Unveiling the Microbes of the World's Largest Flowers

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ABSTRACT

The plant family Rafflesiaceae is known for producing the largest flowers in the world. They are solely parasitic to the plant genus *Tetrastigma* of the grape family. Interestingly, members of Rafflesiaceae are the only plants we know so far that have lost their chloroplast genome. Rafflesiaceae is comprised of three genera: *Rafflesia, Sapria,* and *Rhizanthes.* Members of Rafflesiaceae are restricted to the fast-disappearing tropical forests of Southeast Asia and are endangered. It is virtually unknown how Rafflesiaceae infects its hosts. This makes Rafflesiaceae very difficult to conserve and propagate. However, bacterial endophytes have shown to play a major role in promoting the growth of plants. In this study, we characterized and analyzed the associated microbiome of Rafflesiaceae, particularly *Rafflesia speciosa* and *Sapria himalayana* to find "microbial markers" of Rafflesiaceae infection. It was hypothesized that *Rafflesia* and its *Tetrastigma* host will have similar microbiomes given their intimate connection, however, results across different taxonomic levels demonstrate that *Rafflesia* buds have a distinct microbial endophyte composition and possessed some unique genera compared to its infected hosts. Its microbiome was also diverse compared to *Sapria*'s. Non-host *Tetrastigma* spp. possessed unique bacteria that may be antagonistic to *Rafflesia* infection. This study unveiled the symbiotic microbes of *Rafflesia* and *Sapria,* which may have potential horticultural application in propagating the world's largest flowers.

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LITERATURE REVIEW

BACKGROUND ON THE FAMILY RAFFLESIACEAE

The plant family Rafflesiaceae is known for producing the largest flowers in the world, up to a meter in diameter, but unlike most plants, members of this family do not have stems, roots nor leaves and lack the ability to photosynthesize. They are solely parasitic to the plant genus *Tetrastigma* of the grape family (Nikolov & Davis, 2017). However, not all *Tetrastigma* species can be parasitized. Interestingly, members of Rafflesiaceae are the only plants we know so far that have lost their chloroplast genome, the set of genes that purportedly defines plants (Molina et al., 2014; Cai et al., 2021). Rafflesiaceae belongs to the order Malpighiales, and it is comprised of three genera: *Rafflesia* R.Br. ex Thomson, *Sapria* Griff, and *Rhizanthes* Dumort. *Rafflesia* contains 41 species, *Rhizanthes* contains 4 species, and *Sapria* contains 4 species as well (Plants of the World Online). The first *Rafflesia* species (*R. arnoldii*) was discovered in 1818 by Sir Stamford Raffles and Dr. Joseph Arnold when they embarked on a journey to explore the Sumatran island in Bengkulu (Brown, 1821). Two of the four *Rhizanthes* (*Rh. lowii* and *Rh. zippelii*) species were found in the early nineteenth century in Indonesia by German botanist Alexander Zippelius and were then described by Karl Ludwig von Blume (Wicaksono et al., 2022). The first *Sapria* species (*S. himalayana* Griff) was described in 1836 by the British botanist William Griffith afrom his north-east Indian collection (Dorji et al., 2022). Unfortunately, all members of Rafflesiaceae are rare, restricted to the fast-disappearing tropical forests of Southeast Asia, and are thus endangered. They are a charismatic icon of conservation, hailed as the "panda of the plant world".

LIFE CYCLE OF RAFFLESIACEAE

As a holoparasite, Rafflesiaceae siphons organic and inorganic nutrients from their hosts and reside inside its host as strands of cells also known as endophytes. It has been observed that a *Rafflesia* endophyte can grow inside its host plant for approximately 2-3 years (Hidayati et al., 2000; Wicaksono et al., 2021). As it transitions to its flowering stage, the strands of cells become a mass of cells called a protocorm that eventually emerges as a flower bud from the inside of the woody host (Nikolov & Davis, 2017). As the flower blooms, it emits a rotting meat type of smell that attracts pollinators such as carrion flies. The pollination process consists of a fly entering a male flower, exiting with pollen grains attached to its body, and transferring it to a female flower (Beaman et al., 1988). After successful pollination, it takes several months for the fruits to form. The fruit then dehisces revealing millions of seeds. The fruit produces a rancid coconut meat odor that attracts multiple insect visitors. Although little is known about the seed dispersal of Rafflesiaceae, it is thought that the seeds can be dispersed by different kinds of wild animals like shrews, squirrels, elephants, and ants (Wicaksono et al., 2021).

PROPAGATION AND CONSERVATION EFFORTS

It is virtually unknown how Rafflesiaceae infects its hosts (Wicaksono et al., 2021). This makes Rafflesiaceae very difficult to conserve and propagate. It has never been cultivated outside of its native range. Since 2015, Molina et al (2017) have been trying to propagate *Rafflesia* species from Southeast Asia at the United States Botanic Garden (USBG) by importing *Rafflesia*-infected host cuttings and *Rafflesia* seeds. Molina et al (2017) have tried to induce germination of *Rafflesia speciosa* seeds with the use of different plant growth regulators (PGRs) including extracts from the host plant *Tetrastigma*. The results showed that there was no germination in the *Rafflesia speciosa* seeds. However, there were four PGRs (GA4+7, KAR1, kinetin, & BRA) that induced germination in wildflower seeds that were used as controls, and these PGRs were combined and applied on the *Rafflesia speciosa* seeds but this treatment was also unsuccessful. Researchers have also tried to stimulate germination on other species such as *Rafflesia patma* Blume, but the *in vitro* germination assays on *Rafflesia* species remains a continuing challenge (Wicaksono et al., 2016). Molina et al (2017) explained that the unsuccessful attempt in germination is a result of not knowing the simulating host chemicals and/or that the seeds tested may be agamospermous (i.e. lacking an embryo), which has been observed in some *Rafflesia* species (Nais, 2001). Recently, Molina et al (2023) identified key genes in the *Rafflesia* seed transcriptome that may be involved in its germination, including genes for response to smoke-related chemical karrikin, as well as genes for laccase enzymes that in the unrelated plant parasite *Striga,* breakdown host tissue to facilitate infection. Thus in the future, it may be worthwhile to incubate *Rafflesia* seeds in laccase and karrikin in the presence of the host to induce germination. In 2017, two uninfected host cuttings (*Testrastigma harmandii* & *Tetrastigma cf. magnum)* that were collected from the Philippines have successfully rooted and are now growing at the USBG. These plants have been used as rootstocks for *Rafflesia*-infected cuttings collected in January 2023. It can take at least a year to know if the graft is completely successful, when a *Rafflesia* bud emerges from the grafted scion.

