'*Candidatus* Phytoplasma ziziphi', a novel phytoplasma taxon associated with jujube witches'-broom disease

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Phylogenetic relationships of five jujube witches'-broom (JWB) phytoplasma isolates from four different districts, and other phytoplasmas, were investigated by 16S rDNA PCR amplification and sequence analysis. The 16S rDNA sequences of any pair of the five isolates of JWB phytoplasmas were >99.5% similar. The JWB phytoplasma 16S rDNA sequences were most closely related to that of the elm yellows (EY) phytoplasma in 16S-group VIII. Phylogenetic analysis of the 16S rDNA sequences from the JWB phytoplasma isolates, together with sequences from most of the phytoplasmas archived in GenBank, produced a tree in which the JWB isolates clustered as a discrete subgroup. The uniqueness of the JWB phytoplasma appears to be correlated with a specific insect vector (*Hishimonus sellatus*) and the host plant (*Zizyphus jujuba*), or with a specific geographical distribution. The unique properties of the JWB phytoplasma sequences clearly indicate that it represents a novel taxon, '*Candidatus* Phytoplasma ziziphi'.

The common jujube (*Zizyphus jujuba*) is widely grown in the temperate zone of the Northern Hemisphere as a commercially important vegetable crop. Jujube witches'-broom (JWB) disease is prevalent in China and Korea and causes serious problems for the industry. JWB disease has been reported in all parts of Korea; yield reductions that range from 30 to 80% have been observed (Lee, 1988); it has also been reported in at least three prefectures in Japan (Ohashi *et al.*, 1996). This disease is a common problem, being found in jujube orchards in different locations, which grow different cultivars and have different management practices.

JWB disease was first described, tentatively, as a graft-transmissible viral disease of jujube trees in Korea (Kim, 1965). However, transmission electron microscopy showed that a phytoplasma was associated with the disease (Yi & La, 1973). Sequence analysis of amplified 16S rDNA from the Korean isolate (Namba *et al.*, 1993a) revealed that the pathogen is distinct from mulberry dwarf (MD) and

paulownia witches'-broom (PaWB) phytoplasmas, both of which are classified in the aster yellows subgroup of 16S-group I (Jung *et al.*, 2002). More recently, additional studies (Zhu *et al.*, 1996; Tian *et al.*, 2000) that used 16S rDNA RFLP and sequence analysis have revealed that the JWB phytoplasma from China is related to elm yellows (EY) phytoplasma, which currently belongs to 16S-group VIII (the EY group) (Jung *et al.*, 2002), and that the pathogen may constitute a distinct subgroup as it is divergent from other 16S-group VIII members, such as EY and flavescentia dorée (FD). To determine whether the phytoplasmas associated with JWB form a discrete, coherent taxon, we analysed JWB phytoplasma isolates from four different areas of Korea and Japan and compared them with other JWB isolates and most other phytoplasmas analysed so far.

More than 20 samples from naturally diseased jujube trees that displayed symptoms of witches'-broom were collected from fields in Kyoto (JWB-Ky), Gifu (JWB-G) and Fukui (JWB-F) prefectures in Japan from 1993 to 1995, and from fields in Gyeongbuk Province, Korea (JWB-Kor), in 1993. As these jujube phytoplasma lines produced indistinguishable symptoms, the JWB phytoplasma isolates were named after their places of origin. Non-symptomatic plants were also collected from the same locations. Healthy tissues were collected from greenhouse-grown jujube seedlings. Total

Abbreviations: EY, elm yellows; FD, flavescentia dorée; JWB, jujube witches'-broom.

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of jujube witches'-broom phytoplasma reported in this paper are AB052875–AB052879.

nucleic acids were extracted from tissues as described elsewhere (Namba *et al.*, 1993b), for use as PCR templates. A pair of primers that consisted of a previously designed primer (SN910610; Namba *et al.*, 1993b) plus SN011119 (5'-TCGCCGTTAATTGCGTCCTT-3') was used to amplify 16S rDNA from each sample tested. The following thermal cycling programs were used: 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 1 min 30 s at 72 °C, with a final elongation step of 7 min at 72 °C. Direct PCR with the primer-pair SN910610/SN011119 amplified an approximately 1.8 kbp fragment of the phytoplasma 16S rDNA gene from all diseased jujube trees examined. Under the same conditions, no products were amplified from asymptomatic plants collected from the same areas or from healthy plants grown in a greenhouse (data not shown). The PCR results indicated that there was an association between phytoplasma and JWB disease.

