Molecular techniques for detection of granary weevil (Sitophilus granarius L.) in wheat and flour
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Molecular techniques for detection of granary weevil (*Sitophilus granarius* L.) in wheat and flour

Abstract

The granary weevil (*Sitophilus granarius* L.) is a stored grain pest, which causes major economic losses. It reduces the quantity and quality of the grain by its feeding and excretion. We have analyzed sequences of *S. granarius* mitochondrial cytochrome oxidase subunits genes: mtCOI and mtCOII and compared them with mtCOI/II sequences available in GenBank. The analyzed genes displayed a high level of homology between corresponding subunits. We have undertaken attempts to develop detection methods for contamination by *S. granarius* in wheat and wheat flour based on the molecular biology techniques: standard and real-time PCR with a TaqMan molecular probe. We applied specific primers designed on the basis of available sequences for mtCOI and mtCOII genes, and established optimal reaction conditions. The specificity of both methods was studied by using a species closely related to *S. granarius* – *S. oryzae* and *S. zeamais*. We showed that the sensitivity threshold was very high – we were able to detect the equivalent of one beetle per 100 kg of flour when the real-time PCR with TaqMan probe method was applied to model samples. The primer sets used turned out to be species specific, and technique was rapid, reliable and very sensitive.

Key words: *Sitophilus granarius*, detection, real-time PCR
Introduction

The granary weevil, *Sitophilus granarius* (L.), and other stored-product insect pests cause extensive economic losses in stored grain. Each year in the USA, stored-product insects may cause from $1 billion (Cuperus and Krischik 1995) to $5 billion in losses (Davis 1991). *S. granarius* reduces not only the amount of grain available to sell, but also the quality of wheat flour. Excrement left by the insects and fragments of insects contaminate the flour milled from infested wheat. Therefore, detecting and removing insects from grain, preventing insect pests from contaminating processed food, and detecting insect fragments in flour are of great importance. At present, there are no specified permissible limits for contamination of food with stored-product insect pests in Poland. According to old Polish norm fewer than 3 insects beetles per kilogram of food were allowed (PN-74/A-74016). In the USA, only fewer than 75 insect fragments per 50 g of flour are allowed (Toews et al. 2006).

Larval and adult insects that live outside grain kernels are easily detected by sieving. However, some primary pests of grain [*Sitophilus granarius* L., *S. oryzae* L., *S. zeamais* Motsch. (Curculionidae) and *Rhizopertha dominica* F. (Bostrichidae)] develop inside grain kernels, causing so called hidden infestation, which is very difficult to detect, particularly just after oviposition and during the early larval stages.

There are number of techniques available for the detection of stored-product insects hidden inside of grain kernels, and they have been recently reviewed (Hagstrum and Subramanyam 2006). They include very old methods such as staining of kernels as described by Frankenfeld (Frankenfeld 1948) and also quite modern methods such as NIR reflectance spectroscopy (Dowell et al. 1998), NMR spectroscopy (Chambers et al. 1984),
acoustical sensors (Hagstrum et al. 1996, Fleurat-Lessard et al. 2006), microwave radar
(Mankin 2004), computed tomography (Toews et al. 2006), X-ray method (Karunakaran et
al. 2003, Fornal et al. 2006), immunoassay analysis (Kitto 1993) and the Single Kernel
Characterization System (SKCS) (Martin 1993).

The accuracy of above-mentioned methods depends on insect species, developmental stage of insects and grain type (soft or hard). Accuracy of acoustical methods is correlated with temperature and activity of insect moving and feeding (Hagstrum and Flinn 1993): the number of sounds increased as the temperature increase from 17.5 to 37.5°C. The automated NIR system was able to detect different stages of live insects (small, medium and large larvae and pupae) with an accuracy of 62%, 84%, 92% and 94%, respectively (Maghirang et al. 2003). The SKCS system had an accuracy of 24.5%, 62.2%, 87.5%, and 88.6%, respectively (Pearson et al. 2003), for the same insect stages. The X-ray method using film took about 2.5 h and had 97% accuracy. Digitizing the film X-ray images for automated detection decreased the error rate below 1% (Haff and Slaughter 2002). These modern electronic and chemical methods are more accurate than flotation and cracking methods (Russel 1988, Brader et al. 2002). Methods of detection vary in their sensitivity and the time needed for analysis, which is very important in commercial application of the technologies. There is a trend towards automatization and shortening of time procedures of insect detection in food processing industries.

