Molecular Techniques for Detection of *Tribolium confusum* Infestations in Stored Products

K. NOWACZYK,[1,2] A. OBREPALSKA-STEPLOWSKA,[1] M. GAWLAK,[3] J. E. THRONE,[4] P. OLEJARSKI,[3] AND J. NAWROT[3]

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ABSTRACT The confused flour beetle, *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae) is a stored-product pest that contaminates a wide range of food products, from flour and cereals to spices. The insect reduces food quality and is responsible for large economic losses every year. Although several methods for detection of stored-product pests are common and widely used, they are time-consuming and expensive. Therefore, establishing molecular methods of detection of stored-product pests could provide a useful alternative method. We have undertaken attempts to establish methods of detection of *T. confusum* based on molecular biology techniques of standard and real-time polymerase chain reaction (PCR). Total DNA of *T. confusum* and red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), used as a negative control, was isolated from insects and used as a template in standard and real-time PCR reactions. Specific primers have been designed on the basis of sequences of internal transcribed spacer (ITS) fragment of rDNA and subunit I of mitochondrial cytochrome oxidase of *T. confusum* available in the GenBank database. Standard PCR reactions with primers specific to the ITS fragment proved to be reliable and sensitive. Real-time PCR reactions with primers specific for mitochondrial DNA are considered to serve as a supplemental detection method for quantitative assessment of the infestation level.

KEY WORDS *Tribolium confusum*, stored-product pests, detection, real-time PCR

The essential aim of attempting to detect insects in stored food is to locate them in the early stage of infestation so that pests will not be present in consumer products. Presence of insects and other filth in food products may lead to destruction of the product and economic losses. Conventional detection methods can prove less sensitive to low population densities. The development of better methods of insect detection in grain, seeds, fruits, and processed food is a very important problem throughout the world.

The confused flour beetle, *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae), is a major secondary stored-product pest, feeding on cereals, flour, bean, spices, pasta, and many other products. It causes major economic losses by contaminating food, lowering its nutritive value, and creating conditions favorable for mold growth. It has been estimated that economic losses caused by stored-product pests can range from 1.25 to 2.5 billion dollars annually in the United States (Flinn et al. 2007). *T. confusum* and its close relative, the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), may elicit an allergic response. Also, the presence of excrement and insect fragments reduces the quality of food products. The importance of *Tribolium* species is evidenced partly by the extensive studies that have been done concerning one of the *Tribolium* species, *T. castaneum*, in the areas of biochemistry and genetics, including commercial support to map its genome (Throne et al. 2003).

Classic methods of detection of hidden contamination with stored-product pests (acoustic detection, carbon dioxide measurement, soft X-ray method, and near-infrared spectroscopy) are routinely and widely used, and they had been reviewed previously (Hagstrum and Subramanyam 2006). However, they do not always meet the requirements of the modern large-scale food industry for rapid and inexpensive detection techniques. Immunochemical methods (e.g., enzyme-linked immunosorbent assay, Krizkova-Kudlikova et al., 2007); flow-injection immunoassay analysis, immunoassay chromatography, and immunosensors) also are used for detection of stored-product pests, but some immunoassays are quantitative methods, whereas others can only be used as screening methods. Larvae and adults of *T. confusum* live

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[1] Interdepartmental Laboratory of Molecular Biology, Institute of Plant Protection–National Research Institute, Wladyslaw Wegorek str. 20, 60-318 Poznan, Poland.

[2] Corresponding author, e-mail: k.nowaczyk@ior.poznan.pl.

[3] Department of Entomology, Institute of Plant Protection–National Research Institute, Wladyslawa Wegorka 20, 60-318 Poznan, Poland.

[4] USDA–ARS Grain Marketing and Production Research Center, Manhattan, KS 66502.

Table 1. Primer and probes sequences used in the study

Primer/ probe name	Sequence 5′–3′	Annealing temp (°C)	Product length (bp)
TconfA	GTGTATCTACACACACACAC	54 (PCR)	150
TconfB	GAACCGCCAATATTGAGTTG		
TconCOIa	CTTCCTGGATTCGGAATAATC	55 (PCR) 60 (qPCR)	100
TconCOIb	CGAACCCTAGTAATCCAATTG		
TcasCOIa	CTTCCAGGATTTGGCATAATC	57 (PCR)	100
TcasCOIb	CAAAACCTAAGAGCCCAATTG		
TaqMan	6FAM-CATTTGGCACCTTAGGAATAA-BHQ	60	

The primers TconfA/B anneal to the rDNA sequence, TconCOIa/b primers and TasCOIa/b primers anneal to the mtCOI gene of *T. confusum* and *T. castaneum,* respectively. TaqMan it is a dual-labeled probe: with FAM and Black Hole Quencher (BHQ) dyes.

loosely among food products, and they can be detected by sieving samples or by pheromone traps. However, remnants of their bodies in processed food are very difficult to remove.

