Revealing of Wheat Products Contamination With Flour Beetles *Tribolium* spp. by Molecular Technique

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**Abstract:** Molecular technique is an accurate, susceptible, and species-specific method for detection of stored-product pests. The closely related flour beetles, *Tribolium* spp.; red flour beetle, *Tribolium castaneum* and confusion flour beetle, *Tribolium confusum* cause extensive economic loss of wheat grains and its products due to the contamination with their fragments and toxic secretions. PCR (Polymerase Chain Reaction) has been used successfully to detect DNA insect fragments in different commercial wheat product samples. Specific primers were designed to amplify elongation factor 1-alpha (EFA1) and beta-tubulingenfor detection of *T. castaneum* and *T. confusum* DNA, respectively. Five types of flour have been examined and two types of local biscuits were tested as final wheat products. The results showed that the two primers, which designed for amplification of the two flour beetles DNA, are effective, sensitive, and species-specific for detection of the insect fragments in all wheat products. It was a pity that bands of the DNA of the two beetles appeared in all types of tested product samples. The contamination with these secondary pests, especially with *T. confusum*, might be started in the early stage of storing whole grains before milling process.

**Keywords:** *Tribolium castaneum*, *Tribolium confusum*, Primers, PCR, Wheat Flour, and Biscuits

I. Introduction

Wheat flour is the most nutrition plant food. It is an essential component in home baking and almost every commercially baked product and pasta. Wheat is distinctive in the production of flour, it is the only cereal grain with sufficient gluten content to make a typical loaf of bread without being mixed with other grains (Kumar et al., 2011). The insect pest that attack the wheat grain are crushed during milling. The result is contamination of wheat products with insect fragments and their toxic secretion (Flinn et al., 2004). *T. castaneum* and *T. confusum* are the two main pests that attack flourmills (Sinha and Watters, 1985). The two beetles cause extensive bad quality of the flour and make it unacceptable to the consumer. Consequently, cause huge economic losses (Hill, 2002). Lakshmi Kantha et al. (2010) recommend that the two *Tribolium* spp. are vectors of many important pathogens. In addition, the *Tribolium* spp. beetle's defensive prothoracic and abdominal glands, which secret benzoquinones (Blum, 1981 and Howard, 1987). Certainly, insect fragments and toxic in flour are unpalatable to consumers and cause many harmful effects to humans (Toews et al., 2007). These toxic secretions are causing carcinogenic effects in animal cells (El-Mofy et al., 1988; 1989 and 1992). However, flour beetles produce large quantities of a dust that prevents seed respiration, and lead to increase the temperature and moisture subsequently, the growth of mycotoxic fungi, bacteria and mites (Lis et al., 2011). Consequently, the food safety and health protection not only required pest control management, but also detection of dead pest bodies, eggs and fragments in the whole grain and its products. The traditional methods are not accurate in detecting in these insect parts (Balasubramanian et al., 2007).

DNA fingerprinting is allegically accurate technique that widely used for identification of organisms (Peng et al., 2003; Saez et al., 2004 and Balasubramanian et al., 2007). The acceptable level of insect fragment in USA is 75 insect fragments per 50 g of wheat (FDA, 1988), but this level is often higher than US mills will tolerate (Flinn and Hagstrum, 2001). Conversely, in Canada, the number of fragments in wheat flour should be less than twenty fragments in samples of 50 g (Bhuvaneswari et al., 2011). Similarly, there is zero or near zero tolerance to insect fragments in food in most European countries (Tremattera et al., 2011). The short life cycle and ease of rearing and maintenance of flour beetle has made this species as an ideal model for studying the genetic information (Haas et al., 2001 and Lorenzen et al., 2002). The aim of our study has been to detect the DNA pattern of *T. castaneum* and *T. confusum* in different types of wheat products by using PCR mechanism. This is of great our concern in this research to serve as an early warning for taking appropriate control measures, for the hygiene conditions of milling products and popular health.

