

Detection of an Elm Yellows-Related Phytoplasma in Eucalyptus Trees Affected by Little-Leaf Disease in Italy

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ABSTRACT

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Eucalyptus trees showing yellowing and decline in central and southern Italy were examined for phytoplasmal infection using fluorescence microscopy (DAPI test) and polymerase chain reaction (PCR) assays. Neither the DAPI test nor direct PCR using universal and group-specific phytoplasma primers detected phytoplasmas. However, when the products obtained in the initial amplification were reamplified with universal or group-specific nested primers, phytoplasmal DNA was detected in about half of the trees. Restriction patterns of the PCR-amplified ribosomal DNA and use of specific primers indicated that the eucalyptus trees were infected by a phytoplasma of the elm yellows group. The name eucalyptus little-leaf (ELL) phytoplasma is proposed for the causal agent. The phytoplasma-infected trees differed from the trees that tested negatively by the expression of little-leaf symptoms and witches'-brooms, which are both typical of the phytoplasma-induced little-leaf diseases of eucalyptus reported from Asia and the Sudan.

Plant-pathogenic phytoplasmas (formerly called mycoplasma-like organisms [MLOs]) induce diseases in several hundred plant species, including several *Eucalyptus* spp. (24). One of the first reports of a eucalyptus phytoplasmosis was of the little-leaf disease described by Sastry et al. (29) from India, a disorder which was then thought to be caused by a virus. Later, a similar disease was observed by other researchers in India, who discovered phytoplasmal infections in affected trees by electron and light microscopy (9,25,26,33). Phytoplasmal diseases of eucalyptus have also been reported to occur in China (34) and the Sudan (6). The symptoms described from the various geographic areas and different *Eucalyptus* spp. are similar. They include, apart from the reduced leaf size, shortened internodes and precocious growth from axillary buds, both giving the shoots a bushy, broomlike appearance. Diseased trees or symptomatic portions of them do not set fruits, are stunted, and die back. Severely affected trees decline (9,33).

All reports on the etiology of eucalyptus little-leaf disease are based on microscopic observations, which do not allow distinction among the phytoplasmas. Thus, the identity of the causal agent(s) was unclear.

Also, the sensitivity of microscopic methods is not always sufficient for detection of low-titer phytoplasmal infection (3).

The amplification of phytoplasmal DNA by the polymerase chain reaction (PCR) has proved to be a highly sensitive technique for phytoplasma detection and identification, depending on the primers used (2,14,16). Sensitivity of PCR detection can further be improved by hybridizing the amplification products with oligonucleotide probes (3) or by reamplifying the DNA fragments obtained in the first amplification using internal primers (nested PCR) (13). We employed both methods to study the etiology of a eucalyptus disease that is widespread in central and southern Italy. Symptoms of the disorder are similar to those described for the little-leaf disease from Asia and the Sudan. According to a preliminary note, the disease has been present in Italy for at least 15 years (28).

MATERIALS AND METHODS

Plant samples and phytoplasma reference strains. Samples from asymptomatic eucalyptus trees and from others showing yellowing, proliferating shoots, stunting, dieback, and decline were collected in the Campania, Basilicata, Latium, and Apulia regions of central and southern Italy. Eucalyptus seedlings grown in an insect-proof greenhouse were used as healthy controls. Plants were identified only to the genus level.

Several strains of the elm yellows (EY) phytoplasma were included in this study for comparison. They comprise strains ULW from European field elm (*Ulmus*

minor Mill.), collected by G. Morvan (Avignon-Montfavet, France), and EY1 from American elm (*U. americana* L.), collected by W. A. Sinclair (Ithaca, NY). Both were maintained in periwinkle (*Catharanthus roseus*). In addition, samples from EY-affected trees of European field elm collected in the Basilicata region were examined. Other reference strains representing major phylogenetic groups established by Seemüller et al. (32) and described elsewhere (22,30) were compared. These strains included the aster yellows, stolbur, apple proliferation, ash yellows, western X-disease, and sunnhemp witches'-broom agents as well as a sugarcane white leaf-related phytoplasma. These strains were also maintained in periwinkle.

