

# Qualitative real-time PCR SYBR® Green detection of Petri disease fungi

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**Summary.** Real-time PCR provides a fast, reliable, and cost-effective method for detecting the presence or absence of Petri disease fungi in grapevines. The primer pairs, Pmo1f + Pmo2r, and Pac1f + Pac2r, were designed for species and genus-specific amplification of *Phaeoconiella chlamydospora* and *Phaeoacremonium* spp. respectively, using real-time PCR with SYBR® Green. The primers were specific and showed no primer-primer dimers until after 35 cycles. *Pa. chlamydospora* was detected in roots, shoots, and young trunks of drill-inoculated vines. *Phaeoacremonium* was detected in trunk cross-sections of naturally infected vines from which *Phaeoacremonium aleophilum* had been isolated. The protocol presented here can be adapted to provide a reliable detection system for research and industry.

**Key words:** *Phaeoconiella*, *Phaeoacremonium*, grapevine decline, clean vine.

## Introduction

A study was conducted to determine the extent and cause of vine decline in the states of Pennsylvania and New York (Stewart and Wenner, 2004). Results obtained from the study showed that Petri disease fungi are commonly associated with mature declining vines and with recently planted grafted vines. *Phaeoconiella chlamydospora* (W. Gams, Crous, M.J. Wingfield & L. Mugnai) Crous & W. Gams and several species of *Phaeoacremonium* W. Gams, Crous & M.J. Wingfield are the causal agents of Petri disease, previously known as black goo (Morton, 1995) of grapevine (Ferreira *et al.*, 1994; Crous and Gams, 2000; Fourie *et al.*,

2000; Sidoti *et al.*, 2000; Fourie and Halleen, 2002; Gubler, 2003).

Conventional PCR-based methods have been published for the detection of *Phaeoacremonium* spp. and *Pa. chlamydospora* (Groenewald *et al.*, 2000; Tegli *et al.*, 2000). However, these methods have not been adapted to real-time PCR, which would afford scientists and industry a rapid, practical technique to detect the presence of Petri disease fungi in symptomatic and asymptomatic vines. This report describes a qualitative real-time PCR technique that does not require a DNA quantification step, providing investigators with a simple method to screen grapevines for *Pa. chlamydospora* and *Phaeoacremonium* spp.

## Materials and methods

### Fungal strains

Cultures of *Phaeoconiella chlamydospora* (Pch), *Phaeoacremonium* spp., and *Cadophora* La-

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gerb. & Melin collected during a grapevine decline survey in Pennsylvania and New York were maintained on malt extract agar (MA) at room temperature and stored long-term in 100% glycerol over liquid nitrogen. The following isolates were used in this study: Pch 129 and 178; *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingfield & L. Mugnai (Pal), 287; *Phaeoacremonium rubrigenum* W. Gams, Crous & M.J. Wingfield (Prub), 322; *Phaeoacremonium mortoniae* Crous & W. Gams (Pmort), 170; *Phaeoacremonium angustius* W. Gams, Crous & M.J. Wingfield, 316; and *Cadophora* cf. *malorum* (Kidd & Beaum) W. Gams, 294. Isolates were identified using standard morphology and confirmed by DNA sequencing of the internal transcribed spacer (ITS) rDNA region and subsequent blast searches. There is uncertainty surrounding the name *P. angustius* because two different sequences, both putatively from the ex-type culture (CBS 249.95) have been deposited in GenBank under this name; AF118138 deposited by Dupont *et al.* (2000) and AF197974 deposited by Groenewald *et al.* (2001). The Dupont *et al.* (2000) GenBank sequence (AF118138) is identical to the sequence deposited from the ex-type culture of *P. aleophilum*. The Groenewald *et al.* (2001) GenBank sequence (AF197974) shares sequence homology with the ex-type sequence deposited for *Phaeoacremonium viticola* J. Dupont. Therefore, isolate 316 is abbreviated (Pang/Pvit).

