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# Next-generation sequencing reveals endosymbiont variability in cassava whitefly, *Bemisia tabaci*, across the agro-ecological zones of Kerala, India

E.R. Harish, ManiChellappan, T. MakeshKumar, Deepu Mathew, M.T. Ranjith, and D. Girija

**Abstract:** Silverleaf whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), is one of the most notorious invasive insect pests, infesting more than 900 species of plants and spreading more than 200 viral diseases. This polyphagous agricultural pest harbours diverse bacterial communities in its gut, which perform multiple functions in whiteflies, including nutrient provisioning, amino acid biosynthesis, and virus transmission. The present exploratory study compares the bacterial communities associated with silverleaf whitefly infesting cassava, also known as cassava whitefly, collected from two different zones (zone P: plains; zone H: high ranges), from Kerala, India, using next-generation sequencing of 16S rDNA. The data sets for these two regions consisted of 1 321 906 and 690 661 high-quality paired-end sequences with mean length of 150 bp. Highly diverse bacterial communities were present in the sample, containing approximately 3513 operational taxonomic units (OTUs). Sequence analysis showed a marked difference in the relative abundance of bacteria in the populations. A total of 16 bacterial phyla, 27 classes, 56 orders, 91 families, 236 genera, and 409 species were identified from the P population, against 16, 31, 60, 88, 225, and 355, respectively, in the H population. *Arsenophonus* sp. (Enterobacteriaceae), which is important for virus transmission by whiteflies, was relatively abundant in the P population, whereas in the H population *Bacillus* sp. was the most dominant group. The association of whitefly biotypes and secondary symbionts suggests a possible contribution of these bacteria to host characteristics such as virus transmission, host range, insecticide resistance, and speciation.

**Key words:** *Arsenophonus*, cassava mosaic, NGS, 16S rDNA, symbiotic bacteria.

**Résumé :** L'aleurode du tabac, *Bemisia tabaci* (Gennadius) (Hemiptera : Aleyrodidae), est l'un des insectes ravageurs envahissants les plus notoires, affectant plus de 900 espèces de plantes et propageant plus de 200 maladies virales. Ce ravageur agricole polyphage abrite diverses communautés bactériennes dans son intestin, lesquelles remplissent de multiples fonctions incluant le provisionnement en nutriments, la synthèse d'acides aminés et la transmission de virus. La présente étude exploratoire visait à comparer, au moyen du séquençage des ADNr 16S, les communautés bactériennes associées aux aleurodes infectant le manioc, aussi connus sous le nom d'aleurode du manioc, échantillonnes au sein de deux zones (P pour les plaines et H pour les hauts massifs) du Kerala en Inde. Les données pour ces deux régions étaient composées de 1 321 906 et 690 661 séquences en paires de haute qualité d'une longueur moyenne de 150 pb. Des communautés bactériennes très diversifiées étaient présentes au sein d'un échantillon comptant 3513 unités taxonomiques opérationnelles (OTU). Une analyse des séquences a montré une préférence marquée dans l'abondance relative des bactéries au sein de ces populations. Au total, 16 embranchements, 27 classes, 56 ordres, 91 familles, 236 genres et 409 espèces bactériennes ont été identifiés au sein de la population P contre 16, 31, 60, 88, 225 et 355 chez la population H. Le genre *Arsenophonus* sp. (Enterobacteriaceae), lequel est important pour la transmission des virus par les aleurodes, était relativement abondant chez la population P, tandis que le genre *Bacillus* sp. était le plus dominant chez la population H. L'association entre des biotypes d'aleurodes et des symbiontes secondaires suggère une possible contribution de ces bactéries aux caractéristiques de l'hôte telles que la transmission des virus, la gamme d'hôtes, la résistance aux insecticides et la spéciation. [Traduit par la Rédaction]

**Mots-clés :** *Arsenophonus*, mosaïque du manioc, NGS, ADNr 16S, bactéries symbiotiques.

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## Introduction

Microorganisms and insects are the two most successful groups of organisms on planet Earth. Ubiquitous microbes are also present inside the body of insects in close association and are known as endosymbionts. Of the symbionts, bacteria perform the most diverse roles and hence are the most studied (Engel and Moran 2013). Less than 1% of endosymbionts are culturable, but techniques such as next-generation sequencing enable researchers to explore more deeply the true complexity of insect-microbe associations and to understand the profound influence of microbial metabolic activity on the other organisms (Schloss and Handelsman 2003).

Endosymbiotic theory (Mereshkowsky 1910) classifies endosymbionts into primary and secondary. Primary endosymbionts (P-endosymbionts) are associated with insect hosts for a long period of time and form obligate associations and co-speciation with their insect hosts, whereas secondary endosymbionts (S-endosymbionts) are more recently developed associates that sometimes get horizontally transferred between hosts and live in the hemolymph of the insects but never obligate. S-symbionts are not confined into specialized S-bacteriocytes found in gut tissues, glands, body fluids, cells surrounding the P-bacteriocytes, or even invading the P-bacteriocytes themselves (Baumann et al. 2006). S-symbionts seem to be the result of multiple independent infections and, although they are usually maternally inherited, their transmission may also occur horizontally across the hosts.

These microbes help in a variety of ways for the dominance of insects. They provide various fitness advantages, such as increased fecundity, increased longevity, female-biased sex ratio, as well as greater immunity against natural enemies and pathogens (Su et al. 2013). S-symbionts are shown to increase resistance of insects to fungal pathogens (Aksoy et al. 1997). In their host insects, endosymbionts play various roles such as nutrition; e.g., *Buchnera* provides essential amino acids that are lacking in the plant sap diet of its aphid host (Xie et al. 2018), detoxification of toxins, plant allelochemicals and insecticides (Kikuchi et al. 2012), source of cues and signals (Dillon et al. 2002), defense toward pathogens and parasites (Oliver et al. 2003), adaptation to environment shock (Montllor et al. 2002), virus–vector interaction (Chiel et al. 2007), population dynamics (Kikuchi et al. 2012), insect–plant interactions (Hosokawa et al. 2007), biotype determinants (Gueguen et al. 2010), and protectants against natural enemies (Oliver et al. 2003). Rosell et al. (2010) described mutualistic and dependent relationships of endosymbionts with other organisms.

Sequencing the 16S ribosomal RNA (rRNA) was the most popular method adopted to identify the bacteria (Petti et al. 2005; Ranjith et al. 2016). Nearly 1500 bp of 16S

rDNA is large enough for bioinformatics analyses (Patel 2001), and additionally, it is present in all bacteria and its function is well defined (Janda and Abbott 2007). But this fails in polymicrobial specimen wherein multiple templates result in uninterpretable Sanger reads (Drancourt et al. 2000). Next-generation sequencing with the primer spanning hypervariable regions (V1–V9) of 16S rRNA gene circumvents these limitations. Metagenomics applies to a suite of genomic technologies and bioinformatics tools to directly access the genetic content of entire communities of organisms (Thomas et al. 2012).

