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Bacterial communities associated with invasive populations of *Bactrocera dorsalis* (Diptera: Tephritidae) in China

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Abstract

The oriental fruit fly *Bactrocera dorsalis* (Hendel) is a destructive insect pest of a wide range of fruits and vegetables. This pest is an invasive species and is currently distributed in some provinces of China. To recover the symbiotic bacteria of *B. dorsalis* from different invasion regions in China, we researched the bacterial diversity of this fruit fly among one laboratory colony (Guangdong, China) and 15 wild populations (14 sites in China and one site in Thailand) using DNA-based approaches. The construction of *16S rRNA* gene libraries allowed the identification of 24 operational taxonomic units of associated bacteria at the 3% distance level, and these were affiliated with 3 phyla, 5 families, and 13 genera. The higher bacterial diversity was recovered in wild populations compared with the laboratory colony and in samples from early term invasion regions compared with samples from late term invasion regions. Moreover, *Klebsiella pneumoniae* and *Providencia* sp. were two of the most frequently recovered bacteria, present in flies collected from three different regions in China where *B. dorsalis* is invasive. This study for the first time provides a systemic investigation of the symbiotic bacteria of *B. dorsalis* from different invasion regions in China.

Keywords: *Bactrocera dorsalis*, bacteria diversity, invasion population, laboratory colony, wild population

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Introduction

The oriental fruit fly *Bactrocera dorsalis* (Hendel) is a significant invasive fruit fly pest in parts of Asia and Africa and can cause considerable economic losses (Clarke *et al.*, 2005; Schutze *et al.*, 2015). The damage caused by this fruit fly can be attributed to its high fecundity and wide host range (Li & Ye, 2000). Much attention has been paid to the population genetics of *B. dorsalis* to uncover the origin and invasion pathways of this pest in China (Aketarawong *et al.*, 2007; Li *et al.*, 2007; Shi *et al.*, 2010). The invasions of *B. dorsalis* in China can be divided into two branches: one originated from South Asia, and the other originated from Southeast Asia. The Southeast Asia-originated fruit flies landed in China at Yunnan Province and then expanded to Sichuan, Chongqing, Guizhou, and Guangxi Provinces. The South Asia-originated flies may have landed at Guangdong and Guangxi Provinces and then expanded to south and east China, including Fujian, Zhejiang, and Jiangsu Provinces and Shanghai city (Li *et al.*, 2007, 2012; Shi *et al.*, 2010).

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Many studies have attempted to investigate the associated bacteria of fruit fly species (Tsiropoulos, 1983; Bextine *et al.*, 2005; Capuzzo *et al.*, 2005; Konstantopoulou *et al.*, 2005; Mazzon *et al.*, 2008, 2010; Sacchetti *et al.*, 2008; Kounatidis *et al.*, 2009; Morrow *et al.*, 2015*a*, *b*). Previous studies have shown that gut bacteria play an important role in the development and reproduction of their host fruit flies (Lauzon, 2003). Firstly, Tephritidae mainly feed on nitrogen-poor fruits and are unable to synthesize essential amino acid by themselves. Associated bacteria can participate in the carbon and nitrogen cycle of the host fruit flies and help synthesize essential amino acids and minerals (Miyazaki *et al.*, 1968; Lauzon *et al.*, 2000, 2003). Associated *Enterobacter agglomerans* of the Mexican fruit fly *Anastrepha ludens* (Loew) can produce the volatile 2, 5-dimethyl pyrazine, which can attract the fly. This attractiveness could be the second function of associated bacteria (Robacker *et al.*, 2004, 2009). Thirdly, *E. agglomerans* isolated from apple maggot fly *Rhagoletis pomonella* (Walsh) can also help flies to degrade the toxic purine compounds of host plants (Lauzon *et al.*, 2000). Last but not least, intestinal associated bacteria can also prevent the colonization of pathogenic bacteria in *Ceratitis capitata* (Wiedemann) and improve its fitness (Behar *et al.*, 2008*b*). Besides associated bacteria, some tephritids have bacterial symbionts that are more tightly associated with their host, which means there exist potential co-evolutionary interactions between these bacteria and its host. The bacteria found in olive fly (*Bactrocera oleae*) and Tephritinae subfamily flies, developing in composite flower heads, is a good example (Mazzon *et al.*, 2008, 2010).

Several reports have analyzed the associated bacteria of *B. dorsalis*. Volatile 2-butanone and amino compounds produced by associated *Citrobacter freundii, Klebsiella pneumoniae* and *Enterobacter cloacae*, can attract *B. dorsalis* to feed on the special host plant (Jang & Nishijima, 1990). The presence of *Wolbachia* infection in *B. dorsalis*, even if with low infection rate, was detected by Sun *et al.* (2007). The gut bacterial diversity from three populations of *B. dorsalis*, including laboratory-reared, laboratory sterile sugar-reared, and field-collected populations were studied and compared (Wang *et al.*, 2011). In order to identify the cultivable bacteria for developing bacterial biocontrol agents, the attractive potential of the cultivable bacteria in the intestine of the three populations was also discussed (Wang *et al.*, 2014). The bacteria associated with different developmental stages of *B. dorsalis* were also identified, and were assigned to 172 Operational Taxonomic Units belonging to six phyla (Andongma *et al.*, 2015). All these reports provided useful information insights into biological control and the development of environmentally friendly pesticides (Wang *et al.*, 2014). However, up to now no studies have compared the associated bacterial diversity between different field populations from different regions within the invasive range of *B. dorsalis*.

