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Development of Three Molecular Diagnostic Tools for the Identification of the False Codling Moth (Lepidoptera: Tortricidae)

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Abstract

Three molecular protocols using qPCR TaqMan probe, SYBR Green, and loop-mediated isothermal amplification (LAMP) methods were set up for the identification of larvae and adults of an African invasive moth, *Thaumatotibia leucotreta* (Meyrick, 1913) (Lepidoptera: Tortricidae). The DNA extracts from larval and adult samples of *T. leucotreta* were perfectly amplified with an average Ct value of 19.47 ± 2.63. All assays were demonstrated to be inclusive for *T. leucotreta* and exclusive for the nontarget species tested; the absence of false positives for nontarget species showed a 100% of diagnostic specificity and diagnostic sensitivity for all assays. With the SYBR Green protocol, the Cq values were only considered for values less than 22 (cutoff value) to prevent false-positive results caused by the late amplification of nonspecific amplicons. The limit of detection (LoD) for the qPCR probe protocol was equal to 0.02 pg/µl while a value equal to 0.128 pg/µl for the qPCR SYBR Green assay and LAMP method were established, respectively. The intrarun variabilities of reproducibility and repeatability in all the assays evaluated as CV%, ranged between 0.21 and 6.14, and between 0.33 and 9.52, respectively; the LAMP values were slightly higher than other assays, indicating a very low interrun variability. In order for an operator to choose the most desirable method, several parameters were considered and discussed. For future development of these assays, it is possible to hypothesize the setup of a diagnostic kit including all the three methods combined, to empower the test reliability and robustness.

Key words: quarantine pest, molecular diagnostics, qPCR TaqMan probe, qPCR SYBR Green, loop-mediated isothermal amplification

Thaumatotibia leucotreta (Meyrick, 1913) (Lepidoptera: Tortricidae), also known as the false codling moth, is a tortricid moth native to the Ethiopian region (Venette et al. 2003), currently widespread throughout Sub-Saharan Africa (EPPO 2018). In South Africa, this highly polyphagous species is a key pest of citrus with fruit losses up to 90% (EPPO 2013), while the economic impact to other agricultural and horticultural industries should not be underestimated (Golding 1946, Venette et al. 2003, Gilligan et al. 2011, Adom et al. 2021). Larval feeding activity causes direct damage on citrus, consisting in early ripening and fruit drop (EPPO 2013) as well as indirect bacterial and/or fungal infections (Gilligan et al. 2011).

Since 1983, *T. leucotreta* larvae has frequently been intercepted in *Citrus sinensis* fruit imported from South Africa at several ports of entry in Europe, including Spain and Italy (for interception list and updates, see EPPO Global Database 2018) (Mazza et al. 2014). The risk of introduction the pest on the fresh citrus pathway increases from April to August when more than 40% of South African citrus

fruit is exported to Europe as the production of the Mediterranean areas ceases (PPECB 2020). *Thaumatotibia leucotreta* was also occasionally intercepted in the United States (Venette et al. 2003). For its harmfulness and phytosanitary risk *T. leucotreta* is listed among quarantine pests in Europe (EPPO 2013; EFSA 2019, 2020) and North America (NAPPO 2016). While the risk of introducing this species from Africa on the fresh fruit pathway remains high, effective control measures were developed to reduce the risk of introduction of *T. leucotreta*, in order to secure the export of fresh produce, especially citrus fruit from South Africa (Hattingh et al. 2020, Adom et al. 2021).

An early detection of concealed insect pests or plant pathogens in commodities at ports of entry, as well as a prompt identification of these organisms are essential to minimize the possibility of establishment and spread in a new environment. The availability of rapid, accurate, and reliable diagnosis kits for different pest species at any stage of their development is therefore crucial in order to avoid possible economic and social impacts resulting from incorrect or misidentification of species (Chua et al. 2010, Blacket et al. 2012).

A great variety of diagnostic techniques and methodologies is available, with different performance relating to sensitivity, flexibility, execution speed, and cost, aspects relevant to biomolecular techniques applied to phytosanitary diagnostics. The most widely applied methods include qPCR with both SYBR Green and TaqMan probe chemistry (Tajadini et al. 2014, Kralik and Ricchi 2017), and loop-mediated isothermal amplification (LAMP) (Notomi et al. 2000, 2015; Wong et al. 2018; Panno et al. 2020). Several diagnostic protocols for the identification of insect pests of economic importance have been developed and applied over time, each relying on one technique (Yu et al. 2004; Dhami et al. 2016; Kim et al. 2016; Ide et al. 2016a, b; Koohkanzade et al. 2018) or, sometimes, on the integration of two different techniques (Harper et al. 2010, Rizzo et al. 2021).

In this study, three alternative techniques of gene amplification, based on different approaches (qPCR probe, qPCR SYBR Green, and LAMP chemistry), were developed and compared to establish a rapid and reliable protocol for the identification of larvae and adult *T. leucotreta*. This procedure can be an effective tool to detect the presence of *T. leucotreta* specimens in commodities imported from African countries. Moreover, the availability of alternative diagnostic methods could allow to perform identification tests in laboratories provided with different equipment and operator expertise.

Materials and Methods

Insect Samples

The target samples used for DNA extraction included 19 specimens of *T. leucotreta*. Eighteen adults (males and females) were obtained from the laboratory population of *T. leucotreta* from the XSIT (Pty) Ltd. mass-rearing facility (Citrusdal, Western Cape, South Africa) while one larva was collected from a batch of infested oranges intercepted at the entry point of the Leghorn port (Italy) in 2014 (Mazza et al. 2014).

Twenty-five nontarget sample species, some taxonomically related (species 7–11 belonging to the same family and/or subfamily), some sharing host plants and/or commodities (species 5, 16, 22 developing on citrus), and others, including quarantine or invasive pests, with no relation to the target species were used in developing the assay. Most of these nontarget specimens were available in the DNA collection of the phytopathological laboratory of the Phytosanitary Service of Tuscany Region (Italy), where the experiments were carried out; others were kindly provided by colleagues of Italian or foreign research institutions or extension services (Table 1).

DNA Extraction

DNA extraction was performed in duplicate for each of the 19 *T. leucotreta* specimens to a total of 38 DNA extracts. The duplicates of the DNA extract coincide with the technical replicates of the same starting lysate. Genomic DNAs were extracted using a modified cetyltrimethylammonium bromide (CTAB) extraction method by Rizzo et al. (2020b) from Li et al. (2008). Quality and quantity evaluation of DNA samples were performed on the QiaExpert instrument (Qiagen, Hilden, Germany). After the elution in 100 µl of nuclease-free water, DNA was used for the qPCR or LAMP reactions immediately or, alternatively, stored at –20°C until use. Positive and negative isolation controls were used during each DNA extraction to monitor possible contamination and to test the extraction effectiveness. To assess the quality of the extracted DNA, 1:50 DNAs/double distilledH_2O were tested in a qPCR reaction using a dual-labeled probe targeting a highly conserved region of the 18S rDNA (Ioos et al. 2009). These amplifiability tests can allow to detect the presence of PCR inhibitors on the basis of both the Cq values and the slope of the relative amplification curves.

