Sensitivity analysis of DNA fingerprinting technique for detecting insect fragments in wheat flour

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Balasubramanian, A., Jayas, D.S., Fernando, W.G.D., Li, G. and White, N.D.G. 2007. **Sensitivity analysis of DNA fingerprinting technique for detecting insect fragments in wheat flour**. Canadian Biosystems Engineering/Le génie des biosystèmes au Canada **49**: 4.1-4.5. *Tribolium castaneum* (Herbst) and *T. confusum* (Jaquelin du Val) are the most commonly occurring insect species in flour mills and in damaged and milled grains. Deoxyribonucleic acid (DNA) fingerprinting, a molecular tool which helps to identify the unique DNA pattern of an organism was used to detect the presence of insect fragments of *T. castaneum* and *T. confusum* at different concentrations in commercial and lab-milled wheat flour. Insect nuclear primers were screened to identify the insect specific primer for *T. castaneum* and *T. confusum*. Two sets of primers were found to be specific for each of the *Tribolium sp*. The primer pair responsible for amplification of elongation factor 1-alpha gene sequence (520 bp) was found to be specific to *Tribolium castaneum* and the primer pair responsible for amplification of beta-tubulin intron gene sequence (500 bp) was found to be specific to *T. confusum*. The primers were verified for their specificity against other commonly occurring stored product pests such as *Cryptolestes ferrugineus* (Stephens), *C. pusillus* (Schonherr), *Rhyzopertha dominica* (Fabricius), and *Sitophilus oryzae* (Linnaeus) and was confirmed to be specific only for the two insect species mentioned above. The crushed insect parts of red flour beetles and confused flour beetles were added at 1.0, 0.5, 0.25, 0.1, and 0.05% by mass to 200 mg of cleaned commercial and lab-milled flour. The DNA fingerprinting technique was able to identify *T. castaneum* and *T. confusum* contamination only at the 1.0% contamination level in both commercial and lab-milled flour. The technique was simple and a large number of samples could be evaluated at once. It helps in the identification of the insect species contaminating the flour. **Keywords:** DNA fingerprinting, *Tribolium castaneum*, *T. confusum*, stored product pests, insect nuclear primers, DNA amplification.

Le *Tribolium castaneum* (tribolium rouge de la farine) et le *T. confusum* (tribolium brun de la farine) sont les espèces d'insectes les plus couramment rencontrées dans les moulins à farine ainsi que dans les grains endommagés et moulus. Le traçage à l'acide désoxyribonucléique (ADN), un outil moléculaire qui permet d'identifier le patron d'ADN unique d'un organisme, a été utilisé pour détecter la présence de fragments d'insectes *T. castaneum* et *T. confusum* à des concentrations différentes dans des échantillons de farine moulue commercialement ou en laboratoire. Les amorces nucléaires des insectes ont été étudiées pour identifier celles spécifiques aux insectes *T. castaneum* et *T. confusum* à différentes concentrations dans des farines moulues commercialement ou en laboratoire. Deux ensembles d'amorces spécifiques pour chaque *Tribolium sp* ont été trouvés. Il a été déterminé que la paire d'amorces responsables pour l'amplification du facteur d'élongation de la séquence génétique 1-alpha (520 bp) est spécifique aux *Tribolium castaneum* et que la paire d'amorces responsable pour l'amplification de la séquence génétique de l'intron beta-tubuline (500 bp) est spécifique au *T. confusum*. La spécificité des amorces a été vérifiée et comparée pour d'autres espèces d'insectes pouvant contaminer des produits entreposés comme le *Cryptolestes ferrugineus* (cucujide roux), *C. pusillus* ('Schonherr'), *Rhyzopertha dominica* (capucin des grains) et *Sitophilus oryzae* (charançon du riz). Il a ainsi été démontré que ces amorces sont spécifiques seulement aux deux espèces d'insectes mentionnées précédemment. Les parties d'insectes broyés provenant des triboliums et mélangées à d'autres insectes qui infestent la farine ont été additionnées à 200 mg de farines nettoyées moulues commercialement et en laboratoire à des proportions de 1,0, 0,5, 0,25, 0,1, et 0,05% sur une base massique. La technique d'empreinte ADN a permis d'identifier la contamination au *T. castaneum* et au *T. confusum* seulement pour le niveau de concentration de 1% dans la farine moulue commercialement et dans celle moulue en laboratoire. La technique s'avère simple et permet d'évaluer un grand nombre d'échantillons en même temps. Ceci aide dans l'identification des espèces d'insectes qui contaminent la farine. **Mots clés:** empreinte d'ADN, *Tribolium castaneum*, *T. confusum*, insectes infestant produits entreposés, amorces nucléaires d'insectes, amplification d'ADN.

