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Bacterial Communityof the Leafhopper *MatsumuratettixHiroglyphicus* (Matsumura) (Hemiptera: Cicadellidae), a Vector of Sugarcane White Leaf Phytoplasma

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Abstract

We investigated the bacterial community in the leafhopper *Matsumuratettixhiroglyphicus*, a vector of sugarcane white leaf phytoplasma.Bacterial diversity was analyzed using V3-V4 16S rRNA metagenomic sequencing. A taxonomic analysis of the sequencing data revealed 43 known bacterial genera belonging to eight phyla, 16 classes, 30 orders, and 39 families. Bacteroidetes and Proteobacteria were the most abundant phyla in all samples, followed by Tenericutes, whereas phyla with relatively low abundances included Actinobacteria, Cyanobacteria, and Firmicutes. The

Most abundant genus was primary symbiont*Sulcia*, followed by unclassified bacteria from family Tremblayaceae.Another abundant genus was and*Phytoplasma*, which was detected exclusively in female leafhoppers. The remainder of the identified genera, including *Acinetobacter*, *Bacillus*, *Agrobacterium*, *Propionibacterium*, *Pseudomonas*,*Sphingomonas*,and *Corynebacterium*, were present relatively low abundances. To the best of our knowledge, this is the first report on the composition of the bacterial community in the leafhopper vector of sugarcane white leaf disease. The results may pave the way for the development of alternative strategies for the management of this insect.

Key words: leafhopper, phytoplasma, bacterial community, 16S rRNA gene sequencing

INTRODUCTION

There has been growing interest in the study of insect-associated microbial communities, particularly bacterial symbionts and gut microbes, which play a crucial role in the growth and development of their insect hosts (Engel and Moran, 2013; Douglas, 2015). Obligate or primary bacterial symbionts, such as *Buchnera*in aphids, *Carsonella* in psyllids, and *Sulcia* in

leafhoppers, provide essential nutrients for host survival and development (Ferrari and Vavre, 2011; Douglas, 2015; Mao et al., 2018; Whittle et al., 2021). In addition to mutualistic associations, insects have been reported to host facultative bacteria, which exert various effects on the hosts. For example, the bacterium *Hamiltonelladefensa* protects its aphid hosts against parasitoid wasps (Oliver and Higashi, 2019), whereas *Burkholderia* bacteria can detoxify insecticidal toxins in bean bugs, rendering them resistant to insecticides (Sato et al., 2021). *Rickettsia*infection promotes plant virus acquisition, retention, and transmission in whiteflies (Kliot et al., 2019). Other bacteria, such as *Wolbachia*,*Carnidium*,and *Spiroplasma*, have been identified as reproductive parasites that can manipulate the reproductive system of their hosts(Landmann, 2019; Sazama et al., 2019). Consequently, insect microbes have attracted attention as potential novel control agents against insect pests (Hoffmann et al., 2015; Gonella and Alma, 2023). Therefore, studying insect microbial communities, their interactions, and biodiversity can facilitate the establishment of effective insect pest control strategies.

The leafhopper*Matsumuratettixhiroglyphicus*(Matsumura)is one of the most devastating sugarcane pests, causing economic losses by transmitting phytoplasmas which cause white leaf disease (Roddee et al., 2019). The disease incidence has increased across Southeast Asia, posing a threat to sugarcane production. In 2021–2022, it affected more than 80 million tons of sugarcane production in Thailand (Office of the Cane and Sugar Board, Ministry of Industry of Thailand 2022). Understanding bacterial diversity associated with *M. hiroglyphicus* may lead to alternative control strategies. To the best of our knowledge, only one study has evaluated bacterial symbionts in *M. hiroglyphicus* based on 16S rRNA gene amplification, cloning, and sequencing, revealing only two types of uncultured primary bacterial symbionts (Wangkeeree et al., 2012). However, this method does not provide information on the actual number of bacteria or their composition. Therefore, the bacterial community of leafhopper symbionts remains largely unknown.

