

The first record in Italy of *Trichogramma cordubense* Vargas & Cabello 1985 (Hymenoptera Trichogrammatidae) emerging from the eggs of *Lobesia botrana* (Denis & Schiffermüller, 1775) (Lepidoptera Tortricidae)

A. LUCCHI¹⁾, P. L. SCARAMOZZINO¹⁾, G. MICHL²⁾, A. LONI¹⁾ and C. HOFFMANN²⁾

¹⁾ Department of Agriculture, Food and Environment, Pisa University, Pisa, Italy

²⁾ Julius Kühn-Institute (JKI), Institute for Plant Protection in Fruit Crops and Viticulture, Laboratory of Zoology and Integrated Production in Viticulture, Siebeldingen, Germany

Summary

This study investigated the egg parasitoids of *Lobesia botrana* (Denis & Schiffermüller, 1775) feeding on *Daphne gnidium* L. (Malvales, Thymelaeaceae) in the San Rossore-Migliarino-Massaciuccoli Nature Reserve (Tuscany, Italy). Four species of egg parasitoids of the genus *Trichogramma* spp were obtained.

The parasitization rate gradually increased over the season, reaching its maximum level in September 2015, with a percentage of parasitized eggs close to 55 %. Three of the species obtained were already known as *L. botrana* parasitoids, whereas the finding of *Trichogramma cordubense* Vargas & Cabello, 1985 represents the first recording in Italy, as well as the first report of this species among the natural enemies of *L. botrana*.

Key words: egg-parasitoids; natural environment; European Grapevine Moth; spurge flax; biocontrol.

Introduction

The European grapevine moth (EGVM), *Lobesia botrana* (Denis & Schiffermüller, 1775) (Lepidoptera Tortricidae) causes serious damage to European wine and table grape production (IORIATTI *et al.* 2011). It has been recently found in Chile (2008), California (2009) and Argentina (2010) and is of great concern to the vine growers there. Applied entomologists are now investigating control strategies, including biological and biotechnical approaches, aimed at eliminating or reducing the negative impact of this pest in the new areas, thus safeguarding the environmental balance (IORIATTI *et al.* 2012, COOPER *et al.* 2014).

Interest in the natural enemies of *L. botrana* has thus increased in relation to the possibility of creating biological control programs in the new areas colonized by the pest, by exploiting the most effective parasitoids native to the Mediterranean region. About ninety species of natural enemies of *L. botrana* have been recorded in Italy, and the list is continuously being updated due to collections in new areas or in natural environments (SCARAMOZZINO *et al.* 2015).

It is believed that *Daphne gnidium* L. (Malvales, Thymelaeaceae) is the original host plant from which EGVM is presumed to have passed to the grapevine in the second half of the nineteenth century (MARCHAL 1912, GRASSÉ 1928, BALACHOWSKY and MESNIL 1935, BOVEY 1966). Despite the great importance attributed to this plant as an initial reservoir of EGVM infestations in vineyards, early research paid little attention to the ecological relationship between the plant, its arthropod pests (including *L. botrana*) and their natural enemies.

In the last few years, we have therefore undertaken a series of observations on the natural enemies of EGVM in a natural environment, where the tortricid feeds exclusively on *D. gnidium* (LUCCHI and SANTINI 2011).

Methods

The surveys were carried out from March to November 2015 in the Migliarino-San Rossore-Massaciuccoli Nature Reserve in Tuscany (central Italy) in several areas hosting a huge number of *D. gnidium* shrubs.

Nine pheromone traps (Biogard Delta traps, CBC (Europe) S.r.l. Biogard Division, 20834 Nova Milanese (MB), Italy <http://www.biogard.it/index.php/en>) were used to monitor EGVM flights. The pheromone lures were replaced monthly and the captures were counted weekly.

