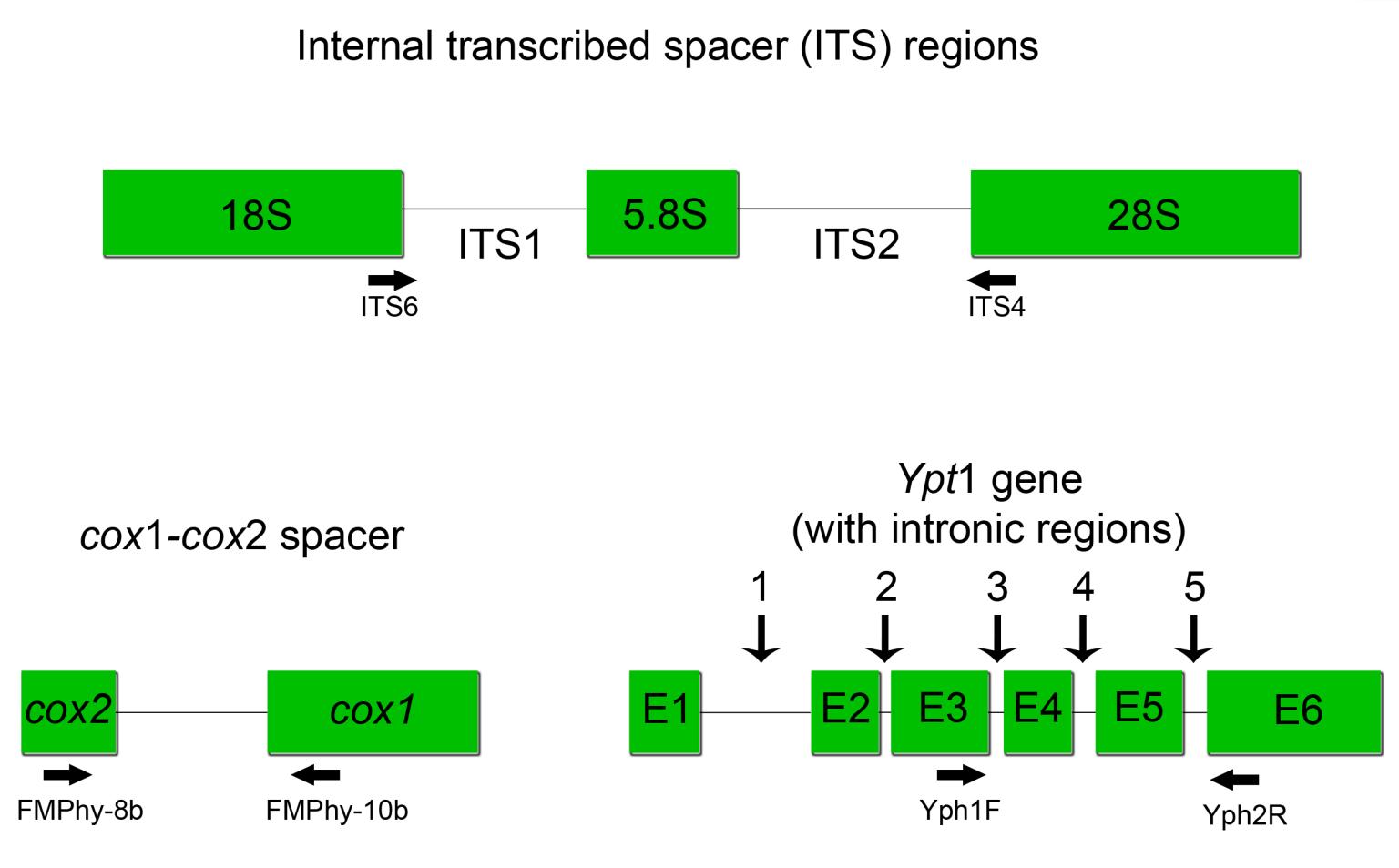


Mitochondrial marker systems for studying *Phytophthora* and *Pythium*

Timothy Miles, California State University-Monterey Bay, Seaside, CA
Frank Martin, Crop Improvement and Protection Station, USDA-ARS

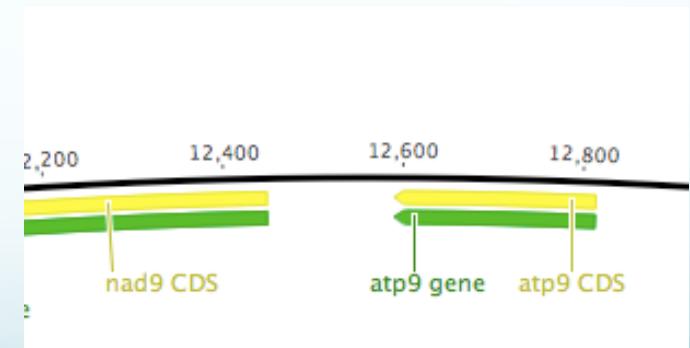
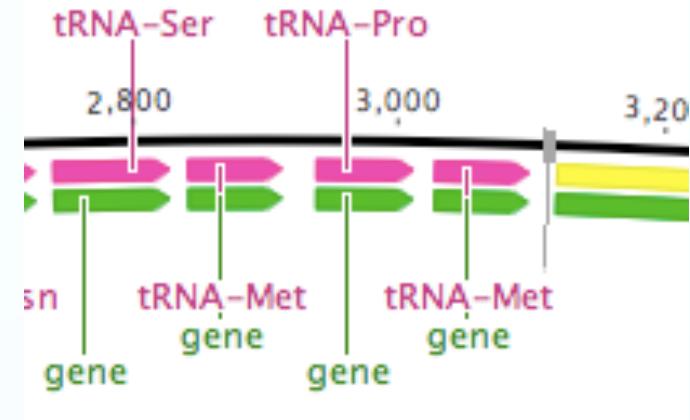
Background information

