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'*Candidatus* Phytoplasma asteris', a novel phytoplasma taxon associated with aster yellows and related diseases

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Aster yellows (AY) group (16SrI) phytoplasmas are associated with over 100 economically important diseases worldwide and represent the most diverse and widespread phytoplasma group. Strains that belong to the AY group form a phylogenetically discrete subclade within the phytoplasma clade and are related most closely to the stolbur phytoplasma subclade, based on analysis of 16S rRNA gene sequences. AY subclade strains are related more closely to their culturable relatives, *Acholeplasma* spp., than any other phytoplasmas known. Within the AY subclade, six distinct phylogenetic lineages were revealed. Congruent phylogenies obtained by analyses of *tuf* gene and ribosomal protein (*rp*) operon gene sequences further resolved the diversity among AY group phytoplasmas. Distinct phylogenetic lineages were identified by RFLP analysis of 16S rRNA, *tuf* or *rp* gene sequences. Ten subgroups were differentiated, based on analysis of *rp* gene sequences. It is proposed that AY group phytoplasmas represent at least one novel taxon. Strain OAY, which is a member of subgroups 16SrI-B, *rp*I-B and *tuf*I-B and is associated with evening primrose (*Oenothera hookeri*) virescence in Michigan, USA, was selected as the reference strain for the novel taxon '*Candidatus* Phytoplasma asteris'. A comprehensive database of diverse AY phytoplasma strains and their geographical distribution is presented.

INTRODUCTION

Phytoplasmas are cell wall-less bacteria that are associated with diseases in several hundred plant species (McCoy *et al.*, 1989; Marwitz, 1990; Lee *et al.*, 2000). A pioneer phylogenetic study based on 16S rRNA and ribosomal protein (*rp*) gene sequences was the first to indicate that an aster yellows (AY) phytoplasma strain was related to *Acholeplasma laidlawii*, a member of the *Mollicutes* (Lim & Sears, 1989). Phylogenetic analyses of 16S rRNA gene sequences revealed that phytoplasmas form a large, discrete, monophyletic clade that belongs to the class *Mollicutes* (Kuske & Kirkpatrick, 1992; Namba *et al.*, 1993; Gundersen *et al.*, 1994; Seemüller *et al.*, 1994). Thus far, 20 distinct subclades have been identified within the phytoplasma clade (Seemüller *et al.*, 1998). Collective RFLP profiles, derived by analyses of 16S rDNA sequences, have delineated 15 distinct phytoplasma 16S rDNA (16Sr) groups that coincide with well-defined subclades (Lee *et al.*, 1993b, 1998b; Montano *et al.*, 2001). Gundersen *et al.* (1994) proposed that the phytoplasma clade should be recognized as a novel genus within the class *Mollicutes* and that each subclade (or 16Sr group) represents at least a species. Thus far, eight '*Candidatus* Phytoplasma' species have been proposed (Zreik *et al.*, 1995; Davis *et al.*, 1997; White *et al.*, 1998; Griffiths *et al.*, 1999; Sawayanagi *et al.*, 1999; Montano *et al.*, 2001; Jung *et al.*, 2002; Verdin *et al.*, 2003).

The AY phytoplasma group (16SrI) comprises AY phytoplasma and numerous related phytoplasmas worldwide, representing the most diverse and widespread phytoplasma group (Lee & Davis, 2000; McCoy *et al.*, 1989). Aster yellows, which attacks the China aster, *Callistephus chinensis* Nees, was widespread throughout the eastern United States at the turn of the 20th century (Kunkel, 1926). It was transmitted primarily by the aster leafhopper *Macrosteles quadrilineatus* Forbes (formerly *Macrosteles fascifrons* Stal.)

Abbreviation: AY, aster yellows.

The GenBank/EMBL/DDBJ accession numbers for the aster yellows phytoplasma strain OAY sequences are M30790 (16S rDNA) and M74770 (*rpl22–rps3*).

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and was later called Eastern aster yellows, in order to distinguish it from another AY disease that occurred in celery in California (Kunkel, 1932). The latter, which is called Western aster yellows, is spread by a wider range of insect vectors, induces different symptoms in plants and is distributed worldwide (Tsai, 1979). Eastern and Western AY diseases are now known to be associated with phytoplasmas that belong to the AY phytoplasma group (16SrI) subgroups A (16SrI-A) and B (16SrI-B), respectively. Today, the term 'AY phytoplasmas' refers generally to phytoplasma strains that belong to subgroups 16SrI-A and 16SrI-B. However, in prior decades, numerous diseases characterized by symptoms similar to those caused by subgroup 16SrI-A and subgroup 16SrI-B strains were reported as AY, but the causal agents of those diseases could not be identified accurately, based on the biological criteria that were used at that time, such as symptoms or insect vectors. Through the use of molecular tools, it has become evident that many diseases are caused by phytoplasmas that are different from, but related closely to, AY phytoplasmas that are classified in subgroups 16SrI-A and 16SrI-B.

Strains in the AY phytoplasma group share $\geqslant$97 % similarity in their 16S rDNA sequences, but substantial genetic variations are evident and they occupy diverse ecological niches (Gundersen *et al.*, 1994; Seemüller *et al.*, 1994, 1998). Thus, the widely diverse AY phytoplasma group may consist of more than one species. Based on composite RFLP patterns of 16S rDNA and the *tuf* gene, the AY group phytoplasmas have been differentiated into at least six distinct subgroups (Marcone *et al.*, 2000), most of which occupy mutually exclusive ecological niches (Lee *et al.*, 1998a). Recently, four additional subgroups that are associated with diseases in soybean, cherry and strawberry were identified (Jomantiene *et al.*, 2002b; Lee *et al.*, 2002; Valiunas, 2003).

This paper reports phylogenetic relationships among strains in the AY phytoplasma group, based on combined analyses using sequences from the 16S rRNA gene and the ribosomal protein operon (genes *rps19* and *rpl22*). We describe the widespread 16SrI-B, *rp*I-B strain OAY (=MIAY), associated with *Oenothera* virescence in Michigan, USA, as a representative of a novel taxon, '*Candidatus* Phytoplasma asteris'.

METHODS

Phytoplasma strains and nucleic acid preparation. Thirty representative AY group (16SrI) phytoplasma strains were used (Table 1). The phytoplasma strains were previously characterized and identified on the basis of RFLP analysis of 16S rRNA or *tuf* gene sequences (Lee *et al.*, 1993b, 1998b; Marcone *et al.*, 2000). Total nucleic acid was extracted, according to Ahrens *et al.* (1993) or Lee *et al.* (1993a), from original natural host plants or experimentally infected periwinkle (*Catharanthus roseus*) plants.

