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Dual-marker amplicon profiling of bacterial and fungal communities associated with the rose aphid *Macrosiphum rosae*

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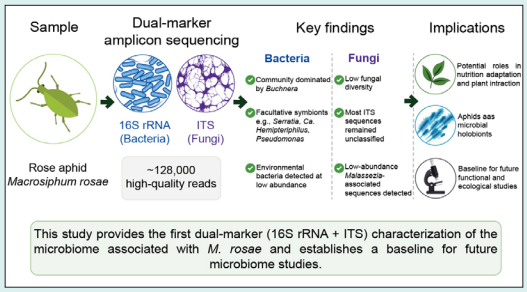
Abstract

Introduction: Microbiome studies of insect organisms are vital in understanding their physiological function and ecological adaptation, yet there is insufficient information on the fungal component in aphids.

Methods: This pilot study provides a preliminary characterization of the bacterial and fungal communities associated with *Macrosiphum rosae* based on 16S rRNA and ITS amplicon sequencing. Amplicon-based microbial profiling using an Illumina sequencing platform was performed based on 16S rRNA gene sequences and ITS markers obtained from a pooled aphid sample. After quality filtering of the reads, analysis was carried out to classify the microbiomes associated with the aphid samples.



Results: The bacterial community was strongly dominated by the obligate symbiont *Buchnera*, whereas lower-abundance taxa included *Serratia*, *Candidatus* Hemipteriphilus, and *Pseudomonas*. Phylogenetic analyses of sequences showed that there were clear phylogenetic clusters corresponding to known bacterial lineages. On the other hand, fungi exhibited low diversity and comprised mostly *Malassezia restricta* and *M. arunalokei*.

Conclusion: These results show that the bacterial community is dominated by obligate and facultative symbionts, while the diversity of fungi is relatively low. The results, therefore, support the concept of aphids as holobionts. These findings provide an initial baseline for future replicated investigations of aphid-associated microbial communities.

Introduction

Rose (genus *Rosa*; family Rosaceae) is one of the most economically valuable flowering plants across the world and is extensively used for landscaping, cut flowers, perfumes, cosmetic, pharmaceutical industries, etc.[1,2] Despite being economically valuable plants, roses are very vulnerable to insect pests, where aphids have been recognized as one of the most damaging pests of roses.

Aphids comprise one of the most diverse and economically important groups of phytophagous insects, with approximately 5,000 described species worldwide.[3] Their ecological and evolutionary success is largely attributed to cyclical parthenogenesis, a high reproductive rate, fast population growth, and extreme phenotypic plasticity.[4,5] Under suitable environmental conditions, aphid populations grow rapidly, resulting in considerable damage to crops across different agricultural systems.[6] Aphids affect plants by feeding on phloem sap and producing honeydew, thus reducing vigor, aesthetics, and economic value.[7,8] In addition to direct feeding damage, aphids are efficient vectors of numerous plant viruses, resulting in substantial economic losses in agriculture, horticulture, and forestry.[9,10]

Among these insects, the rose aphid (*Macrosiphum rosae*) is one of the predominant herbivores of cultivated roses around the world. The colonies of *M. rosae*

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colonize young shoots, flower buds, expanding leaves, and inflorescences, where they feed extensively by ingesting phloem sap through their piercing-sucking mouthparts.[11,12] Feeding by aphids causes leaf curling, chlorosis, shoot stunting, flower bud deformation, and premature leaf senescence.[13,14] Under heavy infestation, prolonged phloem feeding substantially reduces plant vigor and diminishes the commercial and ornamental quality of cut flowers. Furthermore, honeydew excreted by *M. rosae* promotes the growth of sooty mold fungi, which interfere with photosynthesis and reduce overall plant performance.[8,15] Rose aphids have been identified as a vector of several plant viruses, including pea mosaic virus, cauliflower mosaic virus, and cabbage black ring spot virus.[16]

The ecological and evolutionary success of aphids is closely linked to their symbiotic associations with microorganisms. Feeding on phloem sap, which is rich in carbohydrates but deficient in essential amino acids, aphids depend on the obligate intracellular bacterium *Buchnera aphidicola* to compensate for these nutritional deficiencies.[17-19] This primary endosymbiont, a member of the Gammaproteobacteria, resides within specialized host cells (bacteriocytes) and is vertically transmitted with remarkable fidelity across generations.[18,20]

The long-standing partnership between *B. aphidicola* and aphids represents one of the best-characterized examples of insect-microbe coevolution and is marked by extensive metabolic integration and strong interdependence between host and symbiont.[21-23] In addition to *Buchnera*, many aphid species are associated with several facultative secondary symbionts, such as *Serratia symbiotica*, *Hamiltonella defensa*, *Regiella insecticola*, *Rickettsia*, *Wolbachia*, *Spiroplasma*, and *Arsenophonus*.[24,25] The facultative symbionts differ from the obligatory symbionts as they do not occur in all populations of aphids, and their presence is determined by the host genotype and environmental factors.[26] However, these bacteria can significantly increase aphid fitness by conferring ecologically principal traits, including thermotolerance, resistance to parasitoids and pathogens, adaptation to host plants, and reproductive manipulation.[27-30] Collectively, these various microbial associations support the view of aphids as holobionts, in which the host and its related microbiota function as an integrated biological unit rather than as independent organisms.[20,21,24,31]

