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
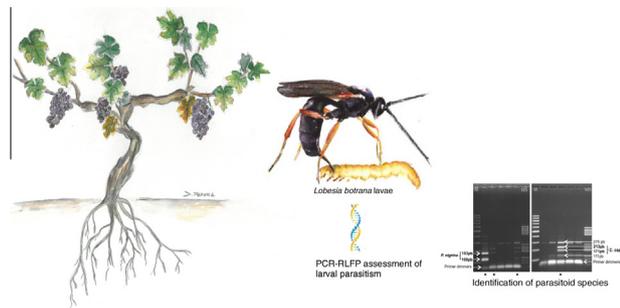
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ABSTRACT

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.

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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

pupation for non infected larvae. The emerging parasitoids were then individually stored in 95% ethanol, at −80 °C, until their identification on the basis of morphological criteria.

2.2. DNA extraction

Total DNA was individually extracted from parasitoid adults already identified on the basis of morphological criteria: *C. capitator* (N = 8), *P. nigrina* (N = 6), *E. tibialis* (N = 2), *Elechertus* spp. (N = 3) and from the five parasitoid-free *L. botrana* larvae, with the DNeasy Blood & Tissue kit (Qiagen Inc, Chatsworth, CA). The extracted DNA was then eluted in 100 μl of 10 mM Tris-HCl-1 mM EDTA, pH 7.8 and stored at −20 °C.

2.3. MtDNA COI gene amplification and sequence analysis

The universal primers C1-J-1859 F (5′-GGAACTGGATGAACAGTA TATCCACC-3′) and C1-N-2191 R (5′-CCAGGTAAAATTAAAATA TAAACTTC-3′) (Simon et al., 1994) were used to amplify a portion of the mtDNA COI gene of the four parasitoid species (*C. capitator*, *E. tibialis*, *Elachertus* spp. and *P. nigrina*) and that of their host, *L. botrana*. PCR was carried out in a final volume of 25 μl containing 5 ng of genomic DNA, 4 mM MgCl$_2$, 150 μM of each dNTP, 6 pmol of each primer, 1× BSA (10 μM) and 0.5 U *Taq* Silverstar DNA polymerase (Eurogentec) in reaction buffer. Reactions were performed with the following program: an initial denaturation step of 3 min at 94 °C, followed by 40 cycles of denaturation for 20 s at 94 °C, annealing for 20 s at 48 °C, elongation for 35 s at 72 °C, and a final elongation step for 5 min at 72 °C. The amplified mtDNA COI fragments for the 24 individuals were then sequenced, in both directions, on a Beckman Coulter Ceq8000 automated sequencer, using the manufacturer's fragment detection chemistry, and the data were analyzed with DnaSP version 3 software (Librado and Rozas, 2009). A multiple alignment was obtained for each consensus sequence, with ClustalW software used with the default settings (Thompson et al., 1997). A neighbor-joining tree was constructed from the different sequences for each species, with *MEGA* version 6 (Tamura et al., 2013). Branch support was estimated by bootstrapping with 10,000 replications.

2.4. Digestion with restriction enzymes

We generated a restriction map for each consensus sequence of the four parasitoid species and their host *L. botrana*, using the online restriction map software Restrictionmapper (http://www.restrictionmapper.org/index.html). Simulations were run to determine the combination of enzymes maximizing the differences in restriction pattern between species. The enzymes selected for subsequent tests were those yielding a potentially legible pattern on agarose electrophoresis. The following protocol was used for enzymatic digestion: 1 μl of PCR product digested with 0.1 U restriction enzyme in 10× enzyme buffer for 16 h at 37 °C. The digested fragments were separated by electrophoresis in a 2% agarose gel at 100 V and visualized instantly by using Fluorescent EZ-vision DNA dye of Amresco VWR Company and a standard U.V. transilluminator.

2.5. Statistical analysis

A generalized linear mixed model (GLMM) with a binomial error distribution was fitted to examine the effect of the method used (traditional or PCR-RFLP-based methods) on the parasitism levels obtained (Zuur et al., 2009). Field site was used as a random effect, to account for repeated measures at the same site. Differences in parasitism levels resulting from the presence of different species were assessed in a Mann-Whitney test for paired samples,

taking site dependence into account. The GLMM was constructed with the R package 'lme4' and the function 'glmer'.

