Population genetic analysis reveals a low level of genetic diversity of ‘Candidatus Phytoplasma aurantifolia’ causing witches’ broom disease in lime

Shaikha Y. Al-Abadi1, Abdullah M. Al-Sadi2*, Matthew Dickinson3, Mohammed S. Al-Hammadi4, Rashid Al-Shariqi4, Rashid A. Al-Yahyai2, Elham A. Kazerooni5 and Assunta Bertaccini6

Abstract
Witches’ broom disease of lime (WBDL) is a serious phytoplasma disease of acid lime in Oman, the UAE and Iran. Despite efforts to study it, no systemic study attempted to characterize the relationship among the associated phytoplasma, ‘Candidatus Phytoplasma aurantifolia’, from the three countries. This study utilized sequences of the 16S rRNA, imp and secA genes to characterize 57 strains collected from Oman (38), the UAE (9) and Iran (10). Phylogenetic analysis based on the 16S rRNA gene showed that the 57 strains shared 98.5–100 % nucleotide similarity to each other and to strains of ‘Ca. P. aurantifolia’ available in GenBank. The level of genetic diversity was low based on the 16S rRNA (0–0.011), imp (0–0.002) and secA genes (0–0.015). The presence of low level of diversity among phytoplasma strains from Oman, the UAE and Iran can be explained by the movement of infected lime seedlings from one country to another through trading and exchange of infected plants. The study discusses implication of the findings on WBDL spread and management.

Keywords: WBDL, Phytoplasma, Acid lime, Population structure

Background
Citrus fruits are among the most important fruits in the world due to their high nutritional value and possibility to be consumed fresh as well as processed. Orange trees are the most widely cultivated citrus species, with Brazil being the largest orange exporter in the world. Lime and lemon trees are also cultivated extensively around the globe. India, with about 16 % of the world’s overall lemon and lime production, tops the production list, followed by Mexico (~14.5 %), Argentina (~10 %), Brazil (~8 %), and Spain (~7 %) (FAO 2015).

Citrus aurantifolia Swingle, known locally as Omani lime, has many other names in other parts of the world, such as Mexican lime, key lime and acid lime. It has been grown in Oman for at least four centuries where it was brought by Arabian sailors (Davies and Albrigo 1994). It was considered as the second most important fruit crop after dates in Oman until the 1970s, and today it is ranked among the top four fruit crops in terms of production. It is also an important crop in the UAE and Iran (FAO 2015).

Witches’ broom disease (WBDL) is the most destructive disease of lime trees in Oman, the UAE and Iran (Chung et al. 2006; Al-Yahyai et al. 2015; Al-Sadi et al. 2012b; Bové et al. 2000) where it killed more than a million lime trees. ‘Candidatus Phytoplasma aurantifolia’ is the phytoplasma associated with WBDL (Zreik et al. 1995). Phytoplasmas are phloem-limited bacteria which can be transmitted by phloem feeding insects such as leafhoppers and psyllids. WBDL was first observed in Shinas and Liwa in the northern coast of Al-Batinah.
governorate in the 1970s, then it spread rapidly to other parts of Oman (Bové et al. 1988). It was reported in the UAE in 1989 and in Iran in the 1990s (Garnier et al. 1991; Bové et al. 2000). WBDL results in the production of a large number of leaves, light green to yellow in color and smaller in size. This is also associated with production of many branches. Leaves on the symptomatic branches usually dry within 1 year of symptom appearance. WBDL symptoms progress on the entire canopy until the whole tree collapses and dies within three to 7 years of first symptom appearance (Al-Yahyai et al. 2015; Al-Sadi et al. 2012b).

The classification system of phytoplasmas is based on RFLP and/or sequencing of 16S rDNA (Schneider et al. 1995), while in the past, phytoplasma strains were differentiated according to their biological properties such as the similarity in symptoms, plant host, and insect vectors. This way of classification was laborious, time-consuming and not reliable. Recently a number of genetic markers have been developed for differentiation among phytoplasma strains, including immunodominant membrane protein (imp) and secA genes (Siampour et al. 2012; Bekele et al. 2011; Hodgetts et al. 2008).