Since members of Rafflesiaceae are only detectable by their flowers and completely hidden within host tissues before they flower, conservation monitoring of populations has been difficult. Fortunately, Osanthanunkul (2019) successfully detected the presence of *Sapria himalayana* at different sites by using the eDNA (environment DNA)-based monitoring method. The eDNA-based monitoring method uses the amplification of specific DNA fragments that organisms release into the environment. Researchers have applied this monitoring method to aquatic animals, amphibians, and even mammals (Osathanunkul, 2019). Species like *Sapria himalayana* have low chances of survival due to their high bud mortality rate (46-67%) and low fruiting rate (8-12%). The study of Osathanunkul (2019) brought to light a successful monitoring method that could be applied towards conservation monitoring of *Rafflesia* and *Rhizanthes.*

PLANT MICROBIOMES AND ENDOPHYTES

Given the difficulty in propagating members of Rafflesiaceae outside of their natural habitat using conventional horticultural methods, we have been exploring the microbiome of *Rafflesia* and its host *Tetrastigma.* Microbes that reside within plant tissues, or microbial endophytes, play a major role in promoting the growth of plants by producing certain growth regulators and/or protecting their plant hosts from pathogens. Bacterial endophytes that reside in the rhizosphere can enter internal plant tissues through primary and lateral root hair cells, root wounds, and the stomata from leaves and young stems (Santoyo et al., 2016). Proteobacteria is the dominant phylum that is generally found as bacterial endophytes. The two classes that are also found as endophytes are Firmicutes and Actinobacteria. Regarding the genera level, *Pseudomonas*, *Bacillus*, *Burkholderia*, *Strenotrophomonas*, *Micrococcus*, *Pantoea*, and *Microbacterium* are often found as endophytes (Santoyo et al., 2016). These bacterial endophytes have been demonstrated to produce hormones that promote plant growth and protect their plant hosts from pathogens and stressful environmental conditions (Santoyo et al., 2016).

Researchers have started to explore the positive impacts of bacterial endophytes and how they can be applied to improve agriculture. In a study, the endophytic community of sugarcane was investigated and researchers discovered that two strains, *Enterobacter and Shinella* were the true endophytes of commercial sugarcane as they were able to show plant growth promoting effects (Taulé et al., 2016). It is possible that there are unique microbial endophytes that may facilitate *Rafflesia* infection and can serve as bioinoculants to help *Rafflesia* grow ex situ. Like *Rafflesia, Phelipanche aegyptiaca* is a plant parasite which infects tomatoes, *Solanum lycopersicum.* Kruh et al (2017) studied the endophytic communities in non-parasitized tomato roots and parasitized tomato roots and found that when parasitized, the parasite and host had similar endophytic composition compared to the non-parasitized tomato root. This clearly indicates that the connection between the parasitic weed and the tomato host plant serves as a passageway for bacterial endophytes. Moreover, the researchers identified a new *Pseudomonas* sp. and found that it was able to reduce germination of the parasite seeds by 80% without harming the tomato host roots (Kruh et al., 2017). Their findings on the *Pseudomonas* strain could foster the use of biocontrol agents in controlling parasitic weeds, which could replace harmful chemical pesticides that are used in agriculture.

In the research study by Felestrino et al (2017), bacterial endophytes were also studied in the holoparasitic plant, *Langsdorffia hypogaea*, its various hosts, as well as from the soil community surrounding them, i.e the rhizosphere. This study led to the discovery of 81 bacterial isolates. 95% of the isolates were capable of fixing nitrogen and this gave the parasitic plants the ability to convert atmospheric nitrogen for their growth when soils were leached or contaminated with heavy metals. 86% of the isolates produced indoleacetic acid (IAA), which is a hormone that increases root growth and 20% of the isolates inhibited the growth of plant pathogens. This research sheds light on the importance of bacterial endophytes even to plant parasites. Thus, investigation of the bacterial endophytes within members of Rafflesiaceae could reveal how their microbiome could possibly benefit the growth and development of Rafflesiaceae for propagation and conservation.

In this study, the goal is to characterize the associated microbiome of Rafflesiaceae, particularly *Rafflesia speciosa* and *Sapria himalayana* to find "microbial markers" of Rafflesiaceae infection. It is hypothesized that these microbial markers will be present in Rafflesiaceae and their infected host plants but not in uninfected host plants. This study could also reveal "core" endophytic bacteria common in different species of Rafflesiaceae which may serve as bioinoculants to facilitate Rafflesiaceae propagation and conservation.

METHODOLOGY

One of us, JM, collected various samples for microbiome characterization: eight (8) samples of host roots infected with *Rafflesia speciosa,* nine (9) *Rafflesia speciosa* buds, five (5) uninfected samples of host roots, and seven (7) samples of nonhosts (*Tetrastigma* spp. never observed to be parasitized by *Rafflesia*) were collected from Miagao, Iloilo, Philippines. In addition, two (2) buds of *Sapria himalayana* were collected

from Chiang Mai, Thailand. All collections were conducted under the appropriate permits and authorizations.

All samples were surface-sterilized with 2% sodium hypochlorite, rinsed, and processed for 16s microbiome sequencing (Illumina) and metagenomic analyses by Zymo Research (Irvine, CA). To deplete plant 16s rDNA chloroplast reads, sequence-specific peptide nucleic acid (PNA) blockers were used by Zymo. In addition, nanopore metagenomic sequencing was performed on seven *Rafflesia* flower buds to characterize their endophytic bacterial composition. The 16S Barcoding kit (SQK-16S024) was used for library preparation, and sequencing was performed on the MinION Mk1b device (Oxford Nanopore). The EPI2ME 16S-BLAST workflow was used for data processing to identify bacterial endophytes at the genus level.

