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RESEARCH ARTICLE

Infestation of Rice by Gall Midge Influences Density and Diversity of *Pseudomonas* and *Wolbachia* in the Host Plant Microbiome

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Abstract: ***Background***: The virulence of phytophagous insects is predominantly determined by their ability to evade or suppress host defense for their survival. The rice gall midge (GM, *Orseolia oryzae*), a monophagous pest of rice, elicits a host defense similar to the one elicited upon pathogen attack. This could be due to the GM feeding behaviour, wherein the GM endosymbionts are transferred to the host plant *via* oral secretions, and as a result, the host mounts an appropriate defense response(s) (*i.e.*, up-regulation of the salicylic acid pathway) against these endosymbionts.

Methods: The current study aimed to analyze the microbiome present at the feeding site of GM maggots to determine the exchange of bacterial species between GM and its host and to elucidate their role in rice-GM interaction using a next-generation sequencing approach.

Results: Our results revealed differential representation of the phylum Proteobacteria in the GM-infested and -uninfested rice tissues. Furthermore, analysis of the species diversity of *Pseudomonas* and *Wolbachia* supergroups at the feeding sites indicated the exchange of bacterial species between GM and its host upon infestation.

Conclusion: As rice-GM microbial associations remain relatively unstudied, these findings not only add to our current understanding of microbe-assisted insect-plant interactions but also provide valuable insights into how these bacteria drive insect-plant coevolution. Moreover, to the best of our knowledge, this is the first report analyzing the microbiome of a host plant (rice) at the feeding site of its insect pest (GM).

Keywords: *Orseolia oryzae,* plant-insect interaction, rice microbiome, insect microbiome, Proteobacteria*, 16S rRNA, ftsZ.*

1. INTRODUCTION

The Asian rice gall midge (GM; *Orseolia oryzae*; Diptera; Cecidomyiidae) is one of the major pests of rice. Infestation by GM at the vegetative stage makes the rice plant sterile, thereby causing severe economic loss to the farmers. Like other members of Cecidomyiidae, GM's life cycle consists of three instars as maggots prior to pupation. The maggot stage lasts for 8-10 days, after which it enters the pupal stage, which lasts for 10-12 days. GM pupa is highly active as it crawls through the elongated gall cavity and drills an exit hole at the apex, protruding out to facilitate eclosion followed by adult emergence [1]. In the life cycle of the GM, the maggot stage is the only feeding stage. During this stage, it feeds actively on the dividing meristematic region and, in the process, damages the rice plant tissues. The interaction of GM with its host (rice) can be categorized as compatible or incompatible. A compatible GM-rice interaction induces the formation of a leaf sheath gall, *i.e.*, a tube-like outgrowth or silver shoot, inside which the insect feeds and completes a major part of its life cycle [2, 3]. Such a gall makes the tiller unproductive and causes a huge reduction in the crop yield. In a compatible gall midge-rice interaction, the GM salivary gland secretions enter the rice plant, enabling continuous feeding while suppressing the rice defense [3-5]. In contrast, in an incompatible interaction, although the gall midge maggots initiate feeding on the resistant plant, they die within 96 h. It is usually characterized by a hypersensitive reaction (HR) and maggot mortality at the feeding site [6], a common phenotypic feature studied extensively in plant-pathogen interactions [7].

Although GM can initially infest all rice varieties, defense mechanisms triggered in the resistant plants prevent the GM larvae from continued feeding, eventually leading to the death of the maggots. Our earlier work on rice-gall

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midge interaction has revealed mechanisms underlying rice defense [1, 6] and counter defense of GM [6]. One of the studies on comparative metabolomics and transcriptomics of rice varieties revealed certain compounds specifically present in the resistant or susceptible rice upon gall midge attack [8]. These have been classified as resistance and susceptibility features in a broader sense. In addition, expression profiling of GM-infested resistant rice plants revealed various defense-related genes that are upregulated upon GM infestation [9]. Besides, several gall midge resistance (R) genes have been mapped and characterized from the resistant rice varieties, conferring resistance against various GM biotypes [10]. However, none of the identified R genes provides resistance to all the GM biotypes, and none of the GM biotypes exhibits virulence against all the known R genes [1, 10]. Therefore, several rice lines pyramided with a combination of resistance genes have been developed to achieve broad-spectrum resistance against GM biotypes [11]. So far, seven distinct GM biotypes have been characterized from different parts of India [12]. Of these, GM biotype 4M is considered to be the most virulent one as only three of the known R genes, namely, *Gm3*, *Gm4,* and *Gm8,* confer resistance against this biotype [13].

Resistance of rice to gall midge is primarily due to antibiosis and is specific to the rice variety and GM genotypes [1]. Generally, plant defense responses against insect herbivores are distinct from those observed against plant pathogens [14]. However, the results of our previous investigations regarding rice-GM interaction indicated that the responses of rice to GM attack were very similar to those observed in rice upon pathogen attack [15]. In addition, previous reports have indicated that pathogenesis-related (PR) proteins, considered biomarkers for a pathogen attack, were upregulated in a hypersensitive-mediated resistance response in rice upon GM attack [16]. Furthermore, differential regulation of the genes (NBS-LRR, cytochrome P450, heat shock proteins, phenylalanine ammonia-lyase, and *OsPR10α*) commonly altered in pathogen infection was observed in a transcriptomic study carried out using suppressive subtraction hybridization technique [16].

