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Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs

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Summary. A new universal oligonucleotide primer pair R16mF2/R1 and a modified universal oligonucleotide primer pair R16F2n/R2 for polymerase chain reaction (PCR) were designed on the basis of comparative analysis of 16S rRNA gene sequences from 19 phytoplasmas (previously called mycoplasmalike organisms), 48 related mollicutes, and other prokaryotes. These primer pairs specifically initiated amplification of 16S rDNA sequences from representatives of all known phytoplasma 16S rRNA groups plus one closely related *Acholeplasma* strain. These primer pairs did not initiate amplification from any healthy plant host or any plant pathogenic bacteria tested. Direct PCR assays using the new primer pair R16mF2/R1 allowed sensitive detection of phytoplasmas from most woody hosts, including ornamental and fruit trees where the associated phytoplasmas had not been readily detected before. Nested-PCR assays using two universal primer pairs, R16mF2/R1 and R16F2n/R2, increased detection sensitivity over 100 fold and readily detected phytoplasmas from all the woody hosts and insect hosts tested. RFLP anlysis of the nested-PCR products allowed identification of the primary phytoplasma(s) associated with each tissue sample.

Introduction

Phytoplasmas (previously called mycoplasmalike organisms) are phloem-limited plant pathogenic prokaryotes. They are associated with disease in several hundred plant species including many economically important ornamental and fruit tree crops (McCoy *et al.*, 1989). Because of the inability to isolate them in pure culture, the detection of phytoplasmas in the past primarily relied on the use of molecular probes in serological (Chen *et al.*, 1992; Chen *et al.*, 1993; Sarindu and Clark, 1993), DNA-DNA hybridization (Kirkpatrick *et al.*, 1987; Bertaccini *et al.*, 1990; Bonnet *et al.*, 1990; Lee and Davis, 1992; Lee *et al.*, 1992; Vibio *et al.*, 1994), or PCR assays (Ahrens and Seemüller, 1992; Davis and Lee, 1993; Deng and Hiruki, 1991; Firrao *et al.*, 1993; Lee *et al.*, 1993a, 1993b, 1994, 1995; Namba *et al.*, 1993; Schaff *et al.*, 1992; Schneider *et al.*, 1993; Seemüller *et al.*, 1994). PCR assays using primers derived from phytoplasma-specific DNA probes or 16S rRNA gene sequences proved to be the most sensitive in detection of phytoplasmas in infected plant or insect hosts.

To date, a few universal primer pairs based on sequences of the conserved 16S rRNA gene have been developed by different laboratories which allowed detection of some or all known phytoplasmas (Ahrens and Seemüller, 1992; Deng and Hiruki, 1991; Lee *et al.*, 1993b; Schnieder *et al.*, 1993). However, the sensitivity and/or specificity of these primers was limited when they were used for detection of phytoplasmas because non-phytoplasma sequences were often co-amplified, making it difficult to unequivocally confirm the presence of phytoplasma(s). Often, direct PCR (single amplification with series of 35 cycles) using the primers failed to detect phytoplasma from woody hosts, such as fruit and ornamental tree crops. The lack of ideal universal primers and assay procedures that can be used for ultrasensitive and specific detection of

phytoplasmas associated with these woody hosts has greatly hindered the epidemiological studies of tree diseases.

In this communication, we have designed a new set of universal primers based on sequences external to the region of 16S rDNA amplified by using the universal primer pair R16F2/R2 previously designed in this laboratory (Lee *et al.*, 1993b). Nested-PCR assays using these two sets of universal primer pairs allowed sensitive detection of all known representative phytoplasma groups associated with herbaceous or woody plant hosts and some representative phytoplasma insect vectors.

