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Direct detection of *Taphrina deformans* on peach trees using molecular methods

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Abstract

The ascomycetous fungus *Taphrina deformans* is the agent of peach leaf curl, a worldwide disease of peach potentially devastating to both crop yields and tree longevity. Conspicuous leaf curl symptoms result from the invasion of host tissue by the strictly parasitic mycelial phase of the *T. deformans* dimorphic life-cycle. Successful isolation of the fungus in pure culture is cumbersome and limited to late spring/early summer (time of ascospore discharge from infected leaves) and only rarely has the asymptomatic yeast phase been isolated from buds. Molecular methods, namely those based on the hybridisation of nucleic acids, are advantageous for diagnostic purposes since they do not require isolation of the fungus on culture media. Direct amplification using the polymerase chain reaction (PCR) and fluorescent *in situ* hybridisation (FISH) were tested for diagnosis of peach leaf curl disease in order to provide a fast and reliable method for disease risk assessment. Specific primers and probes were designed based on available ribosomal DNA sequence data. Positive and specific diagnoses of peach leaf curl were achieved with primer TDITS1, using PCR-detection, and probe TDE634, using FISH, both on infected leaves and in washings of asymptomatic peach buds.

Abbreviations: bp – base pair; Cy3 – 5,5′-disulfo-1,1′-(-γ-carbopentynyl)-3,3,3′,3′-tetramethylindolocarbocyanin-N-hydroxysuccinimidester; ddH_2O – double distilled sterile water; FISH – fluorescent *in situ* hybridisation; ITS – internal transcribed spacer; PBS – phosphate-buffered saline; PCR – polymerase chain reaction; rDNA – nuclear encoded ribosomal DNA; rRNA – ribosomal RNA; SDS – sodium dodecyl sulfate; YM – yeast extract–malt extract agar.

Introduction

Taphrina deformans is a biotrophic phytoparasitic fungus that causes leaf curl on peach trees (*Prunus persica*) (Mix, 1935). The yeast phase of the life-cycle of this dimorphic ascomycete begins with budding of ascospores discharged from curled leaves onto peach twigs and bud surfaces. (Fitzpatrick, 1934; Mix, 1935; Pscheidt, 1995). When overwintered yeast cells (aka bud-conidia) gain contact with young leaves (i.e. undifferentiated tissue) the fungus switches to the parasitic mycelial phase if favourable climatic conditions occur (see below). The bud-conidia initiate the infection producing a short hypha that penetrates the cuticle and continues to invade the host tissue between the epidermal cells until it reaches the parenchyma cells below (Mix, 1935). This phase culminates with the production of asci, mainly on the upper surface of hypertrophied leaf tissues (Pscheidt,

1995). Inoculation experiments (Fitzpatrick, 1934; Mix, 1935; Caporali, 1971) and fungicide tests (Pierce, 1900) have demonstrated that peach leaf curl is monocyclic and that overwintered yeast cells on peach buds are capable of establishing the disease each spring.

Peach leaf curl has a worldwide distribution and all known peach cultivars are susceptible to infection by *T. deformans* (Pscheidt, 1995). This disease is routinely controlled with fungicides since sanitation and cultural practices are largely ineffective. The risk of severe peach leaf curl outbreaks is high when wet and cold conditions prevail during bud opening and/or due to faulty spraying (Fitzpatrick, 1934; Mix, 1935). Serious outbreaks may cause peach leaves to drop prematurely and defoliation for several seasons can lead to the death of peach trees (Pscheidt, 1995).

The mycelial phase is easily recognisable by the typical curl symptoms on leaves, which are present throughout the spring and early summer. In contrast, *T. deformans* cannot be detected visually during the asymptomatic saprobic phase. The classical way to detect the fungus is the isolation of the yeast phase on culture media. Fitzpatrick (1934) and Caporali (1971) successfully isolated *T. deformans* from buds on peach trees heavily infected in the previous spring. However, Mix (1935) and Martin (1940) failed to isolate the fungus because the isolation process is cumbersome and requires some skill since *T. deformans* is frequently overgrown on culture media by saprophytic fungi present on peach tree surfaces. The fungus may go unobserved on isolation plates, leading to erroneous diagnoses (Mix, 1935; Caporali, 1971).

Molecular genetic differences among *Taphrina* spp. from different hosts have been evaluated by sequencing of selected regions of the ribosomal DNA (rDNA) unit (Rodrigues and Fonseca, 2003). However, no studies have focused on molecular techniques for detection of *Taphrina* directly on their hosts. Molecular diagnostic methods based on the polymerase chain reaction (PCR) with species-specific primers have been used to detect fungal pathogens both in culture and in plant tissues (Henson and French, 1993; Trout et al., 1997; Willits and Sherwood, 1999; Lecomte et al., 2000; Lee and Tewari, 2001; Frederick et al., 2002). Genes encoding ribosomal RNA (rRNA) have been used extensively in PCR tests designed to detect fungal pathogens since the high copy number of these genes enhances sensitivity of the PCR detection and allows the amplification of rDNA from mixed DNA preparations (White et al., 1990; Henson and French, 1993).

