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Research

Rapid Identification of *Tropilaelaps* Mite (Mesostigmata: Laelapidae) Species Using a COI Barcode-HRM

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Abstract

Tropilaelaps mite (Mesostigmata: Laelapidae) is an ectoparasite of bees present, to date, only on the Asian continent. In the context of the threat of *Tropilaelaps*'s introduction into new regions, accurate, rapid, and sensitive detection of the *Tropilaelaps* spp. is essential. In the present study, we developed a novel molecular method for bee mite's identification, which consists of a new real-time PCR method. A high-resolution melting analysis (HRM) was then performed on the amplified products to differentiate the species. PCR amplification was applied on the cytochrome c oxidase subunit I gene (580 bp). Short fragments from the most variable regions of this gene were identified in silico to amplify and discriminate among the four *Tropilaelaps* species. Four reference plasmids were synthesized to characterize species by well-distinguished melting curves. The method was then validated in terms of its specificity and sensitivity using a panel of 12 specimens. The results showed that an HRM method can be applied for the intended objective: for rapid and simultaneous identification of *Tropilaelaps* species. To our knowledge, this study reports the first direct HRM assay developed for the genome of a bee mite, specific for *Tropilaelaps* species. This COI barcode-HRM technique could be a promising tool for mite species identification.

Key words: *Tropilaelaps*, real-time PCR, HRM, mtCOI gene

Among the parasites that can infect honey bees, *Tropilaelaps* mites are currently one of the major risks. *Tropilaelaps* is an ectoparasite of the Acari:Laelapidae family that, similar to *Varroa destructor*, another important pest for bees, completes its entire life cycle in the hive. The primary hosts of this acarian are the giant honey bee (*Apis dorsata*) and Himalayan giant honey bee (*Apis laboriosa*). Four species have been described to date: *Tropilaelaps clareae* (Delfinado and Baker 1961), *Tropilaelaps koenigerum* (Delfinado-Baker and Baker 1982), and *Tropilaelaps mercedesae* and *Tropilaelaps thaii* (Anderson and Morgan 2007). They are reported in various Asian countries such as the Philippines, Thailand, Vietnam, China (Laigo and Morse 1968, Stephen 1968, Luo et al. 2011) and in the Indian continent (India, Sri Lanka, Afganistan, and Pakistan; Stephen 1968, Woyke 1984, Delfinado-Baker and Aggarwal 1987, Abrol and Kakroo 1997). European honeybees (*Apis mellifera*) have been reported to be parasitized by the two species *T. clareae* and *T. mercedesae*, after *A. mellifera* was imported in Asia (Burgett et al. 1983, Anderson and Morgan 2007). Therefore, the introduction of these two *Tropilaelaps* species in any territory free of them and where *A. mellifera* is the main bee species could cause large losses in beekeeping sector. On the other hand, *T. koenigerum* and *T. thaii* have only been observed on *A. dorsata* and *A. laboriosa*, respectively (Delfinado-Baker and Baker 1982, Anderson and Morgan 2007), two bee species present exclusively in the Asian continent. Moreover, a distribution study of this parasite shows that *T. mercedesae* is wide spread and could become an important pest in future years (Chantawannakul et al. 2016). However, despite a lower spread of the three other species, the importance of their surveillance on *A. mellifera* remains essential. As an example, *T. koenigerum* has been reported in debris of *A. mellifera* hive in Thailand. The potential adaptation along with climate change and importations explains why *Tropilaelaps* mites are considered an important emerging threat in countries currently free of these parasites.

Regulatory measures concerning the import of bees in Europe have been implemented in 1992 (Council Directive 92/65/EEC) and modified in 2004 (Council Directive 2004/68/EC) for the control of *Tropilaelaps* mite due to its absence in European countries. In fact, infestation with *Tropilaelaps* spp. is a regulated disease listed by the International Office of Epizooties (OIE). Standard procedures for its identification are described in the Terrestrial Manual (OIE 2018) and in a special issue of the *Journal of Apicultural Research* (Anderson and Roberts 2013). Any detection of the parasite must be notified to the competent

authorities. It is therefore essential to have highly suitable tools available for the detection, and rapid and reliable identification, of *Tropilaelaps* spp.

Currently, two identification methods can be used. After visual inspection, the main method is based on microscopic observation using various morphological keys applied to Acari (Delfinado and Baker 1961, Smiley 1991). Although new online tools have emerged over the last decade to help identify adult mites (http://insects.ummz.lsa.umich.edu/beemites/index.html, https://idtools.org/id/mites/beemites), distinguishing between the four species based solely on this morphological method is not routinely possible. This is because morphological identification is time-consuming and requires highly experienced personnel. In some cases, the integrity of the adult samples could be compromised, which may lead to doubts and inability to conclude. In addition, morphological methods are not applicable to immature stages. The development of molecular methods is a good alternative to complete the analysis.

Therefore, to differentiate the species more accurately and consistently, studies on molecular markers have been carried out. The first genetic differences between *T. clareae* and *T. koenigerum* (Tangjingjai et al. 2003), the first species identified, were described through the analysis of internal transcribed spacers (ITS) sequences. With a difference of about 4% on 600 bp, restriction fragment length polymorphism (RFLP) patterns were shown to differentiate the two species. In addition, a random amplified of polymorphic DNA (RAPD) analysis indicated substantial genetic differences. Subsequently, the two species *T. mercedesae* and *T. thaii* were defined by sequencing their ITS sequences and cytochrome c oxidase subunit I (COI) genes, and the molecular analysis was supported by morphological characters (Anderson and Morgan 2007). Thereby, nucleic acid-targeted sequencing appeared to be the most reliable method to determine *Tropilaelaps* species. However, it is not practical to use targeted sequencing for rapid confirmation of diagnosis, even though new portable molecular device systems are now available.