Materials and methods

Sources of phytoplasma-infected tissues and other prokaryotes. Phytoplasma-infected tissues of pear (PE-G10), European elm (*Ulmus carpinifolia*) (ELM-I), rubus (*Rubus fruticosus*) (Rub1), and phytoplasma-infected pear psyllid (*Cacopsylla pyricola*) were collected from the Emilia-Romagna region in northern Italy. Aster yellows leafhopper *Macrosteles fascifrons* infected with Tulelake strain of aster yellows was kindly provided by A.H. Purcell, University of California, Berkeley, California. Representative phytoplasma strains were kindly provided by the following researchers, who provided each strain separately in periwinkle, in other hosts as indicated, or as DNA samples from plant hosts as indicated (Table I). DNA samples of *Acholeplasma laidlawii, Mycoplasma arthritidis, Mycoplasma gallisepticum, Mycoplasma capricolum, Mycoplasma pulmonis, Mycoplasma hominis, Ureaplasma urealyticum, Bacillus cereus, Streptococcus pneumoniae,* and *Escherichia coli* were kindly provided by D.T. Kingsbury, George Washington University, Washington D.C. *Acholeplasma* sp. strain J-233 (A. J-233) [now known as *Acholeplasma palmae* (Tully *et al.*, 1995)] was kindly provided by K.J. Hackett, USDA, ARS, Beltsville, Maryland. Some representative plant pathogenic bacteria: *Pseudomonas syringae* pv. *phaseolicola, Pseudomonas solanacearum, Pseudomonas fluorescens, Erwinia carotovora* pv. *carotovora, Erwinia chrysanthemi, Xanthomonas campestris* pv. *campestris, Xanthomonas campestris* pv. *malvacearum* and *Agrobacterium tumefaciens* were kindly provided by C.-H. Liao, USDA, ARS, Eastern Region Research Center, Philadelphia, Pennsylvania. Periwinkle plants infected with *Spiroplasma citri (S.c.)* were kindly provided by G.N. Oldfield, University of California, Riverside. DNA samples from *Spiroplasma kunkeli* and *Spiroplasma melliferum* were prepared in our laboratory.

Primer pairs and PCR conditions. The universal primer pair R16mF2/R1 was designed on the basis of unique sequences of phytoplasmas by comparative analysis of aligned 16S rRNA gene sequences from 19 diverse phytoplasmas, 48 related *Mollicutes,* and several other prokaryotes. A specific DNA fragment approximately 1.4 kb in size was amplified by using primer pair R16mF2/R1. A second universal primer pair (R16F2n/R2) was designed by modification to the 5' end of the primer R16F2 and use of R16R2, both originally designed by Lee *et al.* (1993b). The oligonucleotide sequences of these primers (numbers in parentheses correspond to 16S rRNA nucleotide positions of strain MIAY as numbered in Lim and Sears, 1989) are:

R16mF2/R1:
R16mF2, 5'-catgcaagtcgaacgga-3' (53-69)
R16mR1, 5'-cttaaccccaatcatcgac-3' (1469-1487)

R16F2n/R2:
R16F2n, 5'-gaaacgactgctaagactgg-3' (149-168)
R16R2, 5'-tgacgggcggtgtgtacaaaccccg-3' (1373-1397)

For PCR, total nucleic acid was extracted from healthy or phytoplasma-infected periwinkle or insect tissue as described elsewhere (Lee *et al.*, 1992). Nucleic acid extractions from woody (elm, chokecherry, walnut, pecan, plum, pear, and apricot) tissues were performed as previously described (Lee *et al.*, 1993a, 1995). Nucleic acid samples were diluted in sterile deionized water to give a final concentration of 20 ng/µl. PCR assays were performed as previously described (Schaff *et al.*, 1992), with 20 ng of total nucleic acid, 200 µM each dNTP and 0.4 to 1.0 µM primer pair. Thirty-five PCR cycles were conducted in an automated thermocycler (Perkin-Elmer Cetus, Norwalk, CT). The following parameters were used: 1 min (2 min for the first cycle) denaturation at 94°C, annealing for 2 min at 60°C (55°C for reactions using R16F2n/R2), and primer extension for 3 min (10 min in final cycle) at 72°C. Tubes with the reaction mixture devoid of DNA templates were included in each experiment as negative controls. PCR products were analyzed by electrophoresis through a 1% agarose gel

TABLE I. - Sources of phytoplasma strains and phytoplasma-infected plants and insects

