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Isolation and characterization of gut bacteria of fruit fly, *Bactrocera tau* (Walker)

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Abstract Gut bacteria of fruit fly, *Bactrocera tau* (Walker) (Diptera: Tephritidae), were isolated and the isolates attractive to *B. tau* adults were characterized using morphological, biochemical and 16S rRNA analyses to determine their taxonomic position. Based upon morphological, biochemical and 16S rRNA sequences (on the basis of closest match), five gut bacterial species of *B. tau* were characterized as *Delftia acidovorans*, *Pseudomonas putida*, *Flavobacterium* sp., *Defluvibacter* sp. and *Ochrobactrum* sp., of which four bacterial isolates, *viz*., *Delftia acidovorans*, *Flavobacterium* sp., *Defluvibacter* sp. and *Ochrobactrum* sp. are new records from guts of the fruit fly species.

Keywords *Defluvibacter* · *Delftia* · *Flavobacterium* · *Ochrobactrum* · Phylogeny · *Pseudomonas* · 16S rRNA

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Introduction

The insect-bacterial association has co-evolved for more than 250 million years and has resulted in multifaceted interactions between insects and bacteria, ranging from pathogenicity to highly sophisticated mutualistic relationships (Douglas & Beard 1997; Oliver *et al*. 2005; Wernegreen 2002); may be extracellular or intracellular and play a role in the nutrition, physiology and reproduction of the host insect (Brune 1998; Douglas *et al*. 2001). On insect gut bacterial associations, the earliest report was published by Petri in 1909 in an insect species, the olive fly, *Bactrocera* (*Dacus*) *oleae* (Rossi).

A range of bacteria belonging to different genera, *viz*. *Acetobacter*, *Agrobacterium*, *Arthrobacter*,

Bacillus, *Citrobacter*, *Enterobacter*, *Erwinia*, *Escherichia*, *Hafnia*, *Klebsiella*, *Kluyvera*, *Lactobacillus*, *Listeria*, *Micrococcus*, *Pantoea*, *Pectobacterium*, *Proteus*, *Providencia*, *Pseudomonas*, *Raoultella*, *Serratia*, *Staphylococcus*, *Streptococcus* and *Xanthomonas*, has been isolated and characterized from the gut of tephritid fruit fly species (Behar *et al*. 2009; Lloyd *et al*. 1986; Prabhakar *et al*. 2009a; Wang *et al*. 2011).

In fruit flies, symbionts act as a natural source of nitrogen, amino acids and vitamins lacking in fruit tissues to their host and are vertically transmitted to the next generation (Drew *et al*. 1983; Gupta & Anand 2003). Moreover, cultivable gut bacteria have several implications in pest management strategies, *e.g*. bacteria were found to be involved in the degradation of the toxic substances ingested by the host insect leading to insecticide resistance (Bousch & Matsumara 1967; Prabhakar *et al*. 2008). Certain components of bacterial odor play a vital role in fruit fly behavior as either feeding or ovipositional stimulants (Lauzon *et al*. 2000) and are being exploited in pest management in the form of baits or traps (Robacker 2007; Sood *et al*. 2010; Thaochan & Chinajariyawong 2011).

The pumpkin fly, *Bactrocera tau* (Walker), infests a wide range of commercially important cucurbit and solanaceous crops and has a wide distribution throughout south Asian countries (India, Sri Lanka, Bangladesh and Bhutan) to the southeast Asian countries (Thailand, Malaysia, Vietnam, Philippines and Indonesia) and the far east Asian region including Taiwan and south China (Prabhakar *et al*. 2009b, 2012; Thanaphum & Thaenkham 2003; White & Elson-Harris 1992). In the previous study, members of the Enterobacteriaceae (*Klebsiella oxytoca and Pantoea agglomerans*) were found to be the dominant microbial population in the gut of laboratory reared *B*. *tau* (Prabhakar *et al*. 2009a). Laboratory studies on bacterial odors (*K*. *oxytoca* and *P*. *agglomerans*) attracting *B*. *tau* adults (males and females) suggested their possible applied role in fruit fly management programmes (Sood *et al*. 2010). However, there is still a dearth of knowledge on the bacterial communities attracting *B*. *tau*. The cultivable gut bacteria in particular will be more useful than non-cultivable bacteria when searching for a good fruit fly attractant. Therefore, in the present study, an attempt has been made to characterize cultivable gut bacteria of *B*. *tau*, a serious pest of cucurbits in southeast Asia, India and China.

