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Phylogenetic and Taxonomic Status of the Phytoplasmas Associated with Water Dropwort (*Oenanthe javanica* DC) Disease in Korea and Japan

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To evaluate the phylogenetic and taxonomic status of the phytoplasmas associated with water dropwort (*Oenanthe javanica* DC) disease in Korea and Japan, their 16S rDNA was analyzed. DNAs extracted from water dropworts collected in Korea (Kyongnam province) and Japan (Chiba prefecture) affected by witches' broom and yellows were subjected to PCR using phytoplasma-specific primers, which amplified a 1.4-kbp fragment that included the 16S rDNA. Phytoplasmas were characterized by RFLP analysis using *Alu*I, *Hae*III, *Hha*I, *Kpn*I, *Mse*I, and *Rsa*I restriction enzymes and by sequence analysis of the PCR products. The water dropwort witches' broom (WDWB) and water dropwort yellows (WDY) 16S rDNA sequences were identical and closely related to onion yellows (OY, 99.9% identity), which belong to the aster yellows (AY) 16S-subgroup. However, the *Kpn*I RFLP analyses clearly distinguished the WDY and WDWB phytoplasmas from the OY phytoplasma. The phylogenetic analysis based on 16S rDNA showed that WDWB and WDY phytoplasmas are members of a relatively homogeneous group that evolved from a common ancestor.

Keywords : phylogeny, phytoplasma, water dropwort witches' broom.

The water dropwort (*Oenanthe javanica* DC) grows wild in freshwater marshes and swampy fields, and along ditches, canals, and streams in many Asian countries. It is also cultivated in these countries and is a very important commercial crop that is grown for local consumption and industry in Korea and Japan. Recently, Lee and Woo (1999), and Woo (2000) reported water dropwort witches' broom (WDWB) disease, documented that it is a serious disease that has spread wherever water dropwort is grown in Korea, and determined that the causal agent was likely a phytoplasma by virtue of evidence provided by fluorescence and electron microscopy and assays employing DNA amplification by PCR. In Japan, a disease associated with phytoplasma (previously termed mycoplasma-like organism, MLO), named water dropwort yellows (WDY) disease, was also reported in Ishikawa Prefecture (Shiomi and Sugiura, 1983). The symptoms of diseased plants included generalized yellowing and stunting, development of witches' broom growths, and leaf malformation.

Phytoplasmas are unique plant pathogens that are wall-less prokaryotes with the ultrastructural characteristics of mollicutes (Kirkpatrick, 1992). In the last thirty years, all attempts to isolate these microorganisms in pure culture have failed; so until recently, their evolutionary origin and genetic diversity remain uncertain. Although molecular techniques using serological procedures and DNA hybridization based on undefined genomic fragments are useful tools for detecting and identifying phytoplasmas, these techniques are not suitable for determining the relative phylogenetic positions of phytoplasmas to each other and to other microorganisms. In contrast, the 16S rDNA is a universal prokaryote gene that contains highly conserved and sufficiently variable regions. In addition, 16S rDNA sequence homologies and DNA polymorphisms have become a reliable approach for identifying, establishing genetic relatedness, and classifying phytoplasmas (Jung et al., 2002; Lee et al., 1998).

This study investigated the phytoplasma associated with water dropwort disease in Korea and Japan using molecular biological methods. This paper reports the results of an extended study involving the PCR amplification of phytoplasma-specific DNA from WDWB and WDY

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The EMBL/DDBJ/GenBank accession number for the 16S rDNA sequence of water dropwort witches' broom phytoplasma reported in this paper is AB078436

phytoplasma templates, and the analysis of amplified DNA. We also report the nucleotide sequence of segments of the WDWB and WDY phytoplasma 16S rDNA for the first time, and present the results of a phylogenetic analysis and the genetic relationship between water dropwort phytoplasma isolates from two different areas.

Materials and Methods

Source of phytoplasma strains. Samples from diseased dropwort plants displaying symptoms of water dropwort witches' broom (WDWB) were collected near Milyang, Kyongnam province, in March 1999. Water dropwort plants naturally infected with water dropwort yellows (WDY) phytoplasma were collected from fields in Chiba prefecture, Japan. Periwinkle plant (*Catharanthus roseus* (L.) G. Don.) infected with WDY phytoplasma originally by transmission through dodder was maintained in periwinkle by side grafting. Additional phytoplasma reference strains (Sawayanagi et al., 1999) were included in this study to help grouping the unknown strains; they were onion yellows (OY), rice yellow dwarf (RYD), and tsuwabuki witches' broom (TWB) phytoplasma. Healthy water dropwort and periwinkle plants grown from seeds under insect-proof cages were used as negative controls.

