A Molecular Approach for the Detection and Quantification of *Tribolium castaneum* (Herbst) Infestation in Stored Wheat Flour

Running Title: qRT-PCR based detection of *Tribolium castaneum* (Herbst) in wheat flour

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SUMMARY

*Research background.* The presence of insect fragments is one of the major constraints in stored food commodities and cause considerable loss in the quality of the produce. The management of the pest is viewed as a huge challenge in food processing industry. Conventionally, the detection of *T. castenaum* in the food processing industry is carried out by acid hydrolysis and staining methods which are not precise and time consuming.

*Experimental approach.* Considering the importance of a quick and effective method, a qPCR-based approach was developed and elucidated in this study. The mitochondrial cytochrome oxidase I (mtCOI) gene was identified as a target due to the abundance in the pest. Specific primers were designed against the target gene by primer premier software and amplified in a qPCR.
Results and conclusions. This developed method is capable of detecting all the ontogenic stages of *T. castaneum* in stored wheat flour. Earlier experiments had demonstrated that about 20 µg of DNA can be obtained from 2.2 mg of insects, to quantify the infestation levels the Ct values obtained from known samples were subjected to regression analysis and expressed as adult equivalents. In the unknown samples tested, the infestation was calculated as 1.74 and 0.046 adult insects in 5 g of wheat flour. The maximum permissible limit of insect fragments in flour is 75 insect fragments or approximately 3 adults per 50 g of flour as per FDA. Hence, by adopting this new method, it is possible for the warehouse operators to arrive at a decision to proceed with efficient management practices were wheat flour is stored. Also, this method can be ratified by government agencies associated with international business to ascertain whether the wheat flour meets the standards set by the respective country before subjecting to foreign trade.

Novelty and scientific contribution. This study is the first of its kind in the detection and quantification for *T. castaneum* in milled products. So far only conventional methods are employed to assess the presence of the pest and manual counting of fragments are practiced to quantify the infestation levels. The developed qPCR method is faster, reliable and can be employed in milling industries, Bakery industries, food processing plants and foreign trade units for critical detection and quantification *T. castaneum* pest infestation.

Key words: insect detection, wheat flour, *T. castaneum*, qRT-PCR, DNA, red flour beetle

INTRODUCTION

Wheat is one of the major cereal crops cultivated in India and the production of wheat in India has reached an all-time high of 99.70 million tonnes in the year 2019 (1). Globally India ranks second in the production of wheat, despite the total post-harvest losses of about 33.5 % in stored wheat and wheat flour (2). The major issue is the presence of insects that is responsible for 5-15 % loss during storage (3). *T. castaneum* is one of the major pests infesting wheat flour after post-harvest processing. Even after high standards of sanitary and handling procedures are practiced, the flour is susceptible to be infested by stored pests (4).

The adults of *T. castaneum* are long-lived, often up to 3 years and undergo complete metamorphosis during its life cycle. The female insect lays up to 300-500 eggs during its life span. Complete life cycle of the insect ranges from 7-12 weeks depending on temperature (35 ºC) and relative humidity (60-80 %) (5). Larva and adults are the active feeding stages of *T. castaneum* and
cause significant damage to food commodities. During post-harvest handling and storage process, the grains seeds are often broken or wounded by other internal feeders, which make it easier for the red flour beetle to be infested (6). The pest infestation causes severe damage to the products, like contamination with faeces (mainly uric acid) that increase the humidity of the flour, promoting hot spots for fungal or mould growth. Besides the contamination with the body fragments and frass, the flour is also exposed to quinones that are secreted by the adult insects as a defence mechanism (7). These quinones are responsible for the unpleasant odour and are known to induce liver and spleen tumours, when tested in rats and other vertebrates (8,9). These quinones also affect humans by causing several complications like jaundice, anaemia, allergic responses etc. Owing to the infestation, the product quality and marketability of the flour is greatly reduced, resulting in the huge economic loss.

In the food industry, the quality and integrity are considered as important criteria for the successful export/import of any produce in the market. The insects present in the broken wheat, get crushed during the milling process and thereby the fragments are blended to the flour and ultimately affect the quality of the flour. In European countries there are no systematic surveillance or any scientific risk assessment programs on storage pests, albeit a zero or nil tolerance for insect or insect fragments has been established in food and food products (10,11). In USA, the Food and Drug Administration (FDA) has a defect action level of 75 insect fragments per 50 g of wheat flour (12), whereas in Canada the defect action level should not exceed more than 25 fragments per 50 g of flour (13).

