

Detection and Differentiation of Plant-Pathogenic Mycoplasma-like Organisms Using Polymerase Chain Reaction

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ABSTRACT

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Specific oligonucleotide primers were synthesized using sequence data of the 16S rRNA gene from three plant-pathogenic mycoplasma-like organism (MLO) groups (I-III). When used as primers in the polymerase chain reaction (PCR), four sets of primer pairs were identified: 1) mollicute-specific, 2) MLO-specific, 3) MLO-group I-specific, and 4) MLO-group III-specific. MLO 16S rRNA genes were selectively amplified by PCR from infected plants and vector insects. Using these primers and thermal cycling conditions, this technique was effective for detecting MLO 16S rRNA genes from diseased plants and infected insect vectors and for differentiating MLO 16S rRNA genes from similar rRNA genes of eukaryotic host organelles and other microorganisms associated with

healthy vector insects. Using group-specific primers, the 16S rRNA gene of each MLO group was also differentially amplified. A modified PCR method (recycled PCR [RPCR]) was developed to perform several different PCR reactions simultaneously in one tube by adding the second primer to the tube containing the first PCR products. RPCR enabled detection of mollicute-specific DNA fragments and MLO-specific or group-specific DNA fragments as multiple bands. Each MLO group could be identified reliably by RPCR. These results show the utility of PCR for diagnosing MLO diseases and the development of a phylogenetically based MLO taxonomy.

Additional keywords: prokaryotes; *Mollicutes*; MLO-groups I, II, and III; diagnosis.

Mollicutes are wall-less prokaryotes which are associated with many important diseases of humans, animals, arthropods, and plants (29). The origin and phylogenetic relationships of *Mollicutes* have been studied by analysis of 16S and 5S rRNA sequences and by oligonucleotide cataloging (21,23,30,36-38). Cloned *Mollicutes*' rRNA genes have been used as sensitive hybridization probes to detect mycoplasma contamination in cell cultures (6,7) and to detect *Mycoplasma fermentans* (*incognitus* strain) from AIDS patients (22). Oligonucleotide probes complementary to the variable regions in the 16S rRNA can distinguish species of *Mollicutes* (5). Polymerase chain reaction (PCR) using primers that amplify a species-specific gene have been used to detect *Mycoplasma pulmonis* contamination in animal cell cultures (15).

Since the nonculturable mollicutes, commonly referred to as mycoplasma-like organisms (MLOs), were first discovered in 1967 (4), they have been implicated as pathogens in more than 300 plant diseases worldwide (24). However, because these unique prokaryotes have not yet been cultured *in vitro*, their evolutionary origin and genetic diversity are largely unknown. Molecular cloning of MLO, chromosomal DNA fragments extracted from MLO-infected plants (2,14,18-20,26,33) and from vector insects (13) has made hybridization probes available for detection of these pathogens in plants and insects and for studies of their genetic relationships. These probes have facilitated comparison and differentiation among MLOs, but not their phylogenetic relationships with other prokaryotes. To address this problem, molecular phylogenetic analyses of the 16S rRNA gene of MLOs have been

reported recently (12,17,19,21,27,33). Oligonucleotide probes that are specific for the MLO 16S rRNA gene but do not recognize rRNA of plant mitochondria and chloroplasts improved the sensitivity of tests to detect nonculturable *Mollicutes* when used in place of cloned chromosomal DNA probes (11). This suggests that the MLO 16S rRNA gene can be useful for identification and differentiation of MLOs. Recent studies on the 16S rRNA sequence of western X MLO, a severe strain of western aster yellows MLO (SAY-MLO) (12,17,19), a virescence MLO associated with the phylloxy of *Oenothera hookeri* (O-MLO) (21,33) in the United States, and six Asian MLOs (27) suggest that these MLOs are most closely related to *Anaeroplasma* and *Acholeplasma* but are evolutionarily distinct from animal mycoplasmas.

Recent studies suggest the utility of PCR as a diagnostic tool for MLO diseases. Deng and Hiruki (3) reported that 16S rRNA genes of MLOs could be amplified by PCR using specific primers. Ahrens and Seemüller (1) established a diagnostic method using restriction fragment length polymorphism (RFLP) analysis of PCR amplified 16S rRNA genes. In this work, we describe the effective detection and differentiation of various MLOs by PCR amplification of the 16S rRNA gene using combinations of MLO-specific primers. We then describe a modified PCR method (recycled polymerase chain reaction [RPCR]) developed to perform multiple PCR amplifications simultaneously in one tube. A preliminary report of this study was presented at the meeting of the Phytopathological Society of Japan, Tokyo, on July 24, 1992.

