CHAPTER 4

Bacterial community structure in *Apis florea* analyzed by denaturing gradient gel electrophoresis and 16S rRNA gene sequencing

4.1 Introduction

The dwarf honeybee, *Apis florea*, is an indigenous honeybee species in tropical Asia, which spans the lowlands of Asia and the Middle East (Otis, 1991). *A. florea* builds a small single-comb nest which is apically attached to tree branches of smaller diameter (Rinderer et al., 1996). Because *A. florea* only stores a small amount of honey, it has drawn less attention from researchers. However, the most important contribution of this honey bee is its valuable pollination of many fruit plants and diversified flora in tropical ecosystems (Soman and Chawda, 1996). The current dramatic decrease in wild native honeybees, which are seriously threatened by unknown pathogens, calls for a comprehensive study of their resident bacterial community, including *A. florea*. Among the four stages of the honeybee life cycle, the larval stage is the target of many major pathogens, including bacteria (Seeley, 1995). Besides introduced microorganisms from outside sources, microbes are mainly transmitted between honeybees in two ways: vertically from the queen to the offspring and horizontally as a result of various social interactions that occur in bee colonies (Möckel et al., 2011). Bacteria are advantageous to many insects by providing nutrition and protecting hosts from pathogens (Dillon and Dillon, 2004; Engle and Moran, 2013). Metagenomic study of the gut microbiota of honeybees found three main functions: pathogen defense, immunity and nutrition, all of which affect honeybee health (Engel et al., 2013).

Previous work regarding to the microbial community in the honeybee has been concentrated on the European honeybee (*Apis mellifera*) and the cavity-nesting Asian honeybee (*Apis cerana*), using culture-dependent (Mohr and Tebbe, 2007; Yoshiyama and

Kimura, 2009) and culture-independent methods (Jeyaprakash et al., 2003; Cox-Foster et al., 2007; Martinson et al., 2011; Disayathanoowat et al., 2012; Moran et al., 2012; Ahn et al., 2012). Only a few studies have documented the bacterial community associated with the dwarf honeybee, including one study of lactic acid bacteria in the crop of *A. florea* (Vasquez et al., 2012)and another reporting on bacteria in the gut of a closely related species, *Apis andreniformis* (Martinson et al., 2011).

In this present study, we used a nested polymerase chain reaction coupled with denaturing gradient gel electrophoresis (PCR-DGGE) and 16S rRNA gene sequencing methods to examine the diversity and distribution of bacterial flora in *A. florea* larvae, followed by a comparison of the bacterial composition among three geographically different locations in Chiang Mai province, Thailand. Based on the assumption that geographic isolation can affect symbiotic bacteria, we hypothesized that *A. florea* larvae from all three sites would share common bacteria in their composition with certain variation between the sampled sites. Our findings provide important insights leading to a better understanding of the characteristics of the overall bacterial community structure that might be beneficial for *A. florea* health.

4.2 Materials and methods

4.2.1 Sample collection

A total of 120 larvae were collected for this study. Twenty larvae were collected randomly from each of six nests. Two nests were collected from the same location. The three locations included Saraphe (18°42′48″N, 99°2′11″E) for colony 1 and 2; Maetang (19°7′19″N, 98°56′37″E) for colony 3 and 4, and Maerim (18°54′50″N, 98°56′42″E) for colony 5 and 6. The three locations are districts in Chiang Mai province, Thailand. The distance between Saraphe and Maerim is ca. 35 km, and between Maerim and Maetang, the distance is ca. 24 km. The three sample sites were located in agricultural environments. Saraphe was a site where longan (*Dimocarpus longan* Lour.) orchards were maintained,

while Maetang and Maerim were surrounded by fields of rice (*Oryza sativa*). All collections were carried out in March 2011, and larvae were immediately stored at -20ºC for further analysis.

4.2.2 DNA extraction

Larvae were surface-sterilized with sodium hypochlorite in sterile plates as described by Inglis et al. (1998). Bacterial DNA of each individual larva was extracted according to a previous protocol (Sharma and Singh, 2005). Briefly, larvae were crushed in tubes containing 500 µl phosphate buffer saline (PBS) (137 mMNaCl, 2.7 mMKCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) and centrifuged at 10,000 g for 10 min. The supernatant was discarded. The sample was washed twice with 500 µl of Tris-EDTA-NaCl (TEN) (10 mMTrisHCl (pH 8.0), 1 mM EDTA, 1M NaCl) and centrifuged at 10,000 g for 1 min. Next, 200 µl of 20% (w/v) sucrose were added into a solution of TEN, followed by 100 µl of 10% (w/v) sodium dodecyl sulphate (SDS), 2 units of lysozyme, 10 units of RNAase and sterile glass beads. After incubation at 37ºC for 90 min, 75 µl of 5 M NaCl were added and mixed. The DNA was extracted inaqueous phase with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1). DNA was precipitated from the aqueous phase with isopropanol and recovered by centrifugation. The pellet was resuspended in 20 µl of TE buffer. DNA concentration was determined using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The bacterial DNA was stored at -20ºC.

4.2.3 PCR amplification

Amplification of the whole bacterial 16S rRNA gene, about 1,500 bp, was performed using universal primers (8F, 5′-GCG GAT CCG CGG CCG CTG CAG AGT TTG ATC CTG GCT CAG-3′; 1492R, 5′-GGC TCG AGC GGC CGC CCG GGT TAC CTT GTT ACG ACT T-3′ (Lane, 1991). Each reaction mixture (a total volume of 50 µl) contained 100 ng of total genomic DNA, 0.2 mM of each deoxynucleoside triphosphate

(dNTP), 50 pmol of primers, 1.5 mM $MgCl_2$, 5 μl of 10 x PCR buffer II, and 2.5 U of *Taq* DNA polymerase (Applied Biosystems, USA). PCR conditions were as follows: initial denaturation at 95ºC for 4 min and 30 cycles consisting of 1 min at 95ºC, 1 min at 55ºC, 2 min at 72ºC, and an additional cycle of 5 min at 72ºC for chain elongation. The PCR product was then used as a template in the second PCR. A set of primers, including 343F (5′-ACT CCT ACG GGA GGC AGC AG-3′) and 534R (5′-ATT ACC GCG GCT GCT GG-3′), were applied to target the V3 hypervariable region (190 bp) of the 16S rRNA gene locus (Nakatsu et al., 2000). Primer 343F at its 5′-end had 40-nucleotide GC-rich sequences (GC clamp). The PCR reaction was carried out with 10 μl of the first PCR product, 0.2 mM of each dNTP, 10 pmol of primers, 1.5 mM $MgCl_2$, 5 μl of 10 x PCR buffer II, and 2.5 U of *Taq* DNA polymerase. PCR conditions were 94ºC for 3 min, followed by 35 cycles of 1 min at 94ºC, 30 sec at 55ºC, 1 min at 72ºC, and then 10 min at 72ºC. The PCR products were evaluated by 1.5% agarose gel electrophoresis.

