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A PCR-based diagnostic assay for detecting DNA of the olive fruit fly, *Bactrocera oleae*, in the gut of soil-living arthropods

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Abstract

Bactrocera oleae (Rossi) (Diptera: Tephritidae) is considered the most devastating pest of the olive tree worldwide. In an effort to develop management and biological control strategies against this pest, new molecular tools are urgently needed. In this study, we present the design of *B. oleae*-specific primers based on mitochondrial DNA sequences of *cytochrome oxidase subunit I* (*COI*) gene. Two pairs of *B. oleae*-specific primers were successfully designed and named as SBo1-F/SBo1-R and SBo2-F/SBo1-R, being able to amplify 108 and 214 bp *COI* fragments, respectively. The specificity of designed primers was tested by amplifying DNA from phylogenetically related (i.e. Diptera order) and other non-pest insects living in olive groves from the Mediterranean region. When using these primers on a PCR-based diagnostic assay, *B. oleae* DNA was detected in the gut content of a soil-living insect, *Pterostichus globosus* (Fabricius) (Coleoptera: Carabidae). The detection of *B. oleae* DNA in the guts of arthropods was further optimized by adding bovine serum albumin enhancer to the PCR reaction, in order to get a fast, reproducible and sensitive tool for detecting *B. oleae* remains in the guts of soil-living arthropods. This molecular tool could be useful for understanding pest–predator relationships and establishing future biological control strategies for this pest.

Keywords: *Bactrocera oleae*, specific primers, diagnostic PCR, feeding assay

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Introduction

Bactrocera oleae (Rossi) (Diptera: Tephritidae) is the most harmful olive pest worldwide. Female flies lay eggs in olive fruits, where the newly hatched larvae feed upon the pulp, reducing the quality of olives and olive oil. Larvae pupate and overwinter on the ground, and then emerge in spring as adult flies. The olive fruit fly only attacks plants of the *Olea* genus, and spreads quickly to nearby olive groves. There are well-reported *B. oleae* outbreaks in the Mediterranean region and other regions have been seriously affected, such as North, Central and South America, Central Asia (Augustinos *et al.*, 2002), or East and South Africa (Copeland *et al.*, 2004). Due to these attacks, economic losses can reach nearly 800

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million US dollars per year (Bueno & Jones, 2002). Therefore, more efficient and safer methods for pest control are required.

For many years, the main method adopted to control this pest has been based on spraying organophosphate insecticides, which have led to the development of *B. oleae* pesticide resistance and enhancement of the risk of pest outbreaks (Bueno & Jones, 2002; Vontas *et al.*, 2002; Hawkes *et al.*, 2005). More recently, there has been an increasing interest for the application of conservation biological control strategies against this pest aiming at conserving and promoting the effectiveness of *B. oleae* natural enemies and decrease the use of pesticides in olive groves. Thus, accurate identification of *B. oleae* natural enemies is required, mainly of those soil arthropods which feed on hibernating pupae and could contribute for maintaining pest density at a low level (Gariepy *et al.*, 2007). PCR-based techniques have been valuable tools for studying trophic relationships of pest species, such as *Bactrocera minax* (Enderlein) and *Bactrocera tsuneonis* (Miyake) (Diptera: Tephritidae) (Jiang *et al.*, 2014), *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) (Monzó *et al.*, 2010) and seven mealybug species found in vineyards (Daane *et al.*, 2011). These tools have been also used in large-scale studies describing food webs in ecosystems (Valentini *et al.*, 2009; Staudacher *et al.*, 2011), as well as in biological control programmes (Agboton *et al.*, 2009; Jenkins *et al.*, 2012). Another helpful molecular approach is DNA barcoding that allows species identification by sequencing a standardized DNA sequence (barcode). Since animal mitochondrial DNA sequences evolve faster than nuclear DNA, they accumulate many nucleotide differences even between closely related species, which has been very useful for phylogenetic studies (e.g., Cameron, 2014). For animal identification, the most suitable barcode has been the 5′ end of the mitochondrial *cytochrome oxidase subunit I* (*COI*) sequence, due to the high sequence divergence found between species and at the same time the availability of conserved regions that allows the use of universal primers (Hebert *et al.*, 2003). This molecular approach has been applied in the identification of insects, including Diptera and several primers have been designed so far for Diptera-specific PCR amplification (Gibson *et al.*, 2011).