With the development of next-generation sequencing, 16S rDNA metagenomics has emerged as an important tool for evaluating the microbiota structure in environmental and biological samples. This technique is convenient, effective, and overcomes the challenge ofthat most symbiotic bacteria cannot be isolated and cultured on artificial media *in vitro*. Several recent studies have used this high-throughput sequencing approach to reportthe bacterial composition and diversity in insects (Douglas, 2018; Malacrinò, 2018).

This study aimed to investigate the composition and diversity of bacteria hosted by *M. hiroglyphicus* leafhoppers infesting sugarcane fields. Bacterial communities were explored by employing V3-V4 16S rRNA gene metagenomic sequencing. The results of this study canimprove our understanding of the microbial community in the leafhopper and provide informationto support the development and establishment of efficient control strategies.

MATERIALS AND METHODS

Leafhopper collection and DNA extraction

Adult *M. hiroglyphicus* infesting sugarcane fields in four provinces of Thailand (Khon Kaen, Sa Kaeo, Kamphaeng Phet, and Kanchanaburi) were collected using light traps (Table 1). The specimens were immediately immersed in absolute ethanol and stored at –20 °C until DNA extraction. For each location, the specimens were separated according to sex, and 20 males and 20 females were pooled into two samplesper locations. Subsequently, the specimens were surface-sterilized with 70% ethanol for 15 min and washed three times with distilled water to remove surface contaminants. Genomic DNA was extracted using the PureLink Genomic DNA

Mini Kit (Invitrogen, Carlsbad, CA, USA). The DNA concentration was measured using a NanoDrop Lite spectrophotometer (Thermo Scientific, Waltham, MA, USA), and DNA quality was visually inspected on a 1% (w/v) agarose gel. The extracted DNA was stored at –80 °C until library construction.

Library construction and sequencing

For gene library construction, the bacterial 16S rRNA gene was PCR-amplified using a set of primers targeting the V3-V4 hypervariable region (V3 forward 341F, 5′-CCTACGGGNGGCWGCAG-3′, and V4 reverse 805R, 5′-GGACTACHVGGGTWTCTAAT-3′). The amplicons were purified using AgencourtAMPure beads (Beckman Coulter Inc., Brea, CA, USA), according to the manufacturer's instructions. The constructed libraries were subjected to quality tests, and qualified libraries were sent to Biomark Technologies (Beijing, China) for metagenomic sequencing. 16S V3-V4 amplicon metagenomics sequencing was conducted on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) per the manufacturer's recommendations, generating 250-bp paired-end reads.

Bioinformatic analysis

Raw data were filtered according to the Quantitative Insights into Microbial Ecology (QIIME) quality control process (Caporaso et al., 2010) to eliminate adapters and low-quality reads, yielding clean reads. Using QIIMEprocess, the tags were clustered into operational taxonomic units (OTUs) at a 97% sequence similarity threshold. Chimeras were removed using UCHIME (Edgar et al., 2011). Unique representative OTU sequences were taxonomically annotated using the Ribosomal Database Project classifier, which was trained on the Greengenes database, with 80% confidence (Cole et al., 2014). Microbial diversity was analyzed using QIIME and visualized using R (version 2.15.3). Different species were identified, and their alpha and beta diversity were determined based on OTUs and taxonomic ranks.

RESULTS

Overall bacterial community composition in *M. hiroglyphicus* leafhoppers

The characteristics of the *M. hiroglyphicus* 16S rRNA metagenomic libraries are shown in Table 1. After sequencing and quality filtering, 20789–32006 clean reads were retained for each of the eight libraries, with read lengths ranging from 465 to 496. In total, 82–138 OTUs (3% cut-off level) were obtained for each of the leafhopper samples, and Simson, Shannon, and Chao1 indexes were determined to evaluate bacterial communitydiversity and richness. The Shannon index values ranged from 0.42 to 0.72, suggesting a relatively high bacterial diversity. Simson's index values ranged from 1.05–1.86 to 0.99. The Chao1 index values were 84.50–131.00. No significant differences among the eight samples were observed.