Primer pairs and PCR conditions. Total nucleic acids for templates in the PCR were extracted from the tissue of healthy plants and plants with phytoplasma-associated disease by a method previously described (Namba et al., 1993a). The extracted nucleic acids were quantified by agarose gel electrophoresis. A pair of previously designed universal primers (SN910610 and SN910502, Namba et al., 1993a) was used in the PCR to amplify the 16S rDNA from each sample tested. The conditions used for the PCR were the same as those described in a previous paper (Sawayanagi et al., 1999). The PCR products were electrophoresed in 0.7% agarose gels in TAE buffer and visualized with an UV transilluminator following ethidium bromide staining.

RFLP analysis of PCR amplification products. PCR products were digested singly with each restriction enzyme (*Alu*I, *Hae*III, *Hha*I, *Kpn*I, *Mse*I, and *Rsa*I) according to the manufacturer's specifications. The digested DNA was electrophoresed in 2.5% agarose gels, stained with ethium bromide, and visualized with an UV transilluminator.

Nucleotide sequence analysis of 16S rDNA. The primers for sequencing the 16S rDNA of OY and other related phytoplasmas reported elsewhere (Namba et al., 1993a, b) were used to sequence the WDWB and WDY phytoplasma 16S rDNA. The PCR-amplified 16S rDNA products were sequenced using Dye

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

Strains	*Associated plant disease and origin*	Accession No.
ACLR	Apricot chlorotic leaf roll; Spain	X 68338
AshY	Ash yellows; NY, USA	X68339
AT	Apple proliferation; Germany	X68375
AUSGY	Australian grapevine yellows; Australia	L76865
AY	Maryland aster yellows; MD, USA	L33767
BAWB	Black alder witches' broom; Germany	X76431
BVK	Blutenverkleinerung; Germany	X76429
CP	Clover proliferation; Canada	L33761
EY	Elm yellows; NY, USA	AF122910
LDG	Ghanaian Cape St. Pauls wilt disease; Ghana	Y13912
LDT	Tanzanian coconut lethal disease; Tanzania	X80117
LfWB	Loofah witches' broom; Taiwan	L33764
LY	Coconut lethal yellowing; FL, USA	U18747
OY	Onion yellows; Japan	D12569
PaWB	Paulownia witches' broom; Korea	AF279271
PPWB	Caribbean pigeon pea witches' broom; USA	U18763
PpYC	Papaya yellow clinkle; Queenland, Australia	Y10097
RYD	Rice yellow dwarf; Japan	D12582
SCWL	Sugarcane white leaf; Thailand	X76432
STOL	Stolbur of *Capsicum annuum*; Serbia	X76427
WBDL	Witches' broom disease of lime; Omen	U15442
WDWB	Water dropwort witches' broom; Korea	AB078436
WDY	Water dropwort yellows; Japan	AB078436
WX	Western X-disease; CA, USA	L04682
Acholeplasma palmae	–	L33734

Terminator Cycle Sequencing Kits (Perkin-Elmer Applied Biosystems, Foster City, CA).

Cladogram construction. Nearly complete 16S rDNA sequences from almost all phytoplasmas reported and from *Acholeplasma palmae* (Table 1) were aligned with that from WDWB phytoplasma using a program CLUSTAL W (Thompson et al., 1994), and base positions were numbered using a previously described system (Namba et al., 1993b). Sequences of other organisms used in this study were obtained from the DNA Data Bank of Japan. Nucleotide substitution rates (K_{nuc} values) were calculated (Kimura, 1980), and a phylogenic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987), with *A. palmae* as the outgroup. The topology of trees was evaluated by bootstrap analysis of the sequence data with CLUSTAL W, based on 100 random samplings.

Nucleotide sequence accession numbers. GenBank accession numbers of the 16S rDNA sequences of WDWB and other phytoplasmas used in this study and of *A. palmae* are listed in Table 1.