In 2003, DNA barcoding was proposed as a molecular approach for species identification (Hebert et al. 2003) in the plant and animal kingdoms. Even though various genes could be used, the mitochondrial COI gene was often chosen to be amplified and is used to classify and discriminate animal species, including arthropods (Miller et al. 2016). Alongside DNA barcoding, high-resolution melting (HRM), a sequencing-free method to detect genetic variations following a PCR reaction, was developed (Ririe et al. 1997). HRM analysis is a post-PCR method based on measuring the rate of double-stranded DNA dissociation to single-stranded DNA, with increasing temperatures. This method was first used to detect single-nucleotide polymorphisms (SNPs) for the rapid genotyping of pathogenic agents and the detection of mutations (Liew et al. 2004, Reed and Wittwer 2004). Recent progress made concerning instrument resolution and precision has led to improved performance of the technique. More recently, DNA barcoding and HRM analysis (Bar-HRM) have increasingly been used in combination for rapid identification of various biological organisms, such as animals (Behrens-Chapuis et al. 2018, Fernandes et al. 2018) and plants (Osathanunkul et al. 2016, Sun et al. 2016).

The main goal of this study was to establish a Bar-HRM method for the rapid confirmation of *Tropilaelaps* mite identification following morphological analysis. For this purpose, the available COI sequences of the four species (*T. mercedesae*, *T. clareae*, *T. koenigerum*, and *T. thaii*) were analyzed in silico to find regions with high levels of variability and short conserved regions. The method's applicability was demonstrated by analyzing four reference plasmids representing the four *Tropilaelaps* species and then by testing a panel of samples. The method was also confirmed through sequencing. Our results will contribute to the proposal of a new alternative in the rapid identification of these mites.

Materials and Methods

Mite Specimen Collection

In total, 12 *Tropilaelaps* specimens originating from Pakistan and the Philippines were kindly provided by the National Agricultural Research Center in Islamabad, Pakistan and CSIRO Ecosystem Sciences in Canberra, Australia, respectively (Table 1). *Tropilaelaps* specimens were stored in 90% ethanol and were washed in phosphate buffer three times before DNA extraction. In addition, DNA extracts from different pests frequently encountered in the hive including *V. destructor*, *Neocypholaelaps apicola*, *Braula coeca*, *Galleria mellonella*, *Achroia grisella*, and *Aethina tumida* were used to evaluate the specificity of the selected primers.

DNA Extraction and Amplification

Total genomic DNA was extracted using a High Pure PCR Template Preparation Kit (Roche Diagnostics, Germany), according to the manufacturer's instructions. Briefly, 120 µl of a suspension, obtained after grinding 1–10 specimens in 200 µl of phosphate buffer, were added to 40 µl of proteinase K and 200 µl of TLB buffer. After incubation for 1 h 30 min at 55°C, the manufacturer's protocol was

Table 1. List of *Tropilaelaps* specimens used for this study, country of origin, accession number (DDBJ), and melting temperature (T_m) values obtained with the three selected primer pairs

Genus	Species	Country	DDBJ no.	Sample identity	T_m (°C) Primer pair 2	Primer pair 7	Primer pair 14
Tropilaelaps	*mercedesae*	Pakistan	LC474405	TC3563	78.60	74.20	75.40
Tropilaelaps	*mercedesae*	Papua New Guinea	LC474394	TC11	78.40	74.20	75.20
Tropilaelaps	*mercedesae*	Indonesia	LC474395	TC29	78.40	74.40	75.20
Tropilaelaps	*mercedesae*	Indonesia	LC474396	TC66	78.20	74.20	75.40
Tropilaelaps	*mercedesae*	Indonesia	LC474397	TC78	78.20	74.20	75.20
Tropilaelaps	*mercedesae*	Indonesia	LC474398	TC95	78.40	73.80	75.00
Tropilaelaps	*mercedesae*	Indonesia	LC474399	TC108	78.80	74.00	75.20
Tropilaelaps	*mercedesae*	Sri Lanka	LC474400	TC150	77.20	74.00	75.00
Tropilaelaps	*mercedesae*	South Vietnam	LC474401	TC169	78.60	73.80	75.00
Tropilaelaps	*clareae*	Philippines	LC474402	TC126	77.20	73.60	74.60
Tropilaelaps	*clareae*	Philippines	LC474403	TC130	76.30	73.40	74.20
Tropilaelaps	*clareae*	Philippines	LC474404	TC144	76.80	73.40	74.00

DDBJ no., DNA Data Bank of Japan.

followed, as recommended. Finally, genomic DNA was eluted in 200 µl of elution buffer and was kept at –20°C until use.

A partial COI sequence (580 bp) of *Tropilaelaps* specimens to be tested was amplified using the following primers designed by Anderson et al. (2007): TCF1 5′-CTATCCTCAATTATTGAAATA GGAAC-3′ and TCR2 5′-TAGCGGCTGTGAAATAGGCTCG-3′. Briefly, the PCR was performed in a total volume of 20 µl containing 2 µl DNA, 10 pmol of each primer, 9 nmol of dNTPs, and 1 U Taq polymerase. The cycling conditions were as follows: 95°C for 5 min, 35 cycles (95°C for 30 s, 58°C for 30 s, and 72°C for 45 s) and a final extension at 72°C for 7 min. PCR products were purified with a QIAquick PCR purification Kit (Qiagen, Germany). The purified products were sequenced by the Sanger method in both directions (Beckman Coulter Genomics, United Kingdom) using the same primers as the amplification reaction. Consensus sequences were produced, aligned, and edited using the Clustal W multiple alignments in BioEdit v7 (Hall 1999). All the consensus sequences of the tested specimens were registered in the DNA Data Bank of Japan (Table 1). A maximum likelihood tree was conducted by MEGA7 (Kumar et al. 2016) with 1,000 bootstrap replicates.

Reference Plasmid Preparation

Two plasmids (*T. mercedesae* and *T. clareae*) were constructed by cloning the COI PCR fragments obtained after the PCR amplification step from the specimens TC3563 and TC130, respectively. Briefly, the PCR fragments were cloned in the pGEMt easy vector (Promega, Madison, Wisconsin), using the TA cloning site. The recombinant plasmids were verified by sequencing of the inserted fragments. The other two plasmids (*T. koenigerum* and *T. thaii*) were obtained by synthetic cloning (GeneCust, France). The DNA fragments of 580 bp were synthetized based on the provided sequences from GenBank database EF025452 and EF025451 and cloned in the pUC57 vector at the *SmaI* cloning site. The recombinant plasmids were verified by restriction enzyme digestion analysis and sequencing. Briefly, DH10B bacteria were transformed with the plasmids to produce the reference material. After overnight culture at 37°C, plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen, Germany) according to the manufacturer's recommendations. The purified plasmidic DNA samples were quantified spectrophotometrically at 260 nm and then kept at –20°C. The four plasmids were HRM tested to establish the reference profiles and to characterize the method.

