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Morphological and Molecular Identification of Novel Green Peach Aphids (*Myzus persicae*) (Hemiptera: Aphididae) and Their Microbiome Diversity in Taif Governorate

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ABSTRACT

Background: The green aphid (*Myzus persicae*) found in Taif Governorate is a sap-sucking insect that occurs globally. These insects target all the plant parts in both their nymphal and adult stages, causing them to discolor and dry out.

Methods: In this study, 250-300 aphids were widely collected from rose plants in different locations in the Taif Governorate. These insects were identified using morphological traits and mitochondrial gene sequencing.

Result: According to morphological traits, these samples belonged to the species *Myzus persicae*. Considering that aphid species are very similar morphologically, the identification of samples that were similar to more than one species was performed using their genetic characteristics. Aphid strain characterization through Sanger sequencing with multilocus sequence typing (MLST) and phylogenetic analysis revealed significant diversity and comparing mitochondrial gene sequences of aphids with extant sequences in GenBank showed high similarity and a new strain was recorded in the NCBI database for the first time in Taif Governorate. For microbiome isolation, five bacterial species were isolated from *M. persicae* (*Bacillus* spp., *Serratia* spp., *Staphylococcus, Micrococcus* spp. and *Escherichia coli*). Our results showed a significant correlation between *M. persicae* and microbial communities. Future research should focus on discovering new strains of *M. persicae* and understanding the eco-evolutionary patterns of aphid-symbiont interactions in the Taif Governorate, particularly in biological control.

Key words: Green aphid, Microbiome, *Myzus persicae*, Taif governorate.

INTRODUCTION

Aphids are commonly known as plant sap-sucking insects and they belong to the order Hemiptera. Whenever aphid infestation occurs, plants suffer from the loss of color and become dry and discolored as a result of aphid infestation (Abd-Ella, 2015). The destructive nature of insects such as these often leads to extensive damage to crops as consequence of them causing extensive damage to the crops (Singh and Singh, 2020). Besides damaging plants, aphids are also responsible for the secretion of honeydew, which causes smoky molds, which harm photosynthesis and the yield of crops (Hawrył *et al*., 2015). There have been approximately 4,000 species of aphids classified within this order, which makes it one of the most destructive pests in agriculture due to the large number of species it contains (Dixon *et al*., 1987; Blackman and Eastop, 1984).

Saudi Arabia relies on the Taif Rose to obtain oil. This is because the Taif rose has an incredibly high oil content and capacity to adapt to its local climate; therefore, aphids are the most damaging insects. There are several parts of a rose plant that can be infested by insects, but buds, leaves and flowers are the most likely to be affected by these pests. Because aphid infestation hinders rose production, particularly the green peach aphids (*Myzus persicae*), which are the most common aphid pests to infest roses, so aphid infestations lead to lowered rose production (Karlik and Tjosvold, 2003). The symptoms of this pest are deformed leaves and new bloom stems, stunted growth, galls and a change in the plant's biochemistry when infected with it (Singh *et al*., 2014). There is a significant reduction in photosynthetic activity and yield when blossoms and leaves secrete honeydew, which, in turn, leads to mold development (Ali Reza *et al*., 2012).

The presence of *M. persicae* in roses, in turn, negatively affects the market value of rose flowers and hurts the ability of roses to flower, resulting in huge losses for the industry (Jayma and Ronald, 1992). Aphids cause damage to plants in several ways, including direct and indirect damage to the plants, as well as the spread of bacteria and viruses, several of which live symbiotically with the aphids (Fuchs, 2010). It has become increasingly apparent that insects have a strong relationship with their microbiota, which has huge implications for their ecology and growth (Lewis and Lizé, 2015).

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According to several studies that have been conducted, there seems to be a link between geographical location, nutrition and insect microbiome composition (Ma *et al*., 2021). Even though many microbes are found in the body of insects, including symbiont bacteria, there is still a lack of understanding about the functions of these microbes. Symbiont bacteria have several benefits due to their presence, including the ability to promote growth and protect against the natural enemies of the organism (Oliver and Perlman, 2020). Besides helping insects cope with environmental challenges, they also assist in enhancing the growth of insects (Dunbar *et al*., 2007). In the last few months, Taif Governorate has been experiencing an increase in the number of green aphids.

