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A PCR-based method for estimating parasitism rates in the olive fly parasitoids *Psyttalia concolor* and *P. lounsburyi* (Hymenoptera: Braconidae)

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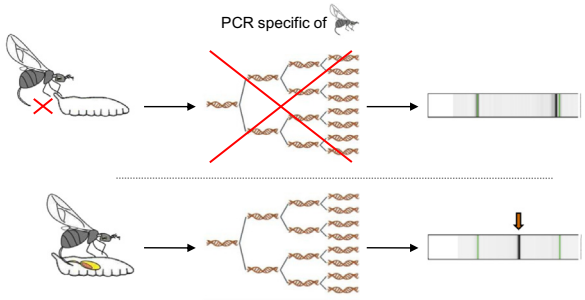
HIGHLIGHTS

- Biological control of *Bactrocera oleae* needs a reliable estimation of parasitoid efficiency.
- We present a specific PCR tool to compare parasitism rates of *Psyttalia* species.
- The PCR method allows early and sensitive detection of *Psyttalia* eggs.
- The PCR method is more reliable than traditional ones (rearing and dissection).

GRAPHICAL ABSTRACT



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ABSTRACT

Several parasitoids of the genus *Psyttalia* have been repeatedly introduced as biological control agents against the principal pest of olive, the fly *Bactrocera oleae*. However, few of the parasitoids released have become established and proved effective against *B. oleae*. It may however still be possible to find effective biological control agents adapted to local environmental conditions among the highly diverse *Psyttalia* species and populations infesting *B. oleae* worldwide. For this purpose, we have developed a rapid, sensitive molecular method based on the polymerase chain reaction (PCR) for estimating and comparing the parasitism success of *Psyttalia* parasitoids through the detection of eggs and larvae within the host. This method was tested and shown to be appropriate for two *Psyttalia* species (*Psyttalia concolor* and *Psyttalia lounsburyi*). The possible detection of DNA was also demonstrated for several populations of these species and for other *Psyttalia* species, namely *Psyttalia humilis* and *Psyttalia ponerophaga*. For *P. concolor* and *P. lounsburyi*, a strong correlation was observed between the parasitism rates estimated by PCR, host larva dissection and counts of emerging parasitoids. No significant difference was found between the rates of parasitism estimated by host larva dissection and PCR, whereas the rates of parasitism estimated by PCR were significantly higher than those estimated from emergence, suggesting occurrence of mortality during the parasitoid development. This PCR method is thus highly reliable and provides an objective criterion for estimating the efficacy of biological control agent candidates from diverse taxa and populations of *Psyttalia*.

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1. Introduction

The fruit fly pest *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) is considered one of the most damaging pests of olive, causing estimated losses of 5% worldwide and of up to 98% in some parts of the Mediterranean Basin (Bueno and Jones, 2002). It can rapidly colonize new areas (Zygouridis et al., 2009), and thus occurs in most places in which cultivated or wild olive trees are present. It damages olives in various ways, from oviposition stings on the surface rendering the fruit unsuitable for table consumption, to a loss of production due to fruits rotting and dropping and a decrease in the quality of the oil, which is acidified by the presence of larvae. This damage results in economic losses of approximately US$ 800 million/year (Bueno and Jones, 2002; Nardi et al., 2005). The main methods for controlling *B. oleae* are mass trapping and insecticide treatments (Daane and Johnson, 2010). However, increasing insecticide resistance in *B. oleae* (Kakani et al., 2010; Vontas et al., 2001) and the need to decrease pesticide use has led to the repeated testing of biological control, with various degrees of success (Daane and Johnson, 2010; for review, see Daane et al., 2011). The main biological control agents used against *B. oleae* in the last 60 years have been the solitary endoparasitoids of the tephritids *Psyttalia concolor*, *Psyttalia humilis* and *Psyttalia lounsburyi* (Braconidae: Opiinae) (Daane et al., 2011), all of which belong to the *P. concolor* species complex (Kimani-Njogu et al., 2001; Rugman-Jones et al., 2009). This complex comprises several closely related taxa, most of which are difficult to distinguish morphologically, and some can hybridize (Billah et al., 2007). A strong genetic differentiation has been observed between geographically distant populations of part of these taxa (Cheyppe-Buchmann et al., 2011; Karam et al., 2008; Rugman-Jones et al., 2009), and local adaptation of some taxa to the host or to environmental conditions may occur (Rugman-Jones et al., 2009). Thanks to this diversity, it may still be possible to find appropriate biological control agents combining high levels of successful parasitism with adaptation to local environmental conditions in the target area. However, this will require accurate estimates of parasitism rate (PR) and parasitism success (i.e. the rate of success for parasitoid development within the initially parasitized host) for the candidate parasitoid species or populations.