4.2.4 DGGE profile analysis

DGGE was performed by the Bio-Rad DCode System (Bio-Rad, USA). A 35% to 60% linear DNA denaturing gradient (100% denaturant is equivalent to 7 mol/L urea and 40% deionized formamide) was formed in 8% (w/v) polyacrylamide gels. PCR products were directly loaded into each lane, and electrophoresis was performed at a constant 55 V at 60ºC for 19 h in 1X Tris-acetate-EDTA buffer (TAE, pH 8.0). The gels were then stained for 15 min in water containing 0.5% μg/ml ethidium bromide, followed by 15 min of destaining. DGGE images were digitally captured and recorded by Alpha Image 4000 (Alpha Innotech Corporation, USA). All DGGE images were analyzed by using BioNumerics version 6.0 (Applied Maths, Belgium). Gels were first normalized according to the reference markers, and then the background was subtracted by use of mathematical algorithms according to the spectral analysis of overall densitometric curves. A minimal profiling setting (5.0%) was applied for the band search for all DGGE gels. DGGE profiles were determined by measuring the PCR amplicons (bands), migration distances, and the

intensities of the bands within each lane. Similarity matrices of pairwise comparison were evaluated using the number of different bands (optimization and tolerance both set to 0.5%). The results were digitally recorded and transferred into a microbial database that allowed us to cross-compare multiple DGGE profiles simultaneously. The final parameters used to analyze the banding patterns included the numbers of the detected band, the band frequency distribution, the Shannon diversity index (H′) for species richness, and similarity of the DGGE patterns among different colonies by using Sorenson's pairwise similarity coefficients (C_s) (Tanaka et al., 2006). Ward's algorithm was used to construct a dendrogram for cluster analysis. To differentiate between resident and transient bacteria by identified bands in each colony, resident bacteria were found in all six colonies, but transient bacteria were found in less than three colonies.

4.2.5 Cloning and 16S rRNA gene sequencing

Distinct bands identified from DGGE gels were excised with sterile blades immediately after staining. The DNA samples were eluted from the gels, purified and amplified with the same set of primers without the GC clamp. PCR products were cloned with a pCR4 TOPO (TOPOTA cloning kit, Invitrogen, USA) according to the manufacturer's instructions and transformed into *Escherichia coli* DH5α. The transformed cells were plated on Luria-Bertani agar plates supplemented with kanamycin (50 µg/ml) and X-gal (40 mg/ml), and the plates were incubated overnight at 37°C. At least ten white colonies per band were selected and screened for insertion by PCR with universal M13 primers. To confirm the correct bands, the vectors with insertion were determined by primer 343F with GC and primer 534R and then run on DGGE gel.PCR products with positive clones were purified using the QIAquick PCR purification kit (Qiagen, USA). Three positive clones per band were sequenced with the M13F primers by sending to Macrogen for sequencing (Macrogen, USA). The bacterial 16S rRNA gene sequences were subjected to NCBI BLAST-N (http://www.ncbi.nlm.nih.gov/blast/) and Ribosomal Database Project (RDP) II software (http://rdp.cme.msu.edu/) to search for closely related

sequences. The degree of sequence similarity among the tested sample sequences and other known organisms was reported as a percent of identity. The nucleotide sequences of the clones retrieved from excised DGGE bands have been deposited in GenBank with accession numbers KF264598 to KF264621.

4.2.6 Statistical analyses

The differences in bacterial diversity were assessed by comparing the DGGE profiles within and among both sample colonies and the three locations. The correlation between bacterial communities was evaluated using analysis of variance (ANOVA) and the least significant difference (LSD) test. The analyses were performed with IBM® SPSS® Statistics version 20.0 (IBM, Armonk, NY, USA).

4.3 Results

In the previous trial experiment, we would like to determine bacteria diversity in the midguts of *A. florea* from four developmental stages (larva, pupa, emerged bee, and worker). Six nests from three locations (Saraphe, Maetang and Maerim) were collected and total samples were 480 bees. After bacterial DNA extraction, DNA concentration was very low as a result of undetected bands in many samples. Eventually we replaced all water volume in the PCR reaction with DNA, the DNA concentration in many samples still less than 100 ng. Furthermore, the DNA volume was not sufficient to repeat or trial with other protocols. Another possible reason to explain unamplified bacteria is the low sensitivity of this protocol such as primers particularly for the second PCR. Overall, most samples from larva stage (120 samples) could be detected bacterial bands then we decided to continue the experiment only this stage.

4.3.1 Comparison of the DGGE profiles of 16S rRNA gene fragments

Based on 120 larvae collected from 6 colonies at 3 locations in northern Thailand, DGGE profiles were successfully obtained from a total of 103 larval samples. Cluster analysis of the DGGE profiles demonstrated that the profiles from each individual colony presented a high percentage of similarity, thus forming a single cluster (Figure4.1). Within the same location, however, one sampled colony was different from another, with the exception of colonies 5 and 6 from Maerim, which shared similar bacterial profiles. Therefore, these two colonies were clustered together (Figure4.1). Three colonies, Saraphe (colony 1) and Maetang (colony 3 and 4), were distinctively different from all other colonies (Figure4.1). Across all samples, pairwise comparison demonstrated an overall low similarity not only between two colonies at the same location but also among the different locations. The similarity index (C_s) of bacterial DNA profiles ranged from 4.9% to 24.5% (Table 4.1).