Root and tuber crops, including cassava, provide a substantial part of the world's food supply (Chandrasekara and Kumar 2016). Cassava whitefly (*Bemisia tabaci*) transmitted cassava mosaic disease causes 30%–40% yield losses on a global scale (Malathi et al. 1985). As a phloem-feeding homopteran, *B. tabaci* harbours various endosymbionts for its nutritional requirements and functionality. These endosymbionts act as carotenoids sources of whiteflies (Sloan and Moran 2012); few endosymbionts such as *Portiera aleyrodidarum* provides B-complex vitamins and amino acids lacking in fly feeds (Lai et al. 1996; Xie et al. 2018). They also assist in virus transmission (Nirgianaki et al. 2003; Chiel et al. 2007) and a GroEL homologue from endosymbionts assists in the circulative transmission of virus by protecting the virus from destruction during its passage through the haemolymph (Morin et al. 1999; Rana et al. 2012). According to Gottlieb et al. (2010), *Hamiltonella* sp. found in B-biotype of whiteflies help them to become successful vectors of *Tomato yellow leaf curl virus*.

Endosymbionts play crucial roles in making whitefly a pest of global importance. Keeping in view the economic losses by cassava whitefly, as the vector of *Cassava mosaic virus*, this study answers the hypothesis that under different agro-ecological zones, at various disease severities, and in genetically varying populations, the endosymbiont population varies. Using next-generation sequencing of 16S rDNA from gut microbiome, variation in the relative abundance of different bacterial endosymbionts in cassava whitefly populations from different agro-ecological zones of Kerala, India, is shown. Further, the pest management application of the understanding of endosymbiont variability under specific physiological conditions is also discussed.

## Materials and methods

### Collection of whiteflies

*Bemisia tabaci* samples were collected from cassava fields of 13 different agro-ecological zones of Kerala (Mohankumar 2007) (latitude: 8.1730°N–12.4740°N; longitude: 74.2747°E–77.3712°E), India (Harish et al. 2016; Table S1<sup>1</sup>; Fig. S1<sup>1</sup>). Whiteflies were collected individually (March 2014 – May 2016) in microfuge tubes containing

<sup>1</sup>Supplementary data are available with the article through the journal Web site at <http://nrcresearchpress.com/doi/suppl/10.1139/gen-2018-0050>.

70% ethanol and stored at -20 °C. Each sample was identified as *B. tabaci* by adopting the morphological identification keys of Martin (1987).

Genetic variability study/barcoding using mitochondrial *Cytochrome oxidase1* gene (primers C1-J2195 and L2-N-3014) showed the presence of two biotypes/genetic groups of *Bemisia*, Asia I and Asia II5, in cassava plants of Kerala, and the biotypes were confirmed by using reference sequences from NCBI database. According to Dinsdale et al. (2010), 3.5% genetic divergence is the minimum requirement to separate two putative species/biotypes/genetic groups of whitefly. In this case, up to 15.96% sequence divergence was observed between the biotypes. Whiteflies collected from plains (elevation less than 150 m asl) belonged to Asia II5 biotype, whereas whiteflies collected from hilly regions (elevation more than 900 m) belonged to Asia I biotype. Composite samples, P and H, were made from the two biotypes identified (biotypes Asia II5 from plains and Asia I from hills) and analysed using a metagenomic approach to compare the endosymbiont variations. Sample P included one whitefly each from 12 agro-ecological zones of plains (<150 m asl) and H had 12 whiteflies from hilly regions of Sulthan Bathery. Also, from the cassava plants surveyed for severity of cassava mosaic disease symptoms, plants in high elevations of Sulthan Bathery region (>900 m asl) had shown very less severity with a score of 1 compared to plants from other areas (severity score 3–5, Ikotun and Hahn 1994).

#### Isolation of metagenomic DNA from adult *B. tabaci*

The insects were surface sterilized with ethanol and household chlorine bleach, as described by Davidson et al. (1994). Metagenomic DNA from samples P and H were isolated through a direct method (Zhou et al. 1996). They were homogenized in 400 µL of extraction buffer (200 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), and 250 mM NaCl, SDS (0.5%)) in a 1.5 mL Eppendorf tube, using liquid nitrogen. The homogenized samples were incubated at room temperature for 1 h and centrifuged at 12 000 rpm for 5 min at 4 °C. The supernatants were collected in fresh tubes, and an equal volume of phenol – chloroform – isoamyl alcohol (24:25:1) was added to the supernatants and again centrifuged at 10 000 rpm for 20 min at 4 °C. The aqueous phase was transferred to a fresh tube, and an equal volume of iso propanol was added and the mixture was incubated at room temperature for 15 min, centrifuged at 13 000 rpm for 5 min at room temperature, and the metagenomic DNA pellet precipitated out. The DNA pellet was washed with ethanol (95%) by centrifugation at 10 000 rpm for 10 min, air dried, dissolved in 25 µL of autoclaved distilled water, and stored in a deep freezer (-80 °C) for future use.

#### Quality checking of metagenomic DNA

The 16S rDNA fragment was amplified by polymerase chain reaction from the metagenomic DNA using the

universal 16S rDNA primers fD1 (5'-GAGTTTGATCCT GGCTCAG-3') and rP2 (5'-ACGGCTACCTTGTACGACTT-3') (Haris et al. 2014). The reaction mixture contained 0.2 µL template DNA (20 ng), 0.1 µL each of the forward and reverse primers, 1 µL of 10 mM dNTP (Genei®), 0.2 µL of Taq DNA polymerase (Genei®), 2.5 µL of Taq DNA buffer A (Genei®), and 15.9 µL of grade I water. Thermal cycling included initial denaturation at 94 °C for 2 min; followed by 29 cycles with denaturation at 94 °C for 45 s, primer annealing at 55 °C for 1 min, and primer extension at 72 °C for 2 min; and final extension at 72 °C for 10 min. The products were electrophoresed on agarose gel (0.8%).

#### Sequencing of 16S ribosomal RNA amplicon

The metagenomic DNA isolated from *B. tabaci* adults were sequenced using Next-Generation Illumina MiSeq™. An amplicon library was prepared with specific primers spanning the hypervariable V3 region of the 16S rRNA gene (Fig. S2<sup>1</sup>) and used for sequencing and subsequent classification.

#### Amplicon PCR

Metagenomic DNA samples were normalized to 5 ng/µL in 10 mM Tris (pH 8.5) and amplicon PCR was carried out using V3 primers (341F, 5'-CCTACGGGA GGCAGCAG-3'; 518R, 5'-ATTACCGCGGCTGCTGG-3') (Bartram et al. 2011). The PCR master mix consisted of 2 µL each of forward and reverse primers (10 pM/µL), 0.5 µL 40 mM dNTPs, 5 µL 5X Phusion HF reaction buffer, 0.2 µL 2U/µL or µL F-540 Special Phusion HS DNA polymerase, 5 ng input DNA, and water to make up the volume to 25 µL. PCR reaction was programmed with initial denaturation at 98 °C for 30 s; followed by 30 cycles of denaturation at 98 °C for 10 s, primer annealing at 55 °C for 30 s, and primer extension at 72 °C for 30 s; and final extension at 72 °C for 5 min. PCR products were quantified using the fluorescence quantitative fluorometer (Qubit 2.0®) with the Qubit dsDNA HS assay kit (Invitrogen, USA).