In this study, we characterized and compared the associated bacterial communities present in geographically diverse wild populations of *B. dorsalis* collected from different invasion areas of China through the construction of *16S rRNA* gene libraries. Moreover, the putative function of associated bacteria of *B. dorsalis* was discussed. The knowledge of the microbial communities associated with wild populations of *B. dorsalis* from different invasion areas will help to understand the impact of the bacterial diversity on the ability of fruit fly to invade new areas.

Materials and methods

Fruit fly sampling

In this study, *B. dorsalis* samples from one laboratory colony and from 15 wild populations were analyzed. The laboratory population was provided by Guangdong Inspecting and Quarantine Technology Centre (IQTC) and reared using an artificial diet at 25°C with a relative humidity of 75%. The wild sampling sites included 14 sites in China and one site in Thailand. Samples were collected in each site at a single collecting time. The Chinese populations were sampled in eight representative south-eastern provinces to cover as much of the invasion area of *B. dorsalis* in China as possible. The only population sampled in Thailand was Sisaket, which is located in the northeast part of Thailand. Detailed information on sample collection is summarized in table 1 and fig. 1.

All of the wild samples were collected by trapping with methyl eugenol (ME). Approximately 3 h later insects were removed from traps and transported to the laboratory and identified by IQTC experts. As the ME is a male-specific attractant only males were collected. The samples of *B. dorsalis* were maintained at −80°C until DNA extraction.

Invasion regions

Flies were sampled from provinces in which they have had different time periods of invasive populations. According to these periods three different regions were considered: the early term invasion region (ET region), the medium term invasion region (MT region) and the late term invasion region (LT region). As shown in table 1, the ET region includes Yunnan, Guangdong, Guangxi and Guizhou provinces in which *B. dorsalis* has been found since 1970s (Bai & Song, 1997). The LT region includes the four sites in Shanghai city, where *B. dorsalis* had been established from 2001 (Zhou *et al.*, 2006), and the other collection sites were attributed to the MT region (Li *et al.*, 2012).

DNA extraction and 16S rRNA gene libraries construction

Before DNA extraction, the whole fruit flies were sterilized in 1% sodium hypochlorite (NaClO) and 97% ethanol for 5 min and then washed five times with sterile distilled water following the procedure described by Tang *et al.* (2010).

The whole body of three adults from each *B. dorsalis* population were processed to construct the gene libraries. The bacterial DNA was extracted using the commercial Tissue/Cell DNA Mini Kit (Tiangen, China) following the manufacturer's protocol. The *16S rRNA* gene was amplified using the universal primers 27F/1492R (Rani *et al.*, 2009). Each polymerase chain reaction (PCR) was conducted in a reaction volume of 50 µl containing 5 µl of 10 × reaction buffer (Mg^{2+}), 1 µl of the extracted nucleic acid DNA (115.8 ng μl^{-1}) as the template, 1U of Taq polymerase (Tiangen, China), 4 µl of the dNTP mixture (2.5 mM), and 1 µl of the forward and reverse primers (10 µM), respectively. The DNA amplification was performed on a Veriti TM 96-well Thermal Cycler (ABI, USA) with the following reaction conditions: 95°C for 3 min, 35 cycles of 94°C for 3 min, 55°C for 1 min, and 72°C for 1 min, and a final incubation at 72°C for 10 min. The PCR products were purified using a DNA Purification Kit (Tiangen, China). The purified PCR products were cloned into the PGMT-19 vector (TaKaRa) and transformed into *Escherichia coli* DH5α cells (TaKaRa).

Table 1. Characteristics of 16 collection sites of *Bactrocera dorsalis* and bacterial *16S rRNA* gene sequence clone libraries.