4.3.2 Comparison of dominant bacteria in *A. florea* larvae

Out of a total 97 distinct PCR amplicons detected in the DGGE profiles, we found several bands that presented high frequency in different colonies and locations, such as bands 16 and 17, 19 and 20. Altogether, 24 representative DGGE bands were selected, excised from the gels cloned, and sequenced for genus identification (Figure 4.3). The sequences were compared to the NCBI BLAST-N and RDP databases. We found that these bands shared more than 97% similarity with known 16S rRNA sequences in the databases (Table 4.3). The identified bands were associated with Alphaproteobacteria (35%), Bacilli (23%), uncultured bacteria (14%), Gammaproteobacteria (12%), Clostridia (8%), and Betaproteobacteria (8%), respectively. Although the 5 groups of bacteria and uncultured bacteria were detected in all locations, the distribution varied among the 6 colonies (Figure 4.4), as well as among the 3 different locations, in which approximately 3% -11% of bacteria were uncultured species (Figure4.5).

Within the phylum of Firmicutes, *Lactobacillus kunkeei* was the most abundant species. In the Alphaproteobacteria and Betaproteobacteria classes, *Saccharibacter* sp. and *Snodgrassella alvi* were highly detected, respectively (Table 4.3). Bands 12 and 15 were identified as transient bacteria which were randomly found among the 6 colonies. It was determined that these transient bacteria belonged to an uncultured bacterium clone and *Saccharibacter floricola*, a bacterium from the environment. Interestingly, the dominant bands were identified as *Snodgrassella alvi* and *Lactobacillus kunkeei*, respectively. Band 19 was closely related to the pathogenic bacterium *Melissococcus plutonius*, which is a causative agent of European foulbrood disease in the European honey bee (*A. mellifera*) larvae. *M. plutonius* (Firmicutes) was detected in all colonies, except colony 5 from Maerim. Band 20 was identified as belonging to *Saccharibacter* sp.

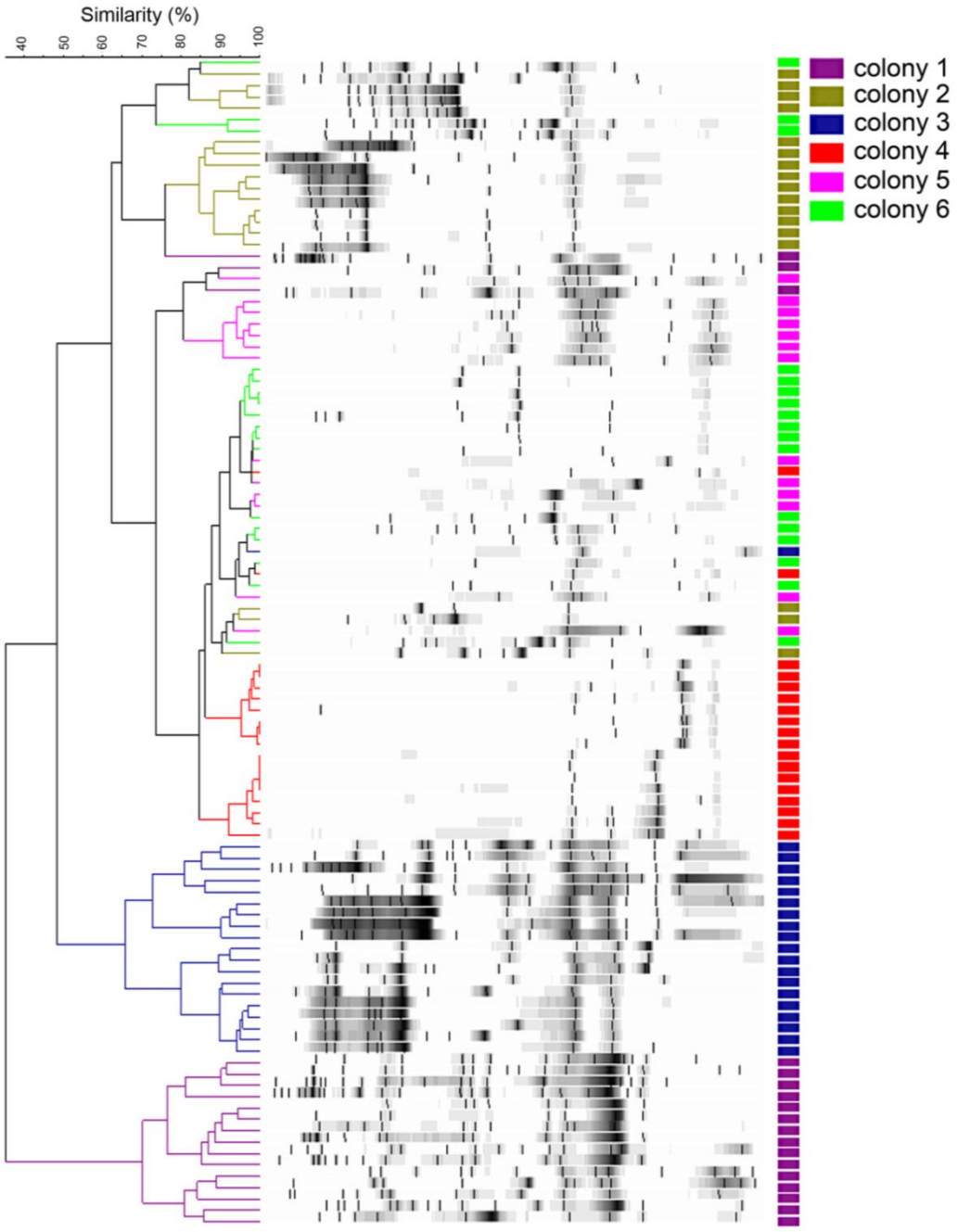

Figure 4.1 Cluster analysis of the bacterial DNA profiles obtained from all *A. florea* larvae samples. The color-coded blocks indicate colonies 1-6 from three locations. The scale shows percent of similarity among the bacterial bands.

