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Identification and evaluation of cultivable gut bacteria associated with peach fruit fly, *Bactrocera zonata* (Diptera: Tephritidae)

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Abstract The aim of the present study was to identify the cultivable gut bacteria associated with peach fruit fly, *Bactrocera zonata*, and evaluate their potential to attract adults of *B. zonata*. Based on culture-dependent characterization methods and *16S rRNA* gene sequence analysis, bacteria were identified as members of family Enterobacteriaceae (BZM1, *Klebsiella oxytoca*), Microbacteriacea (BZM2, *Microbacterium* spp.) and Nocardiaceae (BZM4, *Rhodococcus* spp.). Molecular phylogeny placed *Klebsiella oxytoca* within gram negative γ-proteobacteria whereas, *Microbacterium* spp. and *Rhodococcus* spp. were clustered under gram positive Actinobacteria group in family Microbacteriacea and Nocardiaceae, respectively. *16S rRNA* gene sequence comparison with the available NCBI database sequences further confirmed the characterizations of bacterial symbionts. Population of these bacterial species increased significantly up to the 11th day after emergence of adults and thereafter it remains constant. Among 3 bacterial symbionts, metabolites produced from *K. oxytoca* had the highest attraction to the *B. zonata* adult females over metabolites produced from other bacteria and their combinations in field bioassay. The *B. zonata* adult male flies attracted to metabolites produced from each bacterial symbionts alone and their combinations were less in number with comparison to the *B. zonata* adult females. The present study provides the first description of the attractancy potential of metabolites produced by gut microbial community of *B. zonata* in open field condition. This study results may prompt the development of a female-targeted population control strategy for this fly.

Keywords *Bactrocera zonata* · Bacterial symbionts · *16S rRNA* · *Klebsiella oxytoca* · Attractancy

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Introduction

The class Insecta, composed of over a million species, is regarded as the most diverse group of animals found almost in every habitat (Vilmos and Kurucz 1998; Park *et al*. 2004). Insects are estimated to harbor endosymbionts including viruses, bacteria, fungi, protozoa, nematodes and multicellular parasites (Buchner 1965; Douglas 1998; Moran *et al*. 2005). Associations of insect with micro-organisms are complex and intimate ranging from parasitism to mutualism, with a long period of association and co-evolution history (Dillon and Dillon 2004; Dale and Moran 2006). Interactions between hosts and their microbes can be parasitic, such as the interaction of the bacterium, *Paenibacillus larvae*

(American foulbrood) in honeybees (Schmid-Hempel 1998; Müller *et al.* 2015) and mutualistic, such as the interaction between termites and their gut microbes (Breznak and Brune 1994; Schmitt-Wagner *et al.* 2003). Compared to mammals, there is a lack of literature regarding the functions of microorganisms in insect guts but in several insect species, such as desert locusts and termites gut microorganisms have been extensively studied (Dillon and Charnley 2002; Ohkuma 2003; Hongoh *et al.* 2005).

Tephritid flies are commonly known as "fruit flies" because a number of species infest a wide variety of fruits and vegetables in tropical and subtropical regions of the world. Symbiotic associations between tephritids and their gut bacteria have been studied well particularly for viz., *Anastrepha, Bactrocera, Ceratitis* and *Rhagoletis* (Kuzina et al. 2001; Raghu *et al.* 2002; Petri 1909; Capuzzo *et al.* 2005; Kounatidis *et al.* 2009; Daser and Brandl 1992; Lauzon *et al.* 1998; Lauzon 2003; Prabhakar *et al.* 2009, 2013). Among *Bactrocera*, the olive fly *Bactrocera oleae* (Rossi) (Subfamily Dacinae), was the first species for which a bacterial symbiosis was described (Petri 1909). A range of bacteria belonging to different genera *viz. Acetobacter*, *Agrobacterium*, *Arthrobacter*, *Bacillus, Citrobacter, Defluvibacter, Delftia, Enterobacter, Escherichia, Erwinia, Flavobacterium, Hafnia, Kluyvera*, *Klebsiella, Listeria, Lactobacillus, Micrococcus, Ochrobactrum, Pantoea*, *Pectobacterium*, *Pseudomonas, Proteus, Providencia, Staphylococcus, Stentrophomonas, Streptococcus, Raoultella, Serratia* and *Xanthomonas* has been isolated and characterized from the gut of tephritid flies (Lloyd *et al.* 1986; Drew and Lloyd 1987; Jang and Nishijima 1990; Lauzon *et al.* 1998, 2000; Zinder and Dworkin 2000; Bergey *et al.* 2001; Kuzina *et al.* 2001; Marchini *et al.* 2002; Belcari *et al.* 2003; Behar *et al.* 2005, 2008, 2009; Capuzzo *et al.* 2005; Sacchetti *et al.* 2008; Kounatidis *et al.* 2009; Prabhakar *et al.* 2009; 2013; Crotti *et al.* 2010; Thaochan *et al.* 2010; Wang *et al* 2011; Reddy *et al.* 2014). These associated bacterial symbionts play a very important role in the host's nutrition, development, reproduction, resistance to pathogens, and semiochemicals production (Brand *et al.* 1975; Brune 2003; Moran *et al.* 2005; Dillon and Dillon 2004). They also provides digestive enzymes or vitamins (Nakabachi and Ishikawa 1999) lacking in fruit tissues of their host and improves digestion efficiency which are ultimately manifested in the development, fecundity, and survivability of their hosts. Furthermore, cultivable gut bacteria have also been implemented in pest management strategies; as bacteria were found to be involved in the degradation of the toxic compound ingested by the host insect leading to insecticide resistance (Eutick *et al.* 1978; Fukatsu and Hosokawa 2002; Bousch and Matsumara 1967; Prabhakar *et al.* 2008). Certain components of bacterial odour play an important role in fruit flies behaviour in feeding or ovipositional stimulants (Drew and Lloyd 1987; Lauzon *et al.* 2000) and have also been exploited in the pest management strategies in the form of baits or traps (Sacchetti *et al.* 2007; Robacker 2007; Thaochan and Chinajariyawong 2011; Sood *et al.* 2010).