Currently, various techniques are employed to detect insect and its fragments in stored food commodities are based on (i) sieving, (ii) physical examination, (iii) microscopic methods such as staining and acid hydrolysis, (iv) Enzyme-Linked Immunosorbent Assay (ELISA) (14,15), (v) Near Infrared spectroscopy (NIR) (16) and (vi) filth flotation (17), (vii) soft X-rays (18-22). Although some of these methods are effective for detecting a particular stage of T. castaneum during infestation, yet none of these can detect all the developmental stages of the insect. Notably, detection of T. castaneum at initial stages of its life cycle is difficult. Due to stringent regulations followed by the government during import and export, it is imperative that the flour should be devoid of any insect or insect fragments before subjected to foreign trade. Hence, there is an urgent need to develop a new methodology to detect and quantify T. castaneum infestation at all ontogenic stages present in wheat flour. To the best of our knowledge no study has been conducted utilizing qPCR for identifying insect infestation in stored wheat flour. This is the first of its kind study to use a qPCR technique that
specifically amplifies a portion of mtCOI (mitochondrial cytochrome oxidase) gene to successfully check and assess T. castaneum infestation in stored flour.

MATERIALS AND METHODS

Insect specimen collection and rearing

Major stored-grain pests namely, T. castaneum, Sitophilus oryzae and Lasioderma serricorne cultures were maintained at Indian Institute of Food Processing Technology (IIFPT), Primary processing, storage and handling laboratory; Thanjavur, India. The cultures of S. oryzae were maintained on whole wheat and T. castaneum and L. serricorne in wheat flour at 30 °C and 70 % relative humidity (RH). All stages of the insects’ viz., egg, larva, pupa and adults were maintained separately for the experimental study.

Staining of T. castaneum eggs and fragments

The staining of T. castaneum eggs was done by Iodine method as per the standard procedure described in American Association of Cereal Chemists (23). About 50 g of T. castaneum infested wheat flour was mixed with 500 mL of 5 % HCl (Hi-Media, Nashik, India) and light mineral oil. The sample was boiled for 10 min with constant stirring to ensure complete digestion. The digested sample was transferred to a separating funnel and allowed to stand at room temperature until phase separation was clearly visible. The lower layer was drained off leaving about 2.5 cm of interface. Subsequently, the sample was then washed three times with hot water followed by phase separation in a separating funnel. After the final wash, the sample was filtered through a lined filter paper and examined under a microscope (Leica microsystem Leica S8AP0, CMS GmbH stereo-zoom microscope LAS version 3.8.4, Singapore) (24). The insect fragments present were counted and pictured.

Insect DNA extraction procedures

About 30 mg of T. castaneum (egg, larva, pupa and adults) and adults of L. serricorne and S. oryzae, were ground in liquid nitrogen using a clean glass rod to make a fine powder. DNA isolation from egg, larva, pupa and adults were carried out using HiPurA™ (insect DNA Purification Kit MB529, Hi-Media, Nashik, India) as per manufactures instructions. Finally, the total DNA was eluted in 200 µL of elution buffer (10 mM Tris-Cl, pH=8.5) by brief centrifugation (Tehtnica, Centric 200R, Železnik, Slovenia) at 16128 g. Efficiency and integrity of the isolated DNA was checked by agarose gel electrophoresis and the purity was analyzed spectrophotometrically.
(Shimadzu, UV 1800, Sapporo, Japan) by measuring the absorbance at \(\lambda_{260}\) nm and \(\lambda_{280}\) nm. The extracted DNA was stored at \(-20^\circ\text{C}\) until used for experiments.

**Wheat DNA isolation**

Wheat grains purchased from the market were screened to ensure that there was no visible infestation. Wheat grains were finely ground with a mortar and pestle. The powder was suspended in extraction buffer (100 mM Tris-HCl (pH=8.0), 50 mM EDTA (pH=8.0), 500 mM NaCl, 1% m/V SDS) (Hi-Media, Nashik, India) and vortexed for few minutes. The contents were allowed to settle and 3 M potassium acetate (pH=5.2) (Hi-Media, Nashik, India) was added, vortex and centrifuged (Tehtnica, Centric200R, Železnik, Slovenia) at 15700 g for 10 min. The upper layer was aspirated and pre-chilled isopropanol added to sample and incubated at -20°C. The pelleted DNA was centrifuged and the supernatant was discarded, followed by washing the pellet with ethanol twice and air dried. The pellet was dissolved in 100μL of TE buffer and analyzed by agarose gel electrophoresis (Bio-rad, Irvine, CA, USA) for consistency.

