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ORIGINAL ARTICLE

Molecular characterization and diversity analysis of bacterial communities associated with *Dialeurolonga malleswaramensis* (Hemiptera: Aleyrodidae) adults using 16S rDNA amplicon pyrosequencing and FISH

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Abstract *Dialeurolonga malleswaramensis* Sundararaj (Hemiptera: Aleyrodidae) is a phytophagous sap sucking insect. It infests *Polyalthia longifolia*, an important avenue tree of India, effective in alleviating noise pollution and having immense medicinal importance. Samples of this insect were collected from *Polyalthia longifolia*. The cytochrome *c* oxidase subunit I gene (*mtCO1*) helped in the molecular characterization of the insect. This study reports the bacterial diversity in *D. malleswaramensis* adults by high throughput 16S rDNA amplicon pyrosequencing. The major genera identified were *Portiera* and *Arsenophonus*. Other bacterial genera detected were uncultured alpha proteobacterium, *Sphingopyxis* and *Methylobacterium*. We also employed fluorescence *in situ* hybridization (FISH) in whole mount samples to confirm the presence of dominant endosymbionts *Portiera* and *Arsenophonus* to the bacteriocyte of *D. malleswaramensis*. This study concludes that combining techniques like 16S rDNA amplicon pyrosequencing and FISH reveal both dominant and rare bacteria. The data also predict the evolutionary position of this pest with respect to other whitefly species using a mitochondrial marker.

Key words 16S rDNA amplicon pyrosequencing; bacterial diversity; *Dialeurolonga malleswaramensis*; FISH; *mtCO1*

Introduction

Dialeurolonga malleswaramensis Sundararaj (Hemiptera: Aleyrodidae) is a phytophagous sap sucking insect. It is a pest of an important avenue tree of India, namely *Polyalthia longifolia* (Sundararaj, 2001). It is widely distributed in the Palaearctic (India) region (Sundararaj, 2001). Like all whiteflies, it feeds on the phloem sap of the plant thus damaging its surface and impairing its major metabolic and physiological processes. Aleyrodids have been associated with several ubiquitous microorganisms, thus allowing the host to flourish on a suboptimal diet and endure harsh climatic conditions. For example, *Portiera*, which resides in specialized structures called bacteriocytes (Buchner, 1965; Baumann *et al*., 1995), compensate for nutritional deficiency in whiteflies by producing essential amino acids (Douglas, 1998). *Rickettsia* has been shown to influence thermotolerance in *Bemisia tabaci* (Montllor *et al*., 2002) and *Wolbachia* has been shown to enhance fitness of its host in addition to providing shield against parasitization (Xue *et al*., 2012).

Excessive use of pesticides over a long period of time for eliminating insects has resulted in deterioration of the environment (Ibitayo, 2011). Moreover, insects have become resistant to a majority of insecticides (Elbert & Nauen, 2000). Modern pest management strategies employ microorganisms to control pests. For instance, Cry1Ac toxin protein from *Bacillus thuringiensis* has been used widely to control lepidopteran insects (Wu *et al*., 2008;

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Rajagopal, 2009). Attempts, thus have been made to analyze bacterial population in several hemipterans like *B. tabaci* (Indiragandhi *et al*., 2010), *Acyrthosiphon pisum* (Fukatsu *et al*., 2000; Ateyyat, 2008), *Aleurocanthus woglumi* (Pandey *et al*., 2013), etc. in the hope of getting a wider picture of hemipteran associated microorganisms. Traditional techniques of plating homogenized insects on nutrient-agar media has previously failed to reveal the bacterial community, which is presumed unculturable (Vaughan *et al*., 2000). Moreover, the culture-based techniques are both time consuming and laborious. Sanger-based gene cloning libraries (Sanger *et al*., 1977) has been used successfully to gain insight into the microbial structure of an organism (Eckburg *et al*., 2005). However, this approach is slowly losing its appeal as the depth per sample is limited, usually up to 100 clones due to the high cost involved. With the advancement in technology, massive parallel sequencing techniques like 16S rDNA amplicon pyrosequencing have emerged as powerful tools for revealing the microbiota both rapidly and efficiently (Dowd *et al*., 2008). Using this, we can sequence hundreds of samples with depth that cannot be attained by the traditional Sanger-based clone library. It has been used to understand the microbial system in various environments (Sogin *et al*., 2006; Ley *et al*., 2008; Martinez *et al*., 2009; Benson *et al*., 2010). Additionally, probe-based techniques like fluorescent *in situ* hybridization (FISH) allow rapid visualization of the organism's bacterial communities (Gottlieb *et al*., 2008; Pandey *et al*., 2013).

