'Candidatus Phytoplasma japonicum', a new phytoplasma taxon associated with Japanese Hydrangea phyllody

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A phytoplasma was discovered in diseased specimens of field-grown hortensia (Hydrangea spp.) exhibiting typical phyllody symptoms. PCR amplification of DNA using phytoplasma specific primers detected phytoplasma DNA in all of the diseased plants examined. No phytoplasma DNA was found in healthy hortensia seedlings. RFLP patterns of amplified 16S rDNA differed from the patterns previously described for other phytoplasmas including six isolates of foreign hortensia phytoplasmas. Based on the RFLP, the Japanese Hydrangea phyllody (JHP) phytoplasma was classified as a representative of a new sub-group in the phytoplasma 16S rRNA group I (aster yellows, onion yellows, all of the previously reported hortensia phytoplasmas, and related phytoplasmas). A phylogenetic analysis of 16S rRNA gene sequences from this and other group I phytoplasmas identified the JHP phytoplasma as a member of a distinct sub-group (sub-group Id) in the phytoplasma clade of the class Mollicutes. The phylogenetic tree constructed from 16S rRNA gene sequences was consistent with the hypothesis that the JHP phytoplasma and its closest known relatives, the Australian grapevine yellows (AUSGY), Phormium yellow leaf (PYL), Stolbur of Capsicum annuum (STOL) and Vergilbungskrankheit of grapevine (VK) share a common ancestor. The unique properties of the DNA from the JHP phytoplasma clearly establish that it represents a new taxon, 'Candidatus Phytoplasma japonicum'.

Keywords: Japanese Hydrangea phyllody, 'Candidatus Phytoplasma japonicum'

INTRODUCTION

The hortensia plant (Hydrangea spp.) is native to Japan. It was exported to Europe more than 200 years ago and now is distributed all over the world. Recently, Kanehira et al. (1996) reported a Japanese Hydrangea phyllody (JHP) disease, documented that it is a serious disease and spread wherever hortensia is grown in Japan, and determined that the causal agent was likely a phytoplasma. Although rigorous proof of the pathogenicity of phytoplasmas has been elusive because of the inability to culture these cell-wall-less prokaryotes in a cell-free medium, indirect evidence from electron microscopy, and use of molecular probes support the hypothesis of a phytoplasmal aetiology for these diseases (Kanehira et al., 1996). JHP was such a case as described above (Kanehira et al., 1996).

We used molecular biological methods to classify the phytoplasma associated with JHP disease in Japan. This paper reports the results of an extended study involving the PCR amplification of phytoplasma-specific DNA from JHP phytoplasma templates and the analysis of the amplified DNA. We report the nucleotide sequence of a segment of the JHP phytoplasma 16S rDNA for the first time, present the results of a phylogenetic analysis of the sequences, and
describe sequences that are unique to JHP phytoplasma 16S rDNA. Our data led us to propose that the JHP phytoplasma is a taxonomically distinct phytoplasma.

**METHODS**

**Plant samples and reference phytoplasma strains.** Samples from naturally occurring diseased *Hydrangea macrophylla* (Thunb. ex J. Murr.) Ser. f. macrophylla (Thunb. ex J. Murr.) Ser. f. normalis (E. H. Wils.) Hara, and *Hydrangea serrata* (Thunb.) Ser. f. macrophylla, *H. macrophylla* field from Tochigi [isolate number (No.) 95095], Shizuoka (No. 95224) and Oita (No. 95094) Prefectures in Japan, during 1994 and 1995 (Kanehira et al., 1996). The causal agent was transmitted to periwinkle (*Catharanthus roseus* (L.) G. Don) by graft inoculation and the infected periwinkles were used as samples. *Hydrangea* phyllody phytoplasma isolates other than JHP phytoplasma used for RFLP analyses were an Italian isolate (HyPhl) (Lee et al., 1993), Belgium isolate (HYDP) (Schneider et al., 1993), French isolate (HF) (Cousin & Sharma, 1986), and three Canadian isolates (HC) (Hiruki et al., 1994). Additional samples were taken from healthy, greenhouse-grown hortensia and periwinkle seedlings. The reference phytoplasmas used included onion yellows (OY) in periwinkle or garland chrysanthemum (*Chrysanthemum coronarium* L.) tissues, *tsuwabuki* witches' broom (TWB) in periwinkle or garland chrysanthemum tissues, and rice yellow dwarf (RYD) in rice (*Oryza sativa* L.) tissues.

