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"*Candidatus* Phytoplasma australiense," a New Phytoplasma Taxon Associated with Australian Grapevine Yellows

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A phytoplasma was detected in naturally diseased 'Chardonnay' grapevines exhibiting symptoms of Australian grapevine yellows disease. The use of PCR designed to amplify phytoplasma DNA resulted in detection of phytoplasma DNA in all of the diseased plants examined; no phytoplasma DNA was detected in healthy seedling grapevines. The collective restriction fragment length polymorphism (RFLP) patterns of amplified 16S ribosomal DNA differed from the patterns described previously for other phytoplasmas. On the basis of the RFLP patterns, Australian grapevine yellows phytoplasma was classified as a representative of a new subgroup, designated subgroup 16SrI-J, in phytoplasma 16S rRNA group 16SrI (aster yellows and related phytoplasmas). A phylogenetic analysis in which parsimony of 16S rRNA gene sequences from this and other group 16SrI phytoplasmas was used identified the Australian grapevine yellows phytoplasma as a member of a distinct subclade (subclade xii) in the phytoplasma clade of the class *Mollicutes*. A phylogenetic tree constructed on the basis of 16S rRNA gene sequences was consistent with the hypothesis that there was divergent evolution of Australian grapevine yellows phytoplasma and its closet known relative, European stolbur phytoplasma (subgroup 16SrI-G), from a common ancestor. The unique properties of the DNA from the Australian grapevine yellows phytoplasma clearly establish that it represents a new taxon, "*Candidatus* Phytoplasma australiense."

Australian grapevine yellows is a serious malady that is widely distributed in vineyards in Australia (25). Since the earliest reports of the disease (26–28), considerable effort has been devoted to understanding its spread and determining the nature of its causal agent, which was presumed to be a phytoplasma (formerly mycoplasmalike organism) (26, 28, 29). In its symptomatology, Australian grapevine yellows resembles flavescence dorée, bois noir, Vergilbungskrankheit, and other grapevine yellows diseases also believed to be caused by phytoplasmas (3, 4, 7, 25–30, 33, 34, 36, 37, 42). Although rigorous proof of the pathogenicity of phytoplasmas has been elusive because of an inability to culture these cell wall-less prokaryotes in cell-free medium, indirect evidence from electron microscopy, antibiotic therapy, and use of molecular probes has supported the hypothesis of phytoplasmal etiology (6, 16, 21, 26, 29, 33, 36, 37, 42). In the case of Australian grapevine yellows, both the sensitivity of the disease to the antibiotic tetracycline and electron microscopic observations of phytoplasmas in the phloem of diseased grapevines have provided evidence of phytoplasmal etiology (25, 29). Although current evidence points to genetically diverse phytoplasmas as the probable causes of grapevine yellows diseases in different countries (4, 9, 10, 13, 38), the relationship of the Australian grapevine yellows phytoplasma to the phytoplasmas that cause grapevine yellows diseases elsewhere is not understood.

We and other workers have previously used molecular methods to study grapevine yellows diseases in France, Italy, the United States, and elsewhere; the evidence has revealed an unexpectedly diverse array of phytoplasma taxa associated with these diseases (4, 9, 10, 13, 38). For example, on the basis of collective restriction fragment length polymorphism (RFLP) patterns of 16S rRNA gene (16S rDNA) sequences, flavescence dorée sensu stricto (5) is associated with a phytoplasma that is related to several other phytoplasmas (8) and that is a member of 16S rRNA group 16SrV (elm yellows and related phytoplasmas [23]) (38), which has been proposed as a taxon that represents a distinct phytoplasma species (18). Bois noir in France and grapevine yellows in Spain, Germany, some regions of Italy, and elsewhere are consistently associated with phytoplasmas that are members of group 16SrI and are related to aster yellows and stolbur phytoplasmas (1, 9, 10, 30, 31) and that, in the phylogenetic group system proposed by Seemüller et al. (40), belong to the distinct stolbur phytoplasma subgroup within the aster yellows phytoplasma group (39, 40). According to separate studies (1, 10b), this subgroup corresponds to the 16S rRNA subgroup designated subgroup 16SrI-G by Vibio et al. (43). It is possible that the stolbur phytoplasma represents a *Candidatus* species which can be distinguished from other members of group 16SrI (23) or the aster yellows phytoplasma group (40).

Mounting evidence clearly supports the hypothesis that group 16SrV and subgroup 16SrI-G phytoplasmas have causal roles in two distinct types of grapevine yellows diseases, but the etiology of grapevine yellows diseases in nature may be more complex. Further evidence has emerged which indicates that members of other phytoplasma groups and subgroups also infect grapevines and may induce yellows symptoms in grapevines (2, 13, 38). Although some studies provide few data that indicate the identities of diverse phytoplasmas in grapevine yellows disease (9, 10), together with other work (1, 4, 13, 38, 40) these studies support the concept that the grapevine yellows diseases are a group of diseases that, although similar in symptomatology, are caused by different phytoplasmas. The etiology of these diseases may be perceived as a complex, the precise nature of which depends on several factors, including geographical location.