Thus far, none of the published research reports the phylogenetic position and bacterial communities associated with *D. malleswaramensis*. Keeping this in mind, we have designed the objectives for our current study. In this study, the phylogenetic position of *D. malleswaramensis* based on *mtCO1* gene sequence was determined. Further, we investigated the bacterial consortium of *D. malleswaramensis* adults using high-throughput 16S rDNA based amplicon pyrosequencing. Additionally, we confirmed the presence of major endosymbionts in *D. malleswaramensis* by localizing them to bacteriocytes using fluorescent probes and visualized them under a confocal microscope.

Materials and methods

Sample collection

Adult insect samples were collected individually from *Polyalthia longifolia*, North campus, University of Delhi (718 ft, 077°12.3875′E, 28°41.249′N) in June 2012 and 2013. Samples were preserved in 100% ethanol at -20 °C in separate microfuge tubes. Each sample was identified as *D. malleswaramensis* by Dr. V.V. Ramamurthy and Dr. A.K. Dubey (Division of Entomology, IARI, New Delhi) by adopting all the morphometric identification keys (Sundararaj, 2001).

DNA extraction

Whole insect samples (20) were washed twice with sterile water. Genomic DNA was extracted (whole insect) using HiPurA^{TM} Insect DNA Miniprep Purification Spin Kit (Himedia Labs, India) according to the manufacturer's protocol with minor modifications. The insect samples were homogenized thoroughly with a hand-held homogenizer. The insect homogenate was kept in 200 μL of lysis solution (provided with kit) containing 45 mg/mL lysozyme for 2 h at 37 °C to ensure total extraction of DNA from both Gram-negative and Gram-positive bacteria. The DNA was then extracted according to kit's protocol. Purity and concentration of the DNA was analyzed by using the NanoDrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA).

PCR analysis of mitochondrial genes

Genetic characterization was done by amplifying the *mtCO1* gene. Amplification was carried out using *mtCO1* forward primer F-BQ-2819 (5′-CTGAATATCGRCGAGGCATTCC-3′) and reverse primer R-BQ-2195 (5′-CTGGTTYTTTGGTCATCCRGARGT-3′) (Chu *et al*., 2011). Each PCR reaction contained 20 ng of insect DNA, primers (7.5 pmoles each), dNTP (2.5 mmol/L), 10× *Taq* buffer (2.5 μL), 1U *Taq* DNA polymerase and the mixture was adjusted to a final volume of 25 μL with water. The PCR reaction conditions were 94 °C for 1 min, followed by 35 cycles of 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 50 sec with a final extension at 72 °C for 5 min. A negative control without DNA was also performed. The presence of final PCR products was determined on 0.8% agarose gel. The amplified product was 700 base pairs in length. Gel extraction was carried out by using the Hi yield Gel/PCR DNA (RBC) kit. Purified PCR products from 3 individuals were sequenced (Macrogen, Seoul, South Korea). Resulting sequences were trimmed using Mac Vector software version 11.1.1. Similarity searches were conducted using BLAST algorithm in NCBI (E-value cut-off of 1×10^{-10}, minimum coverage 90% and 80% identity) (Altschul *et al*., 1990). The *mtCO1* sequence from this insect and other sequences retrieved from NCBI database were aligned using the Clustal X algorithm 1.83 (Thompson *et al*., 1997). A maximum Likelihood tree was constructed using the GTRGAMMA model of

nucleotide substitution in RAxML (Version 7.2.8). The ML tree was estimated with 1000 replicates followed by search for best scoring tree (Stamatakis, 2006).