One plausible explanation for these observations is the likely transmission of the GM gut microbes to the rice plant during the feeding process, which induces the host into perceiving the GM infestation as a pathogen attack and consequently triggers the synthesis of various pathogen-specific PR proteins. Furthermore, as it is well established that the insect's gut microbiome affects their insect host [17], the microbiome may be involved in mediating rice-GM interaction and facilitating GM's survival in the rice host.

The transfer of intracellular insect symbionts, such as *Wolbachia*, to the host plant during feeding has been experimentally demonstrated across various plant-insect models. However, the route of bacterial transmission to the host plant depends on the transmitting insect's anatomy, physiology, and feeding mechanism [18]. Plants defend themselves against microbial pathogens and herbivorous insects through the salicylic acid (SA) and jasmonic acid (JA) pathways, respectively [19, 20]. Induction of the JA pathway leads to the production of repellent, antinutritive, or toxic compounds as an attempt to deter herbivory [21]. However, the SA pathway triggered against a pathogen attack involves oxidative bursts, callose deposition, ethylene production, and induction of defensive genes, including antimicrobial peptides [22]. Interestingly, both pathways are antagonistic to each other [20], thereby imposing a trade-off between defense against herbivore attack and bacterial challenge. Hence, by facilitating the symbiont transfer, arthropods can successfully evade host defenses and infest host plants. Considering this, we speculate that GM also manipulates the defense responses induced in rice against the invading maggots, quite similar to what has been previously reported for the Colorado potato beetle and tomato interaction [23]. However, to ascertain the occurrence of such a phenomenon, it is crucial to first establish the transfer of microbes from GM to rice upon infestation.

It is reported that the Gram-negative bacteria suppress the jasmonic acid (JA)-defense response (against the invading phytophagous insects) *via* activation of the salicylic acid (SA) pathway, as has been previously shown for various insect-plant interactions [24]. One of our earlier studies, which is in congruence with these reports and involved an investigation of the GM microbiome, revealed an overabundance of Gram-negative bacteria (particularly those belonging to the phylum Proteobacteria) [25], thereby hinting toward its role in mediating rice-gall midge interaction. Besides, as several complementary and independent studies described above indicate similarities between the response of rice to pathogen and GM attack, therefore, we deemed it pertinent to investigate the relationship between the bacterial communities present in the gall midge, gall midge infestation site, and their likely role in the initiation of rice defense response.

Amongst the overabundant Gram-negative bacteria found in the microbiome of the GM, *Pseudomonas* and *Wolbachia* are two dominant species [25]. *Wolbachia* is a maternally-inherited endosymbiotic bacteria present in the cytoplasm of germ cells and is widespread in arthropods. This bacterium has been well-studied for its role in influencing insect population dynamics and manipulating insect reproduction [26-28]. On the other hand, *Pseudomonas* is a bacterial genus with great metabolic versatility, inhabiting many different environments and hosts with beneficial or pathogenic interactions, especially with insects [29, 30]. The interaction between *Pseudomonas* and its insect host has been reported in several studies where it has been shown that insects likely acquired abilities to survive and compete in their niches due to the bacterial metabolism [31].

Therefore, we investigated whether any exchange of *Pseudomonas* and *Wolbachia* occurs between the rice host and GM. While *Wolbachia* (maternally-inherited bacteria) is over-represented in the pupal and adult stages, *Pseudomonas* (a facultative and environmentally-acquired microbe) is abundantly present in maggots [25]. As GM maggots are confined to rice tissues of growing points of apical or lateral buds, we speculated the host to be the only possible source for acquiring *Pseudomonas*. Hence, we also focused on assessing species diversity of *Pseudomonas* and *Wolbachia* (up to Supergroup level) in GM-infested and -uninfested rice tissues to demonstrate the transfer of these bacteria between host and insect. Taken together, we believe that the

current investigation is a step towards deciphering the rice-GM-microbial associations and is based on the hypothesis that the gall midge microbiome influences rice defense against GM, an aspect that remains largely unexplored.

2. MATERIALS AND METHODS

2.1. Plant Tissue Sampling

Gall midge biotype 1 (GMB1) culture was maintained under standard greenhouse conditions (on TN1 plants grown on puddled soil with temperatures ranging from 27±3°C and ~70% relative humidity) at the Indian Institute of Rice Research (ICAR-IIRR) (formerly Directorate of Rice Research), Hyderabad, India. Infestation by GMB1 insects of 15-day-old seedlings of TN1 rice variety (susceptible to GMB1; compatible interaction) was performed by insect release method as described previously [13]. A tray of TN1 seedlings of the same age was maintained as control. 48 h after insect release, both the trays with infested and uninfested plants were transferred to a high humidity chamber (>90% RH) for egg incubation and maggot establishment (for details, see Sardesai *et al.* [2]). Post gall formation on the plants subjected to insect infestation, the plant tissues (1 cm of the shoot from the root-stem interface) were dissected under a microscope to remove maggots present within the gall chamber (gall tissue-GT). The tissues were stored at -80°C for further processing. Tissue samples from comparable regions (as sampled for the infested rice plants) were also collected from un-infested TN1 plants (control tissue-CT). The infestation and dissection procedures followed were as mentioned previously [8]. In addition, a batch of TN1 plants was also grown on autoclaved (sterile) vermiculite in a BOD chamber (maintained at 28°C with 16 h light and 8 h dark photoperiod) at ICGEB, New Delhi.

