Gut Bacterial Inhabitants of Open Nested Honey Bee, Apis Florea

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Abstract

Honey bees are complex social system, which are highly dynamic having close interactions with their surrounding environment. Gut microbiota of honey bees has a major role in interaction behavior with its environment and health. *Apis florea* is the primitive among all the honey bees and are indigenous to Indian subcontinent. The study reports the identification and analysis of bacteria in the gut of wild species of honey bee, *Apis florea*, by culture-based and culture-independent methods. Cultured bacteria were identified and characterized by MALDI-TOF MS and 16S rRNA sequencing. A comprehensive analysis and identification of non-culturables bacteria were performed by 16S rRNA amplicon next generation sequencing. The sequence analysis approach classified gut bacteria into 5 bacterial phyla, 8 families and 10 genera in major. The dominant bacterial taxa identified in *Apis florea* belonged to Prevotellaceae (52.1%), Enterobacteriaceae (42.7%) and Halobacteriaceae (1.3%). The dominant bacteria belonged to genera of *Prevotella*, *Escherichia-Shigella*, *Natronomonas*, *Methylobacterium*, *Pantoea*, *Bifidobacterium*, *Enterobacter*, *Klebsiella*, *Lactobacillus* and *Nitrobacter* belonging to phyla Bacteroidetes, Proteobacteria, Euryarchaeota, Actinobacteria, and Firmicutes. Many of these bacteria identified herewith are not reported for their occurrence in others species of *Apis* genus making this study of highly relevance with respect to bee microbiome.

Introduction

Honey bees are complex social system, which are highly dynamic having close interactions with their surrounding environment making them superorganisms. Fossil records indicate that among all other living species of *Apis*, *Apis florea* is the most primitive. This is substantiated by their colony size, open nest and behavioral pattern (Kaspi and Shafir 2013; Radloff et al. 2005). The evolutionary divergence of *Apis florea* from the common ancestor is much earlier from the remaining *Apis* clades (Biewer et al. 2016). *Apis florea* is prevalent and restricted to south and southeastern part of Asia. It is commonly known as dwarf bee or little bee and survives temperature up to 50 °C (Biewer et al. 2016). The honey production by these little bees is less in quantity and often consumed by them hence, not reared for commercial purpose. Contribution of *Apis florea* towards pollination is agriculturally and ecologically significant rather than role in producing honey. Depending upon the forage availability, *A. florea* often migrates between plains and adjacent low hills during seasonal variations. The species prefer warmer climate for their foraging activity (Balachandra et al. 1999). *A. florea* species functions as an important pollinator and recent times have experienced a drastic decline in the population. Various reasons for extreme honey bee losses have been proposed, which include indiscriminate use of toxic pesticides, poor nourishment, genetic diversity, parasites and microbial pathogens (Anjum et al. 2018).

Accumulating evidence suggests a crucial role between the host-microbe interactions (Saraithong et al. 2015). Microbes contribute to functional capabilities to the host by providing essential nutrients and immunological defense to the honey bee health. The diversity and sociality affect an organism's physiological and behavioral adaptations. Gut microbiome in the honey bee gut help to protect against attacking pathogens (Kwong and Moran 2016). Some studies showed that relative abundance of core gut species is having a direct impact on the susceptibility to various pathogens (Raymann and Moran 2018). The delineation of functions attributed by the gut bacteria to the host are addressed by studying the interactions between gut bacteria and the host. Gut bacterial diversity in honey bee belonging to *A. mellifera* (Western honey bee) is well demonstrated. Different social corbiculate bees possess highly characteristic gut communities. These distinct gut communities in the host are largely independent of the geographical occurrence of the host (Kwong and Moran 2017; Moran et al. 2012; Martinson et al. 2011). However, high strain level diversity in the gut microbiota of *A. mellifera* and *A. cerana* are reported, opening insights into the adaptability of honey bees to local conditions (Ellegaard et al. 2020).
For majority of the wild bee species, composition and function of microbiome is largely unknown and remains to be elucidated (Engel et al. 2016). The need of such study arises as the gut microbiome has key roles in host health which will extricate the relationship between host fitness in both managed and wild pollinator bees (Engel et al. 2016). Reports on exploring the gut microbiota are surging linearly; however, there is lack of information on the gut microbiota of A. florea. A. florea being an important contributor for crop pollination, the aim of the study was to explore and characterize the gut microbiota in the whole alimentary canal of wild species of A. florea. For the culture dependent method, isolates were subjected to MALDI-TOF-MS and 16S rRNA sequencing analysis and to characterize the non-culturable organisms, high throughput sequencing techniques were used. The study provides insights about this primitive clade of Apis whose composition can be compared with the core bacteria reported for other Apis species. This study adds on to the knowledge of Bee microbiome which is associated to unravel the evolution and ecology of host-microbiome interactions (Engel et al. 2016).

