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Molecular diversity and antibiotic sensitivity of gut bacterial symbionts of fruit fly, *Bactrocera tau* Walker

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ABSTRACT: Random amplified polymorphic DNA–Repetitive extragenic palindromic (RAPD–REP) PCR–restriction length polymorphism (PCR-RFLP) were used to study the genetic similarity among bacterial symbionts of fruit fly, *Bactrocera tau* Walker. RAPD–PCR and REP-PCR placed three bacteria into two groups, *viz*., group A (BC1 and BC3) and group B (BC2). However, PCR–RFLP analysis of *recA* and *rrs* genes of bacterial symbiont placed BC1 and BC2 into group A and BC3 into group B. Studies on antibiotic sensitivity pattern also supported the results of PCR–RFLP as the response of BC1 and BC2 was similar to different antibiotics. All the bacterial symboints were found to be sensitive to co–trimoxazole, gentamycene and nofloxacin. The findings suggest that there may be a possibility of utilising these antibiotics for disruption of fruit fly biology as foliar symbioticides for their management.

KEY WORDS: Bacterial symbionts, *Bactrocera tau*, *recA* gene, *rrs* gene, RAPD and REP–PCR analysis

INTRODUCTION

Fruit flies (Diptera: Tephritidae) are one of the most diverse groups of insects, comprising over 4000 species in 481 genera (Thompson, 1998). The melon fly (*Bactrocera cucurbitae* Coquillett) and pumpkin fly (*B. tau* Walker) have wide distribution throughout South–East Asia and attack fruits of a wide range of plant species including vegetable and fruit crops (White and Elson–Harris, 1992; Sardana *et al*., 2005; Huque, 2006). In India, *B. cucurbitae*, *B. tau* and *Dacus ciliatus* (Loew) are the most serious pests of cucurbits and cause annual estimated losses to the tune of $ 855.40 million (Sardana *et al*., 2005).

About 15% of all insects harbour diverse communities of endosymbionts (Stouthamer *et al*., 1999; Moran *et al*., 2008). Symbiotic associations span a spectrum of types that differ with the effect of the symbionts on the host, *viz*. mutualism, commensalism and parasitism (Werren and O'Neill, 1997; Oliver *et al*., 2005). In Tephritidae, symbionts provide their hosts with certain essential amino acids lacking in fruit tissue (Jang and Nishijima, 1990; Gupta and Anand, 2003). These symbionts may act as a natural source of nitrogen, amino acids and vitamins. Their nitrogenase activity might be involved in nitrogen fixation as in rhizobia of legumes (Murphy *et al*., 1988; Behar *et al*., 2005). Certain components of bacterial odour play a vital role in fruit fly behaviour as either feeding or ovipositional stimulants (Lauzon *et al*., 1998; 2000) and are being exploited in pest management in the form of baits or traps.

Molecular approaches for the detection and characterization of microbes have resulted in a dramatic change in our understanding of microbial diversity. It is now recognized that approximately 99% of the microbes in the environment cannot be cultivated (Amann *et al*., 1995). However, nucleic acid based approaches for the characterization of microbes have provided new information in spite of their own limitations (Head *et al*., 1998). Random amplified polymorphic DNA (RAPD) analysis has been used to compare the strains of bacteria between insects and within the generations (Dillon and Dillon, 2004). Some bacteria in thrips persisted for two years through 50 generations and were therefore indigenous bacteria, whereas transient bacteria ingested with food did not pass to the next generations (de Vries *et al*., 2001a,b). A significant genetic diversity of lactic acid bacterial community in wood and soil feeding termites has been observed using ERIC–PCR (enterobacterial repetitive intergenic consensus) (Bauer *et al*., 2000). Waleron *et al*. (2002) studied the genotypic characteristicsof *Erwinia*

based on the analysis of PCR–RFLP (polymerase chain reaction–restriction fragment length polymorphism) of the *recA* gene fragment. The present studies were undertaken to characterize symbiotic bacteria of *B. tau*, a predominant fruit fly species in North–Western Himalayas, using RAPD, repetitive extragenic palindromic-polymerase chain reaction (REP-PCR) and PCR-RFLP based finger printing and detect genetic similarity among the gut bacterial symbionts.