Collection site	Code	Geographical coordinate	Elevation(m)	Date	Invasion region	No clones[1]	No. OTUs[2]	OTU ID[3]	Chao1 estimate	Coverage[4]	GenBank number[5]
Lab population-female						46	2	1, 2	2	100	JQ918001, JQ918002
Lab population-male						47	4	1, 2, 4, 9	4	100	JQ918003-JQ918006
Thailand-Sisaket	15 TH	104°15E, 15°01 N	132	July 2009		48	6	6, 9, 10, 11, 22, 23	7	85.7	JQ918070-JQ918075
Yunnan-Jinghong	1 JH	100°46E, 21°57 N	575	Nov 2009	Early term	48	7	11, 13, 17, 18, 22, 23, 24	7.33	95.5	JQ918007-JQ918013
Guizhou-Xingyi	2 XY	104°53E, 25°05 N	1337	July 2010	(ET)	45	5	5, 11, 15, 17, 18	5	100	JQ918014-JQ918018
Guangxi-Pingxiang	3 PX	106°45E, 22°05 N	299	May 2009		47	5	11, 14, 15, 23, 24	5	100	JQ918019-JQ918023
Guangxi-Nanning	4 NN	108°22E, 22°47 N	105	May 2009		46	5	2, 7, 15, 17, 21	5	100	JQ918024-JQ918028
Guangdong-Guangzhou	6 GZ	113°17E, 23°22 N	200	June 2010		46	8	1, 2, 4, 5, 7, 11, 12, 13	11	72.7	JQ918032-JQ918039
Hainan-Qionghai	5 QH	110°28E, 19°15 N	22	June 2010	Medium term	50	3	3, 9, 15	3	100	JQ918029-JQ918031
Fujian-Fuzhou	7 FZ	119°13E, 26°05 N	7	July 2010	(MT)	52	6	2, 14, 15, 16, 17, 24	6	100	JQ918040-JQ918045
Fujian-Ningde	8 ND	119°31E, 26°39 N	16	July 2010		45	4	2, 4, 7, 9	4	100	JQ918046-JQ918049
Fujian-Xiamen	9 XM	118°09E, 24°43 N	29	July 2010		45	2	4, 12	2	100	JQ918050, JQ918051
Zhejiang-Ningbo	10 NB	121°24E, 29°39 N	13	July 2010		50	5	7, 8, 14, 17, 18	5	100	JQ918052-JQ918056
Shanghai-Songjiang	11 SJ	121°13E, 31°01 N	5	Sep 2010	Late term	42	5	3, 5, 9, 17, 20	8	62.5	JQ918057-JQ918061
Shanghai-Qingpu	12 QP	121°15E, 31°08 N	10	Sep 2010	(LT)	46	3	7, 9, 15	3	100	JQ918062-JQ918064
Shanghai-Jiading	13 JD	121°08 E, 31°18 N	9	Sep 2010		44	2	9, 19	2	100	JQ918065-JQ918066
Shanghai-Baoshan	14 BS	121°19 E, 31°22 N	7	Sep 2010		42	3	4, 9, 15	3	100	JQ918067-JQ918069
Total							24				

[1]Total number of clones sequenced for each individual PCR libraries.
[2]The OTU was determined based on a 3% distance level.
[3]ID assigned for the recovered OTUs based on a 3% distance level.
[4]Coverage was estimated as the ratio between the OTUs observed and the total number of OTUs estimated in the population (Chao1).
[5]GenBank numbers of the submitted sequences based on a 3% distance level.

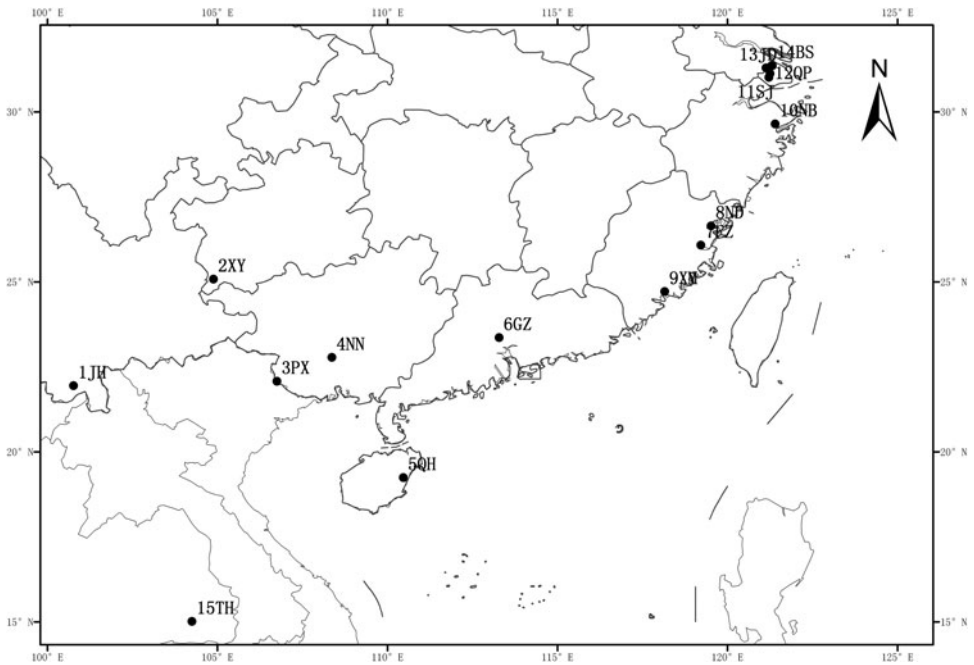

Fig. 1. Fruit flies collection site. Geographical map of the Chinese provinces from where *Bactrocera dorsalis* samples were collected. The site numbers correspond with those shown in table 1.

The transformation was verified using the RV-M/M13-47 universal primers. The amplification products were sequenced using the same primers.

Sequencing and phylogenetic analysis

For each gene library, at least 50 clones were chosen for sequencing. The sequence chromatograms were visually inspected, and the sequences were edited and aligned using MEGA 4.0 (Tamura *et al.*, 2007). The chimeric sequences were removed using Mallard 1.02 (Ashelford *et al.*, 2006), and the remaining sequences were blasted in The National Center for Biotechnology (NCBI; http://www.ncbi.nlm.nih.gov). The distance matrices were constructed using the DNADIST program of PHYLIP 3.69 (Felsenstein, 1989). The operational taxonomic units (OTUs), based on their identity and 3% distance level, were determined by DOTUR 1.53 (Schloss *et al.*, 2006). This program assigns sequences to OTUs based on sequence data by using values that are less than the cut-off level.

In order to estimate the adequacy of sampling, rarefaction curves were generated and the Chao1 richness estimator (Chao, 1984) was calculated using the same software.

