Detection, Identification, and Molecular Characterization of the 16SrII-D Phytoplasmas Infecting Vegetable and Field Crops in Oman

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Abstract

Typical symptoms of phytoplasma infection were observed on 11 important crops in Oman that included alfalfa, sesame, chickpea, eggplant, tomato, spinach, rocket, carrot, squash, field pea, and faba bean. To identify the phytoplasmas in these crops, samples from infected and asymptomatic plants were collected, followed by amplifying and sequencing of the 16S ribosomal RNA, *secA*, *tuf*, *imp*, and SAP11 genes. We found that these sequences share >99% similarity with the peanut witches' broom subgroup (16SrII-D). Whereas some sequence variation was found in the five genes among 11

phytoplasma isolates of different crops, all sequences grouped into one clade along with those of other phytoplasmas belonging to the 16SrII-D group. Thus, 16SrII-D phytoplasmas infect a diverse range of crops in Oman. Phytoplasmas in this group have not been reported to occur in carrot, spinach, rocket, and field pea previously. Within Oman, this is the first report of the presence of 16SrII-D phytoplasmas in tomato, spinach, rocket, carrot, squash, field pea, and faba bean. Sequences of the five genes enabled for better distinction of the 16SrII-D phytoplasmas that occur in Oman.

Phytoplasmas (genus '*Candidatus* Phytoplasma') are bacterial plant pathogens that belong to the class Mollicutes, which are thought to have diverged from gram-positive bacteria through loss of cell walls and reduction in overall genome size (IRPCM Phytoplasma/Spiroplasma Working Team 2004). Phytoplasmas reside intracellularly inside the cytoplasm of phloem sieve cells of the plant vascular tissue (Hogenhout et al. 2008). Phloem-feeding insects, mainly leafhoppers, planthoppers, and psyllids, acquire the phytoplasmas from these cells and transmit them to other plants (Ammar and Hogenhout 2006). Phytoplasmas can cause disease in more than 700 plant species, including cereal and vegetables crops, fruit trees, and ornamental and medicinal plants from over 100 plant families (Bertaccini 2007; Hogenhout et al. 2008). Phytoplasma-infected plants often show dramatic symptoms such as witches' brooms, virescence (nongreen flower organs remain green), phyllody (development of leaf-like structures instead of flowers), sterility, general chlorosis (yellowing) of leaves, overall stunting of the plant, formation of many little leaves, enlarged flower organs (big buds), reduced fruit and seed production, rapid dieback and decline, and eventual death of the plant (Bertaccini 2007; Hogenhout et al. 2008).

Because Koch's postulates cannot be completed for phytoplasma infections, phytoplasmas have been assigned to the '*Candidatus* Phytoplasma' genus (Serena et al. 2016) and their relationships determined by 16S ribosomal RNA (rRNA) gene sequences (Ahrens and Seemüller 1992). Thus far, about 43 '*Candidatus* Phytoplasma' spp. were assigned (Zhao and Davis 2016) and, within these species, phytoplasmas are further divided into subgroups based on 16S ribosomal DNA (rDNA) sequences (Lee et al. 1998). However, some studies suggest that it is not sufficient for describing subgroups within a species (Lee et al. 2010). Therefore, other genes have been used to differentiate closely related phytoplasma strains, including genes for translocase protein A (*SecA*), antigenic/immunodominant membrane protein

(*Amp/Imp*), and translation elongation factor Tu (*Tuf*) (Makarova et al. 2012; Valiunas et al. 2013). The *tuf* gene has been used for classification of 16SrI group phytoplasmas (Marcone et al. 2000). Nucleotide sequences of *secA* and 23S rRNA genes also improved differentiation of some phytoplasma isolates (Hodgetts et al. 2008). The surface membrane proteins *Imp* and *Amp* are variably present among phytoplasmas and are divided into three groups: immunodominant membrane protein (*Imp*), immunodominant membrane protein A (*ImpA*), and antigenic membrane protein (*Amp*) (Kakizawa et al. 2006). Nucleotide sequences of *imp* genes were used for diagnosis of several strains of the 16SrII phytoplasma '*Ca. P. aurantifolia*' in Iran (Siampour et al. 2013), a phytoplasma that is also known as witches' broom disease of lime (WBDL) and that has caused major losses for lime productions in Middle East, including Oman (Al-Yahyai et al. 2012). Genes encoding virulence (effector) proteins are also variably present among phytoplasmas (Bai et al. 2009; Chung et al. 2013; Orlovskis 2017; Sugio et al. 2014) and were shown to be required for the induction of typical phytoplasma symptoms such as witches' brooms by SAP11 (Janik et al. 2016; Lu et al. 2014; Sugio et al. 2011a) and Tengu (Hoshi et al. 2009), and induction of leaf-like flowers by SAP54 (MacLean et al. 2011, 2014).

Phytoplasmas in the 16SrII phytoplasma group, which includes peanut witches' broom (PnWB) and sweet potato little leaf (SPLL) phytoplasmas, are diverse, with at least 21 subgroups (Yang et al. 2016), and are widespread in the Middle East and India. Thus far, the 16SrII subgroup B '*Ca. P. aurantifolia*' (WBDL) has only been found in citrus trees (Bertaccini et al. 2014) and, therefore, may have a host range restricted to this host (Alhudaib et al. 2009; Garnier et al. 1991; Ghosh et al. 1999). However, other 16SrII phytoplasmas are found in alfalfa (Hosseini et al. 2015a; Khan et al. 2002), sesame (Al-Sakeiti et al. 2005), tomato (Alhudaib and Rezk 2011), and sunflower (Salehi et al. 2015).

About 11 host plants belonging to different families have been seriously hampered due to the presence of the 16SrII-D phytoplasma group in Oman. Alfalfa witches' broom was first reported in Oman in the 1990s (Khan et al. 2002). In addition, several phytoplasmas were found associated with sesame (*Sesamum indicum*) (Al-Sakeiti et al. 2005), chickpea (*Cicer arietinum*) (Al-Saady et al. 2006) and eggplant (*Solanum melongena* L.) (Al-Subhi et al. 2011).

During a survey in fields in 2014 and 2015, typical symptoms of phytoplasma infection were observed on 11 crops, including alfalfa (*Medicago sativa*), sesame (*Sesamum indicum*), chickpea (*Cicer arietinum*), eggplant (*Solanum melongena*), tomato (*S. lycopersicum*), spinach (*Spinacia oleracea*), rocket (*Eruca sativa*), carrot (*Daucus carota*), squash (*Cucurbita pepo*), field pea (*Pisum sativum*), and faba bean (*Vicia faba*) in different parts of the country. Symptoms typical of

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phytoplasma included excessive proliferation of shoots and formation of witches' brooms and big bud, phyllody, and virescence, which was associated with stunted growth.

The aim of this study was to detect and characterize phytoplasma associated with these crops and their relationship to phytoplasmas in the 16SrII group. We used *secA*, *tuf*, *imp*, and SAP11 gene sequences of these phytoplasmas to further investigate their relationship with related phytoplasma members.

