Evaluation of the presence of *Paenibacillus larvae* in commercial bee pollen using PCR amplification of the gene for tRNA^{Cys}

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Abstract

American foulbrood (AFB) caused by *Paenibacillus larvae* is the most destructive honeybee bacterial disease and its dissemination via commercial bee pollen is an important mechanism for the spread of this bacterium. Because Mexico imports bee pollen from several countries, we developed a tRNA^{Cys}-PCR strategy and complemented that strategy with MALDI-TOF MS and amplicon-16S rRNA gene analysis to evaluate the presence of *P. larvae* in pollen samples. *P. larvae* was not detected when the tRNA^{Cys}-PCR approach was applied to spore-forming bacterial colonies obtained from three different locations and this result was validated by bacterial identification via MALDI-TOF MS. The genera identified in the latter analysis were *Bacillus* (fourteen species) and *Paenibacillus* (six) species. However, amplicon-16S rRNA gene analysis for taxonomic composition revealed a low presence of Paenibacillaceae with 0.3 to 16.2% of relative abundance in the commercial pollen samples analyzed. Within this family, *P. larvae* accounted for 0.01% of the bacterial species present in one sample. Our results indicate that the tRNA^{Cys}-PCR, combined with other molecular tools, will be a useful approach for identifying *P. larvae* in pollen samples and will assist in controlling the spread of the pathogen.

Keywords *Paenibacillus larvae* · tRNA^{Cys}-PCR · MALDI-TOF MS · Amplicon-16S rRNA

Introduction

The economic importance of bees for the pollination of many high-value crops, fruits, and wild flowers has led to the commercial production of over a million bee colonies per year in Europe, North America, South America, and Asia [1–3]. However, these insects are attacked by numerous pathogens and parasites including viruses, bacteria, fungi, and metazoans [4]. Therefore, maintenance of healthy colonies is crucial not only for apiculture but also for agriculture and food safety, and to reduce the risk that the bees will act as vectors for the dissemination of various pathogens during pollination or through the use of pollen and honey as a foodstuff by humans and insects [3].

Bee pollen is a favorable microhabitat for many spore-forming bacteria, such as *Paenibacillus larvae*, which is one of the most important bacterial pathogens of honeybees [5, 6]. *P. larvae* is the causal agent of American foulbrood (AFB). The larval stage of honeybees becomes infected through the ingestion of *P. larvae* spores, which germinate in the midgut lumen. Vegetative bacteria proliferate within the midgut, breaching the epithelium, and invading the hemocele, ultimately leading to the death of the infected organisms [4, 7–9].

P. larvae detection plays an important role in the efficient control of dissemination of the pathogen during commercial

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production of hive products and many countries have implemented mandatory disease screening for imported materials [4, 10]. However, control measures for this bee pathogen are not implemented in all countries. In Mexico, for example, regulatory requirements are in general not well defined or do not include detailed instructions for their application. Although American foulbrood has been reported in Mexico since 1994 and a few officially acknowledged cases have been detected or isolated by the Mexican Agriculture Ministry [11, 12], there are no available data quantifying disease screening for *P. larvae* or the detection of the pathogen in imported hive products.

Although various sterilization methods have been used to eliminate microorganisms present in pollen, some bacteria survive these treatments because of their ability to form spores [13, 14]. In consequence, several approaches have used gamma radiation as a control method for eradication of spore-forming *P. larvae* [13]. Nevertheless, there is ample reason for concern about the possibility that preparations of commercial bee pollen may be contaminated with pathogenic bacteria such as *P. larvae*, despite the implementation of sterilization methods.

Several approaches have been used to identify *P. larvae*, including molecular typing methods based on 16S rRNA gene sequences [6, 15–19] and methods based on the repetitive-element polymerase chain reaction (PCR) (rep-PCR) using enterobacterial repetitive intergenic consensus (ERIC) primers [6]. Interestingly, ERIC genotypes can also be distinguished via matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [20]. The utilization of multilocus sequence typing schemes (MLST) for *P. larvae* has allowed the establishment of global patterns in the population structure [21]. Another molecular approach, high-throughput sequencing of the 16S rRNA gene, allows recognition of the diversity of bacteria present in a sample, facilitating the identification of pathogens. This methodology has allowed the identification of *P. larvae* in worker honeybees, and thus has a high potential for use in the diagnosis of AFB [22].

While these molecular strategies for detecting *P. larvae* have their applications, their limitations and the fact that Mexico imports bee pollen from a number of different countries led us to consider alternative molecular approaches for the identification of *P. larvae*. In this study, we take advantage of the observation that some tRNAs, such as tRNA^{Cys}, are encoded by a single copy gene in some Firmicutes [23, 24]. The position of these genes and their association with rRNA and tRNA operons or constitutive genes in the *P. larvae* genome recommends their use in molecular approaches for *P. larvae* detection. Thus, we report here the evaluation of the presence of *P. larvae* in commercial bee pollen, based on tRNA^{Cys}-PCR amplification, complemented by MALDI-TOF MS and amplicon-16S rRNA gene analysis. Application of

this approach should help to reduce the risk of the spread of *P. larvae* through commercial bee pollen.

