



RESEARCH ARTICLE

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Specific and sensitive primers for the detection of predated olive fruit flies, *Bactrocera oleae* (Diptera: Tephritidae)

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Abstract

Bactrocera oleae, the olive fruit fly, is a major pest of olive (*Olea europaea* L.) trees worldwide. Its presence can cause important losses, with consequences for the economies of countries that produce and export table olives and olive oil. Efforts to control olive fruit fly populations have, however, been insufficient. Now more than ever, environmentally friendly alternatives need to be considered in potential control programs. Generalist predators could provide a way of managing this pest naturally. However, the identification of candidate predator species is essential if such a management system is to be introduced. The present paper describes a set of species-specific primers for detecting the presence of *B.oleae* DNA in the gut of predatory arthropods. All primers were tested for checking cross-reactive amplification of other fruit fly DNA and evaluated in heterospecific mixes of nucleic acids. All were found to be very sensitive for *B. oleae*. Subsequent feeding trials were conducted using one of the most abundant species of ground dwelling carabids in olive groves in south-eastern Madrid, Spain. These trials allowed determining that 253F-334R and 334F-253R primer pairs had the highest detection efficiency with an ID₅₀ of around 78h. These primers therefore provide a very useful tool for screening the gut contents of potential predators of *B. oleae*, and can thus reveal candidate species for the pest's biological control.

Additional keywords: *Olea europaea*; predation; Carabidae; species-specific and sensitive primers; feeding-trials; cytochrome oxidase subunit I.

Abbreviations used: BOLD (Barcode of Life Database); COI (Cytochrome Oxidase Subunit I)

Authors' contributions: Conceived and designed the study: BM, CC, EL, MDO and SP. Designed the primers: BM. Established the molecular assays: EL. Laboratory work: EL and SP. Analysed the data, compiled tables and figures, and wrote the paper: BM, CC and EL. Revised and improved the manuscript: MDO and SP.

Citation: Lantero, E.; Matallanas, B.; Ochando, M. D.; Pascual, S.; Callejas, C. (2017). Specific and sensitive primers for the detection of predated olive fruit flies, *Bactrocera oleae* (Diptera: Tephritidae). Spanish Journal of Agricultural Research, Volume 15, Issue 2, e1002. <https://doi.org/10.5424/sjar/2017152-9920>

Supplementary material (Table S1) accompanies the paper on SJAR's website.

Received: 09 May 2016. **Accepted:** 03 May 2017

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Funding: Ministerio de Economía y Competitividad of Spain (part of project RTA2013-00039-C03-03, *Biological control of Bactrocera oleae: Effect of landscape structure and importance of predation*) and European Regional Development Fund.

Competing interests: The authors have declared that no competing interests exist.

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Introduction

Olives (*Olea europaea* L.) have been a representative crop of the Mediterranean Basin since they were domesticated some 6000 years ago (Besnard *et al.*, 2013). Currently, the European Union produces 71.7% and 30.5% of the world's olive oil and table olives respectively (<http://www.internationaloliveoil.org/>), with Spain being the main producer and exporter (<http://www.prosodol.gr/>).

The olive fruit fly, *Bactrocera oleae* (Rossi, 1790), is the single most destructive olive pest. Its distribution

is limited to areas with a Mediterranean climate and to where olive trees (domesticated or wild) are found. The females lay their eggs under the olive epidermis, and when the larvae hatch, they feed on the pulp. The galleries they leave behind also provide an environment in which microorganisms can grow. These insults reduce crop yields and the quality of the olive oil that can be produced (Daane & Johnson, 2010).

Unfortunately, controlling *B. oleae* with chemical insecticides can have undesired effects on other members of the arthropod fauna, and even on human health and can result in insecticide resistance (Pereira-

Castro *et al.*, 2015). Alternative, more environmentally friendly control methods are available, such as mass trapping or spraying with processed kaolin, but these methods can also negatively affect the arthropod community (Pascual *et al.*, 2010).