454 amplicon pyrosequencing of 16S rDNA

16S rDNA amplicon pyrosequencing was performed to determine insect's bacterial endosymbionts using the method described by Dowd *et al*. (2008). Briefly, 16S universal Eubacterial primers- 27Fmod (5′-AGRGTTTGATCMTGGCTCAG-3′) and 519R (5′-GTNTTACNGCGGCKGCTG-3′) was used for amplifying the DNA. PCR was performed using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA, USA). The PCR reaction conditions were 94 °C for 3 min, followed by 28 cycles of 94 °C for 30 sec; 53 °C for 40 sec, and 72 °C for 1 min after which a final elongation step at 72 °C for 5 min was performed. After PCR, amplicon products were purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Each insect's bacterial amplicons were sequenced using Roche 454 FLX titanium instruments (MR DNA Lab, TX, USA) and reagents and following manufacturer's protocol. Analyses included water only samples as negative controls to rule out any laboratory contamination.

Sequence processing pipeline and phylogenetic analysis

SFF files produced from 454 sequencing were processed for adapter trimming and quality filtering using the FASTX tool kit (Pearson *et al*., 1997). Specifically, sequences below a quality score 20 (Phred score), minimum read length below 250 base pairs and reads with ambiguous bases (*N*) were excluded from further analysis. The resulting sequences were then depleted of any chimeras using custom software set at default parameters (Gontcharova *et al*., 2010). Of the 17 022 reads obtained initially, only 15 832 remained after quality filtering and removing chimeras. Quality-trimmed sequences were clustered in to OTUs at 97% sequence similarity using the UPARSE pipeline (Edgar, 2013). OTUs with <10 reads were excluded (Morgan *et al*., 2013). Taxonomy was assigned to each candidate OTU using the RDP classifier (80% confidence value scheme) (Wang *et al*., 2007).

Confocal microscopy

Portiera and *Arsenophonus* confirmation was performed using a FAM labeled probe bearing the 5′-TGTCAGTGTCAGCCCAGAAG-3′ sequence and a TYE-665 probe bearing the 5′-TCATGACCACAACCTCCAAA-3′ sequence, respectively (Gottlieb *et al*., 2010). Insect samples were processed according to the standardized protocol for whitefly with minor modifications (Gottlieb *et al*., 2006). Briefly, insect samples collected in acetone were fixed in Carnoy's fixative (chloroform: ethanol: glacial acetic acid, 6 : 3 : 1) and decolorized with 6% H_2O_2 for 24 h. The decolorized insects were hybridized at 42 °C, with 0.6 pmoles of DNA probes, in hybridization buffer (20 mmol/L Tris-Cl, pH 8.0; 0.9 mol/L NaCl; 0.01% SDS; 40% formamide). After overnight incubation, the samples were thoroughly washed in buffer (0.3 mol/L NaCl; 0.03 mol/L sodium citrate; 0.01% SDS) for 5 min and mounted using Fluoroshield (Sigma, St. Louis, MO, United States of America). All the images were acquired with a Nikon A1 confocal microscope.

Nucleotide sequence accession numbers

The *mtCO1* and 16S rDNA pyrosequencing data have been deposited in the NCBI database under accession numbers KJ957167 and SRP043181, respectively.

Results

Phylogenetic analysis of D. malleswaramensis based on mtCO1 gene sequences

All the sequences obtained were identical. A continuous stretch of 530 base pairs was subjected to a similarity search using BLAST. Our sequence (GenBank KJ957167) showed maximum sequence similarity (85%) with *Bemisia atriplex*. The maximum likelihood tree was constructed along with the sequences retrieved from NCBI database. It was observed that *mtCO1* sequence of *D. malleswaramensis* clustered with *mtCO1* sequences corresponding to *A. woglumi* and *Bemisia* sp. (Fig. 1)

Detection and identification of bacterial endosymbiont from D. malleswaramensis

To analyze the broad spectrum of bacterial diversity in *D. malleswaramensis*, 16S rDNA pyrosequencing was performed yielding 15 799 high-quality reads. Reads were clustered in to 5 major bacterial phylotypes (Table 1). None of the relevant bacterial phylotypes were detected in the negative controls (water-only sample). The rarefaction curve tends to saturation indicating that the sampling depth was sufficient to determine the major bacterial phylotypes (Fig. 2). *D. malleswaramensis* is dominated by 2 genera belonging to "Gamma" subclass of proteobacteria namely its primary endosymbiont *Portiera* and secondary endosymbiont *Arsenophonus*. The *Portiera* sequence