The taxonomic assignments for all bacteria based on the sequence data are presented in Table 2. A taxonomic analysis of the V3-V4 16S rRNA gene amplicon reads revealed eight phyla, 16 classes, 30 orders, 39 families, and 43 genera. Additionally, one class, one order, five families and 10 genera were unidentified taxa (Table 2).

Table 1. Summary of *M.hiroglyphicus* 16S rRNA gene sequencing data and alpha diversity indices.

Sex	Sample origin of (province)	Sample ID	Number of reads	Average read length	Number of OTUs	Shannon	Simson	Chao1
Female	Udon Thani	UDF	28900	487	82	1.05	0.43	84.50
	Sa Kaeo	SKF	25784	491	127	1.34	0.47	128.50
	Kanchanaburi	KNF	26117	496	118	1.79	0.67	83.07
	Kamphaeng Phet	KPF	32006	483	109	1.65	0.72	98.00
Male	Udon Thani	UDM	25745	494	138	1.85	0.58	120.55
	Sa Kaeo	SKM	20789	472	104	1.86	0.53	131.00
	Kanchanaburi	KNM	23688	465	98	1.34	0.62	102.56
	Kamphaeng Phet	KPM	21653	476	115	1.56	0.42	119.59

OTU: operational taxonomic unit; UDF (UdonThaai - female), SKF (Sa Kaeo - female), KNF (Kanchanaburi - female), KPF (Kamphaeng Phet - female), UDM (Udon Thani - male), SKM (Sa Kaeo - male), KNM (Kanchanaburi - male), KPM (Kamphaeng Phet - male)

Table 2. Taxonomic characterization of the overall bacterial community in the leafhopper *M.hiroglyphicus.*

Phylum	Class	Order	Family	Genus
Actinobacteria	Actinomycetia	Actinomycetales	ACK-M1	
			Corynebacteriaceae	*Corynebacterium*
			Dermabacteraceae	*Brachybacterium*
				Dermacoccus
			Microbacteriaceae	*Microbacterium*
				Arthrobacter
			Mycobacteriaceae	*Mycobacterium*
			Propionibacteriaceae	*Propionibacterium*
				other
			Streptomycetaceae	*Streptomyces*
Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	*Prevotella*
	Flavobacteriia	Flavobacteriales	Blattabacteriaceae	*Sulcia*
			Flavobacteriaceae	*Flavobacterium*
	Sphingobacteriia	Sphingobacteriales	other	
Cyanobacteria	4C0d-2	MLE1-12	other	
	Chloroplast	Streptophyta	other	
Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*
			Staphylococcaceae	*Staphylococcus*
		Lactobacillales	Streptococcaceae	*Streptococcus*
	Clostridia	Clostridiales	Tissierellaceae	*Finegoldia*
				Peptoniphilus
OD1	other	other		
Planctomycetes	Phycisphaerae	Phycisphaerales	other	
	Planctomycetia	Gemmatales	Gemmataceae	*Gemmata*
		Pirellulales	Pirellulaceae	other
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	other
		Rhizobiales	Bradyrhizobiaceae	*Bradyrhizobium*
			Hyphomicrobiaceae	*Devosia*
			Methylobacteriaceae	*Methylobacterium*

				Other
			Rhizobiaceae	*Agrobacterium*
				Rhizobium
		Rhodobacterales	Rhodobacteraceae	*Paracoccus*
				Rhodobacter
		Rickettsiales	mitochondria	Other
		Sphingomonadales	Sphingomonadaceae	*Blastomonas*
				Novosphingobium
				Sphingobium
				Sphingomonas
	Betaproteobacteria	Burkholderiales	Alcaligenaceae	*Achromobacter*
				Denitrobacter
				Achromobacter
				Denitrobacter
			Comamonadaceae	*Delftia*
				other
				Pelomonas
		Methylophilales	Methylophilaceae	*Methylotenera*
		Rhodocyclales	Rhodocyclaceae	*Methyloversatilis*
		Tremblayales	Tremblayaceae	other
		Bdellovibrionales	Bdellovibrionaceae	*Bdellovibrio*
	Deltaproteobacteria	Myxococcales	0319-6G20	other
	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	*Campylobacter*
	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	other
		Legionellales	other	
		Oceanospirillales	Halomonadaceae	other
		Pseudomonadales	Moraxellaceae	*Acinetobacter*
				Enhydrobacter
			Pseudomonadaceae	*Pseudomonas*
		Xanthomonadales	Sinobacteraceae	other
			Xanthomonadaceae	other
				Stenotrophomonas
Tenericutes	Mollicutes	Acholeplasmatales	Acholeplasmataceae	*Phytoplasma*

