Molecular Identification of a New Phytoplasma Associated with Alfalfa Witches'-Broom in Oman

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ABSTRACT

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Alfalfa (*Medicago sativa*) plants showing witches'-broom symptoms typical of phytoplasmas were observed from Al-Batinah, Al-Sharqiya, Al-Bureimi, and interior regions of the Sultanate of Oman. Phytoplasmas were detected from all symptomatic samples by the specific amplification of their 16S-23S rRNA gene. Polymerase chain reaction (PCR), utilizing phytoplasma-specific universal primer pairs, consistently amplified a product of expected lengths when DNA extract from symptomatic samples was used as template. Asymptomatic plant samples and the negative control yielded no amplification. Restriction fragment length polymorphism profiles of PCR-amplified 16S-23S rDNA of alfalfa using the P1/P7 primer pair identified phytoplasmas belonging to peanut witches'-broom group (16SrII or faba bean phyllody). Restriction

enzyme profiles showed that the phytoplasmas detected in all 300 samples belonged to the same ribosomal group. Extensive comparative analyses on P1/P7 amplicons of 20 phytoplasmas with *Tru9I*, *Tsp509I*, *HpaII*, *TaqI*, and *RsaI* clearly indicated that this phytoplasma is different from all the other phytoplasmas employed belonging to subgroup 16SrII, except tomato big bud phytoplasma from Australia, and could be therefore classified in subgroup 16SrII-D. The alfalfa witches'-broom (AlfWB) phytoplasma P1/P7 PCR product was sequenced directly after cloning and yielded a 1,690-bp product. The homology search showed 99% similarity (1,667 of 1,690 base identity) with papaya yellow crinkle (PapayaYC) phytoplasma from New Zealand. A phylogenetic tree based on 16S plus spacer regions sequences of 35 phytoplasmas, mainly from the Southern Hemisphere, showed that AlfWB is a new phytoplasma species, with closest relationships to PapayaYC phytoplasmas from New Zealand and Chinese pigeon pea witches'-broom phytoplasmas from Taiwan but distinguishable from them considering the different associated plant hosts and the extreme geographical isolation.

Alfalfa (*Medicago sativa* L.) is cultivated as a forage crop throughout the Sultanate of Oman with an average annual production of 480,000 tons worth US\$ 120 million. In recent years, a prokaryote has been found associated with alfalfa witches'-broom (AlfWB) disease (17). The disease is characterized by proliferation of shoots and yellowing of leaves in 1- to 2-year-old plants and tillering of stems in 4- to 5-year-old plants, rendering the crop either infertile or unmarketable. Losses due to witches'-broom disease of alfalfa in Oman result from drastic reduction in forage yield with respect to infected crowns, reduction of an infected field's life span over time as crowns die, inhibition of seed production, and the continuous need to reseed the infected field (A. J. Khan, A. M. Al-Subhi, and A. F. Bertaccini, *unpublished data*). Average annual loss of alfalfa in the Sultanate of Oman due to witches'-broom disease is approximately 25% of green hay, an estimated loss of US\$ 30 million.

Phytoplasmas are a poorly understood group of organisms, largely because they cannot be cultured *in vitro*. Until recently, they were identified based on symptomatology, host range, and vector specificity, but these methods are not suitable to reveal the genetic relatedness among different phytoplasmas. DNA-based molecular techniques, such as DNA-DNA hybridization with cloned probes and polymerase chain reaction (PCR), have been used to identify different phytoplasmas (1,2,18,19). The majority of primers used in PCR are derived from ribosomal gene sequences (9,27), which contain highly conserved and variable regions that

have been used to develop universal and pathogen-specific PCR primers for phytoplasma detection. Based on the PCR-amplified ribosomal DNA, phytoplasmas are classified using restriction fragment length polymorphism (RFLP) and sequence analysis (13, 21,22,32,33). This paper reports results of a PCR-based phytoplasma survey on diseased alfalfa grown in different regions of the Sultanate of Oman. RFLP analysis of PCR-amplified ribosomal DNA and sequencing of the 16S-23S ribosomal gene were used to characterize the Omani phytoplasma associated with this disease, as well as to study its phylogenetic relatedness to other phytoplasmas.

MATERIALS AND METHODS

Plant samples. Alfalfa (*M. sativa* L.) samples showing typical symptoms of witches'-broom disease (Fig. 1A to E) were collected from different regions of the Sultanate of Oman, namely Al-Batinah, Dakhliya, North and South Sharqiya, Muscat, and Al-Bureimi (Fig. 2). A total of 360 samples, symptomatic and asymptomatic, were collected from 40 different agricultural farms in these regions.

Phytoplasma DNA. One representative of the infected alfalfa samples from the field was employed for further characterization and named AlfWB. Nineteen phytoplasma DNAs extracted from periwinkle infected with phytoplasmas belonging to group 16SrII, peanut witches'-broom (21), or faba bean phyllody (FBP) (32), and to other groups were employed in PCR and RFLP analyses (Table 1). All the phytoplasmas belonged to the micropropagated phytoplasma collection maintained in *in vitro* periwinkle shoots in the phytoplasma laboratory of DiSTA (University of Bologna, Italy).

