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Characterization of endosymbionts of cotton mealybug (*Phenacoccus solenopsis* Tinsley) on okra using metagenomics approach

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Abstract
The exotic cotton mealybug, *Phenacoccus solenopsis* Tinsley is highly invasive, cryptic and polyphagous pest inflicting severe crop damage in economically important crops worldwide. *P. solenopsis* is characterized by their ability to thrive in various crop ecosystems due to their high fecundity, capacity to develop resistance against synthetic insecticides and association with diverse range of microorganisms which play crucial role in their life cycle. A study was therefore envisaged to identify endosymbionts of *P. solenopsis* and analysis was done to quantify the major endosymbionts associated, in Kerala during 2016 – 2017. The samples collected from Thrissur and Palakkad districts were subjected to metagenomics analysis. It revealed the presence of 15 bacterial phyla, 25 classes, 40 orders, 63 families, 97 genus and 189 species under 3 bacterial domains.

Keywords: *Phenacoccus solenopsis*, endosymbionts, metagenomics, 16S rRNA library preparation

Introduction
Cotton mealybug, *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae) has been regarded as highly invasive and polyphagous pest infesting vegetable crops, fruit crops, ornamentals and numerous weed plants. Endosymbiotic bacteria play vital role in host range of insects and their survival in various crop ecosystems. Many insects maintain obligate mutualistic symbiosis with more than one bacterial species, so that two evolutionary outcomes are possible: complementation through the establishment of a bacterial consortium or replacement of one endosymbiont by another (Moya *et al.*, 2009) [31]. Endosymbionts have evolved metabolically to complement their host's natural diet (Gosalbes *et al*., 2010) [1]. They help the insect in synthesis of vitamins, digestion and development of resistance to synthetic insecticides. It is widely accepted that up to 99.8% of the microbes present in many environments are not readily culturable, thus 'metagenome technology' tries to overcome this bottleneck by developing and using culture-independent approaches (Streit and Schmitz, 2004) [5]. The advances in genome sequencing and the development of metagenomic methods have been critical for our knowledge of the bacterial world (Lopez-Madrigal, *et al.*, 2015) [3]. Metagenomics (also referred to as environmental and community genomics) is the genomic analysis of microorganisms by direct extraction and cloning of DNA from an assemblage of microorganisms and development of metagenomics stemmed from the ineluctable evidence that as-yet-uncultured microorganisms represent the vast majority of organisms in most environments on earth (Handelsman, 2004) [4]. Next-generation sequencing technologies are increasingly used in metagenomic studies, largely due to the high sequence data throughput capacity and unbiased approach in determining the genetic composition of an unknown environmental sample (Coetzee, 2010) [2].
The main objective of present study is to characterize endosymbionts of cotton mealybug, *P. solenopsis* using metagenomic approach. For this, next generation sequencing technology has been carried out to identify and analyse the bacterial community within the species by isolation and polymerase chain reaction using 16S rDNA primer (16S rDNA f- GAGTTTGATCCTGGCTCAG and 16S rDNA r- ACGGCTACCTTGTTACGACTT).

Materials and Methods
Sample collection and storage of DNA
Mealybug samples were collected from infested okra field for the extraction of DNA.

Collected samples were preserved in 95 per cent ethanol and stored at _20°C.

Isolation of metagenomic DNA from mealybug, *Phenacoccus solenopsis*

Mealybugs were surface sterilized in de-ionized water and was then blotted carefully to remove the moisture. They were grounded in 1.5 ml Eppendorf® tube with sterilized micropistle with addition of 1 ml pre-warmed (65°C for 10 min) extraction buffer [SDS (0.5 %), 200mM Tris-HCl (P^H 8), 25mM EDTA (p^H 8), 250mM NaCl] and vortexed for 5 min. The slurry was transferred to centrifuge tube and incubated at room temperature for 1h, later centrifuged at 10,000 rpm for 8 min and supernatant was collected. An equal volume of phenol: chloroform isoamyl alcohol (25: 24: 1) was added and centrifuged at 9000 rpm for 23 min at 4°C. Supernatant was collected and DNA was precipitated with equal volume of ice cold isopropanol and incubated at room temperature for 30 min. Later the mixture was centrifuged at 10,000 rpm at room temperature for 8 min. Supernatant was discarded and 600 µl of ethanol was added. DNA pellet was washed by centrifugation at 10,000 rpm for 12 min. DNA pellet was air dried and dissolved in 25µl distilled water and stored in deep freezer for future use. The quality of DNA was assessed by one per cent agarose electrophoresis.