Methods

Sample collection and dissection of the bees

To study the cultivable and uncultivable honey bee gut bacteria, a total of eighty two worker honey bees (A. florea) were collected from Mavina Halla Forest, Karnataka, located in Western Ghats forest of India (Latitude:12.55821DMS N 12° 33' 29.556", Longitude:75.95338DMS E 75° 57' 12.167°). On the same day, live bees were transported to the laboratory in small cages; whole bees were cold anesthetized and were surface-sterilized with 7% sodium hypochlorite and 70% ethanol in sterile falcon tubes (Inglis et al. 2012), followed by four times wash with sterile 1xphosphate-buffered saline (PBS), followed by dissection. The whole alimentary canal of bees was aseptically dissected on slides using normal saline (0.9%) by clipping the stinger with sterile forceps. The dissected guts were transferred to 1 ml of PBS and immediately stored at -20 °C until further experiments.

Culturing of bacteria

The bee gut samples (20) were homogenized using micropestle. Different dilutions (i.e., 1/10, 1/100 and 1/1000) of this composite homogenate were made and 100 µl aliquots each of the diluted sample were inoculated into six different media procured from HiMedia, Mumbai, India: Nutrient Agar (NA), MRS Agar (MRS), Brain Heart Infusion Agar (BHI), Eosin-methylene blue (EMB), Luria Bertani (LB) and Gluconobacter agar (GB) and incubated for 24–72 h at 30 °C. The bacterial colonies grown on the plates were enumerated and selected, based on different morphologies. The separated colonies in master plates were repetitively sub-cultured to obtain pure colonies of bacteria.

Culture-Based methods

MALDI-TOF MS Based Characterization

A simple extraction protocol was employed to analyze the bacterial sample. Loopful of bacterial cultures were mixed thoroughly with ethanol (70% v/v) and the suspended cells were centrifuged at 12,000 rpm for 5 min. The pellet was recovered by discarding the supernatant carefully. The pellet was air dried at room temperature and resuspended in formic acid (70% v/v) by vigorous mixing followed by the addition of acetonitrile. The mixture was centrifuged at 12,000 rpm to separate the pellet and 1 µl of clear supernatant was placed on a MALDI target plate. The bacterial smear was overlaid with 1 ml saturated solution of alpha-cyano-4-hydroxycinnamic acid (HCCA) matrix prepared in acetonitrile (50%) and trifluoroacetic acid (2.5%) and allowed to dry at room temperature. The extracted samples were analyzed using Autoflex speed system (Bruker Daltonik GmbH, Germany). Mass spectra were obtained in a mode of linear positive ion extraction at a laser frequency of 1000 Hz within a mass range from 2k to 20kDa. The ion source 1
voltage was 19.5 kV, ion source 2 voltage was maintained at 18.2 kV, lens voltage at 7 kV, and the extraction delay time was 240 ns. Calibration of spectra was done externally by using the standard calibration mixture (E.coli extracts including RNase A and myoglobin as additional proteins, Bruker Daltonics). The MALDI Biotyper software 3.0 (Bruker Daltonik) was used to identify the bacterial isolates and to visualize the mass spectra. Species-level identity has been considered for the isolates with biotyper score value ≥2.0, while the analysis for the isolates with score value ranging from 1.7 to 1.99 was repeated to achieve the higher score values (Kurli et al. 2018). The isolates which were not identified up to species level and with biotyper score value <2.0 were subjected for 16S rRNA sequencing.

