CHAPTER 3

Midgut bacterial communities in the giant Asian honey bee (*Apis dorsata*) across four developmental stages: a comparative study

3.1 Introduction

Recent evidence of a worldwide dramatic decrease in both wild and domestic honeybees has emerged (Potts et al., 2010). Definitive causes of the losses have not yet been clearly identified; however, researchers have begun to look more closely at the indigenous bacterial communities within the digestive tract of honeybees (Moran et al., 2012; Engel et al., 2012). In previous studies, bacteria in the gut of honeybees have been studied using culture-dependent and culture-independent methods in two types of honeybee species: *Apis mellifera* and *Apis cerana*. Surveys of *A. mellifera* gut collected from several regions have shown at least eight distinct core bacterial phylotypes (Martinson et al., 2011; Ahn et al., 2012; Disayathanoowat et al., 2012; Moran et al., 2012). These core bacteria are suggested to be beneficial microbes in domestic honeybees (Engel and Moran 2013). *Alphaproteobacteria, Betaproteobacteria* (e.g., *Snodgrassellaalvi*), and *Gammaproteobacteria* (e.g., *Gilliamellaapicola*) were reported to be the core bacteria in *A. dorsata* gut (Martinson et al., 2011; Koch et al., 2013).

However, little is known about the dynamics of bacterial community structure in the gut during the developmental stages of the giant honeybee *A. dorsata*. This species is found throughout the Indian subcontinent, Southeast Asia, and numerous southwestern Pacific archipelagos (Ruttner, 1988). *A. dorsata* is an important part of the ecology of rainforests because of its high capacity to pollinate diverse plant species. Furthermore, it provides local populations with income from honey and wax harvesting. The present study aimed to determine and compare the diversity of normal bacterial flora in the midgut of *A. dorsata*

from different nest sites at four different developmental stages by using polymerase chain reaction coupled with denaturing gradient gel electrophoresis (PCR-DGGE) and 16S rRNA gene sequencing. Focusing on midgut, the principal site of digestion and absorption, as well as a target for pathogenic infection, our study examined bacterial community structure to determine any differences with respect to developmental stage and nesting site of this *Apis* species.

3.2 Materials and methods

3.2.1 Sample collection

A total of 720 bees were collected for this study. Thirty bees at each stage of the life cycle, including larva, pupa and worker (free-flying bee), were collected directly from six colonies at two locations in the districts of Maerim (18°54′50′N, 98°56′42′E) and Saraphe (18°42′48′N, 99°2′11′E) in Chiang Mai, Thailand. We designated colony 1, 2 and 3 for Maerim and colony 4, 5 and 6 for Saraphe. The two sampling sites were approximately 23 km apart. Sample sites in Maerim were located in the deep northern forest where bees were foraging on wild flowers. Sample sites in Saraphe were located in a populated lowland municipality. The bees collected from Saraphe were maintained on Longan (*Dimocarpus longan* Lour.) orchards. Incubated at 30ºC for 12 h in the dark with humidity, newly emerged bees hatched from late-stage pupae of sealed brood cells were collected the same day. All collections were carried out in March of 2011, and samples were stored at -20ºC for further analysis.

3.2.2 Dissection of honeybee midgut

Individual bees were surface-sterilized with 70% ethanol, 5% sodium hypochlorite, and sterile water for 1 min each in sterile plates. The whole larvae were ground by sterile pestle. Midguts of pupae, emerged bees, and workers were dissected by separating the abdomen from the thorax, cutting open the abdomen with a micro scissor and a sterile blade along both sides, removing the ventral cuticula and transferring the individual midgut to a

sterile microcentrifuge tube. The instruments used in the dissection process were flame-sterilized between each individual. All steps were performed under aseptic conditions. The midgut samples were kept at -20^{o}C.

3.2.3 DNA extraction

Total bacterial DNA was extracted from the midgut of individual bees according to the modified protocol described by Disayathanoowat et al. (2012). Briefly, single guts were thawed and mixed with 500 µl phosphate buffer saline (PBS) (137mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4, 2 mmol/L KH_2PO_4). The tubes were centrifuged at 5,000 g for 10 min, and the supernatant was discarded. The sample was washed twice with 500 µl of Tris-EDTA-NaCl (TEN) (10 mmol/L TrisHCl (pH 8.0), 1 mmol/L EDTA, 1 mol/LNaCl) and centrifuged at 5,000 g for 1 min. After discarding the supernatant, 200 µl of 20% (w/v) sucrose in TEN were added, followed by 100 µl of 10% (w/v) SDS, 2 units of lysozyme, 10 units of RNAase and sterile glass beads. The samples were mixed vigorously by vortexing for 1 min and incubated at 37^{o}C for 90 min. Then, 75 µl of 5 mol/LNaCl were added and mixed. The DNA was extracted into aqueous 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 (pH 8.0). DNA concentration was determined using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The bacterial DNA was stored at -20^{o}C until use.

3.2.4 PCR amplification

Fragments of 16S rRNA (190 bp) corresponding to the V3 hypervariable region of the 16S rRNA gene were amplified via PCR using the universal bacterial primers: 343F with 40-nucleotide GC-rich sequences at the 5’ end (5’-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCC CGG GGG ACT CCT ACG GGA GGC AGC AG-3’) and 534R (5’-ATT ACC GCG GCT GCT GG-3’), as described by Nakatsu et al.(2000). The 50

μl reaction mixture contained 100 ng of the total genomic DNA, 0.2 mM of each deoxynucleoside triphosphate, 10 pmol of primers 343F with GC clamp and 534R, 1.5 mM $MgCl_2$, 5 μl of 10 x PCR buffer II, and 2.5 U of *Taq*DNA polymerase (Applied Biosystems, New Jersey, USA).PCR conditions were as follows: initial denaturation at 94ºC for 3 min, and 35 cycles consisting of 1 min at 94ºC, 30 sec at 55ºC, 1 min at 72ºC, and an additional cycle of 10 minutes at 72ºC for chain elongation. Negative controls without DNA were included in all amplifications. The PCR products were evaluated by using 1.5% agarose gel electrophoresis and ethidium bromide staining. PCR amplicons were stored at -20ºC until DGGE analysis.

