FEMS Microbiology Ecology, 94, 2018, fix160

doi: 10.1093/femsec/fix160
Advance Access Publication Date: 14 November 2017
Research Article

RESEARCH ARTICLE

Bacterial communities associated with the ectoparasitic mites *Varroa destructor* and *Tropilaelaps mercedesae* of the honey bee (*Apis mellifera*)

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One sentence summary: The bacterial communities associated with two parasitic mites (*Varroa destructor* and *Tropilaelaps mercedesae*) of the honey bee (*Apis mellifera*) were significantly different.

Editor: Julie Olson

ABSTRACT

Varroa and *Tropilaelaps* mites have been reported as serious ectoparasites of the honey bee (*Apis mellifera*). In this study, bacterial communities associated with *Varroa destructor* and *Tropilaelaps mercedesae* from northern Thailand were determined, using both culture-dependent and culture-independent approaches. Adult female mites were collected from apiaries in Chiang Mai and Lampang provinces. Culturable bacteria were isolated from individual mites. On average, we observed approximately 1340 and 1140 CFU/mite in *Varroa* and *Tropilaelaps*, respectively. All isolates were assigned to the genus *Enterococcus*. Six samples of genomic DNA from 30–50 mites were extracted and subjected to pyrosequencing of bacterial 16S rRNA amplicons. The resulting 81 717 sequences obtained from *Varroa* were grouped into 429 operational taxonomic units. The most abundant bacteria in *Varroa* mites belonged to the family *Enterobacteriaceae*, especially the genera *Arsenophonus*, *Enterobacter* and *Proteus*. For *Tropilaelaps* mites, 84 075 sequences were obtained and clustered into 166 operational taxonomic units, within which the family *Enterococcaceae* (particularly the genus *Enterococcus*) was predominant. Localization of bacteria in the mites using fluorescence *in situ* hybridization with two universal bacterial probes revealed that these bacteria were in the cecum of the mites. Taxon-specific *Enterobacteriaceae* and *Arsenophonus* probes also confirmed their localization in the cecum of *Varroa*.

Keywords: ectoparasites; pyrosequencing; honey bee

Received: 31 May 2017; **Accepted:** 13 November 2017

INTRODUCTION

The western honey bee, also known as the European honey bee, *Apis mellifera*, originates from Europe and Africa but has subsequently been spread globally by human activity. It was introduced into Thailand around the middle of the 20th century (Wongsiri *et al.* 2000). As honey bees can easily acclimatize to tropical conditions, this species has become widespread and is economically important in Thailand. *Apis mellifera* is the most popular species of bee for apiculture in northern Thailand, Thailand's largest apicultural area (Chantawannakul, Petersen and Wongsiri 2004). Apiculture yields various export products such as honey, pollen, royal jelly, propolis, wax, bee brood and bee venom (Wongsiri *et al.* 2000; Wongsiri, Chanchoa and Kongpitak 2012).

However, the decline of the honey bee population has recently become a serious problem due to a combination of factors including pesticide poisoning (Stanley *et al.* 2015), changes of land-use (Otto *et al.* 2016), low genetic diversity (Mattila and Seeley 2007), diseases caused by viruses, bacteria (Cox-Foster *et al.* 2007; Forsgren 2010; Ebeling *et al.* 2016), fungi and protozoa, and ectoparasitic mites (Chantawannakul *et al.* 2016). Some of the most virulent parasites are ectoparasitic mites in the genera *Varroa* and *Tropilaelaps* (Rosenkranz, Aumeier and Ziegelmann 2010). Both genera were originally found in Asia, but *Varroa* has since spread globally, with the exception of Australia and Newfoundland in Canada (Wilfert *et al.* 2016). Initially, *Apis cerana* and *A. dorsata* were reported as the original hosts of *Varroa* and *Tropilaelaps*, respectively (Oudemans 1904; Laigo and Morse 1968). Later, both mites shifted to the western honey bee, having a devastating effect on this species.

Adult female *Varroa* are reddish-brown and bigger than the lighter colored male mites (Rosenkranz, Aumeier and Ziegelmann 2010). Adult female *Tropilaelaps* are light brown but smaller than *Varroa* (Anderson and Morgan 2007). *Varroa* can be found either in bee brood or attached to adult bees where they feed on the bees' hemolymph. On the other hand, only the brood bees are susceptible to *Tropilaelaps*. Both mites have been implicated in the decline of bee populations. Honey bees of infested colonies are weakened and usually show physical abnormalities, for example deformed legs and wings, resulting in a shorter life span for the majority of surviving bees (Rosenkranz, Aumeier and Ziegelmann 2010). Additionally, *Tropilaelaps mercedesae* reduces honey bee longevity and was also reported as a vector of deformed wing virus (Khongphinitbunjong *et al.* 2016). Viruses associated with the mites have severe consequences for bee health, with deformed wing virus particularly contributing to colony collapse disorder (Di Prisco *et al.* 2016; Khongphinitbunjong *et al.* 2016; Nazzi and Le Conte 2016).

Several methods have been used to control the parasitic mite populations, such as chemical and biological controls (Dietemann *et al.* 2012; Dietemann *et al.* 2013; Zemene *et al.* 2015). However, several chemicals were found to contaminate honey bee products and have negative effects on honey bees (Bogdanov 2006), such as Amitraz® (Rinkevich, Danka and Healy 2017) and rotenone (Satta *et al.* 2008). Biological control methods and other methods, such as smoking and dropping mites, trapping, and heating, have been used (Sammataro, Gerson and Needham 2000).

Many arthropods are associated with microbial symbionts that provide ecologically important benefits to their hosts. In particular, the guts of insects act as reservoirs for diverse microorganisms (Moran, McCutcheon and Nakabachi 2008). Many of these bacteria improve their host's health or life span by aiding in nutrition, digestion, reproduction and pathogen defense (Zheng *et al.* 2017). Thus, microorganisms associated with honey bees might provide novel avenues for *Varroa* control (Chandler *et al.* 2011).

A previous study on microorganisms associated with *Varroa destructor* revealed *Morganella* sp. as the most abundant taxon, followed by *Enterococcus faecalis*, which was isolated from hive debris in the winter (Hubert *et al.* 2015). Genome sequencing of *V. destructor* revealed an abundant actinomycete that was more prevalent in adult female mites than in the males and in other life stages (Cornman *et al.* 2010). Other bacteria associated with *V. destructor* were culturable oxalotrophic bacteria in the *Proteobacteria* and *Actinobacteria* (Maddaloni and Pascual 2015). Gram-positive bacteria (*Bacillus* and *Microbacterium* genera) and Gram-negative bacteria (*Brevundimonas* and *Rhizobium* genera) were also found on the *Varroa* body surface using cultivation-based approaches (Vanikova *et al.* 2015). Despite these previous studies, the bacterial communities of *Varroa* in Thailand and the microbiota of *Tropilaelaps* mites in general have yet to be characterized.

To investigate bacterial communities associated with *Varroa* and *Tropilaelaps* mites in northern Thailand, we combined culture-dependent and culture-independent (454-pyrosequencing) approaches. Furthermore, localization of bacteria was investigated in both genera by fluorescence *in situ* hybridization. Expanding our knowledge of microbes associated with honey bee parasitic mites will not only provide insights into host–symbiont associations, but also reveal novel avenues for biological control of these important honey bee pests.