The Chao1 richness index estimates diversity of a community based on the number of singletons (OTUs represented by only one sequence) and doubletons (OTUs represented by two sequences) found in a sample (Bohannan & Hughes, 2003). The richness estimates are reported for 3% difference between sequences.

To study the phylogenetic relationships among the associated bacteria of *B. dorsalis*, we considered bacteria found in other fruit flies and the closely related bacterial sequences present in GenBank. Sequences of at least 1000 bp were considered for phylogenetic tree construction. Bacteria retrieved in *C. capitata*, *B. oleae*, *Bactrocera zonata*, four taxa of the subfamily Tephritinae (*Campiglossa guttella*, *Dioxyna bidentis*, *Trupanea amoena*, *Tephritis matricariae* and *Noeeta* genus) were selected as the reference sequences (Supplementary table 1). The phylogenetic relationships among these bacteria were estimated using the neighbour-joining method and the Jukes-Cantor model. For each *16S rRNA* gene library, representative sequences for each OTU obtained were submitted to GenBank under the accession numbers JQ918001–JQ918075 (table 1).

Bacterial diversity comparison

We provided three kinds of bacterial diversity comparisons: male and female of the laboratory colony, among 15 wild populations, and among three different invasion regions. Clone percentage, clone frequencies across number of PCR libraries was used to describe the relative abundance of each genus. The bacterial diversity among three different invasion regions was statistically analyzed performing ANOVA analysis with Tukey-Kramer test using CoStat-Statistics Software v6.204 (http://www.cohort.com/costat.html).

Results

Bacterial 16S rRNA libraries and phylogenetic analysis

16S rRNA gene libraries were constructed for 15 wild populations and one laboratory population, and 789 sequences with a length of 1500 bp were obtained. All 789 sequences were grouped into 133 OTUs based on their identity (similarity = 100%) and 24 OTUs based on their 3% distance level (similarity >97%) (table 1). The rarefaction curves for most of the clone libraries tended to be saturated at 3% difference between sequences (Supplementary figure 1). Moreover, except for three populations (GZ, SJ and TH), the coverage was

Table 2. Taxonomical assignment of the cloned *16S rRNA* bacterial amplifications from *Bactrocera dorsalis*

OTU[1]	Representative clone	Class	Family	Genus	Sequence database match[2]	Identity (%)	Clone percentage[3]
1	GDF3	Gamma-proteobacteria	Xanthomonadaceae	*Stenotrophomonas*	*Stenotrophomonas maltophilia* JN208909	99	3.80
2	FZ17	Gamma-proteobacteria	Enterobacteriaceae	*Enterobacter*	*Enterobacter hormaechei* AJ853890	99	**23.45**[4]
3	SJ-36	Gamma-proteobacteria	Enterobacteriaceae	*Enterobacter*	*Enterobacter aerogenes* GU265554	99	5.20
4	ND2	Gamma-proteobacteria	Enterobacteriaceae	*Enterobacter*	*Enterobacter asburiae* HQ242719	99	6.34
5	GD1-7	Gamma-proteobacteria	Enterobacteriaceae	*Morganella*	*Morganella morganii* DQ355171	99	2.53
6	TH4-3	Gamma-proteobacteria	Enterobacteriaceae	*Kluyvera*	*Kluyvera georgiana* AM933755	99	2.28
7	ND9	Gamma-proteobacteria	Enterobacteriaceae	*Providencia*	*Providencia rettgeri* NR_042413	99	5.70
8	NB1	Gamma-proteobacteria	Enterobacteriaceae	*Kluyvera*	*Kluyvera ascorbata* FN813247	99	3.68
9	JD1-56	Gamma-proteobacteria	Enterobacteriaceae	*Klebsiella*	*Klebsiella pneumoniae* CP000964	99	**6.46**
10	TH4-8	Gamma-proteobacteria	Enterobacteriaceae	*Klebsiella*	*Klebsiella* sp. HM165190	97	0.13
11	GD5	Gamma-proteobacteria	Orbaceae	*Orbus*	*Orbus hercynius* strain FJ612598.1	95	5.32
12	GD4	Gamma-proteobacteria	Enterobacteriaceae	*Serratia*	*Serratia marcescens* GQ889261	99	3.04
13	YN8	Gamma-proteobacteria	Enterobacteriaceae	*Citrobacter*	*Citrobacter* sp. KF374709	99	0.76
14	FZ10	Gamma-proteobacteria	Orbaceae	*Orbus*	*Orbus* sp. JN561614	99	2.53
15	FZ18	Gamma-proteobacteria	Enterobacteriaceae	*Citrobacter*	*Citrobacter freundii* AB244451	99	**9.76**
16	FZ7	Gamma-proteobacteria	Enterobacteriaceae	*Erwinia*	*Erwinia billingiae* strain CIP106121 JN175337	96	0.13
17	YN4	Gamma-proteobacteria	Enterobacteriaceae	*Klebsiella*	*Klebsiella oxytoca* AJ871857	99	3.42
18	NB10	Gamma-proteobacteria	Enterobacteriaceae	*Citrobacter*	*Citrobacter farmeri* FN433034	99	1.77
19	JD1-9	Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas*	*Pseudomonas aeruginosa* KM506786	99	4.18
20	SJ3-74	Gamma-proteobacteria	Enterobacteriaceae	*Enterobacter*	*Enterobacter aerogenes* KC990787	99	0.13
21	NN-39	Gamma-proteobacteria	Enterobacteriaceae	*Enterobacter*	*Enterobacter* sp. JX021670	98	0.14
22	YN3	Delta-proteobacteria	Desulfovibrionaceae	delta- proteobacterium	Uncultured delta- proteobacterium AB234538	93	2.28
23	YN21	Bacilli	Streptococcaceae	*Lactococcus*	*Lactococcus lactis* AB572041	99	1.14
24	YN2	Bacilli	Enterococcaceae	*Vagococcus*	*Vagococcus carniphilus* NR_025689	99	5.83

