Systematic development of markers for *Phytophthora* species using mitochondrial loci

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Abstract

A number of *Phytophthora* species are non-native to the United States and are considered potentially devastating if introduced through shipment of plant material. A TaqMan real time PCR genus and species-specific diagnostic assay was developed for their detection based on mitochondrial gene order differences that allows for the systematic development of species-specific TaqMan probes. Previous research validated this marker system for 14 *Phytophthora* spp. (Phytopathology 104: 733-748) and the project was recently expanded by designing over 100 *in silico* species-specific probes as well as optimizing the system by normalizing annealing temperature and probe concentration across all assays. In addition, 36 new species-specific probes targeting primarily invasive species were validated against 135 different taxa in two laboratories. All probes were found to be species-specific and could be multiplexed with a genus-specific probe and a plant internal control. The assays were also validated with multiple thermal cyclers in two labs to identify potential problems with technology transfer. In an effort to simplify identification of multiple species present in a single sample, RFLP and terminal RFLP fragment analysis (primers were fluorescently labeled and fragment sizes determined on a DNA sequencer) of the genus specific amplicon digested with several restriction enzymes was conducted. To facilitate isolate identification in these mixed samples a Java based program was developed. This system represents a comprehensive, hierarchal approach to increase detection capability and provide important tools for investigating the community structure of *Phytophthora*.

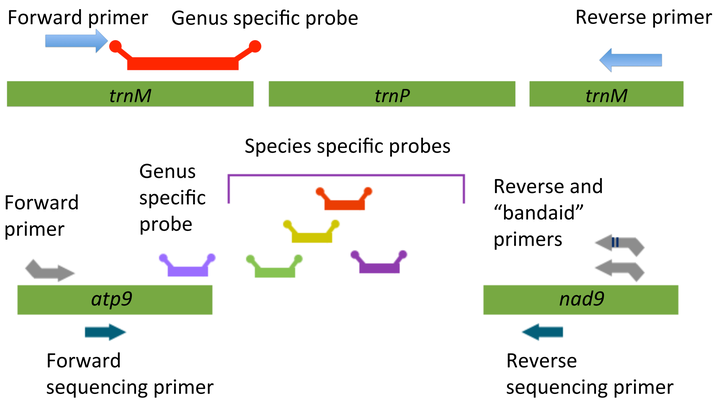

Fig. 1. Graphical representation of the *Phytophthora* TaqMan detection system for the mitochondrial loci *trnM-trnP-trnM* and *atp9-nad9*, originally by Bilodeau et al. 2014 and further refined in this study.

Introduction

Problems for detection

Traditional plating assays from plant samples can take 4-6 weeks to get results.

Several *Phytophthora* species are often present in a single environmental sample and standard surveys often miss the bigger picture by targeting a single species.

The genus *Phytophthora* contains many non-native plant pathogens that currently have no detection assays available. A systematic approach to develop species-specific assays for many species would simplify development of diagnostic assays.

Objectives

1) Develop and validate several species-specific probes for important non-native *Phytophthora* species
2) Optimize the amplification of the *atp9-nad9* assay for all members of the genus *Phytophthora* by standardizing the annealing temperature
3) Develop multiple methods to confirm species identification outside of traditional amplicon sequencing
4) Explore alternative technologies for this locus to make quantification more accurate