	16S rRNA group, subgroup (*b*)	Disease	Source	Researcher who provided organism
Strain (*a*)				
AY1	I-B	Maryland aster yellows	Maryland	R.E. Davis
PnWB	II	Peanut witches'-broom	Taiwan	H.J. Su
CX	III-A	Canadian peach X-disease	Canada	L.N. Chiykowski
LY	IV	Palm lethal yellows	Florida	N.A. Harrison
EY	V	Elm yellows	New York	W.A Sinclair
CP	VI	Clover proliferation	Canada	C. Hiruki
AshY	VII	Ash yellows	New York	W.A. Sinclair
LfWB	VIII	Loofa witches'-broom	Taiwan	H.J. Su
PPWB	IX	Pigeon pea witches'-broom	Florida	N.A. Harrison
AP-A	X	Apple proliferation	Italy	L. Carraro via A. Bertaccini
TLAY-i	I-B	Infected *M. fascifrons*	California	A.H. Purcell
Sample (*c*)				
SGP1	I-V	Strawberry green petal	Canada	N. Nickerson
Gla1	I-B	Gladiolus germs fins	Italy	A. Bertaccini
PeG-10	X-A	Pear decline	Italy	A. Bertaccini
PeW-5	X-A	Pear decline	Italy	A. Bertaccini
Apc2	I	Apricot chlorotic leaf roll	Italy	A. Bertaccini
Apc3	I	Apricot chlorotic leaf roll	Italy	A. Bertaccini
P135	X-B	Plum leptonecrosis	Italy	A. Bertaccini
P136	I	Plum leptonecrosis	Italy	A. Bertaccini
CC-W.A.	III-A	Chokecherry X-disease	North Dakota	Z. Cheng
Rub1	V	Rubus stunt	Italy	
PB1	III-C	Pecan bunch	Georgia	C.J. Chang
WWB1	III-E	Walnut witches'-broom	Georgia	C.J. Chang
LY	IV	Palm lethal yellows	Florida	N.A. Harrison
Elm-I	V	Elm yellows	Italy	
Elm3	V	Elm yellows	W. Virginia	H.M. Griffiths
Elm5	V	Elm yellows	W. Virginia	H.M. Griffiths
C. pyricola	X-A	a vector of pear decline	Italy	
M. fascifrons	I-B	a vector of aster yellows	California	A.H. Purcell

(*a*) Phytoplasma strains except strain LY were maintained in periwinkle (*Catharanthus roseus*) plants. Strain LY was provided as a DNA sample prepared from an infected palm tree.
(*b*) Identities of phytoplasmas were based on RFLP profiles of 16S rDNA PCR products amplified with primer pair R16F2/R2.
(*c*) Field plant tissue samples were collected from associated hosts. The insect *C. pyricola* was collected from a pear orchard and *M. fascifrons* was fed on an aster yellows infected celery in the greenhouse. Strains Elm3 and Elm5 were provided as DNA samples extraced from field collected elm.

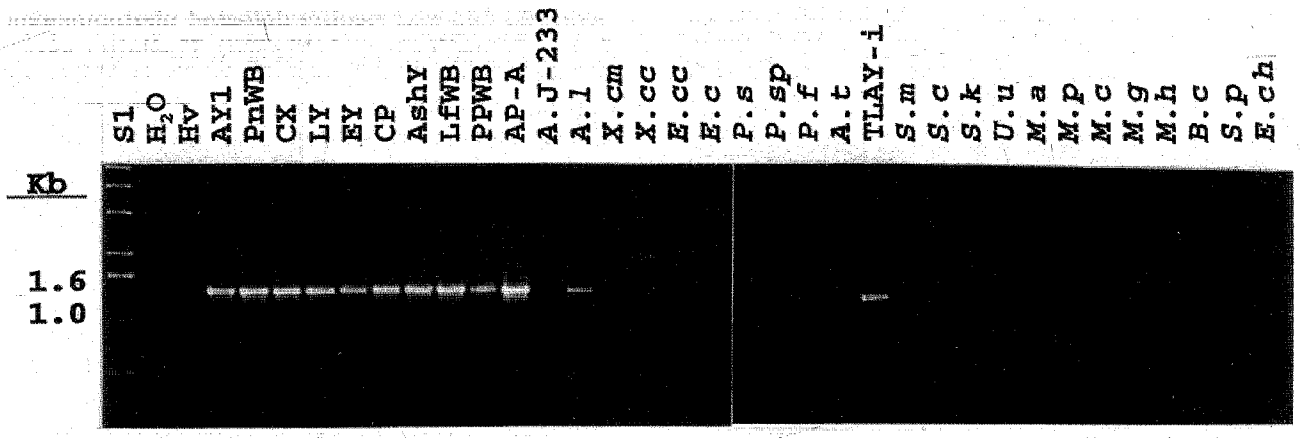

Fig. 1 - Polymerase chain reaction (PCR) amplification of 16S rDNA sequences from various phytoplasmas representing 10 phytoplasma 16S rRNA groups, other related mollicutes, plant pathogenic bacteria, and other bacteria using primer pair R16mF2/R1. PCR products were separated by electrophoresis through a 1% agarose gel. Lane S1, 1-Kb DNA ladder (GIBCO/BRL); lane Hv, healthy *Catharanthus roseus*. Other abbreviations are given in the text and Table I.

followed by staining in ethidium bromide and visualization of DNA bands using a UV transilluminator.