[1]The OTU was determined using the DOTUR program based on a 3% distance level.
[2]Organism; GenBank Accession No. (BLAST using both the type and non-type strains in NCBI).
[3]The clone percentages were calculated from a total of 789 clones in all of the 16 populations.
[4]The bold values represented the clone percentage of the top three OTUs.

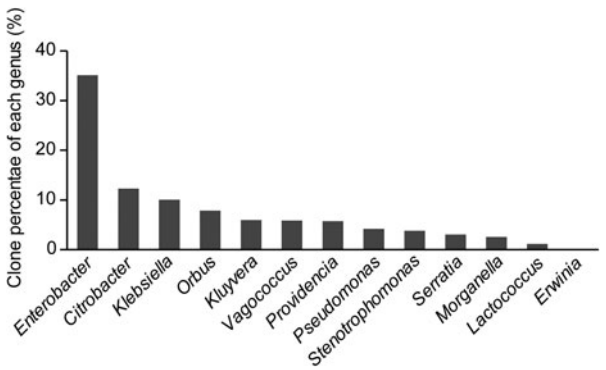

Fig. 2. Clone percentage of symbiotic bacteria of each genus. The percentage was calculated as the percentage of clones corresponding to the indicated genus of the total 789 clones.

higher than 95% (table 1) suggesting that the number of clones sampled was enough to provide an adequate estimation of *B. dorsalis* bacterial diversity.

The 24 OTUs retrieved were affiliated to three classes, namely gamma -Proteobacteria (21 OTUs), delta -Proteobacteria (one OTU), and Bacilli (two OTUs), belonging to seven families (Xanthomonadaceae, Enterobacteriaceae, Pseudomonadaceae, Orbaceae, Desulfovibrionaceae, Streptococcaceae, and Enterococcaceae) and 13 genera (*Stenotrophomonas, Pseudomonas, Enterobacter, Morganella, Kluyvera, Providencia, Klebsiella, Serratia, Citrobacter, Orbus, Erwinia, Lactococcus*, and *Vagococcus*) (table 2). The results show that the Enterobacteriaceae family and *Enterobacter* genus were the most preponderant bacterial taxa of *B. dorsalis* with percentages of approximately 83 and 35% in the 789 clones, respectively (fig. 2).

The 24 OTUs were distributed in seven families and all the seven families were well supported in seven clades, respectively (fig. 3). Sixteen OTUs were grouped in the Enterobacteriaceae clade together with bacteria retrieved in other fruit flies. However, within the Enterobacteriaceae, none of the OTUs found in *B. dorsalis* were present in the highly supported group, which was shared by the specific symbiotic bacteria of Tephritinae subfamily (*Ca. Stammerula* sp.) and *B. oleae* (*Ca. Erwinia dacicola*). Moreover, besides the clade of the family Orbaceae, that were represented by three OTUs (11, 14 and 16), the remaining family clades contained only one OTU (fig. 3).

Bacterial diversity associated with the laboratory colony

In the laboratory population, the bacteria found in female fruit flies belonged to two genera (*Stenotrophomonas* and *Enterobacter*), whereas the bacteria found in males belonged to three genera (*Stenotrophomonas, Enterobacter* and *Klebsiella*), of which *Enterobacter* was the most dominant genus (with a clone percentage of 64 and 88%, respectively) (fig. 4).

Bacterial diversity associated with different wild populations

Among the total 24 OTUs based on 3% distance level, the Guangdong-Guangzhou (GZ) population had the largest number of OTUs ($n = 8$), whereas the Fujian-Xiamen (XM) and Shanghai-Qingpu (QP), had the least number of OTUs ($n = 2$) (table 1).

The genus-level bacterial diversity was analyzed for each population, and the detailed results are shown in fig. 4. An average of 3.9 genera of bacteria per population was present in the wild populations of *B. dorsalis*. Of the 15 wild populations, Guangdong-GZ was the population with the highest number of genera. Seven different genera were found, including *Stenotrophomonas, Orbus, Citrobacter, Morganella, Providencia, Serratia*, and the predominant *Enterobacter* (with a clone percentage of 69%) (fig. 4).

The populations of Shanghai Region (SJ, QP, BS, and JD) showed the fewest number of genera. Particularly in the Shanghai-JD population, the bacteria were affiliated with two genera, namely *Klebsiella* (25% clone percentage) and *Pseudomonas* (75% clone percentage), whereas three genera were present in the SJ, QP, and BS populations. *Enterobacter* was the most predominant genus in SJ (80% clone percentage), whereas *Providencia* and *Citrobacter* were the most predominant genera in QP (50% clone percentage) and BS (53% clone percentage), respectively.

We consider the genera that were recovered in more than five Chinese populations of *B. dorsalis* as the most common genera. Thus, the most frequent genus was *Klebsiella*, which was found in 13 populations, followed by *Citrobacter* (ten populations), *Enterobacter* (nine populations), *Orbus* (eight populations) and *Providencia* (5 populations).