3.2.5 Denaturing gradient gel electrophoresis (DGGE)

DGGE was carried out as described by Li et al. (2007) using a Bio-Rad DCode System (Bio-Rad, California, USA). PCR products (15 μl) were applied in 8% (w/v) polyacrylamide gels in 1X Tris-acetate-EDTA (TAE), pH 8.0, buffer containing a denaturing gradient of urea and formamide varying from 35% to 60% (100% denaturant is equivalent to 7 mol/L urea and 40% deionized formamide). The gels were run for 19 h at 60ºC and 55 V. After electrophoresis, the gels were stained for 15 min with 0.5% μg/ml ethidium bromide, followed by 15 min of destaining. DGGE profile images were digitally captured and recorded by UV Transilluminator (Alpha Innotech Corporation, California, USA).

3.2.6 Cloning and 16S rRNA gene sequencing

A total of 41 dominant and unique bands were identified across all DGGE gels. The bands were excised and placed into a microcentrifuge tube containing 150 μl of deionized water and kept overnight at 4ºC. The elution solution (5 μl) was used for PCR reamplification with the same set of primers, 343F/534R, without the clamp. PCR reaction with expected size (190 bp) was cloned into a pCR4 TOPO (TOPOTA cloning kit; Invitrogen, Carlsbad, California, USA). Vectors with insertion were 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). The plates were then incubated overnight at 37ºC. From each transformation culture, ten white colonies were selected and grown overnight at 37ºC with shaking. Clones containing DNA inserts were screened by universal M13 primers. PCR products were evaluated by electrophoresis on 1% agarose gels, and positive clones were purified using the QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany). At least three positive clones per band were sequenced with the M13F primers in accordance with conventional sequencing by the chain termination method (Macrogen, Rockville, Maryland, USA). All sequences were checked for chimeric artifacts using the check-chimera program of the Ribosomal Database Project (RDP), and partial bacterial 16S rRNA gene sequences were initially aligned with the CLUSTAL W program. All unique sequences were directly compared with sequences available in the Ribosomal Database Project II (RDP II) database. The degree of sequence similarity between the tested sample sequences and other known organisms was reported as a percent of similarity. All DGGE sequence data in this study have been submitted to EMBL under accession numbers HG518581 to HG518621.

3.2.7 DGGE profiles and statistical analyses

All DGGE profiles were analyzed by using BioNumerics version 6.5 (Applied Maths, Sint-Martens-Laterm, Belgium). The profiles were normalized. Minimum profiling and grey zone setting were adjusted to 5% and used uniformly across samples. DGGE profiles with a reference pattern included in all gels were determined by measuring the migration distances and the intensities of the bands within each lane. Each band was considered as at least one operational taxonomic unit (OTU). Bacterial DNA profiles of larva, pupa and emerged bee stages were evaluated using Dice coefficient algorithm based on the means of arithmetic averages (UPGMA) for the hierarchical clustering of pairwise distances. Similarity between bacterial communities of workers was analyzed using similarity analysis based on Pearson curve rank correlation, as followed by Ward. The final parameters used to analyze the banding patterns included the numbers of the detected band,

the band frequency distribution, and the Shannon index (H′) for species richness, i.e., the number of different distinct bands in any individual sample, and evenness. The differences in bacterial diversity were assessed by comparing the DGGE profiles within and among sampled colonies and within and between the two different locations from each stage. The Shannon index, which represents the measure of richness and evenness for all colonies, was calculated by EcoSim software (EcoSim, Colorado, USA). Heat map and principal component analysis (PCA) were generated from DGGE bands by MultiExperiment Viewer version 4.8 (MeV, Massachusetts, USA). Variation among and between microbial groups found in bee gut, i.e., degree of microbial diversity, was evaluated using analysis of variance (ANOVA), the least significant difference (LSD) test and Tukey HSD. The statistical analyses were performed with IBM® SPSS® Statistics version 21.0 (IBM, Armonk, USA).

3.3 Results

Overall, bacterial community structure significantly differed among the developmental stages, six colonies, and two locations (Table 3.1). Among the four developmental stages, the midgut of worker bees and newly emerged bees showed greater variances in bacterial community. The bacterial profiles of pupal samples were tightly clustered (Figure3.1b). The high relative abundance of bacteria in workers, emerged bees and larvae had low GC content (Figure3.1a).All 32 bands were excised, cloned, and sequenced for bacterial identification (Figure 3.2 and Table 3.2). Figure 3.3 shows the distribution of the excised bands among the three developmental stages, revealing that many bacterial species present in the larvae were dramatically decreased in pupae. The most frequent bands in larvae were consistently present among the emerged bees and workers.

Two phyla (Proteobacteria and Firmicutes) and four classes (Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, and Bacilli) were identified in the samples, their distribution varied somewhat among the different developmental stages, six colonies, and

Table 3.1 Comparison of the DGGE profiles of *A. dorsata* at four stages collected from two locations.