Fluorescent *in situ* hybridisation (FISH) with species-specific probes is widely used in ecological studies to detect microorganisms in environmental samples (Amann et al., 1990; Moter and Göbel, 2000), but has been rarely applied to detect plant pathogens in host tissues. Two recent reports have focused on bacterial pathogens of potatoes (Li et al., 1997; Wullings et al., 1998). Probes for FISH usually target the rRNA molecules in intact ribosomes and this technique allows the visualization of whole cells, unlike the PCR-based method.

The current study was undertaken to evaluate two molecular methods for direct detection of *T. deformans* in symptomatic and asymptomatic material, namely leaves and buds from infected peach trees. Two different methods were tested: one based on the PCR technique with specific primers designed in the rDNA internal transcribed spacer (ITS) region and another based on *in situ* hybridisation with a Cy3-labelled specific probe targeted at the 18S rRNA. The sensitivity and specificity of both detection methods were determined and optimised.

Materials and methods

Fungal strains and growth conditions

Taphrina deformans strains used in this study are listed in Table 1. Additional strains for specificity tests represented 12 species in the genus *Taphrina*, two in the genus *Protomyces*, four in the genus *Lalaria* and a few other species usually present on peach trees (Buck et al., 1998) (Table 2). *Protomyces* is the closest relative to the genus *Taphrina* (Sjamsuridzal et al., 1997; Rodrigues and Fonseca, 2003) and *Lalaria* species correspond to yeast-phase anamorphs of *Taphrina* that can occur saprophytically on plant surfaces (e.g. Inácio et al., 2004). The fungi were grown on yeast extract–malt extract (YM) agar at 18–20 °C.

Plant material

Plant samples were collected during the spring and summer of 2001 and autumn and winter of 2001/2002, namely leaves with or without symptoms of

Table 1. Taphrina deformans strains used in the development of PCR-based and FISH detection assays

Strain[a]	Source[b]	GenBank acession N° ITS
CBS 356.35	Fruit of *Prunus persica*, Holland	AF492093
T-470	*Prunus communis* (= *P. dulcis*)	AF492095
AX1	Curled leaves of *Prunus persica*, Lisbon, Portugal	AF492094
P. Soure 3a	Curled leaves of *Prunus persica*, Beira Interior, Portugal	–
DeB1	Curled leaves of *Prunus persica*, Bombarral, Portugal	–
Y17787	*Prunus persica* (?), Japan	–
BTX2	Curled leaves of *Prunus persica*, Lisboa, Portugal	–
CEP A1.1.	Curled leaves of *Prunus persica*, Pegões, Portugal	–
CEP A2.1.	Curled leaves of *Prunus persica*, Pegões, Portugal	–
SaB1	Curled leaves of *Prunus persica*, Santarém, Portugal	–
AlB3	Curled leaves of *Prunus persica*, Alcobaça, Portugal	–
HA1304	*Prunus persica,* Slovakia	AF492092
CBS 353.35	*Prunus persica*, Canada	–

[a] Origin of the strains: CBS, Centraalbureau voor Schimmelcultures (The Netherlands); T or Y, ARS Culture collection (USA); HA, Institute für Angewandte Mikrobiologie (Austria); other numbers correspond to isolates obtained in Portugal by A. Fonseca and M.G. Rodrigues (CREM).

[b] Species names and geographic location of host plants are indicated unless that information is not available for a particular strain, in the latter case a question mark is indicated.

leaf curl and buds of peach trees (cv Tasty Free) in the Prunus orchard of the Agricultural School campus (Instituto Superior de Agronomia) in Lisbon.

DNA extraction

Genomic DNA was extracted from 1 week-old cultures by a simplified method using glass beads for cell disruption (Sampaio et al., 2001). DNA extraction from leaves was based on the protocol of Cennis (1992). Extraction of fungal cells from the surface of peach buds (1g/10ml) was achieved by washing with a solution of SDS (0.15%), with the aid of a vortex (full speed for 2 min). After centrifugation of the washings for 5 min at $11{,}500 \times g$, the resulting pellet was treated according to a protocol modified from Bramwell et al. (1995). The samples were incubated with 500 µl lysis buffer (50 mM Tris; 50 mM EDTA; 250 mM NaCl; 0.3% wt/vol SDS; pH 8) and 20 µl Zymoliase-100T (0.1 mg ml^{-1}, Seikagaku Kogyo, Tokyo, Japan) for 30 min at 37 °C, followed by extraction with chloroform and isoamyl alcohol (24:1). DNA was precipitated with ethanol in the presence of sodium acetate and washed twice with 70% ethanol. DNA was dissolved in double distilled sterile water (ddH_2O).