Fluorescence microscopy and DNA extraction. Microscopic detection of phytoplasmal infections was performed using the DAPI (4'-6-diamidino-2-phenylindole) method (31). Cryotome sections from petioles, shoots, branches, and roots were examined. DNA was extracted from petioles, midribs, or phloem of branches and roots approximately 5 cm in diameter. 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).

Primers and PCR amplification. The primers were derived from ribosomal sequences (16,22), and all except oligonucleotide rRNA have previously been described in some detail. As universal phytoplasma primer pairs, P1 (8) and P7 (12) or fU5/rU3 (16) and fU5/rRNA were used. Primer rRNA (provided by B. C. Kirkpatrick, University of California, Davis) primes in the conserved rRNA^{16S} gene of the 16S/23S rDNA spacer region and amplifies, in combination with fU5, a fragment of approximately 1,230 bp. For group-specific amplification of DNA from the EY agent and closely related phytoplasmas, primer pairs fB1/rULWS (12) and fAY/rEY (1,22) were employed.

Direct and nested PCR amplification and analysis of the amplification products were performed as described (22). Exceptions were that the annealing temperature for primers fAY/rEY was 59°C, and the extension time with primer pairs fU5/rRNA and fAY/rEY was 60 s. For the second round of amplification with nested

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primers, either 3 μ l of undiluted PCR products obtained in the initial amplification or 5 μ l of a 1:40 dilution were used in the reaction mixture.

Hybridization of PCR products. Products obtained with primer pairs P1/P7 and fB1/rULWS in the initial amplification were transferred from agarose gel to nylon membrane (Hybond N⁺, Amersham-Buchler, Braunschweig, Germany) following the procedure described by Maniatis et al. (18). Hybridization with radiolabeled group-specific oligonucleotide rEY was performed as previously described (3), except that the repeated posthybridization washes were carried out at 40°C.

RESULTS

Fluorescence microscopy. Many trees were examined over a period of several years. Most samples collected from the various plant parts scored negative for phytoplasmal infection. In other samples, sparse fluorescent particles were detected in phloem sieve tubes. However, this fluorescence was difficult to interpret and did not allow us to rate these samples as positive.

Phytoplasma detection by nested PCR and RFLP analysis of the amplification products. Samples for PCR diagnosis were taken twice. Aerial parts of 31 symptomatic trees were sampled in September

1994. When template DNA from these trees was amplified with universal phytoplasma primer pairs P1/P7 and fU5/rU3, no visible bands were resolved by agarose gel electrophoresis. However, when the amplification products obtained with primers P1/P7 were reamplified with universal pair fU5/rRNA in nested PCR, phytoplasmal DNA was detected in 14 trees (Fig. 1A). Following separate digestion of the PCR products with *AluI* and *RsaI*, all phytoplasma-positive samples showed the same restriction profile with each endonuclease. These profiles were identical to those of strain ULW (Fig. 2) and the other reference strains of the EY phytoplasma.

After the group identity of the eucalyptus-infecting phytoplasma was revealed, primers specific for the EY agent and closely related phytoplasmas were employed in direct and nested PCR. As with universal primers P1/P7 and fU5/rU3, no visible amplification products were obtained with group-specific primer pairs fB1/rULWS and fAY/rEY by direct PCR. However, when these primers were used in combination with P1/P7 in nested PCR, the same samples that tested positively in the combination of P1/P7 with fU5/rRNA yielded an amplification product (Fig. 1B and C). The same result was obtained when primers fB1/rULWS were followed by pair fAY/rEY in nested PCR assays (data not shown). All trees that tested negatively in nested PCR with universal primers P1/P7 followed by fU5/rRNA also gave negative results when fB1/rULWS and fAY/rEY were used as nested primers. As described above for nested primers fU5/rRNA, the amplification products obtained with pair P1/P7 followed by primers fB1/rULWS in nested PCR showed the same restriction profile for each *AluI* and *RsaI*. These profiles were identical to those of strain ULW (Fig. 2) and the other reference strains of the EY phytoplasma. Similarly, digestion of fB1/rULWS products obtained from eucalyptus samples and the various EY strains with *Sau3AI* resulted in the same restriction profiles.