Molecular biology techniques such as standard and real-time PCR are increasingly popular in detection studies. They are frequently used for detection of viruses (Watzinger 2006), bacteria (Ward and Bej 2006), nematodes (Madani et al. 2005), and other microorganisms. Real-time PCR is also an approved method of detection of GMO components in food. In such analyses, primers that hybridize to sequences introduced into cells together with
transgenes are employed. Examples are the *Cauliflower Mosaic Virus* 35S promoter (CaMV35S) (Akiyama et al. 2005), the α-glucuronidase gene (GUS) (Ding et al. 2005), and the 5′-transgene integration sequence (Yang et al. 2005).

Substantial progress has been made in molecular genetic analysis of insects in the areas of identification, gene structure-function relationships, and use of molecular markers to track insect populations (Handler and Beeman 2003). There has been much research conducted in the field of stored-product pests. Molecular tools have been used to determine the distribution of species (Fields and Philips 2002) and in determination of genetic relationships and molecular diversity between strains and populations of stored-product insect pests using the PCR-RAPD approach (Fields and Philips 2002, Fleurat-Lessard and Pronier 2006). Also diversity of *Sitophilus* spp. was analyzed genetically with RAPD fingerprints (Fleurat-Lessard and Pronier 2006), and molecular markers for diagnosis of *S. oryzae* and *S. zeamais* were used to classify both species to two distinct gene pools using PCR-RAPD and PCR-RFLP approaches (Hidayat et al. 1996). These pests also were analyzed using DNA amplification fingerprinting with primers for aldolase, prolactin receptor, and interleukin-beta genes and using PCR for ITS1/2 fragments of rDNA genes (Peng et al. 2002). The ITS2 fragment of rDNA was also used to develop marker-assisted identification method of *S. oryzae* (Jeong et al. 2006)

Sequences of mitochondrial DNA (mtDNA) and DNA encoding ribosomal RNAs (rDNA) are commonly used for detection, identification or phylogeny studies (Sheppard et al. 2005, Horn et al. 2006). Among insects, these genes were used to identify internal feeders of pome fruits by both standard and real-time PCR reaction (Barcenas et al. 2005). Also, multiplex PCR reaction based on mtCOI and mtCOII was applied for differentiation of western and northern corn rootworm larvae from the genus *Diabrotica* sp. (Roehrdanz...
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The major advantage of this approach is their occurrence of mtDNA in many copies in each cell. Copies exist either in tandem, as rDNA genes or in organelles like mitochondria that are numerous in every cell. This feature enables detection or identification of organisms even if a limited quantity of material is available for analysis.

The objective of our study was to analyze the sequences of mitochondrial oxidase subunits (COI and COII), and to design specific primers using this information and to determine the possibility of detection of S. granarius infestations in food by using the molecular biology techniques: standard PCR and real-time PCR. The specificity of both methods as well as sensitivity and reactions conditions are reported.

Materials and methods

Insects

Adults and larvae of the granary weevil used in experiments came from our laboratory colony maintained on whole wheat kernels (winter variety KANCLER) at 24°C and 65-70% relative humidity. Adults were collected every three days, and larvae were extracted from kernels by manually dissecting them at different stages of larval development. Also three populations from Germany, France and Denmark kindly provided by Dr Cornel Adler, Dr Patrick Ducom and Dr Lise Stengard Hansen, respectively, were tested. In detection studies, samples of DNA isolated from S. granarius L., S. oryzae L., S. zeamais Motsch., and secondary stored-products pests: Tribolium confusum Duv. and T. castaneum Herbst. as well as uncontaminated flour or wheat, and contaminated with S. granarius flour and wheat were analyzed. The flour and wheat samples were purposely contaminated (with

beetles and larvae of S. granarius, respectively) with appropriate amount of insect material,
and then in case of flour mixed thoroughly in homogenizer for equal distribution. Later, several of random-chosen samples of 50 mg were taken for analyzes.