Biological control may offer a solution to manage *B. oleae* populations. Attempts to use natural enemies such as parasitoids have been ineffective up to date (Daane & Johnson, 2010). The use of generalist predators, such as carabids that feed on the pupae of *B. oleae*, could be an alternative and promising approach to handle this pest (Dinis *et al.*, 2015). However, selecting the most appropriate predator is difficult in the field, not only because the food web is complex, but also due to the presence of researchers making observations could alter natural behaviour (Rosenheim *et al.*, 1999). Post-mortem visual examination of the gut contents of candidate predators might appear to provide another method of gathering information on the prey species chosen. Given the feeding habits of arthropods, and the size of any prey remains, such analyses are generally unsuccessful (Symondson, 2002). Currently it is possible to study the predator gut contents using PCR-based techniques. The reliable identification of a prey species requires that only prey DNA sequences are detected. The design of species-specific primers is therefore essential (O'Rourke *et al.*, 2012). Since digestion of the prey results in degradation of its DNA, the mitochondrial cytochrome oxidase I (COI) gene appears as a suitable target for amplification because there are several copies of the gene per organelle and also several organelles per cell. The variability of this gene makes it suitable for distinguishing even very closely related species. This feature makes this gene to be considered as the universal marker for animal species identification (Pentisaari *et al.*, 2016). In addition, the Barcoding of Life Database (BOLD), that contains a large library of eukaryote COI sequences, facilitates the design of species-specific primers (Ratnasingham & Hebert, 2007).

The aim of the present work was to develop a large primer set to detect small COI fragments belonging to *B. oleae* and to test them in specificity and sensitivity trials and also in the gut of carabid predators. This information will be essential to identify future candidate predatory species in the field for controlling this pest.

Material and methods

Materials

To assess the specificity of the primers designed the tephritid species *Bactrocera oleae*, *B. curcubitae*

(Coquillett 1899), *B. dorsalis* (Hendel 1912), *Ceratitidis capitata* (Wiedemann 1824) and *C. rosa* (Karsch 1887) were used along with *Drosophila melanogaster* (Meigen 1830). All specimens were stored at -80°C at the Department of Genetics at the Complutense University of Madrid.

Adult specimens of the carabids *Pterostichus globosus* (Fabricius 1792) and *Orthomus barbarus* (Dejean 1828) were employed. These two species are amongst the most abundant ones captured in olive groves in south-eastern Madrid (unpublished results). Other arthropods such as *Forficula auricularia* (Linnaeus 1758), *Alopecosa cuneata* (Clerck 1757) and *Tapinoma nigerrimum* (Nylander 1856), also from this olive-growing area, were used as well.

Primer design

Since the main objective of this study was to ensure the accurate and reliable detection by PCR of *B. oleae* DNA, species-specific primers (Table 1) were designed considering the most variable interspecific regions of the COI gene, while, at the same time, avoiding amplification of related tephritids species and also of potential predators. To that end, 55 *B. oleae* COI haplotypes from the Iberian Peninsula and 207 COI sequences from other tephritid and carabid species were downloaded from BOLD and GenBank databases (Table S1 [suppl]). The whole set of sequences was aligned with CLUSTAL W algorithm (Thompson *et al.*, 1997) as provided by BioEdit v.7.0.9.0 (Hall, 1999). Visual inspection of the alignment revealed five regions showing suitable interspecific variation for developing species-specific primers.

Table 1. PCR primers designed to amplify seven *B. oleae* COI gene fragments. Primer names refer to their position in the COI gene according to the complete sequence of *B. oleae* mitogenome, GU108472.

Primer name	Primer sequence 5'-3'
166F	GTAAGTCTCACGCTTTCGTA
253F	TTAGGGGCACCAGATATG
334F	CAGCAGCATAGTGGAAAACG
440F	CACTCCATTTAGCAGGTATC
334R	CGTTTTCCACTATGCTGCTG
440R	GATACCTGCTAAATGGAGTG
635R	GTCGAAAAGGAAGTATTTCAGA

DNA extraction and PCR analysis

To check the specificity of designed primers, genomic DNA from *B. oleae*, *B. dorsalis*, *B. curcubitae*, *C. capitata*, *C. rosa* and *D. melanogaster* was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) with some modifications. Genomic DNA from the arthropods was extracted from legs and antennae, to ensure that it was exclusively from the specimen and not from preys remaining in their guts. In all cases, DNA integrity was checked in 0.8% agarose gels stained with ethidium bromide (0.5 mg/mL).