PCR and RFLP analysis. To obtain nearly full-length 16S rDNA, a nested PCR using the primer pair P1/P7 (Deng & Hiruki, 1991; Schneider *et al.*, 1995) followed by P1A/P7A (5′-ACGCTGGCG-GCGCGCCTAATAC-3′/5′-CCTTCATCGGCTCTTAGTGC-3′), was used to amplify a DNA fragment (P1A/P7A PCR products, about 1·8 kbp) that extends from the 5′ end of the 16S rRNA gene, through the intergenic spacer region, to the 3′ end of the 23S rRNA gene. A nested PCR using the primer pair rpF1/rpR1 (Lim & Sears, 1992) followed by rp(I)F1A/rp(I)R1A (5′-TTTTCCCCTACACGTA-CTTA-3′/5′-GTTCTTTTTGGCATTAACAT-3′) was used to amplify a phytoplasma DNA segment (about 1·2 kbp) of the *rp* operon that encompassed genes *rpl22* and *rps3*. For PCR amplification, 38 cycles were conducted in an automated thermal cycler (MJ Research DNA Thermal Cycler PTC-200) with AmpliTaq Gold polymerase. PCR mixtures contained 1 µl undiluted DNA preparation, 200 µM each dNTP and 0·4 µM each primer. The following conditions were used: denaturation at 94 °C for 1 min (11 min for the first cycle), annealing for 2 min at 55 °C (50 °C for *rp* sequence amplification) and primer extension for 3 min (7 min in the final cycle) at 72 °C. Diluted (1 : 30) PCR product from the first amplification (1 µl) was used as the template in the nested PCR. PCR products (5 µl) were electrophoresed through 1 % agarose gel.

PCR products of *rp* sequences were digested with *Alu*I, *Mse*I and *Tsp509*I. The restriction products were then separated by electrophoresis through 5 % polyacrylamide gel (12 % for *Mse*I and *Tsp509*I digests of *rp* products).

Cloning of PCR products and sequencing of DNA. PCR-amplified fragments of the 16S rRNA and *rp* genes were cloned and sequenced. P1A/P7A PCR products and rp(I)F1A/rp(I)R1A PCR products (about 1·2 kbp, containing *rpl22–rps3* gene sequences) were purified by using a Qiaquick PCR Purification kit and cloned in *Escherichia coli* by using a TOPO TA cloning kit (Invitrogen), according to the manufacturers' instructions. Sequencing was performed with an automated DNA sequencer (ABI Prism model 377). The cloned nucleotide sequences were deposited in GenBank; accession numbers are given in Figs 1 and 2.

Phylogenetic analysis. Phylogenetic interrelationships among strains of the AY group and other phytoplasma groups were assessed, based on 16S rRNA gene sequences. Partial sequences of 16S rDNA (1·5 kbp) from members of the AY phytoplasma group, other phytoplasma strains and *Acholeplasma laidlawii* (strain JA1) that were available in GenBank were aligned by using CLUSTAL, version V, and DNASTAR's Lasergene software. Cladistic analyses were performed by using PAUP (Phylogenetic Analysis Using Parsimony), version 4.0 (D. L. Swofford, University of Illinois, IL, USA). Uninformative characters were excluded from analyses. A phylogenetic tree was constructed via random stepwise addition by 100 replicates of a heuristic search, employing the tree bisection–reconnection algorithm, to find optimal trees. *A. laidlawii* was selected as the outgroup to root the tree. Bootstrap analyses (100 replicates) were performed to estimate the stability and support for inferred clades. Finer phylogenetic relationships among members of the AY phytoplasma group were determined based on partial sequences of the *rp* gene operon (1·2 kbp, encompassing the *rpl22* and *rps3* genes) by using a branch-and-bound search. *A. laidlawii* was selected as the outgroup for *rp* gene analysis.

RESULTS AND DISCUSSION

Phylogenetic analysis based on 16S rDNA and *rp* operon sequences

A phylogenetic tree constructed by using nearly full-length 16S rRNA gene sequences indicated that AY group phytoplasmas (30 strains) represent a distinct phylogenetic subclade that is related most closely to the stolbur phytoplasma

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Table 1. Classification of AY group phytoplasmas based on RFLP analyses of 16S rRNA, ribosomal protein and *tuf* gene sequences

–, Not applicable. 16SrI, 16S rRNA gene RFLP group I; *rp*I, ribosomal protein gene RFLP group I; *tuf*I, *tuf* gene RFLP group.