INTRODUCTION

Canada produces about 23 Mt (23 million tones) of wheat annually ranking among the top six countries in wheat production. The majority of the wheat is milled to produce wheat flour, which is made into different products for consumption. The quality of wheat flour depends on the wheat that is milled and one of the common reasons for grain quality loss is insect infestation. The insects found in the bulk grain get crushed during milling. Many also become residents in flour mills. The result is the occurrence of insect fragments in the milled wheat flour (Flinn et al. 2004). In an experiment done with lesser grain borer to find the source of insect fragments obtained, it was found that the adults contribute 28 times as many fragments in flour compared to larva or pupa. Thus 20 kernels per kilogram of wheat infested with the adult insects were sufficient to exceed the Food and Drug Administration (FDA) standard limit (Perez-Mendoza et al. 2005). *Tribolium castaneum* and *T. confusum* are the two most commonly occurring insects in flour mills (Rivers 1997; Sinha and Watters 1985) and inhabit milling equipment, elevator boots, wall and floor habitats (Sinha and Watters 1985), and bulk wheat. They cause considerable quality and financial losses (Hill 2002).

Detection of insect fragments in wheat flour is of great concern to the milling industry due to the stringent regulations by government (Bauer 1984). The Health Protection Branch of the Government of Canada has set standards for the level of insect fragments that occur in wheat flour. In Canada the number of insect fragments (>0.2 mm) in wheat flour after milling should be fewer than 20 in three test samples of 50 g each (CFIA 1999). The FDA in the United States has set the defect action level in wheat flour as 75 or more insect fragments per 50 g of flour (FDA 1988).

The current detection methods include chemical methods (Rivers 1997), immunoassay method (Quinn et al. 1992), and near-infrared spectroscopy (Perez-Mendoza et al. 2003). Though the chemical method based on the separation of fragments due to density difference is the only approved method by Health Protection Branch, Health Canada, none of the methods are precise and simple for detecting the fragments. Thus, we evaluated a molecular detection technique, namely DNA fingerprinting, to detect the insect fragments and also to identify the insect species in cleaned wheat flour.

In addition to the bigger fragments, most insect fragments that are not visible to the naked eye may be found in large quantities in cleaned wheat flour. Deoxyribonucleic acid (DNA) fingerprinting is a molecular tool, which helps to identify the unique DNA pattern of an organism by the genetic polymorphism in the DNA, which constitutes the genetic material. The individual specific DNA patterns render DNA fingerprinting a powerful molecular tool and the technique is being widely used for identification of biological materials (Crawford et al. 1993; Jayarao and Oliver 1994; Peng et al. 2003; Saez et al. 2004).

The adults of two of the most common species of *Tribolium*: *T. castaneum* and *T. confusum* are differentiated by the features of their eyes and the antennae through a hand lens. The adults of *T. castaneum* can fly while the adults of *T. confusum* cannot (Okumura 1984). Thus *T. castaneum* can spread easily around the mill and requires rapid control measures. Spot fumigation can be done for *T. confusum*, which is not the case with the *T. castaneum* for the above mentioned reason (Dawson 1984). The population of *T. castaneum* is found to increase more rapidly compared to other stored-product insects (Hill 2002). The larvae and adults of the *T. castaneum* are found to be ingestant allergens (Bernton and Brown 1967). Thus identification of the insect species would help to take specific control measures in the stored grain or flour mill.

The main objective of this study was to find the insect-specific primer for the two species of *Tribolium (T. castaneum* and *T. confusum)*, and to analyze the sensitivity of the DNA fingerprinting technique for the detection of insect fragments in wheat flour.