Materials and methods

Bacterial strains

Bacterial strains obtained from the LMUAQ-collection (Laboratory of Microbiology, University Autonomous of Queretaro, Mexico) and primers used in this study are described in Table 1. *Paenibacillus* spp. strains were grown in MYPGP medium, consisting of 1.5% yeast extract (Difco Laboratories), 1.0% Mueller-Hinton broth (Difco Laboratories), 0.2% glucose, 0.3% K$_2$HPO4, 0.1% sodium pyruvate, and 2.0% agar [25]. All other bacterial strains were grown in tryptic soy agar medium (Difco Laboratories; Detroit, MI. USA). *P. polymyxa*, *P. odorifer*, and *P. peoriae* were isolated in a screening of pollen samples and identified by analyzing 16S ribosomal deoxyribonucleic acid (rDNA) sequences and by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF MS) mass spectrometry using a MicroFlex LT mass spectrometer (Bruker Daltonics, Bremen, Germany).

tRNA sequences analysis

The tRNA sequences used in our analysis were aligned and extracted from complete *Paenibacillus* genomes using the tRNAscan-SE program [26]. From predicted genes, tRNA counts per iso-acceptor were obtained for each genomic sequence and cluster analyses were done using the software R, version 3.0.2.

Amplification conditions

PCR primers were designed to amplify the tRNA^{Cys} region in the *Paenibacillus larvae* genome. Pan1 and Pan3 were designed to target the hydrolase-like gene, while Pan2 and Pan4 were designed to target the tRNA^{Cys} gene. The hydrolase and tRNA^{Cys} genes are located relatively close to each other in the *P. larvae* genome (See Fig. 3). The various primer combinations were predicted to yield products of the following sizes: Pan1-Pan2, 1268 bp; Pan3-Pan4, 907 bp; Pan1-Pan4, 1218 bp; and Pan2-Pan3, 957 bp. For PCR, a single colony (or in some cases, a pool of several colonies) of each bacterial strain tested (see Table 1) was suspended in 50 μL of distilled water and heated to 95 °C for 10 min and 1 μL of the bacterial suspension was used in a 30-μL PCR mix using Phusion high-fidelity DNA polymerase (Thermo Scientific; Waltham, MA, USA). PCR conditions were as follows: after a hot start (2 min, 95 °C), 35 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and final elongation step at 72 °C for

Table 1 Strains and primers used in the present study

Strain	Source*	Strain	Source
Paenibacillus larvae	ATCC 9545	*Staphylococcus cohnii*	LMUAQ
Paenibacillus polymyxa	This study	*Staphylococcus xylosus*	LMUAQ
Paenibacillus odorifer	This study	*Arthrobacter phenanthrenivorans*	LMUAQ
Paenibacillus peoriae	This study	*Exiguobacterium aurantiacum*	LMUAQ
Escherichia coli	LMUAQ	*Micrococcus luteus*	LMUAQ
Pseudomonas sp.	LMUAQ	*Kocuria* sp.	LMUAQ
Salmonella gaminara	LMUAQ	*Sanguibacter marinus*	LMUAQ
Psychrobacter faecalis	LMUAQ	*Kytococcus sedentarius*	LMUAQ
Bacillus koreensis	LMUAQ	*Citricoccus* sp.	LMUAQ
Bacillus pumilus	LMUAQ	*Planomicrobium chinense*	LMUAQ
Bacillus amyloliquefaciens	LMUAQ	*Microbacterium oleivorans*	LMUAQ
Bacillus thuringiensis	LMUAQ	*Arthrobacter gandavensis*	LMUAQ
Bacillus subtilis	LMUAQ	*Corynebacterium xerosis*	LMUAQ
Bacillus altitudinis	LMUAQ	*Agrococcus lahaulensis*	LMUAQ
Staphylococcus aureus	LMUAQ	*Planococcus plakortidis*	LMUAQ
Primers	Target	Sequence	
Pan1	Hydrolase	AAGAAATGTACATAATTGATGAGGG	
Pan2	5′ Cys	GGCGCCATAGCCAAGTGGTAAGGC	
Pan3	Hydrolase	TTTATTCTGACGAATACAAGACCGG	
Pan4	3′ Cys	GTTCGAATCTGGGTGGCGCCTCCA	

*Bacterial strains were obtained from the Laboratory of Microbiology, University Autonomous of Queretaro, Mexico (LMUAQ)

5 min. PCR products (5 μL) were analyzed by electrophoresis in 1.0% agarose (Sigma-Aldrich; St. Louis, MO, USA) and visualized on a UV transilluminator.