The recent developments in next-generation sequencing (NGS) platforms have considerably enhanced the characterization of microbial communities in insects by providing culture-free and precise analyses of microbiomes.[32] Amplicon sequencing of the bacterial 16S ribosomal RNA (16S rRNA) gene and the fungal internal transcribed spacer (ITS) region enables comprehensive characterization of bacterial and fungal community composition without prior assumptions regarding microbial diversity. It has been shown that the microbial community associated with aphids is much more complex and dynamic than previously recognized using conventional polymerase chain reaction (PCR) methods.[33,34] Although numerous studies on aphid symbiosis have been published recently, current knowledge remains heavily biased toward a few model species, particularly *Acyrthosiphon pisum*.[24,35] In contrast, the microbiomes of many economically important aphid species, including *M. rosae*, remain poorly characterized. Moreover, whereas bacterial symbionts have been extensively investigated, the fungal component of the aphid microbiome has received comparatively little attention. These knowledge gaps highlight the need for integrated characterization of both bacterial and fungal communities in non-model aphid species.

Although the rose aphid, *M. rosae*, is an economically and ecologically important insect, its microbial community has not been well explored, especially in the context of a combined characterization of bacteria and fungi. In light of this, the present exploratory study was conducted to provide an initial examination of the bacterial and fungal communities associated with *M. rosae* via amplicon-based sequencing of bacterial 16S rDNA and fungal ITS regions. Through the characterization of both bacteria and fungi, this study adds to the growing body of knowledge about the microbial community of the rose aphid.

Materials and Methods

Sample collection

Adult specimens of *Macrosiphum rosae* (L.) were collected from rose (*Rosa* spp.) leaves collected from Tehran, Iran, in August 2024. Taxonomic confirmation of the specimen was conducted by an expert entomologist (Dr. Mehrparvar). In order to prevent any contamination of the environment, aphids were obtained using sterile forceps and kept on ice in sterile microcentrifuge tubes. Aphid biomass, weighing around 25 mg, was taken from multiple individuals from the same colony for molecular analyses.

As this experiment was carried out as a pilot study, only one sample was tested to determine the baseline composition of the microbial communities associated with aphids.

DNA extraction

The genomic DNA was extracted from whole aphids using a modified CTAB procedure. About 25 mg of aphid tissue was crushed in 200 µL of the extraction buffer (1 M Tris-HCl, pH 8.0; 10% CTAB; 5 M NaCl; 50 mM EDTA) using a sterile micro-pestle.

Digestion of proteins was done by incubating the homogenate with 20 µL Proteinase K (10 mg/mL) for 1 hour at 60 °C. Removal of RNA was accomplished through the addition of 4 µL RNase solution and incubated at 30 °C for 10 minutes.

After lysis, an equal volume of chloroform: isoamyl alcohol (24:1) was added, and the sample mixture was centrifuged for 10 minutes at 10,000 rpm. The aqueous layer was collected, and the DNA was precipitated with

cold isopropanol at −20 °C for 40 minutes. This DNA pellet was washed with 70% ethanol, air-dried, and dissolved in 50 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Negative controls for DNA extraction and the PCR process were included.

DNA quality assessment

The concentrations and purity of the DNA were measured using a NanoDrop spectrophotometer using the A260/A280 ratio. The DNA quality was checked by agarose gel electrophoresis using 0.8% gels in 1x TBE buffer.

In order to check the suitability of extracted DNA for further amplifications, the COI gene located in the mitochondrial cytochrome c oxidase subunit I was amplified using two primers LCO1490 and HCO2198 (Table 1).

PCR reaction mixtures (20 µL) contained 10 µL of 2×Master Mix (Amplicon), 1 µL each of both primers, 1 µL of genomic DNA, and nuclease-free water. The thermocycler program included initial denaturation at 95 °C for 5 minutes; then 35 cycles of 95 °C for 30 seconds, 45 °C for 30 seconds, and 72 °C for 1 minute, finishing with final extension at 72 °C for 10 minutes.

Library preparation and amplicon sequencing

A high-quality sample of genomic DNA was analyzed at BGI (Shenzhen, China) through the sequencing of the bacterial 16S rRNA and fungal ITS amplicons. Also, library preparation, PCR amplification, and sequencing were performed by BGI (Shenzhen, China) using their standardized commercial amplicon sequencing workflow. Sample processing and quality control followed the provider's internal standard operating procedures. The near full-length sequences of the bacterial 16S rRNA and fungal ITS genes were amplified by a universal set of primers included in the sequencing protocols of the provider. The sequencing was carried out using the Illumina platform under the manufacturer's protocols.