DNA isolation

Insects were ground under liquid nitrogen with a mortar and pestle. The DNA from adult or larval insects was isolated with DNeasy Tissue Kit (Qiagen, Wroclaw, Poland) and from wheat or flour with DNeasy Plant Mini kit (Qiagen, Wroclaw, Poland) according to the manufacturer’s instructions. We used a prolonged incubation time of 3 hours for lysis of the cells. Efficiency and integrity of isolated DNA were checked by electrophoresis in agarose gels, and its purity was analyzed spectrophotometrically by measuring $A_{260}/A_{280}$ ratio.

PCR amplification

The genomic DNA was amplified in vitro with specific primers. All primers were designed with PrimerSelect Software from DNASTAR Lasergene 7.1 package (Madison, USA).

First, primers complementary to the sequence encoding I and II subunits of mitochondrial cytochrome oxidase were applied (Table I). We performed amplification of COI and COII in triplicate. The obtained sequences (see DNA cloning, sequencing and sequence analyses section) were compared with their counterparts from GenBank and together used to design other specific primer sets (giving shorter products) for detection purposes. These primers were utilized for detection of *S. granarius* larvae in wheat flour and wheat using standard and real-time PCR (Table II, primers’ hybridization positions are shown in Figure 1).

PCR reactions were carried out in an Eppendorf Mastercycler in a final volume of 10 µl. The reaction mixtures contained 50 ng DNA, 1 µM of each primer, 200 µM dNTP, 0.2 U of Allegro Taq polymerase (Novazym, Poznan, Poland) in 1x polymerase buffer [70
mM Tris-HCL, 16 mM (NH₄)₂SO₄ and 2.5 mM MgCl₂]. The samples were amplified for 35 cycles. Each cycle consisted of the following steps: denaturation at 95°C for 1 min, annealing at temperatures listed in Tables I or II (depending on the primers used) for 30 sec., and primer extension at 72°C for 1 min. Each food or insect sample was tested in triplicate. Products of amplifications were analysed in 2% agarose gel stained with ethidium bromide.

[Insert Table I about here]

[Insert Table II about here]

[Insert Figure I about here]

DNA cloning, sequencing and sequence analyses

DNA fragments obtained in PCR reactions with primers listed in Table I (three samples for each mitochondrial cytochrome oxidase subunit) were separated on 2% agarose gels, and then extracted and purified with QiaExII Gel Extraction Kit (Qiagen, Wroclaw, Poland). Purified fragments were cloned in pGEM®T-Easy Vector System (Promega, Straszyn, Poland) according to the manufacturer’s instruction. The obtained recombined plasmids were added to 50 µl of DH5α competent cells and transformed by electroporation into using Micro Pulser electroporation system (BIO-RAD, Warsaw, Poland). Then bacterial cells were incubated with shaking in LB medium at 37°C for one hour. Next, transformation cultures were plated onto LB/ampicilin (100 µg/ml)/IPTG (0.5 mM)/X-gal (80 µg/ml) plates and incubated overnight at 37 ºC. The obtained bacterial colonies were screened for the presence of inserted DNA sequence in vector by using PCR amplification with primers from Table 1 as described before (PCR amplification section). Colonies with confirmed recombinant plasmids were grown again in 3 ml of LB/ampicilin medium and eventually plasmids were isolated using Qiaprep Spin Miniprep Kit (Qiagen, Wroclaw,
Poland). Then, 8 clones from each sample were automatically sequenced (DNA Sequencing and Oligonucleotides Synthesis Service, IBB Warsaw) to confirm that nucleotide changes were neither due to polymerase error nor a result of sequencing of one of the alleles from a gene in heterozygous state. The obtained sequences were subsequently analyzed and compared with appropriate sequences from GenBank by using blastn tool (www.ncbi.nlm.nih.gov/blast).