MATERIALS AND METHODS

Collection of adult female mites

Varroa destructor (at least 250 adult female mites) and *T. mercedesae* (at least 350 adult female mites) were collected directly from three hives of *Apis mellifera* in Chiang Mai province (designated Va1, Va3, Va5 and Tr1, Tr3, Tr5, respectively) and three hives in Lampang province (designated Va2, Va4, Va6 and Tr2, Tr4, Tr6, respectively). The sampling was performed in June 2016. Adult female mites were collected with a fine-bristle brush into 1.5 mL microcentrifuge tubes and divided into two portions. In the first portion of each sample, bacteria associated with the mites were immediately isolated from individual animals by a culture-dependent approach. The second portion was placed in 70% (v/v) ethanol and preserved at −20°C for DNA extraction and characterized by a culture-independent approach.

Mite identification

Mite species were identified based on their morphology. Hoyer's mounting was used to preserve and clear their body tissue and mounted on glass microscope slides. Subsequently, the slides were kept at 60°C for 5 min and stored at room temperature for approximately 2 weeks. The morphology was examined using a stereo microscope (Olympus SZX7). Furthermore, 10 individuals of each mite were identified by sequencing parts of the mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene, using primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer *et al.* 1994). DNA extraction was performed using chelating resin (Chelex100, Bio-Rad) as described previously (Boonham *et al.* 2002). The *COI* amplification was performed as reported previously (Hebert, Cywinska and Ball 2003). PCR products were run

on 1.5% (w/v) agarose gels and purified with PureLink™ Quick PCR Purification Kit (Invitrogen). All amplicons were sequenced by Sanger sequencing (Macrogen, South Korea).

Determination of bacterial community using culture-dependent approach

The mite samples were surface sterilized with 7% (v/v) sodium hypochlorite for 1 min, 95% (v/v) ethanol for 1 min and 70% (v/v) ethanol for 30 s, then soaked in sterile water for 3 min and dried on sterile tissue paper. All mites were placed in 1.5 mL microcentrifuge tubes and ground under liquid nitrogen using sterile wooden sticks. Two hundred microliters of sterile phosphate-buffered saline was added to the ground samples. The suspensions were serially diluted from 10^{-1} to 10^{-5}, and 100 μL. A portion was spread onto tryptic soy agar and nutrient agar plates. The agar plates were incubated at 30°C under aerobic conditions for approximately 2 days, and colony forming units per mite (CFU/mite) were counted. Bacterial colonies from each mite were selected and identified using morphological characteristics, Gram straining and 16S rRNA sequencing. All bacterial colonies were kept in 500 μL of 50% (v/v) sterile glycerol with 500 μL of 50% (v/v) sterile normal saline and placed in 1.5 mL microcentrifuge tubes for storage at −20°C.

Amplification, sequencing and analysis of isolated bacteria

DNA of selected bacterial isolates was extracted using a DNA extraction kit (PowerSoil® DNA Isolation Kit, Mo Bio Laboratories, Inc., Carlsbad, CA, USA) and the 16S rRNA was amplified using two universal primers: 27F, 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1492R, 5′-TACGGYTACCTTGTTACGACT-3′. PCR reactions (25 μL) contained 1× PCR buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.8 μM of each primer, 1 unit of *Taq* DNA polymerase (Invitrogen), 1 μL of DNA template and deionized distilled water. The PCR cycling began with an initial denaturation of 3 min at 94°C, followed by 30 cycles of 45 s at 94°C, 30 s at 55°C and 1.30 min at 72°C, and a final extension for 10 min at 72°C. PCR products were analyzed on 1.5% (w/v) agarose gels. The 16S rRNA gene fragment was sequenced bidirectionally by Macrogen (Seoul, South Korea). Resulting sequences were trimmed using Mega 7 (version 7.0.14) and aligned using BioEdit 7 (version 7.1.3.0). Taxonomic identification was done using BLASTn against the sequences in the NCBI database (Disayathanoowat *et al.* 2012). A phylogenetic tree, including the sequences of the isolated bacteria as well as representative sequences from NCBI, was reconstructed with FastTree using the GTR model (Price, Dehal and Arkin 2010) and visualized using NJplot (Perrière and Gouy 1996).

Bacterial community analysis using next-generation sequencing

For each mite species, genomic DNA from six pooled samples of 30 *Varroa* or 50 *Tropilaelaps* adult females was extracted according to the standard β-mercaptoethanol method using the DNeasy blood and tissue kit (Qiagen). The bacterial 16S rRNA gene was amplified with primers 27F, 5′-GAGTTTGATCMTGGCTCAG-3′, and 518R, 5′-WTTACCGCGGCTGCTGG-3′, and sequenced using GS-FLX Titanium 454 pyrosequencing (Roche) by Macrogen (Seoul, South Korea).

The sequence data were analyzed using QIIME 1.9.1. (Caporaso *et al.* 2010). Sequences shorter than 200 bp or longer than 600 bp were discarded, and chimeric sequences were identified and removed using USEARCH61 (Edgar 2010). Sequences were binned into operational taxonomic units (OTUs) based on 97% sequence similarity using cdhit (Li and Godzik 2006). The longest sequence was selected as the representative sequence for each OTU, and taxonomic assignment was performed using the Greengenes (available on http://greengenes.lbl.gov/) (DeSantis *et al.* 2006) and RDP databases (Cole *et al.* 2013). A phylogenetic tree was reconstructed using FastTree with the GTR model (Price, Dehal and Arkin 2010). Representative sequences of OTUs affiliated with *Enterococcus*, as well as sequences of related free-living bacteria as obtained from the NCBI database, were aligned in BioEdit 7. *Streptococcus bovis* was chosen as the outgroup.

Based on the high-quality sequences retained, alpha diversity was calculated using QIIME. Rarefaction curves were constructed using the Shannon index and observed OTUs. Non-metric multidimentional scaling, principal component analysis and non-parametric multivariate analysis of variance were performed to compare microbial communities associated with *Tropilaelaps* and *Varroa*, using the Paleontological Statistics Software Package (PAST) version 3.1 (Hammer, Harper and Ryan 2001).

Detection of *Enterococcus faecalis* in individual *Tropilaelaps* mites

Forward and reverse primers specific for *E. faecalis* were designed based on their 16S rRNA amplicons from pyrosequencing data (5′-CTTCTTTCCTCCCGAGTGCT-3′ and 5′-CCTTTCACTCTTATGCCATGC-3′). Both primers for *E. faecalis* were designed using Primer 3, and specificity was confirmed *in silico* using the probe match option in the Ribosomal Database Project (RDP).

Twenty individual *Tropilaelaps* were washed for 1 min in 7% (v/v) sodium hypochlorite, followed by 1 min in Triton X-100 in phosphate-buffered saline (1:10 dilution) and 1 min in phosphate-buffered saline. The genomic DNA was then extracted with a DNA extraction kit (Epicenter MasterPure). The total DNA was eluted with 10 μL Low TE buffer and stored at −20°C for further analysis. The DNA concentrations were quantified using a NanoDrop spectophotometer. PCR was done in a 25 μL reaction volume containing 1× PCR buffer, 0.24 mM dNTPs, 0.8 μM of each primer, 0.5 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (Peqlab Biotechnologie GmbH, Germany), 1 μL of DNA template and sterile Millipore water. The PCR reaction started with 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 55°C for 30 s and 72°C for 45 s, and a final extension of 72°C for 5 min. The PCR products of 155 bp in length were visualized on 1.6% agarose gel, purified using innuPREP PCRpure kit (Analytik Jena, Germany) and sequenced on an ABI 3130 sequencer.