Materials and methods

Isolation of gut bacteria The bacteria were isolated from 10-day-old adult flies reared from field-collected cucurbit fruits from vegetable fields of Himachal Pradesh (Table 1), as described by Lloyd *et al*. (1986). Flies were cold anaesthetized for 5 min. and surface sterilized with alcohol (70%) for 30 s. followed by sodium hypochloride (0.25%) for 1 min. and then washed three times with sterilized distilled water (SDW) to remove external contaminations. The surface sterilized flies (n=5 adult flies from each *B*. *tau* isolate collected from the same location and host plant; N=45 adult flies from nine *B*. *tau* isolates) were dissected open in physiological saline to remove fly gut. Gut content was streaked separately on Peptone Yeast Extract Agar (PYEA) and Brain Heart Infusion Agar (BHIA) for bacterial growth at 30±1°C for 48–72 h. A single colony of each isolate was used for establishment of pure culture with respective medium and then maintained on PYEA slants and/or plates at 4–8°C.

Selection of gut bacteria for characterization Initially, the isolated bacteria were evaluated for their attractancy to fruit fly, *B*. *tau* (choice method). Thirty gut bacterial isolates, which were frequently isolated from different fruit fly samples across the locations, were used to study their attractiveness to *B*. *tau*. Pure culture (72 h old) of different bacterial isolates was grown on PYE (peptone yeast extract) broth medium. The bacterial isolates were taken in separate petri plates and kept inside the cage (45 cm×45 cm×55 cm) with un-inoculated PYE broth as control. Twenty-five pairs of 5-day-old fruit flies were released in the cage and flies visiting each treatment were recorded for 30 min. The experiment was repeated six times for *B*. *tau* and data obtained were analyzed statistically by using the technique of analysis of variance for completely randomized design as described by Gomez & Gomez (1984). The treatment means were compared at 5% level of significance by least significance difference test described by Gomez & Gomez (1984). Five gut bacteria having maximum fruit fly attractiveness were selected for further characterization.

Characterization of gut bacteria

Morphological and biochemical characterization Morphological (shape, Gram's staining, mobility), cultural

Table 1 Isolation of gut bacteria from different populations of *Bactrocera tau* from Himachal Pradesh (India)

B. tau isolates (n=5) used for bacterial isolation			Culture media used					
Isolate No. of *B. tau*	Location (District)	Host	Peptone Yeast Extract Agar (PYEA)			Brain Heart Infusion Agar (BHIA)		
			Bacterial colonies isolated	Bacterial isolates selected for attractancy screening	Bacterial isolate number	Bacterial colonies isolated	Bacterial isolates selected for attractancy screening	Bacterial isolate number
P1	Nadaun (Hamirpur)	Bottle gourd	4	4	P1A, P1B, P1C, P1D	4	2	B1A, B1B
P2	Nadaun (Hamirpur)	Bitter gourd	3	2	P2A, P2B	4	1	B2A
P3	Sundernagar (Mandi)	Cucumber	3	1	P3A	3	2	B3A, B3B
P4	Palampur (Kangra)	Cucumber	2	1	P4A	5	2	B4A, B4B
P5	Nauni (Solan)	Summer squash	4	2	P5A, P5B	4	1	B5A
P9	Mandi (Mandi)	Cucumber	2	1	P9A	3	1	B9A
P10	Ghumarwin (Bilaspur)	Cucumber	4	2	P10A, P10B	4	2	B10A, B10B
P15	Jawalamukhi (Kangra)	Bitter gourd	2	1	P15A	5	2	B15A, B15B
P18	Shahpur (Kangra)	Bitter gourd	4	2	P18A, P18B	3	1	B18A
Total			28	16		35	14	

(pigment production, growth in broth medium) and biochemical (citrate, methyl red, VP, TSI, catalase, oxidase and carbohydrate fermentation tests) characterization of pure culture was done by standard techniques and isolates were identified using *Bergey's Manual of Determinative Bacteriology* (Holt *et al*. 2000).

