For: Genetics

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 (STS) markers, and single-strand conformational polymorphism (SSCP) markers. Resistance QTL in three linkage groups (LG3, LG6, and LG8) were each mapped to intervals less than 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 *TIWI* and the susceptible *cSM* strains. Based on transcription data, eight genes were selected for RNA interference (RNAi) analysis to investigate their possible roles in *H. diminuta* resistance, including cytochrome P450 (LOC657454) and toll-like receptor 13 (TLR 13, LOC662131). The transcription of P450 and TLR 13 genes in the resistant *TIWI* strains was reduced more than nine-fold relative to the control. Moreover, the effects of gene knockdown of P450 and TLR 13 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.
Introduction

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; Zhong et al. 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 amplifying host when ingested, but cannot be horizontally or vertically transmitted between beetles.

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[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 resistant *TIWI* strain. In contrast the gene action at QTL hds[6, L1A16.141] was underdominance or recessive in resistant *TIWI* strain (Zhong et al. 2003; Zhong et al. 2005). Fine-mapping of quantitative trait loci (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 increasing the accuracy of assigning QTL genotypes (Nezer et al. 2003). QTL should be defined by informative, codominant markers within a 1–2 cM range or less in order 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.

In order 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; Jagodic et al. 2004; Wang et al. 2003). 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), now 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 (G15) 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 *Tribolium 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 intra-strain variability in beetle susceptibility to parasites. The two strains have been reared in the laboratory for over 15 years. The origin of the two strains and their F1 and F2 segregation populations have been published (ZHONG et al. 2003; ZHONG et al. 2005). The first population was generated from pairwise mating between a *TIW1* male and a cSM female and an F1 intercross (as cross 1). The second was from pairwise mating between a cSM male and a *TIW1* female and an F1
intercross (as cross 2). Two AIL G₁₅ (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 (25mm×95mm) containing about 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°C 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, North Carolina, USA). Carolina has maintained rats infected with *H. diminuta* for the past 40 years. In order 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 COI gene sequence were identified with one to two mutations for the 957bp PCR fragment (GenBank access no. KC990401-KC990403) and seven unique ITS2 sequences were detected with three microsatellite polymorphic regions for the PCR fragment 756~769bp (GenBank access no.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 linkage group 3 (*LG3*) (*hds*[3, *L1B1.69*]), *LG6* (*hds*[6, *L1A6.141*]) 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) were used to screen for polymorphism between *TIW1* and *cSM* parent strains surrounding the three
QTL regions of approximately 10MB. 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/prime3/). The sequences of forward and reverse primers are listed in Table S1. Marker polymorphism was screened using the method described previously (ZHONG et al. 2004; ZHONG et al. 2006), and the Li-Cor Model 4300 automated DNA analyzer (Li-Cor, Lincoln, NE). The Gene ImagIR 4.33 software (Licor) 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; ZHONG et al. 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, Lincoln, NE). Genotypes were evaluated manually and double-checked by the same individual.
Double-stranded RNA (dsRNA) 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 *) for double-stranded RNA synthesizing and the size of the dsRNA are listed in Table 1. Total RNA was extracted from cSM and TIWI adult beetles. The dsRNA of genes on LG3 and LG8 were synthesized from the total RNA extract from the TIWI 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 TIWI 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°C 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 TIWI 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 *TIWI* beetles and 20 susceptible *cSM* beetles (two 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 polyA⁺ 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 iScript™ 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 cDNA sequences obtained from Genebank. 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 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 (CIs) for QTL were defined as the region within 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 G15 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 pair-wise Student’s t-tests to detect 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 TIWI parental and the resultant F1 progeny were reported previously, with the cSM and F1 populations being significantly more susceptible to tapeworm parasite infection than the TIWI (ZHONG et al. 2003; ZHONG et al. 2005). In the current study, the individuals of two G15 AIL populations were exposed to feces obtained from H. diminuta infected rats. A total of 400 G15 AIL beetles in each cross were infected with tapeworm parasites. The average infection intensity of Cross 1 AIL populations was 5.3 ± 0.7 (range 0-28; n = 400) and 3.85 ± 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 (DEMUTH et al. 2007; ZHONG et al. 2004), were used to screen for polymorphism between cSM and TIWI parent strains. Of the 42 markers previously reported on LG3, nine 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, ten of which 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 S1 A and Table S1). The QTL region was narrowed to a genomic region of approximately 0.13 cM (46kb) within a 95% CI. Based on the Tribolium genomic database, this region contains four predicted genes, including a candidate gene, cytochrome P450 (Genbank acc. XM_963914) (Figure S1 A), 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), nine markers identified polymorphisms in the parental strains, and 11 new markers were then developed which indentified 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 S1 B and Table S1). The QTL region was narrowed to a genomic region of approximately 0.55 cM (192kb) within a 95% CI. Based on the Tribolium genomic database, this region contains 11 predicted genes of unknown function (Figure S1 B).

