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

Toshimi Sawayanagi,¹ Norio Horikoshi,² Tsutomu Kanehira,² Masayuki Shinohara,² Assunta Bertaccini,³ M.-T. Cousin,⁴ Chuji Hiruki⁵ and Shigetou Namba¹

Author for correspondence: Shigetou Namba. Tel: +81 424 69 3125. Fax: +81 424 69 8786.
e-mail: snamba@ims.u-tokyo.ac.jp

¹ Laboratory of Bioresource Technology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku 113-8657, Japan

² College of Bioresource Sciences, Nihon University, Fujisawa, Kanagawa 252-0813, Japan

³ Istituto di Patologia vegetale, Università degli Studi, Bologna 40126, Italy

⁴ Unite de Pathologie Vegetale, Centre de Versailles, Institut National de la Recherche Agronomique, F78026 Versailles Cedex, France

⁵ Department of Agricultural, Food, and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada T6G 2P5

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 patho-

genicity 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

Abbreviations: AUSGY, Australian grapevine yellows; JHP, Japanese *Hydrangea* phyllody; OY, onion yellows; PYL, *Phormium* yellow leaf; RYD, rice yellow dwarf; STOL, Stolbur of *Capsicum annuum*; TWB, tsuwabuki witches' broom; VK, Vergilbungskrankheit of grapevine.

The EMBL/DBJ/GenBank accession number for the 16S rDNA sequence of Japanese *Hydrangea* phyllody phytoplasma is AB010425.

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*, *H. macrophylla* (Thunb. ex J. Murr.) Ser. f. *normalis* (E. H. Wils.) Hara, and *Hydrangea serrata* (Thunb.) Ser. f. exhibiting symptoms of JHP, which include phyllody and proliferation of the flower organs as well as stunting and dieback, were collected in the field from Tochigi [isolate number (No.) 95095], Shizuoka (No. 95227) 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 *Dra*I, *Eco*RI, *Eco*RII, *Hae*III, *Hind*III, *Hinf*I, *Kpn*I, *Sau*3AI (Nippon Gene), *Alu*I, *Hha*I, *Hpa*II, *Rsa*I, *Taq*I (Toyobo), *Acc*II, *Hpa*I (Takara Shuzo) and *Tru*9I (Boehringer Mannheim) according to the manufacturers' instructions.

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

Phytoplasma	Associated disease	GenBank no.
AshY	Ash yellows	X68339
ACLR	Apricot chlorotic leafroll	X68338
AT	Apple proliferation	X68375
AUSGY	Australian grapevine yellows	L76865
BB	Tomato big bud	L33760
BVK	Leafhopper <i>Psammotettix cephalotes</i>	X76429
CP	Clover proliferation	L33761
Cph	Clover phyllody	L33762
FD	Flavescence dorée	X76560
JHP	Japanese <i>Hydrangea</i> phyllody	AB010425
LfWB	Loofah witches' broom	L33764
LY	Coconut lethal yellowing	U18747
OY	Onion yellows	D12569
PPWB	Pigeon pea witches' broom	U18763
PYL	<i>Phormium</i> yellow leaf	U43569
RYD	Rice yellow dwarf	D12571
SAY	Western aster yellows	M86340
STOL	Stolbur of <i>Capsicum annuum</i>	X76427
TWB	Tsuwabuki witches' broom	D12570
VK	Vergilbungskrankheit of grapevine	X76428
WBDL	Witches'-broom disease of lime	U15442
<i>Achleoplasma laidlawii</i>		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 rDNA 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 GCT 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_{unc} 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* (Weisberg *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 *Hind*III; HyPhl, Italian isolate of hortensia phytoplasma; HYDP, Belgium isolate of hortensia phytoplasma; OY, onion yellows phytoplasma; TWB, tsuwabuki 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.

Fig. 2. For legend see facing page.

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 (HyPhl) (Lee *et al.*, 1993), a Belgium isolate (HYDP) (Schneider *et al.*, 1993), a French isolate (HF) (Cousin & Sharma, 1986) and

Fig. 2. RFLP analysis of 16S rDNA using the PCR products shown in Fig. 1. (a) DNA fragments digested with the restriction enzymes *AccII* (*Thal*), *AluI*, *DraI* and *EcoRI*. (b) DNA fragments digested with the restriction enzymes *EcoRII*, *HaeIII*, *HhaI* and *HindIII*. (c) DNA fragments digested with the restriction enzymes *HinfI*, *HpaI*, *HpaII* and *KpnI*. (d) DNA fragments digested with the restriction enzymes *RsaI*, *Sau3AI*, *TaqI* and *Tru9I* (*MseI*). M, marker DNA mixture with λ DNA digested by *EcoRI* and *HindIII*, and ϕ X174 DNA digested by *HinfI*. Other abbreviations are the same as described in Fig. 1.