2.2. Genomic DNA Extraction and Quality Control

DNA was isolated from dissected tissues, obtained from five rice seedlings for both gall (GT) and control tissue (CT) samples using the QIAamp DNA Mini kit (Qiagen, USA), and quality control checks were performed using the PicoGreen (Invitrogen, USA) method on a Qubit 3.0 fluorometer (Invitrogen, USA). Thereafter, genomic DNA pools were prepared for GT and CT samples in equal amounts for library preparation and sequencing. The quality of the pooled DNA samples was assessed by gel electrophoresis on 0.8% TBE agarose gel [32].

2.3. Library Construction and Illumina-MiSeq Sequencing

The V3-V4 hypervariable region of the 16S rRNA gene was PCR amplified using 25ng of the total genomic DNA (obtained from the gall (GT) and control tissue (CT)) as a template. The most promising primer pair (V3-341F 5′-CCTACGGGNGGCWGCAG-3′ and V4-805R 5′-GACTACHVGGGTATCTAATCC-3′) for microbiome profiling, as suggested by Klindworth *et al.* [33], was used for amplification. A total of 25µl of PCR reaction contained 200µM dNTPs, 0.5U Taq DNA polymerase (Bangalore Genei, (India) Pvt. Ltd.), and 13µM of each primer. The PCR cycling conditions were 95°C for 2 min, 25 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension of 72°C for 5 min. The amplified fragments were approximately 460bp, and these were gel purified using the QIAquick Gel Extraction kit (Qiagen, Germany). The gel-purified DNA was further quantified using the NanoVue Plus spectrophotometer (GE Healthcare, UK). These PCR samples were sent for library preparation and sequencing to M/s Macrogen Inc. South Korea. Standard Illumina workflow for 16S Metagenomic sequencing library preparation (Illumina 16S sample preparation guide ver # 15044223 Rev. B) was followed. In brief, 2ng DNA from each sample was used to re-amplify the V3-V4 region with primers possessing a tag sequence complementary to the adapter and index primers of XT Index kit V2 (Illumina, Cat # FC-131-2002). Furthermore, a quality control check of the library was performed using a Bioanalyzer Chip. For size verification, amplicons were run on the Bioanalyzer Chip. Next, dual indices and Illumina sequencing adapters were further attached to the amplicons using the Nextera XT Index Kit (New England Biolabs, USA) for the final library preparation. Afterward, the library quality check was carried out using Bioanalyzer (Agilent Technologies, USA) before loading onto the MiSeq flow cell for sequencing on the Illumina MiSeq platform (Illumina, San Diego, CA) using 300 paired-end chemistry.

2.4. Generation of Raw Data, Pre-processing, and Clustering

Real-Time Analysis v1.18, an integrated analysis tool in Illumina MiSeq control software v2.2, was used to perform the base calling and quality scoring. Furthermore, the conversion of the base call binary into FASTQ was performed by Illumina package bcl2fastq conversion software (v2.8.4). Following adapter trimming, the sequences were assembled using the FLASH tool [34]. rDnaTools software and CD-HIT-OTU ([35]; http://weizhongli-lab.org/cd-hit-otu/) were employed for pre-processing and clustering of the generated raw reads. Reads shorter than 100bp were filtered out, and A-tails were trimmed. Homology-based clustering was performed at 100% cut-off. Chimeric reads were identified and removed. Non-chimeric reads were clustered using the average neighbour algorithm into OTUs with a 97% cut-off at the species level.

2.5. Taxonomic Assignment, Diversity Estimation, and Statistical Analyses

QIIME ([36]; www.qiime.org) was used to study the diversity and taxonomy of the identified OTUs. Taxonomic classification was carried out using QIIME-UCLUST software, where the taxonomic units were assigned to their representative sequences from each operational taxonomic unit (OTU) using the RDP-16S rDNA database (http://rdp.cme.msu.edu/). Next, the rarefaction analysis was performed to evaluate the species richness in each sample and calculate the community diversity index (Shannon and Simpson's diversity index, Chao1;[37]) for estimating the differences in microbial communities between CT and GT samples. The rarefaction curves and diversity indices were also plotted using QIIME. Furthermore, the aligned and filtered sequences of OTUs were used to construct a phylogenetic tree using UPGMA, and finally, OTU heatmaps were created using QIIME.

2.6. PCR Amplification and Cloning of *Wolbachia*- and *Pseudomonas*-specific *16S rRNA* Fragments from the GM-infested Rice Samples

PCR was performed (in duplicate) using 25ng of the pooled genomic DNA isolated from the plant tissues. For amplification of the *Wolbachia*-specific *16S rRNA* fragment, the primers used were Wol-F 5'-TTGTAGCCTGCTATGG TATAACT-3', Wol-R 5'-GAATAGGTATGATTTTCATGT-3' [38], and the PCR amplification profile used consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30s, 55°C for 1 min and 72°C for 1 min, and a final extension of 72°C for 5 min. To amplify the *16S rRNA* fragment from *Pseudomonas*, the primers used were Pseudo-S2-F 5'-GACGGGTGAGTAATGCCTA-3', Pseudo-S2-R 5'-CACTGGTGTTCCTTCCTATA-3' [39], and the PCR amplification profile consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30s, 58°C for 1 min, and 72°C for 45s, and a final extension of 72°C for 5 min. The PCR amplified products were run on 0.8% agarose gel. The constituents of the PCR mix were as described before.