Materials and Methods

Sample collection and nucleic acid extraction. Field surveys were carried out throughout all regions of the Sultanate of Oman

during 2014 and 2015 to collect samples showing typical symptoms of witches' broom disease (WBD). Samples of alfalfa, sesame, chickpea, tomato, spinach, rocket, carrot, squash, field pea, faba bean, and eggplant showing typical WBD symptoms were collected from Al-Dahira, Al-Dakhilia, Al-Wousta, and Dhofar governorates (Table 1) and stored at -80°C until used. Total nucleic acids were extracted from symptomatic and asymptomatic plants samples following the method of Doyle and Doyle (1987), with some modifications.

Phytoplasma detection using the 16S rRNA gene and sequencing.

Direct and nested polymerase chain reactions (PCR) were conducted to amplify the 16S rRNA gene sequence of phytoplasmas from the 11 crops. DNA samples extracted from symptomatic plants were used as

Table 1. List of phytoplasma strains of the 16Sr-II group used in this study with their geographical origins, and corresponding 16S ribosomal DNA (rDNA), *secA*, *imp*, *tuf*, and SAP11 gene accession numbers

Isolate	Phytoplasma strain	Acronym	Origin	Accession numbers				
				16S rDNA	<i>SecA</i>	<i>tuf</i>	<i>imp</i>	SAP11
1	Alfalfa witches' broom	AIWB	Southern Oman	KX358564	KX358576	KX358588	KX358600	KX358612
2	Sesame phyllody	SEWB	Northern Oman	KX358563	KX358575	KX358587	KX358599	KX358611
3	Chickpea phyllody	CEPH	Northern Oman	KX358573	KX358585	KX358597	KX358609	KX358621
4	Tomato big bud	TOBB	Northern Oman	KX358566	KX358578	KX358590	KX358602	KX358614
5	Spinach phyllody	SPPH	Northern Oman	KX358567	KX358579	KX358591	KX358603	KX358615
6	Rocket virescence	ROVI	Northern Oman	KX358569	KX358581	KX358593	KX358605	KX358617
7	Carrot phyllody	CAPH	Northern Oman	KX358568	KX358580	KX358592	KX358604	KX358616
8	Squash virescence	SQVI	Central Oman	KX358565	KX358577	KX358589	KX358601	KX358613
9	Field pea phyllody	FIPH	Northern Oman	KX358571	KX358583	KX358595	KX358607	KX358619
10	Faba bean phyllody	FAPH	Northern Oman	KX358570	KX358582	KX358594	KX358606	KX358618
11	Eggplant big bud	EGBB	Northern Oman	KX358572	KX358584	KX358596	KX358608	KX358620
12	Lime witches' broom	WBDL	Northern Oman	KX358574	KX358586	KX358598	KX358610	KX358622

Table 2. Universal and specific primers used in this study for polymerase chain reaction (PCR) amplification and sequencing of the 16S ribosomal RNA, *secA*, *imp*, *tuf*, and SAP11 genes of group-II Omani phytoplasmas^a

Primer sets	Specificity	Location	PCR product size (bp)	Reaction	Sequence (5' to 3')	References
P1/P7	Universal	16S/23SR	1,800	Direct PCR	AAGAGTTTGATCCTGGCTCAGGATT/CGTCTTCATCGGCTCTT	Deng and Hiruki 1991; Schneider et al. 1995
R16F2n/R16R2	Universal	16S/IS	1,240	Nested PCR	TGACGGGCGGTGTGTACAAACCCCG/GAAACGACTGCTAAGACTGG	Gundersen et al., 1996
P1/P6	Universal	16S	...	Seminested PCR	AAGAGTTTGATCCTGGCTCAGGATT/CGGTAGGGATACCTTGTTACGACTT	Deng and Hiruki 1991
P4/P7	Universal	16S/23SR	...	Seminested PCR	GAAGTCTGCAACTCGACTT/CGTCTTCATCGGCTCTT	Schneider et al. 1995
SecA-II-F1/SecA-II-R1	16SrII	<i>secA</i> gene	2,141	Direct PCR	AAAGATGAAGATTTTCTTAAAGA/TCCATATCATTTATATGACGTTGA	This study
SecA-II-F1/SecA-II-R4	16SrII	<i>secA</i> gene	1,511	Seminested PCR	AAAGATGAAGATTTTCTTAAAGA/ACAAAAAATTTAGTATAACCAGGATC	This study
SecA-II-F2	16SrII	<i>secA</i> gene	...	Sequence used	TGACTCGTGAATATGGTTATGT	This study
IMP-II-F1/IMP-II-R1	16SrII	<i>imp</i> gene	786	Direct PCR	GTTATAATTGAAGCGATATTG/ATAGAGGAGAAGAAAAAGTTTCT	This study
IMP-II-F2/IMP-II-R1	16SrII	<i>imp</i> gene	717	Seminested PCR	GATCATATTTGGTTTATAGGAG/ATAGAGGAGAAGAAAAAGTTTCT	This study
TUF-II-F1/TUF-II-R1	16SrII	<i>tuf</i> gene	1,490	Direct PCR	GCTTTTGTCTTTTAGCAGAA/AGACTATACACTAGTCTTCT	This study
TUF-II-F2/TUF-II-R1	16SrII	<i>tuf</i> gene	1,094	Seminested PCR	CGCAAAGATATTTAACTTTAG/AGACTATACACTAGTCTTCT	This study
SAP11-IID-F1/SAP11-IID-R1	16SrII-D	SAP11 gene	820	Direct PCR	CGGCAAAATAAAAGTTCAAATCA/AATCGAAACCAACCACTTATAG	This study
SAP11-IID-F2/SAP11-IID-R2	16SrII-D	SAP11 gene	465	Nested PCR	TTCTCAATTAACGAACCTTACG/AAAAAGACCCTTCAGAAAGGGTC	This study
SAP11-WBDL-F1/SAP11-WBDL-R1	16SrII-B (WBDL)	SAP11 gene	1,050	Direct PCR	CTTCAGCCACAAATAGAATCTTT/CAAATACAAATCGCTGCATAAA	This study
SAP11-WBDL-F2/SAP11-WBDL-R2	16SrII-B (WBDL)	SAP11 gene	550	Nested PCR	TTCTTTTATGAAATCACCTCAG/GCGCATATTATTAACCTCTTT	This study

^a WBDL = witches' broom disease of lime.

templates for PCR amplification of the 16S-23S ribosomal gene of the phytoplasma. Negative controls (DNA from asymptomatic plants and sterile water) and a positive control (phytoplasma DNA from WBDL) were used. The phytoplasma universal primer pair P1 (Deng and Hiruki

1991) and P7 (Schneider et al. 1995) (Table2) was used for the direct amplification of the 16S-23S rRNA gene. The product of direct PCR was a template for nested PCR after 1:20 dilution using primer pair R16F2n/R16R2, as described by Gundersen and Lee (1996) (Table 2).