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TABLE 1. Oligonucleotide primers and primer pairs used in PCRs in this study

Primer pair or primer	Nucleotide sequence	Size of amplified product (kbp)	Specificity of PCRs primed	Reference
16R758F-16R1232R		0.5	Phytoplasmas (universal)	15
16R758F	5′-GTCTT TACTG ACGCT GAGGC-3′			
16R1232R	5′-CTTCA GCTAC CCTTT GTAAC-3′			
R16F1-R16R0		1.4	Phytoplasmas (universal)	
R16F1	5′-AAGAC GAGGA TAACA GTTGG-3′			14
R16R0	5′-GGATA CCTTG TTACG ACTTA ACCCC-3′			23
R16F2n-R16R2		1.2	Phytoplasmas (universal)	
R16F2n	5′-GAAAC GACTG CTAAG ACTGG-3′			17
R16R2	5′-TGACG GGCGG TGTGT ACAAA CCCCG-3′			23
R16(I)F1-R16(I)R1		1.1	Group 16SrI (aster yellows and related strains)	22
R16(I)F1	5′-TAAAA GACCT AGCAA TAGG-3′			
R16(I)R1	5′-CAATC CGAAC TGAGA CTGT-3′			
fStol-rStol		0.5	Stolbur (subgroup 16SrI-G)	31
fStol	5′-GCCAT CATTA AGTTG GGGA-3′			
rStol	5′-AGATG TGACC TATTT TGGTG G-3′			
G35p-G35m		1.2	Some subgroups in group 16SrI	11
G35p	5′-TAACA CTGTG GAAGC TCA-3′			
G35m	5′-CGTCA ATGGC TAATC GAT-3′			

In the present study, we investigated the phytoplasma associated with grapevine yellows disease in Australia. Our results defined the relationship of the Australian grapevine yellows phytoplasma to phytoplasmas associated with grapevine yellows and other diseases around the globe. Previously published data indicated that the Australian grapevine yellows phytoplasma was not closely related serologically to the flavescence dorée phytoplasma (21). Other work indicated that phytoplasmas in naturally yellows-diseased grapevines in Australia were not identical to other phytoplasmas, including the phytoplasma of flavescence dorée (35). Here we report results from an extended study involving the priming of phytoplasma-specific DNA amplification from Australian grapevine yellows phytoplasma templates in PCRs and the analysis of amplified DNA. The results include the results of RFLP analyses of amplified 16S rDNAs and placement of the findings in the context of a comprehensive classification scheme (23) that contains no less than 11 16S rRNA gene groups, each of which represents at least one putative *Phytoplasma* species (18). We also report for the first time the nucleotide sequence of a segment of the Australian grapevine yellows phytoplasma 16S rRNA gene and the results of a phylogenetic analysis of the sequence and describe unique sequences in Australian grapevine yellows phytoplasma 16S rRNA. Our data led us to propose that the Australian grapevine yellows phytoplasma is taxonomically unique among the known phytoplasmas.

MATERIALS AND METHODS

Plant samples and reference phytoplasma strains. Samples from naturally diseased grapevines (*Vitis vinifera* L. 'Chardonnay') exhibiting symptoms of grapevine yellows were collected in the field in South Australia during 1993. Additional samples were taken from healthy, greenhouse-grown grapevine or periwinkle (*Catharanthus roseus* (L.) G. Don) seedlings. The reference phytoplasmas used included strain AY (= AY1 = MDAY) that had been collected previously in a naturally diseased periwinkle plant growing in a field in Beltsville, Md., and the following other phytoplasma strains in tissues of periwinkle or in other hosts or as DNA samples: clover phyllody strain CPh; clover yellow edge strain CYE, in ladino clover (*Trifolium repens* L.); Canada peach X-disease strain CX; Italian periwinkle virescence strain IPVR (11, 12); stolbur strain STOL; elm yellows strain EY1 (=EY); ash yellows strain AshY (=AshY); tomato big bud strain BB; potato witches'-broom strain PWB; beet leafhopper-transmitted virescence strain VR; apple proliferation strain AP-A; western X-disease strain WX; peanut witches'-broom strain PnWB; sweet potato witches'-broom strain SPWB; loofah (*Luffa* sp.) witches'-broom strain LfWB; paulownia witches'-broom strain PaWB; maize bushy stunt strain MBS, in corn (*Zea mays* L.); lethal yellowing of palms strain LY (DNA sample from diseased coconut); Mexican periwinkle virescence strain MPV; pigeon pea witches'-broom strain PPWB; Florida periwinkle witches'-broom strain FlPerWB; rice yellow dwarf strain RYD, in rice (*Oryza sativa* L.); sugarcane grassy shoot strain SCGS, in sugarcane (*Saccharum officinarum* L.); and European grapevine yellows (bois noir) strain GA1, in grapevine (*V. vinifera* L.).

Primer pairs and conditions for PCRs. Six pairs of previously designed oligonucleotide primers were used in PCRs. The sequences of the primers, the specificities of PCRs primed by the primer pairs, and the approximate sizes of amplified DNAs are given in Table 1. The R16F2n-R16R2 primer pair is nested within the positions of annealing (on 16S rDNA) of R16F1 and R16R0. The 16R758F-16R1232R and R16(I)F1-R16(I)R1 primer pairs are nested within the positions of annealing of primers R16F2n and R16R2 along the length of phytoplasma 16S rDNA. An additional primer pair (see below) was designed on the basis of the nucleotide sequence of the 16S rDNA of Australian grapevine yellows phytoplasma determined in the present work.