The PCR amplified fragment of the appropriate size was cut and eluted from the gel using a nucleic acid extraction kit (Vivantis GF-1, Malaysia). The eluted product was thereafter cloned into a pCR4–TOPO vector using TOPO TA Cloning Kit (Invitrogen, USA). The cloned fragments were transformed into *E. coli* (DH5α) competent cells, which were then plated onto LB + antibiotics (Kanamycin; 50µg/ml) plates for the selection of transformants. Plasmid DNA was isolated from the positive clones (5 to 10) using QIAprep Spin Miniprep Kit (Qiagen, USA) and subsequently sent to M/s Macrogen Inc. (Seoul, Korea) for sequencing. The chromatograms obtained for each sequencing reaction were evaluated using the MacVector suite of Sequence Analysis Programmes (MacVector Inc., USA; version 15.5).

2.7. Cross-verification and Identification of *Wolbachia*-Supergroups in the GM-infested Rice Samples using *ftsZ* Gene-specific Primers

To re-verify and define *Wolbachia*-Supergroups present in the rice samples, *ftsZ* gene-specific universal primers were used [40]. The primers were ftsZ(uni)F 5'-GG(C/T)AA(A/G)GGTGC(A/G)GCAGAAGA–3' and ftsZ (uni)R 5'-ATC(A/G)AT(A/G)CCAGTTGCAAG–3' and the PCR amplification profile consisted of an initial denaturation at 94°C for 2 min followed by 30 cycles at 94°C for 30s, 55°C for 30s, and 72°C for 2 min with a final extension of 72°C for 5 min.

The PCR amplified fragment of the expected size was gel-purified and cloned into a pCR4–TOPO vector. Furthermore, transformation, screening for positive clones, and plasmid DNA isolation were carried out as described earlier and sequenced by M/s Macrogen Inc. The raw sequences were analyzed using the MacVector suite of sequence analysis programs.

3. RESULTS AND DISCUSSION

This study compared the microbiome of GM-infested gall tissue (GT) with that of un-infested control tissue (CT) of rice seedlings using NGS (next-generation sequencing) protocols. More than 2,00,000 reads were obtained for each sample. Processed reads post clustering (cut off: 97%) were more than 40,000 for both the samples and these were utilized for further analysis (Table **1**). Subsequent annotation of processed sequences revealed major representation from the phylum Cyanobacteria, which accounted for more than 50% of the counts in each sample. This was followed by the phylum Actinobacteria. However, Proteobacteria with the third-highest representation in the samples showed a differential presence between gall tissue (GT) and control tissue (CT) samples (Fig. **1**). Moreover, this study also provided insights into the microbial diversity of both CT and GT samples. The diversity of bacterial species within CT and GT samples was measured using species richness estimator (Chao1) and diversity indices (Shannon and Simpson). The species richness values were estimated to be 62.5 and 74 for CT and GT samples, respectively. Furthermore, the Shannon and Simpson's diversity index values reflecting the proportion of species abundance (evenness) within samples differed between CT and GT samples (Table **2**). Collectively, it was observed that the microbial diversity in CT samples was less than in the GT samples, as indicated by both Shannon and Simpson's indices [41, 42] (Fig. **2**, Table **2**). In addition, the rarefaction curve analysis (Fig. **3**) confirmed that the total number of reads used to derive this inference was sufficient to identify species or operational taxonomic units (OTUs).

Furthermore, owing to a significantly higher representation of bacteria belonging to the phylum Proteobacteria in the gall tissue, it became pertinent to identify the different members of this group present at the feeding site of the GM

Table 1. Read and quality statistics of the sequencing data obtained for CT and GT samples (using Illumina MiSeq paired-end chemistry) after adapter trimming and assembly.

Sample	Total Bases[1]	Total Reads[2]	GC (%)[3]	N (%)[4]	Q20 (%)[5]	Q30 (%)[6]
CT	95,287,686	215,445	54.87	0.0253	96.51	89.1
GT	102,840,496	231,895	54.68	0.0252	96.57	89.3

[1]: Total number of unambiguous bases identified in the reads.

[2]: Number of assembled reads.

[3]: Percent GC content of reads.

[4]: Percentage of ambiguous bases.

[5]: Percentage of bases with phred score >20.

[6]: Percentage of bases with phred score >30.

