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Pyrosequencing Analysis of the Bacterial Communities in the Guts of Honey Bees *Apis cerana* and *Apis mellifera* in Korea[§]

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The bacterial communities in the guts of the adults and larvae of the Asian honey bee *Apis cerana* and the European honey bee *Apis mellifera* were surveyed by pyrosequencing the 16S rRNA genes. Most of the gut bacterial 16S rRNA gene sequences were highly similar to the known honey bee-specific ones and affiliated with *Pasteurellaceae* or lactic acid bacteria (LAB). The numbers of operational taxonomic units (OTUs, defined at 97% similarity) were lower in the larval guts (6 or 9) than in the adult guts (18 or 20), and the frequencies of *Pasteurellaceae*-related OTUs were higher in the larval guts while those of LAB-related OTUs in the adult guts. The frequencies of *Lactococcus*, *Bartonella*, *Spiroplasma*, *Enterobacteriaceae*, and *Flavobacteriaceae*-related OTUs were much higher in *A. cerana* guts while *Bifidobacterium* and *Lachnospiraceae*-related OTUs were more abundant in *A. mellfera* guts. The bacterial community structures in the midguts and hindguts of the adult honey bees were not different for *A. cerana*, but significantly different for *A. mellifera*. The above results substantiated the previous observation that honey bee guts are dominated by several specific bacterial groups, and also showed that the relative abundances of OTUs could be markedly changed depending on the developmental stage, the location within the gut, and the honey bee species. The possibility of using the gut bacterial community as an indicator of honey bee health was discussed.

Keywords: honey bee, gut, bacterial community, 16S rRNA gene, pyrosequencing

Introduction

Honey bees are greatly valued as honey producers for human consumption, and even more importantly, as pollinators in natural and agricultural ecosystems. However, there is clear evidence of recent declines in both wild and domestic pollinators, including honey bees (Potts *et al.*, 2010). The suggested drivers for this phenomenon include changes in land use, agrochemicals, pathogens, foreign species, climate change, and the interactions between them. Thus, research on honey bee health is increasing, especially with respect to both the beneficial and harmful symbionts harboured by honey bees, including bacteria, fungi, yeasts, protozoans, mites, and viruses (Gilliam, 1997; Cox-Foster *et al.*, 2007; Evans and Schwarz, 2011).

The gut bacteria of insects are known to be essential for host nutrition and defence against pathogens (Dillon and Dillon, 2004), and therefore, it is thought that the health status of the host can be monitored by observing the microbial communities in the guts, as revealed by the human gut microbiome studies (Turnbaugh *et al.*, 2008; Armougom *et al.*, 2009). Recent studies have shown that several characteristic bacterial groups occupy most of the bacterial communities in the guts of honey bees and bumble bees (Mohr and Tebbe, 2006; Koch and Schmid-Hempel, 2011a; Martinson *et al.*, 2011), and some of the groups may contribute to the host defence against known bee pathogens (Koch and Schmid-Hempel, 2011b). The simple bacterial communities in the honey bee guts contrast with those in the human guts (Rajilić-Stojanović *et al.*, 2007; Nam *et al.*, 2011), and this may be attributed to their simple food requirements of nectar and pollen (Winston, 1987). The former provides carbohydrates in the form of sugars, and the latter, protein, lipids, vitamins, and minerals.

Two honey bee species are reared in Korea: the Asian honey bee, *Apis cerana*, and the European honey bee, *Apis mellifera*. In 2008, the number of *A. cerana* hives in Korea accounted for only 22.7% of all hives (Lee *et al.*, 2010). This is because *A. cerana* produces less honey, has a high rate of swarming and absconding, and lacks established beekeeping methods. However, the honey produced by *A. cerana* sells at a higher price than that by *A. mellifera* due to its rarity in Korea.

In this study, we investigated the bacterial communities in the guts of the adult honey bee and the larvae of the two honey bee species collected in Korea, by pyrosequencing their 16S rRNA genes. For adult honey bees, the bacterial communities in both the midguts and hindguts were surveyed. Most digestion and absorption occur in the midgut (ventriculus), and solid wastes are then passed through the intestine to the hindgut (rectum) for excretion (Winston, 1987). Because most of the studies on the bacterial symbionts of honey bees have focussed on *A. mellifera*, this study will provide new insights into the symbionts of the Asian counterpart. In addition, the ultra-deep sequencing enabled by pyrosequencing is expected to provide more comprehensive

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and quantitative information about the beneficial or harmful bacterial symbionts of honey bees than that provided by previous studies based on terminal restriction fragment length polymorphism (T-RFLP) (Babendreier *et al.*, 2007; Disayathanoowat *et al.*, 2012), single-strand conformation polymorphism (SSCP) (Mohr and Tebbe, 2006), or 16S rRNA gene clone library analysis (Martinson *et al.*, 2011).

Materials and Methods

Sample collection and DNA extraction

The adult honey bees (n=40 for *A. cerana* and n=30 for *A. mellifera*) and the larvae (n=20 for each species) were collected from an apiary in the Rural Development Administration, Suwon, Korea on June 2, 2011. The hives of *A. cerana* and *A. mellifera* were separated by about 10 m and sampling was performed on one hive per honey bee species. Each sample was collected into 100% ethanol, and then immediately transferred to a nearby laboratory for DNA extraction. For surface sterilisation, samples were suspended in 70% ethanol, 5% sodium hypochlorite, and then sterile water for 1 min each. The guts of the adult honey bees were isolated from the abdomen with sterile forceps, and the midguts and hindguts were separated with a sterile scalpel. The larvae were dissected with sterile forceps, and the whole gut was isolated. The gut samples of the same type (larval guts, adult midguts or adult hindguts from each honey bee species) were immediately transferred to a Lysing Matrix E tube provided in the FastDNA SPIN Kit for Soil (MP Biomedicals), and DNA was extracted according to the manufacturer's instruction.