Disease caused (strain)	Natural host	Place of isolation	RFLP subgroup classification			Reference
			16SrI*	*rp*I	*tuf*I	
Aconitum proliferation (AcP)	*Aconitum napellus* L. (aconite)	Lithuania	A	–	–	Valiunas *et al.* (2001)
Alberta aster yellows (AY27)	*Callistephus chinensis* (China aster)	Canada	A	A	A	Lee *et al.* (1993b)
Canola yellows (CanY)	*Brassica* spp. (canola)	Canada	A	–	–	Wang & Hiruki (2001)
Carrot proliferation (CarrP)	*Daucus carota* (carrot)	Lithuania	A	–	–	Valiunas *et al.* (2001)
Carrot yellows (Btsv2CarD1)	*Daucus carota* (carrot)	Texas, USA	A	A	–	Unpublished
Carrot yellows (Btsv2CarD5)†	*Daucus carota* (carrot)	Texas, USA	A	A	–	Unpublished
Chrysanthemum yellows (CHRY)†	*Chrysanthemum frutescens* (marguerite)	Germany	A	A	A	Marcone *et al.* (2000)
Chrysanthemum yellows (CHRYM)†	*Chrysanthemum frutescens* (marguerite)	Germany	A	A	A	Marcone *et al.* (2000)
Dandelion yellows (DanY)	*Taraxacum officinale* (dandelion)	Canada	A	–	–	Wang & Hiruki (2001)
Dill yellows (DillH2)	*Anethum graveolens* (dill)	Texas, USA	A	A	–	Unpublished
Eastern aster yellows (NAY)	*Lactuca sativa* (lettuce)	Canada	A	A	–	Lee *et al.* (1993b)
Erigeron yellows (ErY4)	*Erigeron canadensis* (horsweed)	Maryland, USA	A	A	–	Lee *et al.* (1998b)
False ragweed (RdwdD1)	*Parthenium hysterophorus* (false ragweed)	Texas	A	A	–	Unpublished
Grey dogwood stunt (GD1)†	*Cornus racemosa* (grey dogwood)	New York, USA	A	M	–	Griffiths *et al.* (1994), Lee *et al.* (1998b)
Hyacinth yellows (HyaY)	*Hyacinthus orientalis* (hyacinth)	Lithuania	A	–	–	Valiunas (2003)
Hydrangea phyllody (HYDP)†	*Hydrangea macrophylla* (hydrangea)	Belgium	A	A	A	Schneider *et al.* (1993)
Lazy daisy yellows (LDD1)	*Aphanostephus skirrhobasis* (lazy daisy)	Texas, USA	A	A	–	Unpublished
Lettuce yellows (OKAY1)	*Lactuca sativa* (lettuce)	Okalahoma, USA	A	A	–	Lee *et al.* (1993b)
Limonium yellows (LimY)	*Limonium sinuatum* (statice)	Lithuania	A	–	–	Valiunas (2003)
New Jersey aster yellows (NJAY)	*Lactuca sativa* (lettuce)	New Jersey, USA	A	A	A	Lin & Chen (1985), Lee *et al.* (1993b)
Oat proliferation (OatP)	*Avena sativa* (oat)	Lithuania	A	–	–	Jomantiene *et al.* (2002a)
Onion yellows (OnionD2)†	*Allium cepa* (onion)	Texas, USA	A	A	–	Unpublished
Periwinkle little leaf (CN1)	*Catharanthus rosea* (periwinkle)	Connecticut, USA	A	A	–	Lee *et al.* (1993b), Davis *et al.* (1990)
Parsley yellows (ParsD3)	*Corinadrum sativum* (parsley)	Texas, USA	A	A	–	Unpublished
Plantago virescence (PVM)†	*Plantago coronopus* (plantago)	Germany	A	A	A	Schneider *et al.* (1993)
Prickly lettuce yellows (PLD1)	*Sonchus asper* (prickly lettuce)	Texas, USA	A	A	–	Unpublished
Primrose virescence (PRIVB)	*Primula* sp. (primrose)	Germany	A	A	A	Schneider *et al.* (1993)
Purple coneflower yellows	*Echinacea purpurea* (purple coneflower)	Wisconsin, USA	A	A	–	Stanosz *et al.* (1997)
Tomato big bud (BB)†	*Lycopersicon esculentum* (tomato)	Arkansas, USA	A	A	A	Lee *et al.* (1993b)
Alfalfa stunt (AlfS)†	*Medicago sativa* (alfalfa)	Lithuania	B	–	–	Jomantiene *et al.* (2000)
American aster yellows (AAY)	*Catharanthus rosea* (periwinkle)	Florida, USA	B	–	–	Seemüller *et al.* (1994)
Broccoli phyllody (IBP)	*Brassica oleracea* var. *italica* (sprouting broccoli)	Italy	B	–	B	Marcone *et al.* (1997b)
Cabbage proliferation (CabD3)†	*Brassica oleracea* (cabbage)	Texas, USA	B	B	–	Unpublished
Carrot yellows (Btsv2CarD3)†	*Daucus carota* (carrot)	Texas, USA	B	B	–	Unpublished

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Table 1. cont.

Disease caused (strain)	Natural host	Place of isolation	RFLP subgroup classification			Reference
			16SrI*	*rp*I	*tuf*I	
Carrot yellows (OKAY3)	*Daucus carota* (carrot)	Oklahoma, USA	B	B	–	Lee *et al.* (1993b, 1998b)
Cactus virescence (CC)	*Opuntia* sp. (cactus)	UK	B	–	–	Keane *et al.* (1996), Vibio *et al.* (1996)
Catharanthus virescence (CVL)	*Catharanthus rosea* (periwinkle)	Peru	B	–	B	Schneider *et al.* (1993)
Catharanthus virescence (CVT)	*Catharanthus rosea* (periwinkle)	Thailand	B	–	B	Schneider *et al.* (1993)
Chayote witches'-broom (CWB)	*Sechium edule* (chayote)	Costa Rica	B	–	–	Villalobos *et al.* (2002)
Chrysanthemum yellows (CY)	*Chrysanthemum frutescens* (marguerite)	Italy	B	–	–	Bertaccini *et al.* (1990), Lee *et al.* (1993b)
Chrysanthemum yellows	*Chrysanthemum frutescens* (marguerite)	Japan	B	–	–	Okuda *et al.* (1997)
Chrysanthemum witches'-broom (GCWB)	*Chrysanthemum coronarium* (crown daisy)	Japan	B	–	–	Okuda *et al.* (1997)
Cosmos phyllody (CosmPh)	*Cosmos bippinatus* (cosmos)	Mexico	B	–	–	Unpublished
Cyclamen virescence (GCV)	*Cyclamen persicum* (cyclamen)	Germany	B	–	B	Schneider *et al.* (1997)
Delphinium Virescence (DEV)	*Delphinium* hybrid (larkspur)	Germany	B	–	B	Schneider *et al.* (1993)
Dill yellows (DillD2)	*Anethum graveolens* (dill)	Texas, USA	B	B	–	Unpublished
Diplotaxis virescence (DIV)	*Diplotaxis eucoides* (white wall rocket)	Spain	B	–	B	Schneider *et al.* (1993)
Dog-fennel yellows	*Eupatorium capillifolium* (dog-fennel)	Florida, USA	B	–	–	Harrison *et al.* (1996b)
Dwarf aster yellows (DAY)	*Trifolium* sp. (clover)	California, USA	B	B	–	Lee *et al.* (1993b)
Eggplant dwarf (ED)	*Solanum melongena* (eggplant)	Japan	B	–	–	Okuda *et al.* (1997)
European aster yellows (EAY)	*Callistephus chinensis* (China aster)	Germany	B	–	–	Keane *et al.* (1996), Vibio *et al.* (1996)
Epilobium phyllody (EpPh)	*Epilobium* sp. (fireweed)	Lithuania	B	–	–	Alminaite *et al.* (2002)
Gladiolus witches'-broom (GLAWB)	*Gladiolus* hybrid (gladiolus)	Belgium	B	–	B	Van Slogteren *et al.* (1974)
Gladiolus witches'-broom (GLAWC)	*Gladiolus* hybrid (gladiolus)	France	B	–	B	Albouy (1966)
Hydrangea phyllody (HyPH1)†	*Hydrangea macrophylla* (hydrangea)	Italy	B	K	B	Lee *et al.* (1998b)
Hydrangea phyllody (HYDB)	*Hydrangea macrophylla* (hydrangea)	Belgium	B	–	B	Schneider *et al.* (1993)
Hydrangea phyllody (HYDF)	*Hydrangea macrophylla* (hydrangea)	France	B	–	B	Cousin *et al.* (1986)
Italian cabbage yellows (ICY)	*Brassica oleracea* var. *capitata* (cabbage)	Italy	B	–	B	Bertaccini *et al.* (1996), Marcone *et al.* (1997b)
Italian lettuce yellows (ILY)	*Lactuca sativa* (lettuce)	Italy	B	–	B	Marcone *et al.* (1997b), Vibio *et al.* (1994)
Kale phyllody (SIKP)	*Brassica oleracea* var. *palmifolia* (kale)	Italy	B	–	B	Marcone *et al.* (1997b)
Marigold phyllody (MgPh)	*Tagetes erecta* (marigold)	Mexico	B	–	–	Unpublished
Marigold virescence (SICV)	*Calendula officinalis* (marigold)	Italy	B	–	B	Marcone *et al.* (1997a)
Maize bushy stunt (MBS)†	*Zea mays* (corn)	Mexico	B	L	B	Harrison *et al.* (1996a), Lee *et al.* (1998b)
Maryland aster yellows (AY1)†	*Catharantus rosea* (periwinkle)	Maryland, USA	B	B	–	Lee & Davis (1988), Lee *et al.* (1993b)
Mitsuba witches'-broom (JHW)	*Cryptotaenia japonica* (Japanese honewort)	Japan	B	–	–	Okuda *et al.* (1997)
Mulberry dwarf (MD)	*Morus bombycis* (mulberry)	Japan, China	B	B	–	Lee *et al.* (1998b)
Olive witches'-broom	*Olea europaea* (olive)	Italy	B	–	–	Danielli *et al.* (1996)