Real-time PCR

Qualitative real-time PCR reactions were performed using MxPro 3005P (Stratagene, Warsaw, Poland). Analysis and interpretation of results were done with the software provided by the manufacturer. Reactions were carried out in a 10 µl mixture containing: AmpliLight Mix (Novazym, Poznan, Poland), 0.5 µM primers, 1 pmol of Taqman probe (5’FAM-TGACGGAACACCTGGTC-TMR-3’) designed in Beacon Desinger, PREMIER Biosoft International, linked with FAM as a fluorophore and TAMRA as a quencher (TibMolBiol, Poznan, Poland) and DNA template.

The real-time PCR reactions were performed under the following conditions: initial denaturation at 95°C for 2 minutes followed by 40 cycles consisting of denaturation at 95°C for 20 sec., and annealing at 60°C for 30 sec., with single measurement of fluorescence emitted by FAM dye released from TaqMan probe hydrolysis due to 5’ exonuclease activity of DNA polymerase.

Analysis of the sensitivity and specificity of real-time PCR

We evaluated also the absolute sensitivity of the real-time PCR reaction. We prepared several concentrations of contaminated flour: 100 beetles/1 kg, 10 beetles/1 kg, 1 beetle/1 kg of flour. From the last concentration, samples were taken and diluted with
sterile water to obtain concentrations corresponding to equivalent of 1 beetle/10 kg (=0.1 beetle/kg, see Figure 4) and 1 beetle/100 kg of flour (=0.01 beetle/kg).

To assess the specificity of the reaction as negative control beetles two closely related species belonging to primary stored-products pests: *S. oryzae* and *S. zeamais*, and two species belonging to secondary stored-products pests: *T. confusum* and *T. castaneum* were tested.

All samples were analyzed in triplicate.

**Results**

*Analysis of the DNA sequences for mtCOI and mtCOII*

To achieve our goal, we used the sequences encoding subunits of mitochondrial cytochrome oxidase (COI and COII). We compared DNA sequences encoding I and II subunits of mitochondrial cytochrome oxidases by using PCR amplification with appropriate primer sets (Table I). We analyzed the sequences of DNA of *S. granarius* beetles available in our laboratory and compared them with sequences already deposited in GenBank (AY131101 and M83970). We assumed that there could have been nucleotide changes in these genes for *S. granarius* populations that have been geographically isolated. Therefore, the amplified fragments were cloned, multiplied and sequenced. We performed amplification of COI and COII in triplicate. These obtained sequences are available in GenBank under accession numbers: DQ453486 for mtCOI and DQ462235 for mtCOII. The comparison of sequences originating from our laboratory with those already deposited in GenBank showed a very high level of sequence similarity (about 99%). The comparison of mtCOII sequence from *S. granarius* with sequences derived from other *Sitophilus* species (*S. oryzae*: AY014880, *S. zeamais*: AY014881) (Fig. 1) allowed us to design distinct primer pair that specifically detect only *S. granarius* in standard and real-time PCR reaction.
The species specificity of the PCR methods were evaluated using samples of *Sitophilus oryzae* (L.) and *Sitophilus zemais* (Motsch.) a close relatives of *S. granarius* L., as well as *Tribolium confusum* (Duv.) and *Tribolium castaneum* (Herbst.) as the negative controls. We established the optimal conditions for the PCR method, and the sensitivity and reaction conditions of the real-time PCR reaction for the primers and biochemicals also were determined.

**PCR detection**

For detection of *S. granarius*, we used a primer pair specific for the sequence of II subunit of mitochondrial cytochrome oxidase (COIISG1/2). The following samples were used: DNA from uncontaminated flour and wheat, from contaminated flour and wheat, from *S. granarius* as a positive control and from *S. oryzae* as a negative control. *S. oryzae* was also a control of the specificity of primers and method. PCR reaction under conditions described in the materials and methods and Table II gave single and distinct products in the positive control and contaminated samples (Figure 2). There was no reaction product in either the uncontaminated samples nor in the sample containing DNA from *S. oryzae*.

