116-PC, isoform C
7.87	2755664	LOC663354	13248	mRNA	similar to CG31116-PD, isoform D
7.89	2759979	LOC663367	1178	mRNA	similar to CG1227-PA
7.91	2769936	LOC663421	4322	mRNA	similar to CG17248-PA, isoform A
7.93	2776190	LOC663441	7675	mRNA	similar to CG1512-PB, isoform B
7.96	2785243	LOC663455	843	protein	similar to CG13618-PA
7.96	2787678	LOC663474	882	mRNA	similar to CG13618-PA
7.98	2793592	LOC663534	4834	protein	similar to CG1443-PA
8.10	2835857	LOC663587	190827	ab initio	similar to CG2052-PB, isoform B



C

Position (cM)	Start	Symbol	Size (bp)	Function	Description
10.78	3771637	LOC661897	1122	ab initio	similar to CG2713-PA
10.83	3791374	LOC661941	1654	mRNA	similar to CG4767-PA
10.87	3804037	LOC661984	27225	protein	similar to CG10889-PA
10.96	3835748	LOC662022	8261	mRNA	similar to CG4636-PA
10.98	3844344	LOC662059	401	protein	similar to LSM3 homolog, U6 small nuclear RNA associated
11.02	3856854	LOC662131	4959	mRNA	similar to toll-like receptor 13
11.04	3864695	LOC662165	22296	protein	similar to calpain 11
11.24	3932373	LOC662235	27566	mRNA	similar to CG17839-PB, isoform B
11.34	3969669	LOC662271	1120	mRNA	hypothetical protein LOC662271
11.35	3973123	LOC662311	1144	mRNA	hypothetical protein LOC662311
11.37	3980803	LOC662348	90308	protein	similar to CG8503-PA
11.64	4075128	LOC662383	6211	protein	similar to CG11160-PA, isoform A

Figure S1 Fine mapping of QTL on linkage group 3, 6 and 8. Results were scaled according to the physical position of markers on the *Tribolium castaneum* (red flour beetle) genome database. Tick marks appear at marker positions. **(A)** Linkage group 3 showing four protein coding genes located in the QTL region; **(B)** Linkage group 6 showing 11 genes located in the QTL region; and **(C)** Linkage group 8 showing 12 genes located in the QTL region.

Table S1 Primer sequences of microsatellite, sequence-tagged site (STS), and single-strand conformational polymorphism (SSCP) markers used in the study for fine mapping beetle tapeworm parasite susceptibility QTL in *Tribolium castaneum*.

No.	Marker [a]	*LG*	*cM*	Forward primer	Reverse primer	Motif (Repeats)	Size(bp)	Note [b]
1	Tca3.2267	3	6.48	GCCAGAACGCCAAATAAAAC	TACGGTATGTTGCGGATTGA	SSCP	191	AC191[L]
2	Tca3.2331	3	6.66	GGCCAACAATATAACAACCGA	GAATCAGAGGCTGCTAACGTC	A(12)	232	This study
3	Tca3.3038	3	8.69	TGTGATTTTCTTTGGTTCAACG	CAAATTGCATTTGTCGCATC	SSCP	290	26.M03t[L]
4	Tca3.3800	3	10.86	GACCGAATGGCGTCTTACTC	ACAATAAAAGCCTAGCTGGAGG	T(12)	232	This study
5	Tca3.4657	3	13.31	GCCGTCGTTTTATTTCCAGA	CTGGTTCGGTCTGTGGATTT	SSCP	258	This study
6	Tca3.4771	3	13.63	GCCAACAGACGCGCTTTCATT	ACGAGTTACCGCATCAGATTCCTTTAT	CGG(5)	281	S1031[L]
7	Tca3.4821	3	13.77	CCAATGGTGAGAGGTCCCTA	CATTCCGTAATCCGCAAACT	SSCP	278	This study
8	Tca3.4924	3	14.07	GGACTCGGACTTTCAAGCAG	GTCGACGAAATGGGAAAGAA	SSCP	219	This study
9	Tca3.4970	3	14.20	GCCTGGGTGTCATCACTGTA	TGGAGCACGATCAAAGAGTG	SSCP	213	This study
10	Tca3.5286	3	15.10	TTGCTGCTTCCGAATGTATG	CCCATATTTGCACTCAAGCA	SSCP	317	This study
11	Tca3.5465	3	15.61	GAATATTCCGTCTGGCCGTA	TGTGATTGCGAAACTCGAAG	GGC(6)	202	L2A8.377s[Z]
12	Tca3.5912	3	16.89	GTATATTGCGGTTCGCTGGT	GTCATCGTGAACGTTGTTGG	SSCP	321	This study
13	Tca3.5953	3	17.01	CGCAACTGCAAGAAAAATTG	TTGGTTTTGCGTGTTACGAG	SSCP	204	25E11[L]
14	Tca3.6143	3	17.55	ATCCGGCCAGTAAAAGTGTG	CGCGCATAAATAAACCGAAT	SSCP	228	This study
15	Tca3.6763	3	19.31	TGTTTTTGATTTTCTCTTTGCAT	AAGCACAATTGGTCAAAACAAA	T (16)	271	Tca-3.45[D]
16	Tca3.6901	3	19.69	ACCAATCGACCACGTTTTTC	ACCGAGGTGGGCTTTAAACT	SSCP	211	32.D14s[L]
17	Tca3.6939	3	19.83	TTCAATCAGTTTTCTTTCTGTCAA	TACGATGCATTGGATTTTGG	TAT (6)	239	Tca-3.22[D]
18	Tca3.6972	3	19.92	CCTCCTGAAAGGACACAGGA	GGTGCAACTCGCTTCTTCAT	TCA (5)	228	Tca-3.11[D]