Table 3. 16S ribosomal DNA (rDNA) sequences of different phytoplasma strains obtained from GenBank for phylogenetic analysis

Accession numbers	16S rDNA groups	Acronym	Phytoplasma strains
NC_007716	I-A	AYWB	Aster yellows witches' broom (AYWB)
AF222066	I	CPPb	Clover phyllody P. (strain CPh rmB)
L33760	I-A	BB	Tomato big bud
M30790	I-B	MAY	Aster yellows (MAY) ' <i>Candidatus</i> Phytoplasma asteris'
NC_005303	I-B	OY-M	Onion yellows mild strain (OY-M)
AF222065	I-C	CPh	Clover phyllody (CPh)
Y10097	II	PYC	<i>Mollicutes</i> sp.(Papaya yellow crinkle)
GI:471234556	II-A	PnWB	Peanut witches' broom
EF186828	II-B	LWB	' <i>Ca. .</i> Phytoplasma aurantifolia' strain LWB
U15442	II-B	WBDL	Lime witches' broom ' <i>Ca. P. aurantifolia</i> '
X83432	II-C	FBP	Faba bean phyllody
AB257291	II-D	ScaWB	Scaevola WB
AJ289193	II-A	SPLL	Sweet potato little leaf strain V4
Y10096	II-D	PpM	Papaya mosaic ' <i>Ca. P. australasia</i> '
Y16393	II-E	PEY	Pichris echioides phyllody
EF186827	II-F	CoP	Cotton phyllody
AF056095	III	SCYP-IA	Sugarcane yellows IA, Africa
AF175304	III	CloY	Clover yellows edge
AF495657	III	ChiY	Chinaberry yellows
JQ044392	III-A	PX11CT1	Peach X-disease ' <i>Ca. P. pruni</i> '
AF173558	III-B	CYE	Clover yellow edge
GU004371	III-C	PB	Pecan bunch
AF190226	III-G	WWB	Walnut WB (rmA)
AF302841	III-Q	BRWB7	Black raspberry witches' broom
FJ914654	I-S	COAH10	Mexican potato purple top phytoplasma strain COAH10
AF434989	IV	TexPp	Texas Phoenix palm phytoplasma
AF248957	IX-A	PPWB	Pigeon pea witches' broom
AF515636	IX-B	AIWB	Almond witches' broom ' <i>Ca. P. phoenicium</i> '
AF515637	IX-D	AIWB	Almond witches' broom
AY197655	V-A	EY	Elm yellows ' <i>Ca. P. ulmi</i> '
AB052876	V-B	JWB-G1	Jujube witches' broom ' <i>Ca. P. ziziphi</i> '
AY390261	VI-A	CP	Clover proliferation ' <i>Ca. P. trifolii</i> '
AF190224	VI-B	MC	Strawberry multiplier disease
AF409069	VI-C	EY-IL1	Illinois elm yellows
AF092209	VII-A	AshY	Ash yellows ' <i>Ca. P. fraxini</i> '
AY034608	VII-B	ErWB	Erigeron witches' broom
AY147038	VII-C	ArAWB	Argentinian alfalfa witches' broom
AF086621	VIII-A	LufWB	Loofah witches' broom
AJ542541	X-A	AP	Apple proliferation ' <i>Ca. P. mali</i> '
AJ542544	X-B	ESFY	European stone fruit yellows ' <i>Ca. P. prunorum</i> '
AB052873	XI-A	RYD	Rice yellow dwarf ' <i>Ca. P. oryzae</i> '
L76865	XII-B	AUSGY	Australian grapevine yellows ' <i>Ca. P. australiense</i> '
AJ243045	XII-C	StrawLY	Strawberry lethal yellows
AF248960	XIII-A	MPV	Mexican periwinkle virescence
AJ550984	XIV-A	BGWL	Bermudagrass white leaf ' <i>Ca. P. cynodontis</i> '
AB054986	XIX-A	...	Chestnut witches' broom ' <i>Ca. P. castaneae</i> '
AF147708	XV-A	HibWB	Hibiscus witches' broom ' <i>Ca. P. brasiliense</i> '
HQ258882	XV-B	GWB	Guazuma witches' broom
AY725228	XVI-A	...	Sugarcane yellow leaf syndrome ' <i>Ca. P. graminis</i> '
AY725234	XVII-A	...	Papaya bunchy top ' <i>Ca. P. caricae</i> '
DQ174122	XVIII-A	...	American potato purple top wilt ' <i>Ca. P. americanum</i> '
X76431	XX-A	...	Rhamnus witches' broom ' <i>Ca. P. rhamni</i> '
AJ310849	XXI-A	PinP	Pinus phytoplasma ' <i>Ca. P. pini</i> '
KF751387	XXII-A	LYDM	Lethal yellow disease Mozambique ' <i>Ca. P. palmicola</i> '
AY083605	XXIII-A	...	Buckland valley grapevine yellows
AF509322	XXIV-A	...	Sorghum bunchy shoot
EF666051	XXIX-A	CaWB	Cassia witches' broom (CaWB) ' <i>Ca. P. omanense</i> '
AF521672	XXV-A	...	Weeping tea witches' broom
AJ539179	XXVI-A	...	Sugarcane phytoplasma strain D3T1
AJ539180	XXVII-A	...	Sugarcane phytoplasma strain D3T2
AY744945	XXVIII-A	...	Derbid phytoplasma
FJ432664	XXX-A	...	Salt cedar witches' broom ' <i>Ca. P. tamaricis</i> '
HQ225630	XXXI-A	SoyST1c1	Soybean stunt isolate SoyST1c1 ' <i>Ca. P. costaricanum</i> '
EU371934	XXXII-A	MaPV	Malaysian p. virescence (MaPV) ' <i>Ca. P. malaysianum</i> '

The PCR mixture and reactions for the direct and nested PCR are as described by Al-Sadi et al. (2012).

The phytoplasma 16S rRNA gene amplified by P1 and P7 primers was purified from agarose gel or PCR product using the Qiagen PCR Purification Kit (Qiagen GmbH). Both forward and reverse strands of a purified P1/P7 fragment from phytoplasma-infected crops were sequenced (Macrogen) with primers P1, P6 (Deng and Hiruki 1991), P4 (Schneider et al. 1995), and P7 (Table 2).

