FINAL REPORT

Validation of the morphological and molecular identification protocols for
Thaumatotibia leucotreta (Meyrick, 1913)

EPPO PM 7/137 (1) *Thaumatotibia leucotreta*
and
EPPO PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests
and
Rizzo *et al.* (2021). Development of Three Molecular Diagnostic Tools for the Identification of the False Codling Moth (Lepidoptera: Tortricidae)

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1. Introduction

The European Reference Laboratory for Insects and Mites has to select, adapt or develop reliable identification protocols for European Union regulated insect and mite species (included in the Commission Delegated Regulation (EU) 2019/1702 and in the EURL for Insects and Mites working programmes). One of the tasks of the EURL is to validate diagnostic protocols before recommending their use to the National Reference Laboratories of the European Union.

The Entomology and Botany Unit of ANSES Plant Health Laboratory (Montpellier, France) and the Institute for Sustainable Plant Production of AGES (Vienna, Austria) are in charge of the activities of the EURL for Insects and Mites. The consortium performs validation studies for morphological and molecular identification tests.

According to the ISO/IEC 17025 standard, the validation of a test is defined as the "confirmation by examination and the provision of objective evidence that the particular requirements for a given intended use are met". In fact, this confirmation consists of comparing the values of the performance criteria determined during the test characterization study with those expected or assigned beforehand (limits of acceptability, objectives to be achieved), then declaring the analytical test valid or invalid. In the field of entomology, identification tests are qualitative, meaning that they allow the identification at a given taxonomic level, providing a response in terms of presence/absence.

The EURL for Insects and Mites focuses on the validation of tests published in international or regional standards, such as those issued by the International Plant Protection Convention (IPPC) or the European and Mediterranean Plant Protection Organization (EPPO).

Thaumatotibia leucotreta (Meyrick, 1913) (Lepidoptera: Tortricidae) is native of Sub-Saharan Africa. It has only successfully established in two regions where it is not indigenous, which are the Western Cape of South Africa (Giliomee and Riedl, 1998; Hofmeyr *et al*., 2015) and Israel (Wysoki, 1986). It has a poor dispersal and colonisation ability, confirmed by the fact that it has not spread further in the Middle East than Israel, despite being established there since 1986. At present, no established populations have been reported in the EU territory, although the pest has occasionally been recorded in Europe. However, these have been isolated recordings, linked to imported fruits from the countries where it is present (CABI, 2022). In 2009, an incursion of *T. leucotreta* was detected in the Netherlands on *Capsicum chinense* in glasshouse, but was subsequently eradicated (EPPO, 2010; Potting and van der Straten, 2011).

Thaumatotibia leucotreta is a European Union regulated species, listed among the EU quarantine pests (Annex II of the Commission Implementing Regulation (EU) 2021/2285) and among the EU priority pests (Commission Delegated Regulation (EU) 2019/1702). This species in included in the EPPO A2 list.

2. Scope of validation and tests

2.1 Scope

The scope of this validation study was to provide objective evidence that the selected protocols are suitable to perform routine identification of *Thaumatotibia leucotreta* by the staff of the EU National Reference Laboratories.

2.2 Description of the tests under validation

The tests evaluated are based on two diagnostic protocols for the morphological and molecular identification of *Thaumatotibia leucotreta*, as well as on a paper published in a peer reviewed journal:

- EPPO PM 7/137 (1) *Thaumatotibia leucotreta* (EPPO, 2019a)
- EPPO PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests (EPPO, 2021a), which includes tests for the DNA barcoding of arthropods
- Rizzo *et al.* (2021). Development of Three Molecular Diagnostic Tools for the Identification of the False Codling Moth (Lepidoptera: Tortricidae)

The validation study focused only on the **identification of the pest at the larval stage**, due to the fact that this species is mainly intercepted as larva.Validation was conducted according to the EPPO PM 7/98 (5) Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity (EPPO, 2021b).

2.2.1 Morphological identification of larvae

Protocol: EPPO PM 7/137 (1) *Thaumatotibia leucotreta* (EPPO, 2019a)

The reliable identification at species level for *Thaumatotibia leucotreta* requires morphological examination of adult moths. However, the **most likely stage to be detected during inspection of imported commodities is the larva** and time required for rearing to adult is too long to make it a viable option in case the identification is urgent. Mature larvae (L3-L5), provided that host commodity and country of origin are known with certainty, can be reliably identified by well experienced staff. The use of a stereomicroscope with magnification 20X or higher is needed.
The protocol provides guidance for the identification at species level of *Thaumatotibia leucotreta*:

- **paragraph 4.1.2.2 Late instars (L3-L5, page 252).** Description of late instars larvae morphology (L3-L5): a list of diagnostic characters that, combined, allow the positive identification of that species (number 7 to 13, some of which being shared by other Olethreutinae species) and a list of other characters that are not unique for that species (number 14 to 18).
- **4.1.4 Adult**. A list of characters is given for the morphological identification of adults.
- **Appendix – Table of characteristics that separate larvae of *Thaumatotibia* from some other Lepidoptera taxa (page 258).** A table provides characters that separate larvae of *Thaumatotibia* from some other Lepidoptera taxa, specifically closely related taxa and taxa frequently found on the same commodities as *T. leucotreta*.

This validation study took into account the **list of diagnostic characters at paragraph 4.1.2.2 (from 7 to 18)** and not the table of characteristics in Appendix. This table of characteristics allows a preliminary screening of specimens and the identification of a larva suspected to be *T. leucotreta* must be accomplished with the full description given in the main document (paragraph 4.1.2.2). The decision not to include the table of characteristics in the validation is justified by the fact that each specimen of the sample panel will be carefully checked against the list of character in the description, making the validation of the table of characters in Appendix unnecessary.
Descriptions of eggs, first instars larvae (L1-L2) and pupae are also provided in the diagnostic protocol, but it is stated that, for these life stages, *T. leucotreta cannot be identified to the species level using morphology only and rearing to later instars or molecular identification is needed for reliable identification* (page 251).
Identification of the adult stage (male and female) is not considered in this study.

2.2.2 Molecular identification

Molecular tests can support morphological identifications of *T. leucotreta*, especially of early instar larvae. The EPPO barcoding protocol (EPPO PM 7/129 (2) was validated, as well as two pest-specific real-time PCRs (Rizzo *et al*., 2021). Pest-specific real-time PCRs (a TaqMan and a SYBR Green qPCR) and a LAMP test are included in this publication. The LAMP test was not further considered in this validation. Reasons for that were that the LAMP test targets the same genetic region as one of the validated real-time PCR tests and that the practicability of this LAMP for onsite diagnostics bases on rather erratic visual (color changes) interpretation. Real-time LAMP fluorescence reading would however require advanced molecular laboratory facilities (real-time PCR cycler) counteracting the advantages of such methods like onsite detection.

Protocol: EPPO PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests (EPPO, 2021a), Appendix 1

- DNA barcoding of arthropods. DNA barcoding is used to identify the arthropods at a certain taxonomic level. DNA barcoding is described as reliable tool to identify all life stages of *T. leucotreta* to the species level. The chosen marker region is the mitochondrial cytochrome c oxidase I (*COI*) gene. Two different primer sets (LCO1490/HCO2198 and LepF/LepR), targeting this gene, were validated.

Protocol: Pest-specific real-time PCRs according to Rizzo *et al.* (2021).

- Pest-specific real-time PCRs (a TaqMan and a SYBR Green qPCR) were validated. The primer set for SYBR Green qPCR (Tleuco_266F/Tleuco_359R) targets the (*COI*) gene, the TaqMan primer set (Tleuco_1001F/Tleuco_1070R/ Tleuco_1041P) targets the internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence.

2.3 Composition of the sample panel

A panel of samples of 46 mid-to-late instar larvae was used. It consisted of larval specimens belonging to target and non-target species (14 taxa). Table 1 provides a summary of the sample panel. Not all samples were used for both the validation of the morphological test and the molecular tests. For the detailed composition and use of the panel of samples, see Appendix 1 of this document. For the validation of some performance characteristics with the molecular tests additional, adapted sample panels were prepared including some adult Tortricidae specimens (see 3.3.2 – Molecular tests).
Target specimens originated from 4 different countries (Israel, South Africa, Togo, Zimbabwe). Most of the non-target specimens belonged to the family Tortricidae and were selected primarily based on the close similarity to the target species ("look-alikes") and the availability in the reference collections of ANSES and AGES. In addition, some non-target specimens belonged to the families Crambidae, Gelechiidae and Pyralidae and were selected as they share with *T. leucotreta* either a host plant or the geographic distribution or both. The origin of the non-target specimens was variable, including African, American, Asian, and European countries. After randomization, each sample was re-labelled (coded) with numbers from 1 to 46 by supervisors. Original codification of samples was available only to supervisors. For uniformity, all samples were preserved in single tubes, filled with either 70 or 96% ethanol.
The composition of the set was chosen to allow the evaluation of sensitivity, specificity, repeatability, reproducibility and accuracy of the tests.

Table 1: Summary of the composition of the sample panel

Species	Total Number of specimens	Country of origin	Host plant
Thaumatotibia leucotreta	10 [A, B]	Israel, South Africa, Togo, Zimbabwe	*Citrus paradisi, Citrus reticulata, Citrus sinensis, Fortunella, Punica granatum, Solanum aethiopicum*
Cryptophlebia peltastica	8 [A, B]	Réunion Island, South Africa	*Litchi sinensis*
Apomyelois ceratoniae	4 [A, B]	Israel, Morocco, South Africa, Tunisia	*Citrus paradisi, Citrus reticulata, Punica granatum*
Cydia pomonella	3 [A, B]	France	*Juglans regia, Malus sp.*
Epinotia thapsiana	3 [A, B]	Tunisia	*Ferula communis*
Gymnandrosoma aurantianum	3 [A, B]	Brazil	*Citrus sinensis, Psidium guajava*
Anarsia sp.	2 [A, B]	Tunisia	*Prunus armeniaca*
Cryptoblabes sp.	2 [B]	Israel	*Punica granatum*
Grapholita funebrana	2 [B]	France	*Prunus persica*
Grapholita molesta	2 [A, B]	France, Uruguay	*Malus sp., Prunus persica*
Leucinodes orbonalis	2 [A, B]	Cambodia	*Solanum melongena*
Talponia batesi	2 [A, B]	Dominican Republic	*Solanum torvum*
Lobesia botrana	2 [A, B]	France	*Vitis vinifera*
Cacoecimorpha pronubana	1 [A, B]	France	*n.d.*
TOTAL NUMBER	**46**	-	-

[A] validation of morphological test
[B] validation of molecular tests

3. Validation of the tests

3.1 Performance characteristics assessed

According to the guidance given in PM 7/98 (5) (EPPO, 2021b) and the definitions given in PM 7/76 (5) (EPPO, 2018), PM 7/122 (1) (EPPO, 2014) and EPPO PM 7/129 (2) (EPPO, 2021), validation of diagnostic tests relies on the evaluation of the following performance characteristics: sensitivity, specificity, reproducibility, repeatability and accuracy.

Table 2 shows the criteria that were used to calculate the performance characteristics of the tests in this study.

Table 2: Definition and calculation of performance characteristics

Performance criteria	Definition	Calculation
Diagnostic specificity	The proportion of non-target samples (true negatives) testing negative compared with results from an alternative test (or combination of tests) *Comments: as far as possible, the evaluation of specificity must include samples from non-target organisms that can be confused with the target species*	Diagnostic specificity = true negatives/(true negatives + false positives)
Analytical specificity	**Inclusivity:** The performance of a test with a range of target organisms covering genetic diversity, different geographical origin and hosts	-
	Exclusivity: The performance of a test with regards to cross-reaction with a range of non-targets (e.g. closely related organisms)	-
Diagnostic sensitivity	The proportion of target samples (true positives) testing positive compared with results from an alternative test (or combination of tests)	Diagnostic sensitivity = true positives/(true positives + false negatives)
Analytical sensitivity	The smallest amount of target that can reliably be detected. In the case of molecular test, it is referred to as "limit of detection", i.e. the lowest DNA concentration of the target organism that can be reliably detected). For DNA barcoding the limit of detection is the DNA concentration that is sufficient to generate an amplicon which can be sequenced and leading to a HQ consensus sequence of at least 99%	-
Repeatability	The level of agreement between replicates of a sample tested under the same conditions	% level of agreement
Reproducibility	The ability of a test to provide consistent results when applied to aliquots of the same sample tested under different conditions (e.g. time, persons, equipment, location)	% level of agreement
Accuracy	The proportion of target samples (true positives) testing positive and non-target samples (true negatives) testing negative compared with the total number of samples It is worth noting that the accuracy is a global criterion which can be subdivided, to refine the analysis, into three other criteria: sensitivity, specificity and repeatability	Accuracy = (true positives + true negatives)/(true positives + false negatives + true negatives + false positives)

3.2 Performance characteristics already available

EPPO PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests (EPPO, 2021a), performance characteristics:

- Analytical sensitivity: DNA concentration (PCR amplicon) of 4ng/µl sufficient for high quality amplicon sequencing
- Analytical specificity: The interspecific variation of the gene locus was determined to be sufficient for identification at species level
    - Inclusivity: Summary list of identified arthropods in Appendix 1 (Table 1) of the standard
- Diagnostic sensitivity: 98-100%

Performance characteristics for pest-specific real-time TaqMan PCR (Rizzo *et al.* 2021):

- Diagnostic specificity: 100%
- Analytical specificity:
    - Inclusivity: 19 target specimens from South Africa
    - Exclusivity: 24 non-target species from Italy, Ecuador, USA, Afghanistan, Kenya, and South Africa
- Diagnostic sensitivity: 100%
- Analytical sensitivity: The limit of detection (LoD) of the assay was 0.02pg/µl of target DNA

According to the authors, only samples below a Ct of 35 are considered positive.

Performance characteristics for pestspecific real-time SYBR Green PCR (Rizzo *et al.* 2021):

- Diagnostic specificity: 100%
- Analytical specificity:
    - Inclusivity: 19 target specimens from South Africa
    - Exclusivity: 24 non-target species from Italy, Ecuador, USA, Afghanistan, Kenya, and South Africa
- Diagnostic sensitivity: 100%

Analytical sensitivity: The limit of detection (LoD) of the assay was 3.2pg/µl of target DNA (given by the authors for the cut-off at Ct 22).

3.3 Validation protocol

3.3.1 Morphological test

A panel of 30 specimens was analysed by three operators, belonging to the two different institutes (AGES and ANSES). The set composition was defined by the supervisors and known to the supervisors only.
Supervisors provided operators with the Check Lists and Summary Result sheet in Appendix 2, but did not provide operators with origin and host plants data. During the analysis, to be carried out at a stereomicroscope, operators have filled the Check List for each sample and record the identification results on the Summary Result sheet. The results of the identification were expressed as:
- POSITIVE, if **all** the characters of the specimens matched with those of *T. leucotreta*;
- NEGATIVE, if **not all** the characters of the specimens matched with those of *T. leucotreta*.

If the matching of characters was ambiguous, operators were required to highlight which characters lead to the ambiguity and which parts in the protocol are weak (Notes column in the Summary Result sheet).
After the analysis, the Summary Result sheets have been retrieved by the supervisors. In case of deviations of the results from the expected ones, the Check List allowed the supervisors to precisely identify any critical issues within the protocol.
Performance characteristics were assessed according to the following plan:

- Diagnostic sensitivity and specificity were assessed on the basis of the analysis of the whole sample panel carried out by operator 2 (ANSES).
- Repeatability was assessed on the basis of the analysis of the whole sample panel carried out by operator 3 (ANSES) (three repetitions of analysis).
- Reproducibility was assessed on the basis of the analysis of the whole sample panel carried out by operator 1 (AGES), 2 and 3 (ANSES) (first of the three repetitions of analysis).

After the morphological analyses were performed by the three operators, the majority of the specimens used for the morphological validation were transferred to the molecular team in AGES for the validation of the molecular tests.
Figure 1 provides a scheme of the activity.

3.3.2 Molecular tests

DNA extraction

For DNA extraction of whole adult specimens (e.g. reproducibility) the DNeasy Blood & Tissue Kit (Qiagen) was used destructively, whereas larvae (for validation of specificity) were extracted non-destructively using the same kit.

Analytical specificity

Sample panel: An adapted set of specimens as for the morphological analysis was considered for the validation of the molecular tests (Appendix 1).
Inclusivity: 10 targets
Exclusivity: 28 non-targets

The primer sets and PCR parameters are described in Appendix 3.
SANGER sequencing was outsourced to a certified sequencing service provider (EUROFINS Genomics).
Data-analysis: The software Geneious prime® 10.1.3 was used for the consensus sequence preparation. For sequence alignment, the following genetic databases were consulted: NCBI-GenBank, Bold and EPPO Q-bank.

In silico testing: The analytical specificity for the barcoding primer sets (LCO1490/HCO2198 and LepF/LepR) and the primer sets for real-time PCRs (Tleuco_1001F/Tleuco_1070R/Tleuco_1041P; Tleuco_266F/Tleuco_359R) were tested *in silico* by a database alignment (NCBI- GenBank), as well as *in silico* PCRs (see Appendix 6).

Analytical sensitivity and repeatability

Sample panel:
1 larva of *T. leucotreta* from Israel (sample 7)
1 larva of *T. leucotreta* from South Africa (sample 12)
1 larva of *T. leucotreta* from Togo (sample 32)

Analytical sensitivity: 3 samples were prepared in different dilutions. Three experiments were performed with adequate dilutions of this sample panel for each test, respectively.
Dilutions (1:10, 1:100, 1:1.000; 1:10.000; 1: 100.000, 1: 1.000.000, 1: 10.000.000).
To define the limit of detection for DNA barcoding, the two highest dilutions from which amplicons could be generated, were sequenced and analysed.

Repeatability: Three biological replicates of *T. leucotreta* (dilution near by the detection limit) were analysed with 3 technical repetitions to determine the repeatability.

Reproducibility

Sample panel for testing reproducibility of the PCR tests:
Three targets and three non-targets were used to test the reproducibility of the PCR tests (Table 3). The targets were part of the specificity sample panel as well (corresponding to the samples 12, 28, and 32, respectively), whereas the non-targets were additional extracts of adult specimens (destructive DNA extraction, see 6.2).

The tests were performed under different conditions (two operators on different days and using different thermocycler machines).

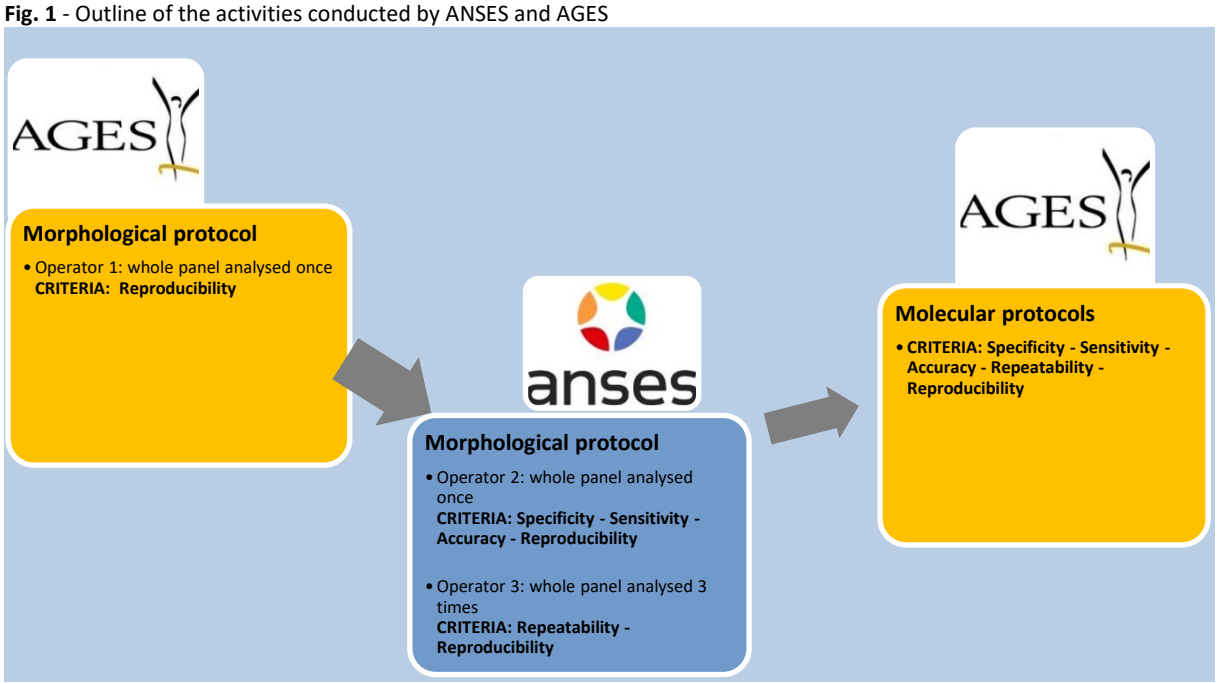
Table 3:–Sample panel for the evaluation of the reproducibility of the molecular *T. leucotreta* identification

Target	**Non target**	**Origin**
Target 1 *Thaumatotibia leucotreta* – larva (sample 12)		South Africa
Target 2 *Thaumatotibia leucotreta* – larva (sample 28)		Israel
Target 3 *Thaumatotibia leucotreta* – larva (sample 32)		Togo
	Non-target 1 *Cydia pomonella* - adult	Austria
	Non-target 2 *Lobesia botrana* - adult	Austria
	Non-target 3 *Eupoecilia ambiguella* - adult	Austria

Sample panel for testing reproducibility of the SANGER sequence analysis:
The reproducibility of the SANGER sequence analysis was tested with the sample panel described above. The sequence analysis was performed by two operators on different days. The alignment of the consensus sequence was performed in three different data bases (NCBI GenBank, Bold, and EPPO Q-Bank).

Specifications and parameters for the molecular tests are provided in Appendix 3.