Quantitative PCR of *Enterococcus faecalis*

The extracted genomic DNA of *E. faecalis*, prepared as above, was also used for estimation of *Enterococcus* 16S rRNA gene copies using quantitative PCR (qPCR). Twenty-five microliters of qPCR contained SYBR green MasterMix, 0.5 μM of each primer (as above), 2.5 μL of DNA template and distilled water. The reaction conditions were 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, 70°C for 15 s and 72°C for 10 s.

Fluorescence *in situ* hybridization of mite sections

Individual mites were washed and sectioned longitudinally (with 0.8 μm thickness) and then subjected to fluorescence *in situ* hybridization as described previously (Kaltenpoth *et al.*

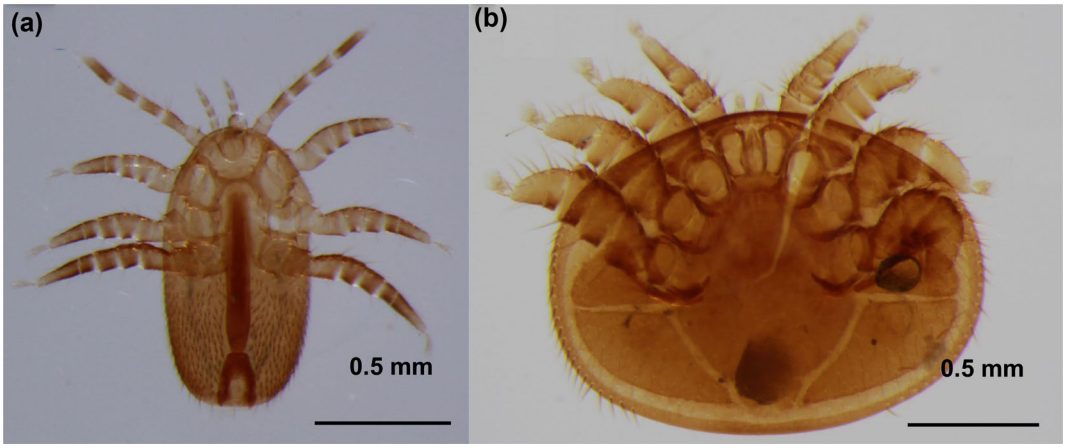

Figure 1. Adult female *Tropilaelaps mercedesae* (a) and *Varroa destructor* (b) mites were mounted under Hoyer's solution and observed using a stereo microscope (Olympus SZX7).

Table 1. Culturable bacteria isolated from *Varroa* and *Tropilaelaps* mites. Closest hits from BLASTn searches against the RDP and NCBI databases are given.

		GenBank		
No.	RDP genus	Match	Accession no.	Identity (%)
Va1	*Enterococcus*	*Enterococcus faecalis*	KX018438.1	99
Va2	*Enterococcus*	*Enterococcus faecalis*	JF772057.1	99
Va3	*Enterococcus*	*Enterococcus faecalis*	JF772057.1	99
Va4	*Enterococcus*	*Enterococcus faecalis*	JF772057.1	99
Va5	*Enterococcus*	*Enterococcus* sp.	KJ210577.1	99
Va6	*Enterococcus*	*Enterococcus* sp.	KJ210577.1	99
Va7	*Enterococcus*	*Enterococcus faecalis*	KX018438.1	99
Tr1	*Enterococcus*	*Enterococcus faecalis*	JF772057.1	99
Tr2	*Enterococcus*	*Enterococcus faecalis*	JF772057.1	99
Tr3	*Enterococcus*	*Enterococcus faecalis*	AM157433.1	99
Tr4	*Enterococcus*	*Enterococcus faecalis*	HQ154556.1	99
Tr5	*Enterococcus*	*Enterococcus faecalis*	KX018438.1	99

2012). The sectioned specimens were hybridized with two universal bacterial probes (EUB338-Cy5 and EUB784-Cy3) and 4′,6-diamidino-2-phenylindole at 50°C for 90 min. Excess probes were washed with a warm washing buffer for 20 min, washed again with water and incubated twice in water for 20 min. The samples were mounted with Vectashield and observed using the fluorescence microscope AxioImager.Z2 with ApoTome (Zeiss, Germany). Moreover, taxon-specific probes of *Enterobacteriaceae*-Cy3 (5′-CTCTTTGGTCTTGCGACG-3′) and *Arsenophonus*-Cy5 (5′-CCTTAACACCTTCCTCACGAC-3′) were adapted from previous studies (Friedrich *et al.* 2003; Bressan, Terlizzi and Credi 2012) and used to confirm the results from pyrosequencing.

RESULTS

Mite species identification

Ten individual mites of the *Varroa* and *Tropilaelaps* genera were identified morphologically (Fig. 1) and by sequencing the *COI* gene and blasting the resulting sequences against the sequences in the NCBI database. They were identified as *V. destructor* and *T. mercedesae*, respectively (accession nos KY865176–KY865185 for *V. destructor* and KY865186–KY865195 for *T. mercedesae*).

Determination of bacterial community using culture-dependent approach

Enumeration of bacteria in each individual *Varroa* and *Tropilaelaps* mite showed average counts of 1360 and 1140 CFUs/mite, respectively. Representative colonies obtained from each mite genus were selected and identified based on their 16S rRNA gene sequences. All bacterial colonies examined were circular, cream-colored with an entire margin and convex elevation. The bacteria were Gram-positive cocci and all isolates were assigned to *E. faecalis* based on comparison of their 16S rRNA gene sequence with those in the GenBank database (Table 1). Despite their close affiliation with *E. faecalis*, the sequences obtained from the two mite genera formed separate monophyletic clades (Fig. 2). Twelve sequences of cultured bacteria were deposited in the NCBI database and were assigned the accession nos KY697081–KY697092.