The peach fruit fly, *Bactrocera zonata* (Saunders) is a serious and polyphagous pest of fruit crops and wild plant species in many parts of the world (Duyck *et al.* 2004). At present, it is widely distributed in Bangladesh, Bhutan, India, Iran, Laos, Myanmar, Nepal, Oman, Pakistan, Saudi Arabia, Sri Lanka, Thailand, United Arab Emirates, Vietnam and Yemen from Asia; and part of Africa (White and Elson-Harris 1992; Kapoor 1993; Choudhary *et al.* 2012; 2015). Reddy *et al.* (2014) reported bacterial population of genus *Bacillus*, *Enterobacter*, *Klebsiella* and *Stenotrophomonas* from the gut of laboratory reared and field collected *B. zonata* (Reddy *et al.* 2014). Laboratory studies on these cultivable bacterial odours suggest their possible implications in fruit fly management programmes (Shi *et al.* 2012; Reddy *et al.* 2014). However, there is still a dearth of knowledge on the bacterial communities attracting *B. zonata* under field conditions. The cultivable gut bacteria in particular will be more useful than non-cultivable bacteria while searching for a good fruit fly attractant. In this study, the gut symbionts of *B. zonata* were isolated and characterized using culture-dependent methods and molecular technique (*16S rRNA*) for their identification and also to establish the phylogenetic position. In this study, we also evaluated the chemo-attraction potential of dominant cultivable gut bacteria of *B. zonata* male and female adult flies under field conditions.

Materials & Methods

Origin and initiation of stock culture of *B. zonata*

Bactrocera zonata adult flies used in this study were reared with infested mango fruits collected from the research farm of ICAR Research Complex for Eastern Region, Research Centre, Ranchi, India (23° 45′ N latitude, 85° 30′ E longitude, elevation 620 m AMSL). Subsequent rearing was carried out at ambient room conditions (25±1°C temperature; 65±5% RH; 12:12 h LD photoperiod) in the laboratory. The infested fruits were kept individually in 20 × 15 cm cage with 5 cm of thick sterile fine sand until emergence of the adults. The adults were identified on the basis of morphological descriptions given by Drew and Raghu (2002), Madhura and Verghese (2004) and Prabhakar *et al.* (2012) and a pair (male and female) of *B. zonata* was released into a smaller rearing cage (30×30×30 cm) provided with their natural host (Mango/Guava) for oviposition. The feeding was supplemented with adult diet [(glucose and protein hydrolyzate (Protinex®, Pfizer Ltd., India) in the ratio of 1:1 in Petri plates)] and water *ad-libitum* through soaked cotton swabs in a 50 ml beaker. The food supplements were replaced weekly. Six inbreeding generations were reared for isolation of predominant and closely associate bacterial symbionts.

Dissection of *B. zonata* and isolation of gut bacteria

The bacteria were isolated from the gut of 11 days old adult flies reared in laboratory as described by Lloyd *et al.* (1986). Before dissection, adults were anesthetized at -20°C for 5 min. Flies were surface sterilized with ethanol (70%) for 30 seconds followed by sodium hypochloride (0.25%) for one minutes and then washed three times with sterilized distilled water (SDW) to remove external contaminations. Five adults of each male and female were dissected aseptically with two pairs of sterilized tweezers in a plate containing physiological saline to remove fly gut under laminar air flow. Gut content was streaked separately on Peptone Yeast Extract Agar (PYEA) and Nutrient Agar (NA) for bacterial growth at 30±1°C for 48-72 h. A single colony of each of the bacterial isolates was separated with the inoculation loop and streaked onto respective PYEA and NA plates for their growth. Predominant bacterial isolates were obtained through repeated sub-culturing to ensure their purity. The purified bacterial isolates with respective medium were maintained on PYEA slants and/or plates at 4-8°C for further use.

Morphological and biochemical characterization of gut bacteria

Morphological (Shape, Gram's staining), cultural (Pigment production, growth in broth medium) and biochemical (citrate, methyl red, Voges–Proskauer (V.P.), triple sugar iron (TSI), catalase, oxidase and carbohydrate fermentation tests) characterization of the pure culture was done by standard techniques and isolates characteristics were compared with *Bergey's Manual of Determinative Bacteriology* (Holt *et al.* 2000).

Molecular characterization

Genomic DNA extraction

Total genomic DNA of each bacterial isolate was extracted following the method of Prabhakar *et al.* (2009). The 48 h old bacterial cultures were multiplied on Peptone Yeast Extract Broth (PYEB) and transferred to 1.5 ml microtube 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 pellets were ground to fine powder immediately using micro pestle. To this, 700 µl of cetyltrimethylammonium bromide (CTAB) extraction buffer was added and incubated at 65° C for 1 h in a water bath. An equal volume (700 µl) of chloroform: isoamyl alcohol (24:1 v/v) was added and contents were mixed thoroughly. Tubes were spun at 10,000 rpm for 12 min in high speed refrigerated centrifuge (REMI India) at 4°C. The 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 per cent ethanol (three times), dried and dissolved in 100 µl of Tris EDTA buffer (10mM Tris HCl and 147 1mM EDTA, pH 8.0). RNAse @ 10 µl/ ml (Hi-media, Mumbai, India) was added and emulsion was incubated for 30 min at 37°C. Genomic DNA obtained was visualized after electrophoresis in a 1.0% agarose gel in 1× TAE and stored at -20°C for further use in PCR amplification.

PCR amplification of 16S rRNA genes

The *16S rRNA* universal bacterial primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3') (Weisburg *et al.* 1991) were used for Polymerase chain reaction (PCR), yielding an amplicon of approximately 1450bp. 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 (Fermentas) and 10X reaction buffer. Amplifications were performed using Flexigene 9700 thermal cycler (QIAGEN India Pvt Ltd.) with an initial denaturation step of 5 min at 94°C followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min 30 sec and a final extension 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, 1mM EDTA). PCR products of *16S rRNA* gene of three gut bacteria obtained through amplification with specific primers were freeze dried (*CHRIST ALPHA I-2LD*) and were custom sequenced (*ABI PRISM 310^{TM} 165 Genetic Analyzer, Applied Biosystems, USA*) using the same upstream and downstream primers (Xcelris Labs Limited, India).

Nucleotide sequence analysis

The sequences of different bacterial isolates were blasted using online NCBI Blastn program (http://www.ncbi.nih.gov/blast). Sequences with ≥98% identity, considered to be from same species and Sequences with ≥97% identity, considered to be form the same genera (Schloss and Handelsman 2005). Twenty-seven *16S rRNA* sequences of different bacteria (free living and insect symbionts including fruit fly symbionts) of high sequence similarity were selected for sequence comparison from GenBank Nucleotide Database, NCBI. The selected sequences along with three submitted bacterial sequences were aligned into the MEGA 6.0 alignment utility (Tamura *et al.* 2013), and a multiple sequence alignment was constructed using ClustalW using default parameters. The phylogenetic tree was inferred using the neighbour-joining (NJ) algorithms (Saitou and Nei 1987) in MEGA 6.0 software with *Burkholderia pseudomallei* kept as outgroup, and the confidence level of the tree topology was tested using 1000 bootstrap replicates (Felsenstein 1985).