Fig. 1 - Outline of the activities conducted by ANSES and AGES



4. Performance adequacy and validation

The final step was the comparison of the performance values obtained by the method with the predetermined, expected performance characteristics. The adequate expected performance characteristics are shown in Table 4. They are also referred to as "limits of acceptability" of the test. These performance characteristics were extrapolated from each publication, respectively. In the case of the analytical sensitivity of the SYBR Green real-time PCR according to Rizzo *et al.* (2021), the described LoD for the cutoff value of 22 (3.2 pg/µl) is not congruent with the values given in Table 4 in this publication. For this validation study, the expected sensitivity of 2ng/µl was selected based on the values for Ct <22 given by the authors in this table.

If the obtained performance characteristics did not reach the expected values, a cause analysis was carried out to identify the critical steps in the test(s) that led to the unexpected results (i.e. false negatives, false positives).

Table 4: Expected performance characteristics (limits of acceptability)

Performance criteria	Expected performance characteristics			
	EPPO PM 7/137 (1) *Thaumatotibia leucotreta* Morphological identification	EPPO PM 7/129 DNA Barcoding[a]	Rizzo *et al.* (2021) Real time PCR - TaqMan probe	Rizzo *et al.* (2021) Real-time PCR – SYBR Green
Diagnostic specificity	100%	100%	100%	100%
Analytical specificity (Inclusivity)	100%	100%	100%	100%
Analytical specificity (Exclusivity)	100%	100%	100%	100%
Diagnostic sensitivity	100%	100%	100%	100%
Analytical sensitivity	1 adult specimen	4ng/µl	0.02pg/µl	2ng/µl
Repeatability	100%	100%	100%	100%
Reproducibility	100%	100%	100%	100%
Accuracy	100%	100%	100%	100%

[a] as from Appendix 1, paragraph 4 of EPPO PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests (EPPO, 2021a)

5. Time schedule and staff

The trial period was from March to April 2022 for the morphological analysis and from June to December 2022 for the molecular analysis and involved staff from the EURL for Insects and Mites.

Participating staff:

- for morphological tests:
  Experts/ Supervisors: Sylvia Blümel, Andrea Taddei
  Technical staff/ Operators: Anna Moyses, Jean-Marie Ramel, Philippe Reynaud

- for molecular tests:
  Experts/ Supervisors: Richard Gottsberger, Helga Reisenzein
  Technical staff/ Operators: Claudia Heiss, Chiara Pohn

6. Results of the validation analysis

6.1 Morphological test

Protocol: EPPO PM 7/137 (1) *Thaumatotibia leucotreta* (EPPO, 2013)

The values obtained for diagnostic specificity, diagnostic sensitivity and accuracy met the expected value of 100% (Table 5). The test was found to be 100% inclusive for target specimens from Israel and South Africa and collected from *Citrus paradisi, Citrus reticulata, Fortunella sp., Punica granatum.* It proved to be 100% exclusive for a range of non-target specimens belonging to the family Tortricidae (*C. pronubana, C. peltastica, C. pomonella, E. thapsiana, G. molesta, G. aurantianum, T. batesi, L. botrana*) and to other Lepidoptera families (Crambidae, Gelechiidae and Pyralidae) that share with *T. leucotreta* either host plant or geographic distribution or both (*Anarsia sp., A. ceratoniae, L. orbonalis*).
The value obtained for repeatability did not meet the expected value of 100% but reached a value of 98.9%. The cause was found in the divergent results obtained by operator 3 for two positive samples, namely one *T. leucotreta* specimen (26) identified as negative in all three repetitions of analysis and one *T. leucotreta* specimen (28) identified as negative in one of the three repetitions. The divergent result obtained for *T. leucotreta* specimen coded 26 affected the reproducibility as well, which obtained a value of 98.9%.
The Check Lists compiled by operators during the performance of the analyses allowed to track back the potential critical steps in the protocol that led to the deviation from the expected result.

Appendix 4 of this document shows the results obtained by the three operators.
Appendix 5 shows the calculations for the performance characteristics.

Table 5: Summary of the results obtained for the morphological test

Performance criteria	Definition	Calculation	Expected performance characteristics	Obtained performance characteristics
Diagnostic specificity	The proportion of non-target samples (true negatives) testing negative compared with results from an alternative test (or combination of tests)	Diagnostic specificity = true negatives/(true negatives + false positives)	**100%**	**100%**
Analytical specificity	**Inclusivity:** The performance of a test with a range of target organisms covering genetic diversity, different geographical origin and hosts	-	**100%**	**100%** [A]
	Exclusivity: The performance of a test with regards to cross-reaction with a range of non-targets (e.g. closely related organisms)	-	**100%**	**100%** [B]
Diagnostic sensitivity	The proportion of target samples (true positives) testing positive compared with results from an alternative test (or combination of tests)	Diagnostic sensitivity = true positives/(true positives + false negatives)	**100%**	**100%**
Analytical sensitivity	The smallest amount of target that can be detected reliably	-	**1 adult specimen**	**1 adult specimen**
Repeatability	The level of agreement between replicates of a sample tested under the same conditions	% level of agreement	**100%**	**98.9%**
Reproducibility	The ability of a test to provide consistent results when applied to aliquots of the same sample tested under different conditions (e.g. time, persons, equipment, location)	% level of agreement	**100%**	**98.9%**
Accuracy	The proportion of target samples (true positives) testing positive and non-target samples (true negatives) testing negative compared with the total number of samples	Accuracy = (true positives + true negatives)/(true positives + false negatives + true negatives + false positives)	**100%**	**100%**

[A] Geographical origin: Israel, South Africa
[B] Non-target species: *Anarsia sp., Apomyelois ceratoniae, Cacoecimorpha pronubana, Cryptophlebia peltastica, Cydia pomonella, Epinotia thapsiana, Grapholita molesta, Gymnandrosoma aurantianum, Leucinodes orbonalis, Talponia batesi, Lobesia botrana*

6.2 Molecular tests

For the goal of species identification in animals and some protists the cytochrome c oxidase subunit 1 (*COI*) gene of the mitochondrial DNA has been introduced as standard marker. DNA sequencing of the *COI* DNA barcode can be applied to distinguish several Tortricidae species.

Pest-specific end-point and real-time PCRs are a powerful tool when identifying unknown specimens, as they allow for rapid identification of certain pests during import control. TaqMan probe-based real-time PCRs can be highly sensitive, detecting target DNA amounts usually in the range of pico- or femtogram.

Sufficient amount and quality of sample DNA is crucial when performing molecular tests. Especially when non-destructive DNA extraction methods are used, or when the samples have been stored for long, the retrieval of such DNA can be difficult. In this validation study, for the specificity sample panel, larvae were extracted non-destructively, after having been stored in ethanol, whereas the non-target adult samples for the reproducibility were extracted destructively (both with the Qiagen DNeasy Blood and Tissue Kit). Additionally, nine already extracted DNA samples (larvae, non-destructive) were included in this study.

Unfortunately, it was not possible to attain sufficient high-quality DNA in the case of sample 42 of the specificity sample panel, which was therefore excluded from the molecular study and all following performance characteristics were calculated accordingly

Protocol: EPPO PM 7/129(2) DNA barcoding as an identification tool for a number of regulated pests (EPPO, 2021a)

EPPO PM7/129(2), Appendix 1 – DNA barcoding of arthropods (sequencing of *COI* locus, LCO1490/HCO2198 and LepF/LepR primer sets).

***In silico* testing of analytical specificity** by a database alignment (NCBI GenBank) was performed with the DNA barcoding primer sets (LCO1490/HCO2198 and LepF/LepR). The search set was limited to "*Thaumatotibia leucotreta*" (taxid:463830). The results showed suitability of both primer sets (see Appendix 6) for identification of *T. leucotreta*, although we must state that barcoding is a generic test including targets and non-targets.

The values obtained for **analytical specificity** (inclusivity and exclusivity) met the expected values (Table 6).
Sequencing of the *COI* locus was able to fully discriminate all listed species, except for two non-target samples, assigned as *Talponia batesi*. At the time of this study, no sequences for this species were available in the databases, except one sequence in BOLD deposited as "Tortricidae sp., probably *Talponia batesi*".
The test was found to be 100% inclusive for *T. leucotreta* from South Africa, Israel, Togo, and Zimbabwe. The following non-targets could be distinguished: *Anarsia sp., Cryptophlebia peltastica, Grapholita molesta, Gymnandrosoma aurantianum, Cydia pomonella, Cacoecimorpha pronubana, Epinotia thapsiana, Leucinodes orbonalis, Lobesia botrana, Apomyelois ceratoniae, Grapholita funebrana, Cryptoblabes sp.* (see Appendix 7).

The **analytical sensitivity** with both primer sets also easily met the expected value of 4ng/µl.
The **reproducibility of the PCR** tests using two different primer sets and **reproducibility of the SANGER sequence analysis** were 100% in all cases. The same is true for the **repeatability**, reaching 100% (Table 6).

The **diagnostic sensitivity and specificity** as well as **accuracy** were 100% for both validated tests (Table 6).

Appendix 6 displays the results of the *in silico* testing of analytical specificity.
Appendix 7 of this document shows the detailed results for analytical specificity.
Appendix 8 shows the results for analytical sensitivity, repeatability and reproducibility.
Appendix 9 shows the calculations for the diagnostic sensitivity, diagnostic specificity and accuracy.

Table 6: Summary of the results obtained for the molecular protocol – EPPO PM7/129 (2), Appendix 1, *COI* gene locus

Performance criteria	Definition	Calculation	Expected performance characteristics PM 7/129 (EPPO, 2021a)	Obtained performance characteristics for sequencing of *COI* (primer set LCO1490/HCO2198)	Obtained performance characteristics for sequencing of *COI* (primer set LepF/LepR)
Diagnostic specificity	The proportion of non-target samples (true negatives) testing negative compared with results from an alternative test (or combination of tests)	Diagnostic specificity = true negatives/(true negatives + false positives)	**100%**	**100%**	**100%**
Analytical specificity	**Inclusivity:** The performance of a test with a range of target organisms covering genetic diversity, different geographical origin and hosts	-	**100%**	**100%**	**100%** [A]
	Exclusivity: The performance of a test with regards to cross-reaction with a range of non-targets (e.g. closely related organisms)	-	**100%**	**100%**	**100%** [B]
Diagnostic sensitivity	The proportion of target samples (true positives) testing positive compared with results from an alternative test (or combination of tests)	Diagnostic sensitivity = true positives/(true positives + false negatives)	**100%**	**100%**	**100%**
Analytical sensitivity	The smallest amount of target that can be detected reliably	-	**4ng/µl**	**4pg/µl**	**42pg/µl**
Repeatability	The level of agreement between replicates of a sample tested under the same conditions	% level of agreement	**100%**	**100%**	**100%**
Reproducibility	The ability of a test to provide consistent results when applied to aliquots of the same sample tested under different conditions (e.g. time, persons, equipment, location)	% level of agreement	**100%**	**100%**	**100%**
Accuracy	The proportion of target samples (true positives) testing positive and non-target samples (true negatives) testing negative compared with the total number of samples	Accuracy = (true positives + true negatives)/(true positives + false negatives + true negatives + false positives)	**100%**	**100%**	**100%**

[A] Geographical origin: Israel, South Africa, Togo, Zimbabwe.

[B] Non-target species: *Anarsia sp., Apomyelois ceratoniae, Cacoecimorpha pronubana, Cryptoblabes sp., Cryptophlebia peltastica, Cydia pomonella, Epinotia thapsiana, Grapholita funebrana, Grapholita molesta, Gymnandrosoma aurantianum, Leucinodes orbonalis, Talponia batesi, Lobesia botrana*.

Pest-specific real-time PCRs according to Rizzo *et al.* 2021

Two *Thaumatotibia leucotreta*-specific real-time PCRs (TaqMan and SYBR Green) according to Rizzo *et al.* 2021 were included in this validation study.

a. TaqMan real-time PCR

The *Thaumatotibia leucotreta*-specific TaqMan real-time PCR according to Rizzo *et al.* was included in this validation study and performed according to the parameters given in Appendix 3.

***In silico* testing of analytical specificity** by a database alignment (NCBI GenBank) was performed with the primer/probe set for the TaqMan real-time PCR (Tleuco_1001F/Tleuco_1070R/Tleuco_1041P). They were aligned with restricting the search set to Tortricidae. Additionally, in silico PCRs were performed. *In silico* specificity could be shown (see Appendix 6).

Analytical specificity:
Inclusivity: the tests were found to be 100% inclusive for *Thaumatotibia leucotreta* from South Africa, Israel, Togo, and Zimbabwe. The Ct values ranged from 15.51 to 20.27.
Exclusivity: in total, 27 non-targets from 13 species were tested (*Anarsia sp., Cryptophlebia peltastica, Grapholita molesta, Gymnandrosoma aurantianum, Cydia pomonella, Cacoecimorpha pronubana, Epinotia thapsiana, Leucinodes orbonalis, Lobesia botrana, Apomyelois ceratoniae, Talponia batesi, Grapholita funebrana, Cryptoblabes sp.*).

Surprisingly, most of the larval non-target samples showed Ct values ranging from 29.66 to 38.39, only four samples (samples 21, 43, 44, and 46) were clearly negative, respectively (see Appendix 7, Table B). Of the nine tested DNA samples two showed Ct values (32.19 and 37.42), while all others were negative. Six of the non-target samples (sample 6, 8, 16, 19, 23, and 39) showed Ct values even below a Ct of 35, meaning they would have been suspected to be positive by the original authors (Rizzo *et al.* 2021).
However, the Ct values of these samples were still much higher compared to the positive samples, indicating a contamination with low amounts of *Thaumatotibia leucotreta* DNA. Despite this, these samples were unambiguously identified as non-targets by barcoding in the previous experiments. Therefore, these contaminations were not considered for the calculation of the diagnostic specificity.
To gain further insights, spike and recovery experiments were performed (see chapter 7 analysis of critical issues, 7.2).

The performance characteristics for the TaqMan real-time PCR are described in Table 7.
The expected **analytical sensitivity** values (0.02pg/µl) were met.
The expected analytical specificity (inclusivity as well as exclusivity) was met.
Reproducibility and repeatability of the real-time PCRs met the expected 100% , respectively.

The calculated values for the **diagnostic specificity**, as described above, met the expected values.
The values obtained for **diagnostic sensitivity and accuracy** met the expected values .

Appendix 6 displays the results of the *in silico* testing of analytical specificity.
Appendix 7 of this document shows the detailed results for analytical specificity.
Appendix 8 shows the results for analytical sensitivity, repeatability and reproducibility.
Appendix 9 shows the calculations for the diagnostic sensitivity, diagnostic specificity and accuracy.

Table 7: Summary of the results obtained for the molecular test real-time TaqMan PCR according to Rizzo *et al.* 2021

Performance criteria	Definition	Calculation	Expected performance characteristics	Obtained performance characteristics
Diagnostic specificity	The proportion of non-target samples (true negatives) testing negative compared with results from an alternative test (or combination of tests)	Diagnostic specificity = true negatives/(true negatives + false positives)	**100%**	**100%***
Analytical specificity	**Inclusivity:** The performance of a test with a range of target organisms covering genetic diversity, different geographical origin and hosts	-	**100%**	**100%** [A]
	Exclusivity: The performance of a test with regards to cross-reaction with a range of non-targets (e.g. closely related organisms)	-	**100%**	**100%** [B]
Diagnostic sensitivity	The proportion of target samples (true positives) testing positive compared with results from an alternative test (or combination of tests)	Diagnostic sensitivity = true positives/(true positives + false negatives)	**100%**	**100%**

Performance criteria	Definition	Calculation	Expected performance characteristics	Obtained performance characteristics
Analytical sensitivity	The smallest amount of target that can be detected reliably	-	**0.02pg/µl**	**20fg/µl**
Repeatability	The level of agreement between replicates of a sample tested under the same conditions	% level of agreement	**100%**	**100%**
Reproducibility	The ability of a test to provide consistent results when applied to aliquots of the same sample tested under different conditions (e.g. time, persons, equipment, location)	% level of agreement	**100%**	**100%**
Accuracy	The proportion of target samples (true positives) testing positive and non-target samples (true negatives) testing negative compared with the total number of samples	Accuracy = (true positives + true negatives)/(true positives + false negatives + true negatives + false positives)	**100%**	**100%**

* The identified contaminations were not considered for the calculation of the diagnostic specificity.
[A] Geographical origin: Israel, South Africa, Togo, Zimbabwe.
[B] Non-target species: *Anarsia sp., Apomyelois ceratoniae, Cacoecimorpha pronubana, Cryptoblabes sp., Cryptophlebia peltastica, Cydia pomonella, Epinotia thapsiana, Grapholita funebrana, Grapholita molesta, Gymnandrosoma aurantianum, Leucinodes orbonalis, Talponia batesi, Lobesia botrana*.

b. SYBR Green real-time PCR

The *Thaumatotibia leucotreta*-specific SYBR Green real-time PCR according to Rizzo *et al.* was included in this validation study and performed according to the parameters given in Appendix 3. For this test, the cut-off of Ct 22 (as described in the publication) was applied.

***In silico* testing of analytical specificity** by a database alignment (NCBI GenBank) was performed with the primer set for the SYBR Green real-time PCR (Tleuco_266F/Tleuco_359R). They were aligned restricting the search set to Tortricidae. Additionally, *in silico* PCRs were performed. These *in silico* data analysis revealed possible specificity issues of this test: for example, only two mismatches for *Gymnandrosoma aurantianum* on the 5´ end of the reverse primer as well as only one mismatch on the first base of the 5´ end of the forward primer. There are indications that such unspecific bindings may occur with other species from the Tortricidae group as well (see Appendix 6).

Analytical specificity:
The melting peak temperature was not suitable to discriminate target from non-targets (data not shown). Therefore, only Ct values were considered.
Inclusivity: the tests were found to be 100% inclusive for *Thaumatotibia leucotreta* larvae from South Africa, Israel, Togo, and Zimbabwe. The Ct values ranged from 12.93 to 19.08.
Exclusivity: in total, 27 non-targets from 13 species were tested (*Anarsia sp., Cryptophlebia peltastica, Grapholita molesta, Gymnandrosoma aurantianum, Cydia pomonella, Cacoecimorpha pronubana, Epinotia thapsiana, Leucinodes orbonalis, Lobesia botrana, Apomyelois ceratoniae, Talponia batesi, Grapholita funebrana, Cryptoblabes sp.*).
All non-targets except for *Gymnandrosoma aurantianum* resulted in Ct values higher than 22. One out of two *G. aurantianum* samples had a Ct value below the cut-off (20.4) and was assigned as a false positive value. The second *G. aurantianum* sample showed a Ct value of 26.90. Due to the applied cut-off value (Ct 22) this sample was considered as negative.
It has to be be stated that the contamination of the non-targets with low amounts of *T. leucotreta* DNA did not have an influence on the performance characteristics (Appendix 7, Table B).

The performance characteristics for the SYBR Green real-time PCR are described in Table 8.

The expected **analytical sensitivity** values (2ng/µl) were met.
Diagnostic sensitivity, reproducibility and repeatability of the SYBR Green real-time PCR met the expected 100% .

The values obtained for **analytical specificity (exclusivity), diagnostic specificity and accuracy** did not meet the expected values .

Appendix 6 displays the results of the *in silico* testing of analytical specificity.
Appendix 7 of this document shows the detailed results for analytical specificity.
Appendix 8 shows the results for analytical sensitivity, repeatability and reproducibility.

Appendix 9 shows the calculations for the diagnostic sensitivity, diagnostic specificity and accuracy.

Table 8: Summary of the results obtained for the molecular test real-time SYBR Green PCR according to Rizzo *et al.* 2021

Performance criteria	Definition	Calculation	Expected performance characteristics	Obtained performance characteristics
Diagnostic specificity	The proportion of non-target samples (true negatives) testing negative compared with results from an alternative test (or combination of tests)	Diagnostic specificity = true negatives/(true negatives + false positives)	**100%**	**96%**
Analytical specificity	**Inclusivity:** The performance of a test with a range of target organisms covering genetic diversity, different geographical origin and hosts	-	**100%**	**100%** [A]
	Exclusivity: The performance of a test with regards to cross-reaction with a range of non-targets (e.g. closely related organisms)	-	**100%**	**96% *** [B]
Diagnostic sensitivity	The proportion of target samples (true positives) testing positive compared with results from an alternative test (or combination of tests)	Diagnostic sensitivity = true positives/(true positives + false negatives)	**100%**	**100%**
Analytical sensitivity	The smallest amount of target that can be detected reliably	-	**2ng/µl**	**65pg/µl**
Repeatability	The level of agreement between replicates of a sample tested under the same conditions	% level of agreement	**100%**	**100%**
Reproducibility	The ability of a test to provide consistent results when applied to aliquots of the same sample tested under different conditions (e.g. time, persons, equipment, location)	% level of agreement	**100%**	**100%**
Accuracy	The proportion of target samples (true positives) testing positive and non-target samples (true negatives) testing negative compared with the total number of samples	Accuracy = (true positives + true negatives)/(true positives + false negatives + true negatives + false positives)	**100%**	**97%**

* One *G. aurantianum* sample tested false positive.

[A] Geographical origin: Israel, South Africa, Togo, Zimbabwe.

[B] Non-target species: *Anarsia sp., Apomyelois ceratoniae, Cacoecimorpha pronubana, Cryptoblabes sp., Cryptophlebia peltastica, Cydia pomonella, Epinotia thapsiana, Grapholita funebrana, Grapholita molesta, Gymnandrosoma aurantianum, Leucinodes orbonalis, Talponia batesi, Lobesia botrana*.

7. Analysis of critical issues

7.1 Morphological identification of T. leucotreta

When performing the analyses, operator 3 had doubts on the interpretation of the character (11) *L-Pinaculum on prothorax enlarged and extending beneath and beyond the spiracle* for samples 26 and 28, claiming that the posterior margin of the pinaculum was not clearly visible. This lead operator 3 to consider these two positive samples as negative. Operator 2, despite identifying samples 26 and 28 as positive, made the same remark in the notes concerning the doubtful interpretation of this character on these two specimens (dubious criteria 11). All target specimens were checked by supervisor. It was observed that that L-pinaculum on prothorax of samples 26 and 28 was not evenly pigmented on at least one side, making it more difficult to define its posterior margin with respect to the spiracle (Fig. 2 and 3). It should be noted that the spinules that cover the integument are not present on the pinacula surface, which is smooth. This characteristic can be useful to determine the extent of the L-pinaculum when it is not strongly and evenly pigmented (Fig. 4).