Sensitivity and universality of detection using universal primer pair R16mF2/ and R16F2n/R2. Primer pairs R16mF2/R1 and R16F2n/R2 were evaluated separately in the PCR assays for sensitivity in detection of phytoplasmas. Primer pair R16mF2/R1 was evaluated for its ability to sensitively amplify phytoplasma and *A. laidlawii* DNA sequences. Nucleic acid samples from AY phytoplasma-infected periwinkle and *A. laidlawii* were diluted in sterile water and used as template in amounts ranging from 0.001 pg to 10 ng for PCR assays using R16mF2/R1 as described.

Nested-PCR assays with two universal primer pairs. Nucleic acid samples extracted from field-collected phytoplasma-infected plants and leafhoppers, *M. fascifrons* and *C. pyricola* (Table I) were used as template for PCR assays. In the nested-PCR assay, PCR products initially amplified using the universal primer pair R16mF2/R1 were diluted (1/40) with sterile deionized water and used as template for a subsequent series of 35 PCR cycles (Lee *et al.*, 1995) in which reaction mixtures contained the universal primer pair R16F2n/R2.

RFLP analyses of PCR products. Phytoplasma 16S rDNA sequences amplified by nested-PCR using the primer pair R16F2n/R2 were analyzed by restriction endonuclease digestion. For analyses of nested-PCR products, 2-4 µl of each PCR product was digested separately with two restriction endonucleases, *Rsa*I (GIBCO/BRL, Gaithersburg, Md) and *Mse*I (New England Biolabs, Beverly, MA) selected based on their ability to distinguish phytoplasma strains as shown in Lee *et al.* (1993b). The restriction products were then separated by electrophoresis through a 5% polyacrylamide gel followed by staining in ethidium bromide and visualization of DNA bands using a UV transilluminator. The patterns derived from R16F2n/R2-PCR products is essentially the same as those from R16F2/R2-PCR products (Lee *et al.*, 1993b).

Results

Direct (single amplification) PCR assays using each of the two universal primer pairs, R16mF2/R1 and R16F2n/R2, primed amplification of 16S rDNA sequences from representative strains for all ten distinct phytoplasma 16S rRNA groups previously identified (Gundersen *et al.*, 1994; Lee *et al.*, 1993b) (Fig. 1). The phytoplasma identities were revealed by separate RFLP analyses of the amplified 16S rDNA (data not shown). The primer pair R16mF2/R1 (Fig. 1) also weakly primed

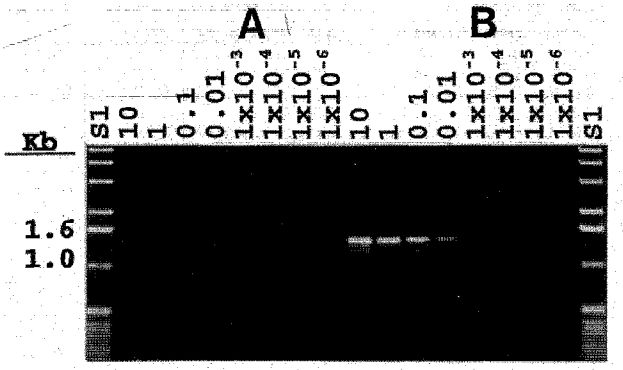

Fig. 2 - Sensitivity of 16S rDNA amplification using universal primer pair R16mF2/R1. Decreasing quantities (given in nanograms) of template DNA from **a**, *Acholeplasma laidlawii* and **B**, aster yellows (AY) phytoplasma were amplified by polymerase chain reaction (PCR) using primer pair R16mF2/R1. PCR products were separated by electrophoresis through a 1% agarose gel. Lane S1, 1-Kb DNA ladder (GIBCO/BRL).