Bacterial composition and proportions from Zymo's metagenomic analyses were used to make bar charts (Fig. 1-3) to better understand microbiome patterns. EPI2ME analysis of the nanopore reads for the *Rafflesia* buds produced a phylogeny of the bacterial endophytes (Fig. 4). Ecological characteristics of noteworthy bacterial taxa (Table 1) were investigated in the literature (Table 2) to gain insights on how these bacterial endophytes possibly facilitate infection and development of Rafflesiaceae.

RESULTS

Figures 1-3 are bar charts showing bacterial proportions and compositions at phylum, class, and genus level. M-Raffbud, M-Infec, M-Uninfec, and M-Nonhost are *Rafflesia* speciosa-associated samples from Miagao (M), Philippines and T-Sapbud represents the *Sapria* himalayana buds from Chiang Mai, Thailand (T). Figure 4 shows the phylogeny of seven *Rafflesia* buds. Table 1 represents the different bacterial genera that were shared across the samples and Table 2 describes the ecological roles of these genera from the literature.

DISCUSSION

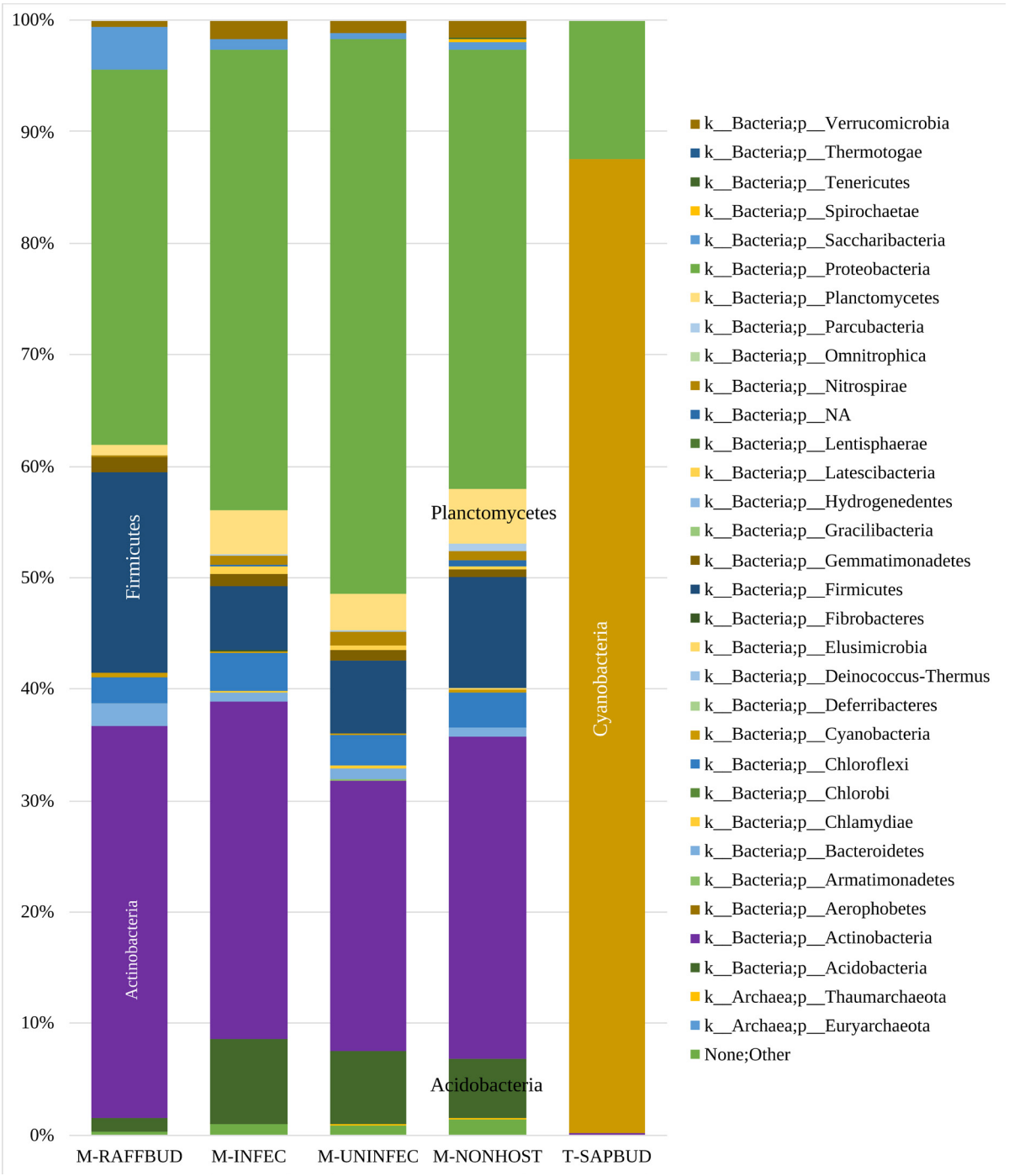
In this study, the goal was to characterize the associated microbiome of Rafflesiaceae, particularly *Rafflesia speciosa* and *Sapria himalayana* to find "microbial markers" of Rafflesiaceae infection, which may have growth-promoting properties that can facilitate Rafflesiaceae propagation and conservation. It was also hypothesized that *Rafflesia* and its host will have similar microbiomes given their intimate connection, similar to what was observed in *Phelipanche* and its tomato host (Kruh et al., 2017). However, results across different taxonomic levels (phylum Fig. 1; class Fig. 2; genus Fig. 3) demonstrate that *Rafflesia* buds have a distinct microbial endophyte composition and possessed some unique genera (Table 1) compared to its infected hosts. The *Tetrastigma* samples, whether nonhost or host, infected or not, visually have similar microbial composition and proportion especially at the Phylum and Class levels (Fig. 1-2). Interestingly, *Rafflesia* and *Sapria* differed dramatically in their microbiomes with *Rafflesia* having much more generic diversity (Fig. 3).