While performing the analyses, operators identified some other characters that deserve commenting. It has to be noted that the way in which these characters are currently described in the diagnostic protocol did not affect the correct identification (expressed in its qualitative form as positive/negative) of all samples in the panel of samples. These characters are listed below:

- **size of fully-grown larvae**: two positive larvae (sample code 18 and 30) are significantly larger than described in the diagnostic protocol (i.e. 7-10 mm); it seems that the length range given underestimates the actual size of fully-grown larvae, but this has to be confirmed on a larger number of individuals;
- **setae MD1, MSD1, MSD2 and MV** are difficult to see, as it is already stated in the text, and are not marked in the setal map (Fig. 19), which makes it necessary for a lesser experienced entomologist to check their position in other bibliographic resources, e.g. Stehr (1987), Fig. 5;
- L-Pinaculum on prothorax: the position of L-pinaculum might be better defined with respect to the spiracle, e.g. *extending beneath and beyond (**posterad of**) the spiracle*, as from Lepintercept fact sheet (Gilligan & Passoa, 2014) and not only *extending beneath and beyond the spiracle*;

As for other general remarks, it was observed that

- the order of the diagnostic characters could be better chosen to facilitate the analysis, starting from the head to the last abdominal segment, and grouping them based on position on the body. It is suggested that the information that diagnostic characters (14) to (18) are not unique for *T. leucotreta* is added in the note above Fig. 13 in the diagnostic protocol. A possible new order is proposed here below as example (in brackets the original order in the DP is shown):
    - (7) **Prothorax** with three L-setae, all on the same pinaculum
    - (11) L-Pinaculum on **prothorax** enlarged and extending beneath and beyond the spiracle. This characteristic will separate it from the larva of many other Tortricidae
    - (13) **Abdominal segments** with SD2 seta present and anteroventral (in front and below) of SD1 on the same pinaculum (magnification 30X or higher; easiest to see on segments 5–7). In *Cryptophlebia* (e.g. C*. peltastica*) SD2 on at least abdominal segments 5–7 is on a separate pinaculum
    - (14) Usually the numbers of SV-setae on **abdominal segments** A1, 2, 7, 8 and 9 are, respectively, 3, 3, 2, 2 and 1 (in C. peltastica usually 3, 3, 3, 2, and 2, but some specimens have 3, 3, 2, 2 and 1, like T. leucotreta).
    - (8) Crochets on ventral **prolegs** in a full circle
    - (16) Abdominal **prolegs** with 29–48 crochets, irregularly tri-ordinal, slightly shorter and uni-ordinal on the lateral
    - (15) SD pinaculum is in front of the spiracle on abdominal **segment 8**
    - (9) Abdominal **segment 9** with D2 setae on shared saddle pinaculum
    - (10) Abdominal **segment 9** with D1 and SD1 setae on the same pinaculum
    - (17) L-group on A9 (= abdominal **segment 9**) usually trisetose (with all setae usually on the same pinaculum, but L3 can be on a separate pinaculum as well)
    - (18) **Skin** densely covered with short spinules (magnification 30X or higher)
    - (12) **Anal comb** normally present, with 4–10 teeth (occasionally up to 13), but usually with 5–8 teeth. In some larvae the anal comb is not well developed, and the teeth are reduced in number or length, or the anal comb can be completely absent. If the anal comb is absent, characteristic 13 should be checked;
- in Appendix, the sign used for the last footnote below the table (‡‡) is not linked to any item in the table, while it should probably replace the asterisk (*) at the right of "Yes" at the crossing between *Anal comb present* and Thaumatotibia *spp*. In addition, the asterisk (*) at the crossing between *L-pinaculum on T1* and *Crambidae* should probably be removed as it is not referred to the note * *For example,* Helicoverpa armigera *and* Spodoptera *spp*.;
- in Fig. 15, the writing *SD1 + D1 op 1pinaculum* should probably be corrected to *SD1 + D1 **on** 1 pinaculum.*

Fig. 2 – Sample 26 (*T. leucotreta*), prothorax in lateral view: (A) right side and (C) same picture with L-pinaculum highlighted, (B) and (D) blow-ups of (A) and (C) respectively; (E) left side and (G) same picture with L-pinaculum highlighted, (F) and (H) blow-ups of (E) and (G), respectively.

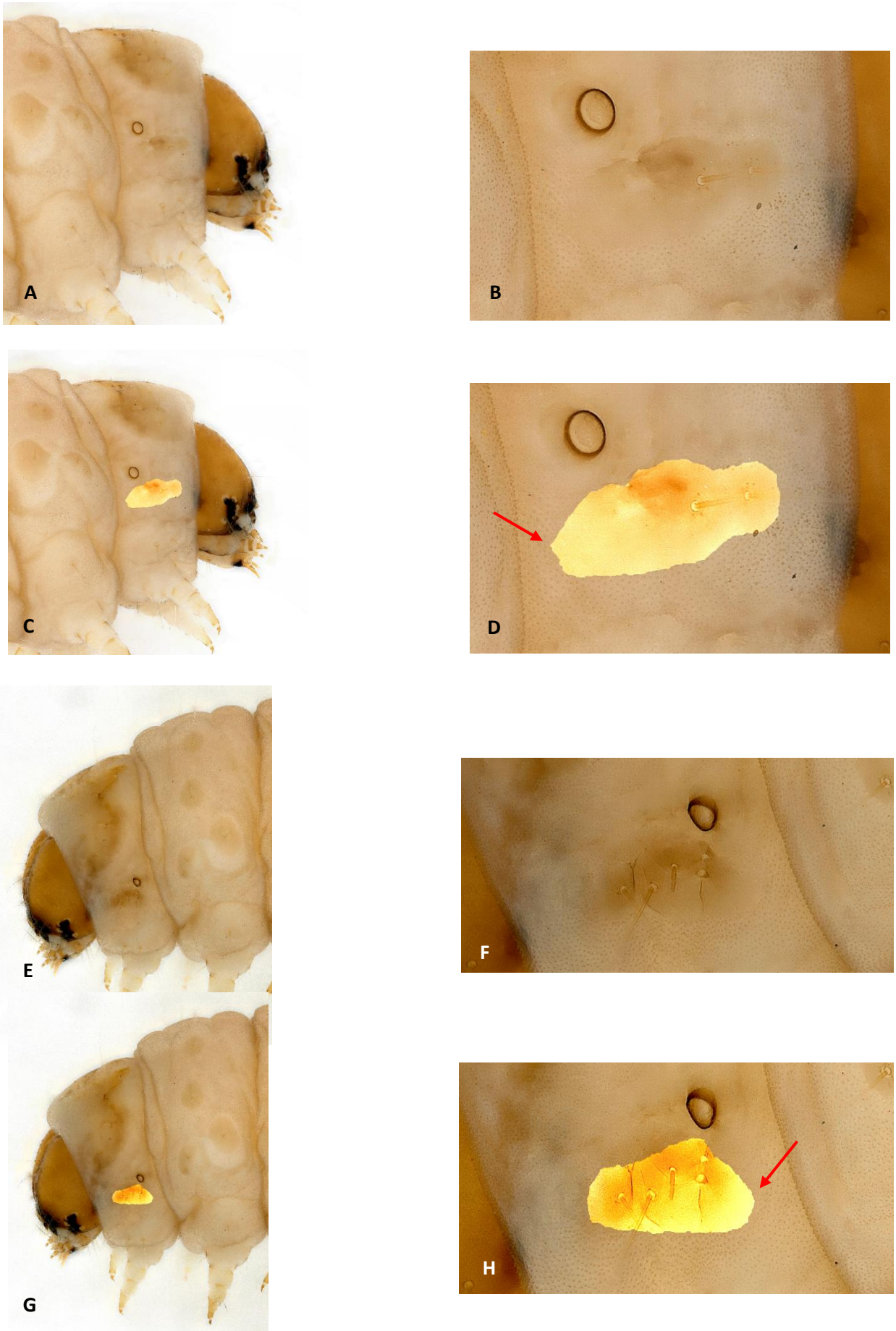


Fig. 3 – Sample 28 (*T. leucotreta*), prothorax in lateral view: (A) right side and (C) same picture with L-pinaculum highlighted, (B) and (D) blow-ups of (A) and (C) respectively; (E) left side and (G) same picture with L-pinaculum highlighted, (F) and (H) blow-ups of (E) and (G), respectively.

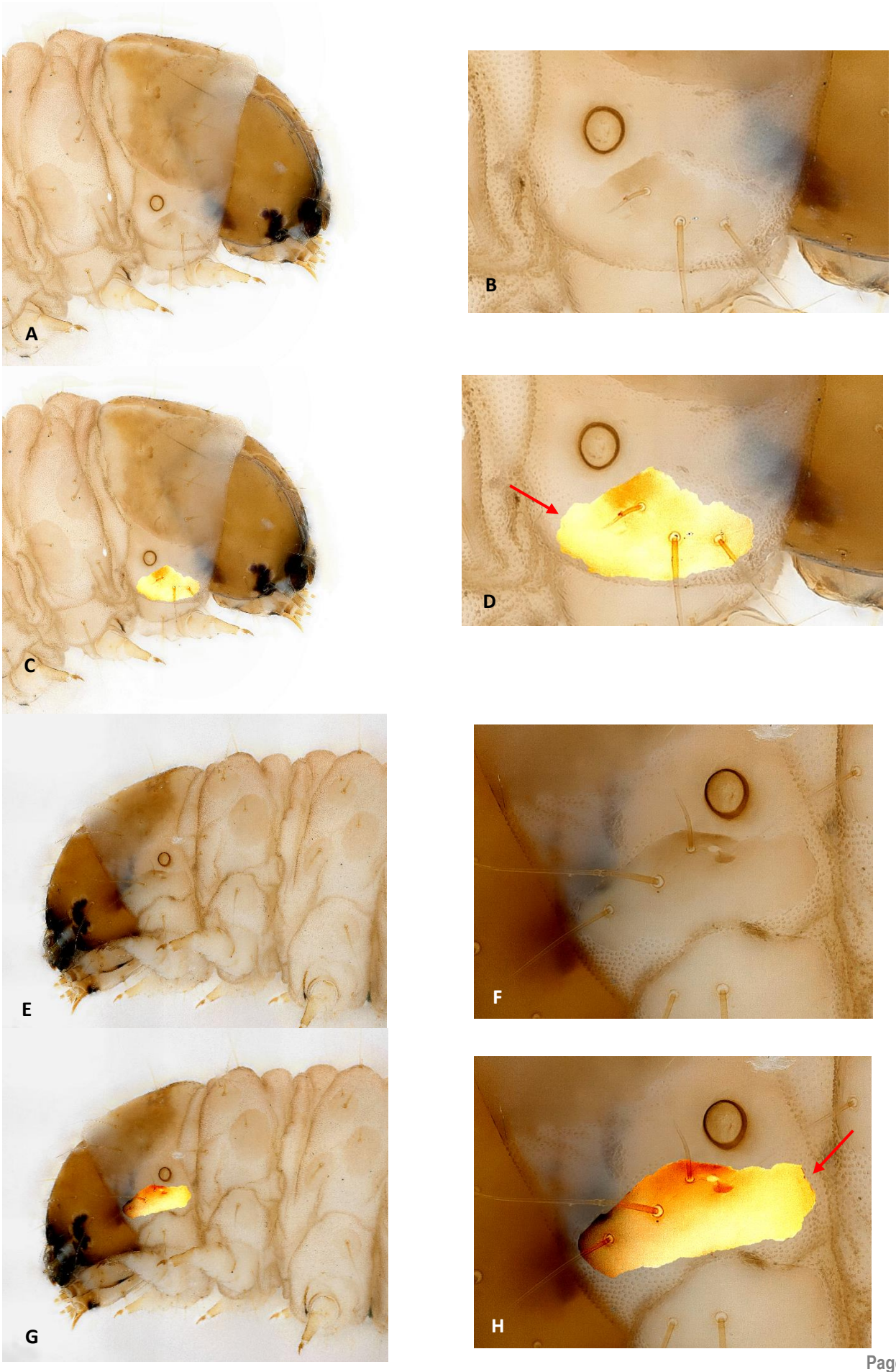


Fig. 4 – *T. leucotreta* prothorax in lateral view, head on the left: (A) in sample 7, L- pinaculum is well pigmented so that the posterior margin is clearly distinguishable; (B) in sample 26, L- pinaculum is less pigmented and its contour less visible, making it more difficult to distinguish posterior margin. In this case, the posterior margin of L-pinaculum can be deduced as the boundary between the tegument with and without spinules.

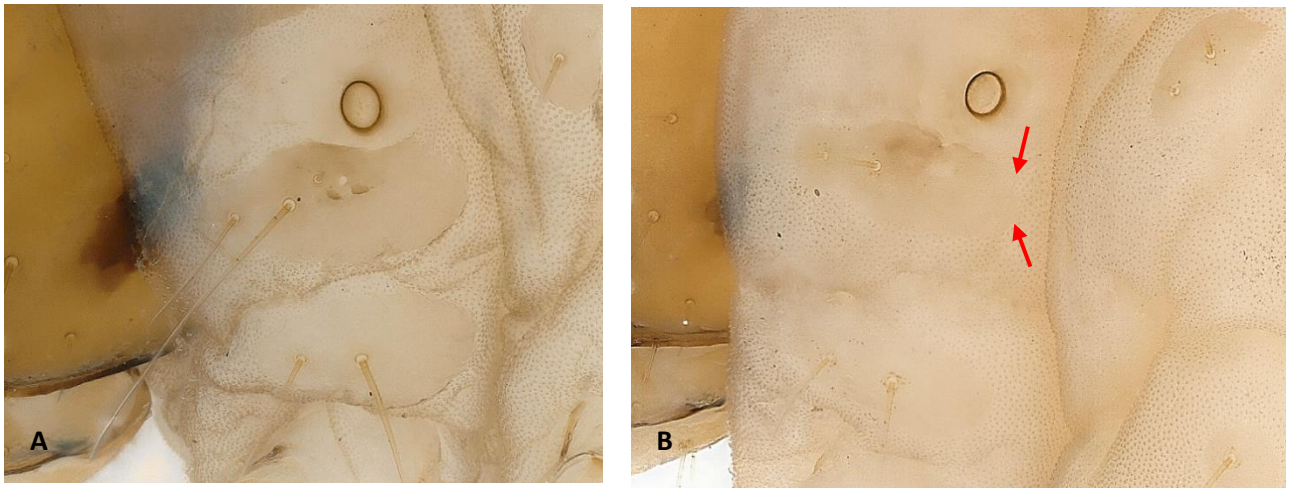
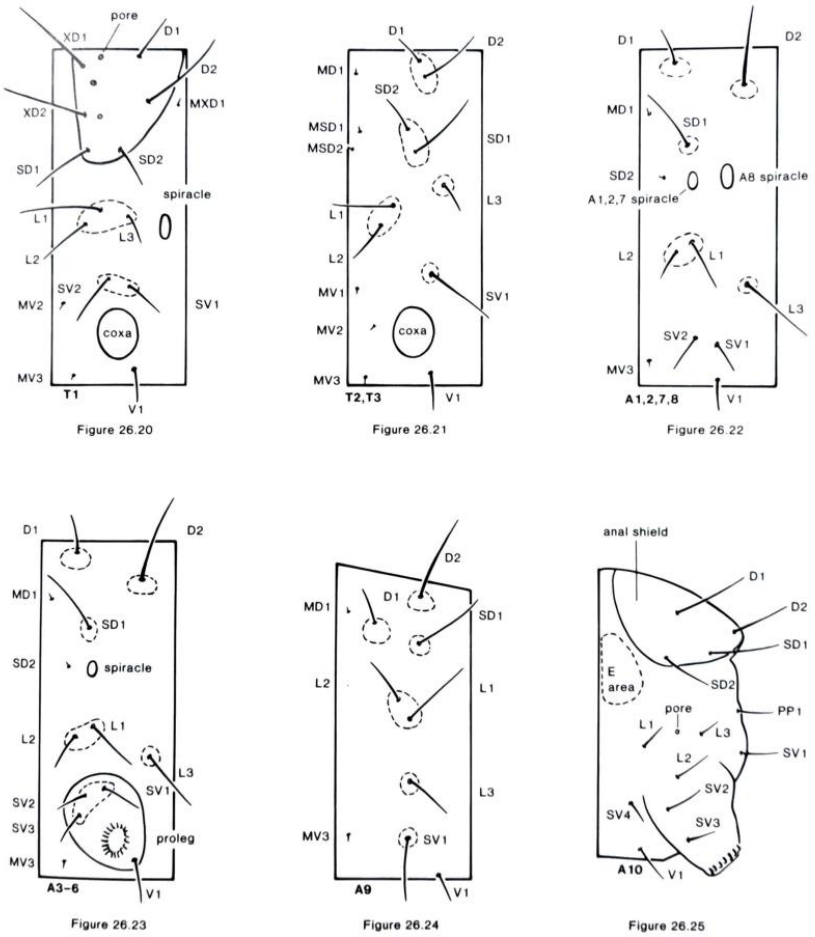


Fig. 5 – Representation of setal maps of a generic larva of Lepidoptera (Stehr, 1987). Micro-setae MD1, MSD1, MSD2 and MV are included in the representation.



Figures 26.20–26.25. Setal maps: **26.20.** T1; **26.21.** T2, T3; **26.22.** A1, A2, A7, A8; **26.23.** A3–6; **26.24.** A9; **26.25.** A10.
See pages 299–304 and tables 26.1 and 26.3–26.5.

7.2 Molecular identification of T. leucotreta

7.2.1 Inconsistencies in the publication according to Rizzo et al. (2021)

During the evaluation of existing performance characteristics in literature, several inconsistencies such as conflicting information or lack of data were seen in the publication according to Rizzo *et al*. (2021).

Sample panel composition

19 targets (one larva and 18 adults from South Africa) and 24 non-target species (8 Lepidoptera, 4 Tortricidae) were used for testing the analytical specificity of the assays in the original publication. All adults were obtained from a mass-rearing facility in South Africa, while the larva was intercepted in Italy. According to the EPPO PM 7/98 (5), this indicates limited intraspecies variability in this samples set. The selection of non-target species was focused on host plants and commodities. This does not reflect an appropriate range of closely related species, which could lead to cross reactions.

Analytical sensitivity – SYBR Green assay

Inconsistencies in terms of analytical sensitivity were also observed in the publication. In the abstract they give the limit of detection as 0.128pg/µl. However, in the section describing the performance characteristics of the assay Rizzo *et al*. state `The LoD was equal to 0.02 pg/µl for the qPCR probe protocol. In qPCR SYBR Green assay, LoD value was determined at 0.128 pg/µl of DNA; if the Cq cut-off value of 22 is adopted, the LoD is 3.2 pg/µl´. This information is not congruent with the data given in table 4, where a 3.2 pg/µl dilution corresponds with a Cq value of 29.58.

Fig 6 - Table 4 of Rizzo *et al*. 2021 depicting the limit of detection assay, relevant information for interpretation of analytical sensitivity and cut-off values are highlighted in yellow.

Table 4. LoD assay using 1:10 serial dilutions (from 10 ng/µl to 0.02 fg/µl) and 1:5 serial dilutions (from 10 to 25.6)

	qPCR probe (1)		qPCR SYBR Green (2)	Real-time LAMP (3)	Visual LAMP (3)
Dilutions 1:10	Cq means ± SD	Dilutions 1:5	Cq means ± SD	Cq means ± SD	positive (+)/negative (–)
10 ng/µl	14.47 ± 0.40	10 ng/µl	18.84 ± 0.06	12.32 ± 0.37	+
1.0 ng/µl	17.28 ± 0.15	2.0 ng/µl	20.82 ± 0.12	13.41 ± 0.39	+
0.02 ng/µl	20.55 ± 0.19	0.4 ng/µl	23.06 ± 0.25	14.35 ± 0.74	+
0.002 ng/µl	23.52 ± 0.08	0.08 ng/µl	25.32 ± 0.32	16.25 ± 1.20	+
2 pg/µl	26.47 ± 0.12	0.016 ng/µl	27.21 ± 0.10	17.48 ± 0.91	+
0.2 pg/µl	29.12 ± 0.09	3.2 pg/µl	29.58 ± 0.12	21.29 ± 4.16	+
0.02 pg/µl	31.85 ± 0.17	0.64 pg/µl	32.04 ± 0.30	24.61 ± 7.76	+
0.2 fg/µl	n/a	0.128 pg/µl	34.80 ± 1.49	28.97 ± 0.78	+
0.02g/µl	n/a	25.6 fg/µl	n/a	n/a	–

Cq value is the mean of the three threshold cycles of each dilution. Cq values above 35 were considered as negative results. (1) qPCR probe (1001F/1070R/1041P); (2) qPCR SYBR Green (266F/359R); (3) Real-time and visual LAMP.

Analytical sensitivity – TaqMan assay

In the section `Assay Conditions of the qPCR TaqMan Probe, SYBRGreen, and LAMP Protocol´ the authors state `Samples were considered positive when the resulting qPCR curves showed an evident inflection point (in addition to increasing kinetics) and Cq values less than 35´. No further clarifications or data are provided for this statement.

7.2.2 Considerations of employing SYBR green real-time PCR and cut-off values for the identification of insect specimens

SYBR green real-time PCRs using melting curve analysis offers the advantage of identification and characterisation of PCR products with respect to their melting characteristics. This was not possible for the validated SYBR green real-time PCR, because there was no significant deviation in melting peak temperature between targets and non-targets. The conclusions had to be drawn on Ct-values/cut-off values only, carrying the risk of deviating results. Ct-values are generally influenced by DNA amount and quality. For identification of insect specimens this is relevant because the DNA amount and quality can be influenced by different developmental stages, different storage conditions of the specimens, sample preparation and subsequent DNA extraction. Therefore, setting a cut-off value without examination of all these parameters can lead to erratic results.