**Designing of Primer sequence and specificity analysis**

Specific primers were designed using primer premier software available online (Primer Primer 6, PREMIER Biosoft, Palo Alto, CA, USA) (25) to target *T. castaneum*, mitochondrial Cytochrome Oxidase I (*mtCOI*) gene; GenBank (26) accession number EU048277.1 and synthesised by Eurofins Genomics India Pvt. Ltd., Bangalore, India. Multiple sequence alignment was performed using Gene runner program (27) and a comparison of *mtCOI* gene between *T. castaneum*, *L. serricorne*, and *S. oryzae* was done to ensure the designed primers does not overlap with genes of other storage pests.

**Internal control/reference gene**

The Ct values of the target gene were normalized with Ct values of an internal control or reference gene. Here we used *T. castaneum* Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene as a reference for this study GenBank (26) accession number is XM_969088. The primer sequence for GAPDH used in the study is 5’-GGACGCCTACGACCCTTCAG-3’ and 5’-GTCATCAACCCTCCACAATCT-3’ for leading and lagging strands respectively (28) and synthesised by Eurofins Genomics India Pvt. Ltd., Bangalore, India.
PCR reaction

PCR amplification (Applied biosystem, Veriti™ 96-Well Thermal Cycler, Foster city, CA, USA) was performed in a reaction volume of 20 μL, containing 10 μL of 2X Master Mix (Aura, Chennai, India), 0.2 μM of forward and reverse primer and the template DNA at a concentration of 100 ng/reaction (29,30). The PCR conditions used for the amplification were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles consisting of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s and final extension at 72 °C for 5 min. The PCR products including non-target controls (NTC) were electrophoresed together on a 1.5 % agarose gel and along with 1Kb ladder. The gel was stained with ethidium bromide and the bands were visualized under UV light.

qRT-PCR reaction

DNA samples isolated from T. castaneum, L. serricorne and S. oryzae were amplified using Roche thermal cycler (Light cycler 96, Roche-Life science, Bavaria, Germany). qPCR Reaction mixture (20 μL) contained 10 μL fast start essential SYBR Green PCR Master Mix (Roche Diagnostics, GmbH, Indianapolis, IN, USA), 2 μL (0.2 μM) of each T. castaneum specific forward and reverse primers and 5 μL of DNA corresponding to 100 ng/reaction served as template for the study. The amplification conditions were followed as described in Amin et al. 2020 (31). Samples without template DNA was used as negative control and DNA isolated from adult of T. castaneum was used as a positive control.

Regression analysis for quantification of T. castaneum

Insect fragments of 10, 5, 2 and 1 were counted and added to 5 g of wheat flour that was equivalent to 10, 1, 0.1, 0.01 and 0.001 adult insects in 5, 50, 500 and 5000 g of wheat flour respectively. Seven different replicates of each sample with a different T. castaneum specimen were performed per addition that accounted for 42 samples. The DNA was isolated from the infested flour and amplified by qPCR as mentioned previously. The Ct values obtained for each infestation were subjected to regression analysis.

Analysis of defect action level of insect fragments in wheat flour

To examine the possibility of application of the developed method in unknown samples, wheat flour was purchased randomly from a commercial store (2 samples from a popular brand viz., Aashirvad, Chennai, India and Naga, Dindigul, India) and a local mill (3 samples) located in the city
of Thanjavur. The DNA extracted from the wheat flour was subjected to qPCR analysis as described earlier.

Data analysis

Statistical analysis was performed using Graphpad Prism program (32) and two tailed t-test was carried out for all stages of *T. castaneum* (egg, larva, pupa and adult), ANOVA was used to compare between the groups and values p<0.05 were considered as statistically significant. The Ct values obtained from qPCR were inversely proportional to the quantity of original template DNA. In order to obtain a regression for the quantification of insect infestation in wheat flour, a regression analysis was performed at 95% confidence level.