Bioinformatic processing

The initial bioinformatics processing was done using the BGI microbial analysis pipeline. This process involved demultiplexing the reads, trimming adapter sequences, and removing low-quality reads with respect to a minimum threshold of quality ($Q \geq 30$).

High-quality reads were aligned and assembled into Operational Taxonomic Units (OTUs) with 97% sequence similarity.

Data analysis

Subsequent analyses were done by utilizing CLC Genomics Workbench version 20 together with the Microbial Genomics Module.

Quality control and preprocessing

The quality of sequencing was judged on the basis of base quality distribution, GC content, and read lengths. Sequences with lengths below 50 bp, ambiguous base sequences, and sequences contaminated with adapters were filtered out from the dataset. The detection and removal of chimeras was carried out as well. A total of 127,925 high-quality sequences were retained for further analysis.

Taxonomic assignment

For the taxonomic classification of 16S rRNA bacterial sequences, the SILVA database (v128) was used, setting the minimal identity level to 97%. Fungal ITS sequences were subjected to taxonomic assignment using the UNITE dynamic database (v9.0) with a 97% identity level.

Diversity analyses

Taxonomic profiling primarily focused on characterizing microbial communities based on their taxonomic composition, as bacteria and fungi are present in association with *M. rosae*. Distributions of relative abundances of microbial communities were assessed at various taxonomic levels to identify abundant as well as rare microbes. Since this was an exploratory study utilizing only one composite sample, no diversity or multivariate analyses were conducted.

Phylogenetic analysis

OTU sequences were aligned by means of multiple sequence alignments through the CLC pipeline. The Neighbor-Joining method for inferring phylogeny was applied through MEGA11. Tree topology stability was tested through bootstrap analysis with 1,000 replications.

Statistical considerations

In light of the exploratory nature of the study, along with the lack of biological replication due to the application of a pooled sample, no statistical comparisons or multivariate ordination were performed since they would have required biological replication. This analysis was centered on the descriptive characterization of the microbial communities sampled and their diversity.

Results

Sequencing output and data overview

A total of around 144,287,012 raw reads were obtained in the sequencing process. After filtering out any reads based on their target specificity, quality, chimeras, and pre-processing, approximately 127,925 high-quality

Table 1. Primers for the COI gene

Name	Sequence	Length (bp)	Tm (ºC)	%GC
LCO1490	GGTCAACAAATCATAAAGATATTGG	25	57.4	32.0
HC02198	TAAACTTCAGGGTGACCAAAAAATCA	26	63.6	34.6

reads corresponding to bacterial 16S rRNA and fungal ITS datasets were retained following preprocessing and quality filtering. Out of these reads, roughly 63,050 reads represented bacterial 16S rRNA sequences, whereas around 64,875 reads represented fungal ITS sequences.

Data quality assessment

Read length distribution and quality evaluation of 16S rRNA

The read-length distribution of trimmed 16S rRNA sequences showed a narrow range of values (1500-1519 bp), suggesting the presence of nearly full-length 16S rRNA amplicons produced in library preparation (Fig. 1a). The assessment of PHRED values showed that the majority of the bases had a quality score within the range of Q25-Q45 (Fig. 1b), suggesting excellent base calling accuracy.

Read length distribution and base quality evaluation of ITS Data

Post-quality assessment, 64,875 ITS reads were acquired. Read lengths varied between 830 bp and 989 bp, where the most common length was between 830 and 839 bp, with a second mode at 980-989 bp. The distribution pattern is expected considering primer design and inherent variation of ITS regions (Fig. 2a). Analysis of PHRED scores demonstrated that the bases had scores between Q25-Q45, which confirmed the quality of the reads. Furthermore, no ambiguous bases were detected, implying high quality of data (Fig. 2b).

Sequencing summary metrics

To illustrate the general quality and dependability of the processed sequencing data, Table 2 summarizes key