According to botanical research, Buchnera contains an unmatched level of amino acids such as methionine and tryptophan, among others (Gündüz and Douglas, 2009). Furthermore, aphids have evolved biosynthetic genes that allow them to provide their symbionts with compounds the symbionts are unable to produce for themselves thanks to their ability to provide them with compounds (Brinza *et al*., 2009).

Aphids carry a variety of secondary symbionts in addition to *Buchnera* and its associated symbionts, in addition to the aphids which carry *Buchnera* and its associated symbionts. Some of these symbionts manipulate host reproduction, whereas others work in a mutualistic manner to increase host survival and fertility, including *Serratia symbiotica, Hamiltonella defensa, Regiella inseticola, Rickettsia, Rickettsiella, Spiroplasma, Wolbachia, Arsenophonus* and *Fukatsuia symbiotic* (Ayoubi *et al*., 2020). The microbial diversity of *M. persicae*, however, has not been studied and most species of aphids do not have well-defined bacterial communities.

As a first study, we describe the morphological and molecular identification of the *M. persicae* green peach aphid and its distinct isolated microbiome in Taif Governorate.

MATERIALS AND METHODS

Collection and morphological identification of *M. persicae*

From ten locations in the Taif Governorate, a total of 250-300 samples of the bacterium *M. persicae* were collected (Al-Howya, Shahar, Al-Waheet, Al-Maween, Al-Roddaf, Omsharam Alolia, Wdy Shaqra, Al-Sar, Al-Hada and Al-Shafa). Aphids were collected and placed in small plastic containers with small holes (1 mm) and the tops were covered with gauze to keep them from escaping; then, they were transferred to the Laboratory of the Faculty of Sciences at King Abdul-Aziz University (KAU), Jeddah Province for further experiments.

In this study, males, females and nymphs of the species *M. persicae* were analyzed along with their adult counterparts. Using standard taxonomic keys based on their morphological characteristics, the species were identified by using standard taxonomic keys. *M. persicae* fixed in 70% ethanol was morphologically examined under a stereomicroscope (OLYMPUS, TOKYO, JAPAN) at a magnification of 10x or 20x using a stereo microscope (for the morphological examination) (Wipfler *et al*., 2016).

Molecular identification of *M. persicae*

A DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) was used to extract genomic DNA. Genomic DNA purity was measured by a nanodrop instrument with a ratio of $A_{260/280}$ ≥1.7. Universal primers (LCO1490 (5′-GGTCA ACAAATCAT AAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTG ACCAAAAAATC-3′) were used in PCR (polymerase chain reaction) with 700-800 bp. The PCR conditions were as follows: initial denaturation: 5 min at 94°C, denaturation: 1 min at 94°C, annealing: 1 min at 58°C, extension: 2 min at 72°C, number of cycles: 35 and final extension: 10 min at 72°C. For size estimation, 10 µl of molecular weight marker was loaded in the first well. The gel was run at 127 V for one hour, attached firmly and connected to the power supply (MOLECULE-ON PS-M-300 V Electrophoresis Power Supply, India). One percent agarose powder was weighed and dissolved in 1x TAE buffer by placing the suspension in a microwave oven for heating. A final concentration of 0.1 µg/ml ethidium bromide (EtBr) from a 10 mg/ml stock in distilled water was added to the agarose. Then, agarose was poured into the gel tray and the comb was placed at one end. The gel was left for one hour to solidify before the comb was removed. The gel was then placed into an electrophoresis tank and submerged in 1x TAE buffer as the running buffer. The 5x loading dye was added to the samples to be loaded according to the volume ratio 5:1 of sample to dye. The gel was run in a horizontal gel apparatus at 127 volts for 60-90 min. DNA fragments were visualized on a UV transilluminator and photographed by using a Viber Lourmat Gel Imaging System. A 700 bp DNA ladder (Promega, USA) was used as a marker. The PCR products were 700 bp. Samples were sequenced using Sanger sequencing at Macrogen, South Korea. It was conducted comparison between nucleotide sequence data and the NCBI database using the Basic Alignment Search Tool (BLAST). By comparing the sequences of the aphids collected from the field with those in the gene bank, the aphids collected from the field were identified.