16S rRNA gene PCR and pyrosequencing

Amplification, purification, and pyrosequencing of partial bacterial 16S rRNA gene sequences were performed at Chunlab, Inc (Seoul, Korea), as previously described (Chun *et al.*, 2010) with the primer pair V1-9F/V3-541R and the following touchdown PCR protocol: initial denaturation at 94°C for 5 min, followed by 10 cycles at 94°C for 30 sec, 60°C for 45 sec, and 72°C for 90 sec, in which the annealing temperature was reduced by 0.5°C/cycle from the preceding cycle, followed by 20 cycles of 94°C for 30 sec, 55°C for 45 sec, and 72°C for 90 sec.

Processing of pyrosequencing data

The removal of PCR and pyrosequencing errors was performed using the PyroNoise algorithm (Quince *et al.*, 2011) implemented in the Mothur software package (version 1.22.1) (Schloss *et al.*, 2009) and the modified otupipe script [version 1.1.9, a pipeline based on USEARCH (Edgar, 2010) and UCHIME (Edgar *et al.*, 2011) (http://drive5.com/otupipe)]. Briefly, the pre-filtered flowgrams of the pyrosequencing reads of each sample were clustered using the PyroNoise algorithm, and the modified otupipe script was run to remove chimeric sequences (minimum size of the cluster=1, identity threshold=98, alignment by the Needleman-Wunsch algorithm, and the final clustering procedure was omitted). Then, the sequences were trimmed to the first 300 bp with reference to the sequencing primer in order to reduce the error rate (Gilles *et al.*, 2011; Schloss *et al.*, 2011). Several chimeric sequences were further identified on the basis on the results of BLAST analysis (Altschul *et al.*, 1997). The qualified sequences from each sample were merged, and the pair-wise distances were calculated using the pairwise.seqs command in the Mothur package, which aligns two sequences by the Needleman algorithm. Sequences were then clustered to operational taxonomic units (OTUs) at the cut-off of 97% similarity by the average neighbour algorithm.

When the above pipeline was applied to a raw pyrosequencing data set (SRR042616 in the NCBI sequence read archive) obtained from a mock community, which consisted of 20 different bacterial genomic DNAs (Schloss *et al.*, 2011), we obtained 26 OTUs, which consisted of 18 Goods (OTUs containing reference sequences and pyrosequencing reads), 2 Misses (OTUs containing only reference sequences), and 6 Noises (OTUs containing only pyrosequencing reads).

Phylogenetic analysis

The representative sequences for each OTU and closely related sequences in GenBank were imported into the ARB software package (version 5.2) (Ludwig *et al.*, 2004) loaded with the SILVA 16S rRNA database (SSURef-108) (Pruesse *et al.*, 2007). Sequences were aligned using the SINA aligner (version 1.1) (Pruesse *et al.*, 2007), if absent from the database. Nearly full-length sequences (>1,300 bases) were used for the construction of the initial tree by using the maximum-likelihood algorithm (Phylip) and the positional variability filter for *Bacteria* provided with the ARB package. The shorter sequences from this study (398–501 bases) were added to this tree by using the ARB parsimony tool, which allowed the addition of short sequences to phylogenetic trees without changing global tree topologies (Ludwig *et al.*, 1998). The alignments of the nearly full-length sequences were exported to the MEGA program (version 5.0) (Tamura *et al.*, 2011), and the bootstrap values were calculated using the maximum-likelihood algorithm. Taxonomic assignment to each OTU was performed on the basis of the above phylogenetic analysis and the classifier tool in the RDP release 10 (http://rdp.cme.msu.edu/classifier). The OTUs assigned to the chloroplasts or mitochondria were removed from subsequent analysis.

Hierarchical clustering using Fast UniFrac

A maximum-likelihood tree was constructed using the representative sequences for each OTU and the MEGA program (version 5.0). The resulting tree and the number of reads per OTU were imported into the Fast UniFrac environment (http://bmf.colorado.edu/Fast UniFrac/) (Hamady *et al.*, 2009). Weighted Fast UniFrac distance measures between the gut samples were calculated, and a UPGMA dendrogram was constructed on the basis of the distance measures. UniFrac significances were also calculated using 1,000 permutations to compare the microbial communities.

DNA sequence data

Raw pyrosequencing data is available in the NCBI Sequence Read Archive under the accession number SRP010401. The

Table 1. Summary of the pyrosequencing data from the honey bee gut samples

	Apis cerana			*Apis mellifera*		
	Larvae	Midgut[a]	Hindgut[a]	Larvae	Midgut[a]	Hindgut[a]
Number of reads	1,999	1,852	2,598	6,164	3,626	5,135
Good's coverage	1.0	1.0	1.0	1.0	1.0	1.0
Number of OTUs	6	18	20	9	20	18

[a] The midguts and hindguts were obtained from the adult honey bees.

representative sequences for each OTU were deposited under the accession numbers JQ437498–JQ437535.

Results

Overview of the bacterial diversity in honey bee guts

Table 1 shows the summary of the pyrosequencing data obtained from the honey bee gut samples. Although the number of pyrosequencing reads differed considerably among the samples (from 1,852 to 6,164), Good's coverage values (Good, 1953) were 1.00 for all the samples, indicating that direct comparison of bacterial diversity was possible without normalization of the read numbers. The number of OTUs was larger in adult honey bee guts (18 or 20) than in the larval guts (6 or 9) for both honey bee species.