Population kinetics of bacterial symbionts within *B. zonata*

The bacterial flora of adult fruit flies as affected by their age and sex was studied by bacterial count of whole fruit flies in different age groups of both sexes by the serial dilution method procedure as followed by Prabhakar *et al.* (2009). Three pairs (male and female) of fruit flies were randomly picked for the study. The adult flies (male and female) were anesthetized using cold treatment at 4°C for 10 min and flies were surface sterilized in sequential washing with 70% (v/v) alcohol for 30 s, 0.25% (v/v) sodium hypochloride for 1 min and sterile distilled water (SDW) for 30 s. The sterilized flies were crushed individually in a test tube with a sterile glass rod in 1 ml SDW. The final volume was made to 10 ml by adding SDW (stock solution). The bacterial population in the whole fruit fly was enumerated on PYEA plates using the serial dilution method. A known quantity (0.1 ml) of bacterial suspension from different dilutions was spread on the PYEA plates with the help of glass spreader. The plates were incubated at 30°C for 48 hrs. The process was repeated three times for 1-,2-,3-,4-,5-,7-,9-,11-,13-, 15-,17-, 19- and 21 day old flies. Data obtained were expressed in colony forming units (cfu's) after emergence of adult fruit flies.

Bioassay for attractancy of adult *B. zonata* flies to bacterial culture

The bacterial culture preparation and bioassay was performed as described by Martinez *et al.* (1994). In brief, bacterial isolates from the gut of *B. zonata* were inoculated to 100 ml of sterile PYE broth. The conical flasks were incubated in a shaker at 150 rpm for 6 days at 30 ±1°C. Bacterial cultures were centrifuged at 10,000 rpm for 15 min to separate pellet and supernatant. The pellet was discarded because most of the bacterial cells contained in the pellet were supposed to be less attractive, as shown in experiments described by Robacker and Garcia (1993).

The field bioassays were conducted in the mango orchard of ICAR Research Complex for Eastern Region, Research Centre, Ranchi, India during the month of June, 2014. Eight treatments were made comprising of three bacterial filtrates, three different combinations of bacterial filtrates, Methyl Eugenol (ME) trap and a control (uninnoculated PYE Broth). To test attraction of *B. zonata* adults under field conditions, 5 ml

filtrate of each bacterium + 1 drop of Dichlorvos (Nuvan®) was soaked onto the cotton balls. These cotton balls were tied with the help of thread and passed through the hole of the trap (plastic container) and lid was closed. Each trap was a cylindrical plastic container with wide holes on the surface to allow the entry of fruit flies inside it, one side having a lid and a small hole on the opposite side to pass the thread holding bacterial filtrates. After that, these traps were placed in the field fastened well on lower branches, positioned 1.0 m above the ground keeping 20 m distance between each other in three replications. The observations were taken after 24 hrs, 48 hrs and 72hrs. The same procedure was repeated three times in the same orchard.

Data analysis

The statistical analyses of the results were performed using the SPSS 16 statistical software (SPSS Inc., Chicago, IL, USA, 2007). The number of fruit flies attracted towards the treatment sources in the field attraction bioassay was converted into percentages and compared by one-way analysis of variance (ANOVA) with square-root transformation to meet the homogeneity of variances. Significant ANOVAs were followed by t-tests for multiple comparisons of mean values ($P \leq 0.05$).

Results

Characterization of gut bacteria

The three dominant cultivable bacterial isolates (BZM1, BZM2 and BZM4) from the mid gut of *B. zonata* were isolated, characterized and analyzed for attractancy towards fruit fly, *B. zonata*. Characterization based on morphological and biochemical tests showed that the isolated colonies were belonging to various genera, including rods of both Gram positive and Gram negative. Bacterial isolates BZM2 and BZM4 were gram positive while BZM1 was gram negative in reaction. With reference to biochemical characteristics, isolates BZM2 and BZM4 showed negative reaction for V.P., indole, cellobiose, raffinose and gas production in glucose medium while, found positive for catalase, growth in 10% NaCl and D-sorbitol tests. Morphological and biochemical characteristics were compared with *the Bergey's Manual of Determinative Bacteriology* (Holt *et al*. 2000) and the three bacterial isolates (BZM1, BZM2 and BZM4) were tentatively identified as *Klebsiella* sp., *Microbacterium* sp. and *Rhodococcus* sp., respectively (Table 1).

16S rRNA gene sequencingof three bacterial isolates *viz.*, BZM1, BZM2 and BZM4, using eubacterial universal primer resulted in the sequences of length 1337, 1336 and 1340bp, respectively. Homology analysis of bacterial symbionts with the blastn program, GenBank, NCBI revealed that BZM1 showed maximum homology with *Klebsiella oxytoca* strain 127 (KF254665, E-value 0.0, Total score 2,379, Maximum score 2,379 and Maximum identity 99.47%) identified as *Klebsiella oxytoca*, whereas, BZM2 with *Microbacterium* sp. (JN867365, E-value 0.0, Total score 2,412, Maximum score 2,412 and Maximum identity 97.00%) and BZM4 with *Rhodococcus* sp. (EU741198, E-value 0.0, Total score 2,304, Maximum score 2,304 and Maximum identity 98.35%) were identified as *Microbacterium* sp. and *Rhodococcus* sp., respectively. *16S rRNA* gene nucleotide sequences of these bacteria were submitted to GenBank database under accession number KR024407 (*Klebsiella oxytoca*), KR024408 (*Microbacterium* sp.) and KR024409 (*Rhodococcus* sp.).

The phylogenetic analysis also confirmed a similar relationship pattern of the three symbionts. The phylogenetic analysis with α-proteobacteria, *Burkholderi pseudomallei* as an outgroup placed *Klebsiella oxytoca* within gram negative γ-proteobacteria. *Microbacterium* sp. and *Rhodococcus* sp. were clustered under gram positive Actinobacteria group within the separate families, Microbacteriacea and Nocardiaceae, respectively (Fig 1).

Population kinetics of bacterial symbiont of the fruit fly

Symbiotic bacteria were found associated with the newly emerged adult *B. zonata* flies and the total bacterial count in one-day-old flies were 7.00×10^6 cfu in male and 9.00×10^6 cfu in female. The cfu values indicated that there was a significant rise in bacterial population in the adult flies with the increase in age up to the 11^{th} day (383.33×10^6 cfu in male and 436.67×10^6 cfu in female). After that, the bacterial population did not vary significantly and fluctuated to around 383.33×10^6 cfu and 436.67×10^6 cfu in male and female, respectively. The bacterial symbionts of adult fruit flies follow a characteristic S- shaped growth curve with respect to age of fruit flies in both male and female.