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Extraction of nucleic acid. For PCR studies, total nucleic acid was extracted from healthy and diseased alfalfa plants as described by Lee et al. (22) and Neimark and Kirkpatrick (28), with some modifications. Leaf samples (3 g) were grinded in liquid nitrogen in a prechilled mortar and pestle followed by extraction of nucleic acid with 50 ml of ice-cold isolation buffer (0.1 M Na_2HPO_4 , 10% sucrose, and 2% PVP-40, pH 7.6, to which 0.15% bovine serum albumin and 1 mM ascorbic acid were added just before use) (7). The extract was filtered through double layers of cheesecloth, and the supernatant was centrifuged ($18,000 \times g$, 5 min, 4°C). The pellet was resuspended in 800 μl of extraction buffer (100 mM Tris-HCl, pH 8.0, 100 mM EDTA, pH 8.0, and 250 mM NaCl) containing proteinase K (100 $\mu\text{g}/\text{ml}$) and sodium dodecyl sulfate (SDS) at a final concentration of 1%. The extract was incubated for 1 to 2 h at 55°C with occasional mixing. Ice-cold isopropanol (0.6 volume) was added to the extract, mixed gently, and left overnight at -20°C . The extract was centrifuged ($7,500 \times g$, 15 min, 4°C), and the pellet was resuspended in 9 ml of Tris-EDTA (TE) buffer, pH 8.0. Proteinase K and SDS were added to give a final concentration of 100 $\mu\text{g}/\text{ml}$ and 0.5%, respectively. The extract was mixed thoroughly and incubated for 1 h at 37°C . To this, 1.575 ml of 5 M NaCl and 1.260 ml of cetyltrimethylammonium bromide (CTAB)/NaCl solution (10% CTAB in 0.7 M NaCl) were added and incubated for 10 min at 65°C . The total DNA was extracted with an equal volume of chloroform-isoamyl alcohol (24:1) followed by extraction with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). The total DNA was precipitated overnight with 0.6 volume of isopropanol at -20°C and pelleted by centrifugation ($8,000 \times g$, 10 min, 4°C). The pellet was washed with chilled 70% ethanol and resuspended in TE buffer.

DNA from periwinkle samples was extracted following the chloroform-phenol procedure described by Prince et al. (30) and stored in TE buffer at -20°C .

PCR. DNA samples extracted from symptomatic alfalfa plants were used as template for PCR. DNA extracted from asymptomatic plants and sterile water was used as negative controls. FBP phytoplasma DNA from periwinkle was used as positive control. The primer pair P1 (9) and P7 (31) was used to prime the amplification of 1.8-kbp product of 16S rRNA gene, the spacer region between the 16S and 23S rRNA gene, and the start of the 23S rRNA gene regions of the phytoplasma genome. Fifty nanograms of each DNA preparation in 1 μl of TE were added to PCR beads (Ready-To-Go PCR beads; Pharmacia Biotech, Uppsala, Sweden) followed by 1 μl of each primer (10 pmol) and 22 μl of sterile deionized water for a final reaction mixture volume of 25 μl . The DNA was amplified by 35 cycles consisting of denaturation at 94°C for 30 s (2 min for cycle 1), annealing at 55°C for 1 min, and primer extension at 72°C for 1.5 min (7.5 min for cycle 35). Reactions were cycled in a thermocycler (PCR-Express; Hybaid, Middlesex, UK). These PCR products were diluted with sterile distilled water (1:40) prior to amplification by nested PCR using R16F2n/R16R2 primer pairs as described by Gundersen and Lee (12). Parameters for 35 cycles of nested PCR were denaturation at 94°C for 1 min (4 min for cycle 1), annealing at 60°C for 1 min, and primer extension at 72°C for 1.5 min (7.5 min for cycle 35). After amplification, a 4- μl aliquot from each sample was electrophoresed in a 1% agarose gel and visualized by staining with ethidium bromide and UV illumination.

RFLP. Ten microliters (approximately 200 ng of DNA) of each positive nested PCR product was separately digested with 2.5 units of restriction endonucleases *Eco*RI, *Hae*III, *Hha*I, *Sau*3AI, and *Taq*I and 5 units of *Tru*9I (Promega, Madison, WI) in buffers supplied by the manufacturer. Digestion mixtures were incubated overnight at 37°C for all the restriction enzymes except *Taq*I and *Tru*9I, which were incubated for at least 16 h at 65°C . Fragments were analyzed by electrophoresis in a 3% NuSieve GTG agarose gel (FMC BioProducts, Riverside, CA) with $0.5\times$ Tris-borate-

Fig. 1. Alfalfa witches'-broom symptoms in **A**, healthy field; **B**, 3 year infected field showing the reduction of yield; **C**, close up of healthy plant; and **D** and **E**, close up of infected plants showing typical symptoms.

EDTA as running buffer and visualized by staining with ethidium bromide and UV illumination.

RFLP analyses on 200 ng of DNA from P1/P7 amplimers obtained from the AlfWB sample and the 19 control samples listed in Table 1 were carried out with *Tru9I*, *Tsp509I*, *RsaI*, *TaqI*, and *HpaII* (Fermentas, Vilnius, Lithuania) for at least 16 h following the instructions of the manufacturer. The restriction patterns were compared after electrophoresis on a 5% polyacrylamide gel followed by ethidium bromide staining and photographed under UV at 312 nm by a transilluminator.

Cloning of PCR products. The rRNA genes of phytoplasma amplified by P1/P7 primer pairs were purified using a PCR DNA purification system (Wizard; Promega). The phytoplasma rDNA was cloned using a cloning kit according the manufacturer's in-

structions (pGEM-T Easy Vector System II; Promega). Recombinant plasmids were screened by blue/white color screening on indicator plates and PCR amplification using P1/P7 primers.

Sequencing and phylogenetic analysis. A cloned P1/P7 fragment from AlfWB phytoplasma was sequenced to verify phylogenetic relationships. The sequence was deposited as GenBank Accession No. AF438413.

Automatic sequencing was performed on an ABI Prism (Model 377; Perkin-Elmer Applied Biosystems, Foster City, CA) with primers P1, P6 (9), P4, and P7 (31) using Big Dye terminator chemistry (14).