Primer and probe design

Oligonucleotides to be used as primers or probes were designed using appropriate software developed by João Inácio (CREM, unpublished data). The species-specific primer TDITS1 was designed based on sequence alignment of the ITS region from a number of species in the genus *Taphrina* (Rodrigues and Fonseca, 2003), as well as from other fungi (sequences retrieved from GenBank, NCBI, USA). The probe TDE634 was designed within the 18S rRNA gene based on the alignment of sequences retrieved from GenBank, most of the sequences of *Taphrina* spp. having resulted from the work of Sjamsuridzal et al. (1997). The selected primer TDITS1 and probe TDE634 (monolabelled at the 5′ end with Cy3) were synthesized by MWG Biotech (Ebersberg, Germany).

PCR amplification

All reaction mixtures had a final volume of 25 µl and contained PCR buffer (1×, Fermentas),

Table 2. Fungal strains used in specificity tests of the direct detection methods and the result of PCR amplification with the primer pair TDITS1/NL4

Species/strain[a]	Source[b]	GenBank accession N°		Amplification primer TDITS1[c]
		18S	ITS	
Taphrina spp.				
T. alni HA872	*Alnus incana*, Austria	AJ495831	AF492077	n.a.
T. betulina T-726	*Betula intermedia*, Sweden	–	AF492080	n.a.
T. caerulescens CBS 351.35	*Quercus alba*, N. America?	–	AF492081	n.a.
T. communis T-755	*Prunus nigra*, N. America	(AB000949)[1]	AF492087	n.a.
T. letifera CBS 335.55	*Acer spicatum*, N. America	AB000952	AF492099	n.a.
T. padi CBS 693.93	*Prunus padus*, Germany	(AJ495833)[2]	AF492103	n.a.
T. populina CBS 337.55	*Populus nigra*, Sweden	D14165	AF492106	n.a.
T. pruni HA1306	*Prunus domestica*, Slovakia	AJ495815	AF492111	n.a.
T. pruni–subcordatae CBS 381.39	*Prunus subcordata*, N. America	AB000957	AF492109	n.a.
T. tormentillae CBS 339.55	*Potentilla canadensis*, N. America	–	AF492120	n.a.
T. virginica CBS 340.55	*Ostrya virginiana*, USA	AB000960	AF492125	n.a.
T. wiesneri CBS 275.28	*Prunus avium*, N. America	–	AF492126	n.a.
Protomyces spp.				
Prot. gravidus Y17093	*Ambrosia trifida*, USA	–	–	n.a.
Prot. inouyei YB4354	*Crepis japonica*, Japan	(D11377)[3]	–	n.a.
Species usually found on the surface of plants				
Lalaria inositophila PYCC 5733	*Acer monspessulanum*, Arrábida, Portugal	–	AY239209	n.a.
Lalaria inositophila Silv. 2.1A	*Prunus persica*, Portugal	–	AY239214	n.a.
Lalaria veronaerambellii PYCC 5734	*Acer monspessulanum*, Arrábida, Portugal	–	AY239216	n.a.
Lalaria carpini 5A3.3	*Quercus pyrenaica*, Caramulo, Portugal	–	AY239215	n.a.
Aureobasidium sp.	*Prunus persica* cv. 'Royal Glory', Portugal	–	–	n.a.
Rhodotorula aurantiaca PYCC 4582	Type strain	–	–	n.a.
Rhodotorula bacarum 6QSF4	Leaf of *Quercus faginea*, Arrábida, Portugal	–	–	n.a.
Rhodotorula minuta PYCC 4790	Type strain	–	–	n.a.
Sporolomyces roseus 4QSF19	Leaf of *Quercus faginea*, Arrábida, Portugal	–	–	n.a.
Cryptococcus albidus PYCC 2409	Type strain	–	–	n.a.

[a] Origin of the strains: CBS, Centraalbureau voor Schimmelcultures (The Netherlands); T or Y, ARS Culture collection (USA); HA, Institute für Angewandte Mikrobiologie (Austria); other numbers correspond to isolates obtained in Portugal by A. Fonseca and M.G. Rodrigues (CREM).

[b] Species names and geographic location of host plants are indicated unless that information is not available for a particular strain, in the latter case a question mark is indicated.

[c] n.a. – no amplification.

