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Molecular characterization of the 16S rRNA gene of phytoplasmas detected in two leafhopper species associated with alfalfa plants infected with witches' broom in Oman

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Summary. Two leafhopper species, *Austroagallia avicula* and *Empoasca* sp., were consistently found in alfalfa fields infected with witches' broom phytoplasma (OmanAlfWB) in the Al-Batinah, Dakhliya, North and South Sharqiya, Muscat, and Al-Bureimi regions of the Sultanate of Oman. Phytoplasmas from both leafhoppers were detected by specific polymerase chain reaction (PCR) amplification of the 16S rRNA gene and the spacer region in direct PCR using P1/P7 primer pairs. Comparative RFLP profiles of the amplified rRNA gene and the spacer region from leafhopper phytoplasmas and from 20 phytoplasma controls yielded patterns referable to phytoplasmas belonging to the peanut witches' broom group (16SrII group). In particular, extensive RFLP analyses with the endonucleases *Hpa*II, *Tru*9I, *Tsp509*I, and *Rsa*I indicated that the phytoplasmas in *A. avicula* and *Empoasca* sp. were identical but showed some differences from OmanAlfWB; however, RFLP patterns obtained with *Taq*I showed the OmanAlfWB and the phytoplasmas from the two leafhoppers to be identical. Direct PCR products amplified from phytoplasma leafhopper DNA using the P1/P7 primer pair were cloned and sequenced yielding 1758 bp and 1755 bp products from *A. avicula* and *Empoasca* sp. respectively; the homology of these sequences with OmanAlfWB and papaya yellow crinkle phytoplasmas was more than 98%. A phylogenetic tree based on the 16S rRNA gene and spacer region sequences from 44 phytoplasmas revealed that the phytoplasmas from the leafhoppers clustered with OmanAlfWB, papaya yellow crinkle, and gerbera phyllody phytoplasmas, all belonging to 16SrII group, but were distinct from lime witches' broom phytoplasma, the most commonly found phytoplasma in the Sultanate of Oman.

Key words: phylogeny, RFLP.

Introduction

Phytoplasmas (previously known as plant-pathogenic mycoplasma-like organisms, MLOs) are wall-less plant pathogenic bacteria. They are found associated with numerous plant diseases affecting many varieties of crops and trees of agricultural importance worldwide (McCoy *et al.*, 1989). The most common phytoplasma disease symptoms are yellowing of leaves, stunting, die-back, small leaves, proliferation of axillary buds (witches' broom), phyllody (leaf-like petals and sepals), virescence (greening of petals), and big buds (Davis *et al.*, 1997). Phytoplasmas have not yet been cultured *in vitro* and they are difficult to purify from infected plants. These constraints

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have greatly hampered research into the biology of phytoplasmas and their interaction with host plants and insects. Because phytoplasmas cannot be cultured, their detection and identification have been largely based on polymerase chain reaction (PCR) assays using universal and pathogen-specific phytoplasma primers (Deng and Hiruki, 1991a; Lee *et al*., 1993; Namba *et al*., 1993; Lee *et al*., 1995); restriction fragment length polymorphism (RFLP) of PCR amplified DNA (Lee *et al*., 1993; Schneider *et al*., 1993; 1995); and phylogenetic analysis of 16S rRNA gene sequences from representative phytoplasma groups (Kuske and Kirkpatrick, 1992; Gundersen *et al*., 1994; Seemüller *et al*., 1994; Lee *et al*., 1998; Seemüller *et al*., 1998). Although phytoplasmas can be transmitted by grafting and dodders, the most common means of transmission in the field is by leafhoppers. Several insect species have been found as vectors or carriers of various phytoplasmas (Nault, 1990; Vega *et al*., 1993).

Alfalfa (*Medicago sativa* L.) is the primary forage crop in the Sultanate of Oman, with an annual average yield of green hay worth US$ 120 million. Recently, a wall-less prokaryote associated with witches' broom of this crop has been found playing an important role in limiting production (Khan *et al*., 2001). Losses due to alfalfa witches' broom associated with phytoplasmas are estimated at 25% of production, or some US$ 30 million annually. The pathogen causes excessive proliferation of the shoots and yellowing of the leaves, followed by tillering of stems in the later stages, rendering the crop either infertile or unmarketable (Khan *et al*., 2001). In the past, traditional methods such as fluorescence, electron microscopy, DNA staining, DNA-DNA hybridization and symptomatology were used for detecting phytoplasmas in plants and leafhoppers (Chen, 1977; Hiruki and Rocha, 1986; Lee and Davis, 1988; Hiruki, 1989; Bertaccini *et al*., 1990; Bertaccini *et al*., 1993). More recently, molecular techniques have facilitated phytoplasma detection and the study of genetic relatedness among phytoplasma isolates from host plants and their insect vectors (Kirkpatrick, 1989; Deng and Hiruki, 1991a, b; Ahrens and Seemüller, 1992; Lee *et al*., 1994). In the Sultanate of Oman, it was reported that leafhoppers are putative vectors of lime witches' broom (Garnier *et al*., 1991). Recent surveys of alfalfa fields in different regions of Oman showed that a high percentage of alfalfa plants have symptoms of witches' broom. Subsequent investigation with molecular techniques found that alfalfa witches' broom phytoplasma is a new species closely related to papaya yellow crinkle phytoplasma and belongs to the 16Sr II-D subgroup (Khan *et al*., 2002). Although witches' broom phytoplasma diseases are common in Oman, there is no report on leafhoppers as vectors of alfalfa witches' broom. Hence, a molecular detection and characterization of the 16S rRNA gene plus spacer region was carried out to identify the phytoplasmas in leafhoppers collected from witches' broom-infected alfalfa fields.

