"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 phytoplasm clad 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 closest 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."
Additional samples were taken from healthy, greenhouse-grown grapevine or grapevine yellows were collected in the field in South Australia during 1993. Australian grapevine yellows phytoplasma is taxonomically periwinkle yellows strain EY1 (=EY); ash yellows strain Ashy (=Ashy); tomato big bud previously in a naturally diseased periwinkle plant growing in a field in Beltsville, strain CYE, in ladino clover unique among the known phytoplasmas.

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 (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 rRNAs 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 RNA. 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 CPF; clover yellow strain CYE; in ladino clover (*Trifolium repens* L.); Canada peach X-disease strain CX; Italian periwinkle virescence strain IPV (11, 12); stoolbur strain StOL; elm yellows strain EY1 (=EY); ash yellow strain AshY (=AshY); tomato big bud strain BB; potato witches'-broom strain PWB; beet leafhopper-transmitted vi-

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

<table>
<thead>
<tr>
<th>Primer pair or primer</th>
<th>Nucleotide sequence</th>
<th>Size of amplified product (kb)</th>
<th>Specificity of PCRs primed</th>
<th>Reference</th>
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<tbody>
<tr>
<td>16R758F-16R1232R</td>
<td>5'-GCTTCT TACTG AGCCT GAGGC-3'</td>
<td>0.5</td>
<td>Phytoplasmas (universal)</td>
<td>15</td>
</tr>
<tr>
<td>16R758F</td>
<td>5'-CTTCA GCTAC CCTTT GTAAC-3'</td>
<td>1.4</td>
<td>Phytoplasmas (universal)</td>
<td>14</td>
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<tr>
<td>R16F1-R16R0</td>
<td>5'-AAGAC GAGGA TAACA GTTTG-3'</td>
<td>1.2</td>
<td>Phytoplasms (universal)</td>
<td>17</td>
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<tr>
<td>R16F1</td>
<td>5'-GGATA CCTTG TTACG ACTTA ACCCC-3'</td>
<td>1.11</td>
<td>Group 16SrI (aster yellows and related strains)</td>
<td>22</td>
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<tr>
<td>R16R0</td>
<td>5'-GCAA GACTG CTAAAG ACTTG-3'</td>
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<td></td>
<td>23</td>
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<tr>
<td>R16F2n-R16R2</td>
<td>5'-TGACG GCGCG TGTGT ACAA CCCCG-3'</td>
<td>0.5</td>
<td>Stolbur (subgroup 16SrI-G)</td>
<td>31</td>
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<td>R16F1</td>
<td>5'-TTAAA GACCT AGCAG TAAA-3'</td>
<td>5'-CAATC CGAAC TGAGA CTGT-3'</td>
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<tr>
<td>R16F1</td>
<td>5'-CGCAT CATTG AGTGT GGAG-3'</td>
<td>5'-AGATG TGACC TATTT TGTTG G-3'</td>
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<td>R16F1-R16F1</td>
<td>5'-TAAAAG CACCC AATCG TTTGC-3'</td>
<td>5'-GAGGA ATACA GTTGG-3'</td>
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<tr>
<td>R16F1-R16F1</td>
<td>5'-GAATC CATTG AGTGT GGAG-3'</td>
<td>5'-AGATG TGACC TATTT TGTTG G-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sStol-rStol</td>
<td>5'-GCGAT CATTG AGTGT GGAG-3'</td>
<td>5'-AGATG TGACC TATTT TGTTG G-3'</td>
<td></td>
<td></td>
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<tr>
<td>sStol</td>
<td>5'-GCGAT CATTG AGTGT GGAG-3'</td>
<td>5'-AGATG TGACC TATTT TGTTG G-3'</td>
<td></td>
<td></td>
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<tr>
<td>G35p-G35m</td>
<td>5'-TAACAT CTGGT GAAGC TCA-3'</td>
<td>5'-CGTCA ATGCG TAATC GAT-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In nested PCRs, the product from a direct PCR primed with primers R16F1 and R16RO or primers R16F2n and R16R2 was diluted 1:10^6 (for grapevine samples) or 1:10^5 (for C. roseus samples) 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 R16F1-R16F1 and R16F1-R16R1 (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