The abundances and taxonomic affiliations of OTUs are shown in Fig. 1, in which minor OTUs are not labelled for simplicity. The abundances of all OTUs are presented in Supplementary data Table S1. Figure 1 indicated that: (1) *Proteobacteria* and *Firmicutes* dominate honey bee guts (91.7–100.0%); (2) the proportions of *Proteobacteria* are generally large in the larval guts (77.4–97.1%) compared to those in the adult honey bee guts (13.3–81.1%); (3) in the adult honey bee guts, the order *Lactobacillales* generally dominates (17.0–84.2%); (4) the bacterial communities in the midguts and hindguts of *A. cerana* are similar to each other, but they are considerably different in the case of *A. mellifera*; (5) several taxonomic groups are present exclusively, or in much higher frequencies, in only one of the two honey bee species (*Lactococcus*, *Spiroplasma*, *Enterobacteriaceae*, and *Flavobacteriaceae* in *A. cerana*; *Lachnospiraceae* and *Bifidobacterium* in *A. mellifera*).

Figure 2 shows the similarities between the bacterial communities from the different honey bee gut samples based on the weighted Fast UniFrac analysis, which accounts for changes in relative abundance of lineages between different communities. The samples were divided into two clusters, one containing the larval guts of both species and the midgut of adult *A. mellifera*, and the other containing the two gut sections of adult *A. cerana* and the hindgut of adult *A. mellifera*. The UniFrac significance test also indicates that the bacterial communities within one cluster are not different from each other but generally differ from those within the other cluster ($P<0.001$). This result indicates that the bacterial communities in larval guts are significantly different from those in adult guts. It also indicates that the bacterial communities in the midguts and hindguts of *A. cerana* are similar to each other but the bacterial communities in the midguts of *A. mellifera* are more similar to those in the larval guts than to those in the hindguts, as revealed by the taxonomic distributions (Fig. 1). The latter fact suggests that the intestinal system of *A. mellifera* may be more specialised than that of *A. cerana*.

Members of the bacterial communities in honey bee guts

The phylogenetic positions of each OTU are presented in Figs. 3 and 4. Among the total of 38 OTUs obtained from the six gut samples, 25 OTUs showed high 16S rRNA gene similarities (≥ 97%) with clones or isolates found in honey bees and bumble bees in previous studies (indicated by asterisks in Figs. 3 and 4). In addition, all eight phylotypes defined as representative bacterial sequences from honey bee guts (Martinson *et al.*, 2011) were recovered in this study (indicated at the corresponding nodes in Figs. 3 and 4). Tentative cluster names were given based on shared taxonomic assignments to OTUs within the cluster.

Ten OTUs were assigned to cluster 1 and clustered with the bacterial isolates and clones from honey bees, bumble bees, aphids, beetles, and fruit flies found in previous studies (Fig. 3). Pair-wise similarities between these OTUs ranged from 93% to 98%. Among these OTUs, OTU1 was recovered in all gut samples of both honey bee species in relatively high frequencies (4.4–45.1%), while OTU7 and OTU8 occurred mainly in the guts of *A. cerana*, and OTU4 and OTU10 in the guts of *A. mellifera* (Fig. 1). Taken together, the OTUs within this cluster occupied 55.6–76.9% in the larval guts and 5.1–48.9% in the adult guts. The phylogenetic analysis showed that this cluster is related to the family *Pasteurellaceae* (Fig. 3). Members of the family *Pasteurellaceae* are obligate parasites in vertebrates (primarily mammals and birds), and some are important human and animal pathogens (Korczak *et al.*, 2004). In contrast, the members of cluster 1 appear to prefer insects as their hosts, as previously suggested (Martinson *et al.*, 2011). Both respiratory and fermentative forms of metabolism are found among the members of the family *Pasteurellaceae* (Garrity *et al.*, 2005), including the only type strain within cluster 1, *Orbus hercynius*. A recent study has shown that strain W08.001, affiliated with this cluster, may play a role in protecting bumble bees against an intestinal parasite (Volkmann *et al.*, 2010; Koch and Schmid- Hempel, 2011b).

Four *Enterobacteriaceae*-related OTUs (Fig. 3, cluster 2) occurred mainly in the guts of adult *A. cerana* (Fig. 1). They were clustered with the bacterial clones or isolates from honey bees, stink bugs, plant, and humans. The *Pseudomonadaceae*-related OTU15 (Fig. 3, cluster 3) was detected only in the guts of adult *A. mellifera* (~0.6%). The close relatives (≥99%) to OTU15 in GenBank were only the sequences obtained from *A. mellifera* (Babendreier *et al.*, 2007), suggesting its close association with honey bees. The *Neisseriaceae*-related OTU16 (Fig. 3, cluster 4) occupied 5.3% and

