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Characterisation of *Phoma tracheiphila* by RAPD-PCR, microsatellite-primed PCR and ITS rDNA sequencing and development of specific primers for *in planta* PCR detection

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Abstract

Thirty six isolates of *Phoma tracheiphila* from Italy, the causal agent of the "mal secco" disease on *Citrus* species, were characterised by different molecular tools in comparison with representative isolates of other phytopathogenic *Phoma* species. These included analysis of the distribution of RAPD and microsatellite markers and sequencing of the internal transcribed spacer (ITS) region of the nuclear rRNA genes. The results obtained with 12 RAPD primers (92 markers) and 7 microsatellite primers (56 markers) suggest that Italian isolates of *P. tracheiphila* are genetically homogeneous, leading to identical patterns upon amplification with all the tested primers. Accordingly, ITSI-5.8S-ITS2 sequences were highly conserved (98–100% identity along a 544-characters alignment) among all the isolates of *P. tracheiphila*. A neighbor-joining analysis of ITS sequences of *P. tracheiphila* in comparison with those of other *Phoma* species, as well as with alignable sequences from anamorphic and teleomorphic taxa retrieved in BLAST searches, revealed a close relationship between *P. tracheiphila* and *Leptosphaeria congesta*. A pair of *P. tracheiphila*-specific primers was designed on the consensus sequence (555 residues) obtained from the alignment of the newly generated *P. tracheiphila* ITS sequences. A PCR-based specific assay coupled to electrophoretic separation of amplicons made it possible to detect *P. tracheiphila* in naturally infected *Citrus* wood tissue collected from both symptomatic and symptomless plants. The limit of detection was 10 pg of genomic DNA and 5 fg of the ITS target sequence.

Introduction

The mitosporic fungus *Phoma traccheiphila* (Petri) Kantschaveli *et* Gikachvili is the incitant of "mal secco", a serious disease of lemon (*Citrus limon*) and other *Citrus* species throughout the Mediterranean region, including the Black Sea area, (Punithalingam and Holliday, 1973; Perrotta and Graniti, 1988). The pathogen penetrates the host *via* wounds, and possibly through stomata (Perrotta and Graniti, 1988). The fungus invades the xylem and causes impairment of the water-transport system of the plant, leading to wilting of branches and eventually plant death. Typical symptoms consist of vein chlorosis, shedding of leaves and die-back of twigs and branches and a

salmon-pink discoloration of wood. Chronic infections on adult trees may cause a browning of the hard wood (''mal nero'') without any external symptoms. Infected trees collapse suddenly when the fungus reaches the functional xylem. A rapid lethal *facies* of the disease (''mal fulminante'') leads to a sudden wilting of branches of the whole tree. In most cases, this form is associated with root infection (Scrivani, 1954; Solel, 1976). When grown in liquid substrate, the fungus produces a complex of phytotoxic glycoproteins, which are able to reproduce some of the disease symptoms when injected into plant leaves or shoots (Nachmias et al., 1977, 1979; Fogliano et al., 1998). Despite the considerable efforts devoted to the identification of resistant *Citrus* clones by conventional breeding, clonal and somaclonal selection, or protoplast fusion (Gentile et al., 1993; Deng et al., 1995; Reforgiato Recupero et al., 1997; Tusa et al., 2000), the pathogen still represents a major threat to citriculture in the Mediterranean basin.

The high infecting potential through rain-borne or wind-borne conidia and the lack of effective curative treatments, still make the use of pathogen-free propagative material the most efficient means to control this pathogen, which is included in the current (approved September 2003) version of the European and Mediterranean Plant Protection Organization (EPPO) A2 list of quarantine pests. Furthermore, *P. tracheiphila* is of quarantine concern to most other regional plant protection organizations, such as APPPC, CPPC, COSAVE, IPSC, and NAPPO (see Data Sheets on Quarantine Pests http://www.eppo.org/QUARANTINE/fungi/Deuterophoma_tracheiphila/DEUTTR_ds.pdf). This emphasizes the need for tools to allow rapid and reliable detection of *P. tracheiphila* within the host tissue.

A dot-blot assay and a polymerase chain reaction (PCR) test were developed by Rollo et al. (1987, 1990), who matched a 102-bp randomly cloned fungal DNA sequence. While the level of sensitivity of the PCR approach was demonstrated, the specificity of the primer pair A + B was only tested with a single isolate of *P. lingam* (Rollo et al., 1990) and deserves further investigation.

Our aims were firstly, to evaluate the level of genetic diversity among Italian isolates of *P. tracheiphila,* and secondly, to develop a reliable PCR-based method for the detection of this pathogen in culture and *in planta*. Accordingly, the distribution of RAPD and microsatellite markers were analysed within a collection of Italian isolates of the pathogen; based on the sequences of the Internal Transcribed Spacer (ITSI-5.8S-ITS2; ITS) region of the nuclear rRNA genes, a pair of *P. tracheiphila*-specific primers was designed and compared for specificity and sensitivity in comparison to primer pair A + B developed by Rollo et al. (1990).

Materials and methods

Fungal strains and storage conditions

Isolates of different *Phoma* species, including *P. tracheiphila, P. betae, P. cava, P. exigua, P. fimeti, P. glomerata, P. lingam and P. medicaginis,* and other fungi commonly associated with *Citrus* species were used (Table 1). All the 36 Italian isolates of *P. tracheiphila* were previously characterised on the basis of conventional criteria. They showed different colony morphology and included chromogenic and non-chromogenic isolates, *sensu* Baldacci and Garofalo (1948). Furthermore, all isolates were different in virulence in previous pathogenicity tests (Magnano di San Lio et al., 1992). A collection of the tested isolates is maintained at the Department of Plant Protection, University of Sassari (Italy) on potato dextrose agar (PDA, Merck, Darmstadt, Germany) at 4 °C and in 50% glycerol at −80 °C.