Distribution of the bacterial community

The taxonomic analysis revealed eight classified phyla, including Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, OD1, Planctomycetes, Proteobacteria, and Tenericutes (Table 1). The relative abundance of phyla differed among the samples. The three most abundant phyla were Bacteroidetes, Proteobacteria, and Tenericutes. Among these, Bacteroidetes had a 29.30–55.09% relative abundance and was detected in all leafhopper samples(Figure 1A). This phylum was represented by three genera belonging to three families, three orders, and three classes (classes Bacteroidia, Flavobacteriia, and Sphingobacteriia) (Table 1). The second most abundant phylum was Proteobacteria, with a 19.83–47.87% relative abundance and present in all samples. It contained the highest number of bacteria, represented by 26 genera belonging to 23 families, 18 orders, and five classes, including Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria, and Gammaproteobacteria (Table 1). The phylum Tenericutes contained only one bacterial genus, had a 7.75–36.50% relative abundance, and was only present in female leafhoppers ((Figure 1A).

The low-abundant phyla included Actinobacteria, with a 2.51–4.46% relative abundance; this phylum contained seven genera belonging to five families and one order in the class Actinomycetia. The phylum Cyanobacteria had a 1.56–4.58% relative abundance; it contained unclassified genera and families belonging to two orders and two classes, including 4C0d-2 and chloroplast. The remaining two phyla, OD1 and Planctomycetes, also had a low relative abundance (Table 1 and Figure 1A).

The nine most abundant classes included Flavobacteriia, Betaproteobacteri, Gammaproteobacteria, Alphaproteobacteria, Mollicutes, Bacilli, Actinobacteria,Clostridia, and Bacteroidia (Figure 1B). Among these, the top three included Flavobacteriia(relative abundance: 8.76–55.06 %) >Betaproteobacteria (14.03–30.93%)>Mollicutes (7.75–36.50%). Flavobacteriia and Betaproteobacteria were present in all samples, whereas Mollicutes was present only in female leafhoppers.Figure 1C presents the top ten most abundant orders, includingFlavobacteriales, Caulobacterales, Rhizobiales, Tremblayales, Legionellales, Oceanospirillales, Acholeplasmatales, Bacillales, Pseudomonadales, and Actinomycetales. The top three order includedFlavobacteriales (relative abundance: 28.76–55.06 %) >Tremblayales(9.80–30.3%) >Acholeplasmatales(7.75–36.50%).Flavobacteriales and Pseudomonadales were obtained from all samples, whereas Acholeplasmatales was only reported from female leafhoppers samples.The top tenmost abundant families in leafhopper sample are shown in Figure 1D, including Blattabacteriaceae, Corynebacteriaceae,Bacillaceae, Caulobacteraceae, Rhizobiaceae, Tremblayaceae, Halomonadaceae, Acholeplasmataceae, Moraxellaceae and Propionibacteriaceae. Among these, the most abundant family was Blattabacteriaceae, followed by Tremblayaceae and Acholeplasmataceae which had a relative abundance as mentioned in their order.