RESULTS AND DISCUSSION

Primer specificity and Multiple sequence alignment (MSA)

The main aim of this study was to develop a molecular technique to detect and quantify the presence of red flour beetle in wheat flour. qRT-PCR is a well known technique for rapid detection and quantification of *T. castaneum* based on gene expression in food samples. The mtCOI gene of *T. castaneum* is used as a target, due to its abundance and availability in the cells as multi-copies (33), thus greater sensitivity can be achieved if it were used as a candidate for identifying infestation. The primer for *mtCOI* gene was designed using primer premiere application with options set in the primer probe design and multiplex PCR mode. Several forward and reverse primers were generated from the program that were sorted for suitability based on the recognition site, the annealing site corresponding template sequence, the Tm value (melting temperature), GC ratio, no primer-dimer formation (self- annealing and self-complementarity) and no possibility of hairpin-loop formation. The primer sequence is, 5'-GGGCCCACCACATATTACCGT-3' complementary to leading strand to serve as forward primer and 5'-GAGTGCCGTGAAGAGTGGCT-3' complementary to lagging strand to serve as reverse primer (Fig. 1a).

As the stored wheat flour is prone to harbour many pests it becomes important to ascertain that the designed primer does not cross react with *mtCOI* gene of other stored pests. Hence, a multiple sequence alignment of *mtCOI* gene from all three major stored pest was carried out which indicated that the red flour beetle gene shares 75% and 43.4% homology with *S. oryzae* (internal feeder) and *L. serricorne* (external feeder) respectively. It was observed that *T. castaneum* primers could not anneal to *mtCOI* gene (Fig. 1b) of other major pests of wheat flour. Additionally, to reaffirm that the
primers does not cross react with closely related species within the same genus, MSA comparison was made between *T. castaneum* and *T. confusum*. The results indicated that the primer is specific only to *T. castaneum* (Supplementary Fig. S1) and thus it is proved that mtCOI gene is the ideal candidate for the present study Elbrecht *et al.* reported that ideal primer sets with optimal annealing temperatures play a significant role in minimizing the amplification bias among the taxa to maximize species recovery (34).

**Primer specificity and sensitivity**

To analyse the sensitivity of the primer and its ability to detect *T. castaneum* at all ontogenic stages, DNA was purified from egg, larva, pupa and adults that were subjected to PCR amplification (Supplementary Fig. S2). The results indicated that the sensitivity of the primer is high, that amplification is observed in DNA samples purified from different ontogenic states of *T. castaneum*, while no amplification is noticed in DNA sample from *L. serricorne* (Fig. 2 and Supplementary Fig. S3a and S3b). To increase the stringency level of detection and to avoid false positives, several qPCR assays were performed from DNA isolated from different sets of samples. The Ct values obtained were tight and single peak melt curve is observed after every run. In our study, samples with Ct value equal or above 30 for internal control or samples were considered as negative. The amplification curve appeared at 12th cycle for pupal stage at 15th cycle for egg stage, the Ct values for larva and adult were in between the two stages (Supplementary Fig. S4a). The amplification curves obtained appeared as a single bundle indicating that there was no off-target amplification at any stages of *T. castaneum* along the whole run. Melt curve analysis also showed specific melting temperature (Tm) 78.96±0.09 for all mtCOI amplified products of *T. castaneum* and 81.37±0.130 for GAPDH (Supplementary Fig. S4b and Table. 1). A single sharp peak was observed in melt curve of control sample and there were no non-specific products and primer dimer observed in the qPCR reaction. Similarly, no amplification was detected in negative samples as well as other non-target amplification in pest DNA revealing specificity of designed primer for real time assay. Previous studies on qPCR data analysis reveals that a single melt curve ensures that the signal detected is genuinely the target of interest (35).

**Quantification of *T. castaneum* infestation by regression analysis**

Serial dilutions of DNA from adult *T. castaneum* were mixed with wheat DNA at different concentrations ranging from 200 ng to 0.125 ng. We used 0.125 ng of DNA as the minimum detection level were Ct value above 30 were considered as undetectable or negative and further loss of linearity is also observed (Fig. 3a and 3b) (Table 2). To increase the reproducibility of obtained results in each
qPCR run, 200 ng of adult *T. castaneum* DNA was added as positive control for which the Ct value was around 16.8±0.02.

