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

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3 1 **Molecular techniques for detection of granary weevil (*Sitophilus***
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6 2 ***granarius* L.) in wheat and flour**
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11 4 **Abstract**
12

13 5 The granary weevil (*Sitophilus granarius* L.) is a stored grain pest, which causes major
14 6 economic losses. It reduces the quantity and quality of the grain by its feeding and
15 7 excretion. We have analyzed sequences of *S. granarius* mitochondrial cytochrome oxidase
16 8 subunits genes: mtCOI and mtCOII and compared them with mtCOI/II sequences available
17 9 in GenBank. The analyzed genes displayed a high level of homology between
18 10 corresponding subunits. We have undertaken attempts to develop detection methods for
19 11 contamination by *S. granarius* in wheat and wheat flour based on the molecular biology
20 12 techniques: standard and real-time PCR with a TaqMan molecular probe. We applied
21 13 specific primers designed on the basis of available sequences for mtCOI and mtCOII genes,
22 14 and established optimal reaction conditions. The specificity of both methods was studied by
23 15 using a species closely related to *S. granarius* – *S. oryzae* and *S. zeamais*. We showed that
24 16 the sensitivity threshold was very high – we were able to detect the equivalent of one beetle
25 17 per 100 kg of flour when the real-time PCR with TaqMan probe method was applied to
26 18 model samples. The primer sets used turned out to be species specific, and technique was
27 19 rapid, reliable and very sensitive.
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51 21 **Key words:-** *Sitophilus granarius*, detection, real-time PCR
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2 Introduction

3 The granary weevil, *Sitophilus granarius* (L.), and other stored-product insect pests cause
4 extensive economic losses in stored grain. Each year in the USA, stored-product insects
5 may cause from \$1 billion (Cuperus and Krischik 1995) to \$5 billion in losses (Davis
6 1991). *S. granarius* reduces not only the amount of grain available to sell, but also the
7 quality of wheat flour. Excrement left by the insects and fragments of insects contaminate
8 the flour milled from infested wheat. Therefore, detecting and removing insects from grain,
9 preventing insect pests from contaminating processed food, and detecting insect fragments
10 in flour are of great importance. At present, there are no specified permissible limits for
11 contamination of food with stored-product insect pests in Poland. According to old Polish
12 norm fewer than 3 insects beetles per kilogram of food were allowed (PN-74/A-74016). In
13 the USA, only fewer than 75 insect fragments per 50 g of flour are allowed (Toews et al.
14 2006).

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

20 There are number of techniques available for the detection of stored-product insects
21 hidden inside of grain kernels, and they have been recently reviewed (Hagstrum and
22 Subramanyam 2006). They include very old methods such as staining of kernels as
23 described by Frankenfeld (Frankenfeld 1948) and also quite modern methods such as NIR
24 reflectance spectroscopy (Dowell et al. 1998), NMR spectroscopy (Chambers et al. 1984),

1
2
3 1 acoustical sensors (Hagstrum et al. 1996, Fleurat-Lessard et al. 2006), microwave radar
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5 2 (Mankin 2004), computed tomography (Toews et al. 2006), X-ray method (Karunakaran et
6
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8 3 al. 2003, Fornal et al. 2006), immunoassay analysis (Kitto 1993) and the Single Kernel
9
10 4 Characterization System (SKCS) (Martin 1993).

11
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13 5 The accuracy of above-mentioned methods depends on insect species,
14
15 6 developmental stage of insects and grain type (soft or hard). Accuracy of acoustical
16
17 7 methods is correlated with temperature and activity of insect moving and feeding
18
19 8 (Hagstrum and Flinn 1993): the number of sounds increased as the temperature increase
20
21 9 from 17.5 to 37.5°C. The automated NIR system was able to detect different stages of live
22
23 10 insects (small, medium and large larvae and pupae) with an accuracy of 62%, 84%, 92%
24
25 11 and 94%, respectively (Maghirang et al. 2003). The SKCS system had an accuracy of
26
27 12 24.5%, 62.2%, 87.5%, and 88.6%, respectively (Pearson et al. 2003), for the same insect
28
29 13 stages. The X-ray method using film took about 2.5 h and had 97% accuracy. Digitizing the
30
31 14 film X-ray images for automated detection decreased the error rate below 1% (Haff and
32
33 15 Slaughter 2002). These modern electronic and chemical methods are more accurate than
34
35 16 flotation and cracking methods (Russel 1988, Brader et al. 2002). Methods of detection
36
37 17 vary in their sensitivity and the time needed for analysis, which is very important in
38
39 18 commercial application of the technologies. There is a trend towards automatization and
40
41 19 shortening of time procedures of insect detection in food processing industries.

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44 20 Molecular biology techniques such as standard and real-time PCR are increasingly popular
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46 21 in detection studies. They are frequently used for detection of viruses (Watzinger 2006),
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48 22 bacteria (Ward and Bej 2006), nematodes (Madani et al. 2005), and other microorganisms.
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50 23 Real-time PCR is also an approved method of detection of GMO components in food. In
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52 24 such analyses, primers that hybridize to sequences introduced into cells together with
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1 transgenes are employed. Examples are the *Cauliflower Mosaic Virus* 35S promoter
2 (CaMV35S) (Akiyama et al. 2005), the α -glucuronidase gene (GUS) (Ding et al. 2005),
3 and the 5'-transgene integration sequence (Yang et al. 2005).

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

19 Sequences of mitochondrial DNA (mtDNA) and DNA encoding ribosomal RNAs
20 (rDNA) are commonly used for detection, identification or phylogeny studies (Sheppard et
21 al. 2005, Horn et al. 2006). Among insects, these genes were used to identify internal
22 feeders of pome fruits by both standard and real-time PCR reaction (Barcenas et al. 2005).
23 Also, multiplex PCR reaction based on mtCOI and mtCOII was applied for differentiation
24 of western and northern corn rootworm larvae from the genus *Diabrotica sp.* (Roehrdanz

1 2003). The major advantage of this approach is their occurrence of mtDNA in many copies
2 in each cell. Copies exist either in tandem, as rDNA genes or in organelles like
3 mitochondria that are numerous in every cell. This feature enables detection or
4 identification of organisms even if a limited quantity of material is available for analysis.