In Silico DNA Barcode Analysis and Primer Design

In total, 53 partial COI sequences of the four *Tropilaelaps* species were obtained from the NCBI database (source: http://www.ncbi.nlm.nih.gov/). Consensus sequences were determined for *T. mercedesae*, *T. clareae*, and *T. koenigerum*. Only one sequence was available for *T. thaii*. A sequence alignment was performed using BioEdit software to identify the variable and conserved regions between the four species. Primer pairs were designed against the conserved regions and were selected to yield a size between 50 and 220 bp. Primers were synthetized by Eurogentec (Belgium).

Real-Time PCR and High-Resolution Melting Assay

The real-time PCR and the HRM curve analysis were performed on a CFX 96 thermal cycler (Bio-Rad Laboratories). Each PCR mix was prepared in 20 µl of total volume containing 5 µl of extracted DNA, 1× precision melt supermix (Bio-Rad Laboratories) and 200 nM of each primer (Table 2) in the following conditions: 95°C for 2 min; 40 cycles at 95°C for 10 s, and 55°C for 30 s. Optical measurements were collected at the end of each cycle and were analyzed with Bio-Rad CFX Manager software (Bio-Rad Laboratories). After the amplification step, the final PCR products were subjected to 0.2°C/s ramping between 55 and 95°C. Precision melt analysis software (v1.2) and the HRM algorithm provided by Bio-Rad Laboratories were used to analyze the melting profiles of the four reference plasmids, which were assigned as 'species' (*T. mercedesae*, *T. clareae*, *T. koenigerum*, and *T. thaii*). The HRM profiles of the different test specimens were analyzed and compared with those of the four references.

Real-Time PCR and High-Resolution Melting Assay Characterization

Several parameters of the method were evaluated. Analytical specificity and lack of cross-amplification with targets were tested for each set of primers using DNA from other pathogens often encountered in the hive. These included *V. destructor*, *N. apicola*, *B. coeca*, *G. mellonella*, *A. grisella*, and *A. tumida*. Method sensitivity was tested using serial 10-fold dilutions (1×10^8 copies to 1×10^4 copies/reaction) of *T. mercedesae*, *T. clareae*, *T. koenigerum*, and *T. thaii* reference plasmids. Method reproducibility was assessed by testing five repeats of each plasmid standards with the selected primer pairs. Means (± SD) of the melting temperature were calculated.

Results

Primer Pair Experimental Selection

The 538-bp reference sequences of the four *Tropilaelaps* species were aligned to define the conserved and variable regions (Fig. 1). Seven conserved regions were identified and 16 primers pairs were defined to allow amplification of fragments with size ranges from 41 to 198 bp (Table 2). Selection of the primer pairs yielding the best amplification and HRM profiles was carried out for the four reference plasmidic DNAs.

Preliminary selection was based on the differences in melting temperatures (ΔT_m) and the differences in relative fluorescence unit values (ΔRFU), which must be greater than 0.25 between the standard plasmids of the two most commonly found species: *T. mercedesae* and *T. clareae*. Of the 16 primer pairs selected in silico, 10 were preselected.

The second step consisted in testing these 10 primer pairs on the four reference plasmids. Based on the same selection criteria, three of them were chosen for their ability to meet these criteria. As shown in Table 2, primer pairs nos. 2, 7, and 14 amplified fragments of 185, 149, and 145 bp, respectively. The three amplified fragments have 46, 22, and 16 nucleotide differences, respectively. Of the three selected primer pairs, only one primer (179–198F) showed a one to two nucleotide difference between the four species (Fig. 1). For each amplified fragment, the nucleotide differences therefore resulted only in a slight difference in T_m between the four species (Table 3, Fig. 2), whereas primer pair no. 2 enabled clear differentiation of *T. clareae* specimens from *T. mercedesae* and *T. koenigerum* specimens, with a ΔT_m = 1.76°C and a ΔT_m = 1.60°C, respectively. A ΔT_m = 0.88°C was observed between *T. clareae* and *T. thaii* specimens. The ΔT_m between *T. clareae* and *T. thaii* was greater when using primer pairs nos. 7 or 14 (ΔT_m = 1.20 and ΔT_m = 1.08, respectively).

Regarding the HRM profiles obtained with the three primer pairs, four different or distinct curves were observed for the four *Tropilaelaps* species (Fig. 3). Primer pair no. 2 was able to clearly

Table 2. List of primers tested for this study, sequence of the primers, and size (bp) of the amplified fragments

ID	Primer pair number	Primer	5′–3′ sequences	Amplicon size (bp)	Reference
1-18F	1	Forward	AGGAACAGGATGAACAGT	198	This study
179-198R		Reverse	ACAGATCATACAAATAGGGG		This study
179-198F	**2**	Forward	CCCCTATTTGTATGATCTGT	**185**	This study
341-363R		Reverse	ACTTCAGGGTGACCAAAAAATCA		This study
179-198F	3	Forward	CCCCTATTTGTATGATCTGT	218	This study
378-396R		Reverse	ATAATACCAAATCCTGGTA		This study
341-363F	4	Forward	TGATTTTTTGGTCACCCTGAAGT	56	This study
378-396R		Reverse	ATAATACCAAATCCTGGTA		This study
341-363F	5	Forward	TGATTTTTTGGTCACCCTGAAGT	95	This study
416-435R		Reverse	GGATTTTTTTTTCCTCTATG		This study
341-363F	6	Forward	TGATTTTTTGGTCACCCTGAAGT	116	This study
438-456R		Reverse	ATTATACCTAAATTTCCGA		This study
341-363F	7	Forward	TGATTTTTTGGTCACCCTGAAGT	**149**	This study
468-489R		Reverse	AATCCTAAAATTCCAATAGTTA		This study
378-396F	8	Forward	TACCAGGATTTGGTATTAT	58	This study
416-435R		Reverse	GGATTTTTTTTTCCTCTATG		This study
378-396F	9	Forward	TACCAGGATTTGGTATTAT	79	This study
438-456R		Reverse	ATTATACCTAAATTTCCGA		This study
378-396F	10	Forward	TACCAGGATTTGGTATTAT	112	This study
468-489R		Reverse	AATCCTAAAATTCCAATAGTTA		This study
378-396F	11	Forward	TACCAGGATTTGGTATTAT	183	This study
TCR2		Reverse	TAGCGGCTGTGAAATAGGCTCG		Anderson et al. (2007)
416-435F	12	Forward	CATAGAGGAAAAAAAAATCC	41	This study
438-456R		Reverse	ATTATACCTAAATTTCCGA		This study
416-435F	13	Forward	CATAGAGGAAAAAAAAATCC	74	This study
468-489R		Reverse	AATCCTAAAATTCCAATAGTTA		This study
416-435F	**14**	Forward	CATAGAGGAAAAAAAAATCC	**145**	This study
TCR2		Reverse	TAGCGGCTGTGAAATAGGCTCG		Anderson et al. (2007)
438-456F	15	Forward	TCGGAAATTTAGGTATAAT	52	This study
468-489R		Reverse	AATCCTAAAATTCCAATAGTTA		This study
438-456F	16	Forward	TCGGAAATTTAGGTATAAT	123	This study
TCR2		Reverse	TAGCGGCTGTGAAATAGGCTCG		Anderson et al. (2007)