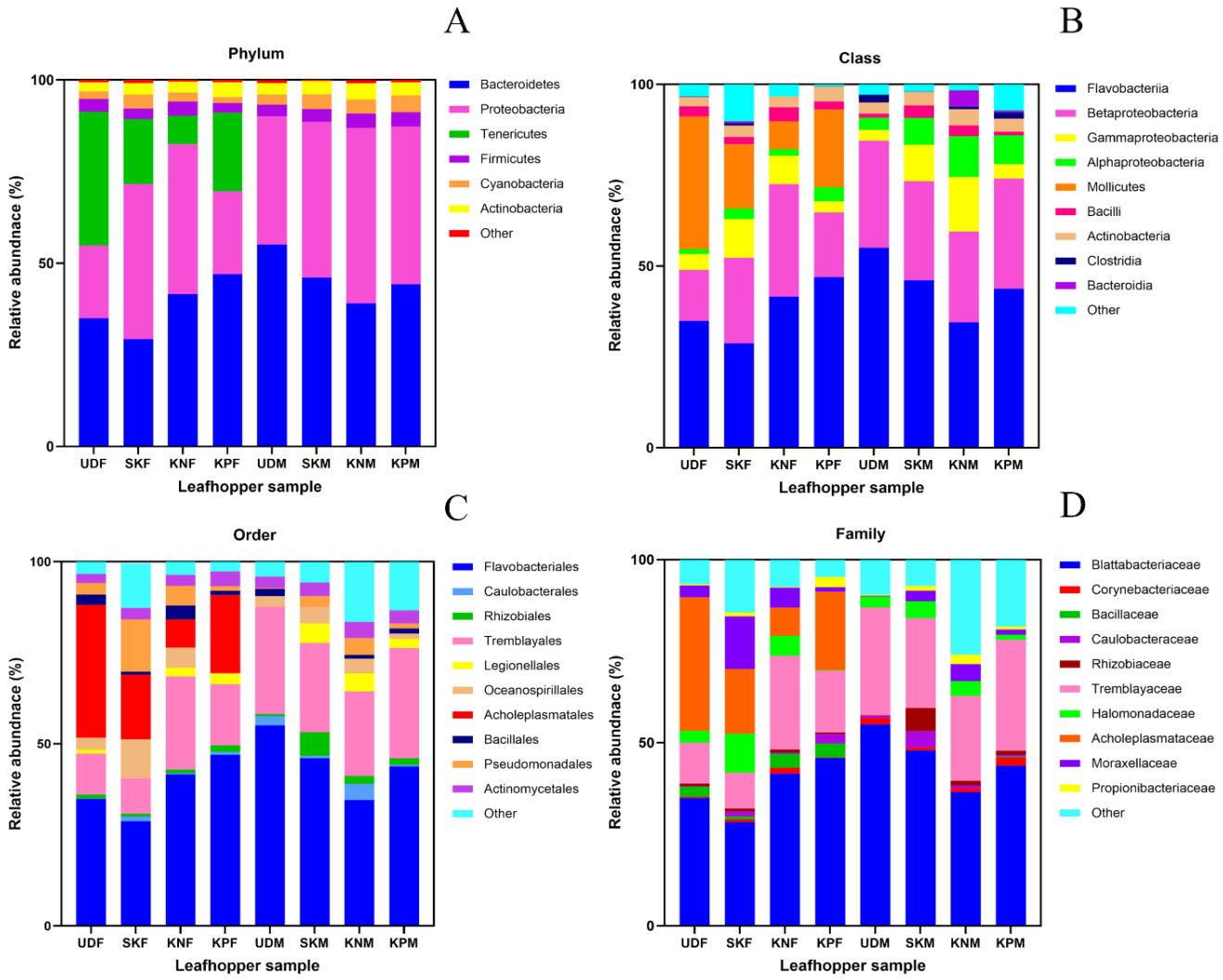

Figure 1 Relative abundance of taxa in the bacterial community (A); phylum level, (B); class level, (C); order level, (D); family level in a natural population of *M.hiroglyphicus* leafhoppers. Remaining reads with a relative abundance and unclassified were grouped under "other." The origin and sex of the leafhopper samples are represented by the sample ID: UDF (Udon Thani - female), SKF (Sa Kaeo - female), KNF (Kanchanaburi - female), KPF (Kamphaeng Phet - female), UDM (Udon Thani - male), SKM (Sa Kaeo - male), KNM (Kanchanaburi - male), KPM (Kamphaeng Phet - male).