Materials and methods

Field survey

Surveys of alfalfa fields were carried out in the Al-Batinah, Dakhliya, North and South Sharqiya, Muscat, and Al- Bureimi regions of the Sultanate of Oman. More than 500 leafhopper specimens were collected from 40 witches' broom-infected alfalfa fields (Fig. 1). Leafhoppers were collected in a power suction trap and were divided in the entomology laboratory into two groups based on their colour and morphological characteristics. About 20 male leafhoppers representative of each group were sent to the University of Turin, Italy, for identification. The remaining leafhoppers, approximately 200 from each group, were kept separately in Eppendorf tubes at -20°C for extraction of total DNA. All experimental analyses were repeated at least twice unless otherwise indicated.

DNA extraction

Total DNA extraction from the leafhoppers was as previously described by Rogers and Bendich (1985), and Vega *et al*. (1993) with some modifications. About 50 leafhoppers from each group and from each of the 40 fields were separately macerated with liquid nitrogen in a pre-chilled mortar and pestle followed by extraction of DNA with 15 ml of ice-cold CTAB extraction buffer (1% CTAB; 1.4 M NaCl; 2% PVP-10; 0.02 M EDTA; 0.1 M Tris-HCl; pH 8.0; 0.1% 2-mercapto-ethanol) containing proteinase K (100 $\mu g\ ml^{-1}$) and 1% sodium dodecyl sulfate (SDS). The extract was incubated for 15 min