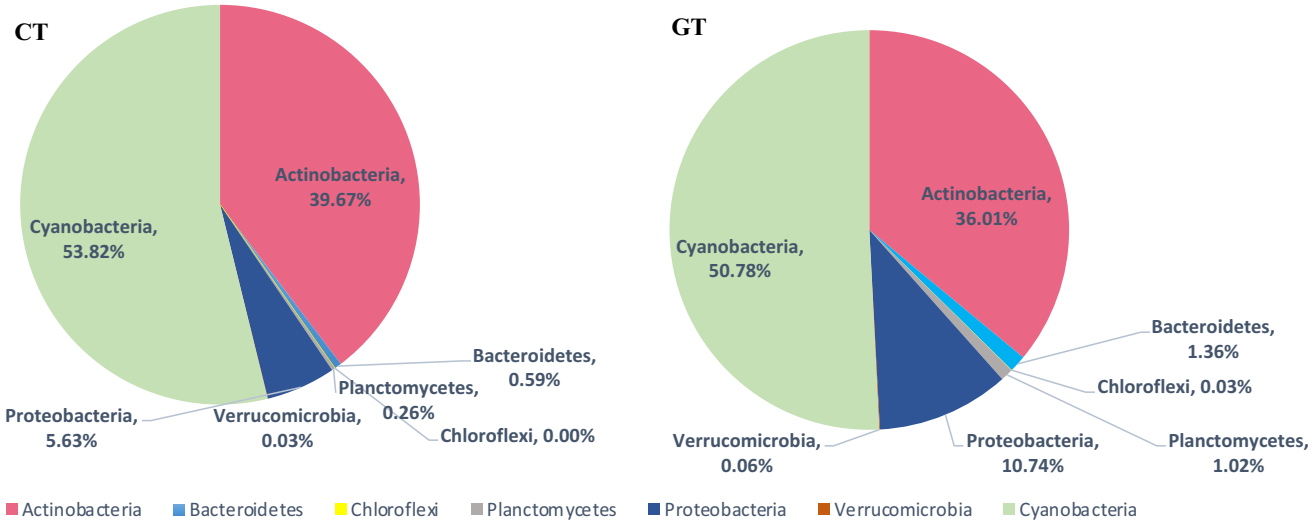

Fig. (1). Differential representation of identified OTUs from rice tissue infested by gall midge (GT) and uninfested rice tissue (CT). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 2. Diversity (Shannon and Simpson) and species richness (Chao1) indices for CT and GT samples as determined using QIIME.

Sample	OTUs[1]	Chao1[2]	Shannon[3]	Simpson[4]	Good's Coverage[5]
CT	60	62.5	1.4416	0.5517	0.9998
GT	74	74	1.844	0.6079	0.9999

[1]: Cluster of similar sequence variants of the 16S rDNA marker gene sequence.

[2]: Chao1 estimates the species richness of a sample.

[3]: Shannon index takes into account the number and evenness of species.

[4]: Simpson index represents the likelihood of two randomly selected individuals in the habitat belonging to the same species.

[5]: Good's coverage (C) is calculated as C=1-(s/n), where s is the number of unique OTUs and n is the number of individuals in the sample.

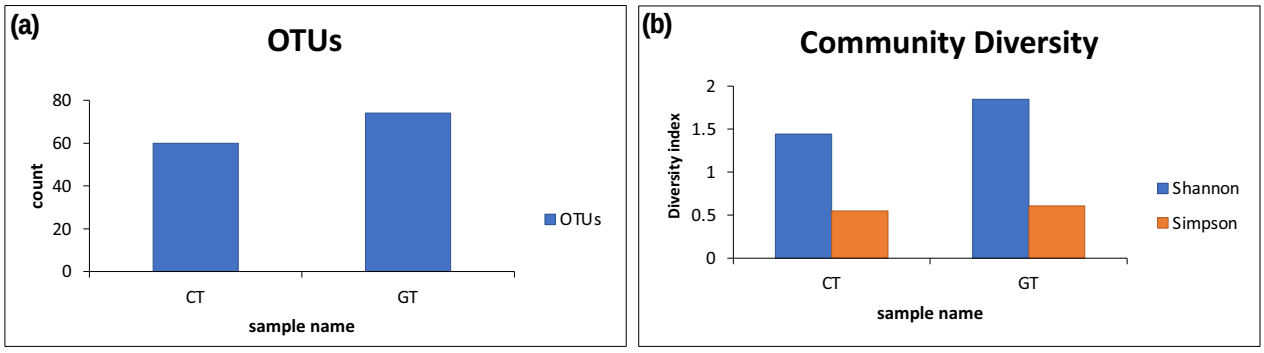

Fig. (2). (**a**) Species richness (Chao1) and (**b**) the diversity indices (Shannon and Simpson) as determined for the CT (control tissue) and GT (gall tissue) samples. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

maggots for a better understanding of their role, if any, in mediating the rice-gall midge interaction. In this regard, the current study focused on assessing the diversity of *Pseudomonas* and *Wolbachia* at the gall midge feeding site. These bacteria were not only present (in high abundance) in the GM, as reported earlier [25], but were also detected in both GT and CT samples during the current investigation. Though we did not observe any significant difference in titres of *Pseudomonas* between GT and CT samples, the density of *Wolbachia* increased by >2% in the GT samples compared to CT (see Supplementary Table **1**, highlighted rows 30 and 51). However, while post infestation by GM, we did observe a change in the density of *Wolbachia* in rice (GT). Therefore, future studies aimed at investigating whether an increase of >2% can have significant biological effects on rice-GM interaction are warranted. Furthermore, a comparison of the GM microbiome with that obtained for the rice host (GT) revealed similarities between the two da-