MATERIALS AND METHODS

Sources of MLOs. Four MLO isolates (onion yellows [OY] [25], tsuwabuki witches'-broom [TW] [9], tomato yellows [TY]

TABLE 1. Identification of mycoplasma-like organisms (MLOs) and plant hosts

Group ^a	MLO (abbreviation)	Plant (latin name) ^b	Vector insect	Literature cited
I	Onion yellows (OY)	Garland chrysanthemum (<i>Chrysanthemum coronarium</i> L.)	<i>Macrostes orientalis</i> Virbaste	25
	Tomato yellows (TY)	Tomato (<i>Lycopersicon esculentum</i> Mill.) Garland chrysanthemum	<i>M. orientalis</i> Virbaste	10
	Mulberry dwarf (MD)	Mulberry (<i>Morus bombycis</i> Koidz cv. Wase Midori)	<i>Hishimonus sellatus</i> Uhler <i>Hishimonoides sellatiformis</i> Ishihara	4
	Paulownia witches' broom (PW)	Paulownia (<i>Paulownia tomentosa</i> (Thunb.) Steud.)	<i>Cyrtopeltis tenuis</i> Reuter <i>Halyomorpha mista</i> Uhler	4
II	Tsuwabuki witches' broom (TW)	Tsuwabuki (<i>Farfugium japonikum</i> Kitamura) Garland chrysanthemum	<i>Scleroracus flavopictus</i> Ishihara	9
III	Rice yellow dwarf (RY)	Rice (<i>Oryza sativa</i> L.)	<i>Nephotettix cincticeps</i> Uhler <i>N. nigropictus</i> Stål <i>N. virescens</i> Distant	28

^a MLO groups classified by the 16S rRNA sequence.

^b Plants used to maintain each MLO.

TABLE 2. Primers for polymerase chain reaction (PCR) amplification and sequencing of 16S rRNA gene of plant-pathogenic mollicutes

Primer	Sequence (5' - 3')	Position ^a	Length (mer)
SN910601 ^b	GTTTGATCCTGGCTCAGGATT	1 - 21	21
9F	GTTTGATCCTGGCTC	1 - 16	15
SN920201	ATCCTGGCTCAGGATTA	6 - 23	18
SN920203 ^c	TTTAAGCAATTAACCTTA	63 - 81	19
120R	GTGTTACTCACCCGT	103 - 89	15
350F	TACGGGAGGCAGCAG	335 - 349	15
350R	CTGCTGCCTCCCGTAG	349 - 334	16
520F	GTGCCAGCAGCCGCGG	501 - 516	16
520R	ACCGCGGCTGCTGGC	517 - 503	15
SN910501	AATATATGGAG(A)GAACA CCA(T)GTA(G)	688 - 709	22
SN920204 ^c	CCTCAGCGTCAGTAA	746 - 732	15
788F	ATTAGATACCCTGGTA	772 - 787	16
920R	GTCAATTCCTTTGAGTTT	902 - 885	18
SN920202 ^c	ATGCACCACCTGTATCC	1031 - 1015	17
1099F	GCAACGAGCGCAACCC	1074 - 1089	16
1100R	AGGGTTGCGCTCGTTG	1090 - 1075	16
SN911201	GATACAATGGCTG	1209 - 1221	13
SN911202	CAGCCATTGTATC	1221 - 1209	13
SN910502 ^b	AACCCCGAGAACGTATTCACC	1368 - 1348	21
SN910602	AGGGATACCTTGTTACGACTT	1494 - 1374	21

^a The numbers of the positions are followed in the 16S rRNA sequence of group I mycoplasma-like organism (MLO) (27).

^b Primers used for PCR.

^c Primers used for PCR and sequencing.

[10], and rice yellow dwarf [RY] [28]) were maintained in greenhouse-grown host plants by serial inoculation with insect vectors. Two MLO isolates (mulberry dwarf [MD] [4] and paulownia witches'-broom [PW] [4]) were collected in fields. MLO isolates and plant hosts used in this study are listed in Table 1. Healthy plants were also grown and used as controls.

The leafhopper *Macrostes orientalis* Virbaste, a vector of the OY-MLO, was maintained on both healthy and OY-MLO-infected garland chrysanthemum (*Chrysanthemum coronarium* L.).

DNA isolation. Total DNA samples from healthy control plants and MLO-infected plants were isolated using a modification of the procedure described by Koller et al (14). A tissue sample of 0.3 g was ground using a mortar and pestle in 0.9 ml of extraction buffer containing 2.5 M NaCl, 0.5% (w/v) PVP-10 (Sigma Chemical Co., St. Louis, MO), 1% (w/v) CTAB (hexadecyltrimethylammonium bromide), 0.5 M Tris-HCl (pH 8.0), 0.25 M ethylenediaminetetraacetic acid (EDTA) (pH 8.0), and 0.2% 1-thioglycerol. The suspension was incubated for 30 min

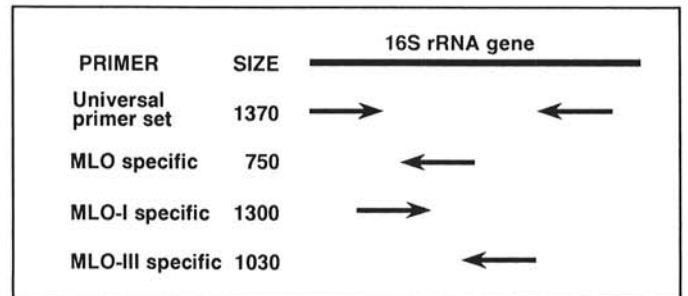


Fig. 1. Location of each primer and expected size of amplified specific DNA fragments. Arrows indicate the direction of synthesis for each primer.

at 65 C and centrifuged for 5 min at 1,200 g. The supernatant was extracted with chloroform-isoamyl alcohol (24:1) and precipitated with isopropanol. The resulting pellet was resuspended in 100 µl of TE (10 mM Tris, 1 mM EDTA, pH 8.0) buffer and stored at 4 C.