Approximately 1.7 kbp of 16S rDNA and spacer gene sequences from 35 representatives of the genus *Candidatus* Phytoplasma (Table 2) were aligned separately by the CLUSTAL

Fig. 2. Map of the Sultanate of Oman showing different administrative regions in which alfalfa samples were collected.

method, version V, with LaserGene software (DNASTAR, Madison, WI). The sequences were visually inspected for logical placement of gaps and manually adjusted where necessary. Cladistic analyses were performed on a Power Macintosh G4 with the computer program phylogenetic analysis using parsimony (PAUP, version 4.0b5 (PPC), written by D. L. Swofford, Sinauer Associ-

ates, Sunderland, MA). Uninformative characters were excluded from analyses. A phylogenetic tree was constructed by a heuristic search via random stepwise addition implementing the tree bisection and reconnection algorithm to find the optimal phylogenetic trees. *Acholeplasma palmae*, a culturable Mollicute that is phylogenetically related to the phytoplasmas, was designated as the out

TABLE 1. Restriction fragment length polymorphism analyses results on 20 phytoplasma strains using five restriction enzymes on 16S plus spacer region amplified by P1/P7^a

Phytoplasma strain	Acronym	Origin	<i>TaqI</i>	<i>Tsp509I</i>	<i>Tru9I</i>	<i>RsaI</i>	<i>HpaII</i>	Group ^b
Cleome phyllody	CLP	Thailand	A	A	A	A	A	16SrII-A
Faba bean phyllody	FBP	Sudan	B	B	B	A	B	16SrII-C
<i>Crotalaria saltiana</i> phyllody	FBPSA	Sudan	B	B	B	A	B	16SrII-C
Sesame phyllody	SEPN	Thailand	A	A	A	A	A	16SrII-A
Sesame phyllody	SEPT	Thailand	A	A	A	A	A	16SrII-A
Soybean phyllody	SOYP	Thailand	B	B	B	A	B	16SrII-C
Sun hemp witches'-broom	SUNHP	Thailand	A	A	A	A	A	16SrII-A
Cotton phyllody	CoWB	Burkina Faso	C	B	B	A	B	16SrII-F
Phyllody from <i>C. juncea</i>	CrP	Thailand	B	B	B	A	B	16SrII-C
<i>Pichris echioides</i> phyllody	PEP	Italy	D	C	A	A	A	16SrII-E
Tomato big bud	TBB	Australia	B	A	A	A	A	16SrII-D
Peanut witches'-broom	PnWB	Taiwan	A	A	A	A	A	16SrII-A
Lime witches'-broom	LWB	Oman	B	A	B	A	B	16SrII-B
Alfalfa witches'-broom	AlfWB	Oman	B	A	A	A	A	16SrII-D
Ash yellows	ASHY	New York	E	D	C	B	C	16SrVII-A
Rubus stunt	RuS	Italy	F	E	D	C	D	16SrV-E
Stolbur	STOL	Serbia	G	F	E	D	E	16SrXII-A
Apple proliferation	AP	Italy	H	G	F	E	F	16SrX-A
Catharanthus phyllody	CPS	Sudan	I	H	G	E	G	16SrVI-C
Beet leafhopper transmitted virescence	VR	California	I	I	G	E	G	16SrVI-A

^a Identical letters indicate identical patterns.

^b Groups are designated in agreement with literature (6,22).

TABLE 2. Phytoplasma strains employed in phylogenetic analyses

Accession number	Phytoplasma strain	Acronym	Origin	16Sr RNA group ^a
AF438413	Alfalfa witches'-broom	AlfWB	Oman	16SrII
AF248958	Apple proliferation	AP	Italy	16SrX-A
AF189215	Ash yellows	ASHY	New York	16SrVII-A
AF222063	Aster yellows	AY	Maryland	16SrI-B
AF248961	Bermuda grass white leaf	BGWL	Thailand	16SrXIV-A
AF228052	Brinjal little leaf	BLL	India	nd
AF200718	Cactus phytoplasma	CactusP	Mexico	nd
AF105315	" <i>Candidatus</i> Phytoplasma brasiliense"	Pbras	Brazil	16SrXV
AF028813	Chinese pigeon pea witches'-broom	CPPWP	Taiwan	16SrII
AF173558	Clover yellow edge	CYE	Canada	16SrIII-B
AJ295330	Cocky apple witches'-broom	CockyAPWB	New Zealand	nd
AY029540	ESFY from apricot	ESFY	Austria	16SrX-B
X83432	Faba bean phyllody	FBP	Sudan	16SrII-C
AB026155	Gerbera phyllody	GPP	Japan	nd
Y16390	Italian alfalfa witches'-broom	ItalAlfWB	Italy	nd
AF305240	Jujube witches'-broom	JJWB	China	16SrV-B
U15442	Lime witches'-broom	LWB	Oman	16SrII-B
AF248956	Loofah witches'-broom	LfWB	Taiwan	16SrVIII-A
AF248960	Mexican periwinkle virescence	MPV	Mexico	16SrXIII-A
Y10096	Papaya mosaic disease	PMD	New Zealand	nd
Y10097	Papaya yellow crinkle	PapayaYC	New Zealand	nd
AF228053	Periwinkle little leaf	PerLL	Bangladesh	nd
X83438	Phytoplasma from <i>Cirsium arvense</i>	CirP	Germany	nd
Y08173	Phytoplasma sp. papaya	PhytPapaya	Australia	nd
Y15863	Phytoplasma sp. strain Boll	BoLL	Australia	nd
Y15865	Phytoplasma sp. strain Gall	Gall	Australia	nd
Y15866	Phytoplasma sp. strain Vill	ViLL	Australia	nd
AF248957	Pigeon pea witches'-broom	PPWB	Florida	16SrIX-A
Y16395	Rubus stunt	RuS	Italy	16SrV-E
AJ289192	Stilosantes little leaf	StiLL	Australia	nd
AF248959	Stolbur	STOL	Serbia	16SrXII-A
AJ243044	Strawberry green petals	AustrSGP	Australia	16SrXII-B
AJ243045	Strawberry lethal yellows	StrLY	Australia	16SrXII-B
AF056095	Sugarcane yellows	SCY	South Africa	nd
AF222064	Big bud	BB	Arkansas	16SrI-A

^a nd = group not available; groups are indicated following published classifications (6,22,25).

group to root the tree. The analysis was replicated 1,000 times. Bootstrap analysis was performed to estimate stability and support for the inferred clades (10).