In Fig. 1, *Rafflesia* bud (M-Raffbud) has a slightly different phylum proportion from the *Tetrastigma* roots (M-Infec, M-Uninfec, and M-Nonhost) as it is enriched in Firmicutes, Saccharibacteria, and Actinobacteria, but it is diminished in Planctomycetes and Acidobacteria. Firmicute species are commonly known to have beneficial traits in plant growth promotion and agriculture (Hashmi et al., 2020). In two studies, Saccharibacteria played a major role in bacterial wilt disease suppression (Shen et al., 2018; Zhang et al., 2017). Similarly, Actinobacteria has a disease suppression ability because they are producers of secondary metabolites, which are products synthesized by bacteria that help with survival functions (Hazarika et al., 2020). Firmicutes, Saccharibacteria, and Actinobacteria could possibly provide plant growth promoting properties and protection against pathogenic bacteria to the *Rafflesia* bud as it transitions into a flower. The seemingly very large proportion of phylum Cyanobacteria in *Sapria* bud compared to *Rafflesia* bud, is probably an artifact, most likely from 16s rDNA chloroplast reads that were not affected by the PNA blockers used by Zymo. PNA clamps/blockers suppress plant host chloroplast DNA amplification and prevent host plant contamination to better analyze the bacterial communities in plants (Fitzpatrick et al., 2018). The large proportion of putative chloroplast reads in *Sapria* bud is interesting because Cai et al (2021) reported that *Sapria himalayana* lost its entire chloroplast genome, but the resulting data here suggest otherwise, or at least a remnant 16s rDNA has been retained by *Sapria*.

In Fig. 2, at the Class level, the bacterial composition of *Rafflesia* bud varies as well from the rest of the samples since it has a larger proportion of Clostridia and Coriobacteriia but a diminished proportion of Deltaproteobacteria and Acidobacteria. Clostridia has been shown to be a nitrogen-fixing endophyte present in the grass *Miscanthus sinensis* (Miyamoto et al., 2004). There is not much known about the plant growth promoting traits of Coriobacteriia but Coriobacteriia belongs to the phylum Actinobacteria, well known for enhancing plant growth through direct actions such as nitrogen fixation, phytohormones production, and siderophores production (Boukhatem et al., 2022). These effects could be potentially found in Coriobacteriia and may offer similar plant growth

FIGURE 1

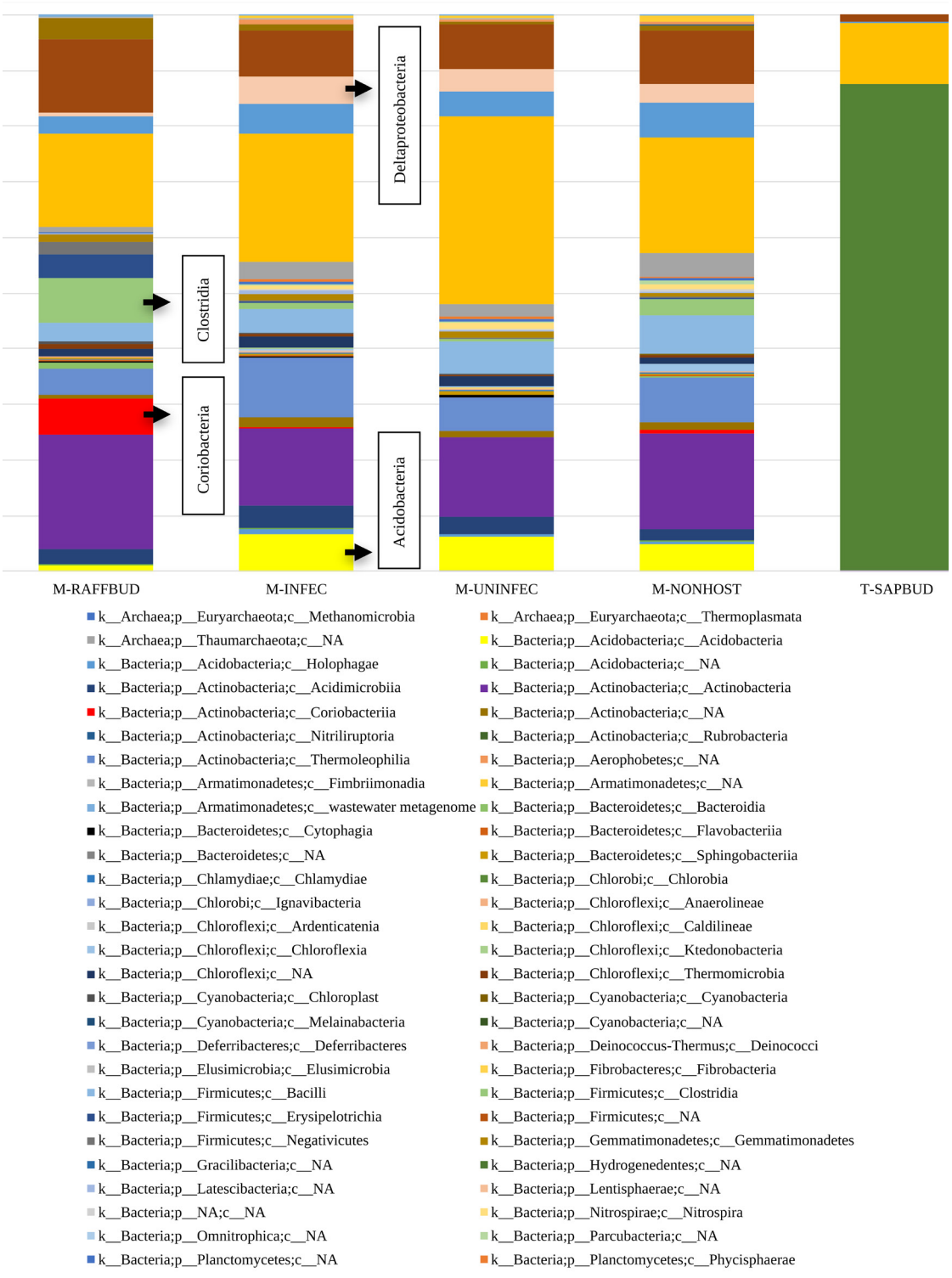
Endophytic Bacterial Proportions and Compositions at Phylum Level of Rafflesiaceae samples from Illumina Sequencing



Miagao *Tetrastigma* roots (M-Infec, M-Uninfec, and M-Nonhost) seem to have similar bacterial compositions and proportions regardless of Rafflesia infection, but M-Raffbud has a slightly different bacterial proportion from the rest of the samples with more Actinobacteria (purple) and Firmicutes (dark blue), yet diminished in Planctomycetes (yellow) and Acidobacteria (dark green). The bacterial compositions of T-Sapbud and M-Raffbud significantly differ from each other. Cyanobacteria largely composed the Sapbud microbiome (>87%, light brown). On the other hand, M-Raffbud only contains 0.34% of Cyanobacteria but has a higher microbial diversity than T-Sapbud.