#### 16S rDNA amplicon library preparation

##### PCR clean-up

PCR clean-up was carried out using AMPure XP beads to purify the 16S V3 amplicon away from free primers and primer dimer species. The reagents consisted of 10 mM Tris (pH 8.5) (52.5 µL per sample), AMPure XP beads tk'1(20 µL per sample), and freshly prepared ethanol (80%) (400 µL per sample). Standard protocol (Amplicon PCR et al. 2013) was followed and the cleaned products were stored at -20 °C.

##### IndexPCR

Illumina™ Truseq adapters and indices were added to the cleaned PCR products. The PCR master mix consisted of 2 µL each of 10 pM/µL forward and reverse primers, 1.0 µL 40 mM dNTP, 10 µL 5X Phusion HF reaction buffers, 0.4 µL 2U/µL F-540 special Phusion HS DNA polymerase, 10 µL (minimum 5 ng) PCR1 amplicon, and water to make

up the total volume to 50  $\mu\text{L}$ . PCR reaction was programmed with initial denaturation at 98 °C for 30 s; 15 cycles with denaturation at 98 °C for 10 s, primer annealing at 55 °C for 30 s, and primer extension at 72 °C for 30 s; followed by final extension at 72 °C for 5 min.

#### PCR clean-up 2

AMPure XP beads were used to clean up the final library before quantification. The reagents consisted of 10 mM Tris (pH 8.5) (27.5  $\mu\text{L}$  per sample), AMPure XP beads (56  $\mu\text{L}$  per sample), and freshly prepared 80% ethanol (400  $\mu\text{L}$  per sample).

#### Library quantification, normalization, and pooling

Libraries were quantified using a fluorometric quantification method, and a concentrated final library was diluted using distilled water. Diluted DNA (5  $\mu\text{L}$ ) from each library was pooled with unique indices.

#### Library denaturing and MiSeq sample loading

In preparation for cluster generation and sequencing, pooled libraries were denatured with NaOH, diluted with hybridization buffer, and then heat denatured before MiSeq® sequencing. Each run included a minimum of PhiX (5%) to serve as an internal control for these low diversity libraries. Denatured library was loaded into the reagent cartridge of Illumina MiSeq™ sequencer. The output files (fastq) generated from the sequencer were analysed.

#### Analysis of NGS data

Total raw reads of samples obtained from Illumina sequencing platform were quality checked for base quality (Phred Score), base composition, adaptor dimer contamination, ambiguous bases, and GC content using Fast QC (Version 0.11.8) tool with default parameter. The 16S rDNA V3 hypervariable region specific primers were checked in the paired-end reads and allowed to merge using Clustal Omega (version 1.2.0) program with minimum overlap length of 10 bp. The merged consensus fasta of all samples were pooled and taken for various downstream analyses.

As a part of pre-processing of sequence reads, singletons, generated due to the sequencing errors and could result in spurious operational taxonomic units (OTUs), were removed before starting OTU clustering, by removing the reads that did not cluster with other sequences (abundances <2). Singleton are the R1+R2 merged consensus of V3 FASTA contig sequence whose frequency is only one, or present only one time. Chimeras were also removed using the de novo chimera removal method UCHIME implemented in the tool USEARCH.

Using Uclust program, pre-processed reads from all samples were pooled and clustered into OTUs based on their sequence similarity (similarity cut off of 0.97). QIIME (Caporaso et al. 2010) and MG-RAST (Meyer et al. 2008) programmes were used in downstream analyses. Representative sequences were identified for each OTU

and aligned against Greengenes core set of sequences using PyNAST program (DeSantis et al. 2006a, 2006b). Further, these representative sequences were aligned against reference chimeric datasets, and taxonomic classification was performed using RDP classifier and Greengenes OTUs database.

The taxonomic categories of bacteria (from phylum to species level) present in both the P and H populations of *B. tabaci* were compared using Jaccard distance (a measure of how dissimilar the sets are) in R software (version 3.6.0). The greater the distance between each taxonomic category, the greater the variation between them.

Community matrices at the phylum, class, order, family, genus, and species levels were prepared for both the populations, and the relative abundances ( $P_i$ ) were prepared based on OTU counts. The data was further analyzed using Shannon–Weiner diversity Index, with the function  $H = \sum[(P_i) \times \ln(P_i)]$ .  $P_i$  is per cent of a particular bacterium compared to the total bacteria identified in that population at that taxonomic level, using the pre-processed total reads. For example, when we compare the variability between the P and H populations at the phylum level in terms of the bacterium Proteobacteria, the P population has a per cent abundance of 87.57, whereas it is 13.40 in the H population. Thus,  $P_i$  values shall be 0.8757 and 0.1340, respectively, and corresponding index values  $-0.116$  ( $0.8757^* - 0.1327$ ) and  $-0.269$  ( $0.1340^* - 2.0099$ ), respectively. Index ratio between the populations indicated the variation across both the populations in each level. Similarly, at each level of taxonomic classification, indices for every bacterial community were calculated and compared.

Rarefaction analysis was carried out to assess species richness of the samples based on the construction of rarefaction curves using MG-RAST software. A phylogenetic tree of bacteria at the family level was also constructed using MG-RAST with Illumina sequencing data set. The RDP database was used as an annotation source, and a minimum identity cutoff (90%) was applied.

#### Sequence Read Archive (SRA) submission

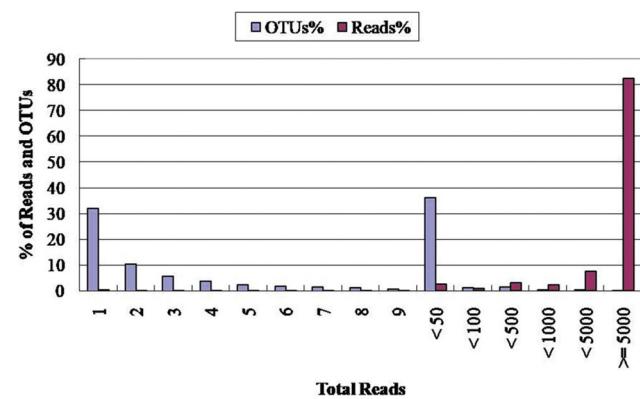
Metagenomic sequences were submitted to Sequence Read Archive (SRA) at <https://submit.ncbi.nlm.nih.gov/subs/sra/>. Experiment ID and Run ID were received for each submission.