amplification from *A. laidlawii*. The modified primer pair R16F2n/R2 has essentially the same specificity as the original primer pair R16F2/R2, but showed increased sensitivity in detection (data not shown). Both universal primer pairs did not prime amplification of DNA from healthy control plants. The primer pair R16mF2/R1 enabled detection of as little as 0.1 ng of *A. laidlawii* nucleic acid and 1 pg of phytoplasma-infected periwinkle nucleic acid (approximately 0.01 pg of phytoplasma DNA) (Fig. 2). It has been suggested that as little as 1% of nucleic acid from *C. roseus* and 0.1% from diseased woody hosts may be phytoplasma DNA (Schaff *et al.*, 1992). But direct PCR assays using the current modified primer pair R16F2n/R2 (data not shown), like primer pair R16F2/R2 (Lee *et al.*, 1995), did not readily detect phytoplasmas from woody field samples. In contrast, direct PCR using R16mF2/R1 detected phytoplasmas from a variety of herbaceous and woody plants including those infected with strawberry green petal (SGP1), gladiolus germs fins (Gla1), pear decline (PeG-10, PeW-5), chokecherry X-disease (CC-wa), rubus stunt (Rub1), palm lethal yellows (LY), and elm yellows (Elm-I, Elm3, Elm5) which are commonly present in America or on the European continent (Fig. 3A). The direct PCR also enabled detection of phytoplasmas from insect vectors (Fig. 3A, lanes *C. pyricola* and *M. fascifrons* Nested-PCR using this primer pair and the internal primer pair R16F2n/R2 increased detection sensitivity over 100 fold and readily detected phytoplasmas from all samples tested (Fig. 3B). The latter assay also proved very effective for detection of phytoplasmas from known insect vectors and can be useful for investigation of potential insect vectors (I.-M. Lee, unpublished). The universality of this assay was confirmed by RFLP analyses of the nested-PCR products (Fig. 4). Predominant phytoplasma(s) associated with these infected hosts were identified as members of 16S rRNA group and subgroup (Lee *et al.*, 1993b) as follows: strawberry, group I-C; gladiolus, group I-B; pear, group X-A; apricot, group I; Japanese plum, group X-B (sample P135), and I (sample P136) (data not shown); chokecherry, group III-A; rubus, group V; pecan, group III-C (Gundersen *et al.*, 1996); walnut, group III-E (Gundersen *et al.*, 1996); palm, group IV; elm (Italy), group V; elm

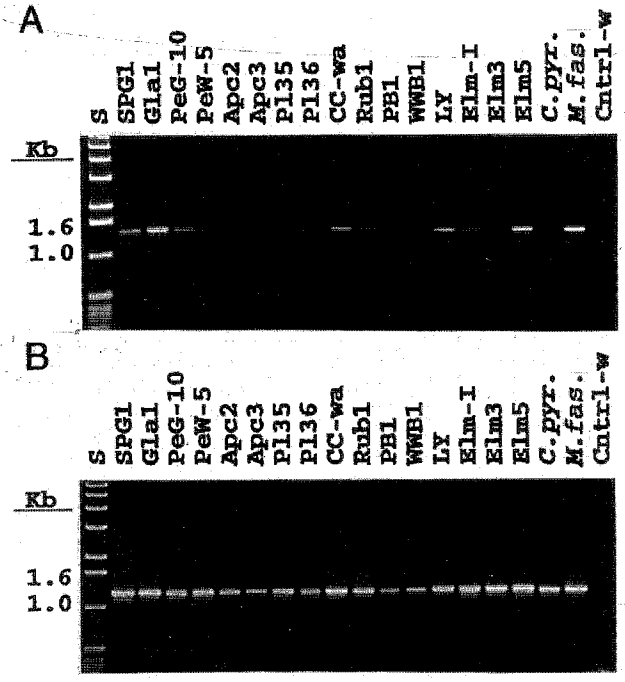

Fig. 3 - Direct and nested-PCR amplification of phytoplasma 16S rDNA sequence from various hosts. Direct PCR amplification **A**, was perfomed (35 cycles) using the universal primer pair R16mF2/R1, while nested-PCR amplification **B**, was performed initially using primer pair R16mF2/R1 for 35 cycles followed by second PCR amplification (35 cycles) using universal primer pair R16F2n/R2. PCR products were separated by electrophoresis through a 1% agarose gel. Lane S1, 1-Kb DNA ladder; lane Hv, healthy *Catharanthus roseus*; lane elm-H, healthy elm *Ulmus americana*. Other abbreviations are given in Table I.

