Divergence of Cultivable Bacteria Associated with Larvae and Adult *Bactrocera dorsalis* (Diptera: Tephritidae) Laboratory-Reared Strains

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Authors’ contributions

This work was carried out in collaboration among all authors. Author CYN conceived research. Author MA designed the protocol, conducted the experiments and wrote the first draft of the manuscript. Authors XR, YW, XQ and SC analyzed data. Authors OT, KMML and RANN edited and critically revised the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Insects entertain intricate and mutualistic relationship with an array of microorganisms, which significantly influence their fitness, ecology and evolution. In recent decades, there has been increasing interest toward studying the effects of microbiome on many host insects (Dipterans, Lepidopterans, and Coleopterans). Studies so far realized indicate that gut microbiome contribute to host nutritional ecology, defense, immunity and lifespan.

*Bactrocera dorsalis* (Tephritidae: Diptera) is a polyphagous fruit fly which attacks a huge variety of fruits and vegetables worldwide and has been placed as a quarantine species by many countries. To
investigate the specific functions of the gut endosymbionts, it is a prerequisite to know the composition of gut bacterial communities whose manipulation will help to decipher their ecological relevance. Here, we used the culture-dependent technique to isolate and identify gut bacteria from *B. dorsalis* at different developmental stages. The results revealed 11 bacterial species from the third instar larvae, 18 and 12 from female and male populations, respectively. These bacteria were assigned to six families, namely, Enterobacteriaceae, Enterococcaceae, Staphylococcaceae, Streptococcaceae, Micrococaceae and Bacillaceae. Bacterial species from these families were differentially represented in various samples, except *Klebsiella oxytoca*, *Enterobacter cloacae*, *Pantoaea dispers* and *Enterococcus faecalis* that were detected at all developmental stages. Overall, Enterobacteriaceae was the most dominant family in females and third instar larvae accounting for 57.89% and 26.32%, respectively, while Enterococcaceae was dominant in males with 75% of the total bacterial taxa. These results suggest that *B. dorsalis* possesses a huge variety of cultivable bacteria that could be used to explore their specific functions on host physiology and fitness.

**Keywords**: *Bactrocera dorsalis*; culture-dependent technique; gut bacteria; symbiosis.

1. INTRODUCTION

Insects harbor a variety of bacteria in their intestinal tracts with which they share multiple and long evolutionary interactions [1,2]. Several previous studies highlighted the importance of gut bacteria in the fitness and reproduction of their host flies in a reciprocal beneficial fashion [3].

The oriental fruit fly *Bactrocera dorsalis* is one of the most invasive, multivoltine and polyphagous fruit flies from the Tephritidae family, which causes considerable loss of cultivated crops in most western and eastern parts [4,5] and attack over 350 hosts worldwide [6]. The recurrence of damage and the continually rising economic losses caused attention around the world to initiate studies for better management of this pest and to rescue crops.

The analysis of the intestinal bacteria from Tephritid fruit flies revealed a huge variety of bacterial communities from Enterobacteriaceae and Enterococcaceae families [4,7] and the advent of high throughput sequencing technology had allowed the characterization and identification of gut bacteria at the taxonomic level. The most commonly described genera are *Klebsiella*, *Citrobacter*, *Enterobacter*, *Pantoaea*, etc. which appear to play important ecological and physiological roles in host biology [4,7,8]. For example, the host flies offer shelter to bacteria, which in turn release readily available sources of nitrogen and essential amino acids (which they are unable to synthesize by themselves) through its metabolic activities (carbon and nitrogen cycle) [9,10]. Their interactions can be mutualistic (termites and their gut-associated bacteria) [11], parasitic (communication of the American foulbrood bacterium with the honeybees) or commensals [12].

Although the molecular sequencing techniques based on the 16s rRNA gene (e.g., polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) & 454-pyrosequencing) have significantly improved our understanding and estimation of insect gut microbiota (composition & diversity), there is still a gap toward understanding the specific function of core or transient bacteria in their interaction with the host. Over the past decades, the use of culture-dependent technique through the isolation and identification of insect gut bacteria yielded conclusive understanding of their functions and contribution to the host’s fitness and physiology. For example, gut bacteria was reported to increase host tolerance to pathogens [13], enhance host resistance to environmental stresses [14], and improve its social cohesions [15]. Bacteria isolates from tephritid fruit flies (*Candidatus Erwinia dacicola*, *Klebsiella oxytoca*, *Citrobacter freundii*, *Enterobacter cloacae*, *Enterococcus faecalis* and *Bacillus cereus*) have been shown to enable the insect to overcome host defense [16], enhance the mating ability of male flies [17,18], contribute in metabolizing pesticides components [19,20] and possess attractive potentials to *B. dorsalis* [2]. Most of these abundant bacterial communities were isolated from wild populations and are inhabitants of natural ecosystem (soil, water, fly feces & decomposing matters). Reports on the diversity of gut cultivable microbiota of laboratory established populations of *B. dorsalis* are fragmentary.