Despite studies conducted on ‘Ca. P. aurantifolia’ in Oman and elsewhere there is a lack of knowledge concerning diversity and genetic relationship among ‘Ca. P. aurantifolia’ strains. This study was conducted to investigate the diversity of the strains infecting acid lime in Oman, the UAE and Iran based on 16S rRNA, secA and imp genes. This way of classification was laborious, time-consuming and not reliable. Recently a number of genetic markers have been developed for differentiation among phytoplasma strains, including immunodominant membrane protein (imp) and secA genes (Siampour et al. 2012; Bekele et al. 2011; Hodgetts et al. 2008).

Despite studies conducted on ‘Ca. P. aurantifolia’ in Oman and elsewhere there is a lack of knowledge concerning diversity and genetic relationship among ‘Ca. P. aurantifolia’ strains. This study was conducted to investigate the diversity of the strains infecting acid lime in Oman, the UAE and Iran based on 16S rRNA, secA, and imp genes, and to determine the ability of the latter two genes to discriminate ‘Ca. P. aurantifolia’ strains from phytoplasmas belonging to other ribosomal groups. Knowledge gained about relationships among the WBDL phytoplasma isolates from the three countries will help in developing plans for management strategies to contain WBDL spreading.

**Results**

**Survey and sample collection**

Surveys in Oman, UAE and Iran showed the presence of typical symptoms of witches’ broom disease in the three countries. Disease symptoms were characterized by appearance of dense growth of shoots, clustering of leaves and branches, and reduction in size and yellowing of leaves (Fig. 1). Dieback symptoms were also observed in one farm in Al-Ain (UAE). Severity of the disease was variable among farms and could not be quantified due to the tendency of many farmers to remove symptomatic branches as soon as they appear. The total samples which were collected during this study were 75 from Oman, 20 from Iran and 20 from the UAE. However, only samples which yielded good quality sequences of the 16S rRNA, imp and secA genes (38 from Oman, 10 from Iran and 9 from the UAE) were included in further analysis.

**Phytoplasma diversity on 16S rRNA, secA and imp genes**

PCR analysis of 57 acid lime samples based on the 16-23S rRNA using P1/P7 and R16R2/R16F2n produced fragments of 1784 base pairs (bp) and 1238–1248 bp, respectively (Table 2). Only fragments of 1238 bp representing the 16S rRNA gene of the strains were used in the phylogenetic analysis. Comparison of these sequences showed that strains shared 98.5–100 % (average 99.7 %) nucleotide similarity to each other and 99.4 % similarity to the reference strain of lime witches’ broom phytoplasma from Oman in GenBank (Accession number: EF186828). All strains from Oman clustered with reference strains of ‘Ca. P. aurantifolia’ (subgroup 16SII-B) from GenBank (Fig. 2). Analysis of genetic divergence among ‘Ca. P. aurantifolia’ strains based on 16S rRNA gene showed that the range of divergence was 0–0.003 for Omani strains, 0–0.002 for UAE strains and 0–0.002 for the Iranian strains (Table 3). The overall level of divergence based on all ‘Ca. P. aurantifolia’ from this study and from reference strains was 0.000–0.011 (avg. 0.001).
The primer pair SecAfor2/SecArev3 resulted in a product of 482 bp in size for the 57 strains analyzed (Table 1). Analysis of secA sequences showed that all strains share 99.8–100 % sequence similarity to each other and to the secA sequence of lime witches’ broom phytoplasma (Accession number: EU168731, Oman). Based on the SecA phylogenetic tree, ‘Candidatus Phytoplasma aurantiofolia’ isolates were separated from 9 other phytoplasmas with a very high bootstrap support (Fig. 3). Analysis of genetic divergence among the 57 ‘Ca. P. aurantiofolia’ based on secA gene sequences showed that the range of divergence is 0-0.010 among Omani strains, 0–0.004 among the UAE strains and 0-0.006 among the Iranian strains (Table 3). The overall sequence divergence in the secA genes from all ‘Ca. P. aurantiofolia’ isolates of this study and the reference strains was 0.000–0.015 (avg. 0.003).