Molecular-based approaches have largely contributed to improve our knowledge of insect pests and biological control auxiliaries. For instance, a large part of the diversity of tephritid pests and of their parasitoids has been characterized (Jenkins et al., 2012). DNA-barcoding and microsatellite genotyping have notably provided insights on the taxonomy and population structure in the *Psyttalia* genus (Cheyppe-Buchmann et al., 2011; Rugman-Jones et al., 2009). In contrast, the evaluation of PR, an important parameter for biological control, still encounters technical limitations. One easy-to-perform method, referred to hereafter as the "rearing method", is based on the counting of parasitoid adults emerging from hosts previously exposed to parasitism. However, this method provides only an apparent PR because parasitism may end in the death of the parasitoid or of both the host and the parasitoid, making it difficult, if not impossible, to determine whether the host was initially parasitized (Gariepy et al., 2005, 2007; Gariepy, 2007; Greenstone, 2003, 2006; Jones et al., 2005; Ratcliffe et al., 2002). Another method for estimating PR gets around this problem by estimating the proportion of hosts containing parasitoid eggs through the dissection of host larvae (the "dissection method"). This approach is time-consuming and tedious, and it may lead to more or less severe underestimations of PR depending on whether eggs are difficult to find in the host tissue (Agustí et al., 2005). Examples of PCR-based detection of parasitoid species, whether inside the host or inside the fruit, have been reported (Jenkins et al., 2012), and PR estimation techniques based on such detection of parasitoid eggs within the host are among the best alternatives (Gariepy et al., 2007; Greenstone, 2006). They are increasingly used, as they are less time-consuming and often more reliable than the "dissection" and "rearing" methods. They are also generally more sensitive, cheaper, and they require less expertise compared to other molecular methods aimed at detecting parasitoid specific proteins, e.g. enzyme electrophoresis and serological assays using monoclonal antibodies (Stuart and Greenstone, 1997; for a complete overview, see Greenstone, 2006). We present here a PCR-based method for the detection of eggs of *Psyttalia* spp. within host larvae, and demonstrate that this method accurately estimates PR from the first day of oviposition, for both *P. concolor* and *P. lounsburyi*. Successful PCR amplification suggests that it may also be appropriate for *P. humilis* and other related taxa. This method will then be useful for estimating the PR of some of the candidate biological control agents of the genus *Psyttalia*, providing an objective criterion of choice for the various taxa and populations. Moreover, it could also be used for quality control on mass-reared biological control agent populations.

2. Materials and methods

2.1. Biological material

The *P. concolor* and *P. lounsburyi* populations used to estimate PRs originate from Sicily, Italy (collection in 2010) and Stellenbosch, South Africa (collection in 2005; Cheyppe-Buchmann et al., 2011), respectively. Since collection, they have been reared in the laboratory as described in Thaon et al. (2009), under controlled conditions (22 ± 1 °C; relative humidity 55%; photoperiod 16L:8D), on the alternative host *Ceratitis capitata*. Briefly, a nutrient media (104 g of brewer's yeast, 112 g of carrot powder, 180 g of potato flakes, 1.8 g of Nipagin (Specialites Chimiques Distribution, Gellainville, France), 1.8 g of sodium Benzoate, 40 mL of hydrochloric acid at 16,5‰ and 900 mL of water) containing one week-old *C. capitata* larvae is used to coat a ping pong ball, which is then wrapped in stretched Parafilm™ (Pechiney Plastic Packaging, Chicago, US) and suspended in a cage containing 400 *Psyttalia* parasitoids to allow parasitism for approximately seven hours. Parasitoid-exposed *C. capitata* larvae are then transferred to a rearing box containing the same nutrient media until parasitoid adults emerge. A more detailed version of the rearing protocol can be found in Benvenuto et al. (2012).

2.2. Design of PCR primers

For PCR amplification purposes, sequences from the mitochondrial cytochrome oxidase subunit I gene (COI – LCO/HCO) and from the ribosomal region between the 5.8S and 28S (including the internal transcribed spacer 2 [ITS2]) were tested. Primers were designed so as to ensure amplification of the target sequence from *Psyttalia* species (*P. concolor* and *P. lounsburyi*), while avoiding amplification from the host species (*B. oleae* and *C. capitata*). The available sequences for the host and parasitoid species (six ITS2 and two COI sequences for the hosts; eight ITS2 and 34 COI sequences for the parasitoids) were recovered from GenBank (ITS2 sequences: EU761063 and EU761064, EU761048 to EU761052, AF276515, AF276516, AY209010, AF332590, AF187102, AF189691, AF307848 and DQ490237; COI sequences: EU761020 to EU761025, GU725008 to GU725031, EU761036 to EU761038, DQ116368 and GQ505009) and aligned with the Clustal W program (Larkin et al., 2007). Primers were designed with Primer 3