The PCR products of JWB phytoplasmas were sequenced using six primers that have been used previously to sequence phytoplasma 16S rDNA (Namba *et al.*, 1993b, c). Primers 1505F (5'-GGTATCCCTACCGGAAG-3') and 1840R (5'-TCGCCGTTAATTGCGTCCTT-3') were used to sequence the 16S–23S rRNA spacer region. The PCR-amplified products of at least three independent JWB-infected plant samples were sequenced by using Dye Terminator Cycle Sequencing kits (Applied Biosystems); similarity levels were calculated among these JWB phytoplasmas and other phytoplasma sequences available in GenBank (see Table 1 for accession numbers). All 16S rDNA sequences of JWB phytoplasma, isolated from four different regions in Japan and Korea, were virtually identical to each other and to the sequences of two isolates, JWB-Kor2 and JWB-Ch, which were deposited in GenBank. The sequence similarity among these strains exceeded 99.5%. The representative JWB phytoplasma line (JWB-G1^T) with the most conserved sequence was compared with other phytoplasma lines that represented the major phylogenetic groups. Sequence comparisons also revealed that the JWB phytoplasma 16S rDNA sequences were most similar to those of the EY strains, with similarities that ranged from 97.9 (JWB-Kor1 vs FD) to 99.0 (JWB-G1^T vs EY2) %.

A search for putative restriction sites in 16S rDNA was conducted by using the program DNASIS (Hitachi Software Engineering). Putative restriction sites were also identified in the 16S rDNA sequences amplified by PCR with primers SN910610 and SN910502 (Namba *et al.*, 1993b), enabling the comparison of 1370 nucleotide positions. Comparative analysis of the putative restriction sites in the sequenced DNA is summarized in Fig. 1. RFLP patterns that used 17 restriction enzymes were identical in all JWB isolates; therefore, we assume that the JWB phytoplasmas are relatively homogeneous. The JWB phytoplasmas can also be distinguished from other phytoplasmas by RFLP analysis of 16S rDNA. Although JWB and other phytoplasmas that belong to 16S-group VIII had most of the restriction sites in common, either the *Hpa*II or *Rsa*I restriction sites clearly

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

Strain	Associated plant disease	GenBank no.
AAY	American aster yellows	X68373
AIY	Alder yellows	Y16387
APS	Apple proliferation	X76426
AshY1	Ash yellows	AF092209
AshY2	Ash yellows	AF189215
AT	Apple proliferation	X68375
AUSGY	Australian grapevine yellows	L76865
BAWB	Black alder witches'-broom	X76431
BGWL	Bermudagrass white leaf	Y16388
BVK	Blutenverkleinerung	X76429
CIRP	<i>Cirsium arvense</i> phyllody	X83438
CnWB	Chestnut witches'-broom	AB054986
CP	Clover proliferation	L33761
CYE	Clover yellow edge	AF175304
EY	Elm yellows	L33763
EY1	Elm yellows	AF122910
EYWV	Elm yellows	AF122911
FD	Flavescence dorée of grapevine	X76560
HD1	Hemp dogbane*	AF122912
HibWB	<i>Hibiscus</i> witches'-broom	AF147708
IBS	Italian bindweed stolbur	Y16391
JHP	Japanese <i>Hydrangea</i> phyllody	AB010425
JWB-Ky	Jujube witches'-broom Kyoto isolate	AB052875
JWB-F	Jujube witches'-broom Fukui isolate	AB052878
JWB-G1 ^T	Jujube witches'-broom Gifu isolate 1	AB052876
JWB-G2	Jujube witches'-broom Gifu isolate 2	AB052877
JWB-Kor1	Jujube witches'-broom Korea isolate 1	AB052879
JWB-Kor2	Jujube witches'-broom Korea isolate 2	AF279272
JWB-Ch	Jujube witches'-broom Chinese isolate	AF305240
LDG	Coconut lethal yellowing	Y13912
LDT	Coconut lethal yellowing	X80117
LfWB	Loofah witches'-broom	L33764
LY	Coconut lethal yellowing	U18747
MIAY	Michigan aster yellows	M30970
OY	Onion yellows	D12569
PnWB	Peanut witches'-broom	L33765
PPWB	Pigeon pea witches'-broom	L33735
PpYC	Papaya yellow clinkle	Y10097
RuS	<i>Rubus</i> stunt	Y16395
RYD	Rice yellow dwarf	D12581
SCWL	Sugarcane white leaf	X76432
SPAR	<i>Spartium</i> witches'-broom	X92869
StLL	<i>Stylosanthes</i> little leaf	Y17055
STOL	Stolbur of <i>Capsicum annuum</i>	X76427
STRAWB1	Strawberry witches'-broom	U96614
TWB	Tsuwabuki witches'-broom	D12580
ULW	<i>Ulms</i> witches'-broom	X68376
VILL	<i>Vigna</i> little leaf	Y15866
WBDL	Witches'-broom disease of lime	U15442
WX	Western X disease	L04682
<i>A. palmarum</i>		L33734

*Host name.