FIGURE 2.

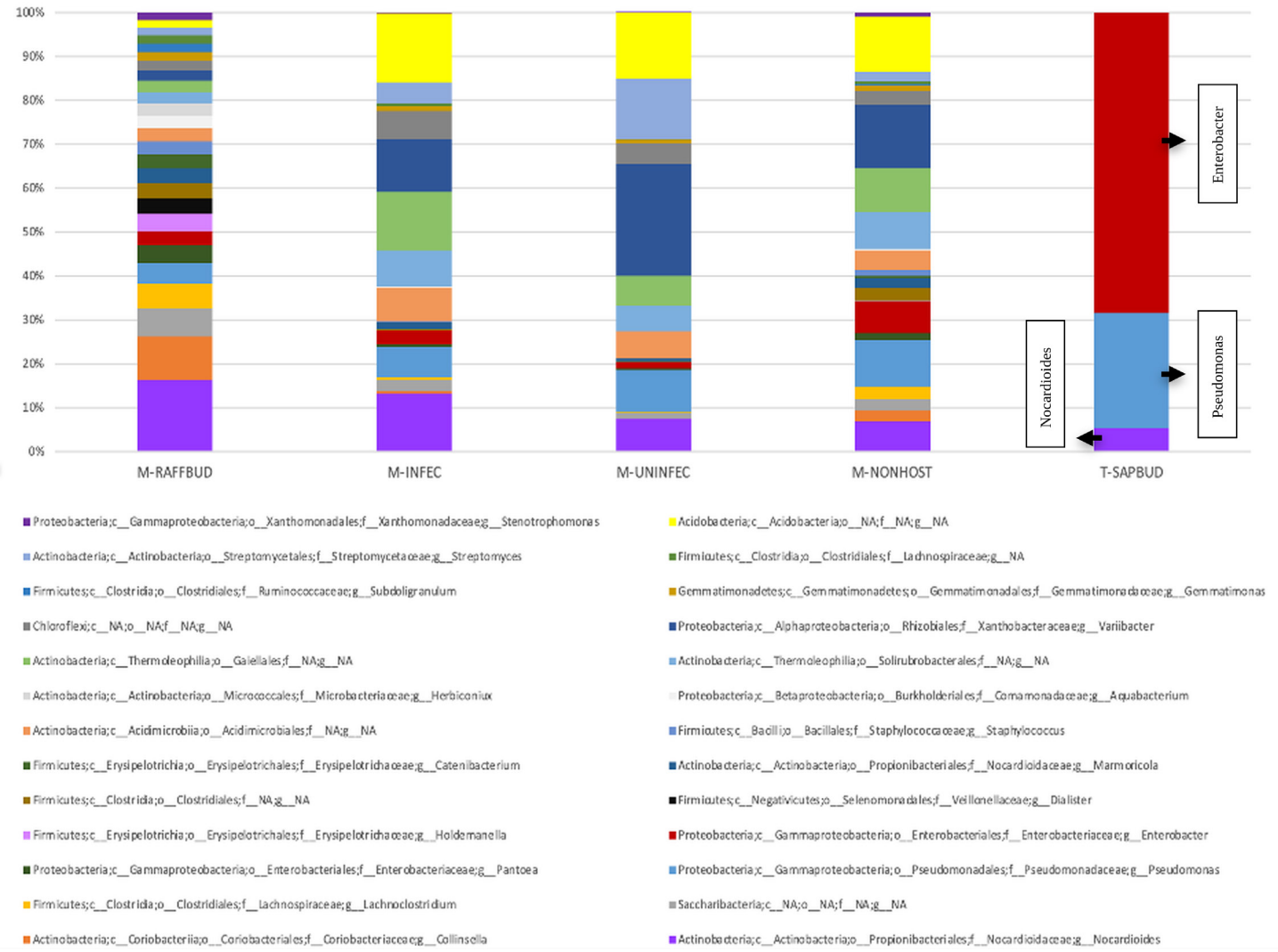
Endophytic Bacterial Proportions and Compositions at Class Level of Rafflesiaceae samples from Illumina sequencing.



Rafflesia buds (M-Raffbud) show bigger proportion of Clostridia (light green), Coriobacteria (red) but diminished proportion of Deltaproteobacteria (beige) and Acidobacteria (yellow) compared to the Miagao *Tetrastigma* roots, whether infected or not. The *Tetrastigma* roots (M-Infec, M-Uninfec, and M-Nonhost) seem to share similar bacterial compositions. The bacterial composition of T-Sapbud is significantly different from M-Raffbud.

FIGURE 3.

Endophytic Bacterial Proportions and Compositions at Genus Level of Rafflesiaceae samples from Illumina sequencing.



This is the top 0.1% genera in *Rafflesia* (M-Raffbud) and *Sapria* (T-Sapbud) buds, which differ dramatically from each other in bacterial genera composition and proportions. *Sapria* bud only had 3 genera: *Enterobacter, Nocardioides, Pseudomonas* which were all present in the other samples as well. M-Raffbud also shows a different composition compared to the *Tetrastigma* samples, infected or not. *Herbiconiux* was among the top 0.1% unique genera in *Rafflesia* bud. Other unique M-Raffbud taxa are listed in Table 1.

TABLE 1.

*Endophytic bacterial genera shared across samples from Ilumina sequencing. (RB=Raffbud, SB=Sapbud, TH=*Tetrastigma *host, NH=*Tetrastigma *nonhost)*

Genus	Unique to RB	Present in both RB/SB	Unique to RB/TH	Unique to NH
Holdemanella (P. Firmicutes)				
Dialister (P. Firmicutes)				
Herbiconiux (P. Actinobacteria)				
Prevotella (P. Bacteroidetes)				
Olsenella (P. Actinobacteria)				
Bifidobacterium (P. Actinobacteria)				
Propionibacterium (P. Actinobacteria)				
Salinibacterium (P. Actinobacteria)				
Buttiauxella or *Lelliottia* (P. Proteobacteria)				
Pseudomonas (P. Proteobacteria)				
Enterobacter (P. Proteobacteria)				
Kluyvera (P. Proteobacteria)				
Rhizobium (P. Proteobacteria)				
Novosphingobium (P. Proteobacteria)				
Klebsiella (P. Proteobacteria)				
Pantoea (P. Proteobacteria)				
Erwinia (P. Proteobacteria)				
Flavobacterium (P. Bacteroidetes)				
Kosakonia (P. Proteobacteria)				
Mucilaginibacter (P. Bacteroidetes)				
Shinella (P. Proteobacteria)				
Rathayibacter (P. Actinobacteria)				
Lactovum (P. Firmicutes)				
Arthrobacter or Glutamicibacter (P. Actinobacteria)				

TABLE 2.