Colony PCR and 16S rRNA sequencing

The 16S rRNA gene sequence was amplified using universal primers (8F: 5’-AGAGTTTGATCCTG GCTCAG -3’ and 1391R: 5’-GACGGGCGGTGTGTRCA -3’) according to the method described by Turner et al. (1999). These primers are specific for conserved regions of bacterial 16S ribosomal RNA. PCR conditions are as follows; Step.1: pre-denaturation-94 °C, 5 min; denaturation-94°C, 5 min; annealing-55 °C, 1 min; extension-72 °C, 1.30 min; Step.2: final extension – 72°C, 7 min; stored at 4 °C. PCR products (3 µl) were subjected to electrophoresis and 22 µl was purified by PEG-NaCl method. The purified PCR products were sequenced using ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing kit on a 3730xl Genetic Analyzer (Thermo Fisher Scientific®, UK).

Sequence analysis

The sequence data obtained were assembled and analyzed using DNA sequence assembling software Lasergene SeqMan Pro (DNASTAR Inc.). The 16S rRNA sequence of each bacterial isolate was compared using BLAST (Camacho et al. 2009) against 16S ribosomal RNA sequences (Bacteria and Archaea) database (a subdivision of GenBank).

Culture- Independent methods

Next generation sequencing

Community DNA extraction

The honey bees were surface sterilized and, the alimentary tract of worker honey bees (60) were collected in 1.5 mL of phosphate buffered saline. To this 200 µL of cell lysis buffer (ATL buffer, Genomic DNA extraction, Qiagen Tissue kit) was added and subjected to homogenization in the presence of glass beads using the temperature controlled (~22 °C) vortex shaker for 30 min. Twenty microlitre of proteinase K was added and further steps followed were according to manufacturer's protocol. The concentration of DNA was measured using Qubit HS DNA kit (Invitrogen, USA) and stored at −20 °C till further processing.

Amplicon sequencing

To investigate bacterial diversity of honey bee gut, NGS library preparation was carried out by targeting V4 region of 16S rRNA gene using primers 515F (F515: 5’-GTGCCAGCMGCCGCGGTAA-3’) and 806R (806R: 5’-GGACTACHVGGGTWTCTAAT-3’) (Kumbhare et al. 2015; Caporaso et al. 2010a). The resultant libraries were purified, pooled in equimolar concentration and sequences using 2 x 250bp v2 Chemistry on Illumina MiSeq platform.

Bioinformatics analysis

The raw reads obtained from the high-throughput sequencing were analyzed using QIIME1 pipeline (Caporaso et al. 2010b). The OTU picking was carried out by reference based OTU picking method against the SILVA (v132) (Edgar
The taxonomic assignment was performed using SILVA (v132) raw taxonomy. Alpha diversity indices were assessed by richness like Chao1, ACE and diversity (Shannon) were calculated via QIIME, which predisposes sample rarefaction to the same sequencing depth (Bokulich 2012).

## Results And Discussion

The global decline in the population of pollinator bees (Potts et al. 2010) has attracted the researchers for a comprehensive study of host microbiome community. As major contributors in pollination, honey bees are very crucial organisms in securing the agricultural produce and the maintaining ecosystem. Unlike western bees, *A. florea* are not reared for commercial honey or wax; rather they occur in wild functioning as major pollinators (Balachandra et al. 1999). These little dwarf bees differ from western bees in their defense against pathogens (Suwannapong et al. 2011). In the present study, Asiatic bees, *A. florea* were selected to study and analyze the gut microbiome. The bee symbionts are likely to play a vital role in self defense and metabolism. *Apis florea*, (dwarf bee) is naturally distributed in Indian subcontinent throughout south-east Asia. The most important contribution of this honeybee is its valuable pollination of many fruit plants and diversified flora in tropical ecosystems (Soman and Chawda 1996).