7.2.3 *Possible risks of employing highly sensitive TaqMan real-time PCR for the identification of insect specimens*

In routine diagnosis, the goal is usually identification of whole insect specimens, whereas detection of trace amounts is not the objective. Highly sensitive methods can imply the risk of false positives, when dealing with contaminations even at very low levels. Such contaminations were seemingly detected during this validation study (see Appendix 7, Table B).
Follow-up experiments were conducted to retrace possible ways of contamination and to provide handling and interpretation guidelines for the application of this highly sensitive identification method in routine diagnosis.

Follow up experiments

a. Testing of samples from the specificity sample panel

As a first step, the storage ethanol from selected samples was tested: a) contaminated *Cydia pomonella* samples, b) non-contaminated non-target samples, c) *Thaumatotibia leucotreta* samples. 70µl ethanol were extracted using the Qiagen DNeasy Blood and Tissue Kit and the DNA extracts analysed via *Thaumatotibia leucotreta*-specific real-time PCR.
Only ethanol from *Thaumatobia leucotreta* samples resulted in positive Ct values ranging from 23.92 to 29.32.

b. Spike and recovery experiment:

In a next step, a spike and recovery experiment with *Cydia pomonella* larvae and contaminated storage ethanol was performed (Appendix 10).

Nine *Cydia pomonella* larvae were contaminated with different amounts of storage ethanol from tubes containing *Thaumatotibia leucotreta* larvae (Fig. 7). After incubation at room temperature for four days, the larval DNA was extracted non-destructively. Additionally, the storage ethanol as well as dH_2O, used to wash the samples before DNA extraction, were extracted to monitor the contamination at different steps.

Fig. 7 - Experimental setup for the contamination experiment.

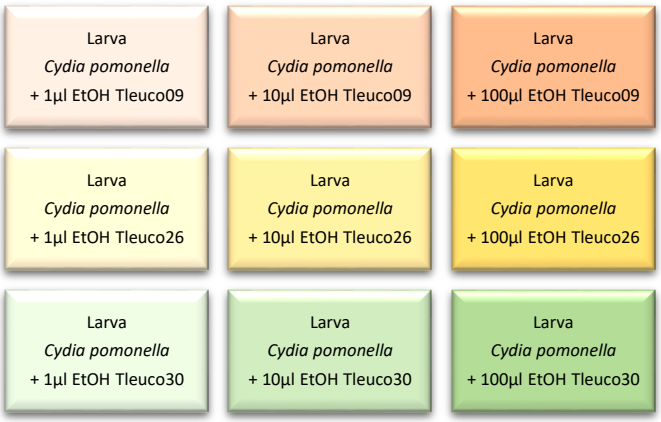
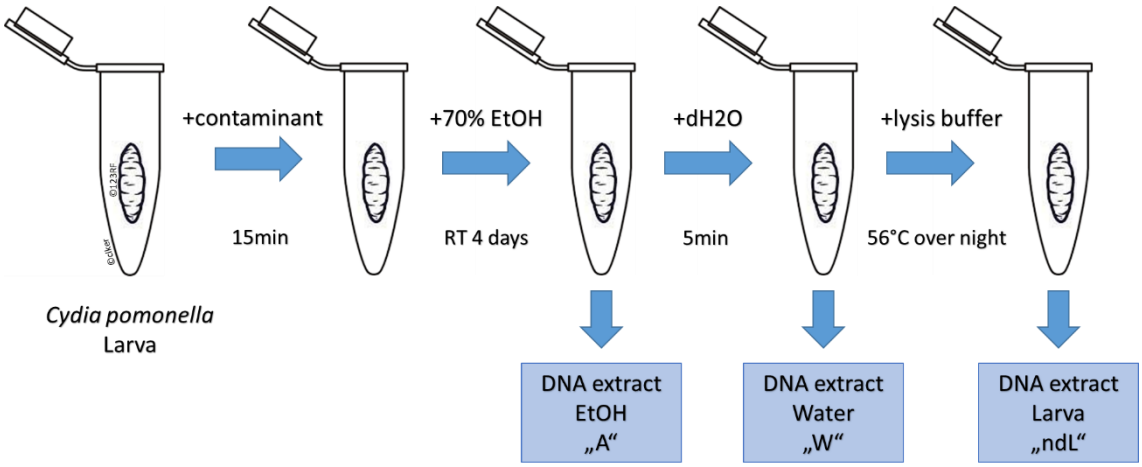


Fig. 8 - Workflow of the contamination experiment.



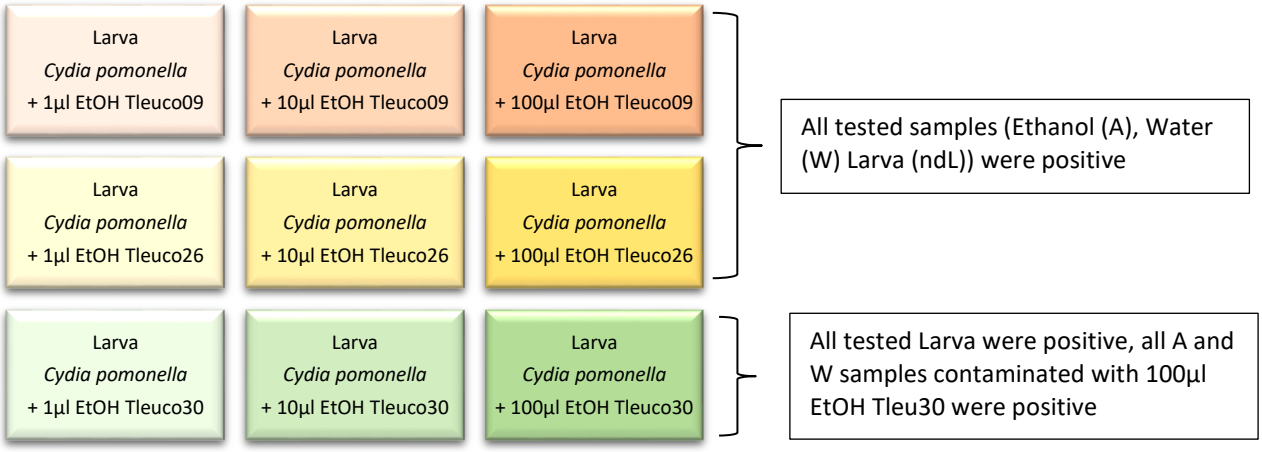
Samples were analysed via *Thaumatotibia leucotreta*-specific TaqMan real-time PCR as well as generic barcoding PCR and resulting amplicons sent for sequencing.

Results of the spike and recovery experiment:

In all larval samples *Thaumatotibia leucotreta* DNA could be detected, with Ct values ranging from 23.38 to 33.00 (depending on the DNA concentration in the contaminated source ethanol). Both the ethanol and the water used in the washing step yielded positive Ct values in most cases (77.78%), but with higher Ct values compared to the corresponding larval samples.

As expected, contamination source ethanol with higher Ct values yielded higher Cts in the contaminated samples, with only ethanol from sample Tleuco30 (Ct 29.32) resulting in contaminated water and ethanol samples without Ct value.

Fig. 9 - Results of the spike and recovery experiment.

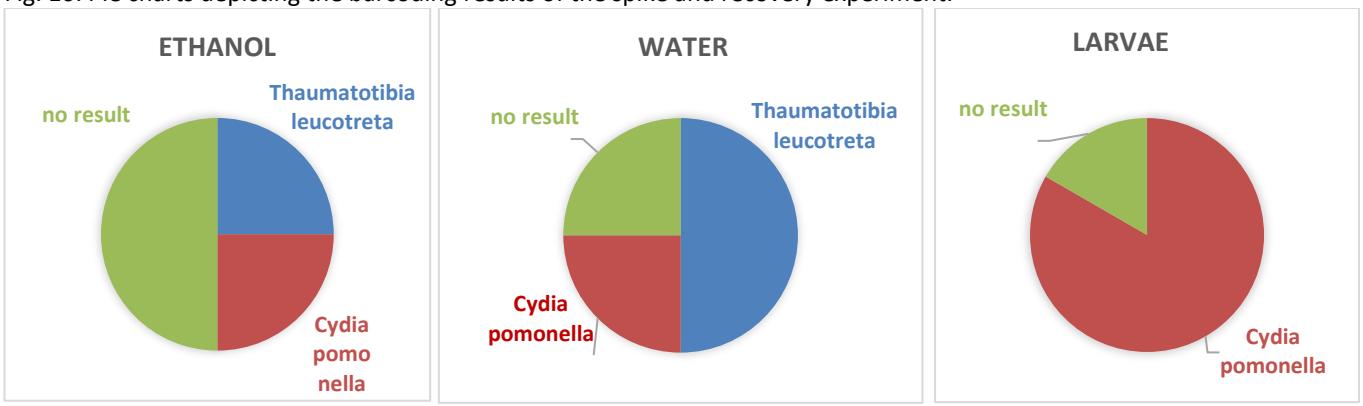


These spike and recovery experiments clearly show the risk of storage ethanol being a source of contamination. Using highly sensitive pest-specific real-time PCRs such as the one published by Rizzo *et al*., can lead to false positive results, especially when employing non-destructive DNA extraction methods.

To evaluate the influence of this kind of contaminations on the barcoding results, all larval extracts, water and ethanol samples were tested with the LepF/R primer set.
7 out of 9 contaminated samples could be identified correctly, despite the contamination with *T. leucotreta* DNA. However, only three samples resulted in consensus sequences of high quality (>95% HQ). One sample resulted in 92.71 percent identity

with “Philodinidae sp.”, which is not a reliable result. Another did not result in any consensus sequence due to low sequence quality.
Sequences from ethanol and water revealed a higher proportion of the contaminant (*T. leucotreta*), but *Cydia pomonella* could be detected as well, but to a lower extend (see Fig. 10).
This indicates, that in our experiment, even highly contaminated *Cydia pomonella* samples could still be identified as such by barcoding, with the contamination at most resulting in lower sequence quality due to insufficient peak separation. This is known to happen and have been observed when barcoding composite samples. It has to be taken into consideration, that particularly in such cases quality criteria need to be met for reliable barcoding results.

Fig. 10: Pie charts depicting the barcoding results of the spike and recovery experiment.



8. Discussion and conclusions

This study aimed at the validation of the EPPO diagnostic protocol PM 7/137 (1) for the morphological identification of *Thaumatotibia leucotreta* at the larval stage. For the molecular identification of *T. leucotreta*, the EPPO PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests, and the pest-specific real-time PCR according to Rizzo *et al.* (2021) were validated. The study has involved staff of the EURL for Insects and Mites from ANSES and AGES and the analytical activities were carried out from March to April 2022 for the morphological and from June to December 2022 for the molecular parts. A main sample panel of 46 Lepidoptera samples, including target and non-target specimens, was used. Additionally, smaller sample panel have been prepared to validate the molecular tests.

Morphological diagnostic test

The morphological identification of larval specimens according to the diagnostic protocol EPPO PM 7/137 (1) achieved the expected value of 100% for the validation criteria diagnostic specificity, diagnostic sensitivity and accuracy. The test was 100% inclusive for *T. leucotreta* specimens originated from South Africa and Israel and collected from different host plants (*Citrus paradisi, Cirtus reticulata, Fortunella* sp.*, Punica granatum*) and 100% exclusive for a number of non-target specimens belonging to the families Tortricidae, Pyralidae, Crambidae and Gelechiidae (see Appendix 1).
Repeatability and Reproducibility reached the slightly lower value of 98.9%. This was due to the divergent results obtained by operator 3 for the samples 26 and 28, which was two *T. leucotreta* specimens identified as negative samples. The cause was detected in the uneven pigmentation of the prothoracic pre-spiracular L-pinaculum, which made it difficult to detect the posterior margin of the pinaculum. The extension of this pinaculum below and beyond the spiracle is one of the key characters for the identification of this pest. Operator 2 also confirmed the dubious presence of this character on samples 26 and 28. In this case, the posterior margin of the pinaculum can be detected by observing the limit between the area of the integument which is covered by spinules and the area which is not. In fact, the surface of all pinacula is smooth in this species, whereas the surrounding integument is not (Fig. 4). It could be useful to add this hint to the diagnostic protocol.

Operators made also other remarks and suggestions for improvement of the diagnostic protocol, namely:

- the size of some fully-grown larvae exceeds the indicated size of 7-10 mm: it seems that the length range given underestimates the actual size of mature larvae, but this has to be confirmed on a larger number of individuals; according to CABI (2022), the size of mature larvae is 15-20 mm. Size is not considered as a diagnostic character for this species, but it can be very useful to estimate the developmental stage;
- setae MD1, MSD1, MSD2 and MV are difficult to see: it could be very useful to mark their position in the setal map at page 253 or to include another illustration, as Fig. 5 (Stehr (1987);
- the relative position of L-pinaculum on prothorax might be better defined with respect to the spiracle, e.g. *extending beneath and beyond (posterad of) the spiracle*, as from Lepintercept fact sheet (Gilligan & Passoa, 2014) and not only *extending beneath and beyond the spiracle*; the addition of *posterad of* would make the interpretation of this character unambiguous;
- the order of the diagnostic characters could be better chosen to facilitate the analysis, following the logical order from head to last abdominal segment, and grouping them based on position on the body; this would allow users a less intensive manipulation of specimens and reduce the risk of damage;
- in Appendix, the use of signs ‡‡ and * should probably be reviewed and corrected;
- in Fig. 15, the writing *SD1 + D1 op 1pinaculum* should probably be corrected to *SD1 + D1* ***on*** *1pinaculum.*

Based on these results, the EURL recommends the use of the EPPO PM 7/ 137 (1) to EU National Reference Laboratories for the morphological identification of *Thaumatotibia leucotreta* larval specimens. Nevertheless, the diagnostic protocol could be improved for a better, stand-alone usability and some minor typing mistakes could be corrected.

Molecular diagnostic tests

Two pest-specific real-time PCRs according to Rizzo *et al*. (2021) were validated. *In silico* testing of the TaqMan real-time PCR showed the suitability of primers and probe for the specific identification of *T. leucotreta*, while revealing uncertainties in regard to the specificity of the SYBR Green real-time PCR. This could be confirmed by the experimental data, resulting in 100% analytical specificity for the TaqMan PCR and only 96% for the SYBR Green PCR, where one *Gymnandrosoma aurantianum* sample in this study was detected as false positive (identical melting peak temperatures, no *T. leucotreta* contamination detected with TaqMan real-time PCR in this sample). The observed cross reaction is especially important, as *T. leucotreta* and *G. aurantianum* have the same host range. The melting temperature not being sufficient for resolution is already indicated in the publication. In the section `Assay Conditions of the qPCR TaqMan Probe, SYBR Green, and LAMP Protocol´ the authors state 'In all the qPCR SYBR Green reactions there were very high values of the diagnostic inclusiveness with Cq values always lower than 22 in *T. leucotreta* samples while in some nontarget samples (*Cicadella viridis, Saperda tridentata*) the Cq values were higher than 27, **with the same melting peak temperature**.' Follow up studies for Tortricidae species that are suspected to give false positive results are necessary for the SYBR Green PCR. In the case of the analytical sensitivity of the SYBR Green PCR the described LoD for the cut-off value of 22 (3.2 pg/µl) is not congruent with the values given in table 4 in this publication. For this validation study, the expected sensitivity of 2ng/µl was chosen based on the values for Ct <22 given by the authors in this table into account.

Another performance characteristic to discuss for the TaqMan real-time PCR is the analytical sensitivity. Already given as highly sensitive in the publication (LoD 0.2 pg/µl), in this study this test was able to detect *T. leucotreta* DNA in the range of femtograms (LoD 20 fg/µl). With such a high sensitivity even miniscule contaminations are detectable, as was the case for some of the specificity samples in this study.
This was replicable in a follow-up artificial contamination experiment, where *T. leucotreta* DNA could be detected in storage ethanol containing *T. leucotreta* larvae. Such storage ethanol was used in a spike and recovery setup to retrace possible ways of contamination.
Even very low amounts of *T. leucotreta* contaminated ethanol could be detected in spiked non-target samples (on *C. pomonella* larvae, in their storage ethanol and water used in a washing step). The highest amount of spiked *T. leucotreta* DNA could be recovered from the larvae. This leads to the assumption that DNA from contaminated ethanol might have a higher affinity for biological tissue, further increasing the risk of contamination.
In the light of these findings, it is crucial to provide handling and interpretation guidelines for the application of highly sensitive methods in routine diagnostics as well as for the performance of validation/verification studies using references specimens stored in ethanol.

Recommendations for routine diagnosis:

For the identification of *Thaumatotibia leucotreta* the TaqMan real-time PCR according to **Rizzo *et al.*, 2021** and / or **EPPO PM 7/129(2)** DNA barcoding as an identification tool for a number of regulated pests (Appendix 1) are recommended.

In routine diagnosis, the goal is usually identification of whole insect specimens. Therefore, it is advisable to establish a control chart for the TaqMan real-time PCR, not only to trace the stability of the PAC, but also to define an intra-laboratory Ct range expected for single target-specimens. This can help to correctly interpret real-time PCR results, especially if Ct values are higher than expected.
COI barcoding is recommended for the identification of *T. leucotreta*, because a sequencing approach usually gives more detailed information on the specificity, even in the case of a composite or contaminated sample. Therefore, barcoding is a useful confirmatory test in the identification procedure.
Due to the uncertainties regarding the diagnostic specificity the SYBR Green real-time PCR is not recommended. However, if the SYBR Green real-time PCR is applied, it should not be used as standalone test and the cut-off value has to be established in each laboratory through extensive testing of targets and non-targets.

Date: 17/07/2023

Philippe Reynaud
EURL Director

Helga Reisenzein
EURL Deputy Director

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Appendix 1 – Composition of the sample panel

Sample	Sample original code	Assigned value	Family	Origin	Host plant	Morphological validation	Molecular validation
1	1800632	*Anarsia sp.*	Gelechiidae	Tunisia	*Prunus armeniaca*	●	●
2	1901903_2	*Cryptophlebia peltastica*	Tortricidae	La Reunion	*Litchi sinensis*	●	
3	1301400_2	*Grapholita molesta*	Tortricidae	France	*Prunus persica*	●	●
4	2100283	*Gymnandrosoma aurantianum*	Tortricidae	Brazil	*Psidium guajava*	●	
5	0701527_8	*Cydia pomonella*	Tortricidae	France	*Malus sp.*	●	●
6	1801876_2	*Cydia pomonella*	Tortricidae	France	*Juglans regia*	●	●
7	**2100001**	***Thaumatotibia leucotreta***	**Tortricidae**	**Israel**	***Punica granatum***	●	●
8	800785	*Cacoecimorpha pronubana*	Tortricidae	France	*n.a.*	●	●
9	**1700951**	***Thaumatotibia leucotreta***	**Tortricidae**	**South Africa**	***Citrus paradisi***	●	●
10	2100474_1	*Epinotia thapsiana*	Tortricidae	Tunisia	*Ferula communis*	●	
11	2101094	*Leucinodes orbonalis*	Crambidae	Cambodia	*Solanum melongena*	●	●
12	**1700915b**	***Thaumatotibia leucotreta***	**Tortricidae**	**South Africa**	***Citrus paradisi***	●	●
13	1700261_3	*Grapholita molesta*	Tortricidae	Uruguay	*Malus sp.*	●	●
14	1900224	*Cryptophlebia peltastica*	Tortricidae	South Africa	*Litchi sinensis*	●	
15	1801876_1	*Cydia pomonella*	Tortricidae	France	*Juglans regia*	●	●
16	2100741_6	*Leucinodes orbonalis*	Crambidae	Cambodia	*Solanum melongena*	●	●
17	1301351	*Tortricidae_1 (Lobesia botrana)*	Tortricidae	France	*Vitis vinifera*	●	●
18	**1801433**	***Thaumatotibia leucotreta***	**Tortricidae**	**South Africa**	***Citrus paradisi***	●	●
19	1700496	*Anarsia sp.*	Gelechiidae	Tunisia	*Prunus armeniaca*	●	●
20	1500826	*Apomyelois ceratoniae*	Pyralidae	South Africa	*Citrus paradisi*	●	
21	2100885_3	*Talponia batesi*	Tortricidae	Dominican Republic	*Solanum torvum*	●	●
22	**1701835**	***Thaumatotibia leucotreta***	**Tortricidae**	**South Africa**	***Fortunella***	●	●
23	1301349_3	*Tortricidae_1 (Lobesia botrana)*	Tortricidae	France	*Vitis vinifera*	●	●

24	2100474_2	*Epinotia thapsiana*	Tortricidae	Tunisia	*Ferula communis*	●	
25	2101678	*Apomyelois ceratoniae*	Pyralidae	Morocco	*Punica granatum*	●	
26	**1700376**	***Thaumatotibia leucotreta***	**Tortricidae**	**Israel**	***Citrus reticulata***	●	●
27	2100885_4	*Talponia batesi*	Tortricidae	Dominican Republic	*Solanum torvum*	●	●
28	**1801455**	***Thaumatotibia leucotreta***	**Tortricidae**	**Israel**	***Punica granatum***	●	●
29	1901903_1	*Cryptophlebia peltastica*	Tortricidae	La Reunion	*Litchi sinensis*	●	
30	**2101368**	***Thaumatotibia leucotreta***	**Tortricidae**	**Israel**	***Punica granatum***	●	●
31	1601390_2*	*Cryptophlebia peltastica*	Tortricidae	La Reunion	*Litchi sinensis*		●
32	**2101378**	***Thaumatotibia leucotreta***	**Tortricidae**	**Togo**	***Solanum aethiopicum***		●
33	2100474*	*Epinotia thapsiana*	Tortricidae	Tunisia	*Ferula communis*		●
34	2001382*	*Gymnandrosoma aurantianum*	Tortricidae	Brazil	*Citrus sinensis*		●
35	2001498*	*Apomyelois ceratoniae*	Pyralidae	Tunisia	*Punica granatum*		●
36	1401847_1*	*Grapholita funebrana*	Tortricidae	France	*Prunus persica*		●
37	1401847_2*	*Grapholita funebrana*	Tortricidae	France	*Prunus persica*		●
38	1901903*	*Cryptophlebia peltastica*	Tortricidae	La Reunion	*Litchi sinensis*		●
39	2000039*	*Apomyelois ceratoniae*	Pyralidae	Israel	*Citrus reticulata*		●
40	**1702058**	***Thaumatotibia leucotreta***	**Tortricidae**	**Zimbabwe**	***Citrus sinensis***		●
41	1601376_2*	*Cryptophlebia peltastica*	Tortricidae	La Reunion	*Litchi sinensis*		●
42	1801406	*Cryptoblabes sp.*	Pyralidae	Israel	*Punica granatum*		●
43	1801414	*Cryptoblabes sp.*	Pyralidae	Israel	*Punica granatum*		●
44	1901903_3	*Cryptophlebia peltastica*	Tortricidae	La Reunion	*Litchi sinensis*		●
45	1900224	*Cryptophlebia peltastica*	Tortricidae	South Africa	*Litchi sinensis*		●
46	2100282	*Gymnandrosoma aurantianum*	Tortricidae	Brazil	*Psidium guajava*		●

* Genomic DNA already available, DNA extraction not performeded in this study

Appendix 2 - Check lists for the morphological analysis

Operator		Date	

List of diagnostic characters for the identification of *Thaumatotibia leucotreta* (modified from paragraph 4.1.2.2 at page 258, EPPO PM 7/137 (1) *Thaumatotibia leucotreta*)

Note: Both sides of the larva should be checked; if the setal arrangement appears to be asymmetric (a different number of setae in a group on each side of the larva), usually the higher number is likely to be the correct number.