MATERIALS AND METHODS

Raising stock culture of fruit flies

Parental stock culture of the fruit fly, *B. tau,* was raised from field collected infested fruits of cucumber in indigenously designed rearing cages (40 x 40 x 45 cm^3) at room temperature (25 ± 2°C) and RH (70 ± 2%). The cage was filled with 5cm layer of sterile fine sand and mixed with saw dust for pupation. The adults were provided with their natural host (cucumber) for oviposition. The feeding was also supplemented with a mixture of dry glucose and protein hydrolyzate (Protinex® Dumex Sciences, India) in the ratio of 1: 1 in a Petri plate following Sood and Nath (1999). The supplement was replaced at weekly intervals. The flies were also provided with water soaked cotton swabs in a 50ml beaker *ad libitum*.

Isolation of gut bacterial symbionts

The bacteria were isolated from adult flies, maggots, eggs as described by Lloyd *et al*. (1986). Flies from the laboratory population were cold anaesthetized for 10 min in a refrigerator at 4°C and dissected open in physiological saline to remove different organs (crop and portion of alimentary canal) aseptically under laminar flow hood. Contents from each part were streaked separately on Peptone Yeast Extract Agar (PYEA) for bacterial growth at 30 ± 1°C for 48–72h. The isolation was repeated five times to get maximum isolates. Single colony of each isolate was used for establishment of pure cultures. Pure cultures were then maintained on PYEA slants and/or plates at 4 – 8°C.

Extraction of genomic DNA

Total genomic DNA of each isolate was extracted following Sharma *et al*. (2005) with minor modifications. The 48h old bacterial culture multiplied on Peptone Yeast Extract Broth (PYEB) was transferred to 1.5ml 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 pellet was ground to fine powder immediately using micro pestle. To this, 700µl of CTAB extraction buffer was added and incubated at 65°C for 1h in a water bath (YORK Scientific Industries, Delhi).

Equal volume (700µl) of chloroform: isoamyl alcohol (24:1) 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 India) at 4°C. Aqueous phase was transferred to new tubes and 450µl 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 thrice, dried and dissolved in 100µl of Tris EDTA (10mM Tris HCl and 1mM EDTA pH 8.0). RNAse @ 10µl 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 UV/VIS spectrophotometer (Bio Rad, SmartSpec 3000). The DNA was stored at –20°C for further use.

RAPD and REP– PCR analysis
Primer used for RAPD and REP- PCR fingerprinting

Initially 30 decamer oligonucleotide primers (Operon Technologies) were screened with the bacterial isolates to select the most polymorphic one. Five exhibiting maximum polymorphism primers, *viz*., OPD–18, OPF–12, OPF–13, OPJ–14 and OPQ–11 were selected for final amplification. For REP–PCR, BOX–AIR (CTA CGG CAA GGC GAC GCT GAC G) and ERIC primer (forward ATG TAA GCT CCT GGG GAT TCA C and reverse AAG TAA GTG ACT GGG GTG AGC G) were used.

PCR amplification

The PCR amplification was carried out in 0.2ml PCR tubes with 25µl reaction volume consisting 20pmol of decamer oligonucleotide primer in 25mM $MgCl_2$, 10mM of each deoxyribonucleoside triphosphate (Fermentas), 5 units of *Taq* polymerase (Life Technologies India, Pvt. Ltd), 10X reaction buffer and 10 – 15ng DNA. Amplifications were performed using thermal cycler (GeneAmp PCR system 9700, Applied Biosystems, USA) with an initial denaturation step of 5min at 94°C followed by 40 cycles at 94°C for 1 min, 37°C for 1 min, 72°C for 2 min and a final elongation step for 72°C for 5 min. The products were separated by electrophoresis on 1% (w/v) agarose gels with ethidium bromide of 10µl /100ml of 1X TAE buffer (40mM Tris–acetate, 1mM EDTA), run at 80 volts in 1X TAE buffer.

PCR-RFLP analysis

PCR–RFLP was done for the endosymbiotic gene (*rrs* gene) (Heddi *et al*., 1998) and *recA* gene (Waleron *et al*., 2002) using specific primers. The base sequences of primers used (presented in Table 1) were custom synthesized (Life Technologies India, Pvt. Ltd.).