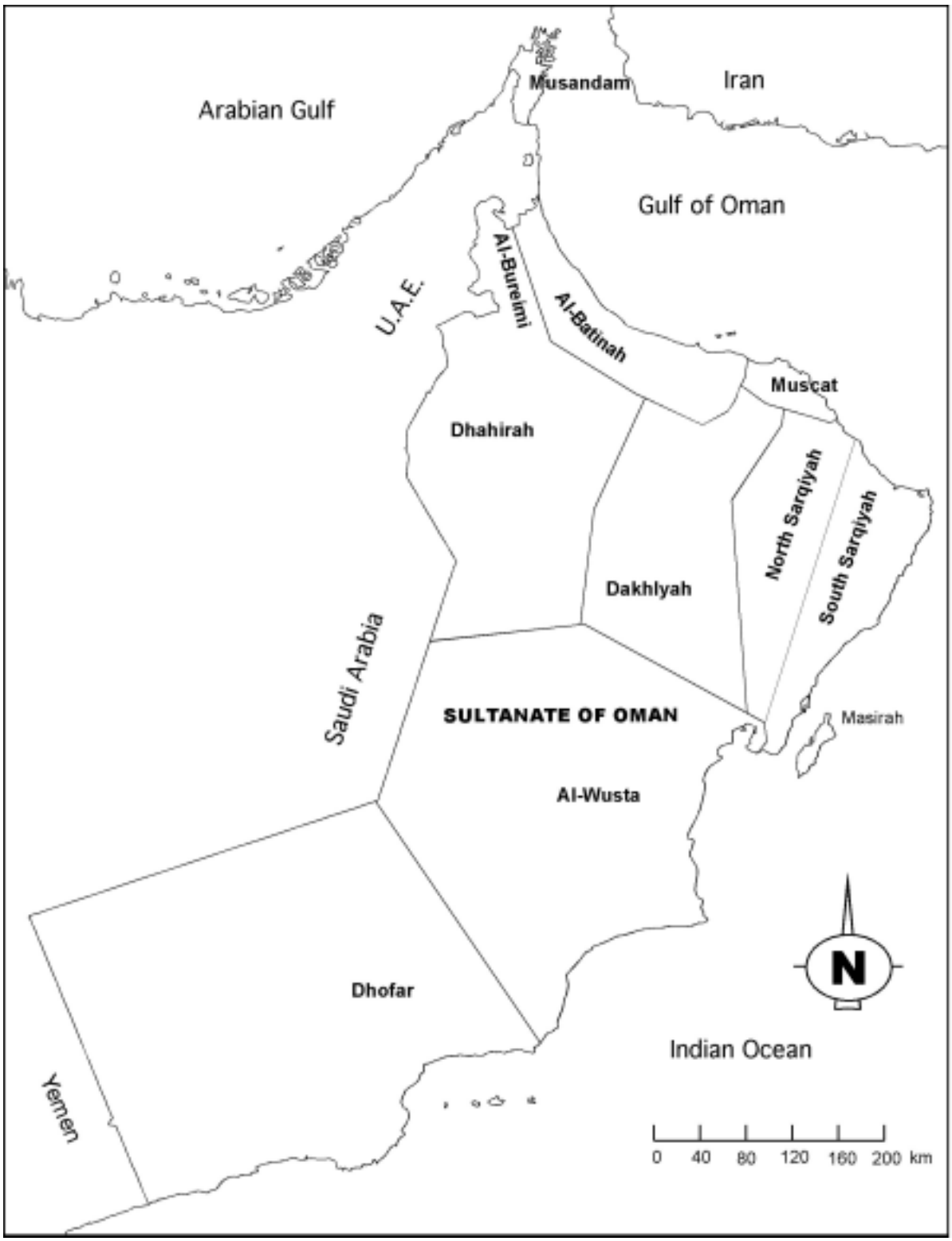

Fig. 1. Map of the Sultanate of Oman showing the regions in which leafhoppers were collected from alfalfa fields.

at 65°C with occasional stirring. 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 nucleic acids were precipitated overnight with 0.6 volume of isopropanol at -20°C and pelleted by centrifugation for 10 min at 800× *g* and 4°C. The pellets were washed two times with chilled 70% ethanol, dried under vacuum, and suspended in TE buffer.

As a positive control, DNA was extracted from symptomatic and asymptomatic alfalfa plants using the method of Khan *et al*. (2002), and from periwinkle samples following the chloroform-phenol procedure described by Prince *et al*. (1993).

Polymerase chain reaction (PCR)

Nucleic acids extracted from leafhopper species were used as template for PCR. DNA extracted from symptomatic alfalfa plants and faba bean phyllody (FBP) phytoplasma maintained on periwinkle plants were used as a positive control. Two primer pairs P1 (Deng and Hiruki, 1991a) and P7 (Schneider *et al*., 1995) universal for phytoplasmas were used to prime the amplification of a 1.8 kb product of the 16S rRNA gene, the spacer region between the 16S and the 23S rRNA gene and the beginning of the 23S rRNA gene of the phytoplasma genome. The PCR reaction mixture (25 µl) consisted of 2.5 *p*mol of each upstream and downstream (P1/P7) primer; 50 ng template DNA; 200 mM of dNTP; 1× polymerase reaction buffer; 2.5 mM $MgCl_2$; 2.5 U Taq DNA polymerase and sterile water to make up the final volume. Amplification was carried out in a PCR-Express thermal cycler (Hybaid, Middlesex, UK) under the following conditions: 35 cycle PCR including denaturation at 94°C for 30 s (2 min for 1st cycle), annealing at 55°C for 1 min and primer extension at 72°C for 1.5 min (7.5 min for cycle 35).

Restriction fragment length polymorphism (RFLP)

P1/P7 positive samples of 1.8 kb of phytoplasma ribosomal DNA sequences from insect, alfalfa and control samples were subjected to analysis by RFLP. Ten-µl aliquots (approximately 200 ng of DNA) of each PCR product were separately digested with endonuclease enzymes *Tsp509*I, *Rsa*I, *Taq*I, and *Hpa*II (Fermentas, Vilnius, Lithuania), following manufacturer's instructions.

Cloning of PCR products

The PCR products of 1.8 kb were amplified by the primer pair P1/P7 using Titanium Taq polymerase with Taq-start antibody (BD Biosciences Clontech, Palo Alto, CA, USA) to reduce primer dimer formation. The products were purified on a PCR-DNA purification system and ligated into pGEM-T easy vector plasmids (Promega Co., Madison, WI, USA) according to manufacturer's instructions. The recombinant plasmids were inserted into JM109 competent cells and plated on SOC medium amended with ampicillin, isopropylthio-ß-D-galactoside (IPTG) and X-gal. Bacterial colonies containing DNA inserts were identified by blue/white screening for recombinants. White bacterial colonies containing recombinant plasmids were transferred to fresh LB agar medium containing ampicillin. Phytoplasma rDNA was amplified from plasmids using the P1/P7 primer pair for sequencing.

Sequencing and phylogenetic analysis

Phytoplasma rDNA fragments amplified by the P1/P7 primer pair from the recombinant plasmids were sequenced on an ABI Prism (Model 377, Applied Biosystems, Foster City, CA, USA) using Big Dye Terminator Chemistry (Heiner *et al*., 1998). To obtain a complete sequence of amplified 16S rDNA (1.8 kb) with the P1/P7 primers, DNA was further amplified with P1 and P6 (Deng and Hiruki, 1991a) primer pairs yielding 1.2 kb, and with P4 (Kirkpatrick *et al*., 1994) and P7 primers, yielding fragments of about 500 bp. A 35 bp forward primer AF1 (AAGTGTTATCCGGAATTATTGGGCGTAAAGGGTGC) was synthesized to amplify the middle region of the 1.2 kb fragment (amplified by P1/P6) using P6 as reverse primer. The sequences were aligned and overlapping regions were removed manually. The rRNA gene sequences were deposited in the NCBI as GenBank Accession No. AY169322 (from *A. avicula*) and AY169323 (from *Empoasca* sp.).