II. Materials and methods

Insect cultures:

*T. castaneum* and *T. confusum* (Family: Tenebrionidae) were collected from stored flour. Then maintained in the laboratory of Entomology Department, Faculty of Science, Benha University, on a mixture...
of wheat flour and brewer’s yeast (95:5 wt:wt). The cultures were grown in two-liter glass jars with vented lid and maintained in an incubator at 30°C and 70% relative humidity.

**Wheat product samples:**
Five types of flour have been examined. Three samples were randomly selected from markets (high quality, all-purpose, and semolina flour). The fourth sample, subsidized flour, was obtained from the bakery. The fifth sample (whole-wheat flour) was obtained from the Institute of the Wheat Research Center Giza as whole-wheat grains and milled in the lab. Furthermore, two types of local biscuits (sample 1 and 2) were selected randomly from markets as final food products of wheat. All products were free from any visible insects or insect fragments.

**Insect DNA extraction:**
The positive controls of the *T. castaneum* and *T. confusum* were prepared by extracting DNA from pure adult insect samples. The insects were cleaned properly by sieving to avoid any mixture of flour or wheat attached to insects and washed with distilled water. We used the Wizard genomic purification kit (Promega, Madison, WI) according to Beeman and Brown (1999) for the DNA extraction of pure insects as a positive control. Samples of the two *Tribolium* spp. insects were ground by using mortar and pestle. 1200 µl of the chilled nuclei lysis solution was added to the samples, then transferred to a sterile 1.5 ml centrifuge tubes. Lysate tubes were incubated in water Bath for 15-30 minutes at 65°C. 200 µl protein precipitation solution was added to precipitate protein at room temperature. Samples were shaken by vortex at high speed for 20 seconds and chilled on ice for 5 minutes. Samples were also centrifuged for 4 minutes at 14000 xg. The precipitated protein formed a tight white pellet. The supernatants containing DNA were carefully removed and leaving the protein pellet behind. The samples were transferred to clean 1.5 ml centrifuge tubes containing 600 µl isopropanol and gently mixed at room temperature. White threadlike strand of DNA formed to visible mass after the inversion of every solution. Centrifugation for 1 minute at 14000 xg at room temperature was performed. The DNA had been visible as a small white pellet. Supernatants were carefully decanted. The DNA pellets were washed by adding 600 µl of 70% ethanol. The samples were centrifuged one more time for a 5 minute at 14000 xg at room temperature. Ethanol was drawn away from DNA pellets by using a Pasteur pipette. The tube was inverted on clean absorbent paper and air dried the pellets for 10-15 min. 50 µl of DNA extractions were added to rehydration solution. DNA samples were rehydrated by incubation at 65°C for 1 hr. The DNA of the two insects was stored at 2-8°C. Gel electrophoresis was performed for 30 minutes after putting the loading dye. The quality of DNA was observed by running it through the gel.

**Extraction of insect DNA from wheat product samples:**
500 mg of each tested flour and ground biscuit samples were used to detect the contamination of wheat products with DNA of the two flour beetles. The same kit, the Wizard genomic purification (Promega, Madison, WI) was used with the same method as previously described. Because of the rigidity of plant cell wall, 1400 µl the plant cell need more lysis buffer solution (Moňková P., Vytřasová J. 2011). 1400 µl of lysis solution was added to all samples instead of 1200 µl as previously described in extraction of DNA from insects.

**Primers**
Insect nuclear primers were designed from GenBank with accession numbers, AY819656 for *T. Castaneum* and AY819657 for *T. confusum*. Life Technologies Company, England, procured these primers from Invitrogen. The two primers were designed to amplify the protein-coding gene, elongation factor 1-alpha (5’ to 3’) CCCAGTC ACTCCAT GATGTT and T TCAGCGACTTGACTTCAG were specific for *T. Castaneum*, and beta-tubulin intron GCTGGACCGTATCA TGTCTAC and GTCGAC TCTTCTGCTTCT were specific to *T. confusum*.