Stage	Location	Colony	DGGE bands* (Mean ± SD)	Shannon index† (Mean ± SD)	DGGE bands‡ (Mean ± SD)	Shannon index§ (Mean ± SD)	DGGE bands¶ (Mean ± SD)	Shannonindex** (Mean ± SD)
Larva (N = 180)	Maerim	1	4.2 ± 3.3	0.9 ± 1.0	5.1 ± 3.7	1.0 ± 1.0	7.7 ± 4.4	1.5 ± 1.1
		2	7.4 ± 3.3	1.4 ± 0.9				
		3	3.5 ± 3.3	0.8 ± 1.0				
	Saraphe	4	10.2 ± 3.9	1.4 ±1.2	10.4 ± 3.4	1.9 ± 0.9		
		5	10.2 ± 2.6	2.1 ± 0.5				
		6	10.8 ± 3.6	2.3 ± 0.5				
Pupa (N = 180)	Maerim	1	3.7 ± 1.3	0.9 ± 0.8	3.7 ± 1.3	0.8 ± 0.8	3.8 ± 1.0	0.9 ± 0.6
		2	4.2 ± 0.6	1.1 ± 0.6				
		3	3.2 ± 1.5	0.5 ± 0.9				
	Saraphe	4	4.1 ± 0.4	1.0 ± 0.2	4.0 ± 0.5	0.9 ± 0.3		
		5	3.8 ± 0.8	0.9 ± 0.3				
		6	4.0 ± 0.0	0.8 ± 0.4				
Emerged bee (N = 180)	Maerim	1	17.4 ± 5.5	2.1 ± 1.2	13.3 ± 6.7	1.9 ± 1.2	11.3 ± 6.0	1.8 ± 1.1
		2	14.3 ± 4.3	2.4 ± 1.0				
		3	8.1 ± 6.4	1.3 ± 1.1				
	Saraphe	4	11.4 ± 4.0	1.7 ± 1.1	9.4 ± 4.6	1.7 ± 1.0		
		5	7.9 ± 4.0	1.8 ± 0.6				
		6	8.9 ± 5.1	1.5 ± 1.1				
Worker (N = 180)	Maerim	1	3.7 ± 5.9	0.7 ± 1.1	8.5 ± 7.5	1.6 ± 1.2	9.3 ± 6.8	1.8 ± 1.1
		2	13.1 ±5.7	2.4 ± 0.7				
		3	8.7 ± 7.8	1.6 ± 1.2				
	Saraphe	4	11.1 ± 5.7	2.2 ± 0.9	10.0 ± 5.9	2.0 ± 1.0		
		5	8.2 ± 6.0	1.8 ± 1.1				
		6	10.7 ± 5.6	2.0 ± 1.0				

*,† The number of DGGE bands and Shannon index were significantly different among the six individual colonies. ANOVA: p <0.001, for both DGGE bands and Shannon index.

‡,§ The number of DGGE bands and Shannon index were significantly different between the two locations. ANOVA: p =0.05 for DGGE bands;
p =0.001 for Shannon index.

¶,**The number of DGGE bands and Shannon index were significantly different among four stages. ANOVA: p <0.001, for both DGGE band and Shannon index.

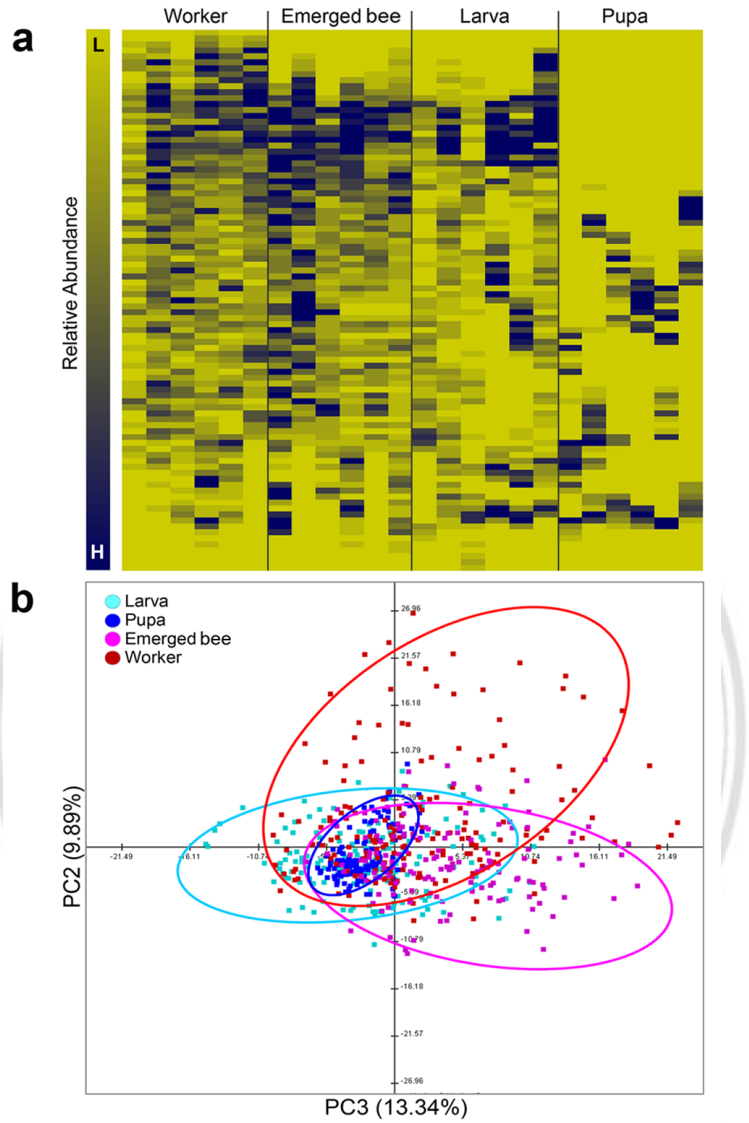

Figure 3.1 Bacterial community profiles of four stages characterized using DGGE bands. (a) Patterns of bacteria profiles were grouped according to stages represented as a heat map. Each lane was a single colony. The color code indicated the relative abundance ranging from light green (low abundance) to dark blue (high abundance). (b) PCA of the bacterial community profiles, as shown in 2D, indicates the correlations among the four stages. Each dot represents an individual bee, and different color-coded blocks indicate each of four stages.

