Nested TaqMan® PCR for Detection of *Phytophthora ramorum* in Environmental Plant Samples

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INTRODUCTION

Diagnosis of *Phytophthora ramorum* was initially attempted via direct isolation from symptomatic plant tissue. However, *Phytophthora* species are often difficult to culture from plants, which can lead to false-negative isolations and misdiagnosis of infected plant material. Polymerase Chain Reaction (PCR) has been used previously to detect other *Phytophthora* species, however a nested PCR approach may be required to detect low levels of pathogen DNA, or when samples contain inhibitors. We have developed 4 specific PCR primers and one real-time probe based on sequences of the internal transcribed spacer of the nuclear rDNA of *P. ramorum*. Colonization of various plant tissues by the pathogen was detected with traditional PCR, followed by a second round of real-time PCR utilizing TaqMan® chemistry.

MATERIALS AND METHODS*

Template DNA

This optimized amplification protocol was used with extracts of DNA from symptomatic plant tissue and culture. Environmental plant extracts were diluted to a minimum of 1:100 before first round PCR.

Specificity of amplification was tested on dilutions of DNA standards (ranging from 0.0015 pg to 1,000 pg) extracted from pure cultures of 18 *Phytophthora* species (Table 1).

Primers and Probes

The first set of primers (**Phyto1** and **Phyto4**) was designed to amplify a 687 bp region of the ribosomal operon of *P. ramorum,* including portions of the ITS1 and ITS2, and entire 5.8S rDNA (FIG.1).

Products from the first amplification were diluted 1:500 in PCR water, then processed for the second amplification.

The second set of primers (**Pram5** and **Pram6**) and fluorogenic probe (**Pram7**) were designed to amplify a 74 bp fragment fully nested within the Phyto1-4 amplicon (FIG.1). Products were visualized using a lightcycler (FIG.2) and TaqMan® chemistry.

Each second round reaction (total volume 15 µl) contained:

1 X	TaqMan® Universal PCR Master Mix (Applied Biosystems)
0.2 µM	each primer
0.2 µM	hybrid oligo (probe)
5 µl	template DNA (diluted 1:500).

PCR was performed in an iCycler thermocycler using the conditions:

1 cycle at	50 C for 10 min,
1 cycle at	95 C for 3 min,
40 cycles at	95 C for 15 sec and
	60.5 C for 1 min.

*The complete protocol can be obtained by request: kivors@nature.berkeley.edu

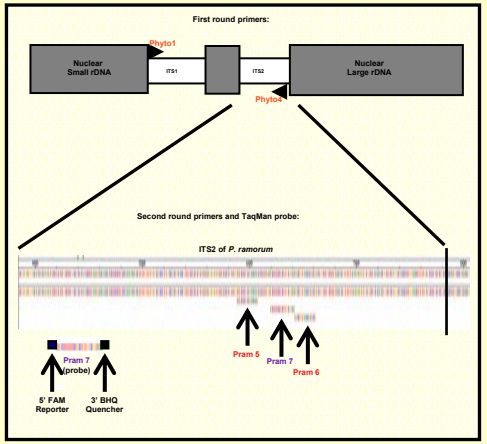

FIG. 1. Maps of ITS region of the nuclear rDNA of *P. ramorum*, indicating location of designed primers and probe.

Table 1. *Phytophthora* species tested with the nested TaqMan® protocol:

P. boehmeriae (1)*
P. cambivora (1)
P. capsici (2)
P. cinnamomi A1 (1)
P. cinnamomi A2 (1)
P. cryptogea (1)
P. erythroseptica (1)
P. gonapodyides (1)
P. hibernalis (3)
P. ilicis (1)
P. lateralis (3)
P. megasperma (1)
P. nemorosa (2)
P. nicotianae (1)
P. palmivora (1)
P. pseudosyringae (1)
P. ramorum (15)
P. syringae (1)

*Number in parentheses indicates number of isolates tested

FIG. 2. BioRad iCycler thermocycler used for real-time PCR.

RESULTS

- This protocol amplified all *P. ramorum* DNA extract dilutions ≥0.015 pg.
- Of the 18 species tested, only *P. ramorum* amplified at concentrations expected in plant tissue.
- Extracts of *P. lateralis* cross-amplified at high concentrations (250 pg), but with detection thresholds >33 cycles. In contrast, *P. ramorum* could be detected at concentrations 4 orders of magnitude lower.
- TaqMan® PCR without first round amplification was significantly less sensitive than nested approach. Amounts of pathogen DNA were rather low in some types of tissue; many sample extracts needed both rounds of amplification before yielding a positive result.
- The nested approach demonstrated repeated successful detection of *P. ramorum* when culturing failed.
- Representative graphs of results from different extracts and dilutions shown below (FIG. 3a & 3b).

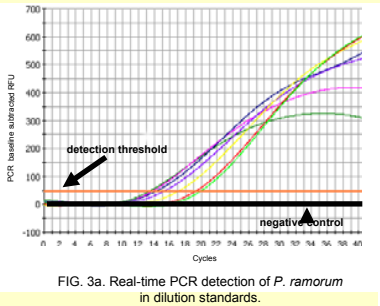

FIG. 3a. Real-time PCR detection of *P. ramorum* in dilution standards.

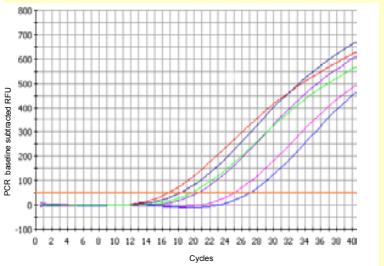

FIG. 3b. Real-time PCR detection of *P. ramorum* in environmental plant extracts.

DISCUSSION

The TaqMan® probe, primers, and nested protocol developed in this study can be used as a rapid screening tool for the detection of *P. ramorum* DNA in pure-culture and environmental plant extracts, without prior isolation and characterization of the organism by traditional microbiological methods. The total assay, from DNA extraction through both rounds of PCR, can be completed in less than 10 hours without the need to run agarose gels or sequencing reactions. This method is extremely sensitive, detecting less than 0.015 pg (15 fg) of *P. ramorum* DNA per reaction. Currently we are using this approach to examine the distribution and spread of this newly described plant pathogen, as this method has allowed us to expand the known host and geographic range of *P. ramorum* within the Western U.S.