Table 4.1 Comparison of the similarity index of the bacterial community in *A. florea* larvae collected from different locations.

colonies	Similarity Index (C_s) value (%)					
	colony1	colony 2	colony 3	colony 4	colony 5	colony 6
colony 1	-	-	-	-	-	-
colony 2	21.3	-	-	-	-	-
colony 3	22.5	19.9	-	-	-	-
colony 4	5.6	4.9	8.7	-	-	-
colony 5	13.0	8.2	10.9	14.0	-	-
colony 6	18.6	24.5	19.3	9.9	12.2	-

4.3.3 Comparison of the distribution and diversity of bacterial 16S rRNA gene fragments

Each band on the DGGE gel was assumed to correspond to a different bacterial phylotype or operational taxonomic unit (OTU) (Simpson et al., 1999). Figure 4.2 demonstrated the frequency distribution of each distinct bacterial 16S rRNA gene fragment detected on DGGE from the samples of the 6 colonies. It should be recalled that 2 colonies from each of 3 different locations were sampled, including Saraphe (colony 1 and 2), Maetang (colony 3 and 4) and Maerim (colony 5 and 6). Overall, the number of detected DGGE bands ranged from 2.6 to 13.3 with a mean ± SD of 7.3 ± 4.8 for the 6 colonies, which was significantly different among the 3 locations (Table 4.2). A significant difference in the number of DGGE bands was also found between colonies 1 and 2 from Saraphe and between colonies 3 and 4 from Maetang, but not between colonies 5 and 6 from Maerim. The number of DGGE bands in Saraphe was 2 to 3 fold higher than that in Maerim. In addition, we found that the diversity of the bacterial community, as measured by the Shannon index, was statistically significant between colonies in the same location, as well as among the three different locations (Table 4.2). Based on the mean number of DGGE bands, the 2 colonies from Maetang and Maerim were similar, but colonies from

Saraphe were significantly different from colonies from both Maetang and Maerim with respect to the mean number of DGGE bands (Table 4.2).

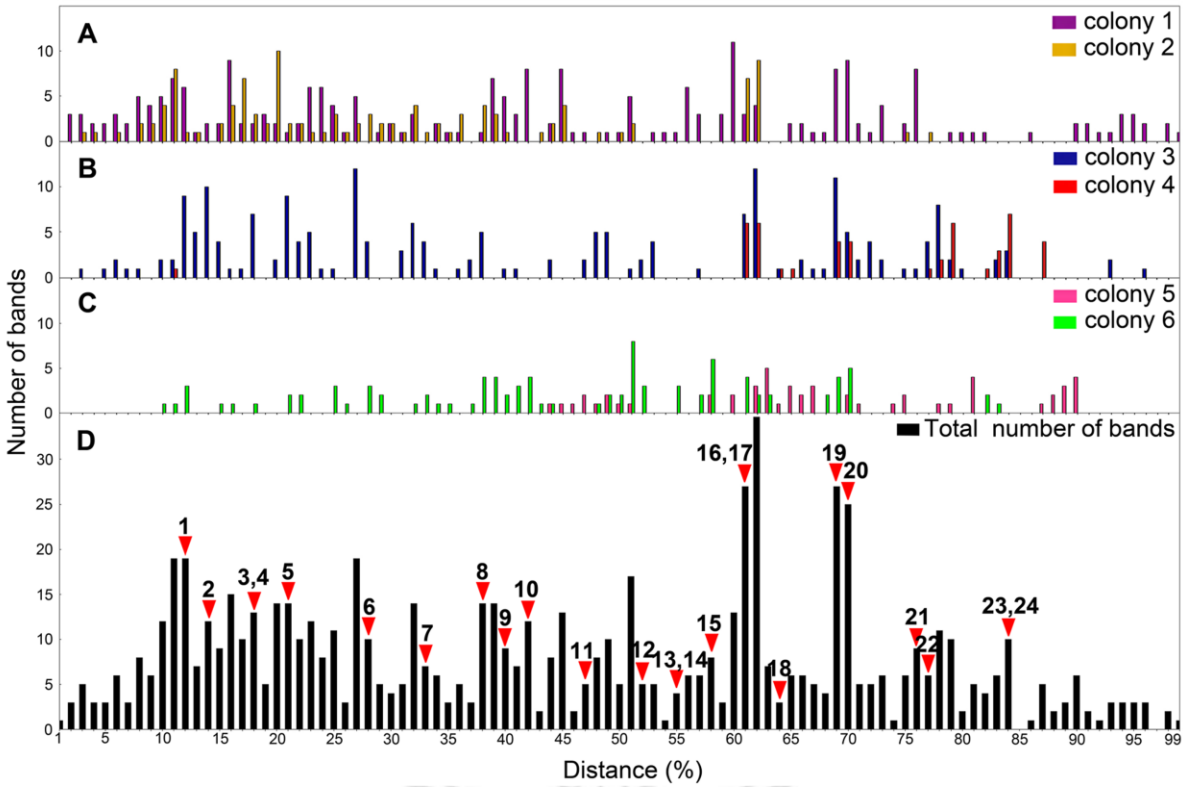

Figure 4.2 Distribution of the amplicons derived from 16S rRNA genes generated by DGGE from the *A. florea* larvae collected from six different colonies. Each bar represents a DGGE band detected in the gel at different migration distances. The arrows represent the bands that were excised and sequenced.

Table 4.2 Comparison of the DGGE profiles of *A. florea* larvae collected from different locations

Location	Colony	No. of DGGE bands (mean ± SD)	Shannon index (mean ± SD)	No. of DGGE bands (mean ± SD)	Shannon index (mean ± SD)
Saraphe	1	13.3 ± 3.6^{a}	2.5 ± 0.3^{a}	10.2 ± 4.4^{a}	2.2 ± 0.5^{a}
N = 35	2	6.8 ± 2.3^{b}	1.9 ± 0.4^{b}		
Maetang	3	10.3 ± 3.5^{a}	2.2 ± 0.5^{a}	6.6 ± 4.7^{b}	1.6 ± 0.8^{b}
N = 38	4	2.6 ± 1.1^{b}	0.9 ± 0.4^{b}		
Maerim	5	4.1 ± 2.1^{ab}	1.2 ± 0.7^{ab}	4.9 ± 3.6^{b}	1.3 ± 0.8^{b}
N = 30	6	5.6 ± 4.3^{ab}	1.4 ± 0.8^{ab}		

For each colony of *A. florea* larvae, mean for the number of DGGE bands and Shannon index followed by different letters are significantly different at $P<0.001$.