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

11 **Materials and methods**

12 *Insects*

13 Adults and larvae of the granary weevil used in experiments came from our laboratory
14 colony maintained on whole wheat kernels (winter variety KANCLER) at 24°C and 65-
15 70% relative humidity. Adults were collected every three days, and larvae were extracted
16 from kernels by manually dissecting them at different stages of larval development. Also
17 three populations from Germany, France and Denmark kindly provided by Dr Cornel
18 Adler, Dr Patrick Ducom and Dr Lise Stengard Hansen, respectively, were tested. In
19 detection studies, samples of DNA isolated from *S. granarius* L., *S. oryzae* L., *S. zeamais*
20 Motsch., and secondary stored-products pests: *Tribolium confusum* Duv. and *T. castaneum*
21 Herbst. as well as uncontaminated flour or wheat, and contaminated with *S. granarius* flour
22 and wheat were analyzed. The flour and wheat samples were purposely contaminated (with
23 beetles and larvae of *S. granarius*, respectively) with appropriate amount of insect material,

1 and then in case of flour mixed thoroughly in homogenizer for equal distribution. Later,
2 several of random-chosen samples of 50 mg were taken for analyzes.

3 *DNA isolation*

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

10 *PCR amplification*

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

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

22 PCR reactions were carried out in an Eppendorf Mastercycler in a final volume of
23 10 μ l. The reaction mixtures contained 50 ng DNA, 1 μ M of each primer, 200 μ M dNTP,
24 0.2 U of Allegro Taq polymerase (Novazym, Poznan, Poland) in 1x polymerase buffer [70

1 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 recombinant 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 °C. 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,

1 Poland). Then, 8 clones from each sample were automatically sequenced (DNA Sequencing
2 and Oligonucleotides Synthesis Service, IBB Warsaw) to confirm that nucleotide changes
3 were neither due to polymerase error nor a result of sequencing of one of the alleles from a
4 gene in heterozygous state. The obtained sequences were subsequently analyzed and
5 compared with appropriate sequences from GenBank by using blastn tool
6 (www.ncbi.nlm.nih.gov/blast).

7 *Real-time PCR*

8 Qualitative real-time PCR reactions were performed using MxPro 3005P (Stratagene,
9 Warsaw, Poland). Analysis and interpretation of results were done with the software
10 provided by the manufacturer. Reactions were carried out in a 10 µl mixture containing:
11 AmpliLight Mix (Novazym, Poznan, Poland), 0.5 µM primers, 1 pmol of Taqman probe
12 (5'FAM-TGACGGAACACCTGGTC-TMR-3') designed in Beacon Desinger, PREMIER
13 Biosoft International, linked with FAM as a fluorophore and TAMRA as a quencher
14 (TibMolBiol, Poznan, Poland) and DNA template.

15 The real-time PCR reactions were performed under the following conditions: initial
16 denaturation at 95°C for 2 minutes followed by 40 cycles consisting of denaturation at
17 95°C for 20 sec., and annealing at 60°C for 30 sec., with single measurement of
18 fluorescence emitted by FAM dye released from TaqMan probe hydrolysis due to 5'
19 exonuclease activity of DNA polymerase.

20 *Analysis of the sensitivity and specificity of real-time PCR*

21 We evaluated also the absolute sensitivity of the real-time PCR reaction. We
22 prepared several concentrations of contaminated flour: 100 beetles/1 kg, 10 beetles/1 kg, 1
23 beetle/1 kg of flour. From the last concentration, samples were taken and diluted with

1 sterile water to obtain concentrations corresponding to equivalent of 1 beetle/10 kg (=0,1
2 beetle/kg, see Figure 4) and 1 beetle/100 kg of flour (=0,01 beetle/kg).

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

7 All samples were analyzed in triplicate.

8 **Results**

9 *Analysis of the DNA sequences for mtCOI and mtCOII*

10 To achieve our goal, we used the sequences encoding subunits of mitochondrial
11 cytochrome oxidase (COI and COII). We compared DNA sequences encoding I and II
12 subunits of mitochondrial cytochrome oxidases by using PCR amplification with
13 appropriate primer sets (Table I). We analyzed the sequences of DNA of *S. granarius*
14 beetles available in our laboratory and compared them with sequences already deposited in
15 GenBank (AY131101 and M83970). We assumed that there could have been nucleotide
16 changes in these genes for *S. granarius* populations that have been geographically isolated.
17 Therefore, the amplified fragments were cloned, multiplied and sequenced. We performed
18 amplification of COI and COII in triplicate. These obtained sequences are available in
19 GenBank under accession numbers: DQ453486 for mtCOI and DQ462235 for mtCOII. The
20 comparison of sequences originating from our laboratory with those already deposited in
21 GenBank showed a very high level of sequence similarity (about 99%). The comparison of
22 mtCOII sequence from *S. granarius* with sequences derived from other *Sitophilus* species
23 (*S. oryzae*: AY014880, *S. zeamais*: AY014881) (Fig. 1) allowed us to design distinct primer
24 pair that specifically detect only *S. granarius* in standard and real-time PCR reaction.

1 The species specificity of the the PCR methods were evaluated using samples of
2 *Sitophilus oryzae* (L.) and *Sitophilus zeamais* (Motsch.) a close relatives of *S. granarius* L.,
3 as well as *Tribolium confusum* (Duv.) and *Tribolium castaneum* (Herbst.) as the negative
4 controls. We established the optimal conditions for the PCR method, and the sensitivity and
5 reaction conditions of the real-time PCR reaction for the primers and biochemicals also
6 were determined.

7 *PCR detection*

8 For detection of *S. granarius*, we used a primer pair specific for the sequence of II subunit
9 of mitochondrial cytochrome oxidase (COIISG1/2). The following samples were used:
10 DNA from uncontaminated flour and wheat, from contaminated flour and wheat, from
11 *S. granarius* as a positive control and from *S. oryzae* as a negative control. *S. oryzae* was
12 also a control of the specificity of primers and method. PCR reaction under conditions
13 described in the materials and methods and Table II gave single and distinct products in the
14 positive control and contaminated samples (Figure 2). There was no reaction product in
15 either the uncontaminated samples nor in the sample containing DNA from *S. oryzae*.
16 Therefore, the primers specificity for only *S. granarius* was confirmed and we can conclude
17 that standard PCR reaction works properly for detection of infestation with granary weevil.