Putative RFLP map. Because nucleic acid was not available from many phytoplasmas that were closely related to AlfWB, based on phylogeny, a virtual RFLP was carried out on five sequences from GenBank in comparison to AlfWB. Phytoplasmas employed were gerbera phyllody (GPP), Chinese pigeon pea witches'-broom (CPPWB), cocky apple witches'-broom (CockyAPWB), papaya yellow crinkle (PapayaYC), and papaya mosaic disease (Table 2). A putative RFLP map was generated on each sequence separately using the MapDraw program of the Lasergene 5.0 DNASTAR suite and manually aligned. Moreover, AlfWB sequence was also compared with the sequences of the other 20 phytoplasmas listed in Table 2 (CPPWB, PapayaYC, phytoplasma sp. papaya [PhytPapaya], Faba bean phyllody [FAB], lime witches'-broom (LWB), GPP, Italian alfalfa witches'-broom [IAWB], CockyAPWB, aster yellows [AY], big bud [BB] from the United States, jujube witches'-broom [JJWB], Rubus stunt [RuS], loofah witches'-broom [LfWB], pigeon pea witches'-broom [PPWB], apple proliferation [AP], ESFY from apricot [ESFY], stolbur [STOL], Mexican periwinkle virescence [MPV], Bermuda grass white leaf [BGWL], and "*Candidatus* Phytoplasma brasiliense" [Pbras]) after alignment using MegAlign of the Lasergene DNASTAR suite, and sequence similarities were estimated (Table 3).

RESULTS

Survey of disease. Forty alfalfa fields grown on farms averaging 4 to 6 ha in size were surveyed in different regions of the Sultanate of Oman. All farms having plants 1 year of age or older were infected with witches'-broom disease. The disease incidence ranged from 20 to 95% in 2- to 6-year-old crops (data not shown).

Symptoms in diseased plants. Typical witches'-broom symptoms were observed on alfalfa grown in different regions of the Sultanate of Oman (Fig. 2). Proliferation of shoots and yellowing of leaves in 1- to 2-year-old plants and tillering of stems in 4- to

5-year-old plants was observed (Fig. 1); plants over 6 years of age did not grow.

Detection of phytoplasma by PCR assays. Three hundred symptomatic samples collected from infected alfalfa fields from various regions in Oman yielded phytoplasma-specific 1.8-kbp products when amplified by PCR using the P1/P7 primer pair (Fig. 3A). A 1.25-kbp product was amplified in nested PCR using the R16F2n/R16R2 primer pairs when amplicons of P1/P7 diluted 1:40 were used as template (Fig. 3B). No PCR products were amplified from the 60 symptomless alfalfa plant tested of the same age and cultivar and collected in the same fields.

RFLP analyses, actual and putative. RFLP patterns of the R16F2n/R16R2-nested PCR products (1.25 kbp) were analyzed after separate digestion with *AluI*, *EcoRI*, *HaeIII*, *HhaI*, *RsaI*, *Sau3AI*, *TaqI*, and *Tru9I* (data not shown). The RFLP profiles indicated that all the AlfWB phytoplasmas from Oman are molecularly indistinguishable from each others, and belong to the 16SrII group (peanut witches'-broom phytoplasma). The AlfWB phytoplasma was distinct from FBP and "*Candidatus* Phytoplasma aurantifolia" associated with LWB in Oman (37). Based on RFLP analyses, all alfalfa samples appeared to contain the same phytoplasma (data not shown).

Extensive comparative analyses among P1/P7 amplimers from 20 phytoplasmas utilizing *Tru9I*, *Tsp509I*, *HpaII*, *TaqI*, and *RsaI* (Fig. 4A to E) clearly indicated that AlfWB is distinct from many of the other phytoplasmas belonging to subgroup 16SrII. In particular, the combination of digestion with *TaqI*, *Tsp509I*, and *Tru9I* shows that AlfWB phytoplasma is only identical to tomato big bud (TBB) from Australia phytoplasma from Australia (Table 1).

Putative RFLP maps (Fig. 5) indicated that AlfWB phytoplasma had slightly differing restriction sites in the 1- to 400-bp region of the sequence than CPPWB and CockyAPWB. The presence of a *HaeIII* restriction site in the 400- to 600-bp region of the AlfWB sequence was unique among phytoplasmas examined. Similarly, the *HinfI* restriction site in the 600- to 800-bp region was unique to AlfWB.

Similarities of 16S rDNA sequence of AlfWB phytoplasma with aligned 16S rRNA gene sequences from CPPWB,

TABLE 3. Sequence similarities among 16S rDNA of selected 16SrII group and phytoplasmas from other groups