Design of Primers and Probes

Different design of primers and probes was applied, depending on the technique assayed.

qPCR using TaqMan and SYBR Green primers and probes

The primer pairs (Table 2) were designed within conserved regions sequence of *T. leucotreta* using the OligoArchitect Primer and Probe Design online software (Sigma-Aldrich, St. Louis, MO), and were synthetized by Eurofins Genomics (Ebersberg, Germany). In particular, for the qPCR TaqMan probe assay, the region ITS was used (internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence), while for qPCR SYBR Green assay the genomic region of COI (cytochrome oxidase subunit 1) was considered.

The in silico specificity of the expected amplicon was tested using the BLAST (Basic Local Alignment Search Tool: http://www.ncbi.nlm.nih.gov/BLAST) software (Altschul et al. 1990), searching the most related nucleotide sequences and aligning them by the MAFFT software package (Katoh and Standley 2016) implemented with the software Geneious 10.2.6 (Biomatters Ltd., Auckland, New Zealand, http://www.geneious.com).

In the in silico alignments of TaqMan probe assay, *T. batrachopa* was the most related species to *T. leucotreta*. In SYBR Green assay, the alignments (Fig. 2) were carried out taking into account also the sequences deposited in BOLD (Barcode of Life Data System). In this way, differences with congeneric species (*T. maculata*, *T. aclyta*, *T. zophophanes*, *T. batrachopa*, *T. salaciae*) were highlighted. Results are shown in Figs. 1 and 2 for TaqMan probe and SYBR Green theoretical amplicons, respectively.

Real-time and Visual LAMP assay

The six LAMP primers consist of two external primers (forward primer, F3, backward primer, B3), two inner primers (forward inner primer, FIP; backward inner primer, BIP), and two loop primers (forward loop primer, FLP; backward loop primer, BLP), as expected from a standard reaction LAMP (Notomi et al. 2000, 2015). The primers were designed (Table 3) using the LAMP Designer software

Table 1. List of target and nontarget specimens (species, life stage, and geographical origin) used for DNA extraction

Order	Family (subf. tribe)	Species	Life stage	Specimens (no.)	Geographical origin of samples
Hemiptera	Cicadellidae	1 *Synophropsis lauri* (Horvath, 1897)	Adult	1	Italy
	Ricaniidae	2 *Ricania speculum* (Walker, 1851)	Adult	1	Italy
Lepidoptera	Pieridae	3 *Pieris napi* (Linnaeus, 1758)	Adult	1	Italy
	Lymantriidae	4 *Lymantria dispar* (Linnaeus, 1758)	Adult	1	Italy
	Pyralidae	5 *Cryptoblabes gnidiella* (Millière, 1867)	Adult	1	Italy
		6 *Euzophera semifuneralis* (Walker, 1863)	Larva	1	USA
	Tortricidae, Olethreutinae Grapholitini	7 *Cydia pomonella* (Linnaeus, 1758)	Adult	2	Italy
		8 *Grapholita (Aspila) molesta* (Busck, 1916)	Adult	1	Italy
		9 *Thaumatotibia leucotreta* (Meyrick,1913)	Larva, adults	19	South Africa
	Tortricidae, Tortricinae Archipini	10 *Cacoecimorpha pronubana* (Hübner, 1799)	Larva	1	Italy
		11 *Archips rosana* (Linnaeus, 1758)	Adult	1	Italy
Coleoptera	Cerambycidae	12 *Stictoleptura cordigera* (Füssli, 1775)	Adult	1	Italy
		13 *Aromia moschata* (Linnaeus, 1758)	Adult	1	Italy
Diptera	Tephritidae	14 *Acanthiophilus helianthi* (Rossi, 1794)	Adult	1	Italy
		15 *Anastrepha fraterculus* (Wiedemann, 1830)	Adult	1	Ecuador
		16 *Anastrepha ludens* (Loew, 1873)	Adult	1	USA[a]
		17 *Anastrepha serpentina* (Wiedemann, 1830)	Adult	1	USA[a]
		18 *Bactrocera dorsalis* (Hendel, 1912)	Adult	1	Afghanistan
		19 *Bactrocera latifrons* (Hendel, 1915)	Adult	1	Kenya
		20 *Bactrocera oleae* (Rossi, 1790)	Adult	1	Italy
		21 *Bactrocera zonata* (Saunders, 1842)	Adult	1	Afghanistan
		22 *Ceratitis capitata* Wiedemann 1824	Adult	1	Italy
		23 *Ceratitis* sp.	Larva	1	South Africa
		24 *Rhagoletis cerasi* (Linnaeus, 1758)	Larva	1	Italy
		25 *Rhagoletis completa* Cresson	Adult	1	Italy

[a]Specimens from a lab colony at the former USDA ARS Kika de la Garza Subtropical Agricultural Research Center (Weslaco, TX).

Table 2. List of the primers and probes designed for qPCR TaqMan and SYBR Green protocols for *Thaumatotibia leucotreta*

Method	Primer/Probe name	Length (bases)	Sequence 5′–3′	Nucleotide position	Product size (bp)	GenBank number
TaqMan probe	Tleuco_1001F	18	CGTCCAAGCATAGCTTTC	1001 to 1019	70	MG264603.1
	Tleuco_1070R	18	GAGGCAGACACGATATCC	1070 to 1052		
	Tleuco_1041P	21	FAM-CGACGACGACCATAACAACGC-BHQ1	1041 to 1020		
SYBR Green	Tleuco_266_F	18	TGGAACAGGATGAACAGT	266 to 284	93	LC055410.1
	Tleuco_359_R	19	TGCTAGGTGAAGAGAGAAA	359 to 340		

(OptiGene Limited, Horsham, United Kingdom) based on the same sequence used for the qPCR probe protocol (accession number MG264603.1), and were synthetized by Eurofins Genomics. As for the other assays, the in silico analytical specificity of the LAMP assay was tested aligning the most related sequences present in GenBank using the MAFFT software implemented in Geneious 10.2.6. Results of alignments are shown in Fig. 3. In the same genomic region of reference (internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence) no similar alignments with other deposited sequences have been highlighted (in particular, with the sequences of the species taxonomically or genetically related). The alignments demonstrate that the *T. leucotreta* sequence does not overlap to the chosen reference for the real-time LAMP protocol, supporting the specificity of the method. For both the LAMP and qPCR TaqMan probe protocols, the same gene region has been used for primers, although the internal distribution of the amplicons was well distinct between the two methods, as shown in Fig. 4.

qPCR With TaqMan Probe and SYBR Green Optimization

DNA amplification for the two methods was carried out using a CFX96 (Bio-Rad, Hercules, CA) thermal cycler. In the optimization protocol, primer annealing temperatures, the reaction mixture, and optimal primer concentrations were included. The optimal primer annealing temperature of the amplification reaction in qPCR TaqMan as well as in SYBR Green protocol was determined by the DNA amplification of two *T. leucotreta* specimens with a temperature gradient ranging from 52 to 60°C. Oligos were also tested at different concentrations (0.2, 0.3, and 0.4 µM) to determine the optimal one.

