Detection of the Apple Proliferation and Pear Decline Phytoplasmas by PCR Amplification of Ribosomal and Nonribosomal DNA

K.-H. Lorenz, B. Schneider, U. Ahrens, and E. Seemüller

Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für Pflanzenschutz im Obstbau, Postfach 1264, D-69216 Dossenheim, Germany.

The work was supported by grants from the European Commission (CAMAR project CT90-0009 and AIR project CT92-0659) and the Deutsche Forschungsgemeinschaft.

We thank L. Carraro, M. F. Clark, M. T. Cousin, F. Dosba, L. Giunchedi, R. Marwitz, and G. Palm for providing phytoplasma sources. We also thank C. Saillard for supplying phytoplasma DNA clones pH184 and pH196.

Accepted for publication 13 April 1995.

ABSTRACT

Lorenz, K.-H., Schneider, B., Ahrens, U., and Seemüller, E. 1995. Detection of the apple proliferation and pear decline phytoplasmas by PCR amplification of ribosomal and nonribosomal DNA. Phytopathology 85: 771-776.

Ribosomal and nonribosomal sequences were analyzed to design polymerase chain reaction (PCR) primers for detection and identification of the phytoplasmas that cause apple proliferation (AP) and pear decline (PD). A ribosomal primer pair (fU5/rU3) was developed that initiated amplification of the target DNA from all 42 samples from PD-infected pear trees and 36 samples from AP-infected apple trees. These primers also amplified rDNA in all other taxonomically different phytoplasma strains that were tested. A pair of group-specific primers (fO1/rO1) derived from the 16S rRNA gene was identified for detection of the closely related phytoplasmas associated with AP, PD, and European stone fruit yellows. PCR detection of the PD agent with primer pairs fU5/rU3 and fO1/rO1 was considerably more sensitive than microscopic detection using the 4′-6-diamidino-2-phenylindole fluorescence method. A more specific ribosomal primer pair (fPD/rO1) amplified phytoplasma rDNA in all samples from infected pear trees but showed some cross-amplification of AP rDNA. Restriction enzyme analysis of the PCR products obtained with primer pairs fO1/rO1 and fPD/rO1 distinguished the AP and PD phytoplasmas. One pair of ribosomal (fPD/rPDS) primers specifically amplified DNA of the PD phytoplasma but from only about 80% of the infected pear trees. Three pairs of nonribosomal primers amplified phytoplasmal DNA from AP- or PD-infected trees or from both phytoplasmas but failed to detect all strains of either of the two pathogens. These results show that is not possible to detect all strains of the AP and PD phytoplasmas, respectively, with pathogen-specific primers.

Additional keywords: restriction fragment length polymorphism, stone fruits.

Apple proliferation (AP) is the most important graft-transmissible and vector-borne disease of apple in the southern half of Europe (16). Pear is affected by a similarly serious disease, pear decline (PD), that occurs mainly in Europe and North America but that seems to occur in most or all areas of the world where the domestic European pear (*Pyrus communis* L.) is grown (25). Both diseases are caused by nonculturable mycoplasmalike organisms, for which the trivial name phytoplasmas has been proposed (12). Recent phylogenetic studies based on sequence analysis of the conserved 16S ribosomal RNA gene showed that the AP and PD agents are closely related organisms that show a 16S rDNA sequence similarity of 98.5% (26). They form, together with the European stone fruit yellows (ESFY) agent and the buckthorn (*Rhamnus catharticus*) witches' broom (BWB) phytoplasma, the AP strain cluster (26). A close interrelatedness of the phytoplasmas of this cluster also has been found by sequence analysis of the spacer region between the 16S and 23S rRNA genes (14). However, based on spacer sequences, two types of phytoplasmas were found in samples from PD-diseased pears collected in California; one was similar to the European type, while the other was closely related to the western X-disease agent (14).

For detection and identification of phytoplasmas, polymerase chain reaction (PCR) technology is increasingly employed. It offers several advantages, including versatility, relative simplicity, specificity, and high sensitivity. PCR is more sensitive than microscopic, serological, and hybridization methods (3,11,19) and, therefore, is suitable for the detection of low-titer phytoplasma infections in woody plants (3). There are several reports on the detection of the AP and PD agents using PCR primers derived from ribosomal and nonribosomal fragments of the phytoplasma chromosome (2,6,9,10,13). The ribosomal primers used were not specific for the AP and PD pathogens but amplified either rDNA of all phytoplasmas or all phytoplasmas plus other prokaryotes. For that reason, the identity of the amplified fragments had to be determined by restriction site analysis (2,6,10) or hybridization with pathogen-specific probes (9). The nonribosomal primers were specific for the phytoplasmas of the AP strain cluster that infect pome and stone fruit trees and allowed some differentiation within this group (13). However, for a clear distinction of the different types of fruit phytoplasmas, restriction fragment length polymorphism (RFLP) analysis was used with these primers as well. The usefulness of primers was only assessed on a small number of samples. In the work presented here, we compared the specificity of various ribosomal and nonribosomal primers for the detection of the AP and PD phytoplasmas. One of the goals of this study was to determine whether PCR primers could be identified that specifically detect all strains of the two pathogens.