**Primer pairs and conditions for PCR.** A pair of previously designed oligonucleotide primers (SN910601 and SN910502) (Namba et al., 1993a) was used in the PCR to amplify the 16S rDNA in each sample tested. Three DNA samples isolated from three different places described above were used to amplify the 16S rDNA.

For the direct PCR, the template consisted of the total nucleic acids extracted from tissues of hortensia, periwinkle, garland chrysanthemum and rice plants using previously described methods (Namba et al., 1993a). Each PCR mixture (total volume, 50 µl) contained 20 ng (for hortensia) or 50–100 ng (for periwinkle, garland chrysanthemum or rice plants) of total nucleic acids extracted from healthy or diseased plant tissue. The PCR conditions were: each primer 0.5 µM, 1.5 mM MgCl₂, 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 0.001 % gelatin, 1.25 U *Taq* DNA polymerase (Takara *Taq*; Takara Shuzo), and 200 µM each dNTP. The reaction mixtures were overlaid with mineral oil, and the PCR was performed in a thermal cycler (Perkin-Elmer Cetus). An initial 2 min denaturation at 94 °C was followed by 40 cycles, each consisting of 2 min denaturation at 94 °C, 2 min annealing at 60 °C, and 3 min extension at 72 °C. In the last cycle, the extension step at 72 °C was 7 min. A 2 µl aliquot of each PCR product was analysed by electrophoresis in a 1.0 % agarose gel, which was stained with ethidium bromide (0.5 µg ml⁻¹) and visualized with a UV transilluminator.

**RFLP analysis of PCR-amplified DNA.** The PCR products were digested individually with each of the restriction enzymes Dral, EcoRI, EcoRII, HaeIII, HindIII, Hinfl, KpnI, Sau3AI (Nippon Gene), AflII, HhaI, HpaII, RsaI, TaqI (Toyobo), AccII, HpaI (Takara Shuzo) and Tnu91 (Boehringer Mannheim) according to the manufacturers’ instructions.

### Table 1. Strains of the phytoplasmas and acholeplasma used in this study, associated diseases, and accession numbers of their 16S rDNA sequences

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<th>Phytoplasma</th>
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<tr>
<td>ACLR</td>
<td>Apricot chlorotic leafroll</td>
<td>X68338</td>
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<td>AT</td>
<td>Apple proliferation</td>
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<td>BB</td>
<td>Tomato big bud</td>
<td>L33760</td>
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<td>BVK</td>
<td>Leaffopper <em>Psamnotettix cephalotes</em></td>
<td>X76429</td>
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<td>CP</td>
<td>Clover proliferation</td>
<td>L33761</td>
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<tr>
<td>Cph</td>
<td>Clover phyllody</td>
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<td>FD</td>
<td>Flavescence dorée</td>
<td>X76560</td>
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<td>Japanese <em>Hydrangea</em> phyllody</td>
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<td>L33764</td>
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<td>U18747</td>
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<td>Vergilbungskrankheit of grapevine</td>
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<tr>
<td>WBDL</td>
<td>Witches' broom disease of lime</td>
<td>U15442</td>
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Acholeplasma laidlawii | M23932
The digested DNA was analysed by electrophoresis in a 2.5% agarose gel, followed by staining with ethidium bromide and visualization of the DNA bands with a UV transilluminator. The RFLP patterns of JHP phytoplasma DNA were compared with those of amplified DNA from the reference strains and previously published data (Ahrens & Seemüller, 1992; Lee et al., 1993; Prince et al., 1993; Schneider et al., 1993). The group names 'I'-'V' were followed from the previous report (Seemüller et al., 1994). However, the classification of sub-groups was basically followed by the reports of Davis et al. (1997) and Gundersen et al. (1994). This classification system was conveniently used to analyse the taxonomic position of JHP phytoplasma among the representative phytoplasmas. The relationship between the classification system in this paper and those reported previously are shown in Fig. 3.