Taking into account future applications in ecological studies about pest–predator interactions, the main goal of this work was to design-specific primers within the *COI* gene to be used in a PCR-based diagnostic method for detecting *B. oleae* DNA. The effectiveness of such a diagnostic molecular tool was validated in this work by detecting *B. oleae* DNA in the guts of *Pterostichus globosus* (Fabricius) (Coleoptera: Carabidae) adults. This species is a generalist predator that has been described as one of the most abundant carabid beetles on the ground of olive groves (Dinis *et al.*, 2016*a*), displaying high predatory activity on *B. oleae* pupae. In non-choice laboratory experiments, a single *P. globosus* specimen was able to ingest up to a maximum of 30 pupae in 24 h (Dinis *et al.*, 2016*b*).

Materials and methods

Insect collection

Bactrocera oleae pupae were obtained from infested olive fruits collected in an olive grove (October/November 2013), while *Rhagoletis cerasi* (L.) (Diptera: Tephritidae) pupae were obtained from cherries collected in an orchard (June 2015), both located in Mirandela (Northeastern Portugal). *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) pupae were obtained from a laboratory culture maintained at the University of Minho (UM). *Calliphora vicina* Robineau-Desvoidy (Diptera: Calliphoridae) adults were collected in the *Campus* of University of Minho (Portugal). Soil insect adults, including *P. globosus* specimens, were collected by hand in an organic olive grove in the region of Mirandela (Northeastern Portugal), between September of 2013 and May of 2014. All insects were initially identified to species using a binocular stereomicroscope, followed by a molecular identification using the methods described in subsection 'DNA extraction and amplification of mitochondrial *COI* gene'. Insects were preserved in absolute ethanol and stored at −80°C to avoid deterioration of DNA.

DNA extraction and amplification of mitochondrial COI gene

For insect molecular identification and to prevent the interference of DNA from ingested preys in the gut content, genomic DNA was extracted from legs, antennae and wings of adult insects using the Ron's Tissue DNA Mini Kit (Bioron GmbH, Germany). The barcode region of mitochondrial *COI* gene was amplified using the universal primers LCO1490/UEA10 (Folmer *et al.*, 1994; Lunt *et al.*, 1996). The reactions were performed using the *FastStart High Fidelity PCR System* (Roche Diagnostics GmbH, Germany) in a final volume of 25 µl containing 1× buffer, 100 µM of each dNTP, 2 mM $MgCl_2$, 200 nM of each primer and 0.05 U *Taq* DNA polymerase. PCR was performed in a *MJ Mini BioRad*® thermocycler with the following protocol: preheating (94°C, 5 min), 35 cycles at 94°C for 60 s, 55°C for 60 s, 72°C for 90 s, and a final extension at 72°C for 10 min. PCR products (∼1500 bp) were run on a 1% (v/v) agarose gel, stained with Green Safe Premium (NZYTech, Portugal) and visualized under UV light. The target amplicons were purified and sequenced (STAB VIDA sequencing services, Portugal).

Design of B. oleae*-specific primers and diagnostic PCR*

Primers were designed to ensure the amplification of the target sequences from *B. oleae*, while avoiding cross-reactivity. Available *COI* sequences for *B. oleae* and other insects were recovered from GenBank and Barcode of Life Data Systems databases and aligned together with *COI* sequence of *B. oleae* specimens collected in Mirandela, using the MegAlign program (DNASTAR, Lasergene Version 7). Two forward primers, SBo1-F (5′CAG TAG TAC TAA CAG CCC TAC T 3′) and SBo2-F (5′TTA GCA GGT ATC TCC TCA ATC 3′) and one reverse primer, SBo1-R (5′CTG GGT CGA AAA AGG AAG TAT′3), were selected as *B. oleae*-specific primers. The target specificity of these primers was checked by using the Primer-BLAST tool of NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast).