**Thermal cycler**
The amplified PCR product was purified with a TC96 k Gradient thermal cycler. PCR was performed in a total volume of 25 µL using a Promega wizard Genomic DNA purification Kit cat# A1120. One microliter each of forward and reverse primer was added to make the final volume to 25 µL mix containing Master Mix 12.5 µL, DNA template 1 µL, and free nuclease H2O 9.5 µL. The thermal cycler program used for the specific primers was 55.9 to 60.5°C followed by 35 cycles for 10 Sec at 98°C and for 30 Sec at 57.5°C and for 40 Sec at 72°C. The above program was used for all the samples throughout the study.

### III. Results
The genomic DNA of pure insects, *T. castaneum* and *T. confusum*, was extracted by using Promega wizard genomic DNA extraction kits (Fig. 1) as a positive control for the comparison with tested wheat product samples to detect the pest contamination. The same kits were used to extract the genomic DNA of flour beetles from tested wheat flour samples. Five types of commercial flour, high quality, all purpose, semolina, subsidizing and whole-wheat flour as well as two biscuit samples (1 and 2) as a final product of wheat flour were examined (Fig. 2 and 3).

Two sets of primers, the elongation factor 1-alpha sequence (AY819656) and beta-tubulin (AY819657), were found to be specific for *T. castaneum* and *T. confusum*, respectively. The quality of DNA was observed by running it through the gel. The size of the bands was approximately 261 and 256 bp for *T. confusum* and *T. castaneum*, respectively (Fig. 4).

The DNA was then amplified by using the two paired primers of the two beetles at low annealing different temperature to increase the stringency and to confirm the specificity of the bands. PCR reaction increased up to 60.5°C. It is clear from the obtained data that the primer pair designed to amplify elongation factor 1-alpha gene efficiently amplified *T. castaneum* DNA (Fig. 5). Similarly, the elongation factor sequence beta-tubulin was found to be very specific for *T. confusum* DNA (Fig. 6).

The DNA was then amplified using the two primers at a low annealing temperature of 57.5°C on the different samples of wheat flour and biscuits to detect each pest separately. We noted that the *T. castaneum* DNA bands appear brightly in whole-wheat, subsidizing and all purpose flours as well as sample 1 of biscuit (Fig. 7). The same results were obtained for *T. confusum* except for all-purpose flour, the DNA band (c band) was low bright than others in an electrophoresis gel (Fig. 8).

The results in Fig. (9) showed that DNA bands of *T. castaneum* specific primer, appear in semolina and sample 2 of biscuit and not appear clearly in whole-wheat flour (a band). While DNA bands of *T. confusum* specific primer appear brightly in all these products (Fig. 10).

### IV. Discussion

To minimize goods loss, reduce the risk of food contamination with insect pests and maintain the consumer health, it is important to develop an effective method for detecting the fragments of flour beetles *T. castaneum* and *T. confusum*. The traditional methods are not applicable to detect unseen insect fragments in wheat flour products. In the current study, we used the molecular technique as a promising effective method for detection of stored product pests.

When the two beetles were crushed with mortar and pestle a viscous mass of polysaccharides formed (Porebski et al., 1997). On the other hand, Fang et al. (1992) found that a high amount of gluten and polysaccharides in the flour inhibit the Tag polymerase activity during PCR. The contamination of DNA samples with polysaccharides can affect manipulation; inhibit enzyme reactions (Schlink and Reski, 2002). DNA extraction method of pure insect and all tested wheat products was successfully done by using the Promega wizard genomic kit. This Kit could overcome the obstacle of high levels of polysaccharide in insects and all wheat products.

The results showed that the two primers, which designed for amplification of the two flour beetles DNA, are effective, sensitive, and species-specific for detection of the insect fragments in all wheat products. Even when the stringency of the reaction was increased by increasing the annealing temperature to 60.5°C it's a pity that DNA bands of *T. confusum* and *T. castaneum* were detected by PCR amplification in all tested wheat products. Except for *T. castaneum* not appear clearly in whole-wheat flour, which obtained by milling the wheat grains in the laboratory. The whole-wheat flour was chosen to detect the early infestation with the two pests. From these results we can state that the wheat flour might be infested with *T. confusum* earlier than *T. castaneum* in early stage of storage as a whole grain. This infestation might be due to oviposition of the female pests on the grain, dusts or dockage. The flour beetles are secondary pests that cannot feed on whole, undamaged grain; they are, however, feed on dust and broken grains (Raney, 1987).