LAMP Assay

Real-time LAMP

Real-time LAMP reactions have been performed and optimized on a CFX96 (Bio-Rad) thermal cycler. The DNA samples listed

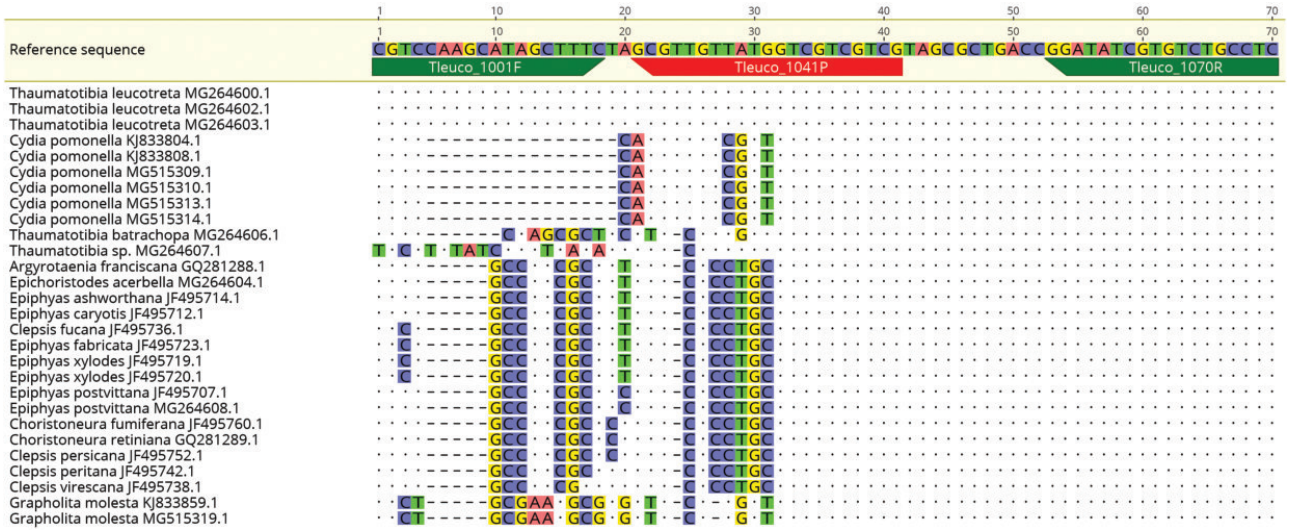

Fig. 1. Alignments resulting from the in silico theoretical TaqMan amplicon and sequences of the related organisms present in GenBank. The forward and reverse primers and the TaqMan probe built in this study are reported on the GenBank number of *Thaumatotibia leucotreta* MG264603.1.

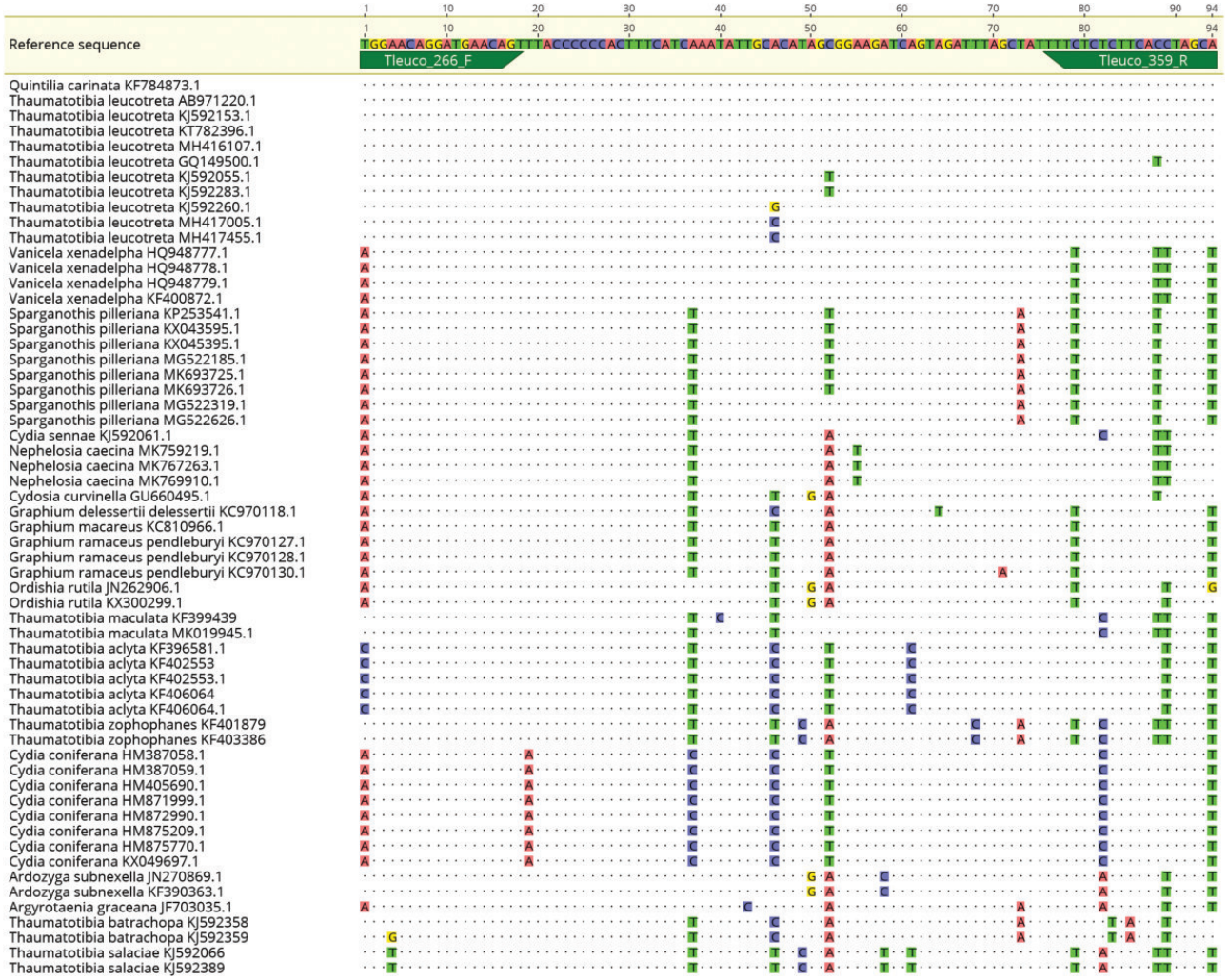

Fig. 2. Alignments resulting from the in silico theoretical SYBR Green amplicon and sequences of the related organisms present in GenBank. The forward and reverse primers built in this study are reported on the GenBank number of *Thaumatotibia leucotreta* LC055410.1.