Therefore, this work aims at isolating and identifying cultivable gut bacterial communities
from *B. dorsalis* laboratory-reared strains using the culture-dependent technique with a particular focus on their variation throughout the developmental stages (larval and adult stages). The data generated from this study will help to initiate a comprehensive survey of specific functional properties of gut bacterial isolates from *B. dorsalis* and develop novel bio-control strategies bacterially oriented against this pest.

## 2. MATERIALS AND METHODS

### 2.1 Insect Collection and Maintenance

Laboratory-reared flies were used for the analysis of cultivable bacteria colonizing the gut of larvae and adult *Bactrocera dorsalis*. Third instar larvae were collected from fallen fruits at the citrus orchard of Huazhong Agricultural University, Wuhan, China (30°49’ N and 114°39’ E). The collected larvae were allowed to pupate in sterile sand and incubated in laboratory conditions at 27±2°C, 70±5 % relative humidity & 12:12 Light: Dark photoperiod. The emerging flies were housed in cubical mesh cages (45 x 30 x 30 cm³) and reared in the laboratory for ten generations. The adult flies were maintained with artificial diet composed of tryptone: 60 mg/ml of water; yeast extract: 40 mg/ml of water; sugar 300 mg/ml of water; agar powder: 12 mg/ml of water; methyl-p-hydroxybenzoate: 1.6 mg/ml of water; L-Ascorbic acid: 3.2 mg/ml of water; compound vitamin B tablets: 4 mg/ml of water & choline chloride: 1.8 mg/ml of water. Water which was provisioned ad libitum in a cotton wool. All individuals were maintained under the incubation conditions as described elsewhere [21]. Thirty (30) third instar larvae from the tenth generation and 30 adult males and 30 females aged 8-10 days were removed and anesthetized at -20 °C for 5 min before dissection and isolation of own guts [4].

### 2.2 Insect Dissection

The 90 flies and larval samples previously anesthetized were individual washed in 70% ethanol for 2 min and rinsed three times in sterile distilled water before dissection. The dissection was carried out aseptically with two pairs of sterilized forceps on a sterilized glass slide spread with 50 µL of sterile distilled water under a stereomicroscope [22]. The whole process was done in a laminar flow hood to avoid atmospheric contaminations. The intact guts were individually and separately transferred into Eppendorf tubes containing 750 µL TE buffer (10 mM Tris-Cl, pH 8; 1 mM EDTA, pH 8), disaggregated and manually homogenized in an Eppendorf adapted pestle before being spread onto standard Luria Bertani (LB) agar plates. The composition and preparation of LB agar media are presented in Table 1.

### 2.3 Isolation of Culturable Gut Bacteria

The homogenate previously prepared was serially diluted by 10⁻⁴–10⁻⁸, and 50 µL were spread onto LB agar and incubated at 37°C for 24–48 h. After 48 hours, the representative bacteria colonies were randomly selected based on the size, color, opacity, and morphology of each colony forming unit (CFU). The predominant types were purified through repeated sub-culturing. The method of purification was as follows: at the end of the incubation period, each bacterial colony was aseptically removed by using an inoculation loop, spread onto LB media and incubated aerobically at 37°C for 24–48h. Later, colonies were sub-cultured twice to ensure purity and preserved at 80°C in 50% glycerol (v/v) for future use. All steps in the isolation procedure were performed in a laminar flow hood to avoid airborne contamination. This procedure allowed the distribution of 300 clonal cultures, of which 100 isolates from each isolated sample were used for total DNA extraction. The extraction and sequencing of bacterial DNA are described in the following sections.