PCR amplification using the primer pair ImpF/ImpR, produced a fragment of 519 bp (Table 1). Analysis based on the imp gene sequences showed that 55 phytoplasmas share 100 % sequence similarity to each other and to lime witches’ broom phytoplasma (Accession number: GU339497, Iran), while they were found to share 99.8 % similarity to strains 8 and 9 from Oman (Table 2). Phylogenetic analysis based on the imp gene sequences showed that all ‘Ca. Phytoplasma aurantiofolia’ isolates were separated with a high bootstrap support from 16 other phytoplasmas (Fig. 4). The genetic divergence among the 57 sequences ranged from 0 to 0.002 for the Omani strains, while there was no divergence among the Iranian and UAE strains (Table 3). The overall sequence divergence in the imp genes from all ‘Ca. P. aurantiofolia’ isolates of this study and the reference strains was 0.000–0.015 (avg. 0.003).

Concatenated sequence analysis of phytoplasma genes
Phylogenetic analysis of the 57 strains based on the concatenated sequence of the 16S rRNA, secA and imp genes (2239 bp) showed clustering of most strains from Oman, the UAE and Iran. There was no relationship between clustering of the strains and the countries of origin or the regions in which they were collected (Fig. 5). The overall divergence among and within the Omani, Iranian and UAE sub-populations was found to be 0.001.

Discussion
Witches’ broom disease symptoms were observed in all the surveyed regions in Oman, UAE and Iran. Analysis of diversity among 57 phytoplasmas from Oman, UAE and Iran based on sequences of the 16S RNA gene showed that all share a high level of nucleotide similarity (mean: 99.7 %).

Previous studies detected limited variation in the 16S rRNA gene sequences among phytoplasma strains belonging to the same group (Bertaccini and Duduk 2009). The findings of this survey indicated the existence of a limited variation among ‘Ca. P. aurantiofolia’ strains from Oman, UAE and Iran, not only based on the 16S RNA gene, but also on secA and imp genes. None of the sequence of the three genes separated strains based on the country from which they were obtained. This finding, together with the overall low level of genetic divergence, may indicate that phytoplasma strains from the three countries have the same origin and could have been moved among the three countries via infected propagation material (Al-Sadi et al. 2012b). It is therefore important to inspect propagative material of acid lime for phytoplasma infection using appropriate detection tools (Duduk et al. 2013; Al-Sadi

### Table 1 Primers used for amplifying Phytoplasma genes

| Gene   | Primer name | 5′-3′ Sequence | Product size (bp) | References                     |
|--------|-------------|----------------|-------------------|-------------------------------|
| 16S rRNA | P1          | AAGAGTGTGACCTGGCTCAGGATT | 1784            | Deng and Hiruki (1991)        |
|        | P7          | CGTCTCCATCGGCTT   | ~1248            | Gundersen and Lee (1996)      |
|        | R16R2       | GAAACGACTGCTAAGCTGG | ~1248            | Hodgetts et al. (2008)        |
|        | R16F2n      | TGAAGGTGTGTCGAAACCCCG | ~1248            | Siampour et al. (2012)        |
| SecA   | SecAfor1    | GARATGAAACTGGGAGG  | 482a             |                                 |
|        | SecAfor2    | GAYGARGSGWAGAACKCT |                 |                                 |
|        | SecArev3    | GTTTRGCAGTTCGGCATNC |                 |                                 |
| imp    | ImpF        | ATGAATCACAAAGAAAAATTTCAC | 519              |                                 |
|        | ImpR        | TTAGTATAATTTAATCTG |                 |                                 |