Polymerase chain reaction with metagenomic DNA

From the DNA sample a 1.5 KB region of bacterial 16S rDNA genome was amplified by using the primers (16S rDNA f – GAGTTTGATCCTGGCTCAG and 16S rDNA r - ACGGCTACCTTGTTACGACTT) in Veriti Thermal Cycler (Applied Biosystems®). The PCR reaction was carried out using, 1µl template DNA, 0.5 µl of forward and reverse primers, 1.5µl of 10mM dNTP (Genei®), 0.4 µl of Taq DNA polymerase (Genei®), 2µl of Taq DNA buffer B (Genei®), 1.5µl of $MgCl_2$ and 12.6µl of Millipore® water. The PCR conditions were programmed as, lid temperature 98°C, initial denaturation 94°C for 4 min, 35 cycles each of denaturation 94°C for 30 seconds, primer annealing 45°C for 1 min and primer extension 72°C for 2 min, followed by 10 min extension at 72°C and storage at 4°C. An intact and clear band was obtained at 1.5 kbp when resoved at 1.2 per cent agarose gel.

The product was sequenced at Sci Genome labs, Cochin. Total raw sequences obtained from sequencer were checked for quality parameters viz., base quality parameters, base parameters, base composition distribution and GC data. After trimming the unwanted sequences from original paired- end data, a consensus V3 region sequence was constructed using Clustal Omega program. Further we applied multiple filters and the highest quality V3 region was taken for downstream analyses. Downstream analysis of sequences was carried out by MG- RAST (Metagenomics Rapid Annotations using Subsystems Technology) program and abundance of endosymbionts was calculated and pie diagrams were constructed.

16S rRNA library preparation and sample loading

Metagenomic DNA (5 ng) was taken and standard protocol was followed for 16S rRNA library preparation and sample loaded to the Illumina MiSeq™ sequencer.

Illumina sequencing data

Total raw sequencing reads (paired end) of 682,772 was obtained from IlluminaMiSeq sequencer. The quality parameters like base quality score distributions, average base content per head and GC distribution in the reads were checked. Base quality of each cycle for all samples is shown in Figure 1-2. The x-axis represents sequencing cycle and y-axis represents percentage of total reads. The quality of left and right end of the paired-end read sequences of the sample is shown in these figures. It showed that nearly 90 per cent of the total reads had Phred score greater than 30 (>Q30; error-probability>= 0.001).

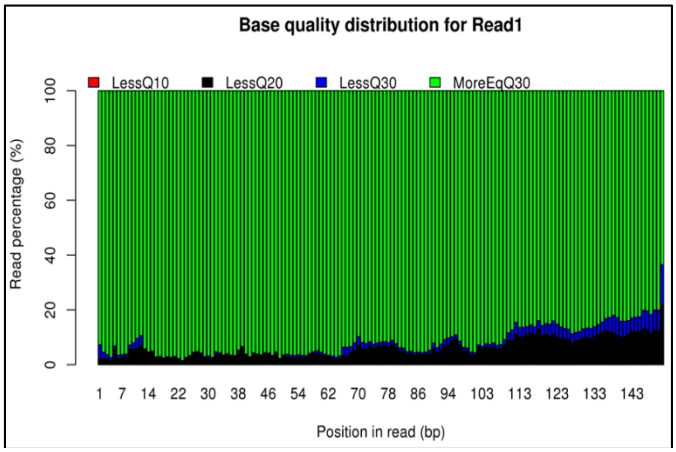

Fig 1: Quality distribution of sample MB-1(R1)

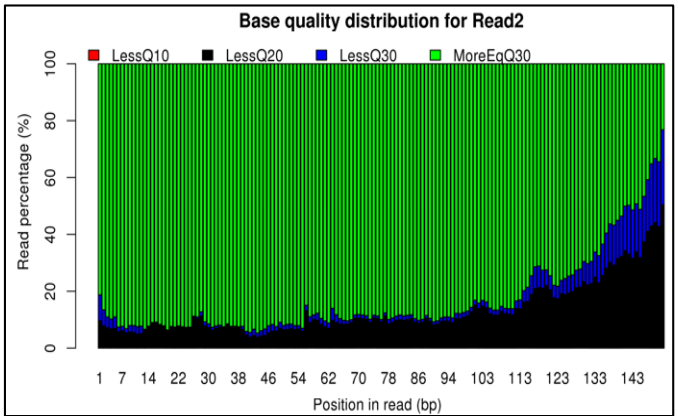

Fig 2: Base quality distribution of sample MB-1 (R2)

The composition of nucleotides in the sequence read for each sample is shown in Figure 3-4. The x-axis represents sequencing cycle and y-axis represents nucleotide percentage.