Ecological characteristics of bacterial genera from Table 1.

Genus	Ecology of the bacteria	References
Holdemanella (RB)	This is an endophyte composing the microbiome of bamboo shoots but its ecological role is unclear.	Cui et al., 2021
Dialister (RB)	This is an endophyte found in watermelon seed embryos but its ecological role is unclear.	Thomas et al., 2021
Herbiconiux (RB)	This is an endophyte found in yerba mate (*Ilex paraguariensis*) and was positive for nitrogen fixation. Amidase, endoglucanase, phosphatase, and superoxidative dismutase were retrieved and classified as gene-clusters with plant growth-promotion (PGP) functions such as IAA-production, cellulose-degradation and anti-oxidant effects.	Pérez et al., 2016
Prevotella (RB)	This is one of the bacterial genera found in the leaves of *Eucalyptus tereticornis* but ecological role is unclear.	Dasgupta et al., 2020
Olsenella (RB)	No ecological information found in the literature.	N/A
Bifidobacterium (RB)	The strain *Bifidobacterium longum subsp. longum* is associated with metabolizing plant-derived poly/oligosaccharides, hemicellulose, and pectin-associated glycans.	Kelly et al., 2021
Propionibacterium (RB)	This is an endophyte found in seeds of *Crotalaria pumila* growing on metal sites in Mexico but ecological role is unclear.	Sánchez-López et al., 2018
Salinibacterium (RB)	*Salinibacterium* was found in *Stevia* leaf and its relative abundance was positively correlated with plant height.	Yu et al., 2015
Buttiauxella or Lelliottia (RB)	The endophytic bacterium, *Buttiauxella* sp. SaSR13 was able to improve the growth of *Sedum alfredii* by 39 and 42% for shoot and root biomass.	Wu et al., 2018
Pseudomonas (RB/SB)	Researchers investigated the effect of the nitrogen-fixing strain, *Pseudomonas stutzeri* A15, and discovered that A15 promoted the growth of rice seedlings. In another research study, the strain, *Pseudomonas brassicacearum* CDVBN10 was selected as a promising candidate to biofertilize rapeseed crops.	Pham et al., 2017; Jiménez-Gómez et al., 2020
Enterobacter (RB/SB)	*Enterobacter* spp. are known to have many plant growth promoting characteristics that involve nitrogen fixation, soil phosphorus solubilisation, production of antibiotics, ability to secrete siderophores, etc.	Jha et al., 2011
Kluyvera (RB/SB)	The bacterial strain EN4 (*Kluyvera ascorbata*) was isolated from cabbage and it was able to reduce *Plutella xylostella*.	Laurentis et al., 2014
Rhizobium (RB/SB)	*Rhizobium* is a major contributor to nitrogen fixation, especially in leguminous plants. Researchers discovered *Rhizobium* species living in the stems of poplar trees, allowing them to grow in nutrient-poor soil.	Zahran, 1999; Doty et al., 2005
Novosphingobium (RB/SB)	*Novosphingobium* sp. PS5 was selected for its plant growth-promoting potential in optimizing rice cultivation.	Rangjaroen et al., 2017
Klebsiella (RB/SB)	The nitrogen-fixing *Klebsiella variicola* strain DX120E was shown to promote sugarcane growth.	Lin et al., 2015