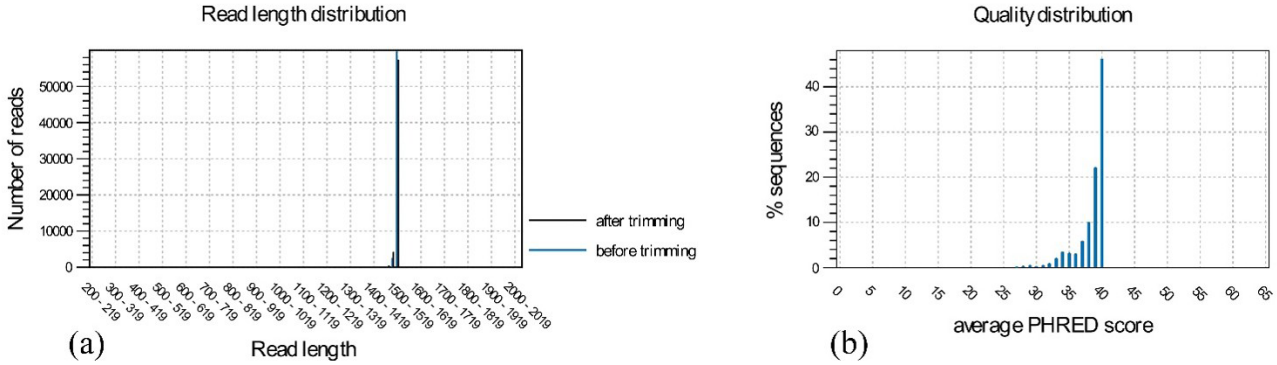

Fig. 1. Quality assessment of the bacterial 16S rRNA sequencing dataset following preprocessing and trimming. (a) Length distribution of quality-filtered 16S rRNA amplicon sequences. The x-axis represents sequence length (bp), and the y-axis indicates the number of retained reads. Most sequences were concentrated within a narrow length range (1500–1519 bp), indicating high consistency among the processed amplicons. (b) Distribution of PHRED quality scores for quality-filtered 16S rRNA reads. The x-axis represents PHRED quality score values, whereas the y-axis indicates read frequency. The predominance of high PHRED scores reflects the overall quality of the sequencing dataset.

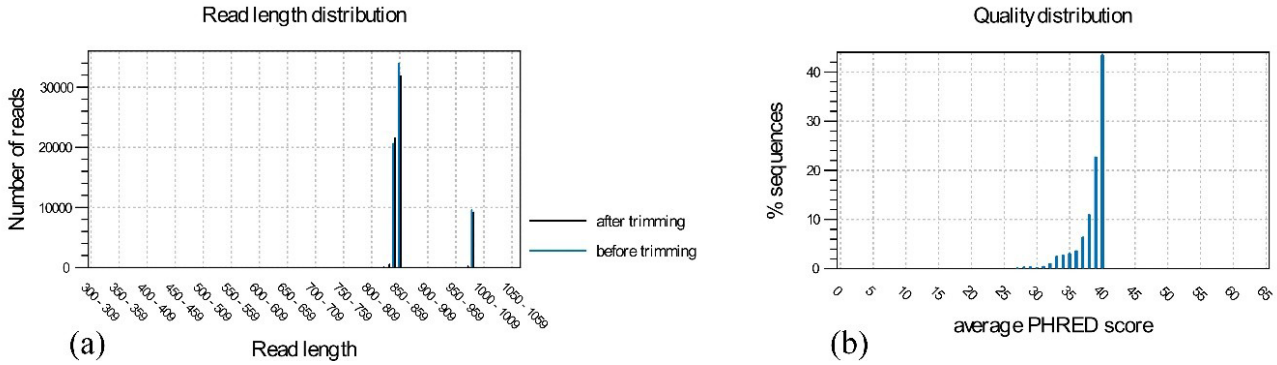

Fig. 2. Quality assessment of the fungal ITS sequencing dataset following preprocessing and quality filtering. (a) Length distribution of quality-filtered ITS amplicon sequences. The x-axis represents sequence length (bp), whereas the y-axis indicates the number of retained reads. The observed distribution reflects the size variation characteristic of fungal ITS amplicons after trimming and filtering. (b) Distribution of PHRED quality scores for quality-filtered ITS reads. The x-axis represents PHRED quality score values, and the y-axis indicates read frequency. The predominance of high-quality scores indicates the overall reliability of the ITS sequencing dataset.

Table 2. Summary of preprocessing and quality-filtering statistics for bacterial 16S rRNA and fungal ITS amplicon sequencing datasets obtained from *M. rosae* samples

Trim	Input reads		Retained Reads		Trimmed Reads		Discarded Reads	
	16S rRNA	ITS	16S rRNA	ITS	16S rRNA	ITS	16S rRNA	ITS
Trim on quality	63050	64875	57148	59770	5902	5105	0	0
Ambiguity trim	63050	64875	63050	64875	0	0	0	0
Filter on length	63050	64875	63050	64855	0	0	0	20

quality-filtering parameters for the bacterial 16S rRNA and fungal ITS sequencing datasets, such as retained read counts and filtering performance.

Bacterial community composition

A taxonomic analysis of high-quality 16S rRNA sequences obtained from the SILVA database confirmed a complex bacterial community associated with *M. rosae*. The identified community consisted of several bacterial genera, namely, *Janthinobacterium*, *Corynebacterium*, *Pseudomonas*, *Cutibacterium*, *Lactococcus*, *Candidatus* Hemipteriphilus, *Streptococcus*, *Serratia*, and *Buchnera*.

The bacterial population was dominated by two genera: *Buchnera* and *Serratia*. *Serratia*-related sequences made up around 50.0% of the total bacterial sequences, whereas *Buchnera*-related sequences contributed roughly 47.3%. Approximately 97.3% of the whole bacterial population was comprised of the two genera. *Lactococcus* (1.0%), *Streptococcus* (1.2%), and *Candidatus* Hemipteriphilus (0.2%) were among the other genera with much lower percentages. Fig. 3 depicts the various taxa in relation to their abundance. Table 3 contains taxonomy data and other information.