Diagnostic characteristics for *T. leucotreta*		mark the decision with Y (present) or N (not present); note any comments									
Morphological character		**Sample code**									
7	Prothorax with three L-setae, all on the same pinaculum	…	…	…	…	…	…	…	…	…	…
8	Crochets on ventral prolegs in a full circle (Fig. 17).	…	…	…	…	…	…	…	…	…	…
9	Abdominal segment 9 with D2 setae on shared saddle pinaculum (Figs 15, 16).	…	…	…	…	…	…	…	…	…	…
10	Abdominal segment 9 with D1 and SD1 setae on the same pinaculum (Figs 15, 16).	…	…	…	…	…	…	…	…	…	…
11	L-Pinaculum on prothorax enlarged and extending beneath and beyond the spiracle (Fig. 14). This characteristic will separate it from the larva of many other Tortricidae (see the second paragraph of section 4.1.2.1 Early instars).	…	…	…	…	…	…	…	…	…	…
12	Anal comb normally present, with 4–10 teeth (occasionally up to 13), but usually with 5–8 teeth (Fig. 18). In some larvae the anal comb is not well developed, and the teeth are reduced in number or length, or the anal	…	…	…	…	…	…	…	…	…	…

	comb can be completely absent. If the anal comb is absent, characteristic 13 should be checked.										
13	Abdominal segments with SD2 seta present and anteroventral (in front and below) of SD1 on the same pinaculum (magnification 30X or higher; easiest to see on segments 5–7) (Figs 19 and 20). In Cryptophlebia (e.g. C. peltastica) SD2 on at least abdominal segments 5–7 is on a separate pinaculum (Fig. 21).	…	…	…	…	…	…	…	…	…	…
Other characteristics, not unique for *T. leucotreta*											
14	Usually the numbers of SV-setae on abdominal segments A1, 2, 7, 8 and 9 are, respectively, 3, 3, 2, 2 and 1 (Fig. 19) (in C. peltastica usually 3, 3, 3, 2, and 2, but some specimens have 3, 3, 2, 2 and 1, like T. leucotreta).	…	…	…	…	…	…	…	…	…	…
15	SD pinaculum is in front of the spiracle on abdominal segment 8 (Fig. 19).	…	…	…	…	…	…	…	…	…	…
16	Abdominal prolegs with 29–48 crochets, irregularly tri-ordinal, slightly shorter and uni-ordinal on the lateral side (Fig. 17)	…	…	…	…	…	…	…	…	…	…
17	L-group on A9 usually trisetose (with all setae usually on the same pinaculum, but L3 can be on a separate pinaculum as well) (Figs 15 and 19).	…	…	…	…	…	…	…	…	…	…
18	Skin densely covered with short spinules (magnification 30X or higher) (Figs 17, 20 and 21)										
Additional information on micro-setae, visible at high magnification in late instar. Some flexibility should be adopted here as micro-setae are sometimes hidden in the folds of the integument	Setae MD1, MSD1 and MSD2 are present on the second and third thorax segment, and MD1 on the abdominal segments (for a fully-grown larva visible at magnification 30X or higher with MD1 and MV1 best seen at abdominal segment 9). At magnification 40X or higher MV-setae can also be seen on the second and third thorax segment, and on the abdominal segments (difficult to see: for a fully grown larva best seen on A9).	…	…	…	…	…	…	…	…	…	…

	Results (POS/NEG)	…	…	…	…	…	…	…	…	…	…
	Comments										

Summary Result sheet for the morphological protocol from EPPO PM 7/137 (1) *Thaumatotibia leucotreta*

Operator	
Stereomicroscope	

Sample code	Identification result (POSITIVE/NEGATIVE)	Date of analysis	Notes
01			
02			
03			
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Appendix 3 – Specifications and parameters for the molecular tests

Specification of the PCR Assay 1 (DNA barcoding - *COI*)

Name of the primer incl. sequence, literature reference, fragment length in bp:

LepF: 5'- ATTCAACCAATCATAAAGATATTGG-3'
LepR: 5'- TAAACTTCTGGATGTCCAAAAAAAATCA-3'

Literature: Hajibabaei, M., Janzen, D. H., Burns, J. M., Hallwachs, W., & Hebert, P. D. (2006). DNA barcodes distinguish species of tropical Lepidoptera. Proceedings of the National Academy of Sciences, 103(4), 968-971.

Fragment length: 709bp

PCR - Parameters:

Thermocyler used: Biometra T3000 Thermal cycler

Mastermix: 5x HOT FIREPol® Master Mix, Solis Biodyne:

Composition:		Final concentration:
	Volume per reaction µl	
Water	6	
Mastermix	2	1x
Primer1:	0,5	0,5µM
Primer2:	0,5	0,5µM
∑	9	
DNA	1	

PCR conditions:

	°C	Duration (min., sec.)	Nr. of Cycles
Start	95	15 min	1
Denaturation	95	45 sec	5
Annealing	44	45 sec	
Extension	72	45 sec	
Denaturation	95	45 sec	35
Annealing	49	45 sec	
Extension	72	45 sec	
Final extension	72	7 min	1
Cooling	15	∞	

Specification of the PCR Assay 2 (DNA barcoding - *COI*)

Name of the primer incl. sequence, literature reference, fragment length in bp:

LCO1490: 5'- GGTCAACAAATCATAAAGATATTGG-3'
HCO2198: 5'- TAAACTTCAGGGTGACCAAAAAATCA-3'

Literature: Folmer O, Black M, Hoeh W, Lutz R & Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine, Biology and Biotechnology 3, 294–299.

Fragment length: 709bp

PCR - Parameters:

Thermocyler used: Biometra T3000 Thermal cycler

Mastermix: 5x HOT FIREPol® Master Mix, Solis Biodyne:

Composition:		Final concentration:
	Volume per reaction µl	
Water	6	
Mastermix	2	1x
Primer1:	0,5	0.5µM
Primer2:	0,5	0.5µM
∑	9	
DNA	1	

PCR conditions:

	°C	Duration (min., sec.)	Nr. of Cycles
Start	95	15 min	1
Denaturation	95	30 sec	5
Annealing	45	30 sec	
Extension	72	1 min	
Denaturation	95	30 sec	35
Annealing	51	1 min	
Extension	72	1 min	
Final extension	72	10 min	1
Cooling	15	∞	

Specification of the PCR Assay 3 (TaqMan real-time PCR)

Name of the primer incl. sequence, literature reference, fragment length in bp:

Tleuco_1001F: 5'- CGTCCAAGCATAGCTTTC-3'
Tleuco_1070R: 5'- GAGGCAGACACGATATCC-3'
Tleuco_1041P: FAM- CGACGACGACCATAACAACGC-TQ2*
* TQ2: Tide Quencher™ 2 phosphoramidite

Literature: Rizzo D., Da Lio D., Bartolini L., Cappellini G., Bruscoli T., Salemi C., Aronadio A., Del Nista D., Pennachio F., Boersma N., Rossi E., Sacchetti, P. (2021). Development of Three Molecular Diagnostic Tools for the Identification of the False Codling Moth (Lepidoptera: Tortricidae). Journal of Economic Entomology, 114(4), 1796-1807.

Fragment length: 70bp

PCR - Parameters:

Analytic Jena qTower^3 G (230 V) with accompanying software, Bio Molecular Systems Magnetic Induction Cycler (MIC) with accompanying software.

Mastermix: PerfeCTa qPCR ToughMix® Quanta Bio. Contains AccuStart II Taq DNA polymerase, AccuVue plate loading dye, MgCl2, dNTPs

Composition:		Final concentration:
	Volume per reaction µl	
Water	2	
Mastermix	5	1x
Primer1:	0.5	0.5µM
Primer2:	0.5	0.5µM
Probe	1	0.1µM
∑	9	
DNA	1	

PCR conditions:

Step	°C	Duration (min., sec.)	Nr. of Cycles
Start	95	10 min	1
Denaturation	95	15 sec	45
Annealing/Extension and fluorescence reading	60	60 sec	

Specification of the PCR Assay 4 (SYBR Green real-time PCR)

Name of the primer incl. sequence, literature reference, fragment length in bp:

Tleuco_266_F: 5'- TGGAACAGGATGAACAGT-3'
Tleuco_359_R: 5'- TGCTAGGTGAAGAGAGAAA-3'

Literature: Rizzo D., Da Lio D., Bartolini L., Cappellini G., Bruscoli T., Salemi C., Aronadio A., Del Nista D., Pennachio F., Boersma N., Rossi E., Sacchetti, P. (2021). Development of Three Molecular Diagnostic Tools for the Identification of the False Codling Moth (Lepidoptera: Tortricidae). Journal of Economic Entomology, 114(4), 1796-1807.

Fragment length: 93bp

PCR - Parameters:

Analytic Jena qTower^3 G (230 V) with accompanying software, Bio Molecular Systems Magnetic Induction Cycler (MIC) with accompanying software.

Mastermix: HOT FIREPol® EvaGreen® qPCR Mix (Solis Biodyne)

Composition:		Final concentration:
	Volume per reaction µl	
Water	6	
Mastermix	2	1x
Primer1:	0,5	0.5µM
Primer2:	0,5	0.5µM
∑	9	
DNA	1	

PCR conditions:

Step	°C	Duration (min., sec.)	Nr. of Cycles
Start	95	12 min	1
Denaturation	95	15 sec	45
Annealing	55	30 sec	
Extension and fluorescence reading	68	30 sec	
Melting curve	68-95	0.1°C/s	Stepwise increment

Appendix 4 – Summary Result sheets with the results from the three operators (morphological analysis)

Operator 1	
Instrument	ZEISS SV11
Date of analysis/identification	10/03/22 – 15/03/22

Sample number	Sample original codification	Analysis/Identification	Notes	Expected result	Assigned value
1	1800632	**Negative**	*Very small larva, hard to identify*	Negative	*Anarsia sp.*
2	1901903_2	**Negative**	*Cryptophlebia spp.*	Negative	*Cryptophlebia peltastica*
3	1301400_2	**Negative**	*Very pale larva, hard to identify*	Negative	*Grapholita molesta*
4	2100283	**Negative**	*Cryptophlebia spp.*	Negative	*Gymnandrosoma aurantianum*
5	0701527_8	**Negative**	/	Negative	*Cydia pomonella*
6	1801876_2	**Negative**	*Grapholita spp.*	Negative	*Cydia molesta*
7	2100001	**Positive**	*Thaumatotibia leucotreta*	Positive	*Thaumatotibia leucotreta*
8	800785	**Negative**	*Tortricinae spp.*	Negative	*Cacoecimorpha pronubana*
9	1700951	**Positive**	*Thaumatotibia leucotreta*	Positive	*Thaumatotibia leucotreta*
10	2100474_1	**Negative**	*Cryptophlebia spp.*	Negative	*Epinotia thapsiana*
11	2101094	**Negative**	*Pyralidae spp.*	Negative	*Leucinodes orbonalis*
12	1700915b	**Positive**	*Thaumatotibia leucotreta*	Positive	*Thaumatotibia leucotreta*
13	1700261_3	**Negative**	/	Negative	*Grapholita molesta*
14	1900224	**Negative**	*Cryptophlebia spp.*	Negative	*Cryptophlebia peltastica*
15	1801876_1	**Negative**	/	Negative	*Cydia molesta*
16	2100741_6	**Negative**	*Pyralidae spp.*	Negative	*Leucinodes orbonalis*
17	1301351	**Negative**	/	Negative	*Lobesia botrana*
18	1801433	**Positive**	*T. leucotreta, but >10 mm!*	Positive	*Thaumatotibia leucotreta*
19	1700496	**Negative**	/	Negative	*Anarsia sp.*
20	1500826	**Negative**	*Pyralidae spp.*	Negative	*Apomyelois ceratoniae*
21	2100885_3	**Negative**	/	Negative	*Talponia batesi*
22	1701835	**Positive**	*Thaumatotibia leucotreta*	Positive	*Thaumatotibia leucotreta*
23	1301349_3	**Negative**	*Lobesia?! Hard to identify and to turn it*	Negative	*Lobesia botrana*
24	2100474_2	**Negative**	*Cryptophlebia spp.*	Negative	*Epinotia thapsiana*
25	2101678	**Negative**	/	Negative	*Apomyelois ceratoniae*
26	1700376	**Positive**	*Thaumatotibia leucotreta*	Positive	*Thaumatotibia leucotreta*
27	2100885_4	**Negative**	/	Negative	*Talponia batesi*
28	1801455	**Positive**	*Thaumatotibia leucotreta*	Positive	*Thaumatotibia leucotreta*
29	1901903_1	**Negative**	*Cryptophlebia spp.*	Negative	*Cryptophlebia peltastica*
30	2101368	**Positive**	*T. leucotreta, but >10 mm!*	Positive	*Thaumatotibia leucotreta*

General comments:

1) two positive larvae (sample code 18 and 30) are significantly larger than described in the EPPO PM7/137 -> p. 49
2) some morphological characters that need to be checked should be listed in a different order:
    a. 11 directly after 7
    b. 15 before 14

   Several characters can be checked without changing the position of the larva constantly
3) It makes a difference whether the larva is wet or dry. Some characters, for example setae, are more visible when the larva is dry. However the larva must not become too dry, otherwise it will collapse
4) Some characters are sometimes difficult to see, it depends on how the larva is shaped (e.g. anal comb, position of L-pinaculum)
5) For me it was not possible to check micro-setae

6) Crochets: sometimes it is hard to define the order/arrangement
7) For determination of larvae I recommend a colourless microscopy bowl and a blunt spring steel tweezer + a fine brush.

Operator 2	
Instrument	LEICA M205 c
Date of analysis/identification	29/03/2022 - 30/03/2022

Sample number	Analysis/Identification	Notes	Expected result	Assigned value
1	**Negative**	/	Negative	*Anarsia sp.*
2	**Negative**	/	Negative	*Cryptophlebia peltastica*
3	**Negative**	/	Negative	*Grapholita molesta*
4	**Negative**	/	Negative	*Gymnandrosoma aurantianum*
5	**Negative**	/	Negative	*Cydia pomonella*
6	**Negative**	/	Negative	*Cydia molesta*
7	**Positive**	/	Positive	*Thaumatotibia leucotreta*
8	**Negative**	/	Negative	*Cacoecimorpha pronubana*
9	**Positive**	/	Positive	*Thaumatotibia leucotreta*
10	**Negative**	/	Negative	*Epinotia thapsiana*
11	**Negative**	/	Negative	*Leucinodes orbonalis*
12	**Positive**	Criteria 11: beneath but not beyond	Positive	*Thaumatotibia leucotreta*
13	**Negative**	/	Negative	*Grapholita molesta*
14	**Negative**	/	Negative	*Cryptophlebia peltastica*
15	**Negative**	/	Negative	*Cydia molesta*
16	**Negative**	/	Negative	*Leucinodes orbonalis*
17	**Negative**	/	Negative	*Lobesia botrana*
18	**Positive**	Spinules hardly visible	Positive	*Thaumatotibia leucotreta*
19	**Negative**	/	Negative	*Anarsia sp.*
20	**Negative**	/	Negative	*Apomyelois ceratoniae*
21	**Negative**	/	Negative	*Talponia batesi*
22	**Positive**	MD1 on T2/T3 not visible	Positive	*Thaumatotibia leucotreta*
23	**Negative**	/	Negative	*Lobesia botrana*
24	**Negative**	/	Negative	*Epinotia thapsiana*
25	**Negative**	/	Negative	*Apomyelois ceratoniae*
26	**Positive**	Dubious criteria 11, beneath but not really beyond. T. leucotreta doubtful	Positive	*Thaumatotibia leucotreta*
27	**Negative**	/	Negative	*Talponia batesi*
28	**Positive**	Dubious criteria 11, beneath but not beyond. T. leucotreta doubtful	Positive	*Thaumatotibia leucotreta*
29	**Negative**	/	Negative	*Cryptophlebia peltastica*
30	**Positive**	/	Positive	*Thaumatotibia leucotreta*

Operator 3	
Instrument	LEICA M216
Date of analysis/identification	04/04/2022 - 05/04/2022

Sample number	Analysis/Identification_1	Notes	Expected result	Assigned value
1	**Negative**	/	Negative	*Anarsia sp.*
2	**Negative**	/	Negative	*Cryptophlebia peltastica*
3	**Negative**	/	Negative	*Grapholita molesta*
4	**Negative**	/	Negative	*Gymnandrosoma aurantianum*
5	**Negative**	/	Negative	*Cydia pomonella*
6	**Negative**	/	Negative	*Cydia molesta*
7	**Positive**	The two sides are not identical	Positive	***Thaumatotibia leucotreta***
8	**Negative**	/	Negative	*Cacoecimorpha pronubana*
9	**Positive**	/	Positive	***Thaumatotibia leucotreta***
10	**Negative**	/	Negative	*Epinotia thapsiana*
11	**Negative**	/	Negative	*Leucinodes orbonalis*
12	**Positive**	/	Positive	***Thaumatotibia leucotreta***
13	**Negative**	/	Negative	*Grapholita molesta*
14	**Negative**	/	Negative	*Cryptophlebia peltastica*
15	**Negative**	/	Negative	*Cydia molesta*
16	**Negative**	/	Negative	*Leucinodes orbonalis*
17	**Negative**	/	Negative	*Lobesia botrana*
18	**Positive**	/	Positive	***Thaumatotibia leucotreta***
19	**Negative**	/	Negative	*Anarsia sp.*
20	**Negative**	/	Negative	*Apomyelois ceratoniae*
21	**Negative**	/	Negative	*Talponia batesi*
22	**Positive**	/	Positive	***Thaumatotibia leucotreta***
23	**Negative**	/	Negative	*Lobesia botrana*
24	**Negative**	/	Negative	*Epinotia thapsiana*
25	**Negative**	/	Negative	*Apomyelois ceratoniae*
26	**Negative**	/	Positive	***Thaumatotibia leucotreta***
27	**Negative**	/	Negative	*Talponia batesi*
28	**Positive**	/	Positive	***Thaumatotibia leucotreta***
29	**Negative**	/	Negative	*Cryptophlebia peltastica*
30	**Positive**	/	Positive	***Thaumatotibia leucotreta***

Operator 3	
Instrument	LEICA M216
Date of analysis/identification	11/04/2022 - 13/04/2022

Sample number	Analysis/Identification_2	Notes	Expected result	Assigned value
1	**Negative**		Negative	Anarsia sp.
2	**Negative**		Negative	Cryptophlebia peltastica
3	**Negative**		Negative	Grapholita molesta
4	**Negative**		Negative	Gymnandrosoma aurantianum
5	**Negative**		Negative	Cydia pomonella
6	**Negative**		Negative	Cydia molesta
7	**Positive**		Positive	Thaumatotibia leucotreta
8	**Negative**		Negative	Cacoecimorpha pronubana
9	**Positive**		Positive	Thaumatotibia leucotreta
10	**Negative**		Negative	Epinotia thapsiana
11	**Negative**		Negative	Leucinodes orbonalis
12	**Positive**		Positive	Thaumatotibia leucotreta
13	**Negative**		Negative	Grapholita molesta
14	**Negative**		Negative	Cryptophlebia peltastica
15	**Negative**		Negative	Cydia molesta
16	**Negative**		Negative	Leucinodes orbonalis
17	**Negative**		Negative	Lobesia botrana
18	**Positive**	Criteria 13 difficult to see Criteria 14: number of setae different on the two sides	Positive	Thaumatotibia leucotreta
19	**Negative**		Negative	Anarsia sp.
20	**Negative**		Negative	Apomyelois ceratoniae
21	**Negative**		Negative	Talponia batesi
22	**Positive**		Positive	Thaumatotibia leucotreta
23	**Negative**		Negative	Lobesia botrana
24	**Negative**		Negative	Epinotia thapsiana
25	**Negative**		Negative	Apomyelois ceratoniae
26	**Negative**	Criteria 11 doubtful (limite en bordure). Likely to be T. leucotreta	Positive	Thaumatotibia leucotreta
27	**Negative**		Negative	Talponia batesi
28	**Positive**		Positive	Thaumatotibia leucotreta
29	**Negative**		Negative	Cryptophlebia peltastica
30	**Positive**		Positive	Thaumatotibia leucotreta

Operator 3	
Instrument	LEICA M216
Date of analysis/identification	19/04/2022 - 20/04/2022

Sample number	Analysis/Identification_3	Notes	Expected result	Assigned value
1	**Negative**		Negative	Anarsia sp.
2	**Negative**		Negative	Cryptophlebia peltastica
3	**Negative**		Negative	Grapholita molesta
4	**Negative**		Negative	Gymnandrosoma aurantianum
5	**Negative**		Negative	Cydia pomonella
6	**Negative**		Negative	Cydia molesta
7	**Positive**		Positive	Thaumatotibia leucotreta
8	**Negative**		Negative	Cacoecimorpha pronubana
9	**Positive**		Positive	Thaumatotibia leucotreta
10	**Negative**		Negative	Epinotia thapsiana
11	**Negative**		Negative	Leucinodes orbonalis
12	**Positive**	Spinules poorly visible	Positive	Thaumatotibia leucotreta
13	**Negative**		Negative	Grapholita molesta
14	**Negative**		Negative	Cryptophlebia peltastica
15	**Negative**		Negative	Cydia molesta
16	**Negative**		Negative	Leucinodes orbonalis
17	**Negative**		Negative	Lobesia botrana
18	**Positive**		Positive	Thaumatotibia leucotreta
19	**Negative**		Negative	Anarsia sp.
20	**Negative**		Negative	Apomyelois ceratoniae
21	**Negative**		Negative	Talponia batesi
22	**Positive**		Positive	Thaumatotibia leucotreta
23	**Negative**		Negative	Lobesia botrana
24	**Negative**		Negative	Epinotia thapsiana
25	**Negative**		Negative	Apomyelois ceratoniae
26	**Negative**	Doubtful	Positive	Thaumatotibia leucotreta
27	**Negative**		Negative	Talponia batesi
28	**Negative**	Doubtful	Positive	Thaumatotibia leucotreta
29	**Negative**		Negative	Cryptophlebia peltastica
30	**Positive**		Positive	Thaumatotibia leucotreta

Sensitivity, specificity, accuracy :

Diagnostic sensitivity, specificity and accuracy is assessed on the basis of the analysis of the whole set carried out by operator 2 (ANSES)

Operator_2

Diagnostic sensitivity = true positives/(true positives + false negatives)

Diagnostic specificity = true negatives/(true negatives + false positives)

		Expected result	
		positive	negative
Operator result	positive	8	0
	negative	0	22

Sensitivity	**100%**
Specificity	**100%**
Accuracy	**100%**

Repeatability : Operator_3_R1, Operator_3_R2, Operator_3_R3

Repeatability is assessed on the basis of the analysis of the whole panel carried out by operator 3 (three repetitions of analysis).