To assess quality of extracted DNA, a first set of PCR reactions was performed using the universal COI primers LCO1490 and HCO2198 (Folmer *et al.*, 1994). Amplifications were undertaken in a final volume of 10 μ L with 1.5 mM of each primer, 0.8 μ M of MgCl₂, 5 μ L of Taq PCR Master Mix (Qiagen), and 10 ng of template DNA. The PCR program included an initial denaturation step at 94°C for 5 min, followed by 35 cycles at 94°C for 45 s, 60°C for 1 min and 72°C for 1 min, and a final extension step at 72°C for 10 min. PCR products were visualized in 2% agarose gels. The aim was to discard, in the subsequent analyses, any false negatives arising from poor quality of DNA.

Next, the specificity of the primers in amplifying only *B. oleae* DNA was verified by PCR reactions using 10 ng of DNA from *B. oleae*, *B. dorsalis*, *B. curcubitae*, *C. capitata*, *C. rosa*, and *D. melanogaster* as well as from *O. barbarus*, *P. globosus*, *F. auricularia*, *A. cuneata* and *T. nigerrimum*. PCR reactions were performed in a final volume of 12.5 μ L with 6.25 μ L Taq PCR Master Mix (Qiagen), 0.8 mM of each designed primer specific for DNA of *B. oleae* and 1.5 μ M of MgCl₂. The PCR program included an initial denaturation of 5 min at 94°C, followed by 10 cycles of 94°C for 45s, 63°C for 1 min and 72°C for 1 min, and then 15 cycles of 94°C for 45 s, 56°C for 1 min and 72°C for 1 min. All PCR products were visualized in 2% agarose gels.

Finally, the sensitivity and the limit of detection of olive fly DNA with the different primer pairs were studied. To that end, *B. oleae* DNA was mixed with *O. barbarus* DNA, assuming that in the predator gut the ratio of prey: predator DNA is small. Samples with olive fruit fly: carabid DNA ratios of 1:1000 (5 pg/ μ L DNA of *B. oleae* to 5 ng/ μ L predator DNA), 1:2000 (2.5 pg/ μ L to 5 ng/ μ L), 1:4000 (1.25 pg/ μ L to 5 ng/ μ L) and 1:5000 (1 pg/ μ L to 5 ng/ μ L) were prepared. PCR reactions were performed as for the specificity assay above. Control samples involved DNA from *O. barbarus* and *B. oleae* (5 ng/ μ L), plus a template negative control.

Feeding trials

Because the carabid *Orthomus barbarus* is an abundant species in the south-east of Madrid olive groves, it was selected for the feeding assays. Fifty six live adult specimens of *O. barbarus* were separated in individual containers and starved for a two-week period at 21°C and 16:8 h (L:D) (water was supplied every day on soaked cotton). The starvation period allowed for the digestion of any other prey consumed before the capture and also adjusted the specimens to a similar hunger level. Pupae of *B. oleae* were chosen for the feeding experiments because this is the overwintering stage of the insect, which is exposed to predation in soil from autumn to spring. Six carabids were selected as negative controls and frozen at -20°C before the trials. The 50 remaining carabids were provided with a single pupa of *B. oleae* and allowed to feed for a maximum of 4 hours. The time for the analysis was set to zero from the moment that preys were completely consumed. Specimens were then frozen at 0, 2, 4, 8, 24, 36, 48 and 72 hours post feeding in order to analyse detection success across time. The predator guts were removed by dissection and DNA was isolated and checked with universal COI primers LCO1490 and HCO2198 as it was described above.

Molecular analysis of feeding trials

The seven specific primer pairs were used for molecular detection of predation at 0, 2, 4, 8, 24, 36, 48 and 72 hours post feeding. The two primer pairs with the highest prey detection success were selected for assessing ID50 (time after the ingestion at which the 50% of the samples tested were still positive) along detection periods.

