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RESEARCH ARTICLE

Use the Molecular Diagnostic in the Early Detection of Insect Pests of Stored Grain

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Abstract

The current study included early detection of insect pests that infect stored grains using the Multiplex-PCR (Polymerase Chain Reaction) technique. The technique included two experiments conducted using specific primers for each species. These two experiments are as follows: In first experiment three species were identified in wheat grain sample. These species are: an internally infected species *Rhyzopertha dominica* and two externally infected species *Tribolium castaneum* and *Oryzaephilus mercator* . As for the second experiment two species were identified and detected one internally infected *Trogoderma granarium* and the other externally infected *Latheticus oryzae* in a red bean sample.

Key word: Molecular diagnostic, Multiplex PCR, grain stored, stored grain pests.

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INTRODUCTION

The increasing concerns and consumer concern for food safety have led to a general trend towards reducing the presence and survival of insects in food (Trematerra, 2013). This situation has led to changes in grain standards in terms of food quality. Such a situation has emphasized the need to organize and arrange approaches, methods or techniques in the commercial chain starting from farmers to consumers. This approach makes changes in the market industrially and politically (Stejskal *et al*., 2014). For example local flour mills have been told not to allow insects at all while the national agency responsible for food safety in the United States, the Food and Drug Administration (FDA) has determined the maximum levels of insects naturally in mills (FDA, 1997). Failure to control insect outbreaks at the beginning of storage or in the field can lead to widespread contamination of stored grains, which affects the quality of food security (Nopsa *et al*., 2015). Therefore, it has become necessary and important to develop methods and strategies for early detection of grain infestation and in order to detect insect infestations tests are routinely conducted on grain samples. These tests include the use of sieves and all methods that lead to breaking the grains in order to detect insects hidden inside the grains in their complete stages and in damaged grains or to detect insect remains. When using such traditional methods and methods used to detect insects present in grains, internal pests in the grains cannot be detected (Brader *et al*., 2002; Hubert *et al*., 2009).

In addition insect remains are not equivalent at each stage of the pest's development. For example, the incomplete stages and the egg contain little or no chitin which shows the need or importance of using methods for early detection of grain infestation with other tests (Brabec *et al*., 2010).

In recent years, molecular techniques and their applications have gained importance in the diagnosis of species present in foods due to their simplicity speed and specificity. DNA-based methods such as PCR have become relevant for the analysis of genetically modified organisms (GMOs) and for the identification of pest species, including insects (Hong and Chuah, 2003), which provides an excellent method for the diagnosis and identification of all forms of complete and incomplete forms, and even for latent species (Correa *et al*., 2013). Among the most common PCR methods Multiplex-PCR is the most convenient and suitable method for the detection and diagnosis of many species of insects in a sample or in a single PCR reaction (Chamberlain *et al*., 1988). Multiplex-PCR was described by Chamberlian and his group and has been applied in many areas of DNA testing including deletion analyses (Chamberlai *et al*.,1992), mutations (Shuber *et al*.,1993), polymorphisms (Mutirangura *et al*., 1993), quantitative measurements (Mansfield *et al*.,1993) or reverse transcription (Crisan, 1994).

MATERIALS AND METHODS

Insects of trial and DNA extraction

DNA extraction was performed three times in two experiments according to the steps mentioned in the protocol prepared by Geneaid (gSYNC TM DNA Extraction kit). DNA was extracted from wheat that was infected with three pest species: *Tribolium castaneum*, *Oryzaephilus mercator* and *Rhyzopertha dominica*. DNA was extracted from two species that infected red beans *Trogoderma granarium* and *Latheticus oryzae*.

The extraction steps are as follows:

1- Tissue Dissociation stage: The mixture of insects and infected grain samples was crushed in three experiments by 0.025 mg for each experiment and transferred to 1.5 ml eppendorf bottles. 200 microliters of GST buffer solution and 20 microliters of Proteinase K enzyme were added to them and shaken using a Vortex shaker. The eppendorf bottles were placed in a water bath for 3 hours with the sample shaken every 10 minutes to mix the added materials with the crushed sample. 100 microliters of Elution buffer were transferred for each experiment into 1.5 ml Eppendorf bottles and also left in a water bath at 60˚C and used in the final step of the extraction process.

2- Cell Lysis Stage: After incubating for 3 hours the eppendorf bottles were placed in a refrigerated centrifuge for 2 minutes at 16000 rpm. The filtrate was transferred to a 1.5 ml eppendorf tube and 200 microliters of GSB buffer solution were added to it and shaken using a shaking device for 10 seconds. This step is essential to mix the GSB buffer with the sample completely.

3- DNA Binding Stage: 200 microliters of absolute ethanol alcohol were added to the sample and mixed for 10 seconds using a shaking device. If a precipitate appears at the bottom of the Eppendrof vial, it is divided using a fine pipette and the GD Column is placed in a 2 ml tube. The mixture is transferred to the separation column and placed in a refrigerated centrifuge at 16000 rpm for one minute. The collection tube is discarded. If the mixture does not

flow through the GD Column membrane the centrifugation is repeated again with increasing time until it passes completely and the separation membrane or separation column is transferred to a new 2 ml collection tube.