Operator_3_R1, Operator_3_R2, Operator_3_R3

Expressed as % level of agreement among repetitions by Operator 3

Sample code	Operator3_R1	Operator3_R2	Operator3_R3	Repetitions	Agreement	Disagreement	Level of agreement %
1	Negative	Negative	Negative	3	3	0	100
2	Negative	Negative	Negative	3	3	0	100
3	Negative	Negative	Negative	3	3	0	100
4	Negative	Negative	Negative	3	3	0	100
5	Negative	Negative	Negative	3	3	0	100
6	Negative	Negative	Negative	3	3	0	100
7	Positive	Positive	Positive	3	3	0	100
8	Negative	Negative	Negative	3	3	0	100
9	Positive	Positive	Positive	3	3	0	100
10	Negative	Negative	Negative	3	3	0	100
11	Negative	Negative	Negative	3	3	0	100
12	Positive	Positive	Positive	3	3	0	100
13	Negative	Negative	Negative	3	3	0	100
14	Negative	Negative	Negative	3	3	0	100
15	Negative	Negative	Negative	3	3	0	100
16	Negative	Negative	Negative	3	3	0	100
17	Negative	Negative	Negative	3	3	0	100
18	Positive	Positive	Positive	3	3	0	100
19	Negative	Negative	Negative	3	3	0	100
20	Negative	Negative	Negative	3	3	0	100
21	Negative	Negative	Negative	3	3	0	100
22	Positive	Positive	Positive	3	3	0	100
23	Negative	Negative	Negative	3	3	0	100
24	Negative	Negative	Negative	3	3	0	100
25	Negative	Negative	Negative	3	3	0	100
26	Negative	Negative	Negative	3	3	0	100
27	Negative	Negative	Negative	3	3	0	100
28	Positive	Positive	Negative	3	2	1	66,7
29	Negative	Negative	Negative	3	3	0	100
30	Positive	Positive	Positive	3	3	0	100
				90	89	1	98,9

Repeatability **98,9%**

Reproducibility : Operator_1, Operator_2, Operator_3_R1

Reproducibility is assessed on the basis of the analysis of the whole panel carried out by operator 1, 2 and 3 (first of the three repetitions of analysis).

Operator_1, Operator_2, Operator_3_R1

Expressed as % level of agreement among repetitions by the three Operators

Sample code	Operator1	Operator2	Operator 3_R1	Repetitions	Agreement	Disagreement	Level of agreement %
1	Negative	Negative	Negative	3	3	0	100
2	Negative	Negative	Negative	3	3	0	100
3	Negative	Negative	Negative	3	3	0	100
4	Negative	Negative	Negative	3	3	0	100
5	Negative	Negative	Negative	3	3	0	100
6	Negative	Negative	Negative	3	3	0	100
7	Positive	Positive	Positive	3	3	0	100
8	Negative	Negative	Negative	3	3	0	100
9	Positive	Positive	Positive	3	3	0	100
10	Negative	Negative	Negative	3	3	0	100
11	Negative	Negative	Negative	3	3	0	100
12	Positive	Positive	Positive	3	3	0	100
13	Negative	Negative	Negative	3	3	0	100
14	Negative	Negative	Negative	3	3	0	100
15	Negative	Negative	Negative	3	3	0	100
16	Negative	Negative	Negative	3	3	0	100
17	Negative	Negative	Negative	3	3	0	100
18	Positive	Positive	Positive	3	3	0	100
19	Negative	Negative	Negative	3	3	0	100
20	Negative	Negative	Negative	3	3	0	100
21	Negative	Negative	Negative	3	3	0	100
22	Positive	Positive	Positive	3	3	0	100
23	Negative	Negative	Negative	3	3	0	100
24	Negative	Negative	Negative	3	3	0	100
25	Negative	Negative	Negative	3	3	0	100
26	Positive	Positive	Negative	3	2	1	66,67
27	Negative	Negative	Negative	3	3	0	100
28	Positive	Positive	Positive	3	3	0	100
29	Negative	Negative	Negative	3	3	0	100
30	Positive	Positive	Positive	3	3	0	100
				90	89	1	98,9

Reproducibility **98,9%**

Appendix 6 – *In silico* testing of analytical specificity with DNA barcoding and real-time primer sets

a. DNA barcoding according to EPPO PM7/129(2)

In silico testing of analytical specificity by a database alignment (NCBI GenBank) was performed (26.09.2022) with the DNA barcoding primer sets (LCO1490/HCO2198 and LepF/LepR). The search set was limited to "*Thaumatotibia leucotreta*" (taxid:463830)". The results showed suitability of both primer sets (see Fig. A-D), although we have to state that barcoding is a generic test including targets and non-targets.

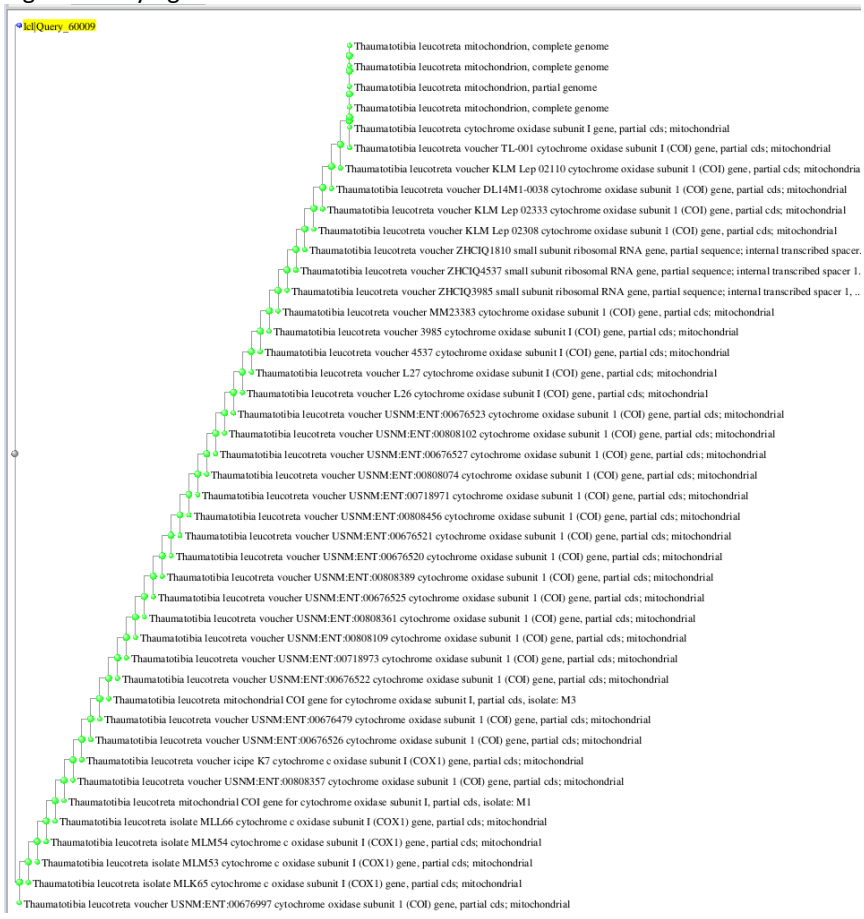
Distance trees of results from BLAST search were created with organism search set to *Thaumatotibia leucotreta* with single primers (LepF, LepR, LCO1490, HCO2198).

Figure A: Phylogenetic tree for LepF constructed with the fast minimum evolution method by blast tree viewer.

Figure B: Phylogenetic tree for LepR constructed with the fast minimum evolution method by blast tree viewer.

Figure C: Phylogenetic tree for LCO1490 constructed with the fast minimum evolution method by blast tree viewer.

Figure D: Phylogenetic tree for HCO2198 constructed with the fast minimum evolution method by blast tree viewer.



b. TaqMan real-time PCR according to Rizzo *et al*. (2021)

In silico testing of analytical specificity by a database alignment (NCBI GenBank) was performed (19.09.2022) with the primer/probe set. The primers (Tleuco_1001F/Tleuco_1070R) and probe (Tleuco_1041P) for the real-time PCR were aligned with restricting the search set to Tortricidae (Fig. E –J). Additionally, an *in silico* PCR was performed with the forward and reverse primers (Fig. K)

Figure E: Phylogenetic tree for Tleuco_1001F constructed with the fast minimum evolution method by blast tree viewer.

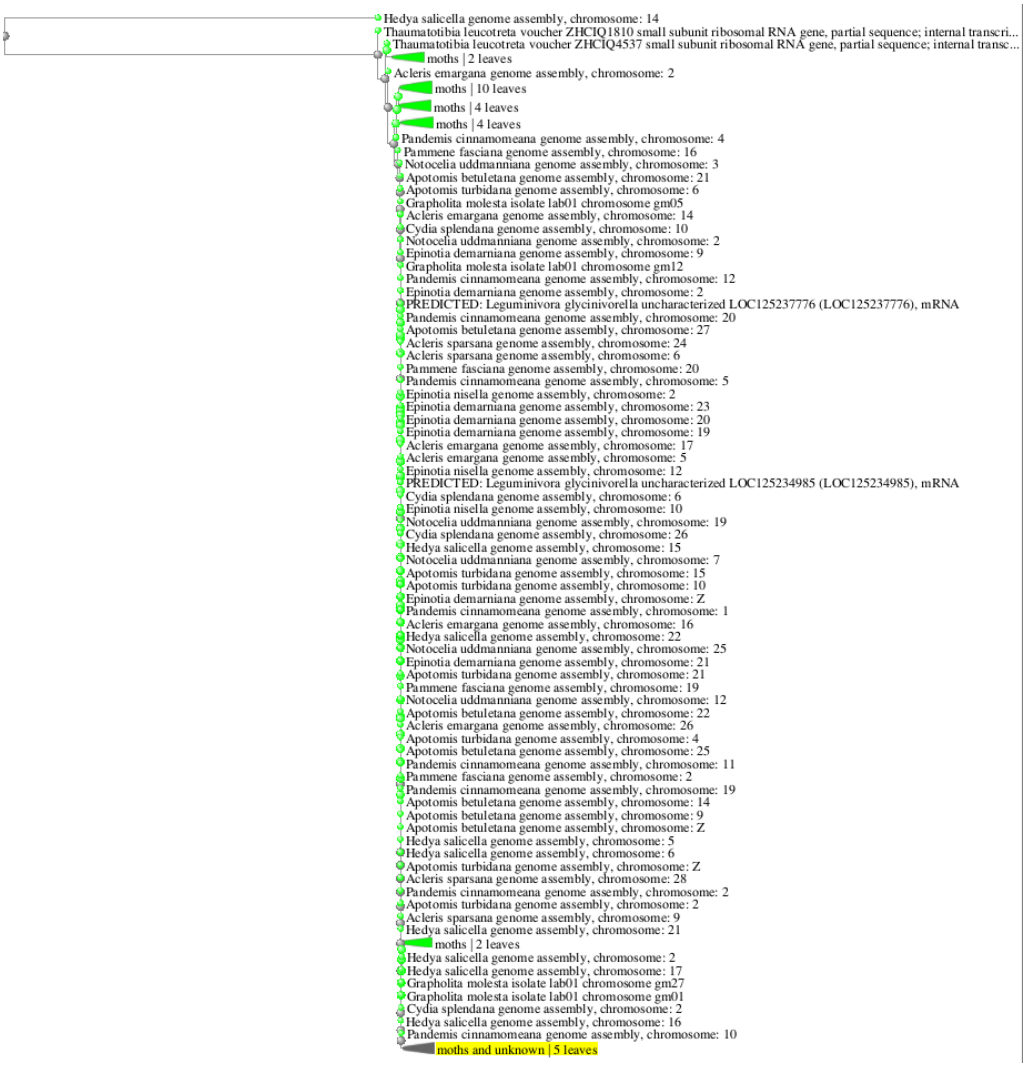


Figure F: NCBI Genbank hits for Tleuco_1001F

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Thaumatotibia leucotreta voucher ZHCIQ1810 small subunit ribosomal RNA gene, partial sequence; internal tra...	Thaumatotibia le...	36.2	36.2	100%	0.42	100.00%	1126	MG264603.1
Thaumatotibia leucotreta voucher ZHCIQ4537 small subunit ribosomal RNA gene, partial sequence; internal tra...	Thaumatotibia le...	36.2	36.2	100%	0.42	100.00%	1121	MG264602.1
Thaumatotibia leucotreta voucher ZHCIQ3985 small subunit ribosomal RNA gene, partial sequence; internal tra...	Thaumatotibia le...	36.2	36.2	100%	0.42	100.00%	1124	MG264600.1
Archips crataeganus genome assembly, chromosome: 6	Archips crataega...	34.2	86.7	94%	1.7	100.00%	23181946	OX402052.1
Eudemis profundana genome assembly, chromosome: 19	Eudemis profund...	34.2	191	100%	1.7	100.00%	18370963	OX344813.1
Archips xylosteana genome assembly, chromosome: 5	Archips xylosteana	34.2	244	94%	1.7	100.00%	24097882	OX387348.1
Hedya salicella genome assembly, chromosome: 20	Hedya salicella	34.2	60.5	94%	1.7	100.00%	17835027	FR990116.1
Pandemis corylana genome assembly, chromosome: 4	Pandemis corylana	32.2	58.5	88%	6.6	100.00%	16927485	OX421854.1
Archips crataeganus genome assembly, chromosome: 25	Archips crataega...	32.2	163	94%	6.6	100.00%	12801476	OX402071.1
Apotomis capreana genome assembly, chromosome: 1	Apotomis caprea...	32.2	222	94%	6.6	100.00%	44926544	OX392499.1
Archips xylosteana genome assembly, chromosome: 18	Archips xylosteana	32.2	111	88%	6.6	100.00%	19668748	OX387362.1

Figure G: Phylogenetic tree for Tleuco_1070R constructed with the fast minimum evolution method by blast tree viewer.

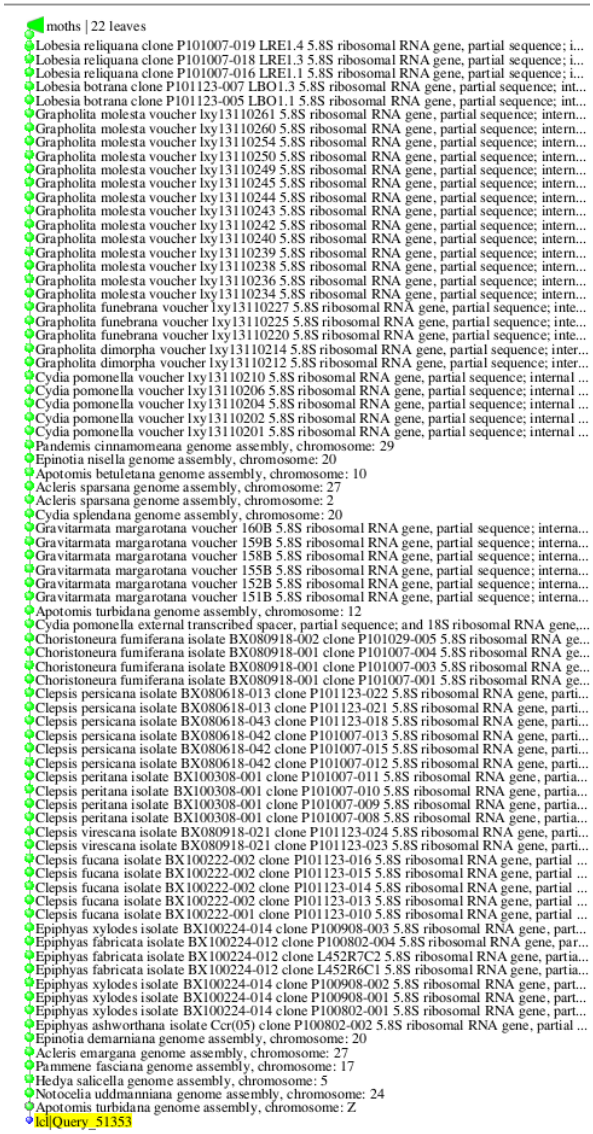


Figure H: NCBI Genbank hits for Tleuco_1070R

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Grapholita molesta isolate apple voucher ZHCIQ7044345 small subunit ribosomal RNA gene, partial sequence; in...	Grapholita molesta	36.2	36.2	100%	0.42	100.00%	1037	MG515319.1
Grapholita molesta isolate peach voucher ZHCIQ6032644 small subunit ribosomal RNA gene, partial sequence; i...	Grapholita molesta	36.2	36.2	100%	0.42	100.00%	1036	MG515318.1
Grapholita molesta isolate pear voucher ZHCIQ6031534 small subunit ribosomal RNA gene, partial sequence; int...	Grapholita molesta	36.2	36.2	100%	0.42	100.00%	1040	MG515317.1
Grapholita molesta isolate Prunus voucher ZHCIQ6025328-A17775 small subunit ribosomal RNA gene, partial se...	Grapholita molesta	36.2	36.2	100%	0.42	100.00%	1035	MG515315.1
Cydia pomonella isolate apple voucher ZHCIQ161012-A17816 small subunit ribosomal RNA gene, partial sequen...	Cydia pomonella	36.2	36.2	100%	0.42	100.00%	1121	MG515314.1
Cydia pomonella isolate apple voucher ZHCIQ161012-A17815 small subunit ribosomal RNA gene, partial sequen...	Cydia pomonella	36.2	36.2	100%	0.42	100.00%	1123	MG515313.1
Cydia pomonella isolate apple voucher ZHCIQ151012-A17814 small subunit ribosomal RNA gene, partial sequen...	Cydia pomonella	36.2	36.2	100%	0.42	100.00%	1135	MG515312.1
Cydia pomonella isolate apple voucher ZHCIQ151012-A17813 small subunit ribosomal RNA gene, partial sequen...	Cydia pomonella	36.2	36.2	100%	0.42	100.00%	1137	MG515311.1
Cydia pomonella isolate guava voucher ZHCIQ59232001-A17818 small subunit ribosomal RNA gene, partial sequ...	Cydia pomonella	36.2	36.2	100%	0.42	100.00%	1129	MG515310.1
Cydia pomonella isolate guava voucher ZHCIQ59232001-A17817 small subunit ribosomal RNA gene, partial sequ...	Cydia pomonella	36.2	36.2	100%	0.42	100.00%	1127	MG515309.1
Epiphyas postvittana voucher ZHCIQ1246 small subunit ribosomal RNA gene, partial sequence; internal transcrib...	Epiphyas postvitt...	36.2	36.2	100%	0.42	100.00%	1013	MG264608.1
Thaumatotibia sp. ZHCIQ6038263 small subunit ribosomal RNA gene, partial sequence; internal transcribed spa...	Thaumatotibia sp...	36.2	36.2	100%	0.42	100.00%	1172	MG264607.1
Thaumatotibia batrachopa voucher ZHCIQ5019726 small subunit ribosomal RNA gene, partial sequence; internal ...	Thaumatotibia ba...	36.2	36.2	100%	0.42	100.00%	1088	MG264606.1
Grapholita funebrana voucher ZHCIQ6019855 small subunit ribosomal RNA gene, partial sequence; internal trans...	Grapholita funebr...	36.2	36.2	100%	0.42	100.00%	1078	MG264605.1
Epichoristodes acerbella voucher ZHCIQ748 small subunit ribosomal RNA gene, partial sequence; internal transc...	Epichoristodes a...	36.2	36.2	100%	0.42	100.00%	1034	MG264604.1
Thaumatotibia leucotreta voucher ZHCIQ1810 small subunit ribosomal RNA gene, partial sequence; internal trans...	Thaumatotibia le...	36.2	36.2	100%	0.42	100.00%	1126	MG264603.1
Thaumatotibia leucotreta voucher ZHCIQ4537 small subunit ribosomal RNA gene, partial sequence; internal trans...	Thaumatotibia le...	36.2	36.2	100%	0.42	100.00%	1121	MG264602.1
Thaumatotibia leucotreta voucher ZHCIQ3985 small subunit ribosomal RNA gene, partial sequence; internal trans...	Thaumatotibia le...	36.2	36.2	100%	0.42	100.00%	1124	MG264600.1
Lobesia fuligana clone P101007-023 LFU1.4 5.8S ribosomal RNA gene, partial sequence; internal transcribed sp...	Lobesia fuligana	36.2	36.2	100%	0.42	100.00%	479	KU530547.1

Figure I: Phylogenetic tree for Tleuco_1041P constructed with the fast minimum evolution method by blast tree viewer.