Calculated ID50 were subjected to a regression analysis using the statistical package SPSS 13.0 (SPSS Inc., Chicago, IL, USA). To test for replicability of prey detection across the post feeding time, PCRs were conducted by independent triplicates using the two primer pairs selected.

Results

All the DNAs extracted from tephritids, carabids and other arthropods present at the olive agroecosystem were firstly used for PCR controls with the universal COI primers. Amplified fragments of around 700 bp confirmed the presence of suitable DNA in all the specimens for the subsequent analyses to be performed in this study.

The different combinations of the primers developed to specifically detect DNA of the olive fly allowed the amplification of seven fragments. Amplicon sizes varied from near 100 bp for the combination 253F-334R, to 300 bp for 334F-635R (Fig. 1). All the primer pair combinations were highly specific in the cross-species amplification tests. They only amplified DNA from *B. oleae* and not from other fruit fly species or potential predators. Fig. 2 shows an illustrative example with the primer combination 334F-440R.

Suitability and sensitivity of the primers were assessed in mixes of heterospecific DNA. Several dilutions of *B. oleae* in *O. barbarus* DNA were used for this purpose. All the primer combinations amplified the *B. oleae* DNA, even when it was present at a very low concentration compared to the predator DNA (1 pg/ μ L of *B. oleae* DNA per 5000 pg/ μ L of *O. barbarus* DNA) (Fig. 3).

Feeding trials revealed that pairs 253F-334R and 334F-440R exhibited the highest percentage of detection success 48 hours after predation and were able to detect *B. oleae* DNA even 72 hours after feeding (Table 2).

These two primer combinations were then tested in triplicate on independent PCR reactions (Table 3) to determine the ID50. The *B. oleae* DNA was amplified at 36 hours post feeding. The percentage of detection was over the 95% (98.98% for primer pair 253F-334R and 96.87% for 334F-440R). After 48 hours, the detection efficiency decreased to 70.3% in the three replicates and for both primer pairs and it was further reduced at 72 hours post feeding, as expected (41.6% and 50% for primer pairs 253F-334R and 334F-440R, respectively). The regression equations for the percentage of positives per replicate against time were $Y_{253F-334R} = -0.719X + 105.96$ ($R^2=0.673$, $p=0.001$) and $Y_{334F-440R} = -0.693X + 104.255$ ($R^2=0.556$, $p=0.001$).

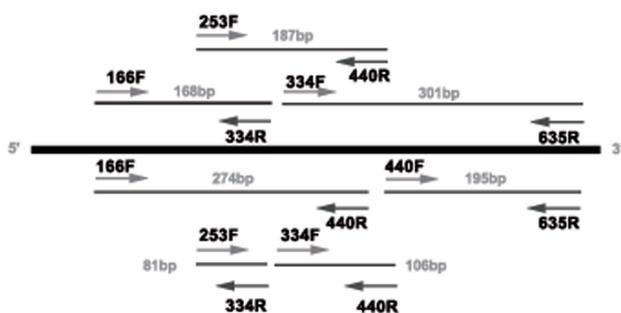


Figure 1. The COI region selected for the present study, showing primer combinations and PCR product sizes. Grey arrows indicate the designed primers (names in bold); light grey numbers indicate amplicon size in base pairs.

Calculated ID50 were around 78 hours for both primer pairs.

Discussion

Understanding the soil food web of olive orchards is essential to detect the potential of different arthropods as natural olive fruit fly control agents. The studies describing the insect fauna in the olive agroecosystem have inferred potential predators of *B. oleae* mainly based on trophic guild classification and relative abundance (Dinis *et al.*, 2015; Gkissakis *et al.*, 2016), and only recently a molecular method has been reported (Rejili *et al.*, 2016).

