Genetic analysis of ‘Candidatus Phytoplasma aurantifolia’ associated with witches’ broom on acid lime trees

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ABSTRACT

“Candidatus Phytoplasma aurantifolia” is associated with witches’ broom disease of lime in Oman and the UAE. A previous study showed that an infection by phytoplasma may not necessarily result in the physical appearance of witches’ broom symptoms in some locations in Oman and the UAE. This study investigated whether phytoplasma strains belonging to “Ca. P. aurantifolia” (based on the 16S rRNA gene analysis) in locations where disease symptoms are expressed are different from phytoplasma in locations where disease symptoms are not expressed. About 21 phytoplasma strains (15 from areas and trees with disease symptoms and six from areas and trees without disease symptoms) were included in the analysis. The study utilized sequences of the imp and SAP11 genes to characterize the 21 strains. Phylogenetic analysis of both genes showed that the 21 strains are similar to each other and to reference strains in GenBank. The study shows that there is a low level of diversity among all phytoplasma strains. In addition, it shows that phytoplasma in places where witches’ broom symptoms are not expressed are similar to phytoplasma in places where disease symptoms are expressed. This may suggest that disease expression is not linked to the presence of different phytoplasma strains, but may be due to other factors such as weather conditions.

INTRODUCTION

Phytoplasmas are prokaryotic gram-positive bacteria that are difficult to be cultured in artificial media (Contaldo et al., 2012, 2016). They are phloem limited and transmitted by phloem-sucking insects of the order Hemiptera, mostly leafhoppers (Cicadellidae), planthoppers (Fulgoroidea), and psyllids (Psyllidae) (Frost et al., 2013; Rashidi et al., 2014; Queiroz et al., 2016). They have a wide range of host plants from over 100 plant families, including many citrus species (Hogenhout et al., 2008).

Symptoms produced by “Candidatus Phytoplasma aurantifolia” on acid lime trees include excessive production of shoots with very small, light green leaves and short internodes, no flowers or fruits, and the general decline of the tree leading to a final dieback. Witches’ broom symptoms progress rapidly from the time of symptom expression.
appearance until the final stage of tree death where the tree collapses within four to five years after first symptom appearance (Al-Sadi et al., 2012; Al-Yahyai et al., 2015).

A previous study showed that the association of “Ca. P. aurantifolia” with acid lime results in witches’ broom symptoms in some geographical locations but not in others (Al-Ghaithi et al., 2017). The study found the symptoms were apparent in most areas in Oman except in monsoon areas or areas with extreme desert conditions. Although this may be in part related to differences in climatic conditions between these areas, it is necessary to characterize the phylogenetic association and diversity of phytoplasma that are present in locations which are conducive and less conducive to witches’ broom symptom expression.

The identification and characterization of phytoplasma has long relied on the use of the 16S rRNA gene (Mehdi et al., 2012). However, other molecular markers have been developed which include the Tuf, SecA, SecY, SAP11, imp, and other genes (Sugio et al., 2011; Bekele et al., 2011; Dickinson & Hodgetts, 2013; Al-Abadi et al., 2016). Immunodominant membrane proteins (imp) genes are more variable than their surrounding genes in phytoplasma. They are located on the external surface of phytoplasma cell membrane (Siampour et al., 2012). SAP11 is an effector protein that targets plant cell nuclei (Bai et al., 2009) and induces stem proliferation, changes in leaf shape, and the down regulation of jasmonic acid production.

Our previous work compared phytoplasma across countries (Al-Abadi et al., 2016) using three genes but only from one area (subtropical). In addition, a recent study by our group compared phytoplasma across different conditions using one gene (16S rRNA) but from three areas (semitropical, subtropical, and desert) (Al-Ghaithi et al., 2017). The aim of our current study was to investigate the genetic relatedness of phytoplasma across different conditions (semitropical, subtropical, and desert) using two additional genes (imp and SAP11) that were not used in 2017.

The study helped to determine whether the difference in witches’ broom symptom expression in different geographical regions could be related to the presence of different phytoplasma strains.

**MATERIALS AND METHODS**

**Sample collection**

Fifteen samples positive for phytoplasma from trees developing WBD symptoms and six samples collected from asymptomatic trees from areas with no apparent WBD symptoms were included in the study. The choice of samples was based on our previous findings (Al-Ghaithi et al., 2017). The 21 samples were collected from 11 different locations, two samples from a semitropical area (Salalah), four samples from desert areas (Najed and UAE), and 15 samples from subtropical areas (Fig. 1). Samples were collected from young leaves developing WBD symptoms.

**Nucleic acid extraction and polymerase chain reaction**

Total nucleic acids were extracted following the method of Doyle & Doyle (1987) in CTAB extraction buffer. Ground leaves mixed with CTAB buffer were incubated at 65 °C for
10 min. This was followed by adding an equal amount of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifugation (this step was repeated twice). Then 0.6 volume of isopropanol and 0.3M NaAc (pH 5.2) was added to the supernatant. The DNA pellets were washed with 70% ethanol, dried and then resuspended in 100 µl sterilized distilled water and stored at −80 °C.