Therefore, the primers specificity for only *S. granarius* was confirmed and we can conclude that standard PCR reaction works properly for detection of infestation with granary weevil.

[Insert Figure 2 about here]

**Real-time PCR detection**

For real-time PCR, the primers qCOIISG1/2 (Table II) and TaqMan probe were used. Detection of contamination with *S. granarius* with real-time PCR was performed using DNA samples isolated from contaminated with insect beetles flour and from contaminated with insect larvae wheat, from uncontaminated flour, and from uncontaminated wheat. DNA isolated from *S. granarius* was used as a positive control of reaction. DNA from *S.
oryzae was used as a negative control and, simultaneously, as control of detection specificity. The control sample and contaminated samples gave positive results entering the logarithmic phase with fewer than 40 cycles of amplification. The sample containing S. granarius DNA exceeded the Ct value after 16 cycle. The contaminated flour and grain exceeded the threshold after 31 and 36 cycle, respectively. The rest of samples did not enter logarithmic phase before 40 cycle (Figure 3, Table 3).

Analysis of the sensitivity of real-time PCR reaction

The sensitivity of the reaction was analyzed using several decreasing concentration of S. granarius in flour and then their dilutions in water (as described in Materials and Methods section). We found that sensitivity of detection is very high, and even very small quantities of genetic material of granary weevil are possible to detect using the real-time PCR technique. The analysis revealed that it is possible to detect S. granarius contamination even when DNA isolated from contaminated flour at the concentration of 1 beetle/1 kg was diluted 100 times in water (Figure 4). The lower concentrations were not detectable.

To check the specificity of real-time PCR method we analyzed samples containing DNA isolated from two closely related species of S. granarius – S. oryzae and S. zeamais as well as two secondary stored-products pests: T. confusum and T. castaneum. Only samples of four populations of S. granarius were detected, confirming specificity of the reaction (Fig. 5).

Discussion
Food must meet certain quality standards to qualify for human consumption. Quality is determined by not only meeting contamination limits for various organic and inorganic substances, but also by presence of microorganisms or insect pests. In the case of raw commodities, some stored-product insect pests and other contaminants are removed by cleaning prior to processing.

The traditional method – so called ‘filth test’ is simple and currently used for detection of insect fragments in grain. It is based on flotation and separation of extraneous matter from grain followed by counting. This is useful for detection of contamination in grain, but for detection of insect fragments in processed food is not applicable.

In our study, we have evaluated the usefulness of molecular biology techniques (standard and real-time PCR) for detection of one of the most common stored-product pests, *S. granarius*. After establishing optimal conditions of detection reactions we then analyzed the specificity of the investigated methods using species closely related to *S. granarius* – *S. oryzae* and *S. zeamais* as well as secondary stored-products pests: *Tribolium castaneum* and *T. confusum* as negative controls. The threshold sensitivity of real-time reaction under the described conditions is corresponding to DNA concentration in the sample equivalent to 1 beetle per 100 kg of flour.

Detection methods using sequences of genes occurring in numerous copies in each cell increases the sensitivity (Hsu et al. 2003, Cheng et al. 2003, Guha and Kashyap 2005). The number of mitochondria depends upon the metabolic requirements of the cell and may range from a few up to a few thousand.

Our earlier analysis of COI and COII genes showed that differences in sequences between *S. granarius* populations (our sequences and those previously deposited in GenBank) are not a concern, so we may assume that our primers will be useful for
detection of any populations of \textit{S. granarius}. However, both primers and reaction conditions have to be designed very carefully to ensure their species specificity. As previous analyses had shown (Peng et al. 2002), even relatively variable sequences, serving for phylogenetic or identification studies, such as ITS1 and ITS2 share a very high level of homology (96-97\%) between closely related species of laboratory colonies of \textit{S. oryzae} (L) and \textit{S. zeamais} Motschulsky. The application of more conservative genes, such as aldolase, prolactine receptor, or interleukin-beta genes using the DNA fingerprinting approach did not result in the ability to distinguish between these species.

