Rapid on-site identification of the biocontrol agent of the Asian chestnut gall wasp

This is the author's manuscript

Original Citation:

Availability:
This version is available http://hdl.handle.net/2318/1601009 since 2016-10-12T11:13:52Z

Published version:
DOI:10.1080/09583157.2016.1195335

Terms of use:
Open Access
Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)
This is the author's final version of the contribution published as:


The publisher's version is available at:
http://www.tandfonline.com/doi/full/10.1080/09583157.2016.1195335

When citing, please refer to the published version.

Link to this full text:
http://hdl.handle.net/
Rapid on-site identification of the biocontrol agent of the Asian chestnut gall wasp

Colombari F.\textsuperscript{1}, Villari C.\textsuperscript{1,2}, Simonato M.\textsuperscript{1}, Cascone P.\textsuperscript{3}, Ferracini C.\textsuperscript{4}, Alma A.\textsuperscript{4}, Guerrieri E.\textsuperscript{3}, Battisti A.\textsuperscript{1}

\textsuperscript{1} Department of Agronomy Food Natural resources Animals and Environment (DAFNAE), University of Padova, Agripolis, 35020 Legnaro (PD), Italy; \textsuperscript{2} Department of Plant Pathology, The Ohio State University, Columbus, OH 43210, USA; \textsuperscript{3} Institute for Sustainable Plant Protection, National Research Council of Italy, 80055 Portici (NA), Italy; \textsuperscript{4} Department of Agricultural, Forest and Food Sciences (DISAFA), University of Torino, 10095 Grugliasco (TO), Italy.

CONTACT Fernanda Colombari \textsuperscript{1} fernanda.colombari@unipd.it
Rapid on-site identification of the biocontrol agent of the Asian chestnut gall wasp

Abstract In classical biocontrol programs a rapid and correct identification of the introduced antagonist is a key issue during both the release and establishment monitoring phases. It is often difficult to distinguish morphologically cryptic species or immature stages, and, in this case, an accurate diagnosis can be provided by molecular diagnostic methods. Among the conventional and real-time PCR based methods, loop-mediated isothermal amplification (LAMP) is a particularly suitable technique as it allows a rapid amplification of target DNA directly in the field. During the program implemented in Italy against the Asian Chestnut Gall Wasp (ACGW) Dryocosmus kuriphilus, we developed a real-time LAMP assay, combined with a simple DNA extraction, for rapid in-field identification of larvae, pupae and adults of the biocontrol agent, the parasitoid Torymus sinensis. Validation of the assay comprised adults as well as preimaginal stages of parasitoids obtained from ACGW galls collected from different localities and results confirmed the effectiveness of the LAMP assay to rapidly and specifically identify the target parasitoid in the field. This assay will be a valuable tool for quick on-site checking of the parasitism rate.

Key words: Torymus sinensis, Dryocosmus kuriphilus, LAMP (Loop Mediated Isothermal Amplification), ACGW, biocontrol, parasitism rate,

Introduction

The measure of the success of classical biocontrol programs is largely determined by whether and to what extent the target pest’s density changes after the establishment of introduced natural enemies (Guit, Wratten, & Barbosa, 2000; Hoddle, 2004; Mahr, Whitaker, & Ridgway, 2008). Identification, preliminary safety testing and release of control agents are undoubtedly matters of great concern (Van Driesche & Hoddle, 2000). Nonetheless, once the agent is approved for release, an important issue is quantifying post-release the effectiveness of parasitoids and predators in reducing pest abundance (Stiling & Cornelissen, 2005; Furlong & Zalucki, 2010). To objectively assess the effectiveness of biocontrol in terms of costs and benefits relative to conventional control, standardized measures of success are needed in post-release
surveys repeated over time (Sweetman, 1935; Van Driesche & Hoddle, 2000). The evaluation of parasitism rate can be performed following ‘rearing’ or ‘dissection’ methods applied to field-collected insects or plant material if host species feed in concealed or semi-concealed situations (i.e. leaf miners, gallmakers, borers). The former method can take a long time and could be delayed by many factors (i.e. diapause, weather conditions), whereas the latter is relatively quick and can guide decisions on whether more or different agents should be released (Day, 1994; Guit et al., 2000).