* Using SecAfor2/SecArev3 primer combinations in a semi-nested PCR (Hodgetts et al. 2008)
| No. | Sample code | Country | Governorate/province | Year of collection | GenBank accession numbers |
|-----|-------------|---------|----------------------|-------------------|------------------------|
|     |             |         |                      |                   | 16S rRNA | secA | imp |
| 1   | Iran 1      | Iran    | Hormozgan            | 2013              | LN872963  | LN873084 | LN873020 |
| 2   | Iran 2      | Iran    | Hormozgan            | 2013              | LN872966  | LN873087 | LN873023 |
| 3   | Iran 3      | Iran    | Hormozgan            | 2013              | LN872969  | LN873090 | LN873026 |
| 4   | Iran 4      | Iran    | Hormozgan            | 2013              | LN872972  | LN873093 | LN873029 |
| 5   | Iran 5      | Iran    | Hormozgan            | 2013              | LN872975  | LN873096 | LN873032 |
| 6   | Iran 6      | Iran    | Kerman               | 2013              | LN872978  | LN873099 | LN873035 |
| 7   | Iran 7      | Iran    | Kerman               | 2013              | LN872981  | LN873102 | LN873038 |
| 8   | Iran 8      | Iran    | Kerman               | 2013              | LN872984  | LN873105 | LN873041 |
| 9   | Iran 9      | Iran    | Kerman               | 2013              | LN872986  | LN873107 | LN873043 |
| 10  | Iran 10     | Iran    | Hormozgan            | 2013              | LN872989  | LN873110 | LN873046 |
| 11  | UAE 1       | UAE     | Al‑Ain               | 2013              | LN872965  | LN873086 | LN873022 |
| 12  | UAE 2       | UAE     | Al‑Ain               | 2013              | LN872968  | LN873089 | LN873025 |
| 13  | UAE 3       | UAE     | Al‑Ain               | 2013              | LN872971  | LN873092 | LN873028 |
| 14  | UAE 4       | UAE     | Al‑Ain               | 2013              | LN872974  | LN873095 | LN873031 |
| 15  | UAE 5       | UAE     | Al‑Ain               | 2013              | LN872977  | LN873098 | LN873034 |
| 16  | UAE 6       | UAE     | Al‑Ain               | 2013              | LN872980  | LN873101 | LN873037 |
| 17  | UAE 7       | UAE     | Al‑Ain               | 2013              | LN872983  | LN873104 | LN873040 |
| 18  | UAE 9       | UAE     | Al‑Ain               | 2013              | LN872988  | LN873109 | LN873045 |
| 19  | UAE 10      | UAE     | Al‑Ain               | 2013              | LN872991  | LN873112 | LN873048 |
| 20  | Oman 1      | Oman    | Musandam             | 2013              | LN872964  | LN873085 | LN873021 |
| 21  | Oman 2      | Oman    | Musandam             | 2013              | LN872967  | LN873088 | LN873024 |
| 22  | Oman 3      | Oman    | Musandam             | 2013              | LN872970  | LN873091 | LN873027 |
| 23  | Oman 4      | Oman    | Musandam             | 2013              | LN872973  | LN873094 | LN873030 |
| 24  | Oman 5      | Oman    | Musandam             | 2013              | LN872976  | LN873097 | LN873033 |
| 25  | Oman 6      | Oman    | Musandam             | 2013              | LN872979  | LN873100 | LN873036 |
| 26  | Oman 7      | Oman    | Musandam             | 2013              | LN872982  | LN873103 | LN873039 |
| 27  | Oman 8      | Oman    | Musandam             | 2013              | LN872985  | LN873106 | LN873042 |
| 28  | Oman 9      | Oman    | Musandam             | 2013              | LN872987  | LN873108 | LN873044 |
| 29  | Oman 10     | Oman    | Batinah              | 2014              | LN872990  | LN873111 | LN873047 |
| 30  | Oman 11     | Oman    | Batinah              | 2014              | LN872992  | LN873113 | LN873049 |
| 31  | Oman 12     | Oman    | Batinah              | 2014              | LN872993  | LN873114 | LN873050 |
| 32  | Oman 13     | Oman    | Batinah              | 2014              | LN872994  | LN873115 | LN873051 |
| 33  | Oman 14     | Oman    | Batinah              | 2014              | LN872995  | LN873116 | LN873052 |
| 34  | Oman 15     | Oman    | Batinah              | 2014              | LN872996  | LN873117 | LN873053 |
| 35  | Oman 16     | Oman    | Batinah              | 2014              | LN872997  | LN873118 | LN873054 |
| 36  | Oman 17     | Oman    | Batinah              | 2014              | LN872998  | LN873119 | LN873055 |
| 37  | Oman 18     | Oman    | Batinah              | 2014              | LN872999  | LN873120 | LN873056 |
| 38  | Oman 19     | Oman    | Batinah              | 2014              | LN873000  | LN873121 | LN873057 |
| 39  | Oman 20     | Oman    | Batinah              | 2014              | LN873001  | LN873122 | LN873058 |
| 40  | Oman 21     | Oman    | Batinah              | 2014              | LN873002  | LN873123 | LN873059 |
| 41  | Oman 22     | Oman    | Batinah              | 2014              | LN873003  | LN873124 | LN873060 |
| 42  | Oman 23     | Oman    | Batinah              | 2014              | LN873004  | LN873125 | LN873061 |
| 43  | Oman 24     | Oman    | Batinah              | 2014              | LN873005  | LN873126 | LN873062 |
| 44  | Oman 25     | Oman    | Batinah              | 2014              | LN873006  | LN873127 | LN873063 |
| 45  | Oman 26     | Oman    | Batinah              | 2014              | LN873007  | LN873128 | LN873064 |
| 46  | Oman 27     | Oman    | Dakhlia              | 2014              | LN873008  | LN873129 | LN873065 |
| 47  | Oman 28     | Oman    | Dakhlia              | 2014              | LN873009  | LN873130 | LN873066 |
| 48  | Oman 29     | Oman    | Dakhlia              | 2014              | LN873010  | LN873131 | LN873067 |
et al. 2012b). Trade is very active among the three countries and the exchange of agricultural material and products is very common owing to globalized market (Al-Sadi et al. 2012a, 2013). It is possible that the phytoplasma moved from the northern part of Oman to the UAE due to trade activities and germplasm exchange between families living on either side of the countries’ borders. In addition, the active trade between the northern part of Oman and the southern part of Iran could have contributed to moving infected material between the two countries. Additionally, the insect vector of WBDL (*Hishimonas phycitis*) and its close relationship with lime (or with some other citrus plants) may have provided a unique and narrow ecological niche for the WBDL phytoplasma resulting in its low genetic divergence.