Table 1. Base sequences of *rss* and *recA* gene primers

Name of the Primer	Sequence (5' to 3')
rrs gene F	5' AGA GTT TGA TCA TGG CTC AG 3'
R	5' TAC CTT GTT ACG ACT TCA CC 3'
recA gene F	5' GGT AAA GGG TCT ATC ATG CG 3'
R	5' CCT TCA CCA TAC ATA ATT TGG A 3'

Polymerase chain reaction (PCR) was performed with eubacterial primers targeting the 16S rRNA gene (*rrs* gene) (Heddi *et al*., 1998). The PCR amplification was carried out in 0.2ml PCR tubes with 25µl reaction volumes consisting 20pmol of each primer in 25mM $MgCl_2$, 10mM of each dNTPs (Fermentas), 5 units of *Taq* polymerase (Life Technologies India, Pvt. Ltd), 10X reaction buffer and 10 – 15ng DNA. Amplifications were performed using thermal cycler (GeneAmp PCR system 9700, Applied Biosystems, USA) with an initial denaturation step of 5min at 94°C followed by 35 cycles at 94°C for 45 sec, 53°C for 45 sec, 72°C for 30 sec and a final elongation step for 72°C for 5 min whereas, *recA* gene amplification was performed with an initial denaturation step of 3 min at 94°C followed by 32 cycles at 94°C for 1 min, 47°C for 1 min, 72°C for 2 min and a final elongation step for 72°C for 5 min.

The amplified product of *rrs* gene and *recA* gene from three symbiotic bacterial isolates was digested with four restriction endonucleases, *viz., Hind III, Pst I, BamH I* and *EcoR I* (Bangalore Genei) according to manufacturer's instructions. Restriction was performed in 0.2ml PCR tubes with 10µl digestion reaction mixture containing 8µl of amplicon, 1µl enzyme buffer and 1µl of restriction enzyme (10U μl^{-1}). The tubes were vortexed, microfuged and incubated at 37°C for 12h in an oven (Bio Rad). Digestion was stopped by raising the temperature of oven to 65°C for five minutes. The digested products were separated by electrophoresis on 3 % (w/v) agarose gels with ethidium bromide of 10µl / 100ml of 1X TAE buffer (40mM Tris–acetate, 1mM EDTA), run at 80 volts in 1X TAE buffer.

Fingerprint analysis

RAPD, REP–PCR and PCR– RFLP profiles generated by primers were used to determine genetic diversity among the isolates. DNA bands that could be scored univocally for presence and absence were included in analysis using GENE PROFILER V 6.0 software package (Alpha Infotech Corporation, USA). Binary matrices were analysed by NTYSIS – pc v 2.0 and Jaccard's coefficient was used to construct dendrogram using SHAN clustered program, selecting the un–weighted pair–group arithmetic mean method (UPGMA) in NTYSIS – pc v 2.0 (Rohlf, 1993). The dendrogram with best fit to similarity matrix based on cophentic value (COPH) and matrix comparison (MXCOMP) was chosen.

Antibiotic sensitivity assay

Antibiotic sensitivity of symbiotic bacteria was tested in PYEA plates, inoculated with 0.1ml of 24h old culture of the test bacterium in PYEB. Antibiotic impregnated discs (Octodisc, Hi-Media Laboratories, Mumbai) with Ampicillin (1mg), Cephalothin (30mg), Co-Trimoxazole (25mg), Gentamycin (10mg), Nalidixic acid (30mg), Nitrofurantoin (300mg), Norfloxacin (10mg) and Tetracyclin (25mg) were placed in the centre of the inoculated PYEA plates with the help of sterile forceps. The plates were incubated at 30°C for 48h. Antibiotic sensitivity was assayed from the diameter of zone of inhibition. As per the manufacture's instructions based on the diameter of inhibition zone, the bacteria were categorized as sensitive, moderately sensitive, highly sensitive and resistant.

RESULTS AND DISCUSSION

Three different isolates of bacteria (BC1, BC2 and BC3) were detected from the gut of fruit fly, *B. tau* on the basis of morphological characterization after repeated isolations. The pure cultures of these bacteria were maintained and used for further studies.