Results

Detection of phytoplasma in diseased water dropwort. A direct PCR with primers SN910610 and SN910502 was used to amplify the phytoplasma 16S rDNA, and this detected one fragment approximately 1.4 kbp in size representing the phytoplasma gene in all diseased water dropworts examined. The target DNA was also amplified from reference strain WDY phytoplasma maintained in periwinkle. Under the same conditions, no amplification products were obtained from nonsymptomatic plants collected in the same areas and from healthy periwinkle plants (Fig. 1).

RFLP analysis of amplified 16S rDNA. RFLP analysis of PCR-amplified 16S rDNA indicated that the diseased water dropworts contained strains of a phytoplasma belonging to aster yellows (AY) 16S-subgroup. All of the DNA samples from phytoplasma strains WDWB and WDY yielded mutually indistinguishable RFLP patterns. The *Alu*I, *Hae*III, *Hha*I, and *Rsa*I RFLP patterns observed with DNA from the two strains from water dropworts corresponded to RFLP patterns that have only been found in phytoplasma strains belonging to Group I (Lee et al., 1998; Sawayanagi et al., 1999). Phytoplasma strains WDWB and WDY were also mutually indistinguishable based on collective RFLP patterns following analysis using eight additional restriction enzymes (data not shown). These results lead us to conclude tentatively that these are strains of the same phytoplasma. The collective RFLP patterns using the 14 restriction enzymes were similar or identical to the patterns for 16S rDNA of OY phytoplasma (data not shown). However, the *Kpn*I RFLP patterns distinguished both WDWB and WDY from OY phytoplasma. The *Alu*I, *Hae*III, *Hha*I, *Kpn*I, *Mse*I, and *Rsa*I RFLP patterns clearly distinguished WDWB

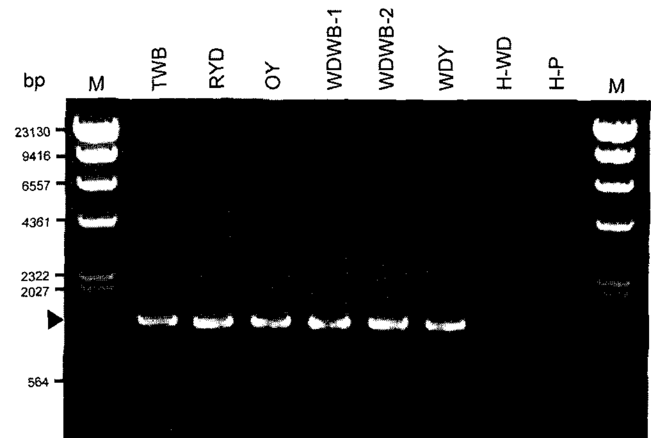

Fig. 1. PCR amplification of a 16S rDNA sequence from diseased water dropwort and a previously described Japanese phytoplasma strain using the primer pair SN910601 and SN910502. PCR products were separated by electrophoresis through a 1% agarose gel. The 16S rDNA is indicated by an arrowhead. Lane M, λDNA digested by *Hind*III; TWB, tsuwabuki witches' broom phytoplasma; RYD, rice yellow dwarf phytoplasma; OY, onion yellows phytoplasma; WDWB, water dropwort witches' broom phytoplasma; WDY, water dropwort yellows phytoplasma; H-WD, healthy water dropwort; H-P, healthy periwinkle.

(WDY) phytoplasma from both RYD and TWB phytoplasmas (Fig. 2).

Nucleotide sequence and phylogenetic analysis. The sequences of the WDWB and WDY phytoplasma 16S rRNA gene PCR products were identical (data not shown) and have been deposited in the GenBank database (accession No. AB078436). Sequence similarities among the water dropwort phytoplasma and aster yellows 16S-subgroup phytoplasmas ranged from 98.6 to 99.9% (data not shown). The expected fragment sizes based on the analysis of putative restriction sites corresponded to the fragment sizes obtained in the RFLP analysis of the amplified 16S rDNA. For the phylogenetic analysis, the water dropwort 16S rDNA phytoplasma sequences were aligned with the 16S rDNA sequences listed in Table 1. Phylogenetic analysis of the 16S rDNA sequences from 30 diverse phytoplasmas, including water dropwort phytoplasma and *Acholeplasma palmae*, were used to construct a neighbor-joining tree. The phylogenetic distance tree was revealed to be the most reliable tree by bootstrap analysis (Fig. 3) and was also in excellent agreement with a tree constructed previously (Jung et al., 2002).