Molecular biology techniques used for detection have proved to be rapid and reliable and allow for large-scale analysis of multiple samples. They are widely used in microbiology for detection of viruses (Agindotan et al. 2007), bacteria (Chomvarin et al. 2007), and fungi (Guglielmo et al. 2007). Real-time polymerase chain reaction (PCR) is an approved method of detection of genetically modified organism components in food (Ciabatti et al. 2006). Molecular biology is being used in entomology as well as in ecological research, and it has found extensive applications in these fields, including functional genomics, basics of insect pheromone biosynthesis and reception, and plant–insect interactions (Tittiger 2004). Molecular biology techniques have also been used in studies of stored-product insect pests. The random amplified polymorphic DNA technique was used to describe molecular markers for differentiation of *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) and *Sitophilus granarius* (L.) Coleoptera: Curculionidae) (Hidayat et al. 1996) and for inter- and intraspecific differentiation of stored-product pests (Fleurat-Lessard and Pronier 2006).

Because rRNA genes as well as mitochondrial genes occur in the cell in many copies, the sensitivity of detection could be increased by the use of mitochondrial genes as a target for specific oligonucleotides in standard and real-time PCR reaction. Both genes have been used for phylogeny studies (Marvaldi et al. 2002) and for detection of stored-product insect pests (Hidayat et al. 1996).

Recently, we have described a real-time PCR-based approach for detection of *S. granarius* (Obrepalska-Steplowska et al. 2008). Here, we have undertaken an attempt to detect *T. confusum* infestation in comestibles by using two methods: standard PCR reaction with primers complementary for rDNA and subunit I of mitochondrial cytochrome oxidase (mtCOI) genes, enabling detection of that insect as well as differentiation between *T. confusum* and *T. castaneum,* and real-time PCR reaction with TaqMan probe for quantitative detection of *T. confusum* in food.

The purpose of this article is to describe this new method for food inspection in manufacturing, packaging, and throughout marketing channels.

Materials and Methods

Insects. The populations of *T. confusum* and *T. castaneum* used in our study came from the laboratory colony maintained in the Department of Entomology in the Institute of Plant Protection–National Research Institute, Poznan, Poland. Insects were reared on oat flakes at 25°C and 65–70% RH.

DNA Isolation. Total DNA from whole adult insects and from contaminated food products was isolated with DNeasy Blood and Tissue Kit (Qiagen, Wroclaw, Poland), after freezing in liquid nitrogen, grinding with mortar and pestle, and overnight digestion with proteinase K. After the DNA isolation, a series of dilutions was prepared. Plant DNA used as a negative control was isolated from oat flakes with DNeasyPlant Mini Kit (Qiagen).

The oat flakes samples were purposely contaminated and mixed for equal distribution. Ten insects were put in 100 g of flakes and mixed thoroughly in homogenizer. Then, a 10-g sample of contaminated flakes was added to 90 g of uncontaminated flakes and mixed as described previously. The procedure was repeated once more to obtain a contamination level of one insect per kg. From the last samples DNA was isolated and, for the purpose of sensitivity analysis, further diluted with water, to obtain concentration of insect DNA corresponding to infestation level of one insect per 10 kg and one insect per 100 kg. Efficiency and integrity of isolated DNA were checked by electrophoresis in agarose gels.

PCR Amplification. The genomic DNA was amplified in vitro with specific primers (Table 1) complementary to the sequence encoding subunit I of mitochondrial cytochrome oxidase and rRNA. All primers were designed with PrimerSelect Software from DNASTAR, Lasergene 7.1 package (GATC Biotech, Konstanz, Germany), based on available *rDNA* sequences of *T. confusum* and *mtCOI* sequences of *T. confusum* and *T. castaneum* obtained from the GenBank database. The conditions of the PCR reaction were optimized for each primer set. Standard PCR was used for differentiation between *T. confusum* and *T. castaneum* and as a preliminary specificity test before real-time PCR analysis. PCR reactions were carried out in an Eppendorf Mastercycler in the final volume of 10 μl. The reaction mixtures contained 50 ng of DNA, 1 μM of each primer, 200 μM dNTP, and 0.2 U of Allegro *Taq* polymerase (Novazym, Poznan,