Table 1 Morphological, biochemical and molecular characteristics of predominant gut bacterial symbionts of peach fruit fly, *Bactrocera zonata*

Characteristics	Bacterial isolates		
	BZM1	BZM2	BZM4
Morphological			
Shape	Rod shape	Rod shape	Rod shape
Motility	-	+	-
Gram's reaction	-	+	+
Colonies Colour	White colonies	Yellow colonies	Orange Colonies
Growth in broth medium	Sediment	Sediment	Pellical formation
Biochemical			
Citrate test	+	+	-
Methyl red test	-	-	-
V.P test	+	-	-
Indole test	+	-	-
TSI test	-	-	-
Catalase test	+	+	+
Oxidase test	-	-	-
Growth in 10% NaCl	+	+	-
Carbohydrate fermentation (Biochemical)			
D-Glucose	+	+	+
D- Glucose (Gas production)	+	-	-
Sucrose	+	+	+
Lactose	+	+	-
Maltose	+	+	-
Raffinose	+	-	-
D-Mannose	+	+	-
Trehalose	+	-	+
Celliobiose	+	-	-
D-Sorbitol	+	+	+
Inositol	+	-	+
D-Xylose	+	+	±
Molecular			
16S rDNA gene sequence blast similarity	99.47% with *Klebsiella oxytoca* (KF254665)	97.00% with *Microbacterium* spp. (JN867365)	98.35% with *Rhodococcus* spp. (EU741198)
Bacteria Characterized as	*Klebsiella oxytoca*	*Microbacterium* spp.	*Rhodococcus* spp.

+, positive reaction; -, negative reaction; ±, doubtful reaction

The population of gut bacterial isolates of *B. zonata* i.e. *K. oxytoca*, *Microbacterium* sp. and *Rhodococcus* sp. were increased significantly upto the 11th day and thereafter fluctuated between 168.00 x 10^6 to 182.33 x 10^6, 133.00 x 10^6 to 137.00 x 10^6, 53.67 x 10^6 to 58.00 x 10^6 cfu upto the 21st day, respectively. The characteristic S- shaped growth curves were obtained for the population of different bacteria with respect to the age of *B. zonata* (Fig 2).

Attractancy bioassay

The results of field evaluation of bacterial supernatant showed a considerable decrease in the number of

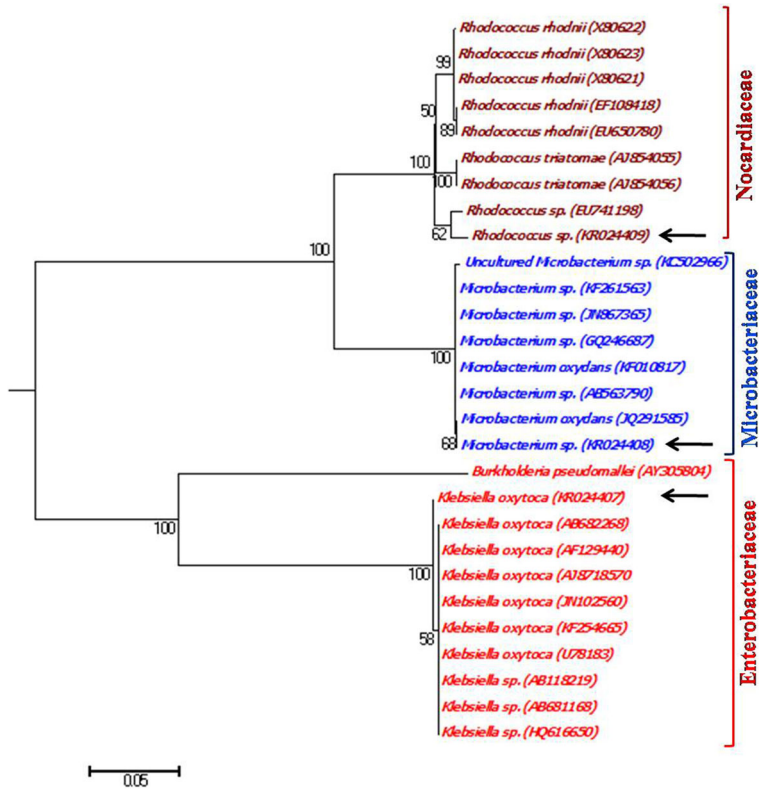

Fig 1 Neighbor-joining tree of bacterial symbionts of *Bactrocera zonata* based on *16S rRNA* gene sequences. GenBank accession numbers are followed by names of bacteria. *Burkholderia pseudomallei* was taken as an outgroup. The arrows indicate gut symbionts of *B. zonata* in present study

B. zonata flies captured to bacterial attractants from 24 h to 72 h of observations. A significant difference was observed in the attractancy of male and female fruit flies at different time intervals under field conditions (Table 2). As described in the methods, the effect of this change in population size was eliminated by transforming the actual counts of flies captured in traps into percentages of flies captured after every 24 hrs. Bacterial isolate BZM1 (*K. oxytoca*) attracted the highest number of females (8.33±0.33%, 5.67±0.33% and 3.33±0.33%) and males (6.67±0.33%, 3.67±0.33% and 1.33±0.33%) at 24, 48 and 72 h intervals, respectively. Whereas, the maximum number of adult male flies were trapped on ME traps (36, 42 and 34 males at 24, 48 and 72 hrs interval, respectively). Combination of BZM1+BZM2 also showed similar trend in the

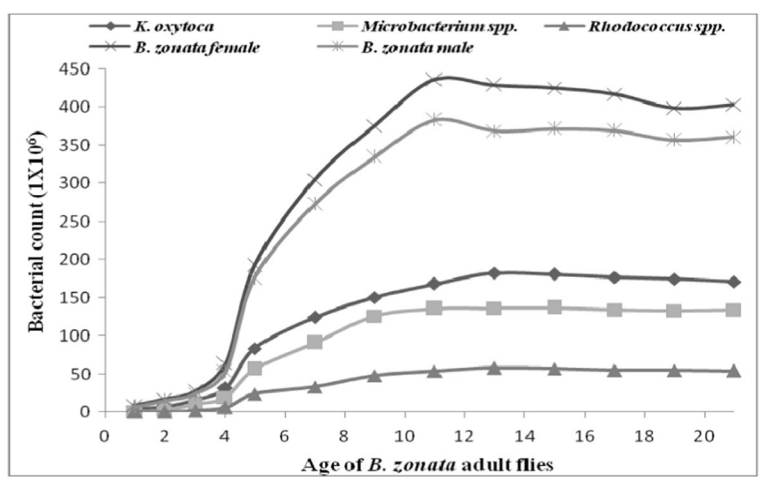

Fig 2 Growth curve of three bacterial symbionts and total bacterial population within the adult male and female *B. zonata* against the age of adult *B. zonata*