[1] CBS 352.35;

[2] CBS 683.93;

[3] IFO6898 (CBS 222.57)

3.5 mM $MgCl_2$ (Fermentas), 0.25 mM (each) deoxynucleoside triphosphate (Promega), 0.8 μM (each) primer, 1 U of *Taq* polymerase (Fermentas) and 5 μl of DNA sample. PCR amplification was performed with a Uno II thermal cycler (Biometra) using the following programme: initial denaturation for 2 min at 94 °C, followed by 35 cycles of 94 °C for 30 s, a varying annealing temperature of 59–65 °C for 30 s and 72 °C for 1 min, and a final extension at 72 °C for 5 min. Amplified DNA fragments were visualized under UV light after electrophoresis at 100 V on 1% (wt/vol) agarose gels stained with ethidium bromide and run in 0.5× Tris–borate–EDTA buffer. The quality (e.g. absence of PCR inhibitors) of each DNA sample was checked with the universal primers NL1 and NL4, which amplify a ca. 600 bp region at the 5′ end of the 26S rDNA (White et al., 1990). Positive (DNA from pure culture of *T. deformans*) and negative (ddH_2O) amplification controls were included in each experiment. All PCR reactions were performed, at least, twice.

PCR sensitivity tests

The sensitivity of the PCR detection was determined by testing a dilution series of yeast cells from a pure culture of *T. deformans*. Initial cell concentration was adjusted to 1×10^6 cells ml^{-1} as determined by counting with a haemocytometer and a serial 10-fold dilution was performed down to 100 cells ml^{-1}. Genomic DNA from each dilution (100 μl) was extracted using the protocol described for peach buds and dissolved in 100 μl of sterile ddH_2O. Therefore, for the initial suspension the resulting 100 μl of DNA was extracted from the equivalent of 1×10^5 cells and the ensuing PCR reaction corresponded to the amplification of DNA from 5×10^3 cells.

FISH

Pure cultures for *in situ* hybridisation were grown in liquid YM medium at 18 °C for 2 days. 1 Cell suspension (1 ml) was centrifuged for 5 min at $11{,}500 \times g$ and the pellet was fixed for 2 h at 4 °C by resuspending in an equal amount of a freshly prepared 4% (wt/vol) paraformaldehyde solution in phosphate-buffered saline (PBS) (10 mM phosphate buffer, pH 7.4, and 140 mM NaCl). After fixation, the samples were washed twice with PBS and resuspended in 1 volume of PBS and 1 volume of cold ethanol (100%). Asci and ascospores of *T. deformans* were obtained by scraping the surface of infected leaves that showed mature symptoms (presence of asci). The extract was fixed using the same method for cells from pure culture. Fungal cells were extracted from asymptomatic peach buds (1 g peach buds/10 ml) by washing with ddH_2O, with the aid of a vortex (full speed for 2 min). The washings were centrifuged for 5 min at $11{,}500 \times g$ and the pellet was fixed in the same manner as the cells from pure culture. About 10 μl (pure culture) and 100 μl (pellet from the washings) of cell suspensions were centrifuged in 1.5 ml tubes; the resulting pellet was incubated at 46 °C for 2 h, with 9 μl (pure culture) and 27 μl (bud washings) of hybridisation solution (containing 5 M NaCl; 20 mM Tris–HCl pH 7; 1% (wt/vol) SDS; 25% (wt/vol) formamide) and 1 μl of probe (50 ng ml^{-1}) per each 9 μl of hybridisation solution. Excess of probe was removed by incubation for 30 min at 46 °C with 1 ml of washing solution (containing 5 M NaCl; 0.5 M EDTA pH 8; 1 M Tris–HCl pH 8; 10% (wt/vol) SDS). After the washing step the tubes were centrifuged for 5 min at $11{,}500 \times g$ and the supernatant was discarded, leaving a small quantity of liquid in the tube. After resuspension of the pelleted cells, 10 μl of suspension were spotted on a microscope slide (Superior Marienfeld, ref. 12164201) and dried at room temperature. Slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA), a coverslip was applied and slides were observed with a light microscope (OLYMPUS BX50) fitted for epifluorescence microscopy with a U-ULH 100W mercury high pressure bulb and a U-MA 1007 filter set for the fluorochrome Cy3 (OLYMPUS). Microphotographs were obtained using a digital camera (OLYMPUS C3030) and edited using standard software (Adobe Photoshop 6.0, Adobe). In FISH experiments each sample was hybridized with the universal probe EUK516 (positive control) (Amann et al., 1990) and with the probe DAN651 (specific for the ascomycetous yeast species *Dekkera anomala*; negative control) (J. Inácio, unpublished data).