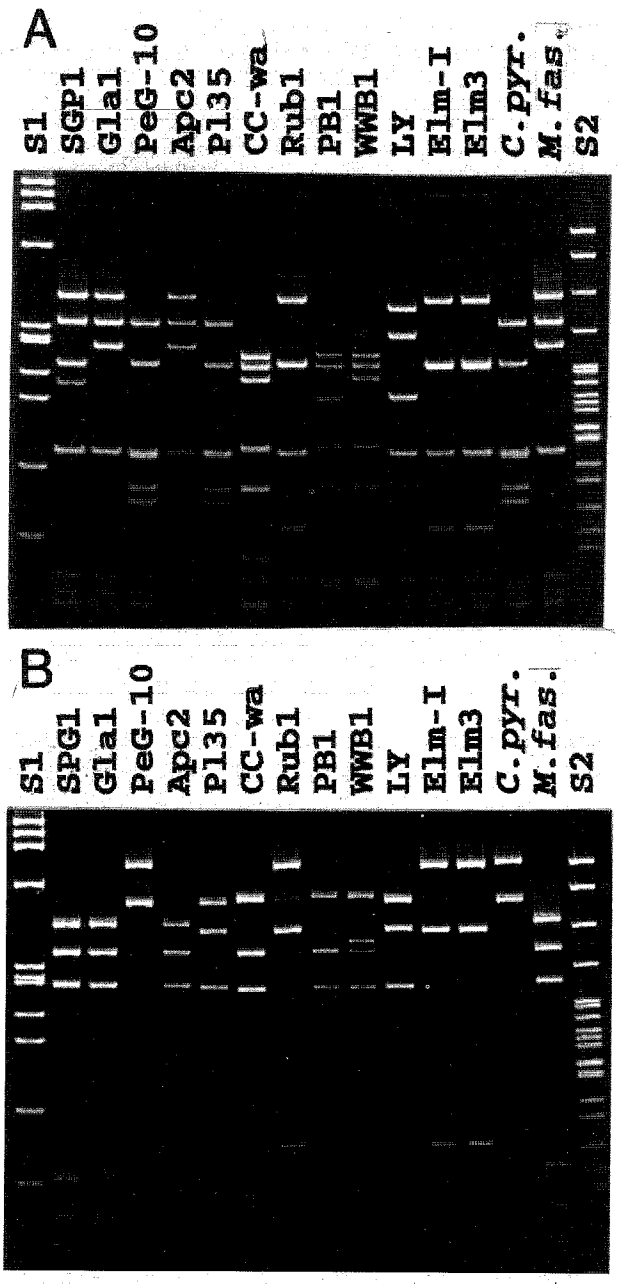

Fig. 4 - Restriction fragment length polymorphisms of phytoplasma 16S rDNA amplified by nested-PCR using the universal primer pair R16mF2/R1 followed by preamplification using the universal primer pair R16F2n/R2. DNA products were digested with restriction endonucleases (**A**, *Mse*I and **B**, *Rsa*I) and separated by electrophoresis through a 5% polyacrylamide gel. Lane S1, ϕ X174 RF I DNA *Hae*III digest, fragment sizes in base pairs from top to bottom are 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118, and 72; lane S2, pBR322 DNA *Msp*I digest, fragment sizes in base pairs from top to bottom are 622, 527, 404, 307, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110, 90, 76, 67, 34, 26, 15, and 9. Other abbreviations are given in Table I.

(America), group V; pear psyllid (*C. pyricola*), group X-A; and aster leafhopper (*M. fascifrons*), group I-B (Table I, Fig. 4).

Discussion

The major deficiencies associated with previous universal primer pairs designed by us and other research groups (Ahrens and Seemüller, 1992; Deng and Hiruki, 1991; Namba *et al.*, 1993) were lack of specificity, sensitivity, or universality. In particular, non-phytoplasma (healthy host or other prokaryote DNA) or non-specific DNA (residual) fragments were amplified by PCR assays using these primers, making it difficult to identify the associated phytoplasma(s) with absolute certainty. This was particularly true when the phytoplasma was associated with some woody hosts. The universality and sensitivity of the universal primer pair R16mF2/R1 in detection of the whole spectrum of phytoplasmas made it very suitable for routine detection of phytoplasmas associated with various sources of plants. The direct PCR assay using this primer pair successfully detected phytoplasmas from many woody hosts including several ornamental and fruit trees where sometimes residual DNA was amplified using other PCR primer pairs (Ahrens and Seemüller, 1992; Lee *et al.*, 1995) designed previously).

Nested-PCR assays using the external primer pair R16mF2/R1 and the nested-primer pair R16F2n/R2 greatly increased the sensitivity as well as specificity in detection of phytoplasmas in woody plant hosts in which phytoplasma titers are usually very low and in which the phytoplasmas are often unevenly distributed. Increased yield of specific PCR products by the nested-PCR assay provided sufficient 16S rDNA for further evaluation or confirmation of phytoplasma identities by RFLP analyses of the amplified 16S rDNA sequences. The nested-PCR assays using two universal primer pairs indiscriminately amplified the constituent phytoplasmas associated with a host. The yield of each specific PCR product presumably was proportional to the titer of each original template DNA (phytoplasma) present in the sample. Predominant phytoplasma strain(s) present in a single host, therefore, could be evaluated by this type of assay. To detect all the possible constituent phytoplasmas (some of which might be cryptic) associated with a single host, however, it was necessary to perform a series of nested-

PCR assays using the universal primer pair R16mF2/R1 and one of the phytoplasma group-specific primer pairs (Lee *et al.*, 1994, 1995).