The defensive and metabolic capabilities of bees are highly correlated with gut microbiome interaction and to further add knowledge on role and interaction of microbial community with host, the analysis of gut bacteria of *Apis florea* honey bees was carried out. Healthy bees were obtained directly from single hive across Western Ghats forest area of Kodagu district, which is recognised as a global biodiversity hotspot, India. The entire thorax and abdomen was processed for analysis, thus including gut microbes and organisms attached to hemolymph or tissues. The analysis of gut bacteria was performed by culture dependent and culture-independent technique from a total of 80 worker dwarf honey bees.

In culture dependent method, the identification and characterization of culturable diversity of bee gut bacteria was done by MALDI-TOF-MS and 16S rRNA gene sequencing analysis. A total of 91 aerobic and facultative anaerobic bacteria were isolated from guts of worker *A. florea* bees. Based on colony characteristics, the bacterial isolates were initially subjected to MALDI-TOF-MS. Fifty six isolates were identified up to species level. The isolates with biotyper score value <2.0 could not be identified up to species level. Remaining thirty-five such isolates which were not identified up to species level from MALDI-TOF-MS were further subjected to 16S rRNA sequencing. Collectively from both MALDI-TOF-MS and 16S rRNA sequencing of culture dependent analysis, the gut bacterial isolates belonging to three bacterial phyla/classes were identified; alpha-Proteobacteria (1%), Firmicutes (19%) and Gamma-Proteobacteria (80%) (Fig. 1). The percentage of isolates belonging to genera, *Klebsiella*, *Enterobacter*, *Bacillus*, *Citrobacter*, *Staphylococcus* and *Lactobacillus* are represented in Fig. 2. These isolates were found to be common in *Apis* clade as reported in previous work (Martinson et al. 2012; Yoshiyama and Kimura 2009; Khan et al. 2017). The presence of a diverse group of bacteria including the phyla Firmicutes, alpha and beta-Proteobacteria suggests their ecological importance (Kwong and Moran 2017). Most of these bacteria belonging to these phyla are facultative anaerobes, ferment sugars and tolerant to acidic environment. The bacterial isolates identified are common in soil, water and few of them are organisms of clinical relevance in humans. The occurrence of such bacteria in insect gut is not unusual and is described before. *Staphylococcus*, *Enterobacter* and *Klebsiella* found in abundance in gut of dwarf bee are typical inhabitants in human. These bacteria are considered as beneficial healthy gut microbiota in humans, insects and other animals as they are the fermenters of sugar and involved in the host defense mechanism (Anderson et al. 2011). *Klebsiella oxytoca*, a prominent organism considered as probiotic, positively affects the health of host by suppressing parasite colonization (Engel et al. 2013). In termite, the role of *Staphylococcus* probably is involved in degradation of cellulose (Sarkar et al. 1986). *Klebsiella* spp (*K. oxytoca*) and *Pantoea agglomerans* were predominantly found in our analysis. *Pantoea agglomerans* is significantly present in desert locust, *Schistocerca*
gregaria, and are involved in breakdown of dietary components leading to synthesis of aggregation pheromones that function in swarming behaviour of locusts (Dillon et al. 2002). It is reported that these organisms gain entry into the bee gut during foraging activity (Loncaric et al. 2009). Bacillus safensis, B. kochii and B. halotolerans were identified in the gut of dwarf bee. Bacillus safensis, B. kochii and B. halotolerans are reported to exist and survive in extreme environment and under stress conditions (Lateef et al. 2013, Zhang et al. 2018, Seiler et al. 2012). In two separate studies on profiling of bacterial community carried out in the gut of flesh flies and floral nectar, Bacillus safensis was identified and isolated (Gupta et al. 2014, Fridman et al. 2012). Desert locust Schistocerca gregaria, inhabits Bacillus safensis which possess high cellulolytic activity indicating the metabolic significance of this bacteria (Nelson et al. 2021). Strains of Bacillus safensis is reported to produce many industrially relevant enzymes, such as amylase, protease, lipase, inulinase and chitinase. The occurrence of Brevundimonas nasdae was prominent in our study. The pH of honey bee midgut is around 8 and this pH favors the optimal growth of Bacillus species and Brevundimonas nasdae and these may aid in degrading of carbohydrate fed by the bees. In addition to Brevundimonas nasdae (Phylum: Proteobacteria), Bacillus species and, Solibacillus silvestris (Phylum: Firmicutes) were predominant in the gut of A. florea. These bacterial species are rarely or no where reported in the available literature on the Apis gut microbiota of other species, whereas interestingly occur in Apis mellifera. The differences in the occurrence of these bacteria might be due to geographical location or existing characteristics of the environment or may be due to the feature of A. florea species itself, which requires further studies.