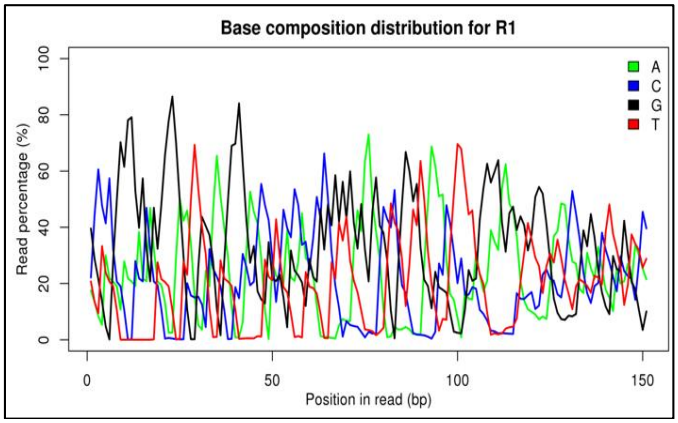

Fig 3: Base composition distribution of sample MB-1(R1)

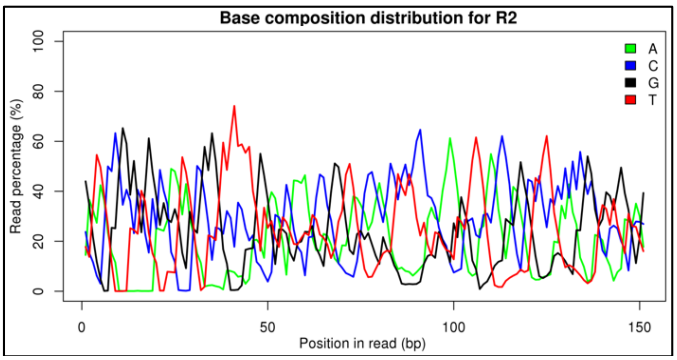

Fig 4: Base composition distribution of sample MB-1(R2)

The base composition distribution of samples were adenine (23.39%), cytosine (25.81%), guanine (27.73%) and thiamine (23.07%).

Table 1: Base composition distribution of the sample

Sample Name	Base Composition (%)			
	A	C	G	T
MB-1	22.28	25.52	29.07	23.13

The average GC content distribution of the sequenced read of the samples is shown in Figure 5. The x-axis represents average GC content in the sequence and y-axis represents percentage of sequences.
It was observed that the average GC content of each sequence reads ranged from 40-50%.

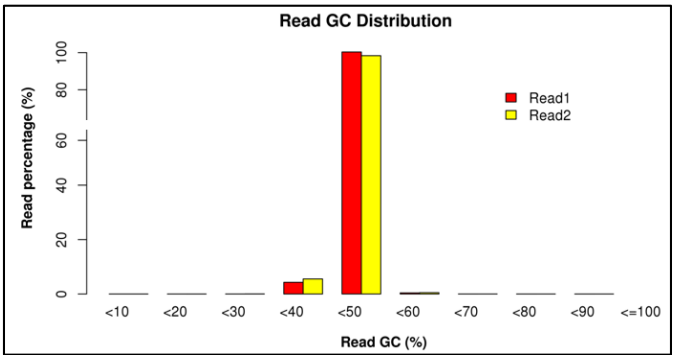

Fig 5: GC distribution of sample

Analysis of NGS data

A propriety wet-lab approach is followed to sequence 16S rRNA V3 region of bacteria. Usually a paired-end sequence from V3 Metagenomics contains some portion of conserved region, spacer and V3 region. As a first step we remove the spacer and conserved region from paired-end reads. After trimming the unwanted sequences from original paired-end data a consensus V3 region sequence is constructed using clustalo program. Apply multiple filters such as, conserved region filter, spacer filter and mismatch filter are performed to take further only the high quality V3 region sequences for various downstream analyses, which resulted in 625,491, 625,006 and 288,857 respectively. While making consensus V3 sequence, more than 40% of the paired-end reads aligned to each other with 0 mismatches with an average contig length of ~135 to ~165bp.

