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Revealing ofWheat 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, Triboliumspp;red flour beetle, Triboliumcastaneum and confused flour beetle,Triboliumconfusum 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-tubulingenefor detection of T. castaneumand T. confusumDNA, respectively.Five types of flour have been examined and two types of local biscuitswere 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 thatbands of the DNA of the two beetles appeared in all types of tested product samples. The contamination with these secondary pests, especially with T. confusm,might be started in the early stage of storing whole grains before milling process*

Keywords:*Triboliumcastaneum,Triboliumconfusum, 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 peststhat attack the wheat grain arecrushed 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 mainlypests that attackflourmills (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). Lakshmikantha et al. (2010) recommend that the two *Tribolium* spp. are vectors of many important pathogens. In addition, the *Tribolium* spp. beetleshave defensive prothoracic and abdominal glands, which secret benzoquinones(Blum, 1981and 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-Mofty et al., 1988; 1989 and 1992). However, flour beetles produce large quantities of a dust that prevents seed respiration, andlead 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 ahighly accurate technique that widely used foridentification 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 (FlinnandHagstrum, 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(Trematerra et al., 2011). The shortlife cycle and ease of rearingand 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^{o}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 Centerat 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 insectsas a positive control**.** Samples of the two *Tribolium*spp. insects were grinded by using mortar and pestle. 1200µl of the chilled nuclei lysis solution was added to the samples, then transferred to a sterile1.5 ml centrifuge tubes. Lysate tubes were incubated in water Bath for 15-30 minutes at 65^{o}C. 200 µl protein precipitation solution was added to precipitate protein at room temperature. Samples were shacked by Vortex at high speed for 20 seconds and chilled on ice for5 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 1minute 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 5minute 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 10-15min. 50 µl of DNA extractionswere added to rehydration solution. DNA samples were rehydrated by incubation at 65^{o}C for 1hr. The DNA of the two insects was stored at 2-8 ^{o}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 type and grinded 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 (Moťková 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 GenBankwith 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′ to3′) CCCAGTCACTCCATGATGTT and TTCGACGGACTTGACTTCAGwere specific for *T. Castaneum*, and beta-tublin intron GCTGGAGCGTATCAATGTCTAC and GTCGCATCCTTCTGCTTCTT 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 Mix12.5µl, DNA template 1µl, and free nuclease H_2O 9.5 µl. The thermal cycler program used for the specific primers was 55.9 to 60.5°C followed by35 cycles for 10 Secat 98ºC and for 30 Sec at 57.5 ºC and for 40Sec at 72ºC.The above program was used for all the samples throughout the study.

III. Results

The genomicDNAof pureinsects,*T.castanuem* 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 fromtested 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 bpfor *T. confusum and T. castaneum*, respectively (Fig. 4).

The DNA was then amplified byusing the two paired primers of the two beetles at low annealing different temperaturesto 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 andbiscuits to detect each pest separately. We noted that the *T. castaneum*DNA bands appear brightly in whole-wheat, subsidizing and all purpose floursas well as sample 1 of biscuit (Fig. 7). The same results were obtained for*T. confusum* except for all-purposeflour,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 appearclearly 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 minimizegoods 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 of 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 polysaccharidesin 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, areeffective, 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 thatDNA 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 thatcannot 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 &Arbogast, 2004 Athanassiouet al., 2005).

In general, the presence of insectsDNA bands in flour and biscuit samplesmightbe very dangerous signswhich could cause a big hazard to the human health.The infestation of wheatflour by these two beetles for more than 3 months able to induce high damage and change in specification of 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 secrets up to 0.5 mg of quinones into the stored grain and seed products (Yezerski et al., 2000

and2004). 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. Theyfound a lower activity level of the antioxidant 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* fragmentswere appeared inall testedwheat productsexceptthe absence of *T. castaneum* DNA bands from whole wheat flour. These productsstill 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.