Figure J: NCBI Genbank hits for Tleuco_1041P.

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Thaumatotibia leucotreta voucher ZHCIQ1810 small subunit ribosomal RNA gene, ...	Thaumatotibi...	42.1	42.1	100%	0.010	100.00%	1126	MG264603.1
☑	Thaumatotibia leucotreta voucher ZHCIQ4537 small subunit ribosomal RNA gene, ...	Thaumatotibi...	42.1	42.1	100%	0.010	100.00%	1121	MG264602.1
☑	Thaumatotibia leucotreta voucher ZHCIQ3985 small subunit ribosomal RNA gene, ...	Thaumatotibi...	42.1	42.1	100%	0.010	100.00%	1124	MG264600.1
☑	Apotomis capreana genome assembly, chromosome: 24	Apotomis ca...	36.2	88.7	85%	0.63	100.00%	15869760	OX392522.1
☑	Apotomis capreana genome assembly, chromosome: 22	Apotomis ca...	36.2	36.2	85%	0.63	100.00%	17670323	OX392520.1
☑	Apotomis betuletana genome assembly, chromosome: 23	Apotomis be...	36.2	62.4	85%	0.63	100.00%	15741175	OW026321.1
☑	Apotomis turbidana genome assembly, chromosome: 25	Apotomis tur...	36.2	62.4	95%	0.63	100.00%	16130032	LR990304.1
☑	Tortricodes alternella genome assembly, chromosome: 21	Tortricodes a...	34.2	147	85%	2.5	100.00%	11989812	OX401989.1
☑	Tortricodes alternella genome assembly, chromosome: 10	Tortricodes a...	34.2	270	95%	2.5	100.00%	17851949	OX401978.1
☑	Tortricodes alternella genome assembly, chromosome: 2	Tortricodes a...	34.2	272	95%	2.5	100.00%	33106409	OX401969.1
☑	Thaumatotibia sp. ZHCIQ6038263 small subunit ribosomal RNA gene, partial sequ...	Thaumatotibi...	32.2	32.2	76%	9.8	100.00%	1172	MG264607.1

Fig. K: *In silico* PCR with the forward and reverse primer of the ITS TaqMan real-time PCR (Tleuco_1001F/Tleuco_1070R).

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CGTCCAAGCATAGCTTTC	18	53.57	50.00	6.00	2.00
Reverse primer	GAGGCAGACACGATATCC	18	54.28	55.56	6.00	4.00

Products on target templates

>MG264603.1 Thaumatotibia leucotreta voucher ZHCIQ1810 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

```
product length = 70
Forward primer  1     CGTCCAAGCATAGCTTTC  18
Template        1001  ..................  1018

Reverse primer  1     GAGGCAGACACGATATCC  18
Template        1070  ..................  1053
```

>MG264602.1 Thaumatotibia leucotreta voucher ZHCIQ4537 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

```
product length = 70
Forward primer  1    CGTCCAAGCATAGCTTTC  18
Template        996  ..................  1013

Reverse primer  1     GAGGCAGACACGATATCC  18
Template        1065  ..................  1048
```

>MG264600.1 Thaumatotibia leucotreta voucher ZHCIQ3985 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

```
product length = 70
Forward primer  1     CGTCCAAGCATAGCTTTC  18
Template        1001  ..................  1018

Reverse primer  1     GAGGCAGACACGATATCC  18
Template        1070  ..................  1053
```

>OX276375.1 Alsophila aescularia genome assembly, chromosome: 2

```
product length = 39
Forward primer  1         CGTCCAAGCATAGCTTTC  18
Template        27161639  AC..................  27161656

Forward primer  1         CGTCCAAGCATAGCTTTC  18
Template        27161677  AC..............T  27161660
```

>LR990972.1 Craniophora ligustri genome assembly, chromosome: 18

```
product length = 68
Forward primer  1        CGTCCAAGCATAGCTTTC  18
Template        8407061  ATA................  8407078

Forward primer  1        CGTCCAAGCATAGCTTTC  18
Template        8407128  ATA................  8407111
```

c. SYBR green real-time PCR according to Rizzo *et al*. (2021)

In silico testing of analytical specificity by a database alignment (NCBI GenBank) was performed (19.09.2022) with the primer set (Tleuco_266F/Tleuco_359R). The search set was restricted to Tortricidae (Fig. L-M). Additionally, an *in silico* PCR was performed. (Fig. N)

Figure L: Phylogenetic tree for Tleuco_266F constructed with the fast minimum evolution method by blast tree viewer.

Figure M: Phylogenetic tree for Tleuco_359R constructed with the fast minimum evolution method by blast tree viewer.

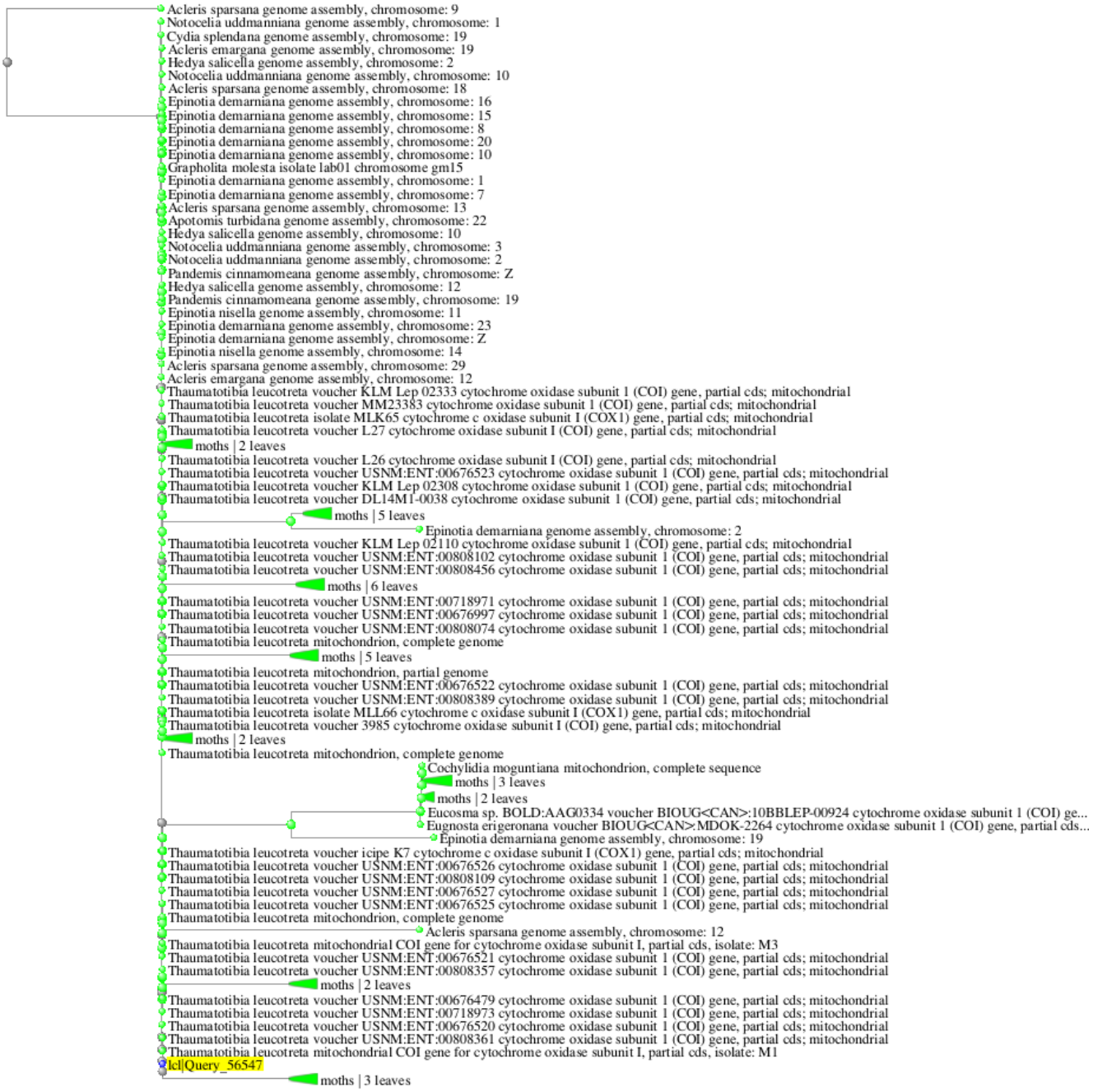


Fig. N: *In silico* PCR with the primer set Tleuco_266F/Tleuco359R.

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TGGAACAGGATGAACAGT	18	52.81	44.44	3.00	3.00
Reverse primer	TGCTAGGTGAAGAGAGAAA	19	52.62	42.11	4.00	0.00

Products on target templates

>NC_064065.1 Thaumatotibia leucotreta mitochondrion, complete genome

```
product length = 94
Forward primer  1     TGGAACAGGATGAACAGT  18
Template        1873  ..................  1890

Reverse primer  1     TGCTAGGTGAAGAGAGAAA  19
Template        1966  ...................  1948
```

>MW755978.1 Thaumatotibia leucotreta mitochondrion, complete genome

```
product length = 94
Forward primer  1     TGGAACAGGATGAACAGT  18
Template        1807  ..................  1824

Reverse primer  1     TGCTAGGTGAAGAGAGAAA  19
Template        1900  ...................  1882
```

>MW755977.1 Thaumatotibia leucotreta mitochondrion, partial genome

```
product length = 94
Forward primer  1     TGGAACAGGATGAACAGT  18
Template        1714  ..................  1731

Reverse primer  1     TGCTAGGTGAAGAGAGAAA  19
Template        1807  ...................  1789
```

>MW755976.1 Thaumatotibia leucotreta mitochondrion, complete genome

```
product length = 94
Forward primer  1     TGGAACAGGATGAACAGT  18
Template        1807  ..................  1824

Reverse primer  1     TGCTAGGTGAAGAGAGAAA  19
Template        1900  ...................  1882
```

>MT212522.1 Thaumatotibia leucotreta voucher icipe K7 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial

```
product length = 94
Forward primer  1    TGGAACAGGATGAACAGT  18
Template        266  ..................  283

Reverse primer  1    TGCTAGGTGAAGAGAGAAA  19
Template        359  ...................  341
```

Additional Tortricidae species with possible cross reactions when *in silico* PCR tested using the primer set Tleuco_266F/Tleuco359R: Indication of mismatch numbers and geographical occurence

Epiblema desertana 2 mismatches reverse (N- America)
Coeloptera gyrobathra 2 mismatches reverse (Australia)
Eucosma sp. 1 mis forward, 1 mismatches reverse (BOLD:AAG0334)
Epiblema scudderiana 3 mismatches reverse (N- America)
Grapholita mesoscia 3 mismatches reverse (East Africa, Madagascar)
Homona sp. 3 mismatches reverse
Pammene aurana 3 mismatches reverse (W-Europe to East Asia)
Thaumatotibia batrachopa 3 mismatches reverse (West, East and southern Africa, islands of São Tomé and Madagascar)

Examples of *in silico* PCR results using the primer set Tleuco_266F/Tleuco359R

>MG364227.1 Epiblema desertana voucher BIOUG22572-G08 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial

```
product length = 94
Forward primer  1    TGGAACAGGATGAACAGT  18
Template        318  ..................  335

Reverse primer  1    TGCTAGGTGAAGAGAGAAA  19
Template        411  .....AA............  393
```

>KJ592218.1 Grapholita mesoscia voucher USNM:ENT:00676707 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial

```
product length = 94
Forward primer  1    TGGAACAGGATGAACAGT  18
Template        319  ..................  336

Reverse primer  1    TGCTAGGTGAAGAGAGAAA  19
Template        412  .....AA........A...  394
```

Primer set Tleuco_266F/Tleuco359R alignment with T. leucotreta and the cross reacting *Gymnandrosoma aurantianum*. *Gymnandrosoma aurantianum* with 1 mismatch forward, 2 mismatches reverse primer

```
34Gymnandrosoma_aurantianum      AATTGTAGAAAACGGAGCCGGAACAGGATGAACAGTTTA
Tleuco_359_R                     ---------------------------------------
Tleuco_266_F                     ------------------TGGAACAGGATGAACAGT---
34Gymnandrosoma_aurantianum      TGCTCATAGTGGTAGTTCTGTTGATTTAGCTATTTTCTCTCTTCATTTAGCAGGA
Tleuco_359_R                     --------------------------------TTTCTCTCTTCACCTAGCA---
```

Appendix 7 – Results of the analytical specificity with DNA barcoding and real-time PCR

Table A: Qualitative result of molecular identification methods used. True positive samples are marked in green. Excluded samples are marked in yellow. One false positive sample is marked in red.

		EPPO PM7/129(2)				Rizzo *et al.* 2021	
		LCO1490/HCO2198		LepF/LepR		COI SYBR Green	ITS TaqMan
Sample	Assigned value	Result	Note	Result	Note	Result	Result
1	*Anarsia sp.*	negative	*Anarsia sp.*	negative	*Anarsia sp.*	negative	negative
3	*Grapholita molesta*	negative	*G. molesta*	negative	*G. molesta*	negative	negative
5	*Cydia pomonella*	negative	*C. pomonella*	negative	*C. pomonella*	negative	negative
6	*Cydia molesta*	negative	*C. pomonella*	negative	*C. pomonella*	negative	negative
7	*Thaumatotibia leucotreta*	positive	*T. leucotreta*	positive	*T. leucotreta*	positive	positive
8	*Cacoecimorpha pronubana*	negative	*C. pronubana*	negative	*C. pronubana*	negative	negative
9	*Thaumatotibia leucotreta*	positive	*T. leucotreta*	positive	*T. leucotreta*	positive	positive
11	*Leucinodes orbonalis*	negative	*L. orbonalis*	negative	*L. orbonalis*	negative	negative
12	*Thaumatotibia leucotreta*	positive	*T. leucotreta*	positive	*T. leucotreta*	positive	positive
13	*Grapholita molesta*	negative	*G. molesta*	negative	*G. molesta*	negative	negative
15	*Cydia molesta*	negative	*C. pomonella*	negative	*C. pomonella*	negative	negative
16	*Leucinodes orbonalis*	negative	*L. orbonalis*	negative	*L. orbonalis*	negative	negative
17	*Lobesia botrana*	negative	*L. botrana*	negative	*L. botrana*	negative	negative
18	*Thaumatotibia leucotreta*	positive	*T. leucotreta*	positive	*T. leucotreta*	positive	positive
19	*Anarsia sp.*	negative	*Anarsia sp.*	negative	*Anarsia sp.*	negative	negative
21	*Talponia batesi*	negative	*Tortricidae sp.*	negative	*Tortricidae sp.*	negative	negative
22	*Thaumatotibia leucotreta*	positive	*T. leucotreta*	positive	*T. leucotreta*	positive	positive
23	*Lobesia botrana*	negative	*L. botrana*	negative	*L. botrana*	negative	negative
26	*Thaumatotibia leucotreta*	positive	*T. leucotreta*	positive	*T. leucotreta*	positive	positive
27	*Talponia batesi*	negative	*Tortricidae sp.*	negative	*Tortricidae sp.*	negative	negative
28	*Thaumatotibia leucotreta*	positive	*T. leucotreta*	positive	*T. leucotreta*	positive	positive
30	*Thaumatotibia leucotreta*	positive	*T. leucotreta*	positive	*T. leucotreta*	positive	positive
31	*Cryptophlebia peltastica*	negative	*C. peltastica*	negative	*C. peltastica*	negative	negative
32	*Thaumatotibia leucotreta*	positive	*T. leucotreta*	positive	*T. leucotreta*	positive	positive
33	*Epinotia thapsiana*	negative	*E. thapsiana*	negative	*E. thapsiana*	negative	negative
34	*Gymnandrosoma aurantianium*	negative	*G. aurantianum*	negative	*G. aurantianum*	positive	negative
35	*Apomyelois ceratoniae*	negative	*A. ceratoniae*	negative	*A. ceratoniae*	negative	negative
36	*Grapholita funebrana*	negative	*G. funebrana*	negative	*G. funebrana*	negative	negative
37	*Grapholita funebrana*	negative	*G. funebrana*	negative	*G. funebrana*	negative	negative
38	*Cryptophlebia peltastica*	negative	*C. peltastica*	negative	*C. peltastica*	negative	negative
39	*Apomyelois ceratoniae*	negative	*A. ceratoniae*	negative	*A. ceratoniae*	negative	negative
40	*Thaumatotibia leucotreta*	positive	*T. leucotreta*	positive	*T. leucotreta*	positive	positive
41	*Cryptophlebia peltastica*	negative	*C. peltastica*	negative	*C. peltastica*	negative	negative
42	*Cryptoblabes sp.*	excluded		excluded		excluded	excluded
43	*Cryptoblabes sp.*	-	-	negative	*C. gnidiella*	negative	negative
44	*Cryptophlebia peltastica*	negative	*C. peltastica*	negative	*C. peltastica*	negative	negative
45	*Cryptophlebia peltastica*	negative	*C. peltastica*	negative	*C. peltastica*	negative	negative
46	*Gymnandrosoma aurantianium*	negative	*G. aurantianum*	negative	*G. aurantianum*	negative	negative

Table B: Detailed results (Ct values) of real-time PCRs according to Rizzo *et al.* 2021.True positive samples are marked in green. One false positive sample for SYBR Green real-time PCR is marked in red. TaqMan real-time PCR results below Ct 35 are marked in orange.
Ct values in yellow, while above the SYBR Green cut-off of 22 and above a Ct of 35 for the TaqMan real-time PCR, might indicate possible contaminations or non-specific signals. SYBR Green results above Ct 30 are marked in grey.

Sample Nb.	Assigned value	Ng/µl	ITS TaqMan PCR		COI SYBR Green PCR	
			Ct value undiluted	Ct value 1:20 dilution	Ct value undiluted	Ct value 1:20 dilution
1	*Anarsia sp.*	0.85	36.65	38.03	32.93	33.00
3	*Grapholita molesta*	1.65	36.54	-	32.68	32.48
5	*Cydia pomonella*	2.00	36.44	-	31.12	32.64
6	*Cydia molesta*	2.15	33.04	36.44	31.12	33.24
7	*Thaumatotibia leucotreta*	4.63	17.55	21.74	14.16	17.89
8	*Cacoecimorpha pronubana*	4.45	32.02	36.51	28.1	32.17
9	*Thaumatotibia leucotreta*	9.20	15.51	19.71	14.07	17.66
11	*Leucinodes orbonalis*	5.43	36.17	-	30.53	32.47
12	*Thaumatotibia leucotreta*	2.35	16.00	20.34	12.93	17.33
13	*Grapholita molesta*	5.37	35.24	-	30.34	31.67
15	*Cydia molesta*	2.00	35.38	-	31.23	37.62
16	*Leucinodes orbonalis*	3.80	33.12	36.65	30.00	32.44
17	*Lobesia botrana*	-	36.94	-	31.37	32.63
18	*Thaumatotibia leucotreta*	1.50	17.90	22.4	16.44	20.91
19	*Anarsia sp.*	-	34.96	-	35.03	33.07
21	*Talponia batesi*	2.00	-	-	34.14	33.10
22	*Thaumatotibia leucotreta*	1.95	19.54	24.5	14.57	18.77
23	*Lobesia botrana*	1.13	29.66	34.22	26.02	30.70
26	*Thaumatotibia leucotreta*	6.47	16.82	21.33	14.52	18.53
27	*Talponia batesi*	1.85	36.59	-	33.21	33.66
28	*Thaumatotibia leucotreta*	1.45	18.14	22.56	14.16	18.42
30	*Thaumatotibia leucotreta*	4.00	19.25	24.29	17.20	21.54
31	*Cryptophlebia peltastica*	0.67	-	-	34.27	34.07
32	*Thaumatotibia leucotreta*	1.25	20.27	24.61	17.42	22.02
33	*Epinotia thapsiana*	18.23	-	-	33.20	32.77
34	*Gymnandrosoma aurantianium*	37.13	-	-	20.40	21.30
35	*Apomyelois ceratoniae*	17.33	-	-	33.39	33.11
36	*Grapholita funebrana*	8.57	-	-	32.23	33.88
37	*Grapholita funebrana*	10.47	-	-	33.24	33.48
38	*Cryptophlebia peltastica*	30.23	-	-	32.25	32.19
39	*Apomyelois ceratoniae*	86.93	32.19	-	-	32.51
40	*Thaumatotibia leucotreta*	3.13	20.01	24.49	19.08	23.05
41	*Cryptophlebia peltastica*	4.10	37.42	-	33.50	33.52
42	*Cryptoblabes sp.*	-	-	-	33.39	33.20
43	*Cryptoblabes sp.*	0.15	-	-	33.04	34.13
44	*Cryptophlebia peltastica*	9.83	-	-	33.91	34.37
45	*Cryptophlebia peltastica*	3.90	38.39	-	34.17	34.46
46	*Gymnandrosoma aurantianium*	4.90	-	-	26.90	29.81

Appendix 8 – Summary result sheets for analytical sensitivity, repeatability, and reproducibility (molecular tests)

Sample panel analytical sensitivity and repeatability:
1 larva of *T. leucotreta* from Israel
1 larva of *T. leucotreta* from South Africa
1 larva of *T. leucotreta* from Togo

Three experimental replicates were performed with this sample panel

Measurement of DNA concentration:
Quantity of DNA was determined using the Thermo Scientific Nanodrop 2000 Spectrophotometer, samples were measured three times (technical replicates), mean and standard deviation were calculated.