18 [Insert Figure 2 about here]

19 *Real-time PCR detection*

20 For real-time PCR, the primers qCOIISG1/2 (Table II) and TaqMan probe were used.
21 Detection of contamination with *S. granarius* with real-time PCR was performed using
22 DNA samples isolated from contaminated with insect beetles flour and from contaminated
23 with insect larvae wheat, from uncontaminated flour, and from uncontaminated wheat.
24 DNA isolated from *S. granarius* was used as a positive control of reaction. DNA from *S.*

1
2
3 1 *oryzae* was used as a negative control and, simultaneously, as control of detection
4
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6 2 specificity. The control sample and contaminated samples gave positive results entering the
7
8 3 logarithmic phase with fewer than 40 cycles of amplification. The sample containing *S.*
9
10 4 *granarius* DNA exceeded the Ct value after 16 cycle. The contaminated flour and grain
11
12 5 exceeded the threshold after 31 and 36 cycle, respectively. The rest of samples did not enter
13
14
15 6 logarithmic phase before 40 cycle (Figure 3, Table 3)

16
17
18 7 [Insert Figure 3 about here]

19 8 *Analysis of the sensitivity of real-time PCR reaction*

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21
22 9 The sensitivity of the reaction was analyzed using several decreasing concentration of *S.*
23
24 10 *granarius* in flour and then their dilutions in water (as described in Materials and Methods
25
26 11 section). We found that sensitivity of detection is very high, and even very small quantities
27
28 12 of genetic material of granary weevil are possible to detect using the real-time PCR
29
30 13 technique. The analysis revealed that it is possible to detect *S. granarius* contamination
31
32 14 even when DNA isolated from contaminated flour at the concentration of 1 beetle/1 kg was
33
34 15 diluted 100 times in water (Figure 4). The lower concentrations were not detectable.

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38
39 16 [Insert Figure 4 about here]

40
41 17 To check the specificity of real-time PCR method we analyzed samples containing DNA
42
43 18 isolated from two closely related species of *S. granarius* – *S. oryzae* and *S. zeamais* as well
44
45 19 as two secondary stored-products pests: *T. confusum* and *T. castaneum*. Only samples of
46
47 20 four populations of *S. granarius* were detected, confirming specificity of the reaction (Fig.
48
49 21 5)

50
51
52 22 [Insert Figure 5 about here]

53 54 55 56 57 23 58 24 **Discussion**

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

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

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

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

22 Our earlier analysis of COI and COII genes showed that differences in sequences
23 between *S. granarius* populations (our sequences and those previously deposited in
24 GenBank) are not a concern, so we may assume that our primers will be useful for

1 detection of any populations of *S. granarius*. However, both primers and reaction
2 conditions have to be designed very carefully to ensure their species specificity. As
3 previous analyses had shown (Peng et al. 2002), even relatively variable sequences, serving
4 for phylogenetic or identification studies, such as ITS1 and ITS2 share a very high level of
5 homology (96-97%) between closely related species of laboratory colonies of *S. oryzae* (L)
6 and *S. zeamais* Motschulsky. The application of more conservative genes, such as aldolase,
7 prolactine receptor, or interleukin-beta genes using the DNA fingerprinting approach did
8 not result in the ability to distinguish between these species.

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

14 Molecular techniques have very big advantages over other methods used at present
15 for detection of stored-product pests. They are very rapid, sensitive, and species specific.
16 Our results have shown that is possible to readily distinguish between infestations of
17 closely related species, such as *S. granarius* and *S. oryzae*. However, these methods also
18 have disadvantages. The price of appropriate kits for PCR and equipment, especially for
19 real-time PCR, are still relatively high, but tend to decrease along with rising popularity and
20 accessibility of real-time PCR systems. Because DNA is the target for detection, the
21 reaction is very specific however, DNA can degrade under very long storage, which can be
22 a drawback, especially because in the case of stored products where problems occur either
23 because of larvae or remains of adults inside the grains. However, the primers designed for

1 PCR and real-time PCR give short product and hybridize to the genes existing in many
2 copies per cell, which may overcome these obstacles.

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

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

15 A very important aspect of appropriate assessment of the infestation level is the
16 method of sampling as well as sample size. To obtain credible results it is crucial to obtain
17 a sample with maximum of homogeneity in the analyzed material. Therefore, flour should
18 be mixed thoroughly, or in the case of grain this should be crushed beforehand. Afterwards,
19 several random-chosen samples should be taken for analysis. In our case we were limited
20 by small-scale laboratory capacity and our tests were carried out on a small sample size of
21 50 mg that in turn was limited by applied kit possibilities. In our experience, however, the
22 sample size though small was sufficient to detect infestation level of the equivalent of 1
23 beetle per kg (as it was allowed in some countries), or lower, from the 50 ng of DNA taken
24 out from that 50 mg sample. The results were comparable when tested in triplicate. To

1 enhance the reliability of detection and infestation assessment it is suggested to use more
2 samples to analyzes as well as increase the sample size, when possible. Then statistically
3 the obtained results will be more accurate.

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

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19 populations of *S. granarius* for this study.

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23 **Abbreviations used**

1 mtDNA – mitochondrial DNA, COI/COII – I/II subunit of mitochondrial cytochrome
2 oxidase,

3 rDNA – DNA encoding rRNA

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

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

9 **References**

10 Akiyama H, Watanabe T, Wakabayashi K, Nakade S, Yasui S, Sakata K, Chiba R,
11 Spiegelhalter F, Hino A, Maitani T. 2005. Quantitative detection system for maize sample
12 containing combined-trait genetically modified maize. *Analytical Chemistry* 77: 7421-
13 7428.

14 Barcenas NM, Unruch TR, Neven LG. 2005. DNA diagnostics to identify internal feeders
15 (Lepidoptera: Tortricidae) of pome fruit quarantine importance. *Journal of Economical*
16 *Entomology* 98: 299-306

17 Brader B, Lee RC, Plarre R, Burkholder W, Kitto GB, Kao C, Polston L, Dorneanu E,
18 Szabo I, Mead B, Rouse B, Sullins D, Denning R. 2002. A comparison of screening
19 methods for insect contamination in wheat. *Journal of Stored Products Research* 38: 75-86.