Both a single (direct, nonnested) PCR and two sequential reactions (nested PCRs) were used. In the direct PCR, the template consisted of the DNA in the total nucleic acid extracted as described previously from grapevine and periwinkle tissues (38). Each PCR mixture (total volume, 50 μl) contained 20 ng (when the template was from *C. roseus*) or 30 to 50 ng (when the template was from grapevine) of total nucleic acid extracted from healthy or diseased plant tissue, each primer of a pair at a concentration of 0.4 μM, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.001% gelatin, 1.25 U of *Taq* DNA polymerase (Amplitaq DNA polymerase; Perkin-Elmer, Norwalk, Conn.), 200 μM dATP, 200 μM dCTP, 200 μM dGTP, and 200 μM dTTP. Each reaction mixture was overlaid with mineral oil, and the PCR was performed in a thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) as follows. An initial denaturation for 2 min at 94°C was followed by 35 cycles, each of which consisted of denaturation for 1 min at 94°C, annealing for 2 min at 50°C (55°C when primers fStol and rStol were used), and extension for 3 min at 72°C. In the last cycle the extension step at 72°C was 10 min long. A 5-μl aliquot of each PCR product was analyzed by electrophoresis in a 1% agarose gel, which was stained with 0.5 μg of ethidium bromide per ml and visualized with a UV transilluminator.

In nested PCRs, the product from a direct PCR primed with primers R16F1 and R16R0 or primers R16F2n and R16R2 was diluted 1:50 (for grapevine samples) or 1:100 (for *C. roseus* samples) with sterile deionized distilled water, and 1 μl was used as the template in a second (nested) PCR. The nested PCR mixtures were primed with primers R16F2n and R16R2 (when the first PCR was primed with primers R16F1 and R16R0) or with primers 16R758F and 16R1232R or primers R16(I)F1 and R16(I)R1 (when the primers for the first PCR were primers R16F2n and R16R2). The products of nested reactions were analyzed as described above.

TABLE 2. Phytoplasmas and acholeplasmas used in this study, associated diseases, and accession numbers of the 16S rDNA sequences

Phytoplasma	Associated plant disease (source)	Accession no.	Reference
AY1	Maryland aster yellows (Maryland)	L33767	18
SAY	Western severe aster yellows (California)	M86340	20
AAY	American aster yellows (Florida)	X68373	40
MIAY	*Oenothera hookeri* virescence (Michigan)	M30970	24
ACLR	Apricot chlorotic leafroll	X68383	40
BB	Tomato big bud (Arkansas)	L33760	18
CPh	Clover phyllody (Canada)	L33762	18
STOL	Stolbur of *Capsicum annuum* (Serbia)	X76427	40
VK	Vergilbungskrankheit of grapevine (Germany)	X76428	40
EY1	Elm yellows (New York)	L33763	18
AshY1	Ash yellows (New York)	L33759	18
PPWB	Pigeon pea witches'-broom (Florida)	L33735	18
CX	Canada peach X-disease	L33733	18
AT	Apple proliferation (Germany)	X68375	40
WBDL	Witches'-broom of lime (Oman)	U15442	45
CP	Clover proliferation (Canada)	L33761	18
RYD	Rice yellow dwarf (Japan)	D12581	32a
PnWB	Peanut witches'-broom (Taiwan)	L33765	18
LfWB	Loofah witches'-broom (Taiwan)	L33764	18
LY	Coconut lethal yellows (Florida)	U18747	18
AUSGY	Australian grapevine yellows	L76865	This paper
A. palmae (formerly *Acholeplasma* sp. strain J233)		L33734	18
A. laidlawii		M23932	44

RFLP analyses of PCR-amplified DNA. Products from PCRs were singly digested with restriction endonucleases *Alu*I (GIBCO-BRL, Gaithersburg, Md.), *Mse*I, *Kpn*I, *Rsa*I, *Hha*I, *Taq*I, *Hae*III, *Hpa*II, *Sau*3A1, *Hin*fI, *Tha*I, and *Hpa*I (New England Biolabs, Beverly, Mass.) according to the manufacturers' instructions. The digested DNAs were analyzed by electrophoresis of the digestion products in a 5% polyacrylamide gel, followed by staining with ethidium bromide and visualization of DNA bands with UV transilluminator. The RFLP patterns of Australian grapevine yellows phytoplasma DNA were compared with the patterns of amplified DNAs from reference strains and patterns published previously (23).

Nucleotide sequencing, sequence alignment, and cladogram construction. The PCR-amplified 16S rRNA gene products were sequenced by using standard dideoxy termination methods. Complete or nearly complete 16S rRNA gene sequences from 22 phytoplasmas and acholeplasmas were aligned and base positions were numbered as previously described (18). The resulting alignments were visually inspected for logical placement and were manually adjusted, when necessary, to retain patterns of conserved sequences for secondary structure. Cladistic analyses, construction of a phylogenetic tree, and a bootstrap analysis were performed as previously described (18). The nucleotide sequence of the Australian grapevine yellows phytoplasma 16S rRNA gene determined in this study was deposited in the GenBank data library. The sequences of the other organisms used in this study were obtained from GenBank (National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md.).