All trees in which phytoplasmas were detected were resampled in January 1995. At this time, not only petioles and phloem tissue of branches, but also root phloem were examined. As in the first examination, all trees gave a negative result in the initial amplification with primer pairs P1/P7 and fB1/rULWS. However, all trees tested again positively in nested PCR assays with the various primer combinations described above. In all phytoplasma-positive trees, the causal agents were detected in both stem and roots (data not shown).

All nonsymptomatic field-grown trees as well as the healthy greenhouse-grown eucalyptus seedlings and periwinkle plants gave negative results in both direct and nested PCR assays. However, universal

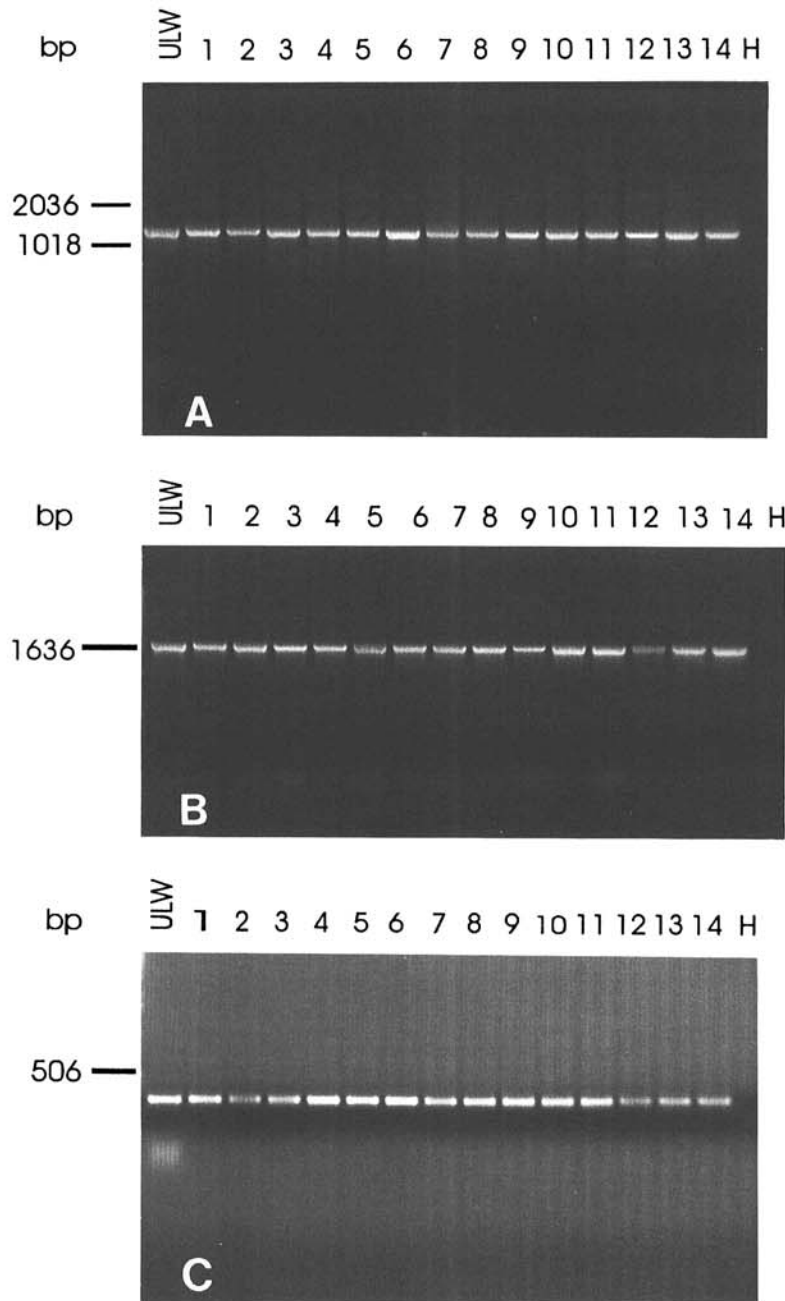


Fig 1. Nested polymerase chain reaction (PCR) amplification of ribosomal DNA fragments obtained with DNA from diseased eucalyptus trees using the following primer pair combinations: (A) P1/P7 followed by fU5/rRNA, (B) P1/P7 followed by fB1/rULWS, and (C) P1/P7 followed by fAY/rEY. ULW, strain of EY phytoplasma; 1 to 14, samples from diseased eucalyptus trees; H, healthy eucalypt.

primer pairs P1/P7, fU5/rU3, and fU5/rRNA amplified the target DNAs in direct PCR from all phytoplasma reference strains maintained in periwinkle. Group-specific primer pair fB1/rULWS amplified the target sequence in both direct and nested PCR only from strains EY1 and ULW of the EY phytoplasma, whereas primers fAY/rEY additionally amplified phytoplasmal DNA from the ash yellows phytoplasma (Fig. 1, data not shown).

Hybridization of PCR products. Amplification products obtained by direct PCR with primer pairs P1/P7 and fB1/rULWS from DNA samples from the 14 eucalyptus that tested positively in nested PCR and eight samples that were negative were hybridized following Southern transfer with group-specific oligonucleotide rEY as probe. Hybridization signals were obtained from 10 of the phytoplasma-positive samples when amplified with primers fB1/rULWS but only from five of these samples when amplified with primers P1/P7. Also, the hybridization signals were stronger with products obtained using primers fB1/rULWS. No hybridization signals were obtained with the samples that tested negatively in nested PCR or with samples from nonsymptomatic and healthy plants (Fig. 3, data not shown).