In culture-independent studies, a comprehensive microbial diversity analysis using high throughput sequencing approach was carried out. Bacterial community profile of honey bee gut as revealed by 16S rRNA gene amplicon sequencing, yielded 15 bacterial phyla representing collective phyla in the gut of A. florea (Fig. 3a). Bacterial phyla distributions were as follows; Bacteroidetes (51.3%), Proteobacteria (45.1%), Euryarchaeota (1.3%), Actinobacteria (1.1%), Firmicutes (0.9%) and the rest (0.3%) constituted the minor phyla. The abundance of ten minor phyla (Acidobacteria, Tectomicrobia, Chloroflexi, Lentisphaerae, Verrucomicrobia, Cyanobacteria, Planctomycetes, Nitrospirae, Tenericutes and Saccharibacteria) was very less; hence the percentage of composition is not shown in Fig. 3a. At family level, OTUs with ≥ 0.3% abundance were filtered (Fig. 3b). The family level distribution of bacteria in the gut of A. florea were Prevotellaceae (52.1%), Enterobacteriaceae (42.7%), Halobacteriaceae (1.3%), Methylobacteriaceae (1.2%), Bifidobacteriaceae (0.9%), Orbaceae (0.4%), Lactobacillaceae (0.4%) and Halomonadaceae (0.4%). Composition percentage of bacterial family which was less than 0.9% is not shown in Fig. 3b.

At the genus level, OTUs with ≥ 0.3% abundance were filtered and distribution of bacterial genera in the gut of A. florea is represented as diagram in Fig. 4 and are as follows; Prevotella (59.3%), Escherichia-Shigella (33.3%), Natronomonas (1.5%), Methylobacterium (1.4%), Pantoea (1.1%), Bifidobacterium (1%), Enterobacter (0.9%), Klebsiella (0.6%), Lactobacillus (0.5%) and Nitrobacter (<0.3%). The composition percentage of bacterial genera which was less than 0.3% was not shown in Fig. 4. Amongst these, Bacteroidetes and Proteobacteria were the predominant phyla. Euryarchaeota are highly diverse and are often found in intestine, which are rarely mentioned in literature on bee gut microbiota. Along with Euryarchaeota, Tectomicrobia, Chloroflexi, Lentisphaerae and Verrucomicrobia are few of the gut symbionts, which are found in A. florea, unmentioned elsewhere in the literature on honey bee gut microbiota.