Total DNAs from *Mycoplasma orale* culture (0.5-mg bacterial pellet for each extraction) and insects (five insects for each extraction) were prepared by the extraction procedure given above for plant tissue. The pellet from the insect sample was resuspended in 2 µl of TE buffer.

DNA was also isolated from an MLO-enriched fraction that was prepared from herbaceous and woody plant tissues using a previously reported procedure (16). DNA extracted from healthy plants using the MLO-enrichment procedure served as the experimental control.

Primers for PCR and sequencing. A search was made for published sequence data of 16S rRNA genes of the O-MLO (21), SAY-MLO (17), and other culturable *Mollicutes*, rickettsias, *Escherichia coli*, chloroplast, plant mitochondria, and the 16S-like rRNA sequences of eukaryotes in the GenBank database (R. 68.0, June, 1991) using a Hitachi Software Engineering DNASIS system (Version 7.01; Hitachi, Japan). On the basis of these aligned 16S rRNA sequences, a universal primer set (SN910601 and SN910502) (Table 2; Fig. 1) highly homologous to all genera of the class *Mollicutes* was synthesized and used for PCR (27).

Using the universal primer set, the 16S rRNA genes of MLOs were amplified by PCR from MLO-enriched fractions of plants infected with each of six Asian MLOs (27), and these DNA fragments were sequenced. A phylogenetic tree constructed using the sequence data of these Asian MLOs and USA MLOs (O-MLO and SAY-MLO) showed that these MLOs consist of

phylogenetic diverse microorganisms and could be classified into three groups: I (OY, TY, MD, PW, O-MLO, and SAY-MLO), II (TW), and III (RY).

Three primers (SN920202, SN920203, and SN920204; Table 2) were synthesized and used for both PCR and sequencing reactions, according to their specificity. The primer SN920204 (Table 2) is specific and universal for all previously sequenced MLOs. Primers SN920203 and SN920202 (Table 2) are specific for MLO groups I and III, respectively. Fifteen additional primers (Table 2) specific for the 16S rRNA gene of six Asian MLOs (27) were synthesized and used for sequencing with the above primers. These primers were synthesized using a DNA synthesizer (Type 391, ABI Japan, Tokyo) (34).

PCR amplification. Total DNA samples of 1 μ l each from OY-, TW-, TY-, RY-, MD-, and PW-MLO-infected and healthy plants; total DNA from vector insect tissue; and total DNA from *M. orale* were used as PCR templates. The amplification was performed in a 20- μ l PCR reaction mixture containing 250 μ M each of dATP, dGTP, dCTP, and dTTP, 1 μ M of each upstream and downstream primer (SN910601 and SN910501, respectively), 2 μ l of 10 \times PCR reaction buffer (Promega Corp., Madison, WI), 0.1 U *Taq* DNA Polymerase (Promega Corp.), and 100 μ l paraffin liquid (light) (261-32GR, Nacalai Tesque Corp., Tokyo, Japan). Using the universal primer set, PCR was carried out for 50 cycles in a Thermo Processor TR-100 (Taitec Corp., Saitama, Japan) under the following conditions: the first cycle, denaturation 90 s at 94 C; ramping over 40 s to 60 C, annealing 2 min at 60 C, ramping 30 s to 72 C, extension for 3 min at 72 C; the continuing cycles, denaturation 2 min at 94 C; the final cycle, extension for 7 min at 72 C. Denaturation and annealing conditions were the same as for the first cycle (8,31).

Thermocycling was done for 20 cycles with the MLO-specific primer set (SN910601, SN920204) and for 10 cycles with the group I-specific primer set (SN920203, SN910502) and group III-specific primer set (SN910601, SN920202). The annealing temperature was 40 C for these three primer sets; other conditions were as mentioned above.

Recycled PCR (RPCR). In our experiments, PCR was initially done using total DNA extracts of plant tissues by the universal primer set (allowing amplification of the 16S rRNA genes of *Mollicutes*). For positive samples, another amplification was done for confirmation and identification of the MLO group. However,

because of the labor and expense required to repeat the PCR for each sample, the PCR method was modified to simultaneously amplify the MLO or MLO group-specific sequences. The modified method consisted of two consecutive steps of the amplification reaction (i.e., step 1 and step 2) (Fig. 2). The amplification was performed in a 50- μ l PCR reaction mixture containing the same ratio of each component as in the original PCR described above. Thermocycling conditions were as given above at step 1 for 10 cycles and at step 2 for 10 cycles (at an annealing temperature of 40 C). This method could be performed continuously for each independent amplification reaction in one tube without any subsequent additions, except for the proper primer. The first PCR reaction (step 1) amplified the 16S rRNA gene using the universal primer set. Then another primer, specific for MLO, group I, or group III, was added, and the novel DNA fragments were amplified in a second PCR reaction (step 2) (Fig. 2). PCR amplification of 10 cycles for each step was enough to detect target DNA fragments by agarose-gel electrophoresis. The DNA fragments amplified by two-step PCR were detected by agarose-gel electrophoresis as composite bands consisting of the two reaction products. Because the PCR reaction components were recycled, except for the second-cycle primer, we refer to this technique as the recycled polymerase chain reaction (RPCR).