**High resolution mapping of the QTL(hds[8, \textit{L6B2.100}]) region on Linkage Group \textit{LG8}**

A 9.93 cM (~ 3.48 Mb) region of the QTL \textit{LG8} (hds[8, \textit{L6B2.100}]) was selected for high resolution mapping. Of 20 markers previously reported in the region (DEMUTH \textit{et al.} 2007), seven markers identified polymorphisms in the parental strains, and eight newly developed markers indentified additional polymorphisms (Table S1), resulting in a total of 15 markers which were used to genotype the two AIL populations. 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 S1 C and Table S1). The QTL region was narrowed to a genomic region of approximately 0.70 cM (245kb) with a 95% CI. This region contains 12 predicted genes, including a candidate gene, Toll-like receptor 13 (TLR 13, GenBank acc. XM_968248) (Figure S1 C), which has been implicated in innate immunity and endotoxin susceptibility in mice (MISHRA \textit{et al.} 2008; ROACH \textit{et al.} 2005; Shi \textit{et al.} 2009).
Gene expression differences between resistant and susceptible strains

The transcription profiles of genes associated with the loci identified in the resistant TIWI and susceptible cSM strains (two 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 eight genes on LG8 (Table 1, Figure 2). The range of expression level between the resistant and susceptible strain were 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 out of the 21 selected genes showed significant differences in gene expression (P < 0.05) between resistant and susceptible strains. The resistant TIWI 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 over dominance of QTL hds[3, L1B1.69] on LG3 and hds[8, L6B2.100] on LG8 and under dominance or recessive of QTL hds[6, L1A16.141] on LG6 in resistant TIWI strain (ZHONG et al. 2003; ZHONG et al. 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 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 as 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 susceptible strains (Table 3). Both genes (LOC657454 and LOC100141631) on LG3 had significantly reduced transcription (10-13 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 knock down effect (3-9 fold changes, P < 0.01). The TLR 13 gene (LOC662131) had the highest gene knock down 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 to genome regions of approximately 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 (CYPIXF2), a family of enzymes involved in insecticide activation and detoxification. The QTL on LG8 contain gene toll-like receptor 13 (TLR 13), 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 over-expressed in the insecticide resistant phenotype,
causing enhanced metabolic detoxification of insecticides (Feyereisen 2005; Zhu et
al. 2008). In *Tribolium*, more than 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 greater than 200-fold increase in
expression in the deltamethrin-resistant QTC279 strain, when compared with a
deltamethrin-susceptible Lab-S strain.