Paenibacillus larvae detection

Commercial bee pollen samples destined for production facilities for bumblebee rearing in Mexico were obtained. We analyzed five samples from Europe, one sample from Mexico, and one sample from Chile. Each replicate of 1 g of bee pollen sample was homogenized in 10 mL of peptone and treated at 80 °C for 10 min to select spore-forming bacteria. Serial dilutions were then inoculated onto MYPGP agar medium and incubated at 37 °C during 48 h. Bacterial colonies obtained by this procedure (> 1000) were tested by tRNA^{Cys}-PCR. Additionally, bacterial colonies were analyzed by MALDI-TOF MS using a MicroFlex LT mass spectrometer (Bruker Daltonics, Bremen, Germany) for species identification. The MALDI-TOF mass spectrometry method uses colonies directly after their treatment with 2 μL of MALDI matrix (a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid). Spectra were analyzed by using the Bruker Biotyper 2.0 software and library (version 2.0, 3740 entries; Bruker Daltonics). The identification score criteria used were those recommended by the manufacturer: a score ≥ 2.0 indicated species-level identification, a score between 1.7 and 1.9 indicated identification at the genus level, and a score < 1.7 was interpreted as no identification.

For amplicon-16S rRNA gene analysis, DNA was extracted from the pollen samples themselves or from microbial enrichment cultures obtained from those samples. Enriched cultures were used to select spore-forming bacteria from bee pollen. To prepare enrichment cultures, 1-L flasks containing 200 mL of MYPGP medium were inoculated with 0.2 g of the pollen samples from Europe, Mexico, and Chile previously treated at 80 °C for 10 min as described above. Culture flasks were incubated at 37 °C for 48 h. DNA was extracted using a ZymoBIOMICS DNA Mini Kit (Zymo Research; Irvine, CA. USA).

Amplicons for 16S rRNA genes were sequenced using the Illumina MiSeq platform at Macrogen Inc. (Seoul, Republic of Korea). Amplicon sequencing data were downloaded in Fastq format and each library was analyzed with the FastQC program (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to validate library quality. All libraries were trimmed in Geneious v.9 with the Trim Ends tool and sequences with an error probability limit of 0.05 were removed in regions with average quality lower than Q30. Reads with sizes larger than 50 bases were merged in paired reads. For the generation of contigs, consensus sequences were used to search the 16S Microbial Database from NCBI (downloaded 05-2017) with Megablast using the following

parameters: E-value 0.000001; word size 64; gap cost linear. The Megablast output was then used as the input for the classify sequences tool in Geneious v.9 to perform the taxonomic assignments and the descriptive analysis. The sequences merged in paired reads were used for analysis of bacterial diversity in environmental samples with the Naive Bayesian classifier and the Ribosomal Database Project (RDP) Classifier in the 16S biodiversity tool in Geneious v.9 [27]. DNA sequences of bacteria in bee pollen samples were deposited in GenBank under the accession numbers; SRP132301 (Chile sample), SRP132302 (Mexico sample), and SRP132303 (Europe samples).

DNA sequences from the amplicon-16S rRNA gene analyses were compared with sequences of 16S rRNA from sixteen species of *Paenibacillus* (31 strains), three species of *Fontibacillus* and one of *Cohnella* using the NCBI databases (http://www.ncbi.nlm.nih.gov/genbank/). The 16S rRNA sequence of *Aneurinibacillus aneurinilyticus* was designated as the outgroup taxon. Sequence data were analyzed using Bioedit v7.0.9.0 [28] and aligned using the Clustal W algorithm in Clustal X v2.1 [29]. Alignments were edited in Seaview v4.6 [30]. Identification of species was performed by phylogenetic inference methods of maximum likelihood using the online platform PhyML (http://www.atgc-montpellier.fr/phyml/) [31]. A best fit nucleotide substitution model was calculated using Smart Model Selection (SMS) [32]. Phylogenetic trees were constructed and edited using Geneious v.9 [27].

Results

tRNA sequences analysis

In the present study, we aimed to establish a reliable method to detect the presence of *P. larvae* in commercial bee pollen and thus, to avoid the dissemination of this pathogen in pollen samples. The initial approach employed a PCR strategy based on tRNA genes present in single copy in the genome of *P. larvae*. The organization of tRNA genes is expected to be relatively stable through evolutionary time, so that tRNA gene counts were predicted using tRNAscan-SE and compared with sequence data from 58 *Paenibacillus* species (Fig. 1).

It is apparent from Fig. 1 that some tRNA iso-acceptors displayed relatively low copy numbers consistently across all genomes analyzed, such as tRNA^{Cys}, tRNA^{Ile}, and tRNA^{Trp}. These tRNAs are represented by a light green color in Fig. 1. Other tRNAs displayed relatively high copy numbers consistently across all genomes analyzed, e.g., tRNA^{Arg}, tRNA^{Gly}, tRNA^{Leu}, tRNA^{Met}, tRNA^{Ser}, and tRNA^{Val} group. Particularly relevant to the present study, there were tRNAs decoding triplets for a given amino acid in *P. larvae* with a single copy gene, for example, tRNA^{Cys}, tRNA^{Ile}, and

tRNA^{Trp}. We decided to use the *trnC* gene (tRNA^{Cys}) to develop a diagnostic method for *P. larvae* detection.