Results

Selection of species-specific oligonucleotides for detection of Taphrina deformans

The design of the *T. deformans*-specific primer for PCR detection, TDITS1, was based on multiple sequence alignment of the highly variable rDNA ITS1 region (Figure 1a). TDITS1 (5′-TCTCCGGATGGGTTTCAA-3′) was used as forward primer in conjunction with the universal primer NL4 located toward the 5′ end of the 26S rRNA gene. The predicted length of the amplified fragment was 1132 bp (Figure 1b). Preliminary experiments with the reverse universal primer ITS4 produced a shorter amplicon (ca. 500 bp), but amplification was possible only up to 62 °C whereas the pair TDITS1/NL4 could be used at higher annealing temperatures. A second specific primer was found in the ITS2 region, but it did not produce the predicted amplicon when used in conjunction with TDITS1, probably due to formation of a hairpin structure (data not shown).

On the other hand, the design of the FISH probe was based on sequences from the less variable 18S rRNA coding region of the rDNA unit, since its targets are the rRNA molecules in the ribosomes. Only a few sequence differences were found between the 18S rDNA sequence of *T. defomans* and those of the other species in the genus *Taphrina*, but it was possible to design a probe TDE634 (5′-AAA-GCCTCGGTCAAGCCA-3′) fully complementary to the rDNA sequence depicted in Figure 1c.

Primer TDITS1 specificity

The optimal annealing temperature determined for the primer pair TDITS1/NL4 was 63 °C; this pair gave rise to a single amplicon of the expected size when used for PCR amplification with DNA extracted from pure cultures. A ca. 1132 bp DNA fragment was amplified from all *T. deformans*

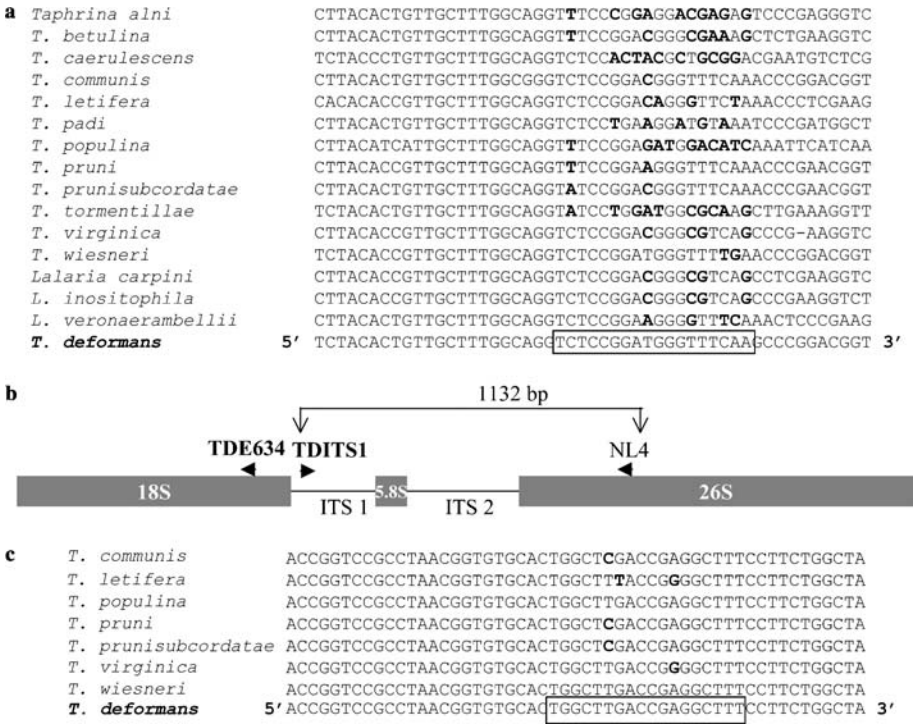

Figure 1. Details of the alignment of rDNA sequences for the design of species-specific oligonucleotides. (a) ITS sequences of *T. deformans* and other species in the genera *Taphrina* and *Lalaria* and design of the primer for PCR-based detection; sequence of primer TDITS1 is highlighted by a box and the mismatches are indicated in bold type. (b) Location of the PCR-detection primers and of probe TDE634 in the rDNA unit. (c) 18S rDNA sequences of *T. deformans* and other species of *Taphrina* and design of the FISH probe; sequence of probe TDE634 is highlighted by a box and the mismatches are indicated in bold type.