Analytical sensitivity and repeatability:
3 samples were prepared in different dilutions (1:100, 1:1000; 1:10.000; 1:100.000, 1:1.000.000, 1:10.000.000) and PCRs with both barcoding primer sets, as well as the real-time PCRs according to Rizzo *et al.* (2021) were performed in three technical repetitions per sample.
Barcoding amplicons at the detection limit and the last dilution step before the detection limit were sent for SANGER sequencing.

Reproducibility:
3 targets and 3 non-targets were analysed with all four tests. The PCR tests were performed by two operators on different cyclers on different days. The sequence analysis was performed by two operators on two different days.

Table A: Sample panel reproducibility:

Targets	Non-targets
Thaumatotibia leucotreta larva, South Africa	*Cydia pomonella* adult, Austria
Thaumatotibia leucotreta larva, Togo	*Lobesia botrana* adult, Austria
Thaumatotibia leucotreta larva, Israel	*Eupoecilia ambiguella* adult, Austria

Results for analytical sensitivity (DNA barcoding):

Table B: Extracted DNA concentration and PCR sensitivity/repeatability for T. *leucotreta* sample panel used for sensitivity and repeatability testing (DNA barcoding)

		LepF/R			**LCO1490/HCO2198**			
Sample	**Dilution**	**Repetition 1**	**Repetition 2**	**Repetition 3**	**Repetition 1**	**Repetition 2**	**Repetition 3**	**DNA conc.**
7	1:10	+	+	+	+	+	+	0.463ng/µl
	1:100	+	+	+	+	+	+	46.3pg/µl
	1:1.000	+	+	+	+	+	+	4.63pg/µl
	1:10.000	+	+	+	+	+	+	0.463pg/µl
	1:100.000	-	-	-	+	-	+	46.3fg/µl
	1:1.000.000	-	-	-	-	-	-	
	1:10.000.000	-	-	-	-	-	-	
12	1:10	+	+	+	+	+	+	0.235ng/µl
	1:100	+	+	+	+	+	+	23.5pg/µl
	1:1.000	+	+	+	+	+	+	2.35pg/µl
	1:10.000	+	+	+	+	+	+	0.235pg/µl
	1:100.000	-	-	-	-	-	+	
	1:1.000.000	-	-	-	-	-	-	
	1:10.000.000	-	-	-	-	-	-	
32	1:10	+	+	+	+	+	+	0.125ng/µl
	1:100	-	+	+	+	+	+	12.5pg/µl
	1:1.000	-	-	-	-	-	-	
	1:10.000	-	-	-	-	-	-	

	1:100.000	-	-	-	-	-	-	
	1:1.000.000	-	-	-	-	-	-	
	1:10.000.000	-	-	-	-	-	-	

Calculation of the analytical sensitivity: average of the last dilution that led to an amplicon throughout all three technical repetitions for all three biological replicates.

Table C: Calculations of the analytical sensitivity for LepF/R

Sample	Last reliably detectable dilution	DNA concentration	Calculation of analytical sensitivity value	Result
7	1:10.000	0.463 pg/µl	$\frac{sensitivity(sample\ 7) + sens.(sample\ 12) + sens.(sample\ 32}{Nr.of\ samples\ (3)}$ = $\frac{0.463 + 0.235 + 125}{3}$	41.9 pg/µl
12	1:100	0.235 pg/µl		
32	1:10	125 pg/µl		

Table D: Calculations of the analytical sensitivity for LCO1490/HCO2198

Sample	Last reliably detectable dilution	DNA concentration	Calculation of analytical sensitivity value	Result
7	1:10.000	0.463 pg/µl	$\frac{sensitivity(sample\ 7) + sens.(sample\ 12) + sens.(sample\ 32}{Nr.of\ samples\ (3)}$ = $\frac{0.463 + 0.235 + 12.5}{3}$	4.4 pg/µl
12	1:100	0.235 pg/µl		
32	1:10	12.5 pg/µl		

Table E: Results for analytical sensitivity (Real-time PCR TaqMan):

Sample	Dilution	DNA conc.	Repetition 1	Repetition 2	Repetition 3	Mean	SD
7	1:1.000	4.63pg/µl	24.43	27.7	27.5	26.54	1.50
	1:10.000	0.463pg/µl	30.75	31.05	30.93	30.93	0.13
	1:100.000	46.3fg/µl	34.05	34.43	33.96	34.15	0.20
	1:1.000.000		36.65	38.16	-		
	1:10.000.000		-	37.34	37.98		
12	1:1.000	2.35pg/µl	26.12	26.29	26.28	26.23	0.08
	1:10.000	0.235pg/µl	29.64	29.53	29.18	29.45	0.20
	1:100.000	23.5fg/µl	32.95	32.14	32.82	32.64	0.36
	1:1.000.000	2.35fg/µl	36.66	36.41	36.37	36.48	0.13
	1:10.000.000		38.00	-	-		
32	1:1.000	1.25pg/µl	30.36	30.40	30.17	30.31	0.10
	1:10.000	0.125pg/µl	34.05	33.90	34.55	34.17	0.28
	1:100.000	12.5fg/µl	38.79	39.91	35.51	38.07	1.87
	1:1.000.000		-	-	38.45		
	1:10.000.000		-	-	-		

Table F: Calculation of the analytical sensitivity: average of the last dilution that led to a signal throughout all three technical repetitions for all three biological replicates.

Sample	Last reliably detectable dilution	DNA concentration	Calculation of analytical sensitivity value	Result
7	1:100.000	46.3 fg/µl	$\frac{sensitivity(sample\ 7) + sens.(sample\ 12) + sens.(sample\ 32}{Nr.of\ samples\ (3)} = \frac{46.3 + 2.35 + 12.5}{3}$	20.38 fg/µl
12	1:1.000.000	2.35 fg/µl		
32	1:100.000	12.5 fg/µl		

Table G: Results for analytical sensitivity (Real-Time PCR SYBR Green):

Sample	Dilution	DNA conc.	Repetition 1	Repetition 2	Repetition 3	Mean	SD
7	1:10	0.463ng/µl	16.94	17.01	16.94	16.96	0.03
	1:100	46.3pg/µl	20.33	20.18	20.10	20.20	0.10
	1:1.000	4.63pg/µl	23.87	23.72	23.76	23.78	0.06
	1:10.000	0.463pg/µl	27.61	26.84	27.70	27.17	0.32
	1:100.000	46.3fg/µl	29.86	31.93	30.54	30.78	0.86
	1:1.000.000		35.34	31.03	33.46	33.28	1.76
	1:10.000.000		-	36.68	35.19		
12	1:10	0.235ng/µl	16.06	15.88	15.93	15.96	0.08
	1:100	23.5pg/µl	18.99	19.16	19.01	19.05	0.08
	1:1.000	2.35pg/µl	22.43	22.54	22.49	22.49	0.04
	1:10.000	0.235pg/µl	26.01	26.20	26.16	26.12	0.08
	1:100.000		29.27	29.61	29.13	29.34	0.20
	1:1.000.000		31.00	33.55	32.17	32.24	1.04
	1:10.000.000		34.35	35.31	41.35	37.00	3.10
32	1:10	0.125ng/µl	20.33	20.39	20.50	20.41	0.07
	1:100	12.5pg/µl	23.63	26.56	23.83	23.67	0.11
	1:1.000	1.25pg/µl	27.19	27.86	27.26	27.44	0.30
	1:10.000		31.09	30.19	31.19	30.82	0.45
	1:100.000		39.20	32.09	33.40	34.90	3.09
	1:1.000.000		33.01	33.48	-		
	1:10.000.000		33.17	39.75	33.50	35.47	3.03

Table H: Calculation of the analytical sensitivity: average of the last dilution that led to a signal below Ct22 throughout all three technical repetitions for all three biological replicates.

Sample	Last reliably detectable dilution	DNA concentration	Calculation of analytical sensitivity value	Result
7	1:100	46.3 pg/µl	$\frac{sensitivity(sample\ 7) + sens.(sample\ 12) + sens.(sample\ 32}{Nr.of\ samples\ (3)} = \frac{46.3 + 23.5 + 125}{3}$	64.9 pg/µl
12	1:100	23.5 pg/µl		
32	1:10	125 pg/µl		

Results for PCR reproducibility of both barcoding tests:

The tests were performed with three technical replicates and under different conditions (two operators on different days and using different thermocycler machines). The results are shown in Tables I and J.

Table I: Reproducibility of the PCR tests operator 1

Operator:	Pohn
Date of performance:	19.09.2022
Thermocycler machine:	BiometraT3000 (I)

	EPPO PM 7/129 (LCO1490/HCO2198)			**EPPO PM 7/129 (LepF/LepR)**		
Sample	**Repetition 1**	**Repetition 2**	**Repetition 3**	**Repetition 1**	**Repetition 2**	**Repetition 3**
Target 1 *T. leucotreta*	Amplicon	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon
Target 2 *T. leucotreta*	Amplicon	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon
Target 3 *T. leucotreta*	Amplicon	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon
Non-target 1 *C. pomonella*	Amplicon	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon
Non-target 2 *L. botrana*	Amplicon	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon
Non-target 3 *E. ambiguella*	Amplicon	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon

*Sequenced

Table J: Reproducibility of the PCR tests operator 2

Operator:	Heiss
Date of performance:	20.09.2022
Thermocycler machine:	BiometraT3000 (II)

	EPPO PM7/129 (LCO1490/HCO2198)			**EPPO PM7/129 (LepF/LepR)**		
Sample	**Repetition 1**	**Repetition 2**	**Repetition 3**	**Repetition 1**	**Repetition 2**	**Repetition 3**
Target 1 *T. leucotreta*	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon
Target 2 *T. leucotreta*	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon
Target 3 *T. leucotreta*	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon
Non-target 1 *C. pomonella*	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon
Non-target 2 *L. botrana*	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon
Non-target 3 *E. ambiguella*	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon

*Sequenced

Results for PCR reproducibility of the SANGER sequence analysis:

The reproducibility of the SANGER sequence analysis was tested with the same sample panel. The sequence analysis was performed by two operators on different days. The alignment of the consensus sequence was performed in three different data bases (NCBI GenBank, Bold, EPPO-Q-Bank). Tables K and L depict the results of reproducibility.

Table K: Reproducibility of the SANGER sequence analysis operator 1

Operator:	Pohn
Date of performance:	21./26.09.2022
Software:	Geneious prime® 10.1.3

	EPPO PM7/129 (LCO1490/HCO2198)			**EPPO PM7/129 (LepF/LepR)**		
Sample	**NCBI GenBank**	**Bold**	**Q-Bank**	**NCBI GenBank**	**Bold**	**Q-Bank**
Target 1 *T. leucotreta*	*T. leucotreta*	*T. leucotreta*	*T. leucotreta*	*T. leucotreta*	*T. leucotreta*	*T. leucotreta*
Target 2 *T. leucotreta*	*T. leucotreta*	*T. leucotreta*	*T. leucotreta*	*T. leucotreta*	*T. leucotreta*	*T. leucotreta*
Target 3 *T. leucotreta*	*T. leucotreta*	*T. leucotreta*	*T. leucotreta*	*T. leucotreta*	*T. leucotreta*	*T. leucotreta*
Non-target 1 *C. pomonella*	*C. pomonella*	*C. pomonella*	*C. pomonella*	*C. pomonella*	*C. pomonella*	*C. pomonella*
Non-target 2 *L. botrana*	*L. botrana*	*L. botrana*	-*	*L. botrana*	*L. botrana*	-*
Non-target 3 *E. ambiguella*	*E. ambiguella*	*E. ambiguella*	-*	*E. ambiguella*	*E. ambiguella*	-*

* no sequences available for these species in EPPO Q-Bank

Table L: Reproducibility of the SANGER sequence analysis operator 2

Operator:	Gottsberger
Date of performance:	29.09.2022
Software:	Geneious prime® 10.1.3

	EPPO PM7/129 (LCO1490/HCO2198)			**EPPO PM7/129 (LepF/LepR)**		
Sample	**NCBI GenBank**	**Bold**	**Q-Bank**	**NCBI GenBank**	**Bold**	**Q-Bank**
Target 1 *T. leucotreta*	*T. leucotreta*	*T. leucotreta*	*T. leucotreta*	*T. leucotreta*	*T. leucotreta*	*T. leucotreta*
Target 2 *T. leucotreta*	*T. leucotreta*	*T. leucotreta*	*T. leucotreta*	*T. leucotreta*	*T. leucotreta*	*T. leucotreta*
Target 3 *T. leucotreta*	*T. leucotreta*	*T. leucotreta*	*T. leucotreta*	*T. leucotreta*	*T. leucotreta*	*T. leucotreta*
Non-target 1 *C. pomonella*	*C. pomonella*	*C. pomonella*	*C. pomonella*	*C. pomonella*	*C. pomonella*	*C. pomonella*
Non-target 2 *L. botrana*	*L. botrana*	*L. botrana*	-*	*L. botrana*	*L. botrana*	-*
Non-target 3 *E. ambiguella*	*E. ambiguella*	*E. ambiguella*	-*	*E. ambiguella*	*E. ambiguella*	-*

* no sequences available for these species in EPPO Q-Bank

Appendix 9 – Calculations of the performance characteristics diagnostic sensitivity, diagnostic specificity and accuracy

Calculations of the applicable performance characteristics (diagnostic sensitivity, diagnostic specificity and accuracy) for the two EPPO PM7/129(2) barcoding primer sets (EPPO 2021a) and the real-time PCRs (Rizzo *et al.* 2021). Numbers are given without sample 42, which was excluded from the study.

		EPPO PM7/129		**Rizzo *et al.* 2021**	
Target Species	**Criteria**	**LCO1490/HCO2198**	**LepF/LepR**	**ITS TaqMan**	**COI SYBR Green**
Thaumatotibia leucotreta	Number of Positive Agreements	10	10	10	10
	Number of Negative Agreements	26[a]	27	27	26
	Number of Negative Deviations	0	0	0	0
	Number of Positive Deviations	0	0	0	1
	Diagnostic sensitivity	**100**	**100**	**100**	**100**
	Diagnostic specificity	**100**	**100**	**100**	**96**
	Accuracy	**100**	**100**	**100**	**97**

a) sample 43 did not yield an amplicon, reducing the total number of samples.

Appendix 10 – Spike and recovery experiments

The aim of the spike and recovery experiments was to retrace possible ways of contamination and to provide handling and interpretation guidelines for the application of this highly sensitive real-time PCR identification method in routine diagnosis.

As a first step, the storage ethanol from selected samples was tested:
70µl ethanol from previously positive specificity *Cydia pomonella* samples (Tleuco05, Tleuco06, Tleuco15), *Thaumatotibia leucotreta* samples (Tleuco09, Tleuco26, Tleuco30) and previously negative non-target samples (Tleuco21, Tleuco44, Tleuco46) was extracted using the DNeasy Blood and Tissue Kit. The DNA extracts were analysed via *Thaumatotibia leucotreta*-specific real-time PCR.
Only ethanol from positive samples resulted in positive Ct values, ranging from 23.92 to 29.32 (see Table A).

Spike and recovery experiment:
Nine *Cydia pomonella* larvae were contaminated with different amounts (1µl, 10µl, 100µl) of storage ethanol from three tubes containing *Thaumatotibia leucotreta* larvae, respectively (Tleuco09, Tleuco26, Tleuco30). Three larvae were used as negative controls (dH_2O). After incubation at room temperature for 15 minutes, 1000µl 70% ethanol were added and the samples left at room temperature for four days. The ethanol was then removed and 70µl of it used for DNA extraction. The larvae were washed with 400µl dH_2O, 70µl of which were used for DNA extraction as well, to monitor the contamination at different steps. Finally, 400µl of lysis buffer containing proteinase K were added to the larvae before incubating at 56°C over night. The lysis buffer was removed and from here on, the manufacturer's instructions for the DNeasy Blood and Tissue Kit were followed with all 400µl of the buffer.
Samples were analysed via *Thaumatotibia leucotreta*-specific real-time PCR as well as generic barcoding PCR (results see Table B) and resulting amplicons sent for sequencing. Sequence analysis results see Table C.

Results:
In all larval samples *Thaumatotibia leucotreta* DNA could be detected, with Ct values ranging from 23.38 to 33.00 (depending on the DNA concentration in the contaminated source ethanol). Both the ethanol and the water used in the washing step yielded positive Ct values in most cases (77.78%), but with higher Ct values compared to the corresponding larval samples.

As expected, contamination source ethanol with higher Ct values yielded higher Cts in the contaminated samples, with only ethanol from sample Tleuco30 (Ct 29.32) resulting in contaminated water and ethanol samples without Ct value. The control samples with dH2O resulted in no Ct value (1µl, 10µl) or very high values (38.18, 38.21).
These spike and recovery experiments clearly show the possibility of storage ethanol being a source of contamination. Using highly sensitive pest-specific real-time PCRs such as the one published by Rizzo *et al*., this can lead to false positive results,

especially when employing non-destructive DNA extraction methods. To evaluate the influence of this kind of contaminations on the barcoding results, all larval extracts, water and ethanol samples were tested with the LepF/R primer set.

7 out of 9 contaminated samples could be identified correctly, despite the contamination with *T. leucotreta* DNA. However, only three samples resulted in consensus sequences of high quality (>95% HQ). One sample resulted in 92.71 percent identity with Philodinidae sp., which is not a reliable result. Another did not result in any consensus sequence due to low sequence quality.
Sequences from ethanol and water revealed a higher proportion of the contaminant (*T. leucotreta*), but *Cydia pomonella* could be detected as well, but to a lower extend (see Fig. A).
This indicates, that even highly with *T. leucotreta* DNA contaminated *Cydia pomonella* samples could still be identified as such by barcoding, with the contamination at most resulting in lower sequence quality due to insufficient peak separation.This is known to happen when barcoding composite samples. It has to be taken into consideration, that particularly in such cases quality criteria need to be met for reliable barcoding results.

Table A: *Thaumatotibia leucotreta*-specific real-time PCR Ct values for DNA extracted from ethanol in tubes containing specificity samples

Sample	**Assigned value**	**Ct undiluted**	**Ct 1:20 dilution**
Tleuco05	*Cydia pomonella*	-	-
Tleuco06	*Cydia pomonella*	-	-
Tleuco15	*Cydia pomonella*	-	-
Tleuco09	*Thaumatotibia leucotreta*	23.92	28.14
Tleuco26	*Thaumatotibia leucotreta*	28.41	32.50
Tleuco30	*Thaumatotibia leucotreta*	29.32	34.41
Tleuco21	*Tortricidae sp. (Talponia batesi*)	-	-
Tleuco44	*Cryptophlebia peltastica*	-	-
Tleuco46	*Gymnandrosoma aurantianum*	-	-

Table B: Results of the spike and recovery experiments analysed via *Thaumatotibia leucotreta*-specific TaqMan real-time PCR as well as generic barcoding PCR (LepF/LepR)

Sample	**Contaminant**	**Ethanol (A)**		**Water (W)**		**Larva (ndL)**	
Spiked *Cydia pomonella*	Ct storage ethanol	Ct value samples	Barcoding amplicon	Ct value samples	Barcoding amplicon	Ct value samples	Barcoding amplicon
TlC 1 1µl	Tleuco 09 Ct 23.92	32.19	-	32.59	-	26.82	**+**
TlC 2 10µl		31.27	-	28.04	**+**	25.15	**+**
TlC 3 100µl		25.5	**+**	24.8	+	23.38	**+**
TlC 4	Tleuco 26 Ct 28.41	34.37	(+)	32.42	(+)	27.22	**+**
TlC 5		33.28	-	32.27	**+**	28.05	**+**
TlC 6		25.73	**+**	24.44	+	24.71	**+**
TlC 7	Tleuco 30 Ct 29.32	39.28	**+**	-	+	33	**+**
TlC 8		-	+	37.05	**+**	27.7	**+**
TlC 9		29.47	+	27.66	+	25.53	**+**

Amplicons in bold were sent for sequencing

Table C: Sequence analysis results for the spike and recovery experiment. Results marked in red are not reliable.

Sample	**Primer**	**Contig**	**Length**	**%HQ**	**Result**	**% identity**
TlC 3 A	LepF	yes	658	99.7	*Thaumatotibia leucotreta*	100
TlC 3 A	LepR					
TlC 6 A	LepF	no	686	98.6	*Thaumatotibia leucotreta*	99.38
TlC 6 A	LepR		153	0	-	
TlC 7 A	LepF	yes	664	99.4	*Malassezia globosa**	100
TlC 7 A	LepR					
TlC 2 W	LepF	yes	658	99.7	*Thaumatotibia leucotreta*	100
TlC 2 W	LepR					
TlC 5 W	LepF	yes	658	99.7	*Thaumatotibia leucotreta*	100

TlC 5 W	LepR					
TlC 8 W	LepF	yes	658	99.7	*Cydia pomonella*	99.85
TlC 8 W	LepR					
TlC 1 ndL	LepF	yes	659	85.6	*Cydia pomonella*	99.85%
TlC 1 ndL	LepR					
TlC 2 ndL	LepF	yes	700	94.7	*Cydia pomonella*	99.70%
TlC 2 ndL	LepR					
TlC 3 ndL	LepF	yes	658	95.6	*Cydia pomonella*	99.85%
TlC 3 ndL	LepR					
TlC 4 ndL	LepF	yes	658	90.4	*Cydia pomonella*	99.70%
TlC 4 ndL	LepR					
TlC 5 ndL	LepF	yes	645	36.4	*Cydia pomonella*	96.12%
TlC 5 ndL	LepR					
TlC 6 ndL	LepF	yes	661	94.7	*Philodinidae sp.*	92.71
TlC 6 ndL	LepR					
TlC 7 ndL	LepF	yes	658	99.7	*Cydia pomonella*	100.00%
TlC 7 ndL	LepR					
TlC 8 ndL	LepF	yes	658	99.7	*Cydia pomonella*	99.85%
TlC 8 ndL	LepR					

**specimen probably covered with this zoophilic fungi*

Figure A: Pie chart diagrams depicting the barcoding results of the spike and recovery experiment.