**Nucleotide sequencing, sequence analysis, and PCR specific for JHP phytoplasma.** The primers for sequencing the 16S rRNA of OY and other related phytoplasmas reported in a previous paper were also used for sequencing the JHP phytoplasma 16S rDNA (Namba et al., 1993a, b). These primers were 350F (TAC GGG AGG CAG CAG), 350R (CTG CTG CCT CCC GAT G), 520R (ACC GCG GCT GTC GGC), 788F (ATT AGA TAC CCT GGT A), 1099F (GCA ACG AGC GCA ACC C) and 1100R (AGG GTT GCGCTC GTT G). Three PCR-amplified 16S rRNA gene products were sequenced by using Dye Terminator Cycle Sequencing Kits (Perkin-Elmer Applied Biosystems). Using the JHP phytoplasma 16S rDNA sequence, a pair of oligonucleotides was designed to specifically amplify DNA by nested PCR from this phytoplasma. Two sequential reactions (nested PCRs) were used. In the nested PCRs, ca. 1-4 kbp product from a direct PCR using the universal primers SN910601 and SN910502 was diluted 1:100 with sterile deionized distilled water, and 1 μl was used as the template for the second (nested) PCR. An initial denaturation for 2 min at 94 °C was followed by 30 cycles of denaturation for 1.5 min at 94 °C, annealing for 1 min at 60 °C, and extension for 1.5 min at 72 °C. In the last cycle the extension step at 72 °C was 7 min long.

**Sequence alignment and cladogram construction.** Complete or nearly complete 16S rDNA sequences (no. 1–1361 for JHP phytoplasma; 1361 nt) from 20 phytoplasmas and Acholeplasma laidlawii were aligned using the Hitachi Software Engineering DNAsis program (version 7.01) and the base positions were numbered using a previously described system (Namba et al., 1993b). The resulting alignments were visually inspected for logical placement and were manually adjusted, when necessary, to retain patterns of conserved sequences that contribute to secondary structure. Multiple alignments were examined using the software CLUSTAL v (version 5.1) (Higgins & Sharp, 1989). A distance matrix and phylogenetic tree were constructed with PHYLIP version 3.5 by the neighbour-joining method of Saitou & Nei using Acholeplasma laidlawii as a root organism (Saitou & Nei, 1987). The genetic distances of the sequences were estimated by the K_\text{nick} value (Kimura, 1980). Each of the confidence values (%) was estimated by the bootstrap sampling method (100 replications) (Felsenstein, 1985). The sequences of the other organisms used in this study were obtained from DDBJ.

**Putative restriction site analysis of 16S rDNA.** Putative restriction site analysis of 16S rDNA was done using sequence data of eight phytoplasmas. To confirm that putative fragment sizes digested by each restriction enzyme are identical to those from corresponding RFLP profiles and to compare with putative RFLP profiles of the STOL/VK phytoplasmas and AUSGY/PYL phytoplasmas of which DNA samples were not used in this experiments, each putative restriction site was predicted with DNAsis.

**Nucleotide sequence accession numbers.** The GenBank accession numbers of the 16S rDNA sequences of JHP phytoplasma, other phytoplasmas and Acholeplasma laidlawii (Wesberg et al., 1989) used in this study are listed in Table 1.

**RESULTS**

**Detection of phytoplasmas in diseased hortensia and periwinkle plants**

A direct PCR with primers SN910601 and SN910502 detected phytoplasma DNA, an approximately 1400 bp fragment, in all three JHP diseased plants tested and other phytoplasma-infected plant samples including hortensia diseases from other countries (Fig. 1; data not shown for HF and HC). This primer pair universally amplifies DNA from all of the phytoplasmas that occur in Japan and other countries so far reported (Namba et al., 1993a) (Table 1).

