Detection and identification of five common internal grain insect pests by multiplex PCR

Mireia Solà, Jordi Riudavets, Nuria Agusti*

IRTA, Ctra. Cabrils km 2, 08348 Cabrils, Barcelona, Spain.

*Corresponding author: IRTA, Ctra. Cabrils km 2, 08348 Cabrils, Barcelona, Spain. E-mail address: nuria.agusti@irta.cat

Abstract

Consumer demands for better quality food have led to research on new tools aimed at early detection of insect pests in agro food industries. In these industries, internal grain feeders are the most concerning pests because of being the first colonizers of stored grain and transmitting harmful micro-organisms, such as fungi and bacteria, which affect both food quality and human health. The immature stages of these cosmopolitan pests develop and feed inside the grain kernels, easily evading visual analysis in food industries. To avoid the consequent underestimation of contamination by internal pest species, a multiplex PCR approach for the detection and identification of the five most concerning primary pests that develop and feed hidden inside the grain kernels (*Rhyzopertha dominica*, *Sitophilus granarius*, *S. oryzae*, *S. zeamais* and *Sitotroga cerealella*) has been developed. Results have demonstrated that the designed protocol can be used for the diagnosis of grain contamination with high sensitivity (0.1 pupa/kilo of rice, except for *R. dominica* 10 pupae/kilo). This tool proved to be specific when 46 other species potentially present in grain commodities were tested, and to detect all developmental stages of *S. zeamais* in different kinds of grain (barley, maize, oat, spelt, rice and wheat) and pasta (macaroni). Detection was even possible when grain was treated with CO_2.

Finally, in order to confirm its applicability in food industries, this method has also been tested in real commercial grain samples from a pasta mill. The multiplex PCR method presented here could be of great help when making commercial decisions aimed at satisfying the current market demands.

Keywords: insect pests, internal feeders, grain cereals, detection, identification, multiplex PCR.

1. Introduction

Cereal grain, either as raw or processed material, constitutes 80% of consumed food (Pimentel et al., 1997). Unfortunately, since the routine procedures before food consumption harbor several pest species, the safety and security of this food are susceptible to being affected when grain is stored, transported and processed (Hagstrum, Reed, & Kenkel, 1999; Nopsa et al., 2015; Stejskal, Hubert, Aulicky, & Kucerova, 2015). Phillips and Throne (2010) estimated post-harvest losses due to stored-product insects of between 9% and 20% or more in developed and developing countries, respectively.

Among insect pests, internal feeders, which are primary pest species that develop and feed inside the grain kernels, have generally been regarded as the most damaging pests of stored cereals (Toews, Campbell, Arthur, & Ramaswamy, 2006). These species not only consume large quantities of grain, but are hidden inside the grain kernels during their preimaginal development. Furthermore, these insects facilitate grain contamination by secondary pests, which might increase the damage to the food by depositing faeces and cast skins. This all causes localized increases in heat and moisture that might lead to accelerated mold growth and mycotoxin production threatening the grain quality and human health (Beti, Phillips, & Smalley, 1995; Phillips & Throne, 2010; Shah & Khan, 2014).

Because these internal feeders are not easily detected and removed during routine cleaning or processing practices, a situation where contamination is underestimated can often occur (Perez-Mendoza, Throne, Maghirang, Dowell, & Baker, 2005; Toews, Campbell, Arthur, & Ramaswamy, 2006). Hence, Storey, Sauer, Ecker, & Fulk (1982) reported that 12% of wheat samples from export loads contained hidden internal insects in the United States. Consequently, it is not surprising that primary pests are mainly present in filth contamination of finished cereal products (Trematerra, Stejskal, & Hubert,

2011). The most concerning internal feeders in grain worldwide are the following five species: *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae); three species of the genus *Sitophilus* (*S. granarius* (L.), *S. oryzae* (L.) and *S. zeamais* (Motschulsky) (Coleoptera: Curculionidae)) and *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae) (Castañé & Riudavets, 2015; Toews et al., 2007; Trematerra, Ianiro, Athanassiou, & Kavallieratos, 2015). Also, *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae), which is an important internal feeder of stored maize and cassava, has also become a serious pest in tropical and subtropical areas (CABI, 2017).

The increased consumer concerns about food safety and wholesomeness have produced a general trend toward a decrease in tolerance of live insects in food (Hagstrum, Reed, & Kenkel, 1999; Trematerra, 2013). This situation has brought changes in grain standards in terms of food quality, which has emphasized the need for regulative approaches in the commercial sequence from the growers to consumers, driving market changes, politically and industrially (FDA, 1997; Stejskal, Aulicky, & Kucerova, 2014). For example, domestic flour millers generally report zero tolerance for live insects, while the national agency in charge of food safety in the US, the Food & Drug Administration (FDA), has produced administrative guidelines that set maximum levels for natural or unavoidable defects in food for humans (FDA, 1997). Because failure to control insect infestations when they initially occur in storage (or in the field) can lead to extensive contamination of the stored grain that could affect food security (Nopsa et al., 2015), the importance of establishing strategies for early diagnosis of insect contamination is evident.

With the purpose of detecting insect contamination, hazard analyses are routinely conducted in grain industries. At the moment, grain is inspected with sieves and all sorts of methods to crack kernels for the identification of insect adults, damaged kernels or

insect fragments. However, when those visual methods are used alone, internal infestations are not evident (Brader et al., 2002; Hubert, Nesvorna, & Stejskal, 2009). Additionally, insect fragments produced are not equivalent at each development stage of the pest (immature stages and eggs have low to no chitin content, respectively), highlighting the need for other analysis approaches (Brabec, Pearson, Flinn, & Katzke 2010; Perez-Mendoza, Throne, Maghirang, Dowell, & Baker, 2005).

Nowadays, there is a panoply of techniques available for insect detection (Hagstrum & Subramanyam, 2014; Neethirajan, Jayas, & White, 2007; Parkin, 1956; Phillips & Throne, 2010; Trematerra, 2013). Unfortunately, although acoustic emissions, ELISA, NIR and X-ray are diagnostic techniques that are capable of detecting hidden infestations (Chen & Kitto, 1993; Fleurat-Lessard, Tomasini, Kostine, & Fuzeau, 2006; Fornal et al., 2007; Maghirang et al., 2003; Perez-Mendoza, Throne, Maghirang, Dowell, & Baker, 2005), they also present some limitations. Among their main drawbacks, some of these approaches do not accomplish the cost-time compromise, while others are less sensitive to low population densities (Neethirajan, Jayas, & White, 2007; Nowaczyk et al., 2009).