Bacterial diversity in different invasion regions

As is shown in table 3, the bacteria obtained from the flies collected in the ET region were assigned to three classes and grouped into 10 genera: *Stenotrophomonas, Enterobacter, Klebsiella, Orbus, Citrobacter, Providencia, Morganella*, Unknown Delta-proteobacteria, *Lactococcus*, and *Vagococcus*. The bacteria of the flies collected in the MT region were attributed to two classes and eight genera. Interestingly, the bacteria found in *B. dorsalis* populations collected from the LT region were attributed only to the Gamma-Proteobacteria class and classified into six genera, four of which are affiliated with the family Enterobacteriaceae (table 3). Four genera, including *Enterobacter, Klebsiella, Citrobacter* and *Providencia* were found in all three invasion regions.

The result associated with three invasion regions showed that, considering OTUs based on a 3% distance level, the bacterial diversity in the ET region was statistically different from that in the LT regions (ANOVA, $F = 5.15$, df = 2,11, $P = 0.036$). However, there was no significant difference between the ET and MT regions or between the MT and LT regions (fig. 5).

Discussion

Bacterial diversity of B. dorsalis

The *16S rRNA* gene libraries from the single laboratory and 15 wild populations indicate that the associated bacteria of *B. dorsalis* are affiliated with the classes Gamma-proteobacteria, Delta-proteobacteria, and Bacilli, of which Gamma-proteobacteria is the most predominant class with a percentage of 90.68%. Gamma-proteobacteria include taxa of important symbiotic bacteria of many insects, such as other fruit flies, mealybugs, mosquitoes, and beetles (Thao *et al.*, 2002; Capuzzo *et al.*, 2005; Schloss *et al.*, 2006; Mazzon *et al.*, 2008; Rani *et al.*, 2009). Within the Gamma-proteobacteria, our