Whichever method is adopted, a correct identification of the parasitoids obtained is the critical step in the evaluation phase of the biocontrol program implemented, as it is a decisive factor when assessing the efficacy and host specificity of control agents and their possible interactions with the pest/native natural enemy complex (Delucchi, Rosen, & Schlinger, 1976; Agustí et al., 2005; Gariepy, Kuhlmann, Gillott, & Erlandson, 2008). Strong support in systematics and taxonomy is essential to correctly identify parasitoids at the species level (Van Driesche & Hoddle, 2000). However, using morphological features to distinguish closely related taxa or members of cryptic species complexes is often difficult, if not impossible, especially for immature stages (Dawah & Rothfritz, 1996; Agustí et al., 2005; Mathé-Hubert, Gatti, Poirié, & Malausa et al., 2013). Several molecular diagnostic methods that have been developed and largely implemented over the last 20 years can assist, complement and even replace morphologically based approaches (Agustí et al., 2005; Gariepy et al., 2008; Jenkins, Chapman, Micallef, & Reynolds, 2012).

In particular, the loop-mediated isothermal amplification (LAMP) is based on specific amplification of target DNA without the need for thermal cycling steps, thus allowing reactions to be performed in a portable heating block (Notomi et al., 2000; Tomlinson, Barker, & Boonham, 2007). The possibility of using LAMP directly in the
field, with short reaction time and no need for a long training period for the staff involved in the survey, gives considerable advantages compared to other conventional or real-time PCR based methods (Jenkins et al., 2012; Tomlinson, Dickinson, & Boonham, 2010; Tomlinson et al., 2013). As the DNA polymerase in LAMP reactions is not influenced by the co-presence of inhibitors or non-target DNA (Kogovšek et al., 2015; Lenarčič, Morisset, Mehle, & Ravnikar, 2013; Notomi et al., 2000) crude DNA extracts can be used, thus increasing the usefulness and portability of the method in the field (Danks & Boonham, 2007).

The Asian Chestnut Gall Wasp (ACGW) *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae), native to China is an invasive species and included in the quarantine list of European Union (EC, 2000). After being accidentally introduced into Japan, Korea, North America, and Nepal (in the forties, fifties, seventies, and nineties, respectively), it arrived in north-western Italy in 2002 and then spread rapidly throughout Italy and several European countries (Slovenia: 2005; France: 2005; Hungary and Switzerland: 2009; Spain, Croatia and the Netherlands: 2010; Czech Republic: 2012; Austria and Germany: 2013; Portugal and Turkey: 2014; United Kingdom: 2015; Belgium: 2016) (EPPO, 2015; EPPO, 2016a, 2016b). ACGW represents a very serious threat to chestnut stands in all the invaded countries as high numbers of galls on leaves and shoots reduces tree vigour and severely affects nut and timber productivity (Kato & Hijii, 1997; Maltoni, Mariotti, & Tani, 2012; Battisti, Benvegnì, Colombari, & Haack, 2014). Management of ACGW infestations, after the unsuccessful attempts to reduce pest densities by other measures, currently relies only on classical biological control methods (Moriya, Inoue, & Mabuchi, 1989). The release of the parasitoid *Torymus sinensis* Kamijo (Hymenoptera: Torymidae) from the native region of the gall wasp has been shown to reduce damage below a tolerable threshold.
level in Japan (less than 30%; Gyoutoku & Uemura, 1985; Moriya, Shiga, & Adachi, 2003) as well as in the USA, where a decline of pest population density has been also reported (Cooper & Rieske, 2007). In Italy, adults of *T. sinensis* were released in the first introduction site of ACGW starting in 2005, and later in all the other invaded areas (Quacchia, Moriya, Bosio, Scapin, & Alma, 2008; MiPAAF, 2010).

We developed a real-time LAMP assay combined with a crude DNA extraction for the identification directly in the field of larvae, pupae and adults of *T. sinensis*. The method was developed in order to quickly identify the preimaginal stages of the parasitoid and to evaluate the parasitism rate, so as to aid prompt management decisions regarding possible further releases of the parasitoid. Moreover, it may help the quality assessment of the parasitoid rearing program.