Table 2 continues on page 13

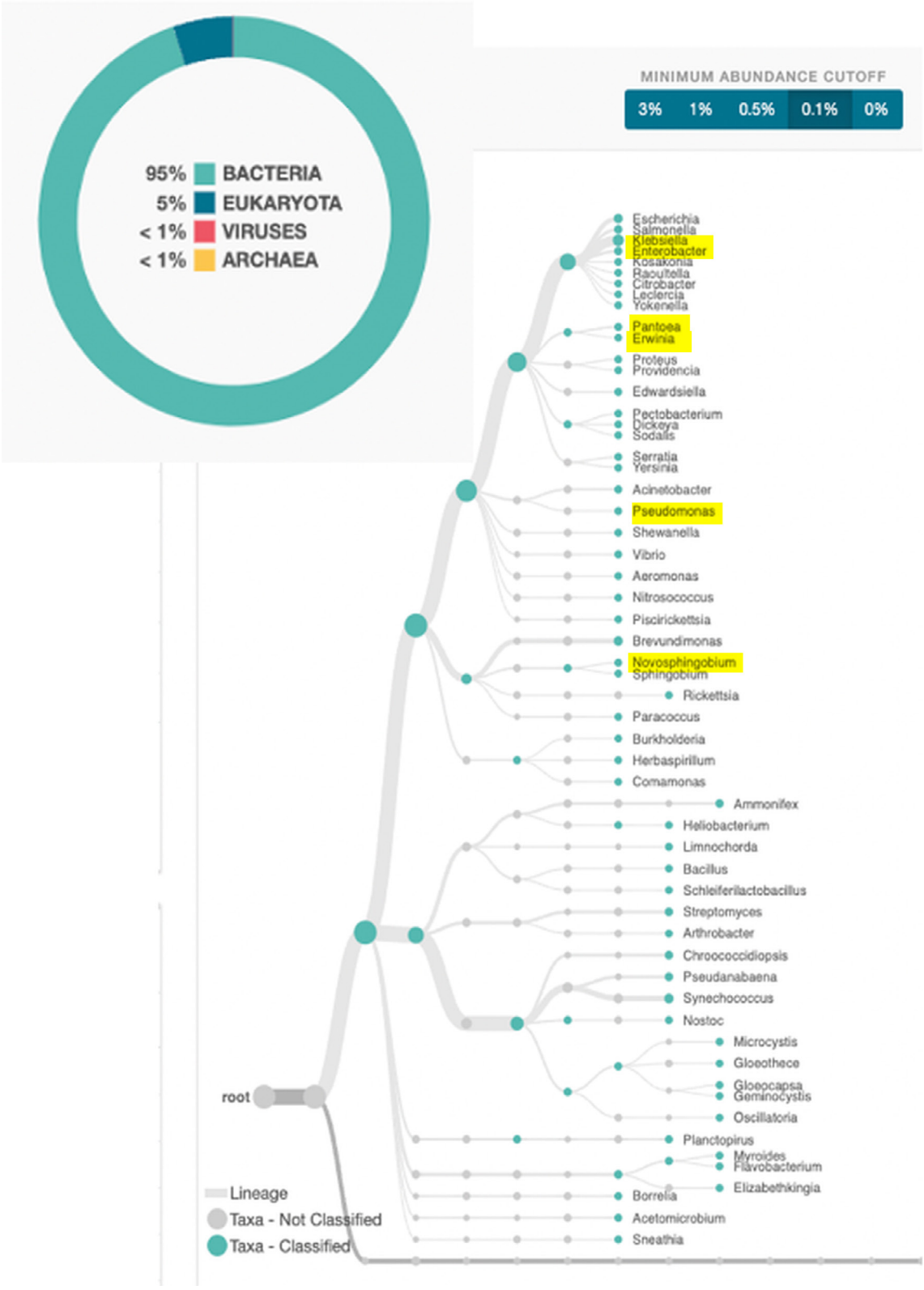
Table 2 continued from page 12

Genus	Ecology of the bacteria	References
Pantoea (RB/SB)	In a study, *Pantoea agglomerans* MK-29 significantly reduced *Meloidogyne incognita* nematode infestation on tomato roots.	Feng et al., 2006
Erwinia (RB/SB)	The endophytic bacterium, *Erwinia gerundensis* strain (A4) was tested for plant growth-promoting ability and the results showed that A4 had a positive effect growth on *Arabidopsis thaliana* as it increased its root hair length.	Guzmán et al., 2021
Flavobacterium (RB/TH)	The genus *Flavobacterium* harbors many extra-cellular macromolecular-degrading enzymes. Some strains have been found to possess H2-oxidizing and ACC (1-aminocyclopropane-1-carboxylate) deaminase activities, which have been correlated to plant growth promotion.	Kolton et al., 2016
Kosakonia (RB/TH)	*Kosakonia* sp. UYSO10 was able to promote sugarcane growth.	Taulé et al., 2019
Mucilaginibacter (RB/TH)	*Mucilaginibacter* is known to have many roles in plant growth promotion. In a study, *Mucilaginibacter* was shown to alleviate salt stress in the plant model, *Arabidopsis thaliana.* In addition, they are bacteria that are capable of degrading pectin, xylan, laminarin and some other polysaccharides.	Fan et al., 2020; Broccanello et al., 2022; Pankratov et al., 2007
Shinella (RB/TH)	In 2012, *Shinella* sp.UYSO24 was identified as a potential endophytic diazotroph for sugarcane (*Saccharum officinarum L.*) In 2016, the researchers identified strain *Shinella* sp.UYSO24 as a true endophyte of sugarcane plants.	Taulé et al., 2012; Taulé et al., 2016
Rathayibacter (RB/TH)	In a study, researchers observed plant growth for pine seedlings that were inoculated with *Rathayibacter tanaceti* AN2r.	Padda et al., 2019
Lactovum (NH)	Cells of the genus *Lactovum* are chemoorganotrophic and characterized by an aerotolerant anaerobic metabolism that yields lactate.	Drake et al., 2014
Arthrobacter or Glutamicibacter (NH)	*Arthrobacter* was isolated from tomato rhizosphere and demonstrated plant growth promoting activities such as indole acetic acid (IAA) production. *Glutamicibacter* sp. FBE-19 was isolated based on its strong antagonism to the cucurbit bacterial blight pathogen *Erwinia tracheiphila* on plates. Members of the *Glutamicibacter* genus can promote plant growth under saline conditions and antagonize fungi via chitinolytic activity.	Banerjee et al., 2010; Fu et al., 2021

(RB=Raffbud, SB=Sapbud, TH=*Tetrastigma* host, NH=*Tetrastigma* nonhost). If there are two bacterial genera in the column, the taxonomic identity was ambiguous based on the database.

FIGURE 4.

Phylogeny of bacterial endophytes in Rafflesia buds.



This phylogeny accounts for the top 0.1% in 7 *Rafflesia* buds with a total of 1,792,958 reads from Nanopore. The highlighted genera are also found in results from Illumina sequencing (cf. Table 1).

promoting benefits to *Rafflesia* bud.

At the genus level (Fig. 3), *Rafflesia* bud is quite different from the other samples in terms of microbial composition. It was hypothesized that *Rafflesia* buds will have a similar bacterial proportion and composition as its infected *Tetrastigma* hosts (M-Infec) but this was not the case with *Rafflesia* bud harboring some unique bacterial genera such as *Holdemanella, Dialister, Herbiconiux*, *Prevotella*, *Olsenella*, *Bifidobacterium*, *Propionibacterium*, *Salinibacterium* and *Buttiauxella-Lelliottia* (Table 1 and Table 2). There was no ecological role found for *Holdemanella* or *Olsenella* but *Holdemanella* is under phylum Firmicutes and as mentioned earlier, Firmicutes species are commonly known for having plant growth promoting traits (Hashmi et al., 2020). As for *Dialister*, there was no sufficient information on its plant growth promoting benefits but *Dialister* also belongs to phylum Firmicutes, and it was found as one of the abundant genera in watermelon seed embryos (Thomas et al., 2021). Many of the unique genera in *Rafflesia* bud that were found in the literature have plant-growth promoting properties such as nitrogen fixation and increase in plant biomass. In a research study, the endophyte *Herbiconiux* was positive for nitrogen fixation (Pérez et al., 2016). Another endophytic bacterium, *Buttiauxella* was able to improve the shoot and root biomass of *Sedum alfredii* by 39 and 42% respectively. There were unique genera in *Rafflesia* bud that were not detected in its associated host, which is surprising. It is possible that these were vertically transmitted via the *Rafflesia* seed when it germinated inside the host, and may be bacteria that do not readily cross between the host and parasite. These unique genera may be important to the germination of *Rafflesia* seeds and may facilitate propagation attempts from seed.