References/Acknowledgements

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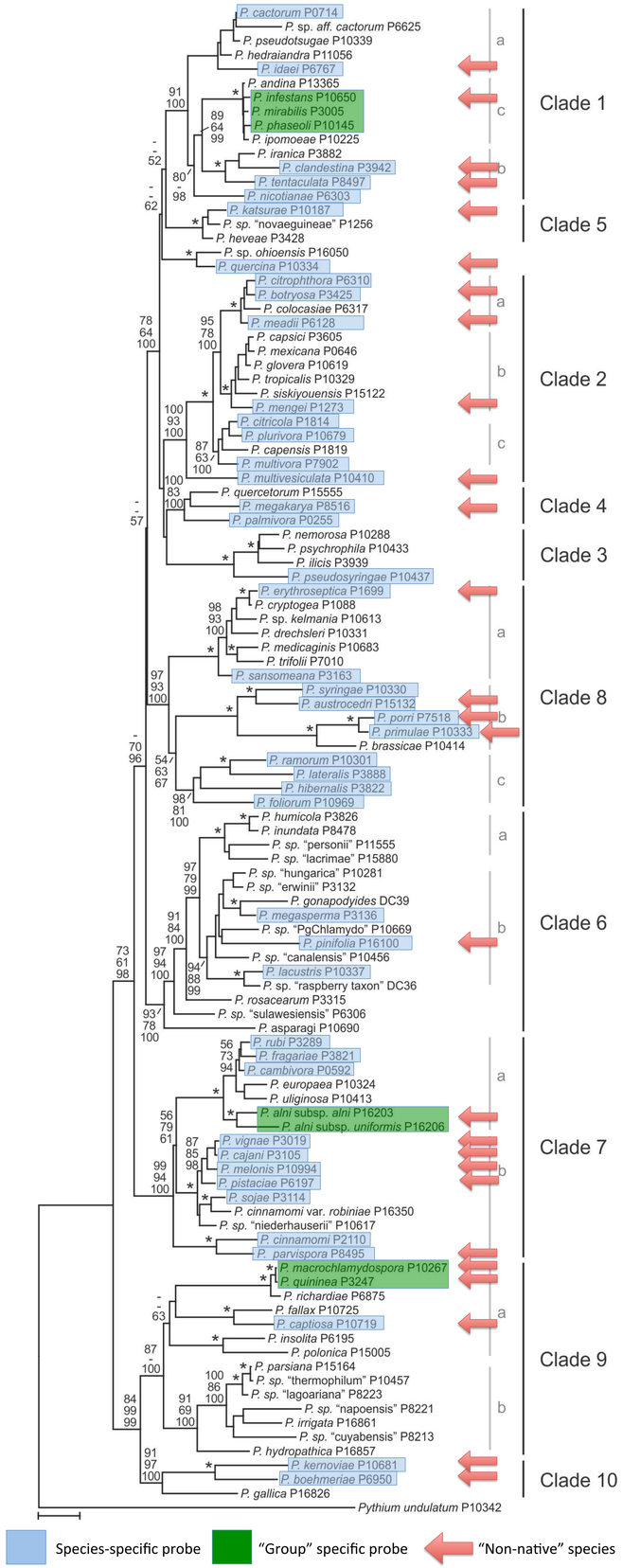


Fig. 2. Phylogram of *Phytophthora* spp. based on mitochondrial DNA markers (Martin *et al*., 2014) showing validated species-specific probes for the *atp9-nad9* region (validated probes denoted by shaded boxes). These probes have been tested for specificity against all of the listed species as well as *in vitro* sensitivity. "Non-native" species identified from Swartzburg *et al*., 2009.

Optimization

All assays use the same annealing temperature by using a "bandaid" primer

5 *Phytophthora* spp. contain SNPs in the original reverse primer, the bandaid primer improves amplification efficiency

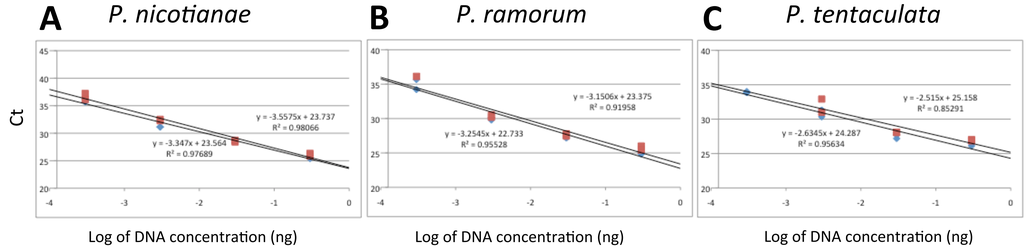


Fig. 3. Standard curve plots of sensitivity for three important *Phytophthora* species detected using the *atp9-nad9* mitochondrial locus with genus (blue diamonds) and species specific (red squares) probes. A) *P. nicotianae*. B) *P. ramorum* and C) *P. tentaculata*. All amplifications were performed at the same annealing temperature.