3. Results

3.1. Parasitoid identification on the basis of COI mtDNA

The universal primer set used in this study (C1-J-1859 F and C1-N-2191) targets the mitochondrial COI gene. It yielded efficient amplification across all four parasitoid species and their host *L. botrana*. The mean size of the amplified fragment was 364 bp (range: 315–386 bp): 315 bp for *Elachertus* spp., 351 bp for *P. nigrina*, 384 bp for *C. capitator* and *E. tibialis* and 386 for *L. botrana*. The nucleotide sequences are available in GenBank under accession numbers KU729004 to KU729018.

Multiple alignments revealed that the number of parsimonious informative sites was relatively high in the amplified region: 169 sites, corresponding to a frequency of 38.6%. Molecular species identification results were consistent with morphological taxonomic findings. All members of a given species were found to cluster within the same clade on neighbor-joining trees (Fig. 1). The COI mitochondrial gene can therefore be used to distinguish clearly between the four parasitoid species and their hosts.

3.2. PCR-RFLP successfully detects four parasitoid species within L. botrana larvae

For the creation of a reliable molecular test for identifying hymenopteran (*C. capitator*, *E. tibialis*, *Elachertus* spp.) and dipteran (*P. nigrina*) parasitism within *L. botrana* larvae, we obtained restriction maps from the COI mitochondrial DNA sequences of these parasitoid species and their host. We tested several different combinations of restriction endonucleases *in silico*, and ApoI was identified as the enzyme providing the restriction pattern best distinguishing between parasitoid species and between parasitoids and their host: *C. capitator* was easily identified on the basis of the presence of two bands close together, at 213 and 171 bp, *E. tibialis* was identified on the basis of three bands, at 202, 111 and 71 bp, *Elachertus* spp. displayed three bands at 118, 113 and 84 bp and *P. nigrina* had a profile characterized by three band at 153, 109 and 99 bp. Another two bands (275 and 111 bp) identified the *L. botrana* profile (Fig. 2). These patterns corresponded to the predictions based on restriction maps and were highly consistent with the morphological species classification.

3.3. PCR-RLFP sensitivity and the occurrence of larval parasitism

PCR-RLFP analyses to estimate the mean level of parasitism of the 252 *L. botrana* individuals revealed that parasitoids were present in all eight vineyards sampled (see Fig. 3).

General linear mixed model analysis revealed a significant effect of the method used on the estimated level of parasitism, with significantly lower levels of parasitism recorded with the traditional method based on morphological criteria (P < 0.001) than with our molecular approach (Table 1).

PCR-RFLP showed that the mean level of parasitism was 24.9% over all sites (range: 3.2–50%) (Table 2). Two parasitoid species were detected, with significantly different levels of parasitism: *C. capitator*, which was detected in 91.2% of the infected larvae, and *P. nigrina*, detected in 8.8% (Mann-Whitney test, P = 0.03).