20 Chambers J, McKevitt NJ, Stubbs MR. 1984, Nuclear magnetic resonance spectroscopy for
21 studying development and detection of the grain weevil (*Sitophilus granarius* (L.)
22 (Coleoptera: Curculionidae) within wheat kernels. *Bulletin of Entomological Research* 74:
23 702-724.

- 1
2
3 1 Cheng YH, Wen CM, Ding ST, Kaoi CC, Kuo TY. 2003. Detecting meat-and-bone meal in
4
5 2 ruminant's feeds by species-specific PCR. Journal of Animal and Feed Science 12L 851-
6
7 3 860
8
9
10 4 Cuperus G, Krischik V. 1995. Why stored product integrated pest management is needed.
11
12 5 In: Krischik W, Cuperus G, Galliard D, editors. Stored Product Management. Oklahoma
13
14 6 State University Cooperative Extension Service Circular. Oklahoma Cooperative
15
16 7 Extension Service: Stillwater, Oklahoma: E-912, p.199.
17
18
19 8 Davis R. 1991. Introduction to food-pest ecology and management. In: Gorham JR, editor.
20
21 9 Ecology and Management of Food-Industry Pests. AOOAC: Arlington, Virginia: p 595.
22
23
24 10 Ding J, Jia J, Yang L, Wen H, Zhang C, Liu W, Zhang D. 2004. Validation of a rice
25
26 11 specific gene, sucrose phosphate synthase, used as the endogenous reference gene for
27
28 12 qualitative and real-time quantitative PCR detection of transgenes. Journal of Agricultural
29
30 13 and Food Chemistry 52: 3372-3377.
31
32
33 14 Dowell FE, Throne JE, Baker JE. 1998. Automated nondestructive detection of internal
34
35 15 insect infestation of wheat kernels by using near-infrared reflectance spectroscopy. Journal
36
37 16 of Economical Entomology 91: 899-904.
38
39
40 17 Fields PG, Philips TW. 2002. The distribution and PCR-based fingerprints of *Rhyzopertha*
41
42 18 *dominica* (F.) in Canada. In: Credland PF, Armitage DM, Bell CH, Cogan PM, Highley E,
43
44 19 editors. Proceedings of the 8th international Working Conference on Stored Product
45
46 20 Protection; July 22-26; York, UK: CAB International, Wallingford, UK. pp. 517-522.
47
48
49 21 Fleurat-Lessard F, Pronier V. 2006. Genetic differentiation at the inter- and intra-specific
50
51 22 level of stored grain insects using a simple molecular approach (RAPD). In: Lorini I,
52
53 23 Bacaltchuk B, Beckel H, Deckers D, Sundfeld E, dos Santos JP, Biagi JD, Celaro JC, D'A.
54
55 24 Faroni L, Bortolini L de OF, Sartori MR, Elias MC, Guedes RNC, da Fonseca RG, Scussel
56
57
58
59
60

- 1 VM, editors. Proceedings of 9th International Working Conf. on Stored Product Protection;
2 Oct 15-18; Campinas, Brazil. pp. 446-455.
- 3 Fleurat-Lessard F, Tomasini B, Kostine L, Fuzeau B. 2006. Acoustic detection and
4 automatic identification of insect stages activity in grain bulks by noise spectra processing
5 through classification algorithms. Lorini I, Bacaltchuk B, Beckel H, Deckers D, Sundfeld
6 E, dos Santos JP, Biagi JD, Celaro JC, D'A. Faroni L, Bortolini L de OF, Sartori MR, Elias
7 MC, Guedes RNC, da Fonseca RG, Scussel VM, editors. Proceedings of 9th International
8 Working Conf. on Stored Product Protection; Oct 15-18; Campinas, Brazil. pp 476-486.
- 9 Fornal J, Jeliński T, Sadowska J, Grundas S, Nawrot J, Niewiada A, Warchalewski JR,
10 Błaszczak W. 2006 Detection of granary weevil *Sitophilus granarius* (L.) eggs and internal
11 stages in wheat grain using soft X-ray and image analysis. Journal of Stored Products
12 Research 43: 142-148.
- 13 Frankenfeld JC. 1948. Staining methods for detecting weevil infestation in grain. USDA
14 Agricultural Research Administration, Bureau of Entomology and Plant Quarantine Series
15 No. ET 256.
- 16 Guha S, Kashyap VK. 2005. Development of novel heminested PCR assays based on
17 mitochondrial 16s rRNA gene for identification of seven pecora species. BMC Genetics 11:
18 6-42.
- 19 Haff RP, Slaughter DC. 2002. Real-time X-ray inspection of wheat for infestation by the
20 granary weevil, *Sitophilus granarius* (L.). Transactions of the ASAE 47: 531-537.
- 21 Hagstrum DW, Flinn PW. 1993. Comparison of acoustical detection of several species of
22 stored-grain beetles (Coleoptera:Curculionidae, Tenebrionidae, Bostrichidae, Cucujidae)
23 over a range of temperatures. Journal of Economical Entomology 86: 1271-1278.