Each primer is identified by its position within the 538 bp of the COI sequence. Primer pairs chosen for the study are shown in bold.

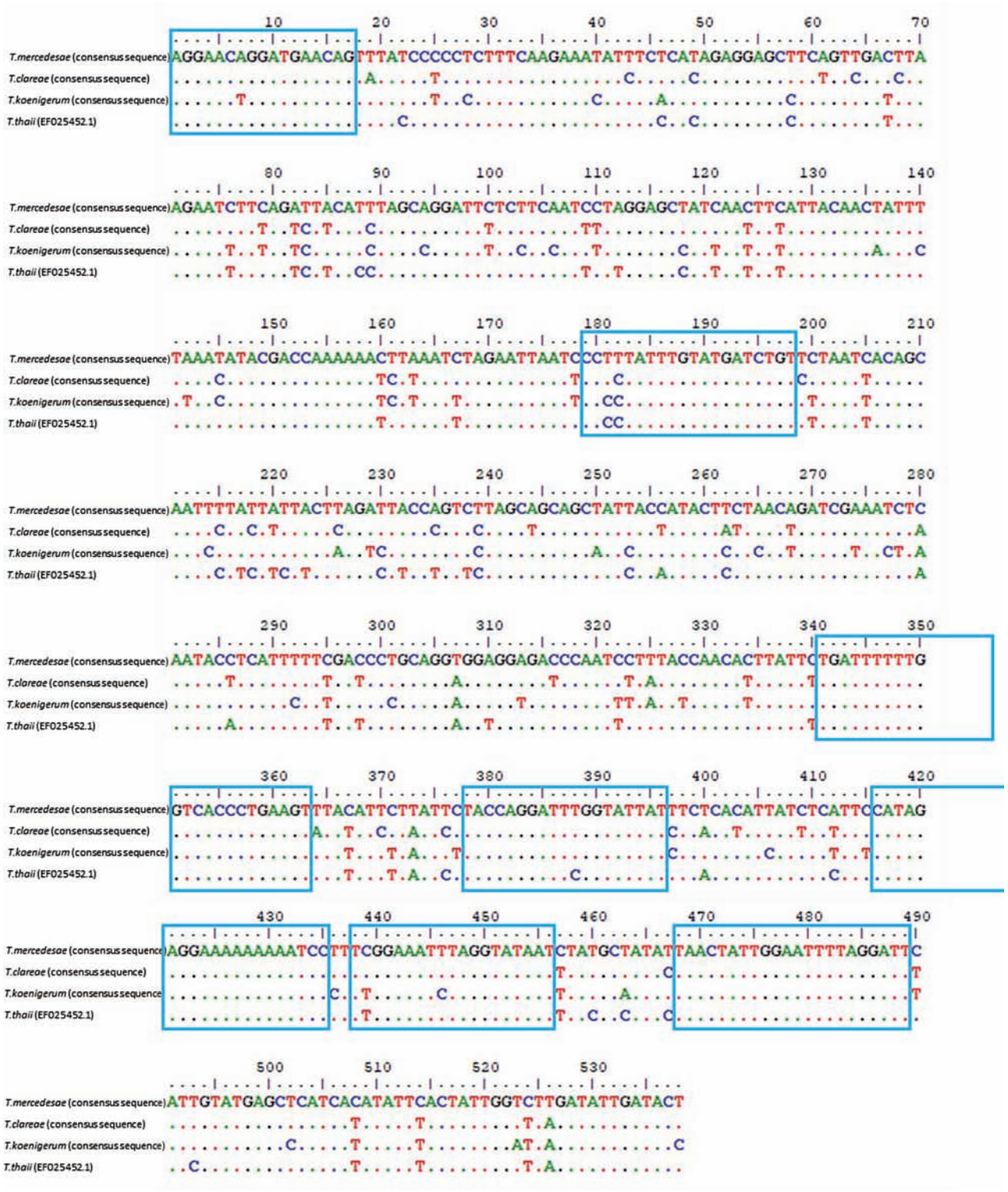

Fig. 1. Sequence alignment of the 538-bp COI fragments showing nucleotide differences between the four *Tropilaelaps* species. The blue boxes represent conserved regions between the species based on a difference of less than three nucleotides.

distinguish *T. clareae* from *T. mercedesae* and *T. koenigerum* reference plasmids, which both have a similar profile, with a ΔRFU = 7 and a ΔRFU = 8, respectively. A ΔRFU = 4 was observed between *T. clareae* and *T. thaii* reference plasmids. When using primer pair no. 7, the largest difference was obtained between *T. clareae* and *T. thaii* with a ΔRFU = 7. Finally, to distinguish *T. mercedesae* from *T. koenigerum*, primer pair no. 7 or 14 could be used.