#### DNA extraction and ITS rDNA sequencing

ITS rDNA sequence data was generated for Pch and *Phaeoacremonium* isolates used as positive controls. Mycelia from positive controls were lyophilized and extraction of genomic DNA was carried out using the phenol and chloroform method outlined in Stewart *et al.* (1999). A 50  $\mu$ l polymerase chain reaction (PCR) was used for ITS amplification following Chaverri *et al.* (2001) using the primer pairs: ITS1 (5'-TCCG TAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990). The ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit with Amplitaq DNA Polymerase FS (PE Applied Biosystems, Foster City, CA, USA) was utilized for sequencing cleaned PCR products for each primer direction as per PE Applied Biosystems instructions. Performa<sup>®</sup> DTR gel filtration cartridges from Edge BioSystems were used for cleaning cycle se-

quencing products. Sequence data were generated at the Nucleic Acid Facility (Life Science Consortium, The Pennsylvania State University).

#### Designing PCR primers

Primers were developed for species-specific and genus level detection of *Phaeoacremonium* and *Phaeoacremonium*, respectively. PCR primers were designed using the primer design software program MacMolly Tetra<sup>®</sup> Lite (the freeware version of Soft Gene's MacMolly Tetra<sup>®</sup> software package). ITS rDNA sequences obtained from GenBank for *Phaeoacremonium inflatipes* W. Gams, Crous & M.J. Wingfield, Pin (AF118140); *Phaeoacremonium parasiticum* (Ajello, Georg & C.J.K. Wang) W. Gams, Crous & M.J. Wingfield, Ppar (U31841); *P. viticola*, Pvit (AF118137); Pal (AF017651); Pmort (AF295328; ex-type CBS 101585) in GenBank as *P. inflatipes*; Pang (AF197974); Prub (AF118139); and Pch (AF197973) were utilized in the design of genus and species-specific primers. The following primers were designed for *Phaeoacremonium* spp.: ITS forward primer Pac1f (5'-ACC CTT TGT GAA CAT ACC TG-3'), ITS reverse primer Pac2r (5'-TAC TGC GCT CGG AGT CCT G-3') (Fig. 1). The following species-specific primers were designed for Pch: ITS forward primer Pmo1f (5'-GTT ACA TGT GAC GTC TGA ACG-3'); ITS reverse primer Pmo2r (5'-CAG TGT ATG CTT GAT TGC TCG-3') (Fig. 1).

#### Sample preparation and primer evaluation

The primers Pmo1f, Pmo2f and Pac1f, Pac2r were used to amplify target DNA and test specificity against DNA from non-target fungi using isolates 129, 178, 287, 322, 170, 316, and 294. Pch-infected vines were generated from two-bud cuttings. One-hundred cuttings were taken from one-year-old 'Vidal Blanc' ('Vidal') wood during the month of April 2003, from the Galen Glen vineyard in Andreas, Pennsylvania. Cuttings were surface disinfested by soaking in 0.5% Chinosol for 24 h and rooted in Fafard No. 2 potting mix in a cutting box for 2 months. Five cuttings, with shoots 8 cm long, were drill-inoculated (Larignon and Dubos, 1997) with a 20  $\mu$ l suspension of Pch 129 conidia ( $1 \times 10^6$  conidia ml<sup>-1</sup>) and transplanted into 11.4-cm-diam. pots. DNA was extracted from the roots, shoots, and young trunks of three 10-month-old inoculated vines. Trunks were sampled 2 cm