Analysis of divergence among 57 isolates from the three countries showed that *imp* is highly conserved compared to the 16S rRNA and secA genes. Siampour et al. (2012) reported variation based on the *imp* gene and the presence of three differentiable subgroups when 18 *Ca. P. aurantifolia*-related strains were analyzed (*imp*-A, *imp*-B and *imp*-C). In their study, the strains were obtained from a range of different host plant species such as alfalfa, tomato, eggplant, carrot, periwinkle, pear, peanut and others, together with only one samples from lime (Siampour et al. 2012). The very low variability of the *imp* gene found in this work is in agreement with *imp* gene reported feature as a phytoplasma gene subjected to positive environmental selection (Kakizawa et al. 2009). It also indirectly confirms the possible spreading mainly through propagation materials (Al-Sadi et al. 2012b). SecA gene has been reported to give high resolution among the different ribosomal groups (Bekele et al. 2011), however also in this gene sequences a very low level of divergence among phytoplasma strains was detected.

**Conclusion**

The genetic diversity of *Ca. P. aurantifolia* in Oman, UAE and Iran was analyzed for the first time based on three genes (16S rRNA, *imp* and secA). Data provided evidence of low genetic diversity among all strains based on analysis of the three genes. Future studies should target the association of other *Ca. P. aurantifolia* genes with geographical locations and any symptoms that are not typical of WBDL.

**Methods**

**Survey and collection of samples**

Acid lime leaf samples were collected in Oman, UAE and Iran from trees with typical WBDL symptoms (Figs. 1, 6). Samples were collected over November 2013 to March 2014. Each sample consisted of at least 30 g of leaves which were kept in a plastic bag and stored in a cool box. All sampling details including location of the farm and age of trees were recorded.