DNA sequence analysis of amplified DNAs. A part of each PCR-amplified sample DNA was purified by agarose-gel electrophoresis as previously described (32). The sequence of each DNA was determined with Sequenase Kit Version 2.0 (U.S. Biochemical Corp., Cleveland, OH) using synthetic primers (Table 1) and 35 S dATP (Fig. 1). Manganese was used for the sequencing buffer to improve the extension reaction according to the commercially supplied protocol (35).

RESULTS AND DISCUSSION

Detection of MLO 16S rRNA gene from plants by PCR. Using the universal primer set, the 1,370-bp DNA fragment of the 16S rRNA gene was expected to be amplified by PCR (Fig. 1). In agarose-gel electrophoresis, a band was detected at about 1.4 kbp from both the DNA preparation of the MLO-enriched samples (Fig. 3, lane 3) and the total DNA extract of various MLO-infected plants (Fig. 3, lanes 5,7,9) and *M. orale* (Fig. 3, lane 10). PCR was also done using each DNA template extracted from MLO-infected plant tissue and the MLO-specific or cor-

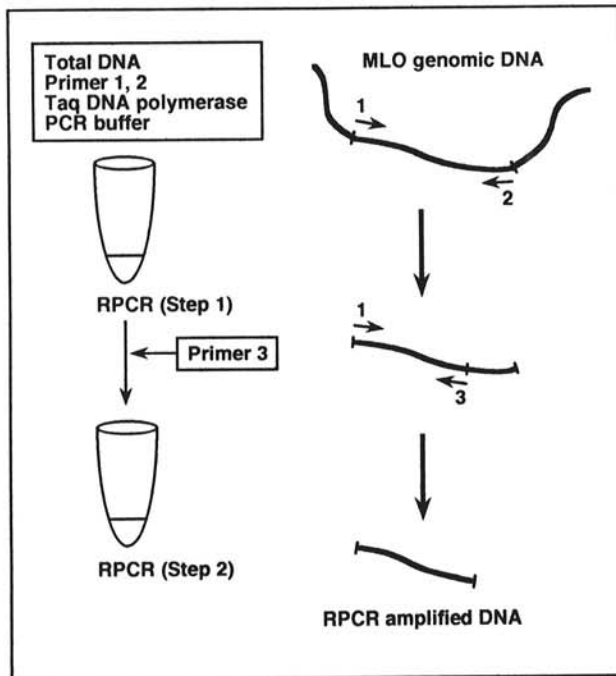


Fig. 2. Recycled polymerase chain reaction strategy. Each numbered arrow indicates a primer and its direction of synthesis.

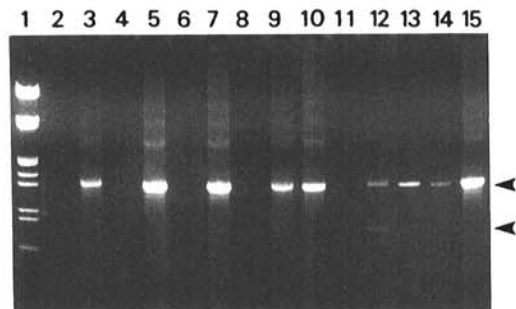


Fig. 3. Agarose-gel electrophoresis of polymerase chain reaction (PCR)-amplified 1.4-kbp 16S rRNA gene fragments (upper arrowhead) from mycoplasma-like organism (MLO)-enriched (lanes 2,3) or total DNA samples (lanes 4-10,14,15) using the universal primer set (SN910601 and 910502), and RPCR-amplified 1.4-kbp (upper arrowhead) and 0.75-kbp (lower arrowhead) 16S rRNA gene fragments from total DNA samples using the universal primer set (step 1) and MLO-specific primer (SN920204; step 2; see text; lanes 11-13). Lane 1 contains λ -EcoRI+HindIII markers. Lanes 2-15 contain PCR products from healthy (lanes 2,4,6,11), OY-infected (lanes 3,5,12), and TW-infected (lane 7) garland chrysanthemum, PCR products from healthy (lane 8) and RY-infected (lane 9) rice, PCR products from a *Mycoplasma orale* culture (lanes 10,13), PCR products from MLO-free (lane 14) and OY-MLO-infected (lane 15) *Macrostelus orientalis* leafhopper. A 4- μ l sample from a total of 20 μ l of each reaction mixture was run on a 1.0% agarose gel. The gel was stained with ethidium bromide and photographed under UV light.

responding group-specific primer set (Fig. 1). By the use of MLO-specific primer, the 750-bp band from MLO-infected plants was expected to be amplified (Fig. 1). The group I-specific primer was expected to amplify the approximate 1,300-bp band from plant tissues infected with group I MLOs (Fig. 1). The group III-specific primer was expected to amplify the approximate 1,000-bp band from plant tissues infected with group III MLOs (Fig. 1). Each corresponding band of the expected size was detected from these samples (Fig. 4, lanes 3–5; Fig. 5, lane 3). In these PCRs, no band was amplified from healthy samples (Fig. 3, lanes 2,4,6,8; Fig. 4, lane 2; Fig. 5, lanes 2,4,6), a *M. orale* culture (Fig. 3, lanes 10; Fig. 4, lanes 6; Fig. 5, lane 8), or diseased samples infected with MLOs, each of which group is different from that of primer pair (Fig. 5, lanes 3,5).