- 1
2
3 1 Hagstrum DW, Flinn PW, Shuman D. 1996. Automated monitoring using acoustical
4
5 2 sensors for insects in farm-stored wheat. *Journal of Economical Entomology* 89: 21-217.
6
7
8 3 Hagstrum DW, Subramanyam BH. 2006. *Fundamentals of Stored-Product Entomology*;
9
10 4 AACC International: St Paul, Minnesota
11
12 5 Handler AM, Beeman RW. 2003. United States Department of Agriculture – Agricultural
13
14 6 Research Service: advances in the molecular genetic analysis of insects and their
15
16 7 application to pest management. *Pest Management Science* 59: 728-735.
17
18
19 8 Hidayat P, Ffrench-Constant RH, Phillips TW. 1996. Molecular and morphological
20
21 9 markers for diagnosis of *Sitophilus oryzae* and *S. zeamais* (Coleoptera: Curculionidae). In:
22
23 10 Highley E, Wright EJ, Banks HJ, Champ BR, editors. *Proceedings of the 6th International*
24
25 11 *Working Conference on Stored Product Protection, 1996 April 6-11; Canberra (Australia).*
26
27 12 CAB International, Wallingford, UK, pp. 528-532.
28
29
30 13 Horn A, Roux-Morabito G, Lieutier F, Kerdelhue C. 2006. Phylogeographic structure and
31
32 14 past history of the circum-Mediterranean species *Tomicus destruens* Woll. (Coleoptera:
33
34 15 Scolytinae). *Molecular Ecology* 15:1603-1615.
35
36
37 16 Hsu MC, Chen KW, Lo HJ, Chen YC, Liao MH, Lin YH, Li SY. 2003. Identification of
38
39 17 medically important fungi by use of real-time LightCycler PCR. *Species Journal of Medical*
40
41 18 *Microbiology* 52: 1071-1076
42
43
44 19 Jeong HJ, Oh J-E, Hong SW, Ryoo MI, Kim KD, Lee H. 2006. Molecular marker-assisted
45
46 20 detection of rice weevil in stored rice. *International Journal of Pest Management* 52: 325-
47
48 21 331
49
50
51 22 Karunakaran C, Jayas DS, White ND. 2003. X-ray image analysis to detect infestation
52
53 23 caused by insects in grain. *Cereal Chemistry* 80: 553-557.
54
55
56
57
58
59
60

- 1
2
3 1 Kitto GB, Quinn FA, Burkholder WE. Development of immunoassays for quantitative
4
5
6 2 detection of insects in stored products. In: Highley E, Wright EJ, Banks HJ, Champ BR,
7
8 3 editors. Proceedings 6th International Working Conference on Stored- product Protection;
9
10 4 April 17-23; Canberra: pp. 415-420.
11
12 5 Madani M, Subbotin SA, Moens M. 2005. Quantitative detection of the potato cyst
13
14 6 nematode, *Heterodera schachtii*, using Real-Time PCR with SYBR green I dye. Molecular
15
16 7 and Cellular Probes 19: 81-86.
17
18 8 Maghirang EB, Dowell FE, Baker JE, Throne JE. 2003. Automated detection of single
19
20 9 wheat kernels containing live or dead insects using near-infrared reflectance spectroscopy.
21
22 10 Transaction of the ASAE 46: 1277-1282.
23
24
25 11 Mankin RW. 2004. Microwave radar detection of stored-product insects. Journal of
26
27 12 Economical Entomology 97: 1168-1173.
28
29
30 13 Martin CR, Rousser R, Brabec DL. 1993. Development of a single-kernel wheat
31
32 14 characterization system. Transaction of the ASAE 36: 1399-1404.
33
34
35 15 Nicholas KB, Nicholas HB Jr, and Deerfield DWII. 1997. GeneDoc: Analysis and
36
37 16 Visualization of Genetic Variation. EMBNEW News 4: 14.
38
39
40 17 Pearson TC, Brabec DL, Schwartz CR. 2003. Automated detection of intenal insect
41
42 18 infestations in whole wheat kernels using a Perten SKCS 1400. Applied Engineering in
43
44 19 Agriculture 19: 727-733.
45
46
47 20 Peng WK, Lin HC, Chen CN, Wang CH. 2002. DNA identification of two laboratory
48
49 21 colonies of the weevils, *Sitophilus oryzae* (L.) and *S. zeamays* Motschulsky (Coleoptera:
50
51 22 Curculionidae) in Taiwan. Journal of Storage Products Research 39: 225-235.
52
53
54
55
56
57
58
59
60

- 1
2
3 1 Roehrdanz RL. 2003. Multiplex polymerase chain reaction method for differentiating
4
5 2 western and northern corn rootworm larvae (Coleoptera: Chrysomelidae). Journal of
6
7 3 Economical Entomology 96: 669-672.
8
9
10 4 Russel GE. 1988. Evaluation of four methods to detect weevils in wheat: granary weevil,
11
12 5 *Sitophilus granarius* (L.), in soft white wheat. Journal of Food Protection 51: 547-553.
13
14
15 6 Sheppard SK, Bell J, Sunderland KD, Fenlon J, Skervin D, Symondson W. 2005. Detection
16
17 7 of secondary predation by PCR analyses of the gut contents of invertebrate generalist
18
19 8 predators. Molecular Ecology 14: 4461-4468.
20
21
22 9 Toews MD, Pearson TC, Campbell JF. 2006. Imaging and automated detection of
23
24 10 *Sitophilus oryzae* (Coleoptera: Curculionidae) pupae in hard red winter wheat. Journal of
25
26 11 Economical Entomology 99: 583-591.
27
28
29 12 Ward LN, Bej AK. 2006. Detection of *Vibrio parahaemolyticus* in shellfish by use of
30
31 13 multiplexed real-time PCR with TaqMan fluorescent probes. Applied and Environmental
32
33 14 Microbiology 72: 2031-2042.
34
35
36 15 Watzinger F, Ebner K, Lion T. 2006. Detection and monitoring of virus infections by
37
38 16 real-time PCR. Molecular Aspects of Medicine 27: 254-298.
39
40
41 17 Yang L, Xu S, Pan A, Yin C, Zhang K, Wang Z, Zhou Z, Zhang D. 2005. Event specific
42
43 18 qualitative and quantitative Polymerase Chain Reaction detection of genetically modified
44
45 19 MON863 maize based on the 5'-transgene integration sequence. Journal of Agricultural
46
47 20 Food Chemistry 53: 9312-9318.
48
49
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Figures

Figure 1

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.