Chimeras were also removed using the *de novo* chimera removal method UCHIME implemented in the tool USEARCH. From 288,857 consensus reads singletons and chimeric sequences were removed and thus obtained 272,993 high quality pre-processed reads. Pre-processed reads from all samples were pooled and clustered into Operational Taxonomic Units (OTUs) based on their sequence similarity using Uclust program (similarity cutoff = 0.97). A total of 1,762 OTUs were identified from 272,993 reads (Losupone *et al*., 2013; D'Argenio *et al*., 2014). QIIME program was used for the entire downstream analysis (Caporaso *et al*., 2010). Representative sequence was identified for each OTU and aligned against Greengenes core set of sequences using PyNAST program (DeSantis *et al*., 2006; DeSantis *et al*., 2006). Further we aligned this representative sequences against reference chimeric data sets.

Results

Composition of bacteria present in the gut of mealybug, *P. solenopsis* was analyzed and grouped them into each taxonomic category from phyla to species level. The abundance of 10 major bacterial groups in each taxonomic category is given in table. We detected altogether 15 bacterial phyla in our sample. Among the phyla, *Proteobacteria* was the most dominant which consisted of 95.93 per cent of total bacterial community, followed by some unclassified bacteria 2.65 per cent (Table 2). Bacteria belongs to *Firmicutes* consist of (1.14%) which was followed by *Bacteroidetes* (0.15%) and *Actinobacteria* (0.05%). Bacteria belongs to *Chlorobi* consist of (0.04%) followed by Streptophyta (0.01%). Bacteria belong to phyla, *Gemmatimonadetes*, *Planctomycetes*, *Nitrospirae*, *Deinococcus* and *spirochaetes* were also recorded in the sample (Fig. 6).

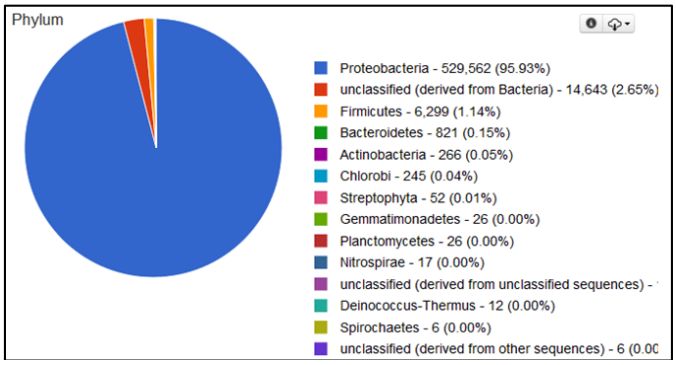

Fig 6: Abundance of gut bacterial community at phylum level

Total 25 bacterial classes were recorded and identified from the sample of *P. solenopsis*. Among them the most dominant group was *Betaproteobacteria* (71.19%) followed by *Gammaproteobacteria* (24.69%), unclassified (derived from bacteria) (2.65%), *Bacilli* (1.11%), *Bacteroidia* (0.12%), *Actinobacteria* (0.05%), *Chlorobia* (0.05%), *Deltaproteobacteria* (0.04%), *Negativicutes* (0.03%), *Flavobacteria* (0.02%), *Sphingobacteria* (0.01%), *Alphaproteobacteria* (0.01%) and *Coniferopsida* (0.01%) (Fig. 7).