Molecular characterization

Extraction of genomic DNA Total genomic DNA of each isolate was extracted following the method of Prabhakar *et al*. (2009a). The 48-h-old bacterial culture multiplied on Peptone Yeast Extract Broth (PYEB) was transferred to 1.5 ml microtubes and spun at 10,000 rpm for 12 min. After discarding the supernatant, the microtubes containing bacterial pellets (approx. 50 mg) were immersed in liquid nitrogen container for one min and the pellet was ground to fine powder immediately using a micro pestle. To this, 700 μl of cetyltrimethyl ammonium bromide (CTAB) extraction buffer was added and incubated at 65°C for 1 h in a water bath (York Scientific Industries, Delhi, India).

An equal volume (700 μl) of chloroform: isoamyl alcohol (24:1 v/v) was added and the contents were mixed thoroughly. Tubes were spun at 10,000 rpm for 12 min in a high speed refrigerated centrifuge (Remi Elektrotechnik, India) at 4°C. Aqueous phase was transferred to new tubes and 450 μl pre-chilled isopropanol was added and kept at −20°C for 20–30 min to precipitate the DNA. Tubes were then spun at 10,000 rpm for 12 min and supernatant was decanted. The DNA pellet was washed with 70% ethanol (three times), dried and dissolved in 100 μl of Tris EDTA buffer (10 mM Tris HCl and 1 mM EDTA, pH 8.0). RNAse @ 10 $\mu l ml^{-1}$ (MBI Fermentas) was added and the emulsion was incubated for half an hour at 37°C. The amount of DNA was quantified by recording the absorbance at 260 nm wavelength using an UV/VIS spectrophotometer (Bio Rad, SmartSpec 3000). DNA was stored at −20°C for further use.

PCR amplification and sequencing Polymerase chain reaction (PCR) was performed with eubacterial primers pair forward 5′AGAGTTTGATCATGGCTCAG3′ and reverse 5′ TACCTTGTTACGACTTCACC 3′ targeting

the 1,400 bp *rrs* (16S rRNA) gene (Heddi *et al*. 1998) The PCR amplification was carried out in 0.2 ml PCR tubes with 25 µl reaction volume containing 10 ng of DNA template, 20 pmol of each primer in 25 mM $MgCl_2$, 10 mM of each deoxyribonucleoside triphosphate (Fermentas), 5 units of *Taq* polymerase (Life Technologies India, Pvt. Ltd.) and 10X reaction buffer. Amplifications were performed using a thermal cycler (GeneAmp PCR system 9700, Applied Biosystems, Carlsbad, CA, USA) with an initial denaturation step of 5 min at 94°C followed by 35 cycles at 94°C for 45 s, 53°C for 45 s, 72°C for 30 s and a final elongation step at 72°C for 5 min. The product was separated in a 1% (w/v) agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). PCR products (1,450 bp) of 16S rRNA gene of five gut bacteria obtained through amplification with specific primers were first purified with ethanol precipitation of amplified DNA, freeze dried (Christ *Alpha I-2LD*), and then custom sequenced (ABI PRISM 310^{TM} Genetic Analyzer, Applied Biosystems, USA) using the same upstream and downstream primers (Life Technologies India).

Nucleotide sequence analysis The sequences of different bacterial isolates were blasted using online NCBI Blastn program (http://www.ncbi.nih.gov/blast). For the purpose, 42 sequences of 16S rRNA of different bacteria of high sequence similarity were selected for sequence comparison from GenBank Nucleotide Database, NCBI. The pair-wise genetic distance between five gut bacterial isolates of *B. tau* and other selected bacterial sequences was determined.