In recent years, the application of molecular techniques has gained importance in food diagnostics because of their simplicity, speediness and specificity (Obrepalska-Steplowska, Nowaczyk, Holysz, Gawlak, & Nawrot, 2008; Solà, Lundgren, Agusti, & Riudavets, 2017). DNA-based approaches such as PCR have become relevant for the analysis of genetically modified organisms (GMOs) in food (Ciabatti, Froiio, Gatto, Amaddeo, & Marchesi, 2006; Datukishvili, Kutateladze, Gabriadze, Bitskinashvili, & Vishnepolsky, 2015), as well as for identifying insect species (Barcenas, Unruh, & Neven, 2005; Zhang et al., 2016), providing an excellent method for both adult and immature forms even for sibling species (Correa, de Oliveira, Braga, & Guedes, 2013;

Hidayat, Phillips, & FrenchConstant, 1996; Peng, Lin, Chen, & Wang, 2002). Among PCR approaches, the multiplex is the most suitable technique for screening multiple species because it is able to simultaneously identify all species present in a sample within a single PCR reaction (King et al. 2011; Solà, Agusti, & Riudavets, 2015). It also offers simplicity of execution, a reduction of carryover errors and time saving, compared to the traditional singleplex PCR (Bai et al., 2009).

A multiplex PCR approach was here developed and described as a reliable molecular method for routine detection and identification of the five main internal feeders in grain samples, namely: the lesser grain borer (*R. dominica*), the three grain weevils species (*S. granarius*, *S. oryzae*, and *S. zeamais*) and the Angoumois grain moth (*S. cerealella*). One major consideration was to perform a large specificity test covering a wide range of species potentially present in stored grain facilities. The sensitivity of this protocol has been determined taking into consideration all developmental stages of the insect pests (egg to adult), the post-mortem time, different grain types and the potential of a grain treatment with modified atmospheres. Finally, some real commercial samples have been analyzed using the developed method.

2. Material and methods

2.1. Biological material

Five target pest species (*R. dominica*, *S. granarius*, *S. oryzae*, *S. zeamais* and *S. cerealella*) were maintained in laboratory cultures at IRTA (Barcelona, Spain). Coleopteran species were grown on organic rice (Eco-Salim, Maquefa, Spain), while the lepidoptera species was reared on maize (Crit d'or, Granollers, Spain). All insect cultures were maintained in climatic chambers at 28 ºC, 70% RH, and 16L: 8D.

130 Forty-six species were tested in the specificity test of the designed primers. The
131 specimens of these non-target species were found in alimentary factory surveys since
132 1997 or came from laboratory colonies (Table 1). Identification of all species was
133 performed using morphological keys before storing the specimens in alcohol 96º or frozen
134 at -20 ºC until DNA extraction.

135 The following insect-free grain and pasta were also tested for the characterization
136 of the protocol: brown rice and wheat (Eco-Salim, Maquefa, Spain), maize (Crit d'or,
137 Granollers, Spain), spelt (Biogrà, Polinyà, Spain), barley and oat (Celnat, Saint-Germain-
138 Laprade, France) and macaroni pasta (Castagno Bruno, Giaveno TO, Italy). In order to
139 ensure that the food samples used in the analyses were insect-free, a sample of 125 g of
140 each grain and pasta was maintained at 28 ºC, and 70% RH for three months and checked
141 for insect adult presence by sieving it with a 2 mm mesh. Also, for the same purpose,
142 three samples of 5 g of each grain type and pasta were first ground with a laboratory
143 grinder (Laboratory Mill 3303, Perten Instruments, Hägersten, Sweden) to be then
144 analyzed for insect presence with the multiplex PCR described below.

145 *2.2. DNA extraction and multiplex PCR*

146 Two different DNA extraction protocols were performed: one for the insect DNA
147 extraction and another for the grain (infested or not). Insect DNA was extracted from
148 whole individuals using a SpeedTools Tissue DNA extraction kit (Biotools, Madrid,
149 Spain) and eluted in 100 µl of AE buffer. In addition, 5 g (or 10 g in the case of the
150 sensitivity test) of homogenized infested grain and pasta DNA was extracted with the
151 Extragen Alimentos extraction kit (Sistemas Genómicos, Valencia, Spain) following the
152 manufacturer's instructions and eluted in 1 ml of purified water. One negative control
153 was included in each DNA extraction group. DNA was stored at -20 ºC until PCR.

Multiplex PCR reaction volumes (10 µl) contained 5 µl of 2x Multiplex PCR Master Mix (Qiagen), 2 µl of primer mix, 2 µl of DNA template, 1 µl of purified water and 0.05 µl of BSA [100 mg/ml]. Primer concentrations in the primer mix were different depending on the species (see Table 2). Samples were amplified in a 2720 thermal cycler (Applied Biosystems, CA, USA) for 35 cycles at 94 °C for 30 s, 60 °C for 90 s and 72 °C for 60 s. An initial denaturation step was carried out at 95 °C for 15 min and a final extension step was performed at 72 °C for 10 min. Targeted DNA and water were always included as positive and negative control in the PCR, respectively. Obtained PCR products were run by electrophoresis in 1.5% agarose gel stained with ethidium bromide and visualized under UV light.

2.3. Primer design and specificity

Four pairs of species-specific primers were designed to target the mitochondrial cytochrome oxidase I (COI) region of the three *Sitophilu*s species (*S. granarius*, *S. oryzae* and *S. zeamais*) and the moth *S. cerealella.* For that purpose, we first searched all the sequences present in the GenBank for each target species. When more than one sequence was found, they were aligned with ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2). Since all sequences showed a 100% of homology, we chose the largest one. Sequences selected for primers design of the four target species corresponded to the accession numbers: DQ200131, AY131101, AY131099 and AY131100 for *S. cerealella*, *S. granarius*, *S. oryzae* and *S. zeamais*, respectively. These sequences, together with the one of *R. dominica* (JQ989165) were aligned and compared for non-conserved regions. In the case of *R. dominica*, a previously developed pair of primers (RdF1/RdR1), which amplified a fragment of 286 bp, was used (Solà, Lundgren, Agusti, & Riudavets, 2017).

177 Specificity was assessed by testing at least 10 individuals of each target species
178 from our laboratory rearings. In order to confirm the detection of other populations of the
179 target species, additional analysis of individuals (n=3) from other origins were also
180 performed. Populations tested were: three of *R. dominica* (one from Portugal, one from
181 Rumania and one from Turkey); and four of *S. oryzae* (one from Andalusia (Spain), one
182 from Portugal, one from France and one from Greece). Also, three individuals from the
183 other 46 non-target species, except two individuals in two of them and one individual in
184 one of them were tested (Table 1). To ensure the presence of DNA in those samples that
185 gave a negative result, they were also amplified using universal primers as a positive
186 control. The following universal pairs of primers were used depending on the species (see
187 Table 1): ZBJ-ArtF1C/ZBJ-ArtR2C (Zeale, Butlin, Barker, Lees, & Jones, 2011), Uni-
188 MinibarF1/Uni-MinibarR1 (Meusnier et al., 2008) or LCO1490/HCO2198 (Folmer,
189 Black, Hoeh, Lutz, & Vrijenhoek, 1994). The DNA was amplified following the protocols
190 described in those studies. If the expected fragment obtained using these universal pair of
191 primers was not amplified, the specimen was not considered in the specificity analysis.
192 The designed primers sequences were also compared by performing a BLAST
193 (www.blast.ncbi.nlm.nih.gov/Blast.cgi) in order to find potential cross-reactions with
194 other species.