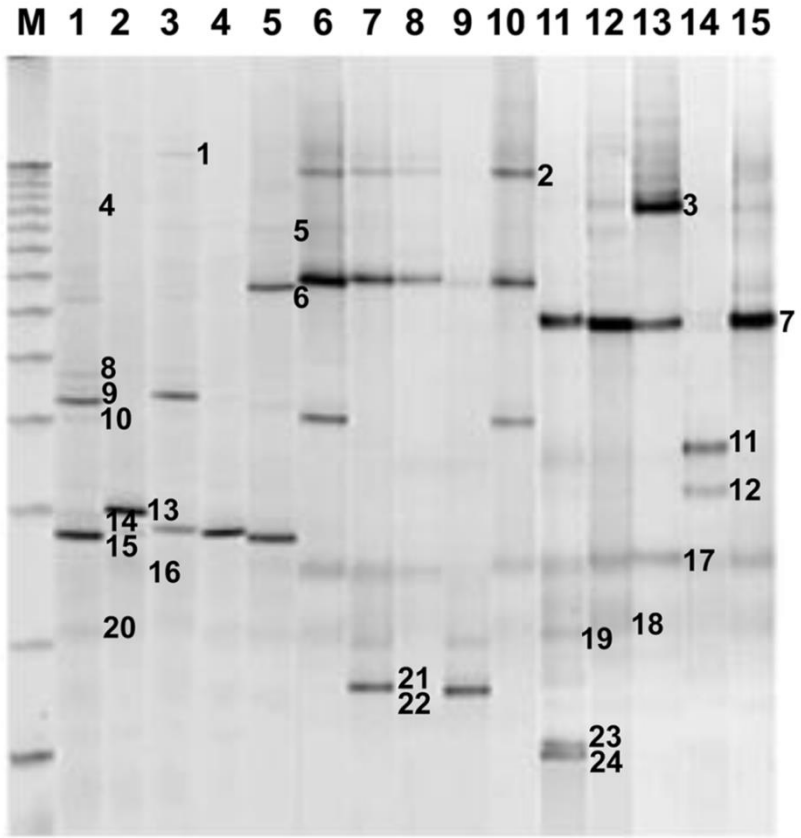

Figure 4.3 Representative DGGE amplicons of 16S rRNA genes from the *A. florea* larvae. A total of 24 DGGE bands were excised for sequencing analysis. The numbers correspond to the sequenced bands listed in Table 3. Lane M DGGE marker; lanes 1-5 are larvae collected from Maerim, and lanes 6-15 are larvae collected from Maetang.

Table 4.3 Closest matches to excised and sequenced 16S rRNA gene-derived DGGE bands isolated from the *A. flo*rea larvae.

DGGE band number	GenBank accession number	16S rRNA sequence phylogenetic classification			Closest Closest relatives	Blast match accession number	BLAST identity (%)	Source
		Phylum	Class	Genus				
1	KF264598	Proteobacteria	Gammaproteobacteria	-	Uncultured gammaproteobacterium clone I10.060Gp	JQ389953	100	corbiculate bee gut
2	KF264599	Firmicutes	Bacilli	Lactobacillus	*Lactobacillus kunkeei* strain 80-30	JQ009344	100	fresh flower, bee hive, bee
3	KF264600	Firmicutes	Bacilli	Lactobacillus	*Lactobacillus kunkeei* strain 80-30	JQ009344	100	fresh flower, bee hive, bee
4	KF264601	Proteobacteria	Alphaproteobacteria	Saccharibacter	*Saccharibacter* sp. Am 22	AJ971906	100	honey bee gut
5	KF264602	Proteobacteria	Gammaproteobacteria	-	Uncultured gammaproteobacterium clone U08.605Gp	JQ389964	100	corbiculate bee gut
6	KF264603	Proteobacteria	Gammaproteobacteria	Gilliamella	*Gilliamella apicola* wkB1	JQ936674	99	honey bee, bumble bee gut
7	KF264604	Firmicutes	Bacilli	Melissococcus	*Melissococcus plutonius* ATCC 35311	NR_074098	99	European foulbrood of the honey bee
8	KF264605	Proteobacteria	Alphaproteobacteria	Saccharibacter	*Saccharibacter* sp. Am 22	AJ971906	100	honey bee gut
9	KF264606	-	-	-	Uncultured bacterium clone WIR31Fastg4_13465	JQ377178	98	environment
		Firmicutes	Clostridia	Clostridium	Uncultured *Clostridium* sp. clone SL29	HQ264089	98	polyphagousledidopteran larva gut
		Firmicutes	Clostridia	-	Uncultured Eubacteriaceae bacterium	AB089002	97	termite gut
10	KF264607	-	-	-	Uncultured bacterium clone WIR31Fastg4_13465	JQ377178	98	environment
		Firmicutes	Clostridia	Clostridium	Uncultured *Clostridium* sp. clone SL29	HQ264089	98	polyphagousledidopteran larva gut
		Firmicutes	Clostridia	-	Uncultured Eubacteriaceae bacterium	AB088975	97	gut of termite

Table 4.3 Closest matches to excised and sequenced 16S rRNA gene-derived DGGE bands isolated from the *A. flo*rea larvae (Cont.).

DGGE band number	GenBank accession number	16S rRNA sequence phylogenetic classification			Closest Closest relatives	Blast match accession number	BLAST identity (%)	Source
		Phylum	Class	Genus				
11	KF264608	-	-	-	Uncultured bacterium clone ncd2583f03c1	JF226657	98	environment
12	KF264609	-	-	-	Uncultured bacterium clone B18-44-B02	JQ088444	100	environment
13	KF264610	Proteobacteria	Betaproteobacteria	Snodgrassella	*Snodgrassella alvi* clone SHOA733	HM112111	100	honey bee, bumble bee
14	KF264611	Proteobacteria	Alphaproteobacteria	-	Uncultured Acetobacteraceae bacterium clone B10.043D3	JQ389885	100	corbiculate bee gut
15	KF264612	Proteobacteria	Alphaproteobacteria	Saccharibacter	*Saccharibacter floricola* strain S-877	NR_024819	100	pollen
16	KF264613	Proteobacteria	Betaproteobacteria	Snodgrassella	*Snodgrassella alvi* clone SHOA733	HM112111	100	honey bee, bumble bee
17	KF264614	Firmicutes	Bacilli	Lactobacillus	*Lactobacillus kunkeei* strain 80-30	JQ009344	100	fresh flower, bee hive, bee
18	KF264615	Firmicutes	Bacilli	Lactobacillus	*Lactobacillus kunkeei* strain 80-30	JQ009344	100	fresh flower, bee hive, bee
19	KF264616	Firmicutes	Bacilli	Melissococcus	*Melissococcus plutonius* ATCC 35311	NR_074098	99	European foulbrood of the honey bee
20	KF264617	Proteobacteria	Alphaproteobacteria	Saccharibacter	*Saccharibacter* sp. Am 22	AJ971906	100	honey bee gut
21	KF264618	Proteobacteria	Alphaproteobacteria	-	Uncultured Acetobacteraceae bacterium clone B10.043D3	JQ389885	100	corbiculate bee gut
22	KF264619	Proteobacteria	Alphaproteobacteria	-	Uncultured Acetobacteraceae bacterium clone B10.043D3	JQ389885	100	corbiculate bee gut
23	KF264620	Proteobacteria	Alphaproteobacteria	Saccharibacter	*Saccharibacter* sp. Am 22	AJ971906	100	honey bee gut
24	KF264621	Proteobacteria	Alphaproteobacteria	-	Uncultured Acetobacteraceae bacterium clone B10.043D3	JQ389885	100	corbiculate bee gut