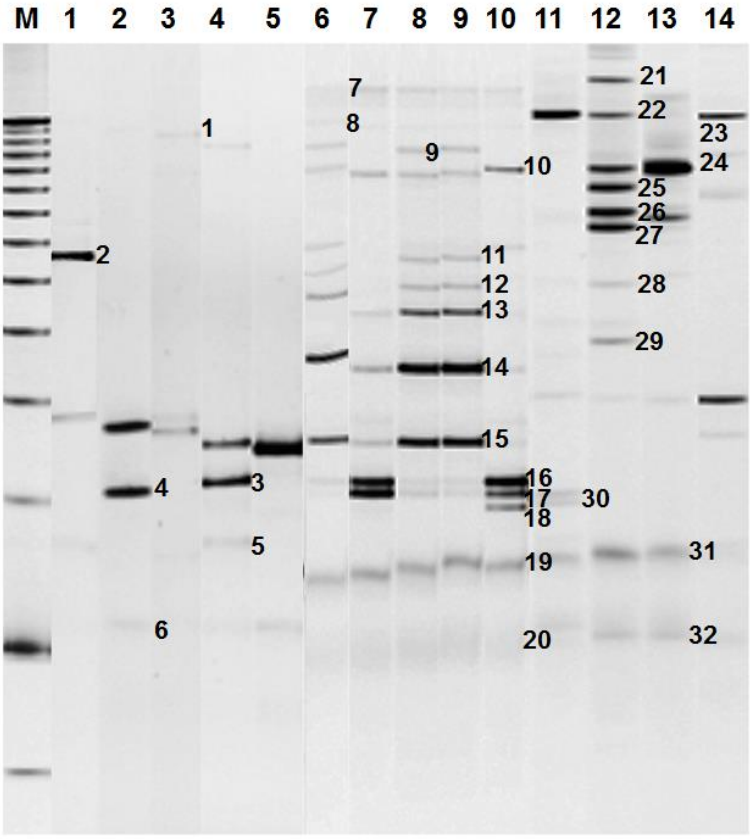

Figure 3.2 Representative DGGE amplicons of 16S rRNA genes from the *A. dorsata* samples. A total of 32 DGGE bands were excised for sequencing analysis. The numbers correspond to the sequenced bands listed in Table 3.2. DGGE marker (lane M), Larvae (lane 1-5), Emerged bees (lane 6-10), and Workers (lane 11-14).

Table 3.2 Bacterial taxa associated with *A. dorsata* midgut of larvae, pupae, emerged bees and workers obtained fromdominant DGGE band.

Stages	Band no.	Accession number	16S rRNA sequence phylogenetic classification			Closest relative	Accession number	Similarity (%)	Source
			Phylum	Class	Genus				
Larva	1	HG518581	Firmicutes	Bacilli	Lactobacillus	*Lactobacillus kunkeei*	Y11374	100	flower, bee hive, bee
	2	HG518582	Proteobacteria	Gammaproteobacteria	Gilliamella	*Gilliamellaapicola*	JQ936675	89	honey bee gut, bumble bee gut
	3	HG518584	Proteobacteria	Betaproteobacteria	-	uncultured Neisseriaceae bacterium	HM111869	100	honey bee gut, bumble bee gut
	4	HG518585	Proteobacteria	Alphaproteobacteria	Saccharibacter	*Saccharibacterfloricola*	AB110421	100	pollen
	5	HG518586	Proteobacteria	Gammaproteobacteria	Gilliamella	*Gilliamellaapicola*	JQ936675	89	honey bee gut, bumble bee gut
	6	HG518587	Proteobacteria	Alphaproteobacteria	Novosphingobium	*Novosphingobium sp.*	JF958135	100	environment
Pupa		HG518617-HG218621	No bacteria band						

Table 3.2 Bacterial taxa associated with *A. dorsata* midgut of larvae, pupae, emerged bees and workers obtained from dominant DGGE bands (Cont.).

Stages	Band no.	Accession number	16S rRNA sequence phylogenetic classification			Closest relative	Accession number	Similarity (%)	Source
			Phylum	Class	Genus				
Emerged bee	7	HG518589	Proteobacteria	Gammaproteobacteria	Enterobacter	*Enterobacter cloacae*	AY787819	100	environment
			Proteobacteria	Gammaproteobacteria	Enterobacter	*Pantoeaagglomerans*	AY941834	100	environment
	8	HG518590	Proteobacteria	Gammaproteobacteria	Enterobacter	*Enterobacteraerogenes*	KF668467	100	environment
			Proteobacteria	Gammaproteobacteria	Raoultella	*Raoultellaornithinolytica*	JCM7251	100	Apid gut
			Proteobacteria	Gammaproteobacteria	Enterobacter	*Enterobacteraerogenes*	KC431784	100	environment
	9	HG518591	Proteobacteria	Gammaproteobacteria	Enterobacter	*Enterobacteraerogenes*	FR773881	100	environment
	10	HG518592	Proteobacteria	Gammaproteobacteria	Enterobacter	*Enterobacteraerogenes*	NCTC10006T	100	environment
			Proteobacteria	Gammaproteobacteria	Raoultella	*Raoultellaterrigena*	ATCC33257T	100	environment
			Proteobacteria	Gammaproteobacteria	Raoultella	*Raoultellaplanticola*	ATCC 33531T	100	environment
	11	HG518593	Proteobacteria	Gammaproteobacteria	Enterobacter	*Enterobacteraerogenes*	NCTC10006T	100	environment
	12	HG518594	Proteobacteria	Gammaproteobacteria	Enterobacter	*Enterobacteraerogenes*	AB244302	100	predatory larvae of the antlion species
			Proteobacteria	Gammaproteobacteria	Enterobacter	*Lelliottiaamnigena*	EF426859	100	environment
	13	HG518595	Proteobacteria	Gammaproteobacteria	Enterobacter	*Enterobacteraerogenes*	NCTC10006T	100	environment
			Proteobacteria	Gammaproteobacteria	Raoultella	*Raoultellaplanticola*	ATCC 33531T	100	environment
			Proteobacteria	Gammaproteobacteria	Raoultella	*Raoultellaterrigena*	ATCC33257T	100	environment
	14	HG518596	Proteobacteria	Gammaproteobacteria	Enterobacter	*Enterobacteraerogenes*	NCTC10006T	100	environment
	15	HG518597	Proteobacteria	Gammaproteobacteria	Enterobacter	*Enterobacteraerogenes*	AF395913	100	environment
			Proteobacteria	Gammaproteobacteria	Enterobacter	*Enterobacterasburiae*	JCM6051	100	environment
			Proteobacteria	Gammaproteobacteria	Enterobacter	*Enterobacterhormaechei subsp. steigerwaltii*	DSMZ 16691	100	environment
			Proteobacteria	Gammaproteobacteria	Enterobacter	*Klebsiellaoxytoca*	AY150697	100	environment
			Proteobacteria	Gammaproteobacteria	Enterobacter	*Klebsiellapneumoniae*	AY369139	100	environment
	16	HG518598	Proteobacteria	Gammaproteobacteria	Enterobacter	*Enterobacter cloacae*	AY787819	100	environment
			Proteobacteria	Gammaproteobacteria	Enterobacter	*Pantoeaagglomerans*	AY941834	100	environment