4- Wash Stage: 400 microliters of the first wash buffer W1 was added to the GD Column membrane separation column and placed in a refrigerated centrifuge at 16000 rpm for 30 seconds. The contents in the collection tube were discarded and the separation column was transferred to a new 2 ml collection tube. 600 microliters of Wash buffer were added to the separation column and placed in a refrigerated centrifuge at 16000 rpm for 30 seconds. The contents in the collection tube were discarded and the separation column was placed in a new 2 ml collection tube and transferred to the refrigerated centrifuge again at 16000 rpm for at least 3 minutes until the column matrix was dry.

5- DNA Elution Stage: Transfer the separation column after drying to a 1.5 ml tube and add 100 microliters of Elution buffer to each tube to the center of the Column Matrix and leave for at least 3 minutes for the Elution buffer to be completely absorbed and place in a refrigerated centrifuge for 30 seconds at 16000 rpm to separate pure DNA.

Electrophoresis of DNA extraction product

Use method (Sambrook *et al*., 1989) to detect the presence of DNA as follows: Take 25 ml of TBE electrophoresis solution and place it in a 100 ml glass beaker and add 0.25 mg of agarose to it to make the final concentration 1%. The mold was prepared by placing the comb in the designated place for the comb at one end and pouring the dissolved agarose into the mold and leaving it in a flat position to ensure the distribution of the agarose and the transport solution evenly and leaving it to solidify at room temperature and after the solution solidifies, the comb is lifted and placed in the transport tank and the transport solution TBE is added to the tank until it completely covers the agarose.

Primers

Perform multiplex-PCR reactions using specific primers (Table 1), for each species for early detection of the five pests that infect stored grains. These primers were prepared by Macrogen in the form of a dried product at concentrations of (10-12 mole = Picomoles).

Table (1) Sequences of the primers for the six species used in the current study

Species name	Gene name	Sequence of nitrogenous bases in the primer	Length of the primer	Size of the product gene	Optimum temperature for binding
T. castenum	COXIF	CCACTCTTCACGGCACTCAA	20	535 bp	51
	COXIR	TGTTCAGCAGGAGGAAGTCT	20		
O. mercator	Uniminibar F	TCYACTAATCATAAAGATATTGGYAC	26	200 bp	51
	Uniminibar R	AAAATTATAATAAARGCRTGRGC	23		
T. granarium	T-gra-1F	TAGACACACGGGCCTACTTC	20	190 bp	54
	T-gra-1R	CCTGTTAGTCCTCCTAGGGTG	21		
L. oryzae	ZBJ-ArtF1c	AGATATTGGAACWTTATATTTTATTTTTGG	30	210 bp	54
	ZBJ-ArtR2c	WACTAATCAATTWCCAAATCCTCC	24		
R. dominica	RDF1	GCTTCTTCCACCCTCCTTAACC	22	286 bp	510
	RdR1	AGATAATAATAAAAGCAAAGC	21		

Polymerase chain reaction - Multiplex program

A Multiplex-PCR Master Mix prepared by Bioneer was used in this reaction. Added 5µL of the extracted DNA of the three species *R. dominica, T. castaneum* and *O. mercator* in the flour and 2µL of Forward Primer and 2µL of Reverse Primer for each primer. This made a total of 12µL of species-specific primers. The volume was completed to 20µL by adding 3µL of Free Water. Extract DNA individually for each species according to the extraction method mentioned above. Add to the master mix tube 5µL of DNA extracted from each of *R. dominica, T. castenum,* and *O. mercator* separately, 2µL of Forward Primer and 2µL of Reverse Primer for each species individually. Complete the volume by adding 11µL of Free Water before placing the samples in the thermo cycler. Shake the master mix tube vigorously by hand or with a Vortex device and use the following program in the device mentioned as in the Table (2).

Table (2) The first experiment program used in the reaction

Steps	Temperature	Time	Number of cycles
Initial denaturation	94 °C	3 min	1
Denaturation	94 °C	1min	1
Annealing	51 °C	1min	30
Extension	72 °C	1min	1
Final Extension	72 °C	7min	1

The above program is for the first experiment. In the second experiment 5 µL of DNA extracted from each of *T. granarium* and *L. oryzae* in red bean were added to one master mix tube. 8 µL of primers specific to each species were added to this tube and the volume was completed to 20 µL by adding 7 µL of Free Water. The master mix tube was shaken well using a Vortex device and placed in a thermo cycler and the following program was used as in the Table (3):

Table (3) The second experiment program used in the reaction

Steps	Temperature	Time	Number of cycles
Initial denaturation	94 °C	3 min	1
Denaturation	94 °C	1min	1
Annealing	54 °C	1min	30
Extension	72 °C	1min	1
Final Extension	72 °C	7min	1

Electrophoresis of Multiplex-PCR

Electrophoresis was performed after the amplification process was completed to ensure the presence of the bands. The electrophoresis of Multiplex-PCR was similar to that of DNA extraction except that 0.5 mg of agarose was dissolved in 25 ml of TBE solution to give a final volume of 2% after removing the comb. A DNA ladder of 100-1500 bp (3µL) was added to the first well as a standard sample and the Multiplex-PCR product was added to the second, third, fourth, and fifth wells. The normal PCR product of each species was added in which the species-specific primer was used with the same degree of binding to the Multiplex-PCR.