Phytoplasma infection in relation to symptoms. The symptoms in all trees examined were assessed at both sampling dates. Symptoms in phytoplasma-infected trees differed from symptoms in trees that tested negatively. While all trees were affected by yellowing, stunting, and die-back, all phytoplasma-positive trees showed in addition dwarfed leaves and precocious growth from axillary buds that was in some trees combined with shortened internodes and witches'-broom formation (Fig. 4).

DISCUSSION

In this work, eucalyptus trees grown in Italy and showing yellowing and decline symptoms were examined for phytoplasmal infection. About half of the trees also expressed little-leaf symptoms and proliferating shoots. A phytoplasma closely related to the EY agent was detected from these trees, and the name eucalyptus little-leaf (ELL) phytoplasmas is proposed for this organism. The symptoms observed in Italy are similar to those of the phytoplasma-associated little-leaf disease described from India (9,25,26,33), China (34), and the Sudan (6). However, the similarity of symptoms observed in Italy and elsewhere does not allow the conclusion that the diseases occurring in other countries are caused by the ELL phytoplasma. Similar symptoms in a given plant may be induced by genetically different phytoplasmas, as observed in grapevine (7,17,27). No phytoplasmas were detected in trees that did not show little-leaf and witches'-brooms. It is possible that these trees were suffering from other diseases.

The ELL phytoplasma apparently occurred in such low numbers that detection by fluorescence microscopy or direct PCR amplification using universal or group-specific primers was not possible. However, infections could be identified through nested PCR assays or hybridization of the products obtained by direct PCR with an

oligonucleotide probe. This result is in contrast to the work of others who detected phytoplasmas in diseased eucalyptus trees by either light (Dienes' stain) (9) or electron microscopy (6,9,26,33,34). Both methods are less sensitive than the DAPI fluorescence test, which again is less sensitive than detection by direct PCR