**RFLP analyses of amplified 16S rDNA**

In the RFLP analyses of the phytoplasma DNA from diseased hortensia and periwinkle plants, we analysed the products of the direct PCR reactions (Namba et

Fig. 1. PCR amplification of a 16S rDNA sequence from diseased hortensia and a previously described Japanese phytoplasma strain using the primer pair SN910601 and SN910502. PCR products (30 cycles) were separated by electrophoresis through a 1% agarose gel. M, λ DNA digested by HindIII; HyPhi, Italian isolate of hortensia phytoplasma; HYDP, Belgium isolate of hortensia phytoplasma; OY, onion yellows phytoplasma; TWB, tsuabuki witches'-broom phytoplasma; RYD, rice yellow dwarf phytoplasma; the three JHP phytoplasmas were obtained from different locations, 95094 from Oita Prefecture, 95095 from Tochigi Prefecture and 95227 from Shizuoka Prefecture; H-P, healthy periwinkle; H-H, healthy hortensia.
al., 1993b) (Fig. 2). For each restriction enzyme, the RFLP patterns of all three JHP isolates were repeatedly shown to be identical (data not shown). The Shizuoka isolate of JHP phytoplasma was used as a representative isolate for the following analysis. In this experiment, hortensia phytoplasma isolates from several countries other than Japan, were tested. These included an Italian isolate (HyPhI) (Lee et al., 1993), a Belgium isolate (HYDP) (Schneider et al., 1993), a French isolate (HF) (Cousin & Sharma, 1986) and
Canadian isolates (HC) (Hiruki et al., 1994), which are reported to belong to sub-group Ia. Since HF and HC gave the same RFLP pattern as HyPhl and HYDP in all of the RFLP analyses (described below), the band pattern of those are not shown in the Fig. 2.

Although three fragments (474, 470 and 424 bp; 470 and 474 bp bands were not distinguished) were detected in KpnI RFLP analysis from these foreign hortensia phytoplasma isolates and OY phytoplasma (sub-group Ia phytoplasma), only two fragments were observed in JHP phytoplasma (Fig. 2c). These sub-group Ia phytoplasmas showed a same RsaI RFLP pattern (Fig. 2d). However, the JHP RsaI RFLP pattern was different. EcoRI RFLP analyses clearly distinguished the JHP phytoplasma from OY (sub-group Ia) phytoplasma, TWB (sub-group IIIb) phytoplasma and RYD (sub-group IVc) phytoplasma (Lee et al., 1993; Namba et al., 1993a) (Fig. 2a). Two HindIII fragments (1238 and 123 bp) were observed in JHP phytoplasma (Fig. 2b), but only one fragment (1368 bp) was detected in OY and foreign hortensia (sub-group Ia) phytoplasmas.

Nucleotide sequence and putative restriction sites in amplified 16S rDNA from JHP phytoplasma

The sequences of all three JHP phytoplasma 16S rRNA gene products by PCR were identical and have been deposited in the GenBank database (accession no. AB010425). Results of a comparative analysis of putative restriction sites in the sequenced DNA are shown in Table 2. The expected fragment sizes based on the analysis of putative restriction sites agreed very well with the fragment sizes obtained in the RFLP analysis of the amplified 16S rDNA. The JHP phytoplasma could be distinguished from other phytoplasmas by the restriction site analysis data. Although two KpnI sites were detected in sub-groups Ia, Ib and Ic except for ACLR phytoplasma which has one KpnI site, only one KpnI site was found in JHP phytoplasma. All three group I sub-groups have similar RsaI sites. However, the JHP RsaI sites were different. One HindIII site has been detected in sub-groups Ib, Ic and JHP phytoplasma, but was absent in sub-group Ia phytoplasmas. Phytoplasmas in sub-group Ia (SAY, ACLR and OY), Ib (STOL and VK) and Ic (AUSGY and PYL) and all other phytoplasmas (belonging to groups II–V) have a single EcoRI site producing similar 16S rDNA fragments (Lee et al., 1993). However, no EcoRI site was detected in the JHP phytoplasma.