1
2
3 1 Figure 2
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5 2 Electrophoresis of PCR products on 2% agarose gel. Products of amplification from DNA
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7 isolated from: Lane 1 – positive control - *S. granarius larvae*, 2 – negative control – *S.*
8
9 *oryzae*, 3 – flour contaminated with *S. granarius larvae*, 4 – grain contaminated with *S.*
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11 *granarius larvae*, 5 – uncontaminated flour, 6 – uncontaminated grain, 7 – negative control
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13 – no DNA added to PCR mixture, 8 – molecular weight marker.
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18 7 Figure 3
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20 8 Real-time PCR detection of *S. granarius* with qCOIISG1 and qCOIISG2 primers. For
21
22 analysis, DNA isolated from *S. granarius*, *S. oryzae*, uncontaminated flour, uncontaminated
23
24 grain, and contaminated flour (FlSG) and grain (GrSG) were used. Only the positive
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26 control (*Sg*) and contaminated samples entered the logarithmic phase before 40 cycle of
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28 reaction.
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32 13 Figure 4
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34 14 Analysis of the real-time PCR reaction sensitivity of the *S. granarius* detection. Decreasing
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36 concentrations of granary weevil in sample were prepared, corresponding to 100 beetles/1
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38 kg (100), 10 beetles/1 kg (10), 1 beetle/1kg (1) of flour, and dilutions of the last
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40 concentration with water: 10 times (described as *0,1), and 100 times (described as *0,01
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42 beetle/kg). Amplification curves show samples of decreasing concentration gradually
43
44 entering the logarithmic phase (A). Table contains Ct values obtained in real-time reaction
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48 (B)
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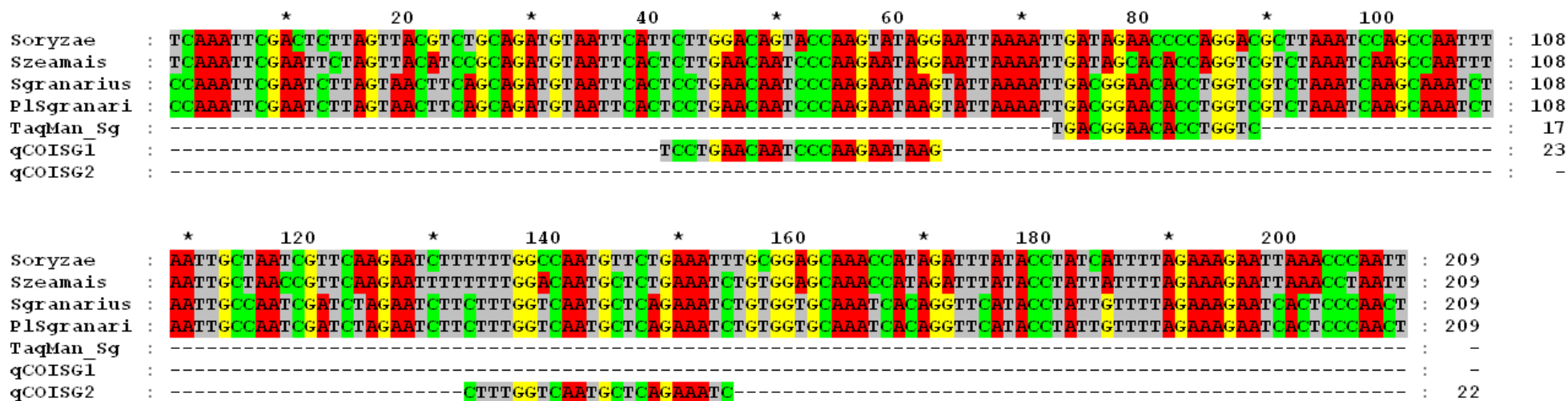
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51 21 Figure 5
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53 22 Analysis of the specificity of the real-time PCR reaction. Four populations of *Sitophilus*
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55 *granarius* beetles coming from Poland (SgPo), Denmark (SgDe), France (SgFr) and
56
57 Germany (SgGe) were tested. As negative controls *S. oryzae*, *S. zeamais* and *T. castaneum*,
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3 1 *T. confusum* beetles were used. Only samples containing DNA from *S. granarius*
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6 2 populations entered logarithmic phase before 30 cycle (A). The Ct values are shown in the
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8 3 table (B).
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Fig. 1



