Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2020; 8(1): 429-439

Received: 08-11-2019
Accepted: 12-12-2019

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Metagenomic exploration of the bacterial endosymbiotic microbiome diversity of papaya mealybug *Paracoccus marginatus* from different host plants

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Abstract
Papaya mealybug (PMB) *Paracoccus marginatus* host several endosymbionts to have nutritional benefits like enhancing the synthesis of essential amino acids and protection over natural enemies. In this study using metagenomic analysis, we characterized the bacterial endosymbiotic diversity of this notorious pest from four different host plants such as papaya (*Carica papaya* L.), brinjal (*Solanum melongena* L.), cassava (*Manihot esculenta* Crantz) and congress grass (*Parthenium hysterophorus* L.). Results revealed that the primary endosymbiont of mealybugs Candidatus Tremblaya was reported from the PMB of all four different host plants *viz*., papaya, brinjal, cassava and parthenium with 79.83, 5.46, 29.01, and 46.66 per cent in abundance, respectively. Secondary endosymbionts like Pseudomonas (4.03 per cent) and Acinetobacter (3.32 per cent); Sphingomonas (8.81 per cent) and Pseudomonas (12.76 per cent); Enterococcus (64.57 per cent); Erwinia (40.27 per cent) and Pseudomonas (4.39 per cent) were identified in papaya, brinjal, cassava and parthenium respectively.

Keywords: Papaya mealybug, endosymbionts, metagenomic analysis, *Tremblaya*, *Sphingomonadaceae*, *Enterococcaceae*

Introduction
Endosymbionts (an organism that lives mutually within the body or cells of another organism) are thought to help the host either by providing nutrients that the host cannot obtain itself or by offering defense against natural enemies. Bacteria benefit from the reduced exposure to predators and competition from other bacterial species, the ample supply of nutrients and relative environmental stability inside the host [1]. Many instances of endosymbiosis are obligate *viz.*, mitochondria and chloroplasts which are originated by symbiogenesis as bacterial endosymbionts. Endosymbionts are categorized into two groups, 'Primary' and 'Secondary' [26]. Primary endosymbionts (P-endosymbionts) have been associated with their insect hosts for many millions of years. They form obligate associations and co-evolve with their host insects. They live in specialized insect cells called bacteriocytes and are maternally-transmitted. Attacking obligate bacterial endosymbionts may pave way to manage their insect hosts [2]. Secondary endosymbionts are more recently developed association, are sometimes horizontally transferred between hosts, lives in the hemolymph of the insects and are not obligate. Researchers have been unable to cultivate the endosymbiotic bacteria in lab conditions outside of the insect. Unfortunately, traditional microbiology and microbial genome sequencing and genomics rely upon cultivated clonal cultures; early environmental gene sequencing cloned specific genes (often the 16S rRNA gene) to produce a profile of microbial diversity in a natural sample. Hence through traditional microbiological work, the vast majority of microbial biodiversity may miss from documenting. Metagenomics is the study of genetic material recovered directly from environmental samples and use either "shotgun" or PCR directed sequencing to get mostly unbiased samples of all genes from all the members of the sampled communities. Because of its ability to reveal the previously hidden diversity of microscopic life, met genomics offers a powerful lens for viewing the microbial world that has the potential to revolutionize understanding of the entire living world [28].
The papaya mealybug (PMB) *Paracoccus marginatus* Williams and Granara de Willink (Hemiptera: Pseudococcidae) was recorded for the first time in Tamil Nadu, during July, 2008 in Coimbatore district on papaya and severe infestation (80 to 90%) was observed on the

crops *viz*., papaya, mulberry, tapioca, brinjal, tomato, bhendi and flower crops [21]. Throughout their life, PMB feeds only on plant sap, which is a nutritionally unbalanced food. Phloem sap is virtually devoid of lipids and proteins; however, most lipids can be synthesized from the carbohydrates, but proteins cannot in the absence of nitrogenous precursors such as essential amino acids. Amino acids present in plant sap are nonessential ones; hence PMB depends on endosymbiotic microorganisms for the supply of essential amino acids and other nutrients, whereby they can live solely on the specialized food source [16]. PMB in their abdomen it carries a structure called bacterium that is packed with bacteriocytes whose cytoplasm is densely populated by endosymbiotic bacteria [4]. Since endosymbionts play a vital role in the physiology of their host, revealing the types of bacteria associated with mealybug will give basic information, which may throw light on the management of this pest. The present study deals with the identification of endosymbionts diversity profile of PMB from different host plants like papaya (*Carica papaya*), brinjal (*Solanum melongena* L.), cassava (*Manihot esculenta*) and congress grass (*Parthenium hysterophorus*) using metagenomic analysis through sequencing of V3-V4 region of 16S rRNA.

Materials and Methods

Insect Rearing

PMB from different host plants *viz*., papaya, tapioca, brinjal and parthenium was collected in September 2017 from farmer field located at 11°37'35.9"N 78°28'41.1"E. Hosts plants *viz*., papaya, tapioca, brinjal, and parthenium were raised inside the metallic cages under laboratory condition at 33 ± 2°C, 40–50% relative humidity and a 14 h light/10 h dark photoperiod. An ovisac from each collected samples was released on to the respective host plants inside the cages and observed for emergence. Mealybugs were allowed to complete three generation and samples for metagenomic analysis were drowned from subsequent generation.

DNA Extraction

Well grown adults (five individuals from each host plant) of papaya mealybug were surface sterilized in sodium hypochlorite (0.1%) for 30 sec and ethanol (70%) for 30sec to remove the adhering contaminants, primarily external microflora. Removal of external micro flora was confirmed by plating the final ethanol solution on Nutrient Agar medium. Genomic DNA was isolated from the surface sterilized mealybugs using c-TAB and Phenol: chloroform extraction method. The isolated DNA was quantified using Nanodrop by determining the A260/280 ratio.