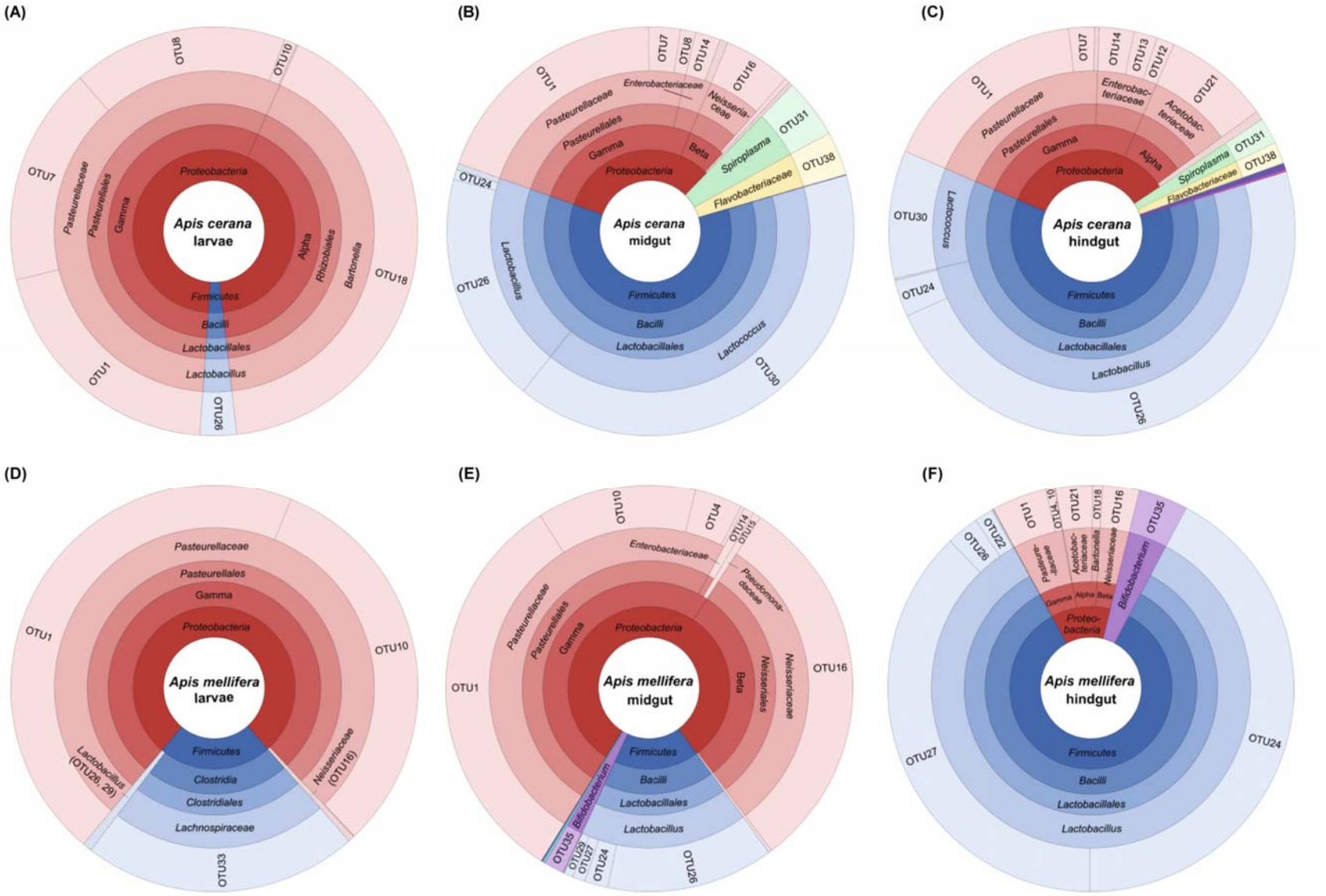

Fig. 1. Taxonomic distributions of the bacterial communities in the gut of *A. cerana* larvae (A), the midgut of adult *A. cerana* (B), the hindgut of adult *A. cerana* (C), the gut of *A. mellifera* larvae (D), the midgut of adult *A. mellifera* (E), and the hindgut of adult *A. mellifera* (F). Classifications were performed on the basis of phylogenetic analysis (Figs. 3 and 4) and RDP classifier. Some taxonomic levels were collapsed for simplicity. Minor OTUs were not labelled. Pie charts were constructed using Krona 2.0 (Ondov *et al.*, 2011).

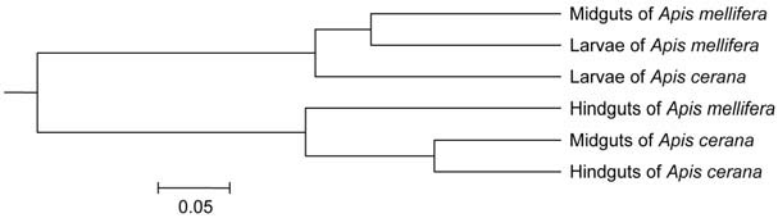

Fig. 2. A UPGMA dendrogram based on the weighted pair-wise Fast UniFrac distances between the bacterial communities of the honey bee gut samples.

30.5% in the midguts of *A. cerana* and *A. mellifera*, respectively; their abundance decreased to 0.7% and 2.9% in the hindguts of both species, respectively (Fig. 1). This observation indicates that the OTU16 may prefer the environmental condition of the midgut to that of the hindgut.

OTU18 was assigned to the genus *Bartonella* (Fig. 3, cluster 6) and occupied the highest proportion in the larval gut of *A. cerana* (41.4%), but was only a minor member in the other gut samples (<0.8%) (Fig. 1). *Bartonella* spp. are facultative intracellular pathogens in a variety of mammals (Minnick and Anderson, 2006). However, OTU18 appeared to be non-pathogenic to honey bees because the closely related sequences (≥99%) were frequently found in seemingly healthy honey bees with high proportions (about 10–30%) in previous studies (Jeyaprakash *et al.*, 2003; Babendreier *et al.*, 2007; Martinson *et al.*, 2011).