Purification of nucleic acids

Fungal genomic DNA was purified from lyophilized mycelium by following a standard method (Aljanabi and Martinez, 1997). DNA extraction from *Citrus* tissue was performed by following the same protocol slightly modified as follows: approximately 500 mg of fresh wood tissue was ground in a mortar with liquid nitrogen; then 450 µl of extraction buffer (0.4 M NaCl, 10 mM Tris–HCl pH 8.0, 2.0 mM EDTA pH 8.0, 400 µg ml^{-1} proteinase K, 2% sodium dodecyl sulfate) was added to 100 mg of ground tissue and mixed well. Samples were incubated for 1 h at 65 °C. After adding 300 µl of 6,0 M NaCl, samples were briefly vortexed and centrifuged at $10{,}000 \times g$ for 30 min, the supernatant was

Table 1. List of fungal species and isolates

Isolate	Species	Source	Location	Year	PCR assay
Pt VIII	*Phoma tracheiphila*	Unknown	–[1]	1982	+[2]
Pt 42	*P. tracheiphila*	*Citrus limon*	Bagheria (PA)[3]	1983	+
Pt 44	*P. tracheiphila*	*Citrus limon*	Bagheria (PA)	1983	+
Pt 49	*P. tracheiphila*	*Citrus limon*	Bagheria (PA)	1983	+
Pt 52	*P. tracheiphila*	*Citrus limon*	Bagheria (PA)	1983	+
Pt 53	*P. tracheiphila*	*Citrus limon*	Bagheria (PA)	1983	+
Pt 54	*P. tracheiphila*	*Citrus limon*	Bagheria (PA)	1983	+
Pt 55	*P. tracheiphila*	*Citrus limon*	Bagheria (PA)	1983	+
Pt 56	*P. tracheiphila*	*Citrus limon*	Bagheria (PA)	1983	+
Pt 60	*P. tracheiphila*	*Citrus limon* Femminello	C.da Baroni Noto (SR)	1988	+
Pt 61	*P. tracheiphila*	*Citrus limon*	C.da Bonavia Cassibile (SR)	1983	+
Pt 62	*P. tracheiphila*	*Citrus limon* Femminello	Balatelle Acireale (CT)	1983	+
Pt 63	*P. tracheiphila*	*Citrus limon* Femminello	Balatelle Acireale (CT)	1983	+
Pt 64	*P. tracheiphila*	*Citrus limon* Femminello	Balatelle Acireale (CT)	1983	+
Pt 71	*P. tracheiphila*	*Citrus limon* Monachello	Balatelle Acireale (CT)	1983	+
Pt 73	*P. tracheiphila*	*Citrus limon* Monachello	Balatelle Acireale (CT)	1983	+
Pt 75	*P. tracheiphila*	*Citrus limon* Monachello	Acireale (CT)	1985	+
Pt 77	*P. tracheiphila*	*Citrus limon* Femminello	Giardini (ME)	1985	+
Pt 79	*P. tracheiphila*	Air sampling	Ognina (CT)	1985	+
Pt 80	*P. tracheiphila*	Unknown	Giardini (ME)	1985	+
Pt 81	*P. tracheiphila*	Air sampling	Giardini (ME)	1985	+
Pt 83	*P. tracheiphila*	Air sampling	Giardini (ME)	1985	+
Pt 84	*P. tracheiphila*	*Citrus limon* Femminello	C.da Scorsonello Savoca (ME)	1988	+
Pt 86	*P. tracheiphila*	Air sampling	Ognina (CT)	1985	+
Pt 87	*P. tracheiphila*	Air sampling	Ognina (CT)	1985	+
ITEM 2338	*P. tracheiphila*	*Citrus limon* fruit	–	–	+
Pt C	*P. tracheiphila*	*Citrus microcarpa*	–	1983	+
Pt V	*P. tracheiphila*	*Citrus volkameriana*	–	1992	+
Pt 20	*P. tracheiphila*	Unknown	–	–	+
Pt Ad1	*P. tracheiphila*	*Citrus limon*	Altofonte (PA)	–	+
Pt Ad2	*P. tracheiphila*	*Citrus aurantium*	Altofonte (PA)	–	+
Pt Ad3	*P. tracheiphila*	*Citrus limon*	Parco d'Orleans (PA)	–	+
Pt Ad4a	*P. tracheiphila*	*Citrus aurantium*	Mazzarà Sant'Andrea (ME)	–	+
Pt Ad4b	*P. tracheiphila*	*Citrus aurantium*	Mazzarà Sant'Andrea (ME)	–	+
ISPaVe ER 1139	*P. tracheiphila*	*Citrus limon*	Cisterna (LT)	2000	+
PVS Pt S1	*P. tracheiphila*	*Citrus limon*	Capoterra (CA)	2004	+
ITEM 201	*P. glomerata*	*Laurus nobilis*	Italy	1981	–
ITEM 203	*P. exigua*	*Vitis vinifera*	Italy	1981	–
ITEM 243	*P. betae*	*Beta vulgaris*	The Netherlands	1966	–
ITEM 244	*P. cava*	*Castanea sativa*	The Netherlands	1966	–
ITEM 246	*P. fimeti*	Greenhouse soil	The Netherlands	1970	–
ITEM 2077	*P. lingam*	*Brassica napus*	Italy	1990	–
ISPaVe ER 693	*P. medicaginis*	*Medicago sativa* (seed)	Foggia (FG)	1991	–
PVS LB 3-2	*Diplodia aurantii*	*Citrus limon*	Sicily	2003	–
PVS A 3	*Phomopsis* sp.	*Citrus aurantium*	Bauladu (OR)	2003	–
PVS Fu A4	*Fusarium semitectum*	*Citrus aurantium*	Bauladu (OR)	2003	–
FS 2 B	*Fusarium solani*	*Citrus aurantium*	Gerbini (CT)	2001	–
FS R 2 B	*Fusarium solani*	*Citrus aurantium*	Gerbini (CT)	2001	–
LAT	*Fusarium solani*	*Citrus sinensis*	Gerbini (CT)	2001	–
Fox R 1 A	*Fusarium oxysporum*	*Citrus aurantium*	Gerbini (CT)	2001	–
Fox R 2 A	*Fusarium oxysporum*	*Citrus aurantium*	Gerbini (CT)	2001	–
FL	*Fusarium lateritium*	*Olea europaea*	Palermo, Sicily	1999	–
C2	*Colletotrichum gloeosporioides*	*Citrus* sp.	Calabria	1992	–
8 (JMO 94-22)[4]	*Colletotrichum gloeosporioides*	*Citrus* sp.	California, USA	–	–