In *P. larvae*, the gene for tRNA^{Cys}, is located within an operon consisting of 18 tRNA genes, 23S rDNA, and 16S rDNA genes (genome region plv: ERIC2_c30780–plv: ERIC2_c30970). For the development of our PCR strategy, we used the hydrolase-like gene located upstream from tRNA^{Cys} (genome region plv: ERIC2_c30770). After the alignment of the selected sequences (from the hydrolase-like gene to tRNA^{Cys}), DNA sequences were found to be specific for *P. larvae* and used for the design of specific primers (Fig. 2).

tRNA^{Cys}-PCR analyses revealed the presence of specific amplicons for *P. larvae* (Fig. 2, lanes 1, 2, 3, and 4) according to the predicted sizes. The amplicon of primers Pan1 and Pan2 in *P. larvae* was sequenced and it was found that the amplicon is unique to the *P. larvae* species (Fig. 3). To test further for the presence of the tRNA^{Cys}-PCR region in other bacteria for cross-reactions, we tested both Gram-negative and Gram-positive bacteria (Table 1) using the PCR conditions used for *P. larvae*. No other bacterial strain generated any similar amplicon under the specified PCR conditions.

Paenibacillus larvae detection

tRNA^{Cys}-PCR analysis

To test for the presence of *P. larvae* in commercial bee pollen from Europe, Mexico, and Chile, we recovered more than one thousand colonies of spore-forming bacteria from MYPGP agar medium and analyzed them by tRNA^{Cys}-PCR with the Pan1 and Pan2 primers. No specific PCR product was obtained from any of these bacterial colonies (data not shown). Thus, by this measure, *P. larvae* was absent from the commercial pollen samples we analyzed.

MALDI-TOF MS analysis

To validate these negative results, the bacterial colonies obtained from the commercial bee pollen were identified by MALDI-TOF MS (Table 2). This analysis identified members of the genus *Bacillus* as the most common species found in association with bee pollen from Europe, followed by *Paenibacillus* species. In the samples from Mexico, the genus *Bacillus* presented eight species and *Paenibacillus* just one species. The samples from Chile presented eight species for the genus *Bacillus*. Confirming the results of the PCR analyses, *P. larvae* was not observed among the bacterial species associated with the bee pollen samples. This result could mean either that *P. larvae* was absent from these samples or was present in amounts too low to be detected by these methods. Therefore, we decided to obtain massive amplicon-16S rRNA

Fig. 1 Distribution and copy number of tRNA genes in genomes of *Paenibacillus* spp. The tRNA genes were mapped and extracted using the tRNAscan-SE program [26]. From predicted genes, tRNA counts per iso-acceptor were obtained and cluster analyses were done using the software R package, version 3.0.2. The color key refers to numbers of copies of the tRNA genes
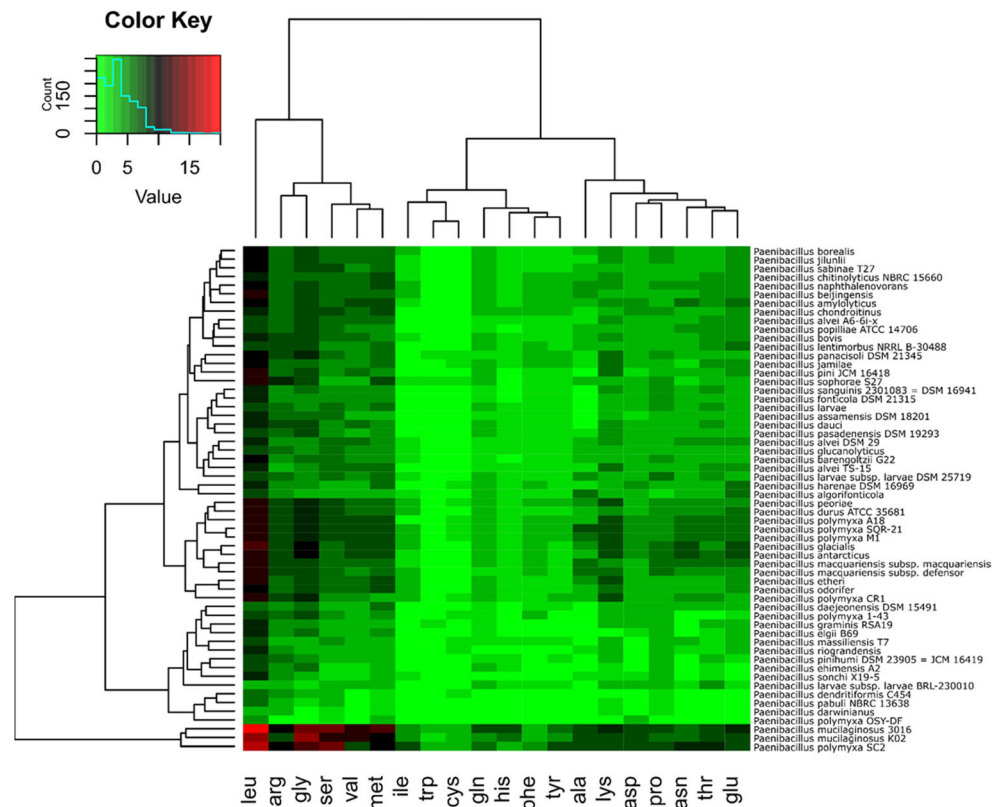
gene sequencing data using the pollen samples themselves and liquid cultures enriched to select spore-forming bacteria.