Method Sensitivity and Specificity

The performance of the method was defined in terms of sensitivity and specificity. First, to assess PCR efficiency, 10-fold serial diluted reference DNA plasmids (1×10^8 copies to 1×10^4 copies/reaction) were amplified by real-time PCR using the three primer pairs selected (nos. 2, 7, and 14). The four real-time PCR assays demonstrated good performance, presenting PCR efficiency

Table 3. Melting temperatures (T_m) determined for the four *Tropilaelaps* reference plasmids, using the three defined primer pairs

Primer pair number	T_m (°C)			
	T. mercedesae (*n* = 5)	*T. clareae* (*n* = 5)	*T. thaii* (*n* = 5)	*T. koenigerum* (*n* = 5)
2	78.68 ± 0.11	76.92 ± 0.11	77.80 ± 0.00	79.40 ± 0.00
7	74.20 ± 0.00	73.60 ± 0.00	74.80 ± 0.14	73.80 ± 0.00
14	75.40 ± 0.00	74.20 ± 0.00	75.28 ± 0.11	74.60 ± 0.00

The T_m represent the mean of five repeats and SD are indicated.

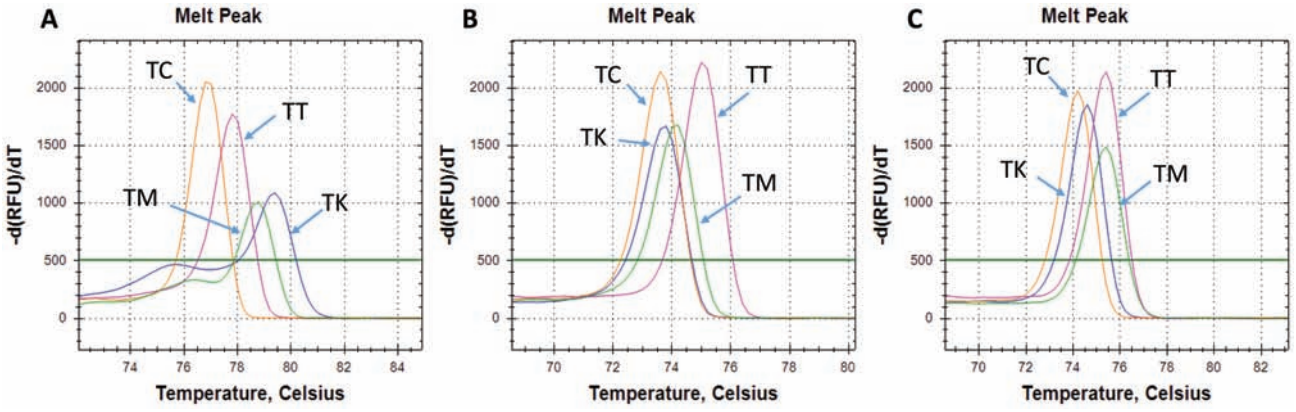

Fig. 2. Melting curve analysis of real-time PCR amplification products. PCR amplifications using primer pair 2 (A), primer pair 7 (B), and primer pair 14 (C) on the four reference plasmids (TM = *T. mercedesae*, TC = *T. clareae*, TK = *T. koenigerum*, TT = T. *thaii*).

values ranging from 85.6 to 99.4%, 87.5 to 94.3%, and 82.6 to 96.7% for primer pairs nos. 2, 7, and 14, respectively. The lowest efficiency was observed for the condition *T. clareae*/primer pair no. 14 (82.6%). To define the minimum number of specimens needed to perform the HRM analysis, different DNA preparations obtained from grindings of 1, 2, 5, or 10 *T. mercedesae* specimens were tested (Fig. 4). The amount of DNA extracted from an only one specimen was sufficient to obtain an HRM profile likely to lead to identification.

To further characterize the method, specificity was tested on biological DNA extracts from *Varroa destructor* (four different origins), *N. apicola*, *B. coeca*, *G. mellonella*, *A. grisella*, and *A. tumida*. No amplification, and thus no melting curve, was produced from these organisms using the three primer pairs (data not shown). These results showed that this assay had good specificity with respect to the target sequences of the four *Tropilaelaps* spp.

HRM Result Confirmation Using Sequencing

To demonstrate the applicability of the method on field samples, 12 DNA extracts from different specimens were tested: nine *T. mercedesae* specimens and three *T. clareae* specimens from different countries. First, all were amplified using the three primer pairs. The data from the observed melting temperature curves are presented in Table 1. Two groups were clearly distinguished. However, one specimen (TC150) showed a melting temperature different from that expected for primer pair no. 2. In fact, the TC150 specimen, identified as *T. mercedesae*, demonstrated a Tm close to the *T. clareae* reference plasmid.

The HRM curve analysis was carried out by overlaying the obtained HRM curves from samples with those obtained with the reference plasmids. The HRM results from the 12 specimens, identified by morphology, showed that out of the nine specimens defined as *T. mercedesae*, one specimen (TC150) demonstrated an HRM profile close to the *T. clareae* species with primer pair no. 2 (Fig. 5). In contrast, the other two profiles obtained with primer pair nos. 7 and 14 were consistent with those of the *T. mercedesae* reference profiles. The HRM profiles are related to the various melting temperatures obtained. The three *T. clareae* specimens were all consistent with the reference profiles.

To confirm our results and to understand the observed discrepancies, the COI gene region (560 bp) of the 12 samples was sequenced in both directions (DDBJ accession nos.: LC474394–LC474405). The phylogenetic tree inferred from these 560 bp was in agreement with the morphological species identification (Supp Fig. 1 [online only]). The alignment of the three amplified regions (namely, regions 2, 7, and 14) against the reference sequence is presented in Fig. 6. The sequences of the two specimens TC95 and TC108 are strictly identical to the *T. mercedesae* reference plasmid, and their HRM profiles overlapped. Four *T. mercedesae* specimens (TC11, TC29, TC66, and TC78) showed the same sequence containing eight SNPs distributed within the three regions (4, 3, and 1 SNP in regions 2, 4, and 7, respectively). However, these differences did not affect their HRM profiles. The atypical profile observed for the TC150 specimen in region 2 can be explained by a nucleotide (nt) difference of 12 nt compared with the reference plasmid, and 13 nt compared with the other *T. mercedesae* specimens. Therefore, the sequence of region 2 is closer to the *T. clareae* sequence. Moreover, the differences also observed in the other two regions did not affect the melting temperature and the HRM profiles. A molecular phylogenetic analysis was then performed separately on these three regions (Supp Fig. 2 [online only]). Compared with the 41 sequences of other specimens from different Asian regions (Anderson and Morgan 2007), the variability of these three regions was clearly shown. The phylogeny confirmed that the 12