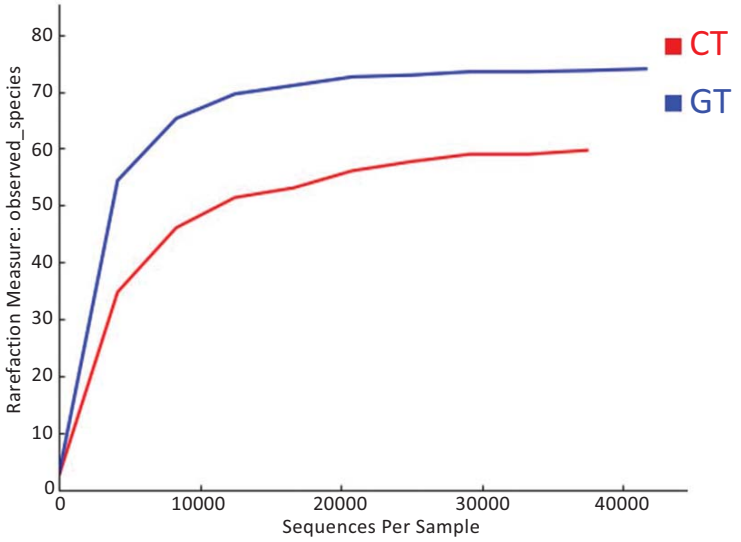

Fig. (3). Rarefaction curve analyses for the observed microbial OTUs in rice tissue samples CT and GT. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

tasets, thereby suggesting the possibility of the exchange of microbes between GM and rice.

In addition, the occurrence of *Wolbachia* in both GT and CT tissues and its absence in TN1 plants grown on sterile vermiculite (Fig. **4a**) also point towards the likely transmission of *Wolbachia* from GM to rice during the feeding process. However, while GM feeding can explain the occurrence of *Wolbachia* in GT, it cannot explain its presence in CT (uninfested rice plants), thereby implying that CT plants likely acquired these bacteria through another route. We believe that as the CT plants were grown in puddled soil, the source of *Wolbachia* in CT plants could be the arthropods present in the soil [43]. However, to test this hypothesis, it was pertinent to differentiate the *Wolbachia* present in GM from the one taken up from the soil.

With this view and to further establish the exchange of bacterial species between GM and rice, we assigned the *Wolbachia* present in CT and GT tissues to Supergroups based on nucleotide variations in the *ftsZ* fragments obtained for CT (GenBank accession numbers MW091359-MW091363) and GT samples (GenBank accession numbers MW091364-MW091378). Interestingly, our results indicated the presence of *Wolbachia* Supergroup B in CT and both Supergroup A and Supergroup B in the GT samples. Moreover, an earlier report [18], indicating the presence of both Supergroup A and Supergroup B *Wolbachia* in GM, further corroborated that both GM and GT are similar in this aspect. Additionally, the occurrence of two major phylogenetic subdivisions (A and B) of *Wolbachia* has already been reported in arthropods [44]. Taken together, these results indicated that while the source of *Wolbachia* belonging to Supergroup B in both CT and GT tissues could be soil, the presence of *Wolbachia* belonging to Supergroup A in GT samples is the likely consequence of GM infestation.

Moreover, the absence of a PCR-amplifiable product (using two different *Wolbachia*-specific primer pairs, targeting *ftsZ* and *16S rRNA*) in TN1 plants grown in sterile vermiculite (Fig. **4a** and **b**) implied that rice plants do not harbour *Wolbachia* but acquire them through GM infestation (Supergroup A) or from soil (Supergroup B). In this regard, earlier studies have already shown that intracellular symbiotic bacteria, such as *Rickettsia, Wolbachia,* and *Cardinium*, can be transmitted from herbivorous insects to plants, get localized within the plant tissues, and infect or contaminate the naïve insects feeding on these plants [45-48].

In addition to *Wolbachia*, the current study also focussed on analysing the diversity of *Pseudomonas* species in CT, GT, and TN1 plant samples with the view to determine the exchange of bacterial species between GM and rice. We chose *Pseudomonas* based on the results of our previous studies, where a differential representation of *Wolbachia* and *Pseudomonas* in GM was observed at different stages of its lifecycle [25]. While *Wolbachia* was over-represented in the pupal and adult stages, *Pseudomonas* was abundantly present in maggots, representing the only feeding stage of the GM. Here, it is worth noting that *Wolbachia* is a maternally inherited bacteria present in the GM microbiome [27], whereas *Pseudomonas* has to be acquired from the environment [49]. Since GM maggots reside within the rice tissue, the host becomes the only possible source for acquiring *Pseudomonas* during feeding. Moreover, a holometabolous insect that undergoes complete metamorphosis requires that it re-establishes its microbiome at each moult [50]. As GM is a holometabolic insect, the likely source of *Pseudomonas* in its maggots is the rice plant. This is further corroborated by the observation that *Pseudomonas* is over-represented in the maggot stage [25]. Therefore, the present study focused on assessing the species diversity of *Pseudomonas* in CT and GT tissues.

In this regard, although the NGS results obtained for CT and GT samples did not reveal a significant difference in the number of OTUs obtained for *Pseudomonas*, we did observe an increase in bacterial diversity in the GT samples (Supplementary Table **1**; Fig. **2b**). Additionally, and in contrast to what was previously observed for *Wolbachia*, *Pseudomonas* was ubiquitously present in all the rice samples (CT, GT, and TN1 rice variety) under investigation in this study.