In the reaction, we applied a TaqMan probe in addition to the specific primer pairs. Using a specific probe allows one to avoid the problem of an unspecific fluorescence signal arising from competitive reactions, such as primer-dimer formation. The presence of such a probe enables direct reading of results because only the specific product of reaction arising \textit{de novo} is detected, and no melting point analysis is needed.

Molecular techniques have very big advantages over other methods used at present for detection of stored-product pests. They are very rapid, sensitive, and species specific. Our results have shown that is possible to readily distinguish between infestations of closely related species, such as \textit{S. granarius} and \textit{S. oryzae}. However, these methods also have disadvantages. The price of appropriate kits for PCR and equipment, especially for real-time PCR, are still relatively high, but tend to decrease along with rising popularity and accessibility of real-time PCR systems. Because DNA is the target for detection, the reaction is very specific however, DNA can degrade under very long storage, which can be a drawback, especially because in the case of stored products where problems occur either because of larvae or remains of adults inside the grains. However, the primers designed for
PCR and real-time PCR give short product and hybridize to the genes existing in many copies per cell, which may overcome these obstacles.

In the present work we are not presenting a ready-to-use protocol for quantitative analysis of *S. granarius*. We describe the method, appropriate sequences, specific primers, and a molecular probe, as well as optimized reaction conditions. The method is good for detection, but for quantitative assessment we suggest preparing a standard curve with material that has been stored for a comparable time with known amounts of granary weevil remains. Then, one should analyze randomly chosen samples and compare them to the standard curve. Our concentration curve was prepared using genetic material from relatively fresh *S. granarius* beetles and served to present the sensitivity of real-time PCR with TaqMan probe under the described reaction conditions.

Nonetheless, the methods have great potential for application in the future. We think that these results will be useful for rapid and sensitive detection of live or dead stored-product pests.

A very important aspect of appropriate assessment of the infestation level is the method of sampling as well as sample size. To obtain credible results it is crucial to obtain a sample with maximum of homogeneity in the analyzed material. Therefore, flour should be mixed thoroughly, or in the case of grain this should be crushed beforehand. Afterwards, several random-chosen samples should be taken for analysis. In our case we were limited by small-scale laboratory capacity and our tests were carried out on a small sample size of 50 mg that in turn was limited by applied kit possibilities. In our experience, however, the sample size though small was sufficient to detect infestation level of the equivalent of 1 beetle per kg (as it was allowed in some countries), or lower, from the 50 ng of DNA taken out from that 50 mg sample. The results were comparable when tested in triplicate.
enhance the reliability of detection and infestation assessment it is suggested to use more
samples to analyzes as well as increase the sample size, when possible. Then statistically
the obtained results will be more accurate.

Based on our results, we can assume that the real-time reaction should be
sufficiently sensitive to meet even the most restrictive detection requirements. In addition,
standard and real-time PCR reactions significantly reduce the time required for detection
without compromising sensitivity and reproducibility. Our approach has shown that both
the primers and protocols used for amplification of subunits of mitochondrial cytochrome
oxidase result in reliable, sensitive, and species-specific detection of insects.

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Abbreviations used
mtDNA – mitochondrial DNA, COI/COII – I/II subunit of mitochondrial cytochrome oxidase,

rDNA – DNA encoding rRNA

Ct – the cycle threshold – parameter defined as the cycle at which PCR enters the exponential phase and the fluorescence emission exceeds the assigned threshold.

TaqMan® probes are dual labeled hydrolysis probes and are a registered trademark of the Roche Molecular Systems, Inc.