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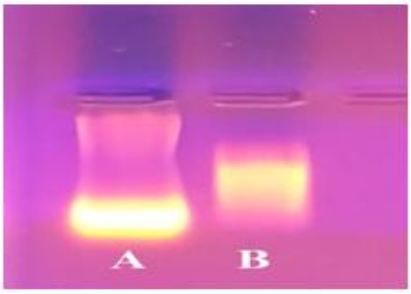

Fig. (1): Gel electrophoresis image of DNA extraction of *T. castaneum* (A) and *T. confusum*(B)

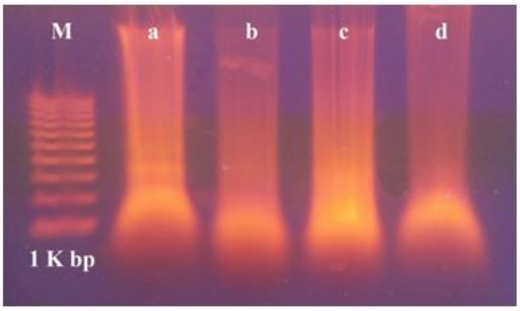

Fig. (2): Genomic DNA extraction image of (a) high quality flour, (b) subsidizing flour, (c) all- purpose flour and (d) sample 1 of local biscuit

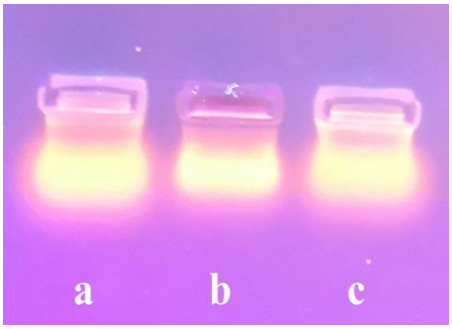

Fig. (3): Genomic DNA extraction image of (a) whole-wheat flour,(b) semolina flour and (c) sample 2 of local biscuit.

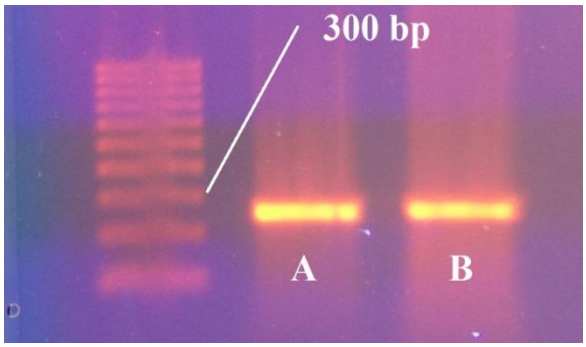

Fig.(4): Gel electrophoresis picture of 1kb ladder , (A) PCR amplified product of pure *T.castaneum* and using elongation1-alpha sequence (AY819656) (B) PCR amplified product of pure *T. confusum* using elongation factor beta-tubulin primers (AY819657).

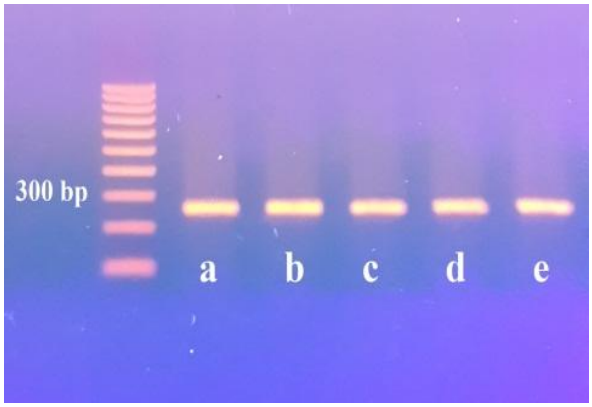

Fig.(5): Gel electrophoresis image of Primer specific to *T.castaneum* at different temperature degrees. a) 55.9,^{o}C b) 57.5^{o}C, c) 58.8^{o}C, d) 59.5^{o}C and e) 60.5 ^{o}C.

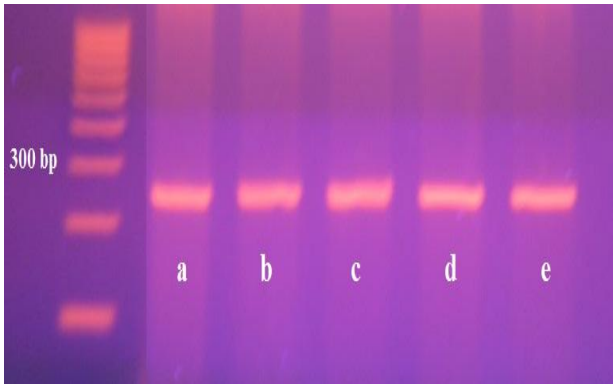

Fig. (6): Gel electrophoresis image of Primer specific to *T.confusum* at different temperature degrees. a) 55.9,^{o}C b) 57.5^{o}C, c) 58.8^{o}C, d) 59.5^{o}C and e) 60.5 ^{o}C.