### 2.4 Total Genomic DNA Extraction (CTAB Method)

Individual isolates were grown in LB broth for about 8 hours in a shaker, and the cell pellets were completely re-suspended with 557 µl TE buffer in a 1.5ml tube. Approximately 10 µL lysozyme was added, (from this point, do not vortex). The solution was incubated at 37°C for 10-60 minutes before 3 µL Proteinases K (20mg/ml) and 30 µL SDS (10-20 %) were added to the solution. Then, the whole was incubated again at 56°C until the solution becomes relatively viscous and clear. 100 µL NaCl (5M) was added and mixed thoroughly (do not vortex). The suspension was incubated at 65 °C for 2 minutes before adding 80 µL CTAB/NaCl solutions (preheated at 65 °C for 10 minutes). The mixture was thoroughly (do not vortex) hand-shaken and the resulting suspension was incubated at 65 °C for 10 minutes. Equal volumes (approximately 800 µL) of tris-phenol/chloroform/isoamyl alcohol (500:480:20)
solution and the suspension were extracted, then centrifuged at 12,000 rpm for 5 minutes and transferred into new Eppendorf tubes. Extract supernatant 1 with equal volumes of chloroform: isoamyl alcohol (96:40) solution. Centrifuge at 12000 rpm for 5 minutes and transfer the upper phase (supernatant 2), containing nucleic acids into a separate 2 ml Eppendorf tube. Extract supernatant 2 with equal volumes of chloroform: isoamyl alcohol (96:40) solution. Centrifuge at 12000 rpm for 5 minutes and transfer the upper phase (supernatant 3), containing nucleic acids into a separate 2 ml Eppendorf tube before adding 0.7% volumes (approx. 36 µL) isopropanol into the precipitate nucleic acid. Mix gently by inverting the tube several times. The DNA should appear as a white viscous precipitate and let sit for 30 minutes at -20°C. Note: Pre-cool the centrifuge machine at 4°C before the next step before centrifuging (12,000-15,000 g) at 4°C for 20 minutes. The DNA should be visible as a pellet on the side of the Eppendorf tube. Remove the isopropanol carefully to avoid disturbing the pellets. Then, wash the pellets with 500 µL ethanol (70%), by inverting the tube several times. Centrifuge at 12,000-15,000 g for 5 minutes at 4 °C. Carefully remove the alcohol and blot the rim of the tube with a paper towel to get rid of excess liquid and re-suspend each pellet in 20 µL TE buffer [24]. The quantity and quality of the DNA were checked on 1.5% agarose gel and determined with a Nanodrop spectrophotometer.

2.5 Polymerase Chain Reaction (PCR)

A master mix of the following PCR reagents (except the template DNA) was prepared in a 1.5 ml Eppendorf tube and the equal volumes were distributed into microfuge tubes after which the template DNA was added. Extracted DNA from bacteria isolates were amplified in a polymerase chain reaction (PCR) using the universal primers for bacteria, 27F 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 1492R 5'-TACGGCTACCTTGTTACGACTT-3' [25]. The composition of PCR solution was made according to the procedures of high fidelity (HiFi) PCR as follows: 1µL of DNA template, Forward and Reverse primers each, 5µL of Buffer I, 4µL of dNTP, 0.5 µL of Taq Polymerase and 37.5 µL of deionized distilled water. The PCR reactions were performed in a programmed thermal cycler under the following conditions: Initial denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 1 min, annealing at 53, 54, 55 or 58 °C for 1 min 30 sec, 72°C for 1 min and a final extension at 72°C for 5 min.

2.6 Agarose Gel Electrophoresis

After the PCR reaction, aliquots of PCR products were examined by electrophoresis in 1% agarose gel as follows. A 1% agarose gel was made by dissolving 0.3g of agar in 30 ml 1xTAE. This was microwaved and allowed to cool to a temperature of approximately 50°C before pouring into a clean gel tank. Then the combs were inserted to create the required wells. After solidification, PCR products were mixed with 6 x loading buffer dye, and the different samples were separately loaded into different wells. Gel electrophoresis was carried out for about 40 minutes (I = 100mA, U = 130V). The targeted fragment bands (~1,500 bp) were stained with ethidium bromide (EB), washed in distilled water and photographed under UV light.