Preparation of 2 × 300 Miseq library

The amplicon libraries were prepared using the Next era XT Index Kit (Illumina Inc.) as per the 16S Metagenomic Sequencing Library preparation protocol (Part # 15044223 Rev. B). Primers used in the present study were 16S rRNA F (GCCTACGGGNGGCWGCAG) and 16S rRNA R (ACTACHVGGGTATCTAATCC). Primers used for the amplification of the 16S rDNA gene sequences were designed and synthesized at Yaazh Xenomics Bioinformatics lab. Amplification of the 16S rDNA gene targeting V3-V4 region specific for bacteria was carried out. Three microliters of PCR products were resolved on 1.2 per cent agarose gel at 120V till the samples reached 3/4th of the gel.

The amplicons with the Illumina adaptors were amplified by using i5 and i7 primers that add multiplexing index sequences as well as standard adapters required for cluster generation (P5 and P7) as per the standard Illumina protocol. The amplicon library was purified by 1×AMPureXP beads and quantified using Qubit fluorometer. The mean of the library fragment size is 594bp, 606bp, 597bp and 606bp for host plants Brinjal, Tapioca, Parthenium and Papaya.

Quantity and quality check (QC) of library

The amplified libraries were analyzed in 4200 Tape Station system (Agilent Technologies) using D1000 Screen tape as per manufacturer instructions.

Cluster Generation and Sequencing

After obtaining the mean peak size from tape station profile, libraries were loaded onto MiSeq at an appropriate concentration (10-20p M) for cluster generation and sequencing. Paired-End sequencing allows the template fragments to be sequenced in both the forward and reverse direction on MiSeq. The kit reagents were used in the binding of samples to complementary adapter oligos on the paired-end flow cell. The adapters were designed to allow selective cleavage of the forward strands after re-synthesis of the reverse strand during sequencing. The copied reverse strand was then used to sequence from the opposite end of the fragment. Bioinformatics analysis was done using QIIME software.

Results

Metagenomic Library Sequencing

Metagenomic DNA from the whole body content of PMB was successfully extracted, and the purity was tested as described and sequenced on Illumina MiSeq using 2×300 bp chemistry (Fig. 1; Tab. 1). All the sequences from the sample were clustered into Operational Taxonomic Units (OTUs) based on their sequence similarity (Fig. 2). High-quality data statistics obtained through sequencing of the metagenomic DNA is presented in Table 2. This metagenome was then mined to determine the whole microbiome profile, along with the functional and metabolic capabilities of the microbiome.

Table 1: Library profile of PMB from different host plants on Agilent Tape Station using D1000 Screen Tape

Figure 1	From [bp]	To [bp]	Average size [bp]	Concentration [ng/µl]	Region Molarity [nm]
A	507	696	594	21.1	54.9
B	502	736	606	51.2	131
C	502	694	597	37.5	97.0
D	516	707	606	31.4	80.1

Table 2: High-quality data statistics of the whole microbiome community in PMB

Sl. No.	Host plants	#Reads	Total Bases	Data in MB
1	Brinjal	137774	71154099	~71
2	Papaya	135222	69348464	~69
3	Tapioca	127476	66106862	~66
4	Parthenium	152443	79064342	~79