Corresponding author: E. Seemüller
E-mail address: ba69ed@genius.embnet.dkfz-heidelberg.de

MATERIALS AND METHODS

Sources of phytoplasmas. Shoot samples from the following naturally infected pear trees were examined: 12 trees (PD1 through 12) from the experimental field of the Dossenheim Institute

(southwestern Germany), 5 trees (PI1 through 5) from the Emilia Romagna region of northern Italy, 3 trees (PF1 through 3) from various locations in southern France, and 2 trees (E1 through 2) from Kent, England. These trees were 8 to 20 years old and showed PD symptoms, including foliar reddening, reduced vigor, and decline. Root samples were collected from 1 tree (PBS) at Braunschweig, 8 trees (PJ1 through 8) from the Hamburg area and 7 trees (PB1 through 7) from the Berlin area (all northern Germany) and 4 trees (PH1 through 4) from the Halle area (central Germany). These trees were between 20 and 60 years old and mostly showed less-pronounced symptoms, such as poor terminal growth. Root or shoot samples from 30 naturally infected apple trees were collected at various locations in southwestern Germany. Most of these trees (A1 through A23) were 8 to 20 years old and showed witches' brooms and/or enlarged stipules as specific AP symptoms. Seven of these trees as well as five trees, from which root samples were collected, in central Germany near Kassel were 40 to 70 years old and exhibited poor terminal growth as nonspecific symptoms or were nonsymptomatic. One apple tree, approximately 60 years old and showing enlarged stipules, was sampled in Burgundy, France. Samples from healthy apple, pear, peach, and apricot trees as well as 1 peach and 1 apricot tree infected with the ESFY phytoplasma were taken from trees growing at the Dossenheim Institute.

A number of phytoplasma strains that previously have been transmitted to periwinkle (*Catharanthus roseus*) and maintained in this host by periodic grafting were included in this study. These phytoplasmas have been previously classified and represent all primary clusters and subgroups established by Schneider et al. (22) and Seemüller et al. (26). They comprise strains AAY, ACLR, AV2192, KV, PLN-V6, PRIVB, SAFP, SAS, and SAY from the aster yellows subgroup of the aster yellows strain cluster; strains MOL, STOL, and STOLF from the stolbur subgroup of the aster yellows strain cluster; AP strains AP1 and AT from the AP strain cluster; strains GVX, PYLR, and VAC from the western X-disease strain cluster; strain SUNHP from the faba bean phyllody strain cluster; strain BVK from the sugarcane white leaf strain cluster; strains EY and ULW from the elm yellows subgroup of the elm yellows strain cluster; and strain ASHY from the ash yellows subgroup of the elm yellows strain cluster. Further information on these strains and their classification is given elsewhere (21,22,26).

DNA isolation and detection procedures. From trees either petioles plus midribs or phloem tissue from stem parts or roots were used. Phloem tissue was prepared as described (3). Young shoots including leaves were taken from periwinkle. DNA was isolated from approximately 1.0 g of fresh tissue using a phytoplasma enrichment procedure as described previously (2). PCR was carried out in a 40 µl volume containing 100 to 200 ng of template DNA, 0.5 µM each primer, 100 µM the four dNTPs, 0.2 units of Goldstar polymerase (from a *Thermus* sp. plasmid, Eurogentec, Seraing, Belgium), and 1× polymerase buffer (Eurogentec). The mixture was overlaid with 40 µl of mineral oil and was subjected to 30 or 35 cycles at the following incubations: 30 s of denaturation at 95°C, 75 s of annealing at 55°C (52°C with primer pair P1/P7), and 90 s of extension at 72°C. The amplification products were analyzed by either direct agarose gel electrophoresis or by restriction enzyme digestion followed by agarose or polyacrylamide gel electrophoresis (2,21). Southern blot hybridization of *Hin*dIII-digested DNA from AP-infected apple trees with the two cloned chromosomal DNA fragments IH184 and IH196 (5) of strain AT of the AP phytoplasma was carried out as described (1). Microscopic detection of phytoplasma infections was performed using the DAPI (4′-6-diamidino-2-phenylindole) fluorescence method (24).