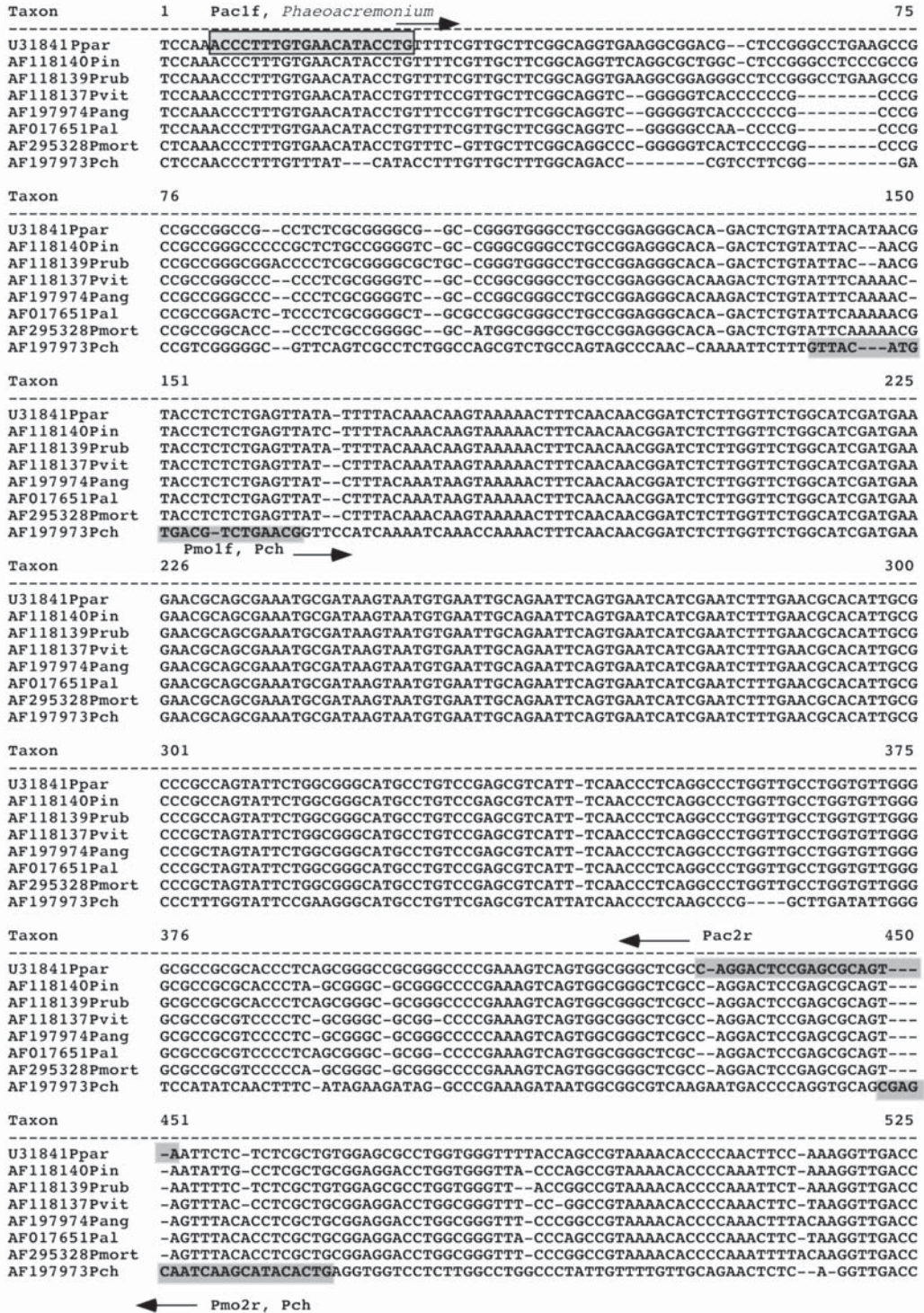


Fig. 1. Multiple alignment of ITS rDNA region. Primer pair locality is indicated by highlighted blocks. The sense of each primer is in accordance with the arrow reported above or below the sequence.

above the site of inoculation. Roots and shoots varied in length and were sampled at their mid-point. Samples were not surface-sterilized prior to DNA extraction. DNA was extracted from three (CG2B, CG3U, CG31U), 6-mm-thick cross-sections of trunks obtained from declining vines from which Pal had previously been isolated.

For all infected vines 30–40 mg of tissue from trunk cross sections, roots, shoots or young trunks were ground in liquid nitrogen and total DNA was obtained using a DNAeasy® Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Positive real-time PCR products were sequenced and compared with sequences in GenBank.