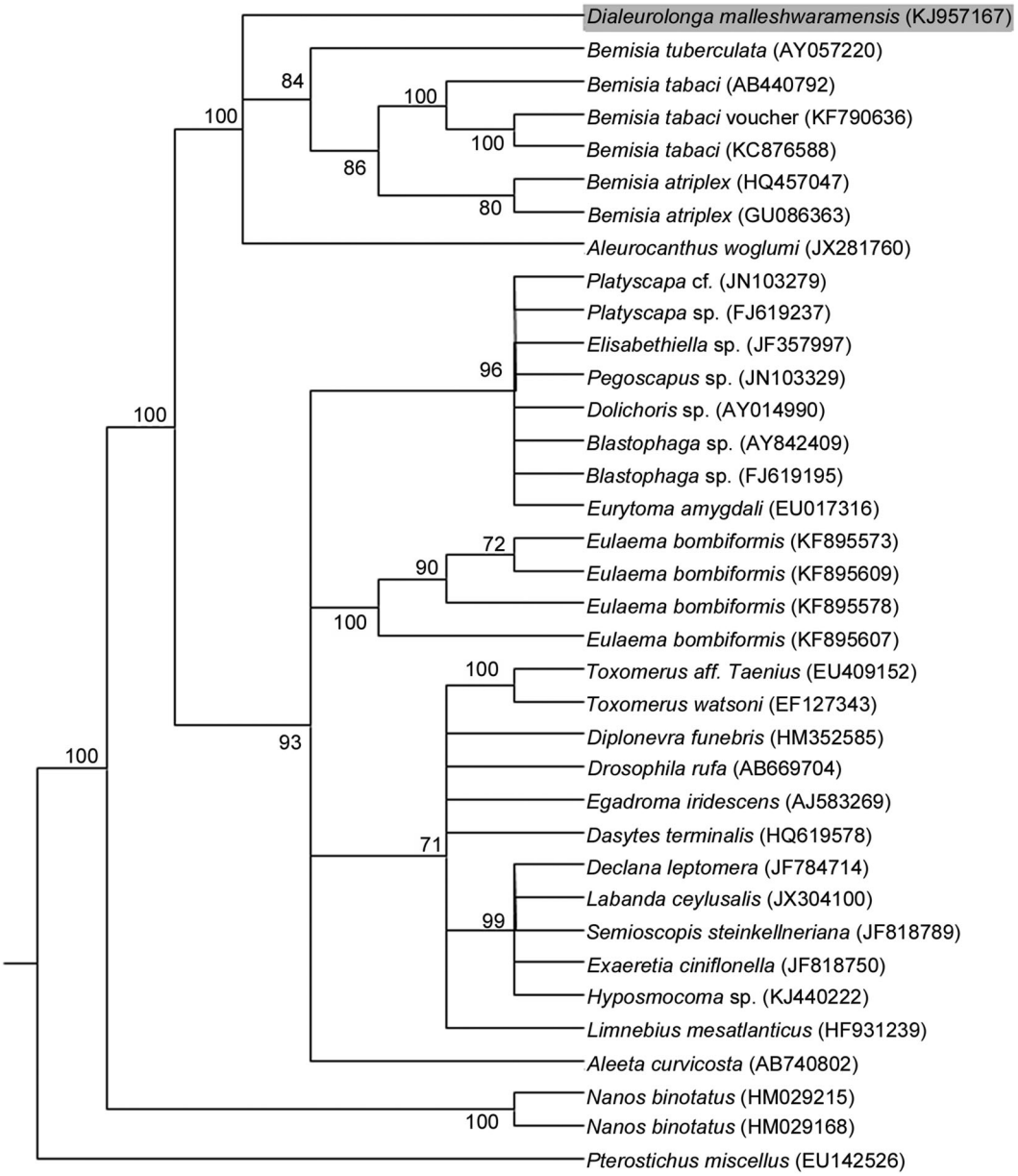

Fig. 1 Phylogenetic tree showing relationship between 526 base pair of *mtCO1* gene sequence of *D. malleswaramensis* with *mtCO1* sequences of its closest relatives taken from NCBI database. Tree was constructed by maximum Likelihood analysis using RAxML version 7.2.8. Only bootstrap higher than 70% are indicated. Sequence generated in this study is in shaded box.