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Alignment of *mtCOII* sequences fragments from *Sitophilus* species available in GenBank under accession numbers: *S. granarius* M83970, *S. granarius* from Poland DQ462235, *S. oryzae* AY014880 and *S. zeamais* AY014881. Visualized in Genedoc (Nicholas et al. 1997)

The origin of sequences as well as primers and probe positions are indicated on the left.
Figure 2
Electrophoresis of PCR products on 2% agarose gel. Products of amplification from DNA isolated from: Lane 1 – positive control - *S. granarius larvae*, 2 – negative control – *S. oryzae*, 3 – flour contaminated with *S. granarius larvae*, 4 – grain contaminated with *S. granarius larvae*, 5 – uncontaminated flour, 6 – uncontaminated grain, 7 – negative control – no DNA added to PCR mixture, 8 – molecular weight marker.

Figure 3
Real-time PCR detection of *S. granarius* with qCOIISG1 and qCOIISG2 primers. For analysis, DNA isolated from *S. granarius*, *S. oryzae*, uncontaminated flour, uncontaminated grain, and contaminated flour (FISG) and grain (GrSG) were used. Only the positive control (Sg) and contaminated samples entered the logarithmic phase before 40 cycle of reaction.

Figure 4
Analysis of the real-time PCR reaction sensitivity of the *S. granarius* detection. Decreasing concentrations of granary weevil in sample were prepared, corresponding to 100 beetles/1 kg (100), 10 beetles/1 kg (10), 1 beetle/1kg (1) of flour, and dilutions of the last concentration with water: 10 times (described as *0,1* ), and 100 times (described as *0,01* beetle/kg). Amplification curves show samples of decreasing concentration gradually entering the logarithmic phase (A). Table contains Ct values obtained in real-time reaction (B).

Figure 5
Analysis of the specificity of the real-time PCR reaction. Four populations of *Sitophilus granarius* beetles coming from Poland (SgPo), Denmark (SgDe), France (SgFr) and Germany (SgGe) were tested. As negative controls *S. oryze*, *S. zeamais* and *T. castaneum*,
T. confusum beetles were used. Only samples containing DNA from S. granarius populations entered logarithmic phase before 30 cycle (A). The Ct values are shown in the table (B).
### Fig. 1

<table>
<thead>
<tr>
<th>Species</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>80</th>
<th>100</th>
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<tbody>
<tr>
<td><em>S. oryzae</em></td>
<td>*</td>
<td></td>
<td>*</td>
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<tr>
<td><em>S. zeamais</em></td>
<td>TAAATTGATTTTAGATGGGTTAAGTTTTTCTTTGAAAATTTGATTTAATTTTTTTTCTTAATTAAATGTTTTTGTGTATT</td>
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<td><em>S. granarius</em></td>
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<td><em>P. longisorbens</em></td>
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<tr>
<td><em>T. australis</em></td>
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<td>TTAGGGTATCTGGGT</td>
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<td>qCOI-SG1</td>
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<td></td>
<td></td>
<td></td>
<td>23</td>
</tr>
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<td>qCOI-SG2</td>
<td>------------</td>
<td></td>
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<table>
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<tr>
<th>Species</th>
<th>120</th>
<th>140</th>
<th>160</th>
<th>180</th>
<th>200</th>
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<tr>
<td><em>S. oryzae</em></td>
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<tr>
<td><em>S. zeamais</em></td>
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<td><em>S. granarius</em></td>
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<td><em>P. longisorbens</em></td>
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<td>209</td>
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<tr>
<td><em>T. australis</em></td>
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<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>qCOI-SG1</td>
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<td>qCOI-SG2</td>
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<td>22</td>
</tr>
</tbody>
</table>

http://mc.manuscriptcentral.com/tfac  Email: fac@tandf.co.uk
Fig. 2

125 bp
Fig. 3

A) Amplification Plots

B) Sample | Sample content | Ct value
---------|----------------|---------
Fl       | Pure flour     | -       
Gr       | Pure grain     | -       
Sg       | *Sitophilus granarius* | 16.11   
So       | *Sitophilus oryzae*   | -       
FISG     | Flour contaminated with *S. granarius* (1 insect/kg) | 31.79   
GrSG     | Grain contaminated with *S. granarius* | 36.79   
H2O      | Water          | -       

Fig. 4

A)

![Amplification Plots](image)