**Detection limit**

Cultures of Pal (287) and Pch (178) were grown on malt extract agar for 30 days under cool fluorescent light. Plates were flooded with 10 ml of autoclaved double distilled water and gently agitated with a sterile glass rod to release conidia. The total concentration of conidia ml<sup>-1</sup> was determined using a hemacytometer. Woody grape tissue (30 mg) was autoclaved and placed in sterile grinding dishes. Concentrations of 200, 100 and 20 conidia of Pal were added to individual grinding dishes followed by grinding and extraction of total DNA. The process was repeated for Pch, with 372, 74, and 37 conidia. Real-time PCR reactions were completed using 1 µl of template with 11.25 µl of sterile H<sub>2</sub>O, and 12.25 µl of 1:25 and 1:50 dilutions.

**Real-time PCR assay for *Phaeoconiella* and *Phaeoacremonium* spp.**

For a single real-time PCR reaction, 12.25 µl of a 1:50 dilution of DNA from pure cultures of fungi, trunk cross-sections, roots, shoots, or young trunk material was added to 12.5 µl Quanti Tect™ SYBR® Green reaction mix (Qiagen, Hilden, Germany), and 0.125 µl of each primer was added from a 100 mM stock solution (0.5 mM final concentration), for a total reaction volume of 25 µl. The following primer pairs were utilized in separate reactions for amplification of the target genus: Pac1f, Pac2r primer pair (*Phaeoacremonium*); the Pmo1f, Pmo2r primer pair (*Phaeoconiella*). All real-time PCR reactions were performed in 25 µl tubes (Cepheid, Sunnyvale, CA, USA) using a Cepheid Smart

Cycler®. All real-time PCR reactions were run as follows: 95°C for 15 min (hot start), followed by 35–40 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 40 s (optics on).

**Results**

An alignment showing the locations where the primers anneal is in Fig. 1. Primers designed for Pch (Pmo1f, Pmo2r) and *Phaeoacremonium* spp. (Pac1f, Pac2r) were specific (Fig. 2 and 3). The real-time PCR product for Pch was approximately 320 base pairs, and identity was confirmed by sequencing. The PCR products for *Phaeoacremonium* species differed slightly in length because of insertions or deletions in the ITS rDNA region between species, with the sequences from Prub and Pal being the largest at approximately 428 base pairs. In general, the real-time PCR prod-

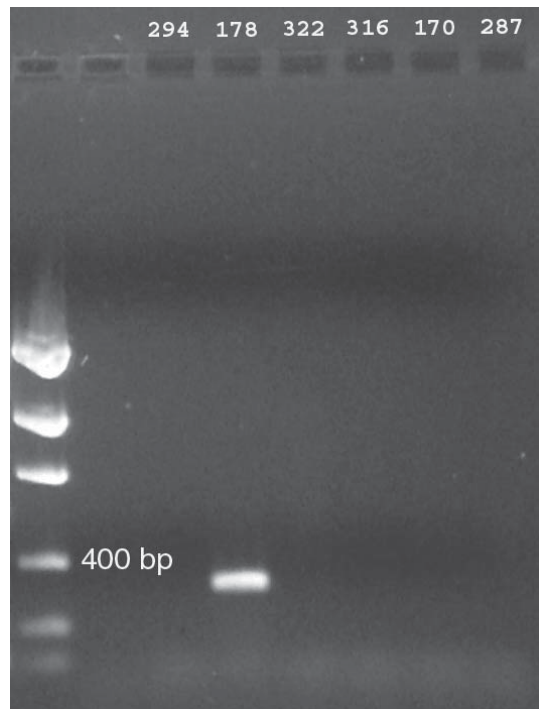


Fig. 2. Gel electrophoresis of real-time PCR products for *Phaeoconiella chlamydospora* (Pch) primer verification: 287 (*P. aleophilum*), 170 (*P. mortoniae*), 316 (*P. angustius/P. viticola*), 322 (*P. rubrigenum*), 178 (Pch), 294 (*Cadophora cf. malorum*). Note that non-target DNA 287, 170, 316, 322, and 294 were not amplified. The real-time PCR product was approximately 320 bp for Pch.