<table>
<thead>
<tr>
<th>Phytoplasma</th>
<th>Associated plant disease (source)</th>
<th>Accession no.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AY1</td>
<td>Maryland aster yellows (Maryland)</td>
<td>L33767</td>
<td>18</td>
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<tr>
<td>SAY</td>
<td>Western severe aster yellows (California)</td>
<td>M86340</td>
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<td>AAY</td>
<td>American aster yellows (Florida)</td>
<td>X68373</td>
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<td>MIAY</td>
<td>Oenothera hookeri virescence (Michigan)</td>
<td>M30970</td>
<td>24</td>
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<td>ACLR</td>
<td>Apricot chlorotic leafroll</td>
<td>X68383</td>
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<td>BB</td>
<td>Tomato big bud (Arkansas)</td>
<td>L33760</td>
<td>18</td>
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<tr>
<td>CP</td>
<td>Clover phylloyd (Canada)</td>
<td>L33762</td>
<td>18</td>
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<tr>
<td>STOL</td>
<td>Stolbur of Cupricum annuum (Serbia)</td>
<td>X76427</td>
<td>32</td>
</tr>
<tr>
<td>VK</td>
<td>Vergilbungkrankheit of grapevine (Germany)</td>
<td>X76428</td>
<td>40</td>
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<td>EY1</td>
<td>Elm yellows (New York)</td>
<td>L33763</td>
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<td>AshY1</td>
<td>Ash yellows (New York)</td>
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<td>PWPB</td>
<td>Pigeon pea witches'-broom (Florida)</td>
<td>L33735</td>
<td>18</td>
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<td>CX</td>
<td>Canada peach X-disease</td>
<td>L33733</td>
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<tr>
<td>AT</td>
<td>Apple proliferation (Germany)</td>
<td>X68375</td>
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<tr>
<td>WBDL</td>
<td>Witches'-broom of lime</td>
<td>U15442</td>
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<tr>
<td>CP</td>
<td>Clover proliferation (Canada)</td>
<td>L33761</td>
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<tr>
<td>RYD</td>
<td>Rice yellow dwarf (Japan)</td>
<td>D12581</td>
<td>32a</td>
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<td>LY</td>
<td>Loofah witches'-broom (Taiwan)</td>
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<td>18</td>
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<tr>
<td>AUSGY</td>
<td>Australian grapevine yellows</td>
<td>L76865</td>
<td>18</td>
</tr>
<tr>
<td>A. palmae (formerly Acholeplasma sp. strain 233)</td>
<td></td>
<td>L33734</td>
<td>18</td>
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</table>

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 16Sr1, 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 16Sr1 (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). 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 16Sr1, it is distinct from other group 16Sr1 phytoplasmas, including STOL, since primers G35p and G35m primed amplification of DNAs from STOL and the related Italian periwinkle virescence phytoplasma (Table 3).

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 polygenetic 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 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 polygenetic 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.).

RFLP analyses of PCR-amplified DNA. Products from PCRs were singly digested with restriction endonucleases Alu1 (GIBCO-BRL, Gaithersburg, Md.), MerI, KpnI, RsrI, HhaI, TaqI, HaellIII, HpaII, Sau3A1, Hinfl, Thal, and HpaI (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 transillumination. 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).

Phytoplasma 16S rDNA was amplified in PCRs primed with primers R16F2n and R16R2, and in PCRs primed with oligonucleotides R16(I)F1 and R16(I)R1, which indicated that all yellows-diseased phytoplasma DNAs amplified in PCRs primed by oligonucleotides R16F2n and R16R2 because priming of DNA 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 16Sr1.

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 KpnI 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 16Sr1.
since KpnI 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 AciI and MseI clearly 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'-ACCTTTAAAGACCTCGCAAG-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 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).

<table>
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<th>Source of DNA</th>
<th>PCR with primers R16F2n and R16R2</th>
<th>PCR with primers R16F1 and R16R0</th>
<th>PCR with primers R16F2n and R16R0</th>
<th>PCR with primers R16F1 and R16R0</th>
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<tbody>
<tr>
<td>Australian grapevines</td>
<td>+</td>
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<td>+</td>
<td></td>
</tr>
<tr>
<td>AUSGY1</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>AUSGY2</td>
<td>+</td>
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<td>Healthy periwinkle</td>
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<td></td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

*DNA amplification in * indicates the presence of amplified DNA; †, no amplified DNA was observed.
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 AluI, MseI, and E;pnI. (A) Products digested with AluI and MseI. (B) Products digested with MseI and KpnI. Lane S1 contained a 6X74 FFI DNA HueIII 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 MseI, AluI, Hpyll, KpnI, TaqI, HaeIII, and HhaI.
AUSGY phytoplasma and distinguished this organism from mal. For example, amplification of DNA fragments characteristic of phytoplasmas in other subclades. Two sequences (at positions 191 to 215 in AUSGY differed from the corresponding sequences in the STOL and CP phytoplasmas at three base positions, and the sequence at positions 999 to 1013 of the 16s rDNA of sequences unique to phytoplasmas established that the sequence at positions 1025 to 1029 differed at five positions from corresponding sequences of phytoplasmas and other phytoplasmas. For example, stolbur strain STOL, and Vergilbungskrankheit phytoplasmas, formed a distinct subclade, which we designate subclade xi and which differs from the members of subclade i (aster yellows and related phytoplasmas) identified in previous studies (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 doree, caused by a phytoplasma classified in 16s rRNA RFLP group 16SrV (elm yellows and related phytoplasmas) (8, 38); and subclade x (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 phytoplasma, along with the stolbur and Vergilbungskrankheit phytoplasmas, a distinct subclade identified in this study. The branch lengths are proportional to the numbers of inferred character state transformations. The values on the branches are bootstrap (confidence) values.