The sequence formed by the 16S rRNA gene, the spacer gene and the beginning of the 23S gene (approximately 1.7–1.8 kb) from 44 representative phytoplasma strains of the genus *Candidatus* (Table 1) along with the two sequences from the leafhoppers were aligned by the CLUSTAL method using the MegAlign suite of LaserGene 5.01 software (DNASTAR, Madison, WI, USA). Where necessary, the sequences were manually adjusted for

Table 1. 16S rDNA sequences of different phytoplasma strains obtained from GenBank, which were used for phylogenetic analysis.

Accession No.	Phytoplasma strain	Abbreviations	Origin	16Sr RNA group
AF438413	Alfalfa witches' broom	OmanAlfWB	Oman	16SrII-D
AF248958	Apple proliferation	AP	Italy	16SrX-A
AF189215	Ash yellows	ASHY	USA	16SrVII-A
AF222063	Aster yellows	AY	USA	16SrI-B
AF248961	Bermuda grass white leaf	BGWL	Thailand	16SrXIV-A
AF222064	Big bud	BB	USA	16SrI-A
AF228052	Brinjal little leaf	BLL	India	n.a.
AF200718	Cactus	CactusP	Mexico	n.a.
AF105315	'*Candidatus* Phytoplasma brasiliense'	Pbras	Brazil	16SrXV
AF515637	'*Candidatus* Phytoplasma phoenicium'	CPP	Lebanon	16SrIX
AB054986	Chestnut witches' broom	CnWB	S. Korea	16SrIV
AY081817	China tree decline	ChTDIII	Argentina	16SrIII
AF028813	Chinese pigeon pea witches' broom	CPPWP	Taiwan	16SrII
AF495882	Chinaberry yellows	CbYP	Bolivia	16SrXIII
X83438	*Cirsium arvense*	CirP	Germany	n.a.
AF173558	Clover yellow edge	CYE	Canada	16SrIII-B
AJ295330	Cocky apple witches' broom	CockyAPWB	New Zealand	n.a.
AY029540	ESFY from apricot	ESFY	Austria	16SrX-B
AY101386	Epilobium phyllody	EPP	Estonia	16SrI
AF411592	Erigeron witches' broom	EWBP	Brazil	16SrVII
X83432	Faba bean phyllody	FBP	Sudan	16SrII-C
AB026155	Gerbera phyllody	GPP	Japan	n.a.
Y16390	Italian alfalfa witches' broom	ItalAWB	Italy	n.a.
AF305240	Jujube witches' broom	JJWB	China	16SrV-B
Y18052	Knuatia phyllody	KPP	Italy	16SrIX
AF515638	Lactuca serriola	LsP	Lebanon	16SrIX
U15442	Lime witches' broom	LWB	Oman	16SrII-B
AF248956	Loofah witches' broom	LfWB	Taiwan	16SrVIII-A
AF248960	Mexican periwinkle virescence	MPV	Mexico	16SrXIII-A
Y10097	Papaya yellow crinkle	Papaya YC	Australia	n.a.
AF228053	Periwinkle little leaf	PerLL	Bangladesh	n.a.
Y08173	Phytoplasma sp. papaya	PhytPapaya	Australia	n.a.
Y15863	Phytoplasma sp. strain BoLL	BoLL	Australia	n.a.
Y15865	Phytoplasma sp. strain GaLL	GaLL	Australia	n.a.
Y15866	Phytoplasma sp. strain Vill	ViLL	Australia	n.a.
AF248957	Pigeon pea witches' broom	PPWB	USA	16SrIX-A
AJ310849	Pine	PinP	Germany	n.a.
U89378	Rape phyllody	RPP	Czech Rep.	n.a.
Y16395	Rubus stunt	RuS	Italy	16SrV-E
AJ289192	Stilosantes little leaf	StilLL	Australia	n.a.
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	n.a.
AF521672	Weeping tea tree witches' broom	WttWBP	Australia	n.a.

n.a., not available.

logical placement of the gaps. Cladistic analyses were performed with Phylogenetic Analysis Using Parsimony (PAUP 4.0 b10) on G4 Power Mac (Swofford, 2002). A phylogenetic tree was constructed under criterion parsimony by heuristic search via random stepwise addition implementing the tree bisection and reconnection algorithm to obtain the optimal phylogenetic tree. A culturable Mollicute phylogenetically related to phytoplasmas, *Acholeplasma palmae*, was designated as the out-group taxon to root the tree. Bootstrap analysis was performed and replicated 1000 times to estimate stability and support of the inferred clades (Felsenstein, 1987).

In order to compare the rRNA gene sequences from the 16SrII group and phytoplasmas from other groups, sequences from leafhoppers were compared with sequences from 17 phytoplasmas (OmanAlfWB, PapayaYC, CPPWP, LWB, FBP, EPP, CYE, ChTDIII, JJWB, EWBP, LfWB, KPP, AP, STOL, CbYP, BGWL, Pbras) listed in Table 1. The sequences were aligned by the Clustal W method using the MegAlign suite of LaserGene 5.01 software (DNASTAR) and percent identity of the sequences was determined (Table 2).