Table 3. List of the primers designed on purpose for LAMP protocol used for *Thaumatotibia leucotreta*

Primer name	Length (nt)	Sequence 5′–3′	Nucleotide position	Product size (bp)	GenBank number
Tleuco_B3	20	AGTCCGCCTACTTTTCATTC	975 to 955	170	MG264603.1
Tleuco_BIP(B1c+B2)	43	ATACGACATCCACCGTTCGC–CGCACGTTCACACCTTAT	851 to 871 and 929 to 911		
Tleuco_F3	18	CGCTTACGCTATTGGAGG	711 to 729		
Tleuco_FIP(F1c+F2)	43	ACACGCACACGAACGGAA–TTTCACCGGGAGTCCTTT	837 to 819 and 760 to 778		
Tleuco_LoopB	22	CGTGCGTACACTCTGTGT	872 to 890		
Tleuco_LoopF	21	CACGAACGTCGCTATAATTGC	819 to 798		

Fig. 3. Alignments of the in silico theoretical LAMP amplicon and other sequences of *Thaumatotibia leucotreta* present in GenBank. The six LAMP primers are reported on the GenBank number of *T. leucotreta* MG264603.1.

in Table 1 were amplified in 0.2-ml real-time PCR tubes (Starlab, Milan, Italy). The thermal cycle was set up at 65°C for 1 s, followed by the record of fluorescence after 59 s. The amplification time (t_{amp}) was set to 30 min. A melting curve was generated by heating the samples at temperatures between 65 and 95°C, with 10-s intervals every 0.5°C at the end of the LAMP reaction (Abdulmawjood et al. 2014). Each isothermal reaction was performed in duplicate, in a final volume of 20 µl. The isothermal amplification time (from 20 to 40 min), the amount of 10× LAMP primer mixture (2.0–5.0 µl), and the concentration of each group of LAMP primers (0.2–0.4 µM of F3 and B3, 0.4–0.8 µM of the LoopF and LoopB, and 0.8–1.2 µM of the FIP and BIP) were accounted for LAMP protocol optimization. To find the optimal temperature for the LAMP amplification, the reactions were performed under a thermal gradient from 60 to 70°C.

Visual LAMP

The DNA samples itemized in Table 1 were also used in real-time LAMP. The LAMP reactions were carried out in duplicate using the Bst 3.0 DNA polymerase (New England Biolabs, Ipswich, MA) in a total volume of 20 µl. The protocol was performed using an endpoint MyCycler thermal cycler (Bio-Rad). The optimization of the Visual assay was obtained as already described in a previous work (Rizzo et al. 2021). Products of LAMP reactions were observed by naked eye under natural light and were photographed using a common smartphone camera. A color change in light blue indicated positive samples, while the negative ones appeared purple.

The Visual LAMP protocol was carried out on the DNA of *T. leucotreta* as well as on nontarget specimens. The reaction was performed at 65°C for 30 min, followed by an additional cycle at 80°C for 2 min.

Performance Characteristics for the qPCR Probe, SYBR Green, and Real-Time LAMP

To evaluate the handling of the tests for routine diagnostics, performance criteria such as analytical sensitivity, analytical specificity, repeatability, and reproducibility were determined. Validation was performed according to EPPO PM7/98 (4) (EPPO 2019).

To test the specificity, the diagnostic sensitivity, and the relative accuracy, an internal blind panel was prepared using 16 samples of the target species and 18 of the nontarget ones. The protocol was already described in a previous work (Rizzo et al. 2021), according to the EPPO standard (EPPO 2019). Reactions were run in triplicate for each of the developed protocols. For qPCR probe the analytical sensitivity was also estimated in triplicate, using 10-fold 1:10 serial dilutions of *T. leucotreta* DNA diluted to 5 ng/µl. These different dilutions were applied due to the best performance obtained in qPCR probe protocol respect to the 10-fold 1:5 serial dilutions. The evaluation range for both protocols was from 10 ng/µl to 0.02 fg/µl.

For both the qPCR SYBR Green and LAMP protocols, the analytical sensitivity (limit of detection [LoD]), was estimated in triplicate, using 10-fold 1:5 serial dilutions of *T. leucotreta* DNA diluted to 5 ng/µl. The evaluation range for both protocols was from 10 ng/µl to 25.6 fg/µl. As far as possible, the same serial dilutions have been tested, to make a comparison among the two different techniques.

The intrarun variation (repeatability) and the interrun variation (reproducibility) were assessed through standard parameters such as the average standard deviation (SD) Cq and the percentage coefficient of variation (% CV). Eight DNA extracts of *T. leucotreta* adult specimens were tested in triplicate, diluted to a concentration equal to 5 ng/µl, in two separate series. In order to estimate qualitatively the repeatability, and the reproducibility, the mean values, SD, and % CV were calculated for each sample and for each series of