MATERIALS and METHODS

Insect cultures

Tribolium castaneum cultures were collected in Argyle, Manitoba from stored wheat in 1995 and *T. confusum* cultures were collected from the Canadian Grain Commission flour mill, Winnipeg, Manitoba in 1994. These cultures were maintained on a mixture of wheat flour and brewer's yeast (95:5 wt:wt). *Cryptolestes ferrugineus* cultures were collected from stored wheat in Argyle, Manitoba in 1994 and maintained on a mixture of whole wheat and wheat germ (20:1 wt:wt). *Cryptolestes pusillus* cultures were collected at Glenlea, Manitoba from stored wheat in 1995 and maintained on wheat flour and brewer's yeast (20:1 wt:wt). *Rhyzopertha dominica* and *Sitophilus oryzae* cultures were collected at Coaldale, Alberta in 1992 and Oakbank, Manitoba in 1995, respectively, and maintained on whole wheat. The cultures were all grown in 4-L glass jars with vented lids and were maintained at 30°C and 70% relative humidity in dark growth chambers.

Commercial flour sample

White Hudson cream flour was procured from Stafford County Flour Mills Company, Hudson, Kansas. The wheat was milled from a mixture of hard red spring wheat cultivars grown in Manitoba. Additives such as ascorbic acid, amylase, niacin, iron, thiamine, mononitrate riboflavin, L-cystine hydrochloride, azodicarbonamide, and folic acid were added to the wheat flour for fortification. The milled flour was sifted and visually checked for insect contamination before taking each sample.

Lab-milled flour sample

The lab-milled flour was milled from hard red spring wheat, "AC Barrie" in a Buhler mill (Buhler Inc., Uzwil, Switzerland). There were no additives added to this flour. The wheat used for milling was free from any visible insects or insect fragments.

Preparation of controls

The positive controls of the *T. castaneum* and *T. confusum* were prepared by extracting DNA from pure insect samples. The insects were cleaned properly by sieving to avoid any mixture of flour or wheat on them. The negative controls were the DNA extracted from pure, cleaned commercial and lab-milled flour.

Preparation of a mixture of flour and insects

The insects, *T. castaneum* and *T. confusum,* were crushed with liquid nitrogen using mortar and pestle. They were added at 1.0, 0.5, 0.25, 0.1, and 0.05% by mass to 200 mg of commercial and lab-milled flour. Three sample replicates were prepared for each of the single insect species mixed at different concentrations to the commercial and lab-milled flour. Four replications were done for each of the concentrations.

DNA extraction

DNA was extracted from the pure cultures of insects and the flour with a slight modification of a very simple extraction method called the high salt extraction method (Aljanabi and Martinez 1997). Forty milligrams of the sample were crushed using 400 µL of sterile salt homogenizing buffer containing Tris, NaCl, and EDTA. Forty microliters of SDS (20%) and 8 µL of Proteinase K (20 mg/mL) were added and the samples were incubated at 65ºC overnight when 300 µL of 6M NaCl were added. DNA was recovered using isopropanol at half the volume of the supernatant and centrifuging at maximum speed for 10 s. The DNA was washed using 70% ethanol. For the commercial and lab-milled flour, the quantity of proteinase K was increased to 12 µL. The DNA was tested for protein contamination and was then quantified using a GeneQuant pro spectrophotometer (Pharmacia Biotech, Uppsala, Sweden).

DNA extraction from a mixture of flour and insects

The Wizard genomic purification kit (Promega, Madison, WI) (Beeman and Brown 1999) was used for the DNA extraction of the mixture of flour and insects. The mixture of flour and insects

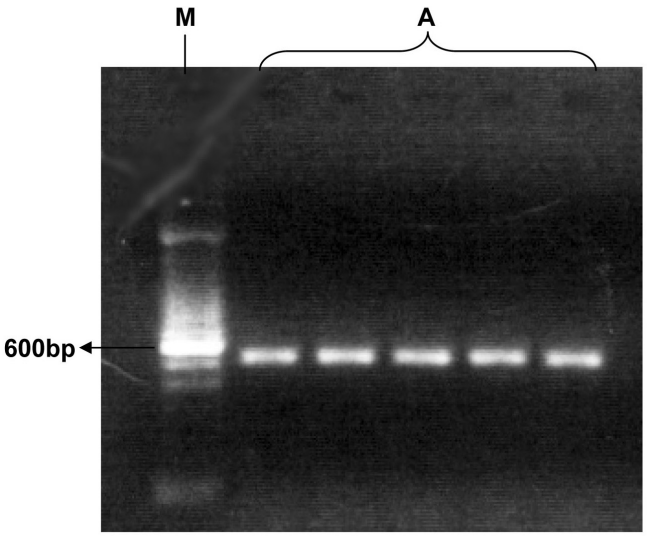

Fig. 1. Gel electrophoresis picture of (M) 100 bp ladder and (A) replications of the PCR amplified product of pure red flour beetle DNA using elongation factor 1- alpha primers (EFS599; EFA923).