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, foreign hortensia (sub-group Ia) phytoplasma, TWB (sub-group IIb) 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 JHPF1, 5'-GTGTAGCCGGC-TGAGAGGTCA-3' (corresponding to nucleotides 277–298 in the JHP 16S rDNA sequence); and primer JHPR1, 5'-TCCAACTCTAGCTAAACAGTTTC-TG-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 JHPF1 and JHPR1, 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.

	Ia (SAY, ACLR, OY)	Ib (STOL, VK)	Ic (AUSGY, PYL)	Id (JHP)	IIIb (TWB)	IVc (RYD)
<i>AccII</i>	101, 393, 512, 1329, 1345	104, 395, 514, 1328, 1343	103, 395, 514, 1329, 1344	103, 395, 514	388, 507	388, 507
<i>AluI</i>	223, 634, 986, 1042, 1233	223, 635, 987, 1043, 1232, 1244	225, 636, 1044, 1233, 1245	217, 628, 1036, 1227, 1239	275, 1037, 1228	275, 980, 1036, 1227
<i>DraI</i>	188	189	191	182	182	182
<i>EcoRI</i>	659	659	660	*	654	654
<i>EcoRII</i>	8, 781, 962, 1193	782, 963, 1192	10, 783, 964, 1193	8, 783, 964, 1195	8, 776, 959	8, 776, 959
<i>HaeIII</i>	257, 301, 323	257, 301, 323	259, 303, 325	295, 317	252, 318	252, 318
<i>HhaI</i>	1081, 1240	1083	1084	610, 1075, 1234	596, 1076	1075
<i>HindIII</i>	*	1242	1243	1238	*	*
<i>HinfI</i>	905, 1255, 1315, 1332	111, 906, 1254, 1314, 1331	907, 1255, 1316, 1332	899, 1249, 1309, 1326	902, 1312, 1329	902, 1310, 1327
<i>HpaI</i>	839	841	842	833	834	834
<i>HpaII</i>	282, 489, 541	281, 489, 541, 715†	283, 490, 542	276, 483, 535	484, 536, 823	484, 536
<i>KpnI</i>	470, 944‡	474, 948	475, 949	938	*	*
<i>RsaI</i>	418, 473, 810, 854, 870, 874, 947	417, 472, 809, 853, 869, 873, 946	418, 473, 810, 854, 870, 874, 947	412, 804, 848, 864, 868, 941	405, 468, 805, 828, 849, 865, 869	805, 828, 849, 865, 869
<i>Sau3AI</i>	5, 923	922§	5, 923	5, 917	5, 918, 1186	5, 918, 1157, 1185
<i>TaqI</i>	53, 940, 1299	53, 295, 656, 938, 1295	53, 940, 1297	53, 934, 1293	53, 935, 1294	53, 198, 935, 1292

* 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 (Seemüller *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

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, GGAGGAACACCAG (nt 695–707) has an A at position 696, and TCTGCAACTCGACTTCATGAG (nt 1290–1311) has a T at position 1305. However, in other phytoplasmas, these sequences are conserved.

Although the sequence TTGG at positions 642–645 was previously thought to be unique to group II (apple

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 AUSGY. The sequences unique to 'STOL and VK' and 'PYL and AUSGY' are highly conserved in their respective sub-groups, compared to JHP phytoplasma.

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), IIIa, IIIb and

Fig. 4. Presumed secondary structure at position ca. nt 50–90 of the 16S rRNA molecule deduced from the secondary structure model for prokaryotic 16S rRNA (Neefs *et al.*, 1990).

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 phytoplasmal. 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 phytoplasmal 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

easily differentiated by RFLP analysis from those of aster yellows and related phytoplasmas belonging to sub-group Ia (Davis *et al.*, 1997; Schneider *et al.*, 1993). *EcoRII*, *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; Liefing *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 previously reported in Europe and North America (Ceranec-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* (*MseI*) 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 sub-groups 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, 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; Liefing *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; Liefing *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).