Primer design, PCR, and sequencing for multiple gene analyses. The 11 phytoplasma strains were further characterized using the *secA*,

imp, *tuf*, and *SAP11* genes. For *secA*, *imp*, and *tuf* (Table 2), primers were designed after multiple alignment of the *secA*, *imp*, and *tuf* gene sequences from draft genome sequences of PnWB (Chung et al. 2013), SPLL (unpublished), and ‘*Ca. P. aurantifolia*’ (WBDL) (unpublished), which all belong to the 16SrII group phytoplasma, using CLUSTALW (Thompson et al. 1994). The most highly conserved parts of each candidate gene (complementary to all three draft genome sequences of phytoplasmas) were designated by visual assessment of the multiple alignments. Primers’ sequences were designed from those conserved complementary sequences with Primer-3 (v. 0.4.0) (Rozen and Skaletsky

Table 4. Representative phytoplasma 16S ribosomal RNA (rRNA), *tuf*, *secA*, and *imp* gene sequences from GenBank for phylogenetic analysis

Phytoplasma strain	16Sr group	Abbreviation	Gene accession number			
			16S rRNA ^a	<i>tuf</i>	<i>secA</i>	<i>imp</i>
Chrysanthemum yellows	I-A	CHRYM	AY265214	JQ824240	KJ462009	GU214179
New Jersey aster yellows	I-A	NJ-AY	HM590622	JQ824265	KJ462010	AB469011
Maryland aster yellows	I-B	AY-1	AF322644	JQ824205	KJ462011	AB469008
Aster yellows AY-J 24126	I-B	AY-J	HM590616	JQ824215	KJ462012	AB469007
Atypical aster yellows	I-B	VUT	...	JQ824285	...	...
Clover phyllody	I-C	KVE	AY265217	JQ824248	KJ462014	...
Carrot yellows	I-C	CA	HM448473	JQ824226	KJ462015	...
Apricot chlorotic leaf roll	I-F	A-AY	...	JQ824251	...	...
Peanut witches’ broom	II-A	PnWB	GI:471234556	GI:471234556	GI:471234556	GI:471234556
Witches’ broom disease of lime	II-B	WBDL	EF186828	JQ824276	KJ462017	JQ745272
Faba bean phyllody	II-C	FBP	...	JQ824262	...	...
Primula blue yellow	II-C	PrBY	JQ868438	JQ824229	KJ462018	JQ745278
Tomato big bud	II-D	TBB-KG	AF494527	JQ824250	KJ462021	JQ745285
Sweet potato little leaf	II-A	SPLL	AJ289193	Draft genome	Draft genome	Draft genome
Pichris echioides phyllody	II-E	PEP	Y16393	JQ824244	KJ462023	...
Peach X disease	III-A	CX	HQ589202	JQ824211	KJ462024	GU247985
X disease	III-A	BF	HQ589203	JQ824233	KJ462025	...
Green valley X disease	III-A	GVX	...	JQ824252	...	...
Plum leptonecrosis	III-B	LNI	JQ868444	JQ824230	KJ462027	...
Goldenrod yellows	III-D	GRI	GU004372	JQ824232	KJ462028	...
Spirea stunt	III-E	SPI	HQ589206	JQ824281	KJ462029	...
Vaccinium witches’ broom	III-F	VAC	HQ589201	JQ824260	KJ462030	...
Milkweed yellows	III-F	MW1	HQ589200	JQ824206	KJ402031	...
Poinsettia branching factor	III-H	JRI	...	JQ824267	...	...
Elm yellows	V-A	EY1	AY197655	JQ824225	KJ462034	KJ402359
Elm witches’ broom	V-A	ULW	...	JQ824259	...	...
Jujube witches’ broom	V-B	JWB	AY197661	JQ824203	KJ462036	...
Rubus stunt	V-E	RuS	AY197648	JQ824210	KJ462043	...
Potato witches’ broom	VI-A	PWB	...	JQ824282	...	...
Clover proliferation	VI-A	CP1	AY390261	JQ824231	KJ462045	...
Brinjal little leaf	VI-A	BLL	...	JQ824268	...	...
Catharanthus phyllody	VI-C	CPS	...	JQ824293	...	...
Apple proliferation	X-A	AT	CU469464	JQ824224	KJ462047	CBI70451
Apple proliferation	X-A	AP15	...	JQ824216	...	...
Plum leptonecrosis	X-B	LNp	JQ868450	JQ824235	KJ462048	AF400587
Napier grass stunt	XI	NGS-BS	JQ868440	JQ824249	KJ462053	AB469012
From unknown insect	XII-A	BA	JQ868436	JQ824228	KJ462058	...
Australian grapevine yellows	XII-B	AGY	NC_010544	JQ824254	KJ462054	...
Coconut lethal yellowing	IV-A	...	...	JQ824292	...	...
Ash yellows	VII-A	ASHY 4	...	JQ824219	...	...
Bermudagrass white leaf, ‘ <i>Candidatus</i> Phytoplasma cynodontis’	XIV-A	...	AJ550984	JQ824264	KJ462061	...
Stolbur, ‘ <i>Ca. P. solani</i> ’	XII-A	STOL11	...	JQ797670	...	...
Suriname virescence -	XV-A	SUV	...	JQ824234	...	...
Rhamnus cathartica stunt -	XX	RhCa	...	JQ824207	...	...
Cotton phyllody	II-F	CoP	...	JQ824204	...	...
Flavescence dorée, FD-C Serbia 86/09	V-C	FD-C	...	JQ824291	...	...
Flavescence dorée, FD-D Veneto 107/06	V-D	FD-D	...	JQ824294	...	...
Naxos yellows, NAXOS	IX-C	NAXOS	...	JQ824245	...	...
Pear decline, PD	X-C	PD	...	JQ824247	...	...
Paulownia witches’ broom	I-D	...	...	KP662171	...	...
Yellows diseased strawberry, ‘ <i>Ca. P. fragariae</i> ’	XII-E	...	...	KJ144900	...	...
Loofah witches’ broom	VIII-A	...	...	AF086617	...	...

^a GenBank accession strains of 16S rRNA gene were used in combined phylogenetic tree only.

2000) using the following parameters: minimum primer length = 21 bp and annealing temperature of at least 53°C. Two to four forward and reverse primers were chosen for synthesis from different regions of the candidate gene in order to improve the success of PCR amplifications and to get the maximum sequence size from each gene (Table 2). The draft genome sequences of PnWB (Chung et al. 2013) and SPL (unpublished) were used to design specific primers for the SAP11 gene, which were used for 16SrII-D group phytoplasmas (Table 2). The draft genome sequences of 'Ca. P. aurantifolia' (WBDL) (unpublished) were used to design specific primers for SAP11 (16SrII-B group) (Table 2). The design methods for these primers were described above.

PCR of the *secA* gene were performed by using the forward primer SecA-II-F1 and the reverse primer SecA-II-R1 as direct PCR, resulting in PCR fragments of approximately 2,140 bp. Primer pair SecA-II-F1/SecA-II-R4 was used for the semidirect PCR of *secA* (about 1,510 bp). The PCR products from semidirect PCR of *secA* were sequenced with primers SecA-II-F1, SecA-II-F2, and SecA-II-R4 (Table 2). For sequencing of the *imp* gene, direct and seminested PCR were performed by using the IMP-II-F1/IMP-II-R1 (about 785 bp) and IMP-II-F2/IMP-II-R1 (about 715 bp) primer pairs, respectively. The seminested PCR products of *imp* were sequenced with primers IMP-II-F2 and IMP-II-R1 (Table 2). The *tuf* gene was amplified with primer pairs TUF-II-F1/TUF-II-R1 (about 1,490 bp) and TUF-II-F2/TUF-II-R1 (about 1,090 bp) as direct and seminested PCR, respectively. The PCR products from semidirect PCR of *tuf* were sequenced with primers TUF-II-F2 and TUF-II-R1 (Table 2).