was vortexed with 3 ml of nuclei lysis buffer. The samples were then incubated at 65°C overnight. The protein was precipitated by 1 mL of protein precipitation solution. The supernatant was extracted with an equal volume of isopropanol and washed with 70% ethanol. The DNA was tested for protein contamination and quantified using the GeneQuant pro spectrophotometer. The quality of DNA was observed by running it through a gel.

Primers

Insect nuclear primers were procured from the University of British Columbia, Vancouver, BC (http://www.michaelsmith.ubc.ca/services/NAPS/Primer_Sets/Primers_Oct2006.pdf) to identify insect specific primers. The primer combinations were tried as a screening process to randomly identify insect specific primers. There were 50 primers in the set. About 58 combinations were screened on all six insect species based on the details given about the primers and the genes they amplify. The initial conditions for the PCR for screening were maintained as: 94°C for 1 min, followed by 30 cycles of 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C. Final extension was done at 72°C for 5 min after the 30 cycles. Once specific bands were obtained at low annealing temperature (56°C), the annealing temperature was then increased to 60°C to increase the stringency and to confirm the specificity of the bands. It was found that the primers designed to amplify the protein-coding gene, elongation factor 1-alpha, ATC TCC GGA TGG CAC GG (CT) GAC AA (EFS599); ACG TTC TTC ACG TTG AA (AG) CCA A (EFA923), were specific to *T. castaneum* and the primers for beta-tublin intron, CAG GCT GGT CAA TGT GG (CT) AA (CT) CA (Tub1-5'); ATA CGG TCT GGG TAC TC (CT) TC (AGCT) CG (Tub4-3'), were specific to *T.confusum.* The amplified PCR product was purified with a QIAquick PCR purification kit (Qiagen ®). The purified product was then sent to the University of Calgary for sequencing as a premix. The *T. castaneum* and *T. confusum* sequences were sent to GenBank. The assigned accession numbers were AY819656 (*T. castaneum*) and AY819657 (*T. confusum).*

Thermal cycler

PCR was performed on a total volume of 25 µL using a Techne thermocycler (Techne, Burkhardtsdorf, Germany). One and half microliters of DNA (20 ng/µL) were used with 19.5 µL of PCR mix containing 2.5 µL of 10X PCR buffer, 2 µL of DNTP's (each at 10 mM), 0.2 µL of Taq polymerase 5 units/µL and 0.75 µL of magnesium chloride 50 mM. Two microliters each of forward and reverse primer (20 pmoles/25 µL) were added to make the final volume to 25 µL. The thermocycler program used for the specific primers was 94°C for 1 min, followed by 30 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C. Final extension was done at 72°C for 5 min after the 30 cycles. The above program was used for all the samples throughout the study, once the insect specific primers were identified.

RESULTS and DISCUSSION

The high salt extraction method (Aljanabi and Martinez 1997) was adopted to extract DNA from the pure insect and flour to avoid the hindrance due to high levels of polysaccharide in both insects and wheat flour. *Tribolium castaneum* and *T. confusum* have tanned cuticle which may contain secondary metabolites (Erlandson and Gariepy 2005). *T. castaneum* and *T. confusum* when crushed formed a viscous mass and this may be due to the presence of polysaccharides (Porebski et al. 1997). The polysaccharides inhibit the Taq polymerase activity during PCR (Fang et al. 1992). The flour has a high amount of gluten and polysaccharides. High salt helps to remove the polysaccharides (Lodhi et al. 1994). DNA extraction from a mixture of flour and insect was done using the promega wizard genomic DNA extraction kit which extracted DNA from a single beetle that was successfully amplified by the insect-specific primer.