RAPD and REP-PCR analysis

Among thirty RAPD primers screened to know the diversity among three bacterial symbionts of *B. tau*, five RAPD and two REP–PCR primers (BOX–AIR and ERIC) showing maximum polymorphism were used for analysis. Number of scorable and polymorphic bands obtained with each primer is presented in Table 2 and RAPD and REP–PCR profiles generated by RAPD, BOX–AIR and ERIC primers are depicted in Fig 1. Five RAPD primers generated a total of 23 polymorphic bands whereas BOX–AIR and ERIC primers gave 17 and 6 bands, respectively of which 14 and 6 were polymorphic. On the basis of RAPD

and REP–PCR analysis of bacterial genomic DNA, three bacterial isolates were placed in two main group (Fig. 2) *viz.,* Group A (BCI BC3) and Group B (BC2). The two groups showed a minimum similarity coefficient of 0.27 indicating high diversity. With in group A, BC1 and BC3 diverged at 0.44 similarity coefficient showing 56 per cent genetic diversity among the two isolates. Diversity analysis using 43 polymorphic RAPD and REP–PCR loci clearly indicated the variation between bacterial isolates. Overall 93.47 per cent polymorphism was detected. No earlier report pertaining to fruit flies could be traced, however, de Varies *et al.* (2001a, b) applied RAPD technique to characterize symbiotic bacteria at DNA level and their transmission to next generation in thrips, whereas Bauer *et al.* (2000) used REP–PCR (ERIC–PCR) to study the genetic diversity within the lactic acid bacteria.

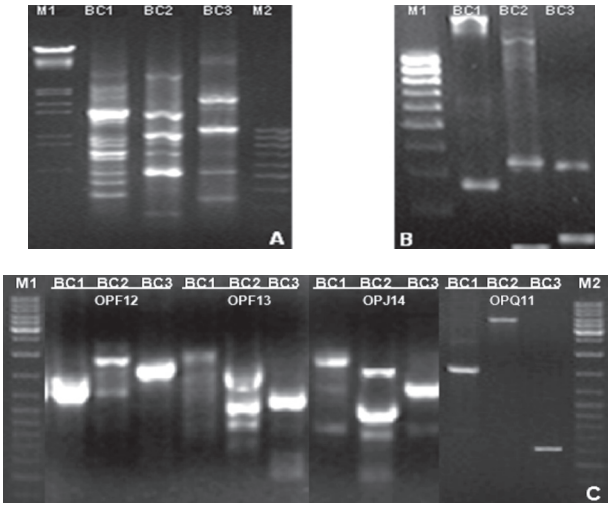


Fig. 1. Gel photograph of amplified DNA with: A– BOX–AIR primer, B– ERIC primer and C– RAPD primers

PCR-RFLP analysis

Digestion of *recA* and *rrs* gene products with four endonucleases, *viz*., *BamH* I, *Hind* III, *Pst* I and *EcoR* I revealed that 700bp amplicon of *recA* gene was restricted/ cleaved by *BamH* I and *Pst* I (Fig 3). The former gave a digested product of 200 and 500bp in BC1 & BC2 amplicon and 200, 500 and 700bp in BC3. *Pst* I yielded three fragments of 200, 500 and 700bp in BC3 and two fragments of 200 and 500bp in BC1 and BC2 amplicon. With BamH I, 1500 bp amplicon of *rrs* gene was not at all restricted in all the bacterial symbionts (Fig 3c) while *Hind* III gave a digested product of 1100 and 400bp in BC3 only. Digestion with *Pst* I yielded a product of 850 and 650bp products in all the three bacterial isolates.

Table 2. Number of scorable and polymorphic RAPD and REP–PCR bands obtained in PCR amplification of genomic DNA of three bacterial symbionts of fruit fly

Name of primer	Number of scorable bands	Number of polymorphic bands
RAPD		
OPF–12	4	4
OPF–13	5	5
OPD–18	4	4
OPJ–14	7	7
OPQ–11	3	3
REP–PCR		
BOX–AIR	17	14
ERIC	6	6
Total	46	43

The PCR–RFLP phylogenetic analysis divided three symbionts into two groups *viz.* Group A comprising of BC1 & BC2 with 100 per cent genetic similarity and Group B having BC3 (Fig. 4). PCR–RFLP pattern of rrs gene gave 1100 and 400bp product unique to BC3 after digestion with Hind III. Many workers had used recA gene for characterization and identification of bacteria belonging to family Enterobacteriaceae (Lloyd and Sharp, 1993; Eisen, 1995; Karlin *et al.,* 1999; Nowak and Kur, 1995; Blackwood *et al.,* 2000; Mahenthiralingam *et al.,* 2000). Waleron *et al.* (2002) used PCR–RFLP analysis of *recA* gene fragment to characterize bacteria belonging to genus *Erwinia*, a member of the family Enterobacteriaceae.