from *D. malleswaramensis* showed maximum sequence similarity to *Portiera* sequence of *Dialeurodes hongkongensis* (AY266107, 98.76% sequence identity, E-value 10^{-5}). On the other side, *Arsenophonus* showed maximum sequence similarity to *Candidatus Arsenophonus arthropodicus* (DQ115536, 99.10% sequence identity, E-value 10^{-5}). The other genera detected were uncultured alpha proteobacterium, *Sphingopyxis* and *Methylobacterium.*

FISH analysis

The 2 dominant genera in *D. malleswaramensis* were also localized with FISH using specific probes. A strong green signal (*Portiera*) and red signal (*Arsenophonus*) was detected inside the bacteriocyte (Fig. 3). Thus, in agreement with the previous reports on hemipterans, our results confirmed the coinfection of bacteriocyte in

Table 1 Pyrosequencing reads distribution of genera identified from adults of *D. malleswaramensis*.

Bacterial division	Nearest match	No. of reads
Gammaproteobacteria	*Candidatus Portiera aleyrodidarum*	15155
Gammaproteobacteria	*Candidatus Arsenophonus arthropodicus*	510
Alphaproteobacteria	Uncultured alpha proteobactrium	60
Alphaproteobacteria	*Sphingopyxis* spp.	52
Alphaproteobacteria	*Methylobacterium* spp.	22

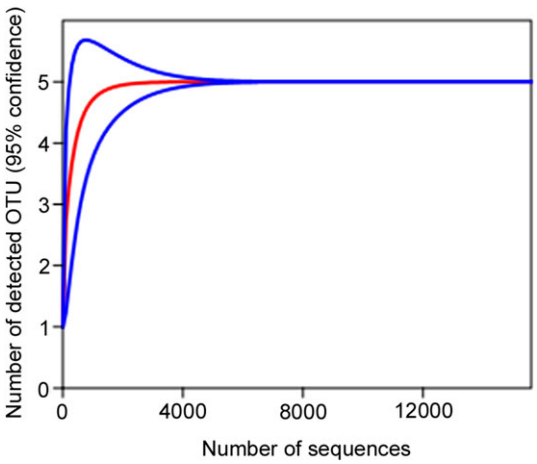

Fig. 2 Rarefaction analysis performed on the number of detected OTUs (at 95% confidence) clustered at 97% sequence similarity.

D. malleswaramensis by its primary and secondary endosymbionts (Gottlieb *et al*., 2008; Priya & Pandey, 2012; Pandey *et al*., 2013).

Discussion

Insect's association with a wide variety of microorganisms varies from being obligatory to facultative in nature (Dillon & Dillon, 2004). Widespread distribution and successful establishment of insects over a broad range of habitats has contributed to its close association with endosymbionts (Dillon & Dillon, 2004). These endosymbionts play a crucial role in various metabolic and physiological processes in insects (Douglas, 1998; Montllor *et al*., 2002; Xue *et al*., 2012). This study examined in detail the *D. malleswaramensis* microbiota using 454 amplicon pyrosequencing and visually confirmed 2 species with FISH. Subsequent to quality filtering, only 5 OTUs were recovered. This insect's microbiota corresponds to the low bacterial diversity also observed in *B. tabaci* (Jing *et al*., 2014). Our study revealed that *Portiera* is the most dominant genus found in *D. malleswaramensis* similar to other whitefly species (Thao & Baumann, 2004; Jing *et al*., 2014). These obligate primary endosymbionts remain inside the bacteriocytes as confirmed by confocal microscopy (FISH) and are transmitted transovarially from mother to offspring (Douglas, 2006). *Portiera* is purported to provide all the essential amino acids to the whitefly which are otherwise lacking in its sugar rich amino acid deficient phloem diet (Thao & Baumann, 2004). The second major genus found in *D. malleswaramensis* was *Arsenophonus*. Recent research has shown its involvement in the transmission of CLCuV (Cotton leaf curl virus) in *B. tabaci* (Rana *et al*., 2012). This secondary endosymbiont is also believed to alter the reproductive capability of its host (Gherna *et al*., 1991). Confocal microscopic observation (FISH) revealed that it coinfects the bacteriocytes along with the primary endosymbiont. Coinfection aids in efficient translocation of the secondary endosymbiont along with bacteriocyte and suggests its functional role in the insect though it may pose constraints like competition for resources among the coinhabitants (Gottlieb *et al*., 2008). Existence and isolation of *Arsenophonus* from *D. malleswaramensis* could be explored further for determining its functional role.