Phytoplasma strain ^a		Similarity with different phytoplasma 16S rDNA (%)																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
AlfWB	1	100	93.2	98.6	97.6	96.8	97.2	93.6	96.7	86.9	86.6	86.9	86.0	87.3	87.7	87.9	85.6	85.6	86.3	86.8	88.2	86.3
CPPWB	2	...	100	99.7	97.4	95.5	97.8	99.4	94.4	89.8	89.6	89.8	87.8	85.7	90.6	91.1	89.2	88.9	89.5	90.2	91.2	88.5
PapayaYC	3	...	...	100	99.2	98.4	98.5	99.5	98.6	87.7	87.8	87.7	86.7	85.3	89.0	89.2	87.8	87.7	88.2	88.7	89.4	88.3
PhytPapaya	4	...	...	...	100	97.6	98.0	97.4	98.0	87.4	87.3	87.4	86.5	84.7	88.5	88.6	85.7	86.2	87.0	87.4	89.0	87.9
FBP	5	...	...	...	...	100	99.1	95.4	98.0	87.4	87.3	87.4	86.9	86.6	88.5	88.8	85.7	86.5	87.3	87.5	88.8	87.5
LWB	6	...	...	...	...	...	100	97.5	97.9	88.1	88.1	88.1	86.3	85.4	89.1	89.0	87.2	87.2	87.9	88.2	89.4	88.4
GPP	7	...	...	...	...	...	...	100	94.2	89.1	89.1	89.1	87.3	85.6	90.0	90.3	88.1	88.1	88.8	89.3	90.3	88.2
ItalAlfWB	8	...	...	...	...	...	...	...	100	87.8	87.7	87.8	87.4	87.4	88.8	88.8	86.2	86.8	87.6	88.0	89.5	87.8
CockyAPWB	9	...	...	...	...	...	...	...	...	100	98.9	100	87.3	86.2	89.4	87.9	91.2	91.4	95.8	95.7	88.7	89.0
AY	10	...	...	...	...	...	...	...	...	...	100	98.9	87.0	85.9	89.3	88.2	91.3	91.4	96.2	96.1	88.8	88.7
BB	11	...	...	...	...	...	...	...	...	...	...	100	87.3	86.2	89.4	87.9	91.2	91.4	95.8	95.7	88.7	89.0
JJWB	12	...	...	...	...	...	...	...	...	...	...	...	100	97.4	92.1	90.4	87.2	86.9	85.1	85.8	90.7	94.5
RuS	13	...	...	...	...	...	...	...	...	...	...	...	...	100	92.1	89.7	86.4	86.3	84.4	85.0	89.9	92.9
LfWB	14	...	...	...	...	...	...	...	...	...	...	...	...	...	100	91.5	91.4	91.2	89.7	90.3	92.8	93.7
PPWB	15	...	...	...	...	...	...	...	...	...	...	...	...	...	...	100	90.3	90.0	88.8	89.6	92.5	91.1
AP	16	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	100	98.0	90.8	91.7	91.3	88.1
ESFY	17	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	100	91.1	91.6	91.1	88.2
STOL	18	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	100	96.2	89.5	87.6
MPV	19	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	100	91.0	87.9
BGWL	20	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	100	92.5
Pbras	21	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	100

^a Alfalfa witches'-broom (AlfWB); Chinese pigeon pea witches'-broom (CPPWB); papaya yellow crinkle (PapayaYC); phytoplasma sp. papaya (PhytPapaya); faba bean phyllody (FBP); lime witches'-broom (LWB); gerbera phyllody (GPP); Italian alfalfa witches'-broom (ItalAlfWB); cocky apple witches'-broom (CockyAPWB); aster yellows (AY); big bud (BB); jujube witches'-broom (JJWB); Rubus stunt (RuS); loofah witches'-broom (LfWB); pigeon pea witches'-broom (PPWB); apple proliferation (AP); ESFY from apricot (ESFY); stolbur (STOL); Mexican periwinkle virescence (MPV); Bermuda grass white leaf (BGWL); and "*Candidatus* Phytoplasma brasiliense" (Pbras).

PhytPapaya, FAB, LWB, GPP, IAWB, CockyAPWB, AY, and TBB (group 16SrI), JJWB and RuS (group 16SrV), LfWB (group 16SrVIII), PPWB (group 16SrIX), AP and ESFY (group 16SrX), STOL (group 16SrXII), MPV (group 16SrXIII), BGWL (group 16SrXIV) (22), and Pbras (group 16SrXV) (26) were 93.2, 97.6, 96.8, 97.2, 93.6, 96.7, 86.9, 86.6, 86.9, 86.0, 87.3, 87.7, 87.9, 85.6, 85.6, 86.3, 86.8, 88.2, and 86.3%, respectively (Table 3).

Phylogenetic analyses. Results of phylogenetic analyses on 16S plus spacer region (Fig. 6) revealed that AlfWB is a new phytoplasma, having the closest relationships to phytoplasmas associated with PapayaYC disease from New Zealand and CPPWB disease from Taiwan.

These results support the conclusion that AlfWB phytoplasma from Oman is closely related to phytoplasmas belonging to the 16SrII group, but that it represents a distinct phytoplasma, with the suggested name of Omani AlfWB.

DISCUSSION

Phytoplasma diseases of alfalfa have been reported in the United States (24,29), Australia (3), Canada (8,16), China (4), and Italy (23). From molecular data reported, it appeared that these phytoplasmas were molecularly distinguishable because different ribosomal groups were identified in different geographical distributions of the described diseases. However, phytoplasmas closely related to the one associated with PapayaYC (16SrII group) were reported for the first time in Oman (17) and in alfalfa. The amplification of 16S rDNA sequences of the Omani AlfWB phytoplasma in all symptomatic samples followed by preliminary grouping based on the RFLP pattern of the 16S rRNA gene was the first approach to classify this phytoplasma on a molecular basis (21,32).

Previously, the origin of the Omani AlfWB phytoplasma was not clear. In order to achieve more detailed information, the phylogenetic analyses were performed for the first time on 16S plus spacer region sequences of AlfWB and of the more representative phytoplasmas whose sequences were available in GenBank.

Fig. 3. One percent agarose gel of **A**, direct and **B**, nested polymerase chain reaction of symptomatic (samples 1 to 12) and asymptomatic (samples 13 to 17) alfalfa. W, water (negative control); FBP, faba bean phyllody phytoplasma (positive control); and M, marker, 1-kb DNA ladder, fragment sizes in base pairs from top to bottom: 10,000; 8,000; 6,000; 5,000; 4,000; 3,500; 3,000; 2,500; 2,000; 1,500; 1,000; 750; 500; and 250 bp.