Bacterial taxonomic diversity in commercial bee pollen

The amplicon-16S rRNA gene analysis generated in Geneious with RPD yielded the following results. For each direct sequencing sample, we obtained 36,704 to 39,031 high-quality reads representing 6 to 7 operational taxonomic units (OTUs) at a 97% level of similarity. Members of the Firmicutes and Proteobacteria were found in all pollen samples. In all enriched samples, the Firmicutes were the most abundant phylum, with a count of 152,320 high-quality reads. Figure 4 shows the relative abundance of bacterial families belonging to the order Bacillales. The high-quality reads were dominated by Bacillaceae (3.7 to 46.3%), Planococcaceae (7.5 to 48.6%), and Thermoactinomycetaceae (9.9 to 88.1%). The most representative families observed in the enriched cultures from the order Bacillales were Bacillaceae (93 to 94%) and Paenibacillaceae (5 to 7%) (Fig. 4).

Figure 5 shows the classification of genera from the family Paenibacillaceae. In general, 7 OTUs were present: *Oxalophagus*, *Brevibacillus*, *Ammoniibacillus*, *Paenibacillus*, *Fontibacillus*, *Saccharibacillus*, and *Cohnella*. The sequences generated from all the pollen samples for the genus *Paenibacillus* were extracted and compared with the NCBI database with Megablast, identifying the

following species: *P. motobuensis*, *P. aceti*, *P. vini*, *P. telluris*, *P. faecis*, *P. barengoltzii*, *P. xylanilyticus*, *P. typhae*, *P. pini*, *P. wynnii*, *P. odorifer*, *P. taiwanensis*, *P. apiarius*, *P. larvae*, *P. alvei*, *P. kribbensis*, *P. forsythiae*, *P. polymyxa*, *P. jamilae*, and *P. durus*.

Phylogenetic analyses were performed using 16S rRNA from members of the Paenibacillaceae family. The phylogenetic tree was robust with the following species: *P. lautus*, *P. amylolyticus*, *P. chibensis*, *P. glucanolyticus*, *P. validus*, *P. peoriae*, *P. abekawaensis*, *Fontibacillus aquaticus*, *F. panacisegetis*, *F. phaseoli*, *Cohnella thermotolerans*, *P. xylanilyticus*, *Bacillus aporrhoeus*, *P. lautus*, *P. chibensis*, and *P. abekawaensis*. *A. aneurinilyticus* was used as the outgroup. The analysis showed that the three contigs from the Europe5 sample were identified as *Paenibacillus larvae*. The best scoring PhyML tree is shown in Fig. 6 and it is rooted to *A. aneurinilyticus*.

The phylogenetic analysis confirmed the result obtained from the Megablast database classification, where only one pollen sample (Europe5) contained a bacterial species identified as *P. larvae*, with a relative abundance of 0.01%.

Discussion

In order to identify *P. larvae* in commercial pollen samples and as a tool for controlling the spread of the pathogen, we

Fig. 2 Organization of the hydrolase/tRNA^{Cys} region from *P. larvae* (upper panel) and tRNA^{Cys}-PCR products (lower panel). Primers were designed to amplify the hydrolase-tRNA^{Cys} region in *P. larvae*. Lane 1, primer combination Pan1-Pan2 (1268 bp product), lane 2, Pan3-Pan4 (907 bp product), lane 3, Pan1-Pan4 (1218 bp product), and lane 4, Pan2-Pan3 (957 bp product). Lane M contains a Thermo Scientific GeneRuler 100 bp DNA ladder
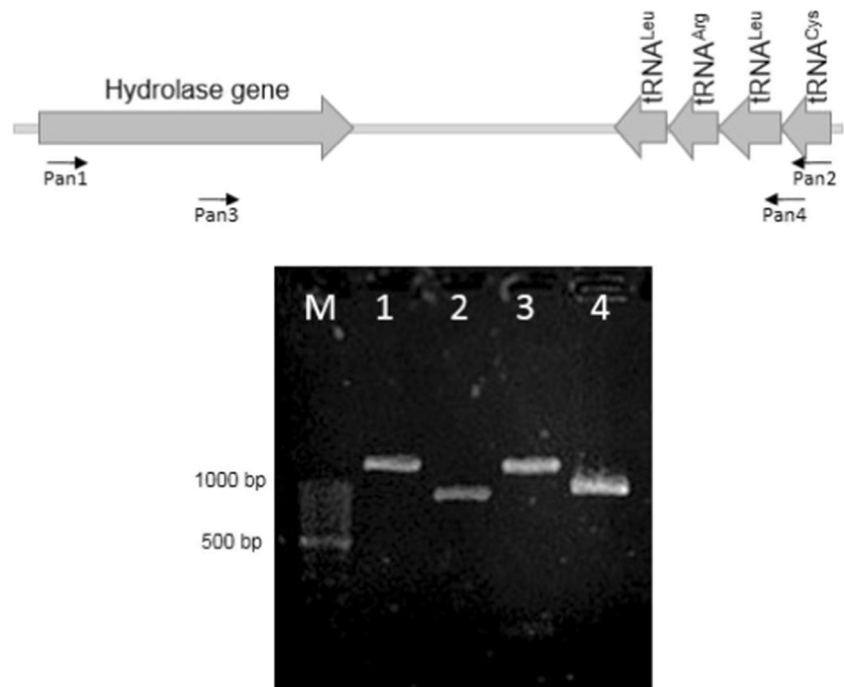
developed the tRNA^{Cys}-PCR approach, based on the amplification of the tRNA^{Cys} gene region. The results presented indicate that consensus tRNA^{Cys} gene, with a single copy gene in the *P. larvae* genome, is an excellent molecular marker associated with an upstream hydrolase gene and could be used as a molecular diagnostic. This property makes the method suitable to the identification of *P. larvae* and recommends tRNA^{Cys}-PCR as a rapid and straightforward approach that complements identification methods that already exist. Moreover, tRNA^{Cys}-PCR is technically less demanding and time-consuming than DNA sequencing when 16S rRNA-PCR is used. tRNA^{Cys}-PCR could be the method of choice when large numbers of commercial bee pollen samples are to be screened or as a first step when identifying species based on genomic sequences.