The *Acetobacteraceae*-related OTU21 (Fig. 3, cluster 8) occupied 8.4% and 3.0% in the hindguts of *A. cerana* and *A. mellifera*, respectively, and was not detected in the midguts of either species (Fig. 1), suggesting that the hindgut provides more favourable conditions for OTU21 than does the midgut. Recent studies have shown close symbiotic relationships between acetic acid bacteria (AAB) of the family *Acetobacteraceae* and insects that rely on sugar-based diets such as nectar and fruit sugars (Crotti *et al.*, 2010). The frequent occurrence of these bacteria in the insect digestive system was attributed to the aerobic environment, acidic pH, and presence of diet-derived sugars in this niche. Because known members of AAB are obligate aerobes (Kersters *et al.*, 2006), the hindguts of the honey bees are thought to be relatively aerobic compared to the midguts.

Eight OTUs were assigned to the genus *Lactobacillus* (Fig. 4, cluster 9) and occupied 0.7–2.9% in the larval guts, 17.0–19.2% in the midguts of adult honey bees, and 50.9–84.2% in the hindguts of adult honey bees (Fig. 1). The pair-wise similarities between these OTUs ranged from 82% to 98%. OTU24, OTU26, and OTU27 occupied 95.8–99.8% of the *Lactobacillus*-related sequences in the adult honey bee guts (Fig. 1). OTU26 and OTU27 showed a close phylogenetic association (Fig. 4, cluster 9), and closely related clones and isolates were frequently found in honey bees and bumble bees, and were present in high proportions in previous studies, indicating that these OTUs are important residents in the guts of honey bees. One strain isolated from *A. mellifera*, Bma5 (97% and 99% similar to OTU26 and OTU27, respectively), showed a strong inhibitory effect against *Paenibacillus larvae*, the causal agent of American foulbrood (AFB) (Olofsson and Vásquez, 2008; Forsgren *et al.*, 2010). Another putative *Lactobacillus* sp., OTU24, occupied 42% of the bacterial communities in the hindguts of *A. mellifera*, and was also detected in the guts of adult *A. cerana* (0.8–2.9%) (Fig. 1). Highly similar sequences (≥99%) were also recovered from honey bees in high frequencies in previous studies; a strain showing a high 16S rRNA gene similarity with OTU24 (99%), Hon2, has also been reported to have a strong inhibitory effect against *P. larvae* (Olofsson and Vásquez, 2008; Forsgren *et al.*, 2010). OTU22 and OTU29 were recovered only in *A. mellifera* guts (Fig. 1). OTU29 showed high 16S rRNA gene sequence similarities (≥99%) to *L. kunkeei* YH-15^T (Y11374) and *L. kunkeei* Fhon2, isolated from wine (Edwards *et al.*, 1998) and honey bees (Olofsson and Vásquez, 2008), respectively.

OTU30 occupied 40.2% and 10.1% in the midgut and hindgut of *A. cerana*, respectively, but was not detected in the guts of *A. mellifera*. OTU30 matched exactly with the 16S rRNA gene sequence of *Lactococcus lactis* subsp. *lactis* NCDO 604^T (AB100803) (Fig. 4, cluster 10). *L. lactis* is the most important organism in the manufacturing of fermented milk products; many strains have been isolated from fermented dairy products, raw milk, bovine intestine, and plant material (Klijn *et al.*, 1995; Teuber and Geis, 2006), and are also found in fish (Itoi *et al.*, 2008, 2009; Pérez *et al.*, 2011) and other insects (Schultz and Breznak, 1978; Bauer *et al.*, 2000; Graber and Breznak, 2005; Morales-Jiménez *et al.*, 2009). A previous study showed that *L. lactis*-related species predominate in the guts of termites (Bauer *et al.*, 2000). However, to our knowledge, this is the first report that *L. lactis*-related sequences have been recovered from the gut of honey bee. Hence, it is interesting how the presence of the close relatives of *L. lactis* in the guts of *A. cerana* sampled in this study affects the nutritional value of the honey and bee bread, because these honey bee products are considered a food product fermented by the lactic acid bacteria (LAB) in the honey bee stomach (Human and Nicolson, 2006; Vasquez and Olofsson, 2009).

OTU31 was affiliated with the genus *Spiroplasma* (Fig. 4, cluster 11). The major habitats of *Spiroplasma*, which belongs to the class *Mollicutes*, a wall-less and obligately parasitic group, are insects and plants (Brown, 2010). OTU31 exhibited 99% 16S rRNA gene similarity to *Spiroplasma apis* B31^T (M23937), which was the causal agent of a lethal infection of honey bees (known as May disease) (Mouches *et al.*, 1984). *S. apis* and *S. melliferum*, another honey bee pathogen, cross the insect-gut barrier, reach the haemolymph, multiply there, and kill the bees (Clark *et al.*, 1985; Bové, 1997). The OTU31 was recovered in higher frequencies from the adult *A. cerana* guts (2.1–4.8%) than from the adult *A. mellifera* guts (~0.2%) in this study (Fig. 1).