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Table 1. cont.

Disease caused (strain)	Natural host	Place of isolation	RFLP subgroup classification			Reference
			16SrI*	*rp*I	*tuf*I	
Oenothera virescence = MI aster yellows (OAY^T = MIAY)†	*Oenothera hookeri* (evening primrose)	Michigan, USA	B	B	–	Lee *et al.* (1993b), Sears *et al.* (1989)
Onion yellows (OY = OAY)	*Allium cepa* (onion)	Japan	B	–	–	Namba *et al.* (1993)
Onion yellows (IOY)	*Allium cepa* (onion)	Italy	B	–	B	Marcone *et al.* (2000)
Papaver virescence (PV)	*Papaver rhoeas* (corn poppy)	Italy	B	–	B	Marcone *et al.* (1997c)
Parsley yellows (ParsD4)	*Corinadrum sativum* (parsley)	Texas, USA	B	B	–	Unpublished
Pear proliferation and decline (PPD)	*Pyrus communis* (pear)	Lithuania	B	–	–	Valiunas (2003)
Periwinkle virescence (FPV)	*Catharanthus rosea* (periwinkle)	Florida, USA	B			Harrison *et al.* (1996b)
Periwinkle witches'-broom (IPWB)	*Catharanthus rosea* (periwinkle)	Italy	B	–	B	Marcone *et al.* (1997b)
Plantago virescence (PVW)	*Plantago major* (plantago)	Germany	B	–	B	Schneider *et al.* (1993)
Primose virescence (PRIVC)†	*Primula* sp. (primrose)	Germany	B	B	B	Schneider *et al.* (1993)
Primrose yellows (IprY)	*Primula* sp. (primrose)	Italy	B	–	B	Marcone *et al.* (2000)
Primula yellows (PY)	*Primula* sp. (primrose)	Germany	B	–	–	Keane *et al.* (1996), Vibio *et al.* (1996)
Poplar witches'-broom (PoWB)	*Populus nigra* 'Italica' (Lombardy poplar)	France	B	–	B	Berges *et al.* (1997)
Purslane yellows (POY)	*Portulaca oleracea* (purslane)	Italy	B	–	B	Marcone *et al.* (1997c)
Ragweed yellows	*Ambrosia artemisifolia* (common ragweed)	Florida, USA	B	–	–	Harrison *et al.* (1996b)
Ranunculus phyllody (IraP)	*Ranunculus asiaticus* (Persian buttercup)	Italy	B	–	B	Marcone *et al.* (2000)
Rape virescence (RV)	*Brassica napus* (rape)	France	B	–	B	Schneider *et al.* (1993)
Safflower phyllody (SAFP)	*Carthamus tinctorius* (safflower)	Israel	B	–	B	Schneider *et al.* (1993)
Salix proliferation (SalP)	*Salix* sp. (willow)	Lithuania	B	–	–	Valiunas (2003)
Sandal spike (SAS)	*Santalum album* (sandalwood)	India	B	–	B	Schneider *et al.* (1993)
Severe aster yellows (Western aster yellows) (SAY)†	*Apium graveolens* (celery)	California, USA	B	B	–	Kuske & Kirkpatrick (1992), Lee *et al.* (1993b)
Tacaco witches'-broom (TacWB)	*Sechium tacaco* (tacaco)	Costa Rica	B	–	–	Villalobos *et al.*, 2002
Tomato yellows (TY)	*Lycopersicon esculentum* (tomato)	Japan	B	–	–	Okuda *et al.* (1997)
Turnip virescence (TPV)	*Brassica rapa* var. *rapifera* (turnip)	Italy	B	–	B	Marcone *et al.* (1997b)
Unknown phytoplasma disease	*Celtis australis* (European hackberry)	Italy	B	–	–	Bertaccini *et al.* (1996)
Watercress witches'-broom	*Nasturtium microphyllum* (watercress)	Hawaii, USA	B	–	–	Borth *et al.* (2002)
Western aster yellows (TLAY)	*Solanum tuberosum* (potato)	California, USA	B	B	–	Lee *et al.* (1993b, 1998b)
Wild radish yellows (WRY)	*Raphanus raphanistrum* (wild radish)	Italy	B	–	B	Marcone *et al.* (1997c)
Anemone virescence	*Anemone coronaria* (poppy anemone)	Italy	C	–	–	Vibio *et al.* (1995)
Clover phyllody (CPh)†	*Trifolium sativum* (red clover)	Canada	C	C	–	Lee *et al.* (1998b)
Clover phyllody (CPh)†	*Trifolium sativum* (red clover)	Canada	C	C	–	Lee *et al.* (1998b)
Clover phyllody (KVG = KV)†	*Trifolium repens* (white clover)	Germany	C	C	C	Schneider *et al.* (1993)
Clover phyllody (KVE)†	*Trifolium repens* (white clover)	France	C	C	C	Marcone *et al.* (2000)
Clover phyllody (KVF)	*Trifolium repens* (white clover)	France	C	–	C	Faivre-Amiot *et al.* (1970)
Olive witches'-broom	*Olea europaea* (olive)	Italy	C	–	–	Bertaccini *et al.* (2000)

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Table 1. cont.