The observations were conducted in an area of 1.5 km², subdivided into nine rectangular plots and delimited by the following four geographical points 43.733642 N, 10.277524 E; 43.712864 N, 10.279648 E; 43.732913 N, 10.292371 E; 43.720101 N, 10.293094 E (DDM). In each plot on a weekly basis we examined the leaves and the tips of five buds on 20 shrubs, which were randomly chosen along a transect of 200 meters, for a total of 100 shoots per plot. We counted the *L. botrana* eggs present on the leaves, distinguishing between the healthy eggs and the dark parasitized eggs. Leaves with parasitized eggs were individually collected in glass vials and stored in the laboratory under controlled environmental conditions until the emergence of the adult parasitoids. The emerged adults of *Trichogramma* were preserved in 96 % ethanol for the taxonomic identi-

Correspondence to: Dr. C. HOFFMANN, Julius Kühn-Institute (JKI), Institute for Plant Protection in Fruit Crops and Viticulture, Laboratory of Zoology and Integrated Production in Viticulture, Geilweilerhof, 76833 Siebeldingen, Germany. Fax: +49-6345-41240. E-mail: christoph.hoffmann@julius-kuehn.de

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fication. Specimens were identified at specific levels with the analysis of the ITS-2 DNA sequences in the Laboratory of Zoology and Integrated Production in Viticulture of the Julius Kühn-Institute (Germany).

For the molecular analysis, we followed SUMER *et al.* (2009). The DNA of individual wasps was extracted with the Chelex method. The ITS 2 region of the internal rDNA was amplified using the primers Tricho for TGTGAACTG-CAGGACACATG and Tricho rev GTCTTGCCTGCTCT-GAG established by SILVA *et al.* (1999). For a 25 µL PCR amplification, we used Dynazyme II DNA Polymerase (Thermo Scientific), applying the conditions provided in the user guidelines with primers in 0.5 M concentration and 50 °C annealing temperature in 30 cycles. RFLP-analysis of the PCR product was performed by restriction digestion with FastDigest Mnl I (Thermo Scientific) and with MseI (Thermo Fisher) in 20 µL reactions containing 13 µL of the PCR product, 1x buffer and 10 units of enzyme, incubated for 25 min at 37 °C and followed by 5 min 95 °C denaturation. PCR and restriction products were electrophoresed in 2 % agarose gel visualized with SERVA DNA stain clear G.

In one case, when MnlI cut differently to (SUMER *et al.* 2009), we sequenced the PCR-product and determined the species with the NCBI-Database.

In this note, the taxonomy of *Trichogramma* spp. at the specific level is expressed in accordance with the Fauna Europea (FUSU 2016) and POLASZEK (2010).

Results and Discussion

In 2015, the flights of *L. botrana* started on March 24 and continued without interruption until December 3. Unlike what usually occurs in vineyards, where frequently there are zero captures in the pheromone traps for 1-2 weeks between the generations, in the natural reserve captures were found throughout the whole trapping period. This prevented a clear definition of the existence of a fourth flight and a fourth generation. Throughout the entire flight period, the eggs of *L. botrana* were found only on the leaves of *D. gnidium* and never on other plant organs (Fig. 1). In most of the cases

eggs are laid singly on the lower leaf surface. Sometimes few eggs (up to six) can be observed on the same leaf.

Eggs parasitized by *Trichogramma* Westwood, 1833 (Hymenoptera Trichogrammatidae) (Figs. 2 and 3) appeared from late June and were observed until the end of September. Their number grew over time and reached its peak on September 10, with a percentage of parasitized eggs close to 55 % (235 out of 428 observed eggs). From 235 parasitized eggs (black colour) only 111 adults of *Trichogramma* emerged of which a representative sample of 48 individuals (43 % of the total sample) was taken randomly to determine the species composition. The rest is kept as reference in the laboratory.

Our first sample of forty-eight *Trichogramma* specimens (PCR-product cut with MnlI) resulted in four species, *T. evanescens* Westwood, 1833, *T. euproctidis* Girault, 1911, *T. cacaeciae* Marchal, 1927 and *Trichogramma cordubense* Vargas and Cabello, 1985 in different proportions (Table and Fig. 3).

In one individual of the species *T. cacaeciae*, we found differing fragment lengths after MnlI digestion compared to SUMER *et al.* (2009). In this case we sequenced the PCR-product and determined the species with the NCBI-Database. Using CLC Main workbench 7, it was possible to cut the PCR product of this species with Mnl I. The experience of SUMER and coauthors that the PCR product of *T. cacaeciae* is not cut by Mnl I could not be confirmed in this individual sample as well as in other individuals reared in German vineyards from *L. botrana* eggs (HOFFMANN and MICHL unpubl.).