PCR specific for JHP phytoplasma

According to the JHP phytoplasma 16S rDNA sequence, a nested primer set was synthesized. The designation and nucleotide sequence of the nested primers are primer JHPFl, 5'-GTGTTAGCGGCCGTCGAGAGGTCA-3' (corresponding to nucleotides 277–298 in the JHP 16S rDNA sequence); and primer JHPRI, 5'-TCCAACTCTAGCTAACAGTTTC-3' (corresponding to nucleotides 623–647 in the JHP 16S rDNA sequence). This primer pair is nested between the annealing sites for the universal primers of SN910601 and SN910502 on the 16S rDNA (Namba et al., 1993a). In PCR mixtures containing primers JHPFl and JHPRI, a 370 bp DNA fragment was
Table 2. Analysis of putative restriction sites of phytoplasma 16S rDNA sequences

Base positions were numbered using a previously described system (Namba et al., 1993b). The numbers shown in the table are actually the position in the 16S rDNA sequence.

<table>
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<th></th>
<th>Ia (SAY, ACLR, OY)</th>
<th>Ib (STOL, VK)</th>
<th>Ic (AUSGY, PYL)</th>
<th>Id (JHP)</th>
<th>IIIb (TWB)</th>
<th>IVc (RYD)</th>
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* No sites were found.
† HpaII site was not found in STOL phytoplasmal 16S rDNA fragment.
‡ Only one KpnI site was found in ACLR phytoplasmal 16S rDNA fragment.
§ 16S rDNA fragments amplified from corresponding phytoplasmas (Seemuller et al., 1994) were slightly shorter than those from other phytoplasmas. It does not include 5'-end restriction site by Sau3AI corresponds to no. 5 of Ia phytoplasmas.

amplified when the template consisted of DNA derived from any of three hortensia plants naturally affected by JHP disease (data not shown). No DNA amplification was observed when the template DNA was from healthy plants.

Phylogenetic analysis

Phylogenetic analysis of the 16S rDNA sequences from 20 diverse phytoplasmas, including JHP phytoplasma and Acholeplasma laidlawii produced four neighbour-joining trees, one of which was revealed to be the most reliable tree by bootstrap analysis and is shown in Fig. 3. This tree is in good agreement with the trees constructed previously (Gundersen et al., 1994; Seemüller et al., 1994), except that it has a new branch for the JHP phytoplasma (sub-group Id). This branch is in the same cluster containing sub-group Ib (STOL and VK) and sub-group Ic [AUSGY ('Candidatus Phytoplasma australiense') and PYL phytoplasmas]. Sub-group Id is most closely related to sub-groups Ib and Ic. Confidence values (> 80%) for each branch of the group I phytoplasmas suggested that the cluster containing group I phytoplasmas is reliable.

Phytoplasma signature sequence and unique 16S rDNA sequences

The 16S rDNA of the JHP phytoplasma has been shown to contain sequences unique to phytoplasmas described as follows. An intensive comparison of these unique sequences with those of other mollicutes revealed six unique phytoplasma sequences: GCTT at positions 224-227 (numbering follows Namba et al., 1993b), GATGTG at positions 273-278, TGGAGG at positions 367-372, ATG at positions 478-480, CCC at positions 487-489 and AGCT at positions 1233-1236. All these sequences are also found in the JHP phytoplasma 16S rDNA.

There are several conserved phytoplasma sequences that have some variation. The sequence TGAAC at
positions 296–300 has a C at position 296 in the 16S rDNA of JHP, STOL, VK, pigeon pea witches' broom (PPWB), leafhopper (Psammotettix cephalotes)-borne and ash yellows (AshY) phytoplasmas. Furthermore, in the JHP sequence the nucleotide at position 297 is A. The sequence GGCCCT at positions 257–261 has an A at position 257 in the 16S rRNA of JHP, PPWB and clover proliferation (CP) phytoplasmas (Namba et al., 1993b).

JHP has several unique sequences compared to the other phytoplasmas studied. In JHP, the sequence GGAATTCC (nt 658–665) has a T at position 664, GGAGGAACCCAG (nt 695–707) has an A at position 696, and TCTGCAACTCGACTTCATGAAG (nt 129–1311) has a T at position 1305. However, in other phytoplasmas, these sequences are conserved.