Phylogenetic relationships among identified bacterial taxa

Phylogenetic analysis using the representative 16S rRNA sequences showed differences in cluster patterns for the identified bacterial groups. The analysis of genetic distances between pairs showed different degrees of divergence in sequences, which reflects the phylogenetic diversity of the microbial community (Table 3).

A phylogenetic tree of the Neighbor-Joining method based on bootstrap values (1,000 replications) showed a strong resolution of clusters, with significant bootstrap support values (Fig. 4).

Based on the taxonomic classification of ITS sequences using the UNITE database (version 9.0; 97% similarity), we can conclude that a relatively low diversity of fungal communities exists among *M. rosae*.

Taxonomic classification of the ITS dataset revealed a predominance of unclassified fungal sequences. The two main unclassified sequences comprised 87.7% and 12.3% of the total ITS reads, accounting for about 99.96% of the total fungal community. A small number of classified sequences were found in which *Malassezia arunalokei* and *M. restricta* were identified as 0.031% and 0.011% of the total reads, respectively. Based on this analysis, a low diversity of fungal communities was found among the studied *M. rosae*. The complete taxonomic data on the above-mentioned species are summarized in Table 4.

Discussion

This investigation provides the first amplicon-based characterization of the bacterial and fungal microbiota associated with *M. rosae*, thereby establishing a baseline for future microbiome investigations in this economically important aphid species. The predominance of symbiotic bacteria observed in the present study further emphasizes the integral role of host-associated microorganisms in aphid biology and ecological adaptation.

One of the most important findings of this study was the extremely high prevalence of *B. aphidicola*, consistent with its established role as the obligate primary endosymbiont of aphids. The predominance of *Buchnera* is biologically expected because this endosymbiont supplies essential amino acids that are absent or scarce in phloem sap, thereby enabling aphids to exploit this nutritionally imbalanced diet. [20,36]

Apart from *Buchnera*, additional facultative symbionts, particularly *Serratia* and *Candidatus Hemipteriphilus* were also detected. Recent studies have shown that these symbionts enhance aphid adaptation to environmental conditions by conferring traits such as resistance to parasitoids, heat shock resistance, and adaptation to host plants.[25,37] For instance, *Serratia symbiotica* has

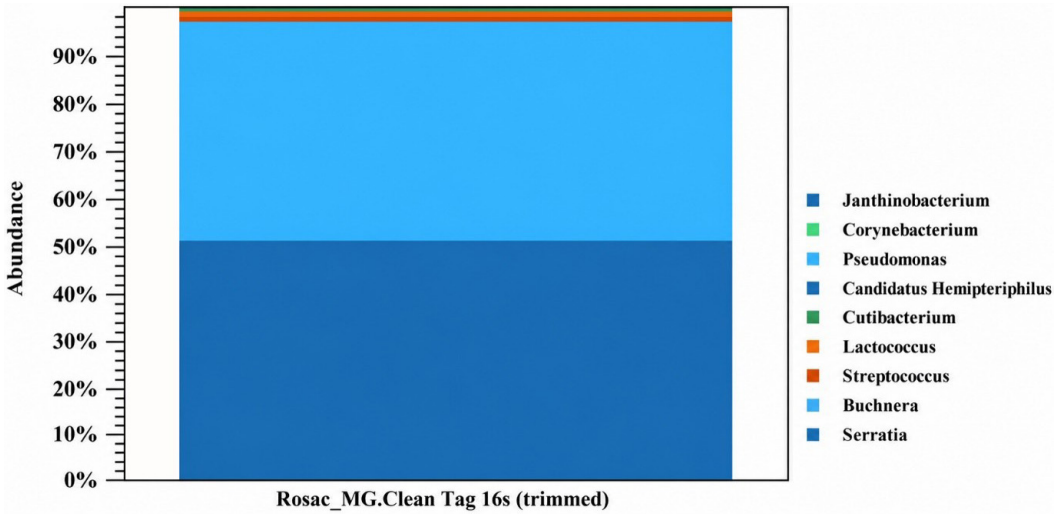

Fig. 3. Taxonomic composition of the bacterial community associated with *M. rosae* based on 16S rRNA amplicon sequencing. Taxa are displayed at the genus level. Relative abundance values represent the percentage contribution of each taxon to the total quality-filtered bacterial reads. The y-axis indicates relative abundance (%), and the x-axis represents the identified bacterial taxa.