Bacterial isolation

Bacteria were isolated from *M. persicae*. Twenty-five aphids from each location (Al-Howya, Shahar, Al-Waheet, Al-Maween, Al-Roddaf, Omsharam Alolia, Wdy Shaqra, Al-Sar, Al-Hada and Al-Shafa) were sterilized by ethanol (70%) and moved to nutrient agar. Then, the hemolymph was extracted, streaked and cultured. To obtain uniform morphologies of bacterial colonies after incubation at 30°C for 48 hours, it was necessary to subculture the colonies after the incubation (Gündüz and Douglas, 2009). After the isolated bacteria were kept in stock cultures for up to three months, the Microbiology Laboratory of King Abdul-Aziz University (KAU), Jeddah province, tested them for the presence of viral and bacterial

pathogens. This study was conducted using an autoclave (OT-40 L-NUVE) for 15-20 minutes at 121°C to sterilize all media used in this experiment.

Gram stain of bacterial isolates

By using forceps, we repeatedly passed a slide with bacteria over a heat source so that it could be "heat-fixed" by staining it with Gram staining and then heating it using forceps again. During the process of passing the slide through the flame, it is necessary to pass it quickly to prevent overheating. Following the fixation of the stain on the staining tray, the slide was placed over the smear and the crystal violet solution was applied over the smear after one minute. After rinsing the slides with either distilled or tap water, slides were then treated with a solution of iodine after being rinsed with an iodine solution. During the process of removing the iodine solution from the slide, the slide is flooded with distilled or tap water for approximately 1-5 seconds, then rinsed with a decolorizer solution. After the slides were soaked in safranin for 30 seconds in an attempt to remove the stain, they were rinsed in tap water or diluted in a solution of tap water and soaked for another 30 seconds. As a part of the process of detecting bacteria, slides were examined using 100×-objective lenses. Those bacteria that are Gram-positive are staining deep violet or blue, whereas those that are Gram-negative are staining pink or red, depending on the strain.

Biochemical reactions of bacterial isolates

It was determined that the bacteria could be identified using Analytical Profile Index Test Strips (API-20E), according to their biochemical and morphological characteristics. Using this test, the enteric-negative rods can be distinguished from the enteric-positive rods. Each of the twenty compartments on each of the strips containing the strips contains a compartment for the dehydrated strips. Each well was then rehydrated with a bacterial suspension that was used as a rehydration agent. Some of the wells showed a change in color as the pH of the solution changed, while others required the addition of reagents. According to codebooks based on the sequences of the profile numbers, the profiles of positive and negative results were correlated with bacterial species using the profiles with positive and negative results.

Molecular identification of isolated bacteria

To determine the DNA of Gram-stained bacteria that were positive and negative, the GeneJET Genomic DNA Purification Kit (#K0721) was used with some modifications. For gram-positive bacteria, 2×10^9 bacterial cells were resuspended in 180 µl of lysis buffer in a 1.5 ml microcentrifuge tube, incubated for 30 min at 37°C, mixed thoroughly by vortexing and then transferred for centrifugation for 10 min at 7000 rpm. For gram-negative bacteria, 2×10^9 bacterial cells were resuspended in 180 µl of digestion solution in a 1.5 ml microcentrifuge tube, mixed thoroughly by vortexing and then transferred for centrifugation for 10 min at 7000 rpm. In the next step, both positive and negative samples were incubated at 56°C while vertexing occasionally using a shaking water bath for 30 min. Then, 20 µl of RNase was added and mixed by vertexing and incubated for 10 min at room temperature. Then, 400 µl of 50% ethanol was added and mixed by vertexing. The prepared lysate was then transferred to a GeneJET Genomic DNA Purification Column inserted in a collection tube and centrifuged for 1 min at 8000 rpm. The flow-through solution was discarded. Then, 500 µl of wash buffer (1) was added and centrifuged for 1 min at 10000 rpm. Then, the flow-through was discarded. Five hundred microlitres of wash buffer (2) was added to the GeneJET Genomic DNA Purification Column and centrifuged for 3 min at 15000 rpm. Two hundred microlitres of elution buffer were added to the center of the GeneJET Genomic DNA Purification Column membrane to elute genomic DNA, incubated for 2 min at room temperature and centrifuged for 1 min at 10000 rpm. Total DNA was stored at -20°C. Genomic DNA purity was measured by a nanodrop instrument ratio of $A_{260/280} \geq 1.7$. The primer sequences used were the universal primer 16S rRNA. The amplification was performed in 25 µl containing 1× GoTaq_Green Master Mix (Promega, USA), 2 µl of DNA template and 1 µl of forward and reverse primer (10 pmol). The amplification was performed by heating the sample at 95°C for 5 min and 35 cycles of 94°C for 30 s, 59°C for 30 s and 72°C for 45 s, followed by a final extension step of 72°C for 10 min. In the final step, the temperature was set at 4°C for an infinite amount of time. Two microlitres of DNA samples were checked using 1.5% agarose gel electrophoreses at 100 V in an electrophoresis system for 25 min in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). A 100 bp DNA ladder (Promega, USA) was used as a marker. The PCR products were approximately 500-600 bp. Samples were sequenced using Sanger sequencing at Macrogen, South Korea. To compare nucleotide sequence data with the NCBI database of nucleotide sequences, we used the Basic Local Alignment Search Tool (BLAST), which is part of the National Centre for Biotechnology Information.