As the detection of *T. castaneum* may be done in DNA isolated from infested wheat flour the exact quantity of the insect DNA present in the total DNA might vary with samples. Our data indicated that a minute quantity of insect DNA is sufficient to observe the amplification. The observed Ct values were so tight (±1 Ct) for each stage of the insect that signifies the conformity of the developed method. Inter-stage comparison was analyzed statistically, that exhibited a significant change in the expression of *mtCOI* gene between all stages of the insect except for larva and pupa. We presume that there may be less developmental changes during the larval and pupal stages, which may be a factor that no significant change in the expression of *mtCOI* (36) was observed.

A standard curve was generated by using different concentrations of *T. castaneum* DNA for quantitative analysis in the wheat flour. Regression analysis was performed that revealed statistically significant relation between the DNA concentration (quantity / infestation dose) and the Ct values obtained (p<0.0016 and $R^2=0.883$) (Fig. 3c) for each concentration. There was an inverse correlation between the infestation and the Ct values, as the rate of infestation decreased, the Ct values increased (the inverse correlation is described by the equation for a slope):

$$y = mx + c,$$

Where $y$ stands for Ct value from adult insect and $x$ represent the insect infestation dose in wheat flour and $c$ is a constant. The details of the statistical analysis are presented in supplementary Table S1.

$$Y=2.817x +21.97$$

$$R^2= 0.883$$

Here $R^2$ is a goodness-of-fit measure for linear regression models. The regression curve was plotted using Ct values, which transform correspondingly into the adult equivalents by using a mathematical equation (Eq./2/). It is understood that the concentration of DNA directly correlates to the biomass of the insect, using this technique shall be used to quantify the infestation level present in wheat flour. As reported earlier, the mean fresh weight of adult *T. castaneum* is about 2.4±0.03 mg, for larva and pupa is 2.3 ± 0.06 mg and egg is about 0.00517±0.00075 mg (37). Our data indicated that 20 µg of DNA directly corresponds to 2.2 mg fresh body mass of *T. castaneum*. Based on regression curve generated, the Ct values were approaching 17 that represent approximately 10
adults, present in 5 g wheat flour. Similarly, the Ct value near 28 will represent 0.001 adult (Supplementary Table S2). Thus, the detection and identification with inverse prediction of the Ct values will help in most of the management decision for stored wheat flour. By using a regression curve, it is possible to determine the minimal defect action level of insect fragments present in unknown samples. Our studies are in-line with result reported in *Rhyzopertha dominica* infestation in rice (38).

Other confounding factors such as high gluten content and polysaccharides present in the wheat flour pose an impediment in the amplification of DNA by interfering with the activity of Taq polymerase in PCR reaction (39,40). Our result suggested that such confounding factors did not significantly affect the qPCR assay. The smallest level of infestation which can be detected by our method is 1 insect fragment in 5 kg of flour which is much lower than the permissible level allowed in countries like the USA and Canada as per the recommendation for minimum defect action level by FDA and CFIA (10).

**Authenticity of T. castaneum detecting in infested wheat flour**

To determine the reliability of the developed method a known number of fragments from *T. castaneum* were blended to a certain quantity of wheat flour. The flour was confirmed for the presence of fragments by staining according to the protocol described in AACC, 2010 (Supplementary Fig. S5). Then whole DNA was extracted from the flour containing the fragments and subjected to qPCR analysis. The results indicated that the developed method is sensitive enough to detect the presence of insect infestation at the level of one fragment in 5 g of wheat flour and the Ct value is 29.03±0.04. As the number of fragments increased, the Ct values decreased from 27.32, 24.27 and 13.25 corresponding to 2, 5 and 10 insect fragments respectively. It clearly indicated that this method is highly sensitive and able to detect one insect fragment in 5 g of wheat flour (Table 3 and Fig. 4a).