**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 MseI and AluI 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 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.

<table>
<thead>
<tr>
<th>Sequence Position</th>
<th>Positiona</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-GAAGC-3'</td>
<td>1025–1029</td>
</tr>
<tr>
<td>5'-GAAAAGATGGTGGAAAAACCATTAT-3'</td>
<td>451–477</td>
</tr>
<tr>
<td>5'-GGTGTTAATGCCATTAAGT-3'</td>
<td>1103–1128</td>
</tr>
<tr>
<td>5'-CCTTATCCCTTAAAGACCTGGCAAGA-3'</td>
<td>999–1013</td>
</tr>
<tr>
<td>5'-TTATCATGGGAAACCCATTTAT-3'</td>
<td>191–215</td>
</tr>
</tbody>
</table>

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 em-
battling all phytoplasma-like organisms 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 phyl-
ogenetic placement of the Australian grapevine yellows phyto-
plasmas, along with the stolbur and Vergilbungskrankheit phy-
toplasmata, 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 con-
taining 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 an-
other chromosomal gene sequence in which PCR primed with
oligonucleotides 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 phyto-
plasmas 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 phyto-
plasmas and other phytoplasmas warrants its delineation as a
new lineage. The phylogenetic analysis data, the results of com-
parisons of the Australian grapevine yellows, STOL, and
Vergilbungskrankheit phytoplasmas by positional inspection of
base identities and the results of an analysis of putative restric-
tion 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 iso-
lation which favored evolution of the distinct AUSGY phyto-
plasma.

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 Phyto-
plasma 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, "Candida-
tus 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'-CGGT
AGAAATATCGT-3' and 5'-TTTATCTTTAAAAGACCTC
GCAAGA-3', P (Vitis, phloem); M].

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REFERENCES

Tessitori. 1996. DNA-based analyses to detect and identify phytoplasma
yellows-diseased grapevines in Sicily. Petria 665-76.
Bertaccini. 1996. Mixed infection of grapevines in northern Italy by phyto-
plasmas including 16S rRNA RFLP subgroup 16SrI-B strains previously
unreptected in this host. Dis. Plant. 86:418-421.
distribution and symptom fluctuation of flavescence doree in 'Chardonnay'
caracterisation of phytoplasmas infecting grapevine in Liguria (Italy). Phyto-
pathol. Mediterr. 34:137-141.
de particules de type "mycoplasme" dans l'etiologie de la flavescence doree
de la vigne. Examen cytologique des plantes malades et des cicadelles infec-
1992. Cloned DNA probes for detection of grapevine flavescence doree
1993. Diversity among mycoplasma-like organisms inducing grapevine yellows
in France. Vitis 32:159-163.
10. Daire, X., D. Clair, J. Larreue, E. Boudon-Padieu, A. Alma, A. Arzone, L.
Carraro, R. Osler, E. Refatti, G. Granata, R. Credi, E. Tanne, R. Pearson,
and A. Caudwell. 1993. Occurrence of diverse MLOs in tissues of grapevine
affected by grapevine yellows in different countries. Vitis 32:247-248.
11. Davis, R. E. Unpublished data.
1992. Polymerase chain reaction detection of Italian periwinkle virescence
mycoplasmalike organism (MLO) and evidence for relatedness with aster yel-
low MLOs. Petria 2183-192.
Carraro, and M. Barba. 1992. Cloned DNA probes for specific detection of
Italian periwinkle virescence mycoplasmalike organism (MLO) and investi-
gation of genetic relatedness with other MLOs. Phytopathol. Medit. 31:
11-12.
Savio, L. Carraro, D. Di Terlizzi, and M. Barba. 1993. Restriction fragment
length polymorphism analyses and dot hybridisations distinguish mycoplas-
ma-like organisms associated with flavescence doree and southern European
grapevine yellows disease in Italy. Phytopathology 83:772-776.
amplification of 16S rDNA sequences for detection and identification of
mycoplasmalike organisms. Phytopathology 83:1008-1011.
little-leaf phytoplasma detected in sweet potato and other plant species
plasma-like organisms in epidemic yellow infected grapevines. Petria 1:171-
175.