Disease caused (strain)	Natural host	Place of isolation	RFLP subgroup classification			Reference
			16SrI*	*rp*I	*tuf* I	
Poa stunt (PoaS)	*Poa pratensis* (common meadow grass)	Lithuania	C	–	–	Valiunas (2003)
Ranunculus phyllody (RPh)	*Ranunculus* sp. (buttercup)	Italy	C	C	–	Lee *et al.* (1998b)
Strawberry green petal (SGP)	*Fragaria x ananassa* (strawberry)	Canada	C	C	–	Gundersen *et al.* (1996)
Yellows-affected grapevine (GY)	*Vitis vinifera* (grapevine)	Germany	C	–	C	Maixner *et al.* (1994)
Paulownia witches'-broom (PaWB)†	*Paulownia* spp. (paulownia)	Taiwan	D	D	B	Lee *et al.* (1998b)
Blueberry stunt (BBS1)†	*Vaccinium* spp.(blueberry)	Arkansas, USA	E	E	D	Lee *et al.* (1993b, 1998b)
Blueberry stunt (BBS3)†	*Vaccinium* spp.(blueberry)	Michigan, USA	E	E	D	Lee *et al.* (1993b, 1998b)
Apricot chlorotic leaf roll (ACLR-AY)†	*Prunus aremeniaca* (apricot)	Spain	F	N	E	Schneider *et al.* (1993)
Leafhopper borne (CVB)†	Leafhopper	Germany	F	N	–	Marcone *et al.* (2000)
Chrysanthemum yellows (CYb)	*Chrysanthemum frutescens* (marguerite)	Italy	H	–	–	Vibio *et al.* (1996)
Strawberry multiplier (STRAWB2)†	*Fragaria x ananassa* (strawberry)	Florida, USA	K	J	F	Jomantiene *et al.* (1998)
Aster yellows (AV2192)†	*Callistephus chinensis* (China aster)	Germany	L	B	B	Schneider *et al.* (1993)
Aster yellows (AV976)†	*Callistephus chinensis* (China aster)	Germany	L	B	B	Marcone *et al.* (2000)
Bermuda grass white leaf symptoms AYBG (BGWL)	*Cynodon dactylon* (bermuda grass)	Thailand	L	–	B	Schneider *et al.* (1993)
Gladiolus yellows (GlY)	*Gladiolus* sp. (gladiolus)	Lithuania	L	–	–	Valiunas (2003)
Primula yellows (PRIVA)	*Primula* sp. (primrose)	Germany	L	B	B	Schneider *et al.* (1993)
Rape phyllody (RapPh)	*Brassica napus* (rape)	Lithuania	L	–	–	Valiunas (2003)
Aster yellows (AVUT)†	*Callistephus chinensis* (China aster)	Germany	M	B	B	Marcone *et al.* (2000)
Cirsium stunt (CirS)	*Cirsium arvense* (Canadian thistle)	Lithuania	M	–	–	Valiunas (2003)
Gladiolus virescence (GlVir)	*Gladiolus* sp. (gladiolus)	Lithuania	M	–	–	Valiunas (2003)
Hyacinthus virescence (HyacVir)	*Hyacinthus orientalis* (common hyacinth)	Lithuania	M	–	–	Valiunas (2003)
Limonium proliferation (LimP)	*Limonium sinuatum* (sinuate sea-lavender)	Lithuania	M	–	–	Valiunas (2003)
Onion phyllody (OnPh)	*Allium cepa* (onion)	Lithuania	M	–	–	Valiunas (2003)
Quercus proliferation (QuerP)	*Quercus robur* (pedunculate oak)	Lithuania	M	–	–	Valiunas (2003)
Saponaria proliferation (SapP)	*Saponaria officinalis* (common soapwort)	Lithuania	M	–	–	Valiunas (2003)
Schizanthus proliferation (SchiP)	*Schizanthus pinnatus* (butterfly flower)	Lithuania	M	–	–	Valiunas (2003)
Symphytum proliferation (SymP)	*Symphytun* L. sp. (comfrey)	Lithuania	M	–	–	Valiunas (2003)
Valeriana yellows (ValY)	*Valeriana officinalis* L. (valerian)	Lithuania	M	B	–	Valiunas (2003)
Ipomoea obscura witches'-broom (IOWB)†	*Ipomoea obscura* (dwarf morning glory)	Taiwan	N	F	G	Marcone *et al.* (2000), this study
Soybean purple stem (SPS)†	*Glycine max* (soybean)	USA	O	–	–	Lee *et al.* (2002)
Unknown potato disease	*Solanum tuberosum* (potato)	Texas, USA	P	–	–	Unpublished
Cherry little leaf (ChLL)	*Prunus cerasus* (sour cherry)	Lithuania	Q	–	–	Valiunas (2003)
Strawberry phylloid fruit phytoplasma (StrawPhF)	*Fragaria x ananassa* (strawberry)	West Virginia, USA	R	–	–	Jomantiene *et al.* (2002b)

*Subgroup 16SrI-G phytoplasmas reclassified in group 16SrXII (stolbur group), subgroup 16SrXII-A (Lee *et al.*, 1998b). Subgroup 16SrI-J phytoplasmas reclassified in group 16SrXIV (Mexican periwinkle virescence group), subgroup 16SrXIV-B (Lee *et al.*, 1998b).
†Denotes strain used in this study.