In our current study, we found that the resistant *TIW1* strain had significantly
higher transcription of CYPIXF2 (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 (PRRs) 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 Lemaître 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 (Aronstein and Saldivar 2005; Imamura and Yamakawa 2002; Tauszig et al. 2000). It has been estimated that most mammalian species have between 10 and 15 types of Toll-like receptors (Roach et al. 2005). Insect Toll receptors 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 (TLR 1-4, TLR 6-10) that encode Toll and Toll homologs. Zhang et al. (2004) demonstrated that TLR11 recognizes urinary pathogenic *E. 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 TLR 13 (MISHRA et al. 2008). In our study, the TLR 13 gene of the resistant TIW1 strain exhibited a significantly higher TLR 13 mRNA level than the susceptible cSM strain in the absence of parasite infection. RNAi analysis showed that injection of dsRNA corresponding to the TLR 13 gene sequences (LOC662131) resulted in a significant gene knock down effect in the resistant TIW1 strain, as evidenced by the presence of a significantly higher number of parasites than the buffer injected controls. Therefore, these data strongly suggest the role of TLR 13 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 (ARONSTEIN and SALDIVAR 2005; BEERNTSEN et al. 1995; MORLAIS et al. 2003; SEVERSON et al. 1995; SEVERSON et al. 1999) 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 Tribolium miRNAs exhibited gender-specific expression patterns upon exposure to environmental. 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 Advanced Intercross Lines and high density molecular markers. Two genes were identified as candidates for resistance genes to H. diminuta infection (CYPIXF2 and TLR 13) using transcription analysis and RNA interference. 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.
Acknowledgments

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Table 1. Genes selected for RNAi assay. Locus name, location, product size and primer sequence of qRT-PCR of the candidate genes in the QTL regions in *Tribolium castaneum*.

<table>
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<th>No.</th>
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<th>LG</th>
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<th>Gene name</th>
<th>Forward primer</th>
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<td>3</td>
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<td>TGGATTCCATCCCTGATGAT</td>
<td>AGCTCTGAGCCACTCTCCAG</td>
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<td>ACTCGCTGAAAGACCGAAAAA</td>
<td>AAGTTGCTTCTCGGCTTTG</td>
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</tr>
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<td>3</td>
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<td>similar to CG5547-PD, isoform D</td>
<td>GGCTTTTTGATCTCTTCCAC</td>
<td>TCAAACTCCAGCTTCTCTT</td>
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<td>8</td>
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<td>CTGTTTTCAGTCCCAGTTTC</td>
<td>CAGAAAGGAAAAAGGTAGG</td>
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<td>CAGAACTGTCCAGATGCTGA</td>
<td>ATGGGGTCTTGTGTTGTGT</td>
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<td>similar to CG2052-PB, isoform B</td>
<td>CACATCTCAAGAAACCAGCA</td>
<td>ATGGGGTCTTGTGTTGTGT</td>
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<td>CCGAAACTAACAACCGACCA</td>
<td>GCACCAATGAAACACATCG</td>
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<td>CTTTGTGCTCTCTTCTTC</td>
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<td>TAACCAGGACGAGCAATACCC</td>
<td>AGTTGGCCGTGATATGTAT</td>
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<td>14</td>
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<td>11.0</td>
<td>similar to toll-like receptor 13</td>
<td>AGGTTGGCTGACATGTTGGA</td>
<td>CTCGAGGTGCTATTCTTGT</td>
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<td>17</td>
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<td>8</td>
<td>11.2</td>
<td>similar to CG17839-PB, isoform B</td>
<td>CTGTTGCTTGGGCCCTTTG</td>
<td>GTTCTCCAGCTGTACCTC</td>
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<td>11.4</td>
<td>similar to CG8503-PA</td>
<td>AGAAGCAGTCTTCTCGCCAAA</td>
<td>TCCTGTTCCATTGACCTGAA</td>
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<td>11.8</td>
<td>similar to Myeloid leukemia factor (Myelodysplasia-myeloid leukemia factor) (dMLF)</td>
<td>ACCGATGAGCACAATCTCCTT</td>
<td>TAATACCGACAGCTGTTG</td>
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<td>8</td>
<td>12.0</td>
<td>similar to Glyceraldehyde-3-phosphate dehydrogenase II (GAPDH II)</td>
<td>CAAAGTTATCCCGGTCTTGA</td>
<td>AAATCGACGAGCTGCTATCC</td>
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<td>12.1</td>
<td>similar to Endothelin-converting enzyme 2 (ECE-2)</td>
<td>TTTTTACCAGCTTCTGCTT</td>
<td>AAAATCAATTCGCGTCTT</td>
<td>237</td>
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</tbody>
</table>
*The dsRNA of genes on LG3 and LG8 were synthesized from the total RNA extract from TIW1 strain (dsRNA-T), while the dsRNA of genes on LG6 were synthesized from the total RNA extract from the cSM strain (dsRNA-C).
Table 2. RNAi effect on parasite infection intensity. Student’s t-test was performed to compare the difference.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment (injection)</th>
<th>LG</th>
<th>N</th>
<th>Infection intensity (Mean ± SE)</th>
<th>Significance*</th>
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<tbody>
<tr>
<td>TTIWI</td>
<td>dsRNA-T</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>LOC657454 (dsRNA)</td>
<td>3</td>
<td>44</td>
<td>5.30 ± 0.93</td>
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<tr>
<td></td>
<td>LOC662131 (dsRNA)</td>
<td>8</td>
<td>44</td>
<td>4.48 ± 0.60</td>
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<tr>
<td></td>
<td>LOC100141631 (dsRNA)</td>
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<td>38</td>
<td>2.42 ± 0.40</td>
<td>B</td>
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<tr>
<td></td>
<td>LOC662348 (dsRNA)</td>
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<td>40</td>
<td>1.85 ± 0.41</td>
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<tr>
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<td>LOC662235 (dsRNA)</td>
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<td>1.58 ± 0.32</td>
<td>BC</td>
</tr>
<tr>
<td></td>
<td>Control (injection buffer)</td>
<td>48</td>
<td></td>
<td>0.60 ± 0.15</td>
<td>C</td>
</tr>
<tr>
<td>cSM</td>
<td>dsRNA-C</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>LOC663441 (dsRNA)</td>
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<td>43</td>
<td>7.74 ± 1.03</td>
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<tr>
<td></td>
<td>LOC663587 (dsRNA)</td>
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<td>7.91 ± 1.10</td>
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<tr>
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<td>Control (injection buffer)</td>
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<td>8.60 ± 1.07</td>
<td>A</td>
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</tbody>
</table>