195 *2.4. Characterization of the multiplex PCR: sensitivity, post-mortem detection, detection*
196 *in different grains and in treated grain*

197 To characterize the multiplex PCR method developed here, four tests were
198 conducted: determination of the sensitivity threshold, determination of the post-mortem
199 detection period, detection of larvae in different grains, and analysis of treated and
200 untreated rice using *S. zeamais* eggs. In all experiments, insects were maintained in
201 climatic chambers at 28 ºC, 70% RH and 16L: 8D. Three replicates consisting of three

202 independent DNA extractions were tested in all experiments and each independent DNA
203 extraction was tested up to three times, being considered positive if at least one of them
204 was positive. In the sensitivity test, only one DNA extraction was conducted, which was
205 also tested three times.

206 The sensitivity threshold of the multiplex PCR developed here was determined by
207 performing artificial infestations with the equivalent of 100, 10, 1 and 0.1 pupae/kg of
208 rice. For that purpose, 20 g of rice infested with two pupae of each species was ground
209 and used as a base for preparing all the insect infestation doses. The highest infestation
210 dose tested (100 pupae/kg of rice) corresponded to a subsample of 10 g of this infested
211 and ground grain. The remaining insect doses were obtained through serial mixtures of
212 90 g of ground insect-free rice homogenized with 10 g of ground infested grain from the
213 preceding infestation dose. Therefore, the highest infestation dose corresponded to a
214 sample of 10 g of infested grain, while the rest consisted in subsamples of 10 g extracted
215 from 100 g of infested grain.

216 In order to determine the post-mortem detection period, five adults (one of each
217 target species) killed by freezing at -80 ºC for 20 minutes were maintained for different
218 periods in small vials with some rice at 28 ºC, 70% RH and 16L: 8D to allow DNA
219 degradation. After, 0, 30, 90, 150, 365, 548 and 760 days, insects were frozen at -20 ºC
220 until DNA extraction to stop their degradation.

221 Insect detection in different kinds of grain was tested by conducting artificial
222 infestations of *S. zeamais* adults in six different grains: barley, maize, oat, spelt, rice and
223 wheat, as well as in pasta (macaroni). For that purpose, 250 g of organic cereal or pasta
224 was infested with 10 adults of *S. zeamais* and maintained for 15 days in the climatic
225 chamber in the same conditions described above. Then, grain was sieved with a 2 mm

mesh to collect the adults and divided into two portions of 125 g; one was ground and frozen for molecular analysis, while the other one was maintained in the climatic chamber (same conditions) for 40 days. The *S. zeamais* adults that emerged from the second portion of grain were counted after sieving as a way to estimate the number of hidden larvae present in the first portion used for molecular analysis.

In order to determine whether the developed multiplex PCR was able to detect *S. zeamais* eggs in treated, as well as in untreated, grain, 1.5 kg of brown rice was infested with 10 *S. zeamais* adults. One week later, the infested rice was sieved to eliminate the introduced adults and divided into three equal parts. Two portions were treated with a modified atmosphere of 90% CO_2 for 12 days before grinding. This CO_2 dose is known to be efficient for killing eggs of these species (Riudavets, Castañé, Alomar, Pons, & Gabarra, 2009). The third portion remained untreated. This one and one of the previously treated portions were ground and frozen at -20 ºC until DNA extraction. The other treated portion was maintained for 40 days under the same controlled conditions as above to check for the presence of adults.

2.5. Analysis of commercial samples

Some commercial grain samples from a real Spanish industry were analyzed for the presence of the five target species using the developed multiplex PCR method. These grain samples came from the routine procedure of this industry when new grain arrives from the field to be processed. This procedure consists in taking a portion of 1 kg of grain and sieving it to check for insect presence. Then, the same 1 kg of grain samples were sent to our laboratory for further analyses. Once in the laboratory, all samples were first sieved with a 2 mm mesh and the obtained insects were counted and identified. Then, each sample was divided into two equal portions of 500 g; one was ground and frozen at

-20 ºC for molecular analysis, while the other one was maintained for at least 40 days in the climatic chamber (same conditions as above) to check for adult insect presence after this period of time. Five commercial samples originally from France (one from 20th May 2015, another one from 29th May 2015, two from different silos from 14th March 2016 and one from 31st May 2016) were analyzed in total. Three replicates consisting of three independent subsamples of 5 g were analyzed by multiplex PCR per each of the samples received, except for one of the samples from 14th March 2016, where only 2 multiplex PCR where carried out. Each sample was considered positive when at least one out of three of these subsamples was positive for insect presence.

2.6. Data analysis

DNA amplification observed in the agarose gels was scored as 1 or 0 according to the presence or absence of the expected band, respectively. Then, the frequency of the positive amplification was calculated. For the sensitivity test and the analysis of post-mortem detection, a logistic regression to the data was performed with JMP® (Version 8.0.1). In the sensitivity test, pest species and infestation dose were used as sources of variation, while in the post-mortem analysis, time and species were the selected factors. The relationship between the results obtained by sieving and by multiplex PCR to diagnose insect presence in commercial samples was studied with a Pearson's correlation using SigmaPlot (Systat Software, San Jose, CA).

3. Results

3.1. Primer specificity

The multiplex PCR developed here using the five species-specific primer pairs successfully amplified the expected amplicons (Fig. 1) when our laboratory rearing specimens were tested. In the case of *S. oryzae*, some specimens amplified two bands, the

274 expected one of 213 bp and a faint one smaller than 151 bp. Nevertheless, the
275 amplification of all *S. oryzae* specimens tested led to the same pattern and did not interfere
276 with the identification of the other four target species. When specimens of *S. oryzae* and
277 *R. dominica* from other origins (different populations) were tested, all of them were also
278 amplified.

279 When the other 46 insect species were tested with the designed multiplex PCR,
280 only the five target species showed the expected band, proving a high specificity for the
281 five target species (Table 1). It is a major consideration that when those 46 species that
282 gave a negative amplification with the designed protocol were tested using insect
283 universal primers, they all gave a positive amplification, thereby demonstrating the
284 presence of insect DNA. When the potential cross-reactivity of the designed primers with
285 sequences of other species was tested by performing a BLAST, the only species identified
286 using both forward and reverse designed primers were the target species with a 100% of
287 matches and an e-value <1. The only exception was the pair of primers of *S. granarius*,
288 which also matched Ichneumonidae sp., which are not pest species of stored products.