Nucleotide sequence accession numbers. The GenBank accession numbers of the 16S rRNA gene sequences of Australian grapevine yellows phytoplasma, other phytoplasma strains, *Acholeplasma palmae* (formerly *Acholeplasma* sp. strain J233 [41]), and *Acholeplasma laidlawii* used in this study are listed in Table 2.

RESULTS

Detection of phytoplasmas in diseased grapevines. The PCR results pointed to association of a phytoplasma with Australian grapevine yellows in all diseased plants studied (Table 3). Initially, direct PCRs with different primer pairs were used in attempts to amplify and detect phytoplasma 16S rDNA in diseased Australian grapevines. Use of primers R16F1 and R16R0 and primers R16F2n and R16R2 in separate reaction mixtures failed to yield detectable phytoplasma DNA amplification. However, a direct PCR with primers fStol and rStol did result in detection of phytoplasma DNA. The use of primers fStol and rStol in a PCR resulted in apparent weak amplification of phytoplasma-specific DNA (Table 3). Priming of DNA amplification by this primer pair was consistent with affiliation of the strains detected with group 16SrI, since these primers were designed based on the sequence of the 16S rDNA of stolbur phytoplasma strain STOL (31), which is a member of group 16SrI (3a, 10c). The use of primers G35p and G35m did not result in PCR amplification of DNA from any of the yellows-diseased Australian grapevine samples (Table 3). Since other work has determined that PCR with primers G35p and G35m is a highly sensitive method for phytoplasma detection (10c), this result is consistent with the hypothesis that although the Australian grapevine yellows phytoplasma is a member of group 16SrI, it is distinct from other group 16SrI phytoplasmas, including STOL, since primers G35p and G35m primed amplification of DNAs from STOL and the related Italian periwinkle virescence phytoplasma (Table 3).

Nested PCR resulted in detectable DNA amplification from templates derived from diseased Australian grapevines. Phytoplasma DNA was amplified in PCRs primed with primers R16F1 and R16R0 and then with primers R16F2n and R16R2, in PCRs primed with primers R16F2n and R16R2 and then with primers 16R758F and 16R1232R, and in PCRs primed with primers R16F2n and R16R2 and then with primers R16(I)F1 and R16(I)R1, which indicated that all yellows-diseased Australian grapevines were infected with phytoplasma. The results of PCRs involving primers R16(I)F1 and R16(I)R1 indicated that the strains detected belonged to group 16SrI.

RFLP analyses of amplified 16S rDNA. In the RFLP analyses of the DNAs of the phytoplasmas detected in diseased grapevines in Australia, we analyzed products from the second of two sequential, nested reactions in which reamplification of DNA was primed with primers R16F2n and R16R2 because previous work that established a comprehensive scheme for phytoplasma classification was based on RFLP analyses of DNAs amplified in PCRs primed by oligonucleotides R16F2n and R16R2 (23). Repeated experiments gave the same results. Sites of *Kpn*I digestion were detected in 16S rDNA amplified from all of the diseased grapevine samples from Australia (Fig. 1) (data from AUSGY not shown), indicating that the Australian grapevine phytoplasma was affiliated with group 16SrI,

TABLE 3. Amplification and detection of phytoplasma target 16S rDNAs from naturally yellows-diseased grapevine (*V. vinifera* L. 'Chardonnay') plants growing in the field in Berri, South Australia, and from reference phytoplasmas by direct (nonnested) and nested PCRs

Source of DNA	DNA amplification in[a]:						
	PCRs with primers R16F2n and R16R2			PCRs with primers R16F1 and R16R0			
	Direct	Nested with primers 16R758F and 16R1232R	Nested with primers R16(I)F1 and R16(I)R1	Direct	Nested with primers R16F2n and R16R2	Direct PCRs with primers fStol and rStol	Direct PCRs with primers G35p and G35m
Australian grapevines							
AUSGY1	−	+	W	−	+	W	−
AUSGY2	−	+	W	−	+	W	−
AUSGY3	−	+	W	−	W	W	−
AUSGY4	−	+	W	−	+	W	−
AUSGY5	−	−	−	−	+	W	−
AUSGY6	−	+	W	−	+	W	−
AUSGY7	−	+	−	−	+	W	−
Healthy grapevine seedling	−	−	−	−	−	−	−
Reference phytoplasmas							
AY	+	+	+	+	+	−	+
CX	+	+	−	+	+	−	−
EY	+	+	−	+	+	−	−
IPVR	+	+	+	+	+	+	+
STOL	+	+	+	+	+	+	+
Healthy periwinkle	−	−	−	−	−	−	−

[a] +, DNA of the expected size was amplified (see Table 1 for sizes of amplified DNAs); W, a faint band of amplified DNA of the expected size was observed on the gel; −, no amplified DNA was observed.

since *Kpn*I sites are characteristic of the 16S rDNAs of strains in this group (23).