Table 3. Taxonomic classification of bacterial genera identified from the 16S rRNA sequence dataset based on sequence IDs, relative abundance, and hierarchical classification

ID	Taxonomy	Combined Abundance	Min
Lactococcus lactis subsp. *cremoris* MG1363; BH771024.532.2058	Bacteria; Bacillota; Bacilli; Lactobacillales; Streptococcaceae; *Lactococcus*; *Lactococcus lactis* subsp. *cremoris* MG1363	12	12
Buchnera sp; BD061520.274044.275571	Bacteria; Pseudomonadota; Gammaproteobacteria; Enterobacterales; Morganellaceae; *Buchnera*; *Buchnera* sp.	245	245
Unidentified; HK241699.10.1514	Bacteria; Actinomycetota; Actinobacteria; Propionibacteriales; Propionibacteriaceae; *Cutibacterium*; Unidentified	2	2
Cutibacterium acnes; AB042287.1.1486	Bacteria; Actinomycetota; Actinobacteria; Propionibacteriales; Propionibacteriaceae; *Cutibacterium*; *Cutibacterium acnes*	5	5
Unidentified; HK241987.3.1530	Bacteria; Bacillota; Bacilli; Lactobacillales; Streptococcaceae; *Streptococcus*; Unidentified	129	129
Buchnera aphidicola (Sitobion miscanthi); KF056924.1.1468	Bacteria; Pseudomonadota; Gammaproteobacteria; Enterobacterales; Morganellaceae; *Buchnera*; *Buchnera aphidicola* (*Sitobion miscanthi*)	2	2
Cutibacterium acnes; AB108477.1.1486	Bacteria; Actinomycetota; Actinobacteria; Propionibacteriales; Propionibacteriaceae; *Cutibacterium*; *Cutibacterium acnes*	5	5
Lactococcus lactis; KC754747.1.1510	Bacteria; Bacillota; Bacilli; Lactobacillales; Streptococcaceae; *Lactococcus*; *Lactococcus lactis*	20	20
Serratia symbiotica; KT175995.1.1534	Bacteria; Pseudomonadota; Gammaproteobacteria; Enterobacterales; Yersiniaceae; *Serratia*; *Serratia symbiotica*	190	190
Lactococcus lactis; JX291541.1.1511	Bacteria; Bacillota; Bacilli; Lactobacillales; Streptococcaceae; *Lactococcus*; *Lactococcus lactis*	15	15
Streptococcus salivarius subsp. thermophilus; LS974444.1032484.1034019	Bacteria; Bacillota; Bacilli; Lactobacillales; Streptococcaceae; Streptococcus; *Streptococcus salivarius* subsp. *thermophilus*	14	14
Pseudomonas poae; KT695820.1.1540	Bacteria; Pseudomonadota; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; *Pseudomonas*; *Pseudomonas poae*	2	2
Secondary symbiont of *Sitobion miscanthi*; KT884618.1.1423	Bacteria; Pseudomonadota; Alphaproteobacteria; Rickettsiales; Rickettsiaceae; Candidatus Hemipteriphilus; Secondary symbiont of *Sitobion miscanthi*	18	18
Serratia symbiotica str. *Tucson*; AENX01000144.4045.5566	Bacteria; Pseudomonadota; Gammaproteobacteria; Enterobacterales; Yersiniaceae; *Serratia*; *Serratia symbiotica* str. *Tucson*	5468	5468
Buchnera aphidicola str. Ak (*Acyrthosiphon kondoi*); CP002645.275145.276683	Bacteria; Pseudomonadota; Gammaproteobacteria; Enterobacterales; Morganellaceae; Buchnera; *Buchnera aphidicola* str. Ak (*Acyrthosiphon kondoi*)	5107	5107
Uncultured bacterium; AY958896.1.1508	Bacteria; Pseudomonadota; Gammaproteobacteria; Burkholderiales; Oxalobacteraceae; *Janthinobacterium*; Uncultured bacterium	2	2
Uncultured bacterium; KF075324.1.1345	Bacteria; Actinomycetota; Actinobacteria; Mycobacteriales; Corynebacteriaceae; *Corynebacterium*; Uncultured bacterium	2	2
Uncultured organism; HQ781522.1.1471	Bacteria; Bacillota; Bacilli; Lactobacillales; Streptococcaceae; *Streptococcus*; Uncultured organism	2	2
Uncultured bacterium; KT851838.1.1486	Bacteria; Actinomycetota; Actinobacteria; Propionibacteriales; Propionibacteriaceae; *Cutibacterium*; Uncultured bacterium	6	6
Uncultured Lactococcus sp; LC055575.1.1532	Bacteria; Bacillota; Bacilli; Lactobacillales; Streptococcaceae; *Lactococcus*; Uncultured *Lactococcus* sp	67	67
Pseudomonas salomonii; FNOX01000039.206.1746	Bacteria; Pseudomonadota; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; *Ps*eudomonas; *Pseudomonas salomonii*	2	2

been shown to improve host tolerance to heat stress, thereby increasing survival under adverse environmental pressure.[38] Interestingly, *Serratia*-associated sequences were discovered in comparable amounts to *Buchnera's*. Although facultative symbionts are usually less common than the primary endosymbiont, some aphid populations have been shown to have higher *Serratia* abundance, which may indicate ecological adaptability or host-symbiont interactions unique to a particular species.