The 21 strains have already been characterized in our previous study based on the 16S rRNA (Table 1) and were found to belong to the 16S rRNA subgroup II-B (Al-Ghaithi et al., 2017). Therefore, polymerase chain reaction (PCR) were conducted for the imp and SAP11 genes as described by Al-Subhi et al. (2017). Imp-R1, Imp-F1, and Imp-F2 primers were used for the amplification of the imp gene using a
two-stage PCR. Imp (F1) and Imp (R1) were used in direct PCR, while Imp (F2)/Imp (R1) were used in seminested PCR (Table 1). Amplification of the SAP11 gene was done using the primers SAP11-W-F1 and SAP11-W-R1 in direct PCR. The primer pair SAP11-W-F2/SAP11-W-R2 was used in nested PCR (Table 1). The PCR conditions for both genes in the direct and nested/seminested PCR were adjusted to the same conditions as follows: 94 °C for 2 min, then 40 cycles (94 °C for 30 s, 53 °C for 45 s, and 72 °C for 1:30 s), followed with final extension at 72 °C for 10 min.

### Table 1 Primers used for amplifying Phytoplasma genes.

| Gene  | Primer name  | 5′-3′ Sequence                  | Product size (bp) | Reference                  |
|-------|--------------|---------------------------------|-------------------|----------------------------|
| imp   | imp-F        | GTTATAATTTGAAGCGATA-TTG         | 519               | Al-Subhi et al. (2017)     |
|       | imp-F2       | ATAGAGGAGAAAGGATGTC             |                   |                            |
|       | imp-R        | GATCATATTTGTTTATAGGAG           |                   |                            |
| SAP11 | SAP11-w-F1   | CTTCAGCCACAATAGAATCTTT          | 1,050             | Al-Subhi et al. (2017)     |
|       | SAP11-w-R1   | CAAATAAATACGCTGCATAAA           |                   |                            |
|       | SAP11-w-F2   | TTCTTTTTATGAAATACCTCAG          | ~550              |                            |
|       | SAP11-w-R2   | GCGCATATTATAAAACCTCCTTT         |                   |                            |

Note: Al-Subhi et al. (2017).
Sequencing and analysis of sequences

Sequencing was carried out at MACROGEN Inc., Korea, using the same primers used in the nested PCR. The forward and reverse sequences for each phytoplasma isolate were aligned and edited using ChromasPro (v. 1.41; Technelysium Pty Ltd., Brisbane, Queensland, Australia). Phylogenetic analysis of the obtained sequences and representative sequences from GenBank for the different subgroups of phytoplasma was carried out using the Kimura 2-parameter evolutionary model in Mega 6 (Tamura et al., 2013). Trees were generated using 1,000 replications and a 50% majority-rule for the bootstrap analysis.

RESULTS

Phylogenetic analysis of phytoplasma strains based on SAP11 gene

Amplification using the primer pair SAP11-w-F2 and SAP11-w-R2 produced a fragment of 339 bp (Fig. 2). Sequencing of the PCR products followed by phylogenetic analysis...
based on SAP11 gene sequences showed that all samples clustered together with the reference isolates belonging to “Ca. P. aurantifolia” (Fig. 3). All strains from Oman clustered with the reference isolate with a very high bootstrap support (100%), and were separated from all other closely related strains.
Phylogenetic analysis of phytoplasma strains based on the *imp* gene

The primer pair ImpF2/Imp R1 resulted in a product of 519 bp in size (Fig. 2). Phylogenetic analysis of the 21 samples and three reference sequences from GenBank showed that all samples clustered with the references strains with a very high bootstrap support (100%) (Fig. 4).

Phylogenetic analysis of 21 isolates based on the combined *Imp* and SAP11 gene sequences showed clustering of all the isolates in one cluster (Fig. 5). GenBank accession numbers for all samples are illustrated in Table 2.

**DISCUSSION**

Phytoplasmas of the taxonomic group 16SrII (peanut witches’ broom phytoplasma group) are associated with diseases affecting crops and wild plants in different geographical areas worldwide. “*Ca. P. aurantifolia,*” from the taxonomic subgroup 16SrII-B, causes a devastating and lethal disease of lime (Lime witches’ broom) in Gulf countries (Oman, UAE, and Saudi Arabia). Phytoplasma inducing a similar witches’ broom disease were reported in different host plants such as alfalfa (*Khan et al., 2002*) and sesame (*Al-Sakeiti et al., 2005*). Furthermore, the 16Sr II group was detected in other crops in the Middle East including Iran and Lebanon (*Weintraub & Jones, 2010*), the Mediterranean region (*Tolu et al., 2006*), Australia (*Aryamanesh et al., 2011*), Mexico...
“Candidatus Phytoplasma aurantifolia” was reported in Oman, the UAE, Iran, and other countries (Bové et al., 2000; Chung, Khan & Brランスky, 2006; Al-Ghaithi et al., 2016). However, our previous findings showed that symptom expression due to WBD is not apparent in some areas, especially deserts and monsoon areas (Al-Ghaithi et al., 2017). Although it was clear that there was a relationship between symptom expression and geography, it was questioned whether phytoplasma infecting trees expressing WBD symptoms could be the same as phytoplasmas from trees not expressing WBD symptoms.