The morphological method yielded a mean level of parasitism about one third (7.3%) that obtained with the molecular method, with the recorded level of parasitism also varying significantly between the geographic sites sampled (the level of parasitoid emergence from infested larvae ranged from 0 to 23.3%). *C. capita-*
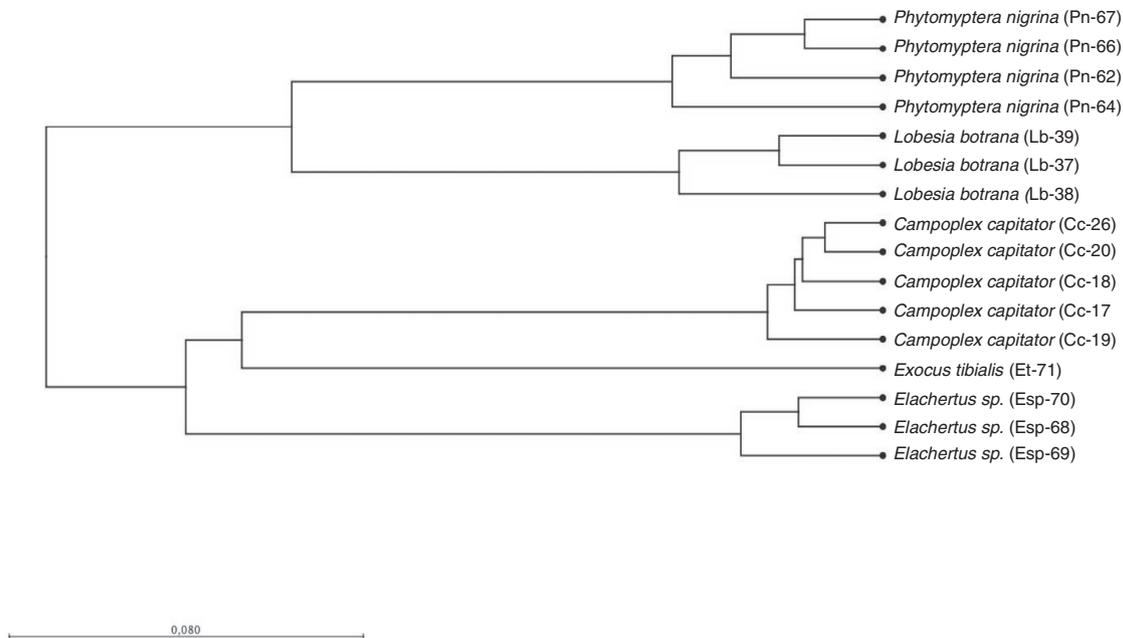
Fig. 1. Neighbor-joining tree based on the analysis of mitochondrial COI gene sequences with the Kishino-Hasegawa model of substitution. It was possible to assign each individual parasitoid or host to the corresponding species. The scale bar denotes the estimated number of substitutions per site.
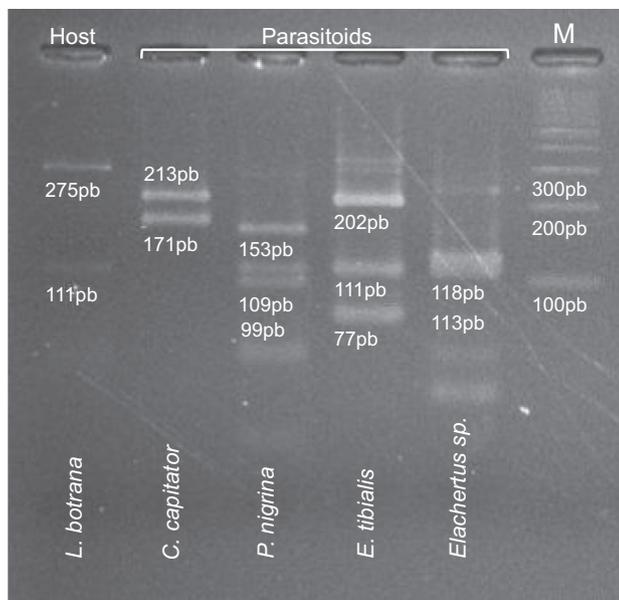

Fig. 2. Specific electrophoretic profiles of the four most abundant parasitoid species (*C. capitator*, *P. nigrina*, *E. tibialis*, *Elachretus* spp.) and their host *L. botrana* obtained after the digestion of COI ribosomal DNA sequences with the *ApoI* restriction endonuclease.

tor was the only parasitoid species identified from infested larvae (Table 2). In two *L. botrana* populations (Pauillac and Bommes) 8.7% and 6.9% of larvae, respectively, died before parasitoid emergence (or before pupation, for the uninfected larvae).

4. Discussion

We report here the development of a molecular method based on PCR-RFLP analysis of the COI gene for the reliable detection of parasitism of the European grapevine moth *L. botrana* by larval parasitoids. This method provided an accurate early-season detection and identification of four parasitoid species within *L. botrana* larvae in a single two-step test. The mitochondrial COI gene used here therefore proved to be sufficiently variable to provide restriction patterns for discriminating between these four parasitoid species, which are morphologically very similar.