Fig. 1. Analysis of putative restriction sites in 16S rDNA sequences from phytoplasmas belonging to 16S-group VIII. Sequences correspond to fragments amplified by PCR, primed by primer-pair SN910601/SN910502. Maps were generated using the MapDraw option of the DNASTAR program and were aligned manually to compare restriction nuclease recognition sites. Nucleotide positions of the fragments are shown at the top. Sequences unique to JWB phytoplasmas are underlined.

distinguished JWB phytoplasmas from all other members of the EY subgroup, supporting the hypothesis that the JWB phytoplasmas represent a distinct novel subgroup.

Based on its 16S rDNA sequence, a pair of oligonucleotides was designed to amplify JWB phytoplasma DNA specifically. PCR conditions were the same as described by Sawayanagi *et al.* (1999), except that the annealing temperature was 54 °C and 30 cycles were run. A primer set was designed from the JWB phytoplasma 16S rDNA sequence to detect JWB phytoplasma specifically. The forward primer was JWB-F1 (5'-TAAAAAGGCATCTTTTGT-3', corresponding to nucleotides 168–177 in the JWB 16S rDNA sequence) and the reverse primer was JWB-R1 (5'-AATCCGGACTA-AGACTGT-3', complementary to nucleotides 1268–1285). PCR using primers JWB-F1 and JWB-R1 amplified a 1119 bp DNA fragment when the template DNA was derived from plants that were naturally infected with JWB phytoplasma. No amplification was observed when the template was derived from healthy plants (data not shown). Conversely, EY-specific primer-pair R16(V)F1/R1, designed to amplify only the 16S rDNA of EY phytoplasma strains (Lee *et al.*, 1994), did not amplify DNA from any of the jujube samples with witches'-broom disease (data not shown). This result supports the hypothesis that this phytoplasma is unique within the EY group.

Nearly complete 16S rDNA sequences from most reported phytoplasmas and *Acholeplasma palmae* (Table 1) were aligned with the JWB phytoplasma sequences by using the

program CLUSTAL W (Thompson *et al.*, 1994). The base positions were numbered by using a previously described system (Namba *et al.*, 1993c). Nucleotide substitution rates (K_{nuc} values) were calculated (Kimura, 1980) and a phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987), with *A. palmae* as the out-group. The neighbour-joining tree constructed by phylogenetic analysis of 16S rDNA sequences from 42 diverse phytoplasmas, three JWB phytoplasmas and *A. palmae* is shown in Fig. 2. Bootstrap analysis revealed that the phylogenetic tree was reliable and in good agreement with a tree constructed previously by using 110 phytoplasma sequences (Jung *et al.*, 2002). In the phylogenetic analysis, the JWB phytoplasma isolates were included in 16S-group VIII (the EY group) and were most closely related to the EY subgroup (Jung *et al.*, 2002). However, the three representative JWB phytoplasma isolates clustered tightly together and constituted a branch that was distinct from all other 16S-group VIII phytoplasmas, with 100 % bootstrap support. This is in good agreement with previous reports (Zhu *et al.*, 1996; Tian *et al.*, 2000), implying that JWB in China is a member of the EY group (16S-group VIII). These data support the notion that the JWB phytoplasmas represent an independent subgroup that is distinct from the other subgroups that belong to 16S-group VIII.

The 16S rDNA sequences of JWB phytoplasmas were compared with sequences from 120 other phytoplasmas. Three sequences previously reported to be unique to phytoplasmas (Namba *et al.*, 1993b; Gundersen *et al.*, 1994),

Fig. 2. Phylogenetic distance tree constructed by the neighbour-joining method, comparing 16S rDNA sequences of JWB phytoplasmas with those of other phytoplasmas from GenBank. *Achleoplasma palmae* was used as the outgroup. Numbers on branches are confidence values obtained from 100 bootstrap replicates (only values above 80% are shown). Corresponding, previously reported phylogenetic group names are shown in the right-hand column (Jung *et al.*, 2002). Phytoplasma abbreviations are described in Table 1. Bar, 1% phylogenetic distance.