Phylogenetic analysis showed that all phytoplasma isolates share the same sequences of the \textit{imp} and \textit{SAP11} genes. This resulted in a lack of clustering of phytoplasma isolates from different climatic conditions, showing that phytoplasma from symptomatic and asymptomatic trees have the same \textit{imp} and \textit{SAP11} gene sequence.

Previous studies using the 16S rRNA gene showed limited variation between phytoplasmas which belong to the same group (Bertaccini & Duduk, 2009). Similarly, low variation was detected between different isolates of “\textit{Ca. P. aurantifolia}” from three different countries (Oman, UAE, and Iran) based on sequences of the 16s rRNA, \textit{secA},

| No. | Sample code | Name of the region | Environment | Year of collection | GenBank accession numbers |
|-----|-------------|-------------------|-------------|-------------------|--------------------------|
| 1   | S2          | Salalah           | Semitropical| 2014              | KX602312 KY829473 KY829493 |
| 2   | S3          | Salalah           | Semitropical| 2014              | KX602309 KY829474 KY829494 |
| 3   | D1          | Najed             | Desert      | 2014              | KX602311 KY829475 KY829495 |
| 4   | D2          | Najed             | Desert      | 2014              | KX602290 KY829476 KY829496 |
| 5   | D3          | UAE               | Desert      | 2015              | KX602307 KY829477 KY829497 |
| 6   | D4          | UAE               | Desert      | 2015              | KX602308 KY829478 KY829498 |
| 7   | W1          | Mahada            | Subtropical | 2014              | KX602293 KY829479 KY829499 |
| 8   | W2          | Mahada            | Subtropical | 2014              | KX602294 KY829480 KY829500 |
| 9   | W3          | Mahada            | Subtropical | 2014              | KX602310 KY829481 KY829501 |
| 10  | W4          | Madha             | Subtropical | 2014              | KX602295 KY829482 KY829502 |
| 11  | W5          | Madha             | Subtropical | 2014              | KX602296 KY829483 KY829503 |
| 12  | W6          | Rustaq            | Subtropical | 2014              | KX602298 KY829484 KY829504 |
| 13  | W7          | Rustaq            | Subtropical | 2014              | KX602313 KY829485 KY829505 |
| 14  | W8          | Barka             | Subtropical | 2014              | KX602299 KY829486 KY829506 |
| 15  | W9          | Barka             | Subtropical | 2014              | KX602300 KY829487 KY829507 |
| 16  | W10         | Sur               | Subtropical | 2014              | KX602301 KY829488 KY829508 |
| 17  | W11         | Sur               | Subtropical | 2014              | KX602302 KY829489 KY829509 |
| 18  | W12         | Nizwa             | Subtropical | 2014              | KX602303 KY829490 KY829510 |
| 19  | W13         | Nizwa             | Subtropical | 2014              | KX602304 KY829491 KY829511 |
| 20  | W14         | Dhank             | Subtropical | 2014              | KX602305 KY829492 KY829512 |
| 21  | W15         | Dhank             | Subtropical | 2014              | KX602306 KY829473 KY829513 |

(Hernandez-Perez et al., 2009), Indonesia (Harling et al., 2009), Europe (Tolu et al., 2006; Davino et al., 2007; Parrella et al., 2008; Harling et al., 2009), and Sudan (Zamora, Acosta & Martinez, 2012).
and *imp* genes. These three genes could not separate strains based on the country from which they were obtained. Findings from our study were in agreement with Al-Abadi *et al.* (2016) who showed limited variation in the *imp* gene.

Although the SAP11 gene is associated with symptom induction (Sugio *et al.*, 2011), no differences were found in the sequence of this gene between isolates obtained from areas with or without WBD expression. Thus, this result and the above results confirm that all phytoplasma isolates are identical and have low genetic diversity. They do not support the possible presence of different phytoplasma strains in the studied locations and trees.

**CONCLUSION**

This study shows that WBD phytoplasma from semitropical areas, subtropical areas, and desert areas share a very high level of genetic similarity based on *imp* and SAP11 genes. This gives indication that acid lime trees in these locations are affected by the same phytoplasma strain, but symptom development is affected by environmental factors rather than by phytoplasma strains. Also, symptom development can be affected by other parameters such as soil moisture or/and plant cultivars or/and cultural practices or even coinfection with other pathogens/strains. Future studies should address the relationship between symptom expression in acid lime and other possible factors.

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**ADDITIONAL INFORMATION AND DECLARATIONS**

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**Competing Interests**

The authors declare that they have no competing interests.

**Author Contributions**

- Aisha G. Al-Ghaithi performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Ali M. Al-Subhi conceived and designed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
• Issa H. Al-Mahmooli performed the experiments, authored or reviewed drafts of the paper, approved the final draft.
• Abdullah M. Al-Sadi conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.

Field Study Permissions
The following information was supplied relating to field study approvals (i.e., approving body and any reference numbers):
Permits are not required for the collection of samples from Oman.

Data Availability
The following information was supplied regarding data availability:
All GenBank accession numbers are provided in Table 2.

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