The developed method was challenged by testing five different blind samples, which were purchased from a local mill and a commercial store. The samples procured from the local mill showed positive results for the presence of insect fragment and the Ct values were 22.65, 25.72 and 32.33 respectively (Table 4, Fig. 4b) whereas the Ct values 30.70 and 30.63 were observed for samples belong to the popular brand. Correspondingly, the Ct values were translated to infestation levels by substituting in equation- Eq./2/. The infestation level was 1.74, 0.046 adults per 5 g of wheat flour for samples S1 and S2, while the rest of the samples exhibited negligible amounts. The amplified product (128bp) was ascertained by agarose gel electrophoresis. The method demonstrated in this study can
be adopted for the identification and / or quantification of *T. castaneum* infestation in an array of grains and flours such as almond, amaranth, barley, buck wheat, cassava, corn, garbanzo, millet, oat, potato, quinoa, rice, rye, sorghum, spelt and coconut (41). Thus, based on our method it is possible to differentiate between infested and non-infested samples with high sensitivity.

**CONCLUSIONS**

The developed qRT-PCR method is highly accurate and species specific that it detects only *T. castaneum* infestation in a given sample. There were no false positive results obtained when tested against a closely related species of the same genus *i.e.* *T. confusum*. Similar results were also obtained when tested with other stored pests such as *S. oryzae* and *L. serricorne*. To the best of our knowledge this is the first study to quantify *T. castaneum* infestation in stored wheat flour using qRT-PCR. Since the method is a rapid technique it can be adapted in warehouses to effectively manage the pest infestation. Further the method can be followed by governmental agencies responsible for import and export of commodities like wheat flour to determine if the commodity has infestation below the defect action level. Simultaneously, small sample size will help to analyze more samples from a single batch of stored flour.

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**CONFLICT OF INTEREST**

All authors of this manuscript declare there is no conflict of interest exists under financial or personal relationship that could possibly influence the work.

**SUPPLEMENTARY MATERIALS**

All supplementary material is available at: www.ftb.com.hr.
AUTHORS’ CONTRIBUTION

Aditi Negi has performed all the experiments, generated data, collected literature and wrote the first draft of the manuscript.

Arunkumar Anandharaj has formulated the concept of study, designed the experiments, reviewed and analysed the data.

Suresh Kumar Kalakandan has done the manuscript draft correction.

Meenatchi Rajamani performed the statistical analysis and supervision of the study.

All the authors read and approved the manuscript.

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REFERENCES


Fig. 1. Multiple sequence alignment of mtCOI gene: (a) The gene sequence of *T. castaneum* mtCOI gene and highlighted is the region of primer binding. (b) Alignment of mtCOI sequence from *Tribolium castaneum*, *Lasioderma serricorne*, *Sitophilus oryzae* available in GenBank (26) under accession numbers: EU048277.1, KU494128.1, and NC_030765.1 respectively. The alignment was done by Gene Runner program (27), that shows no similarity between the same gene of other stored pests, highlighted is the forward primer-binding region specific to *T. castaneum*.
Fig. 2. Electrophoresis of the PCR products on 1.5% agarose gel show amplification product (128 bp) using Primer mtCOI(G). Lane 1: 1.5 kb ladder, Lane 2: *T. castaneum* adults, Lane 3: *T. castaneum* pupa, Lane 4: *T. castaneum* larva, Lane 5: *T. castaneum* egg, Lane 6: *L. serricorne* adult as negative control.
Fig. 3. Quantification of *T. castaneum* infestation by regression analysis and Authenticity of *T. castaneum* detecting in infested wheat flour:

(a) Amplification curves of *T. castaneum* in qPCR reaction. Decreasing concentration of *T. castaneum* adult DNA (ng) from 200, 100, 50, 25, 12.5, 1.25 and 0.125 by serially dilution with
wheat DNA and to emulate number of insects can be detected by this is 10, 5, 2.5, 1, 0.1, 0.01, 0.001 number of insects in 5 g of wheat flour. All the insect samples entered logarithmic phase before 29 cycles of reaction. While wheat DNA used as negative control entered after 33 cycle of amplification. (b) Melt curve analysis of all serially diluted *T. castaneum* DNA show positive single peak for *T. castaneum* mtCOI at 78.96±0.09 °C. (c) Regression plot for *T. castaneum* adult DNA show significant correlation between log number of insects and Ct values in 5g of wheat flour (p<0.0016 and $R^2=0.883$)
Fig. 4. a. The result indicated a sequential increase in the concentration of amplified *mitCOI* gene corresponding to the concentration of insect DNA present in wheat flour. Lane, 1: 1.5 kb ladder; Lane 2: Amplified product from *T. castaneum* adult DNA (Positive control); Lane 3: wheat flour incorporated with 1 insect fragments; Lane 4: wheat flour incorporated with 2 insect fragments; Lane 5: wheat flour incorporated with 5 insect fragments; Lane 6: wheat flour incorporated with 10 insect fragments; Lane 7: *S. oryzae* adult DNA as negative control.
b. DNA isolated from the 5g of wheat flour purchased from a commercial store and amplified with *mtCOI* primer. The qPCR products loaded in Lane1: 1 kb ladder; Lane 2: amplification product from *T. castaneum* DNA (Positive control); - Lane 3: local flour mill sample -1; Lane 4: local flour mill sample – 2; Lane 5: local flour mill sample – 3; Lane 6: commercial store flour sample – 4; Lane 7: commercial store flour sample – 5; Lane 7: *S. oryzae* adult DNA - as negative control