**Materials and methods**

The real-time LAMP assay was first developed using pure DNA extracted from voucher specimens by a salting out protocol. Then, the assay was validated using crude DNA extracted with a simple procedure from adults emerged from the galls (rearing method) and from preimaginal stages obtained by dissecting collected galls from the field.

**Insect material**

For the LAMP protocol development, 13 *T. sinensis*, 29 other chalcid adult parasitoids belonging to the same genus, family, and superfamily (21, 1, and 7 species, respectively; Table 1) as well as two *D. kuriphilus* were used as voucher specimens for molecular characterization. All the specimens were stored in 70% alcohol after their emergence from chestnut or oak galls (i.e. *T. geranii* Walker emerged from galls of *Biorhiza pallida* Olivier) collected in pure or mixed chestnut stands in Veneto (Crespano...
del Grappa - TV), Piemonte (Avigliana - TO), and Campania (Serino - AV, Sicignano
degli Alburni - SA) regions.

Validation of the LAMP protocol was first tested on 30 adults of *T. sinensis* and
15 adults of other native chalcid parasitoids belonging to Torymidae (*Megastigmus
dorsalis* Fabricius and *Glyphomerus stigma* Fabricius), Eurytomidae (*Eurytoma
pistaciae* Rondani and *E. brunniventris* Ratzeburg) and Eupelmidae (*Eupelmus
kuriphilus* were also tested. All these specimens emerged from chestnut galls collected
during the summer and winter at the rearing centre of the Veneto Region (Crespano del
Grappa); *G. stigma* emerged from galls of *Diplolepis rosae* Linnaeus. In addition, a
sample of 10 to 13 adults of *T. sinensis*, obtained from each of 8 Italian and 1 French
population were further tested (N=101), including a few individuals from Korea (N=6)
and Japan (N=3). All the specimens were first identified using various diagnostic
morphological characters (Kamijo, 1982; de Vere Graham & Gijswijt, 1998).

The LAMP assay was then tested on larvae and pupae of the populations for
which they were available (Table 2). At least 1,000 galls were collected during late
winter at 7 Italian release sites in 4 regions (Veneto, Valle d’Aosta, Piemonte, Liguria)
and one site in France. After collection, a subsample of 300 galls for each site was
stored at 4° C to arrest the development of gall inhabitants at larval or pupal stages.
Galls randomly chosen from each subsample were then dissected. Dissections were
carried out until between 12 and 16 immature individual parasitoids were obtained and
the number dissected recorded.

**DNA extraction**

Two types of DNA extractions were performed. For the LAMP assay development, a
pure DNA extraction was carried out on 42 adult parasitoids (Table 1) using a salting
out protocol (Patwary, Kenchington, Bird, & Zouros, 1994). Conversely, for the LAMP assay validation, a crude DNA extraction was performed following a simple protocol intended for applications under field settings on: i) the adult specimens reported in the previous section i.e. 30 adults of *T. sinensis* and 18 adults of other chalcid parasitoids; 110 adults of *T. sinensis* from 11 different populations; ii) the immatures dissected from galls (Table 2). Whole insect bodies were individually placed into 1.5-ml Eppendorf tubes containing 200 μl of double-distilled water and ground with a plastic sterile pestle to obtain a crude homogenate. Tubes were then shaken by hand without vortexing for few seconds before taking the volume to be tested. Approximate DNA concentrations were determined at 260 nm using the Nano-drop 2000 spectrophotometer (Nano-drop Technologies, Wilmington, DE, USA).

**Design of LAMP primers**

Two sets of primers satisfying LAMP requirement criteria were designed (Notomi et al., 2000). A first set of primers, specific for *T. sinensis*, was developed on the sequences of internal transcribed spacer 2 (ITS2) in the nuclear ribosomal region. Primers were designed on the *T. sinensis* sequences retrieved from GenBank (accession numbers AB200273, AB200274, and AB200275), and aligned with homologous sequences of other species of Torymidae and Pteromalidae (i.e., *T. geranii*, GenBank accession number AB200280; *T. flavipes*, GenBank accession numbers HM574233 and HM574237; *Mesopolobus xanthocerus* (Thomson), GenBank accession number HM573972). In preliminary analyses (results not shown), some falsepositives with *T. affinis* were obtained when loop primers only were included in the reactions, although the use of these primers is usually recommended to improve both the specificity and the speed of the reaction (Nagamine, Hase, & Notomi, 2002). These primers were excluded from the primer set.
To avoid falsenegative results (Tomlinson et al., 2010), and to check quality of both DNA and reagents, a set of primers, hereafter referred to as external amplification control (EAC) primer set (D’Agostino, 2013), was designed on the 28S region of other chalcid wasps using sequences retrieved from Munro et al. (2011). Preliminary tests showed a wider inclusiveness of this primer set. Nonetheless, this was not a matter of concern because the purpose of the design was to ensure reliability of the assay by excluding the presence of inhibitors or suboptimal reaction conditions (i.e. not sufficient DNA template).