2.7 DNA- Gel Extraction and Ligation

Nucleic acid amplified by PCR was purified from gel using AxyPrep DNA gel extraction kit (Axygen Scientific, Inc.) according to the manufacturer’s instructions. Briefly, the bands of DNA fragments were excised from agarose gel with a clean, sharp scalpel under ultraviolet light, and a filter paper was used to dry excess moisture. The weight of the gel was estimated. Three times the sample volume of Buffer DE-A was added in a 1.5 ml microfuge tube containing gel (assuming 1 g of sample equals 1 µl). The tubes containing DE-A and DNA samples were heated at 75°C in a water bath until complete dissolution of gel (~6-8 minutes) and the buffer DE-B was added. The volume was equivalent to approximately 0.5 Buffer DE-A volume previously added, and the reagents were mixed thoroughly. The reaction mixture from the previous step was transferred into a miniprep column placed in a 2 ml collection tube and centrifuge for 1min at 12,000 rpm in a microcentrifuge. 500 µL W1 buffer was added into miniprep column, and centrifuge at 12000 rpm for the 30s before 700 µL W2 buffer was added into miniprep column and centrifuge at 12,000 rpm for 1 minute. The process was repeated. After the last wash, miniprep column was centrifuged at 12,000 rpm for 1 min, and tubes were air dried for 5 minutes at room temperature. The DNA was eluted with 25-30µL ddH2O pre-warmed at 65°C and ligation reaction was performed by mixing the reagents (5µL of freshly purified PCR amplicons, 4µL of 10X ligation buffer & 1µL of the pMD18-T vector (25
ng/µL) to a total volume of 10µL in a 0.5ml sterile tube. Tubes with ligation mix were incubated at 4°C overnight or at 16°C for 2-4 hours.

2.8 Molecular Cloning and Transformation

Ligations were performed in 5µl containing 0.1 µL pMD18-T cloning vector, 2.4 µL mix DNA, and the 2.5µL solution I (Takara) and introduced into Escherichia coli DH5α cells (Takara). The recombinants were selected and verified for the correct insert size by vector-targeted PCR with primer M13 F (5’-GTAAAACGACGGCCAGT-3’) (Sigma) and M13 R (5’-CAGGAAACAGCTATGAC-3’) (Sigma) by the following PCR protocol: an initial denaturation at 94°C for 4 min; 35 cycles of 30 sec at 94°C, 40 sec at 53°C and 60 sec at 72°C. Finally, the samples were subjected to 72°C for 10 min. PCR product was transformed into E. coli that were plated on LB amended with IPTG. Overnight white clones were randomly selected and sequenced (Invitrogen China). Only successful sequencing samples were considered; those whose splicing failed or incomplete during the sequencing process was discarded.

2.9 Sequencing and Phylogenetic Analysis

For each gene library, 100 clones were chosen for sequencing. The 16S rRNA gene sequence of each isolate was compared with known 16S rRNA gene sequences in the GenBank database using the BLAST search algorithm [26]. The sequences were trimmed, filtered, edited and aligned using MEGA 6.0 [27]. The chimeric sequences were removed using Mallard 1.02 [28], and the remaining sequences were used for blast analysis in the National Center for Biotechnology (NCBI; http://www.ncbi.nlm.nih.gov).

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [29]. The tree with the highest log likelihood (-5016.6910) is shown. 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 tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 41 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 447 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [27].

3. RESULTS

3.1 Identification of Bacteria

Three hundred individual bacterial colonies were isolated from each sample from which, 100 isolates each were selected for sequencing based on the phenotypic divergence of their colony (morphology, shape, color, and opacity). The average sequence lengths of the samples were 1414±89 bp in females, 1469±20 bp in males and 1463±29 bp in third instar larvae.

The overall samples generated 41 different bacterial phylotypes, 11 from the third instar larvae, 18 and 12 from female and male populations, respectively. These bacterial strains were divided into six families: Enterobacteriaceae, Enterococcaceae, Staphylococcaceae, Streptococcaceae, Micrococcaceae and Bacillaceae which were differentially represented across the various samples. Enterococcaceae and Streptococcaceae families were missing in females (Table 2A), Staphylococcaceae, Streptococcaceae, Micrococcaceae and Bacillaceae were not detected in males (Table 2B), and Micrococcaceae was not found in third instar larvae (Table 2C). In general, Enterobacteriaceae was the most dominant family in females and third instar larvae accounting for 57.89% and 28.32%, respectively (Table 2A & C), while Enterococcaceae was dominant in males with 75% of the total bacterial taxa (Table 2B). Moreover, 99% of bacteria belonging to the Enterobacteriaceae family were gram-negative, whereas 99% of those from the Enterococcaceae family were gram-positive.