Applying the tRNA^{Cys}-PCR method to several samples of commercial bee pollen showed *P. larvae* to be absent from all colonies of spore-forming bacteria analyzed from those samples. This result was verified by MALDI-TOF MS analysis which also identified a number of bacterial species which were present as microbial contaminants of the pollen samples; some of those species were related to *Paenibacillus*. However, our amplicon-16S rRNA gene analysis revealed that Firmicutes and Proteobacteria were the most abundant phyla in pollen samples. Our amplicon-metagenomic analysis did reveal a low abundance (0.01%) of *P. larvae* in one pollen sample from Europe. Additionally, in enrichment culture samples, the Paenibacillaceae family was detected in lower abundance (5 to 7%). This observation establishes a lower limit for the tRNA^{Cys}-PCR method for the detection of *P. larvae*.

Fig. 3 Phylogenetic dendrogram of amplicon Pan1-Pan2. The amplicon was sequenced and compared to the NCBI database using Blast to identify highly similar sequences with E-values < 10^{-40}. Sequences were aligned and a distance tree was generated using the Blast pairwise alignments. The phylogenetic analysis placed the Pan1-Pan2 amplicon in the clade with sequences corresponding to *Paenibacillus larvae* subsp. *larvae*
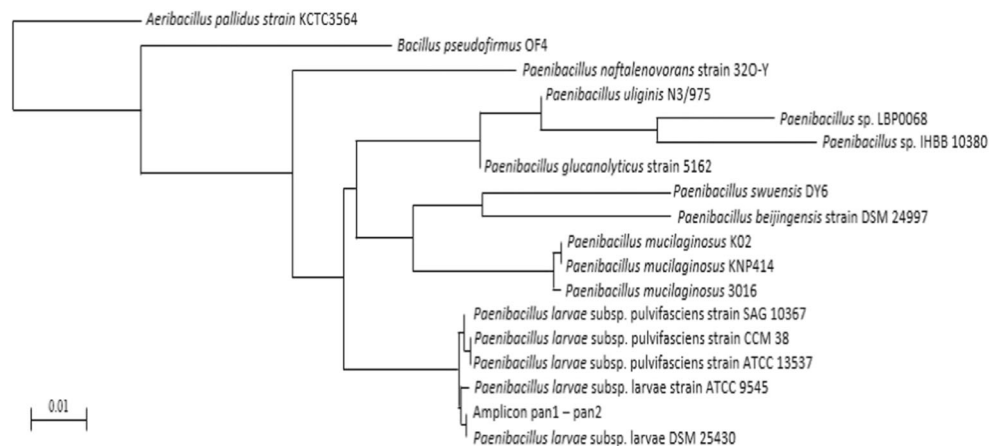
Table 2 Bacterial species identified by MALDI-TOF MS obtained from Europe, Chile, and Mexico commercial bee pollen

Europe	Mexico	Chile
Bacillus licheniformis	*Bacillus licheniformis*	*Bacillus licheniformis*
Bacillus pumilus	*Bacillus pumilus*	*Bacillus pumilus*
Bacillus cereus	*Bacillus cereus*	*Bacillus cereus*
Bacillus mycoides	*Bacillus subtilis*	*Bacillus altitudinis*
Bacillus weihenstephanensis	*Bacillus thuringiensis*	*Bacillus mojavensis*
Bacillus sonorensis	*Bacillus mojavensis*	*Bacillus mycoides*
Bacillus subtilis	*Bacillus amyloliquefaciens*	*Bacillus megaterium*
Bacillus mojavensis	*Bacillus vallismortis*	*Bacillus endophyticus*
Bacillus thuringiensis	*Paenibacillus chitinolyticus*	
Bacillus altitudinis		
Paenibacillus cookii		
Paenibacillus odorifer		
Paenibacillus peoriae		
Paenibacillus polymyxa		
Paenibacillus rhizosphaerae		