Distribution of the bacterial community at the genus level

Forty-three known and 10 unknown genera were identified in all leafhopper samples. The top 10 genera in each leafhopper sample are shown in Figure 2. The majority of the reads was primary bacterial symbiont *Sulcia*, with a 28.35–55.06% relative abundance. Followed by the reads belonged to unclassified bacteria from family Tremblayaceae(relative abundance: 9.80–

30.3%). These two groups present in all leafhopper samples. Another one was *Phytoplasma*, which was detected exclusively in female leafhoppers (all four samples), with a 7.75–36.50% relative abundance.

These three groups, *Sulcia*, unclassifiedTremblayaceae, and *Phytoplasma* represented 70–85% of all genera in females. The *Sulcia* and unclassifiedTremblayaceae represented 60–85% of all genera in male leafhoppers. The remaining genera identified, including *Acinetobacter*, had relatively low abundances (0.07–14.35%). The genus *Bacillus* wasdetected in six out of eight samples, with a 0.02–3.97% relative abundance. The genus *Agrobacterium* was detected in all samples, at 0.1–3.55%. Genera detected in most of the samples included *Propionibacterium*, *Pseudomonas*,*Sphingomonas*,and *Corynebacterium*. Furthermore, several sequences could not be classified.

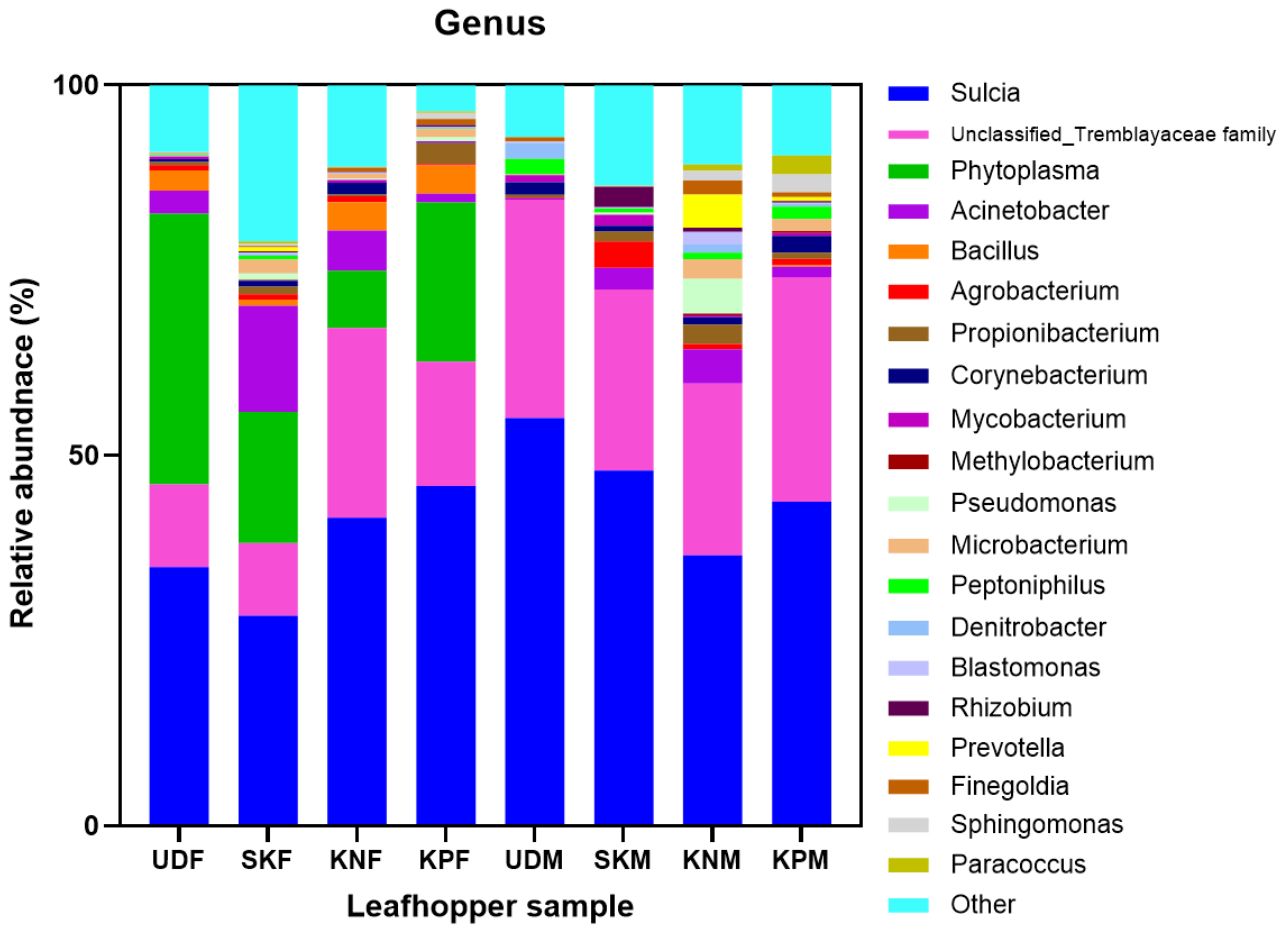

Figure 2 Relative abundance at the genus level of the bacterial community in a natural population of *M. hiroglyphicus* leafhoppers. The top 10–15 genera in each sample are shown. Remaining reads with a relative abundance and unclassified were grouped under "other." The origin and sex of the leafhopper samples are represented by the sample ID: UDF (Udon Thani - female), SKF (Sa Kaeo - female), KNF (Kanchanaburi - female), KPF (Kamphaeng Phet - female), UDM (Udon Thani - male), SKM (Sa Kaeo - male), KNM (Kanchanaburi - male), KPM (Kamphaeng Phet - male).

DISCUSSION

