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Detection and quantification of the insect pest *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae) in rice by qPCR

Mireia Solà [a], Jonathan G. Lundgren [b, 1], Nuria Agustí [a], Jordi Riudavets [a, *]

[a] IRTA, Ctra Cabrils km 2, 08348 Cabrils, Barcelona, Spain
[b] USDA-ARS North Central Agricultural Research Services, Brookings, SD 57006, USA

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ABSTRACT

The early detection of insects during grain storage and processing remains a major issue for the cereal industry, especially when immature stages are hidden inside the grain kernels. For this reason, we developed a qPCR method to detect and quantify one of the main pests of stored products in rice: the coleopteran internal feeder *Rhyzopertha dominica*. For that purpose, a specific primer set was designed to amplify artificial infestations of this pest in rice. Then, using a regression model, a standard curve was generated that correlated individuals to adult equivalent DNA quantity (inverse of the C_t value). Results revealed that the designed primer set was specific for *R. dominica* when tested against the other 4 common internal feeders in grain. The technique showed to be accurated (DNA was detected in more than 73% of the samples) and sensitive to insect presence (i.e. from 0.02 adults, 0.1 3rd instar to pupae or 13 egg to 2nd instar detectable per kg of rice). Moreover, the detection of *R. dominica* was strongly associated with a given infestation size: DNA quantity increased along with the size of the population. The use of the described qPCR protocol in grain and milling factories may enhace the critical detection and quantification of *R. dominica* populations in raw materials and processed food.

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1. Introduction

Cereal grains can be stored for periods longer than one year before being processed or transported. During this interval, grain become susceptible to be attacked by insects, which cause direct quantitative and qualitative losses to the product (HLPE, 2014; Gorham, 1979). As a consequence, significant portions of this food never reaches its final destination and is never consumed. For example, in maize, 30% of grain can be lost due to post-harvest pests (HLPE, 2014). External feeding pests can be removed from grain by sieving before milling. However, grain internal feeders cannot be easily disassociated. Because internal pests are difficult to detect and remove from grain, they are generally regarded as the most pestiferous ones (Flinn et al., 2007; Toews et al., 2007). Storey et al. (1982) reported that 12% of wheat samples from export loads in USA have hidden internal insects which are undetected during the standard grain inspection process.

The lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae) is a grain internal feeder that is a major pest of rice and other cereals around the world (Castañé and Riudavets, 2015). It can be present alone or together with four other common internal feeders: *Sitophilus granarius* (L), *S. oryzae* (L.), *S. zeamais* (Motschulsky) (Coleoptera: Curculionidae) and the moth *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae) (Chanbang et al., 2008). Also, in some tropical areas of America and Africa, *R. dominica* can be found together with the closely related grain internal feeder *Prostephaus truncatus* (Horn) (Coleoptera: Bostrichidae). *Ryzopertha dominica* eggs are laid outside of the grain kernel and newly hatched larvae feed voraciously inside the kernels where they develop until the emergence of the adult (Edde, 2012). Both larvae and adults consume grain-based products resulting in fragmented kernels, powdery residues and a characteristic pungent odour (Toews et al., 2006) which can facilitate the entrance of secondary pests and fungi (Shah and Khan, 2014). Sittisuang and Imura (1987) reported weight losses of up to 40% in brown rice after 14 weeks of artificial exposure to *R. dominica*.

Insect detection, identification and quantification are important to maintain standards of quality for grain. However, the legislation for detecting undesirable insects, frass and exuviae differs

* Corresponding author.
E-mail address: jordi.riudavets@irta.cat (J. Riudavets).
[1] Current address: Ecdysis Foundation, 46958 188th St, Estelline, South Dakota, USA, 57234.

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significantly among countries and industries (Obrepalska-Steplowska et al., 2008; Flinn et al., 2007; Neethirajan et al., 2007; Toews et al., 2007). In the EU there are no systematic surveillance programs or any scientific risk assessment concerning storage pests on the agenda of the European Food Safety Authority (EFSA) panel (Stejskal et al., 2014). In contrast, in the USA, the Food and Drug Administration (FDA) has developed a set of pest action thresholds that serves as a regulatory standard for food quality control. For example, thresholds of 75 insect fragments per 50 g of wheat flour, or 225 insect fragments per 225 g of macaroni and noodle products have been presented. Also, samples with more than 31 insect damaged kernels (IDK) per 100 g of wheat have been classified as unfit for human consumption (FDA, 1997).

Several techniques are currently available to detect insect infestations in stored grain (Neethirajan et al., 2007). Some of these methodologies are based on sieving, grain flotation, Berlese funnel extractions (Hubert et al., 2009), Enzyme-Linked ImmunoSorbent Assay (ELISA) (Kitto et al., 1993), the acoustic emission impact of insect movements within grain (Fleurat-Lessard et al., 2006; Hagstrum et al., 1997), Near Infrared (NIR) (Dowell, 1998) or soft X-rays (Shah and Khan, 2014; Fornal et al., 2007; Karunakaran et al., 2003). Although some of these technologies are effective for detecting insect infestations of stored grain, they are either expensive, time consuming, unable to detect and identify low levels of pests, or not able to quantify internal insect infestations (Trematerra, 2013; Neethirajan et al., 2007). For example, NIR methodology and soft X-ray can discriminate among developmental stages, but the first requires frequent calibration and cannot detect low levels of pests (Perez-Mendoza et al., 2003), while the second is tedious and cost prohibitive (Neethirajan et al., 2007).