In order to specifically detect *B. oleae* DNA, the PCR amplification conditions were optimized, using the designed primers and all basic components as described above (subsection 'DNA extraction and amplification of mitochondrial *COI* gene'). The PCR program was optimized in order to improve the specificity of primers, resulting in the following protocol: an initial preheat for 3 min at 94°C, followed by 30 cycles at 94°C for 30 s, 60°C for 40 s, 72°C for 20 s and a final extension at 72°C for 10 min. The use of bovine serum albumin (BSA) in the diagnostic amplification was tested by adding BSA at a final concentration of 0.5 µg μl^{-1} to the PCR reaction. The specificity

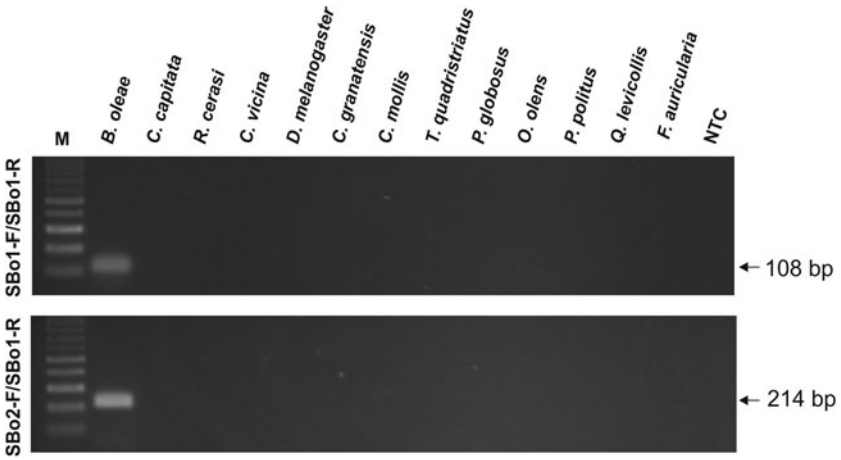

Fig. 1. Designed primers specificity for *Bactrocera oleae* DNA detection. Agarose gel electrophoresis of *COI*-amplified fragments using *B. oleae*-specific primer pairs SBo1-F/SBo1-R and SBo2-F/SBo1-R and template DNA from different insect species (100 or 50 ng for each pair of primers, respectively). The selected species belong to Diptera (Tephritidae: *B. oleae*, *Ceratitis capitata* and *Rhagoletis cerasi*; Calliphoridae: *Calliphora vicina*; Drosophilidae: *Drosophila melanogaster*), Coleoptera (Carabidae: *Calathus granatensis*, *Calathus mollis*, *Trechus quadristriatus* and *Pterostichus globosus*; Staphylinidae: *Ocypus olens*, *Philonthus politus* and *Quedius levicollis*) and Dermaptera (Forficulidae: *Forficula auricularia*). Lane M is a 100 bp DNA ladder and the NTC lane is a no template control.

of the primers to *B. oleae* was evaluated by using genomic DNA (50 ng) extracted from crushed pupae and from legs, antennae and wings of related dipterans and soil insects in a PCR assay, in order to ensure the exclusive amplification of the target sequence from *B. oleae*. The optimal concentration of gut genomic DNA to be used in diagnostic PCR was determined using DNA extracted from the guts of *P. globosus* just after they had been fed on *B. oleae* pupae at different amounts of DNA template amounts (25–150 ng). All PCR products were run on a 2% agarose gel, stained with Green Safe Premium (NZYTech, Portugal) and visualized under UV light.

Feeding assay and digestion of B. oleae

Experiments were conducted by feeding *P. globosus* adults on *B. oleae* pupae. First, to ensure that animals had their guts emptied, they were placed in a climate chamber at 21 ± 1°C, with 70 ± 5% relative humidity, and a photoperiod of 16:8 h (L:D) and starved for 7 days. Each individual was then fed on two pupae of *B. oleae*. After feeding, *P. globosus* adults were sacrificed at different periods (0, 2, 4, 6, 8, 10, 16 h) and their intestinal apparatus were removed. The genomic DNA extraction from insect guts was performed on individuals by using the Ron's Tissue DNA Mini Kit (Bioron GmbH, Germany). DNA pools from three individuals were prepared using equal amounts of DNA and a total of three replicates were prepared. DNA concentrations were measured by a Qubit Fluorometer 3.0 (Invitrogen, USA) using the Quant-iT™ dsDNA high-sensitivity (HS) Assay Kit (Invitrogen, USA). The digestion profile of *B. oleae* was then evaluated by PCR using the designed primers and optimal concentrations, in the presence (0.5 µg µl^{-1}) or the absence of BSA. The DNA extracted from the guts of starved *P. globosus* specimens was used as negative control (C^-) and *B. oleae* DNA was used as positive control (C^+). A PCR reaction without template DNA was used as no template control (NTC).