Some authors reported that the infestation of wheat products by pest revealed to insects come from the contaminated stores, inside kernels and are turned into fragments during the milling process. Insects actively migrate into the mill and semolina from the surrounding resources (Kucerová et al., 2003, Campbell & Arborgast, 2004, Athanassiou et al., 2005).

In general, the presence of insects DNA bands in flour and biscuit samples might be very dangerous signs which could cause a big hazard to the human health. The infestation of wheat flour by these two beetles for more than 3 months able to induce high damage and changes in the specification of the infected flour and other products including external and internal character such as texture, baking, chewing, pulp tested smell etc. (Osama et al., 2012).

The flour beetles (*Tribolium* spp.) contaminate foodstuffs with their secretions. Benzoquinones are major components of defensive secretions used as repellents and irritants (Howard, 1987 and Eisner et al., 1998). An individual beetle secretes up to 0.5 mg of quinones into the stored grain and seed products (Yezerski et al., 2000).
and 2004). These compounds give an unpleasant smell to stored food and may be responsible for liver and spleen tumors in small vertebrates (El-Mofty et al., 1988; 1989; 1992). Lis et al. (2011) concluded that benzoquinones effect may be direct or indirect where, after a series of enzymatic transformations via the metabolic pathways, benzoquinones become secondary metabolites of benzene, suppressing cells and tissues and causing carcinogenic changes. Elhassanen and AbdEl-Moaty (2003) fed rats on flour contaminated with Triboliumconfusum beetles for 10 weeks. They found a lower activity level of the antioxidative enzymes GSH-PX and GSH-R in the red blood cells of rats. In previous study, El Mofty (1989) also reported that the experimental rats fed on biscuits made of flour infested with T. castaneum for 56 weeks, were suffering from tongue carcinomas.

In conclusion, The DNA bands of T. castaneum and T. confusum fragments were appeared in all tested wheat products except the absence of T. castaneum DNA bands from whole wheat flour. These products still material of basic food for the people and it is a source of energy for humans. Also may be contaminated by benzoquinones secreted from flour beetles, which have a toxic effect on humans and animals. Hence, the DNA technique serves as a precise early warning for taking suitable control measures.

Acknowledgments

We are immensely grateful to Prof. Hany A. bdelsalam and his team Amira Hamdy, Sahar Mohamed and Shimaayhia in the Marine Genomic Laboratory, Faculty of Science, Benha University for facilities and efforts they offered throughout this study.

References

Revealing of Wheat Products Contamination With Flour Beetles Tribolium spp. by Molecular...


Fig. (1): Gel electrophoresis image of DNA extraction of T. castaneum (A) and T. confusum (B).

Fig. (2): Genomic DNA extraction image of (a) high quality flour, (b) subsidizing flour, (c) all-purpose flour and (d) sample 1 of local biscuit.

Fig. (3): Genomic DNA extraction image of (a) whole-wheat flour, (b) semolina flour and (c) sample 2 of local biscuit.
Revealing of Wheat Products Contamination With Flour Beetles Tribolium spp. by Molecular..
Fig. 8. Gel electrophoresis picture of (M) 1 kb Ladder and (B) the PCR amplified product of pure *T. confusum* by using DNA and specific primer (a) miller flour, (b) subsidizing flour, (c) all-purpose flour d) sample 1 of biscuit.

Fig. (9): Gel electrophoresis picture using primer of *T. castaneum* a) whole-wheat flour, b) semolina flour and c) sample 2 of biscuit.

Fig. (10): Gel electrophoresis picture using primer of *T. confusum* a) whole-wheat flour, b) semolina flour and c) sample 2 of biscuit.