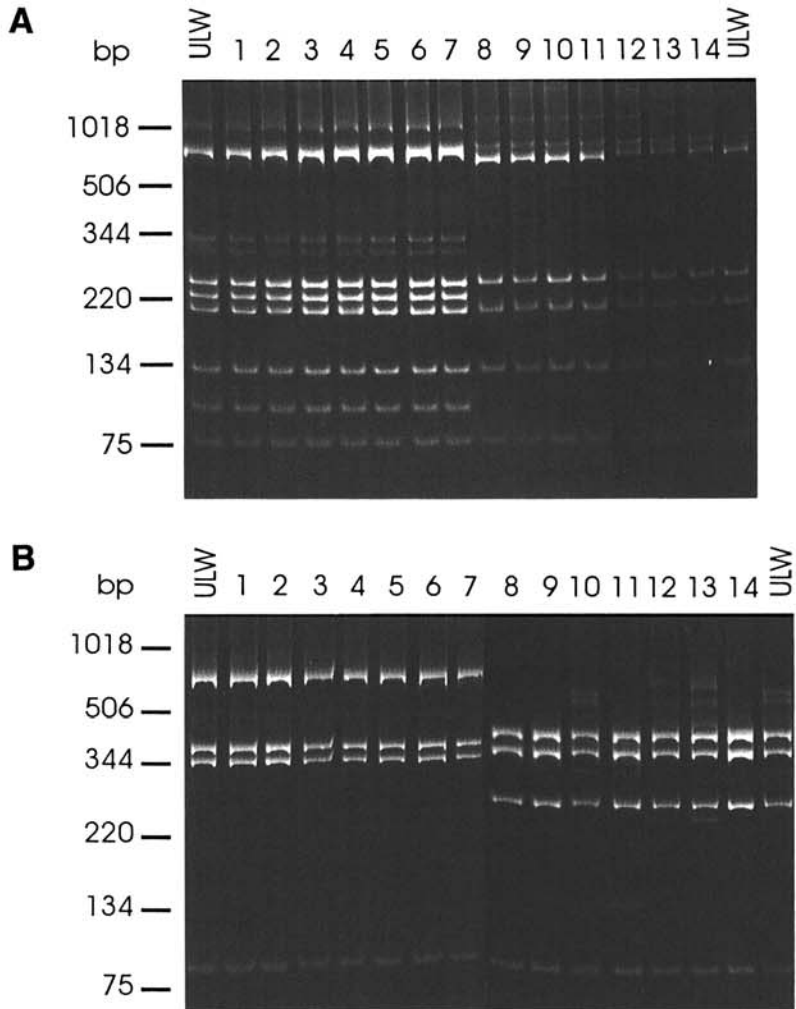


Fig. 2. (A) *AluI* and (B) *RsaI* restriction profiles of ribosomal DNA amplified by nested polymerase chain reaction (PCR) assays from template DNA extracted from diseased eucalyptus. All samples were initially amplified using primer pair P1/P7. Samples 1 to 7 were reamplified with primers fB1/rULWS, and samples 8 to 14 were reamplified with primers fU5/rRNA. Faint bands at about 1 kb and 330 bp in (A) and between 500 and 700 bp in (B) are most likely due to incomplete digestion. ULW, strain of EY phytoplasma.

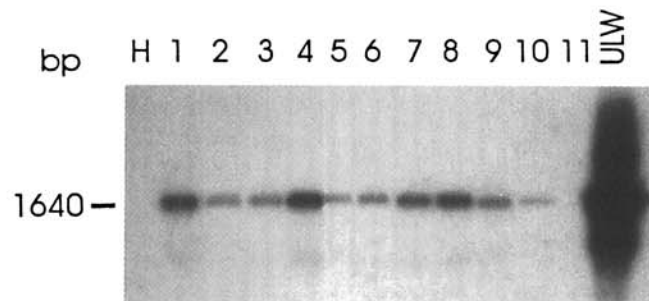


Fig. 3. Hybridization of the blotted polymerase chain reaction (PCR) products obtained with primer pair fB1/rULWS in direct amplification from template DNA extracted from diseased (1 to 11) and healthy (H) eucalyptus trees. Radiolabeled oligonucleotide rEY was used as a probe. ULW, strain of EY phytoplasma.