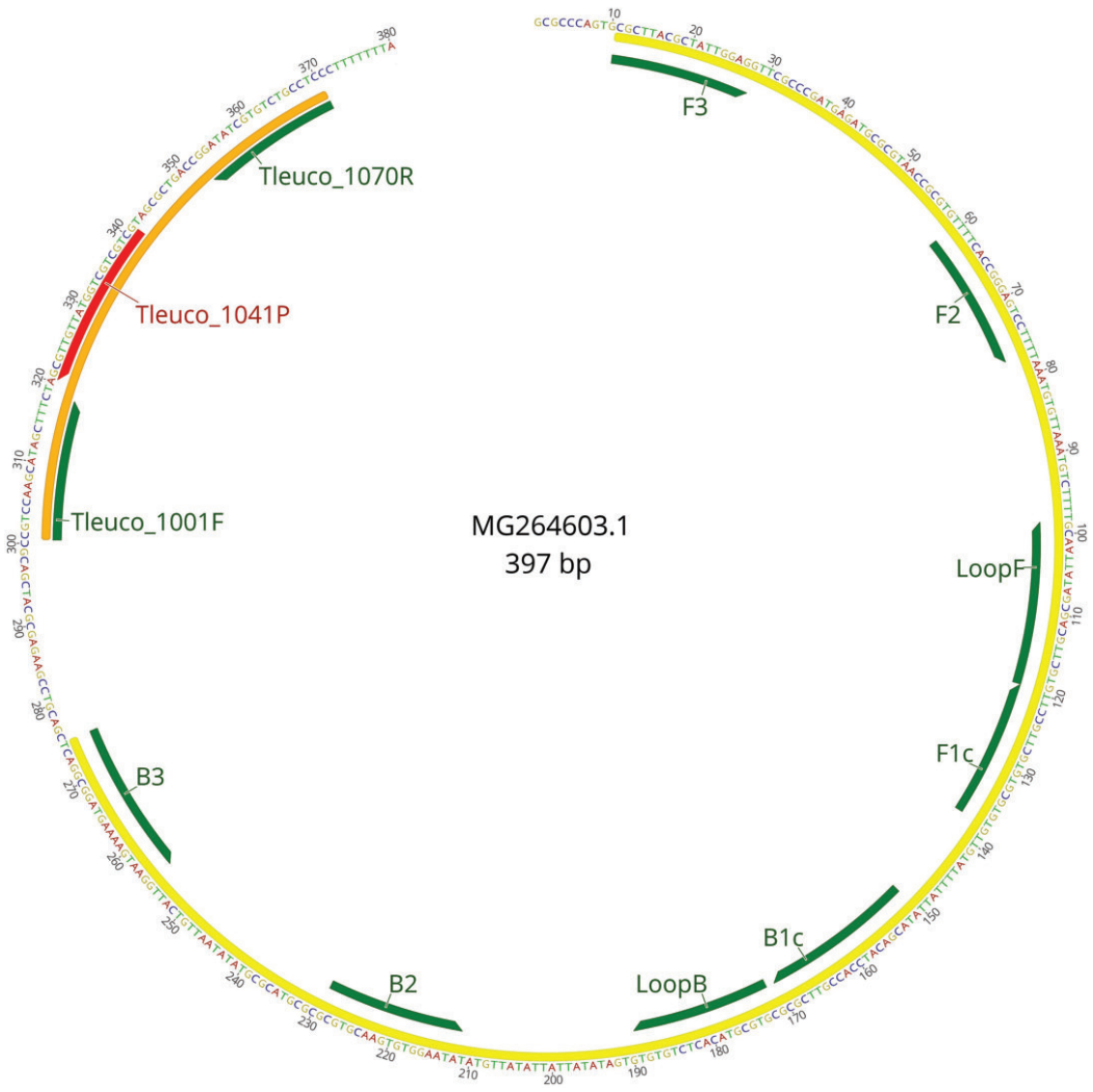

Fig. 4. Position of the primers used for *Thaumatotibia leucotreta* in the LAMP and TaqMan probe protocol. The LAMP amplicon is represented in yellow, from 711 to 975 bp and the probe amplicon is orange, from 1,001 to 1,071 bp. Both methods were built on the same rDNA region using the MG264603.1 sequence retrieved from GenBank.

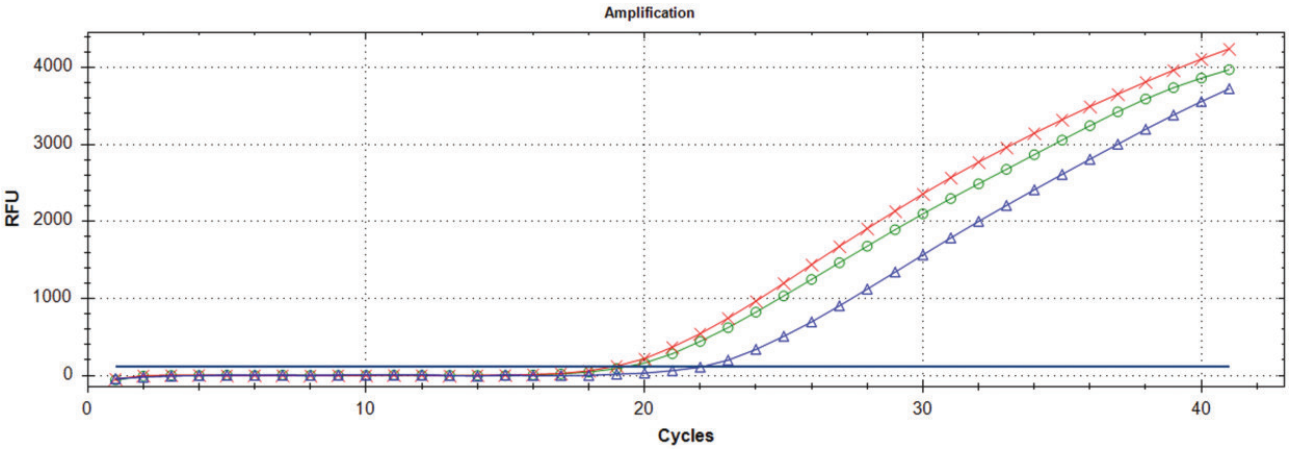

Fig. 5. *Thaumatotibia leucotreta,* qPCR probe protocol. Gene amplification of the larval DNA (circles and crosses) and adult DNA (triangles).