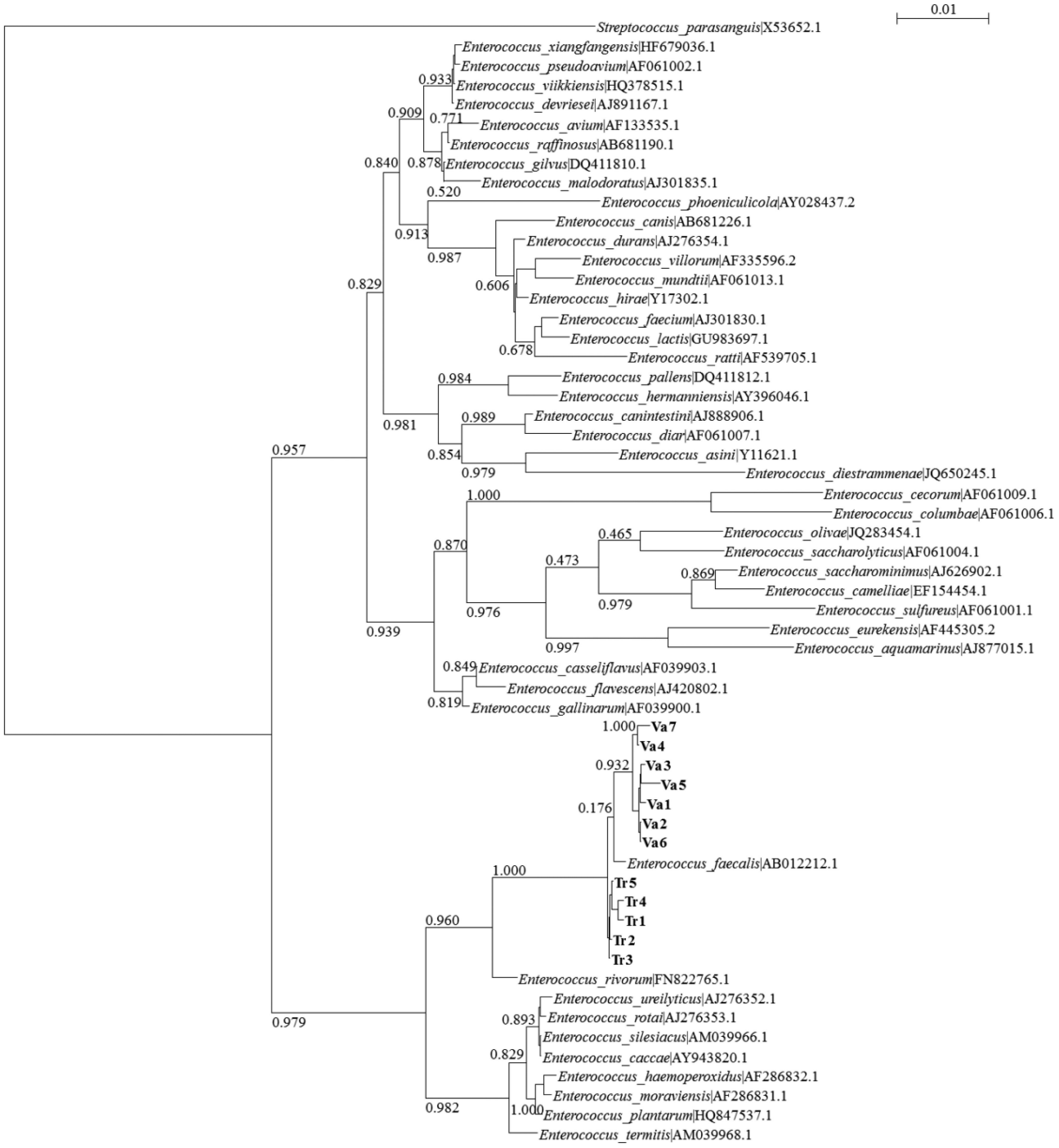

Figure 2. Phylogenetic tree of 16S rRNA from culturable bacteria of *Varroa* and *Tropilaelaps* (highlighted in bold). The phylogenetic tree was reconstructed using FastTree with the GTR model. Numbers at the nodes represent local support values (1000 replicates). *Streptococcus parasanguis* (X53652) was defined as outgroup.

Culture-independent characterization of microbial communities in *Varroa* and *Tropilaelaps* mites

Pyrosequencing of partial 16S rRNA gene resulted in 84 075 sequences across the six samples of *Tropilaelaps* mites, with 55 926 sequences remaining after low-quality sequences were excluded. At the 97% similarity level, these high-quality sequences were assigned to 166 OTUs. The longest sequence of each OTU was selected as the representative sequence. For all six samples, the most abundant bacterium was *Enterococcus* sp., amounting to approximately 88%. The remaining bacteria belonged to other genera in the *Enterococcaceae* family (11%) (Fig. 3). All 28 sequences obtained from the selected *Enterococcus* were most closely related to *E. faecalis* (AB012212) (Fig. 4).

From the six *Varroa* samples, a total of 81 717 sequences were obtained, and 74 347 sequences remained after removing low-quality sequences. The resulting 429 OTUs indicated that

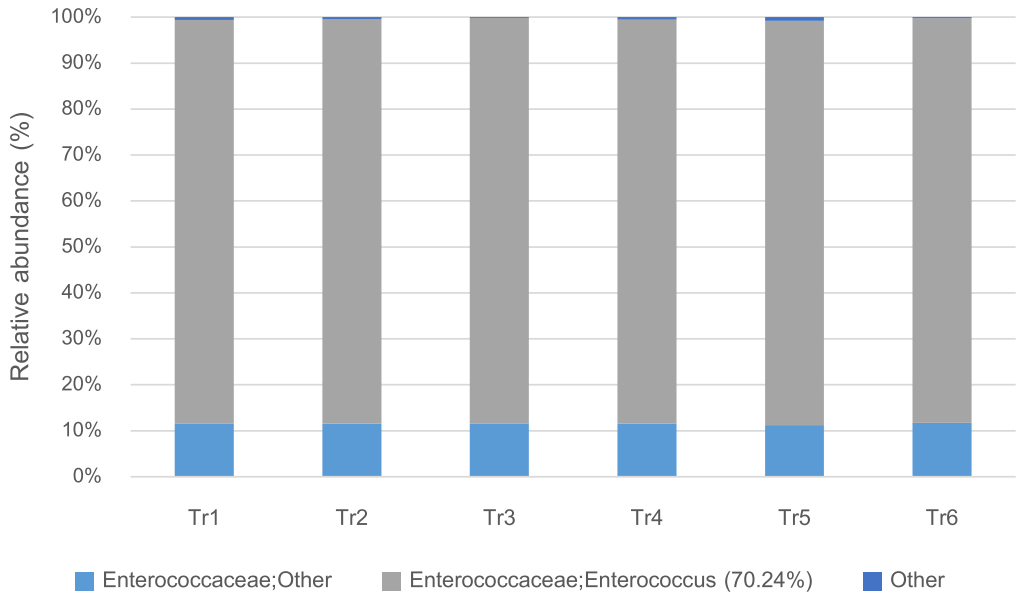

Figure 3. Relative abundance of bacterial communities associated with *Tropilaelaps* mites, as obtained by high-throughput sequencing of bacterial 16S rRNA amplicons. All OTUs that accounted for less than 1% were combined ('Other'). The value in parentheses is average matching with RDP database.

Enterobacteriaceae was the most abundant family. *Arsenophonus* was the predominant genus with 88.38–99.96% relative abundance, in three *Varroa* samples (Fig. 5). The other three samples showed different predominant bacteria: they belonged to genera *Enterobacter* (76.42%), *Pseudomonas* (47.96%) and *Proteus* (38.25%) (Fig. 5).

Alpha diversity of bacterial communities associated with both mites was investigated based on observed OTUs and the Shannon index. In *Varroa*, a higher number of OTUs and a higher variation in diversity of bacterial communities were observed than in *Tropilaelaps* (Supplementary Fig. S1). A multivariate ordination plot of non-metric multidimentional scaling clearly distinguished between bacterial communities in *Varroa* and *Tropilaelaps*, with 95% ellipses by Bray–Curtis distance measurement (Fig. 6a) and principal component analysis ordination plot with Euclidean distance (Fig. 6b). Additionally, a non-parametric multivariate analysis of variance revealed a significant difference between the mite species with Bonferroni-corrected P value = 0.0123. Data derived from 454 pyrosequencing were deposited in the NCBI Sequence Read Archive (SRA) database (accession no. PRJNA378923).

Detection of *Enterococcus faecalis* in individual *Tropilaelaps* mites

To confirm the presence of *E. faecalis* in *Tropilaelaps* mites, 20 selected *Tropilaelaps* mites were assessed using diagnostic quantitative PCR. *Enterococcus* was detected in 6 out of 20 mites (30%). The number of *Enterococcus* gene copies ranged from 14% to 24% of the total eubacterial 16S rRNA gene copies (Fig. 7).

Localization of bacteria by fluorescence *in situ* hybridization

For analysis of the bacterial localization by fluorescence *in situ* hybridization, longitudinal sections of *Varroa* and *Tropilaelaps* were prepared and stained with universal bacterial 16S rRNA probes (EUB338-Cy5 and EUB784-Cy3). The results showed a high prevalence of the bacteria in their cecum (Fig. 8). A more detailed analysis of bacterial localization using *Enterobacteriaceae* and *Arsenophonus* probes also revealed that these bacterial groups were also found in the cecum of *Varroa* (Fig. 9).