Table 1. Continued

Isolate	Species	Source	Location	Year	PCR assay
CP 3	*Colletotrichum gloeosporioides*	*Citrus limon*	Capo d'Orlando (ME)	1999	–
Acg	*Colletotrichum* sp.	*Citrus aurantium*	Bauladu (OR)	2003	–
PVS PD	*Penicillium digitatum*	*Citrus limon*	Sardinia	2003	–
PVS PI	*Penicillium italicum*	*Citrus limon*	Sardinia	2003	–
PVS LA 2-3	*Penicillium* sp.	*Citrus limon*	Sicily	2003	–
PVS E	*Epicoccum* sp.	*Citrus aurantium*	Bauladu (OR)	2003	–
GEO	*Geotrichum* sp.	*Citrus* sp.	Serravalle (CT)	2003	–
PVS B	*Botrytis cinerea*	*Malus domestica*	Sardinia	2003	–
PVS T 4-77	*Trichoderma harzianum*	Soil	Aglientu (SS)	2002	–
PVS-4	*Sclerotinia sclerotiorum*	*Foeniculum vulgare*	Sardinia	–	–
C2	*Alternaria* sp.	Tangelo 'Nova'[5]	Serravalle (CT)	2002	–
PVS MM	*Alternaria* sp.	*Citrus* sp.	Muravera (CA)	2002	–

[1] Not known.
[2] "+" and "−" indicate the presence and absence of the expected 378-bp DNA fragment amplified by the primer pair *Pt*-FOR2 + *Pt*-REV2.
[3] Code in brackets indicates the administrative province of Italian location: PA, Palermo; SR, Siracusa, CT, Catania; ME, Messina; LT, Latina; CA, Cagliari; FG, Foggia; OR, Oristano; SS, Sassari.
[4] Forster and Adaskaveg (1999).
[5] Tangelo is a grapefruit (*Citrus paradisi*) × tangerine (*C. reticulata*) hybrid.

extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform, and precipitated with one volume of isopropanol. The pellet was then rinsed with 100% ethanol, resuspended in TE, pH 7.5 (Maniatis et al., 1982) and stored at 4 °C. All reagents were purchased from Sigma.

RAPD PCR conditions

RAPD PCR was carried out in 25 µl of reaction mix containing: 10 mM Tris–HCl, pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl; 200 µM each of dATP, dCTP, dGTP, and dTTP (Promega, Madison, WI, USA); 0.2 µM of 10-mer primer, about 5 ng of template DNA for each isolate, and 1.0 U of Red*Taq* DNA polymerase (Sigma). Twelve 10-mer oligonucleotides were chosen randomly among the series OPA, OPB, OPL, and OPAN (Operon Technologies, Alameda, CA, USA) and tested as primer sequences. Amplification was run in a Gene Amp PCR System 9600 (Applera, Norwalk, CT, USA) programmed for one cycle of 1 min at 94 °C, 40 cycles of 1 min at 94 °C, 1 min at 37 °C, 2 min at 72 °C, with no ramping; one final cycle of 10 min at 72 °C. RAPD analysis was repeated at least twice for each isolate. Half of the amplification product was loaded in a 1.5% electrophoresis grade agarose (Gibco BRL, Carlsbad. CA, USA) gel containing 0.5 µg of ethidium bromide ml^{-1}. Electrophoresis was performed for 2 h 30 min at 3.3 V cm^{-1} in 1 × TAE (Maniatis et al., 1982) running buffer, and amplimers were observed over a UV light source. Gel images were acquired with a Gel Doc 1000 System (Bio-Rad Laboratories, Hercules, CA, USA).

Microsatellite-primed PCR conditions

Microsatellite-primed PCR was carried out essentially as described for RAPD PCR by using the primers: $(AAC)_8$; $(AAG)_8$; $(AC)_{10}$; $(ATC)_5$; $(GACA)_4$; $(GTG)_5$; 5′-GAGGGTGGCGGTTCT-3′ (matching the phage M13 core sequence) and by adopting the following amplification programme: one cycle of 5 min at 94 °C; 40 cycles of 1 min at 94 °C, 1 min 30 s at 52 °C (for primer M13), at 50 °C [for primers $(AAC)_8$; $(AAG)_8$; $(AC)_{10}$; $(GTG)_5$; $(GACA)_4$] or at 37 °C [for primer $(ATC)_5$], 2 min at 72 °C; one final cycle of 7 min at 72 °C. Amplification was repeated at least twice for each sample.

Cluster analysis of RAPD and microsatellite-primed PCR data

Comparison of each profile for each of the 12 RAPD PCR primers and of 7 microsatellite primers was based on the presence versus absence of amplicons that migrated to the same position in the gel. Bands of the same size obtained by the same primer were scored as identical and only bands repeatable in at least two experiments with the same primer at different times were evaluated. A UPGMA topology of individual isolates using the Nei and Li (1979) distance estimator was generated using Treecon software ver 1.3b (Van de Peer and De Wachter, 1994; http://bioc-www.uia.ac.be/u/yvdp/trecconw.html). The strength of each node of the dendrogram was calculated by bootstrapping over presumed loci with 1000 replicates.

ITS amplification and sequencing

Universal primers ITSI (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990) were used to amplify the ITS regions between the small (18S gene) and large (28S gene) nuclear rDNA, including the 5.8S rDNA of 25 *Phoma* spp. isolates. Amplifications were carried out in a 50 µl volume, containing: 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.1 mM $MgCl_2$, and 0.01% gelatin, 200 µM of each dNTP (Promega), 1.0 µM of each primer, 1.0 U of Red*Taq* DNA polymerase (Sigma) and 30–50 ng of DNA. Cycling parameters were: 5 min at 94 °C; 30 cycles of 1 min at 94 °C, 1 min 30 s at 50 °C, 1 min 30 s at 72 °C; with a final extension at 72 °C for 7 min.