The evolutionary history was inferred using the UPGMA method (Sneath & Sokal 1973). The evolutionary distances were calculated using Maximum Composite Likelihood method with bootstrap test (500 replicates) (Tamura *et al*. 2004). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 298 positions in the final dataset. Phylogenetic analysis was conducted in MEGA 4.1 Software programme (Tamura *et al*. 2007).

Results

Isolation and identification of gut bacterial isolates of fruit fly Gut bacteria were isolated from nine populations of fruit fly, *B. tau* on two culture media, viz. PYEA and BHIA (enriched culture media). It was found that bacteria were associated with all the nine populations of *B. tau* (Table 1). A total of 63 different bacterial colonies were observed on two culture media. Of these, 28 were isolated on PYEA and 35 on BHIA. On the basis of colony morphology (pigmentation, shape and size of colony), 16 and 14 isolates obtained on PYEA and BHIA (which were most common among different fruit fly isolates across the locations), respectively, were evaluated for the attractancy to adult fruit flies under laboratory conditions to select the most promising gut bacteria in terms of their attractancy for further characterization.

The five most promising gut bacterial isolates were selected based on their attractancy to *B. tau* (Table 2) for further characterization. These five gut bacterial isolates: P1B, P3A, P10A, B4A and B10B (P/B-media used for isolation, *i.e*., PYEA and Brain Heart Infusion Agar; 1,3,10,4.10: *B tau* isolate numbers giving information about the location and host; and A,B,C....: different bacterial colonies isolated from the gut of *B. tau*) were gram negative and rod shaped. All five isolates of gut bacteria were producing sediments when multiplied in broth medium. All the five gut bacterial isolates were non-pigment producing and showed mobility except for P10A, which produced yellow pigment on PYEA plates and was non-mobile. With regard to biochemical characteristics, all the isolates showed negative reaction for citrate, VP, TSI and gas production in glucose medium tests, whereas they were found positive for catalase and oxidase test. All isolates except PIB were negative for methyl red test (Table 3). On the basis of cultural, morphological, biochemical and 16S rRNA gene characteristics, the closest match to isolate P1B, P3A, P10A, B4A and B10B was *Delftia acidovorans* (91% DNA identity), *Pseudomonas putida* (97% DNA identity), *Flavobacterium* sp. (95% DNA identity), *Defluvibacter* sp. (98% DNA identity) and *Ochrobactrum* sp. (99% DNA identity), respectively (Table 3).

The 16S rRNA gene nucleotide sequences of these isolates were submitted to GenBank nucleotide database under accession numbers HQ446523 to HQ446527.

Phylogenetic studies of gut bacteria All the sequences of five bacterial isolates were compared with 42 other bacterial sequences available online in GenBank (NCBI) by multiple sequence alignment tools using

Table 2 Attractancy of bacterial isolates against fruit fly, *Bactrocera tau*

Sr. No.	Bacterial isolates (72 h old, 2 ml broth culture)	Fruit flies visited/30 min (n=50)		
		Female*	Male*	Total*
1	P1A	3.17	2.17	5.33
2	P1B**	9.83	7.17	17.00
3	P1C	3.50	3.83	7.33
4	P1D	3.17	3.50	7.17
5	P2A	1.17	1.00	2.17
6	P2B	3.00	2.00	5.00
7	P3A**	10.5	7.67	18.17
8	P4A	3.50	1.33	4.83
9	P5A	4.33	3.00	7.33
10	P5B	3.83	1.83	5.67
11	P9A	5.17	4.50	10.50
12	P10A**	8.67	6.67	15.33
13	P10B	4.67	4.83	9.50
14	P15A	2.17	2.50	4.67
15	P18A	1.67	1.33	2.17
16	P18B	3.50	4.17	7.67
17	B1A	2.33	3.83	6.17
18	B1B	3.00	2.50	5.17
19	B2A	3.50	3.17	9.50
20	B3A	3.33	2.67	6.00
21	B3B	2.17	2.50	4.67
22	B4A**	7.50	6.33	13.83
23	B4B	2.67	2.83	5.50
24	B5A	3.83	1.50	5.33
25	B9A	2.50	3.00	5.50
26	B10A	2.50	1.33	4.00
27	B10B**	6.33	5.83	12.17
28	B15A	4.33	4.17	8.83
29	B15B	3.00	2.83	6.17
30	B18A	2.67	2.17	4.83
31	Control (Un-inoculated PYEA broth)	1.67	1.83	3.50
	$LSD_{0.05}$	1.58	1.48	2.37

*Mean of six replications, LSD test

**Selected for characterization

ClustalW programme. The per cent pair-wise genetic distance of the five promising isolates with other bacterial sequences ranged from 0.00 to 0.70 nucleotide per site.