Results

For the specific detection of *B. oleae* DNA, primers for the variable region of the 5′ end of mitochondrial *COI* sequence were designed taking into account the specific sequence of this insect. Forty-eight available *COI* sequences for *B. oleae* were aligned together with *COI* sequences from *B. oleae* collected in Mirandela, for detecting possible nucleotide polymorphisms in *COI* gene among *B. oleae* sequences. The *B. oleae* conservative sequence was then aligned with 120 *COI* sequences from phylogenetically related insects. *B. oleae*-specific primers were selected by comparing the highly variable *COI* regions among this fruit fly and other species, while avoiding intra-specific single nucleotide polymorphisms. Primers were checked for unfavorable secondary structures and compared with other DNA sequences using a BLAST search in the NCBI database to make sure they do not hit other genomic locations in target organisms. All designed primers (SBo1-F, SBo2-F and SBo1-R) amplified *B. oleae* DNA, resulting in a single DNA fragment. The use of both pairs of primers, SBo1-F/SBo1-R and SBo1-F/SBo2-R, generated a specific amplification with the predicted PCR products of 108 and 214 bp, respectively. Sequence analysis of amplified products indicated that both fragments shared 100% identity with *B. oleae COI* gene. No amplification was detected when using DNA extracted from legs, antennae and wings of other insects, which reveals the high specificity of the designed primers (fig. 1).

The efficiency of this PCR-based diagnostic test was then validated by the detection of *B. oleae* remains in the guts of a soil-living insect, *P. globosus*, as confirmed by the sequencing of the amplified fragments. Taking into account that insect guts could contain several PCR contaminants, and gut genomic DNA should be in sufficient amounts to allow the detection of even limited remains of *B. oleae*, the template DNA amount to be used in diagnostic PCR was optimized using insects that had just been fed on *B. oleae* pupae (fig. 2). The optimal DNA template amount was 100 ng for the primers pair SBo1-F/

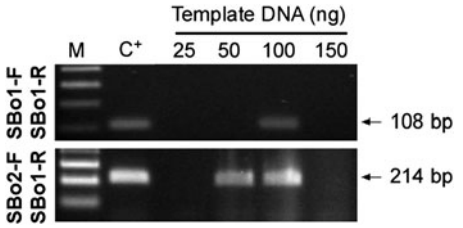

Fig. 2. Gut genomic DNA concentration optimization for detection of *Bactrocera oleae* DNA in feeding assay experiments. *Pterostichus globosus* insects just after been fed on *B. oleae* pupae were sacrificed and their intestinal apparatus used for genomic DNA extraction. Both *B. oleae*-specific primer pairs (SBo1-F/SBo1-R and SBo2-F/SBo1-R) were used in a diagnostic PCR containing BSA (0.5 µg µl^{-1}) and different amounts of template DNA (25–150 ng). Lane M is a 100 bp DNA ladder and C^{+} corresponds to a positive control (*B. oleae* genomic DNA).