Aliquots of about 35 ng of DNA were purified from agarose gel with a Qiaex II Kit (Qiagen, Hilden, Germany) and automatically sequenced with an ABI Prism 3100 DNA Sequencer (Applied Biosystems, Norwalk, CT, USA) by the sequencing core facility C.R.I.B.I. – Bio Molecular Research at the University of Padova, Italy.

Phylogenetic analysis of ITS sequencing data

Phylogenetic analyses based on the ITS were performed on a dataset comprising newly generated sequences from *P. tracheiphila* and other *Phoma* species, as well as alignable sequences from anamorphic and teleomorphic taxa retrieved in BLAST searches (Altschul et al., 1997), using the consensus sequence of *P. tracheiphila* isolates as query, and other closest taxa available in databases.

The Neighbor-joining analysis (NJ) was performed using PAUP* 4.0b10 (Swofford, 2000), with the Kimura two parameter method for superimposed mutations. Robustness of the internal branches was assayed by bootstrap analysis (1000 runs).

Primer design and PCR assay

Sequences of the ITS region were aligned by using GeneDoc v. R.6.02 (Nicholas and Nicholas, 1997). Primer design was performed by using the Primer3 software (Rozen and Skaletsky, 1998). Two primers, coded *Pt*-FOR2 and *Pl*-REV2, were custom synthesized by Invitrogen Life Technologies (Groningen, The Netherlands).

The primer pair *Pt*-FOR2 + *Pt*-REV2 was tested for specificity in comparison to the primers A + B, previously developed by Rollo et al. (1990). The following amplification conditions were chosen for primers *Pt*-FOR2 + *Pt*-REV2: 25 µl of reaction mix containing: 10 mM Tris–HCl, pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl; 200 µM each of dATP, dCTP, dGTP, and dTTP (Promega); 1.0 µM of each primer, about 10 ng of template DNA, and 0.8 µl of crude recombinant *Taq* DNA polymerase prepared according to Desai and Pfaffle (1995); initial denaturation at 94 °C for 5 min; 30 cycles of 30 s denaturation at 94 °C, 60 s annealing at 65 °C, 90 s extension at 72 °C; and 5 min final extension at 72 °C. Amplification conditions as proposed by Rollo et al. (1990) were adopted for primers A + B. Amplified products (10 µl) were analysed by agarose gel electrophoresis as described previously. Primer specificity was tested against genomic DNAs (approximately 10 ng of template) purified from 36 isolates of *P. tracheiphila* collected from different Italian *Citrus*-growing regions in different years, from 7 *Phoma* spp., and from 23 representative isolates of 15 other fungal species associated with *Citrus* spp. (Table 1).

The level of sensitivity of primers *Pt*-FOR2 + *Pt*-REV2 was measured by performing the PCR as described previously with a 10-fold serial dilution of purified total genomic DNA of *P. tracheiphila* isolate Pt 42, and with a 10-fold serial dilution of ITS PCR product of the same isolate, ranging from 10 ng to 1 fg.

Detection of Phoma tracheiphila *in Citrus wood tissue*

During the 2003 growing season, twigs were collected from naturally-infected lemon plants (cv Monachello) showing symptoms of "mal secco" disease, including the salmon-pink discoloration of the wood, from naturally infected symptomless plants (i.e., infected plants not showing wood discoloration) and from healthy plants in a commercial orchard at Giarre (Catania, Italy). Both culturing and PCR methods were used to detect *P. tracheiphila* on these samples.

For the culturing method, small fragments of wood (0.2 × 0.2 cm) were cut from each sample using a sterile surgical blade, surface disinfested with 0.5% sodium hypochlorite for 1–1.5 min, and rinsed with sterile distilled water. Fragments were placed on Petri plates of PDA containing 100 mg l^{-1} each of oxytetracycline hydrochloride and streptomycin sulphate (Sigma). After incubation at 25 °C for 7 days, *P. tracheiphila* colonies growing from these samples were identified based on morphological characters and tested individually by PCR assay.

For the PCR method, 100 mg of wood tissue was cut from infected (both symptomatic and symptomless) twigs and from healthy samples and total DNA was extracted as described previously. Three replicate samples were analysed in each experiment and the experiment was repeated two times.

Results

Characterization of Phoma *spp. isolates by RAPD PCR and microsatellite-primed PCR*

The results obtained with 12 RAPD primers (92 markers) and 7 microsatellite primers (56 markers) indicate that the isolates of *P. tracheiphila* are genetically homogeneous, leading to identical patterns upon amplification with all the tested primers. Representative results obtained with 16 isolates of *P. tracheiphila* are shown in Figure 1. Amplification profiles of all 36 *P. tracheiphila* isolates were distinguishable from those generated by the same primers with DNA from isolates of other *Phoma* species (not shown). To represent the relationship between different *Phoma* species, as shown for other organisms (Sun et al., 1999; Simioniuc et al., 2002; Balmas et al., in press), the two datasets were combined and analysed simultaneously by UPGMA. The resulting dendrogram (Figure 2) confirms the genetic homogeneity of the *P. tracheiphila* isolates, which clustered separately from the tested *Phoma* species: *glomerata, exigua, betae, cava, fimeti, lingam,* and *medicaginis*.