3.2 Abundance at the Taxonomic Level

The relative abundance of different microbial phyla reveals three taxa: Proteobacteria accounting for 54.55%, 34.29% and 25% in third instar larvae, female and male flies, respectively; Firmicutes accounting for 45.45%, 57.14% and 75% in third instar larvae, female and male flies, respectively, and Actinobacteria exclusively detected in female flies (Fig. 1). The immature stage (BD3L) was enriched in Proteobacteria but devoid of Actinobacteria, while females and males were highly abundant in Firmicutes.
Table 1. Ingredients and preparation of the standard Luria Bertani (LB) agar media [23]

| Ingredients (g) | Amounts | Preparation procedures |
|----------------|---------|------------------------|
| 1 Yeast extract | 2.5     | Put ingredients 1-4 in a graduated Erlenmeyer containing 250 mL of dH2O. Then mix the powder well to bring into solution. Add dH2O to a total volume of 500 mL and transfer to 1 L flask. Autoclave at the liquid setting for 20 minutes at 115°C. Let agar cool to ~55°C (you should be able to pick up the jar without a glove) and pipette 25 mL into Petri dish plate. |
| 2 Tryptone | 5 | |
| 3 NaCl | 5 | |
| 4 Agar powder | 7.5 | |
| 5 Water | 500 | |

Note: The preparation of LB broth follows the same procedures but excluding the agar powder.

Table 2A. The identity of gut bacterial isolates from Bactrocera dorsalis female based on 16S rRNA gene sequences

| Isolate labels | Sequence length (bp) | Best tblastn hit species | GenBank accession No. | Identity (%) | Family |
|----------------|---------------------|--------------------------|-----------------------|--------------|--------|
| Females        |                     |                          |                       |              |        |
| BDF-1          | 1408                | Arthrobacter globiformis | HQ455822.1            | 100          | Micrococcaceae- |
| BDF-2          | 1280                | Bacillus cereus          | KC428751.1            | 99           | Bacillaceae+   |
| BDF-3          | 1457                | Bacillus megaterium      | KT986111.1            | 99           | Bacillaceae+   |
| BDF-4          | 1378                | Bacillus subtilis        | MG231261.1            | 100          | Bacillaceae+   |
| BDF-5          | 1399                | Enterobacter cloacae     | CP030347.1            | 99           | Enterobacteriaceae- |
| BDF-6          | 1447                | Enterobacter hormaechei  | CP031726.1            | 99           | Enterobacteriaceae- |
| BDF-7          | 1360                | Enterobacter ludwigii    | KT153616.1            | 100          | Enterobacteriaceae- |
| BDF-8          | 1120                | Enterobacter sp.         | EF523425.1            | 99           | Enterobacteriaceae- |
| BDF-9          | 1492                | Enterobacter xiangfangensis | CP017183.1          | 99           | Enterobacteriaceae- |
| BDF-10         | 1467                | Enterococcus faecalis    | MG543815.1            | 99           | Enterobacteriaceae+ |
| BDF-11         | 1452                | Klebsiella oxytoca       | NR_141452.1           | 99           | Enterobacteriaceae- |
| BDF-12         | 1446                | Klebsiella pneumoniae    | KU199000.1            | 99           | Enterobacteriaceae- |
| BDF-13         | 1453                | Lactococcus lactis      | MG754545.1            | 99           | Streptococcaceae+ |
| BDF-14         | 1433                | Pantoaea dispersa       | AY227805.1            | 99           | Enterobacteriaceae- |
| BDF-15         | 1488                | Proteus pinneri         | MF185213.1            | 96           | Enterobacteriaceae- |
| BDF-16         | 1493                | Staphylococcus equorum subsp. Equorum | KP745578.1  | 97           | Staphylococcaceae+ |
| BDF-17         | 1438                | Staphylococcus sciuri   | MG706002.1            | 97           | Staphylococcaceae+ |
| BDF-18         | 1449                | Klebsiella sp.          | MG231262.1            | 100          | Enterobacteriaceae- |

