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Early detection and identification of larval parasitoids in *Lobesia botrana* using PCR-RFLP method

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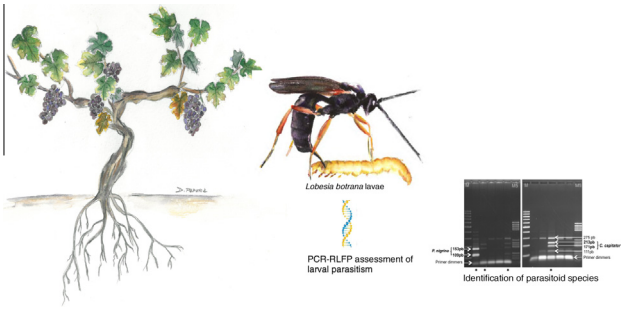
HIGHLIGHTS

- Larval parasitoids like the ichneumonid *Campoplex capitator* control *Lobesia botrana*, a major pest in vineyards.
- PCR-RFLP analysis method developed here allows early detection and discrimination between four larval parasitoid species.
- Such a method will help adapting the control strategy of this pest.

GRAPHICAL ABSTRACT



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ABSTRACT

Several larval parasitoid species are natural enemies of the tortricid moths of European vineyards, including the most damaging of these pests, *Lobesia botrana*. Over the last few years, DNA-based methods have been used for more rapid and accurate detection and identification of parasitoids. In this study, we developed a PCR-RFLP analysis method targeting a mitochondrial cytochrome oxidase I gene fragment after digestion with the restriction enzyme *ApoI*, for discrimination between four parasitoid species of *Lobesia botrana*: *Campoplex capitator*, *Exochus tibialis*, *Elachertus* spp. (Hymenoptera, Ichneumonidae) and *Phytomyptera nigrina* (Diptera, Tachinidae). We assessed the accuracy of this method using populations of *L. botrana* sampled from eight vineyards located in South-West of France. On a total of 547 *L. botrana* larvae collected, 252 were analyzed for parasitism using our molecular method whereas the remaining 295 were reared to assess parasitism rates based on emergence. Our PCR-RLFP method showed a mean parasitism rate of 25%, with values ranging from 3% to 50% across vineyards. The levels of parasitism estimated by this method were about three times those estimated after emergence and identification (7.3%). This difference suggests that mortality may occur during parasitoid development, possibly due to encapsulation. Our method revealed that the two dominating parasitoid species were *Campoplex capitator* (90%) and *Phytomyptera nigrina* (9%), whereas the emergence of parasitoids found only *C. capitator* after taxonomical identification. This study revealed that the PCR-RFLP analysis is an appropriate and reliable tool for estimating the biological control potential of a diverse community of parasitoids on the main tortricid moth of grapevine.

1. Introduction

Assessing the levels of parasitism or predation of pest populations by their natural enemies in agroecosystems, is a challenging

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and complicated task. It requires the accurate detection and identification of each parasitoid or predator species and detailed evaluations of control efficiency (Agusti et al., 2005). The classical method, which is the most employed for quantifying parasitism rates, is based on the identification and quantification of parasitoid adults that emerged from hosts collected in the field. However, the primary technical limitation of this approach relates to parasitoid identification, which is time-consuming and requires accurate skills in systematics. Moreover, host mortality during transport to the laboratory and during the rearing period may lead to an underestimation of biological control potential (Agusti et al., 2005; Traugott et al., 2006). Furthermore, traditional parasitoid identification methods are not compatible with early detection within the hosts, making it impossible to adapt strategies for controlling pests based on first in-field observations of the presence of larvae (Jourdie et al., 2008; Hrcek et al., 2011). Molecular methods are increasingly used by entomologists to detect and identify parasitoids in their hosts, by using primers for a target sequence within a specific gene. Mitochondrial COI gene, which has been shown to display extensive interspecific variation in arthropods, is usually targeted for species-level identifications. This technique requires the availability of a large database of orthologous sequences for comparison. The amount of COI gene sequence information available for hymenopteran and dipteran parasitoids and their hosts has increased in recent years, and now extends to species of agronomic and commercial significance. For instance, primers pairs have been designed to amplify a particular region of COI that was used as a Barcode to delineate the host–parasitoid links between 37 host species within a wide range of lepidopteran families and 46 species of hymenopteran and dipteran parasitoid (Hrcek et al., 2011). Several authors have used this approach to assess levels of parasitism by several parasitoid species, including braconids and ichneumonids (Mowry and Barbour, 2004; Ashfaq et al., 2004; Jourdie et al., 2008; Mathé-Hubert et al., 2013). Therefore, information obtained by traditional methods (i.e. based on rearing of field collected hosts until emergence and identification of parasitoid adults) could now be supplemented and improved by molecular analysis.