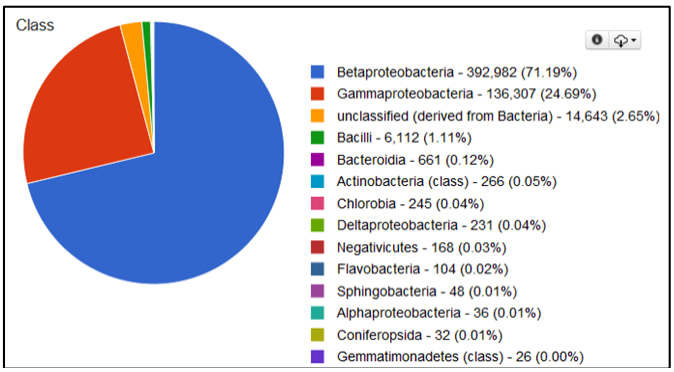

Fig 7: Abundance of gut bacterial community at class level

Later we analyzed the order level which showed altogether, 40 bacterial orders. The most dominant group was unclassified bacteria (derived from *Betaproteobacteria*) (71.15%) followed by *Enterobacteriales* (24.45%), unclassified (derived from bacteria) (2.65%), *Bacillales* (1.08%), *Burkholderiales* (0.16%), *Bacteroidiales* (0.12%), *Vibrionales* (0.06%), *Actinomycetales* (0.05%), *Chlorobiales* (0.04%), *Selenomonadales* (0.03%), *Pseudomonadales* (0.03%), *Lactobacillales* (0.02%) amd *Desulfurellales* (0.02%) (Table 2) (Fig. 8).

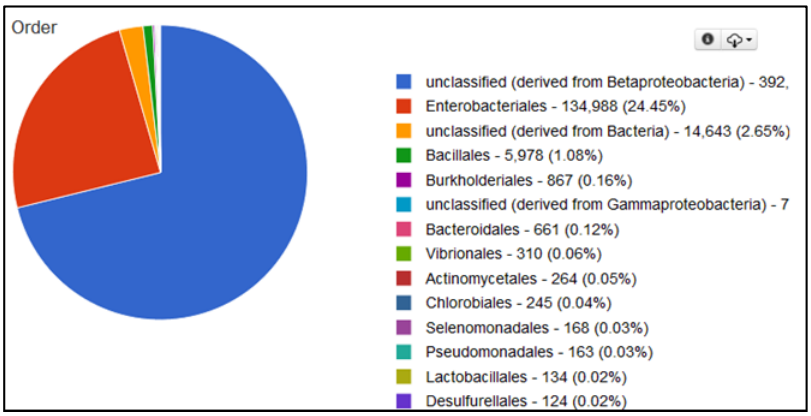

Fig 8: Abundance of gut bacterial community at order level

A total of 63 bacterial families were identified in the sample and unclassified (derived from *Betaproteobacteria*) bacteria (71.19%) was the most dominant among them. Which was followed by *Enterobacteriaceae* (24.45%), unclassified bacteria (derived from bacteria) (2.45%), *Burkholderiaceae* (0.14%), *Bacteroidaceae* (0.11%), *Staphylococcaceae* (0.09%), *Listeriaceae* (0.09%), *Bacilliaceae* (0.09%), *Vibrionaceae* (0.06%), Chlorobiaceae (0.04%), Veillonellaceae (0.03%), *Pseudomonadaceae* (0.03%) and *Desulfurellaceae* (0.02%) (Table 2) (Fig. 9).

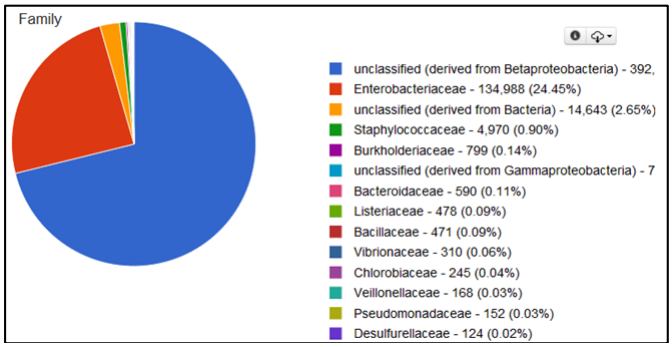

Fig 9: Abundance of gut bacterial community at family level

Analysis at genus level showed 97 genera in the sample. Among them the most dominant was the *Candidatus Tremblaya* (71.03%), followed by *Klebsiella* (12.27%), *Pantoea* (8.30%), unclassified bacteria (derived from Bacteria) (2.65%), *Wigglesworthia* (1.75%), *Kluyvera* (1.55%), *Staphylococcus* (0.09%), *Enterobacter* (0.43%), *Burkholderia* (0.13%), *Bacteroides* (0.11%), *Listeria* (0.09%), *Bacillus* (0.08%) and *Vibrio* (0.05%) (Fig. 10) (Table 2).