DISCUSSION

The bacterial communities in *V. destructor* and *T. mercedesae* mites from northern Thailand were examined using both culture-dependent and culture-independent approaches. Based on 454-pyrosequencing results, the bacterial communities associated with the two mites were distinct, which suggested that each type of mites harbored its own specific microbiota. There was a lower diversity of bacterial communities in *Tropilaelaps* mites than in *Varroa* mites. Across all mite samples, *Enterococcus* was revealed as the most abundant bacterium associated with *Tropilaelaps*, while *Arsenophonus* was the most abundant in *Varroa*. Hubert *et al.* (2015) compared bacterial symbiont sequences in *Varroa* and also reported the detection of *Pseudomonas* and *Arsenophonus*. In addition, *Proteus* and *Enterobacter* were detected in our study. Analysis of a microbial community by next-generation sequencing shows a broader bacterial diversity than cloning and sequencing do, as observed by Hubert *et al.* (2015) and also in our study.

Our study found only one species of culturable bacteria present in both mites. All cultured bacteria were closely related to *E. faecalis*. The pyrosequencing data from *Tropilaelaps* also confirmed this as the most abundant bacterial species. Furthermore, the result from quantitative PCR of 20 individual *Tropilaelaps* mites also revealed 30% prevalence of *Enterococcus* in the samples tested. This result suggested that this bacterium might be harbored in individual mites, not necessarily in all mites of the *Tropilaelaps* genus. In addition, bacterial communities of individual insects can be diverse, depending on the insect's food (Broderick *et al.* 2004) and immune response (Evans and Pettis 2005). From our observation, there was a low diversity of culturable bacteria. Concordantly, Maddaloni and Pascual (2015) observed that bacteria in *V. destructor* were fastidious and only a few types of bacteria could be cultured in rich or selective media.

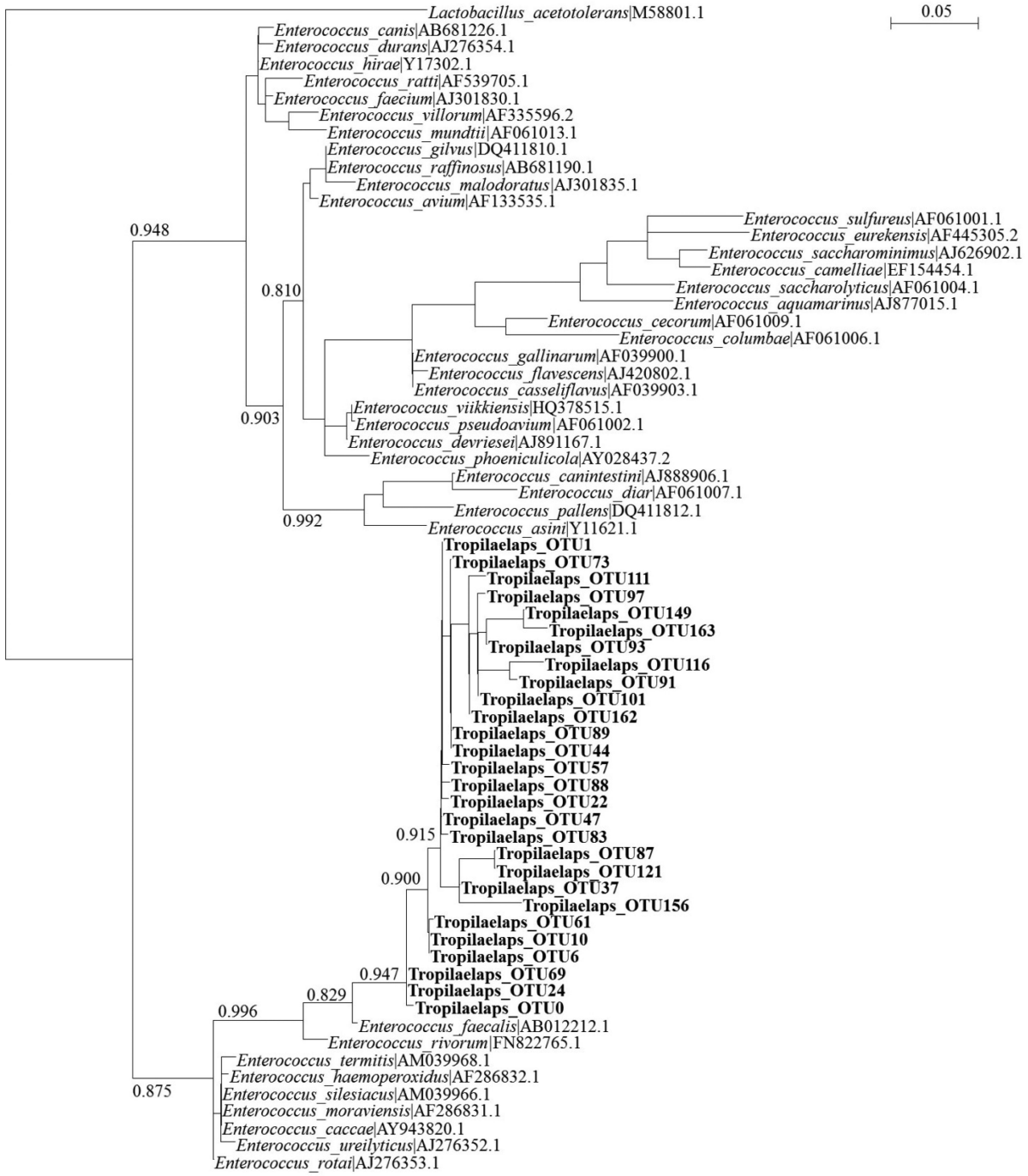

Figure 4. Phylogenetic position of 28 *Enterococcus*-related OTUs obtained by high-throughput sequencing of bacterial 16S rRNA amplicons from *Tropilaelaps* mites. The phylogenetic tree was reconstructed using FastTree with the GTR model. Numbers at the nodes represent local support values (1000 replicates). *Lactobacillus acetotolerans* (M58801) was defined as outgroup.

Enterococcus faecalis is known as a commensal bacterium in the intestinal tract of many animals, including humans. It has also been found in many honey bees as reported in previous studies. For instance, it had a high occurrence in the honey stomach of the giant Asian honey bee (*Apis dorsata*) (Tajabadi *et al.* 2011) and could be isolated from the honey bee's gut (Audisio *et al.* 2011). This bacterium was also found in *V. destructor* (Hubert *et al.* 2015). *Enterococcus* also commonly appeared in honey bee larvae that were infected with *Melissococcus plutonius*, which causes European foulbrood (Forsgren 2010; Gaggìa *et al.* 2015). Moreover, *M. plutonius* and *E. faecalis* were also found in honey bees that are associated with *Varroa* infestation in Kenya (Awino *et al.* 2016). However, the potential functional relevance of *E. faecalis* for the mites remains to be explored.

Localization of bacteria associated with *Varroa* and *Tropilaelaps* mites showed that these bacteria are clearly found in the cecum. Groups of bacteria were also previously detected in the cecum of *Varroa*, although other parts of *Varroa* mites, such as their fat tissue and ventriculus, can also harbor bacteria (Hubert *et al.* 2015).

