

Evaluation of Molecular Markers for *Phytophthora ramorum* Detection and Identification Using a Standardized Library of Isolates¹

F.N. Martin,² M. Coffey,³ R. Hamelin,⁴ P. Tooley,⁵ M. Garbelotto,⁶
K. Hughes,⁷ and T. Kubisiak⁸

Abstract

A number of molecular diagnostic procedures for detection of *Phytophthora ramorum* have been reported in the literature. In an effort to evaluate the specificity of 10 of these techniques a standardized DNA library for 317 isolates was assembled that included 60 described species as well as 22 taxonomically unclassified isolates. These were sent blind at a concentration of ca. 10 ng/μl (a concentration greater than would be encountered in field samples, but was used in an effort to fully evaluate specificity) to collaborators to evaluate the various diagnostic procedures. In general the procedures worked well with varying levels of specificity observed among the different techniques. Low levels of nonspecific amplification were observed for the mitochondrial markers and most of the real-time assays based on nuclear markers. The highest level of false positives was obtained with the conventional nested ITS procedure; however, this technique is not standalone and is used in conjunction with two other assays for diagnostic purposes. The results from the APHIS lab and another lab indicated that using three assays improved the accuracy of the results compared to looking at a single assay alone. The SSCP procedure accurately identified *P. ramorum* and was helpful in classification of other isolates to a species level. Given that one of the objectives of the trials was to determine if there would be any false positives, the DNA concentrations that were tested in all but one of the assays (6 to 10 ng/amplification) was higher than would be expected when processing field samples. As a result false positives for some of the assays (in particular real-time assays that had high C_t values) may not be representative of what might be encountered with field samples. Additional evaluations for these samples with a dilution series of target DNA are needed to evaluate specificity at DNA concentrations more reflective of what would be encountered in field assays. Trials evaluating marker performance with samples recovered from the field are in progress.

Key words: Diagnostics, molecular detection.

¹ A version of this paper was presented at the Sudden Oak Death Third Science Symposium, March 5–9, 2007, Santa Rosa, California.

² USDA-ARS, Salinas, CA, fmartin@pw.ars.usda.gov.

³ University of California, Riverside, CA.

⁴ NRC Canada Forest Service, Ste-Foy, QC, Canada.

⁵ USDA-ARS, Ft. Detrick, MD.

⁶ University of California, Berkeley, CA.

⁷ Central Science Lab, York, UK.

⁸ USDA Forest Service, Saucier, MS.

Introduction

For effective and accurate diagnosis of *Phytophthora ramorum* it is imperative that the techniques employed be fully tested for specificity to ensure that false positives will not be encountered when other species are present in infected plant tissue. Likewise, it is important that isolates of the pathogen from a geographic representation of the pathogens distribution also be evaluated to ensure they can be accurately identified. A number of molecular techniques have been developed for detection of *P. ramorum* from infected tissue. However, there has not been a side by side comparison of the techniques with a broad range of *Phytophthora* spp. using the same DNA samples to fully evaluate specificity.

The objective of this research project was to evaluate the specificity of different molecular detection methods for *P. ramorum* using a standardized library of DNA recovered from a range of *Phytophthora* species recovered from geographically diverse areas. Examples of several *Pythium* spp. were included as well. DNA concentration was intentionally higher than would normally be encountered in field samples in an effort to fully evaluate marker specificity and samples were sent blind to all cooperating labs for analysis using marker systems based on spacer regions (the rDNA ITS region, the spacer region between the *cox* I and II gene) or specific genes (beta tubulin, elicitin) and have been configured for use by both conventional and real-time PCR. A technique based on single strand conformational polymorphism of the ITS region was also evaluated for isolate identification.

Materials and Methods

Marker Systems Evaluated

ITS—

Conventional PCR—

- APHIS approved nested amplification – APHIS lab (Beltsville, MD) using procedure of Garbelotto and others (2002)
- Multiplexed amplification using technique of Winton and Hansen (2001) for detection of *P. lateralis*- APHIS lab (Beltsville, MD)

Real-time PCR—

- Technique of the Central Science Lab (CSL) in York, UK (Hughes and others 2006) – K. Hughes lab, CSL
- APHIS approved CSL technique (some procedural differences from the published CSL procedure) – APHIS lab (Beltsville, MD)
- Nested technique of Hayden and others (2006) – Garbelotto lab
- Technique of Bilodeau and others (2007) – Hamelin lab

Single strand conformational polymorphism—

- Technique of Kong and others (2003) modified by running in automated sequencing unit – Kubisiak lab

Nuclear genes—

Real-time PCR—

- Beta tubulin and elicitin genes using technique of Bilodeau and others (2007) - Hamelin lab

Mitochondrial region (cox spacer region)—

- Conventional PCR – using the nested amplification technique of Martin and others (2003) – Martin lab
- Real-time PCR – using the technique of Tooley and others (2006) – Tooley lab

DNA Samples—

DNA was extracted from 317 isolates representing 60 described species and 22 isolates with ambiguous taxonomic classification. Aliquots were coded and sent blind to all participants (a total of 457 samples were sent). The ITS region for all extracts were also amplified and sequenced to confirm the identity of the samples. Some samples were sent multiple times, when this was done they were recoded.