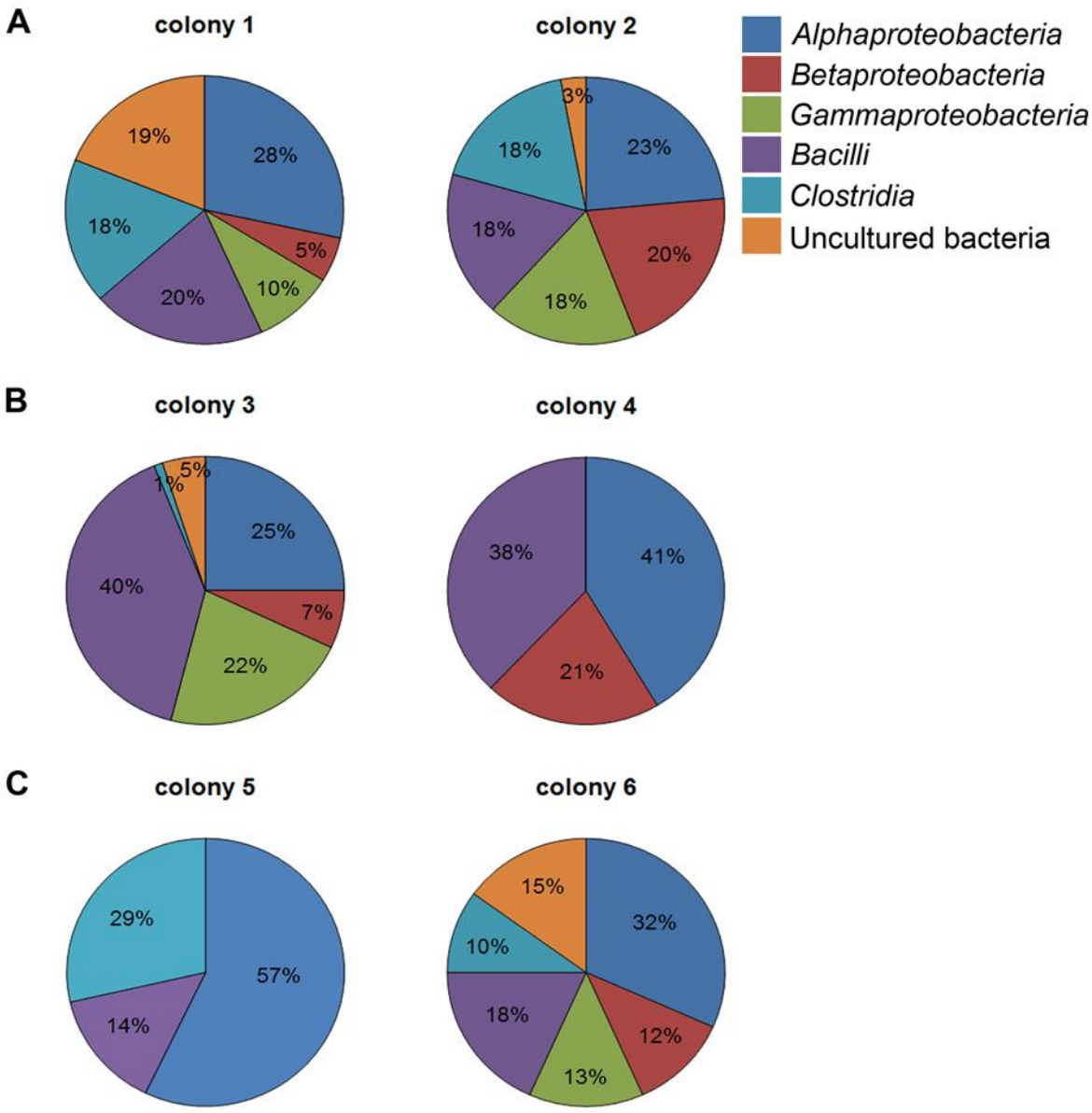

Figure 4.4 Proportion of bacterial groups from six colonies of *A. florea* larvae collected from Saraphe (A), Maetang (B), and Maerim (C).