Table 1. Mean Ct values of all ontogenic stages of *T. castaneum*

<table>
<thead>
<tr>
<th>Name</th>
<th>Mean Ct value±SD</th>
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<tbody>
<tr>
<td></td>
<td>GAPDH</td>
</tr>
<tr>
<td>Egg</td>
<td>29.03±0.03</td>
</tr>
<tr>
<td>Larva</td>
<td>24.34±0.15</td>
</tr>
<tr>
<td>Pupa</td>
<td>22.97±0.23</td>
</tr>
<tr>
<td>Adult</td>
<td>26.37±0.17</td>
</tr>
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</table>

Table 2. Ct values obtained for *T. castaneum* in 5 g of wheat flour for regression analysis

<table>
<thead>
<tr>
<th>Sample</th>
<th>DNA conc(ng)</th>
<th>Insect equivalent</th>
<th>Ct value±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>10</td>
<td></td>
<td>16.8±0.02a</td>
</tr>
<tr>
<td>100</td>
<td>5</td>
<td></td>
<td>19.96±0.11a</td>
</tr>
<tr>
<td><em>T. castaneum</em> adult DNA diluted with wheat DNAs</td>
<td>50</td>
<td>2.5</td>
<td>21.67±0.09a</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>1</td>
<td>22.73±0.23a</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>0.1</td>
<td>26.95±0.17a</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>0.01</td>
<td>27.87±0.32a</td>
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<tr>
<td></td>
<td>0.125</td>
<td>0.001</td>
<td>28.84±0.08a</td>
</tr>
<tr>
<td>Wheat DNA</td>
<td>108</td>
<td>NA</td>
<td>34.44±1.22</td>
</tr>
</tbody>
</table>

*a* Significant difference between all *Tribolium* DNA conc. verses wheat DNA *p*<0.01 level of significance. Each value is represented as mean ± SD (n = 3). NA – Not available
Table 3. Ct values for the detection of insect fragment in wheat flour by qPCR reaction with primer specific for *T. castaneum*

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Mean Ct values ±SD</th>
<th>Threshold samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. castaneum Std.</td>
<td>18.42 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>T1 (1 insect fragment)</td>
<td>29.03 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>T2 (2 insect fragments)</td>
<td>27.32 ± 0.42</td>
<td></td>
</tr>
<tr>
<td>T3 (5 insect fragments)</td>
<td>24.27 ± 0.32</td>
<td></td>
</tr>
<tr>
<td>T4 (10 insect fragments)</td>
<td>13.25 ± 0.41</td>
<td></td>
</tr>
</tbody>
</table>

Each value is represented as mean ± SD (n = 3).

Table 4. Ct values for qPCR reaction with primer specific for *T. castaneum* in the samples obtained from a local mill and a commercial store

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Mean Ct values ±SD</th>
<th>Insect numbers in terms of adult equivalent / 5g of flour</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1 (mill-1)</td>
<td>22.65 ± 0.18</td>
<td>1.74</td>
</tr>
<tr>
<td>S2 (mill-2)</td>
<td>25.72 ± 0.00</td>
<td>0.046</td>
</tr>
<tr>
<td>S3 (mill-3)</td>
<td>32.33 ± 0.33</td>
<td>0.0002</td>
</tr>
<tr>
<td>S4 (local store-4)</td>
<td>30.70 ± 0.21</td>
<td>0.0007</td>
</tr>
<tr>
<td>S5 (local store-5)</td>
<td>30.63 ± 0.06</td>
<td>0.0007</td>
</tr>
</tbody>
</table>