In our study, we present a set of primers to detect the DNA from the olive fly in the gut of its putative predators. All the primers designed successfully amplified the *B. oleae* mitochondrial COI gene fragments, with amplicon sizes ranging from 81 bp to 302 bp. Given the DNA degradation during digestive processes, amplifying small fragments of multi-copy genes improves detection rates of prey DNA in predators' gut (Brown *et al.*, 2015; MacDonald *et al.*, 2014). The amplicons obtained are within the size range for post-digestion amplification products reported in previous surveys with other primers and other pest species: 78-242 bp (Harper *et al.*, 2005), 160-281 bp (Harwood *et al.*, 2007), 101-274 bp (King *et al.*, 2010), 130-330 bp (Monzó *et al.*, 2010), and 150-345 bp (Moreno-Ripoll *et al.*, 2012). The absence of cross reactivity shows that the primer pairs used are highly specific (Fig. 2).

The primers were also very sensitive in heterospecific mixes of DNA. Even though some tests where predator DNA was in great excess, all primer pair combinations returned a detection limit of 1 pg/ μ L of prey DNA (Fig. 3). This outcome surpasses that obtained for the same



Figure 2. Specificity of *B. oleae* DNA detection with the primer combination 334F-440R. M: GeneRuler 100 bp DNA Ladder (Thermo Scientific). The results show the amplicons produced using 10 ng of DNA from: 1, *B. oleae*; 2, *B. dorsalis*; 3, *B. curcubita*; 4, *C. capitata*; 5, *C. rosa*; 7, *D. melanogaster*; 8, *T. nigerrimum*; 9, *A. cuneata*; 10, *F. auricularia*; 11, *O. barbarus*; 12, *P. globosus*; 13, PCR negative control.

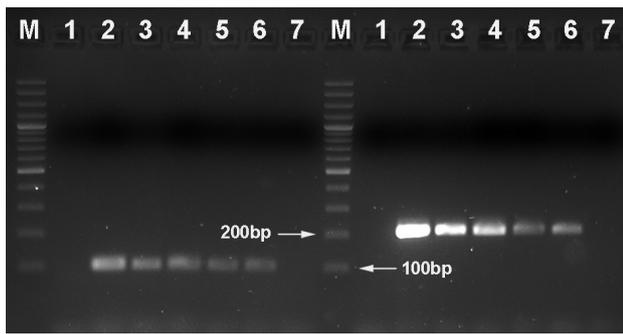


Figure 3. Sensitivity of *B. oleae* detection with primers Bo253F/Bo334R (left) and Bo440F/Bo635R (right). M: GeneRuler 100 bp DNA Ladder (Thermo Scientific). Lanes 1-6 DNA from: 1, *O. barbarus* (10 ng); 2, *B. oleae* (10 ng); 3-6, *B. oleae* - *O. barbarus* DNA dilutions: 3, 1:1000; 4, 1:2000; 5, 1:4000; 6, 1:5000; Lane 7, negative control.

gene in a similar work on sharpshooters (6 pg/ μ L, León *et al.*, 2006).

The specificity and sensitivity tests revealed that all primer combinations were appropriate for molecular detection of predation under laboratory conditions (O'Rorke *et al.*, 2012). However, it was also necessary to test their reliability under real conditions of digestion. Therefore, feeding trials of a single pupa of *B. oleae* were carried using *O. barbarus*, one of the most abundant carabid species in the olive growing area of south-eastern Madrid, Spain. As these analyses were performed with the carabid species *O. barbarus*, post feeding intervals were chosen based on previous works that employed carabids and spanned the 0-72 hours periods (Harper

et al., 2005; King *et al.*, 2010; Sint *et al.*, 2011; Monzó *et al.*, 2011).

After a long time post feeding, the low concentration of prey DNA template compromises the success of its detection by PCR. Likewise, half-time of detection of prey DNA varies considerably among predators due to their feeding habits or the number of preys ingested (King *et al.*, 2008; Gagnon *et al.*, 2011). For ID50 determination, three independent post feeding replicates were performed in order to discard false negatives related to handling errors, small quantities or bad quality of prey DNA. The two primer combinations 334F-440R and 253F-334R were used given their detection efficiency.