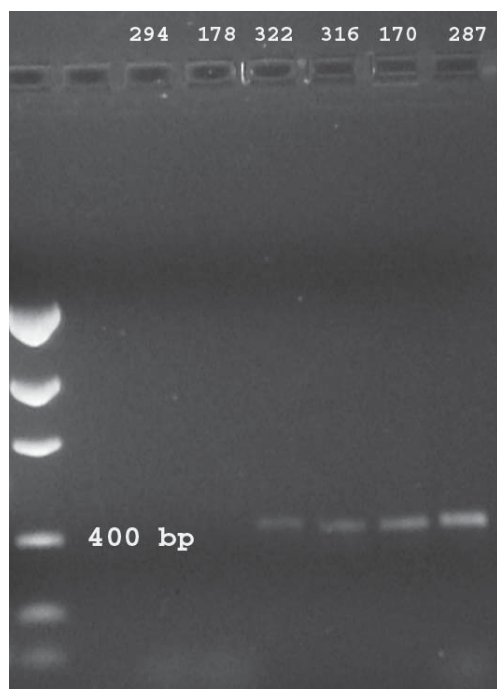


Fig. 3. Gel electrophoresis of real-time PCR products for *Phaeoacremonium* spp. primer verification: 287 (*P. aleophilum*), 170 (*P. mortoniae*), 316 (*P. angustius* / *P. viticola*), 322 (*P. rubrigenum*, Prub), 178 (*Pa. chlamydospora*, Pch), 294 (*Cadophora* cf. *malorum*). Note that non-target DNA 178 (Pch) and 294 (*C. cf. malorum*) were not amplified. Real-time PCR products are 410–430 base pairs. Prub (322) has the largest PCR product, approximately 428 base pairs.

ucts for *Phaeoacremonium* species were 410–428 base pairs. The isolates used for positive controls were identified to species using morphology and sequence data derived from the ITS rDNA region. Isolate numbers and corresponding names are cited in materials and methods. In all cases, the non-target DNA used for negative controls did not amplify (Fig. 2 and 3).

‘Vidal’ cuttings drill-inoculated with Pch tested positive in the roots, shoots, and young trunk regions using the real-time PCR method outlined in this study. The real-time PCR detection from the trunk had a cycle threshold (Ct) score equal to that of the positive control (Table 1, Fig. 4). The shoot and root tissue had the highest Ct scores (35.2 and 33.8, respectively). The 320 base pair real-time PCR product matched GenBank accessions for Pch.

Three pieces of trunk cross-sections stored at 4°C from which Pal had previously been isolated tested positive using real-time PCR, and the identity was confirmed by sequencing (Table 2, Fig. 5). The Pac1f, Pac2r primers are capable of amplifying other *Phaeoacremonium* spp. that may have been present, but were not isolated from the trunk cross-section. However, trace data from sequenced products showed no evidence of multiple products.

#### Detection limit

Conidia from Pch (178) were detected at all concentrations (372, 74, and 37 conidia) when tem-

Table 1. Real-time PCR results from ‘Vidal’ cuttings drill-inoculated with *Phaeoacremonium chlamydospora* (Pch).

Plant tissue sampled	Mean Ct Score	Pch
Young trunk	22.9 SD +/- 0.7	Positive
Root	33.8 SD +/- 0.2	Positive
Shoot	35.2 SD +/- 1.4	Positive
Positive control Pch (178)	23.1 SD +/- 5.5	Positive
No template control (NTC)	Negative	Negative

Table 2. Real-time PCR results from trunk cross-sections in which *Phaeoacremonium aleophilum* (Pal) was isolated.

Trunk cross-sections	Ct scores	Pal
CG2B	33.41	Positive
CG3U	27.93	Positive
CG31U	36.74	Positive
Positive control Pal (287)	33.35	Positive
No template control (NTC)	Negative	Negative
Negative control Pch (178)	Negative	Negative