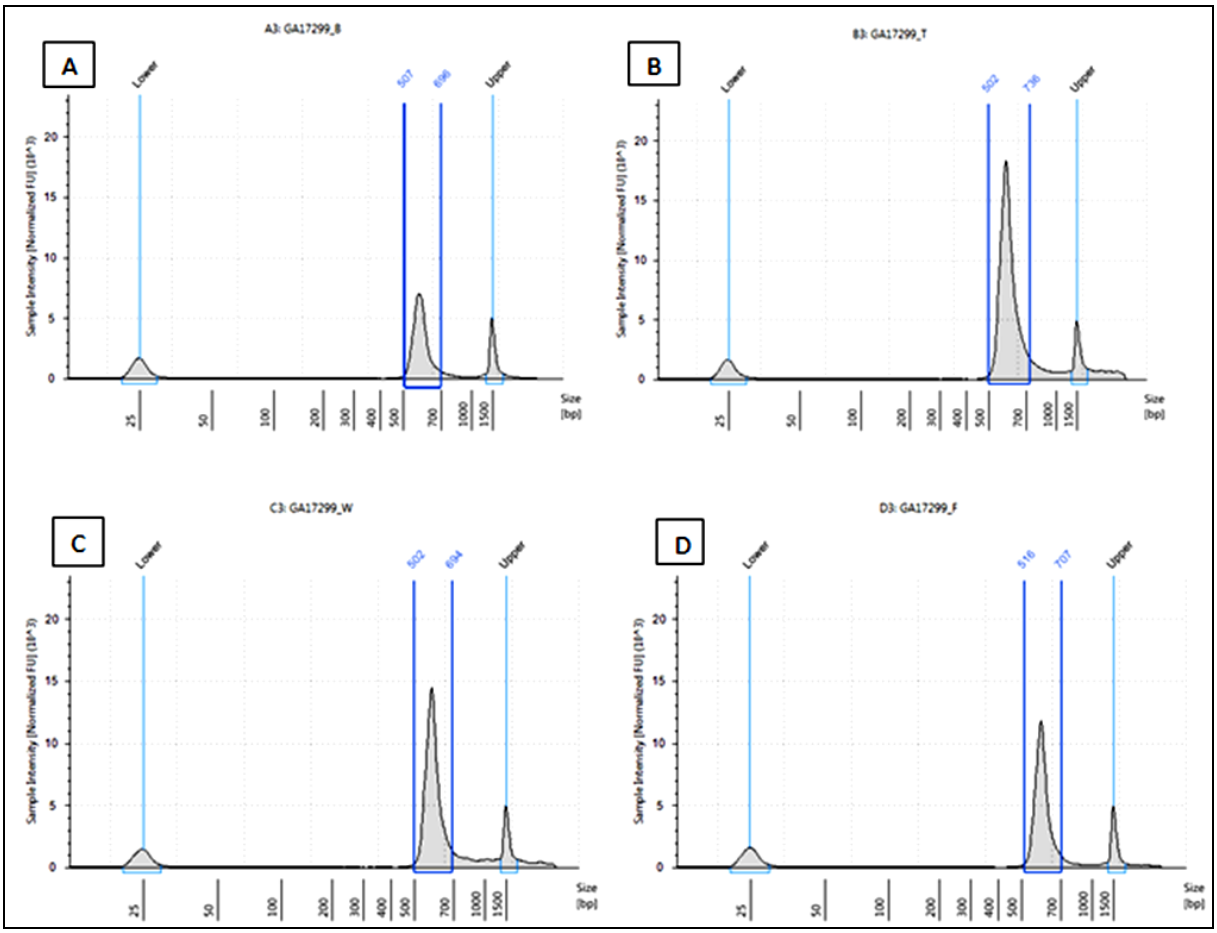

Fig 1: Library profile of PMB from different host plants on Agilent Tape Station using D1000 Screen Tape. A: Brinjal; B: Tapioca; C: Parthenium; D: Papaya

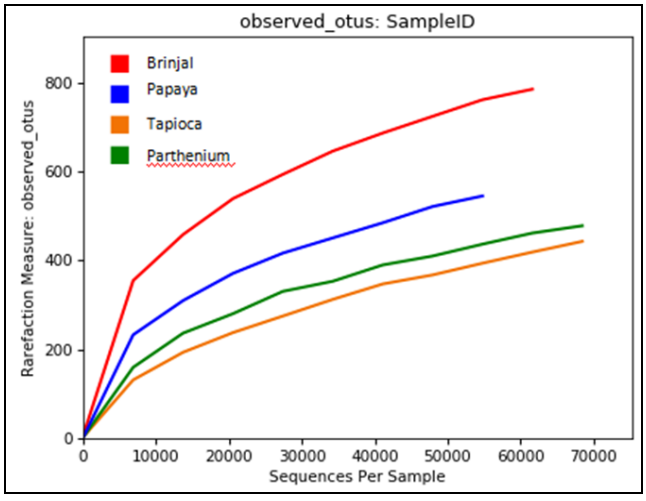

Fig 2: Rarefaction analysis curves of PMB bacterial 16S rDNA sequences spanning the V3-V4 region

Compositional Microbial diversity in PMB from different host plants

Papaya (*Carica papaya* L.)

Metagenomic analysis of PMB of papaya host plants observed that the bacterial phylum Proteobacteria (96.01%) have the upper hand followed by Firmicutes (1.29%) and to a smaller extent phylum Bacteroidetes (0.97%), Cyanobacteria (0.95%), Actinobacteria (0.71%), Planctomycetes (0.02%), TM7 (0.02%), Thermi (0.01%), and Chloroflexi (0.01%) recorded with less than one per cent in abundance. Totally nine phyla and 13 genera of bacterial endosymbionts were identified.

The order Tremblayales (79.83%) found to be more followed by Pseudomonadales (7.62%), Burkholderiales (3.34%), Rhizobiales (1.51%) and Sphingomonadales (1.19%) from the phylum Proteobacteria and others. Family Tremblayaceae (79.83%) was recorded highest in abundance followed by Pseudomonadaceae (4.0%), Moraxellaceae (3.04%), Comamonadaceae (2.96%), Sphingomonadaceae (1.11%) and others. From the complete taxonomic profiling of metagenome of PMB from papaya host plant identified the bacterial genera *viz.*, *Candidatus Tremblaya* (79.83%), *Pseudomonas* (4.03%), *Acinetobacter* (3.32%) and to a lesser extent *Chryseobacterium* (0.75%), *Sphingomonas* (0.38%), *Ochrobactrum* (0.35%), *Mesorhizobium* (0.34%), *Ensifer* (0.32%), *Staphylococcus* (0.26%), *Paracoccus* (0.22%), *Corynebacterium* (0.22%), *Delftia* (0.19%), *Sphingobacterium* (0.18%) and others (Fig. 3; Tab. 3).

Table 3: Taxonomic distribution of bacterial endosymbionts of PMB from host plant papaya

Taxonomy	Abundance (%)
P_ Proteobacteria	96.01
C_ Betaproteobacteria	83.18
O_ Tremblayales	79.83
F_ Tremblayaceae	79.83
G_ *Candidatus Tremblaya*	79.83
O_ Burkholderiales	3.34
F_ Comamonadaceae	2.96
G_ *Delftia*	0.19
C_ Gammaproteobacteria	8.4
O_ Pseudomonadales	7.62
F_ Pseudomonadaceae	4.13
G_ *Pseudomonas*	4.03
F_ Moraxellaceae	3.49
G_ *Acinetobacter*	3.32
O_ Enterobacteriales	0.43
F_ Enterobacteriaceae	0.43
C_ Alphaproteobacteria	4.38
F_ Caulobacteriaceae	0.96
O_ Sphingomonadales	1.19
F_ Sphingomonadaceae	1.11
G_ *Sphingomonas*	038
O_ Rhizobiales	1.51
F_ Brucellaceae	0.35
G_ *Ochrobactrum*	0.35
F_ Phyllobacteriaceae	0.44
G_ *Mesorhizobium*	0.34
F_ Rhizobiaceae	0.37
G_ *Ensifer*	0.32
O_ Rhodobacteriales	0.37
F_ Rhodobacteriaceae	0.37
G_ *Paracoccus*	0.22
C_ Deltaproteobacteria	0.05
P_ Cyanobacteria	0.95
C_ Chloroplast	0.91
P_ Bacteroidetes	0.97
C_ Flavobacteria	0.78
O_ Flavobacteriales	0.78
F_ Weeksellaceae	0.76
G_ *Chryseobacterium*	0.75
C_ Sphingobacteria	0.18
O_ Sphingobacteriales	0.18
G_ *Sphingobacterium*	0.18
P_ Actinobacteria	0.71
C_ Actinobacteria	0.69
O_ Actinomycetales	0.69
F_ Corynebacteriaceae	022
G_ *Corynebacterium*	0.22
P_ Firmicutes	1.29
C_ Bacilli	0.68
O_ Bacillales	0.68
F_ Bacillaceae	0.29
F_ Staphylococcaceae	0.26
G_ *Staphylococcus*	0.26
C_ Clostridia	0.32
P_ Planctomycetes	0.02
P_TM7	0.02
P_ Thermi	0.01
P_ Chloroflexi	0.01