Real-time PCR or quantitative PCR (qPCR) represents a reliable and fast way to detect, identify and quantify all life stages of an organism, based on the presence of its DNA (Mendoza et al., 2006). For this reason, over the last decade this technology has increasingly been used in the field of applied entomology to detect significant quarantine pests (Barcenas et al., 2005).

The objective of the present study was to develop a qPCR method to identify and quantify *R. dominica* individuals in rice using a DNA detection approach, as a model system for detecting rice contaminated with stored grain pests.

2. Material & methods

2.1. Biological material

2.1.1. Insect specimen collection and rearing

We used *Rhyzopertha dominica* from two different origins. For the detection and quantification of *R. dominica* in grain, adults were shared from a colony maintained at the USDA-ARS Stored Pest Insect Research Unit (Manhattan, KS, USA). Then, they were reared at the USDA-ARS North Central Agricultural Research Services on organic brown short rice (Lundberg Family Farms, Richvale, CA, USA) at 30 °C and 70% relative humidity (RH). On the other side, the insects used for the specificity test: *R. dominica* together with the four other internal feeders in our area (*S. granarius, S. oryzae, S. zeamais* and *S. cerealella)* came from a laboratory colony maintained at IRTA Cabrils (Spain). In this case, coleopteran species were reared on organic brown rice (Eco-Salim, Maquefa, Spain) at 28° C and 70% RH and 16L: 8D while lepidopteran were reared on maize (Crit d'or, Spain) at 23 °C and 70% RH and 16L: 8D.

2.1.2. Insect DNA extraction procedures

DNA was extracted from whole adult individuals using the DNeasy Blood & Tissue extraction kit (Qiagen, Valencia, CA, USA; following the protocol for animal tissues). Total DNA was eluted in 400 μl AE buffer (10 mM Tris-Cl 0.5 mM, EDTA; pH 9.0) provided by the manufacturer. However, for the DNA extractions destined to the specificity test, the DNeasy Tissue Kit (QIAGEN, Hilden, Germany; protocol for animal tissues) was used and DNA was eluted in 100 μl of AE buffer.

2.1.3. Rice collection and processing

Rice used in the experiments was from two different origins. For the detection of *R. dominica* in infested grain kernels, we used organic brown short rice (Lundberg Family Farms, Richvale, CA, USA). To detect and quantify *R. dominica* in grain we used the same brown rice as was used for rearing the insects in IRTA Cabrils. In order to ensure that the brown rice was insect-free, a sample of 500 g of brown rice was maintained at 28 °C and 70% RH and three months later, it was sieved in a 2 mm mesh to check for insect adult presence.

2.1.4. Rice DNA extraction

Ten grams of pest-free brown rice was ground in a coffee grinder (Laboratory mill 3303, Perten-Instruments, Hägersten, Sweden) and DNA was extracted using the Extragen® Alimentos extraction kit (Sistemas Genómicos, Valencia, Spain). Total rice DNA was eluted in 100 μl of HPLC-grade water, and 50 μl of this elution was finally added to 150 μl of AE buffer. These DNA rice extractions were carried out in IRTA (Cabrils, Spain) and 50 μl of each were sent to North Central Agricultural Research Laboratory (NCARL)(Brookings, USA) for the qPCR analysis.

A negative control was added to all DNA extraction set (insect, rice and infested rice). Final DNA extractions were stored at −20 °C.

2.2. Primer design and specificity analyses

A *R. dominica*-specific primer set was designed to target part of the Cytochrome Oxidase I (COI) mitochondrial region. For this, COI sequences of *R. dominica* and the mentioned four other common internal grain pests (*S. granarius, S. oryzae, S. zeamais* and *S. cerealella)* were aligned using ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2) and compared for regions specific to *R. dominica* (GenBank accession numbers: JQ989165, DQ200131, AY131101, AY131099 and AY131100, respectively).

DNA samples from all insect strains and rice origins were amplified in a Stratagene MX3000P thermal cycler (Stratagene, La Jolla, CA, USA). Reaction PCR volumes (25 μl) contained 12.5 μl Quantitect SYBR Green PCR Master Mix (Qiagen, Valencia, CA, USA), 1 μl of each *R. dominica*-specific forward and reverse primers (225 nM) and 2 μl of template DNA (whole insect DNA or pest free brown rice DNA). Amplification conditions consisted of a single cycle of 95 °C, followed by 50 cycles of 94 °C for 15 s, 52 °C for 30 s, and 72 °C for 30 s. Each plate also had a positive control series (n = 5 wells) and no-template control series (n = 3 wells). The positive control consisted of a DNA extraction of five *R. dominica* larvae.

The designed *R. dominica* primer set was tested for specificity by amplifying DNA of the four other internal feeder pest species described above under the same qPCR conditions. Also, we ensured that our PCR process did not detect prior infestations with a preliminary set of PCRs where the DNA extraction of the brown rice used, a positive control (five *R. dominica* larvae), a negative control (water) and a mixture of the positive control and the rice, were ran (n = 3 each). To ensure that only the target DNA was amplified, the dissociation temperature of the *R. dominica* qPCR product (melting) temperature was determined by incubating the reaction mix at 95 °C for 60 s and then monitoring the fluorescence every 0.5 °C from 55 to 94 °C.

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