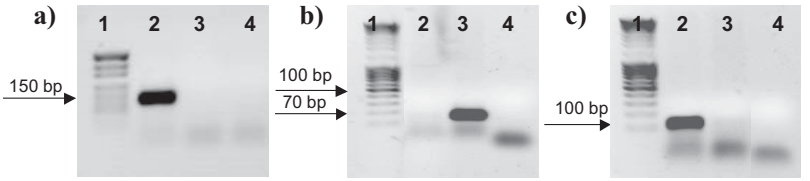

Fig. 1. Results of PCR reaction with PCR primers specific to ITS sequence of *T. confusum* (TconfA/B, see table) (a), mtCOI of *T. confusum* (TconCOIa/b, see table) (b), and mtCOI of *T. castaneum* (TcasCOIa/b, see table) (c). DNA marker in lane 1. The following templates were used: 2, *T. confusum* DNA; 3, *T. castaneum* DNA; and 4, PCR mixture with no DNA template.

Poland) in 1× polymerase buffer [70 mM Tris-HCl, 16 mM $(NH_4)_2SO_4$, and 2.5 mM $MgCl_2$]. The samples were amplified for 30 cycles. Each cycle consisted of the following steps: denaturation at 95°C for 30 s., annealing at temperatures listed in Table 1 for 30 s and primer extension at 72°C for 30 s. Each insect sample was tested in triplicate. Products of amplifications were analyzed in 1% agarose gel stained with ethidium bromide.

Real-Time PCR. In TaqMan based real-time PCR, in addition to primers pair, the reaction includes a linear probe linked with fluorescent dye at the 5′ end and a quencher at the 3′ end. In comparison to DNA binding dyes (e.g., SYBR Green) internal probe (e.g., TaqMan) along with the primers provides a higher level of detection specificity. Another advantage of TaqMan application is that more than one sample can be detected in a single reaction when suitable because probes may be labeled with different fluorophores.

Qualitative and quantitative real-time PCR reactions were performed using MxPro 3005P (Stratagene, Warsaw, Poland). Analysis and interpretation of results were done using software provided by the manufacturer. Reactions were carried out in a 10-μl mixture containing AmpliLight Mix (Novazym), 0.25 μM primers, 0.2 μM TaqMan probe, and DNA template. For sequences of primers and TaqMan probe, see Table 1. TaqMan probes are dual-labeled hydrolysis probes and are a registered trademark of Roche Molecular Systems, Inc.

To assess the sensitivity of detection for the sample of contaminated oat flakes, a series of dilutions were prepared. DNA isolated from contaminated oat flakes dilutions corresponding to infestation levels of 100 insects per kg, 10 insects per kg, one insect per kg, and the DNA dilution of the last sample to obtain the sample with the contamination level equivalent to one insect per 10 kg were used as a template. DNA isolated from *T. confusum* was used as the positive control of reaction. Samples with no DNA template were used as a negative control. For real-time PCR, the same primers specific for mtCOI of *T. confusum* (TcCOIa/TcCOIb) as for standard PCR were used. The TaqMan probe was applied to increase the specificity and sensitivity of reaction (for sequence, see Table 1).

The real-time PCR reactions were performed under the following conditions: initial denaturation at 95°C for 10 min followed by 40 cycles consisting of denaturation at 95°C for 10 s and annealing at 53°C for 10 s. All samples were analyzed in triplicate.

Results

PCR Detection and Differentiation. Specificity of the designed primers toward *T. confusum* rDNA (Fig. 1a) and mtCOI (Fig. 1b) was verified in standard PCR reaction, by using DNA of *T. castaneum* as a negative control. Simultaneously, presence of *T. castaneum* DNA was confirmed in PCR reaction with primers specific for mtCOI of *T. castaneum* with *T. confusum* DNA as a negative control (Fig. 1c).

Figure 2 presents the sequence alignment of mtCOI fragment with oligonucleotides used in the experiment to show the difference between sets of primers and explain the specificity of the reaction with TaqMan probe.