Results

Leafhopper identification

In all three surveys on the 40 farms with a large proportion of alfalfa fields in the different regions of Oman, two leafhopper species were collected, one brown and one green in colour. The brown leafhoppers were larger (3.8–4.2 mm), with four black dots, two on the head and two on the front part of the body; they were identified as *Austroagallia avicula*. The green leafhoppers were smaller (3–5 mm) and were identified as *Empoasca* sp. The population of *Empoasca* sp. was greater than that of *A. avicula* in the surveys performed.

Detection of phytoplasma in leafhoppers

DNA samples from field leafhoppers were used as template to amplify 16S-23S rDNA by direct

Table 2. Analysis of sequence similarities among 16S rRNA gene belonging to selected 16SrII groups and phytoplasmas from other groups.

Phytoplasma strain		Sequence similarity with phytoplasma 16S rRNA gene (%)																		
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
Austroagallia[a]	**1**	100	99.3	98.4	98.4	95.5	96.9	96.9	86.0	89.5	89.1	84.6	87.6	87.4	87.5	85.6	85.8	86.2	87.7	86.1
Empoasca[a]	**2**	...	100	99.1	99.0	96.0	97.5	97.5	86.5	90.4	90.0	85.1	88.3	88.2	88.2	86.1	86.3	86.8	88.5	86.6
OmanAlfWB	**3**	...	...	100	98.9	93.2	97.1	96.8	86.6	89.5	89.0	85.7	87.7	87.3	87.7	85.3	86.3	86.3	87.8	85.9
PapayaYC	**4**	...	...	...	100	99.7	98.4	98.4	87.5	91.0	90.5	86.3	89.1	88.4	86.9	87.6	88.1	88.1	89.1	88.2
CPPWP	**5**	...	...	...	...	100	97.8	95.5	89.6	92.1	91.7	87.5	90.6	90.4	87.7	89.2	89.3	87.5	90.9	88.4
LWB	**6**	...	...	...	...	...	100	99.1	87.9	90.8	90.4	86.1	89.2	88.5	86.6	87.0	87.5	87.8	89.1	88.0
FBP	**7**	...	...	...	...	...	...	100	87.2	90.1	89.6	86.7	88.4	88.2	88.2	86.5	87.0	86.7	88.4	87.2
EPP	**8**	...	...	...	...	...	...	...	100	89.2	88.9	87.2	88.7	89.2	86.1	91.1	96.4	95.8	88.5	88.0
CYE	**9**	...	...	...	...	...	...	...	...	100	99.0	90.1	92.2	91.7	90.4	90.1	89.6	89.9	92.9	91.3
ChTDIII	**10**	...	...	...	...	...	...	...	...	...	100	89.7	92.1	91.9	90.0	89.8	89.3	89.9	92.4	91.3
JJWB	**11**	...	...	...	...	...	...	...	...	...	...	100	93.1	92.2	89.6	87.0	85.2	85.9	90.7	94.4
EWBP	**12**	...	...	...	...	...	...	...	...	...	...	...	100	93.9	89.9	89.6	89.7	90.1	92.6	97.7
LfWB	**13**	...	...	...	...	...	...	...	...	...	...	...	...	100	88.9	91.0	89.7	89.5	92.6	93.6
KPP	**14**	...	...	...	...	...	...	...	...	...	...	...	...	...	100	86.8	85.6	87.2	91.2	90.5
AP	**15**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	100	90.7	90.9	90.8	87.5
STOL	**16**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	100	96.1	89.5	87.3
CbYP	**17**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	100	90.3	87.6
BGWL	**18**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	100	92.5
Pbras	**19**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	100
16Sr group		II	II	II-D	II	II	II	II	I	III	III	V	VII	VIII	IX	X	XII	XIII	XIV	XV

[a] Phytoplasma strains detected in leafhoppers collected from witches' broom infected alfalfa field.

PCR with primer pair P1/P7 yielding a product of 1.8 kb. A DNA of the 16SrII group was used as positive control.

RFLP analysis

Extensive comparative analysis of P1/P7 amplimers from 20 phytoplasmas utilizing *Tsp509*I, *Hpa*II, and *Rsa*I (Fig. 2A, B, C) indicated that the phytoplasmas detected in *A. avicula* and in *Empoasca* sp. were undistinguishable from each other but showed some differences from alfalfa (OmanAlfWB) phytoplasma (Khan *et al*., 2002), while RFLP patterns of these phytoplasmas with *Taq*I were similar without restriction site differences (Fig. 3) making it possible to include the phytoplasmas from the two insects in the ribosomal group 16SrII, where they formed a new subgroup designated as 16SrII-G.