Note: LG, linkage group; N, Number of beetles
* RNAi effect on parasite infection. Treatments not connected by same letter are significantly different in mean infection intensity.
Table 3. RNAi effect on gene expression. The gene expression was compared between RNAi and control groups. ANOVA analysis for significance was listed.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Locus name</th>
<th>LG</th>
<th>Expression changes (RNAi to control)</th>
<th>ANOVA (P value)</th>
</tr>
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<tbody>
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<td>TIWI</td>
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<td>-13.21</td>
<td>0.0002</td>
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<td>LOC662131</td>
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<td>-9.49</td>
<td>0.0008</td>
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<td>LOC100141631</td>
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<td>-10.31</td>
<td>&lt;.0001</td>
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<td>0.0006</td>
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<td>-15.00</td>
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<td></td>
<td>LOC663602</td>
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<td>-5.43</td>
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</tbody>
</table>
Figure legends

Figure 1 Fine mapping QTL associated beetle susceptibility to tapeworm parasite in advanced intercross line (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 *Tribolium castaneum* genome database at [http://www.ncbi.nlm.nih.gov/genome/guide/beetle/index.html](http://www.ncbi.nlm.nih.gov/genome/guide/beetle/index.html).

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

**Supplemental 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*.

**Supplemental 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.

**Data deposition:** Raw genotype and phenotype data used for QTL analysis are deposited in the Dryad Repository: http://dx.doi.org/10.5061/dryad.bk90m
LOD Linkage Group (LG)

Figure 1
Figure 2

Fold changes

<.05  *
<.0001**