A 30-cycle reaction was enough to provide detectable amplification (*data not shown*). Even after 50 cycles, no nonspecific bands were observed (Fig. 3, lanes 3,5,7,9,10; Fig. 4, lanes 3–5; Fig. 5, lane 3). However, high molecular bands were sometimes observed only in the positive samples with use of the universal primer set after 40 cycles of amplification. Ahrens and Seemüller (1) reported a nonspecific band appearing after 40 cycles of amplification from healthy samples. This may be due to lower specificity of the designed primer or lower annealing temperatures. In our case, these high molecular bands were only from diseased samples. However the origin of these bands is not known.

At first, the primer pair of SN910601 and SN910602 did not work well; however, Deng and Hiruki (3) reported that a primer pair of “1 and 6” from the same region worked well. The problem was solved by using SN910502 for the 3' primer and SN910601 for the 5' primer. It is not clear why the results differed in spite of the fact that almost the same primers were used. Of course, there were a few differences between the two experiments, one being that our 3'-primer SN910602 is one nucleotide shorter at the 3' end (3), and the same for the 5' primer. Another is that we used the total DNA extract of MLO-infected plant tissues for PCR templates, while they used the DNA preparation of the MLO-enriched samples. Additional difference is the plant sources. We used plants other than periwinkle, such as paulownia and mulberry, and also herbaceous plants, such as rice, tsuwabuki, onion, garland chrysanthemum, and tomato. More work needs to be done to find the reason for the different results.

Recycled PCR. MLO-specific 16S rRNA gene fragments were effectively amplified by RPCR and detected by agarose-gel elec-

trophoresis as composite bands consisting of the two reaction products (Fig. 3, lane 12; Fig. 4, lanes 11–13; Fig. 5, lane 14). By using the universal primer set and MLO-specific primer, 1.4 kbp- and 0.75-kbp bands were amplified (Fig. 3, lane 12; Fig. 4, lanes 11–13). A large band was expected to be amplified in step 1, and a small band in step 2 (Fig. 1). This was confirmed by checking after each step with agarose-gel electrophoresis (*data not shown*). By the use of the universal primer set and MLO III-specific primer, the 1.4-kbp and 1.03-kbp bands were amplified (Fig. 5, lane 14) and were expected to be amplified in step 1 and step 2, respectively (Fig. 1). Only the 1.4-kbp band was expected to be amplified from a diseased sample infected with MLO of a different group from that of the step 2 primer or other *Mollicutes* bacteria such as a *M. orale* culture (Fig. 1). As expected, only the 1.4-kbp band was amplified from these samples, and no other additional band was amplified in step 2 when either MLO-specific primer or MLO III-specific primer (Fig. 3, lane 13; Fig. 4, lane 14; Fig. 5, lanes 10,12,15) was added. In RPCR, no band was amplified from healthy samples (Fig. 3, lane 11; Fig. 4, lane 10; Fig. 5, lanes 9,11,13).

Recently, Deng and Hiruki (3) reported that nonspecific DNA amplification occurred from healthy control plants at low-stringency annealing conditions. They considered that since 16S rRNA sequences from chloroplasts are similar to MLOs, nonspecific amplification at annealing temperatures below 50 C might be due to the 16S rRNA gene from healthy plant chloroplasts. And they subsequently distinguished MLO-originated PCR fragments from plant-associated PCR fragments by using restriction endonuclease digestion. RPCR could effectively prevent these nonspecific amplifications. In step 1 of RPCR, the 16S rRNA-specific fragment (approximately 1.4 kbp) of *Mollicutes* was amplified by 10 cycles at an annealing temperature of 60 C, which provides enough template for step 2. The quantity of the template for a step-2 primer should be much more than that of other DNAs that would be used for nonspecific amplification. Because the amplification of step 2 did not work well with short primers (SN920202, SN920203, and SN920204) at an annealing temperature above 45 C, it was done at 40 C (*data not shown*). By RPCR, 16S rRNA fragments were effectively and specifically amplified from plants, and each MLO group could be differentiated reliably by the amplified DNA size estimated in agarose-gel using group-specific primers added in step 2 (*unpublished data*).

Detection of the MLO 16S rRNA gene from vector insects by PCR and RPCR. PCR amplification using the universal primer set resulted in amplification of one, similar-size DNA (approx-

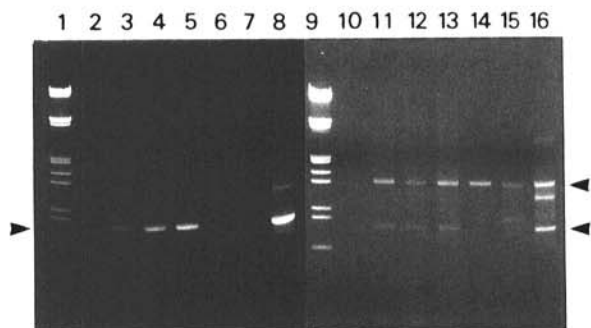


Fig. 4. Agarose-gel electrophoresis of polymerase chain reaction (PCR)-amplified 0.75-kbp 16S rRNA gene fragments (left arrowhead) obtained using mycoplasma-like organism (MLO)-specific primer set (SN910601 and SN920204) (lanes 2–8), and RPCR-amplified 1.4-kbp (right upper arrowhead) and 0.75-kbp (right lower arrowhead) 16S rRNA gene fragments using the universal primer set (step 1) and MLO-specific primer (SN920204; step 2; lanes 10–16) from total DNA of plants and leafhoppers. Lanes 1 and 9 contain λ -EcoRI+HindIII markers. Lanes 2–8 and 10–16 contain a mixture of PCR products from healthy garland chrysanthemum (lane 2) and rice (lane 10), PCR products from OY-infected (lanes 3,11) and TW-infected (lanes 4,12) garland chrysanthemum, RY-infected rice (lanes 5,13), a *Mycoplasma orale* culture (lanes 6,14), MLO-free *Macrostes orientalis* leafhoppers (lanes 7,15), OY-MLO-infected leafhoppers (lanes 8,16). Electrophoresis was done as in Fig. 3.