## Results

#### Isolation and quality checking of metagenomic DNA from adult *B. tabaci*

Metagenomic DNA isolated from the two samples of *B. tabaci* were confirmed with the presence of 16S rDNA fragment in the isolated products by amplification of band of 1500 bp with universal 16S rDNA primers (Fig. S3<sup>1</sup>). The metagenomic DNA was quantified with fluorometer (Qubit 2.0) and the concentrations were 30.6 and 30.4 ng/ $\mu\text{L}$ , respectively, in samples H and P. The

**Fig. 1.** Graphical representation of reads and operational taxonomic unit (OTU) proportion. The blue bar represents the percentage of total OTUs in the read-count groups. The red bar represents the percentage of total reads contributed by the OTUs in the read-count group.



hypervariable V3 region of 16S rDNA was amplified with specific primers (Figs. S4a, S4b<sup>1</sup>) and preceded with 16S rDNA library preparations.

#### Illumina sequencing data

Total raw sequencing reads (paired end) of 1 321 906 and 690 661 with average sequence length of 150 bp was obtained from Illumina MiSeq™ sequencer. The quality of left and right end of the paired-end read sequences of the sample are shown in Figs. S5a, S5b, S6a, and S6b<sup>1</sup>. Nearly 90% of the total reads had phred score greater than 30 (>Q30; error-probability ≤ 0.001).

The base composition distribution of two samples was adenine (24.05%, 23.63%), cytosine (24.06%, 24.64%), guanine (27.46%, 27.78%), and thiamine (24.43%, 23.95%), and the average GC content was 40–50%. Application of multiple filters such as conserved region filter, spacer filter, quality filter, and mismatch filter resulted in 1 240 613, 1 240 116, 1 239 993, and 640 996 reads, respectively, for sample P. For sample H, the corresponding values were 640 923, 640 500, 640 450, and 341 937. While making consensus V3 sequence, more than 48% of the paired-end reads were aligned to each other with zero mismatches with an average contig length of 135–165 bp (Figs. S7a and S7b<sup>1</sup>).

From the 640 996 and 341 937 consensus reads from samples, singletons and chimeric sequences were removed to obtain 611 218 and 334 634 high quality pre-processed reads. These were pooled and clustered into OTUs based on their sequence similarity (similarity cut off = 0.97), and a total of 3513 OTUs were identified from 945 852 reads (Fig. 1).

The rarefaction analysis, carried out to verify the amount of sequencing reflected in the diversity of original microbial community, revealed that the slopes of the curves decline markedly with increasing sequences (Fig. 2). The alpha diversity (6.22 and 3.82 for the P and H populations, respectively) indicated the extent of bacterial species diversity present in *B. tabaci*.

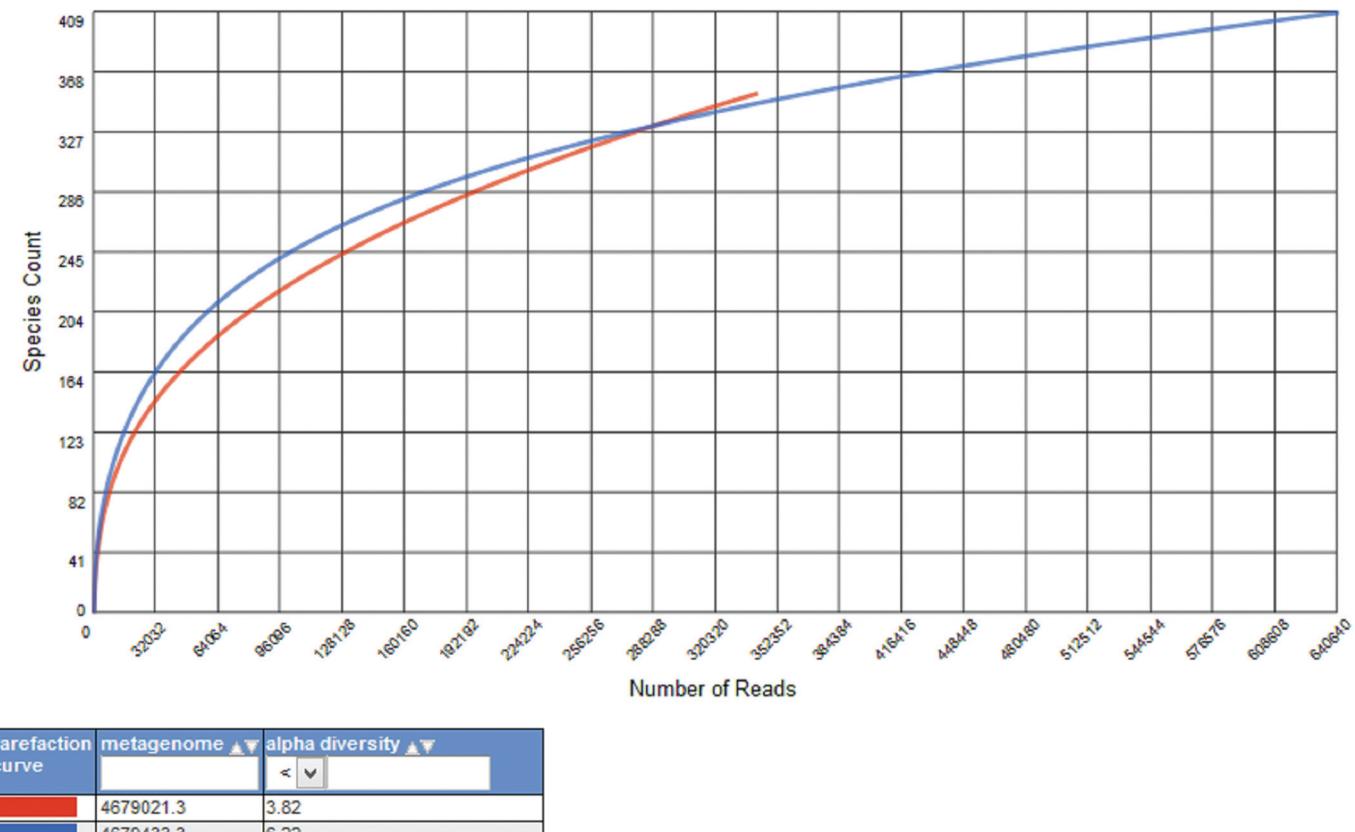
#### Composition of bacterial community of *B. tabaci*

The bacteria present in adult *B. tabaci* were analysed and taxonomically grouped from phyla to species levels using RDP classifier and Greengenes OTUs database. The relative abundance of the 10 major bacterial groups in each taxonomic category is given in Tables 1 and 2. Altogether, 16 bacterial phyla were detected from samples P and H. The most dominant phylum in the P population was Proteobacteria (87.5% of total bacterial community) and in the H population, it was Firmicutes (82.6%). This was followed by Firmicutes (9.3%) in the P population and Proteobacteria (13.4%) in the H population.