The DNA was then amplified using 58 different primer combinations at a low annealing temperature of 56°C. It was found from the bands obtained that the primer pair designed for amplifying elongation factor 1-alpha (EFS599; EFA923) efficiently amplified *T. castaneum*. Even when the stringency of the reaction was increased by increasing the annealing temperature to 60°C, the elongation factor 1-alpha sequence was found to be very specific for *T. castaneum*. The size of the band was approximately 520 bp and the repeatability was 100% (Fig. 1). Similarly, it was found that beta-tubulin intron sequence (Tub1-5'; Tub4-3') was specific to *T. confusum* and the band was missing in *T. castaneum*. The size of the band was approximately 500 bp and the repeatability was 100% (Fig. 2). With the other combinations tried, there were either no amplifications or the amplification was not 100% when the annealing temperature was increased to 60°C or there were no bands. In some cases the primers responsible for the amplification of a gene were common to all the insects. Insect fragments taken from a flour mill may contain many other insects also. So the specificity of the primer was confirmed by amplification with DNA extracted from other stored-product insects that can be found in flour, namely, C. *ferrugeneus, C. puscillus, R. dominica,* and *S. oryzae*. The band was observed only in *T. castaneum* adults when elongation primer was used and similarly it was found only in *T. confusum* adults when beta-tubulin intron primer was used.

The DNA extracted from the mixture was amplified by PCR using the species-specific primers. The insects were detected at

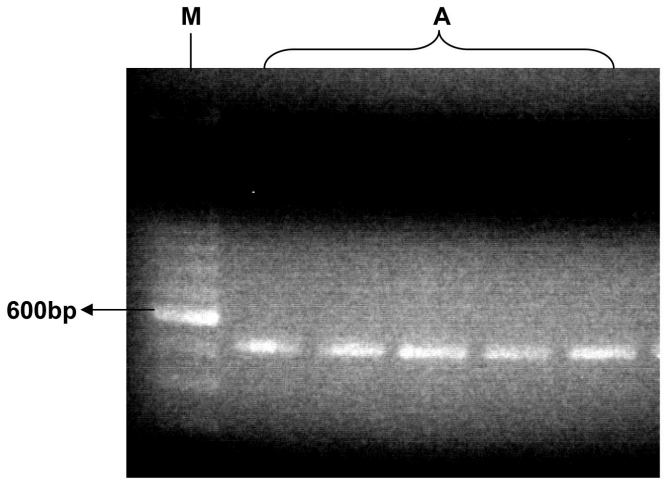

Fig. 2. Gel electrophoresis picture of (M) 100 bp ladder and (A) replications of the PCR amplified product of pure confused flour beetle DNA using beta-tubulin intron primers (Tub1-5'; Tub4-3').

a 1.0% concentration level in all the samples of the mixture of insects and flour amplified using the beta-tubulin intron primer. From Fig.3 it was found that the negative controls, commercial (b) and lab-milled flour (c) had bands of sizes varying from 600-1000 bp but the positive control of the pure insect (a) DNA alone had a PCR product at 500 bp. The 500 bp band was found in all the four replications of the mixture of insect and flour (d and e). Thus the presence of the 500 bp band specific for amplification of beta-tubulin intron gene of *T. confusum* found in the positive control and all the replicates of the mixture and its absence in the negative controls helps to detect the insects at 1% level in the flour. The same trend was observed in all the three samples.

The mixture of *T. castaneum* at different concentrations with commercial and lab-milled flour gave a result similar to that for

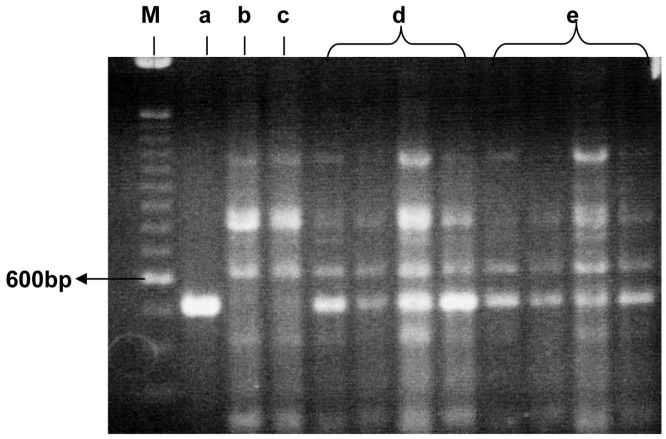

Fig. 3. Gel electrophoresis picture of (M) 100 bp ladder and the PCR amplified product of (a) positive control, negative controls of (b) commercial flour, (c) lab milled flour and four replications of *T. confusum* at 1.0% level mixed with (d) commercial flour and (e) lab milled flour.