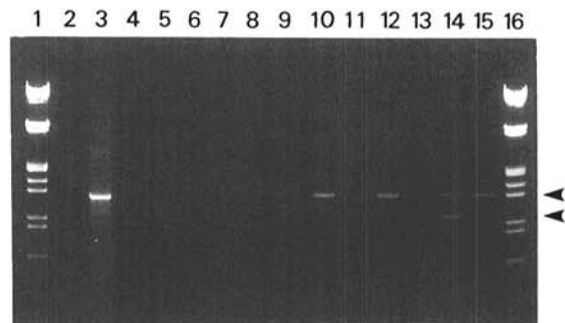


Fig. 5. Agarose-gel electrophoresis of polymerase chain reaction (PCR)-amplified 1.3-kbp (left arrowhead) 16S rRNA gene fragments obtained using mycoplasma-like organism (MLO)-I specific primer set (SN920203 and SN910502) (lanes 2–8), and RPCR-amplified 1.4-kbp (right upper arrowhead) and 1.03-kbp (right lower arrowhead) 16S rRNA gene fragments obtained using universal primer set (step 1) and MLO-III-specific primer (lanes 9–15) (SN920202; step 2) from total DNA extracts of plants and leafhoppers. Lanes 1 and 16 contain λ -EcoRI+HindIII markers. Lanes 2–15 contain PCR products from healthy (lanes 2,4,9,11), OY-infected (lanes 3,10), and TW-infected (lanes 5,12) garland chrysanthemum, PCR products from healthy (lanes 6,13) and RY-infected (lanes 7,14) rice, and from *Mycoplasma orale* (lanes 8,15).

mately 1,400 bp) from total DNA extracts of both MLO-free (Fig. 3, lane 14) and MLO-infected (Fig. 3, lane 15) *M. orientalis* leafhoppers. These DNA fragments were similar in size to the DNA amplified from OY-MLO-infected plants.

RPCR was also done for the same insect samples as above using the universal primer set (step 1) and MLO-specific primer (SN920204) (step 2), and the 750-bp DNA was amplified only from MLO-infected insects (Fig. 4, lane 16). Although several other bands (3,100, 1,370, and 1,200 bp) were observed in this lane, the origins of the 3,100- and 1,200-bp bands are not known. The 1,370-bp band corresponds to the size of the mollicute-specific 16S rRNA gene fragment amplified by the universal primer set (Fig. 1). The 750-bp band corresponds to the size of an MLO-specific band amplified by the SN910601 primer added originally and the SN920204 primer added at step 2 of RPCR (Fig. 1). The 1,370-bp and 750-bp bands were gel purified, excised, reextracted, and sequenced. The sequence of the 1,370-bp band was identical to the group I MLO 16S rRNA gene sequence (positions 1-1,368 (the DNA fragment amplified with primers SN910601 and SN910502). The sequence of the 750-bp band corresponded to a part of this 1,370-bp DNA sequence (positions 1-746 [the DNA fragment amplified with primers SN910601 and SN920204]).

A large band (1,370 bp) and a small band (900 bp) were amplified from the DNA sample of MLO-free insects (Fig. 4, lane 15) by RPCR under the same condition as above. The large band was of the same size as the band amplified from MLO-infected insects by the universal primer set. Both bands from MLO-free leafhoppers were directly sequenced, and the small band was found to be a part of the large-band DNA. The sequence of the large-band DNA was different from that of 1370-bp DNA of MLO-infected insects (i.e., the 16S rRNA sequence of group I MLOs) and also from the 16S rRNA sequences of group II and III MLOs. Homology search indicated that the sequence of this DNA fragment was similar to those of other gram-negative bacteria (*unpublished data*). This indicates that there are prokaryotes associated with some leafhoppers that are unrelated to plant-pathogenic MLOs.

As a further check, PCR was done using the MLO-specific primer set (SN910601 and SN920204) with DNAs from MLO-free and -infected leafhoppers. Although no DNA was amplified from MLO-free insects (Fig. 4, lane 7), the 1,200- and 750-bp bands were detected in MLO-infected leafhoppers (Fig. 4, lane 8). The origin of the 1,200-bp band, which was of the same size as that of the DNA amplified by RPCR (Fig. 4, lane 16), is not known. However, the sequence of the 750-bp band was determined and revealed to be identical to the group I MLO 16S rRNA gene (position 1-746).

These results suggest that MLO-infected vector insects may be detected by RPCR or PCR using the MLO-specific primer. The 750-bp band should be specifically detected only from MLO-infected vector insects by RPCR and PCR. MLO-free vector insects carrying a microorganism which is parasitic or symbiotic and has similar sequences in the 16S rRNA gene may also be distinguished by this method.