The dendrogram constructed by phylogenetic analysis presented in Fig. 1 showed that all the bacterial isolates, *viz*., P1B, P3A, B4A and B10B, clustered with *Delftia*, *Pseudomonas*, *Defluvibacter* and *Ochrobactrum*, respectively (all Proteobacteria), except P10A (HQ446525), which was clustered with *Flavobacterium* (a typical Bacteroidetes). Based on their affinity with known sequences in databank, the isolate P1B belongs to class β-Proteobacteria, P3A to class γ-Proteobacteria, and B4A & B10B to class α-Proteobacteria.

Discussion

Many workers suggested that nucleic acid sequence approaches, particularly 16S rRNA genes, have proved an important tool to settle the taxonomic position of the microbial community of insects (Brauman *et al*. 2001; Wang *et al*. 2011). An immense library of sequence data for 16S rRNA loci and other robust markers, allows the precise identification of many associated species, even those that resist cultivation (Stevenson *et al*. 2004). Over 200,000 bacterial entries exist currently for 16S rRNA, and these sequences can place most surveyed bacterial taxa securely into genera, if not species (Rupp 2004; Ueda *et al*. 2004).

The gut of tephritid fruit flies in general (Behar *et al*. 2009; Lloyd *et al*. 1986; Wang *et al*. 2011) and *B. tau* in particular (Prabhakar *et al*. 2009a; Sood & Nath 2002) is a storehouse of bacterial community. In the present study also 30 different bacterial colonies on the basis of colony morphology were screened for attractancy, but only five isolates which were found promising in terms of their attractancy to adult *B. tau* flies and could have a great implication on their future management programmes were further characterized. All these bacterial isolates except *P. putida* were not reported earlier to be associated with *B. tau* and having any role in fruit fly ecology. The attractiveness of these isolates to fruit flies, however, suggests their possible role in the fruit fly nutrition and physiology. Concrete and concentrated efforts across the fruit fly species are needed to elucidate the complex phenomenon to some conclusion.

Delftia was isolated from *B. tau* for the first time, although its association was earlier reported with cotton bollworm, *Helicoverpa armigera* (Hübner) (Xiang *et al*. 2006); wood borer, *Saperda vestita* (Say) (Delalibera

Table 3 Morphological, biochemical and molecular characteristics of gut bacteria of *Bactrocera tau*

Characteristics	Bacterial isolates				
	P1B	P3A	P10A	B4A	B10B
Morphological					
Shape	Rod	Rod	Rod	Rod	Rod
Gram's reaction	−	−	−	−	−
Pigment production	−	−	Y	−	−
growth in broth medium	Sediment	Sediment	Sediment	Sediment	Sediment
Mobility	+	+	−	+	+
Biochemical					
Citrate test	−	d	−	−	d
Methyl red	+	−	−	−	−
V P test	−	d	−	−	−
TSI	−	−	−	−	−
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
D-Glucose	−	+	+	−	d
Gas production in glucose medium	−	−	−	−	−
Molecular					
16S rDNA sequence blast similarity	91% with *Delftia acidovorans*	97% with *Pseudomonas putida*	95% with *Flavobacterium* sp.	98% with *Defluvibacter* sp.	99% with *Ochrobactrum* sp.
Bacteria–closest match to	*Delftia acidovorans*	*Pseudomonas putida*	*Flavobacterium* sp.	*Defluvibacter* sp.	*Ochrobactrum* sp.