The European grapevine moth, *Lobesia botrana* (Denis and Schiffermüller) (Lepidoptera: Tortricidae) is a major grapevine pest with a broad geographic distribution following a Palaearctic pattern. For more than a century, *L. botrana*, which is of Mediterranean origin (Stoeva, 1982; Maher and Thiéry, 2006), is the current main pest of European vineyards (Delbac and Thiéry, 2016). Ten years ago, its geographic distribution extended to South American vineyards, and it reached California in 2009 (Gilligan et al., 2011). Current methods for controlling grape moths include conventional ovicides or larvicides, mating disruption and Bt toxin (Harari et al., 2007; Louis and Schirra, 2001; Thiéry et al., 2011) but biological control based on parasitoids, predators, nematodes or entomophagous fungi constitutes a promising avenue of research (Thiéry et al., 2011). Parasitism efficiency of trichogramma wasps against *L. botrana* or *Eupoecillia ambiguella* have been measured either in controlled conditions (Pizzol et al., 2012; Moreau et al., 2009) or by inundative releases (Hommay et al., 2002; El-Wakeil et al., 2010) but all show rather diverse results. Biological control is thus currently limited to conserving natural enemies in most vineyards. For some pests, larval parasitoids have yielded promising results (up to 80% parasitism), particularly for the first pest generation (Thiéry et al., 2001; Xuéreb and Thiéry, 2006; Moreau et al., 2010; Marchal, 1912).

Parasitoids of grape tortricid moths have long been known to occur in vineyards (Marchal, 1912), and most vineyards display a high diversity of these species, including egg parasitoids (Barnay et al., 2001), such as *Trichogramma*, and larval parasitoids, such as the ichneumonid *Campoplex capitator*. *C. capitator* is currently the main larval parasitoid in most European vineyards (Thiéry et al., 2011). This solitary endoparasitoid overwinters in its host (pupae) and has strong parasitic activity against the second and third larval stages of the first generation of *L. botrana* (Xuéreb and Thiéry, 2006; Moreau et al., 2010). Other larval parasitoid species associated with *L. botrana* occur in vineyards, but often at a lower frequency (Thiéry et al., 2001; Moreau et al., 2010). These species include the ichneumonid *Exochus tibialis* and the tachinid fly *Phytomyptera nigrina*, which was first detected in France in 2005 (Thiéry et al., 2006) and has a distribution area in southern wine-producing countries like Spain, Italy and Switzerland (Coscolla, 1997; Tschorsnig, 1997).

In this study, we developed and assessed the first PCR-RLFP-based diagnostic tool for detecting larval parasitism of the main pest of grapes, *L. botrana*, and identifying the parasitoid species involved. This molecular diagnostic tool targets four species of parasitoids of the larvae of *L. botrana*. We assessed the utility of this method to identified parasitoids in field-collected *L. botrana* larvae and compared to the parasitism levels obtained with those provided by traditional identification method. The applied goal of this study was to provide a simple and efficient tool for quantifying levels of natural pest control services, which can enable vine growers to decrease pesticide use against grapevine moths.

2. Materials and methods

2.1. Parasitoid collection

The parasitoids used for the development of specific PCR-RFLP profiles came from a large collection sampled between 2009 and 2010 from vineyards located in South-East and South-West France. Nineteen parasitoids emerging from *L. botrana* larvae ($N = 258$) were used in this study. These 19 parasitoids belonged to the four most common species of *L. botrana* larval parasitoids: *Campoplex capitator* ($N = 8$) and *Exochus tibialis* ($N = 2$; Hymenoptera, Ichneumonidae), *Elachertus* spp. ($N = 3$; Hymenoptera, Eulophinae) and *Phytomyptera nigrina* ($N = 6$; Diptera, Tachnidae). These species were identified on the basis of their morphology, according to the taxonomic identification key of (Villemant and Delvarre, 2011) and were stored in 95% ethanol, at −80 °C, until DNA extraction. Five *L. botrana* larvae (third stage) from our laboratory-maintained collection (Thiéry and Moreau, 2005) free from parasitoids, were used as negative controls in all PCR amplifications and PCR-RLFP tests.

We assessed the sensitivity of molecular parasitoid detection within hosts by PCR-RLFP, on naturally occurring *L. botrana* larvae ($N = 252$) collected in June 2013 from eight vineyards in South-West France (Table 2). For each vineyard, we selected one grape cluster per plant on 100 randomly chosen plants. Larval populations were checked before pupation, and when the pupae formed, they were removed from the flower buds and either i) immediately transferred to 95% ethanol, in which they were stored, at −80 °C for subsequent molecular analysis using PCR-RLFP (N = 252), or ii) individually reared in controlled laboratory conditions (22 °C and 60% relative humidity) until parasitoid emergence ($N = 295$) or

Table 1
Summary of the generalized linear mixed model with a binomial error distribution used to test for the effects of identification methods (morphological criteria or molecular approach) on overall parasitism levels. Site was included as a random factor, to account for spatial dependence in the data. The reference level is indicated in brackets.

	Estimate	SD	*z*-Value	*P*
Method (morphological criteria/ molecular approach)	−1.36	0.29	−4.65	<0.001