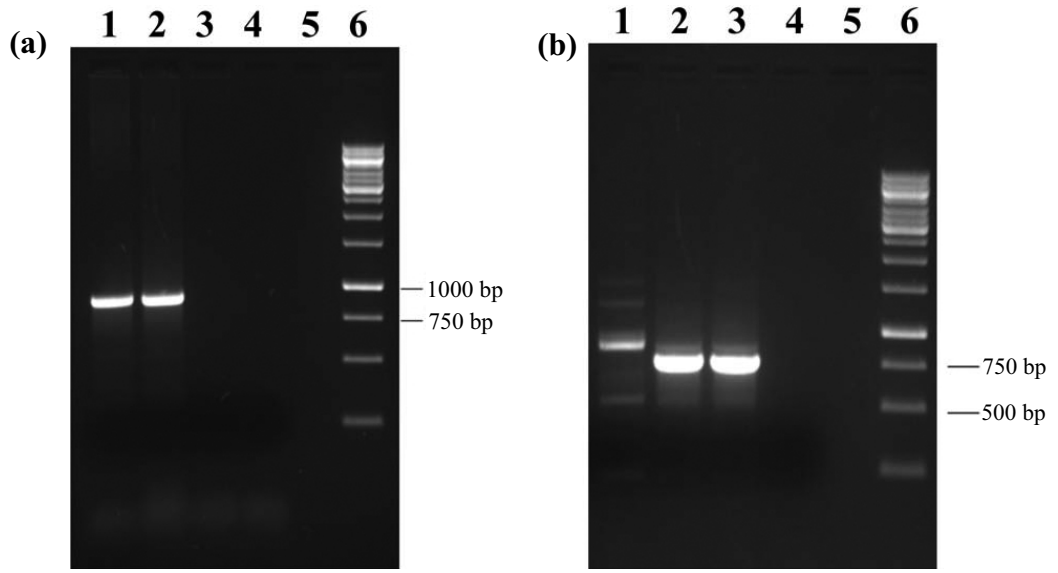

Fig. (4). (a). 0.8% agarose gel showing a ~870bp PCR-amplified fragment that corresponds to a portion of the *Wolbachia 16S rRNA* (see Materials and Methods for details). Lanes (1 – 6) represent control tissue (CT); gall tissue (GT); TN1 rice variety (grown on sterile vermiculite); negative control (no template); blank lane; and 1Kb DNA ladder (Fermentas, Cat No. SMO311), respectively. **(b).** 0.8% agarose gel showing a ~750bp PCR-amplified fragment that corresponds to a portion of the *Wolbachia ftsZ* (see Materials and Methods for details). Lanes (1 – 6) TN1 rice variety grown on sterile vermiculite; control tissue (CT); gall tissue (GT); negative control (no template); blank lane; and 1Kb DNA ladder, respectively. The figures on the right represent molecular weights in base pairs (bp).

Therefore, to distinguish between the *Pseudomonas* present in GM and rice, it became pertinent to identify different species of *Pseudomonas* present in GT and CT. For this, we chose to differentiate the different species of *Pseudomonas* present in CT and GT samples based on the sequence diversity of PCR amplified V3-V4 hypervariable region of the *16S rRNA* of *Pseudomonas* (Fig. **5**). Some of the *Pseudomonas* detected and identified up to the species level from CT and GT are *P. chengduensis*, *P. stutzeri*, *P. guezennei*, *P. glareae, P. indoloxydans,* and *P. mendocina* (Table **3**). Furthermore, owing to the immense diversity of *Pseudomonas* species, we carried out an NGS analysis to identify all the species present in the TN1 rice variety (grown on sterile vermiculite). The sequencing information revealed the presence of over 140 different species of *Pseudomonas* in rice (data not shown). Interestingly, some of these bacterial species were shown to be present in abundance in the GM maggots [25], consequently leading to the possibility of the exchange of bacteria between the insect and its host plant during feeding.

In the light of findings that different species of *Pseudomonas* have remarkably different biology, it was necessary first to identify and then assign the potential role(s) within GM. This coupled with the fact that some species of *Pseudomonas* have pathogenic properties and others share a symbiotic relationship with their host [51]; therefore, it is important to unravel their overarching role(s) in rice-GM interaction [52-54]. When *Pseudomonas* species are present as an endosymbiont within the GM, they can provide a range of benefits to the insect host, such as detoxification of xenobiotic compounds, synthesis of metabolites, and modulation of immune responses [55]. From the data obtained, even though we are unable to assign a role(s) to the different *Pseudomonas* species found in GM maggots, its differential presence in maggots and pupae [25] is likely because maggots acquired it while feeding, whereas, its absence in the pupae, could be due to loss of these microbes during moulting.