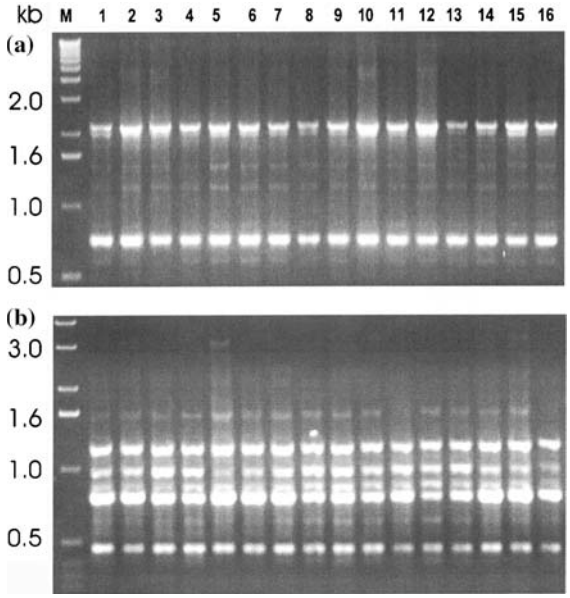

Figure 1. Comparison of amplification patterns obtained (A) by microsatellite-primed PCR with primer $(ATC)_5$ and (B) by random amplified polymorphic DNA with primer OPL-03 from genomic DNAs of *Phoma tracheiphila* isolates (from left to right): 1, Pt VIII; 2, Pt 42; 3, Pt 52; 4, Pt 56; 5, Pt 60; 6, Pt 61; 7, Pt 62; 8, Pt 71; 9, Pt 73; 10, Pt 75; 11, Pt 77; 12, Pt 79; 13, Pt 80; 14, Pt 81; 15, Pt 84; 16, Pt 86; M: molecular weight marker (1 kb DNA ladder; Life Technologies, Gaithersburg, MD, USA). Molecular weight in kb of some marker bands is specified on the left margin.

ITS sequence analysis and primer pair selection

The GenBank accession numbers of the ITS sequences of *Phoma* spp. isolates obtained in this work are: AY531665 through AY531682 and AY531689 (*P. tracheiphila* isolates) and AY531683, AY531684, AY531685, AY531686, AY531687, AY531688 (*P. glomerata, P. exigua, P. betae, P. cava, P. fimeti,* and *P. lingam* isolates, respectively).

P. tracheiphia sequences showed a 98–100% identity along a 544-characters alignment. Since no significant nucleotide differences were detected in sequence fragments useful for primer design, and

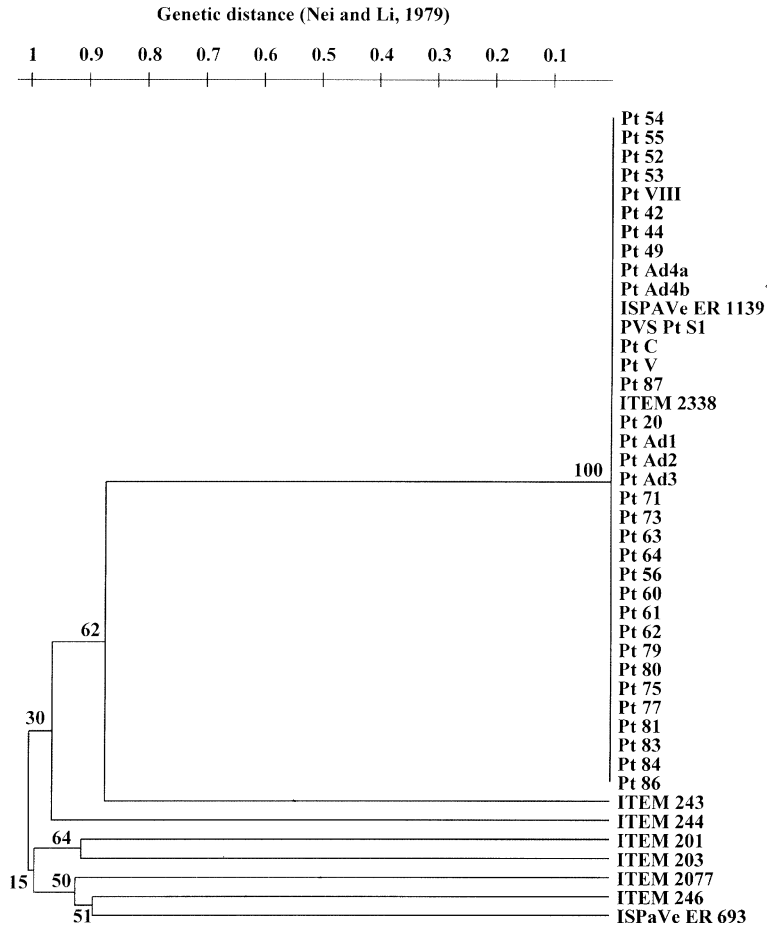

Figure 2. Dendrogram of the genetic distances (Nei and Li, 1979) among 36 isolates of *Phoma tracheiphila* and representative isolates of: *P. betae* (ITEM 243), *P. cava* (ITEM 244), *P. glomerata* (ITEM 201), *P. exigua* (ITEM 203), *P. lingam* (ITEM 2077), *P. fimeti* (ITEM 246), and *P. medicaginis* (ISPaVe ER 693) obtained by combining RAPD and microsatellite-primed PCR molecular data based on the unweighted pair-group method using arithmetic averages (UPGMA). Bootstrap values (%) are represented at each node of the dendrogram.

due to some length differences between newly generated sequences, a consensus sequence (555 residues) was obtained from the alignment (ClustalX, open gap penalties 40, gap extension penalties 15) of the newly generated *P. tracheiphila* sequences with the exception of accession Nos. AY531675, AY531678, AY531681, AY531673. The consensus sequence was identical along a 544-bp character alignment with the previously published sequences of *P. tracheiphila* AF272552, AF272553 and AF272554. When compared with the corresponding fragment from other *Phoma* species sequenced in this work or available in GenBank, the consensus sequence was 55–89% identical to the other species along 346–560 characters alignments.

A pair of oligonucleotides, the 18-bp-long *Pt*-FOR2 (5′-GGATGGGCGCCAGCCTTC-3′) and the 20-bp-long *Pt*-REV2 (5′-GCACAAGGGCAGTGGACAAA-3′), was designed on the consensus sequence and the predicted size of the amplicon was 378 bp. Figure 3 shows the positions and orientations of primers *Pt*-FOR2 and *Pt*-REV2 in the ITS region, with respect to the universal primers ITS3 and ITS4.