Discussion

Phytoplasma-associated disease occurring in water dropwort has not been reported outside Korea and Japan. In Japan, Shiomi and Sugiura (1983) observed phytoplasma in the

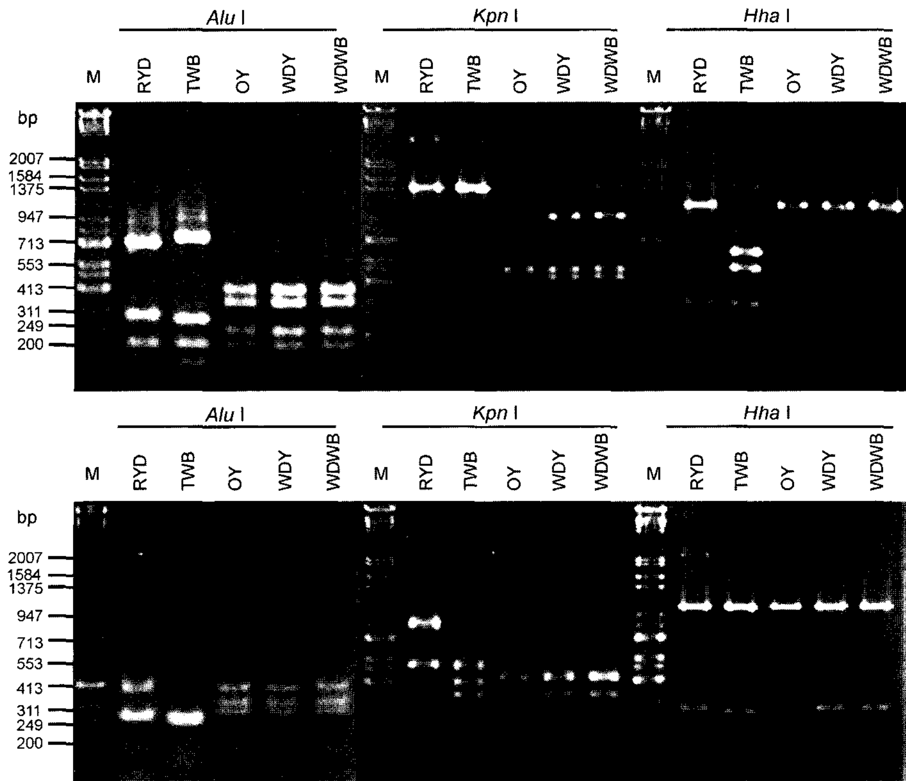

Fig. 2. RFLP analysis of 16S rDNA using the PCR products. Lane M, marker DNA mixtured with λDNA digested by *Eco*RI and *Hind*III and φX174 DNA digested by *Hin*fI. Other abbreviations are the same as described in Table 1.