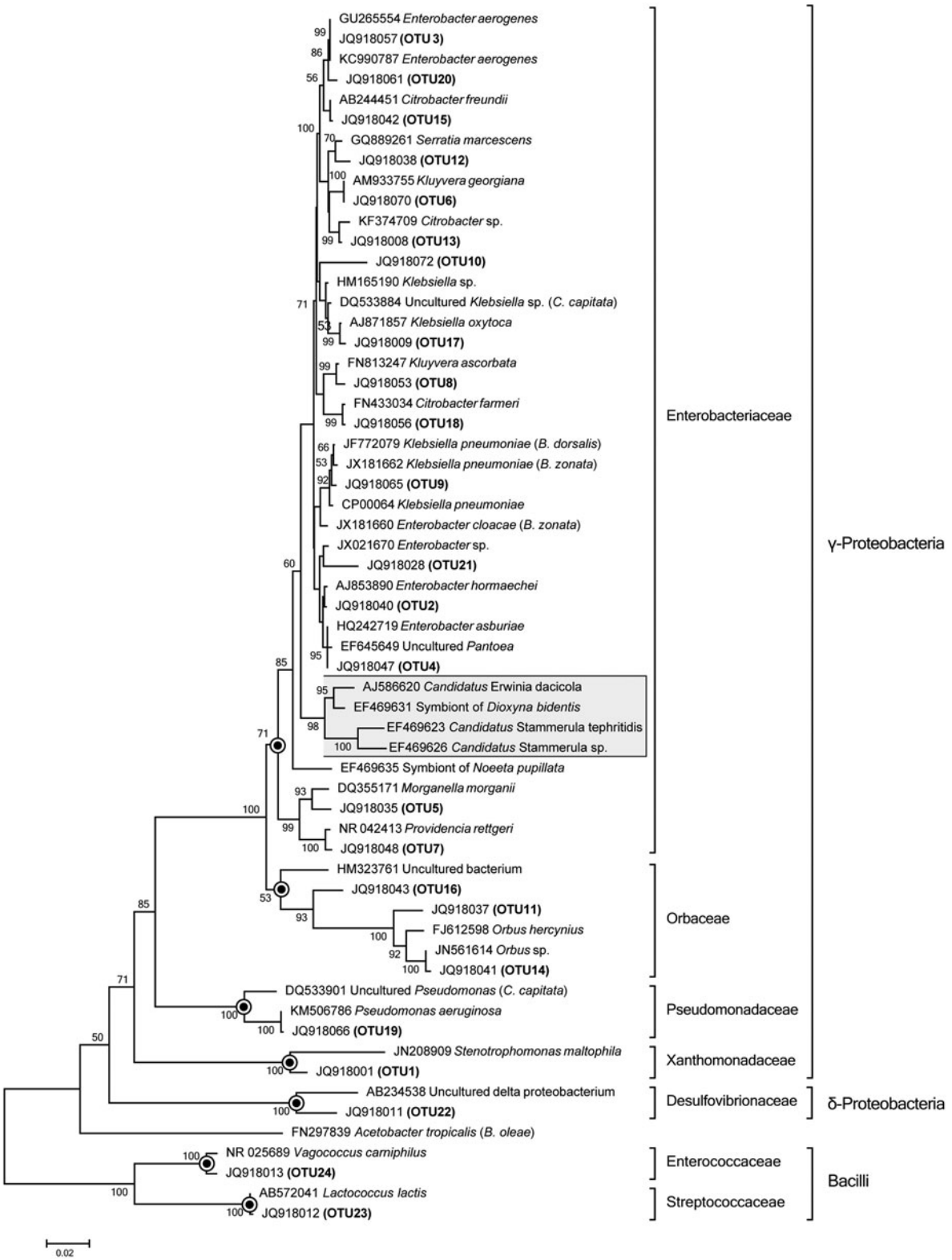

Fig. 3. Neighbour-joining (NJ) phylogenetic tree of bacteria associated with *Bactrocera dorsalis* based on the *16S rDNA* sequences. The NJ tree was constructed using MEGA 4.0 with the NJ method and the Jukes-Cantor model and shows the phylogenetic relationship of symbiotic bacteria affiliated with 24 OTUs, the relative free-living bacteria and other bacteria found in other fruit flies. The grey box contains specific and unculturable symbiotic bacteria of *Bactrocera oleae* and Tephritinae subfamily. The scale bar corresponds to 0.02 substitutions per nucleotide position. The percentage bootstrap values above 50 (1000) replicates are indicated as notes.

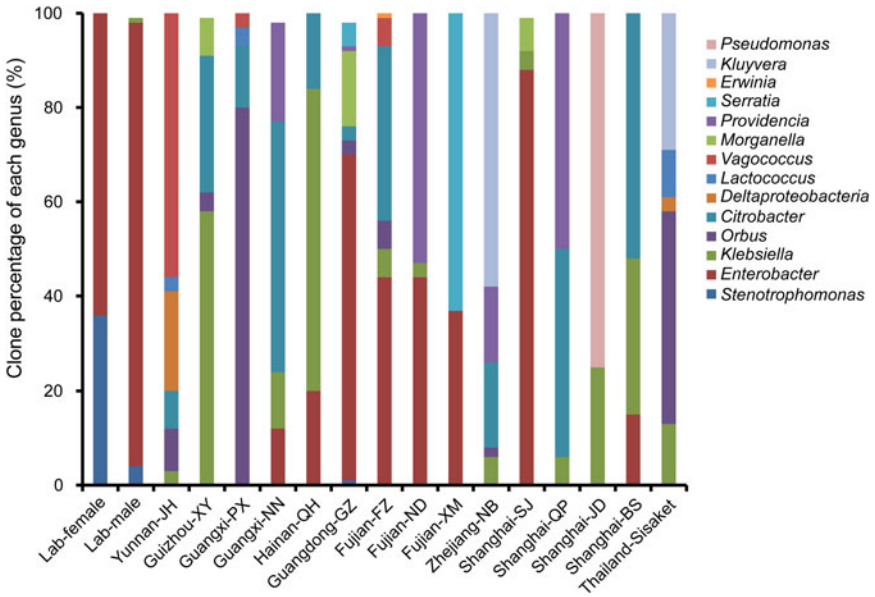

Fig. 4. Relative abundance of the different genera in the associated bacteria from one laboratory and 15 wild populations.

Table 3. Bacteria found in different invasion regions of *Bactrocera dorsalis*

Class	Genus (%)	Detected in different regions		
		ET region	MT region	LT region
γ-proteobacteria	*Stenotrophomonas*	+		
	Enterobacter	+	+	+
	Klebsiella	+	+	+
	Orbus	+	+	
	Citrobacter	+	+	+
	Providencia	+	+	+
	Morganella	+		+
	Serratia		+	
	Kluyvera		+	
	Pseudomonas			+
δ-proteobacteria	*Delta-proteobacteria*	+		
Bacilli	*Lactococcus*	+		
	Vagococcus	+	+	
All		10	8	6

"+" indicates that the genus was detected in this region.

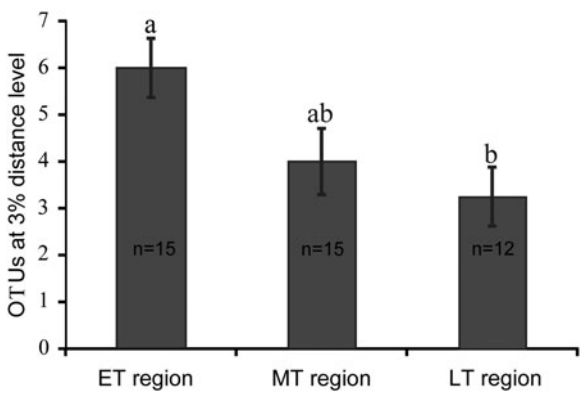

Fig. 5. Bacterial diversity (%) in the three invasion regions. ANOVA followed by a Tukey post-hoc test. Letters above the bars indicate significant differences at $P < 0.05$ (bars are means ± SE). Numbers inside bars represent sample sizes.

results also confirmed that Enterobacteriaceae (in particular with the genera *Enterobacter*, *Klebsiella*, *Citrobacter*, and *Providencia*), are the predominant bacteria family as previously found in *B. dorsalis* (Jang & Nishijima, 1990) and in other tephritid flies (i.e. Behar *et al.*, 2008*b*; Wang *et al.*, 2014) . Similarly, in a single field-collected population of *B. dorsalis*, Wang *et al.* (2011) reported a predominance of Gamma-proteobacteria followed by Firmicutes and few taxa of Delta-proteobacteria, Actinobacteria, Bacteroidetes, Flavobacteria, and Alpha-proteobacteria. Moreover, the analyses showed *Klebsiella*, *Citrobacter*, *Enterobacter*, *Pectobacterium* and *Serratia* the common bacterial phylotypes for all three libraries. On the contrary, Andongma *et al.* (2015) compared the microbiota across the life stage of *B. dorsalis* and came to the conclusion that Enterococcaceae (Bacilli) was the most abundant family in the adult flies.