The 16S rDNAs amplified from all of the Australian grapevine yellows-diseased plants yielded the same collective RFLP patterns, and these patterns were different from those of all other phytoplasmas. RFLP analyses with *Alu*I and *Mse*I clearly distinguished the Australian grapevine yellows phytoplasma from the reference strains, including STOL (Fig. 1), and from known group 16SrI and other phytoplasmas based on comparisons with previously published data (23). Although use of *Rsa*I, *Sau*3AI, *Hpa*II, *Hin*fI, *Tha*I, *Hha*I, *Kpn*I, *Hae*III, and *Hpa*I yielded collective RFLP patterns that were very similar or identical to the patterns of 16S rDNAs of other group 16SrI phytoplasmas, use of *Taq*I also distinguished the Australian grapevine yellows phytoplasma from its apparent closest relative, stolbur phytoplasma strain STOL (data not shown).

Nucleotide sequence and putative restriction sites in amplified 16S rDNA from Australian grapevine yellows phytoplasma. The nucleotide sequence determined for the 16S rDNA amplified in the PCR primed with primers R16F2n and R16R2 has been deposited in the GenBank database. The level of sequence similarity with the aligned 16S rDNA from STOL was about 97%. Results from a comparative analysis of putative restriction sites in the sequenced DNAs are shown in Fig. 2. The expected fragment sizes based on the analysis of putative restriction sites were in excellent agreement with the fragment sizes obtained by enzymatic RFLP analysis of the amplified 16S rDNAs. The Australian grapevine yellows and stolbur phytoplasmas could be distinguished from one another by the restriction site analysis data.

PCR specific for Australian grapevine yellows phytoplasma. On the basis of the sequence of amplified 16S rDNA from Australian grapevine yellows phytoplasma, a pair of oligonucleotides was designed to prime specific amplification of DNA from this phytoplasma. The designations and nucleotide sequences of the primers are as follows: primer AUSGYF1, 5′-ATCTTTAAAAGACCTCGCAAG-3′; and primer AUSGYR2, 5′-AGTTTTACCCAATGTTTAGTACTC-3′. The conditions used for the PCR were the same as those described elsewhere in this paper, except that the temperature of annealing was 55°C in the PCR specific for amplification of DNA from Australian grapevine yellows phytoplasma. A direct (nonnested) PCR was used. In PCR mixtures containing primers AUSGYF1 and AUSGYR2, amplification of a 644-bp DNA was observed when the template consisted of DNA derived from any of seven grapevine plants naturally affected by Australian grapevine yellows disease. No DNA amplification was observed when the template DNA was derived from any of the reference phytoplasmas, including STOL (data not shown).

Phylogenetic analysis. Phylogenetic analysis of 16S rRNA gene sequences from 20 diverse phytoplasmas, including Australian grapevine yellows phytoplasma, and representative *Acholeplasma* species yielded four equally parsimonious trees, one of which is shown in Fig. 3. This tree is in good agreement with the tree constructed previously (18), except that it has a new branch (designated subclade xii) containing the stolbur strain STOL, Vergilbungskrankheit strain VK, and Australian grapevine yellows strain AUSGY phytoplasmas. Subclade xii is most closely related to subclade i (aster yellows and related phytoplasmas).