Table 3.2 Bacterial taxa associated with *A. dorsata* midgut of larvae, pupae, emerged bees and workers obtained from dominant DGGE bands (Cont.).

Stages	Band no.	Accession number	16S rRNA sequence phylogenetic classification			Closest relative	Accession number	Similarity (%)	Source
			Phylum	Class	Genus				
Emerged bee	17	HG518599	Proteobacteria	Gammaproteobacteria	Enterobacter	*Enterobacteraerogenes*	AF395913	100	environment
			Proteobacteria	Gammaproteobacteria	Enterobacter	*Enterobacterasburiae*	JCM6051	100	aphid gut
			Proteobacteria	Gammaproteobacteria	Enterobacter	*Enterobacterhormaechei subsp. steigerwaltii*	DSMZ 16691	100	environment
			Proteobacteria	Gammaproteobacteria	Enterobacter	*Klebsiellaoxytoca*	AY150697	100	environment
			Proteobacteria	Gammaproteobacteria	Enterobacter	*Klebsiellapneumoniae*	AY369139	100	environment
	18	HG518600	Proteobacteria	Gammaproteobacteria	Enterobacter	*Enterobacteraerogenes*	AF395913	100	environment
			Proteobacteria	Gammaproteobacteria	Enterobacter	*Enterobacterasburiae*	JCM6051	100	aphid gut
			Proteobacteria	Gammaproteobacteria	Enterobacter	*Enterobacterhormaechei subsp. steigerwaltii*	DSMZ 16691	100	environment
			Proteobacteria	Gammaproteobacteria	Enterobacter	*Klebsiellaoxytoca*	AY150697	100	environment
			Proteobacteria	Gammaproteobacteria	Enterobacter	*Klebsiellapneumoniae*	AY369139	100	environment
	19	HG518601	Proteobacteria	Gammaproteobacteria	Enterobacter	*Enterobacteraerogenes*	NCTC10006T	100	environment
	20	HG518602	Proteobacteria	Gammaproteobacteria	Cronobacter	*Cronobactersakazakii*	AY752940	100	environment
			Proteobacteria	Gammaproteobacteria	Cronobacter	*Cronobactermuytjensii*	FJ906906	100	environment
			Proteobacteria	Gammaproteobacteria	Trabulsiella	*Trabulsiellaodontotermitis*	DQ453129	100	termite gut

Table 3.2 Bacterial taxa associated with *A. dorsata* midgut of larvae, pupae, emerged bees and workers obtained from dominant DGGE bands (Cont.).

Stages	Band no.	Accession number	16S rRNA sequence phylogenetic classification			Closest relative	Accession number	Similarity (%)	Source
			Phylum	Class	Genus				
Worker	21	HG518603	Proteobacteria	Gammaproteobacteria	Gilliamella	*Gilliamellaapicola*	JQ936674	96	honeybee, bumble bee gut
	22	HG518604	Firmicutes	Bacilli	Lactobacillus	*Lactobacillus kunkeei*	Y11374	100	flower, bee hive, bee
	23	HG518605	Firmicutes	Bacilli	Lactobacillus	*Lactobacillus sp.*	HM534759	100	honeybee gut
	24	HG518606	Proteobacteria	Gammaproteobacteria	Gilliamella	*Gilliamellaapicola*	JQ936675	89	honeybee, bumble bee gut
	25	HG518607	Proteobacteria	Gammaproteobacteria	Frischella	*Frischellaperrara*	JX878306	100	*A. mellifera* gut
	26	HG518608	Proteobacteria	Gammaproteobacteria	Gilliamella	*Pasteurellaceae bacterium*	EF187247	100	*A. mellifera* stomach
	27	HG518609	Proteobacteria	Gammaproteobacteria	Gilliamella	*Gilliamellaapicola*	JQ936674	96	honeybee, bumble bee gut
	28	HG518610	Proteobacteria	Gammaproteobacteria	Aeromonas	*Aeromonassharmana*	DQ013306	90	environment
			Proteobacteria	Gammaproteobacteria	Shewanella	*Shewanellafodinae*	FM203122	90	environment
			Proteobacteria	Gammaproteobacteria	Shewanella	*Shewanelladokdonensis*	GQ245918	90	environment
	29	HG518611	Proteobacteria	Gammaproteobacteria	Aeromonas	*Aeromonassharmana*	DQ013306	90	environment
			Proteobacteria	Gammaproteobacteria	Tolumonas	*Tolumonasosonensis*	GU370947	90	environment
			Proteobacteria	Gammaproteobacteria	Shewanella	*Shewanellafodinae*	FM203122	89	environment
			Proteobacteria	Gammaproteobacteria	Shewanella	*Shewanelladokdonensis*	GQ245918	90	environment
	30	HG518614	Proteobacteria	Gammaproteobacteria	Pantoea	*Pantoeaallii*	AY530795	99	environment
			Proteobacteria	Gammaproteobacteria	Pantoea	*Pantoeaananatis*	GQ497892	99	environment
	31	HG518615	Proteobacteria	Gammaproteobacteria	Gilliamella	*Pasteurellaceae bacterium*	EF187247	100	honeybee stomach
	32	HG518616	Proteobacteria	Gammaproteobacteria	Pantoea	*Pantoeaananatis*	AF364847	100	environment
			Proteobacteria	Gammaproteobacteria	Pantoea	*Pantoea eucalypti*	FM202484	100	environment