This method detected more accurately the larval parasitism in different field samples of *L. botrana*. In all the vineyards sampled, parasitism was detected, at levels ranging from 3.2 to 50%. This range of variation is consistent with the findings of several published studies on *L. botrana* parasitism levels in different French vineyards (Thiéry et al., 2001, 2006; Moreau et al., 2010; Thiéry et al., 2011). Using this molecular method, we were able to detect two parasitoid species in the 252 *L. botrana* larvae: *C. capitator*, which was responsible for about 90% of the parasitism observed, and *P. nigrina*. However, only *C. capitator* was detected by the traditional rearing method. Moreover, we found that the method used had a significant effect on the estimated parasitism level that was higher for the molecular approach. Our molecular tool therefore appears to be more sensitive than the traditional rearing method, and the results of our study highlight the added value of this technique for studying trophic interactions in agroecosystems.

In this study, the mean estimated parasitism levels obtained with the traditional rearing method were always about one third those obtained with the PCR-RLFP-based tool. This difference is of a magnitude similar to that reported in previous studies on the European corn borer *Ostrinia nubilalis* (Agusti et al., 2005) or on *Lygus* spp. in alfalfa fields (Ashfaq et al., 2004). Polydnaviruses DNA, a pseudo-viral entity can be injected by the female parasitoid into the host larvae during oviposition to suppress the host immunity response (Poirié et al., 2009). However, while this DNA is produced by ovaries and/or by the venom gland of parasitoid females and has non-viral origin (Drezen et al., 2006), it is unlikely that the mitochondrial CO1 specific arthropod primers used in this study could amplify this pseudo-viral DNA. Thus it is unlikely that this could have generated false positive in our study. Difference between the two methods could not be attributed to larval mortality following parazitation as was observed by Agusti et al. (2005). We sampled living larvae in the field and early mortality cannot
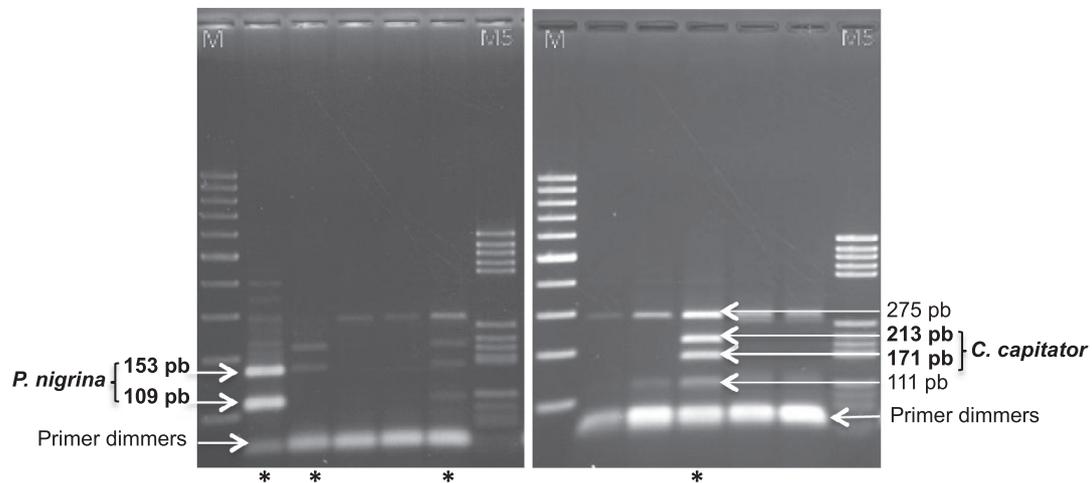
Fig. 3. *ApoI* digestion pattern distinguishing the parsitoids *C. capitator* (213 bp, 171 bp) and *P. nigrina* (153 bp, 109 bp) within the infected *L. botrana* (275 bp, 111 bp) larvae collected in South-West France in 2013. The stars (*) show infected *L. botrana* individuals. M, 100-bp ladder; M5, 1000-bp ladder.

Table 2
Proportions of *L. botrana* larvae parasitized by *C. capitator* and *P. nigrina* in the natural grapevine moth populations collected from eight sites in South-West France, in 2013 (*N* = 547).