(+/-): Gram- or Gram+ bacteria
### Table 2B. The identity of gut bacterial isolates from *Bactrocera dorsalis* male based on 16S rRNA gene sequences

| Sample labels | Sequence length (bp) | Best blastn hit species                  | GenBank accession No. | Identity (%) | Family                |
|---------------|----------------------|-----------------------------------------|-----------------------|--------------|-----------------------|
| **Males**     |                      |                                         |                       |              |                       |
| BDM-1         | 1483                 | *Bacterium* H-9137                      | KC881285.1            | 97           | Enterobacteriaceae-   |
| BDM-2         | 1460                 | *Enterococcus avium*                    | KX674028.1            | 97           | Enterococcaceae+      |
| BDM-3         | 1461                 | *Enterococcus faecalis*                 | MH385355.1            | 97           | Enterococcaceae+      |
| BDM-4         | 1457                 | *Enterococcus gallinarum*               | MG231265.1            | 99           | Enterococcaceae+      |
| BDM-5         | 1484                 | *Enterococcus gilvus*                   | MG231273.1            | 100          | Enterococcaceae+      |
| BDM-6         | 1488                 | *Enterococcus maldoratus*               | MG231264.1            | 96           | Enterococcaceae+      |
| BDM-7         | 1423                 | *Enterococcus pallens*                  | MG231264.1            | 96           | Enterococcaceae+      |
| BDM-8         | 1464                 | *Enterococcus raffinosus*               | MG231274.1            | 100          | Enterococcaceae+      |
| BDM-9         | 1492                 | *Enterococcus sp.*                      | FJ965843.1            | 96           | Enterococcaceae+      |
| BDM-10        | 1471                 | *Klebsiella oxytoca*                    | MF429738.1            | 100          | Enterococcaceae+      |
| BDM-11        | 1456                 | *Morganella morganii*                   | MG209574.1            | 94           | Enterobacteriaceae-   |
| BDM-12        | 1490                 | *Morganella sp.*                        | KT254646.1            | 100          | Enterobacteriaceae-   |

### Table 2C. The identity of gut bacterial isolates from *Bactrocera dorsalis* larvae based on 16S rRNA gene sequences

| Sample labels | Sequence length (bp) | Best tblastn hit species             | GenBank accession No. | Identity (%) | Family                |
|---------------|----------------------|-------------------------------------|-----------------------|--------------|-----------------------|
| **Third instars larvae** |                       |                                     |                       |              |                       |
| BD3L-1        | 1398                 | *Bacillus subtilis*                 | KJ729815.1            | 99           | Bacillaceae+          |
| BD3L-2        | 1447                 | *Enterobacter cloaceae*             | CP027604.1            | 99           | Enterobacteriaceae-   |
| BD3L-3        | 1477                 | *Enterobacter hormaeheli*           | KP318472.1            | 98           | Enterobacteriaceae-   |
| BD3L-4        | 1486                 | *Klebsiella oxytoca*                | MF429738.1            | 100          | Enterococcaceae-      |
| BD3L-5        | 1490                 | *Enterococcus avium*                | KX674028.1            | 97           | Enterococcaceae+      |
| BD3L-6        | 1446                 | *Klebsiella pneumoniae*             | KU199000.1            | 98           | Enterobacteriaceae-   |
| BD3L-7        | 1493                 | *Lactococcus lactis*                | MG754545.1            | 99           | Streptococcaceae+     |
| BD3L-8        | 1433                 | *Pantoea dispersa*                  | AY227805.1            | 99           | Enterobacteriaceae-   |
| BD3L-9        | 1462                 | *Enterococcus faecalis*             | MH628237.1            | 100          | Enterococcaceae+      |
| BD3L-10       | 1495                 | *Staphylococcus sciuri*             | KR476410.1            | 94           | Staphylococcaceae+    |
| BD3L-11       | 1465                 | *Morganella morganii*               | MG209574.1            | 94           | Enterobacteriaceae-   |
3.2.1 Relatedness of bacterial species across life stages of \textit{B. dorsalis}

Only sequences with ≥97% identity with their corresponding species from GenBank were considered in the analysis. The phylogenetic analysis of all identified species three clusters (Fig. 2 A, B & C), representing the closely related bacterial species across the life stages. Cluster A was dominated by Enterococcaceae family, of which 58.82% are \textit{Enterococcus} species (Fig. 2A), while cluster B is abundant in Enterobacteriaceae family, of which 42.86% are \textit{Klebsiella} species (Fig. 2B). Similarly, cluster C was dominated by Enterobacteriaceae family but with highly diversified bacterial communities composed of \textit{Enterobacter}, \textit{Morganella} and \textit{Pantoea} species with 20% of the overall density (Fig. 2C). The majority of bacterial species from Cluster B was isolated from the 3\textsuperscript{rd} instar larvae and is closely related to cluster A (dominantly composed of isolates from males) as compared to that of cluster C (Fig 2). All isolates from 3\textsuperscript{rd} instar larvae were also found in adults.