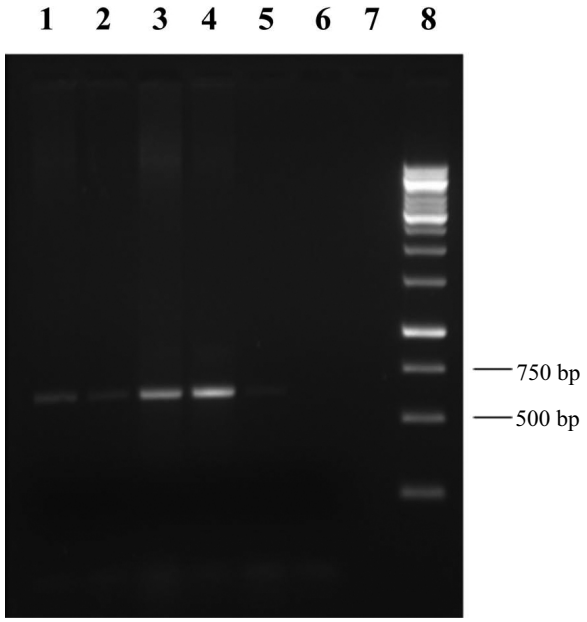

Fig. (5). 0.8% agarose gel showing a ~617 bp PCR-amplified fragment that corresponds to a portion of the *Pseudomonas 16S rRNA* (see Materials and Methods for details). Lanes (1 – 8) represent control tissue 1 (CT-1); control tissue 2 (CT-2); gall tissue 1 (GT-1); gall tissue 2 (GT-2); TN1 rice variety grown on sterile vermiculite; negative control (no template); blank lane; and 1Kb DNA ladder, respectively. (CT-1, CT-2 and GT-1, GT-2 are replicates). The figures on the right represent molecular weights in base pairs (bp).

Table 3. The presence and likely role of a few of the different *Pseudomonas* species identified in rice tissues (CT and GT) and TN1 seedlings grown in sterile vermiculite*

Pseudomonas Species	Samples			Role	Accession No.
	CT	GT	TN1		
P. mendocina	-	+	+	Detoxification of xenobiotic compounds [57]	MW092516, MW092519
P. glareae	-	+	+	Sugar and protein assimilation, Nitrate reduction [58]	MW092517, MW092518
P. guezennei	+	-	+	PHA (polyhydroxy alkanoate) producing bacteria [59]	MW092521, MW092522
P. stutzeri	+	+	+	Pesticides (Parathion) breakdown [60]	MW092515, MW092523, MW092524
P. chengduensis	-	+	+	Nitrate reduction, heavy metal degradation [61]	MW092525, MW092526
P. indoloxydans	-	+	+	Indole-oxidizing bacterium [62]	MW092520

*Although the present study identified more than 150 species of *Pseudomonas* from these rice samples, only the predominant species are listed

+= present; - =absent

This investigation was based on the hypothesis that microbes, present within the plant tissue, are either secreted by the GM (*e.g*., *Wolbachia*) or are inherently present at the feeding site (*e.g*., *Pseudomonas*). If the microbes present within the GM transfer to the plant tissue while feeding, these microbes are likely perceived by the plant. If so, the rice host is likely to initiate a defense response similar to that induced upon pathogen attack [56]. Furthermore, it is equally plausible that there could be microbes secreted by the GM that assist it in gaining access to the host cell and the nutrients within while facilitating the process of gall formation in susceptible hosts. Taken together, data obtained from NGS studies and subsequent comparative analysis of the presence of *Pseudomonas* and *Wolbachia* at the gall midge feeding site and within the GM indicated the exchange of bacterial species between gall midge and its host upon infestation. Future studies for deciphering the exact role of these microbes and the implications of their presence with reference to the rice-GM interaction are underway. Such studies would likely reveal the exact location(s) within the insect/plant body, where the microbes are concentrated along with the major role(s) of these microbes in the interaction.

CONCLUSION

Results presented here provide indirect evidence for the exchange of bacterial species between the insect and its host plant, *i.e*., microbial symbionts present inside GM are selectively transmitted to the rice and probably vice versa during the feeding process. This is likely to be the reason for the differential presence of *Wolbachia* and *Pseudomonas* at the feeding site in the host plant upon GM infestation. The absence of *Wolbachia* Supergroup A in uninfested rice and its presence in infested plants suggested the transmission of bacterial symbionts by GM. However, the ubiquitous occurrence of *Pseudomonas* in both rice and GM and its differential presence in the adult and maggot stages, but not in the pupae of the GM, implies that the bacterial species belonging to this genus are likely taken up from the plant by GM and may aid GM survival. These findings not only add to our current understanding of microbe-mediated insect-plant interaction but also provide an important link to how these bacteria drive insect-plant coevolution.

AUTHORS' CONTRIBUTIONS

DKS and SN designed the study. DKS and AG performed the experiments. DKS, AG, and SN analyzed the data. DKS, AG, and SN wrote the manuscript. APP, JSB, and SN provided supervision. APP and JSB contributed to the collection of reagents/materials and critically revised the manuscript. All authors have read the manuscript and approved the final version of the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

NGS data sets generated during this study (for CT and GT samples) are available as Sequence Read Archive (SRA) files at NCBI and can be accessed using the accession number PRJNA668709. In addition, the nucleotide sequence data for *Wolbachia* Supergroups obtained using *ftsZ* gene-

specific primers have been submitted under the accession numbers [Genbank: MW091359-MW091378], and the sequences for *Wolbachia 16S rRNA* have the accession numbers [Genbank: MW110641-MW110646]. The sequences corresponding to the *Pseudomonas 16S rRNA* have been submitted under the accession numbers [Genbank: MW092515-MW092526].

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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