(16). However, this discrepancy in detection may be due to different host properties among *Eucalyptus* species. In Asia and the Sudan, phytoplasmas were microscopically detected in *E. camaldulensis*, *E. eugenioides*, *E. globulus*, *E. grandis*, *E. microtheca*, *E. multiflora*, and *E. tereticornis*. Although the species we examined were not identified, it is known that *E. camaldulensis*, *E. robustus*, and *E. rudis* are predominantly grown in Italy. It is possible that we examined the latter two species and that they are less suitable hosts than those examined elsewhere. Hosts may vary in susceptibility. Among species of *Malus* that were experimentally inoculated with the apple proliferation phytoplasma, phytoplasma density ranged from moderately high to very low numbers that could not be detected by DAPI fluorescence microscopy. The species showing low-titer infections were still severely affected by the disease (11). Other examples of phytoplasma diseases in which detection of the causal agent with microscopic methods is not possible or is very difficult are flavescence dorée of grapevine (5) and a decline disease of oaks (3). Whereas it is well established that low phytoplasma numbers cause disease in apple and grapevine, inoculation experiments are required to prove the pathological role of low-titer infections in eucalypts and oaks.

Based on restriction fragment length polymorphism (RFLP) analysis of PCR-amplified ribosomal DNA (rDNA) using *AluI* and *RsaI* endonucleases, the phytoplasmas we detected in eucalyptus trees were uniform. Restriction patterns of rDNA obtained with these enzymes and

other frequently cutting endonucleases are reliable characteristics and are, therefore, used to classify phytoplasmas (15,30). In these classification systems, the EY agent and closely related phytoplasmas such as those causing alder yellows, rubus stunt, and flavescence dorée of grapevine, as well as a phytoplasma associated with a witches'-broom disease of *Spartium junceum*, form a distinct group. The existence of this group has been confirmed by sequence analysis of 16S rDNA (10,32).

Following digestion with *AluI* and *RsaI*, the phytoplasmas of the EY group showed the same rDNA restriction profiles (4,22,23,30,32), which were similar to those of the ELL phytoplasma. However, Bertaccini et al. (4) obtained different RFLP profiles when 16S rDNA of the rubus stunt agent and an Italian EY strain were compared with an American EY strain, using *Sau3AI* restriction endonuclease. This was not confirmed in our work, in which American, Italian, and French EY strains showed the same *Sau3AI* profile, which was identical with that of the ELL strain. Differences between phytoplasmas of the EY group showing similar *AluI* and *RsaI* rDNA profiles were also identified by Southern blot analyses, in which the EY phytoplasma was clearly distinguishable from rubus stunt strains (23). However, it is not known if and how the differences detected in restriction profiles are related to host specificity. Thus, it remains to be elucidated if and how the ELL phytoplasma pathologically differs from the EY agent and other closely related phytoplasmas of the EY group. Phytoplasmas of this group appear to be widespread in central and southern Italy. They were recently observed in diseased *U. minor* (20), alder (*Alnus glutinosa*, *A. cordata*) (19), wild blackberry (*Rubus fruticosus*) (21), and Spanish broom (*S. junceum*) (22).

Phytoplasma detection by nested PCR assays bears the risk that contaminations are amplified (C. Marcone and E. Seemüller, unpublished). However, in this work, there is no doubt that the detected phytoplasmal DNA originated in the diseased eucalyptus trees. This is shown by the hybridization experiments, in which most samples from infected trees tested positively when the products of the initial amplification were hybridized with an oligonucleotide probe to reach the required detection sensitivity. By hybridization, the risk of contamination between the first and second amplification round was avoided. Furthermore, by using the primers described here and primers that specifically amplify phytoplasmal DNA from the other phylogenetic groups described by Seemüller et al. (32), only DNA of the ELL phytoplasma but not of other phytoplasmas was detected by either direct or nested PCR (C. Marcone, unpublished). The weak bands shown in Figure 2 seem

not to be due to the presence of DNA of a second phytoplasma in the PCR products, but mainly to incomplete digestion.

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Fig. 4. Phytoplasma-infected eucalyptus branch showing little-leaf and proliferation symptoms. Normal-sized leaves are shown in the upper portion of the picture.

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