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 globo-sus* (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
millon 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 (Danne 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., 2016a), 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., 2016b).

**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* Robinovitch-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 MgCl2, 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 Miranda, using the MegAlign program (DNASTAR, Lasergene Version 7). Two forward primers, SBo1-F (5’CAG TAG TAC TAA CAG CCC TAC T 3’, 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).

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
PCR-based diagnostic assay for Bactrocera oleae DNA

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 using 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 ml⁻¹) or the absence of BSA. The DNA extracted from the guts of starved P. globosus specimens was used as 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/
SB01-R, but only 50 ng for the second primers pair SB02-F/ SB01-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 SB02-F/SB01-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 SB01-F/SB01-R and 16 h for SB02-F/SB01-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. t. teneonis 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 the possibility of potential soil 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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