Y yellow pigment, − negative reaction, + positive reaction, *d* doubtful result

et al. 2005); tobacco caterpillar, *Manduca sexta* (Brinkmann *et al*. 2008); and mosquito, *Aedes albopictus* Skuse (Zouache *et al*. 2009). Whereas *Pseudomonas putida* and members of genera *Pseudomonas* have been reported from many insect species including fruit flies as well as from *B*. *tau* (Brinkmann *et al*. 2008; Sood & Nath 2002). The presence of *Flavobacterium* sp., *Defluvibacter* sp. and *Ochrobactrum* sp. has also been reported in other insect species, *viz*. *Flavobacterium* sp. from ant, *Tetraponera binghami* Forel (van Borm *et al*. 2002), honey bees (Mohr & Tebbe 2007) and tobacco caterpillar, *M*. *sexta* (Brinkmann *et al*. 2008); however, the present findings constitute the first report of their association with fruit flies, particularly *Bactrocera* species.

Defluvibacter sp. is a member of the bacterial family Phyllobacteriaceae from the class α-Proteobacteria and has not been reported from the gut of any insect species. An unassigned bacterium (member Phyllobacteriaceae) has, however, been reported from the gut content of Asian longhorned beetle, *Anoplophora glabripennis* Motschulsky (Geib *et al*. 2009). The bacterial family Phyllobacteriaceae is closely related to the Bradyrhizobiaceae, Methylobacteriaceae and Rhizobiaceae. Bacteria from the families Methylobacteriaceae and Rhizobiaceae have been reported from different insect species, *viz*. *Rhizobium* and *Methylobacterium* from the gut of *Tetraponera* ants (van Borm *et al*. 2002) and *Rhizobium* from the gut content of Asian longhorned beetle, *Anoplophora glabripennis* (Geib *et al*. 2009).

Ochrobactrum sp. belongs to the α-2 subclass of the Proteobacteria (De Ley 1992). The genus was first described by Holmes *et al*. (1988) and the phylogenetic position of *Ochrobactrum* sp. was defined by De Ley (1992) and Yanagi & Yamasato (1993) on the basis of DNA ± rRNA hybridization and 16S rRNA homology studies. Its closest known relative is *Brucella* (De Ley 1992; Yanagi & Yamasato 1993).