289 *3.2. Characterization of the designed multiplex PCR: sensitivity, post-mortem detection,*
290 *detection in different grains and in treated grain*

291 When different artificial infestation doses (100, 10, 1 and 0.1 pupae of each
292 species/kg of rice) were tested to determine the sensitivity of the multiplex PCR, the
293 sensitivity threshold was determined on the doses of 0.1 pupa per kilo of rice for the three
294 *Sitophilus* species and *S. cerealella*, while *R. dominica* was detected up to 10 pupae per
295 kilo of rice (Table 3; Fig. S1). DNA amplification among infestation doses did not present
296 statistical differences (χ^2=5.99, DF=3, *P*=0.112). However, the DNA diagnosis differed
297 among the internal feeder species (χ^2=14.92, DF=4, *P*=0.005).

The analysis of a mixture of five adults (one from each target species) killed at 0, 30, 90, 150, 365, 548 and 760 days showed a post-mortem detection period of 365 days. After 548 days, this molecular method was less able to detect DNA from *S. granarius* and *R. dominica*. However, *S. cerealella*, *S. zeamais* and *S. oryzae* were still detected up to 760 days (more than two years) after insect death (Table 3; Fig. S2). The logistic regression performed showed that the time post-mortem and the insect species affected significantly the insect diagnosis (χ^2=22.23, DF=5, *P*=0.0005 and χ^2=18.28, DF=4, *P*=0.0011, respectively).

The DNA of *S. zeamais* was successfully amplified in all the artificial infestations conducted in 250 g of different grains (barley, maize, oat, spelt, rice and wheat) and pasta (macaroni) with 10 adults of this species for 15 days. These positive results were corroborated when 121, 104, 147, 135 and 156 *S. zeamais* adults were obtained in rice, wheat, oat, barley and spelt, respectively, after sieving the portion maintained under controlled conditions for 40 days. Because no insect adults were obtained in maize, three subsamples of 5 g of a ground mixture of 130 *S. zeamais* adults (the average of the insects' offspring obtained in the other grains) in 125 g of this maize were analyzed by multiplex PCR. The analysis of the maize showed *S. zeamais* DNA amplification (Fig. S3). Similarly, no adults, and only seven small larvae, were obtained in the macaroni pasta. For this reason, we replaced seven insect-free macaroni from the molecular portion with these seven infested macaroni from the climatic chamber portion. The analysis of the portion destined to molecular diagnosis by multiplex PCR confirmed the ability of the technique to detect the immature *S. zeamais* in the artificially infested pasta (Fig. S3). On the other hand, when the non-infested 250 g portion maintained under controlled conditions was sieved, no insect adult was observed. Also, when the non-infested 250 g portion used for the molecular analysis was tested by multiplex PCR, no DNA

amplification was obtained either. This confirmed that before manipulation, cereals and pasta where insect free.

Finally, when brown rice infested with *S. zeamais* and treated with 90% CO_2 for 12 days; and not treated brown rice infested with *S. zeamais* were analyzed by multiplex PCR, *S. zeamais* infestations were detected in both cases. As expected, no *S. zeamais* adults were obtained from the treated grain portion maintained in the climatic chamber for 40 days.

3.3. Analysis of commercial samples

The molecular analysis of the grain samples from a Spanish industry was coherent with the results obtained by sieving in the same industry. The five samples which were received the following dates: 20th May 2015, 29th May 2015, two from 14th March 2016, and 31st May 2016, were again sieved in the laboratory and divided into two portions: one for adult emergence and the other one for molecular analysis. We were informed by the industry that two of them were positive for *Sitophilus* spp. adults. They were the samples from 29th May 2015 and 31st May 2016. After sieving these two samples in the laboratory, one and six *Sitophilus* spp. adults were obtained, respectively. Forty days later, another sieving was performed and two *Sitophilus* spp. adults were observed in both samples. The molecular analysis of these samples showed that they were *S. oryzae*. More specifically, in the first sample, the three subsamples gave a positive result for *S. oryzae*, while in the second sample, two positives were obtained for this species out of three subsamples. The rest of the samples (20th May 2015, 14th March 2016 a and b) were negative for insect presence in the industry, as well as in our laboratory, after sieving twice and after performing the multiplex PCR. Moreover, results obtained by sieving (either in the industry or in the laboratory) were highly correlated with the results obtained by multiplex PCR ($r = 0.86$, $DF = 12$, $P < 0.0001$). In fact, when a sample was considered negative after

348 sieving, was always negative by multiplex PCR. On the other hand, a positive result
349 obtained by sieving, was also positive by multiplex PCR in the 83% of the occasions.

350 **4. Discussion**

351 In the present study, a multiplex PCR protocol has been developed to detect
352 primary pest species in grain, offering significant advantages for routine analysis. This
353 protocol showed high sensitivity by successfully detecting 0.1 pupa from *S. granarius*, *S.*
354 *oryzae*, *S. zeamais* and and *S. cerealella* per 1 kilo of rice (1 pupa per 10 kilos) and 10
355 pupae per kilo of rice in the case of *R. dominica*. This sensitivity threshold is similar to
356 or even overpasses the regulatory standards for insect presence in food factories or
357 commercial trade standards using the most common detection techniques. These defect
358 action levels are commonly based on macro-analytical visual detection of adults, insect
359 fragments or insect-damaged kernels (IDKs) (Chen & Kitto, 1993). In addition, Toews et
360 al. (2007) and Perez-Mendoza, Throne, Maghirang, Dowell, & Baker (2005)
361 demonstrated that near-infrared reflectance spectroscopy (NIRS), one of the techniques
362 used for insect detection, shows an important variability when analyzing samples with
363 fewer than 100 insect fragments per kilo of wheat flour, and was unable to reach the
364 quality standard set by the FDA (75 insect fragments per 50 g of wheat flour) (Brabec,
365 Pearson, Flinn, & Katzke, 2010). On the other hand, X-ray, which is an official standard
366 method in the USA (Fornal et al., 2007), despite appearing to have the greatest potential
367 for being introduced in the food industry for insect detection (Neethirajan, Jayas, &
368 White, 2007), is not sensitive enough to accurately detect eggs and small larvae
369 (Karunakaran, Jayas, & White, 2003). Similarly, note that in the diagnosis of insect
370 presence based on IDKs, the damage caused by insect eggs or small larvae is null or
371 inappreciable.