DNA Concentration—

DNAs were provided at a concentration of 10 ng / μ l and 1 μ l/amplification was used in all procedures with the exception of:

- The nested ITS real-time procedure of Hayden and others (2006) done by the Garbelotto lab - the DNA was diluted 1:100,000 before adding 6.25 μ l to the first round amplification (62.5 fg in 25 μ l final volume). In the second amplification the first round was diluted 1/500 and 5 μ l was added to the master mix (final volume of 15 μ l). Some samples were also run with a dilution of 1/100 (62.5 pg DNA/amplification) for the first round amplification.
- The 3 tests performed by the APHIS lab (APHIS approved ITS nested amplification, the multiplex amplification using the technique of Winton and Hansen (2001), and the APHIS approved CSL real-time ITS technique) - the DNA was diluted 1:10 and 6 μ l of this was added to the amplification reaction (6 ng DNA in 25 μ l final volume); samples were also run with 1:100 dilutions as well.

Notes on Sample Scoring—

Real-time PCR using the techniques of Bilodeau and others (2007; 3 nuclear loci) and the *cox* spacer region using the technique of Tooley and others (2006) - PCR positives were based on a positive result with a C_t cut off of 40. CSL real-time procedure—PCR positives were scored on a C_t cut off of 36. Below 36 they are scored as positive, between 36 to 40 they are retested (if the tests had been done with plant samples they would be re-extracted when material is available), and samples giving C_t of 40 were scored as PCR negative.

APHIS procedures – conclusions on positive samples were based on the results of the three assays that were performed. A retest of the samples would have been triggered by the sample DNA producing a nested PCR band from at least one of the two tested DNA dilutions (1:10 and 1:100 in double distilled H₂O of the provided DNA stock), not producing a *Phytophthora*-specific Multiplex PCR band with the *P. lateralis* primers of Winton and Hansen (2001), and reacting weakly (C_t -values >30) or not at all with the APHIS approved CSL real-time assay. Similar to the CSL scoring noted above, samples with a C_t of 36 and above must be retested or the results confirmed with the nested ITS procedure. When assaying infected plant material retests that were still ambiguous would have amplicons sequenced to confirm the diagnosis.

Procedure for SSCP—

One microliter of each sample was PCR amplified using the oomycete specific primers ITS-6 and ITS-7 (cited in Kong and others 2003). ITS6 was fluorescently 5'-end labeled with 6-FAM and ITS7 with HEX. A 1:10 dilution of the PCR product was run using the default criteria specified in the "High Throughput Fluorescent SSCP Analysis User Bulletin" from Applied Biosystems on an ABI3100. The separation matrix consisted of 5 percent GeneScan polymer containing 10 percent glycerol and ROX500 was used as the internal migration rate standard. Using this procedure many species had only two main peaks in contrast to the four banded pattern previously reported using slab gel systems (Kong and others 2003). For data analysis, only the largest 6FAM and HEX peaks were scored.

Results and Discussion

The general results that were obtained are outlined in the abstract. To evaluate false negatives a total of 67 samples representing 48 isolates of *P. ramorum* from nine locations were sent to the participants and with few exceptions all were correctly identified. The exceptions were for one or two procedures with specific isolates that were tested a single time; before conclusions about the accuracy of these techniques can be drawn the isolates need to be retested.

Literature Cited

- Bilodeau, G.J.; Lévesque, C.A.; De Cock, A.; Duchaine, C.; Kristjansson, G.; Uribe, P.; Martin, F.N.; Hamelin, R.C. 2007.** Molecular detection of *Phytophthora ramorum* by real-time pcr using taqman, sybr green and molecular beacons with three genes. *Phytopathology*. 97:632–642.
- Garbelotto, M.; Rizzo, D.M.; Hayden, K.; Meija-Chang, M.; Davidson, J.M.; Tjosvold, S. 2002a.** *Phytophthora ramorum* and sudden oak death in California. III. Pathogen genetics. In: Standiford, R. and McCreary, D. (eds.) 5th Symposium on California Oak Woodlands. USDA Forest Service, General Technical Report PSW-GTR-184: 765–774.
- Hayden, K.; Ivors, K.; Wilkinson, C.; Garbelotto, M. 2006.** TaqMan chemistry for *Phytophthora ramorum* detection and quantification, with a comparison of diagnostic methods. *Phytopathology*. 96: 846–854.
- Hughes, K.J.D.; Tomlinson, J.A.; Griffin, R.L.; Boonham, N.; Inman, A.J.; Lane, C.R. 2006.** Development of a one-step real-time PCR assay for diagnosis of *Phytophthora ramorum*. *Phytopathology*. 96: 975–981.
- Kong, P.; Hong, C., Richardson, P.A.; Gallegly, M.E. 2003.** Single-strand-conformation polymorphism of ribosomal DNA for rapid species differentiation in genus *Phytophthora*. *Fungal Genet. Biol.* 39: 238–249.
- Levy, L; Mavrodieva, V. 2003.** Evaluation of the PCR detection and DNA isolation methods for use in the *Phytophthora ramorum* national pilot survey, USDA-APHIS. <http://www.ceris.purdue.edu/napis/pests/sod/natplan/nplan02.html>
- Martin, F.N.; Tooley, P.W.; Blomquist, C. 2004.** Molecular detection of *Phytophthora ramorum*, the causal agent of sudden oak death in California, and two additional species commonly recovered from diseased plant material. *Phytopathology*. 94: 621–631.
- Tooley, P.W.; Martin, F.N.; Carras, M.M.; Frederick, R.D. 2005.** Real-time fluorescent PCR detection of *Phytophthora ramorum* and *Phytophthora pseudosyringae* using mitochondrial gene regions. *Phytopathology*. 96: 336–345.
- Winton, L.M.; Hansen, E.M. 2001.** Molecular diagnosis of *Phytophthora lateralis* in trees, water, and foliage baits using multiplex polymerase chain reaction. *Forest Pathology*. 31: 275–283.