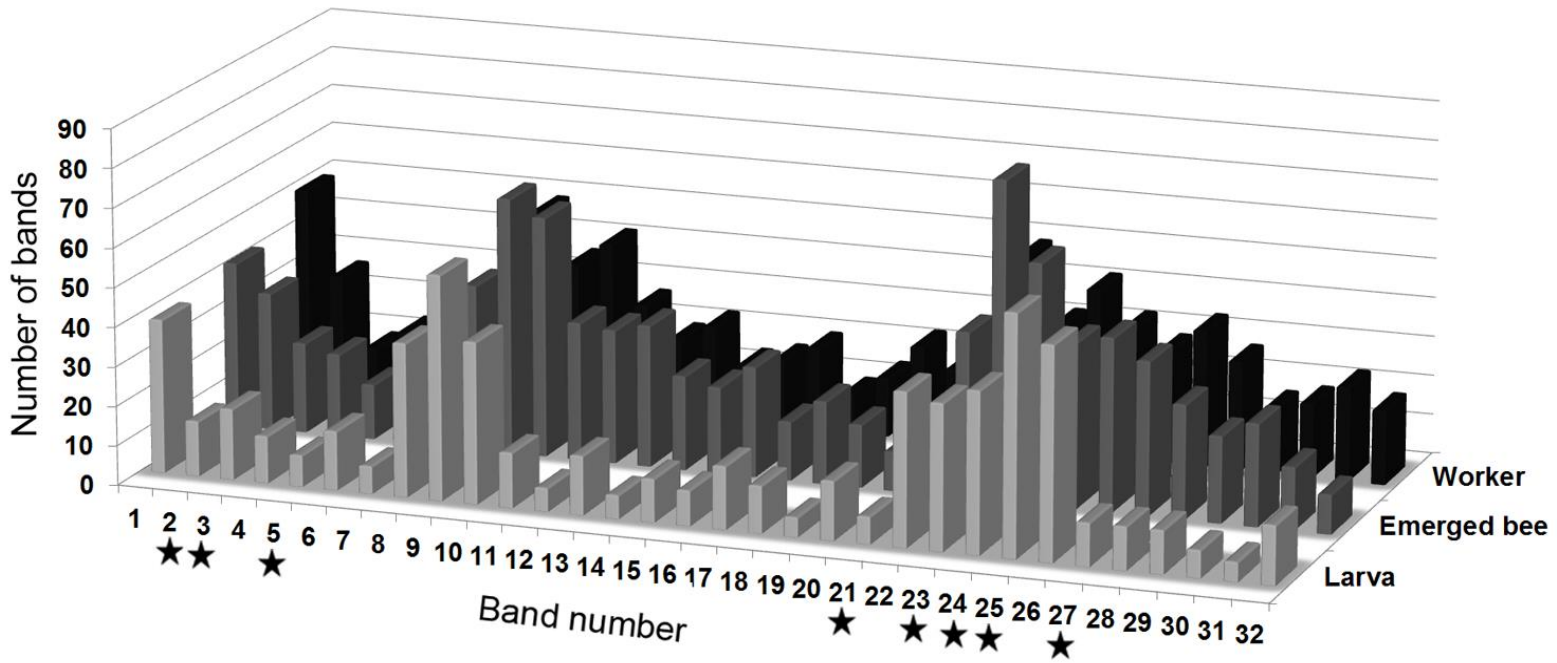

Figure 3.3 Distribution of 32 excised bands at larval, emerged bee and worker stages.Each bar represents a DGGE band detected in the gel at different migration distance. Asterisks indicated that the bands were associated with bee gut.

two locations (Figure 3.4 and Figure 3.5). We found that 31% of identified DGGE bands were closely related to bacteria previously isolated from bees, such as *Lactobacillus kunkeei*, *Gilliamellaapicola*, *Frischellaperrara*, *Pasteurellaceae bacterium* and uncultured *Neisseriaceae bacterium*. The remaining 69% of the identified DGGE bands were closely related to bacteria previously isolated from the natural environment (Table 3.2).Cluster analysis of DGGE profiles consistently showed that the profiles were more likely to be clustered by colony and geographical location for each developmental stage (Figure A1-A4).

3.3.1 Bacterial diversity in larva midgut

The pattern of band distribution of larvae was similar to that of emerged bees and workers (Figure3.1a). The number of distinct bands ranged from 3.5 ± 3.3 to 10.8 ± 3.6 with a mean ± SD of 7.7 ± 4.4 for the six colonies. We observed that the overall mean of Shannon index was 1.5 ± 1.1. The number of bands and the mean Shannon index were significantly greater in Saraphe compared to Maerim (Table 3.1). Six DGGE bands present in larval DNA samples were sequenced, and they were found to be closely related to bacteria belonging to Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, and Bacilli (Table 3.2). Cluster analysis demonstrated that bacterial profiles appeared to be distinct to the two locations (Figure A1). However, the three colonies from Saraphe (colony 4, 5 and 6) and one colony from Maerim (colony 2) shared a common DGGE band, which was closely related to *Gilliamellaapicola*.