Each value is represented as mean ± SD.
SUPPLEMENTARY MATERIALS

**Fig. S1.** Multiple sequence alignment between closely related species *T. castaneum* and *T. confusum* show no species similarities exist in *mtCOI* gene.
Fig. S2. *T. castaneum* egg stained by iodine method (a), Larva (b), Pupa (c) and Adult (d)
Fig. S3. qPCR-based amplification as represented as a Bar diagram showing no amplification in Negative control (NC). (a) L. serricorne and (b) S. oryzae
Fig. S4. qPCR analysis of *T. castaneum* with *mtCOI* and GAPDH primers

(a) Analysis of the qPCR with *mtCOI* primer, GAPDH served as an internal control for normalization of data with all stages of *T. castaneum* (egg, larva, pupa, adult) and adults of *L. serricorne* was used as a negative control. All positive reactions amplified in logarithmic phase before 29 cycles for GAPDH gene (orange) and 16 cycle for *mtCOI* gene (blue). All reactions show single melting peak and no amplification was observed for negative control.

(b) Melt curve analysis of *T. castaneum* DNA with *mtCOI* and GAPDH primers positive single peak obtained for *T. castaneum* with both the primers.
Fig. S5. *T. castaneum* fragments identification in wheat flour by acid hydrolysis method
Table S1. Data obtained from three independent experiments were fed in to Graphpad prism for regression analysis. Correlation has been obtained for the Ct value with the corresponding infestation dose. The slope and intercept values and statistical parameters such as best fit, R2, p value and confidence interval were taken for consideration. The final equation derived from the above analysis is used for the calculation of infestation density present in the flour in terms of adult equivalents.

<table>
<thead>
<tr>
<th>Best-fit values</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope</td>
<td>-2.817 ± 0.4566</td>
</tr>
<tr>
<td>Y-intercept</td>
<td>21.97 ± 0.6825</td>
</tr>
<tr>
<td>X-intercept</td>
<td>7.802</td>
</tr>
<tr>
<td>1/Slope</td>
<td>-0.355</td>
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</tbody>
</table>

**95% Confidence Intervals**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>Slope</td>
<td>-3.990 to -1.643</td>
</tr>
<tr>
<td>Y-intercept</td>
<td>20.22 to 23.73</td>
</tr>
<tr>
<td>X-intercept</td>
<td>5.295 to 13.82</td>
</tr>
</tbody>
</table>

**Goodness of Fit**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>R square</td>
<td>0.8839</td>
</tr>
<tr>
<td>Sy.x</td>
<td>1.675</td>
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</tbody>
</table>

Is slope significantly non-zero?

<p>| | |</p>
<table>
<thead>
<tr>
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<th></th>
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</thead>
<tbody>
<tr>
<td>F</td>
<td>38.06</td>
</tr>
<tr>
<td>DFn,DFd</td>
<td>1,5</td>
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<tr>
<td>p Value</td>
<td>0.0016</td>
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</tbody>
</table>

Deviation from horizontal?

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Significant</td>
<td></td>
</tr>
</tbody>
</table>

Data

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of XY pairs</td>
<td>7</td>
</tr>
</tbody>
</table>

**Equation**

\[ Y = -2.817X + 21.97 \]

Table S2. Frequency of DNA detection at different insect densities in 5 g of stored wheat flour

<table>
<thead>
<tr>
<th>Insect equivalents as adults</th>
<th>Number of T. castaneum beetles</th>
<th>Ct value</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>10 beetle/5 g</td>
<td>16.8 ± 0.02</td>
</tr>
<tr>
<td>1</td>
<td>1 beetle / 5 g</td>
<td>22.73 ± 0.23</td>
</tr>
<tr>
<td>0.1</td>
<td>1 beetle / 50 g</td>
<td>26.95 ±0.17</td>
</tr>
<tr>
<td>0.01</td>
<td>Corresponds to 1 beetle/ 500 g</td>
<td>27.87 ± 0.32</td>
</tr>
<tr>
<td>0.001</td>
<td>Corresponds to 1 beetle/ 5000 g</td>
<td>28.84 ± 0.08</td>
</tr>
</tbody>
</table>

Each value is represented as mean ± SD (N=7)