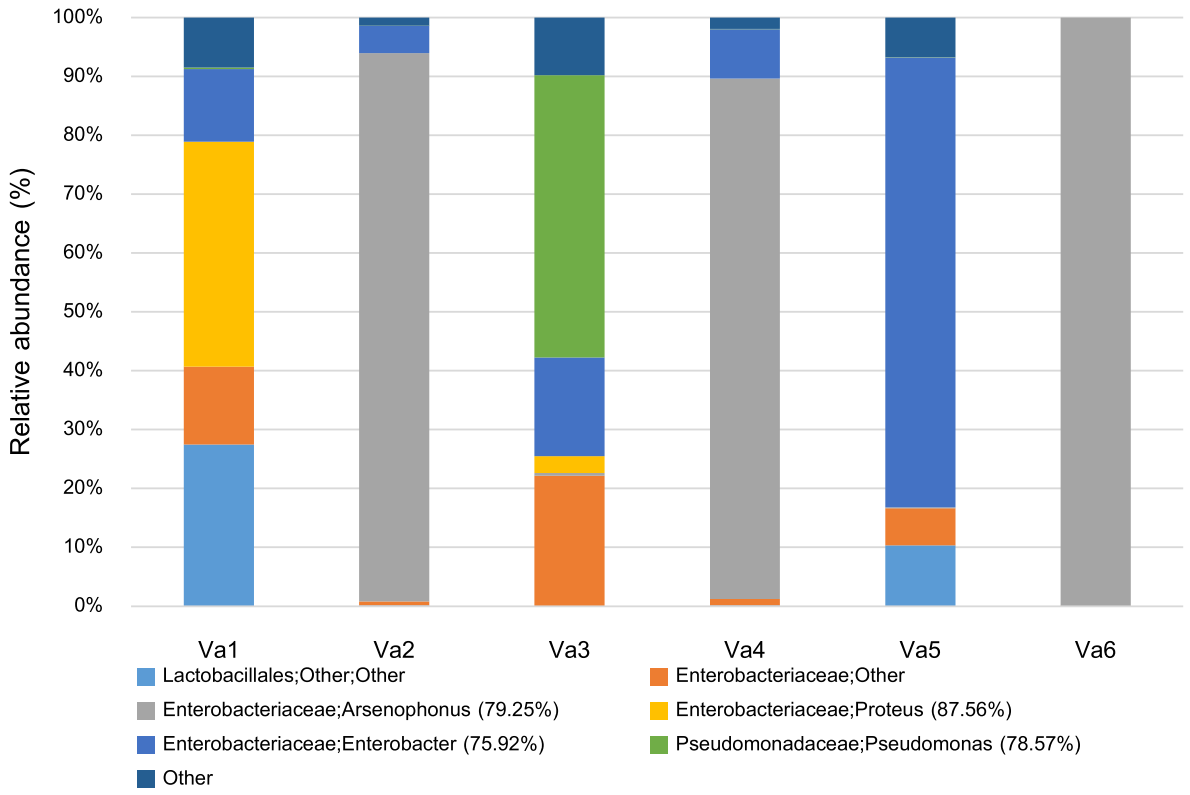

Figure 5. Relative abundance of bacterial communities associated with *Varroa* mites, as obtained by high-throughput sequencing of bacterial 16S rRNA amplicons. All OTUs that accounted for less than 1% were combined ('Other'). The value in parentheses is average matching with RDP database.

Analysis of bacterial communities showed a high level of diversity in *Varroa*. Some abundant genera are *Arsenophonus*, *Enterobacter*, *Pseudomonas* and *Proteus*. Other studies found that the most abundant bacteria associated with *V. destructor* in the winter hive debris were *Morganella*, followed by *Enterococcus*, *Pseudomonas*, *Rahnella*, *Erwinia* and *Arsenophonus* (Hubert *et al.* 2015). The abundance of *Spiroplasma melliferum*, *Arsenophonus nasoniae*, and *E. faecalis* was found to be approximately 31%, 12% and 11%, respectively, in *Varroa* mites in Czechia (Hubert *et al.* 2016). As for our study, the genus *Morganella* was absent. According to Lyapunov *et al.* (2008), *Morganella* was present in *Varroa* and was closely related to a bacterium found in bees during fall and winter. The differences in the bacterial community in *Varroa* observed in previous studies and in our study might be due to the differences in the climate and their environments.

In our study, *Arsenophonus* was found in the cecum of *Varroa*, with frequencies ranging from 88 to 99% across three samples tested. This bacterium is known as an endosymbiont among many insects, and it has previously been found in solitary bees (Gerth *et al.* 2015; Saeed and White 2015) and honey bees (Aizenberg-Gershtein, Izhaki and Halpern 2013; Hubert *et al.* 2015, 2016; Yañez *et al.* 2016). In a previous study, Yañez *et al.* (2016) report that *Arsenophonus* was found in 24% of honey bee workers and that it was also found in the digestive tract of their queens. Its presence was also observed in beehive debris (Hubert *et al.* 2015). This bacterium was found in 73% of beehives in both *V. destructor* and the honey bees within the same hives (Hubert *et al.* 2016). Interestingly, *Arsenophonus* was also observed in honey bees experiencing colony collapse disorder (Cornman *et al.* 2012). Budge *et al.* (2016) reported that *Arsenophonus* was more associated with unhealthy than with healthy honey bees (Budge *et al.* 2016). However, in our study the beehives and the honey bees from which *Varroa* mites were collected were healthy. According to the study of Yañez *et al.* (2016), *Arsenophonus* has been found in some honey bees and this bacterium seems to be acquired horizontally. Individual bees, especially those from different colonies and sites, can harbor different bacterial communities (Moran *et al.* 2012). It is possible that other distinct bacteria found in some samples in our study might be derived from the environment (honey bees or hives).

Potentially pathogenic bacteria of honey bee parasitic mites are of great interest for developing biological control agents for controlling honey bee diseases. Previous studies on pathogenic bacteria in *Varroa* mites found that *Enterobacter cloacae* negatively affected *V. destructor* and increased its mortality (Hrabák 2003). *Bacillaceae* and *Micrococaceae* showed an adverse influence on the *Varroa* population through an increase in *Varroa* mortality (Tsagou *et al.* 2004). From our results, the most abundant bacterial groups belonged to the *Enterobacteriaceae* family. In particular, one sample showed a high abundance of *Enterobacter* in *Varroa*, which may be pathogenic to the mite. However, its influence on parasitism by the mite remains to be elucidated.

Arsenophonus and *Enterococcus* were predominantly found in *Varroa* and *Tropilaelaps*, respectively. Apart from multiple bee species, the genus *Arsenophonus* has also been found in other arthropods, such as wasps, whiteflies, aphids, psyllids and ticks (Thao and Baumann 2004; Nováková, Hypša and Moran 2009; Ahantarig *et al.* 2013). The study of Gherna *et al.* (1991) revealed that *Arsenophonus nasoniae* could infect wasps