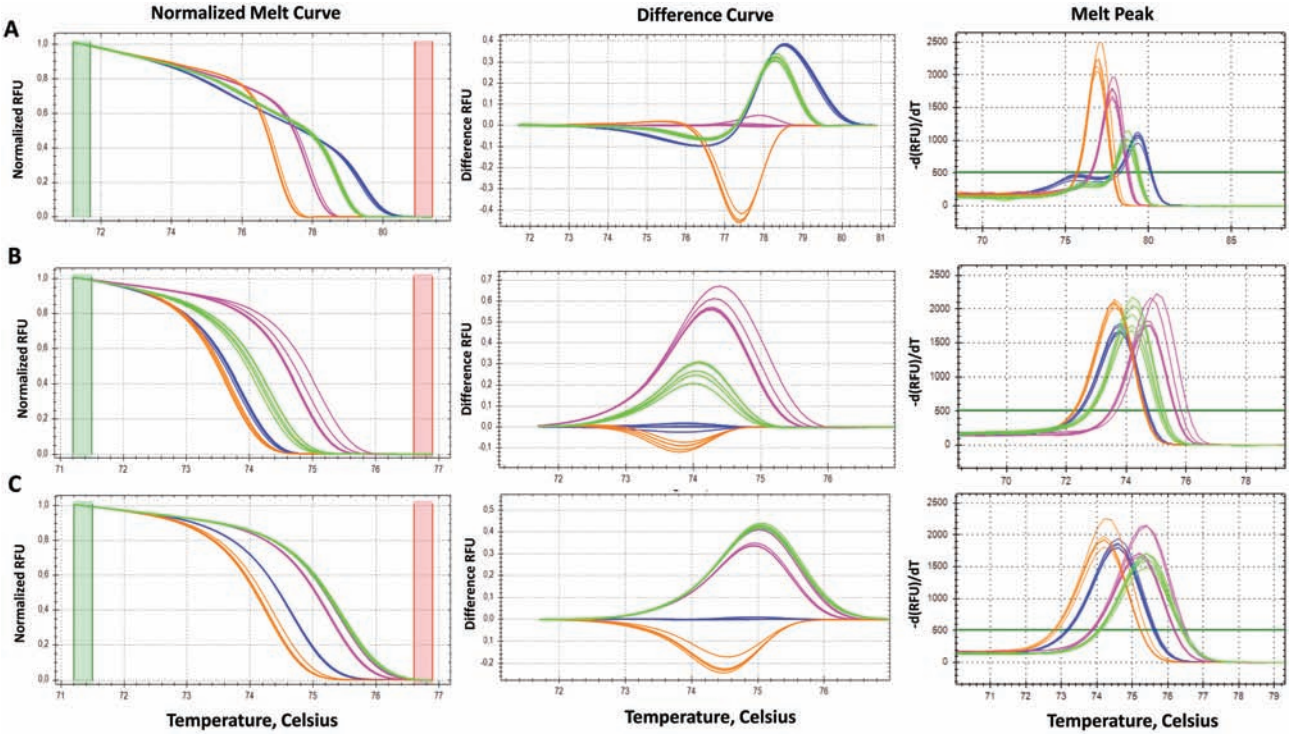

Fig. 3. HRM results obtained for the five replicates of the four reference plasmids (10^6 copies/reaction) using the three selected pairs of primers (pp). Normalized melting curves and peaks for *Tropilaelaps* species: *T. clareae* (orange), *T. mercedesae* (green), *T. thaii* (pink), and *T. koenigerum* (blue) using (A) primer pair 2 with TT as reference; (B) primer pair 7 with TK as reference; (C) primer pair 14 with TK as reference.

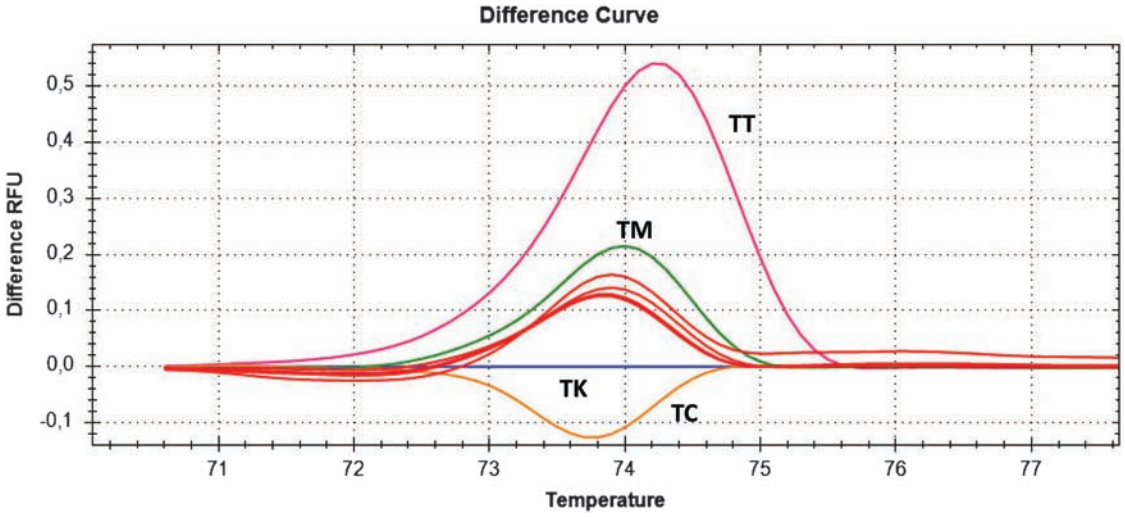

Fig. 4. HRM results obtained for DNA extraction from 1 to 10 *T. mercedesae* specimens. Normalized melting peaks for *Tropilaelaps* species: *T. clareae* reference plasmid (orange), *T. mercedesae* reference plasmid (green), *T. thaii* reference plasmid (pink), and *T. koenigerum* reference plasmid (blue) using the primer pair 7 with TK as reference. Red lines represent the results for the DNA extracts from 1, 2, 5, and 10 specimens.

specimens clustered to *T. mercedesae* (9 specimens) or *T. clareae* (3 specimens) as observed for the 560-bp sequence.