Fig. 4. Polyacrylamide gels (5%) showing the restriction fragment length polymorphism patterns of alfalfa witches'-broom (AlfWB) and 19 additional phytoplasma strain of 16S plus spacer region DNA fragments amplified with P1/P7 primers analyzed with **A**, *Tru9I*, **B**, *Tsp509I*, **C**, *HpaII*, **D**, *TaqI*, and **E**, *RsaI* restriction enzymes. Marker pBR322 *MspI* digest, fragment sizes in base pairs from top to bottom: 622, 527, 404, 307, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110, 90, 76, 67, 34, 26, 15, and 9 bp and marker ϕ X174 *HaeIII* digested, fragment sizes in base pairs from top to bottom: 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118; and 72 bp. Cleome phyllody (CLP); faba bean phyllody (FBP); *Crotalaria saltiana* phyllody (FBPSA); sesame phyllody (SEPN); sesame phyllody (SEPT); soybean phyllody (SOYP); sun hemp witches'-broom (SUNHP); cotton phyllody (CoWB); phyllody from *C. juncea* (CrP); *Pichris echinoides* phyllody (PEP); tomato big bud (TBB); peanut witches'-broom (PnWB); lime witches'-broom (LWB); ash yellows (ASHY); Rubus stunt (RuS); stolbur (STOL); apple proliferation (AP); Catharanthus phyllody (CPS); and beet leafhopper transmitted virescence (VR).

Fig. 6. Phylogenetic tree constructed by parsimony analysis of 16S rRNA and spacer gene sequences from 35 phytoplasma taxa and employing culturable *Acholeplasma palmae* as the out group. Branch lengths are proportional to the number of inferred character state transformations. Bootstrap (confidence) values are shown on the branches. Alfalfa witches'-broom (AlfWB); apple proliferation (AP); ash yellows (ASHY); aster yellows (AY); Bermuda grass white leaf (BGWL); Brinjal little leaf (BLL); cactus phytoplasma (CactusP); "*Candidatus* Phytoplasma brasiliense" (Pbras); Chinese pigeon pea witches'-broom (CPPWB); clover yellow edge (CYE); cocky apple witches'-broom (CockyAPWB); ESFY from apricot (ESFY); faba bean phyllody (FBP); gerbera phyllody (GPP); Italian alfalfa witches'-broom (ItalAlfWB); jujube witches'-broom (JJWB); lime witches'-broom (LWB); loofah witches'-broom (LfWB); Mexican periwinkle virescence (MPV); papaya mosaic disease (PMD); papaya yellow crinkle (PapayaYC); periwinkle little leaf (PerLL); phytoplasma from *Cirsium arvense* (CirP); phytoplasma sp. papaya (PhytPapaya); phytoplasma sp. strain Boll (BoLL); phytoplasma sp. strain Vill (ViLL); pigeon pea witches'-broom (PPWB); Rubus stunt (RuS); Stilosantes little leaf (StiLL); stolbur (STOL); strawberry green petals (AustrSGP); strawberry lethal yellows (StrLY); sugarcane yellows (SCY); and big bud (BB).

Although spacer DNA has only a low significant coding function, the variability found in this region was shown to be highly informative (34). Our studies suggest that many phytoplasmas in Southeast Asia and Australia are phylogenetically related. In particular, PapayaYC, the phytoplasma associated with papaya from Australia, and CPPWB from Taiwan appeared among the most closely phylogenetically related phytoplasmas to the Omani AlfWB.

The alfalfa fields surveyed in this study showed an estimated 20 to 95% of plants of 2 to 6 years of age associated with witches'-broom symptoms. The losses of alfalfa green hay due this disease were considerably high in the Sultanate of Oman. In this study, PCR-based amplification using universal phytoplasma primers directed to ribosomal DNA sequences followed by RFLP analysis was used to identify the phytoplasma causing the destructive witches'-broom disease of alfalfa in all parts of Oman. The DNA extracted from symptomatic alfalfa plants yielded a specific product in direct as well as in nested PCR, the latter technique showing that no detectable latent infection was present in the fields because all asymptomatic samples were negative.

Comparative RFLP analyses among several alfalfa infected samples showed that the phytoplasmas present in the fields were genetically homogeneous. The same analyses carried out with six restriction enzymes among 20 phytoplasmas showed that it was possible to distinguish the Omani AlfWB from all the others, in particular from those belonging to the 16SrII group, with the exception of TBB.

AlfWB was tentatively classified within the phytoplasma subgroup 16SrII-D, with TBB (A. F. Bertaccini, A. J. Khan, S. Botti, A. M. Al-Subhi, and D. E. Gundersen-Rindal, *unpublished data*). RFLP analyses allowed preliminary 16S classification of other phytoplasmas employed as well. In particular, the phytoplasma associated with cotton phyllody from Burkina Faso appeared to be distinguishable from all the other phytoplasmas belonging to 16SrII group and was tentatively assigned to subgroup 16SrII-F. Likewise, Catharanthus phyllody phytoplasma from Sudan was assigned to subgroup 16SrVI-C, because it was possible to distinguish it from the described phytoplasmas belonging to the same group (Table 1) (15,21).

Phylogenetic analyses among Omani AlfWB and 35 other phytoplasmas revealed that the Omani AlfWB phytoplasma fell within the same group as FBP (16SrII or peanut witches'-broom group) and that it was most closely related to PapayaYC phytoplasma from New Zealand (36) and CPPWB phytoplasma from Taiwan. The Omani AlfWB 16S rRNA gene sequence showed greater than 98% similarity compared with PapayaYC phytoplasma from New Zealand; therefore, it could be the same species as PapayaYC from New Zealand and CPPWB from Taiwan because the homology is higher than 97.5%. Following decisions for phytoplasma taxonomy taken by the Phytoplasma Working Team during the 13th International Organization of Mycoplasma held in Fukuoka, Japan (14 to 19 July 2000) in general, a strain can be described as a new "*Candidatus* Phytoplasma species" if its 16S rRNA gene sequence has less than 97.5% identity to any previously described "*Candidatus* Phytoplasma species." However, it was also decided that there are cases of phytoplasmas that share more than 97.5% of their 16S rRNA gene sequence, but clearly represent ecologically separated populations and therefore may deserve description as separate species. It was also decided that the rank of subspecies should not be used. Therefore, considering the different associated plant hosts and the extreme geographical isolation (plus molecular data), there is support to the notion that Omani AlfWB is a distinct species.

The phytoplasma associated with LWB disease, which is widespread in Oman (11,25), was also associated with the 16SrII group, belonging to subgroup 16SrII-B (21). However, the present study showed that Omani AlfWB phytoplasma was distinct from LWB and it was classified into a different subgroup (16SrII-D).