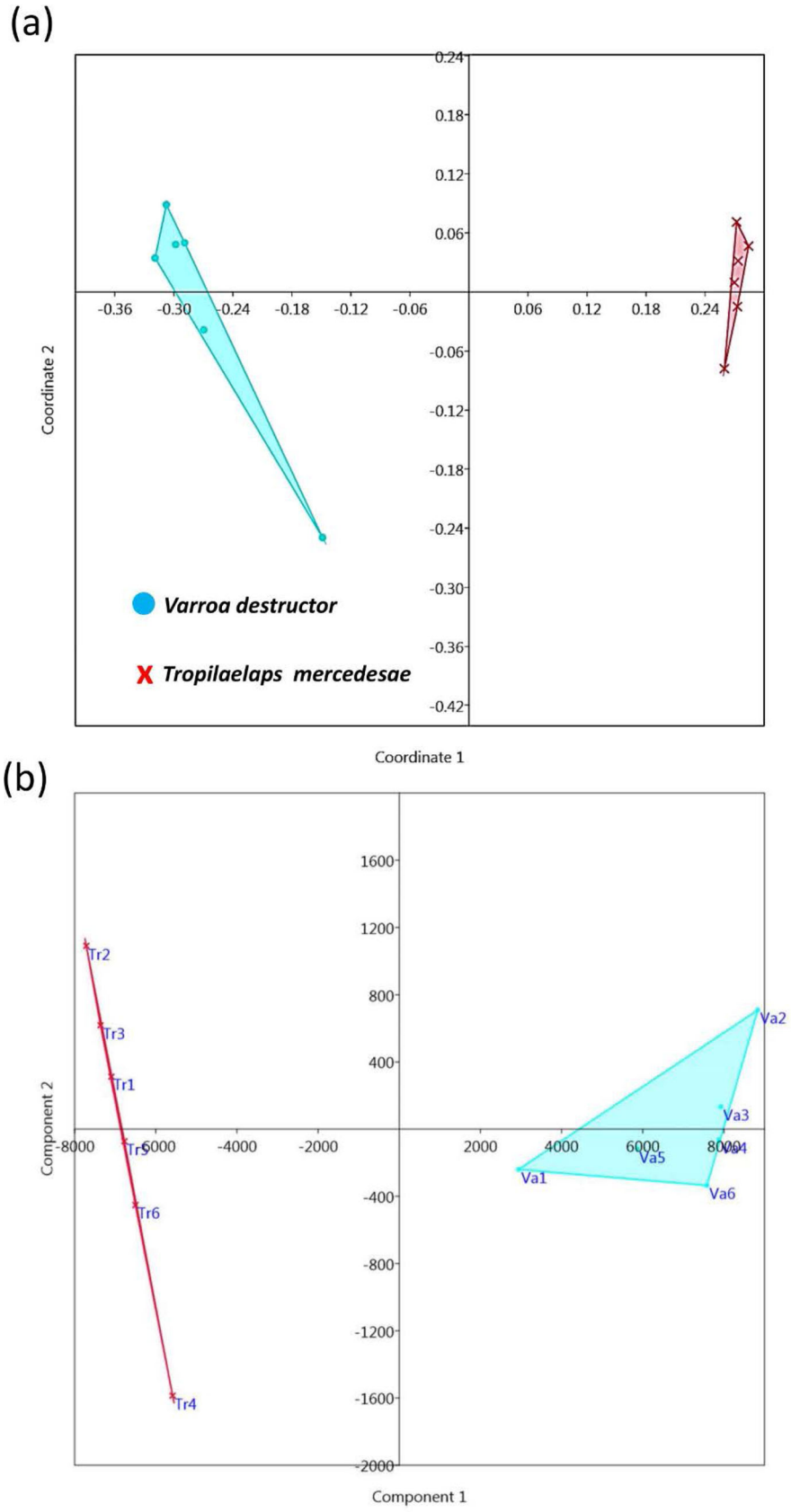

Figure 6. (a) Non-metric multidimentional scaling ordination plot of bacterial communities in *Varroa* (blue circle) and *Tropilaelaps* (red cross) with stress value = 0.03319. (b) Principal component analysis ordintion plot of bacterial communities in both mites with PC1 = 99.138 and PC2 = 0.86208.

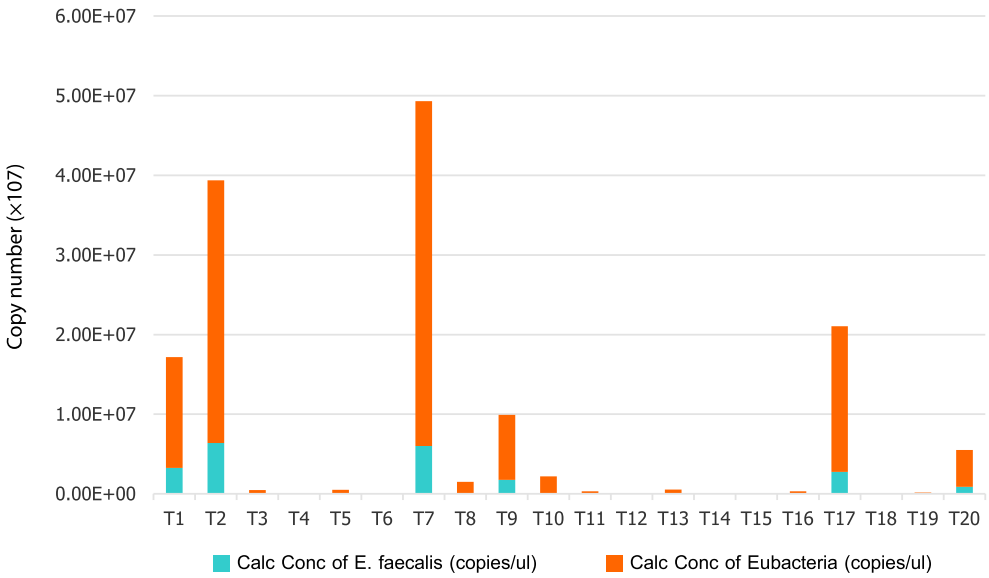

Figure 7. Quantification of general bacterial (orange bars) and *Enterococcus* 16S rRNA (blue bars) copy numbers in 20 individual *Tropilaelaps* mites as revealed by quantitative PCR.