The four pairs of specific primers for gene SAP11 of 16SrII-D group and 16SrII-B group (WBDL) phytoplasmas were used for direct and nested PCR. The direct primer pairs were as follows: SAP11-IID-F1/SAP11-IID-R1 of 16SrII-D group and SAP11-WBDL-F1/SAP11-WBDL-R1 of WBDL; the targets of the primers were 820 and 1,050 bp, respectively, of DNA fragments specific for SAP11. The nested primer pairs SAP11-IID-F2/SAP11-IID-R2 (16SrII-D) and SAP11-WBDL-F2/SAP11-WBDL-R2 (WBDL) were designed to amplify 465 and 550 bp, respectively (Table 2). The nested PCR primers were used in sequencing the nested PCR products of the SAP11 gene of both phytoplasma strains.

The PCR mix of *secA*, *imp*, *tuf*, and SAP11 was as described above. Conditions of direct, semidirect, and nested PCR for the four genes were 94°C for 2 min; followed by 40 cycles of 94°C for 45 s, 53°C for 60 s, and 72°C for 90 s; and a final extension step of 72°C for 10 min. All PCR products of the four genes which were sent for sequencing were purified using a Qiaquick PCR Purification kit (Qiagen GmbH).

Sequence analysis of multiple gene products. The original forward and reverse sequence data of each sample were edited, aligned, and assembled with BioEdit 7.0.4.1 (Hall 1999). The sequences were visually inspected and manually adjusted where necessary. The sequences were verified by BLAST searches within the National Center for Biotechnology Information (NCBI) database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and sequences of representative strains were deposited in the GenBank (NCBI) data library under the accession numbers in Table 1.

Fig. 1. Symptoms of phytoplasmas in different crops in Oman. **A**, Alfalfa showing flower phyllody; **B**, healthy alfalfa field; **C**, field with severely infected alfalfa; **D**, proliferation of shoots, reduction of leaf size, and stem tillering; **E**, infected sesame field; **F**, sesame showing phyllody, virescence, and excess of little leaves and short shoots; **G**, healthy sesame; **H**, infected chickpea plant showing yellows and phyllody; and **I**, healthy chickpea plant.

To study the relationships of the new phytoplasma samples to known phytoplasma groups, multiple sequence alignments of the 16S rRNA gene (approximately 1.15 kb) phytoplasma sequences from this study were aligned with 64 other phytoplasmas (Table 3) from NCBI using CLUSTALW (Thompson et al. 1994). Furthermore, *secA* (536 bp), *imp* (507 bp), and *tuf* (385 bp) gene sequences were aligned with 27, 12, and 51 phytoplasma accessions, respectively, available in GenBank (Table 4). Phylogenetic tree construction and molecular evolutionary analyses were carried out with MEGA 6 software (Tamura et al. 2013) using the neighbor-joining method. Bootstrap analysis based on 1,000 bootstrap replications was performed with the Kimura two-parameter model to obtain confidence estimates for the phylogenetic tree. The trees of 16S rRNA, *secA*, and *tuf* genes were rooted using *Bacillus subtilis* (AB042061), *B. subtilis* (D10279), and *B. subtilis* strain 168 (GCA_000789275), respectively. The phylogenetic analysis was conducted by grouping 16S rRNA, *secA*, *imp*, and *tuf* and just including the phytoplasma strains that have sequences of all four genes available in NCBI GenBank (Tables 3 and 4). In addition, the partition-homogeneity test

(PHT) was implemented in PAUP* 4.0b10 (Swofford 2002) to test whether 16S rRNA *secA*, *imp*, and *tuf* are in concordance to combine into a single phylogenetic tree. The single phylogenetic tree of the four genes was constructed with MEGA 6 software (Tamura et al. 2013) using the same method and model followed in the single-gene tree. Multiple alignment of SAP11 (339 bp) gene sequences of the phytoplasma samples used in this study and six reference phytoplasma species (Supplementary Table S1) as well as the phylogenetic tree of SAP11 were constructed with MEGA 6 software (Tamura et al. 2013). Amino acid sequences of the phytoplasmas used in this study (PnWB, SPLL, and WBDL) were aligned to identify the SAP11 homologs using CLUSTALW (Thompson et al. 1994).

Results

Symptoms in diseased plants. During the 2014 and 2015 field survey, alfalfa (in all Oman) was found to suffer from leaf deformation and phyllody in the initial stages of disease development (Fig. 1A and B). Proliferation of shoots, yellowing and reduction of leaf size, and stem tillering were observed with the progress of the disease

Fig. 2. Symptoms of phytoplasmas in different crops in Oman. **A**, Infected carrot plant showing phyllody; **B** and **C**, close phyllody symptoms in carrot; **D**, healthy carrot flower; **E**, infected eggplant plant showing phyllody; **F**, healthy and infected eggplant flowers; **G**, close phyllody symptoms in eggplant flowers; **H**, infected spinach plants showing flower phyllody; **I**, close phyllody symptoms in spinach; and **J**, healthy spinach flower.

symptoms (Fig. 1C and D). For sesame (in northern Oman), symptoms included phyllody, virescence, and excessive development of short shoots and internodes, resulting in little leaves (Fig. 1F and G). Moreover, samples of chickpea showing yellowing symptoms and phyllody were collected from Al-Dakhilia governorate of Oman (Fig. 1H and I). Carrot, eggplant, spinach, field pea, and faba bean developing phyllody symptoms and shoot proliferation were collected from the northern area of Oman (Fig. 2A to J and A to G). Rocket plants developed virescence symptoms were collected from Al-Dakhilia governorate of Oman (Fig. 3H to J). Symptoms in squash included stunting, yellowing plants, and the female flowers showing an abnormal proliferation and phyllody; in addition, male

flowers showing virescence symptoms were found in Al Wasta governorate of Oman (Fig. 4A to D). Tomato samples showing classical symptoms of phytoplasma diseases were collected from Al-Dakhilia governorate of Oman. The symptoms included witches' brooms, little leaf, big bud, phyllody, and hardened, thick stems (Fig. 4E and F).

Phytoplasma detection and phylogenetic analysis based on the 16S rRNA gene. PCR amplification from three to five samples of symptomatic alfalfa, sesame, chickpea, tomato, spinach, rocket, carrot, squash, field pea, faba bean, and eggplant, plus a sample of WBDL as a positive control, yielded the expected products of 1.8 kb by direct PCR (P1/P7) and a 1.2-kb fragment by nested PCR (R16F2n/R16R2) (data not shown). No amplification was obtained

Fig. 3. Symptoms of phytoplasmas in different crops in Oman. **A**, Infected field pea plant showing phyllody; **B**, close phyllody symptoms in field pea; **C**, healthy field pea plant; **D**, infected faba bean plant showing phyllody flower; **E** and **F**, close phyllody symptoms in faba bean; **G**, healthy faba bean plant; **H**, infected rocket plants showing virescence flower; **I**, close virescence symptoms in rocket; and **J**, healthy rocket plant.

from the asymptomatic samples or the water sample. Successful amplification of the 16S-23S rRNA gene for 11 phytoplasmas from Oman resulted in amplicons of 1,500 to 1,640 bp, and the sequences were deposited in GenBank under the accession numbers listed in Table 1. Sequence similarity searches using BLAST revealed that the phytoplasma 16S rDNA sequences from the 11 crops share >99.5% sequence identity among themselves and >99% similarity with those of sesame phyllody phytoplasma (accession number KT265706), eggplant big bud phytoplasma (accession number JX441321), and alfalfa witches' broom phytoplasma (accession number AY169323). Neighbor-joining phylogenetic tree analyses of partial 16S rDNA sequences (1,151 bp) indicated that the alfalfa, sesame, chickpea, tomato, spinach, rocket, carrot, squash, field pea, faba bean, and eggplant phytoplasmas from Oman clustered in one subclade, with strong (99%) bootstrap support. They were found to be very closely related to Scaevola WB (accession number AB257291), Papaya mosaic 'Ca. P. australasia' (accession number Y10096), and *Mollicutes* sp. (Papaya yellow crinkle) (accession number Y10097) from the 16SrII-D subgroup phytoplasmas (Fig. 5).