In Oman, samples were collected from 4 governorates: Musandam, Batinah North, Dakhilya, and Dhahira (Fig. 6). At least 15 samples were collected from 5 farms in each governorate. Lime leaf samples were also

### Table 2 continued

| No. | Sample code | Country | Governorate/province | Year of collection | GenBank accession numbers |
|-----|-------------|---------|----------------------|-------------------|-------------------------|
|     |             |         |                      |                   | 16S rRNA | secA | *imp* |
| 49  | Oman 30     | Oman    | Dakhila              | 2014              | LN873011 | LN873068 |
| 50  | Oman 31     | Oman    | Dakhila              | 2014              | LN873012 | LN873069 |
| 51  | Oman 32     | Oman    | Dakhila              | 2014              | LN873013 | LN873070 |
| 52  | Oman 34     | Oman    | Dhahira              | 2014              | LN873014 | LN873071 |
| 53  | Oman 35     | Oman    | Dhahira              | 2014              | LN873015 | LN873072 |
| 54  | Oman 36     | Oman    | Dhahira              | 2014              | LN873016 | LN873073 |
| 55  | Oman 37     | Oman    | Dhahira              | 2014              | LN873017 | LN873074 |
| 56  | Oman 38     | Oman    | Dhahira              | 2014              | LN873018 | LN873075 |
| 57  | Oman 39     | Oman    | Dhahira              | 2014              | LN873019 | LN873076 |

(See figure on next page.)

**Fig. 4** Phylogenetic analysis of 57 phytoplasma isolates from Oman, the UAE and Iran with 17 *imp* reference sequences of all representative phytoplasmas available in GenBank. Bootstrap values above 50 % are shown (1000 replications). The circle, triangle and square symbols represent Omani, UAE and Iranian isolates, respectively.
collected from 6 farms in Al-Ain (UAE) in March 2013. In addition, samples were collected from Hormozgan and Kerman (Iran) (Fig. 6). At least 20 samples were collected from each country. All samples were labeled and transferred to Plant Pathology Research Laboratory, Sultan Qaboos University, where they were stored at −80 °C until used.

**DNA extraction**

Lime leaves were washed with tap water and disinfected by 70 % ethanol to remove contaminants. About 1 g of leaf midribs was ground using liquid nitrogen in sterilized mortars and pestles and DNA extraction was carried out by using DNeasy Plant Mini Kit (QIAGEN, GmbH, Hilden, Germany) according to manufacturer’s instructions.

**Polymerase chain reaction (PCR)**

Detection of phytoplasma in the samples was done using the universal primer pair P1 and P7 (Deng and Hiruki 1991; Schneider et al. 1995) (Table 1). PCR was done by using the following conditions: 94 °C for 30 s, then 35 cycles of 95 °C for 2 min, 53 °C for 60 s and 72 °C for 90 s, and final extension of 72 °C for 10 min (Sharmila et al. 2004). The reaction consisted of PuReTaq™ Ready-To-Go PCR™ beads (HVD Life Sciences, Vienna, Austria), 0.4 µM of each primer, ~25 ng DNA and sterilized distilled water up to 25 µl. Nested PCR using the primer pair R16R2 and R16F2n was carried out as described by Gundersen and Lee (1996). PCR products were separated on 1 % agarose gel in TBE buffer containing 1.5 µl of ethidium bromide and visualized under UV light.

The 57 DNA samples positive to phytoplasma were subjected to further PCR analysis to amplify secA gene by direct and semi-nested PCR assays using SecAfor1, SecAfor2, and SecArev3 as explained by Hodgetts et al. (2008). The conditions of PCR were 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 53 °C for 60 s and 72 °C for 90 s and final extension of 72 °C for 15 min. First-round PCR product was diluted 1: 200 with sterilized distilled water and 1 µl of diluted product was used in semi-nested PCR using the primer pair SecAFor2/SecArev3 and the same reaction mixture and PCR conditions described above (Table 1). PCR products were separated as described above.

Amplification of imp gene was done using primers ImpF and ImpR as described by Siampour et al. (2012) (Table 1). The amplification and detection were carried out as described previously.