When the 16S rDNA sequences amplified from *A. avicula* and *Empoasca* sp. were aligned with the

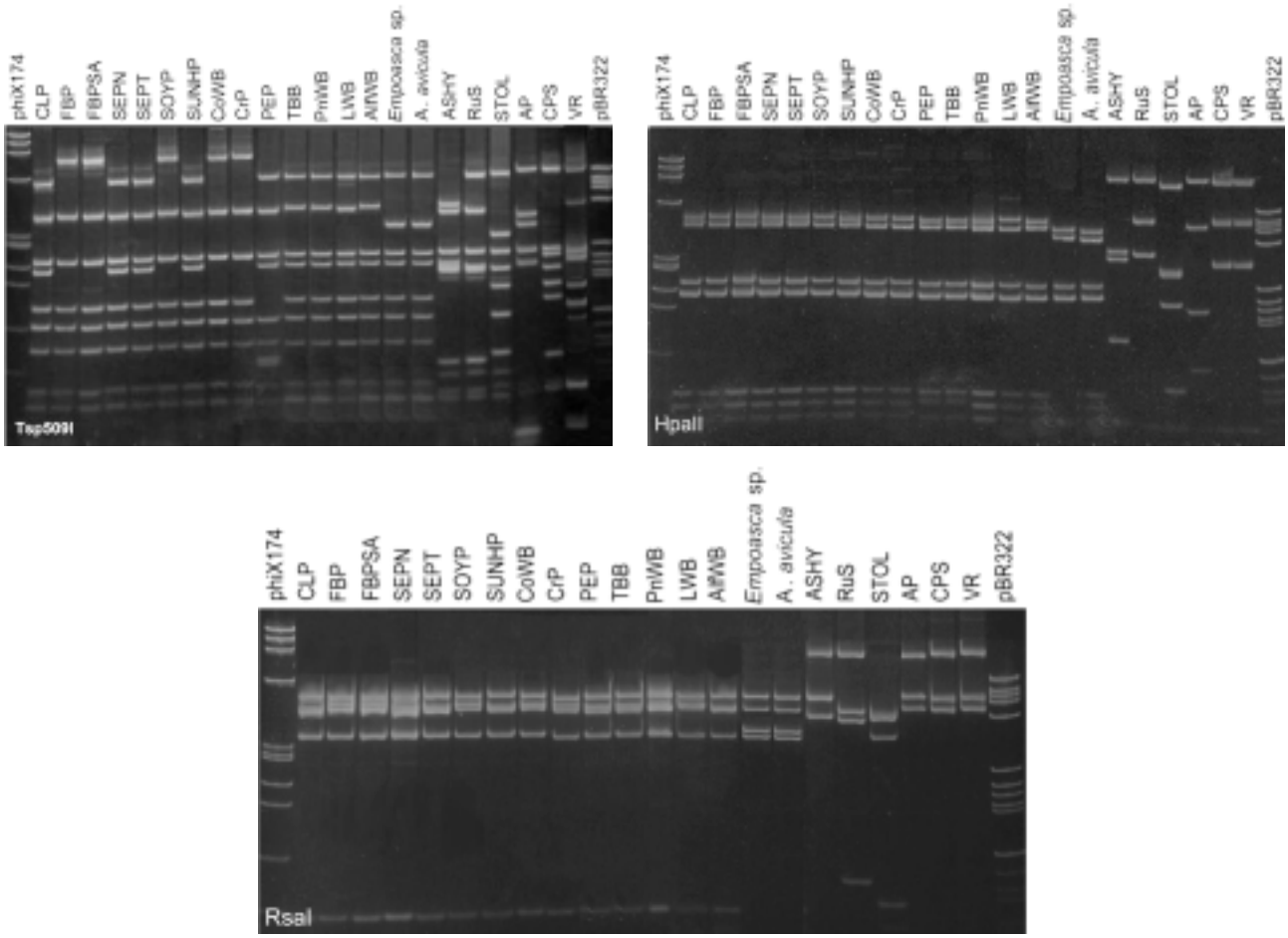

Fig. 2. Polyacrylamide gels (5%) showing the RFLP patterns of phytoplasmas detected in *A. avicula* and *Empoasca* sp., OmanAlfWB and 19 other phytoplasma strains of 16S plus spacer region DNA fragments amplified with P1/P7 primers analyzed with (A) *Tsp509*I, (B) *Hpa*II and (C) *Rsa*I restriction enzymes. Markers pBR322 and ΨX174 *Hae*III digested. Other abbreviations: AlfWB, alfalfa witches' broom from Oman; CLP, cleome phyllody; FBP, faba bean phyllody; FBPSA, *Crotalaria saltiana* phyllody; SEPN, sesame phyllody; SEPT, sesame phyllody; SOYP, soybean phyllody; SUNHP, sun hemp witches' broom; CoWB, cotton phyllody; CrP, phyllody from *C. juncea*; PEP, *Pichris echioides* phyllody; TBB, tomato big bud from Australia; PnWB, peanut witches' broom; LWB, lime witches' broom; ASHY, ash yellows; RuS, rubus stunt; STOL, Stolbur; AP, apple proliferation; CPS, *Catharanthus* phyllody; VR, beet leafhopper transmitted virescence.

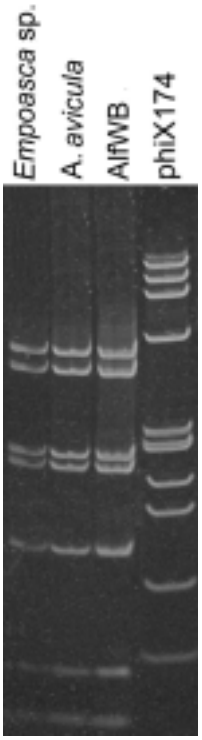

Fig. 3. Polyacrylamide gel (5%) showing RFLP patterns of phytoplasmas detected in *A. avicula*, *Empoasca* sp. and OmanAlfWB phytoplasmas of the 16S plus spacer region DNA fragments amplified with P1/P7 primers analyzed with *Taq*I restriction enzyme. Markers and abbreviations are as in Fig. 2.

rRNA gene sequences (Table 2) they showed respectively 98.4 and 99.1% similarity with OmanAlfWB (16SrII).