DNA sequences and phylogenetic analyses of *secA*, *imp*, and *tuf*. PCR amplification of the 11 phytoplasma-positive crops and WBDL used as the positive control (Table 1) showed products in the direct, nested, and seminested PCR assays of expected size (Table 2), and no amplification was observed in asymptomatic plant samples and water with *secA*, *imp*, *tuf*, and SAP11 genes specific for the 16SrII phytoplasma primers (data not shown).

The total number of nucleotides after alignment and inclusion of gaps were 2,011, 507, and approximately 1,160 bp from the *secA*, *imp*, and *tuf* gene sequences, respectively. BLASTn queries based on the partial *secA* and *tuf* genes indicated that isolates of 16SrII-D subgroup phytoplasmas from the 11 crops in Oman had a high sequence similarity of >99.5% among themselves. The *secA* gene of the 11 phytoplasma showed 99% similarity with tomato big bud phytoplasma (GenBank: KJ462020), SPLL phytoplasma (GenBank: KJ462022), and sesame phyllody phytoplasma (GenBank: JN977041). Also, the *tuf* gene had 99% similarity with tomato big bud phytoplasma

(GenBank: JQ824250). The complete *imp* gene sequences of alfalfa, squash, and faba bean phytoplasma samples showed that there is a mutation in a single nucleotide compared with sesame, chickpea, tomato, spinach, rocket, carrot, field pea, and eggplant samples, which have no mutation. The *imp* gene sequence analysis showed that all strains have relatively >99% similarities among themselves and 99% similarity with the tomato big bud phytoplasma (GenBank: JQ745285) and eggplant big bud phytoplasma (GenBank: JQ745283).

A phylogenetic tree was constructed using 536 bp from the *secA* sequences of the 11 phytoplasmas and the *secA* sequences of PnWB (accession number GI:471234556), SPLL, WBDL, and 27 other phytoplasmas from the GenBank representing different phytoplasma groups (Table 4). It is evident from the results that all 11 phytoplasmas of the 16SrII-D subgroup from Oman are placed in one subclade and clustered with tomato big bud (accession number KJ462021) and SPLL phytoplasmas belonging to the 16SrII-D subgroup. However, PnWB (16SrII-A) phytoplasma formed a separate subclade but sharing the same clade with the 11 phytoplasmas of the 16SrII-D subgroup from Oman. WBDL from Oman formed a separate clade with WBDL phytoplasma (accession number KJ462017) belonging to the 16SrII-B subgroup (Fig. 6).

The phylogenetic tree based on 385 bp of *tuf* sequence of 11 phytoplasmas of the 16SrII-D subgroup from Oman, PnWB (accession number GI:471234556), SPLL, WBDL, and 51 other phytoplasmas from GenBank representing different phytoplasma groups (Table 4) is given in Figure 7. The *tuf* tree placed the 11 phytoplasmas of the 16SrII-D subgroup from Oman, PnWB (16SrII-A subgroup) (accession number GI:471234556), SPLL, tomato big bud (accession number Y08173), and SPLL (accession number JQ824270) within one clade (Fig. 7). In addition, the WBDL isolate clustered with WBDL phytoplasma (accession number JQ824276) of the 16SrII-B subgroup (Fig. 7). The *tuf* gene is highly conserved and the tree is strongly supported by 100% bootstrap analysis.

There is a single mutation in *imp* gene sequences of 11 16SrII-D subgroup phytoplasmas from Oman, which resulted in clustering

Fig. 4. Symptoms of phytoplasmas in different crops in Oman. **A**, Infected squash plant showing stunting and yellowing symptoms; **B**, close male flower showing virescence in infected squash; **C**, proliferation and phyllody of female flowers of infected squash plant; **D**, healthy squash plant; and **E** to **G**, tomato samples showing classical symptoms of phytoplasma diseases symptoms included witches' broom, little leaf, big bud, phyllody and hardened thick stems symptoms.

of the phytoplasmas into two subclades. The phylogenetic tree of complete *imp* gene sequences of eight representative isolates revealed that phytoplasma isolates associated with the 16SrII-D subgroup from Oman, sesame, chickpea, tomato, spinach, rocket, carrot, field pea, and eggplant clustered with the tomato big bud (accession

Fig. 5. Phylogenetic tree of 16S ribosomal RNA gene sequences from the 11 16SrII-D subgroup phytoplasmas and lime witches' broom sample from Oman (black circles). The tree includes 64 phytoplasma strains from previously published sequences while the 16S groups are indicated in parentheses. The tree was rooted using *Bacillus subtilis* (AB042061). The phylogenetic tree was constructed by the neighbor-joining method and Kimura's two-parameter model, and units are the number of base substitutions per site. Bootstrap values are expressed as percentages of 1,000 replications.

number JQ745285) strain of 16SrII-D subgroup phytoplasma. The phytoplasma strains from alfalfa, squash, and faba bean were placed in a separate subclade (Fig. 8). Furthermore, PnWB (16SrII-A subgroup) (accession number GI:471234556) and SPLI were placed in two distinct groups (Fig. 8).

Combined phylogenetic analysis based on 16S rRNA, *secA*, *tuf*, and *imp*. The PHT of the combined 16S rRNA, *secA*, *imp*, and *tuf* gene alignment resulted in a *P* value of 0.001. Therefore, the four sequence datasets were combined, with the total length of the sequence being 2,579 characters. The sequences of the 11 Omani 16SrII-D subgroup phytoplasma isolates were compared with 27 16S rDNA, *secA*, and *tuf* phytoplasma sequences and 12 *imp* phytoplasma sequences retrieved from GenBank (Table 4). The combined tree provided fine resolution of the phylogenetic groups with a strong bootstrap support (Supplementary Fig. S1). The 11 16SrII-D subgroup phytoplasmas from Oman clustered with members of 16SrII-D into two subclusters. In the first subcluster, sesame, chickpea, tomato, spinach, rocket, carrot, field pea, and eggplant phytoplasmas clustered with the tomato big bud strain of 16SrII-D subgroup phytoplasma. On the other hand, the phytoplasmas of alfalfa, squash, and faba bean were in the second subcluster. Furthermore, a clear phylogenetic separation was found between the 11 Omani phytoplasmas

Fig. 6. Phylogenetic tree of *secA* gene sequences from the 11 16SrII-D subgroup phytoplasmas and lime witches' broom sample from Oman (black circles). The tree includes 27 phytoplasma strains from previously published sequences. The 16S groups are indicated in parentheses. The tree was rooted using *Bacillus subtilis* (D10279). The phylogenetic tree was constructed by the neighbor-joining method and Kimura's two-parameter model, and units are the number of base substitutions per site. Bootstrap values are expressed as percentages of 1,000 replications.