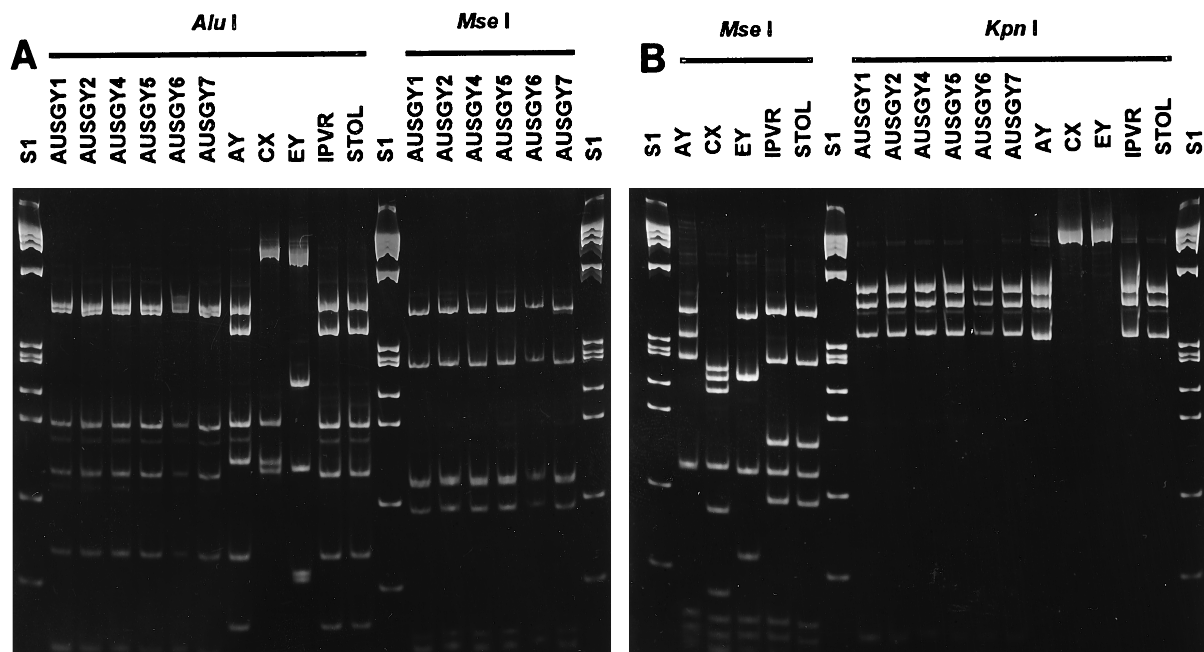

FIG. 1. RFLP analysis of 16S rDNAs amplified in nested PCRs primed with oligonucleotides R16F2n and R16R2 from phytoplasma strains infecting naturally diseased grapevine (*V. vinifera* L. 'Chardonnay') plants in South Australia. The first PCR was primed with primers R16F1 and R16R0, and this was followed by reamplification of target DNA in a nested PCR primed with primers R16F2n and R16R2. DNA products from the second, nested PCR were digested with restriction endonucleases *Alu*I, *Mse*I, and *Kpn*I. (A) Products digested with *Alu*I and *Mse*I. (B) Products digested with *Mse*I and *Kpn*I. Lane S1 contained a ϕX174 RFI DNA *Hae*III digest; the fragment sizes in this lane were (from top to bottom) 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118, and 72 bp. Lanes AUSGY1, AUSGY2, AUSGY4, AUSGY5, AUSGY6, and AUSGY7 contained digests of DNAs amplified from phytoplasmas detected in six separate grapevine yellows-diseased grapevine plants. AY, aster yellows phytoplasma; CX, Canada peach X-disease phytoplasma; EY (=EY1), elm yellows phytoplasma; IPVR, Italian periwinkle yellows phytoplasma; STOL, stolbur phytoplasma.

The phylogeny of subclade xii and the divergence between AUSGY and STOL-related phytoplasmas provide new insight into the evolution of these pathogens. Previously, it has been noted that subclade i (aster yellows and related phytoplasmas) is more closely linked to *Acholeplasma* relatives than are other phytoplasma subclades (18). Inclusion of the strain AUSGY, STOL, and VK phytoplasmas in the present phylogenetic analysis yielded a branching order which indicated that the subclade xii phytoplasmas are also closely linked to the genus *Acholeplasma*.

Phytoplasma signature sequence and unique 16S rRNA sequences. The 16S rRNA of the Australian grapevine yellows phytoplasma contains sequences unique to phytoplasmas. Six sequences previously reported to be unique to phytoplasmas (18), ACUGGA at positions 164 to 169, GUGU at positions 284 to 287, UGGAGG at positions 376 to 381, GGCAAG at positions 662 to 667, AUCAG at positions 1021 to 1025, and AGUU at positions 1321 to 1324, also occurred in the 16S rRNA of the AUSGY phytoplasma. The UAGC sequence at positions 1243 to 1246, another sequence unique to phytoplasmas (18), has a C at position 1243 in the 16S rRNAs of AUSGY and sweet potato witches'-broom phytoplasmas. The sequence corresponding to the previously reported unique sequence 5′-UUUUAAAAG-3′ at positions 196 to 204 (18) is 5′-CUUUAAAAG-3′ only in AUSGY among the phytoplasmas studied; the bases at the corresponding positions in the 16S rRNA of *A. palmae* are the same as the bases in AUSGY.

Whereas the 16S rRNAs of subclade i phytoplasmas have the unique sequence GUUGC at positions 1025 to 1029 (18), the Australian grapevine yellows, STOL, and VK phytoplasmas have GAAGC at these positions, underscoring the hypothesis that these phytoplasmas differ significantly from subclade i organisms. Although the sequence UUGG at positions 653 to 656 was previously found to be unique to subclade ii (apple proliferation and related phytoplasmas) (18), we found that this sequence also occurs at the corresponding positions in the 16S rRNAs of the STOL, VK, and Australian grapevine yellows phytoplasmas.

Several other unique sequences were found in the AUSGY, STOL, and VK phytoplasmas that distinguish these phytoplas-

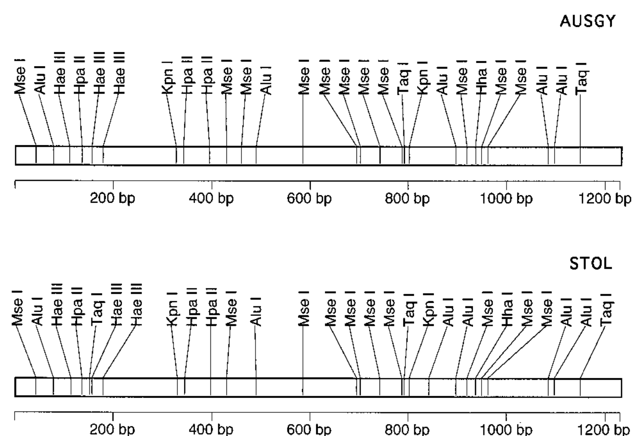

FIG. 2. Analyses of putative restriction sites of phytoplasma 16S rRNA gene sequences. Maps were generated by using the MapDraw option of the DNASTAR program (DNASTAR, Inc., Madison, Wis.) and were manually aligned for comparison of recognition sites for restriction endonucleases *Mse*I, *Alu*I, *Hpa*II, *Kpn*I, *Taq*I, *Hae*III, and *Hha*I.