The 16S rDNA of sub-group Ia phytoplasmas has three unique sequences, TAAATGATGGAAATCATTC (nt 445–465), GTTGC (nt 1014–1018) and ATTTGTTAG (nt 1092–1099) (Davis et al., 1997); however, the JHP, STOL, VK, ATSGY and PYL phytoplasmas have AAGATGGTGGAAAAACATTGAAG and GGTGTTAA in these respective positions.

Although the sequence TTGG at positions 642–645 was previously thought to be unique to group II (apple proliferation and related phytoplasmas) (Gundersen et al., 1994), this sequence was also reported to exist in the 16S rRNA of sub-groups Ib and Ic phytoplasmas (Davis et al., 1997). We also found the same sequence at positions 642–645 in the 16S rDNA of JHP phytoplasmas. However, this sequence is not found in the sub-group Ia phytoplasmas.

There are several differences between the sequences of JHP phytoplasma and the other phytoplasmas of sub-groups Ib and Ic. For example, sequences at positions 63–76, 596–598, 616–624, 984–1000 and 1106–1116, respectively of the 16S rDNA of JHP differed from the corresponding sequences in STOL, VK, PYL and ATSGY. The sequences unique to ‘STOL and VK’ and ‘PYL and ATSGY’ are highly conserved in their respective sub-groups, compared to JHP phytoplasma.

The sequence GTGGAAAAAC at positions 451–460 in sub-groups Ib, Ic and Id (JHP), is different from that in sub-group Ia at positions 451 (G) and 460 (C), is the same as that found in sub-groups IIB, IVa, IVb, Va, Vb, Vc and Vd.

Specific divergence among JHP phytoplasma and previously reported group I phytoplasmas is also supported by an analysis of 16S rDNA secondary structure. The 16S rDNA sequences at positions ca. 50–90 of sub-groups Ia, Ib, Ic, JHP (Id), IIA, IIB and
IVc phytoplasmas were manually aligned following the predicted secondary structure (Neefs et al., 1990) of OAY-phytoplasma (from *Oenothera hookeri*) (Lim & Sears, 1989) (Fig. 4). This region is believed to be a variable region of the 16S rDNA sequence. The sequence GTC GAA CGG AAG TTT AAG CAT TTA AAC TTT AGT GGC at positions 51–86 was previously thought to be unique to group I (Ia, Ib, Ic) phytoplasmas. However, the JHP phytoplasma has GTC GAA CGG AAG CCT TCG GGC TTT AGT GGC at these positions. A comparison of the corresponding secondary structure clearly shows the striking heterogeneity in the length of the branches leading to JHP and sub-groups Ia, Ib and Ic phytoplasmas. The short branch of JHP is similar to those of sub-groups IIIa, IIIb and IVc phytoplasmas.

**DISCUSSION**

Data presented in this paper strengthens the concept that the aetiology of JHP disease is phytoplastic. For example, phyllody is a typical symptom caused by phytoplasma. Reproduction of the disease by graft transmission from diseased hortensia plants to healthy hortensia and different species including periwinkle suggests a phytoplastic causal agent. PCR amplification of DNA fragments characteristic of phytoplasmas points to the constant association of a phytoplasma with the disease, even when the diseased plants were collected from different locations. Additionally, sequences unique to phytoplasmas were found in the 16S rDNA. This information provides strong evidence that the causal agent is a phytoplasma.

The results of the RFLP analyses of PCR-amplified 16S rDNA, the analyses of sequence data for putative restriction sites in the 16S rDNA, and the phylogenetic analyses of 16S rDNA sequences clearly indicate that the JHP phytoplasma is distinct from previously described phytoplasmas.

Phytoplasmas, causal agents of phyllody and virescence disease of hortensia, have been reported in Italy (hydrangea phyllody, HyPhl) (Bertaccini et al., 1992; Welvaert et al., 1975), Belgium (hydrangea phyllody, HYDP, and hydrangea virescence, Bhy) (Schneider & Seemüller, 1994a, b; Vibio et al., 1996), France (HF) (Cousin & Sharma, 1986) and Canada (HC) (Hiruki et al., 1994). RFLP analysis of 16S rDNA (HyPhl, Lee et al., 1993; HYDP, Schneider et al., 1993; HF, Ceranic-Zagorac & Hiruki, 1996) and uncharacterized chromosomal G35p/m DNA (BHy) (Vibio et al., 1996) led to the conclusion that the phytoplasmas detected in these diseases were identical or quite similar to the aster yellows type (sub-group Ia) phytoplasmas (Lee et al., 1993; Schneider et al., 1993).