Additionally, we detected uncultured alpha proteobacterium in *D. malleswaramensis*. Our sequence showed 97% sequence identity at 100% query coverage with uncultured alpha proteobacterium (JQ389881). This uncultured alpha proteobacterium is related to bacterium "*Candidatus Hepatincola porcellionum*" and, to our knowledge, so far has not been recorded from whitefly. It is speculated to facilitate exchange of nutrients between symbionts and host (Wang *et al*., 2004). On the basis of <97% cut off value for determining the novel species by sequencing 16S rRNA gene (Stackebrandt & Goebel, 1994), this uncultured alpha proteobacterium could be proposed as a novel species. However, further taxonomic characterization and microscopic evaluation is necessary to validate it as a novel species.

Thus, current investigation represents the first documented identification of *D. malleswaramensis* associated

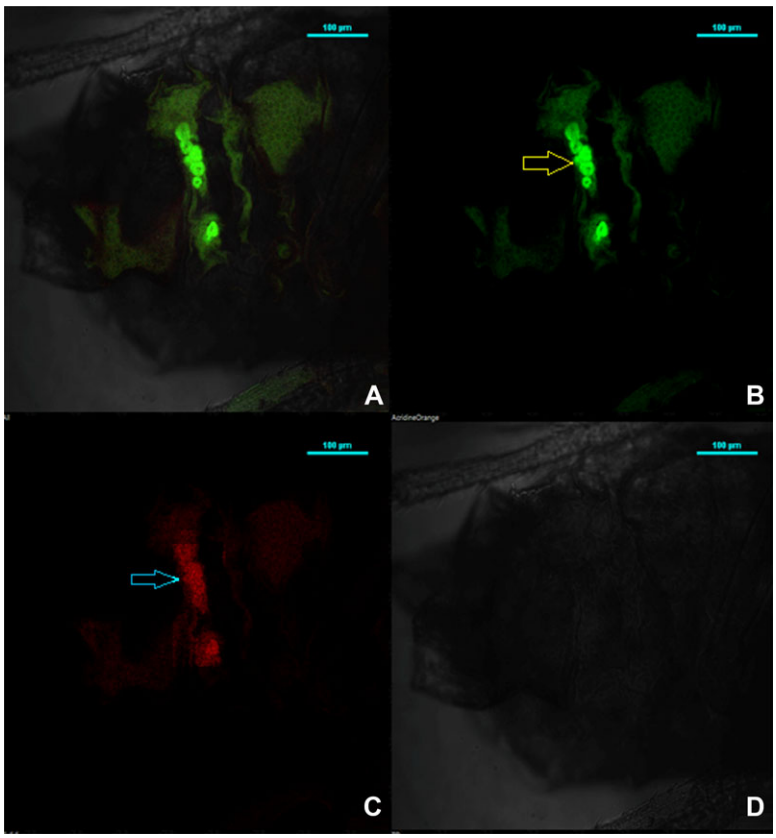

Fig. 3 FISH staining of *Portiera* and *Arsenophonus* 16S rRNA gene in whole mount of *D. malleswaramensis*. FAM and TYE 563 labeled oligonucleotide DNA probes were used to detect *Portiera* (green signal, Panel B) and *Arsenophonus* (red signal, Panel C) respectively in *D. malleswaramensis*. All images were viewed under 20× magnification. Arrows in yellow and blue indicate bacteriocytes. Panels A and D show the merged and DIC image of the respective probe. Scale reference bar = 100 μm.

flora and its *mtCO1* gene phylogeny. We conclude that deep sequencing method like 16S rDNA amplicon pyrosequencing and FISH helped us to gain in-depth insight into the bacterial composition of *D. malleswaramensis*. However, specific functional analysis of all these endosymbionts is needed to develop novel pest management strategies in future.

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Disclosure

The authors have no conflict of interest, involvement, financial or otherwise, that might potentially bias our work.

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