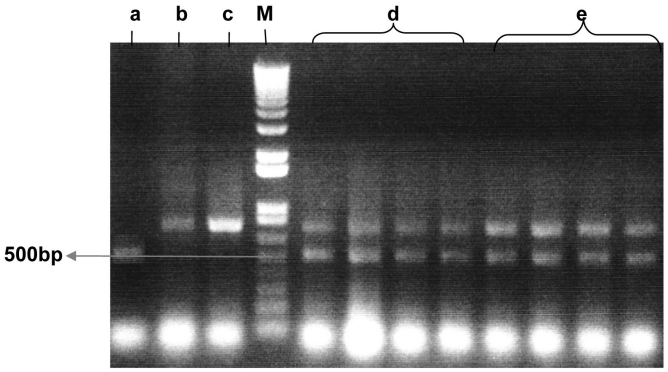

Fig. 4. Gel electrophoresis picture of (M) 1 Kb ladder and the PCR amplified product of (a) positive control, negative controls of (b) commercial flour, (c) lab milled flour and four replications of *T. castaneum* at 1.0% level mixed with (d) commercial flour and (e) lab milled flour.

T. confusum. The insects were detected at a 1.0% concentration level in all the samples of the mixture of insects and flour amplified using the elongation factor 1-alpha primer. From Fig.4, it is seen that there were bands found in the negative controls, commercial (b) and lab-milled flour (c) had bands of sizes varying above 1000 bp but the positive control of the pure *T. castaneum* insect (a) DNA alone had a PCR product at 520 bp. The 520 bp band was found in all the four replications of the mixture of insect and flour (d and e). Thus the presence of the 520 bp band specific for amplification of elongation factor 1-alpha gene of *T. castaneum* found in the positive control and all the replicates of the mixture and its absence in the negative controls helps to detect the insects at 1% level in the flour. The same trend was observed in all the three samples.

The failure for detection of insects at levels lower than 1% may be due to the size of the primer or the current protocol used for detection. The extraction of DNA from a combination of two biological materials, where one of them is 100 to 2000 times more than the other also poses a major problem which requires further research to identify low quantity of one type of DNA in the presence of a large quantity of another type of DNA. The advantage of the technique is its ability to identify the species of the insect. DNA fingerprinting can be used to identify even closely related species. The milled flour can be kept as long as needed and the DNA extracted can also be stored at -20°C for a long time. As the insects were crushed into finer particles, they cannot be detected well by the flotation method. The technique is simple and a large number of samples could be done at the same time. The sample size taken for analysis is very small (200 mg), thus more samples could be analyzed from a batch of flour. The DNA fingerprinting technique could be combined with any other method to do both the quantitative and qualitative analysis. The sensitivity could be increased further by using fluorescently-labeled insect-specific probes that would help in the clear identification of the insect DNA. Enzyme digestion followed by purification of the DNA extracted from the mixture of flour and insect could be done. The enzyme used for digestion should not restrict the insect-specific gene to be amplified. Though these are theoretical possibilities, further research should be done to establish the technique.

CONCLUSION

The primers EFS599 and EFA923, designed to amplify the protein-coding gene, elongation factor 1-alpha were specific to *T. castaneum* (520 bp product) and the primers Tub1-5' and Tub4-3' were specific in amplifying a 500 bp beta-tubulin intron gene of *T. confusum*. The specific primers were then used to identify the species in a mixture of flour and insects using DNA fingerprinting technique. The method was able to identify both *T. castaneum* and *T. confusum* added at a 1.0% level to 200 mg of commercial and lab-milled flour. Thus the technique was found sensitive to 1.0% (2 mg) of insect contamination in 200 mg of wheat flour. The DNA fingerprinting technique helps to identify the type of insect species contaminating the flour. The identification of the insect species would help to take specific control measures by tracing back to the source namely, stored grain or flour mill. This study also identifies a need for further research to develop a finger printing technique to detect a small quantity of DNA (insect) in the presence of a large quantity of second DNA (flour).

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