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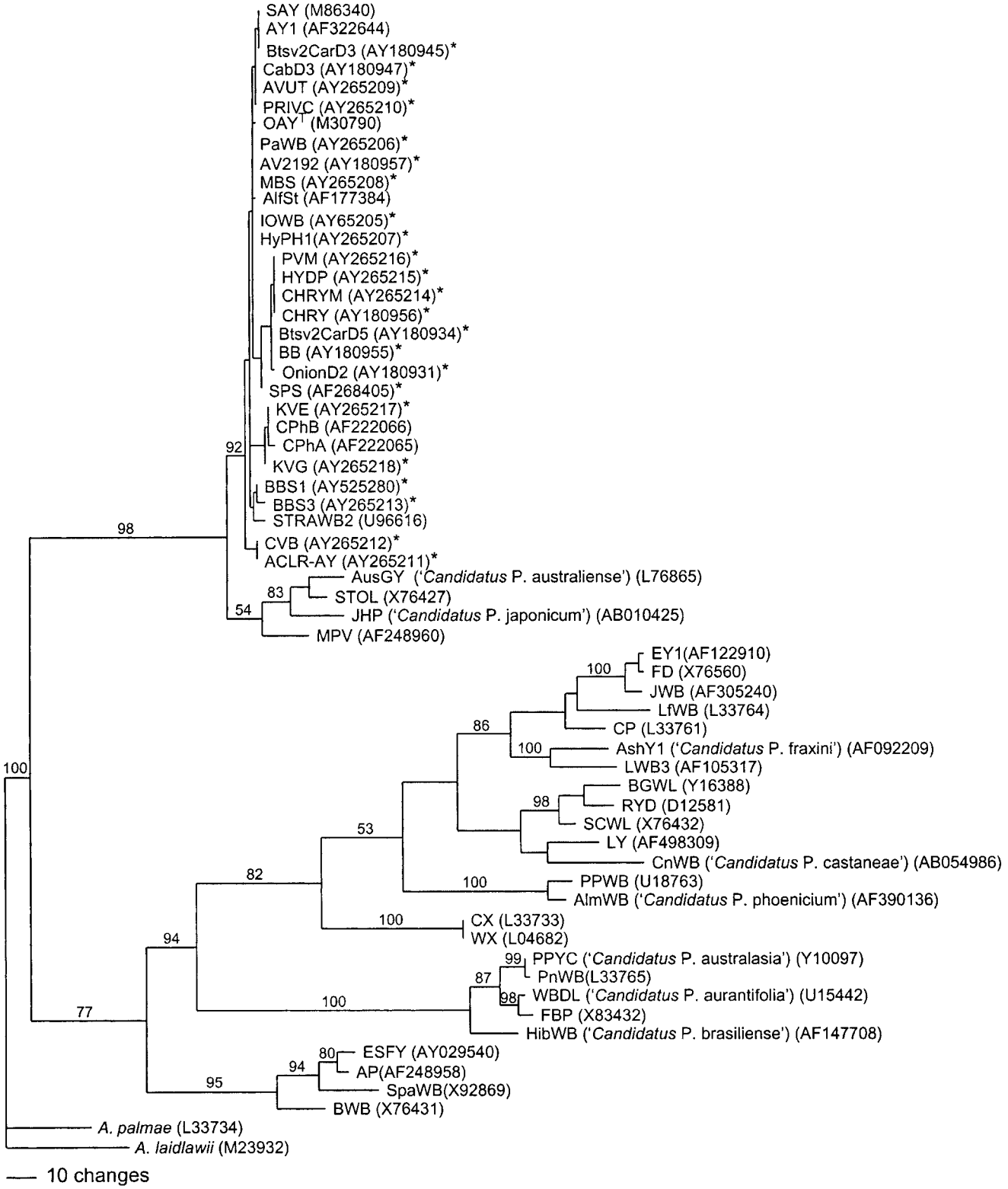

Fig. 1. Phylogenetic tree constructed by parsimony analysis (PAUP version 4.0b; D. Swofford) of nearly full-length 16S rDNA sequences from representative phytoplasma strains in the AY group (16SrI) and other 16Sr phytoplasma groups. Sequences were aligned with CLUSTAL version V (DNAStar Lasergene software). *Acholeplasma laidlawii* (M23932) was employed as the outgroup to root the tree. Branch-lengths are proportional to the number of inferred character state transformations. Bootstrap values >50 % (measures of support for the inferred subclades) are shown on branches. *, Strains sequenced in this study. Strain abbreviations of 16SrI are listed in Table 1. Bar, 10 inferred character state changes.

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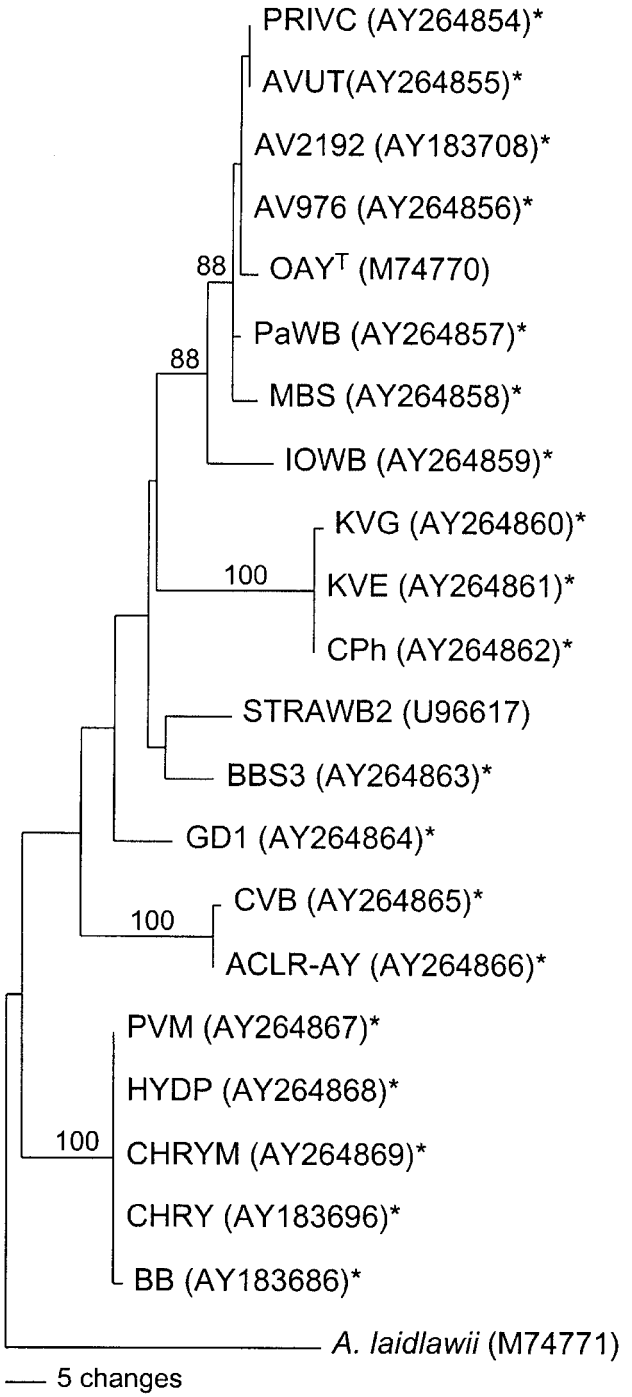

Fig. 2. Phylogenetic tree constructed by parsimony analysis (PAUP version 4.0b, D. Swofford) of partial ribosomal protein operon (*rpl22* and *rps3* genes) sequences from representative phytoplasma strains in the AY group (16SrI). Sequences were aligned with CLUSTAL version V (DNAStar Lasergene software). *A. laidlawii* (M74771) was employed as the outgroup to root the tree. Branch-lengths are proportional to the number of inferred character state transformations. Bootstrap values (measures of support for the inferred subclades) are shown on main branches. *, Strains sequenced in this study. Strain abbreviations are listed in Table 1. Bar, five inferred character state changes.