Previous studies have demonstrated the potential of introducing molecular techniques in grain industries as a tool for diagnosing insect presence. Solà, Lundgren, Agusti, & Riudavets (2017) reached sensitivity thresholds of 13 eggs/small larvae, 0.1 big larvae/pupae and 0.02 adults of *R. dominica* per kilo of rice, using quantitative PCR (qPCR). Alternatively, Obrepalska-Steplowska, Nowaczyk, Holysz, Gawlak, & Nawrot (2008) reached a sensitivity threshold equivalent to 0.01 *S. granarius* adults per kilo of wheat flour using qPCR. This information might help managers from the food industry make decisions about rejecting batches, storing grain, using control measures, processing grain or transporting it to another market outlet with less stringent standards (Brabec et al., 2010; Hagstrum, Reed, & Kenkel, 1999). However, molecular approaches, although they are able to detect all life stages of the target primary pests, are not able to discriminate among life stages in mixed populations. This could be a drawback since stored grain usually has insect pests of mixed ages. Although it is not essential to determine the developmental stage of the pests for grain grading, the precision of insect developmental stages could help to make the most of management decisions on processing the grain (Dowell, 1998).

Degradation of the DNA of dead organisms increases with time post-mortem and this might hamper a successful DNA amplification. For this reason, the detection range of the five target insect species has been determined by analysing several periods after insect death, showing that the developed multiplex PCR was able to detect adults of the five pest species even one year after death. After this period of time, the technique was not able to detect DNA from *S. granarius* or *R. dominica*, but was still able to detect *S. cerealella*, *S. zeamais* and *S. oryzae* even after two years. The bigger size of the amplified amplicons for *S. granarius* and *R. dominica* could be the reason for losing their detection earlier. The detection of immature *S. zeamais* DNA in CO_2-treated grain samples also

shows the ability of the technique to detect dead immature stages of *S. zeamais*, and suggests that this might be the case for the other target species. The fact that dead insects can be detected for long periods of time has positive and negative aspects. On the one hand, the detection of dead insects provides an idea of the contamination in the analyzed grain, even in the past. On the other hand, the inability to discriminate between dead and alive insects could lead to an overestimation of the control measures needed with a consequent overtreatment of the grain (Solà, Lundgren, Agusti, & Riudavets, 2017).

This method enhances the accuracy of the identification of insects based on their specific detection. Because different species have different behaviors and cause different levels of grain loss requiring different approaches to control them (Cao et al., 2015), in this work we have developed a multiplex PCR protocol rather than a singleplex PCR approach (Solà, Agusti, & Riudavets, 2015). This enhanced the specific and simultaneous identification of the five target pest species by easily recognizing the precise bands of different molecular weights in the agarose gels (Fig. 1). The universality of the designed primers is suggested by the positive detection of other populations of *S. oryzae* and *R. dominica* with different origins, as well as for the high homology that presented the sequences of all populations of each target species present in GenBank. Nevertheless, in the case that in future tests other populations of the five target species different from those tested in this study may be present, we recommend to conduct a previous specificity test in order to confirm the correct amplification of the target species population. The cross-reactivity test performed with the 51 species potentially present in stored and agroecosystem environments (Table 1) ensured the specific identification of only the target species. The potential cross-reactivity of the designed primers tested by performing a BLAST also demonstrated their high specificity.

421 The immature stages of some of these species are particularly difficult to
422 recognize, as in the case of the three *Sitophilus* species studied here. Although most
423 identification procedures rely on the morphological characterization of the adults, in the
424 case of sibling species, such as *S. oryzae* and *S. zeamais*, this is tedious and needs the
425 expertise of a taxonomist and the use of microscopy techniques (Hidayat, Phillips, &
426 FrenchConstant, 1996; Peng, Lin, Chen, & Wang, 2002). Using the multiplex PCR
427 designed here, we succeeded not only in distinguishing the *Sitophilus* adults, as achieved
428 in other studies (Correa et al., 2013; Hidayat, Phillips, & FrenchConstant, 1996; Peng,
429 Lin, Chen, & Wang, 2002), but also simultaneously recognizing immature stages of these
430 sibling species in a single PCR reaction.
431 On the other hand, it is well known that *S. oryzae* is more resistant to phosphine,
432 which is one of the most commonly used chemical insecticides in stored grain worldwide,
433 than its sibling species, *S. zeamais* (Hagstrum, Reed, & Kenkel, 1999). Therefore, the use
434 of the present PCR method would help managers use appropriate control measures
435 according to the species present. Usually, only insect eggs or first-instar larvae are present
436 after fumigation (Brabec, Pearson, Flinn, & Katzke, 2010). Thus, since routine analysis
437 techniques are based on visual lures, those infestations may evade diagnosis and then the
438 storability of the grain may be underestimated.
439 We have also demonstrated success in detecting *S. zeamais* eggs in rice after a
440 treatment with CO_2 for 12 days, suggesting that this method would also detect other
441 developmental stages of the other four target species after a treatment of this kind, thereby
442 avoiding future increases of pest populations and therefore decreasing the grain
443 downgrade. In this respect, the use of the present protocol would help managers to
444 fumigate only once infestations reached a critical density (commonly considered to be
445 more than two insects/kg of grain) (Flinn, Hagstrum, Reed, & Phillips, 2010) and avoid

446 unnecessary treatments based on standard calendars (Trematerra, 2013). This would be
447 in line with the increasing public concerns beyond the overuse of agricultural chemicals
448 that are harmful to the environment and human health (Bulathsinghala & Shaw, 2013)
449 and would prevent the development of insect resistance due to continuous fumigation
450 (Hagstrum & Subramanyam, 2014; Phillips & Throne, 2010).

451 The dominant grain crops grown worldwide are rice, wheat, maize, millet, barley
452 and rye (Pimentel et al., 1997), and internal feeders are frequently found in all of them
453 when grain is stored. For this reason, we have tested the detection of the five target insect
454 pests in most of those grains. As a model, we tested the detection of larvae of *S. zeamais*
455 in rice, wheat, maize, oat, spelt and barley. Since these grains are usually processed before
456 consumption, in order to ensure that insects are also detected in processed grain we
457 analyzed the presence of this weevil in macaroni pasta. The results obtained showed that
458 the method was able to detect immature stages of *S. zeamais* in all these grains and in
459 pasta, thereby suggesting that this multiplex PCR method would also detect all
460 developmental stages of the five target species.

461 The fact that no weevil offspring was observed in the infested maize highlights
462 the global effort to select varieties resistant to insect presence in the most valuable grain
463 crops (Abebe, Tefera, Mugo, Beyene, & Vidal, 2009). Nevertheless, the ability of the
464 present method to detect insects in maize was demonstrated with the amplification of the
465 expected band for *S. zeamais* in the agarose gel when analyzing samples consisting in a
466 ground mixture of weevil adults and maize. On the other hand, only seven small larvae
467 were obtained from the macaroni pasta when sieving. A comparison of this number with
468 the others obtained from the rest of the grains (an average of 132 insect adults) reveals
469 that although *S. zeamais* can lay eggs inside pasta, this substrate is not the most suitable
470 for the development of this pest in comparison to the other grain cereals tested.