Alternative uses for these loci

PCR-RFLP analysis

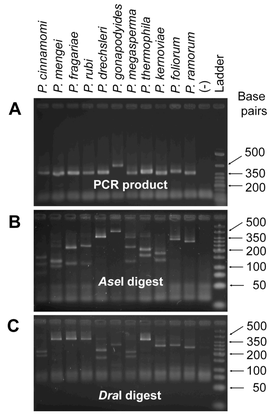
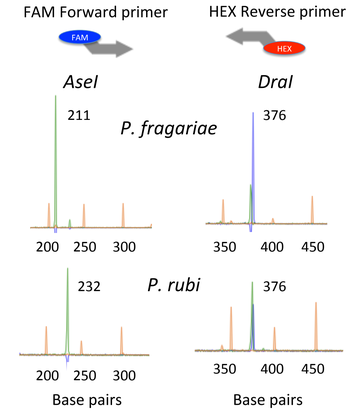


Fig. 4. RFLP analysis of qPCR products from the *atp9-nad9* *Phytophthora* detection system.

Terminal fragment analysis coupled with software



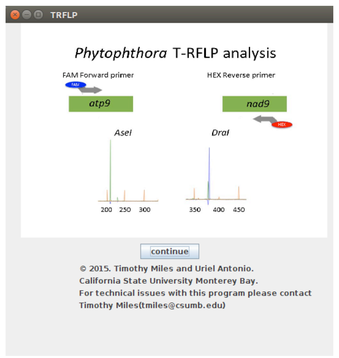


Fig. 5. T-RFLP analysis of *atp9-nad9* PCR products of two closely related *Phytophthora spp*. Followed by analysis with a T-RFLP program (available soon).

Digital Droplet PCR

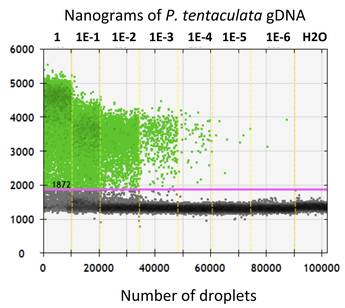


Fig. 6. Digital droplet amplification of the *trnM-trnP-trnM* gene order in *Phytophthora tentaculata* could allow for more detailed quantification.

Results/Conclusions

1) Fifty species specific *Phytophthora* qPCR probes have been validated against over 134 *Phytophthora* taxa, 22 *Pythium* species and a wide range of plant species. This locus has been sequenced from over 800 isolates representing 134 taxa and it is estimated that species specific probes can be developed for approximately 90% of species.
2) The *atp9-nad9* marker system has been optimized so all assays run at the same temperature aiding genus specificity
3) A qPCR-RFLP method has been developed and an *in silico* database has been made to predict various product sizes if sequencing is not available, with conventional methods 44 species can be identified using two different digestions.
4) The RFLP method has been enhanced by exploring terminal RFLP analysis which will allow us to investigate *Phytophthora* communities in environmental samples when multiple species are present. It is estimated that approximately 85-90% of species could be differentiated using this T-RFLP method. The Java based program allows for the rapid identification of these discrete peaks in order to minimize the need for sequencing.
5) The *trnM-trnP-trnM* locus has been adapted to Digital Droplet PCR which will allow for extremely accurate quantification of *Phytophthora* spp.
6) This information can also be transferred to more rapid isothermal based detection systems**. See poster 61**