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Fig. 2

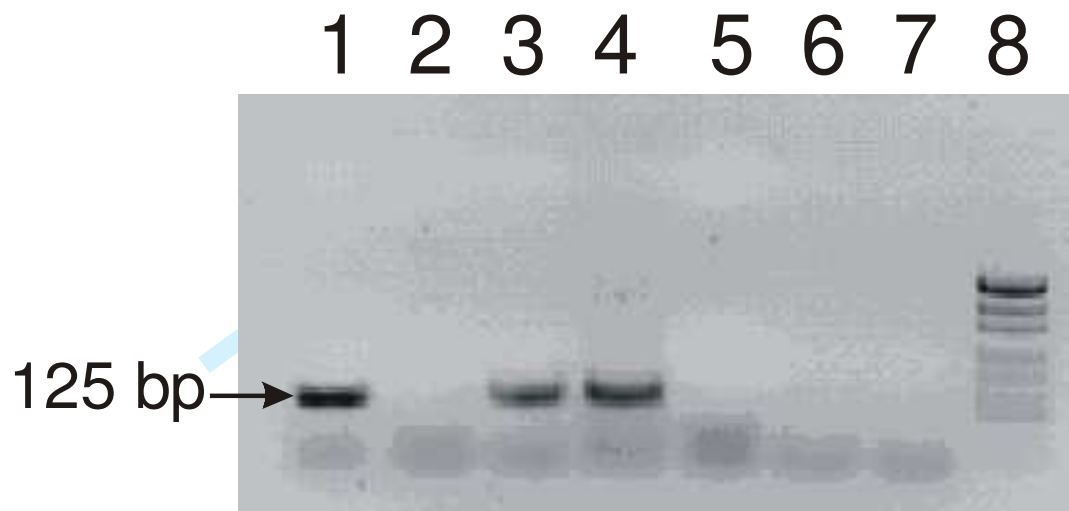
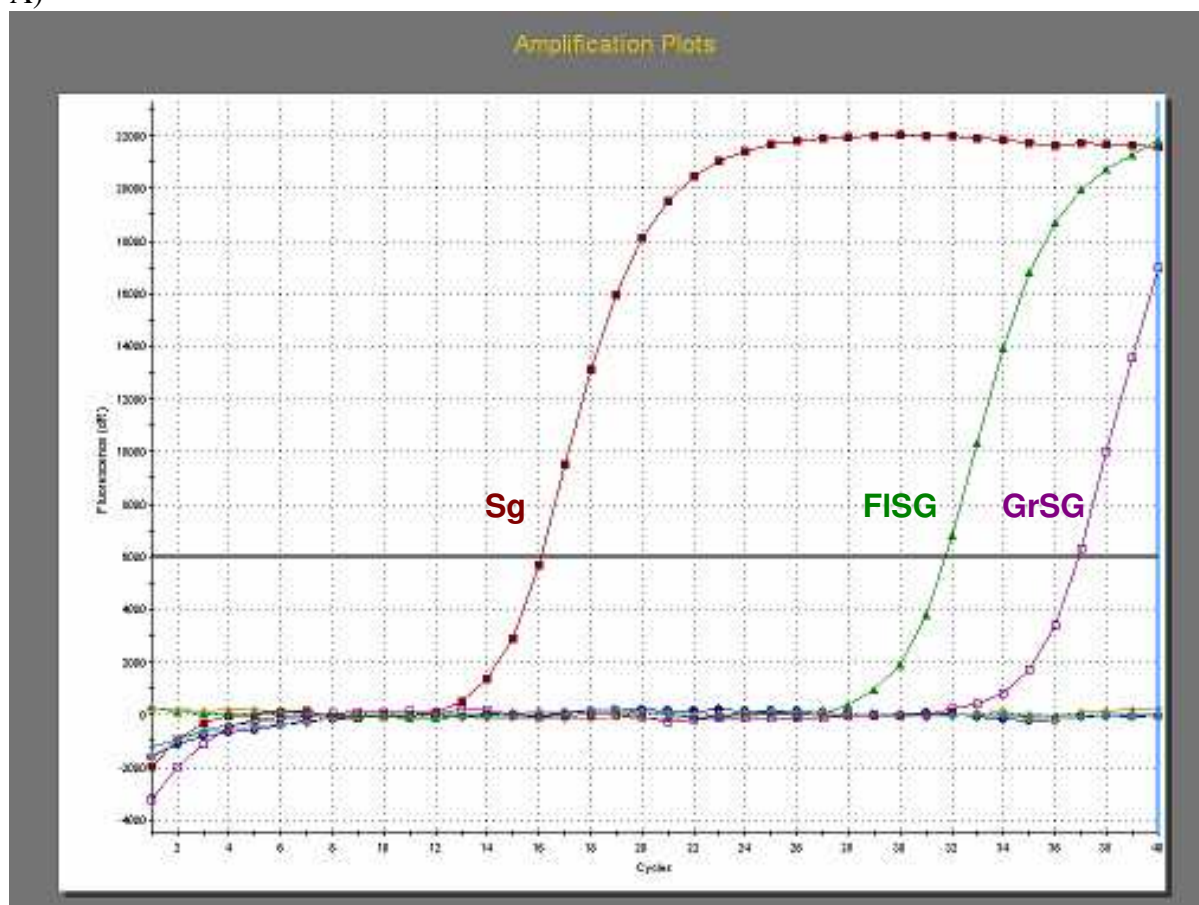


Fig. 3

A)

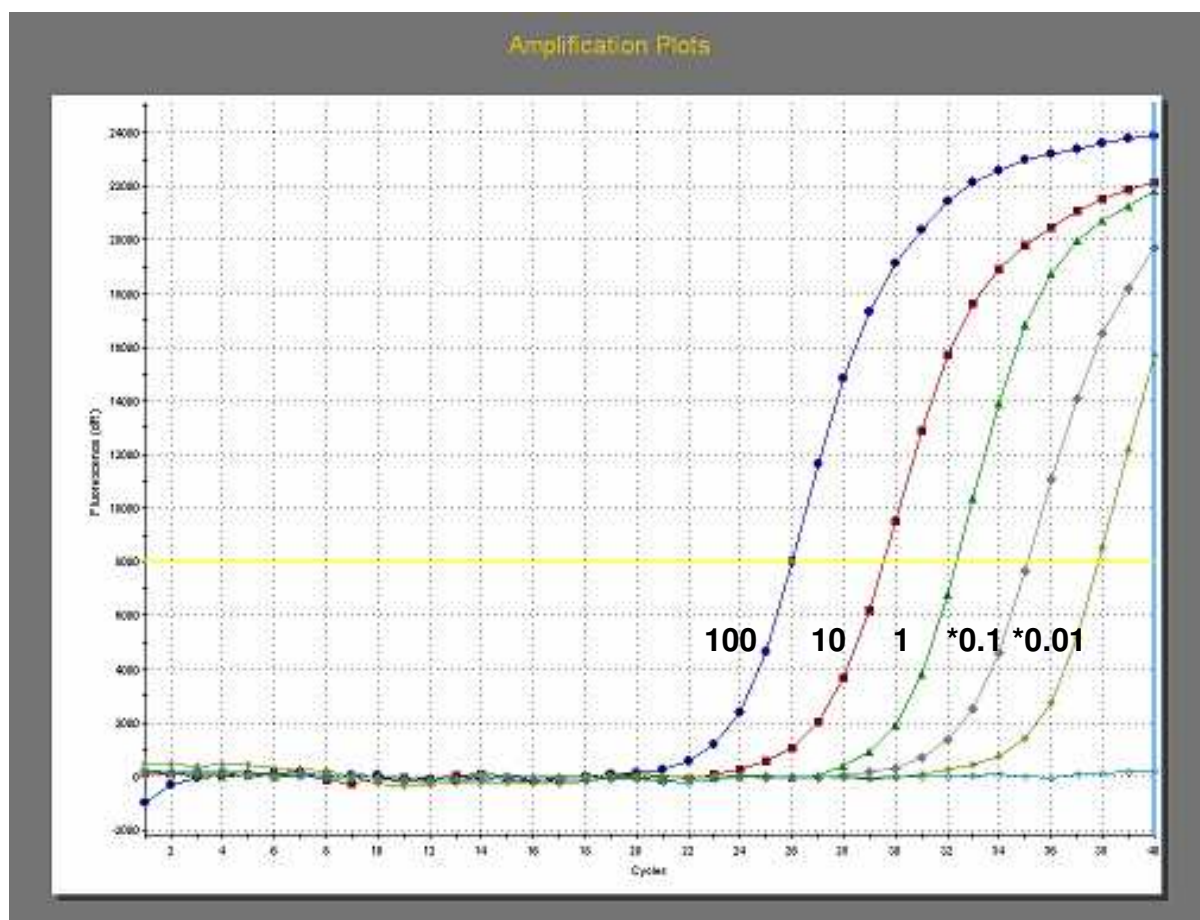


B)

