

Molecular Markers for Identification of *P. ramorum* and Other *Phytophthora* species From Diseased Tissue¹

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Abstract

Molecular techniques have been developed for detection and identification of *P. ramorum* and other *Phytophthora* species that are based on the mitochondrially encoded sequences. One technique uses a *Phytophthora* genus specific primer to determine if a *Phytophthora* species is present, followed by a nested amplification with species-specific primers to determine if the pathogen is *P. ramorum* or one of several other species (such as *P. nemorosa* or *P. pseudosyringe*). This procedure also uses a plant primer pair to serve as a positive control ensuring the ability of the template DNA to amplify. These species-specific and plant markers have been modified for use with real-time PCR. A second technique relies on RFLP analysis of the Cox I and II gene cluster to identify isolates of *Phytophthora* to a species level.

Key words: detection, identification, mitochondrial DNA, *Phytophthora ramorum*

Introduction

The use of molecular methods for detection of plant pathogens can reduce the time required to process samples, as well as improve the accuracy of pathogen identification. Rather than focus on identifying a single species, methods that focus first on identification at the genus level, followed by a nested species-specific detection, could facilitate identification of a broader number of pathogens. For example, while *P. ramorum* is the causal agent of sudden oak death, other species can be recovered from forest samples exhibiting similar symptoms. The diversity of *Phytophthora* species recovered from nursery samples that cause symptoms similar to *P. ramorum* would be even greater. Having a marker system that would first accurately determine if a *Phytophthora* species was present, and then allow identification to a species level, would simplify sample processing, as well as provide additional data on the diversity of *Phytophthora* species present on the hosts under investigation.

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Species-specific PCR Detection

A PCR detection system has been developed that is based on amplification of the spacer region between the mitochondrially encoded Cox I and II genes of *Phytophthora* using genus-specific primers from the conserved regions of the flanking genes (Martin and others 2004). The first round, multiplex amplification contained two primer pairs, one for amplification of plant sequences to serve as an internal, positive control to ensure that extracted DNA was of sufficient quality to allow for PCR amplification, and a second primer pair specific for amplification of sequences from *Phytophthora* species. The products of the first round amplification were amplified with a nested primer pair that was specific for either *P. ramorum*, *P. nemorosa* or *P. pseudosyringae*. Using purified pathogen DNA, the limit of detection for *P. ramorum* using this marker system was approximately 2.0 fg of total DNA. However, when this DNA was spiked with DNA from healthy plant tissue extracted with a commercial miniprep procedure, the sensitivity of detection was reduced by 100- to 1000-fold. This was dependent on the plant species and suggests that the techniques used for DNA extraction did not remove all PCR inhibitors. Using a dilution series of purified DNA from *P. ramorum* the nested mitochondrial marker system was found to have a level of sensitivity comparable to the nested ITS marker system (Garbelotto and others 2002). This marker system was validated with DNA extracted from naturally infected plant samples collected in the field by comparing the sequence of the *Phytophthora* genus-specific amplicon, morphological identification of cultures recovered from the same lesions, and for *P. ramorum*, amplification with a previously published rDNA ITS species-specific primer pair. Results were compared and validated with three different brands of thermocyclers in two different laboratories to provide information about how the described PCR assay performs under different laboratory conditions. The specificity of the *Phytophthora* genus-specific primers suggests they will have utility for pathogen detection in other *Phytophthora* pathosystems, and the variability encountered in the spacer regions should be useful for constructing additional species-specific primers (markers have been developed for five additional species). More recent work has demonstrated that RFLP analysis of the genus-specific amplicons can be used for identification of unknown isolates (Martin, unpublished). The plant primers and species-specific primers for *P. ramorum* and *P. pseudosyringae* from this marker system also have been adopted for use with TaqMan real-time PCR (Tooley and others 2004 and unpublished). Conditions for multiplexed amplification with the plant, *P. ramorum*, and *P. pseudosyringae* primers have been identified and additional work to add *P. nemorosa* to this multiplexed amplification is in progress.

Identification of *Phytophthora* spp. to Species Level by RFLP Analysis

A RFLP method has been developed for identification of *Phytophthora* species based on PCR amplification of a region spanning the mitochondrially encoded Cox I and II genes (Martin and Tooley 2004). Of the 24 species where multiple isolates were examined, intraspecific

polymorphisms were not observed for 16 species, while five species exhibited limited intraspecific polymorphism that could be explained by the addition/loss of a single restriction site. Intraspecific polymorphisms were observed for three species; however, these differences may be a reflection of the variation that has been reported in the literature. The use of the computer program BioNumerics simplified data analysis and identification of isolates. Successful template amplification was obtained with DNA recovered from hyphae using a boiling miniprep procedure, thereby reducing the time and materials needed for conducting this analysis. While the primers were capable of amplifying target DNA from 300 isolates of 31 species in the genus *Phytophthora* tested at the time of publication, additional testing has identified several species that do not amplify. Work is currently in progress to develop primers to amplify all *Phytophthora* species, but not plant DNA, to enable their use for amplification directly from diseased tissue (Martin, unpublished). Additional details on these marker systems can be found at <http://www.ars.usda.gov/Research/docs.htm?docid=8728>.

References

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