All the sequences alignments were edited using the ClustalW algorithm in the software MEGA, version 6, (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). Primers were designed using LAMP Primer Explorer software (version 4; Fujitsu System Solutions Ltd., Tokyo, Japan) and synthesized by Invitrogen.

**LAMP development and diagnostic performance**

The DNA extracted by the salting out method from the specimens listed in Table 1 was used for the evaluation of the sensitivity and specificity of both the primer sets. The reaction mixture for the *T. sinensis* LAMP test contained 15 µl of Isothermal MasterMix 1× (OptiGene), 0.2 µM of each external primer, 2 µM of each internal primer, and 1 µl of template DNA. The same conditions were used for the EAC assay, with a further addition of two loop primers, 1 µM each. Total volume for both reactions was 25 µl. LAMP reactions were performed on a Genie II device (OptiGene).

Optimal conditions for LAMP reaction were determined by performing runs in a range of temperatures for different time periods. The identity of amplification products was evaluated in terms of annealing temperature determined through the measure of fluorescence during a slow annealing step (0.05°C/s) starting at 98°C and ending at 84°C.
The sensitivity of both the assays was tested through 10-fold serial dilutions of template DNA in double-distilled water, starting from a higher concentration of 10 ng μl⁻¹ to a lower concentration of 100 fg μl⁻¹. Each run contained double-distilled water as negative control (Tomlinson et al., 2013). The specificity of both the *T. sinensis* and the *EAC* primer sets was evaluated using the specimens reported in Table 1. Both sensitivity and specificity were tested on pure DNA extracted by the salting out method.

**Validation of LAMP with crude DNA**

Evaluation of the specificity of the LAMP assay on crude DNA was first performed on the 48 specimens (30 adults of *T. sinensis* and 18 adults of other chalcid parasitoids) emerged at the rearing centre. Reactions were repeated three times for each specimen to confirm the diagnosis. For those specimens that were not amplified by the *T. sinensis*-specific set of primers, a *EAC* primer set test was performed in order to ensure quality of both DNA and reagents. The assay was then tested on a further 110 adults of *T. sinensis* belonging to 11 different populations and 80 larvae and 25 pupae obtained by the dissection method from 8 populations (Table 2).

To confirm the results obtained by LAMP assays, 20 specimens (10 immature and 10 adults), randomly selected among the above mentioned 263 samples, were identified by sequencing of the DNA barcode region (Hebert, Cywinska, & Ball, 2003). For each specimen, 100 μl of the crude DNA extract was used for a further DNA extraction using Qiagen DNeasy Plant mini kit (Valencia, CA, USA). The DNA obtained was then eluted in 50 μl of AE buffer and directly used to amplify and sequence the barcode region of the *cox1* gene (Hebert et al., 2003), using the universal primers and the amplification condition as reported in Folmer, Black, Hoeh, Lutz, & Vrijenhoek (1994). PCR products were checked through electrophoresis on 1.0% agarose gels stained with SYBR® (Invitrogen) and then purified using exonuclease and...
Statistical analyses
Throughout the text, temperature values are expressed in degrees Celsius and time values in minutes and seconds. All average values are reported as mean ± standard deviation (SD), unless otherwise specified.

One-way analysis of variance (ANOVA), followed by a Tukey’s HSD (Honest Significant Differences) test, was used to compare the mean annealing temperatures of the 110 T. sinensis adults from the eleven different populations. An alpha level of 0.05 was considered statistically significant. Statistical analyses were performed using STATISTICA, version 8 (Statsoft Inc., Tulsa OK, USA).