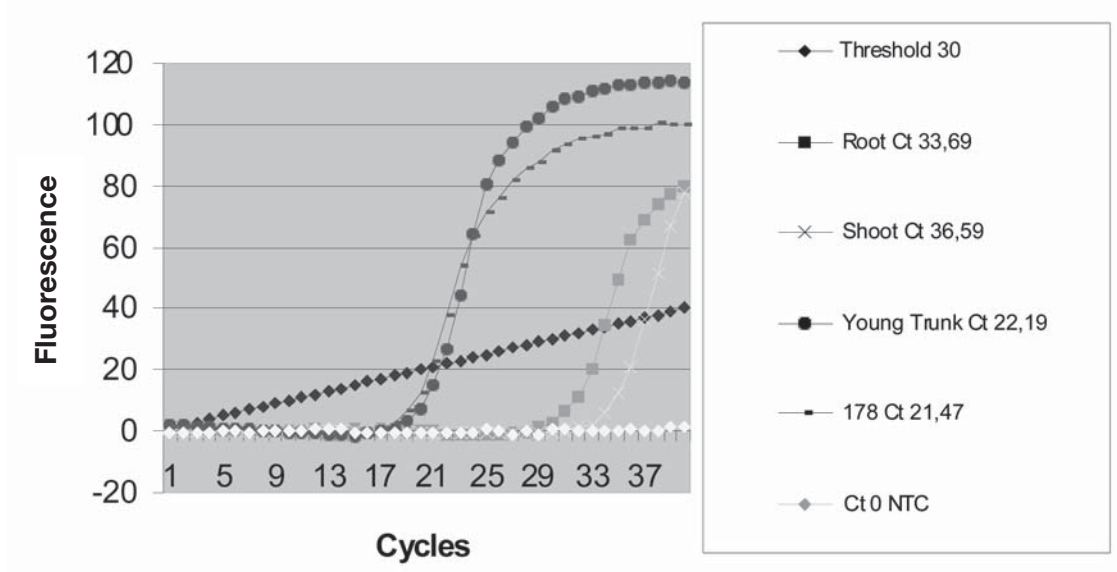


Fig. 4. Real-time PCR data from 1 of 3 replicates for plants drill-inoculated with *Phaeomoniella chlamydospora*. Cycle threshold (Ct) scores were recorded when fluorescence values crossed an original threshold set at 30 fluorescence units.

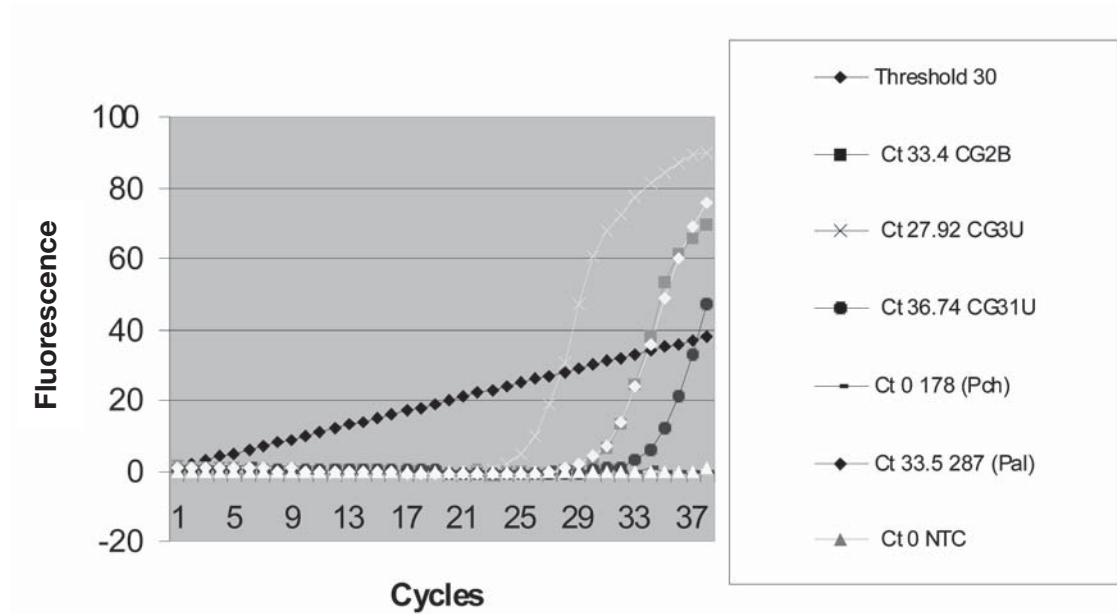


Fig. 5. Real-time PCR run data from trunk cross-sections from which *Phaeoacremonium aleophilum* (Pal) had previously been isolated. Cycle threshold (Ct) scores were recorded when fluorescence values crossed an original threshold set at 30 fluorescence units. Cycle threshold (Ct) scores were consistent with the positive Pal control.

plate DNA, or 1:25 and 1:50 dilutions of extracted DNA were used. Conidia from Pal (287) were detected at all concentrations (201, 100, and 20 conidia) when template DNA or 1:25 dilutions were used. The 1:50 dilution of DNA extracted from 20 conidia failed to consistently yield a positive PCR reaction.