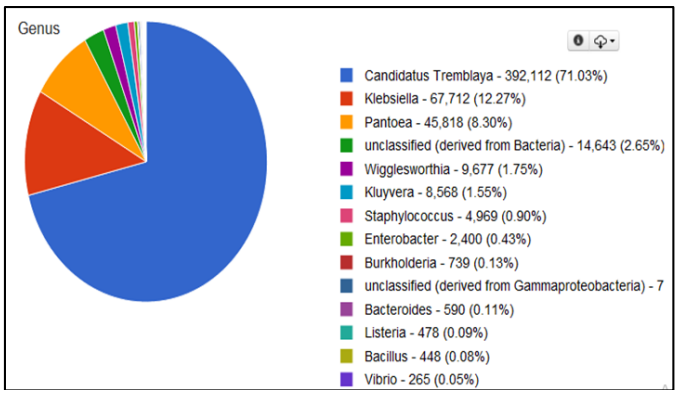

Fig 10: Abundance of gut bacterial community at generic level

A total of 189 species were identified from the sample. *Candidatus Tremblaya princeps* was found as the most dominant (78.28%), followed by uncultured *Klebsiella* sp. (8.83%), *Pantoea agglomerans* (5.98%), *Wigglesworthia glossinidia* (1.93%), uncultured bacterium (1.87%), *Kluyvera ascorbata* (1.14%), *Staphylococcus sciuri* (0.59%), *Enterobacter aerogenes* (0.25%), *Klebsiella pneumoniae* (0.20%), *Pantoea dispersa* (0.13%) and *Listeria grayi* (0.06%) (Fig. 11) (Table 2).

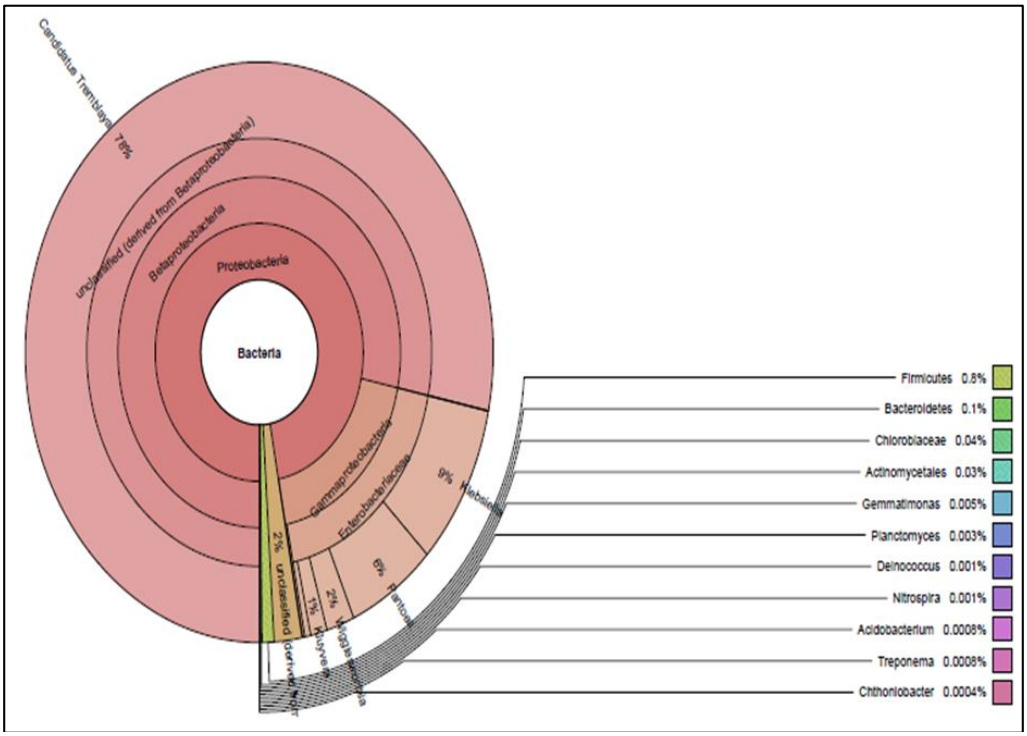

Fig 11: Abundance of gut bacterial community at species level

Table 2: Abundance of major 10 bacterial species from phyla to species occur in the gut of *Phenacoccus solenopsis*