Results
LAMP primers design
The designed T. sinensis primer set contained two external primers (TS_F3 and TS_B3) and two inner primers (TS_FIP and TS_BIP). The EAC primer set was composed of two external (CH_F3 and CH_B3) and two internal primers (CH_FIP and CH_BIP), with the addition of two loop primers (CH_Fl and CH_BL) in order to accelerate the amplification reaction. Primer sequences for both the primer sets are reported in Table 3.

LAMP development and diagnostic performance
Optimal temperature and running time for both the LAMP reactions were an isothermal condition of 67°C maintained for 19 and 23 min. for the T. sinensis and the EAC LAMP assays, respectively. The specificity and the sensitivity of both primer sets and the
diagnostic performance of the LAMP assays were then tested on the DNA extracted by
the salting out method.

Specific primers worked exclusively on *T. sinensis* DNA and did not amplify
any of the non-target sequences. All the 13 *T. sinensis* DNA samples were correctly
identified while EAC primers amplified all the 44 DNA samples analyzed.

Amplification was not observed in the negative control reactions. The serial dilutions of
DNA tested with both the assays, showed an analytical sensitivity of 10 pg μl⁻¹. The
annealing temperatures of the amplification products were 88.82 ± 0.09°C and 87.51 ±
0.18°C for *T. sinensis* and EAC LAMP assays, respectively.

**Validation of LAMP with crude DNA**

Validation of the method on 48 adult parasitoids showed that 30 *T. sinensis* individuals
were positive to the *T. sinensis* assay, whereas all tested insects were positive to the
EAC assay. Average positive reaction times of crude DNA were 16:34 ± 00:42 for *T.
sinensis* assay, and 14:14 ± 1.43 for EAC assay. Annealing temperatures were 88.98 ±
0.06°C and 87.40 ± 0.10°C for *T. sinensis* and EAC assays, respectively. There was a
slight variation in the starting average DNA concentrations of crude extracts (1.17 ±
0.17 ng μl⁻¹), reaction times (16:28 ± 1:47), and mean annealing temperatures (88.84°C
± 0.14°C) among the 11 populations of *T. sinensis*. Interestingly, the mean annealing
temperature of *T. sinensis* adults differed significantly among populations (*F*₁₀, *₉₉* =
2.04, *p* < 0.05), with the Korean specimens showing the highest average value, although
the Tukey's test was not significant for any pair of means.

For preimaginal stages (larvae and pupae) from 8 available populations (Table 2),
average DNA concentrations of crude extracts, reaction times, and mean annealing
temperatures were: 2.72 ± 1.27 ng μl⁻¹, 16:47 ± 1:40, 88.84°C ± 0.17°C for larvae; 2.05
± 0.07 ng μl⁻¹, 15:21 ± 1:52, 88.76°C ± 0.16°C for pupae. At some sites, it was recorded
a low number of larval samples that generated a positive amplification out of the total number of samples tested. DNA barcoding of the 20 samples confirmed the accuracy of the LAMP identification. Positive LAMP results always corresponded to *T. sinensis*, whereas negative results corresponded to species belonging to the genera *Eupelmus* (Hymenoptera: Eupelmidae) and *Mesopolobus* (Hymenoptera: Pteromalidae).

**Discussion**

The LAMP assay enabled real-time detection of *T. sinensis*, the biocontrol agent of the ACGW, through a simple procedure designed to give rapid on-site results for samples without the need for time-consuming analyses or rearing in the laboratory. The use of a quick technique, together with the simple equipment required and the possibility to discriminate insect material obtained from various life stages, make the method valuable for making decisions when evaluating effectiveness of biocontrol programs directly in the field.

The whole procedure developed in this study, from sample preparation to the evaluation of amplification products, was completed in less than 30 minutes. The specific LAMP primer set showed high specificity to *T. sinensis*, with no positive reactions when other species were tested. However, we cannot exclude that the primer set designed specifically for *T. sinensis* works for the closely related *T. beneficus*, which cannot be discriminated on the basis of morphological characters (Yara, 2004). Unfortunately, it has not been possible to retrieve and test any specimen of *T. beneficus* to better validate the *T. sinensis* primer set. For this purpose, it would be interesting to look at the possibility to obtain an annealing temperature specific for the DNA region of the target species, as a way to unambiguously identify the samples and, concurrently, to detect possible variants (Ririe, Rasmussen, & Wittwer, 1997). In our case, the Korean population showed the highest mean annealing temperature, although this result needs
to be supported by a higher number of samples. If confirmed, this result may reveal the occurrence of two different strains of *T. sinensis* in Korea and Japan, as the European populations came from Japan, given their introduction history (Murakami, Ohkubo, Moriya, Gyoutoku, Kim, & Kim, 1995; Yara, 2004; Quacchia et al., 2008).