JHP is a disease that has been observed wherever and whenever hortensia have been grown in Japan. However, it was only recently shown to be caused by a phytoplasma in Japanese hortensia plants. PCR with primers SN910601 and SN910502 allowed us to compare the RFLP patterns including that of HyPhl, HYDP, HF and HC phytoplasmas, sub-group Ia phytoplasmas of phyllody and virescence disease of *Hydrangea* in Europe and North America, with numerous other published phytoplasma RFLP patterns (Lee et al., 1993; Schneider et al., 1993; Ceranic-Zagorac & Hiruki, 1996). The data represented here strongly suggests that the JHP phytoplasma is clearly different from sub-group Ia phytoplasmas (including phytoplasmas of hortensia in other countries) and all other known phytoplasmas. To date JHP phytoplasma has been found only in Japan. The geographical location of Japan may have provided the ecological isolation that perhaps resulted in divergence of the distinct JHP phytoplasma.

Comparisons of the RFLP patterns of the 16S rDNA from the JHP phytoplasma with the patterns of reference strains used in this study and published results (Ahrens & Seemüller, 1992; Lee et al., 1993; Prince et al., 1993; Schneider et al., 1993), led to our classification of the JHP phytoplasma as a member of the group I phytoplasmas (Seemüller et al., 1994). A recent phylogenetic analysis reported a new cluster, within the group I phytoplasmas, that is composed of sub-groups Ib and Ic (Davis et al., 1997; Liefting et al., 1997). Restriction enzyme digestion profiles of phytoplasmas from both sub-groups predicted from the sequence data in the database are clearly different and
have been reported in all of the group I phytoplasmas from the analysis of sequenced 16s rDNA also support the idea. Restriction site profiles deduced from these data clearly establish the phylogenetic position of the JHP phytoplasma, in a distinct group, sub-group Id, alongside the sub-group Ib phytoplasmas (STOL, VK) and sub-group Ic phytoplasmas (PYL, AUSGY) [‘Candidatus Phytoplasma australiense’]. In addition, our phylogenetic analysis of the group I phytoplasmas and the resulting data on the divergence between the JHP phytoplasma and the AUSGY- and STOL-related phytoplasmas provide new insight into the evolution of these pathogens (Fig. 3). Previously, group I was believed to consist of two lineages, one containing sub-group Ia and the other containing sub-groups Ib and Ic (Davis et al., 1997; Liefting et al., 1996). The phylogeny of amplified 16S rDNA sequences confirmed the conclusion that STOL and VK (sub-group Ib) are similar or identical to each other, that PYL and AUSGY (sub-group Ic) are also similar or identical to each other, and that the JHP phytoplasma belongs to a distinct sub-group. Inclusion of the JHP phytoplasma in the phylogenetic analysis yielded a long branch and a branching order that indicated that the JHP phytoplasma is closely related to the genus Acholeplasma but that it has diverged considerably from their common ancestor.