Brinjal (*Solanum melongena* L.)

Taxonomic characterization of metagenomic data reveals that the PMB from brinjal host plant was dominated by the bacterial phylum Proteobacteria (90.47%) followed by Actinobacteria (3.62%), Bacteroidetes (3.38%) and Firmicutes (1.77%). In addition, to a lesser extent phylum Cyanobacteria (0.52%), Thermi (0.07%), Verrucomicrobia (0.06%), Chloroflexi (0.04%), Planctomycetes (0.04%), and TM7 (0.02%) also recorded with less than one per cent composition. At the order level, PMB is dominated by the order Sphingomonadales (32.24%) followed by Rhizobiales (25.87%), Pseudomonadales (16.06%), Burkholderiales

(6.46%) and Tremblayales (5.46%) from the ruling phylum Proteobacteria and others.

Besides, Tremblayales being the primary endosymbiont of PMB was accounted for 5.46 per cent. The family Sphingomonadaceae (32.14%) was recorded higher-up in PMB collected from brinjal host plant followed by Aurantimonadaceae (14.27%), Pseudomonadaceae (13.15%), Methylobacteriaceae (7.74%), Tremblayaceae (5.46%), Comamonadaceae (4.71%), Moraxellaceae (2.92%) and others. The contemporary metagenomic study furnished a complete bacterial microbiome profile of PMB from the brinjal host plant. Altogether ten genera of bacterial endosymbionts *viz.*, Pseudomonas (12.76%), Sphingomonas (8.81%), Candidatus Tremblaya (5.46%), Acinetobacter (2.73%), Chryseobacterium (1.61%) and to a small extent Ochrobactrum (0.91%), Mesorhizobium (0.75%), Methyl bacterium (0.74%), Paracoccus (0.7%), Ensifer (0.64%), and others were recorded (Fig. 4; Tab. 4).

Table 4: Taxonomic distribution of bacterial endosymbionts of PMB from host plant brinjal

Taxonomy	Abundance (%)
P_ Proteobacteria	90.47
C_ Betaproteobacteria	11.97
O_ Tremblayales	5.46
F_ Tremblayaceae	5.46
G_ *Candidatus Tremblaya*	5.46
O_ Burkholderiales	6.46
F_ Comamonadaceae	4.71
C_ Gammaproteobacteria	17.2
O_ Pseudomonadales	16.06
F_ Pseudomonadaceae	13.15
G_ *Pseudomonas*	12.76
F_ Moraxellaceae	2.92
G_ *Acinetobacter*	2.73
C_ Alphaproteobacteria	60.99
O_ Caulobacteriales	1.23
F_ Caulobacteriaceae	1.22
O_ Sphingomonadales	32.24
F_ Sphingomonadaceae	32.14
G_ *Sphingomonas*	8.81
O_ Rhizobiales	25.87
F_ Aurantimonadaceae	14.27
F_ Methylobacteriaceae	7.74
G_ *Methyl bacterium*	0.74
F_ Brucellaceae	0.92
G_ *Ochrobactrum*	0.91
F_ Phyllobacteriaceae	1.03
G_ *Mesorhizobium*	0.75
F_ Rhizobiaceae	0.92
G_ *Ensifer*	0.64
O_ Rhodobacteriales	1.36
F_ Rhodobacteriaceae	1.27
G_ *Paracoccus*	0.7
P_ Bacteroidetes	3.38
C_ Flavobacteria	1.82
O_ Flavobacteriales	1.82
F_ Weeksellaceae	1.61
G_ *Chryseobacterium*	1.61
C_ Cytophagia	1.1
O_ Cytophagales	1.1
F_ Cytophagaceae	1.1
P_ Actinobacteria	3.62
C_ Actinobacteria	3.62
O_ Actinomycetales	3.62
F_ Kineosporiaceae	0.82
P_ Firmicutes	1.77
C_ Clostridia	0.14
O_ Clostridiales	0.14
P_ Cyanobacteria	0.52
P_ Thermi	0.07
P_ Verrucomicrobia	0.06
P_ Chloroflexi	0.04
P_ Planctomycetes	0.04
P_TM7	0.02

Cassava (*Manihot esculenta* Crantz)

Microbial diversity profiling revealed that the PMB from cassava host plants is dominated by the bacterial phylum Firmicutes (64.94%) followed by Proteobacteria (31.81%) and Cyanobacteria (2.36%). In addition, it has the phylum Thermi (0.53%), Actinobacteria (0.19%), Bacteroidetes (0.15) and Chloroflexi (0.01) to a smaller extent. From the phylum Firmicutes order Lactobacillales (64.87%) dominated at the order level followed by the order Tremblayales (29.01%) and Streptophyta (2.35%). Additionally, orders like Pseudomonadales (0.71%), Rhizobiales (0.54%), Deinococcales (0.53%), Burkholderiales (0.38%), Enterobacteriales (0.38%), Sphingomonadales (0.32%), Actinomycetales (0.19%), Rickettsiales (0.16%), Caulobacteriales (0.13%), Cytophagales (0.08), Rhodospirillales (0.07%), Bacillales (0.07%), Rhodobacterales (0.06%), Flavobacteriales(0.04%), Sphingobacteriales (0.02%), Alteromonadales (0.02%) and others were recorded with less than one per cent in composition.