The P population had Bacteroidetes bacteria to the tune of 2.9%. Chlorobi, Actinobacteria, Planctomycetes, Verrucomicrobia, Spirochaetae, Tenericutes, and Acidobacteria were other phyla seen, constituting less than 1%, whereas the H population had only a meager count of Bacteroidetes, Chlorobi, Actinobacteria, Planctomycetes, Verrucomicrobia, Spirochaetae, Tenericutes, and Acidobacteria. A total of 27 and 31 bacterial classes were identified for the P and H populations, respectively. For sample P, class Gammaproteobacteria was most dominant (86.4%) and for H, it was Bacilli (82.6%). In population P, 56 bacterial orders have been detected of which Enterobacterales was dominant (85%). Of the 60 orders seen in population H, Bacillales was most dominant (82.5%). Analyses at the family level revealed a total of 91 and 88 bacterial families in the P and H populations, respectively, with major groups being Enterobacteriaceae, Bacillaceae, Flavobacteriaceae, Vibrionaceae, and Oxalobacteraceae for the P population and Enterobacteriaceae, Bacillaceae, and Alcanivoracaceae for the H population (Fig. 3). Among the 236 genera identified in the P sample, *Bacillus* (35.5%) and *Arsenophonus* (24.6%) were the most dominant. Other important genera in the P population were *Vibrio*, *Riemerella*, *Lysinibacillus*, *Flavobacterium*, *Janthinobacterium*, *Sphingobacterium*, *Bacteroides*, and *Enterococcus*. For the H population, the order of abundance was *Bacillus* followed by *Alcanivorax*, *Staphylococcus*, *Pantoea*, *Lysinibacillus*, *Bacteroides*, *Alistipes*, *Photorhabdus*, *Terribacillus*, and *Enterococcus*. A total of 409 species were identified in sample P and 355 in sample H (Fig. 2). From the RDP database, sequence similarities were observed with *Arsenophonus* spp., *Bacillus* spp., *Riemerella anatipestifer*, *Vibrio harveyi*, *Lysinibacillus sphaericus*, and *Janthinobacterium* spp. for the P population and *Bacillus thuringiensis*, *Staphylococcus* spp., *Bacillus amyloliquefaciens*, *Bacillus megaterium*, *Pantoea dispersa*, and *Bacillus pumilus* for the H population.

Comparison between different taxonomic categories of bacteria present in the P and H populations of *B. tabaci* using Jaccard dissimilarity index shows that at the species level, the bacteria present in both the P and H populations of *B. tabaci* showed a maximum dissimilarity of 99.9%, whereas the bacteria at the genus level recorded the least dissimilarity of 50.5%. At the phylum level, the bacteria showed a dissimilar of 84.9%, whereas 82.7% of

**Fig. 2.** Rarefaction analyses of *Bemisia tabaci* bacterial communities (P population: blue line; H population: red line).



dissimilarity at the class level was observed among bacteria population in the P and H populations of *B. tabaci*. The bacteria at the order and family levels were dissimilar at 88.8%–88.9%. Shannon–Weiner diversity index was used to assess the relative diversity of bacteria in both the populations at each taxonomic level (Table S4<sup>1</sup>). Higher index values had shown higher diversity of the bacterium. At the phylum, class, and order levels, both the populations were found to have equal levels of diversity, though they differed in four bacterial orders. At the family level, population H was distinctly more diverse, but at the genus level, population P showed better diversity. This shows that different genera found in population P belonged to the same family and even though the number of genera was less, bacteria in population H belonged to different family. Diversity index at the species level was high (>1.0) in both the populations, and the species accommodated in both populations varied.

#### SRA submission

SRA submission has generated the experiment ID SRX1592694 and run ID SRR3178391.

#### Discussion

##### Microbial community vary between our study populations

Polyphagous agricultural pests harbour diverse bacterial communities in their gut, which assist diverse functions including polyphagy and general fitness. In the

present study, bacterial communities associated with cassava whiteflies collected from different agro-ecological zones of Kerala, India, were compared. Metagenomic DNA of *B. tabaci* strains were isolated by standard protocol (Zhou et al. 1996) and sequenced in Illumina NGS platform. Analysis of the hypervariable V3 region of 16S rDNA fragment resulted in 1 321 906 and 690 661 high quality paired end sequences with mean length of 150 bp. The number of bacterial species detected was a function of the number of sequences analyzed (Shi et al. 2012). Highly diverse bacterial communities were present in the sample, containing approximately 3513 operational taxonomic units (OTUs). Studies by Chiel et al. (2007) and Gueguen et al. (2010) for identifying bacterial community of *B. tabaci* also used the amplification of 16S rDNA of bacteria. Parallel studies on *B. tabaci* from 14 different locations in northern India, using 16S rDNA clone library sequences, showed that *Portiera* is the primary endosymbiont and the secondary endosymbionts include *Cardinium*, *Wolbachia*, *Rickettsia*, and *Arsenophonus* along with *Bacillus*, *Enterobacter*, *Paracoccus*, and *Acinetobacter* (Singh et al. 2012), but in the present study *Portiera* was not identified, whereas secondary endosymbionts such as *Bacillus*, *Arsenophonus*, *Enterococcus*, and *Bacteroides*, were identified.

Downstream analysis using QIIME (Caporaso et al. 2010) and MG-RAST (Meyer et al. 2008) and statistical analysis