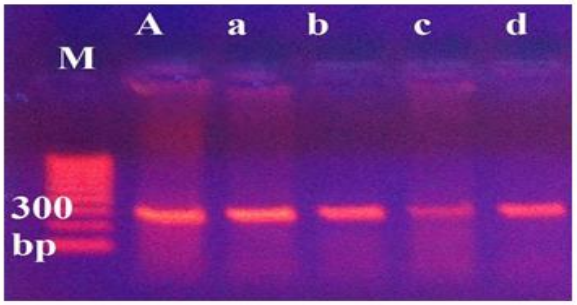

Fig. (7): Gel electrophoresis picture of (M) I kb Ladder and (A) the PCR amplified product of pure *T. castaneum* by using DNA and specific primer (a) whole-wheat flour, (b) subsidizing flour, (c) all purpose flour d) sample 1 of biscuit

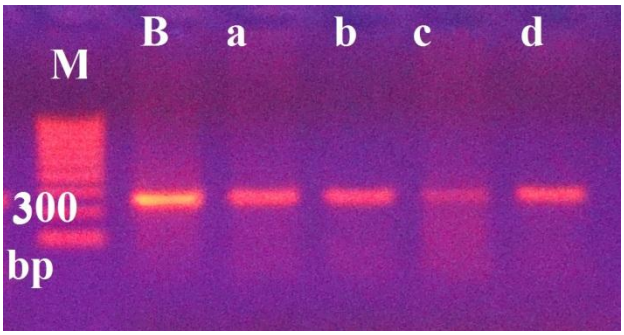

Fig.8. Gel electrophoresis picture of (M) I kb Ladder and(B) the PCR amplified product of pure *T.confusum* by using DNA and specific primer (a) miller flour,(b) subsidizing flour, (c) all-purpose flour d) sample 1 of biscuit

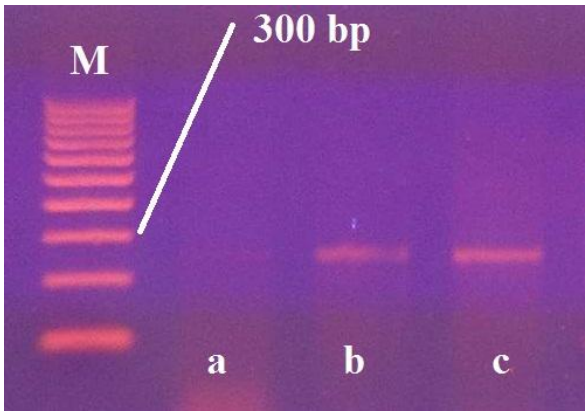

Fig. (9): Gel electrophoresis picture using primer of *T.castaneum* a) whole-wheat flour, b) semolina flour and c) sample 2of biscuit

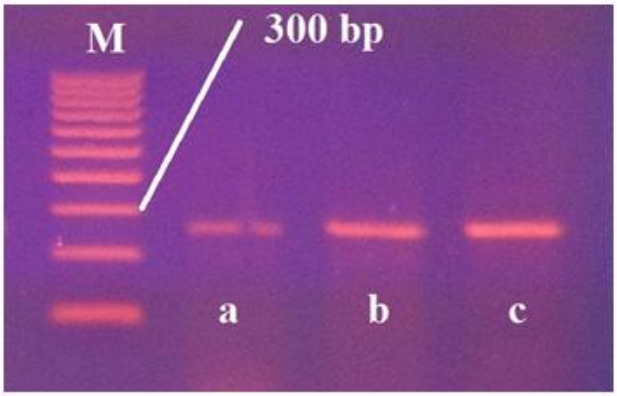

Fig. (10): Gel electrophoresis picture using primer of *T.confusum* a) whole-wheat flour, b) semolina flour and c) sample2 of biscuit