Site	GPS Longitude	GPS Latitude	Date	N	Campoplex capitator		Phytomyptera nigrina	
					MM	MT	MM	MT
Fronsac	0°16′40.5″O	44°55′22.4″N	14/06/2013	65	3,2	0	0	0
Bassens	0°30′37.8″O	44°55′1.5″N	14/06/2013	114	8	1.5	0	0
Pauillac	45°12′22.4″N	0°45′15.3″O	19/06/2013	50	23.3	4.3	13.3	0
Saint-Loubes	0°27′18.8″O	44°54′54.3″N	20/06/2013	117	14.3	1.4	0	0
Fargues	44°32′32.3″N	0°18′47.7″O	24/06/2013	40	41.2	23.3	0	0
Martillac	44°43′49.0″N	0°33′12.9O	24/06/2013	27	0	13.3	7.1	0
Sauternes	0°19′58.0″O	44°33′0.2″N	22/06/2013	79	50	3.9	0	0
Bommes	0°20′23.0″O	44°32′46.7″N	22/06/2013	55	38.7	10.3	0	0
Overall					22.3 ± 19.0	7.3 ± 8.0	2.6 ± 5.0	0

MM, molecular method of parasitoid detection by PCR-RLFP; MT, traditional method based on the rearing of individual *L. botrana* larvae until parasitoid emergence and identification on the basis of morphological criteria.

be considered in this study. Also, few larvae died before parasitoid emergence in only two of the eight populations studied (8.7% and 6.9% of larvae, respectively). Encapsulation of parasitoid eggs may thus have generated this difference, which is a host immune response to foreign bodies (Kohler et al., 2007). The egg is neutralized but is still present in the host larvae and therefore detectable by PCR-RFLP. An encapsulation mechanism and an active immune system were recently discovered in *L. botrana* and its sibling species *Eupoecilia ambiguella* (Vogelweith et al., 2011, 2013). Therefore, we strongly think that encapsulation mechanisms is the main explanation for the observed differences in the estimates of parasitism level obtained with the two methods. However, the most appropriate way to highlight the encapsulation of eggs or early parasitoid instars is to perform dissection of parasitoid infected *L. botrana* larvae (obtained by natural infestations or by infestations induced in laboratory setting) as was previously described for tachnid and diapriid parasitoid species (Agusti et al., 2005; Small et al., 2012). Our PCR-RLFP method could therefore be useful for identification of these early instars or parasitoid eggs. In conclusion, this PCR-RLFP-based method neatly complements the classical method: it can be used to quantify biological control potential, corresponding to all the successful oviposition events within the host, whereas the classical method provides information about the effectiveness of pest control and the next generation of parasitoids.

The accuracy of our method for identifying the four main *L. botrana* larval parasitoids occurring in European vineyards and determining parasitism level demonstrates that this method is useful for characterizing and studying the parasitoid community. The sensitivity of this method should however be tested in order to determine whether very small amounts of parasitoid DNA could be detected when there are in competition with large amounts of host DNA. Future development will be also engaged to validate PCR-RLFP detection for the two other parasitoid species, *E. tibialis* and *Elachertus* spp., on naturally occurring *L. botrana* larvae collected from French vine-growing areas where these two parasitoid species should be more abundant. It might be also worth to adapt this PCR-RLFP tool for detecting other parasitoid complex, which naturally control *L. botrana* in different geographical regions, as in Iran for example, where *Enytus apostate* is the ichneumonid larval parasitoid that exceeds more than 50% of *L. botrana* parasitism (Akbarzadeh-Shoukat, 2012). Therefore, it is likely that new associations with parasitoid species are expected, especially as *L. botrana* has been recently reported in South and North American vineyards (Gilligan et al., 2011). So, in such previously unexplored geographical areas, prior morphological determination of all parasitoids should be done, followed by a quantification of parasitism for the target parasitoid species by this adapted molecular method. Therefore, since it detects parasitism early in the season, this tool can be developed and used to provide relevant information for field management and the optimization of pesticide applications. A combination of the classical and molecular methods provides a reliable picture of the parasitoid community, trophic interactions and natural pest control services in an agroecosystem.

Competing interests

The authors have declared that no competing interests exist.

Acknowledgments

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