Two major limiting factors that affect the sensitivity of a PCR assay are the presence of insufficient target DNA and the presence of reaction inhibitors. The nested-PCR assay using these two universal primer pairs circumvented the effect of the presumed inhibitors through the second amplification, and an increase in specific target DNA, and thus has greatly increased sensitivity in detection of low titer phytoplasmas associated with certain tree crops. Phytoplasmas could also be detected from symptomless trees in which phytoplasma titers were presumably very low (Lee *et al.*, 1995). The ability to detect phytoplasmas from insect hosts has added another dimension to the understanding of the complicated phytoplasma ecology and the disease epidemiology. Although this assay also detected some *Acholeplasma* spp., which are the closest relatives of phytoplasmas (Gundersen *et al.*, 1994; Namba *et al.*, 1993; Seemüller *et al.*, 1994), it does not present a major problem in detection of specific pathogens from plant hosts because no *Acholeplasma* has been reported to be associated internally with living plant tissues. The phytoplasma identities can be unambiguously confirmed by additional RFLP analyses of amplified 16S rDNA sequences. The sensitivity and universality of these primer sets in detection of phytoplasmas make them particularly suitable for etiological studies of unknown diseases that are suspected to be associated with phytoplasmas and for phytosanitary screening processes where a requirement of pathogen-free plant materials is essential.

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Literature cited

Ahrens U. and E. Seemüller, 1992. Detection of DNA of plant pathogenic mycoplasmalike organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. *Phytopathology, 82,* 828-832.

Bertaccini A., R.E. Davis, I.-M. Lee, M. Conti, E.L. Dally and S.M. Douglas, 1990. Detection of chrysanthemum yellows mycoplasmalike organism by dot hybridization and Southern blot anlysis. *Plant Disease, 74,* 40-43.

Bonnet F., C. Saillard, A. Kollar, E. Seemüller and J.M. Bove, 1990. Detection and differentiation of the mycoplasmalike organism associated with apple proliferation disease using cloned DNA probes. *Molecular Plant-Microbe Interactions, 3,* 438-443.

Chen K.H., J.R. Guo, N. Wu, N. Loi, L. Carraro, Y.H. Guo, Y.D. Chen, R. Osler, R. Pearson and T.A. Chen, 1993. Comparison of monoclonal antibodies, DNA probes, and PCR for detection of the grapevine yellows agent. *Phytopathology, 83,* 915-922.

Chen T.-A., J.D. Lei and C.P. Lin, 1992. Detection and identification of plant and insect mollicutes. *In: The Mycoplasmas.* Vol. 5 (R.F. Whitcomb and J.G. Tully, eds.), Academic Press, New York, 393-424.

Davis R.E. and I.-M. Lee, 1993. Cluster-specific polymerase chain reaction amplification of 16S rDNA sequences for detection and identification of mycoplasmalike organisms. *Phytopathology, 83,* 1008-1011.

Deng S. and C. Hiruki, 1991. Amplification of 16S rRNA genes from culturable and non-culturable mollicutes. *Journal Microbiological Method, 14,* 53-61.

Firrao G., E. Gobbi and R. Locci, 1993. Use of polymerase chain reaction to produce oligonucleotide probes for mycoplasmalike organisms. *Phytopathology, 83,* 602-607.

Gundersen D.E., I.-M. Lee, S.A. Rehner, R.E. Davis and D.T. Kingsbury, 1994. Phylogeny of mycoplasmalike organisms (phytoplasmas): a basis for their classification. *Journal Bacteriology, 176,* 5244-5254.

Gundersen D.E., I.-M. Lee, D.A. Schaff, N.A. Harrison, C.J. Chang, R.E. Davis and D.T. Kingsbury, 1996. Genomic diversity and differentiation among phytoplasma strains in 16S rRNA group I (aster yellows and related phytoplasmas) and III (X-disease and related phytoplasmas). *International Journal Systematic Bacteriology, 46,* 64-75.

Kirckpatrick B.C., D.C. Stenger, T.J. Morris and A.H. Purcell, 1987. Cloning and detection of DNA from a nonculturable plant pathogenic mycoplasmalike organism. *Science, 238,* 197-200.