Table 2 Number of adult flies of *Bactrocera zonata* captured in traps baited with bacterial supernatant from cultivable bacterial broth culture

Bacterial isolates	24hrs		48hrs		72 hrs	
	Female	Male	Female	Male	Female	Male
Klebsiella oxytoca (BZM1)	8.33±0.33^{a}	6.67±0.33^{b}	5.67±0.33^{a}	3.67±0.33^{b}	3.33±0.33^{a}	1.33±0.33^{b}
Microbacterium spp. (BZM2)	2.67±1.20^{b}	2.33±0.88^{bc}	2.00±0.58^{b}	1.33±0.33^{c}	1.67±0.33^{b}	1.00±0.00^{b}
Rhodococcus spp.(BZM4)	2.00±0.58^{b}	1.67±0.67^{c}	1.67±0.33^{bc}	1.00±0.58^{c}	1.67±0.33^{b}	0.33±0.33^{b}
Klebsiella oxytoca + *Microbacterium* spp. (BZM1+BZM2)	6.67±0.33^{a}	4.67±0.33^{b}	3.33±0.33^{ab}	2.00±0.00^{c}	2.33±0.33^{a}	1.33±0.33^{b}
Klebsiella oxytoca + *Rhodococcus* spp. BZM1+BZM4	6.33±0.33^{a}	4.33±0.33^{b}	3.33±0.33^{ab}	1.67±0.33^{c}	1.33±0.33^{b}	0.67±0.33^{b}
Microbacterium spp. + *Rhodococcus* spp. (BZM2+BZM4)	3.33±0.33^{b}	1.33±0.33^{c}	2.00±0.00^{b}	1.00±0.00^{c}	1.00±0.00^{b}	0.00^{b}
Methyl Eugenol trap	0.00^{b}	36.00±3.76^{a}	0.00^{c}	42.00±3.76^{a}	0.00^{c}	34±2.65^{a}
Media Blank	1.33±0.33^{b}	0.67±0.33^{c}	0.67±0.33^{bc}	0.33±0.33^{c}	0.33±0.3^{bc}	0.00^{b}

Means value followed by the same superscripting alphabet do not differ significantly at $P \leq 0.05$

attractancy of *B. zonata* flies. Over the course of time during 72 hrs of experiment, all the treatments containing bacterial supernatant, alone or in combinations, attracted more females of *B. zonata* than males (Table 2). This suggests that volatile(s) produced by these bacteria may contain chemical(s) that are more specific for attracting females.

Discussion

In this study, morphological, biochemical reactions and *16S rDNA* sequence analysis were used to identify dominant cultivable gut bacterial populations of the peach fruit fly, *B. zonata*. Earlier, many workers suggested that the nucleic acid sequences, mainly *16S rRNA* genes, have proved to be an important tool to know the taxonomic position of the microbial community of insects (Brauman *et al.* 2001; Toth *et al.* 2001; Thaochan *et al.* 2010; Wang *et al.* 2011). The gut of tephritid fruit flies in general (Lloyd *et al.* 1986; Drew and Lloyd 1987; Belcari *et al.* 2003; Behar *et al.* 2005, 2008, 2009; Capuzzo *et al.* 2005; Sacchetti *et al.* 2008; Kounatidis *et al.* 2009; Crotti *et al.* 2010; Thaochan *et al.* 2010; Wang *et al* 2011) and *B. zonata* in particular (Reddy *et al.* 2014) is a store house of bacterial community. In this study, three dominant bacterial symbionts viz., *K. oxytoca*, *Microbacterium* spp. and *Rhodococcus* spp. were identified. The Gram-negative bacteria i.e. *Klebsiella* belongs to the family *Enterobacteriaceae*, while Gram-positive bacteria (*Microbacterium* sp. and *Rhodococcus* sp.) were from the families *Microbacteriaceae* and *Nocardiaceae*. Earlier, *K. oxytoca*, was also reported from the gut of *B. zonata* and *Bactrocera tau* (Walker) (Sood and Nath 2002; Reddy *et al.* 2014; Prabhakar *et al.* 2009). Moreover, *Enterobacteriaceae* have frequently been identified as the dominant species in the gut of several other tephritids, such as *Dacus* (Drew and Lloyd 1987), *Bactrocera* (Capuzzo *et al.* 2005; Prabhakar *et al.* 2009; Shi *et al.* 2012; Wang *et al.* 2011, 2013), *Anastrepha* (Kuzina *et al.* 2001) and *Ceratitis* (Behar *et al.* 2008). Recently, It has been postulated that gut enterobacteria are dispersed into the female reproductive system, where they are subsequently transferred to the eggs, then to fruit during oviposition and finally passed to the fly offspring (Behar *et al.* 2008; Shi *et al.* 2012).

Microbacterium spp. of phylum Actinobacteria was also identified in the *16S rDNA* cloned libraries from the intestinal tract of laboratory-reared and field-collected populations of oriental fruit fly, *B. dorsalis* (Wang *et al.* 2011). In the present study, *Rhodococcus* spp., which belongs to family *Nocardiaceae*, was first time reported from the gut of tephritids. Ngugi *et al.* (2005) reported, *Rhodococcus opacus* a gram-positive resorcinol degrading bacteria in the gut of termite, *Macrotermes michaelseni* whereas two strain of *Rhodococcus triatomae* were isolated from a blood-sucking bug of the genus *Triatoma* (Yassin 2005). Vertebrate pathogenicity of *Microbacterium* sp. and *Rhodococcus* sp.

cannot be denied as some of these bacterial species have earlier been reported as vertebrate pathogens (Laffineur *et al*. 2003; Muscatello *et al*. 2007). However, the present findings get substantial support from the observation of other workers, who reported the gut association of *K. oxytoca* and *Microbacterium* sp. with different species of tephritids.