Excluding the very high proportion of chloroplast reads, *Sapria* bud had three genera having at least 0.1% proportion: *Enterobacter, Nocardioides, Pseudomonas,* which were also present in other samples *(Rafflesia* and *Tetrastigma)*. Even though *Sapria* bud only had three unique genera, they all demonstrated plant growth promoting benefits that involved production of antibiotics, plant growth under salinity stress, and plant growth on seedlings. *Enterobacter* species are known for their production of antibiotics, nitrogen-fixation ability, and secretion of siderophores. (Jha et al., 2011). In a study, the actinobacterium endophyte, *Nocardioides* sp. NIMME6 had many plant-growth promoting effects that allowed for the growth of wheat plants under salinity stress (Meena et al., 2020). In addition, the nitrogen-fixing strain, *Pseudomonas stutzeri* A15 promoted the growth of rice seedlings (Pham et al., 2017). These genera in *Sapria* bud may also serve as bioinoculants that can facilitate *Sapria* propagation.

There were other genera (not among the top 0.1%) shared by *Rafflesia* bud and *Sapria* bud such as *Kluyvera, Rhizobium, Novosphingobium, Klebsiella, Pantoea, and Erwinia* (Table 1, 2). These taxa were also found in the *Tetrastigma* samples so are not unique to Rafflesiaceae. The plant growth promoting traits of the genera that were shared in *Rafflesia* and *Sapria* bud included nitrogen fixation and plant growth promoting ability. For example, the genera *Rhizobium* is a major contributor to nitrogen fixation, especially to the stems of poplar trees, which are trees that have the ability to grow in nutrient-poor soil (Zahran, 1999; Doty et al., 2005). The nitrogen-fixing *Klebsiella variicola* strain DX120E was able to promote sugarcane growth (Lin et al., 2015). *Rhizobium* and *Klebsiella* are diazotrophic endophytes and they could both potentially provide nitrogen fixation to *Rafflesia* and *Sapria.* The endophytic bacterium, *Erwinia gerundensis* strain (A4) had a positive effect growth on *Arabidopsis thaliana* as it increased its root hair length (Guzmán et al., 2021). These genera were shared by *Rafflesia, Sapria,* and *Tetrastigma* samples. The shared genera between the samples could potentially be common endophytic taxa of plants.

Furthermore, *Rafflesia* buds and its *Tetrastigma* host also share genera, as expected given their intimate association. These shared genera include *Flavobacterium*, *Kosakonia*, *Shinella, Mucilaginibacter* and *Rathayibacter*. *Kosakonia* sp. and *Shinella* sp. were reported to promote sugarcane growth (Taulé et al., 2016; Taulé et al., 2019). *Mucilaginibacter* is known to have many roles in plant-growth promotion, and it was shown to alleviate salt stress in the plant model, *Arabidopsis thaliana* (Fan et al., 2020; Broccanello et al., 2022). *Flavobacterium* strains have been found to possess H2-oxidizing and 1-aminocyclopropane-1-carboxylate deaminase activities (ACC), which have been correlated with plant growth promotion (Maimaiti et al., 2007; Kolton et al., 2016). H2 plays a key role in plant development, with positive effects on seed germination as well as root elongation (Li et al., 2018). Bacteria that produce ACC deaminase activities can directly promote and protect plant growth (Glick, 2014)

Though the genus *Tetrastigma* is the sole host to Rafflesiaceae species, not all *Tetrastigma* species can serve as hosts. In fact, only 11 of 95 *Tetrastigma* species serve as *Rafflesia* hosts (Pelser et al., 2016). There may be unique microbiota in *Tetrastigma* non-host species that could be antagonistic to *Rafflesia* infection such as *Lactovum*, *Arthrobacter*, and *Glutamicibacter* which were identified in non-host species in this study. Cells of the genus *Lactovum* yield lactate; lactic acid bacteria strains have been considered promising candidates in agriculture for their ability to promote plant growth and show antagonistic effects against pathogens (Drake et al., 2014; Raman et al., 2022). *Arthrobacter* demonstrated plant

growth promoting activities such as indole acetic acid (IAA) production and *Glutamicibacter* was able to promote plant growth under saline conditions and antagonize the pathogen, *Erwinia tracheiphila* (Banerjee et al., 2010; Fu et al., 2021). Perhaps these unique bacteria somehow suppress *Rafflesia* infection conferring some immunity to the *Tetrastigma* non-host species.

The phylogeny of endophytic bacteria in *Rafflesia* bud from Nanopore in (Fig. 4) shows six genera that were also characterized by Zymo (Table 1). They are *Klebsiella*, *Enterobacter*, *Pantoea*, *Erwinia*, *Pseudomonas*, and *Novosphingobium*. However, there were also discrepancies between the two datasets when comparing the *Rafflesia* buds. In Zymo, there were 87 genera with at least 0.1% abundance, while in Nanopore sequencing, 57 genera were characterized with 0.1% abundance (Fig 4). An explanation to why Zymo and Nanopore have differences in genera could be because the *Rafflesia* buds samples were collected at different sites. Also, it is possible that Zymo and Nanopore used different 16S primers sets. An issue that arose with Nanopore sequencing was not having sufficient microbial DNA concentration even after experimenting with various extraction kits.

In conclusion, this research has provided insight into the microbiome of Rafflesiaceae species. Microbial taxa present in both *Rafflesia* and *Sapria* bud could be potentially used as bioinoculants in propagation attempts of these horticulturally recalcitrant taxa. The identification of unique bacterial taxa in *Rafflesia* buds despite its close association with its host is interesting and may have been vertically transferred via *Rafflesia* seeds and could thus be vital in stimulating *Rafflesia* seed germination in vitro or in planta. The identification of unique bacteria in *Tetrastigma* non-host species could suggest that there are endophytic microbes that could confer some immunity to non-host species and ecologically antagonize *Rafflesia* infection. This study unveils the symbiotic microbes of *Rafflesia* and *Sapria* and their potential horticultural application in propagating the world's largest flowers.

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