Discussion

This study demonstrated, for the first time, that a real-time PCR method coupled with HRM analysis was able to differentiate the four *Tropilaelaps* species, which are morphologically similar. By using three primer sets, melting profiles showed specific differences. The availability of identification tools capable of providing rapid discrimination of these pests for the purpose of applying appropriate sanitary measures is essential in the context of growing international trade.

In fact, rapid detection and identification of the *Tropilaelaps* mite can contribute to improved surveillance and monitoring in countries free of this parasite, such as in Europe. For example, the introduction of *V. destructor*, another Asian mite, into Europe in the 1970s was disastrous for the beekeeping sector (Colin et al. 1983, Matheson 1995). Its spread could not be contained, and today, few European regions have been spared. Like the *Varroa destructor*

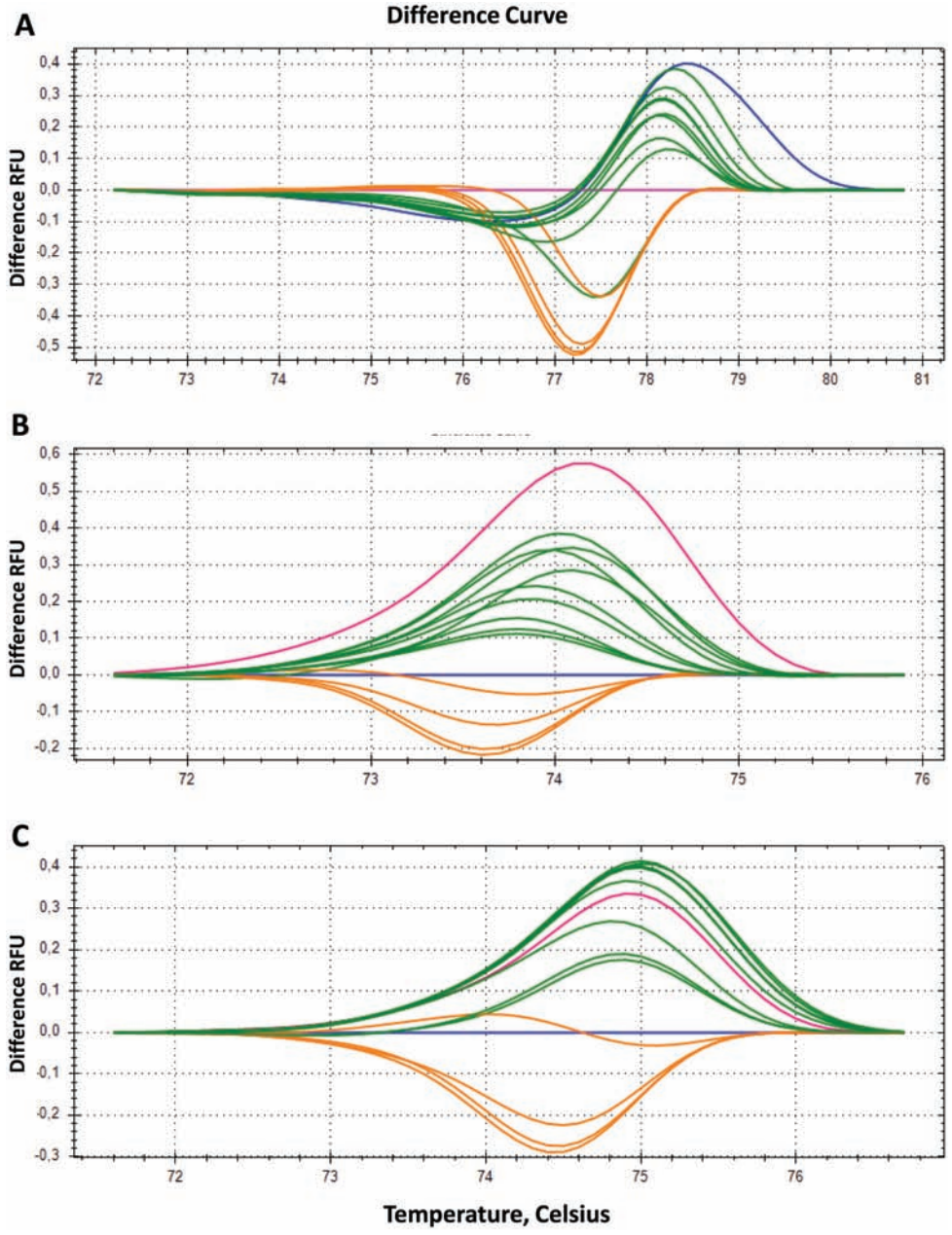

Fig. 5. HRM results obtained for 11 *Tropilaelaps* specimens (Table 1) that had previously been identified by morphology. Normalized melting peaks for *Tropilaelaps* species: *T. clareae* reference plasmid and three *Tropilaelaps* specimens (orange), *T. mercedesae* reference plasmid and eight *Tropilaelaps* specimens (green), *T. thaii* reference plasmid (pink), and *T. koenigerum* reference plasmid (blue) using (A) primer pair 2 with TT as reference; (B) primer pair 7 with TK as reference; (C) primer pair 14 with TK as reference.

mite, *Tropilaelaps* could also become a major threat to *A. mellifera* colonies in Europe (Chantawannakul et al. 2018) and could cause even greater damage. This work proposes an alternative method to the other available methods, which is complementary and faster.