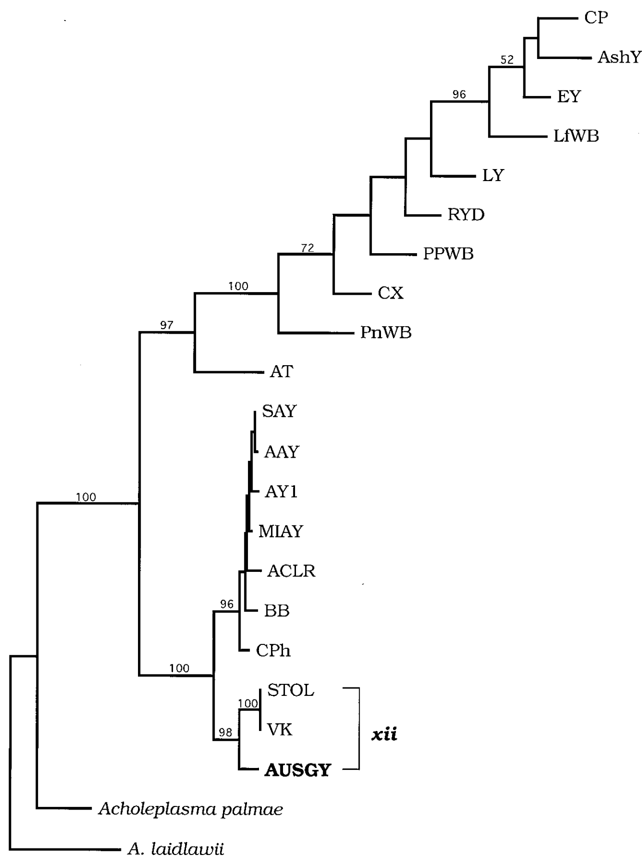

FIG. 3. Phylogenetic tree constructed by parsimony analysis of 16S rRNA gene sequences from 20 phytoplasmas, *A. palmae*, and *A. laidlawii* (used as the outgroup). The phylogenetic subclade identified in this study is indicated. The branch lengths are proportional to the numbers of inferred character state transformations. The values on the branches are bootstrap (confidence) values.

mas from other phytoplasmas (Table 4). For example, the sequence 5′-GAAGC-3′ at positions 1025 to 1029 differed at one to four positions from corresponding sequences of phytoplasmas in other subclades. Two sequences (at positions 191 to 215 and 999 to 1013) were present only in the 16S rDNA of the AUSGY phytoplasma and distinguished this organism from STOL and VK, as well as other phytoplasmas. For example, the sequence at positions 999 to 1013 of the 16S rDNA of AUSGY differed from the corresponding sequences in the STOL and CP phytoplasmas at three base positions, and the sequence at positions 191 to 215 in AUSGY differed from the sequences in STOL and CP at three and eight positions, respectively.

DISCUSSION

Data in the present work strengthen the concept that the etiology of Australian grapevine yellows disease is phytoplasmal. For example, amplification of DNA fragments characteristic of phytoplasmas in PCRs pointed to a constant association of a phytoplasma with the disease. The presence in the 16S rDNA of sequences unique to phytoplasmas established that the agent was a phytoplasma. Our data also indicate that the Australian grapevine yellows phytoplasma is unique. The results of the RFLP analyses of PCR-amplified 16S rDNA, the results of the analysis of sequence data for putative restriction sites in the 16S rDNA, and the results of the parsimony analyses of 16S rRNA gene sequences clearly indicated that the Australian grapevine yellows phytoplasma was distinct from previously described phytoplasmas. On the basis of comparisons of the RFLP patterns of the 16S rDNA from the Australian grapevine yellows phytoplasma with the patterns of reference strains used in this study and with results reported elsewhere (19, 23), Australian grapevine yellows phytoplasma was classified in our study as a member of 16S rRNA RFLP group 16SrI (aster yellows and related phytoplasmas) (19). However, since *Mse*I and *Alu*I RFLP patterns of amplified 16S rDNA clearly distinguished this phytoplasma from all other members of group 16SrI, we propose that the Australian grapevine yellows phytoplasma represents a distinct new subgroup, which we designate 16SrI-J. Our putative restriction site analysis of sequenced 16S rDNAs confirmed the distinctness of the Australian grapevine yellows phytoplasma from other phytoplasmas, including its closest known relative, the subgroup 16SrI-G stolbur phytoplasma (10a). The phylogenetic analysis of 16S rRNA gene sequences indicated that the Australian grapevine yellows phytoplasma, along with the stolbur and Vergilbungskrankheit phytoplasmas, formed a distinct subclade, which we designate subclade xii and which differs from the members of subclade i (aster yellows and related phytoplasmas) identified in a previous study (18). Our results are in agreement with the previous finding of Seemüller et al. (40) that the stolbur phytoplasma is distinct from the aster yellows and related phytoplasmas.