TABLE 1. Oligonucleotide primers designed for phytoplasma detection

Primer	Target	Primer sequence (5′–3′)	Position in 16S rDNA[a]
fU5	16S rDNA	CGG CAA TGG AGG AAA CT	369–386
rU3	16S rDNA	TTC AGC TAC TCT TTG TAA CA	1251–1231
fO1	16S rDNA	CGG AAA CTT TTA GTT TCA GT	65–91
rO1	16S rDNA	AAG TGC CCA ACT AAA TGA T	1136–1115
fPD	16S rDNA	GAC CCG TAA GGT ATG CTG A	204–224
rPDS	16S/23S spacer	CCC GGC CAT TAT TAA TTT TA	
fCAP	Insert AT67	GGT TAC TCA CGA TCA AGA AG	
rCAP	Insert AT67	GTC CCA TCT ATT TTA GAG GC	
fCPD	Fragment PD67	CCA TAG CGA ATG TTT AAA AC	
rCPD	Fragment PD67	CAG TGC GAA AAT TGG TTA AT	
f184	Fragment IH184/13	CAT TGC ATA TTA ACT TAT TAA CC	
r184	Fragment IH184/13	GTG ACA CTT CGT TGC AAC ATA	

[a] All positions correspond to positions in the sequence of aster yellows strain OAY (18).

Primer selection. The primers based on ribosomal sequences (Table 1) were identified by aligning the sequences of the 16S rDNA (sometimes including the 16S/23S rDNA spacer region) of strains AP1, AT, PD1 (PD phytoplasma), PPER (ESFY phytoplasma), and BWB from the AP strain cluster; and strains STOL, VK, ACLR, OAY, SAY, AAY, SUNHP, WX, VAC, BVK, SCWL, FD, ULW, and ASHY from the other major taxonomic clusters established by Schneider et al. (22) and Seemüller et al. (26). The primers fU5/rU3 were designed for universal phytoplasma detection. Of these, the sequence of fU5 was fully homologous with the corresponding sequences of all phytoplasmas that were compared, whereas primer rU3 was fully homologous with most of these strains (differences in one or two bases to strains ACLR, SAY, ULW, and STOL). However, both primers showed considerable differences to corresponding sequences of the other mollicutes and the plant chloroplasts that were examined by Ahrens and Seemüller (2). The primer pair fO1/rO1, designed for the detection of all known European fruit tree phytoplasmas, was fully homologous with the corresponding sequences of AP strains AP1 and AT, PD strain PD1, and ESFY strain PPER but differed in several bases from sequences of strains from other clusters. Primer fPD, identified for specific detection of the PD phytoplasma, differed in three positions from the AP and ESFY strains and had more mismatches with strains from the other clusters. Primer rPDS, also designed for specific amplification of PD phytoplasma DNA, was derived from the 16S/23S rDNA spacer region of strain PD1 and comprised the first two bases from the conserved $tRNA^{Ile}$ gene and 18 bases upstream of this gene. This primer differed in two positions from the corresponding sequences of strains AP1 and AT, and in four positions from strain PPER. The homology with strains from other phytoplasma clusters was lower.

For designing nonribosomal primers, the cloned *Eco*RI DNA fragment AT67 (2.4 kb) from the chromosome of strain AT (23) was selected as target DNA. In Southern hybridization experiments, this fragment hybridized with DNA from PD and ESFY phytoplasmas but not with strains from groups other than the AP strain cluster (23; B. Schneider and E. Seemüller, *unpublished data*). Fragment AT67 was partially sequenced from both ends using the Sequenase kit according to the manufacturer's instructions (U.S. Biochemical, Cleveland). Based on the sequences obtained, the primer pair fCAP/rCAP was synthesized (Table 1). The amplification products obtained with these primers from two pear samples were partially sequenced from both ends by cycle sequencing as described (26), using the fmol DNA sequencing system (Promega Corp., Madison, WI). By comparing the two pear sequences, which were identical, with the corresponding sequences of fragment AT67, two primers were designed for detection of the PD phytoplasma. Of these oligonucleotides, the forward primer, fCPD, differed in four nucleotide positions, and the reverse primer, rCPD, differed in two positions from the corresponding sequences of strain AT (Table 1). Primer pair f184/r184 was derived from the 3.1-kb chromosomal *Hin*dIII fragment IH184 of strain AT (5). This fragment was subcloned in the *Hin*dIII/*Cla*I site of Bluescript M13+. For primer selection, the resulting 1.3-kb fragment was partially sequenced as described above for fragment AT67 (Table 1).

Ribosomal phytoplasma DNA from infected apple and pear trees also was amplified using the primer pair P1 (8) and P7 (14). Of these primers, oligonucleotide P1 primed at the 5′ end of the 16S rRNA gene and P7 primed at the 5′ region of the 23S rRNA gene. The fragment obtained was about 1,800 bp in length.