Metagenomics can provide insights into microbial communities and is particularly useful for uncultivable microorganisms. In the present study, we evaluated the composition of bacterial communities hosted by field-collected adult *M. hiroglyphicus* leafhoppers infesting cultivated sugarcane. Themetagenomic analysis of the *M. hiroglyphicus* bacterial community revealed that Bacteroidetes, Proteobacteria, and Tenericutes were the most abundant phyla. The composition of bacterial communities associated with natural populations of insects exhibits a high degree of complexity and is influenced by multiple factors. Different insect species harbor different bacterial communities. A metagenomic analysis of the bacterial community of the brown planthopper *Nilaparvatalugens* Stal revealed that Proteobacteria, Firmicutes, and Bacteroidetes arethe predominant phyla (Vijayakumar et al., 2018), whereas Proteobacteria and Firmicutes arethe predominant bacterial phyla in the silk moth *Samia ricini* (MsangoSoko et al., 2020). Proteobacteria, Bacteroidetes, and Firmicutes are the predominant bacterial phyla in the whitefly *Bemisiatabaci*(Shah et al., 2020), whereas Firmicutes and Cyanobacteria are common in grasshoppers (Wang et al., 2020). Besides the insect species, the geographical location, environmental factors, agricultural practices, and natural enemies influence the bacterial community composition in insects (Zhao et al., 2016).

Bacterialphyla that had low abundance in some leafhopper samples included Actinobacteria, Cyanobacteria, Firmicutes, OD1, and Planctomycetes. Some of these phyla have been reported in metagenomic studies of other insects, including *B. tabaci*, in which Actinobacteria, Cyanobacteria, and Planctomycetes were detected (Goretty et al., 2019). Actinobacteria, Firmicutes, and Planctomycetes have been identified in the mosquito *Culex pipiens quinquefasciatus* (Wang et al., 2021), whereas Actinobacteria and Firmicutes have been detected in bed bug *Cimex hemipterus* (F.) (Lim and Ab Majid, 2021).