Phylogenetic characterization of Phoma spp. *isolates*

Since many anamorphic *Phoma* species are produced by teleomorphic *Leptosphaeria* or *Phaeosphaeria* species and since sequences from these

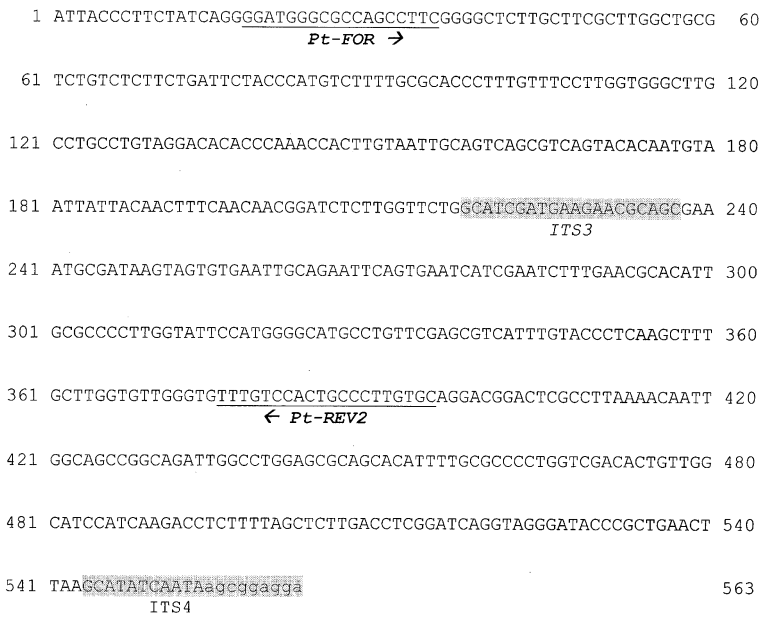

Figure 3. 5′–3′ consensus sequence of the internal transcribed spacer region (ITS1-5.8S-ITS2) of *Phoma tracheiphila* AY531665-AY531682 and AY531689, showing position of the primer pair *Pt-FOR2* and *Pt-REV2* (underlined) with respect to the universal primer ITS3 and ITS4 (shaded).

genera were found among the top-scoring BLAST alignments performed with the consensus *P. tracheiphila* sequence as query, we constructed a dataset comprising prevalently sequences from *Leptosphaeria* and *Phaeosphaeria* species available in databases to resolve phylogenetic relationship of the newly sequenced *Phoma* spp. isolates. *P. tracheiphila* AY531669, AY531671, AY531673, AY531675, AY531678, AY531681, and *P. glomerata* AY531688 were not included in the dataset due to their limited length.

Neighbor-joining analysis (Figure 4) yielded a phylogenetic tree, similar to that published by Câmara et al. (2002), in which three clades are observed. A first clade, supported by an 80% bootstrap value, comprises species of *Leptosphaeria,* including the type species *L. doliolum,* representing *Leptosphaeria sensu stricto*. In this clade, all the newly generated *P. tracheiphila* sequences and the ones previously published are included, clustering together (100% bootstrap) and with *L. congesta* (60% bootstrap), as well as with *P. cava* AY531686, *P. betae* AY531685 and *P. lingam* AY531688. No teleomorphic species have been recognised for *P. cava,* while *P. lingam* has been assigned to *L. maculans* (Sutton, 1980), forming (100% bootstrap) with *Plenodomus wasabiae* (AKA *Phoma wasabiae)* the *Plenodomus* clade within Leptosphaeriaceae (Reddy et al., 1998). *Phoma betae* AY531685 (anamorph *Pleospora betae*) is included in this clade because the dataset does not comprise sequences from family Pleosporaceae.

A second clade, supported by an 64% bootstrap value, includes species of *Phaeosphaeria* and the *Phoma* species *exigua* (AY531684) and *fimeti* (AY531687). Bibliographic evidences supporting or contrasting phylogenetic positioning of *P. cava, P. exigua* and *P. fimeti* obtained in this work are not available.

A third clade (100% bootstrap), including *L. bicolor* and other species isolated from sugarcane, does not contain any of the newly generated sequences.

Specificity and sensitivity of primer pairs

In specificity tests, the primer pair *Pt*-FOR2 + *Pt*-REV2 amplified a 378-bp DNA fragment from all 36 *P. tracheiphila* isolates tested that were collected from *Citrus* orchards at different locations in different years. No fragments were amplified from representatives of