The *Lachnospiraceae*-related OTU33 (Fig. 4, cluster 13) was recovered in a significant proportion (21.9%) only from

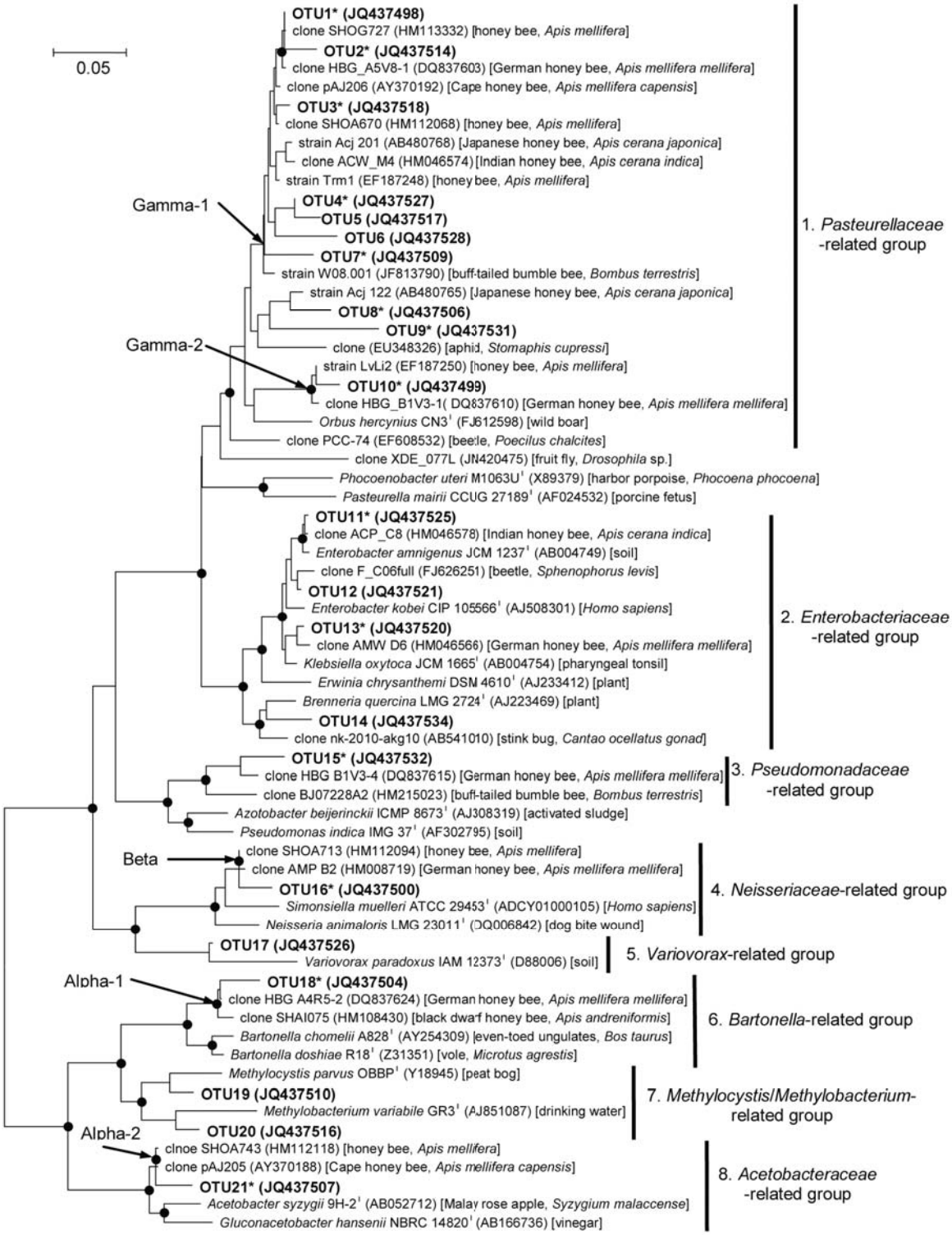

Fig. 3. Phylogenetic tree of sequences obtained in this study (in bold) affiliated with *Proteobacteria*, inferred by maximum-likelihood analysis. Only one representative sequence for each OTU was included in the tree. Asterisks indicate the high similarities (≥97%) to the bacterial 16S rRNA gene sequences obtained from honey bees or bumble bees in previous studies. Eight phylotypes defined in the previous study (Martinson *et al.*, 2011) are indicated at the corresponding nodes with arrows. The black spots on the tree nodes indicate bootstrap support above 90% based on 1,000 iterations. The scale bar indicates a 0.05 estimated change per nucleotide. The isolation sources or host names (common or scientific) are indicated in square brackets, if known.