Prevotella, Escherichia-Shigella, Natronomonas, Methylobacterium, Pantoea, Bifidobacterium, Enterobacter, Klebsiella and Lactobacillus are the dominant genera found in the gut of A. florea worker bees. The bacterial genera Prevotella, Natronomonas etc. are uncultivable under typical laboratory conditions as they require strict anaerobic conditions or haloalkaliphilic conditions. Prevotella, Natronomonas and Methylobacterium are not reported in the available literature of honey bee gut microbiota, however, Methylobacterium and Prevotella are the predominant inhabitants of...
gut in bark beetle, (*Dendroctonus rhizophagus*) (Briones-Roblero et al. 2017). Species of *Prevotella*, are non-cellulolytic carbohydrate degrading bacteria, which bring about digestion of cell wall polysaccharides like xylan (Flint et al. 2012). A relatively lower proportion of *Brevundimonas, Staphylococcus, Streptococcus, Gluconobacter* and *Gilliamella* genera was observed in our culture independent studies, whereas many of these are predominant in *Apis mellifera* (Kwong and Moran 2016). Functional redundancy and crosstalk among the microbes, and host has huge metabolic and physiological impact and, the significance of the presence of these bacterial communities can be untangled by further metagenomic and metatranscriptomic studies.

High throughput sequencing and quality trimming of 16S rRNA gene yielded ~0.118 million quality reads which were used for subsequent analysis. Taxonomic assignment of sequences with the reference database resulted in 589 operational taxonomic units (OTUs). Alpha diversity estimation of gut of *A. florea* using species richness and non-parametric Shannon index suggested higher bacterial diversity in *A. florea* worker bees. The alpha diversity index which is an indicator of bacterial diversity, were calculated for *A. florea* and is given in Table 1. Shannon index for bacterial communities was 3.121 and this observation is suggestive of richness in bacterial diversity in the gut of *A. florea*.

16S rRNA gene sequences of the bacterial isolates of *A. florea* was used to construct the phylogenetic tree showing relationship among the bacteria with reference strains of GenBank (Fig. 5). The bacterial populations in the gut were diverse among forager bees of *A. florea* which belonged to phyla Firmicutes, alpha and beta-Proteobacteria. A plethora of bacterial abundance in any niche suggests their significance in ecological diversity; in *A. florea, Prevotella* was a significant member accounting for 59% of the total gut microbe indicating the possible ecological importance.

Dominant gut inhabitants of *A. mellifera* belong to phyla Firmicutes, Actinobacteria and Proteobacteria (Kwong and Moran 2016; Romero et al. 2019; Ahn et al. 2012). The bee gut microbial communities in *A. mellifera* are specific and are dominated by nine bacterial species clusters viz., *Bartonella apis, Parasaccharibacter apium, Frischella perrara, S. alvi, Gilliamella apicola, Bifidobacterium* spp., *Lactobacillus* Firm-4, *Lactobacillus* Firm-5 and these are believed to impart social behavior among individuals (Kwong and Moran 2016). *Snodgrassella alvi* and *Gilliamella apicola* are ubiquitous in the gut of *A. mellifera* (Kwong and Moran 2012). In our study, *Snodgrassella alvi* was not detected whereas, *Gilliamella apicola* were found in traces. *Acinetobacter* was found in our study similar to the reports of Kim et al (2014). *Acinetobacter apis* spp. nov., was isolated from the intestinal tract of a honey bee, *A. mellifera* (Kim et al. 2014). In the metagenomic survey, the class Alpha Proteobacteria and Gamma Proteobacteria dominated the gut environment of *A. mellifera* (Engel and Moran 2013a). In *A. florea*, Bacteriodetes, Proteobacteria, Euryarcheota, Actinobacteria, Firmicutes and Acidobacteria were the predominant phyla in our study. *Citrobacter* spp., *Providencia vermicola, Planomicrobiu m keanokoites* and *Exiguobacterium acetylicum* were reported for the first time in the genus *Apis* by culture dependent 16S rRNA sequencing (Khan et al. 2017). Pyrosequencing analysis of the bacterial community structure in the midguts and hindguts of the adult honeybees of *A. cerana* and *A. mellifera* were studied. Higher frequencies of *Enterobacteriaceae, Lactococcus, Bartonella, Spiroplasma*, and *Flavobacteriaceae*-related OTUs were found in the guts of *A. cerana* while *Bifidobacterium* and *Lachnospiraceae*-related OTUs were more abundant in guts of *A. mellifera* (Ahn et al. 2012). Anjum et al. (2018) reported Firmicutes (60%), Proteobacteria (26%) and Actinobacteria (14%) in *A. mellifera* gut by 16S rDNA sequencing.