● Alfalfa witches broom (AIWB)
● Sesame witches broom (SEWB)
● Chickpeas phyllody (CEPH)
● Tomato big bud (TOBB)
● Spinach phyllody (SPPH)
● Carrot phyllody (CAPH)
● Rocket virescence (ROVI)
● Squash virescence (SQVI)
● Fieldpeas phyllody (FIPH)
● Faba beans phyllody (FAPH)
● Eggplant big bud (EGBB)
Sweet potato little leaf (IIA)
GI:471234556 (IIA)
JQ824270(IIID)
JQ824250(IIID)
JQ824262(IIIC)
JQ824229(IIIC)
JQ824204(IIIF)
● Lime witches broom (WBDL)
JQ824276(IIIB)
JQ824244(IIIE)
JQ824234(XVA)
JQ824207(XX)
JQ824216(XA)
JQ824224(XA)
JQ824235(XB)
JQ824247(XC)
AF086617(VIIIA)
JQ824249(XI)
JQ824245(IXC)
JQ824292(IVA)
JQ824231(VIA)
JQ824282(VIA)
JQ824268(VIA)
JQ824293(VIC)
JQ824219(VIIA)
JQ824203(VB)
JQ824225(VA)
JQ824259(VA)
JQ824210(VE)
JQ824291(VC)
JQ824294(VD)
JQ824260(IIIF)
JQ824230(IIIB)
JQ824206(IIIF)
JQ824281(IIIE)
JQ824232(IIID)
JQ824211(IIIA)
JQ824233(IIIA)
JQ824252(IIIA)
JQ824267(IIIH)
KJ144900(XIIE)
JQ824228(XIIA)
JQ797670(XIIA)
JQ824254(XIIB)
JQ824251(IIF)
JQ824226(IC)
JQ824248(IC)
JQ824240(IIA)
JQ824265(IIA)
JQ824215(IIA)
KP662171(IIID)
JQ824285(IIB)
JQ824205(IIA)
JQ824264(XIVA)
Bacillus subtilis (GCA000789275)

0.05

DNA sequences, phylogenetic tree, and amino acid alignment based on SAP11. A phylogenetic tree was constructed based on complete sequences of the SAP11 gene from 11 16SrII-D subgroup phytoplasmas from Oman and six phytoplasma strains belonging to three 16Sr phytoplasma groups. Clustering of phytoplasma groups was nearly in agreement with that obtained by 16S rDNA analysis (Supplementary Fig. S2). The 11 16SrII-D subgroup phytoplasmas from Oman showed 100% identity to SAP11, with one mutation with each of the PnWB and SPLL phytoplasmas, which clustered in a separate subclade (16SrII group). The amino acid of SAP11 of all 11 phytoplasma strains from Oman, PnWB, and SPLL phytoplasmas were similar (Fig. 9), indicating the presence of silent mutations. The aster yellows phytoplasma strain, witches' broom phytoplasma (16SrI-A subgroup), and maize bushy stunt phytoplasma (16SrI-B subgroup) clustered in distinct subgroups of 16SrI group clade. Also, '*Ca. P. mali*' (16SrXA group) was placed in a separate clade.

In the last 10 years, extensive surveys in different regions of Oman have resulted in characterizing 25 hosts, including wild and economically important plant species, infected with phytoplasmas belonging to four different phytoplasma groups (16SrII, 16SrVI, 16SrIX, and 16SrXXIX) (Al-Saady et al. 2008; Al-Subhi et al. 2008; Khan et al. 2002). In this study, symptoms typical of phytoplasma were revealed in the following crops in Oman: alfalfa (AIWB), sesame (SEWB), chickpea (CEPH), rocket (ROVI), squash (SQVI), carrot (CAPH), faba bean (FAPH), field pea (FIPH), spinach (SPPH), eggplant (EGBB), and tomato (TOBB). The phylogenetic analyses of PCR amplified 16S rDNA sequences identified in association with 16SrII-D subgroup phytoplasma with 11 crops in Oman (Fig. 5), which is consistent with some previous reports of AIWB (Khan et al. 2002), SEWB (Al-Sakeiti et al. 2005), CEPH (Al-Saady et al. 2006), and EGBB (Al-Subhi et al. 2011) in Oman. To our knowledge, this is the first report of phytoplasmas of the 16SrII-D group

Plant Disease / March 2018 585

associated with tomato, spinach, rocket, carrot, squash, field pea, and faba bean in Oman. Previously, 16SrII-D subgroup phytoplasma strains were detected in tomato and squash in Egypt (Omar and Foissac 2012) and faba bean in Saudi Arabia (Al-Saleh and Amer 2014). Phytoplasmas belonging to the 16SrI group ('*Ca. P. asteris*') have been reported to be associated with diseases in carrot, spinach, and rocket (Hosseini et al. 2015b; Nisbet et al. 2014; Vali-Sichani et al. 2014). Also, stolbur phytoplasma (16Sr XII-A subgroup) has been reported associated with field pea (Zwolińska et al. 2012). Our study showed that the Omani ROVI, CAPH, SPPH, and FIPH phytoplasma isolates associated with diseases in carrot, spinach, rocket, and field pea crops, respectively, are members of the 16SrII-D group, which is the first record worldwide.

Sequencing and restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene is a common method for classification of phytoplasmas into groups or subgroups (Hogenhout et al. 2008). Because it is a highly conserved gene, 16S rRNA is not sufficient for describing subgroups within the existing 16S group (Duduk and Bertaccini 2011). Multiple gene sequence analysis is a tool for finer differentiation of closely related subgroups of phytoplasmas, and has already been successfully used for finer classification of several phytoplasma groups (Hodgetts et al. 2008; Valiunas et al. 2013). The ability to identify a specific subgroup within a given group of phytoplasmas is important in epidemiological and management studies (Duduk and Bertaccini 2011).

The 11 phytoplasma isolates from Oman shared >99.5% sequence similarities with respect to 16S rRNA, *secA*, *tuf*, and *imp*, and more than 99% similarities with tomato big bud phytoplasma strain (16SrII-D). These results suggest that the 11 Omani phytoplasma strains examined in this study belong to the 16SrII-D subgroup. Moreover, the four gene sequences of Omani WBDL phytoplasma

have >99.5% similarity with WBDL ('*Ca. P. aurantifolia*') phytoplasma belonging to the 16SrII-B subgroup. The distinct phylogenetic position among phytoplasma 16Sr groups lineage inferred from the 16S rRNA gene (Fig. 5) in this study is similar to that in previous studies (Lee et al. 1998; Martini et al. 2007). In addition, the distinct phylogenetic position of the phytoplasma taxa was also supported by analysis of the *secA*, *tuf*, and *imp* gene sequences, which are similar to that inferred by 16S rRNA gene-based phylogeny in the present and previous studies (Figs. 6, 7, and 8) (Hodgetts et al. 2008; Kakizawa et al. 2009; Siampour et al. 2013).