B)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of <em>S. granarius</em> beetles</th>
<th>Ct value</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>100 beetles/ kg</td>
<td>26.00</td>
</tr>
<tr>
<td>10</td>
<td>10 beetles/kg</td>
<td>29.60</td>
</tr>
<tr>
<td>1</td>
<td>1 beetle/kg</td>
<td>32.39</td>
</tr>
<tr>
<td>0.1</td>
<td>Corresponds to 1 beetle/ 10 kg</td>
<td>35.13</td>
</tr>
<tr>
<td>0.01</td>
<td>Corresponds to 1 beetle/ 100 kg</td>
<td>37.88</td>
</tr>
</tbody>
</table>
Fig. 5

A) Amplification Plots

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample content</th>
<th>Ct value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SgPo</td>
<td><em>Sitophilus granarius</em> from Poland</td>
<td>18.72</td>
</tr>
<tr>
<td>SgGe</td>
<td><em>Sitophilus granarius</em> from Germany</td>
<td>18.07</td>
</tr>
<tr>
<td>SgFr</td>
<td><em>Sitophilus granarius</em> from France</td>
<td>18.39</td>
</tr>
<tr>
<td>SgDe</td>
<td><em>Sitophilus granarius</em> from Denmark</td>
<td>18.02</td>
</tr>
<tr>
<td>So</td>
<td><em>Sitophilus oryzae</em></td>
<td>-</td>
</tr>
<tr>
<td>Sz</td>
<td><em>Sitophilus zeamais</em></td>
<td>-</td>
</tr>
<tr>
<td>Tcas</td>
<td><em>Tribolium castaneum</em></td>
<td>-</td>
</tr>
<tr>
<td>Tc</td>
<td><em>Tribolium confusum</em></td>
<td>-</td>
</tr>
<tr>
<td>H2O</td>
<td>water</td>
<td>-</td>
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</tbody>
</table>
Table I

List of primers used for comparative analysis of DNA sequences encoding subunits of mitochondrial cytochrome oxidases.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
<th>Region of amplification</th>
<th>Product length</th>
<th>Temperature of annealing</th>
</tr>
</thead>
<tbody>
<tr>
<td>SgCOI1</td>
<td>AAACCACAAAGATATCGGCA</td>
<td>mtCOI gene of S. granarius</td>
<td>299 bp</td>
<td>53ºC</td>
</tr>
<tr>
<td>SgCOI2</td>
<td>TTAAAGATGGGGGAAGTAGT</td>
<td>mtCOI gene of S. granarius</td>
<td>803 bp</td>
<td>57ºC</td>
</tr>
<tr>
<td>SgCOI3</td>
<td>GAGCCCCAGATATAGCCTTC</td>
<td>mtCOI gene of S. granarius</td>
<td>450 bp</td>
<td>50ºC</td>
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</table>
Table II

List of specific primers used for detection *S. granarius* in standard and real-time PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
<th>Region of amplification</th>
<th>Product length</th>
<th>Temperature of annealing</th>
</tr>
</thead>
<tbody>
<tr>
<td>COIISG1</td>
<td>TGATAACCGAACACCAATTCCC</td>
<td><em>mtCOII</em> gene of <em>S. granarius</em></td>
<td>125 bp</td>
<td>68ºC</td>
</tr>
<tr>
<td>COIISG2</td>
<td>TTAGACGACCAGGTT GCCGTC</td>
<td><em>mtCOII</em> gene of <em>S. granarius</em></td>
<td>114 bp</td>
<td>60ºC</td>
</tr>
<tr>
<td>qCOIISG1</td>
<td>TCCTGAACAATCCCAAGAATAAG</td>
<td><em>mtCOII</em> gene of <em>S. granarius</em></td>
<td>114 bp</td>
<td>60ºC</td>
</tr>
<tr>
<td>qCOIISG2</td>
<td>GATTTCTGAGCCATTGCCAAAG</td>
<td><em>mtCOII</em> gene of <em>S. granarius</em></td>
<td>114 bp</td>
<td>60ºC</td>
</tr>
</tbody>
</table>