Insect infestations can occur during the storage process in manufacturing facilities, warehouses, general stores and retail shops, but insects can colonize food at any processing step, providing situations where insects might reach consumers (Jayas, White, & Muir, 1995). The stability of DNA, which can withstand temperatures of pasteurization and sterilization (Laube et al., 2007), suggests that the use of molecular approaches as a diagnostic technique in food factories would enable unambiguous identification of insects in food at any processing point. However, further studies should be conducted in order to corroborate this statement, particularly after the manufacturing process of pasta. This might be an advantage, particularly ahead of approaches based on proteins such as ELISA, where false-positive situations can occur due to the denaturation of proteins at temperatures above 56 ºC (Velebit, Markovic, Jankovic, & Borovic, 2009).

After analyzing commercial samples from a grain industry, results obtained by multiplex PCR were in accordance with those obtained by the operator of the industry. This demonstrates the potential of this molecular method for being introduced in processing industries for diagnosing insect presence. PCR-based methods are commonly accepted and recommended for food quality control, such as the detection of GMOs or for food traceability (Bai et al., 2009; Laube et al., 2007). In this sense, the detection of non-desired insects in food by molecular tools is suitable as a food control measure as has already been suggested by Obrepalska-Steplowska, Nowaczyk, Holysz, Gawlak, & Nawrot (2008), Solà, Riudavets, & Agusti (2015) and Solà, Lundgren, Agusti, & Riudavets (2017).

The determination of the correct sampling of the grain in order to detect infestations is often inaccurate because insect infestations are not homogeneous in grain facilities. Nevertheless, the present method identifies insect infestations with high accuracy and sensitivity when grain is thoroughly homogenized. However, in order to

ensure that the obtained information is representative of the real grain contamination, it is important to establish an adequate number of samples of a determinate size (Jian, Jayas, & White, 2014a, b). Once samples are defined, the transmission of this information to grain managers in food industries should help them to implement IPM practices, develop economic thresholds and set up decision-making strategies aimed at using pesticides more selectively and thus be more environmentally friendly while at the same time preventing the undesired presence of insects in food. Additionally, the combination of the present multiplex PCR with a qPCR protocol for the detection of particular pest species, such as those developed by Solà, Lundgren, Agusti, & Riudavets (2017) and Obrepalska-Steplowska, Nowaczyk, Holysz, Gawlak, & Nawrot (2008), would provide an improved screenshot of the grain status. Therefore, further work is needed to combine simultaneous identification of concerned primary pests with the quantification of real populations.

5. Conclusions

In this study, a multiplex PCR approach is described for the detection and identification of the five main primary pests that develop inside grain cereals (*R. dominica*, *S. granarius*, *S. oryzae*, *S. zeamais* and *S. cerealella*). This method has demonstrated the ability to detect internal stages of the target pests, a characteristic that the most common techniques such as sieving lack. This approach is a reliable technique for simultaneously and specifically identifying the five concerned internal feeders with high sensitivity (0.1 pupa per kilo of rice, or 10 pupae in the case of *R. dominica*), even one year after death. With the detection of hidden immature stages of *S. zeamais* in different kinds of grain (barley, oat, spelt, rice, wheat) and pasta, even when the grain is treated it is expected that this method will detect all target species present. The results

obtained for the analysis of some real commercial samples with the developed multiplex PCR method suggest that the use of the developed multiplex PCR in food control analyses for insect detection and identification would improve the quality of food and satisfy most consumer concerns.

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Table 1. Insect species potentially present in stored grain products and tested for specificity with the developed multiplex PCR protocol. In **bold**, the five target species (n>10). The order, family, origin, collection year and universal primer pair set used as positive control for the presence of DNA are indicated. Three specimens of each non-target species were tested, except for *Trogoderma glabrum* (n=2), *Dinerella agra* and *Alphitobius laevigatus* (n=1).

Order	Family	Species	Origin	Collection year	Universal primer set*
Coleoptera	Anobiidae	*Lasioderma serricorne* (Fabricius)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ZBJ-ArtR2c
		Stegobium paniceum (Linnaeus)	field sample, Spain	2002	ZBJ-ARTF1c/ZBJ-ArtR2c
	Bostrychidae	*Lyctus brunneus* (Stephens)	field sample, Spain	2003	Uni-MinibarF1/Uni-MinibarR1
		Dinoderus minutus (Fabricius)	field sample, Vietnam	2002	Uni-MinibarF1/Uni-MinibarR1
		***Rhyzopertha dominica* (Fabricius)**	lab colony (IRTA), Spain**	2013	ZBJ-ARTF1c/ZBJ-ArtR2c
		Prostephanus truncatus (Horn)	field sample, Mexico	2010	ZBJ-ARTF1c/ZBJ-ArtR2c
	Bruchidae	*Acanthoscelides obtectus* (Say)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ZBJ-ArtR2c
		Zabrotes subfasciatus (Boheman)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ZBJ-ArtR2c
	Chrysomelidae	*Callosobruchus maculatus* (Fabricius)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ZBJ-ArtR2c
	Cleridae	*Necrobia rufipes* (Fabricius)	field sample, Spain	2010	ZBJ-ARTF1c/ZBJ-ArtR2c
	Curculionidae	***Sitophilus granarius* (Linnaeus)**	lab colony (IRTA), Spain**	2013	ZBJ-ARTF1c/ZBJ-ArtR2c
		***Sitophilus oryzae* (Linnaeus)**	lab colony (IRTA), Spain**	2013	ZBJ-ARTF1c/ZBJ-ArtR2c
		***Sitophilus zeamais* (Motschulsky)**	lab colony (IRTA), Spain**	2013	ZBJ-ARTF1c/ZBJ-ArtR2c
	Dermestidae	*Dermestes haemorrhoidalis* (Küster)	field sample, Spain	2006	ZBJ-ARTF1c/ZBJ-ArtR2c
		Dermestes maculatus (DeGeer)	field sample, Spain	2011	ZBJ-ARTF1c/ZBJ-ArtR2c