3.3.2 Bacterial diversity in pupal midgut

All pupae possessed a simple bacterial community structure with one or more DGGE bands (3.8 ± 10, mean ± SD) and a low diversity by Shannon index (0.9 ± 0.6, mean ± SD) in all six colonies (Table 3.1). The number of DGGE bands and Shannon index were slightly higher in Saraphe compared to Maerim. Bacterial profiles of pupae were tightly clustered together (Figure3.1b and Figure A2). Five bands obtained from pupal samples

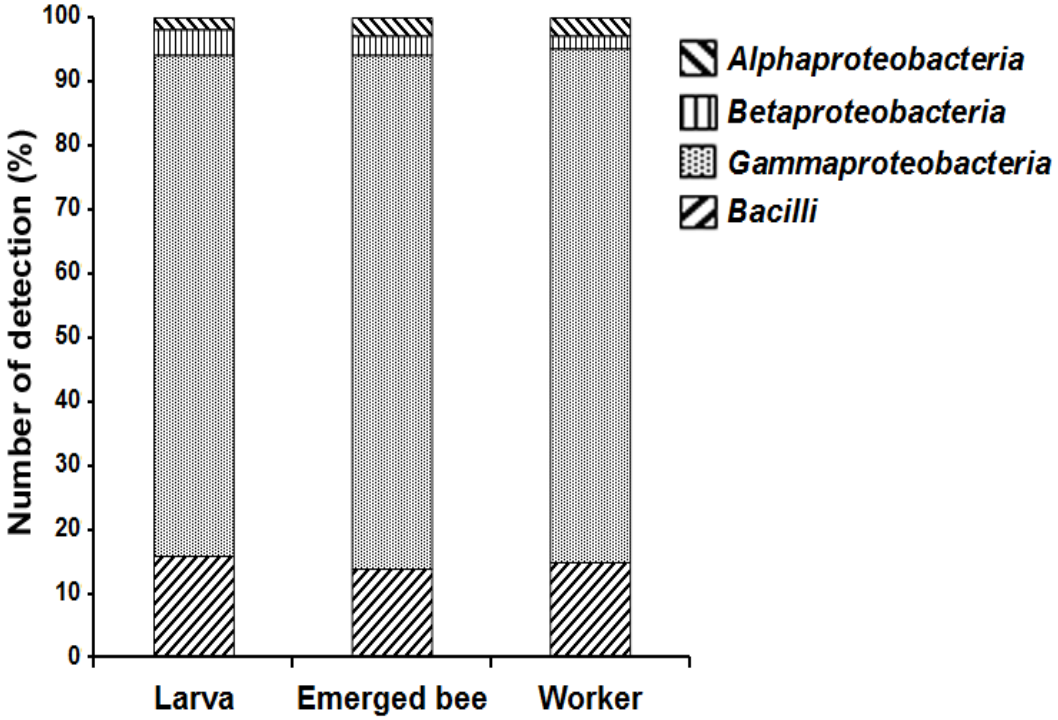

Figure 3.4 Percent detection bands of different bacterial groups found at three stages. Each bacterium is indicated by a different symbol.

showed no similarity to any bacterial sequences deposited in the Ribosomal Database Project (RDP II).

3.3.3 Bacterial diversity of newly emerged bee midgut

We observed a significant increase in midgut bacterial diversity following eclosion from the pupal stage to newly emerged bees. A three-fold increase was observed in the mean number of DGGE bands (11.3 ± 6.0) and a two-fold increase in the mean of the Shannon index (1.8 ± 1.1) in the six colonies compared to those values reported for pupae. We also observed a significant difference among the colonies, as well as between the two locations (Table 3.1). Interestingly, emerged bees from Maerim (colony 3) showed fewer bands and a Shannon index with a bacterial profile similar to that of the pupal stage (Figure A3). The increased bacterial profiles in individual emerged bees were also evidenced in

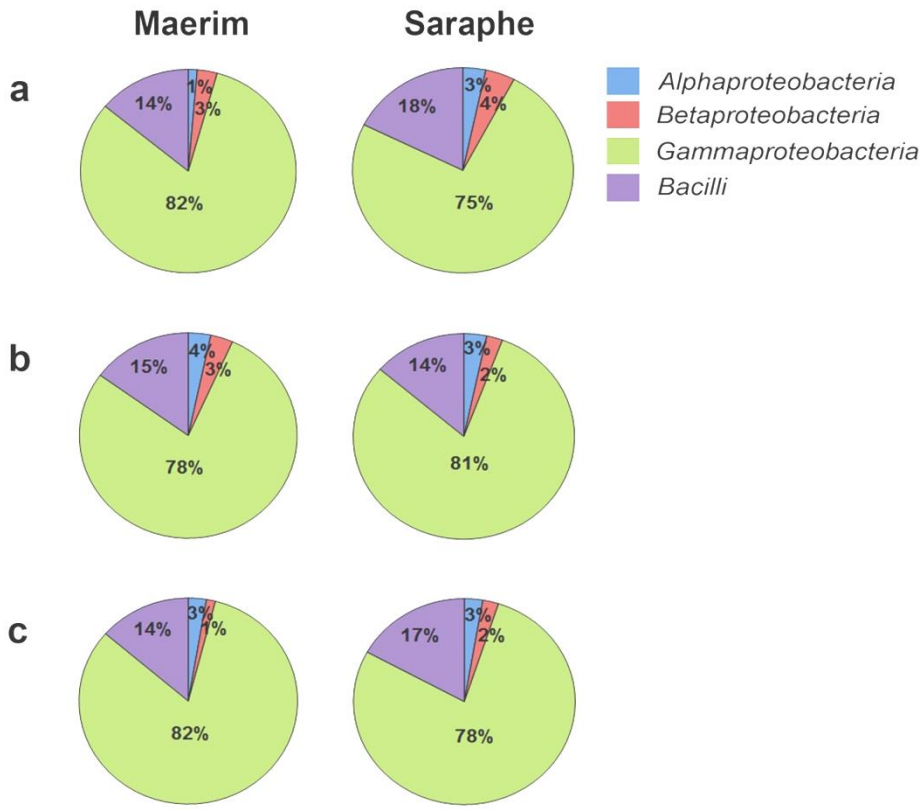

Figure 3.5 Proportion of the groups of bacteria at three stages from two locations. Larva (a), Emerged bee (b), and Worker (c).

principal component analysis (PCA)(Figure3.1b). These 14 bands from the newly emerged bees fell into one bacterial class: Gammaproteobacteria. All sequences were closely related to bacteria isolated from the natural environment (Table 3.2). The percentage of four detected bacterial groups was similar to that of larval and worker developmental stages. Gammaproteobacteria and Bacilli predominated in both locations (Figure 3.5). Clustering analysis showed a high degree of similarity in bacteria profiles in each colony and specific patterns in each location. Bacterial profiles of emerged bees from the same location tended to cluster together (Figure A3).