The significant rise in total bacterial population within the adult flies shows up to the 11th day (383.33 x 10^6 cfu in male and 436.67 x 10^6 cfu in female) and did not differ significantly thereafter. The bacterial population reached its maximum on the 11th day, coinciding with the reproductive maturity of fruit flies (gonad development), indicating a possible role in meeting the protein requirements of the flies. Bacterial symbionts have a vital role in the adult survival (Capuzzo *et al*. 2005), nitrogen fixation and in pectin degradation (Behar *et al*. 2005). The bacterial cells may also be consumed as food, providing amino acids, supplementary nitrogen compounds and other nutrients which are scarce in fruits. The exact role of gut bacteria in the life-cycle of different fruit fly species is not well understood and further study is required.

According to the results of field studies, significant higher numbers of female flies were captured than male flies in all the bacterial based baits. The attractiveness of these isolates to fruit flies however suggests their possible role in the fruit fly nutrition and physiology. However, concrete and concentrated efforts across the fruit fly species are needed to elucidate the complex phenomenon, to draw conclusions. The present findings are similar with earlier reports where attraction of female flies was higher than male flies towards metabolites from bacterial isolates (Wang *et al*. 2012). Male *B. zonata* flies were strongly attracted by methyl eugenol (ME). ME is widely used as an attractant to control fruit flies (Hee and Tan 2004). ME attracted only males while in present study, metabolites produced by the cultivable bacteria of *B. zonata* trapped both females and males. The bacterial species are also known to produce volatiles as a metabolic by-product when cultured on medium (Kai *et al*. 2009). Thus, it can be drawn that bacteria emitting volatile chemical constituents play an important role in luring female flies in the field. To identify the bioactive chemicals in the metabolites of bacterial symbionts further, gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC) is required. With improvement of the emitted volatile compounds, novel compounds supplement to methyl eugenol even for female targeted trapping can be developed. The behavioral responses of *B. zonata* were documented by the field bioassays in this study. Consequently, the current study significantly supplements to the available information on bacterial isolates and also illustrates the potential to develop a female-targeted strategy to control this polyphagous pest as well as the expansion of our understanding of insect-bacteria symbiosis.

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References

Behar, A., Yuval, B., & Jurkevitch, E. (2005). Enterobacteria-mediated nitrogen fixation in natural populations of the fruit fly, *Ceratitis capitata. Molecular Ecology, 14*, 2637–2643.

Behar, A., Jurkevitch, E., & Yuval, B. (2008). Bringing back the fruit into fruit fly-bacteria interactions. *Molecular Ecology, 17*, 1375–1386.

Behar, A., Ben-Yosef, M., Lauzon, C. R., Yuval, B., & Jurkevich, E. (2009). Structure and function of the bacterial community associated with the Mediterranean fruit fly. In K. Bourtzis & T. Miller (Eds.), *Insect symbiosis* (pp. 251–271). Boca Raton: CRC.

Belcari, A., Sacchetti, P., Marchi, G., & Surico, G. (2003). The olive fly and associated bacteria. *Informatics Fitopatologia, 53*, 55–59.

Bergey, D. H., Holt, J. G., & Krieg, N. R. (2001). *Bergey's manual of systematic bacteriology*. Baltimore: Williams and Wilkins.

Bousch, G. M., & Matsumara, F. (1967). Insecticidal degradation by *Pseudomonas melophthora*, the bacterial symbiote of the apple maggot. *Journal of Economic Entomology, 69*, 918–920.

Brand, J. M., Bracke, J. W., Markovetz, A. J., Wood, D. L., & Browne, L. E. (1975). Production of verbenol pheromone by a bacterium isolated from bark beetles. *Nature, 254*, 136–137.

Brauman, A., Dore, J., Eggleton, P., Bignell, D., Breznak, J. A., & Kane, M. D. (2001). Molecular phylogenetic profiling of prokaryotic communities in guts of termites with different feeding habits. *FEMS Microbiology and Ecology, 35*, 27–36.

Breznak, J. A., & Brune, A. (1994). Role of microorganisms in the digestion of lignocellulose by termites. *Annual Review of Entomology, 39*, 453–487.

Brune, A. (2003). Symbionts aiding digestion. In V. H. Resh & R. T. Cardé (Eds.), *Encyclopedia of insects* (pp. 1102–1107). Academic Press.

Buchner, P. (1965). *Endosymbiosis of animals with plant microorganisms*. New York: Wiley.

Capuzzo, C., Firrao, G., Mazzon, L., Squartini, A., & Girolami, V. (2005). '*Candidatus Erwinia dacicola*', a coevolved symbiotic bacterium of the olive fly *Bactrocera oleae* (Gmelin). *International Journal of Systematic Evolutionary Microbiology, 55*, 1641–1647.

Choudhary, J. S., Kumari, A., Das, B., Maurya, S., & Kumar, S. (2012). Diversity and population dynamic of fruit flies species in methyl eugenol based parapheromone traps in Jharkhand region of India. *The Ecoscan, 1*, 57–60.

Choudhary, J. S., Naaz, N., Prabhakar, C. S., Srinivasa Rao, M., & Das, B. (2015). The mitochondrial genome of the peach fruit fly, *Bactrocera zonata* (Saunders) (Diptera: Tephritidae): complete DNA sequence, genome organization, and phylogenetic analysis with other tephritids using next generation DNA sequencing. *Gene, 569*, 191–202.

Crotti, E., Rizzi, A., Chouaia, B., Ricci, I., Favia, G., Alma, A., Sacchi, L., Bourtzis, K., Mandrioli, M., Cherif, A., Bandi, C., & Daffonchio, D. (2010). Acetic acid bacteria, newly emerging symbionts of insects. *Applied and Environmental Microbiology, 76*, 6963–6970.

Dale, C., & Moran, N. A. (2006). Molecular interactions between bacterial symbionts and their hosts. *Cell, 126*, 453–465.

Daser, U., & Brandl, R. (1992). Microbial gut floras of 8 species of Tephritids. *Biological Journal of the Linnean Society, 45*, 155–165.

Dillon, R., & Charnley, K. (2002). Mutualism between the desert locust *Schistocerca gregaria* and its gut microbiota. *Research in Microbiology, 153*, 503–509.

Dillon, R. J., & Dillon, V. M. (2004). The gut bacteria of insects: nonpathogenic interactions. *Annual Review of Entomology, 49*, 71–92.

Douglas, A. (1998). Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. *Annual Review of Entomology, 43*, 17–37.

Drew, R. A. I., & Lloyd, A. C. (1987). Relationship of fruit flies (Diptera: Tephritidae) and their bacteria to host plants. *Annals of the Entomological Society of America, 80*, 629–636.