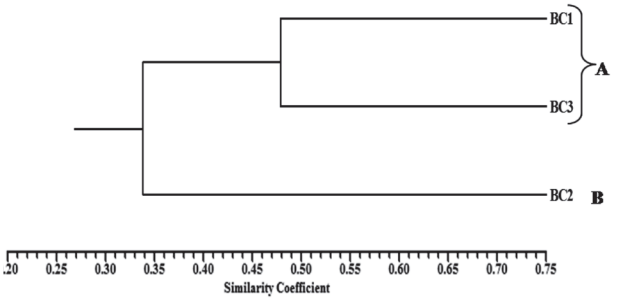
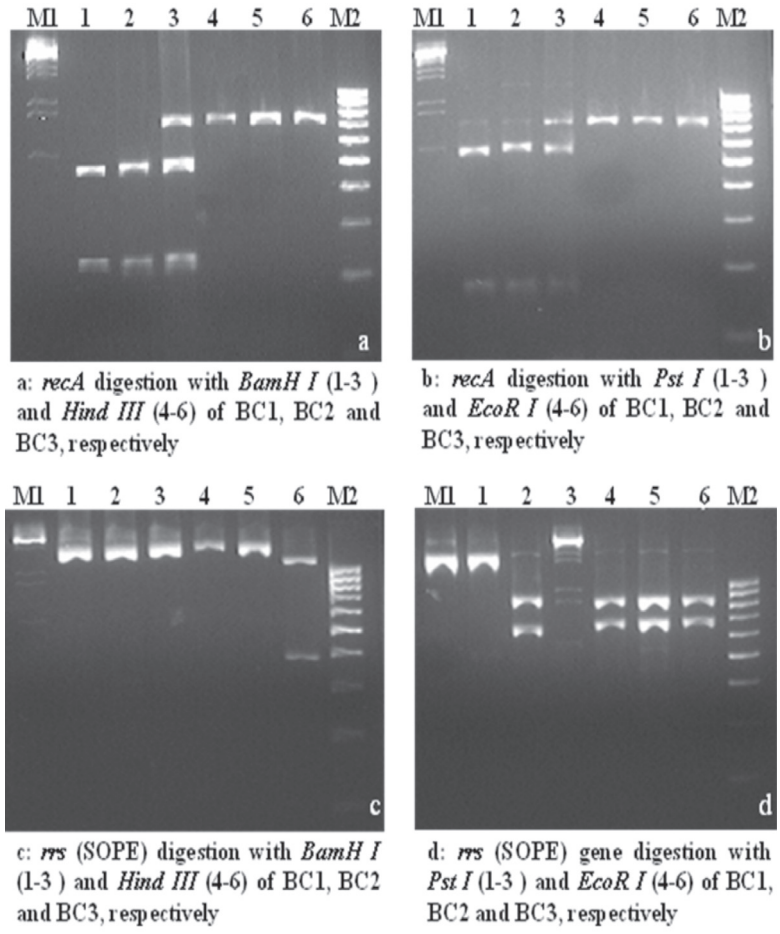


Fig. 2. Dendrogram showing RAPD and REP–PCR analysis of three bacterial symbionts of fruit fly using UPGMA method



a: *recA* digestion with *BamH I* (1-3) and *Hind III* (4-6) of BC1, BC2 and BC3, respectively

b: *recA* digestion with *Pst I* (1-3) and *EcoR I* (4-6) of BC1, BC2 and BC3, respectively

c: *rrs* (SOPE) digestion with *BamH I* (1-3) and *Hind III* (4-6) of BC1, BC2 and BC3, respectively

d: *rrs* (SOPE) gene digestion with *Pst I* (1-3) and *EcoR I* (4-6) of BC1, BC2 and BC3, respectively