Real-Time PCR Detection. Detection of contamination by *T. confusum* with real-time PCR was per-

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                        *        20         *        40         *        60         *        80         *       100
T_confusum : GAAGTTTACATTCTAATTCTTCCTGGATTCGGAATAATCTCCCATATTATTAGACAAGAAAGAGGAAAAAAAGAAGCATTTGGCACCTTAGGAATAATTTA : 101
T_castaneu : GAGGTGTACATTCTAATTCTTCCAGGATTTGGCATAATCTCCCACATTATTAGACAAGAAAGAGGAAAGAAAGAAGCATTTGGAACACTAGGAATAATTTA : 101
TconCOIa   : ------------------CTTCCTGGATTCGGAATAATC------------------------------------------------------------ :  21
TconCOIb   : ------------------------------------------------------------------------------------------------------ :   -
TcasCOIa   : ------------------CTTCCAGGATTTGGCATAATC------------------------------------------------------------ :  21
TcasCOIb   : ------------------------------------------------------------------------------------------------------ :   -
PROBE      : ------------------------------------------------------------------------FAM-CATTTGGCACCTTAGGAATAA-BHQ :  27

                        *       120         *       140         *       160         *       180         *       200
T_confusum : TGCCATAATAGCAATTGGATTACTAGGGTTCGTTGTATGAGCACACCACATATTCACTGTAGGAATAGATGTTGACACTCGAGCCTATTTCACATCAGCAA : 202
T_castaneu : TGCAATAATAGCAATTGGGCTCTTAGGTTTTGTTGTATGGGCCCACCACATATTTACCGTAGGAATAGACGTTGATACTCGAGCCTATTTCACTTCAGCCA : 202
TconCOIa   : ------------------------------------------------------------------------------------------------------ :   -
TconCOIb   : -----------CAATTGGATTACTAGGGTTCG--------------------------------------------------------------------- :   1
TcasCOIa   : ------------------------------------------------------------------------------------------------------ :   -
TcasCOIb   : -----------CAATTGGGCTCTTAGGTTTTG--------------------------------------------------------------------- :   1
PROBE      : ------------------------------------------------------------------------------------------------------ :   -
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Fig. 2. Alignment of mtCOI fragments of *T. confusum* and *T. castaneum* with primers and probe used in the experiment. The sequences of reverse primers are shown as reverse compliment. Nucleotides conserved in all sequences are shaded.

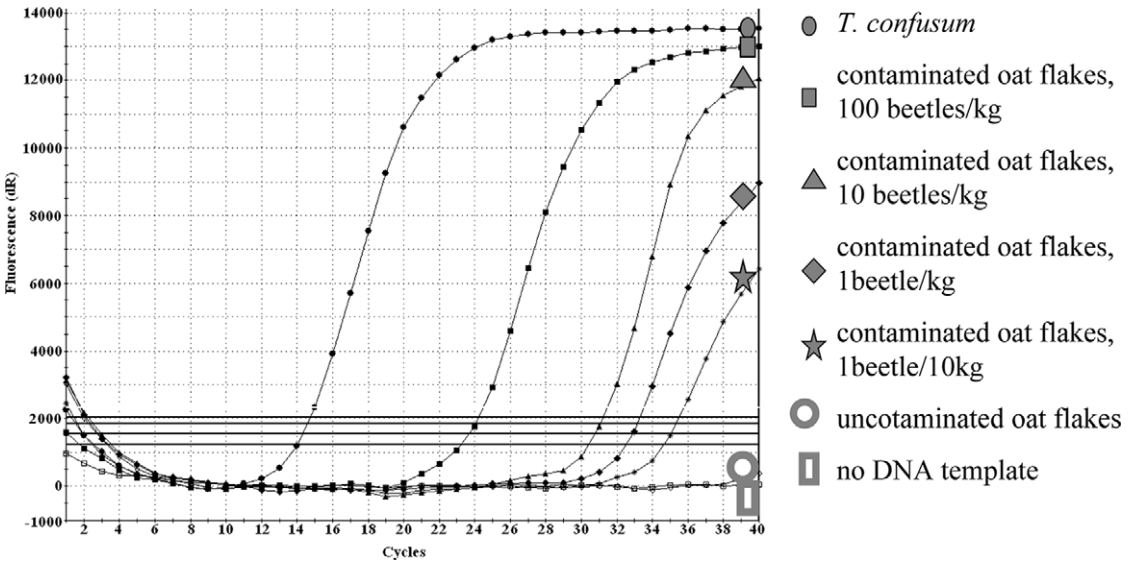

Fig. 3. Amplification plot of real-time PCR reaction with primers and probe specific for COI of *T. confusum.*

formed using DNA samples isolated from contaminated oat flakes and uncontaminated oat flakes. This approach makes it possible to assess the presence of infestation with *T. confusum* quantitatively. The analyzed samples—positive control and other contaminated samples—gave positive results entering the logarithmic phase between 14 and 35 cycles of amplification (Fig. 3). We were able to detect even the sample with the amount of *T. confusum* DNA equivalent to the infestation level of one insect in 10 kg of oat flakes. For that sample, we have obtained the cycle threshold (Ct) value of 24.71 (Table 2). The Ct is defined as the cycle at which PCR enters the exponential phase and the fluorescence emission exceeds the fixed threshold.