Primers P1 and P7, employed in all possible combinations with four AlfWB-specific primers designed based on comparison of AlfWB and LWB phytoplasmas 16S and spacer region sequences (AlfWB1f, AlfWB1r, AlfWB2f, and AlfWB2r) (A. F. Bertaccini, A. J. Khan, and S. Botti, *unpublished data*), showed a specific or no amplification unless the pair P1/AlfWB1r was employed in seminested PCR; in the latter case, an 838-bp expected product was obtained only from the AlfWB-infected samples.

RFLP analyses with *Tsp509I* showed that this alfalfa phytoplasma was distinct from the phytoplasma associated with infected alfalfa in Italy (23). A putative restriction site analysis further revealed AlfWB to be distinct from other phytoplasmas that were closely related by phylogenetic analyses.

AlfWB disease is widespread in all regions of Oman. The disease symptoms start as yellowing of leaves, reduction in leaf size, and proliferation of shoots in 1- to 2-year-old plants. As the disease progresses, tillering of stems becomes visible in 3- to 5-year-old plants with drastic reduction in number and size of shoots. The economic life expectancy of alfalfa infected with witches'-broom disease is reduced to 4 to 5 years compared with 8 to 10 years for disease-free plants.

Two leafhoppers belonging to the genera *Agallia* and *Empoasca* (A. Alma, *personal communication*) have been consistently collected from different alfalfa fields in Oman. DNA extracted from these insects and amplified using P1/P7 primer pair showed amplification of a 1.8-kbp DNA product similar to that of Omani AlfWB phytoplasma (data not shown). This suggests that leafhoppers, as putative vectors, may play an important role in transmission of disease however there is no evidence to suggest that the amplicon is from the same AlfWB phytoplasma, therefore further work will clarify this issue. Transmission of phytoplasmas by leafhoppers has been suggested by several workers based on PCR studies (16,35) despite the fact that other insect species, such as psyllids, cixiids (20), and flatids (5), could not be excluded as potential vectors. Because the phytoplasmas might have been acquired during feeding and might still have been present in the gut lumen at the time of the insect's collection, it will be necessary to perform specific transmission experiments on the PCR positive insects in order to verify their vector role; only after this information is available will it be possible to control the spreading of this dangerous disease in Oman alfalfa cultivations.

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LITERATURE CITED

- Bertaccini, A., Davis, R. E., Lee, I. M., Conti, M., Dally, E. L., and Douglas, S. M. 1990. Detection of Chrysanthemum yellows mycoplasma-like organisms by dot hybridization and southern blot analysis. *Plant Dis.* 74:40-43.
- Bonnet, F., Saillard, C., Kollar, A., Seemüller, E., and Bové, J. M. 1990. Detection and differentiations of mycoplasma-like organism associated with apple proliferation disease using cloned DNA probes. *Mol. Plant-Microbe Interact.* 3:438-443.
- Bowyer, J. W., Artherton, J. G., Teakle, D. S., and Ahern, G. A. 1969. Mycoplasma-like bodies in plants affected by legume little leaf, tomato big bud, and Lucerne witches broom diseases. *Aust. J. Biol. Sci.* 22:271-274.
- Chen, Y., Chen, Z. Y., Shen, C. Y., and Peng, B. Z. 1996. Research of causal agents of Lucerne witches broom disease in Xinjiang of China. Page 20 in: *Proc. Int. Workshop Phytoplasmas*, 5th. Chin. Acad. For., Beijing.
- Danielli, A., Bertaccini, A., Vibio, M., Mori, N., Murari, E., Posenato, P., and Girolami, V. 1996. Detection and molecular characterization of