Fig. 3. Gel photograph of PCR– RFLP

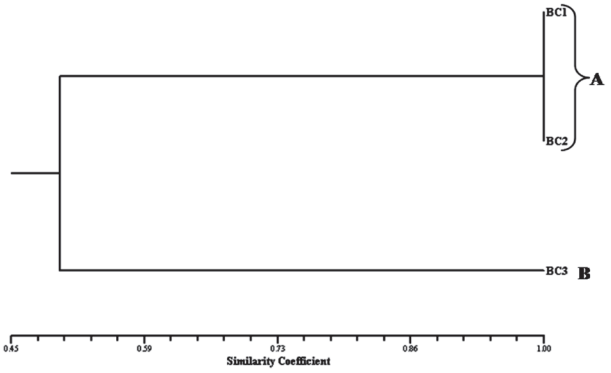


Fig. 4. Dendrogram showing PCR-RFLP analysis of three bacterial symbionts of fruit fly using UP-GMA method

Antibiotic sensitivity assay

All the bacterial isolates exhibited moderate to high sensitivity to co-trimoxozole, gentamycin, norfloxacin and tetracycline. BC1 was found resistant to ampicillin and cephalothin, while BC2 and BC3 were resistant to cephalothin and nalidixic acid, respectively (Table 3).

Natural and semi–synthetic peptide antibiotics of beta lactam series such as ampicillin and cephalothin (first generation antibiotic of cephalosporin) are normally effective against gram positive bacteria which act as an inhibitor of polypeptidoglycan synthesis. Quinolones antibiotics such as nalidixic acid and norfloxacin (floroquinolone) are most effective against gram negative bacteria. Resistance to quinolones antibiotics is common in gram positive cocci. All the bacterial symbionts of fruit fly were observed sensitive against co–trimoxazole, gentamycin, norfloxacin and tetracycline. These are last line of defence in antibiotic regime (Thakur *et al*., 2005). It is to be noted that experiments conducted with large collection of bacteria between 1917 and 1954 showed that although antibiotics resistant genes were present during the pre–antibiotic era, but at a significantly lower frequency than today (Hughes and Datta, 1983).

Table 3. Antibiotic sensitivity pattern of bacterial symbionts of fruit fly

Antibiotics	Bacterial symbionts		
	BC1	BC2	BC3
Ampicillin (A)	R	+	+
Cephalothin (Ch)	R	R	+ +
Co-trimoxazole(Co)	+ + +	+ +	+ +
Gentamycin (G)	+ +	+ +	+ + +
Nalidixic acid (Na)	+ +	+ +	R
Nitrofurantoin (Nf)	+	+	+
Norfloxacin (Nx)	+ + +	+ + +	+ +
Tetracycline (T)	+ +	+ +	+ +

Symbol	Categories	Diameter range
R	Re sistant	Full growth
	Sensitive	
+	Poorly	10–17 mm
	sensitive	18–27 mm
++	Moderately	18–27 mm
	sensitive	
+++	Highly	28–37 mm
	sensitive	

Overall the similarity pattern of three symbionts generated on the basis of RAPD–REP–PCR, PCR–RFLP of *recA* & *rss* gene and antibiotic sensitivity, BC1 and BC2 were found most similar to each other with PCR–RFLP of *recA* & *rss* gene and antibiotic sensitivity analysis. PCR–RFLP profile of three symbionts was more authentic than RAPD–REP–PCR profile as PCR–RFLP profile was based on the specific bacterial gene than profile generated through RAPD–REP–PCR where, amplification of DNA can occur anywhere in the genome. The PCR–RFLP profile of three symbionts of fruit fly was also supported by the antibiotic sensitivity pattern of these symbionts where a close similarity was observed between BC1 and BC2 (Table 3). Hence the studies conclude that BC1 and BC2 were most similar to each other and could be placed in one group/ family while BC3 to a separate group. The sensitivity pattern of bacterial symbionts could also be useful in framing alternate management strategies against fruit flies. Belcari and Bobbio (1999) used copper as a symbioticide in destroying the fruit fly associated bacteria and thus managing the first and second instar larvae of olive fruit fly (*Bactrocera oleae* Gmelin). The antibiotics to which the symbionts are most sensitive can be used as foliar symbioticides to hamper the normal development of fruit flies on the host. However, lack of sufficient molecular data on the fruit fly bacterial symbionts calls for attention to generate such information on fruit fly–bacteria symbiont relationship which in turn will increase our understanding of these bacteria for eco–friendly management of fruit flies in the near future.

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