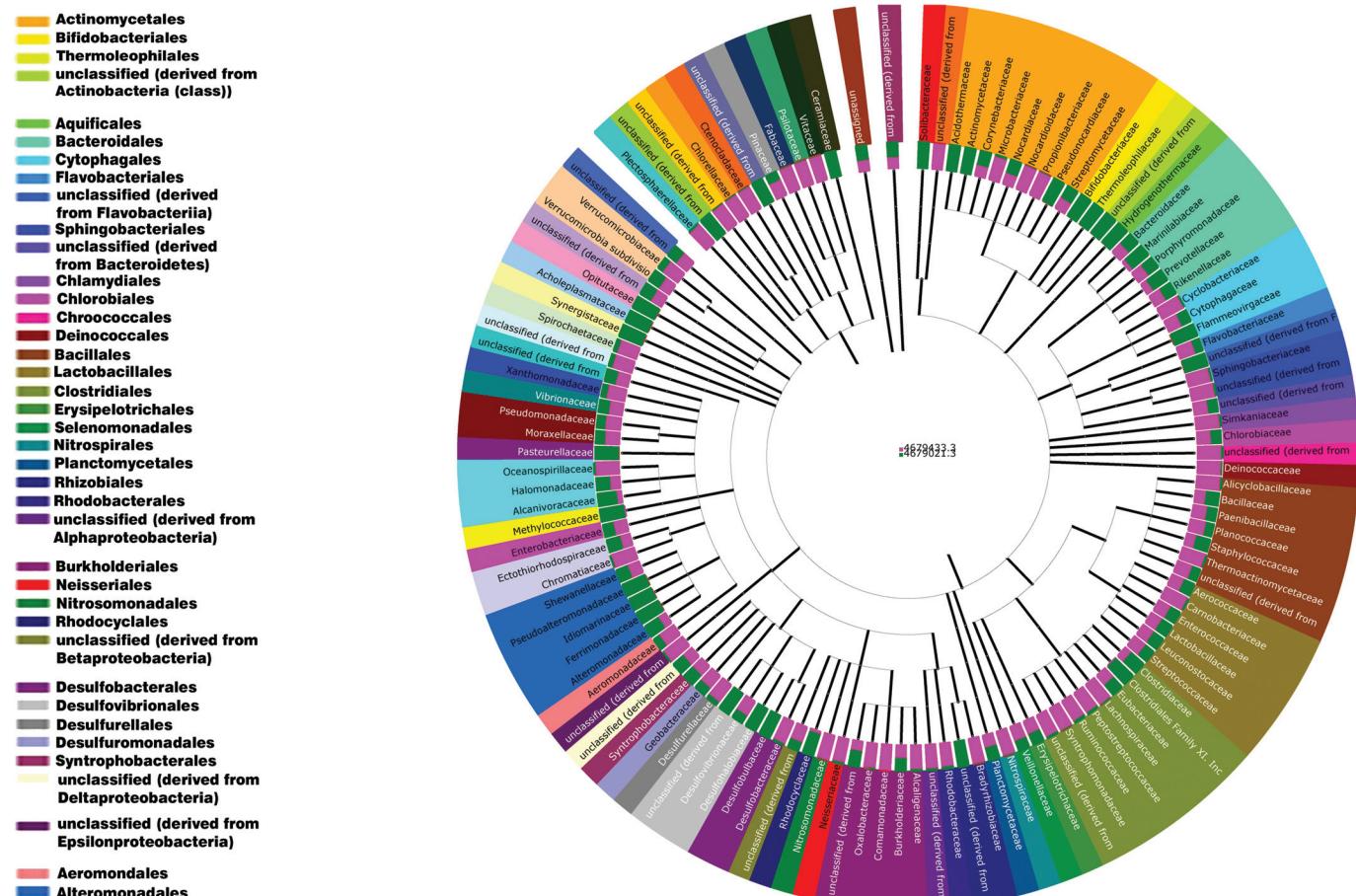
**Table 1.** Ten major bacteria in each taxonomic category, recorded from the P population. Values in parentheses are percent of particular taxonomic category of the bacteria compared to the total identified, when the pre-processed total reads were taken for downstream analyses.

Sl. No.	Phylum	Class	Order	Family	Genus	Species
1	Proteobacteria (87.57)	Gammaproteobacteria (86.47)	Enterobacteriales (85.00)	Enterobacteriaceae (85.01)	Bacillus (35.57)	Secondary endosymbiont of <i>Bemisia tabaci</i> [non-specified] (70.38)
2	Firmicutes (9.29)	Bacilli (9.14)	Bacillales (8.70)	Bacillaceae (8.25)	Arsenophonus (24.69)	Arsenophonus endosymbiont of <i>Bemisia tabaci</i> (7.19)
3	Bacteroidetes (2.91)	Flavobacteriia (1.70)	Flavobacteriales (1.71)	Flavobacteriaceae (1.71)	Vibrio (5.83)	<i>Bacillus cereus</i> (4.07)
4	Chlorobi (0.16)	Betaproteobacteriia (0.94)	Vibrionales (1.30)	Vibrionaceae (1.30)	Riemerella (4.53)	<i>Bacillus megaterium</i> (3.78)
5	Actinobacteria (0.02)	Bacteroidia (0.59)	Burkholderiales (0.69)	Oxalobacteraceae (0.65)	Lysinibacillus (4.20)	<i>Bacillus flexus</i> (1.70)
6	Planctomycetes (0.01)	Sphingobacteriia (0.59)	Bacteroidales (0.60)	Sphingobacteriaceae (0.58)	Flavobacterium (2.87)	<i>Riemerella anatipestifer</i> (1.32)
7	Verrucomicrobia (0.007)	Chlorobia (0.16)	Sphingobacteriales (0.59)	Bacteroidaceae (0.39)	Janthinobacterium (2.94)	<i>Vibrio harveyi</i> (1.30)
8	Spirochaetae (0.005)	Deltaproteobacteriia (0.16)	Lactobacillales (0.47)	Enterococcaceae (0.38)	Sphingobacterium (2.79)	<i>Lysinibacillus sphaericus</i> (1.22)
9	Tenericutes (0.004)	Negativicutes (0.15)	Pseudomonadales (0.38)	Pseudomonadaceae (0.37)	Bacteroides (1.90)	<i>Janthinobacterium</i> sp. J3 (0.86)
10	Acidobacteria (0.002)	Cytophagia (0.03)	Chlorobiales (0.17)	Staphylococcaceae (0.24)	Enterococcus (1.83)	<i>Bacillus pumilus</i> (0.69)

**Table 2.** Ten major bacteria in each taxonomic category, recorded from the H population. Values in parentheses are percent of particular taxonomic category of the bacteria compared to the total identified, when the pre-processed total reads were taken for downstream analyses.

Sl. No.	Phylum	Class	Order	Family	Genus	Species
1	Firmicutes (82.67)	Bacilli (82.65)	Bacillales (82.58)	Bacillaceae (77.42)	Bacillus (82.27)	<i>Bacillus thuringiensis</i> (72.62)
2	Proteobacteria (13.40)	Gammaproteobacteriia (16.28)	Enterobacteriales (8.34)	Enterobacteriaceae (8.34)	Alcanivorax (8.19)	Alcanivorax sp. EPR 6 (7.58)
3	Bacteroidetes (0.84)	Bacteroidia (0.71)	Oceanospirillales (7.62)	Alcanivoraceae (7.62)	Staphylococcus (5.58)	SBR proteobacterium (6.97)
4	Actinobacteria (0.07)	Flavobacteriia (0.12)	Bacteroidales (0.71)	Staphylococcaceae (5.19)	Pantoea (1.13)	<i>Staphylococcus pasteuri</i> (3.31)
5	Chlorobi (0.05)	Deltaproteobacteriia (0.09)	Vibrionales (0.23)	Bacteroidaceae (0.41)	Lysinibacillus (0.76)	<i>Bacillus amyloliquefaciens</i> (1.75)
6	Planctomycetes (0.04)	Actinobacteria (class) (0.05)	Lactobacillales (0.16)	Vibrionaceae (0.23)	Bacteroides (0.44)	<i>Staphylococcus sciuri</i> (1.63)
7	Verrucomicrobia (0.04)	Betaproteobacteriia (0.03)	Flavobacteriales (0.12)	Rikenellaceae (0.20)	Alistipes (0.22)	<i>Bacillus megaterium</i> (1.10)
8	Spirochaetae (0.02)	Chlorobia (0.03)	Actinomycetales (0.05)	Enterococcaceae (0.09)	Photorhabdus (0.21)	<i>Pantoea dispersa</i> (1.02)
9	Tenericutes (0.01)	Negativicutes (0.02)	Chlorobiales (0.03)	Prevotellaceae (0.07)	Terribacillus (0.16)	<i>Lysinibacillus sphaericus</i> (0.70)
10	Acidobacteria (0.01)	Cytophagia (0.01)	Pseudomonadales (0.03)	Paenibacillaceae (0.04)	Enterococcus (0.10)	<i>Bacillus pumilus</i> (0.34)