Results and Discussion

Morphological identification of aphids

Taif Governorate is known as the city of Aphid because of the climate. Large samples of green aphids were collected from ten places in the Taif Governorate, with a total of 250-300 samples. One species of aphid was identified based on its morphological characteristics of green color. The species can be found throughout the Taif Governorate and on a wide variety of plants, including peppers, cucumbers, tomatoes, eggplants and many others, as well as many fruit trees. In our study, we found *M. persicae* in rose plants, which are distributed all over the Taif Governorate. The wingless aphids are yellowish or greenish in color and they measure approximately 0.5 to 1.0 mm in length, as shown in Fig 1 (a and b). The head has a long setaceous antenna with five to six segments as shown in Fig 2 (a and b). The compound eye was clear with a lateral black spot. Mouthparts belong to the piercing-sucking type, as shown in Fig 3.

The thorax is composed of three segments with six legs and no wings, as shown in Fig 4. The abdomen has seven segments, as shown in Fig 5 and ends with cornicles that are moderately long and dark in color, as shown in Fig 6 (a and b).

Wingless males and females can be distinguished by the reproductive system and are only found in females and their blades, as shown in Fig 7 (a and b) and 8 (a and b).

Winged adults are larger with dark heads capsule and long antennae, as shown in Fig 9, dark thorax with long wings, as shown in Fig 10 and long and black segmented cornicles, as shown in Fig 11.

Fig 1: a) Dorsal view of the immature aphid, b) Ventral view of the immature aphid.

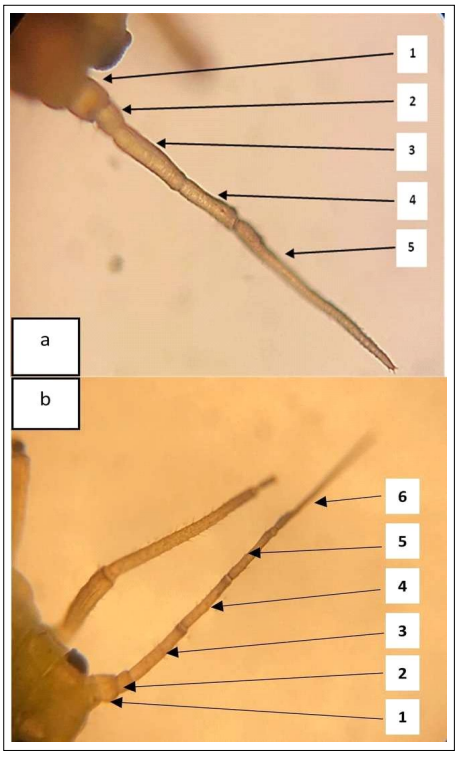


Fig 2: a) Setaceous antenna with five segments, b) Setaceous antenna with sex segments.

Molecular identification of *M. persicae*

To identify aphids using molecular techniques, PCR was used for amplification using universal primers. The samples were detected and the length of the DNA was 700 bp as in

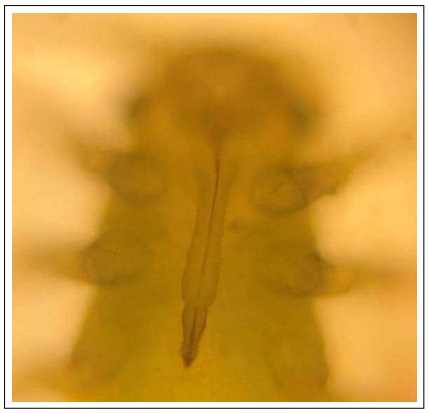


Fig 3: Piercing sucking mouthparts.