## Discussion

Based on the grapevine decline survey reported by Stewart and Wenner (2004) and the results published by Halleen *et al.* (2003), the grape industry should be concerned with detecting the presence or absence of these fungi in foundation block plantings and nursery stock. Conventional methods used to identify *Pa. chlamydospora* and *Phaeoacremonium* spp. from grape involve a combination of recognizing symptoms, fungal isolation, and morphological examination. Symptoms can be unreliable, with some plants appearing asymptomatic for long periods, and distinguishing species based on morphology is difficult without extensive training in fungal taxonomy. Therefore, there is a need for an accurate, rapid, and affordable test to detect Petri disease fungi. The advantages of SYBR<sup>®</sup> Green real-time PCR detection over conventional methods are specificity, sensitivity and speed, but as with all PCR methods, the extraction protocol and primer design are basic factors influencing the efficiency of the reaction.

The DNAeasy<sup>®</sup> Plant Mini Kit from Qiagen was used to establish a standard method for extracting initial target DNA without a quantification step. The extraction method and concentration of initial target DNA are important for detection and sensitivity if quantification of the target pathogen is desired. However, quantification of initial target DNA extracted, or of the pathogen from multiple samples in an industrial setting would be cost-prohibitive and laborious. The extraction method described here, provided target-DNA concentrations that yielded consistent positive results. It was possible to detect as few as 37 conidia of *Pa. chlamydospora* using 1  $\mu$ l of extracted DNA, and 12.5  $\mu$ l of 1:25 and 1:50 dilutions of extracted DNA. However, for *P. aleophilum*, 20 conidia could not be consistently detected when a 1:50 dilution was used, but gave consistent posi-

tives when a 1:25 dilution or 1  $\mu$ l of extracted DNA was used.

Species-specific and genus-specific primers have been published for the detection of *Pa. chlamydospora* and *Phaeoacremonium* using standard PCR (Groenewald *et al.*, 2000; Tegli *et al.*, 2000). The primer pairs; Pch1, Pch2 for Pch, and Pal1N, Pal2 for *Phaeoacremonium* (Tegli *et al.*, 2000) did not consistently amplify target DNA when adapted to real-time PCR in this study. Two of the primers designed herein for real-time PCR share homology with those previously published. The Pmo1f primer designed in this study is almost identical to the PCL1 primer designed by Groenewald *et al.* (2000). The Pac2r primer reported in this study overlaps the Pal2 primer designed by Tegli *et al.* (2000).

The real-time PCR method reported here was used for the detection of Petri diseased fungi in shoots and roots from 10-month old cuttings that had been drill-inoculated, and from trunk cross-sections of naturally infected vines. The genus-specific primers Pmo1f and Pmo2r selectively amplified positive controls of *Pa. chlamydospora* but did not amplify *Phaeoacremonium* or *Cadophora* cf. *malorum* negative controls. The *Phaeoacremonium* specific primers Pac1f, Pac2r selectively amplified positive controls of Pal, Prub, Pmort, and Pang/Pvit, but did not amplify *Pa. chlamydospora* or *Cadophora* cf. *malorum*. In both cases, primer-primer dimers were observed in later cycles (38–40), when the annealing temperature was set to 55°C. The Cepheid<sup>®</sup> Smart Cycler melt curve analysis program was not required to distinguish primer-primer dimers from actual positive reactions. Therefore, this detection method could be used in research and adopted by industry as a diagnostic tool. The Cepheid<sup>®</sup> Smart Cycler is portable, meaning multiple users could share in the use and cost of the equipment.

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