RESULTS

Homogeneity of material examined. RFLP analysis of ribosomal DNA was carried out to investigate the homogeneity of the phytoplasma strains examined. DNA from 22 shoot samples from infected pear trees and from 26 root and shoot samples from infected apple trees was PCR-amplified using primer pair P1/P7. After digestion with the restriction enzymes *Alu*I and *Rsa*I, all samples and strains of AT and AP1 of the AP phytoplasma showed the same restriction profile. Examples of the profiles are shown in Figure 1.

Phytoplasma detection using ribosomal primers. With primer pair fU5/rU3 the target DNA was amplified from all 36 samples taken from infected apple and all 42 samples collected from infected pear trees. Also, DNA from the diseased apricot and peach trees as well as all phytoplasma strains that were maintained in periwinkle was amplified (Table 2; Figs. 2 and 3A). Primer pair fO1/rO1 amplified the target DNA in all samples from infected apple and pear trees as well as in templates from the diseased apricot and peach trees. The amplification product also was obtained from strains AT and AP1 of the AP agent (Table 2; Figs. 3B and 4). Primer pair fPD/rO1 amplified the target DNA in all samples from infected pear trees; 58% of the infected apple trees also were identified, although some of the signals were relatively weak (Fig. 3C). No amplification was obtained with primer pair fPD/rO1 with DNA from the diseased apricot and peach trees (Table 2). Primer pair fPD/rPDS was, as intended, specific for the PD phytoplasma and did not amplify DNA from the plants infected with the AP phytoplasma and the diseased apricot and peach trees. However, only 81% of the diseased pear trees tested positively with these primers (Table 2; Fig. 3D). Primer pairs fO1/rO1, fPD/rO1, and fPD/rPDS did not amplify DNA from phytoplasma strains from groups other than the AP strain cluster, and none of the ribosomal primers amplified DNA from healthy apple, pear, apricot, peach, and periwinkle (Figs. 2, 3, and 4; data not shown).

Phytoplasma detection with nonribosomal primers. Primer pair fCAP/rCAP primed amplification of the target DNA from both apple and pear samples but failed to detect all infected trees of the two species. Although derived from AP phytoplasma sequences, these primers showed only a slight preference for this organism, which they detected in 67% of the samples from infected apple trees. The target sequence also was amplified in sample DNA from strains AT and AP1 of the AP agent but not from the other periwinkle-maintained strains examined. The PD phytoplasma was detected in 60% of the infected pear trees. Some samples from both apple and pear yielded only weak PCR signals with these primers (Table 2; Fig. 3E). Primer pair fCPD/rCPD amplified the target DNA in 76% of the samples from infected pear trees, whereas no observable PCR products were obtained with template DNA from diseased apple trees or strains AT and AP1. However, the diseased apricot and peach trees tested positively with these primers, and weak PCR signals were obtained from a few periwinkle-maintained strains (ACLR, PLN-V6, MOL) of groups other than the AP strain cluster (Table 2; Fig. 3F). Some of the infected pear trees (PJ1, PJ4, PJ5, PJ6, PJ8, and PF2), which were not detected with the nonribosomal primer pairs fCAP/rCAP and fCPD/rCPD, were not detected with the ribosomal primer pair fPD/rPDS.

Primer pair f184/r184 from strain AT amplified AP phytoplasma DNA in only 47% of the infected apple trees, whereas no amplification products were obtained from the pear samples (Table 2). Southern blot analysis revealed that the apple trees that tested positively with these primers were infected with AP phytoplasma strains that showed a *Hin*dIII restriction fragment of the same size as probe IH184 (3.1 kb). The majority of AP phytoplasma strains that exhibited a smaller *Hin*dIII fragment (2.2 kb) hybridizing with this probe were not detected with primer pair f184/r184 (data not shown). The detection results obtained with the nonribosomal primers were similar when the annealing tem-

TABLE 2. Detection of apple proliferation, pear decline, European stone fruit yellows (ESFY), and other phytoplasmas by polymerase chain reaction using primers with different specificities

Primer pair	Detection in pear samples (%)	Detection in apple samples[a] (%)	Detection of ESFY phytoplasma[b]	Detection of other phytoplasmas[c]
fU5/rU3	100	100	Yes	Yes
fO1/rO1	100	100	Yes	No
fPD/rO1	100	58	No	No
fPD/rPDS	81	0	No	No
fCAP/rCAP	60	67	No	No
fCPD/rCPD	76	0	Yes	Few
f184/r184	0	47	No	No

[a] Including periwinkle-maintained apple proliferation (AP) strains AT and AP1.
[b] One sample from diseased apricot and one sample from diseased peach.
[c] Periwinkle-maintained phytoplasma strains from groups other than the AP strain cluster.