Sl. No.	Phylum	Class	Order	Family	Genus	Species
1	*Proteobacteria*	*Betaproteobacteria*	*unclassified (derived from* Betaproteobacteria)	*unclassified (derived from Betaproteobacteria)*	*Candidatus Tremblaya* (71.03)	*Candidatus Tremblaya princeps* (78.28)
2	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Klebsiella* (12.27)	*uncultured Klebsiella sp.* (8.83)
3	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Pantoea* (8.30)	*Pantoea agglomerans* (5.98)
4	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Wigglesworthia* (1.75)	*Wigglesworthia glossinidia* (1.93)
5	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Kluyvera* (1.15)	*Kluyvera ascorbata* (1.14)
6	*Firmicutes*	*Bacilli*	*Bacillales*	*Staphylococcaceae*	*Staphylococcus* (0.09)	*Staphylococcus sciuri* (.59)
7	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Enterobacter* (0.43)	*Enterobacter aerogenes* (0.25)
8	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Klebsiella* (0.20)	*Klebsiella pneumonia* (0.20)
9	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Pantoea* (.13)	*Pantoea dispersa* (0.13)
10	*Firmicutes*	*Bacilli*	*Bacillales*	*Listeriaceae*	*Listeria* (0.09)	*Listeria grayi* (0.06)

Discussion

An attempt was made to explore the bacterial communities associated with *P. solenopsis*, as the feeding bahaviour of *P. solenopsis* was influenced by the gut bacterial communities. Isolation of gut metagenomic DNA of *P. solenopsis* was done by SDS based metagenomic DNA extraction procedure explained by Zhou *et al.* (1996) [6]. Illumina Next Generation Sequencing platform was used to reveal the total bacterial community present in the gut. Analysis of hypervariable V3 region of 16S rRNA fragment resulted in large bacterial community with 1762 OTUs per sample with 92 per cent identity detection.

The analysis of bacterial community at phyla level revealed that *Proteobacteria* was the most dominant in the gut of *P. solenopsis*, which was followed by some uncultured bacteria, *Firmicutes*, *Bacteroidetes* and *Actinobacteria*. Similarly, Szabo (2017) [7] reported that most of the pseudococcinae mealybugs harbor a unique symbiosis setup with betaproteobacterial symbionts which is coming under *Proteobacteria*. Parkinson (2016) [8] also reported the nested symbiosis in citrus mealybug, *Planococcus citri* (Risso), in which phylum *Proteobacteria* was the dominant one. Douglas, (2009) [9], reported the dominance of *Proteobacteria* in haemocoel of sap feeding aphids and psyllids, however he found that *Bacteroidetes* was dominant along with *Proteobacteria* in glassy-winged sharpshooter, *Homalodisca vitripennis* (Germar). *Firmicutes* was the dominant phylum in *Glossina fuscipes fuscipes* as reported by Lindh and Lehane (2011) [10]. Bacterial communities belonged to *Bacteroidetes* and *Firmicutes* were dominant in the gut of termites (Xiang *et al.*, 2012) [11].

Proteobacteria was associated with diverse metabolic and physiological properties such as, cell envelope biogenesis, synthesis of essential amino acids and B vitamins in insects (Benett *et al.*, 2014). Kikuchi (2012) [13] reported that *Proteobacteria* in insects helped in pesticide detoxification. *Proteobacteria* assisted in carbohydrate metabolism of insect body which helped in cellulose degradation in insects (Dalalibera *et al.*, 2005) [14]. According to Brown *et al*. (2012) [15] *Firmicutes* in insects were highly beneficial as they helped in cellulose and hemicellulose degradation in insects. Present study revealed the presence of entomopathogens, *Bacillus*

thuringiensis and *Bacillus cereus* which were coming under the *Firmicutes* in the gut of *P. solenopsis*. Gruwell (2007) [16] found that *Bacteroidetes* in Diaspididae promoted the evolution of paternal genome elimination and thereby increased feminization in insect populations. Schrempf, (2001) [17] reported that *Actinobacteria* helped in production of extracellular enzymes and wide variety of secondary metabolites.