		Trogoderma glabrum (Herbst)	lab colony (IRTA), Spain	2013	Uni-MinibarF1/Uni-MinibarR1
		Trogoderma granarium (Everts)	lab colony (IRTA), Spain	2013	LCO1490/HCO2198
		Trogoderma inclusum (Leconte)	lab colony (IRTA), Spain	2013	Uni-MinibarF1/Uni-MinibarR1
	Laemophloeidae	*Cryptolestes ferrugineus* (Stephens)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ZBJ-ArtR2c
		Cryptolestes pusillus (Schonherr)	lab colony (JKI), Germany	2015	ZBJ-ARTF1c/ZBJ-ArtR2c
		Cryptolestes turcicus (Grouvelle)	lab colony (JKI), Germany	2015	ZBJ-ARTF1c/ZBJ-ArtR2c
	Latridiidae	*Dinerella arga* (Reitter)	field sample, Spain	2000	ZBJ-ARTF1c/ZBJ-ArtR2c
		Dinerella fillum (Aubé)	field sample, Russia	2004	ZBJ-ARTF1c/ZBJ-ArtR2c
	Silvanidae	*Ahasversus advena* (Waltl)	field sample, Spain	2006	Uni-MinibarF1/Uni-MinibarR1
		Oryzaephilus mercator (Fauvel)	field sample, Spain	2004	Uni-MinibarF1/Uni-MinibarR1
		Oryzaephilus surinamensis (Linnaeus)	lab colony (IRTA), Spain	2013	Uni-MinibarF1/Uni-MinibarR1
	Ptinidae	*Niptus hololeucus* (Faldermann)	field sample, Spain	2015	ZBJ-ARTF1c/ZBJ-ArtR2c
	Tenebrionidae	*Alphitobius diaperinus* (Panzer)	field sample, Spain	2007	Uni-MinibarF1/Uni-MinibarR1
		Alphitobius laevigatus (Fabricius)	field sample, Spain	2013	ZBJ-ARTF1c/ZBJ-ArtR2c
		Gnathocerus cornutus (Fabricius)	field sample, Spain	2006	Uni-MinibarF1/Uni-MinibarR1
		Latheticus oryzae (Waterhouse)	lab colony (JKI), Germany	2015	ZBJ-ARTF1c/ZBJ-ArtR2c
		Tenebrio molitor (Linnaeus)	lab colony (JKI), Germany	2015	ZBJ-ARTF1c/ZBJ-ArtR2c
		Tribolium confusum (Jaqueline du Val)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ZBJ-ArtR2c
		Tribolium castaneum (Herbst)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ZBJ-ArtR2c
	Trogossitidae	*Tenebroides mauritanicus* (Linnaeus)	field sample, Spain	1999	ZBJ-ARTF1c/ZBJ-ArtR2c
Hymenoptera	Bethylidae	*Cephalonomia spp.*	field sample, Spain	2015	LCO1490 and HCO2198
	Braconidae	*Habrobracon hebetor* (Say)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ZBJ-ArtR2c

	Ichneumonidae	*Venturia canescens* (Gravenhorst)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ZBJ-ArtR2c
	Pteromalidae	*Anisopteromalus calandrae* (Howard)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ZBJ-ArtR2c
		Lariophagus distinguendus (Förster)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ZBJ-ArtR2c
Lepidoptera	Pyralidae	*Ephestia cautella* (Walker)	lab colony (JKI), Germany	2015	ZBJ-ARTF1c/ZBJ-ArtR2c
		Ephestia elutella (Hübner)	lab colony (JKI), Germany	2015	ZBJ-ARTF1c/ZBJ-ArtR2c
		Ephestia kuehniella (Zeller)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ZBJ-ArtR2c
		Plodia interpunctella (Hübner)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ZBJ-ArtR2c
	Gelechiidae	***Sitotroga cerealella* (Olivier)**	lab colony (IRTA), Spain**	2013	ZBJ-ARTF1c/ZBJ-ArtR2c
	Tineidae	*Nemapogon granella* (Linnaeus)	field sample, Spain	1999	ZBJ-ARTF1c/ZBJ-ArtR2c
Mesostigmata	Ascidae	*Blattisocius tarsalis* (Berlese)	lab colony (IRTA), Spain	2001	Uni-MinibarF1/Uni-MinibarR1
Pseudoscorpionida	Withiidae	*Withius piger* (Simon)	field sample, Spain	2011	ZBJ-ARTF1c/ZBJ-ArtR2c
Psocoptera	Liposcelididae	*Liposcelis botrichophila* (Badonnel)	field sample, Spain	1997	LCO1490/HCO2198
Sarcoptiforme	Acaridae	*Tyrophagus perniciosus* (Zakhvatkin)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ZBJ-ArtR2c
		Tyrophagus putrecentiae (Schrank)	field sample, Spain	1997	LCO1490/HCO2198

* ZBJ-ArtF1c/ZBJ-ArtR2c (Zeale et al., 2011); Uni-MinibarF1/Uni-MinibarR1 (Meusnier et al., 2008); LCO1490/HCO2198 (Folmer et al., 1994).

** The species coming from laboratory colonies (IRTA) were originally from Tarragona, Spain.

Table 2. Specific primer pairs designed for each target species. The corresponding primer concentration (µM) used in the primer mix and the number of base pairs (bp) amplified are also indicated.

Target species	Primer name and sequence (5'-3')	Primer concentration (µM)	Amplicon size (bp)
S. cerealella	**SCF4:** GATACTTATTACGTAGTTGCTC	0.4	93
	SCR4: TAAGGGGTATCAATGAATG	0.4	
S. zeamais	**SZF2:** CTCCCTCCATCATTAATTC	0.6	151
	SZR3: TACCTGCTATATGAAGAC	0.6	
S. oryzae	**SOF4:** TGGAAACTGATTAATCCCAT	0.1	213
	SOR2: CTGAAAATGGCCAGATCAAC	0.1	
R. dominica	**RDF1:** GCTTCTTCCACCCTCCTTAACC	0.6	286*
	RDR1: AGATAATAATAAAAGCAAAGC	0.6	
S. granarius	**SGF1:** CGTTACTGCTCACGCATTT	0.2	452
	SGR1: TAGTAATTGCTCTAGCTAAG	0.2	

*Designed by Solà et al. (2017).

Table 3. Frequencies (%) of multiplex PCR amplification per each insect species at four different infestation doses (pupae/Kg of rice) and six post-mortem periods of time (days). Two replicates have been done for infestation dose, and three for time post mortem.

	Infestation dose (p/Kg)				**Time post-mortem (d)**					
Species	**100**	**10**	**1**	**0.1**	**30**	**90**	**150**	**365**	**548**	**760**
Sitotroga cerealella	100	100	100	100	100	100	100	100	100	100
Sitophilus zeamais	100	100	100	100	100	100	100	100	100	100
Sitophilus oryzae	100	100	100	100	100	100	100	100	100	100
Sitophilus granarius	100	100	100	100	100	100	100	100	67	0
Rhyzopertha dominica	100	100	0	0	100	100	100	100	33	0

Figure 1. Agarose gel electrophoresis of the PCR products amplified with the designed multiplex PCR. **M**: molecular marker (100 bp ladder). **C+**: Positive control of the 5 target species (mixture of 1 µl of DNA extraction of each of the target species in 15 µl of purified water). **SC**: *S. cerealella*, **SZ**: *S. zeamais*, **SO**: *S. oryzae*, **RD**: *R. dominica*, **SG**: *S. granarius*, **C-**: Negative control (purified water).