In group I, analyses of phytoplasma signature sequence and unique 16S rDNA sequences provided evidence for the genetic divergence of the JHP phytoplasma from the aster yellows (and closely related phytoplasmas), STOL, VK, PYL and AUSGY phytoplasmas. This divergence appears to be correlated with the geographic separation of the four phytoplasma populations.

To facilitate reference to a unique phytoplasma lineage such as that of JHP, it is desirable to have a name for the phytoplasma. Although it has not been possible to culture any phytoplasmas in cell-free medium, a means to describe and name putative taxa of prokaryotes such as phytoplasmas has been proposed (Murray & Schleifer, 1994; Namba *et al.*, 1993b). Already, the names '*Candidatus* Phytoplasma australiense' and '*Candidatus* Phytoplasma aurantifolia' have been proposed for the phytoplasmas associated with the AUSGY and witches'-broom disease of limes, respectively (Davis *et al.*, 1997; Zreik *et al.*, 1995). We

propose that the JHP phytoplasma be designated as a new, distinct '*Candidatus*' species, '*Candidatus* Phytoplasma japonicum', with the following description: '*Candidatus* Phytoplasma japonicum' [(*Mollicutes*) NC; NA; O; NAS (GenBank no. AB010425), oligonucleotide sequences of unique regions of the 16S rRNA gene 5'-GTGTAGCCGGGCTGAGAGGTCA-3' and 5'-TCCAACCTAGCTAAACAGTTTCTG-3', P (*Hydrangea*, phloem); M].

ACKNOWLEDGEMENTS

We gratefully acknowledge Mr Shigeru Hatano for excellent technical assistance and Drs S. Iwanami and S. Kato for kindly providing phytoplasma strains used as references. This research was supported by the grants from Bio-oriented Technology Research Advancement Institution, Japan.