Family Enterococcaceae (64.68%) was reported to be highly abundant in PMB of cassava host plant followed by Tremblayaceae (29.01%). Besides, families like Deinococcaceae (0.53%), Pseudomonadaceae (0.5%), Enterobacteriaceae (0.38%), Sphingomonadaceae (0.32%), Comamonadaceae (0.32%), Methylobacteriaceae (0.23%), Moraxellaceae (0.21%), Mitochondria (0.16%), Rhizobiaceae (0.16%), Caulobacteriaceae (0.13%), Carnobacteriaceae (0.12%), Microbacteriaceae (0.09%), Cytophagaceae (0.07%), Acetobacteraceae (0.06%), Phyllobacteriaceae (0.06%), and others are also reported to be present in less than one per cent. Taxonomic profiling of metagenome of PMB resulted in 12 different genera *viz.*, *Enterococcus* with 64.57 per cent abundance, *Candidatus Tremblaya* with 29.01 per cent abundance and to a lesser extent *Deinococcus* (0.53%), *Pseudomonas* (0.48%), *Erwinia* (0.19%), *Acinetobacter* (0.15%), *Agrobacterium* (0.14%), *Sphingomonas* (0.13%), *Nelumbo* (0.12%), *Granulicatella* (0.12%), *Methylobacterium* (0.1%), *Vagococcus* (0.08%), and others (Fig. 5; Tab. 5).

Table 5: Taxonomic distribution of bacterial endosymbionts of PMB from host plant cassava

Taxonomy	Abundance (%)
P_ Proteobacteria	31.81
C_ Betaproteobacteria	29.39
O_ Tremblayales	29.01
F_ Tremblayaceae	29.01
G_ *Candidatus Tremblaya*	29.01
O_ Burkholderiales	0.38
F_ Comamonadaceae	0.32
C_ Gammaproteobacteria	1.11
O_ Enterobacteriales	0.38
F_ Enterobacteriaceae	0.38
G_ *Erwinia*	0.19
O_ Pseudomonadales	0.71
F_ Pseudomonadaceae	0.5
G_ *Pseudomonas*	0.48
F_ Moraxellaceae	0.21
G_ *Acinetobacter*	0.15
C_ Alphaproteobacteria	1.29
O_ Caulobacteriales	0.13
F_ Caulobacteriaceae	0.13
O_ Sphingomonadales	0.32
F_ Sphingomonadaceae	0.32
O_ Rhizobiales	0.54
F_ Rhizobiaceae	0.16
G_ *Agrobacterium*	0.14
F_ Methylobacteriaceae	0.23
G_ *Methylobacterium*	0.1
P_ Cyanobacteria	2.36
C_ Chloroplast	2.36
O_ Streptophyta	2.35
P_ Firmicutes	64.94
C_ Bacilli	64.94
O_ Lactobacillales	64.87
F_ Enterococcaceae	0.38
G_ *Enterococcus*	64.57
G_ *Vagococcus*	0.08
F_ Carnobacteriaceae	0.12
G_ *Granulicatella*	0.12
P_ Actinobacteria	0.19
C_ Actinobacteria	0.19
O_ Actinomycetales	0.19
F_ Microbacteriaceae	0.09
P_ Thermi	0.53
P_ Bacteroidetes	0.15

Congress grass (*Parthenium hysterophorus* L.)

Taxonomic profiling of metagenomic data obtained from PMB of host plant congress grass resulted that the phylum Proteobacteria (98.87%) found to be dominant among other phyla which are less than one per cent in abundance *viz.*, Actinobacteria (0.38%), Firmicutes (0.35%), Cyanobacteria (0.21%), Bacteroidetes (0.09%), Planctomycetes (0.03%), Acidobacteria (0.03%), Fusobacteria (0.01%), Thermi (0.01%), and Chloroflexi (0.01%). Order Tremblayales from the phylum Proteobacteria accounted for 46.66 per cent in abundance and order Enterobacteriales with 43.63 per cent in abundance stands second, succeed by order Pseudomonadales (4.8%), Burkholderiales (1.16%), Sphingomonadales (1.05%). In addition, to a lesser extent Rhizobiales (0.91%), Caulobacteriales (0.4%), Actinomycetales (0.38%), Streptophyta (0.21%), Bacillales (0.2%), Lactobacillales (0.11%), Flavobacteriales (0.05%), Rhodobacteriales (0.05%), Rickettsiales (0.05%), Alteromonadales (0.04%), Oceanospirillales (0.04%), Clostridiales (0.04%), Cytophagales (0.02%) and others were also recorded. Overall, 17 families of microbiome were identified where the dominance was shared by both Tremblayaceae with 46.66 per cent abundance and Enterobacteriaceae with 43.63 per cent abundance.

Other families like Pseudomonadaceae (4.43%), Comamonadaceae (1.06%), Sphingomonadaceae (1.05%) and to a lesser extent Methylobacteriaceae (0.46%), Caulobacteriaceae (0.4%), Moraxellaceae (0.37%), Aurantimonadaceae (0.21%), Microbacteriaceae (0.15%), Bacillaceae (0.12%), Rhizobiaceae (0.09%), Staphylococcaceae (0.07%), Enterococcaceae (0.06%), Phyllobacteriaceae (0.06%), Corynebacteriaceae (0.06%), Rhodobacteriaceae (0.05%) and others were also recorded. Genera identified through metagenomic analysis are *Candidatus Tremblaya* (46.66%), *Erwinia* (40.27%), *Pseudomonas* (4.39%), *Sphingomonas* (0.49%), *Acinetobacter* (0.29%), *Methylobacterium* (0.21%), *Pantoea* (0.11%), *Cronobacter* (0.09%), *Staphylococcus* (0.07%), *Enterococcus* (0.06%), *Corynebacterium* (0.06%) and others (1.52%) (Fig. 6; Tab. 6).