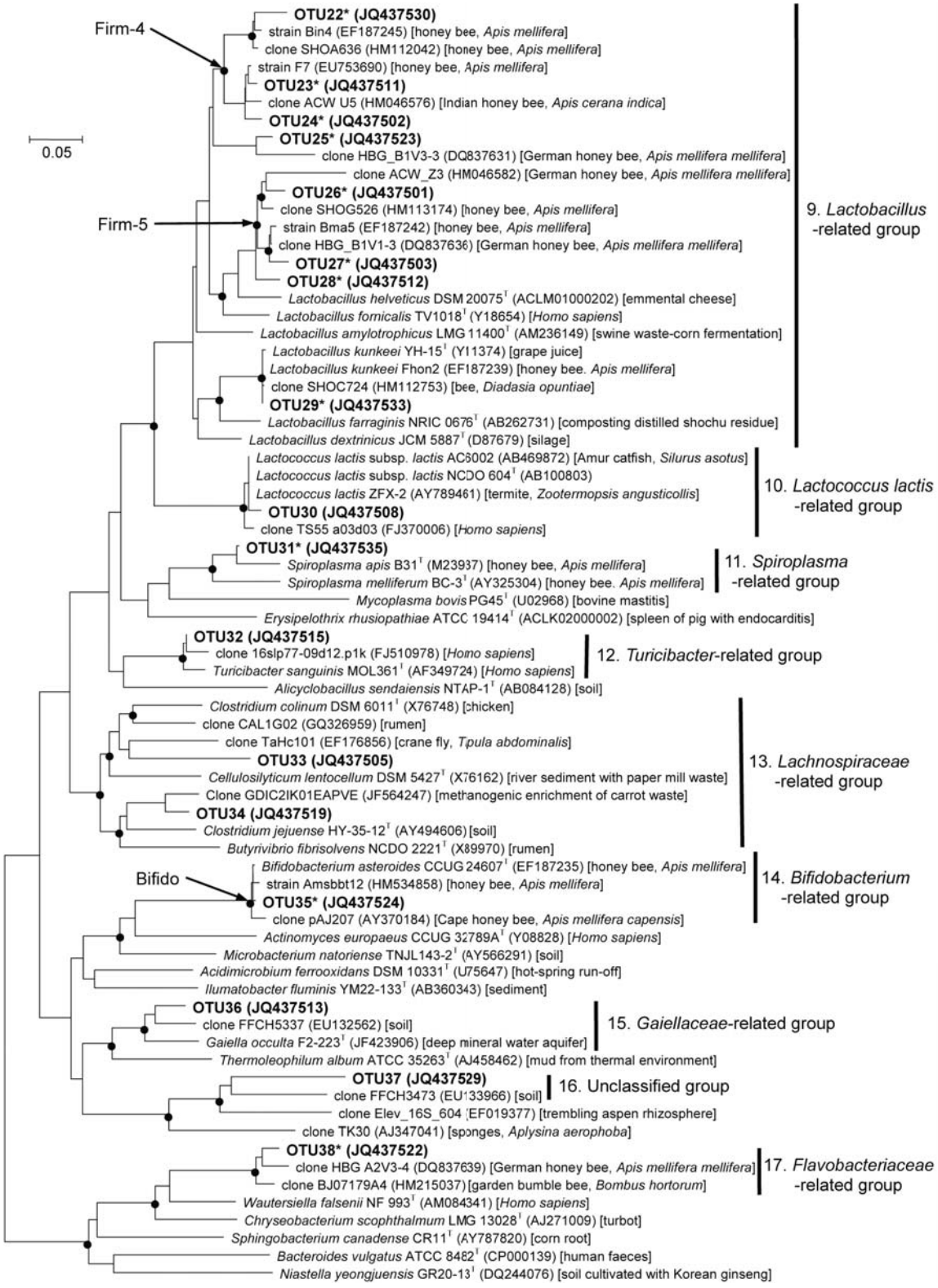

Fig. 4. Phylogenetic tree of sequences obtained in this study (in bold) affiliated with *Firmicutes*, *Tenericutes*, *Bacteroidetes*, and *Actinobacteria*, inferred by maximum-likelihood analysis. For further details, see the legend in Fig. 3.

the larval gut of *A. mellifera* (Fig. 1). The species of this family are obligate anaerobes (Rainey, 2009), and the environmental sequences affiliated with this family were found almost exclusively in the guts (Tamames *et al.*, 2010). The excretory tubules and the midguts of honey bee larvae are closed off until feeding is completed, and open just shortly before pupation for defecation (Winston, 1987). This may provide a relatively anaerobic condition in the larval gut compared to the adult gut.

OTU35 showed high 16S rRNA gene sequence similarities (≥99%) to *Bifidobacterium* spp. isolated from honey bees (Fig. 4, cluster 14) and occurred in higher frequencies in the guts of adult *A. mellifera* (1.6–3.9%) than in the guts of adult *A. cerana* (0.1–0.4%) (Fig. 1). Three *Bifidobacterium* species were isolated from the guts of honey bees, and *B. asteroids* (99% similar to OTU35) were the only species found in the *A. mellifera* gut (Biavati and Mattarelli, 2006). *Bifidobacterium* spp. are anaerobic, fermentative, and lactic acid-producing bacteria, although some species are tolerant to oxygen.

The *Flavobacteriaceae*-related OTU38 (Fig. 4, cluster 17) was recovered mainly from the guts of adult *A. cerana* (1.7–3.5%, Fig. 1). The close relatives to OTU38 (≥96%) in GenBank were recovered only from honey bees and bumble bees through culture-independent studies (Babendreier *et al.*, 2007; Koch and Schmid-Hempel, 2011a). Most members of the family *Flavobacteriaceae* are aerobic, although several species prefer microaerophilic conditions (Bernardet and Nakagawa, 2006).

The OTUs affiliated with *Variovorax*, *Methylocystis*, *Methylobacterium*, *Turicibacter*, or *Gaiellaceae* (Figs. 3 and 4) were recovered in low frequencies (≤0.4%) from the honey bee gut samples, and had no close relatives associated with honey bees in GenBank. Therefore, it is thought that these OTUs are not stable members in the guts of honey bees, but only transiently introduced with food or during the experimental procedure.

Discussion

All eight representative bacterial phylotypes harboured by the guts of honey bees from Australia, Germany, South Africa, Sweden, Switzerland, and the United States (Martinson *et al.*, 2011) were identified in the bacterial communities of the honey bees sampled in this study, most of them as dominant members. This result supports the previous hypothesis that honey bees have a co-evolved symbiotic relationship with a few specific bacterial groups (Martinson *et al.*, 2011).