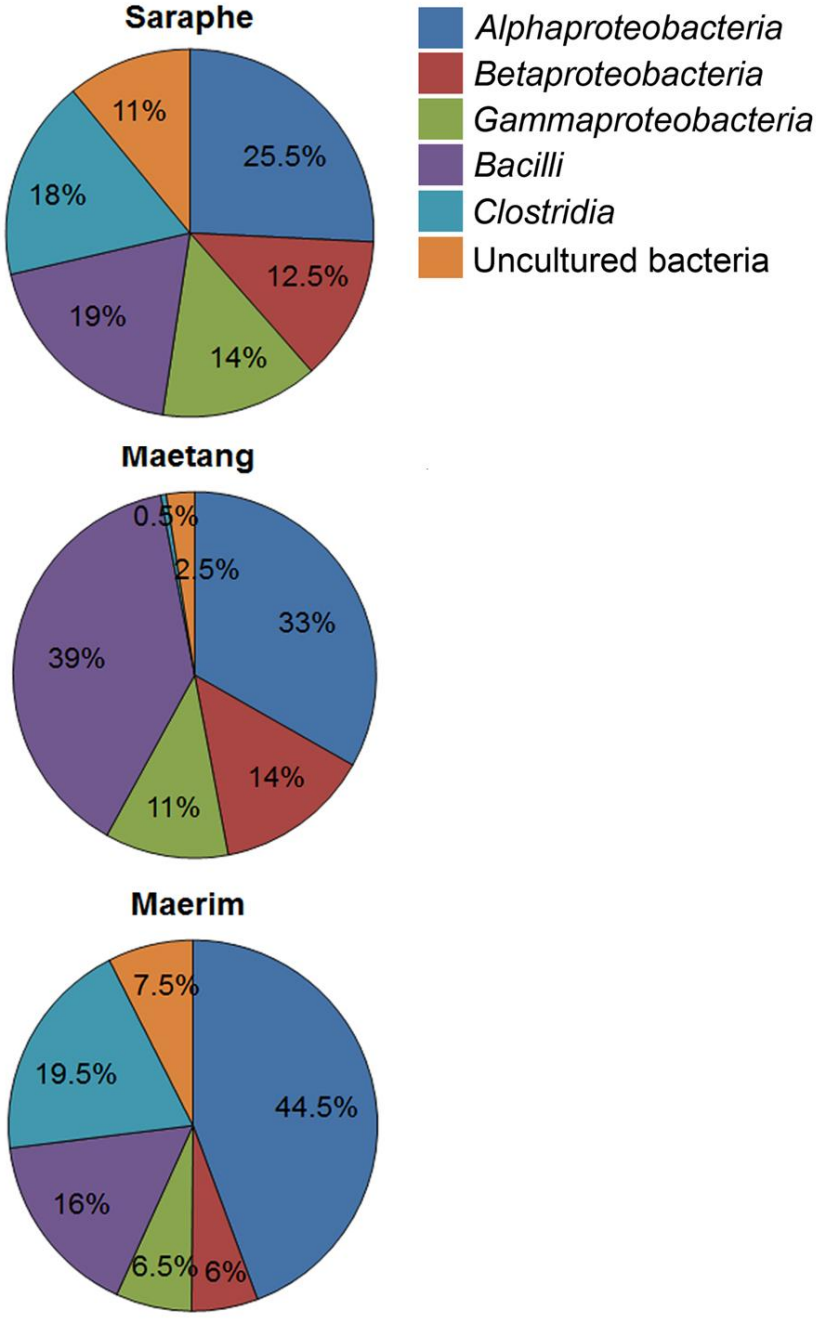

Figure 4.5 Average proportion of five groups of bacteria from three locations.

4.4 Discussion

This study is the first report of the bacterial community structure in *A. florea* larvae. In this study, nested PCR DGGE and 16S rRNA gene sequence analysis were employed to compare bacterial diversity in *A. florea* larvae from 3 locations in northern Thailand. Our results demonstrate that *A. florea* larvae harbor 2 phyla, (Firmicutes and Proteobacteria), 5 dominant classes of bacteria (Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Bacilli, and Clostridia) and an uncultured bacterium clone. Transient bacteria from the environment, including the uncultured bacterium clone and *Saccharibacter floricola*, were commonly found. Although different groups and numbers of bacterial species were detected from different geographic locations, individual colonies showed high levels of similarity based on the bacterial DNA profiles. The dissimilarity in bacterial diversity may have resulted from factors within each colony, as well as unique environmental factors affecting the different geographical locations of the 6 honeybee colonies studied. This study is the first report on bacteria species closely related to *M. plutonius* in *A. florea* larvae.

Although bacterial community profiles of individual *A. florea* larva were heterogeneous, we found that each colony displayed high similarity. Likewise, it has been reported that *A. mellifera* bees worldwide maintain bacteria communities in their hindguts, irrespective of environmental, geographical and subspecies differences (Martinson et al., 2011). The minor variations of bacterial profile in the same colony could be a reflection of age or short-term differences in the physiology or health status of individual bees (Moran et al., 2012). The presence of transient microbes may have been the result of environmental transmission (horizontal transmission). Transient bacteria are present only a short time in insect gut and therefore cannot provide evidence of coevolution with hosts (Sachs et al., 2011). The variation of transient bacteria from previous studies indicated that several ecological factors, such as geographical location, food sources and seasons, have been found to influence the bacterial populations in insect midguts (Haynes et al., 2003; Vasanthakuma et al., 2006), although some core species of bacteria remain present.

In previous studies with the European honey bee, *A. mellifera* larvae in early development were found to contain no, or few, bacteria, with the number of bacteria rising with increasing larval age (Martinson et al., 2012; Vojvodic et al., 2013). We collected random larval samples representing the average of larval age. The results showed that *A. florea* larvae have a less complex bacterial community than other insect larvae, such as grass grub larvae (*Costelytra zealandica*) (Zhang and Jackson, 2008), butterfly larvae (*Pieris rapae*) (Robinson et al., 2010)and black chafer larvae (*Holotrichia parallela*) (Huang and Zhang, 2013), reflecting the limited bacterial transmission in honey bees. *A. florea* larvae from Maetang and Maerim showed no significant difference in either the number of bacterial profiles or Shannon index, perhaps because these bees share a similar environmental niche. However, the number of bacteria profiles and Shannon index were both higher in Saraphe, suggesting a more complex bacterial community in this area, which was distinctively separated from Maetang and Maerim. More specifically, Saraphe was a site where longan (*Dimocarpus longan* Lour.) orchards were maintained, while Maetang and Maerim were surrounded by fields of rice (*Oryza sativa*). These findings strongly suggest that different geographical locations could, by the corresponding differences in environmental factors, directly affect bacteria community composition in *A. florea* larvae. Bacterial diversity and the groups of bacteria in *A. florea* larvae in our study were similar to previous reports in *A. mellifera*, *A. cerana*, and Africanized honey bee larvae (Disayathanoowat et al., 2012; Moran et al., 2012; Ahn et al., 2012; Vojvodic et al., 2013), as well as a related species, *A. andreniformis* workers. A report on *A. florea* crops found lactic acid bacteria belonging to the phylum Firmicutes (Vasquez et al., 2012). A high frequency of four bacteria was reported in *A. mellifera* larval guts: *Lactobacillus* sp., Acetobacteraceae, *Snodgrassella* sp. (previous name *Simonsiella* sp.) and Bifidobacterium (Anderson et al., 2011). Our study could detect the first 3 species. Recently, a culture-dependent study of Africanized and European honey bee larvae presented groups of bacteria which were also found in the adult honey bee, such as *Lactobacillus* sp. and Acetobacteraceae (Vojvodic et al., 2013). Transient bacteria in *A. mellifera* larval guts (*Bacillus* sp., *Serratia* sp. and *Leuconostoc* sp.) were different from our results which were

based on colonies in different geographical locations and, hence, affected by different environmental factors, such as diet, and seasons. Furthermore, a high frequency of Pasteurellaceae (Ahn et al., 2012) has been reported for *A. cerana* which was not detected in *A. florea* larvae.