Robust ID50 was obtained for both primer combinations, specifically 77.82 hours for 253F-334R and 78.29 hours for 334F-440R. These values were similar to those obtained in other works using a different kind of predators such as spiders: 78 h (Monzó *et al.*, 2010) or 79.2 h (Sint *et al.*, 2011). Nevertheless, they were higher than the estimated value for other species of carabids like *Calathus fuscipes*, 23.8-39 h (Boreau de Roince *et al.*, 2012); *Oreonebria castanea*, 30 h (Sint *et al.*, 2011) or *Pseudophonus rufipes*, 32.33 h (Monzó *et al.*, 2011). Our amplicon sizes were small (106 bp and 81 bp, respectively), which may explain their longer survival in the gut, consistent with previous studies (King *et al.*, 2008).

A work closely related to ours has been published recently (Rejili *et al.*, 2016). Three specific primers of *B. oleae* to amplify 108 and 214 bp COI fragments were developed, and only one of them, Sbo1R, overlaps with one of our primers, 635R. However, given the success of the pairs 334F-440R and 253F-334R, 635R

Table 2. Detection success at different post-feeding times using designed primer pairs; bp, amplicon size (on base pairs); T0-T72, percentage of detection after T0-T72 hours post-feeding; Mean, global success of detection. Shaded area, primer combinations selected for further analyses.

Primer pair	bp	T0	T2	T4	T8	T24	T36	T48	T72	Mean (%)
253F-334R	81	100	100	100	100	83	100	87	37	88.37
334F-440R	106	100	100	100	100	100	100	62	25	85.87
166F-334R	168	100	100	100	100	100	100	62	0	73.50
253F-440R	187	100	100	100	83	83	66	22	0	69.25
440F-635R	195	100	100	100	100	66	66	22	0	70.62
166F-440R	274	100	100	83	100	66	66	22	0	67.12
334F-635R	301	100	100	100	100	100	66	22	0	73.50

Table 3. Detection efficiency of *B. oleae* DNA with the primer pairs selected. T0-T72, percentage of detection after 0-72 hours post feeding; numbers in parenthesis, number of individuals tested.

Primer pair		T0(6)	T2(6)	T4(6)	T8(6)	T24(6)	T36(3)	T48(9)	T72(8)
253F-334R	Replicate1	100	100	100	100	83	100	66.6	12.5
	Replicate2	100	100	100	100	100	100	66.6	50
	Replicate3	100	100	100	100	100	100	77.7	62.5
	Mean	100	100	100	100	94.4	100	70.3	41.6
334F-440R	Replicate1	100	100	100	100	83.3	100	66.6	75
	Replicate2	100	100	100	100	83.3	100	55.5	0
	Replicate3	100	100	100	100	83.3	100	90	75
	Mean	100	100	100	100	83.3	100	70.3	50

was not further employed after the specificity and sensitivity assays. Diagnostic PCR assays on the guts of the carabid *P. globosus* at 0, 2, 4, 6, 8, 10 and 16 hours after feeding two *B. oleae* pupae in Rejili *et al.* (2016), determined an optimal DNA level of 100 ng for primers SBo1F-SBo1R and 50ng for the combination SBo2F-SBo1R. In contrast, the sensitivity of our whole primer set allowed amplifying at very low template concentrations (1pg/ μ L). In fact, our study goes a step further regarding the limits of detection and ID50 values. This notwithstanding, all reports about predation of *B. oleae* by soil arthropods are very welcome for creating an effective synergy towards reducing insecticide and pesticide treatments.

The aim of integrated pest management programmes is to find new, environmentally friendly approaches to control pest populations. In this context, natural enemies can be a good complement to the use of insecticides. Despite the big efforts made studying the fauna at the olive agroecosystem until now, it is only possible to determine if one species is a potential predator or not, according to its trophic guild. The proposed PCR-based method can overcome this issue. The two primer pairs selected are highly specific and sensitive, ensuring the reliable detection of *B. oleae* prey remains in predator guts. Thus, possible candidacies of soil arthropods as biological agents for control of the olive fruit fly can now be reliably assessed and confirmed.

Acknowledgments

The authors thank Sonia A.P. Santos and Sergio Pérez-González for the taxonomic identification of the carabids and Manuel González, Marta Ortega

and Ismael Sánchez for their efforts sampling in the field.

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