Phylogenetic analysis

The 16S-23S spacer regions of the rRNA gene sequences from *A. avicula* and *Empoasca* sp. leafhoppers were compared with 44 sequences of phytoplasmas from the GenBank database and analyzed using criterion parsimony via random heuristic search. The phytoplasmas from the insects were closely related to the OmanAlfWB phytoplasma from witches' broom-infected alfalfa plants and belonged to the FBP (peanut witches' broom, 16SrII) group. The phytoplasmas from the two leafhoppers showed the greatest homology to alfalfa witches' broom (OmanAlfWB), papaya yellow crinkle (Papaya YC), gerbera phyllody (GPP), Chinese pigeon pea witches' broom (CPPWP) and cocky apple witches' broom (CockyApWB) phytoplasmas (Fig. 4).

Discussion

Alfalfa witches' broom disease was associated with phytoplasma presence in various parts of the world (Menzies, 1946; Bowyer *et al*., 1969; Chen *et al*., 1996; Khadhair *et al*., 1997; Marcone *et al*., 1997; Peters *et al*., 1999; Khan *et al*., 2001). In Oman, although there are few reports on witches' broom phytoplasma on sour lime (Garnier *et al*., 1991) and alfalfa (Khan *et al*., 2001), adequate information on disease incidence and vectors is lacking.

Molecular techniques can be used to detect and classify *Mollicutes*. In this study, DNA samples from the two leafhopper species, *A. avicula* and *Empoasca* sp., that are consistently found in association with witches' broom in alfalfa were amplified with phytoplasma-specific primer pairs, providing evidence for the presence of phytoplasmas in the leafhoppers.

The genetic relatedness data obtained with RFLP and the sequence analysis of the 16S rRNA genes showed that the phytoplasmas detected in both leafhoppers were very similar to each other and belonged to a new ribosomal subgroup, 16SrII-G. Phylogenetic analysis of the 16S rRNA genes and 44 other phytoplasmas showed that phytoplasma 16S rDNA from the insects clustered with the faba bean phyllody phytoplasma group (16SrII) and showed a high homology with OmanAlfWB (+98%), and also with CockyAPWB, CPPWP, GPP and Papaya YC, all phytoplasmas closely related to OmanAlfWB (Khan *et al*., 2002). The percentage of identity with the phytoplasma sequences from *A. avicula* and *Empoasca* sp. suggested that these phytoplasmas were molecularly near to those in alfalfa witches' broom (OmanAlfWB): according to the International Organization for Mycoplasmology meetings in Fukuoka, Japan, 2000 and in Vienna, Austria, 2002, phytoplasmas with more than 97.5% of 16S rDNA homology should be considered the same organism, or at least not a different species. The other phytoplasmas belonging to the same phylogenetic group, lime witches' broom, subgroup 16SrII-B, associated with a disease of sour lime in Oman (Garnier *et al*., 1991; Zreik *et al*., 1995) is molecularly distinguishable from alfalfa witches'