Table 6: Taxonomic distribution of bacterial endosymbionts of PMB from host plant parthenium

Taxonomy	Abundance (%)
P_ Proteobacteria	98.87
C_ Betaproteobacteria	47.81
O_ Tremblayales	46.66
F_ Tremblayaceae	46.66
G_ *Candidatus Tremblaya*	46.66
O_ Burkholderiales	1.16
F_ Comamonadaceae	1.06
C_ Gammaproteobacteria	48.56
O_ Pseudomonadales	4.8
F_ Pseudomonadaceae	4.43
G_ *Pseudomonas*	4.39
F_ Moraxellaceae	0.37
G_ *Acinetobacter*	0.29
O_ Enterobacteriales	43.63
F_ Enterobacteriaceae	43.63
G_ *Erwinia*	40.27
G_ *Pantoea*	0.11
G_ *Cronobacter*	0.09
P_ Cyanobacteria	0.21
C_ Chloroplast	0.21
O_ Streptophyta	0.21
P_ Actinobacteria	0.38
C_ Actinobacteria	0.38
O_ Actinomycetales	0.38
F_ Corynebacteriaceae	0.06
G_ *Corynebacterium*	0.06
F_ Microbacteriaceae	0.15
P_ Firmicutes	0.35
C_ Bacilli	0.32
O_ Lactobacillales	0.11
F_ Enterococcaceae	0.06
G_ *Enterococcus*	0.06
O_ Bacillales	0.2
F_ Staphylococcaceae	0.07
P_ Bacteroidetes	0.09
P_ Planctomycetes	0.03
P_ Acidobacteria	0.03
P_ Fusobacteria	0.01
P_ Thermi	0.01
P_ Chloroflexi	0.01

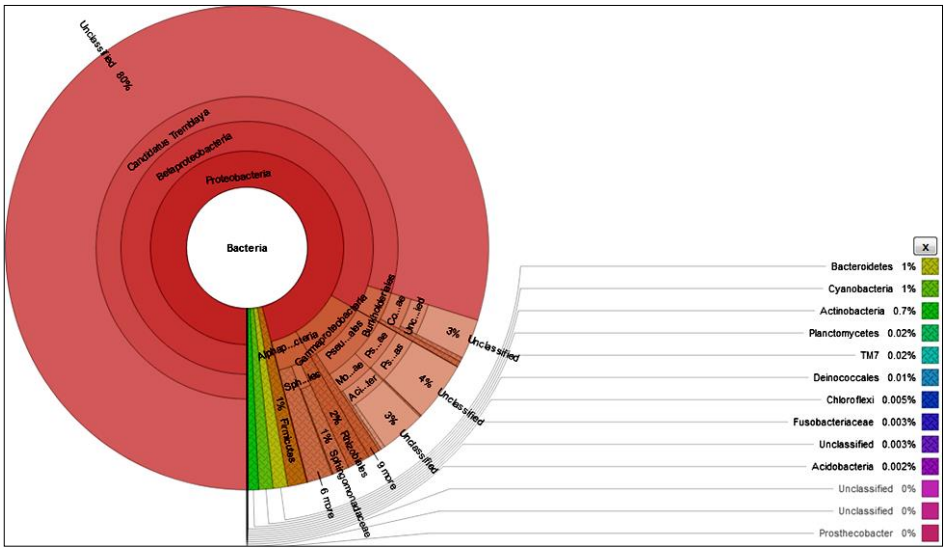

Fig 3: Interactive metagenomic visualization of hierarchical data of bacterial endosymbionts in PMB of papaya host plant through Krona diagram

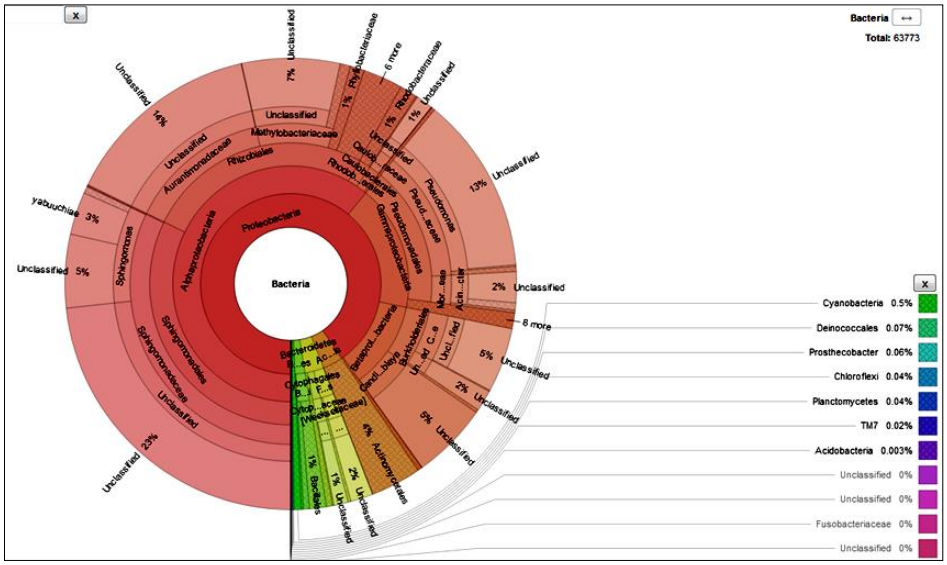

Fig 4: Interactive metagenomic visualization of hierarchical data of bacterial endosymbionts in PMB of brinjal host plant through Krona diagram