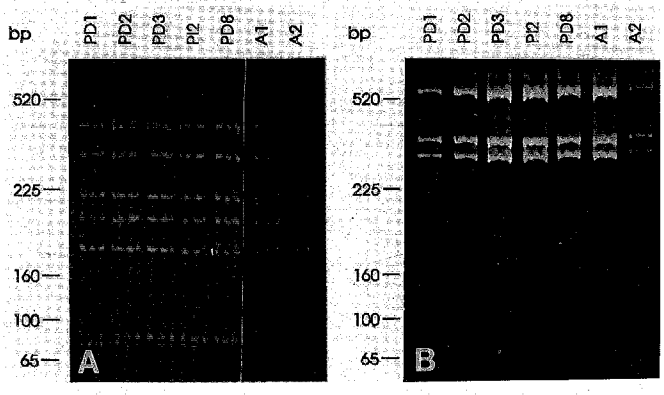

Fig. 1. A, *Alu*I and **B,** *Rsa*I restriction profiles of ribosomal DNA amplified using primer pair P1/P7. The template DNA was from decline-diseased pear trees (PD1, 2, 3, and 8 and P12) and proliferation-diseased apple trees (A1 and A2).

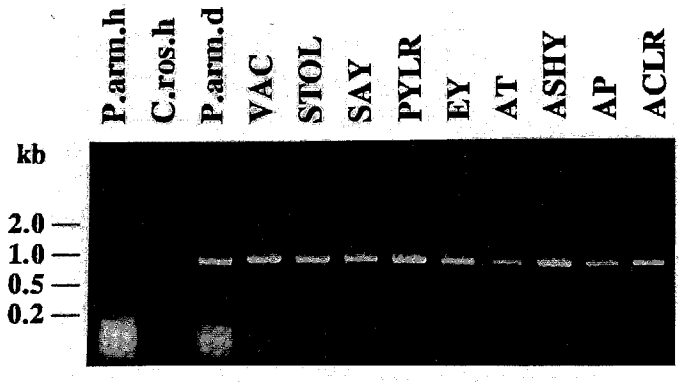

Fig. 2. Polymerase chain reaction amplification of a 16S rDNA fragment from phytoplasmas representing several phylogenetic clusters with universal primer pair fU5/rU3. The templates consisted of DNA extracted from healthy *Prunus armeniaca* (*P.arm.*h, apricot), healthy *Catharanthus roseus* (*C.ros.*h, periwinkle), infected *P. armeniaca* (*P.arm.*d), or from periwinkle plants infected with the following phytoplasma strains: VAC, vaccinium witches' broom; STOL, stolbur; SAY, severe American aster yellows; PYLR, western X-disease; EY, elm yellows; AT, apple proliferation (strain AT); ASHY, ash yellows; AP, apple proliferation (strain AP1); and ACLR, an aster yellows-type phytoplasma from apricot.

perature was lowered to 52°C. None of these primers amplified DNA from the healthy test plants (Fig. 3; data not shown).

Distinction of the AP and PD phytoplasmas by RFLP analysis. Because none of the primer pairs employed allowed the specific detection of all strains of the AP and PD phytoplasmas, we evaluated restriction enzymes that cleave the amplification product obtained with group-specific primer pair fO1/rO1. Enzymes *Ssp*I and *Sfe*I were suitable for the differentiation of AP and PD strains. Endonuclease *Ssp*I digested AP phytoplasma rDNA at position 419, whereas the PCR fragment from the PD phytoplasma was not digested. *Sfe*I digested the PD phytoplasma PCR product at positions 630 and 998, whereas the AP phytoplasma fragment was cut only at the latter position. The restriction patterns resulting from these cleavage sites were shown by all 15 samples from diseased apple and pear trees that were examined. Restriction profiles of the fragment amplified with primer pair fPD/rO1 were similar to those obtained with the amplification product of primer pair fO1/rO1. Examples of the RFLP profiles are shown in Figure 5. The AP and PD phytoplasmas also were distinguished by digesting the PCR fragments obtained with primer pair fCAP/rCAP with *Alu*I and *Rsa*I; there was no variation within the AP or PD samples (data not shown).

Sensitivity of detection. Although all infected pear trees were detected with primer pairs fU5/rU3, fO1/rO1, and fPD/rO1, only 57% of the pear samples tested positively when DAPI fluorescence microscopy was employed. Unclear results were obtained with 12% of the pear trees, and 31% were negative using DAPI staining.