**Fig. 3.** Phylogenetic tree of bacteria at the family level constructed in MG-RAST with an Illumina sequencing data set. Tree is present with orders (colored slices), and families belonging to each orders are given inside colored slices. Magenta boxes inside the colored slice indicate the P population and green boxes indicate the H population. The RDP database was used as an annotation source, and a minimum identity cutoff (90%) was applied.



using Shannon–Weiner diversity index (Shannon and Weaver 1949) showed a marked difference in relative diversity of bacteria in the populations at various taxonomic levels. Altogether, 16 bacterial phyla were detected from the P and H samples. Among the phyla from the P population, Proteobacteria was most dominant followed by Firmicutes and Bacteroidetes and for the H population, it was Firmicutes, Proteobacteria, and Bacteroidetes. Su et al. (2016) identified 27 different phyla of bacterial community associated with *B. tabaci*, from different crops, in which Proteobacteria (94.0%–98.0%) was the most dominant, followed by Bacteroidetes (0.5%–4.5%) and Firmicutes (0.2%–2.0%), and the present study showed similar results.

#### Importance of endosymbionts for insect function

Bacterial endosymbionts are essential for survival, spread, and evolution of *B. tabaci* (Thao and Baumann 2004; Himler et al. 2011). Even though they are known to perform a variety of roles in whiteflies (Rana et al. 2012; Xie et al. 2018), functions of many of these endosymbionts remain still unknown. Detailed examination of their presence and functions in other insects with the

help of literature can provide an idea about their possible roles in whiteflies (Tables S2<sup>1</sup> and S3<sup>1</sup>). Proteobacteria associated with insects aid in carbohydrate degradation (Delalibera et al. 2005), synthesis of B vitamins and essential amino acids (Bennett et al. 2014), and pesticide detoxification (Werren 2012). Osei-Poku et al. (2012) and Jones et al. (2013) found that Proteobacteria is typically the predominant bacterial taxon in the gut of mosquitoes. Proteobacteria followed by Firmicutes and Actinobacteria were the major bacterial phyla detected in the midgut of *H. armigera* larvae (Priya et al. 2012), gut and reproductive organs of both the male and female fruit fly *Bactrocera minax*, and gut of ground beetles (Lundgren et al. 2007) and desert locust, *Schistocerca gregaria* (Dillon et al. 2010). However, Bacteroidetes and Firmicutes were dominant in the gut of termites (Xiang et al. 2012) and bees (Mohr and Tebbe 2006). Some members of Firmicutes assist insects in cellulose and hemicellulose digestion (Brown et al. 2012). Higher termites harbour Bacteroidetes, in their hindgut, to degrade lignocellulose, with the host enzymes acting on the amorphous regions of cellulose and the symbiotic enzymes targeting the crystalline re-

gions (Brune 2014). These bacteria also induce cytoplasmic incompatibility in the parasitoid wasp *Encarsia pergandiella* (Hunter et al. 2003).

In the present study on whiteflies, for both the P and H populations, reads for phyla Chlorobi, Actinobacteria, Planctomycetes, Verrucomicrobia, Spirochaetes, Tenericutes, and Acidobacteria were also seen. Chlorobi is a salivary-associated unique bacterial community in *Anopheles culicifacies* (Sharma et al. 2014), and their role in the insect is unknown. In insects, Actinobacteria exhibit diverse physiological and metabolic properties such as production of extracellular enzymes and formation of a wide variety of secondary metabolites (Schrempf 2001). In termites, they assist in nutrient acquisition from polysaccharides including cellulose (Pasti and Belli 1985; Watanabe et al. 2003) and hemicellulose (Schafer et al. 1996). According to Douglas (2015), Actinobacteria is the most dominant phylum of bacteria in whitefly, followed by Bacteroidetes, Firmicutes, and Proteobacteria. This variation can be attributed to the host variations.

The extreme alkalinity in some compartments of termite guts supports the growth of specialized alkaline-tolerant symbiotic bacteria from Planctomycetes (Köhler et al. 2008; Bignell 2010). Beetles and termites feeding on wood or detritus have higher populations of Verrucomicrobia in their gut (Colman et al. 2012), and in the hindgut of the wood-feeding termites, Spirochaetae are present in abundance (Köhler et al. 2012). The Tenericutes are present in termites and cockroaches also (Sabree and Moran 2014). Acidobacteria were identified from the larvae of the cerambycid *Leptura rubra* feeding on rotten softwood (Grünwald et al. 2010). Acidobacteria uses plant polymers, including xylan and cellulose, and degrade these polymers in the larval gut (Eichorst et al. 2011).

Using 16S rDNA clone library sequences, Singh et al. (2012) identified more than 300 bacterial genera from whiteflies, including secondary endosymbionts such as *Cardinium*, *Wolbachia*, *Rickettsia*, *Arsenophonus*, *Bacillus*, *Enterobacter*, *Paracoccus*, and *Acinetobacter*. Secondary endosymbionts were not uniformly distributed in different locations. In the present study, 236 and 225 bacterial genera were present in the P and H populations, respectively. For the P population, *Bacillus* was the most dominant group followed by *Arsenophonus*, *Vibrio*, *Riemerella*, *Lysinibacillus*, *Flavobacterium*, *Janthinobacterium*, *Sphingobacterium*, *Bacteroides*, and *Enterococcus* and for the H population, the order of relative abundance was *Bacillus*, *Alcanivorax*, *Staphylococcus*, *Pantoea*, *Lysinibacillus*, *Bacteroides*, *Alistipes*, *Photobacterium*, *Terribacillus*, and *Enterococcus*. At the species level, a total of 409 species were identified in sample P, and a total of 355 species were identified in sample H. Secondary endosymbiont of *Bemisia tabaci* (unspecified/unidentified by the software and identified as a single species group), *Arsenophonus*, *Bacillus cereus*, *Bacillus megaterium*, *Bacillus flexus*, *Riemerella anatipestifer*, *Vibrio harveyi*, *Lysinibacillus*

*sphaericus*, *Janthinobacterium* sp., and *Bacillus pumilus* were the 10 major species identified for the P population. For the H population, the major species identified were *Bacillus thuringiensis*, *Alcanivorax* sp., SBR proteobacterium, *Staphylococcus pasteuri*, *Bacillus amyloliquefaciens*, *Staphylococcus sciuri*, *Bacillus megaterium*, *Pantoea dispersa*, *Lysinibacillus sphaericus*, and *Bacillus pumilus*.