Fig 4: Thorax in wingless stage.

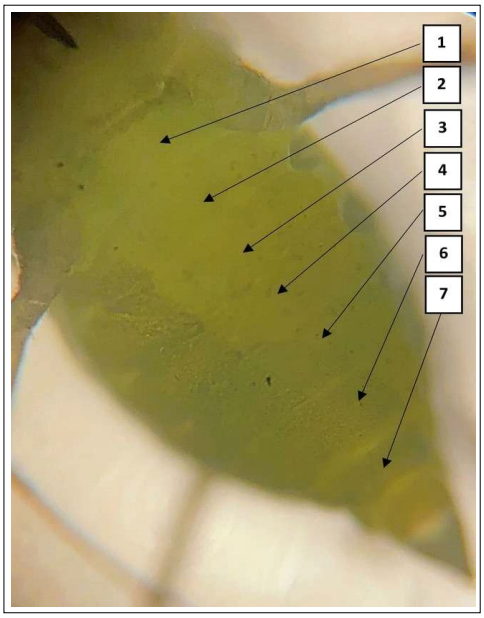


Fig 5: Abdominal segments.

Fig 12. In the sequencing reaction, samples were sent to Macrogen, South Korea and the nucleotide sequence of the samples was obtained by Sanger sequencing. Phylogenetic analysis was carried out based on sequences obtained from aphids with other reference sequences of NCBI through BLAST. The interesting fact of our results is that the similarity was ranging between 97-98% and the sequences of the new aphid have been deposited in GenBank with new accession numbers for the first time in Saudi Arabia and data were recorded and published in the NCBI database.

Fig 6: a) Wingless aphid cornicles (10x), b) Wingless aphid cornicles (20x).

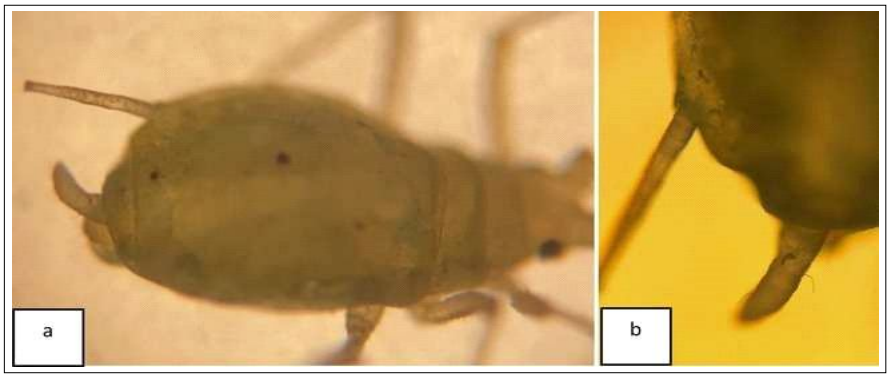


Fig 7: a) Wingless female adult (10x), b) Wingless female adult, (20x).

Fig 8: a) Wingless male adult (10x), b) Wingless male adult (20x).

Fig 9: Black head capsule in winged adult.

Fig 10: Long wings in winged adult.

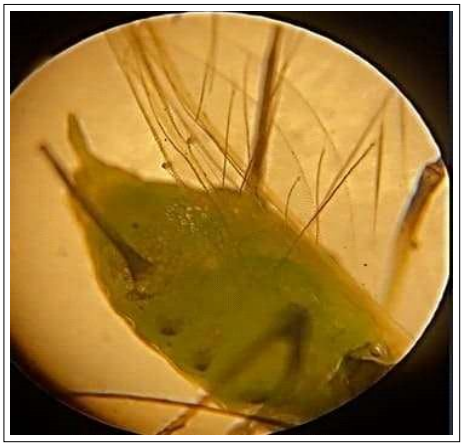


Fig 11: Long and black segmented cornicles in winged adult.