Lee I.-M., A. Bertaccini, M. Vibio and D.E. Gundersen, 1995. Detection of multiple phytoplasmas in perennial fruit trees with decline symptoms in Italy. *Phytopathology, 85,* 728-735.

Lee I.-M. and R.E. Davis, 1992. Mycoplasmas which infect plants and insects. *In:* Mycoplasmas: Molecular Biology and Pathogenesis (J. Maniloff, R.N. McElhansey, L.R. Finch and J.B. Baseman, eds.). American Society for Microbiology, Washington, D.C., 379-390.

Lee I.-M., R.E Davis, T.-A. Chen, L.N. Chiykowski, J. Fletcher, C. Hiruki and D.A Schaff, 1992. A genotype-based system for identification and classification of mycoplasmalike organisms (MLOs) in the aster yellows MLO strain cluster. *Phytopathology, 82,* 977-986.

Lee I.-M., R.E. Davis, W.A. Sinclair, N.D. DeWitt and M. Conti, 1993a. Genetic relatedness of mycoplasmalike organisms detected in *Ulmus* spp. in U.S.A. and Italy by means of DNA probes and polymerase chain reactions. *Phytopathology, 83,* 829-833.

LEE I.-M., D.E. GUNDERSEN, R.W. HAMMOND and R.E. DAVIS, 1994. Use of mycoplasmalike organism (MLO) group-specific oligonucleotide primers for nested-PCR assays to detect mixed-MLO infections in a single host plant. *Phytopathology, 84,* 559-566.

LEE I.-M., R.W. HAMMOND, R.E. DAVIS and D.E. GUNDERSEN, 1993b. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. *Phytopathology, 83,* 834-842.

MCCOY R.E., A. CAUDWELL, C.J. CHANG, T.A. CHEN, L.N. CHIYKOWSKI, M.T. COUSIN, G.T.N. DALE DE LEEUW, D.A. GOLINO, K.J. HACKETT, B.C. KIRCKPATRICK, R. MARWITZ, H. PETZOLD, R.H. SHINA, M. SUGIURA, R.F. WHITCOMB, I.L. YANG, B.M. ZHU and E. SEEMÜLLER, 1989. Plant diseases associated with mycoplasmalike organisms. *In: The Mycoplasmas.* Vol. 5. (R.F. WHITCOMB and J.G. TULLY, eds.). Academic Press, New York, 545-560.

NAMBA S., S. KATO, S. IWANAMI, H. OYAIZU, H. SHIOZAWA and T. TSUCHIZAKI, 1993. Detection and differentiation of plant-pathogenic mycoplasmalike organisms using polymerase chain reaction. *Phytopathology, 83,* 786-791.

SARINDU N. and M.F. CLARK, 1993. Antibody production and identity of MLOs associated with sugar-cane whiteleaf disease and bermuda-grass whiteleaf disease from Thailand. *Plant Pathology, 42,* 396-402.

SCHAFF D.A., I.-M. LEE and R.E. DAVIS, 1992. Sensitive detection and identification of mycoplasmalike organisms by polymerase chain reactions. *Biochemical and Biophysical Research Communications, 186,* 1503-1509.

SCHNEIDER B., U. AHRENS, B.C. KIRKPATRICK and E. SEEMÜLLER, 1993. Classification of plant-pathogenic mycoplasma-like organisms using restriction-site analysis of PCR-amplified 16S rDNA. *Journal General Microbiology, 139,* 519-527.

SEEMÜLLER E., B. SCHNEIDER, R. MÄURER, U. AHRENS, X. DAIRE, H. KISON, K.-H. LORENZ, G. FIRRAO, L. AVINENT, B.B. SEARS and E. STACKEBRANDT, 1994. Phylogenetic classification of phytopathogenic mollicutes by sequence analysis of 16S ribosomal DNA. *International Journal Systematic Bacteriology, 44,* 440-446.

TULLY J.G., R.F. WHITCOMB, D.L. ROSE, J.M. BOVE, P. CARLE, N.L. SOMERSON, D.L. WILLIAMSON and S. EDEN-GREEN, 1995. *Acholeplasma brassicae* sp. nov. and *Acholeplasma palmae* sp. nov., two non-sterol-requiring mollicutes from plant surfaces. *International Journal Systematic Bacteriology, 44,* 680-684.

VIBIO M., A. BERTACCINI, I.-M. LEE, R.E. DAVIS, C. MINUCCI and R.G. MILNE, 1994. Etiology of lettuce yellows in Italy: genetic characterization of associated mycoplasmalike organisms. *Phytopathologia mediterranea, 33,* 179-186.

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