The diagnostic sensitivity level of the assay was high enough to correctly identify the parasitoid in any of the life stages considered, without the need for complex DNA extraction and quantification. Successful outcomes were easily obtained by simply grinding the insects in double distilled water and by directly adding these crude homogenates to pre-prepared strips containing isothermal field stable reagents. Results were positive when specimens both stored in alcohol and freshly collected were used, demonstrating that even DNA of relatively low purity gives positive amplification products (Huang, Hsu, Haymer, Lin, & Wu, 2009). We found that the negative results were successfully amplified by the EAC primer set, minimizing the risk of false negative results.

The samples tested were not sufficient to give reliable estimates of parasitism. However, the proportion of positive larval samples roughly reflects the parasitoid success at the different sites. A higher occurrence of *T. sinensis* was indeed recorded where the parasitoid was released first. Alternatively, the high proportion of positive pupal samples can be explained by the period of gall collection (i.e. late winter). In this period only *T. sinensis* occupies the galls as the other parasitoids generally use other hosts for overwintering (Luo, Huang, & Liao, 1987; Shiga, 2009). A proper identification of *T. sinensis* using a reliable on-site molecular technique is of great importance as the release of the parasitoid is, at present, the only viable long-term management option against *D. kuriphilus* in the invaded areas (Yara, 2006; Gibbs et al., 2011). Although the biocontrol of ACGW is successful in those areas where *T. sinensis*
has been introduced (Gyoutoku & Uemura, 1985; Moriya et al., 2003; Cooper &
Rieske, 2007; Ferracini et al., 2015a), many recently invaded regions have now to deal
with this problem (EPPO, 2014; EPPO, 2015). Consequently, a correct identification of
both preimaginal and adult stages of the parasitoid is fundamental during all the phases
of the biological control program (Danks, 1988; Gordh & Beardsley, 1999). This is
particularly true if we consider that other parasitoid species, such as local oak gall wasp
parasitoids, are rapidly recruited as enemies by ACGW once it arrives in a new area and
that *T. sinensis* may cause unwanted non-target effects (Aebi et al., 2006; Quacchia et
al., 2012; Matošević & Melika, 2013; Panzavolta et al., 2013; Palmeri et al., 2014;
Ferracini et al., 2015b).

The LAMP assay we developed is suitable for diagnostic as well as research use
(Lenarčič et al., 2013) as it can be used both for a rapid quality check of the released
stock in new areas and a quick in-field monitoring of the parasitism rate of the
biological control agent. Moreover, this method bypasses the need for specialist
knowledge or a long period of training for the staff involved (Jenkins et al., 2012)
avoiding, in particular, the long procedure in the morphological identification of
preimaginal stages.

Acknowledgements

This work relies on the contribution of several persons. G. Narduzzo (Veneto Region’s
Forest Service), P. Paolucci and P. Dall’Ara (DAFNAE Padova), C. Salvadori
(Fondazione Mach Trento), the staff of the rearing area Centro Polifunzionale Onè
(Crespano del Grappa), Veneto Region’s Phytosanitary and Forest Services provided
precious support during field activities. E. Ferrari, E. Gonella, M. A. Saladini (DISAFA
Torino) and L. Iodice (IPP Napoli) provided kind help for collection and shipment of
insect specimens. Myron Zalucki carefully revised and edited the text. Two anonymous
reviewers significantly improved the quality of the manuscript. Funding
The research was funded by a grant of the Ministero delle Politiche Agricole Alimentari e Forestali of the Italian government and by the Regione del Veneto Servizi Fitosanitari.