A notable observation in our study was the apparent abundance of putative *Lactobacillus kunkeei* of the phylum Firmicutes, which has been isolated from flowers, bee hives, fresh honey and guts of 9 species of *Apis* in South Africa, Malaysia and Sweden (Olofsson and Vasquez, 2008; Vasquez et al., 2012; Neveling et al., 2012). *L. kunkeei* is a lactic acid bacterium (LAB). This clade of Gram-positive, acid-tolerant bacteria is well recognized as a beneficial host-associated member of the microbiota community of humans, animals and insects (Hammes and Hert el, 2006). *L. kunkeei* has antimicrobial potency against such honey bee pathogens as *M. plutonius* (Vasquez et al., 2012). It has been reported that an *L. kunkeei*-type strain (YH-15), which was originally isolated from wine production, inhibited alcoholic fermentation by *Saccharomyces bayanus* and *Saccharomyces cerevisiae* (Huang et al., 1996) and possessed spoilage-related effects on honey (Olofsson and Vasquez, 2008).

Our study revealed another member of the phylum Firmicutes, class Clostridia, in the guts of *A. florea* larvae. Clostridia were recently reported in halictid bees, thus deserving further study for host relationship (McFrederick et al., 2014). Furthermore, *M. plutonius*, which showed high frequency in *A. florea* larvae, is known to cause European foulbrood (EFB). This is a widespread and economically important disease in many honey-producing countries throughout the world (Matheson, 1993). *M. plutonius* was isolated from *A. mellifera*, *A. cerana* (Bailey, 1974) and the giant honey bee, *A. laboriosa* (Allen et al., 1990). Generally, *M. plutonius* is not ubiquitous in healthy honey bee larvae; however, this bacterium can be present in larvae without symptoms of disease (Bailey, 1982) and persist during every developmental stage (Forsgren et al., 2005). In addition, *A. mellifera* infected with EFB represented multiple bacterial species, including the common secondary

invader *Paenibacillus alvei* (Forsgren, 2010). However, we did not find any *P. alvei* in the *A. florea* samples. To the best of our knowledge, the present study is the first to report that these bacteria, which are closely related to *M. plutonius*, were found in *A. florea* larvae sampled from healthy active colonies. Further studies to confirm its prevalence should be carried out.

The resident bacteria detected in *A. florea* larvae consisted of Alphaproteobacteria, such as *Saccharibacter* sp., and an uncultured Acetobacteraceae bacterium clone. Acetic acid bacteria have previously been found to be common in the guts of insects relying on a sugar-based diet, including honey bees, fruit flies, and mealy bug (Crotti et al., 2010), as well as in flowers (Jojima et al., 2004). Taken together, the findings suggest the possibility of environmental transmission to foraging bees from food sources (Koch et al., 2013). *Saccharibacter* sp. has been identified from honey bee(Mohr and Tebbe, 2007) and solitary bee guts (Martinson et al., 2011), particularly *Saccharibacter floricola*, an osmophilic bacterium first isolated from pollen (Jojima et al., 2004). Acetic acid bacteria have been suggested to play a large role in the bee gut community (Crotti et al., 2010).

Among the frequently detected bands in *A. florea* larvae, we detected bands frequently associated with Betaproteobacteria. In particular, a novel clade, *Snodgrassella alvi*, has been found in the gut of several species of the *Apis* and *Bombus*, as well as other corbiculate bees, along with *Gilliamella apicola* (Gammaproteobacteria) (Moran et al., 2012; Martinson et al., 2012; Kwong and Moran, 2013). Both *S. alvi* and *G. apicola* were present in every individual *A. mellifera* collected from different colonies and sites, indicating that these species were the core bacteria in *A. mellifera* guts (Moran et al., 2012), as well as in the *A. florea* larvae examined in this study. Metagenomic analysis of bacterial genes revealed that these 2 species encoded a relatively high number of functions related to biofilm formation and host interaction, such as pili, outer membrane proteins and secretion (Engel et al., 2012). *G. apicola* also harbors pectin degradation activity, which is involved in food processing (Engel et al., 2012). Strains corresponding to *Snodgrassella* and

Gilliamella were found in bumblebees in which they were linked to protection against protozoan parasites (Koch and Schmid-Hempel, 2011). These bacteria are acquired by vertical transmission from the mother colony to young bees. However, *Gilliamella* may be transmitted horizontally outside of the colony more often than *Snodgrassella* (Koch et al., 2013). The distribution of *G. apicola* infection depends on environmental transmission by geographical distance (Koch et al., 2013). The presence of these bacteria might benefit the honey bee by increasing their resistance to Nosema disease, which is less prevalent in *A. florea* (Chaimanee et al., 2010).

In summary, the use of DGGE and 16S rRNA gene sequencing was adequate to investigate and compare microbial communities from *A. florea* larvae. Although bacterial community profiles were similar in individual colonies, differences were observed in replicate colonies from the same location and different locations. Two phyla, (Firmicutes and Proteobacteria), 5 dominant classes of bacteria (Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Bacilli, and Clostridia), and an uncultured bacterium clone were identified, as well as closely related *M. plutonius*. Bacterial DNA profiles by the DGGE method revealed the most dominant phylotypes, indicating the suitability of this method for studying overall microbial diversity in the investigated environmental samples, and it has also proved useful for exploring bacterial communities in arthropods. By understanding the microbiome of wild honey bees, we can distinguish transient from resident bacterial populations and gain a better understanding of the interaction between bacteria and host under various environmental conditions. The findings of the present study should be applicable for future program development to protect native honey bees from the transmission of pathogens and, hence, ensure sustainable pollination service in the ecosystem.