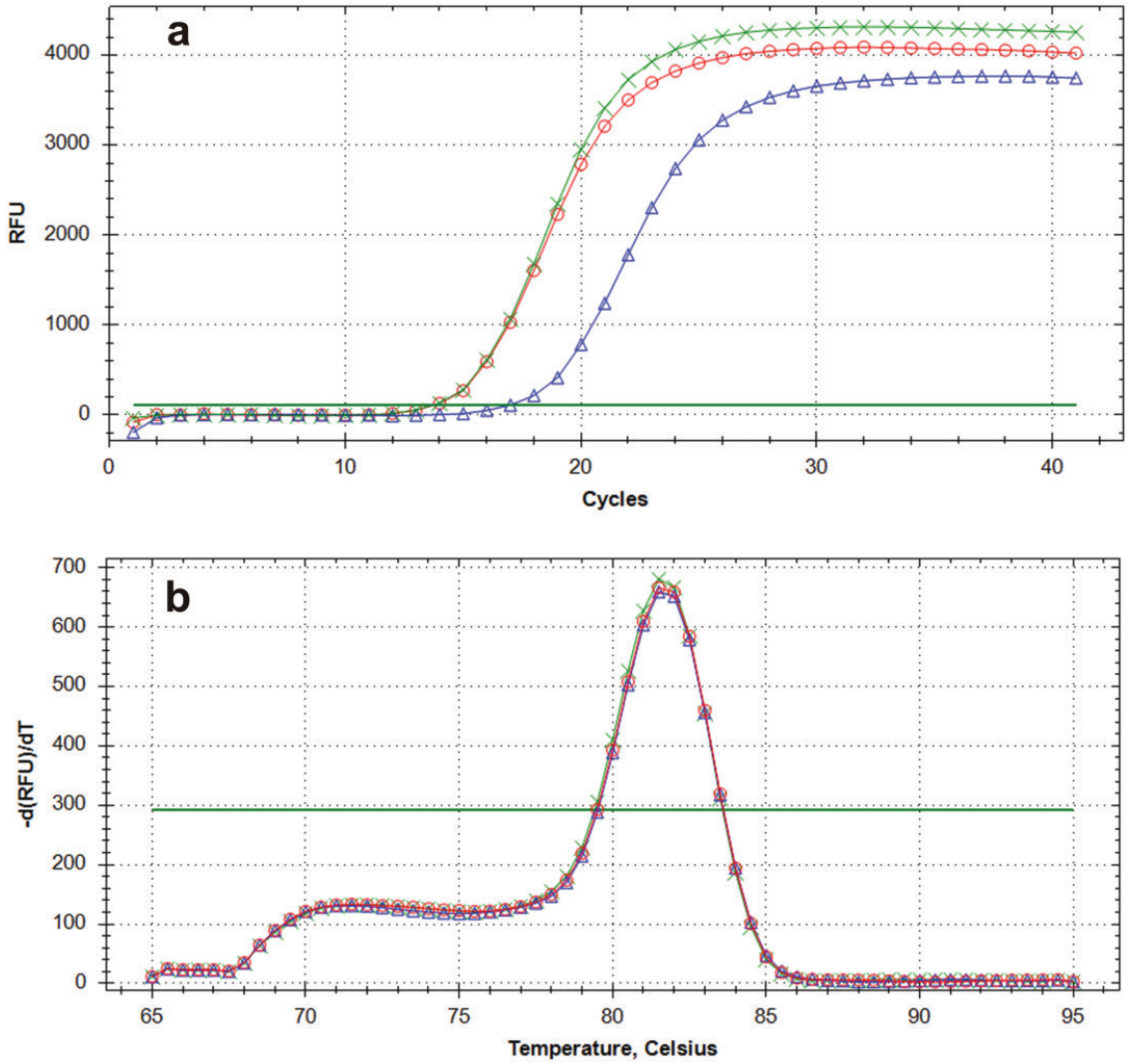

Fig. 6. *Thaumatotibia leucotreta*, qPCR SYBR Green protocol. (a) Gene amplification of the larval DNA (circles and crosses) and adult DNA (triangles). (b) Melting peaks of the resulting amplification products for larval and adult samples.

samples (Dhami et al. 2016, Koohkanzade et al. 2018). The reproducibility of the developed assays was tested by two different operators on different days. Data obtained for the two series of samples were compared.

Overall Qualitative Evaluation

After the development of the three molecular protocols for the identification of *T. leucotreta*, some functional features of the proposed methods were qualitatively evaluated. This was done on the basis of our own experience, rating a concise score to provide a tool for operators in choosing the most appropriate assay for the specific laboratory conditions.

Results

DNA Extraction

The values of average concentration (ng/µl), absorbance (A260/280), SD, and coefficient of variation in percentage of the DNA extracted from the target samples were calculated and grouped according to the developmental stage of specimens of *T. leucotreta*, larva or adult. The average DNA concentrations extracted were found to be 620 ± 16.2 and 52 ± 6.8 ng/µl, for larval and adult samples, respectively. The average absorbance values were 2.01 ± 0.4 for the larval and 1.78 ± 0.16 for adult samples.

The DNA extracts from larval and adult samples of *T. leucotreta* were perfectly amplified (data not shown) with the qPCR probe targeting the 18S rDNA (Ioos et al. 2009) with an average Cq value of 19.47 ± 2.63.

Assay Conditions of the qPCR TaqMan Probe, SYBR Green, and LAMP Protocols

The optimization of the qPCR TaqMan probe protocol stated as the optimal concentration was 10 µl of 2× QuantiNova PCR Master Mix Probe (Qiagen) with primers and probe concentrations at 0.3 and 0.1 µM, respectively. The optimal annealing temperature was 60°C. No significant differences in the Cq values of all *T. leucotreta* samples were observed between the annealing temperatures of 58, 60, and 62°C, or the different concentrations of primers (300 and 500 nM) and probes (150 and 250 nM). The qPCR conditions consisted of an initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, and 60°C for 40 s (Fig. 5). Samples were