RPCR offers the following advantages: 1) It is a more sensitive method than the original PCR for detecting specific DNAs, because it can amplify detectable DNA fragments in fewer amplification cycles; 2) Only a single primer is needed, and the results are reproducible; and 3) After step 1, the amount of template for step 2 can be checked to decide whether it is necessary to continue step 2 (i.e., step 1 can be used as a control reaction). Since plant pathogenic MLOs are nonculturable and restricted to phloem tissues (usually in low concentrations), it is very difficult and time consuming to isolate and clone 16S rRNA genes using standard cloning procedures. With proper control of the thermocycling conditions and selection of specific primers, PCR can be used to isolate the 16S rRNA gene of MLOs and other *Mollicutes* without separation of eukaryotic and organelle genomic DNAs.

We have shown that the MLO 16S rRNA gene is easily amplified by PCR from plant DNA. The gene was also amplified from vector insects and could be differentiated from the corresponding

gene of other leafhopper-associated prokaryotes. The 16S rRNA gene of each MLO group was differentially amplified using group-specific PCR primers. Ahrens and Seemüller (1) reported the detection of MLOs by PCR amplification of a 16S rRNA gene fragment and differentiation of representative MLO groups by RFLP using restriction endonucleases. We developed a newly modified PCR method (RPCR) to increase the reliability and simplify the classification of MLOs without having to analyze PCR products by RFLP analysis. Other than *Taq* DNA polymerase, RPCR needs no additional enzymes, such as restriction endonucleases used for RFLP. Thus, RPCR allows quick and accurate MLO detection and diagnosis. These results should facilitate MLO diagnosis as well as phylogenetic and taxonomic studies.

LITERATURE CITED

- Ahrens, U., and 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.
- Davis, R. E., Lee, I.-M., Douglas, S. M., and Dally, E. L. 1990. Molecular cloning and detection of chromosomal and extrachromosomal DNA of the mycoplasma-like organism associated with little leaf disease in periwinkle (*Catharanthus roseus*). *Phytopathology* 80:789-793.
- Deng, S., and Hiruki, C. 1991. Amplification of 16S rRNA genes from culturable and nonculturable Mollicutes. *J. Microbiol. Methods* 14:53-61.
- Doi, Y., Teranaka, M., Yora, K., and Asuyama, H. 1967. Mycoplasma or P.L.T. group-like microorganisms found in the phloem elements of plants infected with mulberry dwarf, potato witches'-broom, aster yellows or paulownia witches'-broom. *Ann. Phytopathol. Soc. Jpn.* 33:259-266.
- Göbel, U. B., Geiser, A., and Stanbridge, E. J. 1987. Oligonucleotide probes complementary to variable regions of ribosomal RNA discriminate between *Mycoplasma* species. *J. Gen. Microbiol.* 133:1969-1974.
- Göbel, U. B., and Stanbridge, E. J. 1984. Cloned mycoplasma ribosomal RNA genes for the detection of mycoplasma contamination in tissue cultures. *Science* 226:1211-1213.
- Harasawa, R., Mizusawa, H., and Koshimizu, K. 1986. A reliable and sensitive method for detecting mycoplasmas in cell cultures. *Microbiol. Immunol.* 30:919-921.
- Innis, M. A., and Gelfand, D. H. 1990. Optimization of PCRs. Pages 3-12 in: *PCR Protocols: A Guide to Methods and Applications*. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego, CA.
- Kato, S., and Iwanami, S. 1990. A new MLO disease, tsuwabuki witches' broom in Nichinan. *Ann. Phytopathol. Soc. Jpn.* 56:394.
- Kato, S., Shiomi, T., Wakibe, H., and Iwanami, S. 1988. Tomato yellows transmitted by the leafhopper vector, *Macrostelus orientalis* Virbaste. *Ann. Phytopathol. Soc. Jpn.* 54:220-223.
- Kirkpatrick, B. C., and Fraser, J. D. 1989. Phylogenetic relationships of the western X-disease mycoplasma-like organism (X-MLO) as established by 16S rRNA sequences. (Abstr.) *Phytopathology* 79:1138.
- Kirkpatrick, B. C., Kuske, C. R., Lim, P. O., and Sears, B. B. 1990. Phylogeny of plant pathogenic mycoplasma-like organisms. (Abstr.) 8th IOM Congr.:45-46.
- Kirkpatrick, B. C., Stenger, D. C., Morris, T. J., and Purcell, A. H. 1987. Cloning and detection of DNA from a nonculturable plant pathogenic mycoplasma-like organism. *Science* 238:197-200.
- Kollar, A., Seemüller, E., Bonnet, F., Saillard, C., and Bové, J. M. 1990. Isolation of the DNA of various plant pathogenic mycoplasma-like organisms from infected plants. *Phytopathology* 80:233-237.
- Kunita, S., Terada, E., Ghoda, A., Sakurai, Y., Suzuki, H., Takakura, A., and Kagiya, N. 1989. A DNA probe for specific detection of *Mycoplasma pulmonis*. *Exp. Anim.* 38:215-219.
- Kuske, C. R., and Kirkpatrick, B. C. 1990. Identification and characterization of plasmids from the western aster yellows mycoplasma-like organism. *J. Bacteriol.* 172:1628-1633.
- Kuske, C. R., and 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.
- Kuske, C. R., Kirkpatrick, B. C., Davis, M. J., and Seemüller, E. 1991. DNA hybridization between western aster yellows mycoplasma-like organism plasmids and extrachromosomal DNA from other plant pathogenic mycoplasma-like organisms. *Mol. Plant-Microbe*