ACTGGA at positions 155–160, TTTTAAAAG at positions 187–195 and GCTT at positions 224–227, were conserved in the JWB phytoplasma 16S rDNA sequence. Whereas the 16S rDNA of EY subgroup phytoplasmas has the unique sequence CTTCAAAA at positions 63–70, the JWB phytoplasmas have CCTCAAAA at these positions, underscoring the difference between JWB and EY subgroup members. Several other unique sequences that also distinguish JWB phytoplasmas from other EY subgroup phytoplasmas were found. For example, the sequence ATAAAAAG at positions 167–174 differed at two positions from the corresponding sequences of other EY subgroup phytoplasmas. Three sequences (at positions 823–825, 982–987 and 1096–1102) were present only in the 16S rDNA of the JWB phytoplasmas. Additionally, the sequences at positions 1220–1223 and 1268–1279 in JWB differed from the sequences in the other EY subgroup phytoplasmas.

In nature, JWB phytoplasmas have been identified only from jujube trees and not from related tree species. This apparent host limitation of JWB phytoplasmas may be due to insect vector feeding preferences rather than to the resistance of particular plants, as these phytoplasmas can be transmitted experimentally into periwinkle plants with the help of a dodder (La & Woo, 1980). The disease is transmitted experimentally by only one species, the leafhopper *Hishimonus sellatus* (La & Woo, 1980). This ecological property supports the concept of JWB phytoplasmas as a relatively homogeneous taxon, distinct from other phytoplasmas.

According to our survey, JWB phytoplasma appears to be restricted to far-eastern Asia, including China, Japan and Korea, although jujube trees also grow in the Americas and Europe. By contrast, EY and closely related phytoplasmas

appear to be restricted to the Americas and Europe. In fact, some results have revealed that many phytoplasmas tend to be distributed locally rather than uniformly, over all continents (Lee *et al.*, 1994; Seemüller *et al.*, 1998). For example, the apple proliferation, peanut witches'-broom, ash yellows and clover proliferation group phytoplasmas each appear to be localized to specific regions (Lee *et al.*, 1998). The geographical isolation of these phytoplasmas may be correlated with the distribution of their host plants and the insect vectors that are native to a particular region. As mentioned before, JWB phytoplasma is transmitted only by one species of leafhopper, *H. sellatus*, and the EY phytoplasma in America is transmitted by the monophagous or oligophagous vector *Scaphoideus luteolus*, which feeds only on a few plant species, mostly in the genus *Ulmus* (Lee *et al.*, 1992). These factors may contribute to the diversification of two related phytoplasma populations, such as the JWB and EY subgroups.

Our findings are consistent with other data and support recognition of the JWB phytoplasma as a unique novel taxon. The degree of divergence of JWB from EY and other phytoplasmas warrants its delineation as a separate lineage. The phylogenetic analysis, the results of base-by-base comparisons of the JWB and EY phytoplasma sequences and the results of an analysis of putative restriction sites in their 16S rDNA sequences, are consistent with the hypothesis that two distinct gene pools have evolved: the EY and JWB phytoplasma. The geographical distribution of JWB phytoplasma in far-eastern Asia may have provided the ecological isolation that favoured the evolution of the distinct JWB phytoplasma.

In a PCR approach that used a primer-pair specific to JWB

(JWBF1/JWBR1), specific amplification provided evidence for a novel phytoplasma species, JWB phytoplasma. The presence of JWB-specific signature sequences and sequences unique to JWB phytoplasma in the 16S rDNA sequence also support this proposition and provide evidence for the genetic divergence of this pathogen from other phytoplasmas.

Therefore, we propose that the JWB phytoplasma should be designated as a novel, distinct '*Candidatus*' species, namely '*Candidatus* Phytoplasma ziziphi', with the following description: '*Candidatus* Phytoplasma ziziphi' [(Mollicutes) NC; NA; O; NAS (GenBank no. AB052875–AB052879), oligonucleotide sequences of unique regions of the 16S rRNA gene 5'-TAAAAAGGCATCTTTTGT-3' and 5'-AATCCGGACTAAGACTGT-3', P (jujube, phloem); M].

Acknowledgements

We thank Mr Shigeru Hatano for his excellent technical assistance. This work was supported partly by grants-in-aid from the Ministry of Education, Science and Culture of Japan (nos 09460155 and 13306004) and by the program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) of the Ministry of Agriculture, Forestry and Fishers of Japan.

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