Since STOL, VK, PYL and AUSGY represent two unique phylogenetic sub-groups (sub-groups Ib and Ic), they probably represent at least one or two distinct species, in agreement with a previous interpretation (Davis et al., 1997; Gundersen et al., 1994; Liefting et al., 1996). Recently, ‘Candidatus Phytoplasma australiense’ was defined for AUSGY (Davis et al., 1997). The phylogenetic diversity of 16S rDNA sequences underscores the genetic diversity exhibited by sub-groups within a group (Gundersen et al., 1994; Namba et al., 1993b; Seemüller et al., 1994). Within this genus, the taxonomic rank of species has been proposed for each of the 16S rDNA sequence sub-groups (Davis et al., 1997; Gundersen et al., 1994; Zreik et al., 1995). Our data clearly establish the phylogenetic position of the JHP phytoplasma, in a distinct group, sub-group Id, and apricot chlorotic leafroll (ACLR). Since the KpnI, EcoRI, HinfI, HpaII and Sau3AI sites are characteristic of the 16s rDNA group I strains, including sub-group Ia [apricot chlorotic leafroll (ACLR), Western aster yellows (SAY) and onion yellows (OY)] (Kuske & Kirkpatrick, 1992; Namba et al., 1993b; Seemüller et al., 1994), sub-group Ib (VK and STOL) (Seemüller et al., 1994) and sub-group Ic (AUSGY and PYL) (Davis et al., 1997; Liefting et al., 1996), indicating that the JHP phytoplasma is affiliated with group I but not with sub-group Ia, Ib or Ic. Sub-group Ia also includes hortensia phytoplasmas that have been reported in Europe and North America (Ceranic-Zagorac & Hiruki, 1996; Cousin & Sharma, 1986; Lee et al., 1993; Schneider et al., 1993). The HindIII RFLP patterns suggest a close relationship between the JHP phytoplasma and sub-groups Ib and Ic. However, the KpnI and EcoRI RFLP patterns indicate that JHP phytoplasma is clearly different from previously reported phytoplasmas. Two KpnI sites have been reported in all of the group I phytoplasmas (Davis et al., 1997; Lee et al., 1993) except for JHP and apricot chlorotic leafroll (ACLR). Since the KpnI, EcoRI, HaeIII and Tru9I (Msel) RFLP patterns of amplified 16S rDNA clearly distinguished the JHP phytoplasma from all other members of group I phytoplasmas, we propose that the JHP phytoplasma represents a distinct new sub-group, which we designate sub-group Id. Restriction site profiles deduced from the analysis of sequenced 16S rDNA also support this idea.

Furthermore, the phylogenetic analysis of 16S rDNA sequences confirmed the composition of sub-groups Id (JHP), Ib (STOL and VK) and Ic (PYL and AUSGY). The discovery of yet another distinct phyllody disease of hortensia, JHP, caused by a new phytoplasma, in addition to HyPhl phytoplasma (and several other identical or similar hortensia phytoplasma isolates), will undoubtedly have significant implications for the understanding of these diseases and the complexity of phytoplasma diseases. The ability to use molecular approaches to specifically identify JHP and HyPhl phytoplasmas will facilitate efforts to understand the national and international movement of these pathogens.

There have been several reports on diseases of hortensia in different parts of the world (Hiruki et al., 1994; Hearon et al., 1976; Lawson & Smith, 1980). It would be very interesting to determine to which subgroups the causal phytoplasmas of these diseases belong and to know if there are any other unidentified phytoplasmas responsible for these diseases.

Recently, ‘Phytoplasma’ was suggested as the name for a new genus-level taxon that would be a monophyletic clade including all the mycoplasma-like organisms that descended from an Acholeplasma-like ancestor, within the class Mollicutes (Gundersen et al., 1994; Zreik et al., 1995). Within this genus, the taxonomic rank of species has been proposed for each of the 16S rDNA sequence sub-groups (Davis et al., 1997; Gundersen et al., 1994; Zreik et al., 1995). Our data clearly establish the phylogenetic position of the JHP phytoplasma, in a distinct group, sub-group Id, and apricot chlorotic leafroll (ACLR). Since the KpnI, EcoRI, HinfI, HpaII and Sau3AI sites are characteristic of the 16s rDNA group I strains, including sub-group Ia [apricot chlorotic leafroll (ACLR), Western aster yellows (SAY) and onion yellows (OY)] (Kuske & Kirkpatrick, 1992; Namba et al., 1993b; Seemüller et al., 1994)
propose that the JHP phytoplasma be designated as a new, distinct \textit{Candidatus} species, \textit{Candidatus Phytoplasma japonicum}, with the following description: \textit{Candidatus Phytoplasma japonicum} [\textit{Mollicutes}]


\textit{Candidatus Phytoplasma japonicum} (NC; NA; O; NAS (GenBank no. AB010425), oligonucleotide sequences of unique regions of the 16S rRNA gene 5′-GTGTAGGCCGACTGAGGCTCA-3′ and 5′-TTCACTCTAGCTAAACAGTTTCG-3′, P (Hydrangea, phloem); M).

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