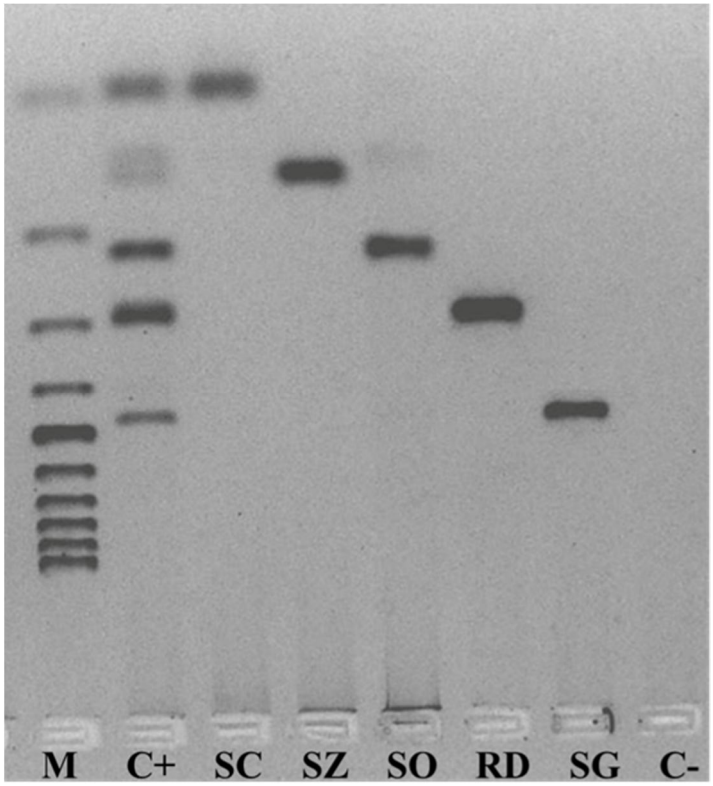

Figure S1. PCR products obtained when testing the sensitivity of the designed multiplex PCR with the five internal feeders (*S. cerealella*, *S. zeamais*, *S. oryzae*, *R. dominica*, *S. granarius*) in different artificial pupae infestation doses in rice (**100:** 100 pupae/Kg, **10:** 10 pupae/Kg, **1:** 1 pupae/Kg, **0.1:** 0.1 pupae/kg, **C+:** positive control of the 5 target species (mixture of 1 µl of DNA extraction of each of the target species in 15 µl of purified water), **C-:** negative control (purified water), **M:** molecular marker (100 bp ladder)).

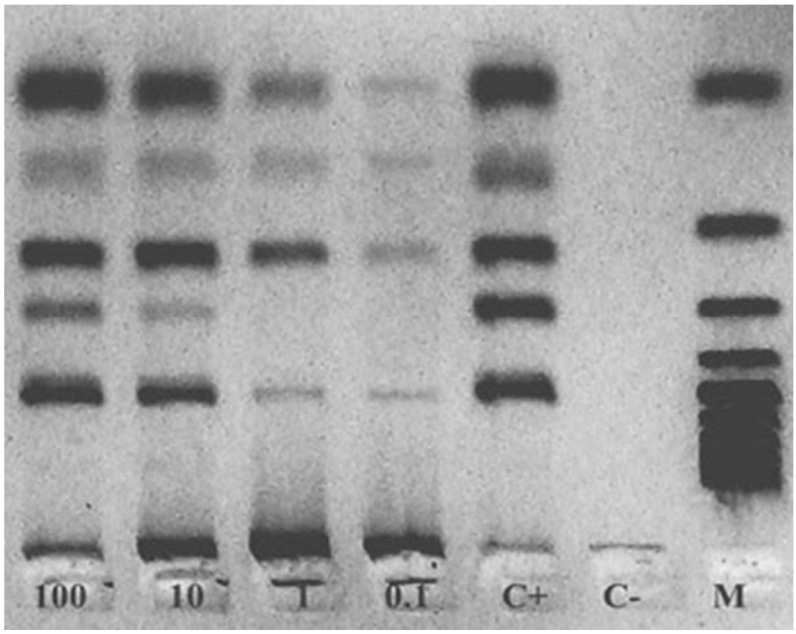

Figure S2. PCR products amplified from the five internal feeders (*S. cerealella, S. zeamais, S. oryzae, R. dominica, S. granarius*) after different post-mortem periods (**M:** molecular marker (100 bp ladder); **C+:** positive control of the 5 target species (mixture of 1 µl of DNA extraction of each of the target species in 15 µl of purified water); **C-:** negative control purified water); **30, 90, 150, 360, 548** and **760**: number of days post-mortem).

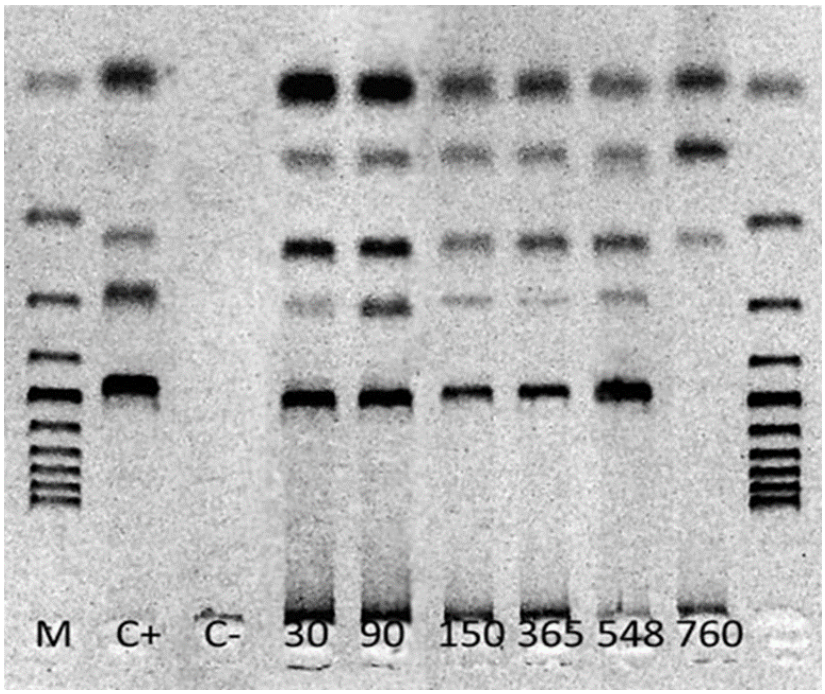

Figure S3. Agarose gel electrophoresis of the DNA amplification of *Sitophilus zeamais* larvae in different cereals (**M**: molecular marker (100 bp ladder); **C-**: negative control (purified water); **1:** spelt, **2:** wheat, **3:** rice, **4:** oat, **5:** barley, **6:** pasta, **7:** maize).