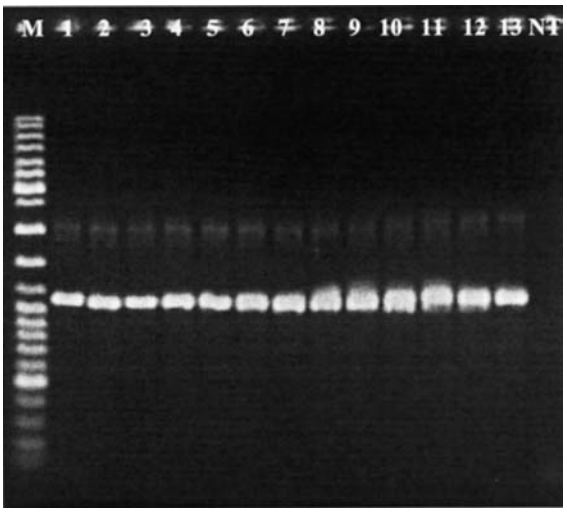

Figure 2. PCR products obtained with DNA extracted from pure cultures of strains of the species *T. deformans*. Primers used were TDITS1 and NL4 (universal primer). M: molecular weight marker 1 Kb; Lane 1: CBS 356.35; Lane 2: T-470; Lane 3: AX1; Lane 4: P. Soure 3a; Lane 5: DeB1; Lane 6: Y17787; Lane 7: BTX2; Lane 8: CEP A1.1.; Lane 9: CEP A2.1.; Lane 10: SaB1; Lane 11: AlB3; Lane 12: HA1304; Lane 13: CBS 353.35; NT: no template, (ddH2O).

strains tested, but not from the other species in Table 2 using the same primers (Figure 2 and Table 2). The quality of all DNA samples was suitable for PCR amplification, as evaluated with the primer pair NL1/NL4 (data not shown).

PCR sensitivity test

DNA extracted from a 10-fold dilution series of *T. deformans* yeast cells was used for PCR detection with TDITS1 and NL4 primers. DNA fragments, amplified using 5 µl of each DNA extraction, were seen down to the equivalent of 5 yeast cells per PCR reaction (corresponding to the 10^3 cells ml^{-1} dilution), while only a faint band was visible with the 100 cells ml^{-1} dilution (data not shown).

PCR detection on peach leaves and buds

DNA extracted from leaves (with or without symptoms) was suitable for PCR, since amplification with the universal primers NL1 and NL4 resulted in positive reactions (Figure 3). Positive reactions were obtained with DNA extracted from leaves with peach leaf curl symptoms using the specific primer TDITS1 in combination with NL4 (Figure 3, lanes 1–3). DNA extracted from the washings of peach buds from trees heavily infected with peach leaf curl in the previous spring appeared not to contain any PCR inhibitors (Figure 3, lanes 6–10 with primer combination NL1/NL4). PCR amplification with the pair TDITS1/NL4 detected *T. deformans* in peach buds collected during the autumn of 2001 and

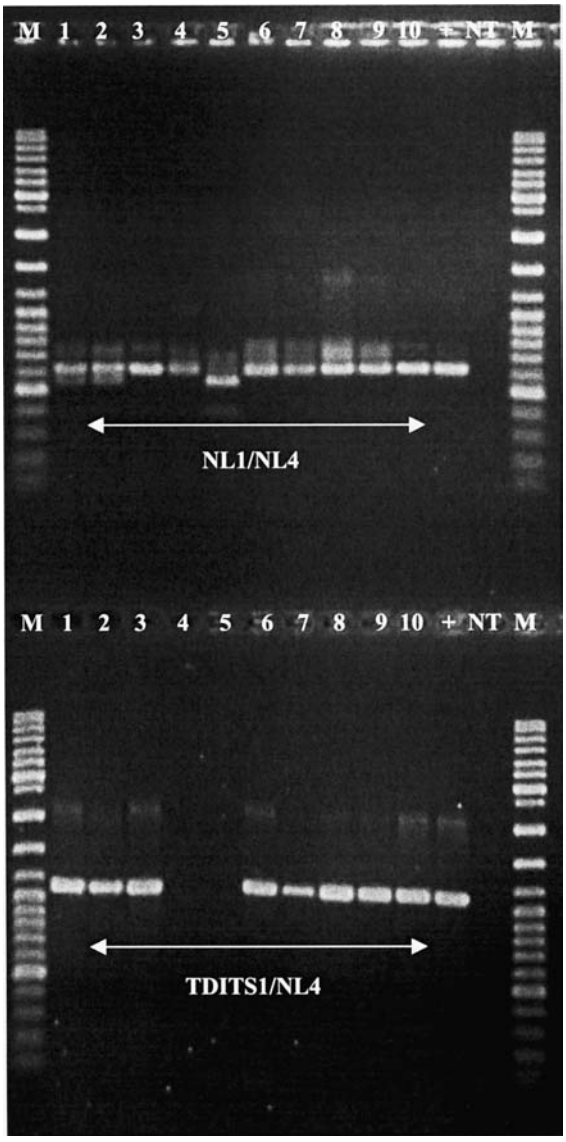

Figure 3. PCR detection of *T. deformans* in natural samples, with the primer pairs NL1/NL4 and TDITS1/NL4. M: molecular weight marker 1 Kb; Lanes 1–3: DNA from peach leaves with symptoms collected in the spring of 2001; Lanes 4, 5: DNA from peach leaves without symptoms collected in the spring of 2001; Lanes 6–10: DNA from washings of peach buds collected during the autumn and winter of 2001/2002; +: DNA from pure culture of *T. deformans*; NT: no template (ddH2O).