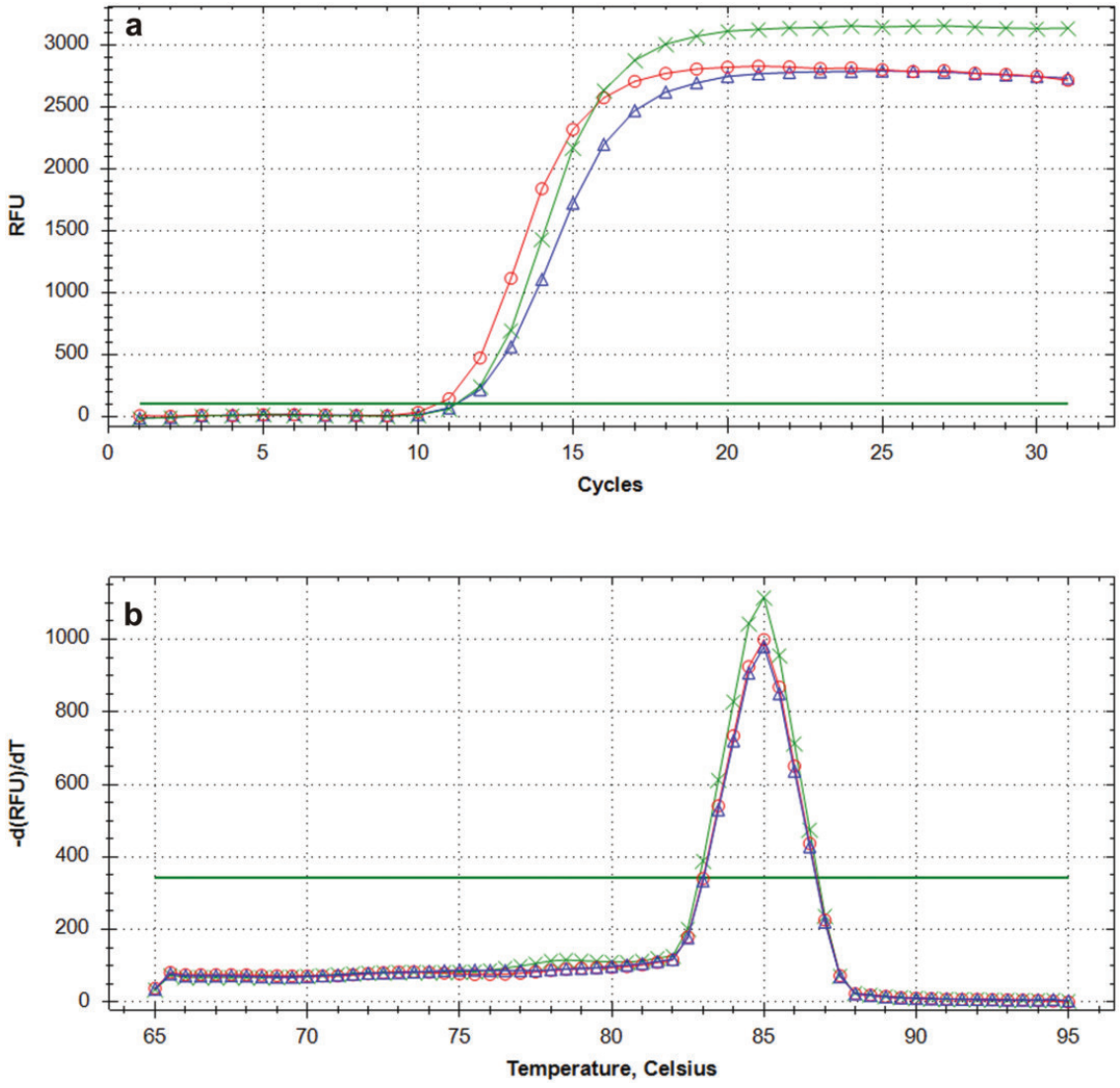

Fig. 7. *Thaumatotibia leucotreta,* real-time LAMP protocol. (a) LAMP amplification curves of the larval DNA (circles and crosses) and adult DNA (triangles); (b) melting peaks.

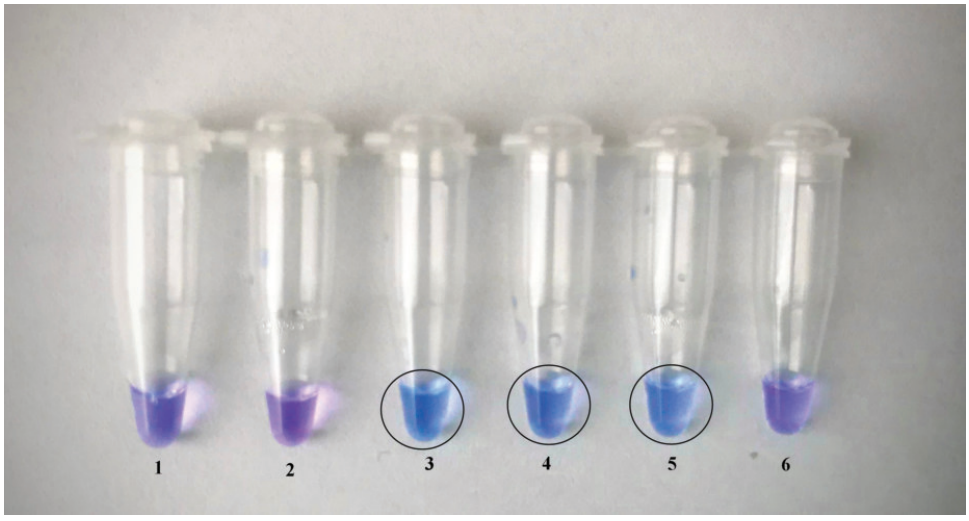

Fig. 8. Visual LAMP assay reaction: Tube 1: *Rhagoletis completa*; Tube 2: *Cydia pomonella*; Tubes 3–5 (with circles): *Thaumatotibia leucotreta*; Tube 6: nontarget control.

considered positive when the resulting qPCR curves showed an evident inflection point (in addition to increasing kinetics) and Cq values less than 35.

For qPCR based on the SYBR Green chemistry, the optimal conditions consisted of an initial denaturation at 95°C for 2 min, followed by 25 cycles of 95°C for 10 s, and 55°C for 40 s. The melting peak was equal to 76.5°C ± 0.5°C (Fig. 6). The Cq values were only considered if assessed lower than 22 (cutoff value) to prevent false-positive results caused by late amplification of nonspecific amplicons.

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.

In real-time LAMP assay, the optimal reaction mix contained 10 µl Isothermal Master Mix (ISO-001) (OptiGene Limited), 2.0 µl LAMP primer mixture 10× (at final concentrations of 0.2 µM of each F3 and B3, 0.4 µM of each of the LoopF and LoopB, and 0.8 µM of each of the FIP and BIP), and 2 µl of template DNA 1:10 diluted using sterile water (about 10 ng/µl).

The LAMP reaction was carried out at 65°C for 30 min, resulting in an average Cq (Ta = time amplification) of 12.65 ± 0.2. The amplification step was followed by an annealing analysis from 65 to 95°C with ramping of 0.5°C to determine the melting peak, which was achieved at 85 ± 0.5°C (Fig. 7).

The optimal Visual LAMP reaction mixture consisted of 20 µl:2 µl Isothermal Buffer 10×, 0.6 mM dNTPs, 2 mM $MgSO_4$, 0.15 mM HNB, 0.2 M Betaine; final concentrations of the LAMP primers were 0.2 µM for F3/B3, 0.4 µM for LoopF/LoopB, 0.8 µM for FIP/BIP, 0.32 U/µl Bst 3.0, and 2 µl of template DNA (5 ng/µl).

Visual LAMP assay results are shown in Fig. 8.

Performance Characteristics of the Assays

All assays developed in this study were inclusive for *T. leucotreta* and exclusive for the nontarget organisms tested. All target specimens were identified using the specific tests while no false positives were obtained for nontarget organisms, resulting in a 100% diagnostic specificity and diagnostic sensitivity for all the tests.

The tests showed the coefficient of determination (r^2) value for each technique assayed equal to 99.80, 99.65, and 97.35% for qPCR probe, qPCR SYBR Green, and real-time LAMP, respectively.

The analytical sensitivity values of the assays are shown in Table 4. 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 cutoff value of 22 is adopted, the LoD is 3.2 pg/µl. In the case of LAMP method, the LoD is 0.128 pg/µl of DNA, as determined in the SYBR Green assay.