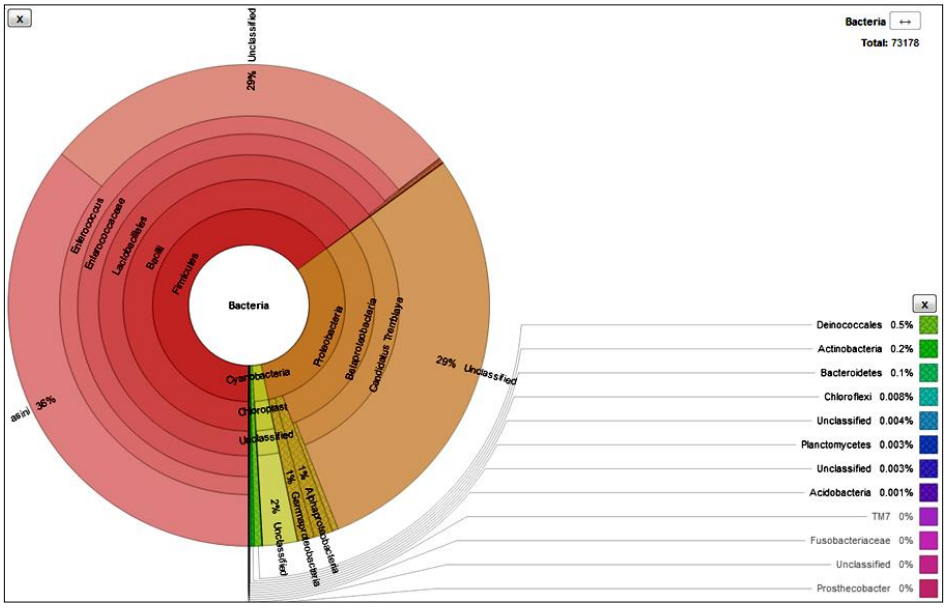

Fig 5: Interactive metagenomic visualization of hierarchical data of bacterial endosymbionts in PMB of cassava host plant through Krona diagram

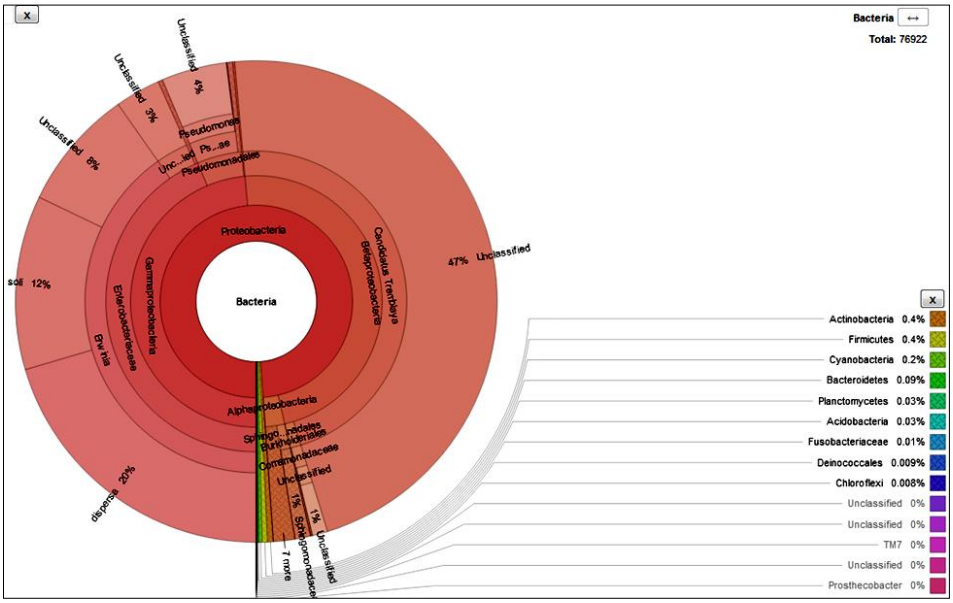

Fig 6: Interactive metagenomic visualization of hierarchical data of bacterial endosymbionts in PMB of parthenium host plant through Krona diagram

Discussion

"Endosymbionts provide novel biochemistry and metabolic traits to host insects that allow insects to exploit otherwise inaccessible niches," says Alex Wilson of the University of Miami, Flaw. Comparative analysis of the diversity of endosymbionts from the PMB of different host plants revealed that microbiome diversity of PMB varies based on its host plant. Previous studies revealed that different lineages of mealybugs are associated with distinct lineages of bacterial endosymbionts. For example, many species of the subfamily Pseudococcinae harbour a betaproteobacterial endosymbiont *Tremblaya princeps* and an additional gammaproteobacterial endosymbiont [26, 9, 13, 7].

The primary endosymbionts *T. princeps* are found in almost all pseudococcine species and exhibit host-symbiont co-speciation [1, 6], whereas the secondary gammaproteobacterial endosymbionts, including *Moranella endobia* whose genome was determined recently [13], are of polyphyletic evolutionary origins [26]. In a like manner, the primary endosymbiont of mealybugs *Candidatus Tremblaya* was also reported from the PMB of four different host plants *viz*., papaya, brinjal, cassava and parthenium with 79.83, 5.46, 29.01, and 46.66 per cent in abundance, respectively. These primary endosymbionts are very much essential for the synthesis of essential amino acids which is low in the plant sap on which mealybugs are feeding [22]. The variation in the abundance per centage is due to the influence of host plants through the dominance of secondary endosymbionts like Sphingomonadaceae in brinjal, *Enterococcus* in cassava and *Erwinia* in parthenium hosted PMB. Subsequently, it was found that PMB also hosts several secondary endosymbionts that are of either alpha, beta or gamma subdivisions of proteobacteria.