Grapevine yellows diseases attributed to phytoplasmas may now be divided into the following three distinct types on the basis of the presumed causal agents: flavescence dorée, caused by a phytoplasma classified in 16S rRNA RFLP group 16SrV (elm yellows and related phytoplasmas) (8, 38) and subclade x (18); bois noir (Vergilbungskrankheit or southern European grapevine yellows), caused by a phytoplasma classified in group 16SrI subgroup G (1, 10d, 30, 31); and Australian grapevine yellows, caused by a phytoplasma classified in group 16SrI subgroup J (this paper). Our findings confirm and extend recent results (35) which indicated that the Australian grapevine yellows phytoplasma is not identical to several other phytoplasmas on the basis of the results of RFLP analyses of PCR-amplified DNA. Our use of primers R16F2n and R16R2 in PCRs allowed us to perform comparisons of RFLP patterns with the RFLP patterns of DNAs amplified similarly from numerous other phytoplasmas, as described previously (1, 4, 23, 28). The finding that there is yet another distinct grapevine yellows disease will undoubtedly have significant implications for understanding this malady and controlling its spread. A means to refer specifically to Australian grapevine yellows phytoplasma based on its molecular properties would facilitate

TABLE 4. Presence of sequences unique to phytoplasmas in the 16S rRNA gene of Australian grapevine yellows phytoplasma strain AUSGY

Sequence	Position[a]
5′-GAAAAGATGGTGGAAAAACCATTAT-3′[b]	451–477
5′-GAAGC-3′[b]	1025–1029
5′-GTTGTTAATTGCCATCATTAAGTT-3′[b]	1103–1128
5′-CGGTAGAAATATCGT-3′[c]	999–1013
5′-TTTATCTTTAAAAGACCTCGCAAGA-3′[c]	191–215

[a] Position numbers correspond to the position numbers in the 16S rRNA gene sequence of Michigan aster yellows phytoplasma strain MIAY (24).
[b] Unique to strain AUSGY, stolbur strain STOL, and Vergilbungskrankheit strain VK among the phytoplasmas.
[c] Sequence which distinguishes AUSGY from STOL and VK.

efforts to regulate national and international movement of the pathogen.

Recently, "Phytoplasma" was suggested as the name for a new genus level taxon to represent a monophyletic clade embracing all mycoplasmalike organisms which descended from an *Acholeplasma*-like ancestor within the class *Mollicutes* (18, 45). Within this clade, the taxonomic rank of species has been proposed for each of the several distinct subclades, each of which corresponds to a separate 16S rRNA gene RFLP group or subgroup (18, 45). Our data clearly establish the phylogenetic placement of the Australian grapevine yellows phytoplasma, along with the stolbur and Vergilbungskrankheit phytoplasmas, in a distinct subclade, subclade xii. In addition, our phylogenetic analysis confirmed the conclusion, based on an RFLP analysis of amplified 16S rDNA, that the stolbur strain STOL and Vergilbungskrankheit strain VK phytoplasmas are similar or identical to one another and that the Australian grapevine yellows strain AUSGY phytoplasma represents a 16S rRNA subgroup that is distinct from the subgroup containing the STOL and VK phytoplasmas. The phylogeny inferred from the parsimonious tree (Fig. 3) indicates that whereas STOL, VK, and AUSGY have a common ancestor, AUSGY represents a lineage distinct from that to which STOL and VK belong.

Since AUSGY, STOL, and VK together represent a unique phylogenetic subclade (subclade xii), they probably represent at least a single distinct species, in accordance with a previous interpretation (18). Polymorphisms observed in 16S rDNA and chromosomal sequences other than the 16S rRNA gene have underscored the genetic diversity exhibited by subgroups within group 16SrI (and subclade i) (19). In subclade xii, analyses of 16S rRNA gene sequences, as well as analyses of another chromosomal gene sequence in which PCR primed with oligonucleotides and G35p and G35m was used, provided evidence of the genetic divergence of the Australian grapevine yellows phytoplasma from the European stolbur and grapevine Vergilbungskrankheit phytoplasmas. This divergence appears to be correlated with the geographic separation of the two phytoplasma populations.

The present findings are consistent with other data (35) and support recognition of the Australian grapevine yellows phytoplasma as a unique organism and recognition of AUSGY as a representative of a new taxon. The degree of divergence of AUSGY from the stolbur and Vergilbungskrankheit phytoplasmas and other phytoplasmas warrants its delineation as a new lineage. The phylogenetic analysis data, the results of comparisons of the Australian grapevine yellows, STOL, and Vergilbungskrankheit phytoplasmas by positional inspection of base identities and the results of an analysis of putative restriction sites in the 16S rDNAs at numerous sites are consistent with the hypothesis that two distinct gene pools evolved, one represented by the STOL and VK phytoplasmas and the other represented by the AUSGY phytoplasma. The geographical location of Australia may have provided the ecological isolation which favored evolution of the distinct AUSGY phytoplasma.

To facilitate reference to a unique phytoplasma lineage such as that of Australian grapevine yellows, it is desirable to have a name by which the phytoplasma can be known. Although it has not been possible thus far to obtain any phytoplasma in culture in cell-free medium, a means to describe and name putative taxa of prokaryotes such as phytoplasmas has recently been described (32). Already, the name "*Candidatus* Phytoplasma aurantifolia" has been proposed for the phytoplasma associated with the witches'-broom disease of lime (45). Thus, we propose that the Australian grapevine yellows phytoplasma be designated a new, distinct "Candidatus" species, "*Candidatus* Phytoplasma australiense," with the following description: "*Candidatus* Phytoplasma australiense" [(*Mollicutes*) NC; NA; O; NAS (GenBank number L76865), oligonucleotide sequences of unique regions of the 16S rRNA gene 5′-CGGTAGAAATATCGT-3′ and 5′-TTTATCTTTAAAAGACCTCGCAAGA-3′, P (*Vitis*, phloem); M].

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