subclade (xii) [corresponding to group 16SrXII of Lee *et al.* (1998b)] (Fig. 1). Among the subclades within the phytoplasma clade, the AY subclade is related more closely to *Acholeplasma palmae* and *A. laidlawii*, members of the *Anaeroplasma* clade, than any other. The AY group strains shared 97–100 % 16S rDNA sequence similarity. The phylogenetic tree constructed by parsimony analysis of nearly full-length 16S rDNA sequences resolved at least six distinct phylogenetic lineages. Six are consistent with RFLP subgroups 16SrI-A (strains BB, PVM, HYDP, CHRYM, CHRY, Btsv2CarD5 and OnionD2), 16SrI-C (strains CPhA, CPhB, KVE and KVG), 16SrI-E (strains BBS1 and BBS3), 16SrI-F (strains ACLR-AY and CVB), 16SrI-K (strain STRAWB2) and 16SrI-O (strain SPS). One contained subgroups 16SrI-B (strains OAY, SAY, AY1, CabD3, AlfSt, Btsv2CarD3, PRIVC and HyPH1), 16SrI-D (strain PaWB), 16SrI-L (strain AV2192), 16SrI-M (strain AVUT) and 16SrI-N (strain IOWB). A previous study that used *tuf* gene sequences for phylogenetic analysis resolved the same six distinct lineages (Marcone, 2000).

The *rp* operon (*rpl22* and *rps3* genes) was more variable in sequence among members of the AY phytoplasma group than 16S rDNA. Some strains shared as little as 95·1 % sequence similarity with other members of the AY group. A phylogenetic tree, derived by analysis of the two *rp* gene sequences, delineated ten distinct phylogenetic lineages among 21 strains analysed (Fig. 2). Six corresponded to 16SrI subgroups 16SrI-C (strains KVG, KVE and CPh), 16SrI-D (strain PaWB), 16SrI-E (strain BBS3), 16SrI-F (strains ACLR and CVB), 16SrI-K (strain STRAWB2) and 16SrI-N (strain IOWB), respectively. One lineage contained subgroups 16SrI-B (strains OAY and PRIVC), 16SrI-L (strains AV2192 and AV976) and 16SrI-M (strain AVUT), whereas strain MBS (a member of subgroup 16SrI-B) formed a lineage that was divergent from the other members of 16SrI-B. Members of 16SrI-A (strains PVM, HYDP, CHRYM, CHRY, BB and GD1) were delineated into two distinct lineages (strain GD1 alone representing one). Phylogenetic analysis based on amino acid sequences of the two *rp* genes resolved the same lineages as those delineated by analysis of nucleotide sequences (data not shown).

The ten distinct lineages that were delineated by phylogenetic analysis of *rp* gene sequences were differentiated readily by RFLP analyses of *rp* sequences, which provided a better molecular tool than the 16S rRNA gene for differentiation among closely related phytoplasma strains. Composite profiles obtained from digests with the restriction enzymes *Mse*I, *Tsp509*I and *Alu*I differentiated the AY group into ten distinct *rp* subgroups (Fig. 3 and Table 1), consistent with the ten phylogenetic lineages that were delineated by *rp* gene sequence-based phylogeny.

Phylogenetic analysis, based on both 16S rRNA and *rp* gene sequences in the present study and on the *tuf* gene (Marcone *et al.*, 2000), reinforced the notion that most subgroups that were identified by RFLP analysis of 16S

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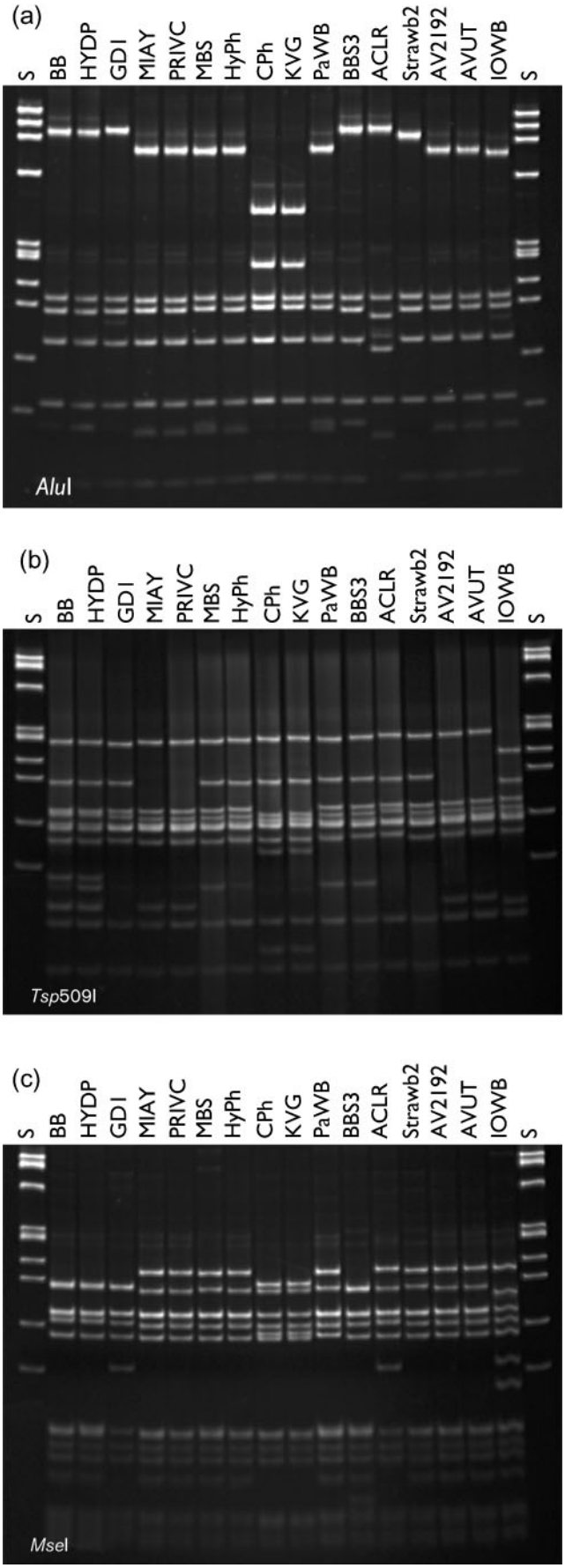

rDNA sequences represent distinct phylogenetic lineages. For example, strains PaWB (subgroup 16SrI-D) and MBS (subgroup 16SrI-B), which share >98 % 16S rRNA gene sequence similarity with members of subgroup 16SrI-B, were clustered with 16SrI-B strains in the 16S rRNA based-phylogeny, but the *rp*-based phylogeny indicated clearly that they represented two distinct lineages (bootstrap values, 99–100 %). In nature, strain PaWB is associated with paulownia witches'-broom disease in south-eastern Asia and strain MBS is associated with maize bushy stunt in the Americas. These two strains share neither insect host nor plant host, and their specific hosts differ from those of the other members of subgroup 16SrI-B. In light of such substantial differences in biological properties, it seems that members of group 16SrI represent more than one biological species. For example, members of subgroups 16SrI-D, 16SrI-E, 16SrI-F, 16SrI-K and 16SrI-N occupy uniquely different ecological niches in nature; they are associated with specific plant and insect hosts and/or are restricted to isolated geographical regions. For practical diagnostic and quarantine purposes, it would be advantageous if such biologically divergent organisms could be designated as distinct taxonomic entities. Indeed, they can be identified readily and defined based on RFLP patterns of the 16S rRNA, *rp* and *tuf* gene sequences. Such distinctions are consistent with relative homology of total genomic DNA (Lee *et al.*, 1992).