19	Tca3.7226	3	20.65	GGCAACCGCACTAAACACTT	AAGTTGGCGCTTTAGGAACA	SSCP	348	This study
20	Tca6.1708	6	4.90	AAGGACAGGTTGTGGTTTTAGG	TCCCATTATAGCCCACTTCACT	T(13)	108	This study
21	Tca6.2427	6	6.93	CCAAAACCACAGAACTTGCATA	AGCTAGACTTCGCCTCCTCATT	AAT(6)	118	This study
22	Tca6.2445	6	6.99	CAGAAGCTATGAGAGCTGCAGTA	TTAACAGAATTGCGGGAAAAA	ATT (9)	187	Tca-6.12 [D]
23	Tca6.2726	6	7.79	GACACGCTCCAGCAAGTTTAC	CGTAGCTCCAATAACAACTCCC	A(16)	159	This study
24	Tca6.2926	6	8.34	AATTCAGCGTTTGCTGCT	TGTTTGCATCGCTTGTTCT	AATT(4)	157	This study
25	Tca6.3003	6	8.67	CACAGGCGTAGATTGGTTGTT	TCAGGCTTACTTGGGTTAGCA	TA(26)	215	This study
26	Tca6.3352	6	9.58	AATTGGAAGGAAGTGTTTGGTG	AAAAGTGGCGATGATTACGTCT	A(12)	226	This study
27	Tca6.3393	6	9.69	GTCCCATAAGCTGCATTCGT	TGGCGCAATTTTGTAACTGA	SSCP	236	This study
28	Tca6.3527	6	10.08	TGGCCTTAAATCGTCAATTTTT	CAGAAATGCCAGCTGTTCTTC	ATTTA(5)	255	This study
29	Tca6.3661	6	10.46	GAAAACGCCAAATCGACATT	TCGTTTATGTAGCCGGTGAA	ATT(5)	222	Tca-6.7 [D]
30	Tca6.3810	6	10.89	ATCATCTGGAGCGACAAACC	GATTTATTTGCGGCGACCT	A(16)	160	This study
31	Tca6.4168	6	11.91	GATAAGGTAGGGCAGCTTCG	GGGTGGGTACGAACAAGAAT	T(13)	243	This study
32	Tca6.4460	6	12.74	TCACCCTTATCTTCCTCCGAT	CATTTGGCCGATCTTCAGTC	A(14)	230	This study
33	Tca6.4509	6	12.89	ACCTGACCTGACCTGACCTG	CGTCAGTTGTTTCTCGCAAA	CCTGA (4)	186	Tca-6.26 [D]
34	Tca6.5044	6	14.37	AGATCCCAATGGGCAAATCT	GCCGAAACTTTGGGTGATAA	TCAAG (5)	200	Tca-6.18 [D]
35	Tca6.5425	6	15.50	TTTTTGCTCAGAACACTCAAAA	CCTTCATATGTGGAAGGAAACA	ATT (5)	194	Tca-6.16 [D]
36	Tca6.5819	6	16.63	AAAATGCGTTTTTCACTCAAAT	CCAACTCGCCCATTATGAAC	T (12)	206	Tca-6.13 [D]
37	Tca6.6324	6	18.07	AACGAGCCCATAACCAGAGA	TTTGGCGAAGAAAACTGAAAA	A (13)	211	Tca-6.4 [D]
38	Tca6.6659	6	19.03	TTGTTTGTTAAGATCAAGGCAAAA	TGGTCACTCTTTTTCGCTATGT	GA (8)	194	Tca-6.36 [D]
39	Tca6.6723	6	19.21	AAATTATAAATCAAGCAGACAGGAG	TCCTGTATAAACGATTATGTTTTCAA	TAA (5)	205	Tca-6.33 [D]