Most notably, the *Pasteurellaceae*- and LAB-related OTUs were the most dominant in all the gut samples, occupying 58.6–89.3% of the gut bacterial communities (Fig. 1). The beneficial effects of LAB, including *Bifidobacterium* spp., on the health of their hosts have been widely studied, including the regulation of the gut microflora, production of antimicrobial substances, enhancement of the immune system, and production of vitamins (Biavati and Mattarelli, 2006), while almost nothing is known about the roles played by the *Pasteurellaceae*-related OTUs in honey bees (Koch and Schmid-Hempel, 2011b). However, these OTUs must have an inseparable association with insects including honey bees, considering that they were recovered almost exclusively from insect guts.

The above result also confirms the previous observation that the bacterial communities of honey bee guts are generally dominated by facultatively aerobic or aerotolerant bacteria, indicating that honey bee guts are not strictly anaerobic (Mohr and Tebbe, 2006). The putative strict anaerobes (*Lachnospiraceae*-related OTU33) or strict aerobes (*Acetobacteraceae*-related OTU21) were present only in specific gut samples, indicating that these gut samples may provide the corresponding conditions, at least within some parts of the gut samples.

Despite this general pattern of dominance, the relative abundances of some OTUs markedly differed from sample to sample, and several OTUs were sample-specific. Many factors such as the presence of specialised structures, pH, redox conditions, digestive enzymes, and type of food can affect the bacterial communities in the gut (Dillon and Dillon, 2004).

It is known that the eggs of honey bees are usually free of microorganisms because of the various antimicrobial systems of bees and their food (Gilliam, 1997). A recent work showed that most of the healthy *A. mellifera* larvae did not yield a PCR product with the universal bacterial primers while most of the larvae infected with European foulbrood yielded one (Martinson *et al.*, 2012). In this study, the bacterial sequences were recovered from the larval guts although the number of OTUs in the larval guts was smaller than in the adult guts (Table 1). One explanation for the difference is that the honey bee colonies used in this study might not be in a good health status and the antimicrobial systems might not work properly (also see below). Otherwise, the larvae might be in process of acquiring the gut microbiota. In such a case, the bacterial diversity in the larval guts is thought to increase as they grow through contact with nurse bees and ingestion of contaminated food.

The abundances of the *Pasteurellaceae*-related OTUs were higher in the larval guts, while those of the LAB-related OTUs were generally higher in the adult guts (Fig. 1). The relatively high abundances of the LAB-related OTUs in adult honey bees in this study are in accordance with previous studies (Mohr and Tebbe, 2006; Disayathanoowat *et al.*, 2012). Mohr and Tebbe (2006) attributed this observation to the difference in the major food source (pollen for larvae and nectar for adults) and more acidic pH conditions in adult guts than in larval guts. The latter hypothesis is supported by the presence of the *Acetobacteraceae*-related OTU21, another acidophilic group, in adult guts and their absence in larval guts (Fig. 1).

It may be important to identify the reason for the low occurrence of LAB in the larval guts because colonisation of the larval midgut is thought to be one of the key steps in the pathogenesis of *Paenibacillus larvae*, and honey bee LAB showed strong antagonistic effects against this pathogen (Forsgren *et al.*, 2010), but some members of the *Pasteurellaceae*-related OTUs did not (Yoshiyama and Kimura, 2009). Chandler *et al.* (2011) observed that the *Pasteurellaceae*-related OTUs and the LAB-related OTUs are also the dominant bacterial members in the guts of *Drosophila* sp., and these

two groups generally show a pattern of reciprocal abundance, suggesting competitive interaction between them.

Ultra-deep sequencing performed in this study also showed the presence of bacterial groups specific for a honey bee species. *Bartonella*-, *Lactococcus lactis*-, *Spiroplasma*-, *Flavobacteriaceae*-, and *Enterobacteriaceae*-related OTUs were detected exclusively, or in much higher frequencies, in the *A. cerana* guts, while the frequencies of *Lachnospiraceae*-, *Neisseriaceae*-, and *Bifidobacterium*-related OTUs were higher in the *A. mellifera* guts (Fig. 1). *L. lactis* and *Bifidobacterium* spp. are generally considered to be non-pathogenic and beneficial to their hosts. The pathogenicities of the other OTUs require more studies. Runckel *et al.* (2011) detected the sequences of *S. apis* and *S. melliferum* in the honey bees, which coincided with the high load of the putative pathogenic microsporidia *Nosema* and the presence of several common honey bee viruses. We observed several symptoms of sacbrood, a viral disease, which was prevalent throughout the country at the sampling time, only in the hive of *Apis cerana*. The higher frequencies of the *Spiroplasma*-related OTU and *Enterobacteriaceae*-related OTUs, and the lower frequency of *Bifidobacterium*-related OTU in adult *A. cerana* guts compared with adult *A. mellifera* guts may be associated with this disease, considering their pathogenic or beneficial effects on the hosts. This suggests that the gut bacterial community structure may be used as an indicator of the health status of honey bees. It will be interesting to study whether the transfer of the putative beneficial bacterial groups to the honey bee species that do not harbour those groups may improve the health status of the honey bee species or the quality of the honey, for example, the transfer of LAB-related OTUs to the larvae.

Although the present study was based on a one-time sampling, it substantiated the previous observation that honey bee guts are dominated by several specific bacterial groups, and also showed that the relative abundances of some OTUs could be markedly changed depending on the developmental stage, the location within the gut, and the honey bee species. Continuous monitoring of the gut bacterial communities of honey bees is needed to explain the community variations, with a particular emphasis on the relationship between the gut bacterial community and honey bee health. In addition, the isolation and characterisation of yet uncultured bacteria in the honey bee guts will be required to use them as a "probiotic" for honey bees.

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