The dominant phyla, Proteobacteria and Firmicutes are reported in eusocial wasps (Order: Hymenoptera) inhabiting eastern and southern Asian region (Suenami et al. 2019). A variety of bacterial phyla are commonly present in insect guts, including Gammaproteobacteria, Alpha-Proteobacteria, Beta-Proteobacteria, Bacteroidetes, Firmicutes including Lactobacillus, and Bacillus species, Clostridia, Actinomycetes, Spirochetes, Verrucomicrobia, Actinobacteria, and others (Colman et al. 2012). Numerous non-culture-based studies show that dominant taxa in *Drosophila*
melanogaster are influenced by diet and vary among laboratories however, certain taxa recur (Broderick and Lemaitre 2012). The gut inhabitants of honey bees are coevolved with bumble bees and comprise a distinctive gut community (Martinson et al. 2011). The wild flies have distinct bacterial communities and more diversification from those of reared species (Chandler et al. 2011), including bee gut bacteria (Kwong and Moran 2016; Engel et al. 2013). In major, gut flora of insects, contribute to metabolism, nutrition, immune modulation and protection against foreign entities (Engel and Moran 2013b). Understanding the bacterial network offers knowledge into bee pathology, host-microbe interaction and aid in improving honey bee health and to discover new sources of biotechnologically potential molecules and enzymes (Romero et al. 2019).

Conclusion

In conclusion, the gut communities of A. florea are more diverse in composition and the sequence analysis approach classified gut bacteria into Bacteroidetes, Proteobacteria, Euryarchaeota, Actinobacteria, and Firmicutes as the major phyla. Most of the isolates found are opportunistic and beneficial gut inhabitants. Prevalence of members of genera Prevotella (59%) is observed in our study which is different in abundance against other species of Apis clade. Sequencing the whole gut using NGS has allowed us to analyze the importance of microbiome role in A. florea host to some extent. Likewise, with other microbiome project (human, animal and insects), this work provides additional information and data pertaining to the microbiome of A. florea, a primitive clade of honey bee, revealing some of the bacterial cobionts not found in earlier reports on honey bee microbiome project. However, further genomic studies are required to study the relevance for diversified microbial occurrence and a comparative analysis of managed and wild species may offer insight about host-microbe interaction.

Declarations

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Conflicts of interest

The authors declare no conflicts of interest.

Ethics statement

Honey bees are invertebrates; hence no ethics approval is required.

Data availability

The sequences obtained from Sanger sequencing is submitted to NCBI, which are available under Bio Project SUB6349615, MN512276:MN512310[accn].

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Table

Table 1. Alpha diversity estimation. Non-parametric alpha diversity was calculated for *A. florea* gut bacteria.

|                      | Chao1 | Observed OTUs | Shannon | ACE   |
|----------------------|-------|---------------|---------|-------|
| Gut bacteria of *A. florea* | 850.84 | 589           | 3.121   | 858.48 |

Figures

Figure 1

Phyla-wise classification of culturable gut bacterial flora of *A. florea*. The gut bacterial isolates were classified based on MALDI-TOF-MS and 16S rRNA gene sequencing data.
Genera-wise classification of culturable gut bacterial flora of A. florea. The gut bacterial isolates were classified based on MALDI-TOF-MS and 16S rRNA gene sequencing data.
Figure 3

Next generation sequencing of culture independent gut bacterial flora of A. florea. Taxa distributions of (a) phylum and (b) family at different phylogenetic level of honey bee gut bacterial flora.
Figure 4

Next generation sequencing of culture independent gut bacterial flora of A. florea. Taxa distribution of genus at different phylogenetic level of honey bee gut bacterial flora

Figure 5

Molecular Phylogenetic analysis by Maximum Likelihood method The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-5509.8000) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 39 nucleotide
sequences. All positions containing gaps and missing data were eliminated. There were a total of 805 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).