Drew, R. A. I., & Raghu, S. (2002). The fruit fly fauna (Diptera: Tephritidae: Dacinae) of the rainforest habitat of the Western Ghats, India. *Raffles Bulletin of Zoology, 50*(2), 327–352.

Duyck, P. F., Sterlin, J. F., & Quilici, S. (2004). Survival and development of different life stages of *Bactrocera zonata* (Diptera: Tephritidae) reared at five constant temperatures compared to other fruit fly species. *Bulletin of Entomological Research, 94*, 89–93.

Eutick, M. L., O'Brien, R. W., & Slaytor, M. (1978). Bacteria from the gut of Australian termites. *Applied and Environmental Microbiology, 35*(5), 823–828.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution, 39*, 783–791.

Fukatsu, T., & Hosokawa, T. (2002). Capsule transmitted gut symbiotic bacterium of the Japanese common plataspid stinkbug, *Megacopta punctatissima*. *Applied and Environmental Microbiology, 68*(1), 389–396.

Hee, A. K. W., & Tan, K. H. (2004). Male sex pheromonal components derived from methyl eugenol in the haemolymph of fruit fly *Bactrocera papayae*. *Journal of Chemical Ecology, 30*, 2127–2138.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., & Williams, S. T. (2000). *Bergey's manual of determinative bacteriology* (pp. 175–533). New York: LIPPNCOTT Williams and Wilkins.

Hongoh, Y., Deevong, P., Inoue, T., Moriya, S., Trakulnaleamsai, S., Ohkuma, M., Vongkaluang, C., Noparatnaraporn, N., & Kudo, T. (2005). Intra and interspecific comparisons of bacterial diversity and community structure support coevolution of gut microbiota and termite host. *Applied and Environmental Microbiology, 71*(11), 6590–6599.

Jang, E. B., & Nishijima, K. A. (1990). Identification and attractancy of bacteria associated with *Dacus dorsalis* (Diptera: Tephritidae). *Environmental Entomology, 19*, 1726–1731.

Kai, M., Haustein, M., Molina, F., Petri, A., Scholz, B., & Piechulla, B. (2009). Bacterial volatiles and their action potential. *Applied Microbiology and Biotechnology, 81*, 1001–1012.

Kapoor, V. C. (1993). *Indian fruit flies (Insecta: Diptera: Tephritidae)* (p. 228). New York: International Sciences Publisher.

Kounatidis, I., Crotti, E., Sapountzis, P., Sacchi, L., Rizzi, A., Chouaia, B., et al. (2009). *Acetobacter tropicalis* is a major symbiont of the olive fruit fly (*Bactrocera oleae*). *Applied and Environmental Microbiology, 75*, 3281–3288.

Kuzina, L. V., Peloquin, J. J., Vacek, D. C., & Miller, T. A. (2001). Isolation and identification of bacteria associated with adult laboratory Mexican fruit flies, *Anastrepha ludens* (Diptera: Tephritidae). *Current Microbiology, 42*, 290–294.

Laffineur, K., Avesani, V., Cornu, G., Charlier, J., Janssens, M., Wauters, G., & Delmée, M. (2003). Bacteremia due to a novel *Microbacterium* species in a patient with leukemia and description of *Microbacterium paraoxydans* sp. nov. *Journal of Clinical Microbiology, 41*, 2242–2246.

Lauzon, C. R. (2003). Symbiotic relationships of Tephritids. In K. Bourtzis & T. A. Miller (Eds.), *Insect symbiosis* (pp. 115–129). Boca Raton: CRC.

Lauzon, C. R., Sjogren, R. E., Wright, S. E., & Prokopy, R. J. (1998). Attraction of *Rhagoletis pomonella* (Diptera: Tephritidae) flies to odor of bacteria: apparent confinement to specialized members of Enterobacteriaceae. *Environmental Entomology, 27*, 853–857.

Lauzon, C. R., Sjogren, R. E., & Prokopy, R. J. (2000). Enzymatic capabilities of bacteria associated with apple maggot flies, a postulated role in attraction. *Journal of Chemical Ecology, 26*, 953–967.

Lloyd, A. C., Drew, R. A. I., Teakle, D. S., & Hayward, A. C. (1986). Bacteria associated with some *Dacus* species (Diptera: Tephritidae) and their host fruits in Queensland. *Australian Journal of Biological Sciences, 39*, 361–368.

Madhura, H. S., & Verghese, A. (2004). A guide to identification of some common fruit flies (*Bactrocera* spp.) (Diptera: Tephritidae: Dacinae). *Pest Management in Horticultural Ecosystem, 10*(1), 87–96.

Marchini, D., Rosetto, M., Dallai, R., & Marri, L. (2002). Bacteria associated with the oesophageal bulb of the medfly *Ceratitis capitata* (Diptera: Tephritidae). *Current Microbiology, 44*, 120–124.

Martinez, A. J., Robacker, D. C., Garcia, J. A., & Esau, K. L. (1994). Laboratory and field olfactory attraction of the

Mexican fruit fly (Diptera: Tephritidae) to metabolites of bacterial species. *Florida Entomology, 77*, 117–126.

Moran, N. A., Degnan, P. H., Santos, S. R., Dunbar, H. E., & Ochman, H. (2005). The players in a mutualistic symbiosis: insects, bacteria, viruses, and virulence genes. *Proceedings of the National Academy of Sciences, USA, 102*, 16919–16926.

Müller, S., Eva, G. S., Elke, G., & Süssmuth, R. D. (2015). Involvement of secondary metabolites in the pathogenesis of the American foulbrood of honey bees caused by *Paenibacillus* larvae. *Natural Product Reports, 32*, 765–778.

Muscatello, G., Leadon, D. P., Klayt, M., et al. (2007). *Rhodococcus equi* infection in foals: the science of 'rattles'. *Equine Veterinary Journal, 39*, 470–478.

`Nakabachi, A., & Ishikawa, H. (1999). Provision of riboflavin to the host aphid, *Acyrthosiphon pisum*, by endosymbiotic bacteria, *Buchnera*. *Journal of Insect Physiology, 45*, 1–6.

Ngugi, D. K., Tsanuo, M. K., & Boga, H. I. (2005). *Rhodococcus opacus* strain RW, a resorcinol degrading bacterium from the gut of *Macrotermes michaelseni*. *African Journal of Biotechnology, 4*(7), 639–645.

Ohkuma, M. (2003). Termite symbiotic systems: efficient biorecycling of lignocellulose. *Applied Microbiology and Biotechnology, 61*, 1–9.