Candidatus Tremblaya princeps was found as the major species of bacteria in the gut of *P. solenopsis*. Parkinson (2016) [8] reported the presence of *Candidatus Tremblaya princeps* in the gut of citrus mealybug, *Planococcus citri* which exhibited nested endosymbiosis with another symbiont namely, *Candidatus Moranella endobia*. *Pantoea agglomerans* was another bacterium associated with the gut of *P. solenopsis*. Dillon and Charnley (1995) [18] reported the presence of *Pantoea agglomerans* (Ewing and Fife) in desret locust, *Schistocerca gregaria* (Forsskal) which helped in production of phenol that inhibited conidia germination of *Metarhizium anisopliae*. *Wigglesworthia glossinidia* was also found in gut of *P. solenopsis*, which were having genes encoding for synthesizing B vitamins *viz.*, pantothenate (Vitamin B_5), biotin (Vitamin B_7), thiamin (Vitamin B_1), riboflavin (Vitamin B_2), pyridoxine (Vitamin B_6), nicotinamide (Vitamin B_3) and folate (Vitamin B^9) in its genome. (Akman *et al*., 2002) [19]. *Acidobacterium capsulatum* was present in the gut of *P. solenopsis*. *Acidobacterium* sp. was also reported by Reid *et al*. (2011) [20] as xylanolytic bacteria in termite gut. Interestingly, gut bacterial community exhibited the presence of bacteria, *Erwinia amylovora*, which is reported to be causing fire blight in apple, pear and other ornamentals (Vanneste, 2000) [21]. *Bacillus pumilus* bacteria was found associated within the gut of mealybug, which was reported to be producing high amount of physiologically active gibberellins (Gutierrez-Manero *et al*., 2001) and cellulose enzyme (Ariffin *et al.*, 2006) [23]. *Candidatus Regiella insecticola*, which was also present in gut of mealybug, was reported to be present in pea aphid, *Acyrthosiphon pisum* that helped to prevent the attack from fungal pathogens. Another bacteria detected in mealybug gut was, *Escherichia coli*, which was reported to elicit effective immunity against lethal and highly virulent insect pathogen, *Photorhabdus luminescens* TT01 in *Manduca sexta* caterpillars (Eleftherianos *et al*., 2006) [24]. *Lactobacillus acidophilus* were present in mealybug gut, which was also reported by Vilela *et al*. (2015) [25] that the presence of *Lactobacillus acidophilus* in *Galleria mellonella* which inhibited the biofilm formation by *Candida albicans*. *Micrococcus* sp. recorded in sample was also recorded by Bulet *et al*. (1999) [26] as it was involved in synthesis of antimicrobial peptides which acted as defensive compounds against insect pathogens. *Paenibacillus* sp. HanTHS1 was found in the bacterial community of mealybug gut. This was reported by Bouraoui *et al*. (2016) [27] as they isolated a multifunctional enzyme named, GH51 arabinofuranosidase from *Paenibacillus* sp. Surprisingly, the entomopathogenic bacteria, *Photorhabdus temperata* was present in gut of *P. solenopsis*. Jung and Kim (2006) [28] reported that the *Photorhabdus temperata* can be used against *Spodoptera exigua* as a synergist with *B. thuringiensis*. *Raoultella ornithinolytica* were associated with the bacterial community in the analysed sample. Kanki *et al*. (2002) [29] reported that the *Raoultella ornithinolytica* could produce histamine poison which might kill fish through histamine fish poisoning. Another bacteria identified in mealybug sample was *Verticillium dahliae*. Bhat and Subbarao (1999) [30] reported that *Verticillium dahliae* exhibited a very specific host range which included cotton, bell pepper, cabbage, egg plant and cauliflower. *Xenorhabdus* sp. were also present in mealybug sample, which was known to be associated with entomopathogenic nematodes (Jung and Kim, 2006) [28]. The uncultured *Burkholderiales* bacterium was found associated with gut bacterial community, which was capable of detoxifying pesticides. Kikuchi (2012) [13] reported that the *Burkholderia* sp. associated with stink bugs utilize organophosphorous compounds as sources of carbon, nitrogen and phosphorous by facilitating detoxification of these compounds.

Conclusion

The metagenomic analysis conducted on *Phenacoccus solenopsis* revealed the presence of 15 bacterial phyla, 25 classes, 40 orders, 63 families, 97 genus and 189 species under 3 bacterial domains. Among the phyla, *Proteobacteria* was the most dominant which consisted of 95.93 per cent of total bacterial community, followed by some unclassified bacteria, 2.65 per cent. The most dominant order identified was unclassified bacteria (derived from *Betaproteobacteria*) 71.15 per cent, followed by *Enterobacteriales* 24.45 per cent. Generic level analysis showed 97 genera in the sample. Among them the most dominant was the *Candidatus Tremblaya* 71.03 per cent followed by *Klebsiella* 12.27 per cent. Endosymbionts play vital role in survival and host range of the invasive mealybug, *Phenacoccus solenopsis*. Metagenomics helps to unveil the microbes in association with the mealybug, which will pave the way for proper management tactics to check the population.

Acknowledgment

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