Recent evidence shows that detection limits of spores of *P. larvae* using a multiplex PCR revealed threshold values of 0.4 spore/mL in samples of honey, 0.8 spore/g in samples of bee pollen, and in samples of royal jelly 1.6 spores/g, these relevant results supporting the idea that PCR is useful to detect spore of *P. larvae* in bee pollen [33]. Due to regulatory restrictions, we were not able to test bee pollen samples from honeybee colonies with AFB symptoms in this study. We feel, nevertheless, that our method will be extremely useful in evaluating the potential for the transmission of AFB via *P. larvae* contamination of pollen samples.

Based on our results, in addition to microbiological and other molecular methods, amplicon 16S-rRNA gene analysis should be useful for detection of *P. larvae* on commercial bee pollen. A metagenomic approach has been used recently to analyze the occurrence of *P. larvae* in affected apiaries, in colonies with clinical signs of AFB infection, in asymptomatic

colonies and control colonies. These studies revealed *P. larvae* relative abundances of 50%, 5%, and 3% respectively [22]. Thus, in the apiaries, this pathogenic bacterium could be transmitted by honeybees or as a component of the microbiota present on pollen. These results are relevant to the interpretation of our metagenomics data which showed a relatively low abundance of *P. larvae* in commercial bee pollen. In both contexts, there is a risk of spread of *P. larvae* during the acquisition of commercial bee pollen and therefore more emphasis should be placed on its detection.

Pollen microbiome studies have increased because of their recognized ecological, commercial, and medical importance. Due to its structure and nutritive composition, pollen provides a unique microhabitat for microorganisms and a reservoir for these microbes in insects that are fed with pollen. Therefore, the diet of an insect and its gut microbiome are tightly linked. Some studies of bacterial community profiles based on 16S rRNA gene sequences show that gut microbiota of *Apis*