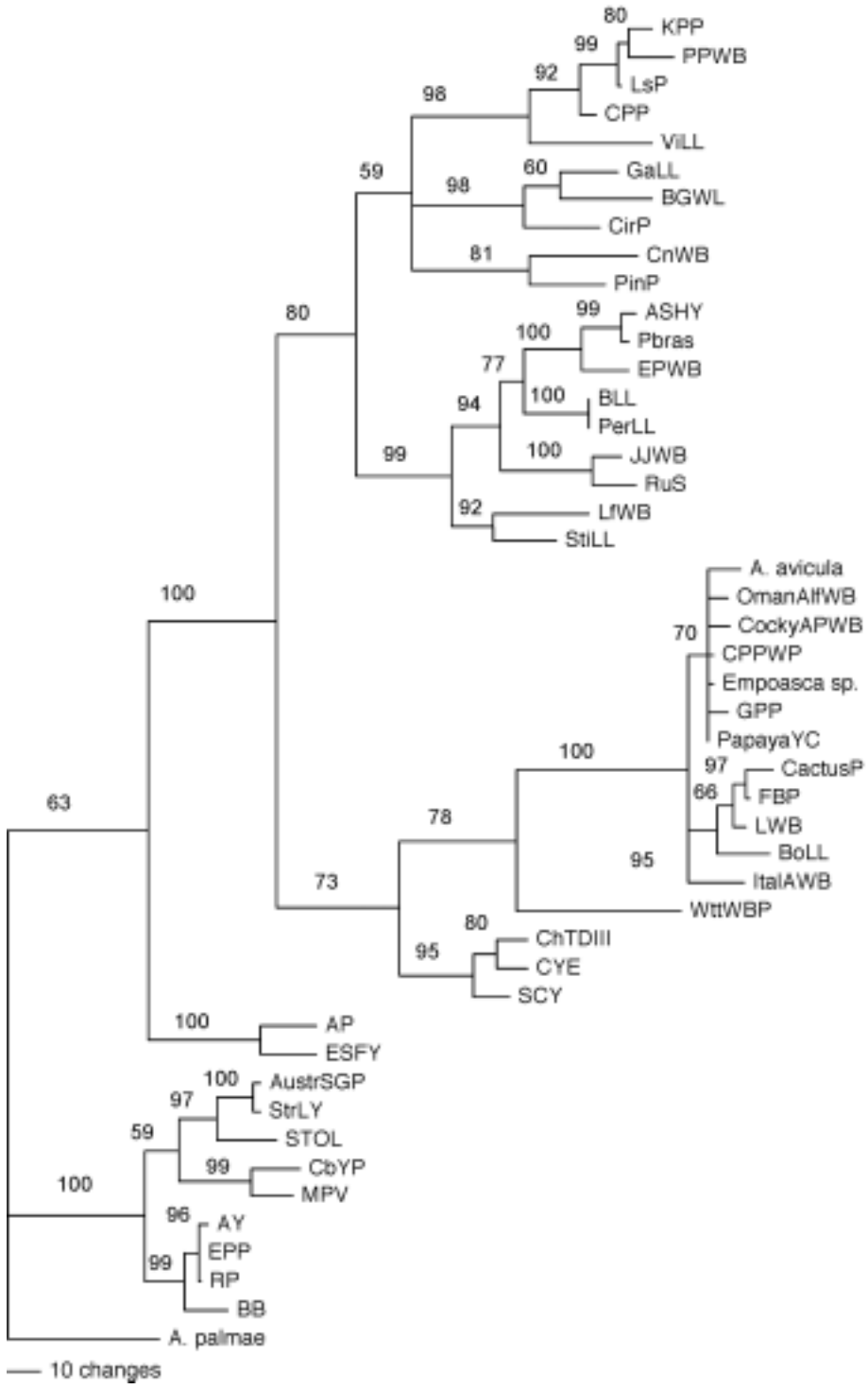

Fig. 4. Phylogenetic dendrogram of the 16S rRNA and spacer gene sequences from 44 phytoplasma taxa from GenBank, 2 phytoplasma sequences from leafhoppers (*A. avicula* and *Empoasca* sp.), generated by parsimony analysis using the PAUP 4.0/b10 program. A culturable mollicute, *Acholeplasma palmae* was used as the outgroup taxon. Branch lengths are proportional to the number of inferred character state transformations and bootstrap values are shown on the branches. Abbreviations: OmanAlfWB, Alfalfa witches' broom from Oman; AP, apple proliferation; ASHY, ash yellows; AY, aster yellows; BGWL, Bermuda grass white leaf; BLL, Brinjal little leaf; CactusP, cactus phytoplasma; Pbras, *Candidatus* phytoplasma brasiliense; CPP, *Candidatus* phytoplasma phoeniceum; CnWB, chestnut witches' broom; CbYP, Chinaberry yellows; ChTDIII, China tree decline; CPPWP, Chinese pigeon pea witches broom; CYE, clover yellow edge; CockyAPWB, cocky apple witches' broom; EPP, epibolium phyllody; EPWB, erigeron witches broom; ESFY, ESFY from apricot; FBP, faba bean phyllody; GPP, gerbera phyllody; ItalAWB, Italian alfalfa witches' broom; JJWB, jujube witches' broom; KPP, knuatia phyllody; LsP, *Lactuca serriola* phytoplasma; LWB, lime witches' broom; LfWB, loofah witches' broom; MPV, Mexican periwinkle virescence; Papaya YC, papaya yellow crinkle; PerLL, periwinkle little leaf; PinP, pine phytoplasma; CirP, phytoplasma from *Cirsium arvense*; BoLL, phytoplasma sp. strain BoLL; GaLL, phytoplasma sp. strain GaLL; ViLL, phytoplasma sp. strain Vill; A. avicula, phytoplasma from leafhopper *Austroagallia avicula*; Empoasca sp., phytoplasma from leafhopper *Empoasca* sp.; PPWB, pigeon pea witches' broom; RP, rape phyllody; RuS, *Rubus* stunt; StiLL, *Stilosantes* little leaf; STOL, Stolbur; AustrSGP, strawberry green petals; StrLY, strawberry lethal yellows; SCY, sugarcane yellows; TBB, tomato big bud; WttWBP, weeping tea tree witches' broom.

broom phytoplasma by both RFLP analysis, with 97.1% rDNA homology, and by RFLP with 96.9 and 97.5% of rDNA homology from phytoplasmas in *A. avicula* and *Empoasca* sp. respectively. Since the mechanisms involved in insect-phytoplasma transmission are unclear, the occurrence of phytoplasmas closely related to OmanAlfWB in both leafhoppers may indicate that they can be harboured in the same insects.

These leafhoppers may not be vectors but carriers, therefore specific transmission studies need to be conducted to verify that they are in fact vectors. Such studies will also provide an approach toward disease management that will prevent the spread of alfalfa phytoplasma in Oman alfalfa cultivations.

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