Phylogenetic analysis of *M. persicae*

Phylogenetic analysis of *M. persicae* was carried out based on universal primers obtained from aphids collected from the Taif Governorate with other reference sequences of NCBI through BLAST. Evolutionary distances were calculated using the Maximum Composite Likelihood method and 1000 bootstrap values were used to construct the phylogenetic. According to the phylogeny, aphids showed a high degree of similarity with *M. persicae* ranging between 97-98%, as shown in Fig 13 and Table 1.

Molecular identification of isolated bacteria

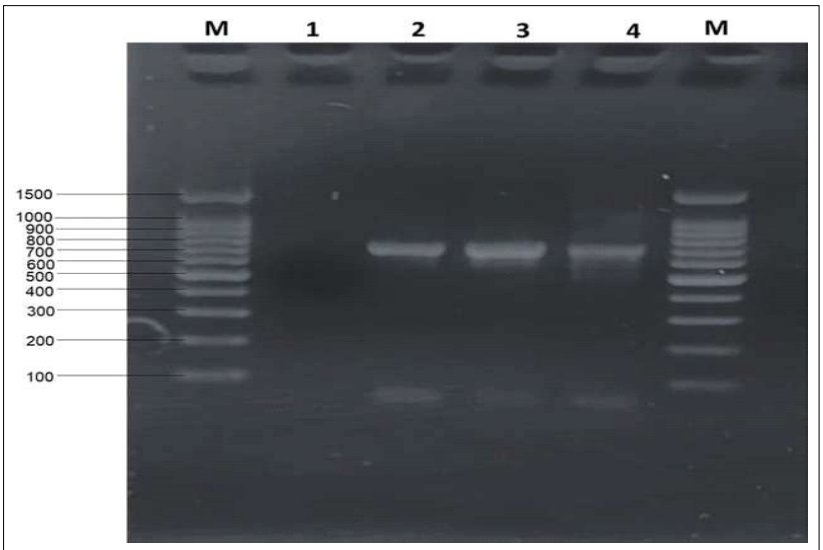
Bacteria were isolated from *M. persicae*. The gram staining of aphids has resulted in the isolation of five species of bacteria that can be found in them. The negative Gram stain bacteria were *Bacillus* spp. and *Micrococcus and* the positive bacteria were *Serratia* spp., *Staphylococcus* and *Escherichia coli*. This study detected Serratia among the isolated species for the first time. For molecular identification, the presence of bacterial DNA was assessed in *M. persicae* using 16S rRNA. Bacterial DNA was extracted and amplified through PCR using 16S rRNA markers. The bacterial DNA was detected in samples and the length of the DNA was 450 bp, appropriate to the 16S rRNA gene length bacterial. An identity comparison with the gene sequence in the GenBank sequence database revealed a 100% similarity to the genome of the GenBank bacteria.

In the present study, morphological and molecular identification of *M. persicae* was examined through the 2022/2023 seasons. Researchers have shown that the morphological characteristics of aphids feeding on rose plants are stable over time and such characteristics were measured in field-collected aphids, which was confirmed by many authors (Mittné *et al*., 2023; Al-Kallabe *et al*., 2023). For molecular identification, phylogenetic analysis of M. *persicae* was carried out based on universal primers obtained from aphids collected from the Taif Governorate with other reference sequences of NCBI through BLAST. According to the phylogeny, aphids showed a high degree of similarity ranging between 97-98% with *M. persicae* and new strains were recorded in the NCBI database with accession numbers. In this study, the diversity of *the M. persicae* microbiome was also examined as part of the study. As part of the study, a population of *M. persicae* was collected from the Al-Taif Governorate and was used as the model. According to Ateyyat (2008), *Bacillus* sp has been found in aphids in a previous study (Ateyyat, 2008). Several antimicrobial compounds, such as phenols, play an important role in preventing bacteria and fungi from colonizing their host (Blackburn, 2008). *Bacillus megaterium*

Table 1: Accession numbers and their links in the NCBI database.