Several studies have validated the application of the PCR coupled with an HRM analysis for organism identification. For the plant kingdom, including fungi, the method involving the 522 bp ITS region is most commonly used (Kalivas et al. 2014, Osathanunkul et al. 2016). Similarly, recent studies on animal parasites such as *Giardia*, *Taenia*, and *Echinococcus* have shown that ITS or other genes can be used to distinguish between species (Tan et al. 2015, Dehghani et al. 2016). However, it is well known that the COI gene shows higher variability in animals, and recently, this COI-gene approach was successfully developed for the identification of fish species (Fernandes et al. 2018). For *Tropilaelaps*, the nucleotide difference on the COI gene was estimated at between 1 and 4% within a single species and between 11 and 15% among the four species (Anderson and Morgan 2007). This genetic variability was used to develop and evaluate this new molecular tool. Three pairs of conserved primers were selected from the available COI gene sequences. These three primer pairs yielded three distinct HRM patterns on the four *Tropilaelaps* species. Thus, the identification of *Tropilaelaps* and its species can be carried out in a single analysis step, based on the combined results of the three HRM profiles obtained. The primer pair no. 2 is able clearly to distinguish *T. mercedesae* from *T. clareae* and *T. thaii*, whereas primer pair no. 7 allows to

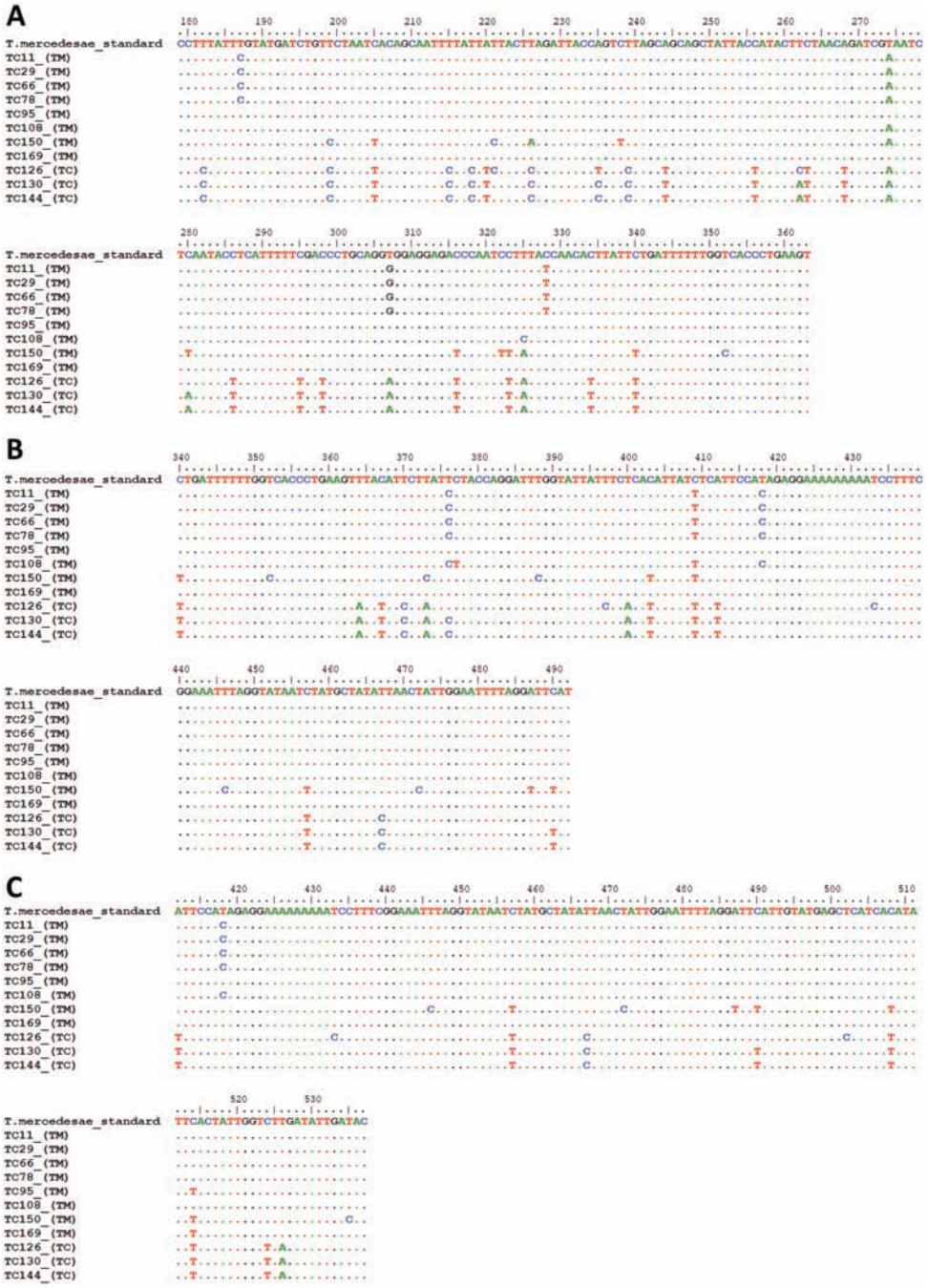

Fig. 6. Alignment of COI sequences of the three amplified regions from the 12 specimens studied (the species is indicated in brackets). (A) Sequences amplified using primer pair 2; (B) sequences amplified using primer pair 7; (C) sequences amplified using primer pair 14.

distinguish *T. koenigerum* and *T. clareae* from *T. mercedesae* and *T. thaii*. Furthermore, the primer pair no. 14 allows to distinguish *T. koenigerum* from the three others species. Moreover, this is supported by the following: 1) the method's specificity for the main external parasites found in the hive was demonstrated, 2) the method's sensitivity indicated that the DNA extracted from a single parasite was enough to enable identification, and 3) the HRM profiles were confirmed by the sequencing and analysis of the COI fragments from a set of 12 selected samples. Although one specimen exhibited an atypical HRM profile when using one of the three primer pairs, the two HRM profiles, obtained with the two other primer pairs, were consistent with the sequence analysis and phylogenetic results. The global analysis of the three profiles makes it possible to orient the identification of the species. Compared with other molecular tools already described, such as RAPD and RFLP or Sanger sequencing, the method developed here showed a reduction in time and cost-effort (Supp. Supp Table 1 [online only]). Furthermore, one important advantage is that HRM is performed immediately after amplification without opening the tube, thus reducing the risk of cross-contamination. Therefore, the method described could provide rapid results to laboratories that do not have an internal sequencing service.

In conclusion, the coupling of real-time PCR and HRM analysis could be a convenient way to confirm the identification of

Tropilaelaps mites, which are a real threat to European bees. To our knowledge, this study describes the first direct HRM assay developed for the genome of a bee mite, and enabling differentiation between *Tropilaelaps* species. However, additional data on a larger sample panel of *Tropilaelaps* mites will be required to confirm these findings. This method provides a promising alternative molecular approach for rapid confirmation of morphology-based identification.

Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

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