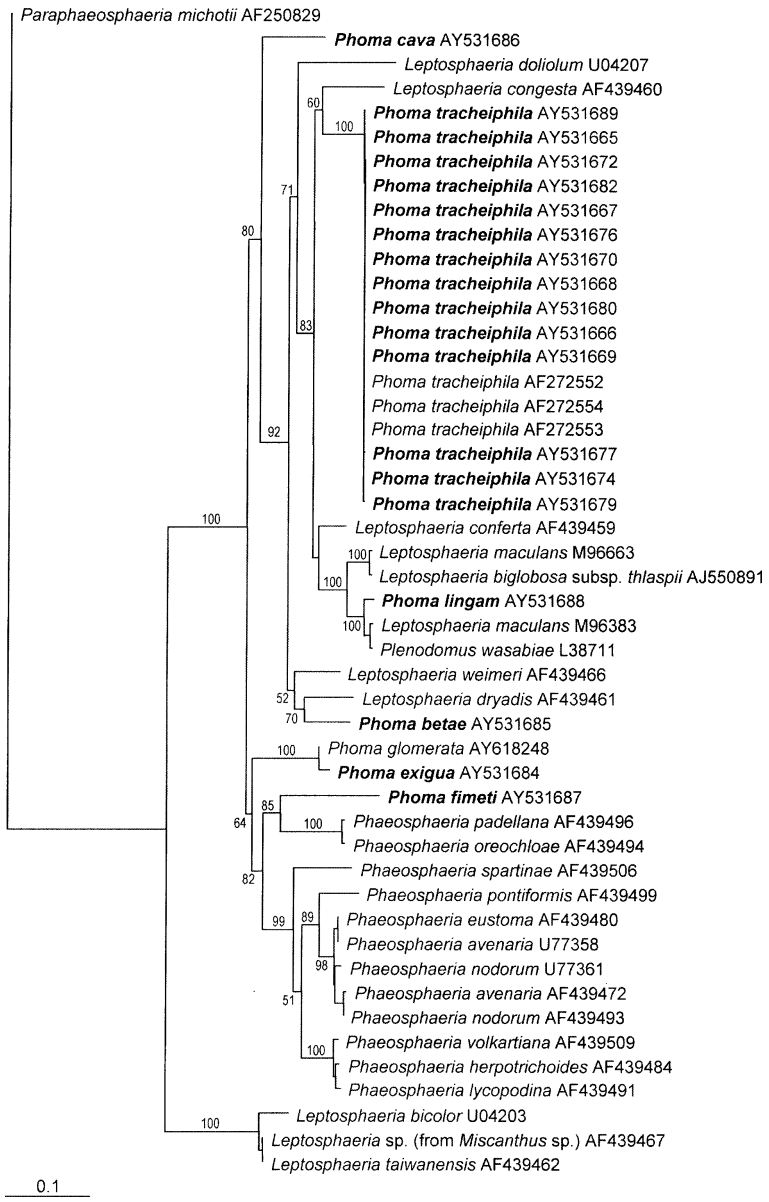

Figure 4. Neighbor-joining tree obtained from the Internal Transcribed Spacer (ITS1-5.8S-ITS2) sequence alignment of newly generated sequences of *Phoma* spp. isolates with sequences of closely related genera (order Pleosporales, subclass Dothideomycetidae, class Ascomycetes) retrieved by BLAST searches or available in sequence databases. Pairwise distance measure was performed with the Kimura two-parameter model for superimposed mutation. Bootstrap values above 50% are indicated (1000 replicates). *Paraphaeosphaeria michotii* AF250829 (family Phaeosphaeriaceae, order Pleosporales, subclass Dothideomycetidae, class Ascomycetes) was used as outgroup taxon to root the tree. Bar, Kimura distance.

other *Phoma* species or from any other fungus associated with *Citrus* spp. (Table 1; Figure 5a).

Amplification of the same genomic DNAs with primer pair A + B (Rollo et al., 1990), while generating the specific 102-bp fragment when *P. tracheiphila* DNA was used as template, gave rise to a series of nonspecific amplimers from several species, including *Phoma* spp., *Alternaria* spp., *Colletotrichum gloeosporioides, Diplodia*

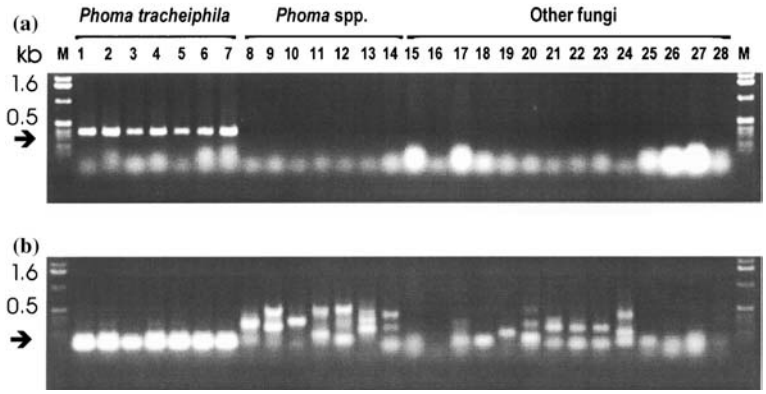

Figure 5. Specificity of (a) the primer pair *Pt*-FOR2 + *Pt*-REV2, and (b) the primer pair A + B (Rollo et al., 1990) for PCR detection of *Phoma tracheiphila*. Amplification was carried out by using 10 ng each of template genomic DNAs of (from left to right): 1–7, *P. tracheiphila* isolates Pt VIII, Pt 49, Pt 52, Pt 60, Pt 63, Pt 77, Pt Ad 3; 8–14, *P. glomerata* ITEM 201, *P. exigua* ITEM 203, *P. betae* ITEM 243, *P. cava* ITEM 244, *P.fimeti* ITEM 246, *P. lingam* ITEM 2077, *P. medicaginis* ISPaVe ER 693; 15, *Diplodia aurantii* PVS LB 3-2; 16, *Phomopsis* sp. PVS A3; 17, *Fusarium semitectum* PVS Fu A4; 18, *F. solani* FS 2B; 19, *F. oxysporum* Fox R1A; 20, *F. lateritium* FL; 21-23, *Colletotrichum gloeosporioides* C2, 8 (JMO 94-22), CP 3; 24, *Colletotrichum* sp. Acg; 25, *Alternaria* sp. PVS MM; 26, *Penicillium digitatum* PVS PD; 27, *P. italicum* PVS PI; 28, *Geotrichum* sp. GEO. M: molecular weight marker. Molecular weight in kb of some marker bands is specified on the left margin; the specific amplification signal for each primer pair (378 bp for *Pt*-FOR2 + *Pt*-REV2 and 102 bp for A + B) is indicated by an arrow.

aurantii, Fusarium oxysporum, F. lateritium, F. semitectum, F. solani, and *Penicillium* spp. (Figure 5b).

Sensitivity assays on *P. tracheiphila* isolate Pt 42 showed that the primer pair *Pt*-FOR2 + *Pt*-REV2 allowed detection of the specific fragment in 10 pg of total genomic DNA (Figure 6a) or in 5 fg of the ITS target sequence (Figure 6b).

Detection of Phoma tracheiphila *on infected* Citrus *by PCR*

PCR assay using the primer pair *Pt*-FOR2 + *Pt*-REV2 for detecting *P. tracheiphila* on lemon tissues was consistently in agreement with the isolation of the pathogen on PDA. The specific amplicon was obtained by PCR assay from all samples infected with *P. tracheiphila* and showing symptoms of "mal secco" disease; samples collected from infected symptomless plants gave rise to a faint, although reproducible, amplification signal. Finally, the pathogen was never detected from the healthy samples by either PCR assay (Figure 7) or the culturing method.