4. DISCUSSION

Insects maintain intricate interactions with diverse bacteria inhabiting gut lumen, homocoel, and exoskeleton [30], and in bacteriocytes which are specialized organs or tissues [31]. Their location on host insects define the type of relationships (parasitic and mutualistic, obligate and transient ones) they have and the inherent functions they exert on the host [32-34]. Irrespective of the status of gut associated bacteria and their locations on host, a large number of previous studies highlighted the roles they play on host nutrition, reproduction, growth and survival [21,23,35-40]. Therefore, it is a necessity to know the composition of gut bacterial communities, before inferring their specific functions, to initiate bacteria-base control strategies against this pest.

Bacteria inhabiting the gut of Tephritidae flies are well documented [41-45] and most of the previously isolated species from \textit{Bactrocera} genera are \textit{Citrobacter freundii}, \textit{Enterobacter cloacae}, \textit{Klebsiella oxytoca}, and \textit{Enterococcus phoeniculicola} [46-48]. As diversity exists among gut bacterial communities in fruit flies, it is important to identify the bacterial species before exploiting them for optimally controlling the host. Therefore, the present study aimed at exploring the diversity of cultivable gut microbiota associated with the 3\textsuperscript{rd} instar larvae, male and female \textit{B. dorsalis} laboratory strains using culture-dependent technique through the construction of 16S rRNA gene libraries.
Fig. 2. Molecular Phylogenetic analysis showing the relationship among cultivable bacterial isolates based on 16S rRNA gene sequences generated from this study and the best blastn hit sequences from the GenBank. BD3L: Bactrocera dorsalis third instars larvae; BDF: Bactrocera dorsalis females; BDM: Bactrocera dorsalis males. The isolate sample names (BD3L, BDF & BDM) are followed by their best match species from NCBI GenBank. The rectangles A, B & C indicate the three main clusters of bacterial isolates.

In our experiments, Enterobacteriaceae and Enterococccaceae were found to be predominant families across the developmental stages. Bacillaceae family was also present but in lesser proportions. Bacterial communities from the Enterobacteriaceae family were reported to be predominantly established in intestinal tracts of many Bactrocera genera [4,8,45], Ceratitis [46, 49] and Anastrepha [42] and play essential roles in host fitness and reproduction [48,50,51]. For example, the supplementation of irradiated Ceratitis capitata male diets with Klebsiella oxytoca was found to significantly increase their mating ability [52]. Lactococcus has capacity
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This study is carried out in the laboratory of

5. CONCLUSION

The occurrence of Klebsiella oxytoca, Enterobacter cloacae, Enterococcus faecalis, Pantoea dispersa and Morganella morganii in all developmental stages (larvae and adults) may be an indication of their ecological importance to B. dorsalis. Although these species have also been identified in other Tephritid fruit flies [17,52,54,57], their prevalence in our insect model could be related to their importance for larval development and adult maintenance. Moreover, most of these bacterial isolates are commonly associated with abiotic niches (soil, water, bird feces and rotting organic matter), and their presence in B. dorsalis laboratory reared strains could certainly be through diet. All the same, the abundant bacteria isolated from this study could be useful in exploring the functions of bacterial isolates, such as that of complementing the missing nutrients in host diet (amino acids and minerals), their effects on foraging behavior and host fruit selection, which may be necessary for the management of B. dorsalis.

5. CONCLUSION

The oriental fruit fly B. dorsalis is intimately associated with gut bacteria, which directly or indirectly influence the host physiology. The bacterial species Klebsiella oxytoca, Enterobacter cloacae, Pantoea dispersa, and Enterococcus faecalis were detected at adult (female and male) and larval stages. The specific functions of these bacteria in host ontogeny, fitness and physiology are subjected to subsequent experiments.

DECLARATION

This study is carried out in the laboratory of Insect Physiology and Biochemistry of the College of Plant Science and Technology, Huazhong Agricultural University (Wuhan, P.R China). The fruit flies Bactrocera dorsalis are not endangered or protected species. Therefore, no permit was required for their collection and manipulation.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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