- phytoplasmas in the planthopper *Metcalfa pruinosa* (Say) (Homoptera: Flatidae). *Phytopathol. Mediterr.* 35:62-65.
6. Davis, R. E., and Dally, E. L. 2001. Revised subgroup classification of group 16SrV phytoplasmas and placement of *flavescence dorée*-associated phytoplasmas in two distinct subgroups. *Plant Dis.* 85:790-797.
7. Dellaporta, S. L., Wood, J., and Hicks, J. B. 1983. A plant DNA miniprep: Version II. *Plant Mol. Biol. Rep.* 1:19-21.
8. Deng, S. J., Forster, R. J., Hiruki, C., and Teather, R. M. 1993. Simultaneous amplification and sequencing of genomic DNA from *Butyrivibrio fibrisolvens*, and detection and genotyping of nonculturable mycoplasma-like organisms directly from total DNA isolated from infected plants. *J. Microbiol. Methods* 17:103-113.
9. Deng, S. J., and Hiruki, C. 1991. Genetic relatedness between two non-culturable mycoplasma-like organisms revealed by nucleic acid hybridization and polymerase chain reaction. *Phytopathology* 81:1475-1479.
10. Felsenstein, J. 1987. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783-791.
11. Garnier, M., Zreik, L., and Bové, J. M. 1991. Witches' broom, a lethal mycoplasma disease of lime trees in the Sultanate of Oman and the United Arab Emirates. *Plant Dis.* 75:546-551.
12. Gundersen, D. E., and Lee, I.-M. 1996. Ultrasensitive detection of phytoplasmas by nested-PCR assay using two universal primer pairs. *Phytopathol. Mediterr.* 35:144-151.
13. Gundersen, D. E., Lee, I.-M., Rehner, S. A., Davis, R. E., and Kingsbury, D. T. 1994. Phylogeny of mycoplasma-like organisms (phytoplasmas): A basis for their classification. *J. Bacteriol.* 176:5244-5254.
14. Heiner, C. R., Hunkapiller, K. L., Chen, S.-M., Glass, J. I., and Chen, E. Y. 1998. Sequencing multimegabase-template DNA with BigDye Terminator Chemistry. *Genome Res.* 8:557-561.
15. Jomantiene, R., Davis, R. E., Maas, J., and Dally, E. L. 1998. Classification of new phytoplasmas associated with diseases of strawberry in Florida, based on analysis of 16S rRNA and ribosomal protein gene operon sequences. *Int. J. Syst. Bacteriol.* 48:269-277.
16. Khadhair, A. H., Hiruki, C., and Hwang, S. F. 1997. Molecular detection of alfalfa witches' broom phytoplasma in four leafhopper species associated with infected alfalfa plants. *Microbiol. Res.* 152:269-275.
17. Khan, A. J., Azam, K. M., Deadman, M. L., Al-Subhi, A. M., and Jones, P. 2001. First report of alfalfa witches broom disease in Oman caused by a phytoplasma of the 16SrII group. *Plant Dis.* 85:1287.
18. Lee, I.-M., and Davis, R. E. 1988. Detection and investigation of genetic relatedness among aster yellows and other mycoplasma-like organisms by using cloned DNA and RNA probes. *Mol. Plant-Microbe Interact.* 1:303-310.
19. Lee, I. M., Davis, R. E., and Dewitt, N. 1990. Non radioactive screening method for isolation of disease specific probes to diagnose plant diseases caused by mycoplasma-like organisms. *Appl. Environ. Biol.* 56:1471-1475.
20. Lee, I.-M., Gundersen-Rindal, D., and Bertaccini, A. 1998. Phytoplasma: Ecology and genomic diversity. *Phytopathology* 88:1359-1366.
21. Lee, I.-M., Gundersen-Rindal, D. E., Davis, R. E., and Bartoszyki, I. M. 1998. Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. *Int. J. Syst. Bacteriol.* 48:1153-1169.
22. Lee, I.-M., Hammond, R. W., Davis, R. E., and Gundersen, D. E. 1993. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasma-like organisms. *Phytopathology* 83:834-842.
23. Marcone, C., Raggozzino, A., and Seemüller, E. 1997. Detection and identification of phytoplasma infecting vegetable, ornamental and forage crops in southern Italy. *J. Plant Pathol.* 79:211-217.
24. Menzies, J. D. 1946. Witches' broom of alfalfa in North America. *Phytopathology* 36:762-774.
25. Moghal, S. M., Shivanathan, P., and Mani, A. 1993. Status of Pest and Diseases in Oman: Series 1. Plant Diseases in the Batinah. Directorate General of Agricultural Research, Agriculture Research Centre, Rumais, Oman.
26. Montano, H. G., Davis, R. E., Dally, E. L., Hogenhout, S., Pimentel, J. P., and Brioso, P. S. T. 2001. "*Candidatus* Phytoplasma brasiliense", a new phytoplasma taxon associated with hibiscus witches' broom disease. *Int. J. Syst. Evol. Microbiol.* 51:1109-1118.
27. Namba, S., Katao, S., Iwanami, S., Oyaizu, H., Shiozawa, H., and Tsuchizaki, T. 1993. Detection and differentiation of plant pathogenic mycoplasma-like organisms using polymerase chain reaction. *Phytopathology* 83:786-791.
28. Neimark, H. C., and Kirkpatrick, B. C. 1993. Isolation and characterization of full-length chromosomes from non-culturable plant-pathogenic mycoplasma-like organisms. *Mol. Microbiol.* 7:21-28.
29. Peters, R. D., Lee, M. E., Grau, C. R., Driscoll, S. J., Winberg, R. M., and Kutzweil, N. C. 1999. First report of aster yellows phytoplasma in alfalfa. *Plant Dis.* 83:488.
30. Prince, J. P., Davis, R. E., Wolf, T. K., Lee, I.-M., Mogen, B. D., Dally, E. L., Bertaccini, A., Credi, R., and Barba, M. 1993. Molecular detection of diverse mycoplasma-like organisms (MLOs) associated with grapevine yellows and their classification with aster yellows, X-disease, and elm yellows MLOs. *Phytopathology* 83:1130-1137.
31. Schneider, B., Seemüller, E., Smart, C. D., and Kirkpatrick, B. C. 1995. Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas. Pages 369-380 in: *Molecular and Diagnostic Procedures in Mycoplasma*. Vol. 2. S. Razin and J. G. Tully, eds. Academic Press, New York.
32. Seemüller, E., Marcone, C., Lauer, U., Raggozzino, A., and Goschl, M. 1998. Current status of molecular classification of the phytoplasmas. *J. Plant Pathol.* 80:3-26.
33. Seemüller, E., Schneider, B., Maurer, M., Ahrens, U., Daire, X., Kison, H., Lorenz, K.-H., Firrao, G., Avinent, L., Sears, B. B., and Stackebrandt, E. 1994. Phylogenetic classification of phytopathogenic *Mollicutes* by sequence analysis of 16S ribosomal DNA. *Int. J. Syst. Bacteriol.* 44:440-446.
34. Smart, C. D., Schneider, B., Blomquist, C. L., Guerra, L. J., Harrison, N. A., Ahrens, U., Lorenz, K. H., Seemüller, E., and Kirkpatrick, B. C. 1996. Phytoplasma-specific PCR primers based on sequences of the 16S-23S rRNA spacer region. *Appl. Environ. Microbiol.* 62:2988-2993.
35. Vega, F. E., Davis, R. E., Barbosa, P., Dally, E. L., Purcell, A. H., and Lee, I.-M. 1993. Detection of a plant pathogen in a non vector insect species by the polymerase chain reaction. *Phytopathology* 83:621-624.
36. Waite, D. T., Blackall, L. L., Scott, P. T., and Walsh, K. B. 1998. Phylogenetic positions of phytoplasmas associated with dieback, yellow crinkle and mosaic diseases of papaya, and their proposed inclusion in *Candidatus* Phytoplasma australiense and a new taxon, *Candidatus* Phytoplasma australasia. *Int. J. Syst. Bacteriol.* 48:941-951.
37. Zreik, L., Carle, P., Bové, J. M., and Garnier, M. 1995. 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.