References


EPPO (European and Mediterranean Plant Protection Organization) (2016b). First
report of _Dryocosmus kuriphilus_ in Belgium. EPPO Reporting Service (Report No. 2). Retrieved March 24, 2016,


Huffaker (Eds.), *Handbook of Biological Control* (pp. 45-55). San Diego: Academic Press.


biologica al *Dryocosmus kuriphilus* del castagno con *Torymus sinensis*.


Yara, K. (2004). Relationship between the introduced and indigenous parasitoids

Table 1. Insect material tested for the development of the LAMP assays with pure DNA extracted by the salting out protocol.

<table>
<thead>
<tr>
<th>Superfamily</th>
<th>Family</th>
<th>Species</th>
<th>Number of tested samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chalcidoidea</td>
<td>Torymidae</td>
<td><em>Torymus sinensis</em></td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>affinis*</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>auratus</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cyaneus*</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>erucarum</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>favardi*</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>flavipes</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>formosus*</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gerani</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>notatus</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>scutellaris</td>
<td>1</td>
</tr>
<tr>
<td>Eupelmidae</td>
<td>Eupelmus</td>
<td>dorsalis</td>
<td>1</td>
</tr>
<tr>
<td>Eurytomidae</td>
<td>Eurytoma</td>
<td>urozonus</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>annulatus</td>
<td>1</td>
</tr>
<tr>
<td>Ichneumonidae</td>
<td>Orthopelma</td>
<td>mediator</td>
<td>1</td>
</tr>
<tr>
<td>Peromalidae</td>
<td>Mesopolobus</td>
<td>tibialis</td>
<td>1</td>
</tr>
<tr>
<td>Ormyridae</td>
<td>Ormyrus</td>
<td>nitidulus</td>
<td>1</td>
</tr>
</tbody>
</table>

* Indicates congeneric species of *T. sinensis* emerged from cynipid galls on oak.
Table 2. Individuals of *T. sinensis* from different populations tested for the validation of the LAMP method with crude DNA. For each sample the number of samples that generated a positive amplification out of the total number of samples tested is reported.

<table>
<thead>
<tr>
<th>Country</th>
<th>Region</th>
<th>Site</th>
<th>Larvae</th>
<th>Pupae</th>
</tr>
</thead>
<tbody>
<tr>
<td>France</td>
<td>St. Dalmas de Tende</td>
<td>8/10</td>
<td>2/2</td>
<td></td>
</tr>
<tr>
<td>Italy</td>
<td>Valle d’Aosta</td>
<td>Forte di Bard</td>
<td>4/7</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>Piedmont</td>
<td>Avigliana</td>
<td>2/8</td>
<td>3/4</td>
</tr>
<tr>
<td></td>
<td>Liguria</td>
<td>Millesimo</td>
<td>7/11</td>
<td>1/1</td>
</tr>
<tr>
<td>Veneto</td>
<td>Cavaso del Tomba</td>
<td>2/14</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>San Mauro di Saline</td>
<td>1/12</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pianezze</td>
<td>2/15</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Seren del Grappa</td>
<td>2/3</td>
<td>11/13</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td><strong>80</strong></td>
<td><strong>25</strong></td>
</tr>
<tr>
<td>LAMP assay target</td>
<td>Primer</td>
<td>Sequence (5’ – 3’)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------</td>
<td>--------</td>
<td>--------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Torymus sinensis</em></td>
<td>TS_F3</td>
<td>CGCAAGATGGATGAGAGAGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TS_B3</td>
<td>GCAAAACAGAGACTCCGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TS_FIP</td>
<td>TCAAAACACTCAAGCGCCTGCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TS_BIP</td>
<td>TACGCACACGCACGCTACGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TS_FL</td>
<td>CGCTACGGACCTCCCATCAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TS_BL</td>
<td>GAACCATCTAGTAGCTGGTTCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chalcid wasps</td>
<td>CH_F3</td>
<td>GGTGAACATATGCTGGTGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CH_B3</td>
<td>TTCGCTTTTACCAGATGAGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CH_FIP</td>
<td>CCGACGATCGCGTTCAGTCTGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CH_BIP</td>
<td>ACTGGTATAGGGGCGAAAGACTAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CH_FL</td>
<td>CGCTACGGACCTCCATCAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CH_BL</td>
<td>GAACCATCTAGTAGCTGGTTCC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>