Fig. 4 Relative abundance of different Families within the order Bacillales obtained from commercial bee pollen from three different regions, indicated along the bottom of the figure (five samples from Europe, indicated by 1 to 5, and one sample from Mexico and Chile). Data from enrichment culture samples are indicated by an asterisk (*). 16S rRNA genes were amplified and sequenced as described in "Materials and Methods"
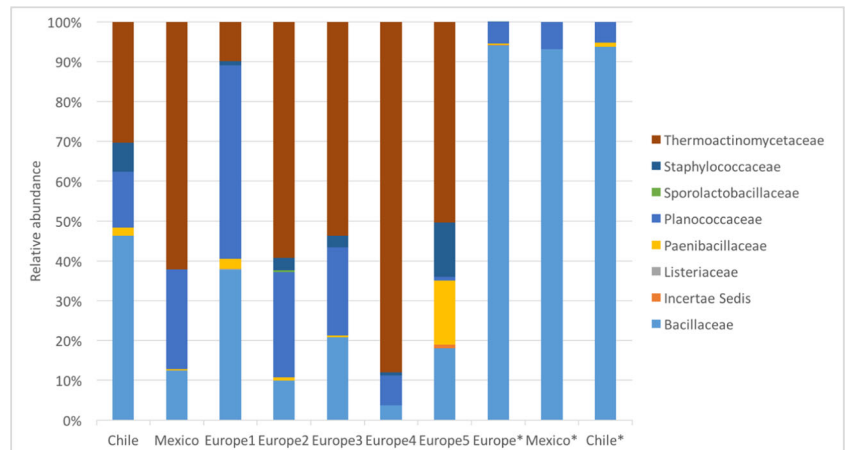
Fig. 5 Relative abundance of different genera from the family Paenibacillaceae observed in commercial bee pollen from three different regions, indicated along the bottom of the figure (four samples from Europe, indicated with number, and one sample from Mexico and Chile). Data from enrichment culture samples are indicated by an asterisk (*). 16S rRNA genes were amplified and sequenced as described in "Materials and Methods"
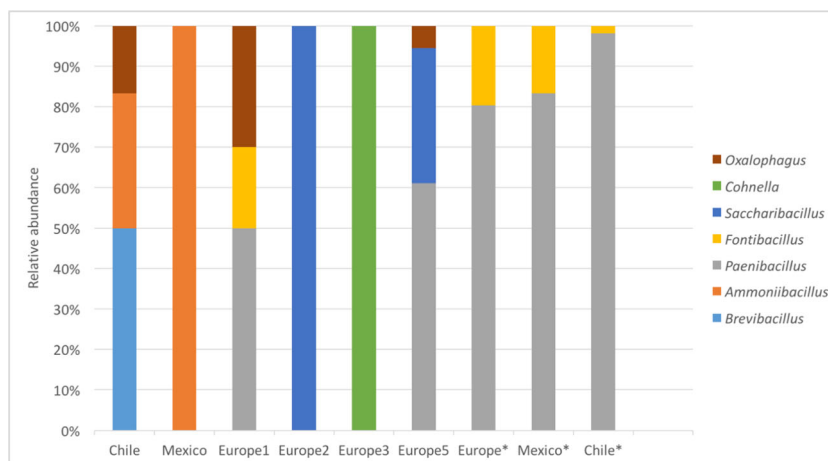
mellifera and some *Bombus* species consistently harbor a specific microbial species. Recent evidence shows that many endogenous bacteria have a mutualistic relationship with their insect host and play a key role in digestion, nutrient production, and pathogen protection [34–36]. It is apparent that additional study is necessary to characterize the microbiomes in
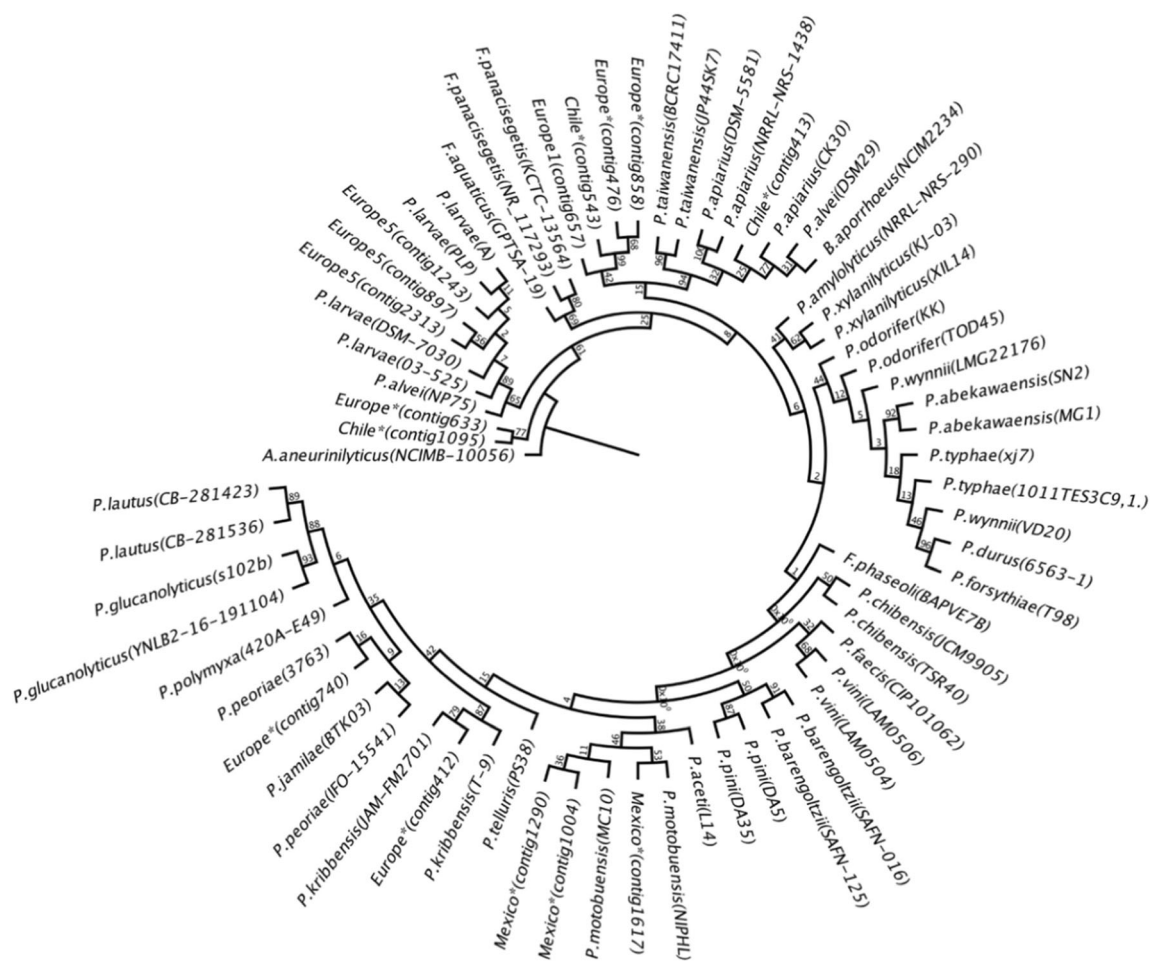

Fig. 6 Phylogenetic inference analysis of the three contigs related to *P. larvae*. Maximum likelihood inference was performed with the GTR nucleotide substitution model, estimated from an analysis of the 16S rRNA sequence alignment. Bootstrap support values from 100 replicates are shown at the nodes only for branches supported by more than 50%. The three contigs generated from the Europe5 sample in this study indicate a phylogenetic relationship with *P. larvae*. The tree was rooted to *A. aneurinilyticus*. The best nucleotide substitution model obtained was GTR. Maximum likelihood analysis (Akaike, −l nL = 3148.09696, Gamma shape parameter 0.304, freqA = 0.23493, freqC = 0.21230, freqG = 0.34438, freqT = 0.20839)

commercial bee pollen, with the specific goal of identifying pathogenic species to avoid their spread.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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