The intrarun variabilities of repeatability and reproducibility in all the assays ranged between 0.21 and 6.14, and between 0.33 and 9.52, respectively (Table 5).

The operational parameters of the three methods evaluated qualitatively, are listed in Table 6 with the respective concise score. The

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.

Table 5. Repeatability and reproducibility values obtained using the three different protocols (TaqMan probe, SYBR Green, and LAMP)

	qPCR TaqMan probe			qPCR SYBR Green			Real-time LAMP		
	Repeatability		Reproducibility	Repeatability		Reproducibility	Repeatability		Reproducibility
Sample (no.)	Cq ± SD	CV%	CV%	Cq ± SD	CV%	CV%	T_{amp} (min:s) ± SD	CV%	CV%
1	13.63 ± 0.05	0.33	0.69	17.41 ± 0.23	1.33	0.21	11.18 ± 0.22	1.99	1.38
2	13.67 ± 0.13	0.94	1.42	17.16 ± 0.49	2.83	0.65	10.66 ± 0.28	2.65	1.80
3	13.65 ± 0.14	1.00	1.22	17.17 ± 0.36	2.08	0.69	10.57 ± 0.18	1.67	1.15
4	13.83 ± 0.17	1.23	0.91	17.27 ± 0.27	1.55	0.71	11.94 ± 1.06	8.91	6.06
5	16.51 ± 1.05	6.33	3.28	16.57 ± 0.84	5.09	4.26	11.27 ± 0.68	6.01	4.09
6	16.20 ± 0.22	1.36	1.84	16.66 ± 0.48	2.89	0.74	11.06 ± 1.05	9.52	5.64
7	15.98 ± 1.63	10.18	2.70	16.57 ± 0.72	4.35	5.73	11.64 ± 1.09	9.38	6.14
8	15.72 ± 0.66	4.17	2.45	16.83 ± 0.73	4.33	2.46	11.19 ± 0.90	5.73	3.75
Avg	14.90 ± 0.50	3.19	1.81	16.95 ± 0.51	3.06	1.93	11.19 ± 0.68	5.73	3.75

Table 6. Qualitative evaluation of some operational parameters of the assays developed in the study

Parameter	qPCR		Real-time LAMP	Visual LAMP
	TaqMan probe	SYBR Green		
Professional skill	+++	++	++	+
Lab equipment	+++	+++	+++	+
Time requirement	+++	++	+	+
Costs	+++	++	++	+

Three crosses: high requirements, one cross: low requirements.

requirement of professional skill was considered relatively low for Visual LAMP assay which was assessed as the easiest to perform in comparison to the other techniques. Moreover, Visual LAMP assay was also the most rapid and the most cost-effective method.

Discussion

International trade is involved in the worldwide spread of insect pests and subsequent establishment of invasive populations outside their native areas (Wu and Zhao 2007, Hulme 2009). The rapid diagnosis of insect pests at entry points or during monitoring activities after their accidental introduction is of paramount importance to avoid their spread in new territories with dramatic ecological and economic consequences. The availability of an easy-to-handle morphological taxonomic key could be a rapid and useful tool for species identification of the larval stage (Gilligan and Passoa 2014). Unfortunately, taxonomic keys undergo different limitations (Walter and Winterton 2007) requiring specific expertise; moreover, very often, the available morphological keys can be unsuitable when suspected insect pests are detected at a very early developmental stage.

On the contrary, biomolecular diagnostic tools can provide a substantial help in species identification (Augustin et al. 2012). In recent years, many of these protocols were developed to recognize different pests under quarantine regulation speeding up the identification process when a rapid response is needed (Blacket et al. 2012; Bouwer et al. 2014; Onah et al. 2016; Rizzo et al. 2020a, b)

The problem with *T. leucotreta* identification was addressed by developing a species-specific, sensitive and effective biomolecular diagnostic tool that can be utilized in all the laboratories involved in pest surveillance activities at entry points, in plant, pest interception, and pest monitoring activities.

Although some nonspecific responses were observed applying the qPCR SYBR Green assay, the cutoff value of Cq, fixed at the value of 22, prevents any possible erroneous attribution. Moreover, in this same assay, the in silico tests showed a homology in the amplicon sequence of a South African cicada, *Quintilia carinata* (Bouwer et al. 2014). On the contrary, *T. leucotreta* demonstrates morphological, biological, and ecological features indisputably different from those displayed by species sharing some homologies (*C. viridis* and *S. tridentata* for SYBR Green assay and *Q. carinata* for the in silico test), so this does not rebut the validity of the diagnostic tests.

The LAMP assays, being very sensitive, appear to be the most flexible techniques, since they can be used both in endpoints (Visual LAMP) and with real-time LAMP. In addition, the LAMP assays are rapid, taking less than 2 h from DNA extraction to results (Koohkanzade et al. 2018).

Repeatability and reproducibility of results were evaluated, indicating the assays in qPCR probe and SYBR Green have a variability less than 5% in almost all measurements (except for some measures in SYBR Green assays) (Teter and Steffer 2017). The LAMP values are slightly higher than other assays, demonstrating a very low interrun variability.

A critical point to newly developed assays is securing a good quality and decent quantity of DNA extraction. The method used in this study, already applied to different insect species and previously proposed (Rizzo et al. 2020b), is fast (the DNA extraction protocol from insects allows to process up to 24 single specimen samples in about 50 min), relatively cheap, and flexible, so that any laboratory equipped only with basic biomolecular apparatus can use it.

The diagnostic tools developed in this study may be used integrated or combined to each other, ensuring the greatest validity of results, either to detect new interceptions, or confirming first assessments, as they are different and alternative techniques, based on amplicons related to different genomic regions. The availability of molecular diagnostic protocols targeting different genomic regions improves validation of samples suspected to be positive, through cross-analyses of these protocols.

The developed protocols could combine advantages provided by the different biomolecular techniques, avoiding negative aftermaths and becoming a helpful tool to detect the presence of false codling moth in imported commodities from African countries to different continents.

For future perspectives, the good analytical performances showed by the developed assays could be used to create a multitechnique diagnostic kit which could ensure highest reliability.

Conclusion

The use of biomolecular diagnostic tools provides the means for the implementation of control and monitoring activities with a new perspective, utilizing the speed, sensitivity, and specificity of innovative diagnostic techniques for a quick identification of potentially harmful insect pests avoiding their introduction into new territories. The protocols developed in this research, both in qPCR (probe and SYBR Green) and LAMP (real-time and Visual), can be applied with rapid and reliable diagnostic results to detect the suspected presence of *T. leucotreta* in commodities exported from African countries to different continents or to monitor recorded hotbeds, providing local authorities with adequate tools, allowing for timely and effective pest eradication.

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