It is evident from the present and other work that many mutually distinct phytoplasma strains fall into a category in which, according to the International Committee of Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes* (ICPRM, 2000), additional biological and genomic criteria are needed to describe a strain that has 16S rRNA gene sequence dissimilarity of <2·5 % with a previously described species as a novel '*Candidatus* Phytoplasma' species. A phylogenetic parameter, such as the *rp* operon, that is more variable than the 16S rRNA gene may aid phytoplasma speciation. Designation of multiple species in group 16SrI is deferred until genetic divergence among the lineages can be evaluated further, in order to improve criteria for distinguishing closely related species in this group. Here, we propose that a novel '*Candidatus*

Fig. 3. RFLP profiles of *rp* operon sequences (1·2 kbp, containing *rpl22* and *rps3* genes), amplified by nested PCR with primer pair rpF1/rpR1 followed by primer pair rp(I)F1A/rp(I)R1A, from representative phytoplasma strains in the AY phytoplasma group (16SrI). PCR products were digested with (a) *Alu*I, (b) *Tsp509*I or (c) *Mse*I. *Alu*I digests were separated by electrophoresis through 5 % polyacrylamide gel and *Tsp509*I and *Mse*I digests were separated through 12 % polyacrylamide gel. Lane S, ΦX174 RFI DNA *Hae*III digest [fragment sizes (bp) from top to bottom for 5 % gel: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72; fragment sizes (bp) from top to bottom for 12 % gel: 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72]. Other abbreviations are defined in Table 1.

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Phytoplasma' species should be designated and that strain OAY (=MIAY) should serve as the reference strain of the AY phytoplasma group.

Properties of '*Candidatus* Phytoplasma asteris'

Biological properties and geographical distribution. The '*Candidatus* P. asteris' concept encompasses all known subgroups within group 16SrI. Strains that belong to subgroups 16SrI-A, 16SrI-B and 16SrI-C are distributed worldwide, are associated with more than 80 plant species and can be transmitted by more than 30 species of insect vectors (Kunkel, 1926; Brcák, 1979; Tsai, 1979). Subgroup 16SrI-B represents the largest and most diverse strain cluster in the group. Subgroups 16SrI-L and 16SrI-M appear to be restricted to the European continent. Experimentally, North American AY phytoplasmas (subgroups 16SrI-A and 16SrI-B) have been transmitted by insect vectors to 191 plant species, belonging to 42 families (McCoy *et al.*, 1989). Some vectors are shared by members of subgroups 16SrI-A, 16Sr-B and 16SrI-C. Leafhoppers, *Macrosteles* spp., *Euscelis* spp., *Scaphytopius* spp. and *Aphrodes* spp. are the major vectors of AY phytoplasma strains (Brcák, 1979; Tsai, 1979; Chiykowski, 1991). Other subgroups in group 16SrI are each associated with a narrow range of plant and insect hosts and the diseases they cause are often restricted to certain geographical areas. For example, the subgroup 16SrI-D phytoplasma associated with paulownia (*Paulownia taiwaniana* and *Paulownia tomentosa*) is found in Asia. The vast majority of strains in the AY phytoplasma group infect herbaceous dicot plant hosts. However, a number of strains that belong to subgroups 16SrI-A, 16SrI-B and 16SrI-C are capable of infecting monocot plants (e.g. corn, onion, gladiolus, oat, wheat and grass). Some strains in subgroups 16SrI-A, 16SrI-B, 16SrI-D, 16SrI-E, 16SrI-F and 16SrI-Q can cause diseases in woody plants (e.g. grey dogwood, sandalwood, blueberry, mulberry, peach, cherry, olive and paulownia).

Symptoms induced in infected plants. Symptoms can vary, depending on the phytoplasma strain. Typical symptoms of AY (caused by members of subgroup 16SrI-A and 16SrI-B) include virescence (greening of flower petals) and phyllody (development of floral parts into leaf-like structures), flower streaking and malformation, yellowing and upright posture of leaves, elongation and etiolation of internodes, excessive branching of axillary shoots, witches'-broom and general stunting of plants. However, some infected plants may exhibit only some of these symptoms. Symptoms induced by subgroup 16SrI-C phytoplasmas generally include virescence and phyllody, without excessive shoot proliferation. Symptoms induced by members of the other 16SrI subgroups include general stunting (little leaves, small flowers, shortening of internodes), leaf curl or rolling, small and faintly coloured flowers and some symptoms that are typical of the AY syndrome. Plants infected with mild strains may show no obvious symptoms.

Description of '*Candidatus* Phytoplasma asteris'. We propose that phytoplasma strain OAY (=MIAY) should be assigned as a reference strain to a novel '*Candidatus*' species to represent the AY group. Based on the guidelines proposed by Murray & Schleifer (1994), the OAY phytoplasma is designated as a novel species with the following description: '*Candidatus* Phytoplasma asteris' [Gr. *aster* star; common name of the first known host]: [(Mollicutes) NC; NA; O, wall-less; NAS (GenBank accession no. M30790, M74770); oligonucleotide sequences of unique regions of 16S rRNA gene: 5′-GGGAGGA-3′ (226–232), 5′-CTGACGGTACC-3′ (476–485) and 5′-CACAGTG-GAGGTTATCAGTTG-3′ (1008–1028); oligonucleotide sequences of unique regions of ribosomal protein genes: *rpl22*, 5′-CCGCGAACAACTT-3′ (218–230) and 5′-AG-TAATAACTTCTAGCACAAACTTGC-3′ (338–363) and *rps3*, 5′-AAAGAAGATTTTTTAATTC-3′ (114–133) and 5′-CTAGAAAATCGTATG-3′ (396–410); P (*Oenothera hookeri*, phloem); M]. OAY is the reference strain.

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