Discussion

Techniques of detection based on molecular biology are used increasingly in several areas of biology because of their rapidity, sensitivity and reliability. In our study, we have undertaken to describe and optimize detection of one of the most important stored-product insect pests, *T. confusum.* To achieve this goal, we have designed specific primers hybridizing to multicopy genes *mtCOI* and *rDNA.* They were used in detection of *T. confusum* and in differentiation between this insect and its close relative *T. castaneum.* Additionally, we designed a specific TaqMan probe for *T. confusum* that enables quantitative assessment of infestation level caused by this insect in food. Using real-time PCR reaction, we are able to detect infestation of stored products with *T. confusum* even if the contamination level was equivalent to that of one insect per 10 kg.

Table 2. Ct values of analyzed samples in real-time PCR reaction with primers and probe specific for COI of *T. confusum*

Sample	Ct value
T. confusum	14.64
Contaminated oat flakes, 100 insects per kg	24.29
Contaminated oat flakes, 10 insects per kg	31.52
Contaminated oat flakes, 1 insect per kg	33.23
Contaminated oat flakes, 1 insect per 10 kg	34.71
Uncontaminated oat flakes	No Ct
No DNA template	No Ct

In the standard PCR reaction, we have shown that primers used are species specific. In real-time PCR reaction, we have shown that the method is very sensitive.

Because the studied insects live loosely in stored grain, it is possible to eliminate them to a significant extent by sieving. However, fragments and remnants of *T. confusum* bodies may remain in processed food. The real-time reaction may serve to check whether food is pure or contaminated with confused flour beetle and to what extent. To quantify level of contamination, a standard curve with known different concentrations as described in the methods is needed. However, we would suggest to prepare this curve independently for each specific real-time PCR machine and stock analyzed because of the differences in Ct readings for both the instrument used and different stored or processed commodities.

The separate standard curve for each stock of material tested is particularly important, especially when quantitative assessment is needed, because DNA (e.g., from dead insects and their fragments) is prone to degradation and the reaction may give slightly different readings for samples stored for different amount of time. However, the present protocol is designed for multicopy genes and the products of both standard and real-time PCR reactions are expected to be very short; hence, the sensitivity of the reaction may drop only insignificantly during storage.

Application of the specific TaqMan probe in the reaction is advantageous because the results can be read directly without the necessity of melting curve analysis. Amount of fluorescence arising during the reaction reflects directly the number of amplifications in real-time gene copies.

The big improvement in sensitivity has been achieved by the application of genes existing in many copies and having also variable regions as in the case of mitochondrial DNA (mtDNA) and internal transcribed spacer (ITS) sequences from rDNA (Cheng et al. 2003, Hsu et al. 2003). Although those genes also share quite high homology between related species as has been shown for related *Sitophilus* species (Peng et al. 2002), other more conservative genes are not useful for distinguishing species at all. Therefore, it is very important to design primer pairs as well as probe in regions with the biggest nucleotide differences even in the case of the aforementioned rDNA and mtDNA sequences.

The biggest drawback of this method is the relatively high price of real-time instruments as well as reaction kits and the TaqMan probe. However, because application of molecular biology techniques gives rapid results without compromising sensitivity and specificity the methods should be beneficial for reliable assessment of food quality.

The first (smallest) level of infestation that is permissible according to the Polish norm PN-69/R-74016, it is one beetle per kilogram. In this light, obtained results indicate that there is possibility to detect even minor infestation in analyzed food. A very important issue for detection purposes by using our approach is the homogeneity of the infestation throughout the tested material that may be obtained by thorough mixing and homogenization. In laboratory experiments, the sample size is small; however, even when the concentration of one beetle per kg was tested in triplicate, results were reproducible. Therefore, in inspection of larger portions, it is important to analyze several randomly chosen samples from previously prepared tested samples for infestation of homogenous commodities.

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