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

Fig. 4

A)

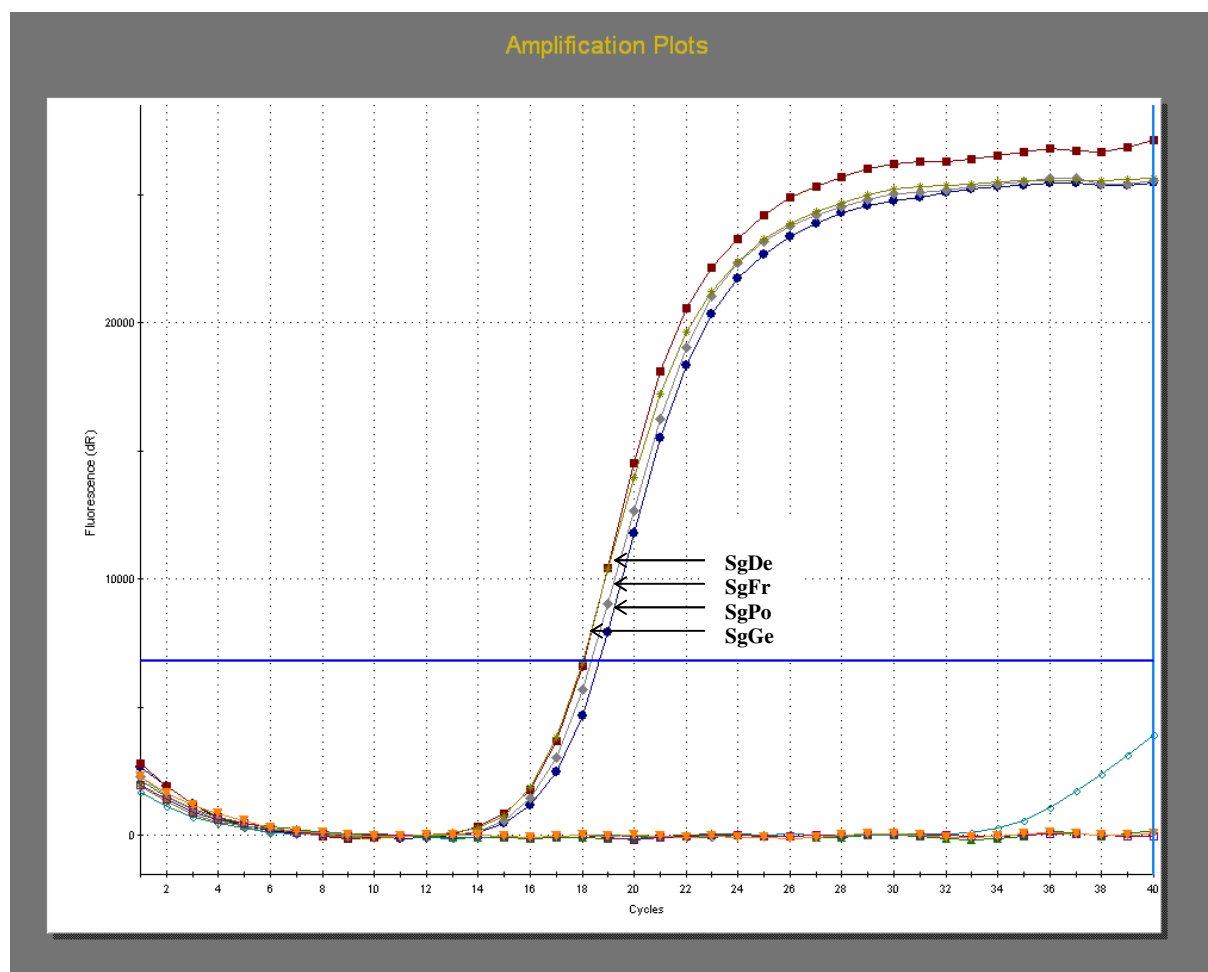


B)

Sample	Number of <i>S. granarius</i> beetles	Ct value
100	100 beetles/ kg	26.00
10	10 beetles/kg	29.60
1	1 beetle/kg	32.39
0.1	Corresponds to 1 beetle/ 10 kg	35.13
0.01	Corresponds to 1 beetle/ 100 kg	37.88

Fig. 5

A)



B)

Sample	Sample content	Ct value
SgPo	<i>Sitophilus granarius</i> from Poland	18.72
SgGe	<i>Sitophilus granarius</i> from Germany	18.07
SgFr	<i>Sitophilus granarius</i> from France	18.39
SgDe	<i>Sitophilus granarius</i> from Denmark	18.02
So	<i>Sitophilus oryzae</i>	-
Sz	<i>Sitophilus zeamais</i>	-
Tcas	<i>Tribolium castaneum</i>	-
Tc	<i>Tribolium confusum</i>	-
H2O	water	-

Table I

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

Primer	Sequence (5'→3')	Region of amplification	Product length	Temperature of annealing
SgCOI1	AAACCACAAAGATATCGGCA	<i>mtCOI</i> gene of <i>S. granarius</i>	299 bp	53°C
SgCOI2	TTAAAGATGGGGGAAGTAGT			
SgCOI3	GAGCCCCAGATATAGCCTTC	<i>mtCOI</i> gene of <i>S. granarius</i>	803 bp	57°C
SgCOI4	CTCCGGTTAGTCCTCCAATA			
SgCOIIa	ATTCCTGCTATTATTCTTATTT	<i>mtCOII</i> gene of <i>S. granarius</i>	450 bp	50°C
SgCOIIb	AGTTGGGAGTGATTCTTTCTA			

Table II

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

Primer	Sequence (5'→3')	Region of amplification	Product length	Temperature of annealing
COISG1	TGATAACCGAACACCAATTCCC	<i>mtCOII</i> gene of <i>S. granarius</i>	125 bp	68°C
COISG2	TTAGACGACCAGGTGTTCCGTC			
qCOISG1	TCCTGAACAATCCCAAGAATAAG	<i>mtCOII</i> gene of <i>S. granarius</i>	114 bp	60°C
qCOISG2	GATTTCTGAGCATTGACCAAAG			