RESULTS AND DISCUSSION

The amplification results in the first experiment (Figure 1) showed that 51˚C is the best temperature for binding primers to three pest species, *R. dominica*, *T. castaneum*, and *O. mercator* when using three primers in the same reaction tube and using a primer specific to each species individually. In the first experiment the first species appeared with a molecular weight of 286 bp the second species appeared with a molecular weight of 535 bp and the third species appeared with a molecular weight of 200 bp. In the second experiment (Figure 2), the temperature of 54 was the best for binding primers as the species *Trogoderma granarium* appeared at a molecular weight of 190 bp while the species *Latheticus oryzae* appeared at a molecular weight of 210 bp.

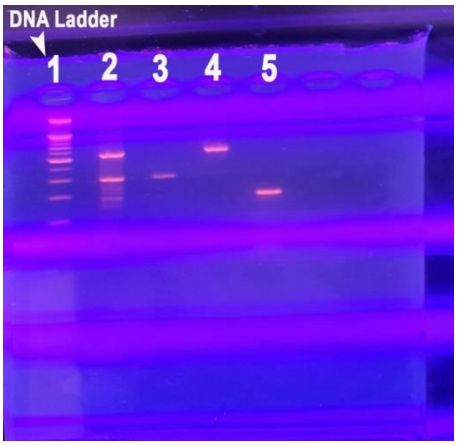

Figure (1) Multiplex PCR electrophoresis results for three species in an infected wheat sample. Sample (1) Ladder DNA and sample (2) the three bands for three pest species *O. mercator*, *T. castenum*, *R. dominica* and sample (3) with a molecular weight of 286bp for the species *R. domonica* and sample (4) with a molecular weight of 535bp for the species *T. castenum* and sample (5) with a molecular weight of 200bp for the species *O. Mercator*.

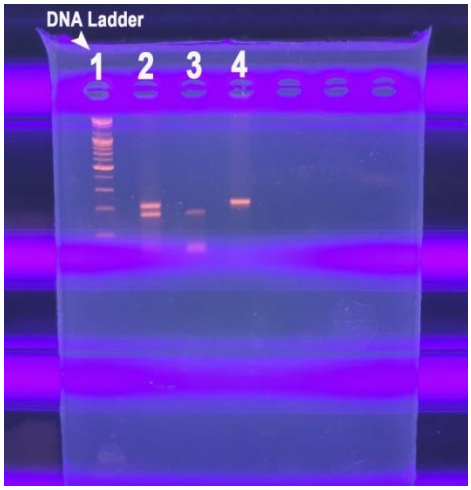

Figure (2) Multiplex PCR electrophoresis results for two species in a red bean sample. Sample (1) Ladder DNA and sample (2) the two bands for two pest species *T. granarium* and *L. oryzae* in red bean sample (3) with a molecular weight of 190bp for *T. granarium* and sample (4) with a molecular weight of 210bp for *L. oryzae*.

Molecular diagnosis is efficient in diagnosing a wide range of species even when the species are undiagnosed or previously unknown (Rugman *et al*., 2009). Among the benefits of molecular diagnosis is that it is also used to

identify undiagnosed species and confirm the diagnosis of phenotypically diagnosed species, when the sample size is small and at different life stages and when the phenotypic diagnosis is insufficient and inconclusive (Packer *et al*., 2009). It is also used when there are large quantities of samples or there are small quantities of samples that need to be diagnosed quickly (Valenzuel *et al*., 2007). PCR technology is generally used in genetic mapping and finding genetic and evolutionary relationships between populations and the reason behind its wide applications is its speed and efficiency in identifying species (Kumar and Gurusubramanian, 2011).

The Multiplex-PCR method in particular which is adopted in our current study is one of the methods that are simple to implement and usually have few errors and save a lot of time compared to other traditional Single plex-PCR methods (Bai *et al*.,2009). It is also one of the reliable molecular methods for detecting five species of internal pests that infect stored grains. One of the main reasons for testing the Multiplex-PCR method as a suitable method is to conduct a large specificity test for a wide range of species expected to be present in grain stores and it works on all stages of growth in pests starting from eggs to adults and even when there are remains of insect species (King *et al*., 2011).

CONCLUSIONS

The results proved the success of Multiplex PCR technology in the early detection of internal infections affecting grains and other stored products, by designing specialized primers for each type.

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