While the cloning method used in this study is optimal for identifying bacteria at the genus level it is limited for the quantitative analysis because of eventual biases of clone frequencies occurring at several levels during the process (e.g. primer bias, amplification efficiency bias, ligation bias etc.). Future studies considering next generation sequencing techniques will help to overcome these limitations.

No bacteria belonging to the genus *Wolbachia* were detected in any of the populations used for this study, even though our sample collecting locations cover almost all the distribution provinces of *B. dorsalis* in China. Similarly, another study reported the absence of *Wolbachia* in Chinese populations of *B. dorsalis* (Yunnan, Fujian and Wuhan population) (Augustinos *et al.*, 2015). In conflict with these results, Sun

et al. (2007), detected *Wolbachia* in four of five Chinese populations but with low infection rates (0.7–3%). As widely reported and discussed by Morrow *et al*. (2015*a*, *b*) for Australian fruit flies, infections of *Wolbachia* at low frequency may be indicative of transient infections that result from spillover events from a yet unknown source, questioning their vertical inheritance.

In our study, we generally found more bacterial diversity, considering the OTUs based on a 3% distance level, in wild populations than in the laboratory population (table 1), and the bacterial diversity in ET regions was statistically higher than that in LT regions. These differences could be influenced by environmental factors such as the habitat and the type of diet (i.e. host plant) that these fruit flies are exposed to as proposed by several authors. Wang *et al*. (2011) reported that the gut bacteria diversity of field collected *B. dorsalis* was higher than that of fruit flies reared on sterile artificial food. They came to a conclusion that different environmental conditions and food supply could influence the diversity of the harboured bacterial communities and increase community variations. Morrow *et al*. (2015*a*, *b*) detected and compared the microbiome associated with field-caught and laboratory-adapted Australian Tephritid fruit fly species, and also came to the conclusion that the environment, species identity and ecology can influence the microbial composition.

Possible function of associated bacteria

The functions of fruit flies' symbionts and free-living associated bacteria have been reported via four aspects, including participating in the carbon and nitrogen cycle of fruit flies to help them synthesize essential amino acids and minerals; attracting the fly to feed on host plant; degrading the toxic compounds from host plants and preventing the colonization of pathogenic bacteria (Robacker & Lauzon, 2002; Lauzon *et al*., 2003; Behar *et al*., 2005, 2008*a*, *b*, *c*; Robacker *et al*., 2009). Many bacteria retrieved in this study have been reported showing beneficial function to host flies such as, *Lactococcus* that has been reported to inhibit the growth of pathogenic bacteria in *Anastrepha ludens* (Kuzina *et al*., 2001). Moreover, it has been reported that some of the bacteria retrieved in this study (*Klebsiella*, *Citrobacter*, *Providencia*, and most *Enterobacter*) have been reported to play a key role in the biological fixation of nitrogen (Robacker & Lauzon, 2002; Lauzon *et al*., 2003). Almost all the bacteria we found in wild male populations belonged to the genus Enterobacter, which can provide advantages to host flies through contributing essential nutrients during the adult life stage (Ben-Yosef *et al*., 2008). *E. cloacae* and *C. freundii* have been reported for their high attractive ability to host flies (Wang *et al*., 2014). The abundant bacteria retrieved in our study could be useful to find more bacteria with attracting ability, which may be important for the biocontrol management of *B. dorsalis*.

One previous report has suggested an important role of the gut microbiota of insects in the host range and host suitability. The Asian longhorn beetle (*Anoplophora glabripennis*), an invasive pest with a broad host range, presents a wide bacterial diversity, whereas the linden borer (*Saperda vestita*), a cerambycid with a more restricted host range, contains only a small subset of the same bacteria (Schloss *et al*., 2006). Recently, collaborative invasion (co-invasion) between the invasive species and its niche related species has also been a hot topic of research, and the most typical examples include the co-invasion of white fly *Bemisia tabaci* (Gennadius) biotype with the gemini viruses and the co-invasion of red turpentine beetle *Dendroctonus valens* (LeConte) with its associated fungus *Leptographium procerum*, (Liu *et al*., 2009; Lu *et al*., 2009; Himler *et al*., 2011). Given the significance of microbial symbionts in the invasion of insects and the important role of associated bacteria (Feldhaar, 2011), the research on the microbiota may help us to uncover the invasion mechanism of invasive insect pests such as *B. dorsalis*. However, the speculated co-invasion between associated bacteria and host flies needs further investigation, including verifying the function of the microbiota through exposing flies to different bacteria. Meanwhile, the bacterial community associated with females from different wild populations and different invasion regions should also be investigated in the future, because the bacterial harboured by females can be transmitted to the larvae and may thus contribute to the invasive potential of the fly since they may determine the ability of larvae to utilize new hosts. Moreover, fruit flies from more instars, more distribution areas, and more genera should also be used in future studies to uncover the co-invasion mechanism of fruit flies and their associated bacteria.

Supplementary Material

The supplementary material for this article can be found at http://dx.doi.org/10.1017/S0007485316000390.

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