**Sequence analysis**

PCR products of the 16S rRNA, secA, and imp genes for the 57 samples collected from Oman, UAE and Iran were directly sequenced at Macrogen, Korea using the same primers employed for their amplification (Table 1). Sequences were aligned using Clustal-W program in Chromas Pro (version 1.41; Technelysium Pty Ltd, Brisbane, QLD, Australia). Construction of UPGMA tree was done based on the matrix of pairwise distances using the Kimura 2 parameter evolutionary model (Mega 6) (Tamura et al. 2013). Sequences of the 16S rRNA, secA and imp genes of reference strains of ‘Ca. Phytoplasma aurantifolia’ and phytoplasmas from other 16Sr groups obtained from National Center for Biotechnology Information (NCBI) were used for comparison with sequence from this study. Bootstrap consensus trees were generated based on 50 % majority-rule using 1000 replications. In addition, genetic divergence was calculated based on the number of base differences per sequence from averaging over all sequence pairs between groups and all ambiguous positions were removed for each sequence pair (Tamura et al. 2013).

**Table 3 Estimation of evolutionary divergence among phytoplasma sequences**

|         | Iran 16S rDNA | imp | secA | UAE 16S rDNA | imp | secA | Oman 16S rDNA | imp | secA |
|---------|--------------|-----|------|--------------|-----|------|---------------|-----|------|
| Mini    | 0.000        | 0.000 | 0.000 | 0.000        | 0.000 | 0.000 | 0.000         | 0.000 | 0.000 |
| Max     | 0.002        | 0.000 | 0.006 | 0.002        | 0.000 | 0.004 | 0.003         | 0.002 | 0.010 |
| Avg.    | 0.000        | 0.000 | 0.002 | 0.001        | 0.000 | 0.001 | 0.001         | 0.000 | 0.003 |

The table provides estimates of evolutionary divergence over sequence pairs within phytoplasma groups from the different countries. The analysis involved 57 nucleotide sequences. Analyses were conducted using the Kimura 2-parameter model (Mega 6)
Authors' contributions

Designed the experiments: AMA, MD, MSH, RS, RAY, AB; Performed the experiments: SYA, AMA, EAK. Analyzed the data: SYA, AMA, AB. Contributed reagents/materials: AMA. Wrote the manuscript: SYA, AMA, MD, MSH, RS, RAY, EAK, AB. All authors read and approved the final manuscript.

Author details

1 Royal Court Affairs, Seeb, Oman. 2 Department of Crop Sciences, College of Agricultural and Marine Sciences, Sultan Qaboos University, PO Box 8, 123 Al Khoudh, Oman. 3 School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, UK. 4 Abu Dhabi Food Control Authority, PO Box 52150, Abu Dhabi, UAE. 5 Islamic Azad University, Shiraz, Iran. 6 Department of Agricultural Sciences, Plant Pathology, Alma Mater Studiorum-University of Bologna, Bologna, Italy.
Acknowledgements

Authors would like to acknowledge Sultan Qaboos University and VALE Oman for the financial support of the study through the projects SR/AGR/CROP/13/01 and EG/AGR/CROP/12/02. Thanks are due to Abu Dhabi Food Control Authority (ADFCA) for support of the study in the UAE and to Issa Al-Mahmooli and growers in Oman, the UAE and Iran for their help in the collection of samples. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 7 March 2016   Accepted: 27 September 2016