There were significant parallels between the bacterial community structure seen in *M. rosae* and the microbiomes previously identified from other aphid species. Specifically, *Buchnera's* predominance is in accordance with its proven function as an obligatory main endosymbiont of aphids, supplying vital nutrients that are absent from phloem sap diets.[20,39] Similarly, the large number of *Serratia*-associated sequences is consistent with earlier research showing that *S. symbiotica* is a prevalent facultative symbiont in a variety of aphid species.[40] Therefore, the presence of *Buchnera* and *Serratia* seems

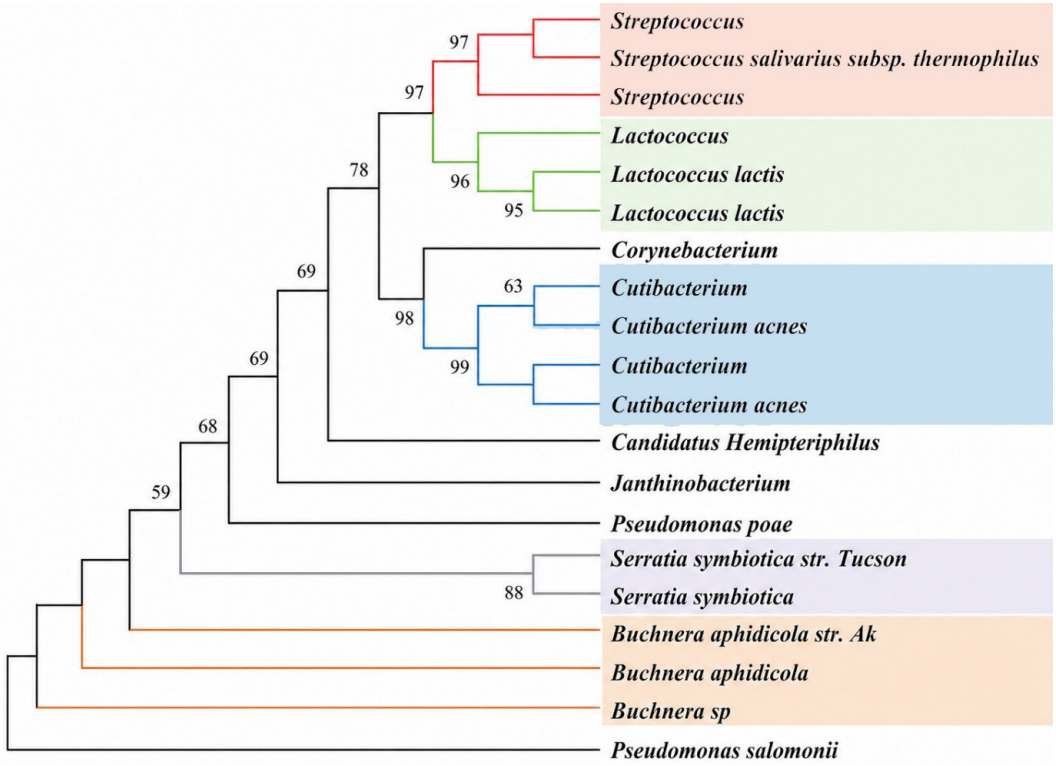

Fig. 4. Neighbor-Joining (NJ) phylogenetic tree based on 16S rRNA sequences. Numbers associated with branches show the percentage of 1000 bootstrap support values for NJ analyses higher than 50. Different groups are shown with different colors.

Table 4. Fungal community structure inferred based on ITS sequencing data; sequence ID, relative abundance, taxonomic affiliation

ID	Taxonomy	Combined Abundance	Min
Malassezia_restricta; SH1102553.09FU_AY743636_refs	Fungi; Basidiomycota; Malasseziomycetes; Malasseziales; Malasseziaceae; *Malassezia*; *Malassezia_restricta*	4	4
Malassezia_arunalokei; SH1102555.09FU_KM235691_refs	Fungi; Basidiomycota; Malasseziomycetes; Malasseziales; Malasseziaceae; *Malassezia*; *Malassezia_arunalokei*	11	11
m84114_251219_112104_s3/262214530/ccs/9583_11155	N/A	30933	30933
m84114_251219_112104_s3/254086597/ccs/4467_6329	N/A	4326	4326

to be a frequent characteristic of microbial communities associated with aphids. However, compared to some other instances where *Buchnera* was the overwhelmingly dominating symbiont, the relative dominance of *Serratia* seen in this study is different. According to Monnin et al[41] and Manzano-Marín et al[42] aphid species, host plants, environmental variables, geographic origin, and microbiome profiling methodology may all contribute to this variance.

Low-abundance genera such as *Pseudomonas*, *Corynebacterium*, and *Cutibacterium* may reflect either transient environmental acquisition or genuine, low-abundance members of the aphid-associated microbiota. It is becoming more evident that insect-associated microbiomes are not static but rather dynamic and influenced by environmental contact, plant microbiomes, and ecology.[43] Because aphids feed directly from plant tissues, they may acquire microorganisms from plant surfaces or internal plant tissues during feeding.[44] However, the functional roles of these low-abundance taxa remain unknown. Some of the taxa that are found to be low in abundance in the current study, such as *Cutibacterium* and *Corynebacterium*, may also be associated with temporary environmental contamination.