Particularly secondary endosymbionts like *Pseudomonas* and *Acinetobacter* from the PMB of the papaya host plant, *Sphingomonas*, and *Pseudomonas* from the PMB of the brinjal host plant, *Enterococcus* from the PMB of the cassava host plant and *Erwinia* and *Pseudomonas* from the parthenium host plant were identified at a higher level in composition. These secondary endosymbionts may reside within the beta-proteobacterial primary endosymbionts or live in the hemolymph, glands and other body tissues of insect [26]. Differences in the composition of secondary endosymbionts of PMB are may be due to the nature of horizontal transmission by secondary endosymbionts, and hence the host plant may influence significantly the composition of different clusters of endosymbionts of different lineages of insects. Bacterial endophytes in the host plants may be horizontally transferred into the insect system and acquired the state of the endosymbiont. Different bacterial endophytes of all four different host plants identified by the researchers, along with the secondary endosymbionts identified from the current study are presented in Table 7.

These facultative secondary endosymbionts may play several roles in its host physiology *viz.*, a) offers defense towards pathogens and parasites, for example, reduced development success following parasitism of parasitoid wasp *Aphidius ervi* in aphid is due to the presence of *Hamiltonella defense* and *Serratia symbiotica* [18], b) influencing insect-plant interaction wherein the food plant use of herbivorous insects are directly influenced by the secondary endosymbionts [29], c) favours the host insect for adaptation to environment like in aphids the secondary endosymbionts *H. defense* and *S. symbiotica* offers tolerance towards high temperature [8], d) impact on population dynamics for example aphids with the facultative endosymbiont *Regiella insecticola* produced fewer number of alate forms upon crowding [11] and e) pesticide detoxification which is reported in stinkbugs where the secondary endosymbiont Burkholderia offers protection against organophosphorus insecticides [3]. Hence, shorting out the actual role of the secondary endosymbionts identified in this study is of future scope.

Table 7: Comparison of the bacterial endophytes previously identified and secondary endosymbionts identified in the present study

Host plant	Bacterial endophytes identified by the researchers	References	Secondary endosymbionts identified in the present study
Papaya	*Bacillus*	Krishnan *et al.* (2012), Thomas *et al.* (2007)	*Pseudomonas Acinetobacter Chryseobacterium Sphingomonas Ochrobactrum Mesorhizobium Ensifer Staphylococcus Paracoccus Corynebacterium Delftia Sphingobacterium*
	Staphylococcus	Krishnan *et al.* (2012)	
	Acinetobacter	Krishnan *et al.* (2012)	
	Enterobacter	Krishnan *et al.* (2012), Thomas *et al.* (2007)	
	Kocuria	Krishnan *et al.* (2012)	
	Pseudomonas	Shi *et al.* (2010)	
	Pantoea	Thomas *et al.* (2007)	
	Brevundimonas	Thomas *et al.* (2007)	
	Sphingomonas	Thomas *et al.* (2007)	
	Methylobacterium	Thomas *et al.* (2007)	
	Agrobacterium	Thomas *et al.* (2007)	
	Microbacterium	Thomas *et al.* (2007)	
Brinjal	*Azospirillum*	Sivagamasundari and Gandhi 2018	*Pseudomonas Sphingomonas Acinetobacter Chryseobacterium Burkholderia*
	Pseudomonas	Sivagamasundari and Gandhi 2018, Ramesh *et al.* (2009), Ramesh and Phadke (2012)	
	Bacillus	Lin *et al.* (2009), Ramesh and Phadke (2012)	
	Burkholderia	Ramesh *et al.* (2009)	
	Enterobacter	Ramesh *et al.* (2009)	
Parthenium	*Acinetobacter*	Mukhtar *et al.* (2010)	*Erwinia Pseudomonas Sphingomonas Acinetobacter Methylobacterium Pantoea Cronobacter Staphylococcus Enterococcus Corynebacterium*
	Ensifer	Mukhtar *et al.* (2010)	
	Streptomyces	Tanvir *et al.* (2013)	
Cassava	*Bacillus*	Melo *et al.* (2009), Teixeira *et al.* (2007)	*Enterococcus Deinococcus Pseudomonas Erwinia Acinetobacter Agrobacterium Sphingomonas Nelumbo Granulicatella Methylobacterium Vagococcus*
	Hyphomicrobium	Chauhan *et al.* (2013)	
	Enterobacter	Melo *et al.* (2009), Teixeira *et al.* (2007)	
	Kluyvera	Melo *et al.* (2009)	
	Bradyrhizobium	Melo *et al.* (2009)	
	Clavibacter	Melo *et al.* (2009)	
	Burkholderia	Melo *et al.* (2009), Teixeira *et al.* (2007)	
	Pseudomonas	Melo *et al.* (2009)	
	Escherichia	Teixeira *et al.* (2007)	
	Salmonella	Teixeira *et al.* (2007)	
	Stenotrophomonas	Teixeira *et al.* (2007)	
	Serratia	Teixeira *et al.* (2007)	
	Paenibacillus	Menpara and Chanda (2013)	

Data availability

Sequence data of the metagenome from this study have been uploaded to NCBI under the accession numbers SAMN12004093 (https://www.ncbi.nlm.nih.gov/biosample/12004093) SAMN12004094 (https://www.ncbi.nlm.nih.gov/biosample/12004094) SAMN12004095 (https://www.ncbi.nlm.nih.gov/biosample/12004095) SAMN12004096 (https://www.ncbi.nlm.nih.gov/biosample/12004096)

Acknowledgements

This study was conducted in partial of the requirement for a Ph.D at the Tamil Nadu Agricultural University, Coimbatore. We acknowledge with gratitude to Yaazh Xenomics, Coimbatore for assistance in the metagenomic analysis.

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