Strains code	Accession number	Accession number in NCBI database
Myzus persicae isolate SHARAWI_1	OQ568964	https://www.ncbi.nlm.nih.gov/nuccore/OQ568964
Myzus persicae isolate SHARAWI_2	OR186347	https://www.ncbi.nlm.nih.gov/nuccore/OR186347

has been isolated from Aphis pomi De Geer, one of the green apple aphids found in the Samsun province of Turkey (Wipfler *et al*., 2016). As we have found out, Serratia species are also found in aphids, where they are frequently found as facultative symbiotic bacteria that live jointly with their hosts (Al-Kallabe *et al*., 2023; Mittné *et al*., 2023). Several benefits can be derived from secondary symbionts for aphids. This provides them with a level of resistance against parasitic wasps and fungi. Apart from improving their tolerance to thermal stress, controlling aphid reproduction, influencing the utilization of the host plant and changing their color, they can also improve their resistance to cold (Manzano-Marín *et al*., 2017). Additionally, studies have found that the gut flora of aphids on field crops contains *Staphylococcus* as well as *Bacillus* species and *Serratia* species (Haynes *et al*., 2003). There have been several *Micrococcus* species isolated from the guts of pea aphids, ranging from immature to apterous adults. It has also been reported that *E. coli* bacteria have been found in aphids raised in a laboratory (Gerardo *et al*., 2010; Moran *et al*., 2005).



M: Marker, 1 negative control sample from 2:4.

Fig 12: Gel electrophoresis of *M. persicae*.

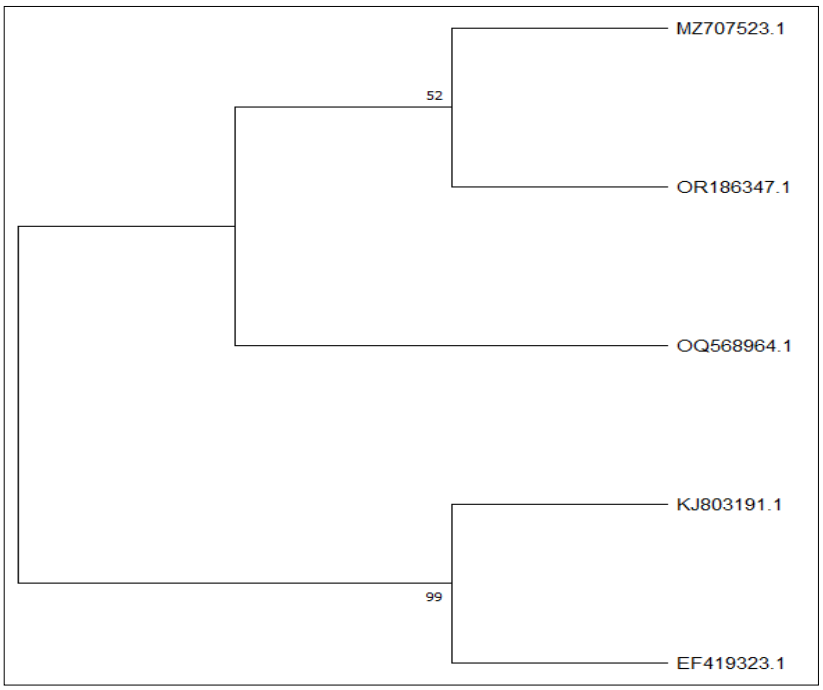


Fig 13: Sequences similarity of tested aphid compared to GenBank using MEGA X software.

2005). A future study should focus on the diversity of bacteria found on aphids obtained from the Taif governorate from the perspective of biological control, especially from the standpoint of understanding the interactions between aphids and their symbionts within the context of eco-evolution.

CONCLUSION

In this study, the microbiome diversity of *M. persicae,* a green peach aphid collected from the Al-Taif Governorate, was examined for its contribution to the microbiome composition. Using mitochondrial gene sequences from the NCBI database for the first time, a new strain of an aphid in Taif Governorate, Saudi Arabia, was identified based on the similarity between the mitochondrial gene sequences in the GenBank and those in the gene bank, ranging from 97-98%. We isolated five bacterial species from *M. persicae* during the isolation of the microbiome. These species include *Bacillus* sp, *Serratia* sp, *Staphylococcus* sp, *Micrococcus* sp and *Escherichia coli* sp. The findings of this study are supported by numerous studies; The fact that aphids are becoming increasingly recognized as pests stems from the need to conduct additional research on them so that we can gain a deeper understanding of how aphids are interacting with their symbionts and how their eco-evolutionary patterns interact.

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Disclaimer

None.

Source of funding

None.

Data availability

The datasets generated during the current study are available in the NCBI database repository, persistent web links or accession numbers to datasets can be found in Table 1.

Conflict of interest: None.

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