Published online: 03 October 2016

References

Al-Sadi AM, Al-Hilali SA, Al-Yahyai RA, Al-Said FA, Deadman ML, Al-Mahmooli IH, Nolasco G (2012a) Molecular characterization and potential sources of Citrus Tristeza Virus in Oman. Plant Pathol 61:632–640
Al-Sadi AM, Al-Moqbali H, Al-Yahyai R, Al-Said F, Al-Mahmooli I (2012b) AFLP data suggest a potential role for the low genetic diversity of acid lime (Citrus aurantifolia) in Oman in the outbreak of witches’ broom disease of lime. Euphytica 188:285–297
Al-Sadi AM, AL-Wehaibi AN, Al-Shariqi RM, Al-Hammadi MS, Al-Hosni IA, Al-Mahmooli IH, Al-Ghaithi AG (2013) Population genetic analysis reveals diversity in Lasiodiplodia species infecting date palm, Citrus, and mango in Oman and the UAE. Plant Dis 97:1363–1369
Al-Yahyai RA, Al-Sadi AM, Al-Said FA, Al-Kalbani Z, Carvalho CM, Elliot SL, Bertaccini A (2015) Development and morphological changes in leaves and branches of acid lime (Citrus aurantifolia) affected by witches’ broom disease. Phytopathol Meditteranea 54:133–139
Bekele B, Abeysinghe S, Hoat TX, Hodgetts J, Dickinson M (2011) Development of specific secA-based diagnostics for the 16SrXI and 16SrXIV phytoplasmas of the Graminaceae. Bull Insectol 64:15–16
Bertaccini A, Duduk B (2009) Phytoplasma and phytoplasma diseases: a review of recent research. Phytopathol Mediterranea 48:355–378
Bové JM, Garnier M, Mjeni AM, Khayrallah A (1988) Witches’ broom disease of small-fruited acid lime trees in Oman: first MLO disease of citrus. In: Proceedings of the 10th conference of the international organization of citrus virologists (ICOV). Riverside, pp 307–309
Bové JM, Danet JL, Banane K, Hassanzadeh N, Taghizadeh M, Salehi M, Garnier M (2000) Witches’ broom disease of lime (WBWL) in Iran. Fourteenth ICOV conference, insect-transmitted procaryotes, pp 207–212
Chung KR, Khan IA, Brlansky RH (2006) Citrus diseases exotic to Florida: witches’ broom disease of lime (WBWL). EDIS Publications, University of Florida, Gainesville, pp 228:1–228:3
Davies FS, Albirigo LG (1994) Citrus. CAB International, United Kingdom
Deng S, Hiruki C (1991) Amplification 16S rRNA genes from culturable and non-culturable mollicutes. J Microbiol Methods 14:53–61
Duduk B, Paltrinieri S, Lee IM, Bertaccini A (2013) Nested PCR and RFLP analysis based on the 16S rRNA gene. Methods Mol Biol (Clifton, NJ) 938:159–171
FAOSTAT (2015) FAO. http://faostat3.fao.org/browse/rankings/countries_by_commodity/E. Accessed 2015
Garner M, Zreik L, Bové JM (1991) Witches’ broom, a lethal mycoplasmal disease of lime trees in the Sultanate of Oman and the United Arab Emirates. Plant Dis 75:546–551
Gundersen DE, Lee IM (1996) Ultrasensitive detection of phytoplasmas by nested PCR assay using two universal primer pairs. Phytopathol Mediterranea 35:144–151
Hodgetts J, Boonham N, Mumford R, Harrison N, Dickinson M (2008) Phytoplasma phylogenetics based on analysis of secA and 23S rRNA gene sequences for improved resolution of candidate species of Candidatus Phytoplasma. Int J Syst Evol Microbiol 58:1826–1837
Kakizawa S, Oshima K, Ishii Y, Hoshi A, Maejima K, Jung H-Y, Yamaji Y, Namba S (2009) Cloning of immunodominantmembrane protein genes of phytoplasmas and their in planta expression. FEBS Microbiol Lett 293:92–101
Schneider B, Seemüller E, Smart CD, Kirkpatrick BC (1995) Phylogenetic classification of plant pathogenic mycoplasmalike organisms or phytoplasmas. In: Raszin S, Tully JG (eds) Molecular and diagnostic procedures in mycoplasmology, vol 2. Academic Press, New York, pp 369–380
Sharmila LB, Bhasker S, Thelley MT, Edwin BT, Mohankumar C (2004) Cloning and sequencing of phytoplasma ribosomal DNA (rDNA) associated with keralal wilt disease of coconut palms. Plant Biochem Biotechnol 13:1–5
Siampour M, Izadpanah K, Galetto L, Salehi M, Marzachi C (2012) Molecular characterization, phylogenetic comparison and serological relationship of the Imp protein of several Candidatus Phytoplasma aurantifolia strains. Plant Pathol 62:452–459
Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729
Zreik L, Carle P, Bové JM, Garnier M (1993) Characterization of the mycoplasma like organism associated with witches’ broom disease of lime and proposition of a Candidatus’taxon for the organism, Candidatus Phytoplasma aurantifolia’. Int J Syst Bacteriol 45:449–453