REFERENCES

- Ahrens, U. & Seemüller, E. (1992). Detection of DNA of plant pathogenic mycoplasma-like organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. *Phytopathology* **82**, 828–832.
- Bertaccini, A., Davis, R. E., Hammond, R. W., Vibio, M., Bellardi, M. G. & Lee, I.-M. (1992). Sensitive detection of mycoplasma-like organisms in field collected and in vivo propagated plants of *Brassica*, *Hydrangea* and *Chrysanthemum* by polymerase chain reaction. *Ann Appl Biol* **121**, 593–599.
- Ceranic-Zagorac, P. & Hiruki, C. (1996). Comparative molecular studies on aster yellows phytoplasmas. *Acta Hort* **432**, 266–276.
- Cousin, M.-T. & Sharma, A. K. (1986). Association of mycoplasma-like organisms (MLOs) with mild type of *Hydrangea* virescence: a study with 60–1000 nm thick sections. *J Phytopathol* **115**, 274–282.
- Cousin, M.-T., Roux, J., Boudon-Padieu, E., Berges, R., Seemüller, R. & Hiruki, C. (1998). Use of heteroduplex mobility analysis (HMA) for differentiating phytoplasma isolates causing witches' broom disease on *Populus nigra* cv *italica* and stolbur or big bud symptoms on tomato. *J Phytopathol* **146**, 97–102.
- Davis, R. E., Dally, E. L., Gundersen, D. E., Lee, I.-M. & Habili, N. (1997). '*Candidatus* Phytoplasma australiense,' a new phytoplasma taxon associated with Australian grapevine yellows. *Int J Syst Bacteriol* **47**, 262–269.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Gundersen, D. E., Lee, I.-M., Rehner, S. A., Davis, R. E. & Kingsbury, D. T. (1994). Phylogeny of mycoplasma-like organisms (phytoplasmas): a basis for their classification. *J Bacteriol* **176**, 5244–5254.
- Hearon, S. S., Lawson, R. H., Smith, F. F., McKenzie, J. T. & Rosen, J. (1976). Morphology of filamentous forms of mycoplasma-like organisms associated with *Hydrangea* virescence. *Phytopathology* **66**, 608–616.
- Higgins, D. G. & Sharp, P. M. (1989). Fast and sensitive multiple sequence alignments on a microcomputer. *CABIOS* **5**, 151–153.
- Hiruki, C., Rong, X. D. & Deng, S. J. (1994). *Hydrangea* virescence, a disease associated with mycoplasma-like organisms in Canada. *Acta Hort* **377**, 325–333.
- Kanehira, T., Horikoshi, N., Yamakita, Y. & Shinohara, M. (1996). Occurrence of hydrangea phyllody in Japan and detection of the causal phytoplasma. *Ann Phytopathol Soc Jpn* **62**, 537–540.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Kuske, C. R. & Kirkpatrick, B. C. (1992). Phylogenetic relationships between the western aster yellows mycoplasma-like organism and other prokaryotes established by 16S rRNA gene sequence. *Int J Syst Bacteriol* **42**, 226–233.
- Lawson, R. H. & Smith, F. F. (1980). Three stable types of *Hydrangea* virescence that differ in severity in foliage and flowers. *Plant Dis* **64**, 659–661.
- Lee, I.-M., Hammond, R. W., Davis, R. E. & Gundersen, D. E. (1993). Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasma-like organisms. *Phytopathology* **83**, 834–842.
- Liefting, L. W., Andersen, M. T., Beever, R. E., Gardner, R. C. & Forster, R. L. S. (1996). Sequence heterogeneity in the two 16S rRNA genes of *Phormium* yellow leaf phytoplasma. *Appl Environ Microbiol* **62**, 3133–3139.
- Lim, P. O. & Sears, B. B. (1989). 16S rRNA sequence indicates that plant-pathogenic mycoplasma-like organisms are evolutionarily distinct from animal mycoplasmas. *J Bacteriol* **171**, 5901–5906.
- Murray, R. G. E. & Schleifer, K. H. (1994). Taxonomic note: a proposal for recording the properties of putative taxa of prokaryotes. *Int J Syst Bacteriol* **44**, 174–176.
- Namba, S., Kato, S., Iwanami, S., Oyaizu, H., Shiozawa, H. & Tsuchizaki, T. (1993a). Detection and differentiation of plant-pathogenic mycoplasma-like organisms using polymerase chain reaction. *Phytopathology* **83**, 786–791.
- Namba, S., Oyaizu, H., Kato, S., Iwanami, S. & Tsuchizaki, T. (1993b). Phylogenetic diversity of phytopathogenic mycoplasma-like organisms. *Int J Syst Bacteriol* **43**, 461–467.
- Neefs, J.-M., Van de Peer, Y., Hendriks, L. & Wachter, R. D. (1990). Compilation of small ribosomal subunit RNA sequences. *Nucleic Acids Res* **18**, 2237–2317.
- Prince, J. P., Davis, R. E., Wolf, T. K., Lee, I.-M., Mogen, B. D., Dally, E. L., Bertaccini, A., Credi, R. & Barba, M. (1993). Molecular detection of diverse mycoplasma-like organisms (MLOs) associated with grapevine yellows and their classification with aster yellows, X-disease, and elm yellows MLOs. *Phytopathology* **83**, 1130–1137.
- Saitou, N. & Nei, M. (1987). Neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Schneider, B. & Seemüller, E. (1994a). Presence of two sets of ribosomal genes in phytopathogenic mollicutes. *Appl Environ Microbiol* **60**, 3409–3412.
- Schneider, B. & Seemüller, E. (1994b). Studies on taxonomic relationships of mycoplasma-like organisms by southern blot analysis. *J Phytopathol* **141**, 173–185.
- Schneider, B., Ahrens, U., Kirkpatrick, B. & Seemüller, E. (1993). Classification of plant-pathogenic mycoplasma-like organisms using restriction-site analysis of PCR-amplified 16S rDNA. *J Gen Microbiol* **139**, 519–527.
- Seemüller, E., Schneider, B., Mäurer, R. & 8 other authors (1994). Phylogenetic classification of phytopathogenic mollicutes by sequence analysis of 16S ribosomal DNA. *Int J Syst Bacteriol* **44**, 440–446.
- Vibio, M., Bertaccini, A., Lee, I.-M., Davis, R. E. & Clark, M. F.

(1996). Differentiation and classification of aster yellows and related European phytoplasmas. *Phytopathol Mediterr* **35**, 33–42.

Weisburg, W. G., Tully, J. G., Rose, D. L. & 9 other authors (1989). A phylogenetic analysis of the mycoplasmas: basis for their classification. *J Bacteriol* **171**, 6455–6467.

Welvaert, W., Sakyn, G. & Lagasse, A. (1975). Recherches sur les

symptomes de la virescence chez l'*Hydrangea macrophylla* Thumb. *Phytopathol Z* **83**, 152–158.

Zreik, L., Carl, P., Bové, J. M. & Garnier, M. (1995). Characterization of the mycoplasma-like organism associated with witches'-broom disease of lime and proposition of a *Candidatus* taxon for the organism, "*Candidatus Phytoplasma aurantiifolia*". *Int J Syst Bacteriol* **45**, 449–453.