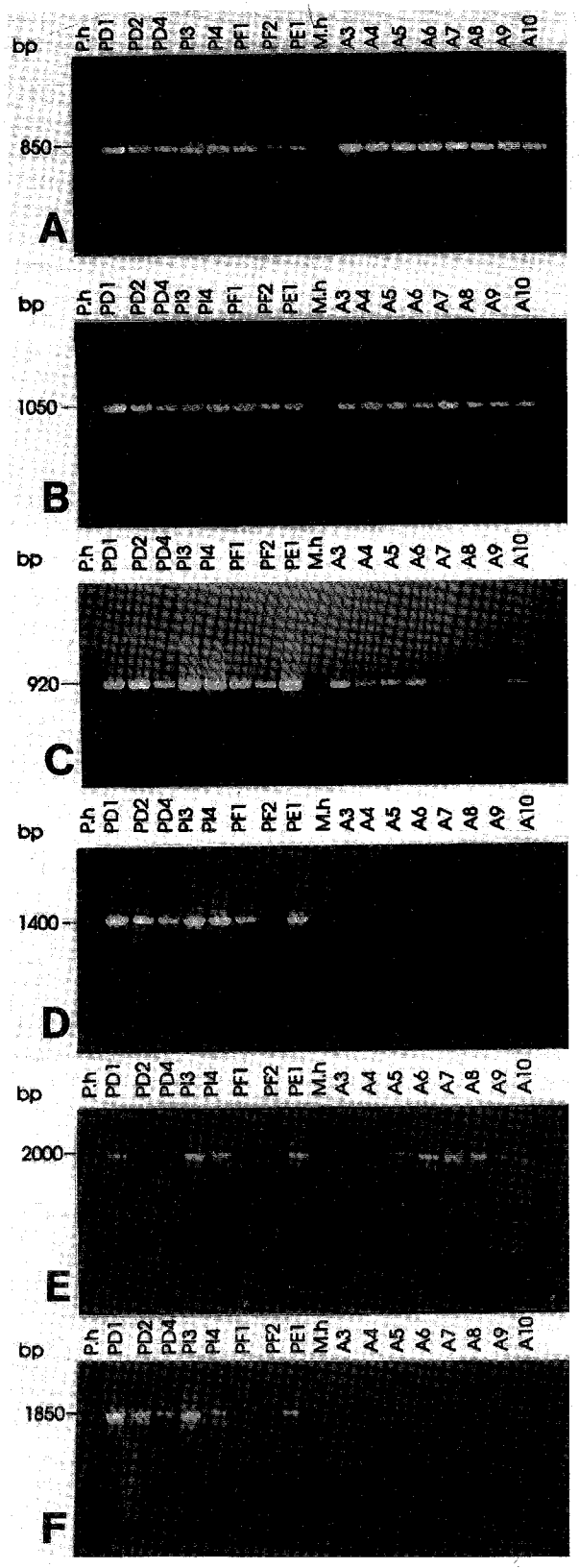

Fig. 3. Examples of the results obtained with ribosomal primer pairs: **A,** fU5/rU3, **B,** fO1/rO1, **C,** fPD/rO1, **D,** fPD/rPDS; and nonribosomal primer pairs: **E,** fCAP/rCAP and **F,** fCPD/rCPD in amplifying template DNA extracted from decline-diseased pear trees (PD1–PE1) and proliferation-diseased apple trees (A3–A10). P.h, healthy pear; M.h, healthy apple.

DISCUSSION

In various attempts to detect phytoplasmas in plant and insect vectors by PCR, primers derived from the 16S rRNA gene were employed most often. In several cases, the amplification products were not specific for a certain organism, so identification had to be achieved by either RFLP analysis or hybridization with pathogen-specific probes (2,8,9,10). In contrast, several strains from the aster yellows group were specifically detected with 16S rDNA-directed primers (7), and in tests with nonribosomal primers, a relatively narrow detection range was observed (4,13,20). Therefore, since their specificity is usually higher than that of most of the 16S rDNA-based primers, nonribosomal primers were included in this study and compared with ribosomal primers for their suitability to detect and distinguish the AP and PD phytoplasmas in Europe.

Sequence analysis of the 16S rRNA gene and the 16S/23S rDNA intergenic region of a few strains of the AP phytoplasma and one strain of the European PD phytoplasma revealed that the two