- Interact. 4:75-80.
19. Kuske, C. R., Kirkpatrick, B. C., and Seemüller, E. 1991. Differentiation of virescence MLOs using western aster yellows mycoplasma-like organism chromosomal DNA probes and restriction fragment length polymorphism analysis. *J. Gen. Microbiol.* 137:153-159.
 20. Lee, I.-M., Davis, R. E., and DeWitt, N. D. 1990. Nonradioactive screening method for isolation of disease-specific probes to diagnose plant diseases caused by mycoplasma-like organisms. *Appl. Environ. Microbiol.* 56:1471-1475.
 21. Lim, P.-O., and 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.
 22. Lo, S.-C., Shih, J. W. K., Newton, P. B. III, Wong, D. M., Hayes, M. M., Bemish, J. R., Wear, D. J., and Wang, R. Y. H. 1989. Virus-like infectious agent (VLIA) is a novel pathogenic mycoplasma: *Mycoplasma incognitus*. *Am. J. Trop. Med. Hyg.* 41:586-600.
 23. Maniloff, J. 1983. Evolution of wall-less prokaryotes. *Annu. Rev. Microbiol.* 37:477-499.
 24. McCoy, R. E., Caudwell, A., Chan, C. J., Chen, T. A., Chiykowski, L. N., Cousin, M. T., Dale, J. L., de Leeuw, G. T. N., Golino, D. A., Hackett, K. J., Kirkpatrick, B. C., Marwitz, R., Petzold, H., Sinha, R. C., Sugiura, M., Whitcomb, R. F., Yang, I. L., Zhu, B. M., and Seemüller, E. 1989. Plant diseases associated with mycoplasma-like organisms. Pages 545-640 in: *The Mycoplasmas. Volume V. Spiroplasmas, Achleplasmas, and Mycoplasmas of Plants and Arthropods*. R. F. Whitcomb and J. G. Tully, eds. Academic Press, New York.
 25. Miyahara, K., Matsuzaki, M., Tanaka, K., and Sako, N. 1982. A new disease of onion caused by mycoplasma-like organism in Japan. *Ann. Phytopathol. Soc. Jpn.* 48:551-554.
 26. Nakashima, K., Kato, S., Iwanami, S., and Murata, N. 1992. Cloning and detection of chromosomal and extrachromosomal DNA from mycoplasma-like organisms that cause yellow dwarf disease of rice. *Appl. Environ. Microbiol.* 57:3570-3575.
 27. Namba, S., Oyaizu, H., Kato, S., Iwanami, S., and Tsuchizaki, T. 1993. Phylogenetic diversity of phytopathogenic mycoplasma-like organisms. *Int. J. Syst. Bacteriol.* 43:461-467.
 28. Nasu, S., Sugiura, M., Wakimoto, S., and Iida, T. 1967. On the etiologic agent of rice yellow dwarf. (Abstr.) *Ann. Phytopathol. Soc. Jpn.* 33:343-344.
 29. Razin, S. 1985. Molecular biology and genetics of mycoplasmas (*Mollicutes*). *Microbiol. Rev.* 49:419-455.
 30. Rogers, M. J., Simmons, J., Walker, R. T., Weisburg, W. G., Woese, D. R., Tanner, R. S., Robinson, I. M., Stahl, D. A., Olsen, G., Leach, R. H., and Maniloff, J. 1985. Construction of the mycoplasma evolutionary tree from 5S rRNA sequence data. *Proc. Natl. Acad. Sci. U.S.A.* 82:1160-1164.
 31. Saiki, R. K., Gelfand, D. H., Stoffe, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239:487-491.
 32. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1982. Plasmid vectors. Pages 1.1-1.110 in: *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
 33. Sears, B. B., Lim, P.-O., Holland, N., Kirkpatrick, B. C., and Klomparens, K. L. 1989. Isolation and characterization of DNA from a mycoplasma-like organism. *Mol. Plant-Microbe Interact.* 2:175-180.
 34. Sommer, R., and Tautz, D. 1989. Minimal homology requirements for PCR primers. *Nucleic Acids Res.* 17:6749.
 35. Tabor, S., and Richardson, C. C. 1989. Effect of manganese ions on the incorporation of dideoxynucleotides by bacteriophage T7 DNA polymerase and *Escherichia coli* DNA polymerase. *Proc. Natl. Acad. Sci. U.S.A.* 86:4076-4080.
 36. Weisburg, W. G., Tully, J. G., Rose, D. L., Petzel, J. P., Oyaizu, H., Yang, D., Mandelco, L., Sechrest, J., Lawrence, T. G., Van Etten, J., Maniloff, J., and Woese, C. R. 1989. A phylogenetic analysis of the mycoplasmas: Basis for their classification. *J. Bacteriol.* 171:6455-6467.
 37. Woese, C. R. 1987. Bacterial evolution. *Microbiol. Rev.* 51:221-271.
 38. Yang, D., Oyaizu, Y., Oyaizu, H., Olsen, G. J., and Woese, C. R. 1985. Mitochondrial origins. *Proc. Natl. Acad. Sci. U.S.A.* 82:4443-4447.