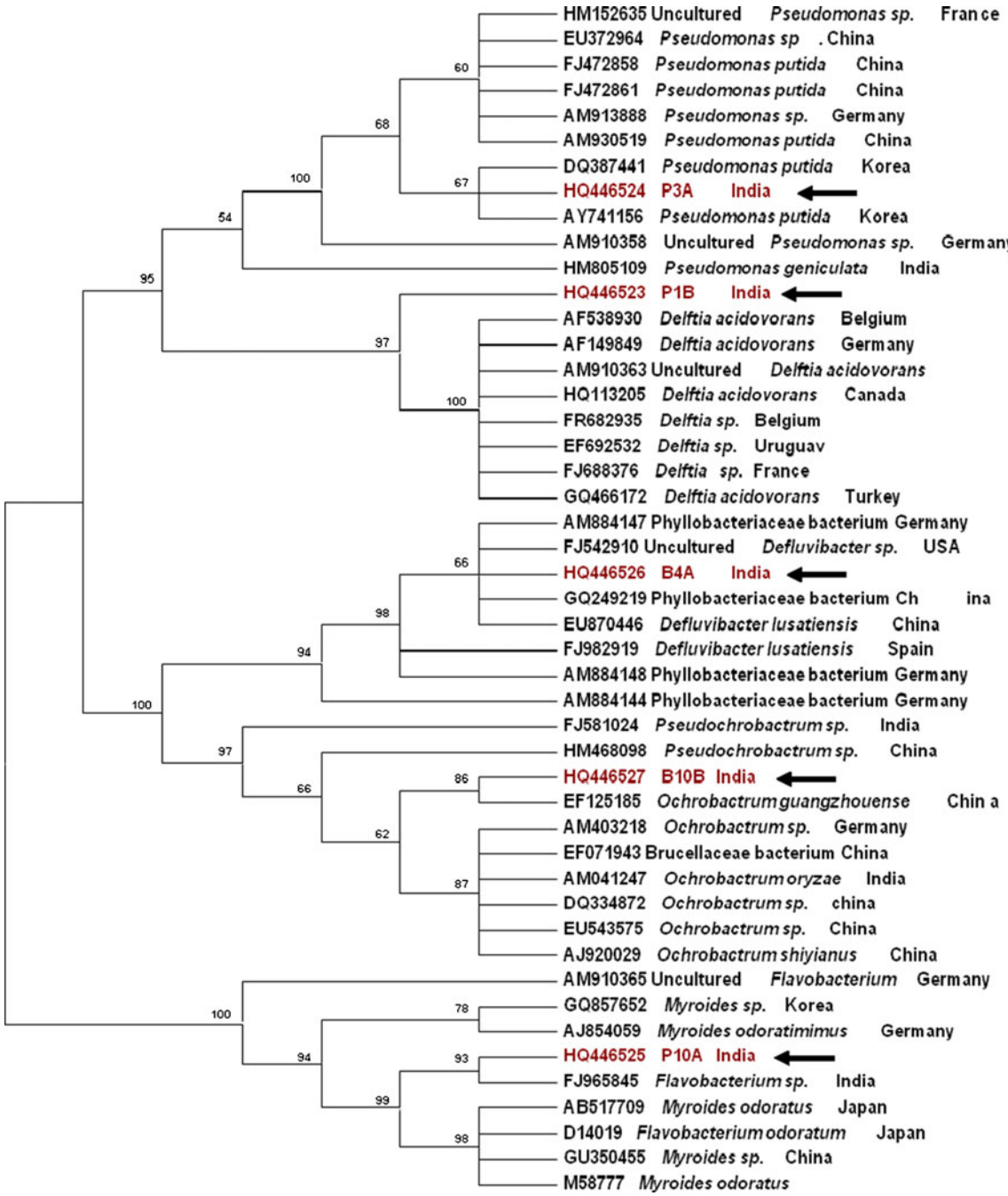

Fig. 1 Phylogenetic tree based on 16S rRNA gene sequences using Unpaired Group Mean Algorithm (UPGMA) method showing the relationships between five gut bacterial isolates of *Bactrocera tau*. Numbers above the nodes are bootstrap values (500 replicates). Phylogenetic analyses were conducted in software MEGA 4.1. *Arrows* indicate gut bacteria of *B. tau* isolated in present study

Ochrobactrum sp. was reported from the insect gut (Asian longhorned beetle, *Anoplophora glabripennis*) by Geib *et al*. (2009); its closest relative, *Brucella* sp., was isolated from the gut of wood borer *Saperda vestita* and identified by 16S rRNA analysis by Delalibera *et al*. (2005).

Earlier *Klebsiella oxytoca*, *Pantoea agglomerans*, *Staphylococcus* sp., *Pseudomonas putida*, *Bacillus* sp.

and *Enterobacter agglomerans* were reported from the gut of fruit fly, *B. tau* (Prabhakar *et al*. 2009a; Sood & Nath 2002). The present finding added a few more genera to this list, but as a whole all these reports suggested that a vast range of gut bacterial diversity exists in the *B. tau* system. Thorough isolation and characterization with culture-dependent and -independent techniques are therefore needed to explore the gut bacterial diversity of *B. tau* to understand the host behavior in relation to gut bacterial community. On the other hand, vertebrate pathogenicity of these bacteria can also not be denied as some species of *Ochrobactrum*, *Delftia*, *Staphylococcus* and *Defluvibacter* were previously reported as human pathogens (Battalgia 2008; Mastroianni *et al*. 1999; Ogston 1984; Preiswerkz *et al*. 2011); this, however, requires further investigations. Detailed investigations are also needed to establish the taxonomic positions of *Delftia*, *Flavobacterium* sp., *Defluvibacter* sp. and *Ochrobactrum* sp. up to species level using chemotaxonomic and molecular approaches to understand the tri-trophic interaction among host–fruit flies–microbes.

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