The digestions with Mse I (Thermo Fisher) confirmed the identification of the four *Trichogramma* species found. The specific cut sizes matched SUMER *et al.* (2009, data not shown).

To date, twelve species of *Trichogramma* have been reported as natural egg parasitoids of EGVM (NOYES 2003). The Universal Chalcidoidea Database lists eleven species of *Trichogramma* Westwood, 1833 and one species of *Trichogrammatoidea* Girault, 1911, tested on *L. botrana* eggs in laboratory trials. Another species of *Trichogramma* was found on EGVM eggs in Iran (EBRAHIMI and AKBARZADEH-SHOUKAT 2008, LOTFALIZADEH *et al.* 2012).



Fig. 1: Left, *Lobesia botrana* female (above) and its egg (beneath) laid on a leaf of *Daphne gnidium*. Right. A shrub of *D. gnidium* in the Migliarino-San Rossore-Massaciucoli Nature Reserve.

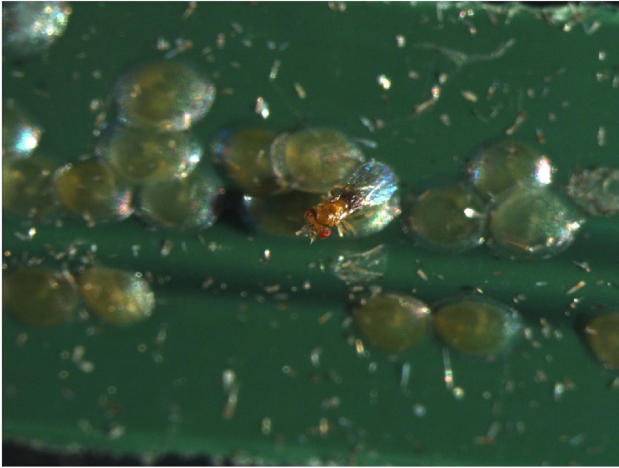


Fig. 2: *Trichogramma* spp. female ovipositing on eggs of *L. botrana*.

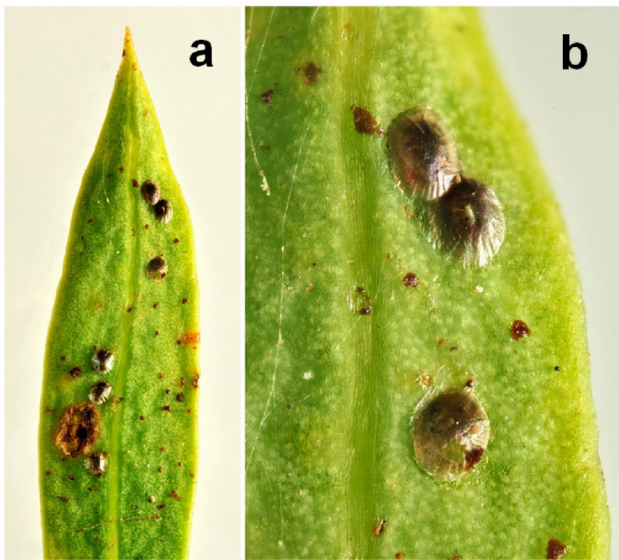


Fig. 3: San Rossore Migliarino Massaciuccoli Nature Reserve (Tuscany): **a**) detail of a leaf of *Daphne gnidium* L. bearing six eggs of *L. botrana* parasitized by *Trichogramma* spp.; **b**) detail of the same leaf (the egg at the bottom is empty and shows the exit hole).

BARNAY *et al.* (2001) and IBRAHIM (2004) briefly summarized the existing knowledge regarding the species of *Trichogramma* found on *L. botrana* and *Eupoecilia ambiguella* Hübner, 1796, (Lepidoptera Tortricidae) in the vineyards of the Old World. Eight species have been found

in France, three species in Germany, two in Ukraine and one species in Russia and Italy. Most of these species were used in inundative biological control programs against the two moths but with limited results (IORIATTI *et al.* 2011, 2012).