Many members of *Bacillus* are present in *B. tabaci* and may contribute in nutrition. According to Chandler et al. (2011), host diet has a greater effect on the bacterial microbiome composition. As *Bacillus* strains have the ability to produce amylase enzyme (Amund and Ogunsina 1987; Oyewole and Odunfa 1992), these amylases may be involved in the initial breakdown of cassava starch into simple sugars. *Bacillus megaterium* isolates were found to produce medium-length sugars from sucrose (Davidson et al. 1994). Also, *Bacillus* spp. associated with *B. tabaci* may produce long-chain sugars that contribute to the stickiness of the honeydew of the insect (Davidson et al. 1994).

Interestingly, *Bemisia* also harbours various entomopathogens such as *Bacillus thuringiensis* (Raymond et al. 2010; Walters and English 1995) and *Bacillus cereus* (Song et al. 2014), which are effective biocontrol agents for whiteflies (El-Assal et al. 2013). *Bacillus pumilus* is effective in reducing second nymphal instar populations of *B. tabaci* (Ateyyat et al. 2010) and entomopathogen *Bacillus megaterium* manages *Aphis pomi* (Aksoy and Ozman-Sullivan 2008). *Bacillus flexus* induces the oviposition of sand fly (*Phlebotomus papatasii*) (Mukhopadhyay et al. 2012), whereas *Bacillus amyloliquefaciens* has strong mosquito larvicidal and pupicidal action, and are used in mosquito control programmes (Geetha et al. 2014).

Endosymbiont, *Arsenophonus* is important in virus transmission by whiteflies (Rana et al. 2012) and are relatively abundant in the P population. GroEL molecular chaperones from *Arsenophonus* sp. are found to be associated with coat proteins of *Cassava mosaic virus* and help them from disintegration in the insect haemolymph. Similar results are reported by Morin et al. (1999) and Gottlieb et al. (2010) in the case of TYLCV (Tomato yellow leaf curl virus), for *Buchnera* GroEL and *Hamiltonella* GroEL, respectively. Since cassava plants from where the P population of whiteflies were collected had shown high severity (Scale 3–5) (Ikotun and Hahn 1994) of cassava mosaic disease, the results are in agreement with the findings of Rana et al. (2012). Our results indicate a possible association of whitefly endosymbiont, *Arsenophonus* with *Cassava mosaic virus* in its transmission. Compared to disease spread by the P population, cassava mosaic disease intensity was negligible (Scale 0–1) in Sulthan Bathery, where *Arsenophonus* was absent in the H population. *Arsenophonus* is also suspected to reduce the fecundity of its host (Ghera et al. 1991; Duron et al. 2008). Raina et al. (2015) observed that the elimination of *Arsenophonus* and

decrease in the diversity of bacterial symbionts by antibiotic treatment leads to increase in fitness of whiteflies.

Luciferases from luminous bacteria, *Vibrio harveyii*, and the presence of *Riemerella* in ant species, *Nylanderia fulva*, were reported by Schmidt et al. (1989) and McDonald (2012), respectively. Santos-Garcia et al. (2014) reported symbiotic association of *Alcanivorax* in moss bugs to fulfil their nutritional requirements, resulting from their unbalanced diet, and their role in marine oil-spill degradation is reported by McGenity et al. (2012). Apart from whitefly management, *Lysinibacillus sphaericus* (El-Assal et al. 2013) can also be used as a biological control agent for insecticide-resistant *Aedes aegypti* (Rojas-Pinzón and Dussán 2017). *Staphylococcus* from *Bemisia* produces medium-length sugars from sucrose and contributes to the stickiness of the honeydew secreted by the host insect (Indiragandhi et al. 2010). McDonald (2012) reported the presence of *Staphylococcus pasteuri* and *Staphylococcus sciuri* from the ant species *Nylanderia fulva* and Ateyyat et al. (2010) reported their potential as biocontrol agents.

Rosenblueth et al. (2012) reported evolutionary relationships of flavobacterial endosymbionts with their scale insect hosts. The endosymbiont *Pantoea* observed in the study may perform semiochemical effects, as it is already reported for *Pantoea agglomerans*, which produces a chemical Guaiacol and helps in the aggregation of desert locust, *Locusta migratoria* (Dillon and Charnley 2002; Davis et al. 2013). *Pantoea dispersa* is reported in the wild mosquito *Aedes albopictus* (Moro et al. 2013). Evidence for the microbial utilization of nitrogenous waste products by *Bacteroides* has been obtained for termites, cockroaches, and hemipterans (Potrikus and Breznak 1981). *Janthinobacterium* strains have been reported to have the capacity to degrade chitin (Gleave et al. 1995; Xiao et al. 2005), and *Janthinobacterium* sp. J3 has been isolated from the gut contents of *Batocera horsfieldi* larvae (Zhang et al. 2011).

*Sphingobacterium griseoflavum* sp. nov. isolated from the insect *Teleogryllus occipitalis* living in deserted crop land (Long et al. 2016) and *Sphingobacterium* isolate exhibiting xylanolytic activity has been isolated from the gut of a cerambycid larva (Zhou et al. 2009). *Alistipes finegoldii* and *Alistipes putredinis* are reported in the gut of medicinal leech (*Hirudo verbena*) (Maltz et al. 2014) and *Alistipes finegoldii* attenuates colitis in mice (Dziarski et al. 2016). *Photorhabdus luminescens* is a bioluminescent entomopathogen that comes under the genus *Photorhabdus*, reported in the study (Schmidt et al. 1989).

An et al. (2007) reported the presence of *Terribacillus halophilus* in various insects, even though their role is unknown. *Enterococcus* sp. found in *Bemisia* was reported to produce cyanide oxygenase and utilize cyanide as a nitrogenous growth substance (Fernandez and Kunz 2005). According to Bressan et al. (2008), SBR proteobacterium is a pathogen associated with the disease syndrome “basses richesses” of sugar beet in France and are

spread by planthoppers — *Cixius wagneri*, *Hyalesthes obsoletus*, and *Pentastiridius leporinus*.

### Concluding remarks

Our study revealed the composition and diversity of the bacterial community associated with *B. tabaci* based on Illumina next-generation sequencing of 16S rDNA amplicons. The study was not extrapolated to know the correlation of endosymbiont bacterium and genetic variability in whitefly, as the study conducted by Singh et al. (2012) already ruled out any such possibility. Mining the diversity of the bacterial community present in the insects has revealed their role in making *B. tabaci* a successful vector and polyphagous pest of global importance. Our analysis has shown that the specific endosymbiont *Asenophonus* is present only in the heavily cassava mosaic disease infested areas. Insecticidal toxin producing opportunistic bacteria such as *Bacillus thuringiensis* and *Bacillus cereus* were also found in *B. tabaci*. Further studies from more regions and detailed analyses are required to determine the trend in endosymbiont variations based on genetic as well as agro-ecological zone variations. Functional roles of endosymbionts in making *B. tabaci* as a successful vector and invasive plant pest have to be thoroughly studied. Elaborated understanding on endosymbionts could very well be utilized not only for planning alternative pest management strategies but also for enhancing efficiency of beneficial insects.

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