At the genus level, *Sulcia* (Bacteroidetes) had the highest abundance among the bacterial community. Similar to other phloem-feeding insects, *M. hiroglyphicus* leafhoppers are dependent on primary symbionts for the provision of essential nutrients lacking in the phloem sap (Skidmore and Hansen 2017).*Sulcia*synthesizes most essential amino acids as well as vitamins and cofactors (Mao et al., 2018).We speculate that geographical and environmental factors do not account for the prevalence of primary bacterial symbionts. Typically, in insects such as the aphid *Aphis gossypii* (Zhou et al., 2016), whitefly *B. tabaci* (Goretty et al., 2019), and mealybug *Paracoccusmarginatus* (Megaladevi et al., 2020), which harbor primary symbionts, these symbionts constitute more than half of the total bacterial community, probably because of an ancient symbiotic relationship that is crucial for host survival.

In addition to primary symbiont, another abundant genus detected exclusively in females was *Phytoplasma* (phylum Tenericutes), which causes white leaf disease in sugarcane. The exclusive detection in females can be explained by their larger size than that of males, enabling them to carry more *Phytoplasma*. This suggests that the sex of the leafhopper directly affects the bacterial community, particularly the presence of *Phytoplasma*. Female leafhopper is likely the main vector transmitting this pathogen to sugarcane plants, providing a basis for enhancing the effectiveness of pest management strategies. While *Phytoplasma* is a well-known sugarcane pathogen, its functional role in *M. hiroglyphicus* remains unknown. The fact that these bacteria were detected in all female samples suggests that they are vertically transmitted, which may guide studies on its role in the leafhopper.

In addition to the above three genera, many other genera, such as *Acinetobacter, Bacillus, Agrobacterium*,*Methylobacterium*,*Microbacterium, Pseudomonas*,and *Rhizobium* were detected.

Propionibacterium, *Pseudomonas*,*Sphingomonas*,and *Corynebacterium* showed low relative abundances with variation among the samples. Some genera, such as *Acinetobacter*, *Bacillus*,*Microbacterium*,and *Methylobacterium*,have been reported as sugarcane bacterial endophytes (Taulé et al., 2016; Rocha et al., 2021), whereas others, including *Methylobacterium*, are found in natural environments and possess plant growth-promoting properties. We speculate that the bacterial communities in sugarcane plants substantially influence the composition of bacteria inside *M. hiroglyphicus* leafhoppers. Bacterial endophytes may be transferred horizontally to insects via feeding (Lòpez-Fernàndez et al., 2017), supporting the claim that the composition of bacterial communities in insects also depends on their host plants. The genera *Pseudomonas*, *Rhizobium*,*Sphingomonas*,and *Corynebacterium* detected in *M. hiroglyphicus*are typically found in broader environments (e.g., in plants and soils). Environmental factors may act as one of important drivers of the differences in bacterial community composition among natural populations of insects (Jones et al., 2019). Some of these genera were shared among most samples, suggesting that they have important functions in leafhoppers. Furthermore, known insect pathogens, such as *Bacillus* (Saxena et al., 2020), were detected at extremely low abundances (<1% of the overall bacterial communities).

In conclusion, our metagenomic sequencing analysis revealed that the composition of the bacterial community in the leafhopper *M. hiroglyphicus* is dominated by the primary symbiont *Sulcia* species. Subsequently, *Phytoplasma* was detected only in females, suggesting that its prevalence is influenced by the sex of leafhoppers. Other bacterial taxa varied in abundance among the leafhopper samples and may have originated from sugarcane plants, the soil, or other environments. This study provides insights into the microbial community associated with *Phytoplasma* transmission and fundamental information for future research on the functional roles of bacterial species in *M. hiroglyphicus*. Furthermore, the results provide useful informationfor the development of alternative strategies for the control of this leafhopper speciesand a basis for future studies focused on the identification of functions of representative bacterial species and their potential value as biocontrol agents.

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