3.3.4 Bacterial diversity of foraging worker midgut

The number of distinct bands ranged from 3.7 to 13.1 with a mean ± SD of 9.3 ± 6.8 for the six colonies with a Shannon index of 1.8 ± 1.1. On average, the total number of detectable bands and Shannon index were significantly different between the two locations (Table 3.1). DGGE patterns from individual workers were clustered separately, which indicates that they were from different groups of bacteria (Figure A4). The broad range of the relative abundance that was found at the adult stage also reveals more diversity than that found in the other three stages (Figure3.1a). In the midgut of *A. dorsata* workers, the 32 DGGE bands were distributed in a pattern similar to that of emerged bees (Figure 3.3). Twelve identified bands from workers were closely related to bacteria isolated from both bees and the natural environment (Table 3.2).For cluster analysis, the bacterial profiles were categorized into three distinct clades. In the first clade, 88% of workers sampled were from Maerim. In the other two clades, 87% of the workers sampled were from Saraphe. More specifically, 97% of workers in the second clade were sampled from Saraphe (colony 4 and, partially, colony 5) (Figure A4).

3.4 Discussion

This is the first report examining the dynamics of bacterial community structure in *A. dorsata* midgut during the four developmental stages. Bacterial DNA profiles were studied by PCR-DGGE and 16S rRNA sequencing. DGGE patterns showed significant difference in the number of bands, Shannon index, bacterial profiles among the four stages and individual colonies, as well as between the two locations. During the developmental period, a total of four bacterial classes, including Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, and Bacilli, were detected. The two predominant bacterial classes were Gammaproteobacteria and Bacilli.

Most of the 32 bands were closely related to bacteria identified in honeybee guts and the natural environments as previously reported for bees. *Gilliamella apicola* and

Snodgrassella alvi have been detected in *A. dorsata* midgut (Koch et al., 2013). *Lactobacillus kunkeei* was also identified in *A. dorsata* stomach (Vasquez et al., 2012). By metagenomic analyses, these groups of bacteria may perform necessary functions, such as food processing, nutrient supplementation, and host protection (Vasquez et al., 2012; Engel and Moran, 2013; Lee et al., 2014). Our results were similar to those of Martinson et al. (2011), who found four bacterial groups, including Alpha-1, Alpha-2.2, Betaproteobacteria, and Gamma-1, in the gut of *A. dorsata* workers sampled from Malaysia (Martinson et al., 2011). However, workers in our study did not possess Alpha-1, which is related to the genus *Bartonella*.

Twelve genera from the three bacterial classes (Alphaproteobacteria, Gammaproteobacteria, and Bacilli) found in our results were also closely related to bacteria originating from the natural environment. *Saccharibacterfloricola* is a bacterium isolated from pollen and has previously been reported in honeybees and solitary bees (Mohr and Tebbe, 2007). A family of Enterobacteraciae detected in our study (genus *Enterobacter*) was also reported in the guts of *A. mellifera* and *A. cerana* in Korea (Ahn et al., 2012). Most of the bands (69%) had high sequence similarities to bacteria isolated from the natural environment which varied less within individual colonies, but were highly different among colonies, particularly from the two different geographical locations. This finding strongly suggests that environmental factors could influence bacterial persistence since both locations provided different food sources. Many studies strongly support the idea that environmental factors influence gut bacteria, especially food sources, such as nectar and pollen (Babendreier et al., 2007).

Studies of *A. mellifera* larvae using culture-based and non culture-based techniques have disclosed several characteristic phylotypes within their gut (Disayathanoowat et al., 2012; Vojvodic et al., 2013). Critical changes at the pupal stage result in fewer numbers of bands and less diversity than found at other stages, possibly because pupa is a non-feeding stage, growing under the brood seal, thus demonstrating low bacterial profiles. In *A.*

mellifera pupae, gut intimae were shed, obviously resulting in the absence of gut bacteria at that stage (Martinson et al., 2012). In our experiment, we incubated pupae for 12 h in order to collect newly emerged bees on the same day (day 1). After bees emerged from brood cells, they might have come into contact with bacteria resident on the comb or associated with other aspects of hive matrix. When pupae become emerged bees, most organs fully develop, especially the gut. It was reported that newly emerged *A. mellifera* bees contained few, or no, bacteria and that the numbers of bacteria increased with age (Martinson et al., 2012). Our results are consistent with previous studies of *A. mellifera* and *A. cerana* development. These studies reported that workers possessed greater bacterial diversity (7-12 core bacteria) than earlier developmental stages in which few, or no, bacteria were found (Ahn et al., 2012; Disayathanoowat et al., 2012; Martinson et al., 2012). Characterization of honeybee microbiota has been attempted using multiple methods for sample collection and DNA extraction, as well as detection and analytical methods, and such studies revealed very similar results; however, some data differed among studies (Sabree et al., 2012).

In conclusion, bacterial community structure changes with the stages of a honeybee's life cycle, possibly resulting from different host-bacteria interactions, as well as normal changes in gut physiology at each stage. Adult workers harbored more bacterial diversity than emerged bees and larvae, respectively. Our study indicated differences among the four bacterial groups in each of the three stages and the two geographical locations. Bacterial profiles were homogeneous among colonies, but heterogeneous within the different colonies and between the two locations, likely highlighting environmental factors that might influence the persistence of bacteria in the midgut. Further studies, including the quantification of bacteria in bee midgut, would provide information enabling a better understanding of the relevance of these bacterial communities.