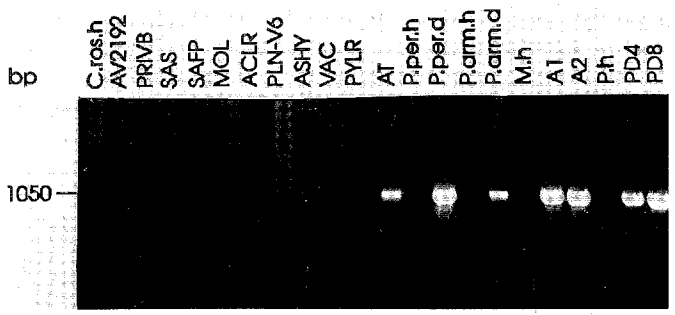

Fig. 4. PCR amplification of a 16S rDNA fragment from phytoplasmas representing several phylogenetic clusters using group-specific primer pair fO1/rO1. The templates consisted of DNA extracted from healthy *Catharanthus roseus* (*C.ros.*h, periwinkle) and from periwinkle plants infected with the following phytoplasma strains: AV2192, aster yellows; PRIVB, aster yellows from *Primula* sp., SAS, sandal spike (aster yellows); SAFP, safflower phyllody (aster yellows); MOL, Molières disease (stolbur); ACLR, aster yellows-type phytoplasma from apricot; PLN-V6, aster yellows-type phytoplasma from Japanese plum; ASHY, ash yellows; VAC, vaccinium witches' broom; PYLR, western X-disease; AT, apple proliferation (strain AT). *P.per.*h, healthy *Prunus persica* (peach); *P.per.*d, infected peach; *P.arm.*h, healthy *P. armeniaca* (apricot); *P.arm.*d, infected apricot; *M.*h., healthy *Malus domestica* (apple); A1 and A2, infected apples; *P.*h., healthy *Pyrus communis* (pear); and PD4 and PD8, infected pears.

pathogens are closely related (14,26). We investigated relatedness and homogeneity of several AP and PD phytoplasma strains by RFLP analysis of PCR-amplified rDNA using the restriction enzymes *Alu*I and *Rsa*I, a method suitable for phylogenetic characterization and classification of plant-pathogenic phytoplasmas (17,21). All strains of both pathogens showed the same restriction profile, confirming that the phytoplasmas infecting apple and pear were closely related.

The similarity of the AP and PD phytoplasmas made it impossible to differentially detect all strains of each of the two pathogens by PCR amplification. Apart from the universal primer pair fU5/rU3, which initiated amplification of the target DNA from all phytoplasmas that were tested, detection of all AP and PD phytoplasma strains also was achieved with primer pair fO1/rO1. Primer pair fO1/rO1 was specific for the fruit tree phytoplasmas of the AP strain cluster, amplifying the target DNA from all strains of the AP and PD agents and two strains of the ESFY phytoplasma and also all other strains of the ESFY agent we examined (K.-H. Lorenz, B. Schneider, and E. Seemüller, *unpublished data*). None of the primer pairs tested specifically amplified DNA from all strains of both the AP and PD phytoplasmas. Primer pair fPD/rO1, which amplified DNA from all strains of the PD phytoplasma, also amplified AP phytoplasma DNA. One primer pair (fPD/rPDS) was, as intended, specific for the PD phytoplasma but did not detect all strains. Similarly, primer pair fCPD/rCPD amplified the target DNA in most of the samples from infected pear trees and did not detect the AP phytoplasma. On the other hand, primer pair f184/r184 specifically amplified AP phytoplasma DNA but only from those strains of the AP phytoplasma that had the 3.1-kb *Hin*dIII fragment present in strain AT. The strains detected with primer pair f184/r184 represent only some of the restriction profiles that were detected in the AP phytoplasma (15; B. Schneider and E. Seemüller, *unpublished data*).