VIGGIANI and LAUDONIA (1989) illustrated the species of *Trichogramma* Westwood recorded for Italy. *Trichogramma evanescens* Westwood, 1833 is the only species emerging from *L. botrana* eggs collected from Italian vineyards (BAGNOLI and LUCCHI 2006). Three other species, *T. brassicae* Bezdenko, 1968, *T. cacaeciae* Marchal, 1927 and *T. embryophagum* Hartig, 1838 were released for the biological control of EGVM in Emilia Romagna, Lombardy and Lazio (BAGNOLI and LUCCHI 2006, BARBIERI *et al.* 1992, LOZZIA and RIGAMONTI 1991, NOBILI *et al.* 1988, DE CRISTOFARO and VITA 1992).

More than a century ago, SILVESTRI (1912) referred to two other species, *T. semblidis* Aurivillius, 1898 and *T. minutum* Riley, 1871, which he did not directly obtain from *L. botrana*. He obtained *T. semblidis* - known in France as a parasitoid of *L. botrana* (MARCHAL and FEYTAUD 1911) - from *Mamestra brassicae* L. (Lepidoptera Noctuidae), whereas he mentioned *T. minutum* on the basis of host data provided by GIRAULT (1912). Such old findings could be misleading because the taxonomy at that time was different from today's.

Interestingly, our collection shows the simultaneous natural occurrence of four different species of *Trichogramma* on *L. botrana* eggs, laid on a group of nearby plants of *D. gnidium* in the Natural Reserve. As mentioned above, *T. evanescens*, was the only species emerging from eggs of *L. botrana* laid on the grapevine in Italy (BAGNOLI and LUCCHI 2006). *T. cacaeciae* was artificially released for the biological control of the moth in Emilia Romagna (Central Italy) (BARBIERI *et al.* 1992), however it has never been found in natural conditions, and nor has *T. euproctidis*. The detection of *T. cordubense* has never been found before on EGVM eggs, and represents the first Italian record of this species, which was previously known in the Iberian Peninsula, the Azores, Madeira and Iran (NOYES 2003). We believe that *T. cordubense* is probably not a newly-established species in Italy, but has only been detected and correctly determined now because of its morphological similarity to *T. evanescens* (see VARGAS and CABELLO 1985). *T. cordubense* is mainly known from Noctuid-, Pyralid-species but also Papilionid-species and the green lacewing *Chrysopa carnea* are mentioned as hosts (NOYES 2003) and is obviously a polyphagous species.

Table

Four species of *Trichogramma* found in the San Rossore Migliarino Massaciuccoli Natural Reserve 2015. Predicted restriction fragment sizes when the various PCR products of the ITS2 are cut by the restriction enzyme *Mnl I* (according to SUMER *et al.* 2009) and actual number of individuals of each fragment pattern

	<i>Trichogramma</i> sp.	Size of ITS 2 PCR Product	Sizes of cuts with <i>Mnl I</i>	No. found 2015
Lane 1 and 5	<i>T. euproctidis</i>	489	270/137/82	2
Lane 2 and 6	<i>T. cordubense</i>	529	296/94/82/57	28
Lane 3 and 7	<i>T. evanescens</i>	548	297/169/82	17
Lane 4 and 8	<i>T. cacaeciae</i>	578	500/78*	1

*differs to SUMER *et al.* (2009), cut confirmed by sequence analysis.

In more than 20 years dedicated to the study of *L. botrana* and its parasitoids in several Tuscan vineyards, we have only rarely observed eggs parasitized by *Trichogramma* spp.

Undoubtedly, the unspoiled environment of the Migliarino-San Rossore-Massaciuccoli Nature Reserve is the perfect location for egg parasitoid insects, which, due to their minute size, are affected more than other parasitoids by anthropic activities. According to THOMSON *et al.* (2000) the widely-used sulfur in the vineyards against the powdery mildew has an especially detrimental effect on all stages of *Trichogramma* sp.

The high parasitization rate expressed by *Trichogramma* spp., particularly by *T. cordubense*, on EGVM eggs analyzed to date, confirms their possible role in an effective natural control of the moth in vineyards. It also highlights the need for applied entomologists and wine growers to set up conservation biological control programmes in vineyards to foster natural enemies' activity.

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