Park, Y., Kim, Y., Tunaz, H., & Stanley, D. W. (2004). An entomopathogenic bacterium, *Xenorhabdus nematophila*, inhibits hemocytic phospholipase A2 (PLA2) in tobacco hornworms, *Manduca sexta*. *Journal of Invertebrate Pathology, 86*, 65–71.

Petri, L. (1909). *Ricerche Sopra i Batteri Intestinali della Mosca Olearia*. Roma: Memorie della Regia Stazione di Patologia Vegetale di Roma.

Prabhakar, C. S., Sood, P., & Mehta, P. K. (2008). Protein hydrolyzation and pesticide tolerance by gut bacteria of *Bactrocera tau* (Walker). *Pest Management and Economic Zoology, 16*, 123–129.

Prabhakar, C. S., Sood, P., Kapoor, V., Kanwar, S. S., Mehta, P. K., & Sharma, P. N. (2009). Molecular and biochemical characterization of three bacterial symbionts of fruit fly, *Bactrocera tau* (Tephritidae: Diptera). *Journal of General and Applied Microbiology, 55*, 213–220.

Prabhakar, C. S., Sood, P., & Mehta, P. K. (2012). Pictorial keys for predominant *Bactrocera* and *Dacus* fruit flies (Diptera: Tephritidae) of north western Himalaya. *Arthropods, 1*(3), 101–111.

Prabhakar, C. S., Sood, P., Kanwar, S. S., Sharma, P. N., Kumar, A., & Mehta, P. K. (2013). Isolation and characterization of gut bacteria of fruit fly, *Bactrocera tau* (Walker). *Phytoparasitica, 41*, 193–201.

Raghu, S., Clarke, A. R., & Bradley, J. (2002). Microbial mediation of fruit fly-host plant interactions: is the host plant the "centre of activity"? *Oikos, 97*, 319–328.

Reddy, K., Sharma, K., & Singh, S. (2014). Attractancy potential of culturable bacteria from the gut of peach fruit fly, *Bactrocera zonata* (Saunders). *Phytoparasitica*. doi:10.1007/s12600-014-0410-9.

Robacker, D. C. (2007). Chemical ecology of bacteria relationships with fruit flies, Integrated Protection of Olive Crops. *IOBC/WPRS Bulletin, 30*, 9–22.

Robacker, D. C., & Garcia, J. A. (1993). Effects of age, time of day, feeding history, and gamma irradiation on attraction of Mexican fruit flies (Diptera: Tephritidae), to bacterial odor in laboratory experiments. *Environmental Entomology, 22*, 1367–1374.

Sacchetti, P., Landini, S., Granchietti, A., Cama, A., Rosi, M. C., & Belcari, A. (2007). Attractiveness to the olive fly of *Pseudomonas putida* isolated from the foregut of *Bactrocera oleae*. Integrated Protection of Olive Crops. *IOBC/WPRS Bulletin, 30*, 37–42.

Sacchetti, P., Granchietti, A., Landini, S., Viti, L., Giovannetti, L., & Belcari, A. (2008). Relationships between the olive fly and bacteria. *Journal Applied Entomology, 132*, 682–689.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution, 4*, 406–425.

Schloss, P. D., & Handelsman, J. (2005). Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Applied and Environmental Microbiology, 71*, 1501–1506.

Schmid-Hempel, P. (1998). *Parasites in social insects*. Princeton: Princeton University Press.

Schmitt-Wagner, D., Friedrich, M. W., Wagner, B., & Brune, A. (2003). Phylogenetic diversity, abundance, and axial distribution of bacteria in the intestinal tract of two soil feeding termites (*Cubitermes* spp.). *Applied and Environmental Microbiology, 69*, 6007–6017.

Shi, Z., Wang, L., & Zhang, H. (2012). Low diversity bacterial community and the trapping activity of metabolites from cultivable bacteria species in the female reproductive system of the oriental fruit fly, *Bactrocera dorsalis* Hendel (Diptera: Tephritidae). *International Journal of Molecular Sciences, 13*, 6266–6278.

Sood, P., & Nath, A. (2002). Bacteria associated with *Bactrocera* sp. (Diptera: Tephritidae) – isolation and identification. *Pest Management and Economic Zoology, 10*, 1–9.

Sood, P., Prabhakar, C. S., & Mehta, P. K. (2010). Eco-friendly management of fruit flies through their gut bacteria. *Journal of Insect Science, 23*, 275–283.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution, 30*, 2725–2729.

Thaochan, N., & Chinajariyawong, A. (2011). Attraction of *Bactrocera cucurbitae* and *B. papayae* (Diptera: Tephritidae) to the odor of the bacterium *Enterobacter cloacae*. *Philippines Agricultural Scientist, 94*, 1–6.

Thaochan, N., Drew, R. A. I., Hughes, J. M., Vijaysegaran, S., & Chinajariyawong, A. (2010). Alimentary tract bacteria isolated and identified with API- 20E and molecular cloning techniques from Australian tropical fruit flies, *Bactrocera cacuminata* and *B. tryoni*. *Journal of Insect Science, 10*, 131.

Toth, E., Kovacs, G., Schumann, P., Kovacs, A. L., & Steiner, U. (2001). *Shineria* larvae gen. nov. isolated from the 1st and 2nd larval stages of *Wohlfahrtia magnifica* (Diptera: Sarcophagidae). *International Journal of Systematic Evolution and Microbiology, 51*, 401–407.

Vilmos, P., & Kurucz, E. (1998). Insect immunity: evolutionary roots of the mammalian innate immune system. *Immunology Letters, 62*, 59–66.

Wang, H., Jin, L., & Zhang, H. (2011). Comparison of the diversity of the bacterial communities in the intestinal tract of adult

Bactrocera dorsalis from three different populations. *Journal of Applied Microbiology, 110*, 1390–1401.

Wang, H., Jin, L., Peng, T., Zhang, H., Chen, Q., & Hua, Y. (2013). Identification of cultivable bacteria in the intestinal tract of *Bactrocera dorsalis* from three different populations and determination of their attractive potential. *Pest Management Science*. doi:10.1002/ps.3528.

Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology, 173*, 697–703.

White, I. M., & Elson-Harris, M. (1992). *Fruit flies of economic significance: Their identification and bionomics*. Wallingford: International Institute of Entomology: CAB International.

Yassin, A. F. (2005). *Rhodococcus triatomae* sp. nov., isolated from a blood-sucking bug. *International Journal of Systematic Evolution and Microbiology, 55*(4), 1575–1579.

Zinder, D. E., & Dworkin, M. (2000). Morphological and physiological diversity. In M. Dworkin et al. (Eds.), *The prokaryotes*. New York: Springer Verlag.