Only a few extremely numerous taxa, namely *Buchnera* and *Serratia*, dominated the bacterial community seen in this investigation; all other genera were found at comparatively low abundances. According to other studies[21,45] aphids have a reduced variety of microorganisms compared to other insects due to their exclusive diet and usage of symbionts inside cells. The diversity reduction in this case might result from ecological filtering that retains only those microbes capable of persisting within the specialized aphid host environment.[43]

Phylogenetic analysis provides additional evidence for the existence of structure within the bacterial community. The distinct clusters and strong bootstrap support found in the phylogenetic tree suggest that the classified species belong to evolutionary lineages. This is in accordance with previous research findings suggesting that phylogenetically structured communities are common in host-associated microbiomes due to the process of

coevolution.[46] Therefore, the combination of phylogenetic analysis with taxonomic profiling constitutes an effective approach to microbial community characterization.

Malassezia restricta and *M. arunalokei* were found at extremely low relative abundances (0.011% and 0.031% of total ITS reads, respectively) among the small number of taxonomically classified fungal sequences. Although species of the genus *Malassezia* have occasionally been reported from a range of environmental and host-associated ecosystems, including maritime habitats, plant surfaces, and diverse invertebrates, the ecological link between them and aphids is still unclear. These findings should be viewed cautiously and should not be regarded as proof of dominant fungal symbionts in *M. rosae* due to their incredibly low quantity and the majority of unclassified ITS sequences.

Nearly 99.96% of the retained ITS reads were unclassified fungal sequences, which was a notable finding of the ITS investigation. Furthermore, only a very small part of the fungal population was represented by taxonomically assigned sequences. This finding implies that the fungal microbiome linked to *M. rosae* is still poorly understood and may contain species that are either underrepresented in reference databases or cannot be reliably classified using ITS amplicon sequencing alone. Studies of low-biomass or poorly characterized microbial communities have similar limitations, where taxonomic resolution is significantly reduced due to insufficient reference databases.

The fungal community detected in the present study should be interpreted with caution. Only a very small percentage of the preserved reads in the ITS dataset were taxonomically assigned to fungi, whereas unclassified fungal sequences predominated. Among the classified sequences, *Malassezia restricta* and *M. arunalokei* were detected at very low relative abundances. Although species of the genus Malassezia have been found in a variety of host-associated and environmental habitats, like plant surfaces, marine environments, and certain invertebrates, little is known about their ecological relationships with aphids.[47] Therefore, the presence of sequences linked to *Malassezia* in *M. rosae* does not necessarily indicate a persistent biological connection. As a result, the fungal taxa described here should be considered preliminary findings that need to be confirmed using independent validation techniques, contamination-controlled procedures, and repeatable sampling. However, the regular identification of sequences linked to *Malassezia* is an intriguing finding that merits more research in subsequent investigations.

The low diversity of taxonomically classified fungi observed in the present study may partly reflect biological characteristics of aphids as well as methodological limitations. Unlike some insect groups in which fungal symbionts play important nutritional roles, aphids primarily depend on the bacterial endosymbiont *B. aphidicola* to compensate for nutritional deficiencies associated with a phloem-based diet.[45] Alternatively, technical issues with ITS amplicon sequencing, such as amplification bias and inadequate reference databases, might possibly be the cause of the poor taxonomic resolution.[48] However, the overall quality of the sequencing shows that the ITS reads that were kept were of excellent technical quality. Nevertheless, the fungal results should be taken cautiously due to the prevalence of unclassified fungal sequences and the lack of contamination controls.

Since this is an exploratory pilot research, several limitations should be noted. Because microbiome profiling was carried out using a single pooled aphid sample without biological replication, statistical comparisons between populations or environmental circumstances were not feasible, and the current findings should be viewed as a first characterization. Furthermore, given that the majority of ITS sequences were still unclassified, the fungal dataset should be evaluated cautiously. Notwithstanding these drawbacks, the constant identification of prominent bacterial taxa and the excellent quality of the sequencing data offer a solid basis for further repeated microbiome research of *M. rosae*. However, because of the high quality of the sequencing and the accurate identification of the prominent bacterial taxa, the overall characterization of the bacterial microbiota is confident.

In conclusion, the microbiome of *M. rosae* was dominated by obligate and facultative bacterial symbionts, whereas only a limited number of fungal taxa could be confidently classified. Despite its exploratory nature, this study provides the first integrated characterization of both the bacterial and fungal microbiota associated with *M. rosae*, thereby establishing a valuable baseline for future replicated and contamination-controlled microbiome studies.

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Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Declaration of AI-assisted Tools in the Writing Procedure

The authors declare that no artificial intelligence (AI)-assisted tools were used in the preparation of this manuscript, including writing, revision, editing, or generation of figures, graphical abstracts, or other artwork.

Ethical Approval

This study did not involve human participants or vertebrate animals. Collection and handling of *M. rosae* specimens complied with institutional and national guidelines applicable to insect research.

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