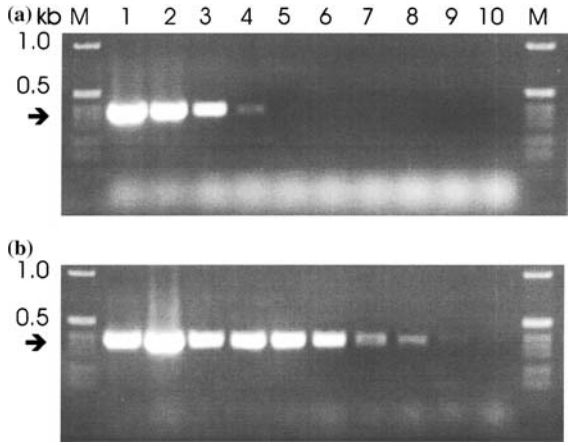

Figure 6. PCR amplification of decreasing amounts (from left to right: 1, 10 ng; 2, 1 ng; 3, 100 pg; 4, 10 pg; 5, 1 pg; 6, 100 fg; 7, 50 fg; 8, 10 fg; 9, 5 fg; 10, 1 fg) of (A) genomic DNA and (B) target ITS sequence of *Phoma tracheiphila* isolate Pt 42 using the specific primer pair *Pt*-FOR2 + *Pt*-REV2. M: molecular weight marker. Molecular weight in kb of some marker bands is specified on the left margin; the specific amplification signal (378 bp) is indicated by an arrow.

Discussion

The results suggest that populations of *P. tracheiphila* in Italy are genetically homogeneous, leading to identical patterns upon amplification with all the assayed RAPD and microsatellite primers and sharing highly conserved ITS1-5.8S-ITS2 sequences among the isolates analysed. This evidence is a prerequisite for developing a reliable diagnostic tool, as the risk of false negatives

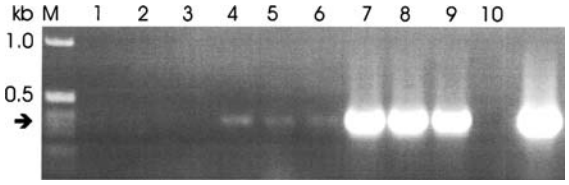

Figure 7. PCR detection of *Phoma tracheiphila* in naturally infected lemon (cv Monachello) tissue by using the primer pair *Pt*-FOR2 + *Pt*-REV2. Amplification was carried out by using approximately 10 ng each of template DNAs of (from left to right): 1–3, healthy plants; 4–6, naturally infected symptomless plants; 7–9, infected plants showing symptoms of "mal secco" disease; 10, negative control (water); 11, positive control (genomic DNA of *P. tracheiphila* isolate Pt 42); M: molecular weight marker. Molecular weight in kb of some marker bands is specified on the left margin; the specific amplification signal (378 bp) is indicated by an arrow.

should be negligible. The genetic homogeneity of *P. tracheiphila* isolates as identified with different molecular markers is in agreement with previous studies in which the variability of Italian isolates was examined with biochemical markers, such as different mycelial isozymes. These features generated identical profiles among isolates of diverse origin (Cacciola et al., 1986), suggesting that the Italian population of the fungus is represented by a clonal lineage. Despite the fact that many authors reported variability of phenotypic characters, such as colony morphology, pigmentation and virulence (Baldacci, 1950; Salerno and Perrotta, 1966; Ciccarone, 1971; Punithalingam and Holliday, 1973; De Cicco and Luisi, 1977; Surico et al., 1981; Rosciglione et al., 1991), it could be inferred that such differences are not correlated to the genetic variability of this fungus as measured with molecular markers.

It is commonly agreed that *P. trachephila* reproduces asexually and that the teleomorph of *P. tracheiphila* has not been identified so far, although a metagenetic relationship with *Leptosphaeria* has been supposed. The phylogenetic analysis performed in this work suggests a close relationship between *P. tracheiphila* and *L. congesta,* but the weak confidence value of the branch node does not lead us to infer anamorphic/teleomorphic connection between these species.

A PCR test was previously developed (Rollo et al., 1990), and more recently applied to reveal the presence of the pathogen on artificially inoculated lemon somaclones (Gentile et al., 2000). While potentially sensitive, this method has never been used on a large scale as a routine diagnostic method, most probably because of the lack of specificity, as shown by the results obtained in the present study using the primer pair A + B which were designed by the cited authors. It has to be emphasized, however, that the paper of Rollo et al. (1990) represents one of the first examples of a PCR-based test applied to the diagnosis of plant pathogenic fungi (Henson and French, 1993).

PCR assay using the primer pair *Pt*-FOR2 + *Pt*-REV2 proved sensitive and specific. It is a very promising tool for the development of a standard diagnostic method, which may be applied both for testing young trees in nurseries for the sanitary certification of propagative material of lemon and for quarantine purposes. Certification is presently achieved by isolating the fungus on agar media (see Data Sheets on Quarantine Pests by CABI and EPPO, 1997). This conventional method, while sensitive and reliable, is time consuming and does not allow an early detection of the "mal secco" disease.

The molecular assay developed in this study could be used to distinguish *P. tracheiphila* from other *Phoma* species, as well as from fungi frequently occurring on the *Citrus* canopy as saprophytes or opportunistic pathogens. This feature is particularly suitable for the diagnosis of wilted twigs, as it could make it possible to distinguish *P. tracheiphila* from *Colletotrichum* species which have a higher saprophytic ability and can easily overshadow the presence of the primary pathogen. Moreover, it could be used as a more reliable method, compared to the conventional one, for the detection of the fungus both in the symptomless twigs and in the hard wood of trees affected by the "mal nero" *facies*.

Finally, the specific primer pair *Pt*-FOR2 + *Pt*-REV2 could be useful for the detection of the inoculum of *P. tracheiphila* surviving in soil, which is responsible for root infections leading to the "mal fulminante" *facies* of the disease (Salerno and Solel, 1988) However, their specificity has to be further ascertained by testing a broader spectrum of soil microrganisms.

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author carried out statistical analysis of sequencing data; the fifth author contributed to the experimental design and to the critical revision of the manuscript; the sixth author coordinated the research and wrote the manuscript.

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This paper is dedicated to Professor Antonio Graniti for his pioneering studies on Citrus mal secco and for having continuously encouraged research on this disease.

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