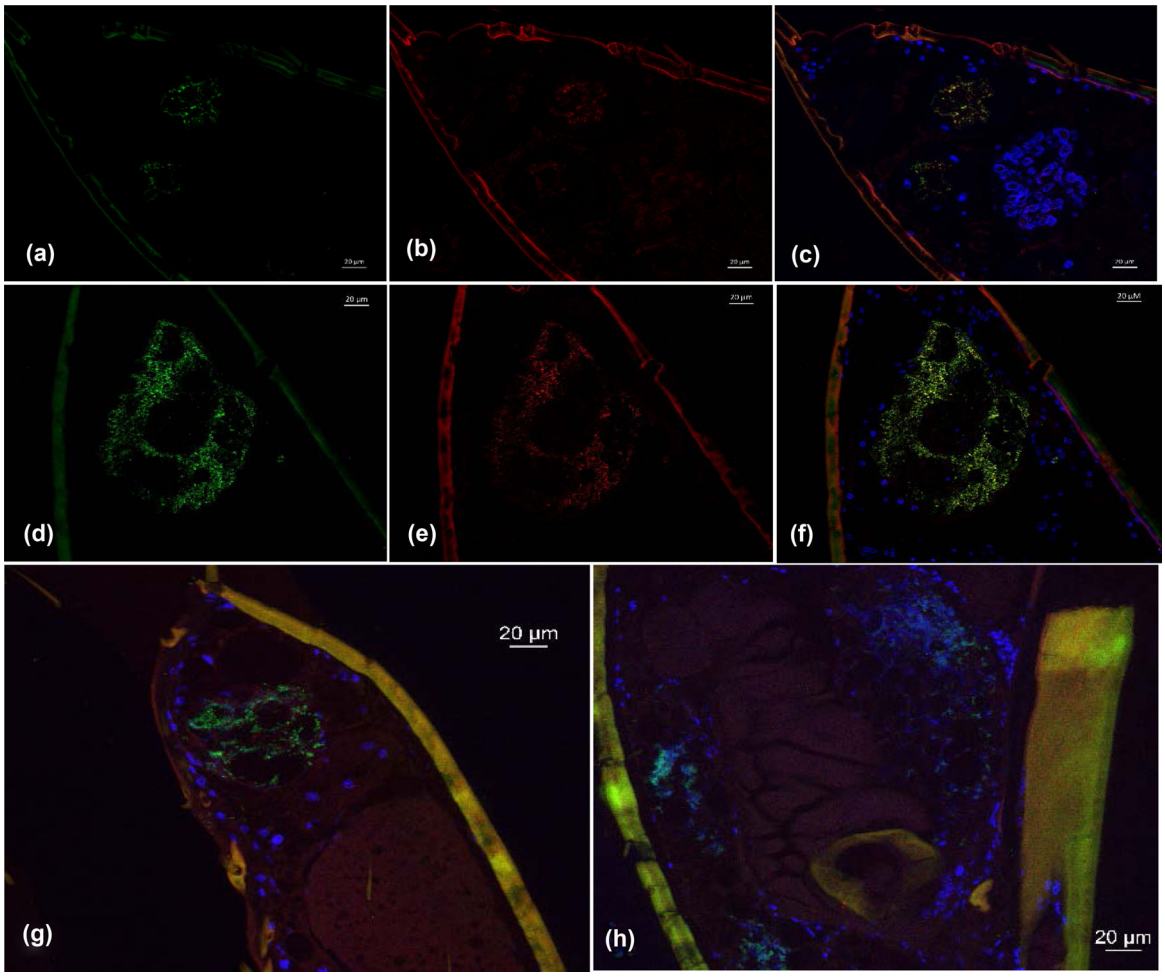

Figure 8. Fluorescence *in situ* hybridization of Eubacteria in longitudinal sections of *Varroa* (a–f) and *Tropilaelaps* (g,h). Bacteria were stained with the general probes EUB784-Cy3 (red) and EUB338-Cy5 (green), and 4′,6-diamidino-2-phenylindole (blue) was used for counterstaining host cell nuclei. Scale bars: 20 μm.

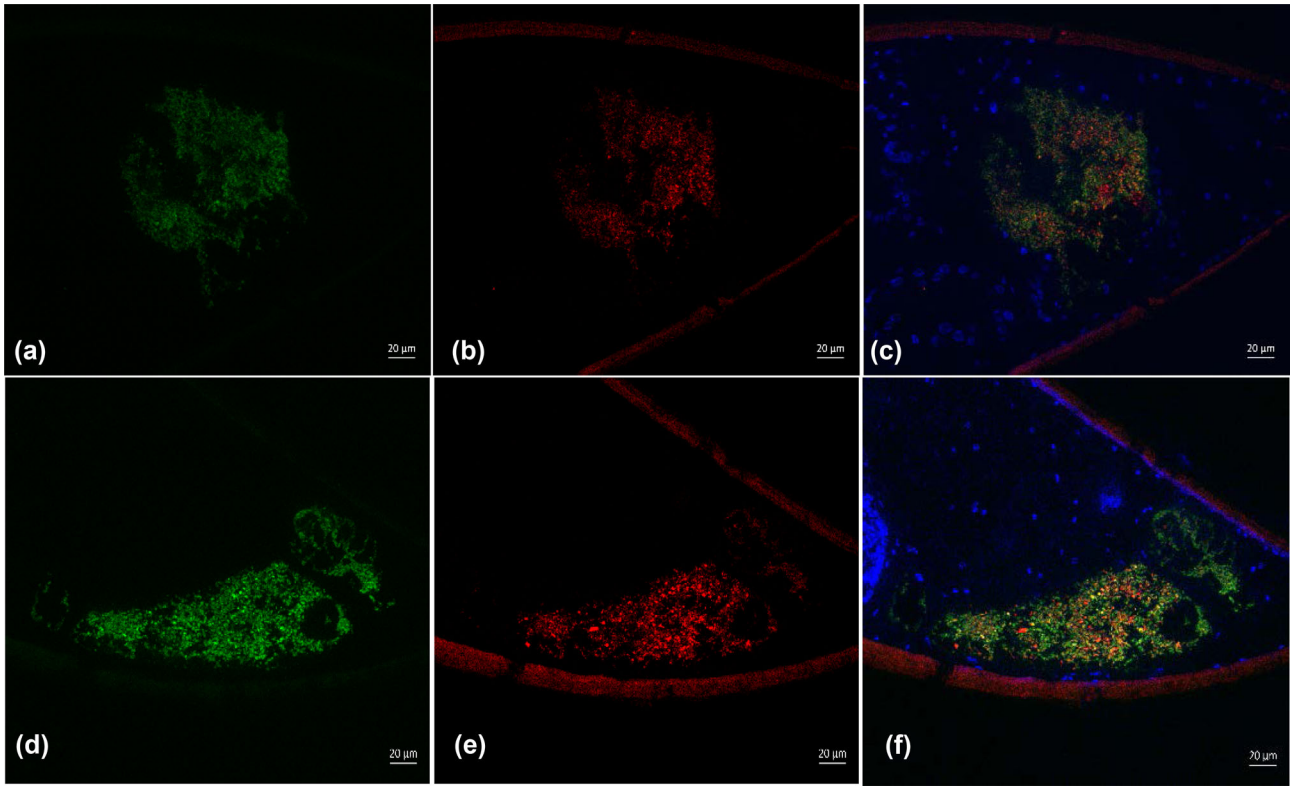

Figure 9. Fluorescence *in situ* hybridization of *Arsenophonus* and *Enterobacteriaceae* in the cecum of *Varroa*. Taxon-specific (a,d) *Enterobacteriaceae*-Cy3 (green) and (b,e) *Arsenophonus*-Cy5 (red) probes were used. (c,f) Overlay of both colors, as well as 4′,6-diamidino-2-phenylindole (blue) for counterstaining of host cell nuclei. Scale bars: 20 μm.

(*Nasonia vitripennis*) causing male-killing. Apart from *A. nasoniae*, the genus *Arsenophonus* is strictly localized inside the bacteriocyte cells of whiteflies (*Trialeurodes vaporariorum* and *Bemisia tuberculata*) (Marubayashi *et al.* 2014) as well as in human lice (*Pediculus humanus caitis* and *P. h. humanus*) (Perotti *et al.* 2007). In addition, the planthopper (*Pentasiridius leporinus*) can harbor an *Arsenophonus* that causes disease in sugar beet (Bressan *et al.* 2009).

Enterococcus, another group of bacteria found in *Tropilaelaps* in our study, has been found in numerous insects, especially in nectar-feeding insects (Martin and Mundt 1972). Moreover, wood-feeding termites harbored an abundance of bacteria in this genus that are involved in carbohydrate utilization in their gut (Tholen, Schink and Brune 1997). Some *Enterococcus* strains have been identified as proteolytic bacteria associated with the order Lepidoptera. These bacteria have been found to play an important role against harmful compounds in plants (Visôtto *et al.* 2009; Vilanova *et al.* 2016).

In conclusion, this study demonstrated the bacterial communities in honey bee ectoparasitic mites in Thailand. It revealed simple communities of bacteria in *Varroa* and *Tropilaelaps*, which were dominated by *Enterobacteriaceae* and *Enterococcus*, respectively. Observation of bacterial localization using fluorescence *in situ* hybridization revealed these bacteria to be located in the cecum of both mites. Further studies should explore the functional significance of the bacteria to the mite hosts.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

ACKNOWLEDGEMENTS

We appreciate the great help from Assist. Prof. Dr Tararat Chitov at Chiang Mai University and Prof. Dr J. Peter W. Young at University of York for their assistance with language correction.

FUNDING

This work was supported by Thailand Research Fund (TRG5880038).

Conflict of interest. None declared.

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