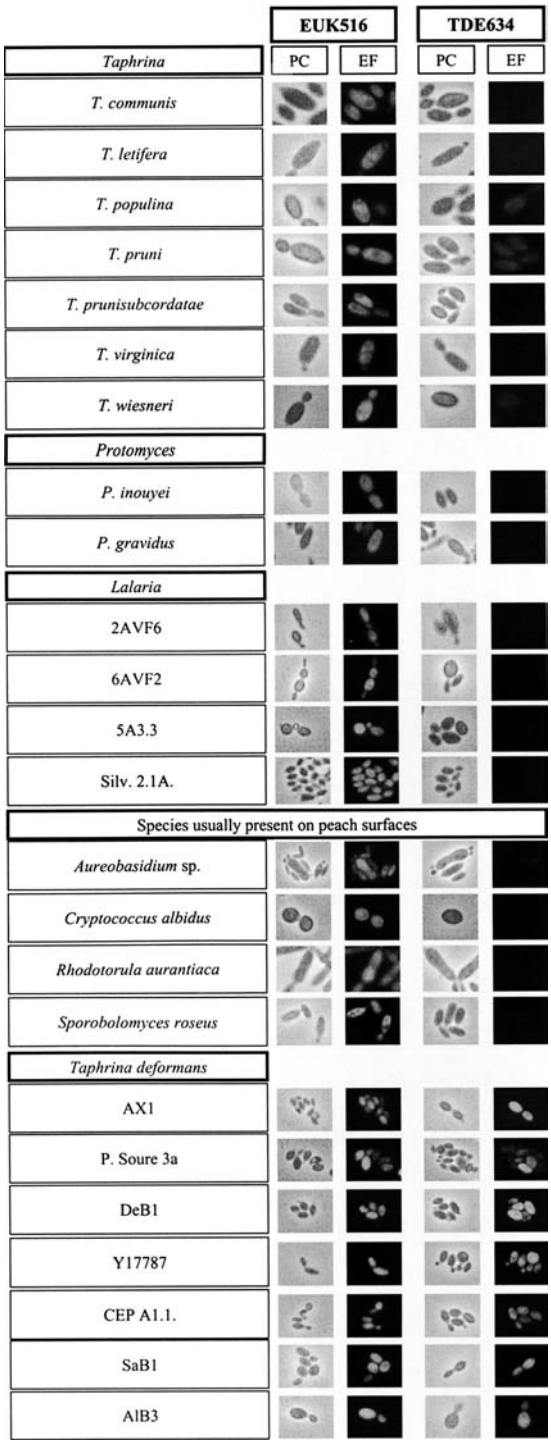

Figure 4. FISH assays with cells from pure cultures of species in the genera *Taphrina*, *Protomyces* and *Lalaria*, as well as species usually found on plant surfaces; hybridisation was performed with the *T. deformans*-specific probe, TDE634 (targeted to the 18S rRNA and labelled with the red excitation stain Cy3) and with the universal eukaryotic probe, EUK516. Microphotographs: PC – Phase Contrast; EF – Epifluorescence.

winter of 2002 (Figure 3, lanes 6–10 with primer combination TDITS1/NL4). The amplicon was identical to that obtained from DNA extracted from pure culture of the fungus and equally strong, thus demonstrating the efficacy and reproducibility of the PCR assay. As expected, DNA from uninfected peach leaves did not produce an amplicon with the specific primer TDITS1 (Figure 3, lanes 4 and 5), confirming that peach DNA is not amplified with this primer.

Probe TDE634 specificity

Specific hybridisation and strong fluorescent signals were observed using probe TDE634 with cells from pure culture of all *T. deformans* strains but not with those of the other species tested (Figure 4). Cells from the species *T. populina*, *T. pruni* and *T. wiesneri* showed a weak signal with the probe TDE634 (Figure 4), as expected according to the sequence alignment (Figure 1). The same result was expected for *T. prunisubcordatae* (Figure 1c) but the cells did not show detectable fluorescent signals. However, the presence of these *Taphrina* species on peach trees is highly improbable (e.g. Mix, 1949). More importantly, species that are normally present on peach trees (Buck et al., 1998; Table 2) were not detectable with the specific probe (Figure 4).

FISH with species-specific probe TDE634

FISH detection was tested in washings from peach buds and scrapings of peach leaves with mature symptoms (Figure 5). Typical *T. deformans* ascospores hybridized with the TDE634 probe and showed strong fluorescence signals (Figure 5a, b), but not with the probe DAN651 (negative control; data not shown). A few cells that resulted from the washing of peach buds also showed strong signals with the specific probe (Figure 5c, d). Background fluorescence from peach leaf debris and bud extracts made the interpretation of the results more difficult, although a few putative *T. deformans* cells could be recognized among autofluorescent plant debris (Figure 5c, d).