Analysis of *secA* gene phylogeny of phytoplasma isolates, including alfalfa, sesame, chickpea, rocket, squash, carrot, faba bean, field pea, spinach, eggplant, tomato, and WBDL phytoplasma from Oman, revealed grouping of these phytoplasma isolates into the 16SrII group clade with two distinct subclades, as noted by Hodgetts et al. (2008). One subclade comprised phytoplasma strains from the 11 crops and the related phytoplasma species, tomato big bud (accession number KJ462021) and SPLL isolates (16SrII-D). However, the WBDL phytoplasma isolate and '*Ca. P. aurantifolia*' phytoplasma (accession number KJ462017) belonging to subgroup 16SrII-B grouped together consistently and with a very strong bootstrap support. The *secA* gene tree provided fine resolution of groups, which supports previous studies on the usefulness of *secA* for fine classification and analysis of phytoplasma diseases (Hodgetts et al. 2008; Valiunas et al. 2015).

The *tuf* gene is a relatively well-conserved gene; it is present as a single copy in phytoplasma genomes and has been used in phylogenetic analysis for other organisms. RFLP analysis of the 16S rRNA gene classified AY group phytoplasmas into 10 16Sr subgroups (-A, -B, -C, -D, -E, -F, -K, -L, -M, and -N) (Marcone et al. 2000). Moreover, Langer and Maixner (2004) used *tuf* to distinguish stolbur

Fig. 9. Multiple sequence alignment of translated SAP11 sequences between the 16SrII-D subgroup phytoplasmas from Oman used in this study along with **A**, peanut witches' broom (PnWB) phytoplasma (GenBank accession number CP000061) and sweet potato little leaf (SPLL) phytoplasma (unpublished draft genomic phytoplasmas) and **B**, alfalfa with witches' broom disease of lime (WBDL) phytoplasma from Oman. Sequences were aligned using CLUSTALW (Thompson et al. 1994).

phytoplasma isolates from grapevine into three genotypes of stolbur. The phytoplasmas of 16SrII groups clustered into one clade and clearly splits into two subclades. One subclade contains the 11 Omani phytoplasma isolates, tomato big bud (accession number JQ824250) (16SrII-D subgroup), PnWB (accession number GI:471234556), and SPLL (accession number JQ824270) (16SrII-A subgroup). WBDL phytoplasma from Oman and 16SrII-B (accession number JQ824276) isolates clustered into the second subclade, supporting findings from a previous study (Makarova et al. 2012).

Phylogenetic relationships among the 11 phytoplasma isolates from Oman were investigated using the complete single-copy *imp* gene sequences (Fig. 8). Phylogenetic analysis of *imp* sequences showed that the 11 phytoplasma isolates from Oman were present within the 16SrII group phytoplasma clade, which supports previous findings (Danet et al. 2007). Phylogenetic *imp* gene-based analysis showed the separation of the isolates into three distinct subclades with very strong bootstrap support, as described by Siampour et al. (2013). All 11 phytoplasma isolates from Oman clustered with the tomato big bud (accession number JQ745285) strain of 16SrII-D subgroup phytoplasma and SPLL (16SrII-A subgroup) phytoplasma. However, PnWB (16SrII-A subgroup) (accession number GI:471234556) was placed in a separate subclade. Moreover, the WBDL isolate and WBDL (16SrII-B subgroup) (accession number JQ745272) groups were located as a third subclade. There is a single-nucleotide polymorphism in *imp* gene sequences (amino acid-altering mutation) of the 11 16SrII-D subgroup phytoplasmas from Oman, which divided the 11 phytoplasma isolates into two distinct subclusters. The surface membrane protein genes are the most variable genes (Kakizawa et al. 2009).

The multiple genes studied (16S rDNA, *secA*, *tuf*, and *imp*) offered a more finely resolved tool than the 16S rRNA gene alone, and should be helpful in studying phylogenetic positions of closely related phytoplasma groups (Hodgett et al. 2008). This phylogenetic tree of multiple genes is coinciding well with the 16S rRNA, *secA*, *tuf*, and *imp* phylogeny trees from this study (Figs. 5, 6, 7, and 8) and with those trees which were reported in previous studies (Danet et al. 2007; Hodgett et al. 2008; Makarova et al. 2012; Siampour et al. 2013). The four combined genes indicate a clear clustering within the 16SrII group, which is identical with the result of the *imp* phylogenetic tree.

During the last few years, many studies showed that phytoplasmas secrete effector proteins, including SAP11, into the host cell cytoplasm that alter plant development and enhance plant susceptibility to the insect vector, therefore favoring phytoplasma transmission and dispersal (Sugio et al. 2011b). SAP11 interferes with plant TEOSINTE BRANCHED1, CYCLOIDEA, PROLIFERATING CELL FACTORS 1 and 2, which are conserved among plant species (MacLean et al. 2011; Sugio et al. 2011a). Transgenic *Arabidopsis thaliana* plants expressing SAP11 show crinkled leaves and produce many stems; these symptoms are usually observed in *A. thaliana* plants infected by AY-WB phytoplasma (Sugio et al. 2011a). Results of this study represent the first attempt to utilize DNA sequence data from the complete SAP11 gene to study the phylogenetic relationships of phytoplasma groups. The SAP11-like sequences have been identified in divergent phytoplasmas; here, we compared those of the 16SrII-D group phytoplasmas using a few SAP11 sequences as outgroups and offer sufficient resolution to distinguish the phytoplasma taxa, which is similar to clustering obtained using the 16S rRNA gene. It placed the 11 phytoplasma isolates from Oman into subgroup 16SrII-D in the same subclade with subgroup 16SrII-A (PnWB and SPLL), with two silent single-nucleotide polymorphisms in the SAP11 nucleotide sequence of PnWB and SPLL phytoplasmas (Fig. 9).

In the present study, all 11 Omani phytoplasma isolates causing diseases of alfalfa, sesame, chickpea, rocket, squash, carrot, faba bean, field pea, spinach, eggplant, and tomato crops are members of the 16SrII-D phytoplasma subgroup. The 16SrII-D subgroup phytoplasmas are dominant phytoplasma types and widespread in the most important crops in Oman, in contrast to the 16SrII-B subgroup phytoplasma, which has been reported in citrus crops only. More studies are needed to understand phytoplasma spread and dissemination between 11 Omani crops by studying the insect vectors and alternative plant hosts from all infected areas in Oman. Findings from

this study showed that multiple gene analyses are useful to understand the phylogenetic relationships among and within phytoplasma groups. This study showed that the SAP11 gene sequence can be used in differentiating phytoplasma lineages. SAP11 sequences are limited in GenBank, and further analysis by including a larger number of phytoplasma groups would be helpful in finding relationships between phytoplasma groups and enrichment of phytoplasma classification.

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