40	Tca8.2341	8	6.69	TTTGTAGATTTGTATTTTGGCAAT	GGAATCCTGGAATCTTAGAACG	TTA (20)	194	Tca-8.8 [D]
41	Tca8.2668	8	7.62	TAGGGTGATCCTGCTAAAAGTACC	TGGATCTAAACTTCGCCCATA	TAA (21)	188	Tca-8.35 [D]
42	Tca8.3441	8	9.83	TTTGGCATTTCTCAAGGTCA	GGTAGATGCGCTGGAATTTT	AAT(8)-ATA(5)	244	This study
43	Tca8.3671	8	10.49	TTTGAAATGGTTCAACACGC	CTCCGCCTGTTTGTTGGTAT	AAT (7)	145	Tca-8.14 [D]
44	Tca8.3781	8	10.80	GGTTTAGCCAAAGGAACTTCG	AAAGTTAACAACGCGAATGGA	CCA(5)-CAA(4)	232	This study
45	Tca8.4007	8	11.50	ATCTGTATTCCGTTTGGTAAGC	AGCTAGATTTTGCCTCTTCACA	TAA(6)-TAA(15)	195	This study
46	Tca8.4236	8	12.10	TGGGTTAAGTTGAAGTAGCCTTG	GAAACGAAGTGAGAAAACGGA	TTA(7)	181	This study
47	Tca8.4398	8	12.57	TGTTTTGTTTGATTTTGAGTTTGTC	TTCGCTTAATGAATGCCTCC	TTA (23)	205	Tca-8.16 [D]
48	Tca8.4517	8	12.85	TGCATTCAGGCGCTTAATTT	TCAATGTACAGGCTGGTGTG	A (15)	196	Tca-8.48 [D]
49	Tca8.5815	8	16.61	GGTTAAATAGGACAAAGTTGCG	AGAACGCCTTGGAAAAGGTA	A(14)	157	This study
50	Tca8.6428	8	18.34	TGTGAGGTCCCATGGTGTAA	ACATTTATTTGGTCCCACAAGC	T (15)	195	Tca-8.22 [D]
51	Tca8.6819	8	19.49	TAAACACACGTCCGGTTCCT	TTTGTTGTCGAGATACTGTTAGACG	CA(8)	180	This study
52	Tca8.7101	8	20.30	AAAGCGCTGTTGCAAAATTC	ACCCACTCAGTAGCCAGTGC	TTA(9)-TTA(5)	178	Tca-8.32 [D]
53	Tca8.7305	8	20.87	CCGGATAATGGAGGTTTCA	TCTCTTTGCATGTGGGTTTT	TTAA(4)	211	This study
54	Tca8.7359	8	21.03	CCGAGCGAGGAGTATATGTTG	GAACGCAAAACGACCAAATC	CA(9)	215	This study

Note: a: Markers are designated by the beetle species name *Tribolium castaneum* (Tca), genetic linkage group and location on linkage group. For example, marker Tca3.2267 represents the marker located at the 2267kb position on linkage group 3, whereas Tca6.1708 represents the marker located at the 1708kb position on linkage group 6. b: Reference *L:* Lorenzen et al 2005; *D*: Demuth et al 2007; *Z*: Zhong et al 2004. *LG*:Linkage Group; *cM*: centiMorgan.