Discussion

We tested two molecular methods aimed at the rapid identification and detection of *T. deformans*

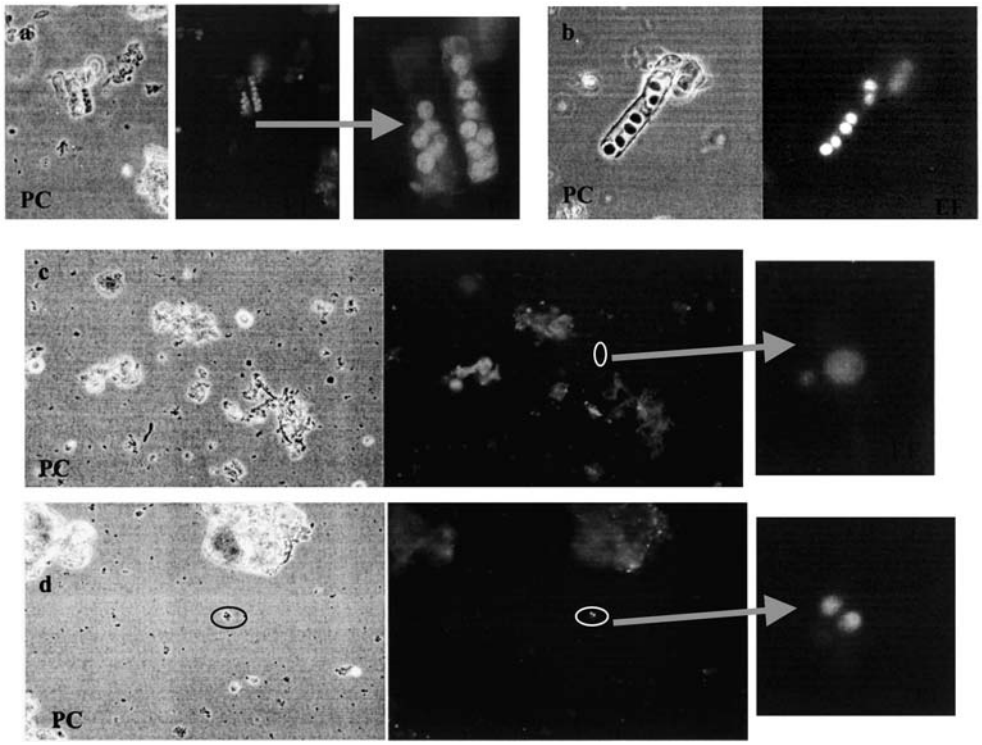

Figure 5. FISH assays with scrapings from the surface of curled peach leaves (a,b) and with peach bud washings (trees infected in the previous spring) (c,d). Hybridisation was performed with the *T. deformans*-specific probe, TDE634 and with the universal eukaryote probe, EUK516. Microphotographs: PC – Phase contrast; EF – Epifluorescence.

that was both specific and sensitive. The newly designed primer TDITS1 was specific for *T. deformans* since it amplified a fragment of 1132 bp from 13 *T. deformans* isolates with different origins, when used in combination with the universal primer NL4. No amplification was observed using DNA from other species in the genera *Taphrina*, *Lalaria* and *Protomyces*, or from other fungi present on peach surfaces (Table 2). Using the same primer combination, *T. deformans* was detected in infected plant tissues, during the symptomatic and asymptomatic phases of the life-cycle of the fungus (Figure 3).

PCR detection using the *T. deformans*-specific primer designed herein could be used to monitor latent infections and colonization of peach trees by *T. deformans* (ascospores and/or yeast cells). The early detection of the fungus is important to help in deciding whether to apply fungicides, and to prevent peach tree nurseries from distributing trees contaminated with *T. deformans*. This method may also be useful for monitoring the efficacy of fungicide treatments.

The *T. deformans*-specific FISH probe detected the fungus in pure culture, as well as in natural samples, i.e. washings from peach buds and scrapings of the surface of diseased leaves. The FISH technique may be valuable not only for the detection of the fungus but especially for studying the epidemiology of the pathogen and tracing the ecological niches where the fungus can survive. Our observations confirmed the results of previous studies, which involved the isolation of the pathogen on culture media (Fitzpatrick, 1934; Caporali, 1971), that the fungus is present and overwinters in peach buds. Application of FISH has the additional advantage of being completed in 1 day. However this technique's major drawback results from the background fluorescence of chlorophyll in plant tissues (Moter and Göbel, 2000).

In conclusion, the PCR-based detection method proved to be useful for disease risk assessment

providing a rapid, specific and sensitive detection of *T. deformans* directly on peach trees.

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