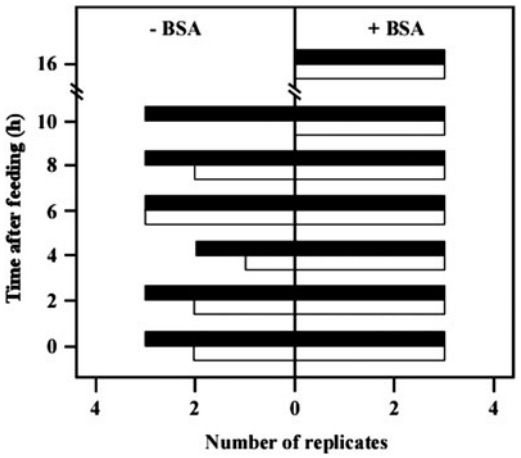

Fig. 3. BSA effect on diagnostic PCR consistency in detecting *Bactrocera oleae* DNA on feeding assays. The number of PCR replicates that have resulted in an amplified product are represented in columns. *Pterostichus globosus* adults were fed on *B. oleae* pupae, after which they were sacrificed at different digestion periods (0–16 h) and their intestinal apparatus used for genomic DNA extraction. Diagnostic PCR was performed using *B. oleae*-specific primers [SBo1-F/SBo1-R (white columns) and SBo2-F/SBo1-R (black columns)] and template DNA amount (100 ng for SBo1-F/SBo1-R primers and 50 ng for SBo2-F/SBo1-R primers), in PCR reactions that contained BSA (0.5 µg µl^{-1}) or not. Three replicates of *P. globosus* gut DNA were tested at each time period (0, 2, 4, 6, 8, 10, 16 h).

SBo1-R, but only 50 ng for the second primers pair SBo2-F/SBo1-R. These optimized concentrations were used in order to follow the digestion of *B. oleae* pupae by *P. globosus* using the designed diagnostic PCR. For testing the enhancer effect of BSA in amplification, all reactions were performed with and without BSA (0.5 µg µl^{-1}). The absence of BSA affected the amplification consistency, since *B. oleae* DNA was not regularly detected in every replicate (fig. 3). This was more evident when using the primers pair SBo2-F/SBo1-R, resulting in an increased irregular amplification in replicates. Also, the feeding period where *B. oleae* DNA ceased to be detected in *P. globosus* was different in the absence of BSA for each: 10 h for SBo1-F/SBo1-R and 16 h for SBo2-F/SBo1-R (fig. 3). On the contrary, PCR amplifications performed in the presence of BSA always resulted in consistent amplifications up to the end of the feeding experiment (16 h).

Discussion

This study developed a new PCR-based diagnostic assay for *B. oleae* using specific PCR primers for the *COI* DNA barcode region (Hebert *et al.*, 2003). This gene displays high variable regions among different animal species, from which species-specific primers have been designed for performing species identification (Gariepy *et al.*, 2007). The use of *COI*-specific primers in diagnostic PCR has been applied with success for the identification of *Bactrocera* spp., such as *B. minax*, *B. tsuneonis* and *B. correcta*, through the amplification of DNA extracted from either part or the entire body of an adult, larva or pupa (Jiang *et al.*, 2013, 2014). The PCR-based diagnostic assay proposed in this work, not only presented new designed primers for the successful diagnosis of *B. oleae*, even in an early stage such as the larva or pupa, but also optimizes the PCR assay for detecting *B. oleae* remains in the guts of potential soil predators, which will be very useful for describing *B. oleae*-related food webs in olive grove ecosystems. Primer's specificity was tested to detect target and non-target species, since generalist predators can feed on a wide variety of prey items in olive groves. This reduces the likelihood of getting false positives due to the cross-reactivity of primers (Admassu *et al.*, 2006).

The feeding activity of *P. globosus* adults against *B. oleae* pupae was successfully followed by applying the conceived diagnostic PCR assay on the guts of the predator. The choice of the method used for DNA extraction of this kind of sample revealed to be very important to obtain a high quality DNA for downstream analysis. Insects are very rich in polysaccharides, one of the contaminants that can inhibit the PCR reaction, especially when the goal is to check the intestinal content (Staudacher *et al.*, 2011). The use of BSA can improve PCR amplification in samples rich in contaminants acting as a PCR enhancer (Farell & Alexandre, 2012). When DNA was extracted from the intestinal content of *P. globosus*, BSA proved to increase the PCR yield in such low purity templates as the intestinal content of arthropods. BSA had an enhancer effect and should be used in these types of samples to get more accurate and robust results.

The designed *B. oleae*-specific primers and optimized PCR-based diagnostic assay could provide an effective, fast and inexpensive method for detecting *B. oleae* predators, which will help to manage a more sustainable olive production system by the future identification of potential predators of *B. oleae*. Further studies will be performed in order to study predation of *B. oleae* by soil arthropods. For olive growers, this will represent the production of high-quality olives by reducing the application of insecticides and improving ecosystem services provided by soil arthropods such as biological control of pests.

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