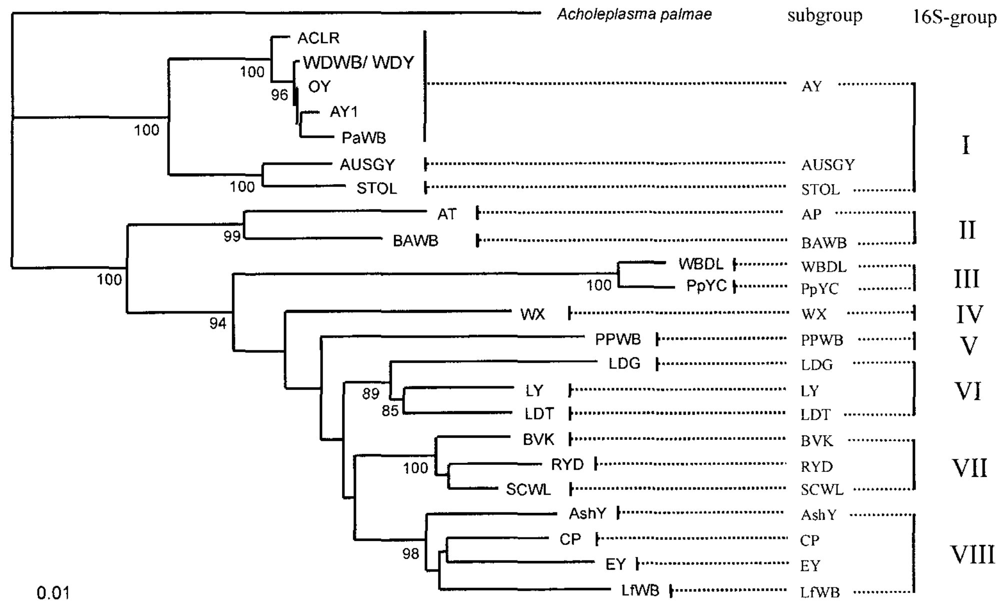

Fig. 3. Phylogenetic distance tree constructed by neighbor-joining method using *Acholeplasma palmae* as the outgroup, comparing the partial 16S rDNA sequences of WDWB/WDY phytoplasma with other phytoplasmas from GenBank. The numbers on branches are the confidence values obtained for 100 replicates (only values above 80% are shown). Corresponding phylogenetic group names previously reported are shown in the right-hand column. [Jung et al. (2002)]. Abbreviations for phytoplasmas are described in Table 1.

phloem of diseased plants and in the leafhopper vector (*Macrosteles orientalis* Virbaste) by electron microscopy, and determined the etiology of this disease. To date, the phylogenetic position of phytoplasma occurring in water dropwort has not been properly determined. This is the first phylogenetic analysis of WDWB and WDY phytoplasmas using their 16S rDNA sequences.

Recently, considerable progress has been made in detecting, identifying and classifying phytoplasmas using DNA-based methods. In particular, restriction site and sequence analyses of 16S rDNA have been used to distinguish and phylogenetically classify many phytoplasmas. The same approaches were used to elucidate the phylogenetic position of the water dropwort phytoplasmas. The RFLP analysis of 16S rDNA could not distinguish the phytoplasmas associated with witches' broom and yellows. The close relationship of the strains collected in Korea and Japan was confirmed by sequencing the 16S rDNA.

The RFLP results presented here suggest that the same phytoplasma is associated with two different disease phenotypes: witches' broom and yellows. In Korea, water dropworts with witches' broom grew alongside the same cultivars with yellows, which suggest that the differences are probably not due to cultivar differences. It is also possible that this phytoplasma is associated with witches' broom and the different disease phenotypes reflect the age of the host plant at infection or the level of inoculum at infection. Other approaches, such as the use of randomly cloned probes in DNA hybridization assays, may allow differentiation of the phytoplasmas from water dropworts with witches' broom and yellows.

The results revealed that these phytoplasmas are most closely related to the OY phytoplasma, which represents the aster yellows 16S-subgroup. Following digestion with *Kpn*I, however, the water dropwort phytoplasma RFLP was similar but not identical to that of OY from Japan. Interestingly, we found that WDWB and WDY phytoplasmas have two rRNA operons. One 16S rRNA gene of the water dropwort phytoplasma seems to lack the *Kpn*I site at position 470, which results in the 970-bp fragment seen at the top of the profile shown in Fig. 2. However, the other rRNA gene seems to possess an additional *Kpn*I site in the 470-bp fragment, which results in the two fragments (470 bp, 400 bp) below the largest fragment (970 bp) from the top (Fig. 2). The difference in the restriction profiles that distinguishes WDY and WDWB from OY phytoplasma can probably be explained by sequence heterogeneity of the two rRNA operons that seem to be present in all phytoplasmas (Schneider and Seemüller, 1994). Such sequence heterogeneity was first suggested for clover phyllody phytoplasma on the basis of RFLP profiles (Lee et al., 1993) and was described by Liefting et al. (1996) as occurring in the 16S rRNA genes of the *Phormium* yellow leaf phytoplasma.

In this study, we used sequence analysis to show that WDWB phytoplasma has the same 16S rDNA sequence as WDY phytoplasma isolated from Japan, and that both are closely related to the OY phytoplasma, a member of the AY phytoplasma 16S-subgroup. The leafhopper species *Macrosteles orientalis* Virbaste is a reported vector of WDY disease in Japan. This insect is also resident in Korea, although little is known about its distribution and behavior, as it is not regarded as a pest. Further transmission trials are required to elucidate the importance of these molecular differences and to clarify whether they are related to some pathogen characteristics, such as host specificity or geographical distribution.

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