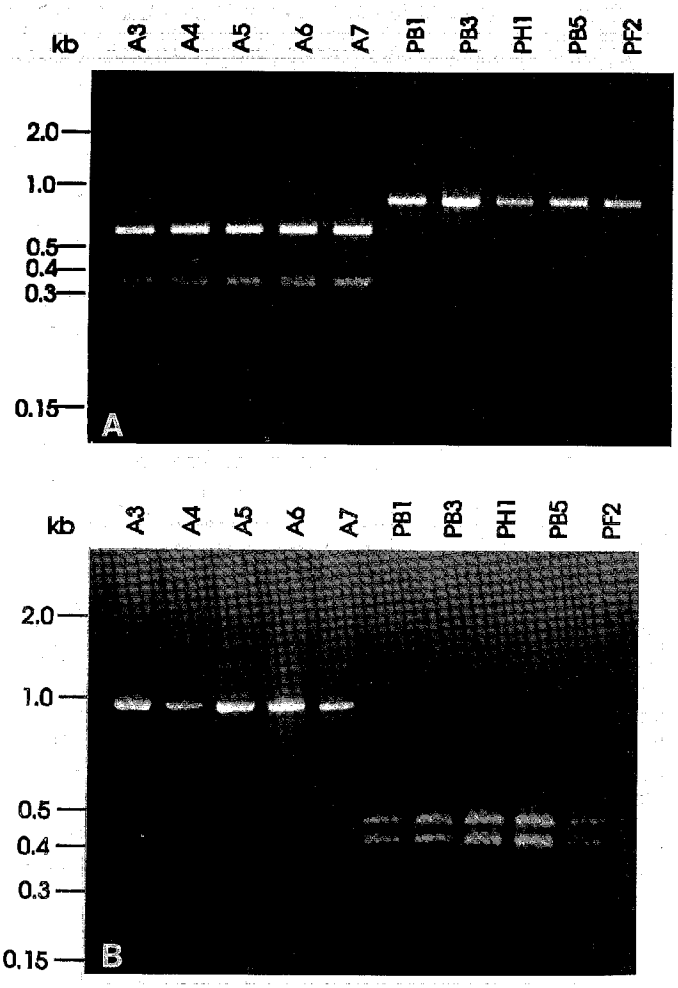

Fig. 5. Agarose gel electrophoresis of polymerase chain reaction products obtained with group-specific ribosomal primer pair fPD/rO1 from template DNA extracted from proliferation-diseased apple trees (A3–A7) and decline-diseased pear trees (PB1–PF2) following digestion with **A**, *Ssp*I or **B**, *Sfe*I. A 138-bp *Sfe*I fragment present in all samples of **B** is not discernible.

The finding that some of the primer pairs developed for specific detection of the PD phytoplasma cross-amplify the AP agent and vice versa supports the similarity of the AP and PD phytoplasmas. However, the PCR amplification also provided evidence that the causal agents are distinct. For instance, ribosomal (fPD/rPDS) and nonribosmal (fCPD/rCPD) primer pairs amplified the target DNA from about 80% of the PD-infected pear trees but did not amplify AP phytoplasma DNA. Since there is more sequence variability in the variable regions of the 16S/23S spacer, from which primer rPDS was derived, than in the variable regions of the 16S rDNA (14), it is possible that the target sequence of primer rPDS differed more among the samples than in the target sequence of the ribosomal primers. This may result in a less efficient amplification of the spacer primers and in the failure to obtain a detectable amplification product from samples with a low phytoplasma titer. That primer pairs fPD/rPDS and fCPD/rCPD are less sensitive than the 16S rDNA-based primer pairs fO1/rO1 and fPD/rO1 is supported by the observation that most of the samples that tested negatively with primer pair fPD/rPDS also were phytoplasma-negative with primer pair fCPD/rCPD and were mostly DAPI-negative.

Evidence that AP and PD are caused by distinct phytoplasmas also was obtained in restriction site analysis. Although digestion of rDNA of the AP and PD agents with *Alu*I and *Rsa*I resulted in similar profiles, the two phytoplasmas were clearly distinguishable by cleaving 16S rDNA with *Ssp*I and *Sfe*I and a nonribosomal DNA fragment with *Alu*I and *Rsa*I. The geographic distribution of the AP and PD phytoplasmas also may indicate they are distinct pathogens. Thus, the limitation of AP to certain parts of Europe and the occurrence of PD in all fruit-growing areas of Europe and North America and probably elsewhere (16,25) would be difficult to explain if the diseases are caused by the same pathogen. A similar etiology would only be possible if the differences in the occurrence are not due to the distribution of the pathogen but to that of the vectors, which are still unknown as far as the AP phytoplasma is concerned. Therefore, further studies on vector-pathogen relationship as well as on pathological and genetic aspects are necessary before firm conclusions about distinction and host specificity of the AP and PD phytoplasmas can be drawn.

Apart from the fact that the AP and PD phytoplasmas are closely related, variability within the pathogens seems to be the major reason for the difficulties in specifically detecting all strains of the causal agents. Such variability of the AP phytoplasma has been observed in Southern hybridization studies using nonribosomal DNA fragments as probes (15). The genetic differences observed in Southern hybridization and PCR amplification are not related to the geographic origin of the samples. Due to variability, detection of all strains of a given pathogen was achieved only with 16S rDNA-directed primers that cross-amplified DNA from the phytoplasma associated with the other host or even from other members of the AP strain cluster, such as the ESFY phytoplasma. The application of such primers requires the use of RFLP analysis of the amplification products for pathogen differentiation. Endonucleases *Ssp*I and *Sfe*I proved suitable for differentiation of the AP and PD phytoplasmas. Similarly, the ESFY phytoplasma can be distinguished using *Bsa*AI digestion (K.-H. Lorenz, *unpublished data*). However, in practical diagnosis, such a differentiation would only be necessary if the phytoplasmas infecting pome and stone fruits in Europe are not host specific. In contrast to the 16S rDNA-directed primers, the primers derived from the 16S/23S rDNA spacer region and from nonribosomal sequences

were too specific to detect all strains of the target organism. The difficulties described here in specifically detecting two closely related phytoplasmas indicate that extensive testing of primers is necessary for successful application of PCR for diagnosis and that substantial numbers of field-collected samples need to be included when assessing the usefulness of putative pathogen-specific PCR primers.

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