- Currently available loci



Mitochondrial gene order differences in *Phytophthora*

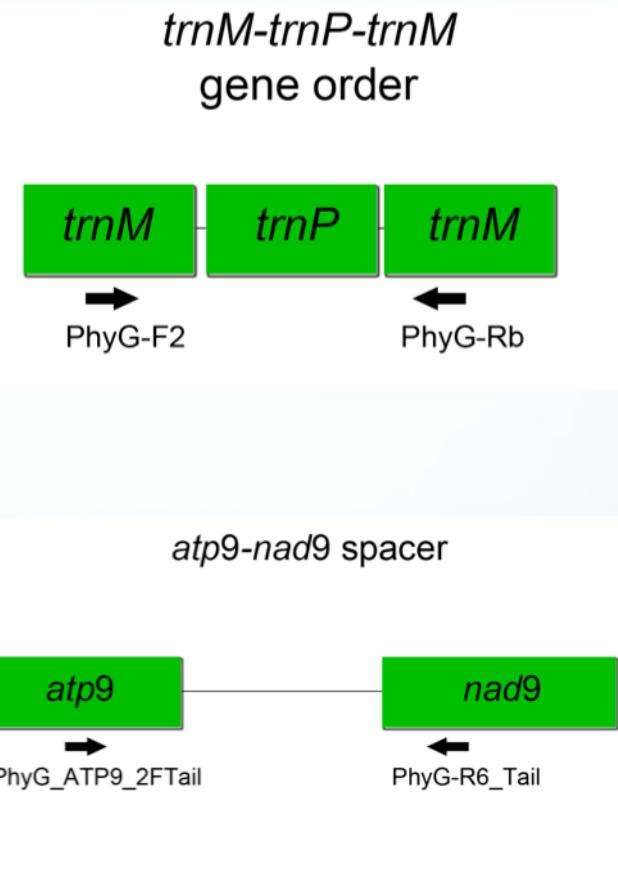
- *trnM-trnP-trnM*
 - In *Pythium* spp., the gene order *trnM-trnP* is conserved but the last *trnM* is located >10 kb away and in the opposite orientation
- *atp9-nad9*
 - In *Pythium* spp., the genes are located 18 to 30 kb apart



P. ramorum gene orders

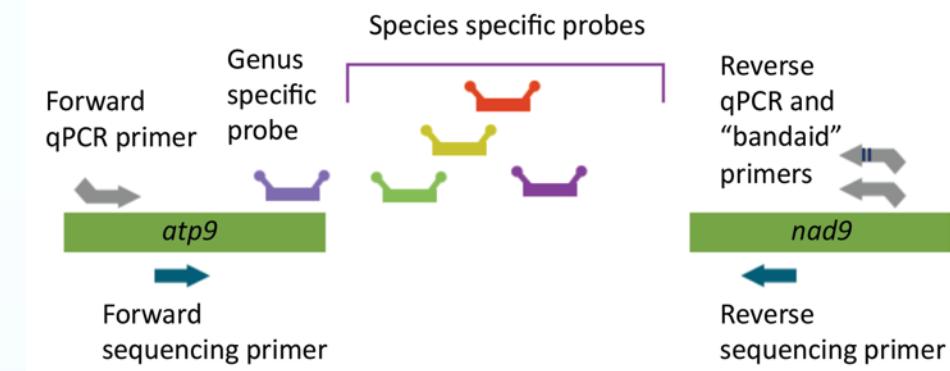
Large database available

- *trnM-trnP-trnM*
 - Sequence data of 250 isolates were collected representing approximately 70 *Phytophthora* taxa
 - Tested by TaqMan PCR on over 130 *Phytophthora* taxa for presence of the gene order
- *atp9-nad9*
 - Sequence data of 900 isolates were collected representing approximately 130 *Phytophthora* taxa
 - Tested by TaqMan PCR on over 130 *Phytophthora* taxa
 - Gene order is present in all species except *P. bisheri* and *P. frigida*



TaqMan diagnostic tools

- *trnM-trnP-trnM*
 - Amplicon size: ~225 bps
 - Genus specific detection capability
 - Sensitivity ~100 fg/μl
 - Specificity tested on over 130 *Phytophthora* taxa and several *Pythium* and *Phytophytum* species
- *atp9-nad9*
 - Amplicon size: 370-400 bps
 - Genus specific detection capability (except *P. bisheri* and *P. frigida*)
 - Over 50 species specific TaqMan probes validated
 - Over 175 *in silico* probes predicted
 - Sensitivity ~100 fg/μl
 - Specificity tested on over 130 *Phytophthora* taxa and several *Pythium* and *Phytophytum* species

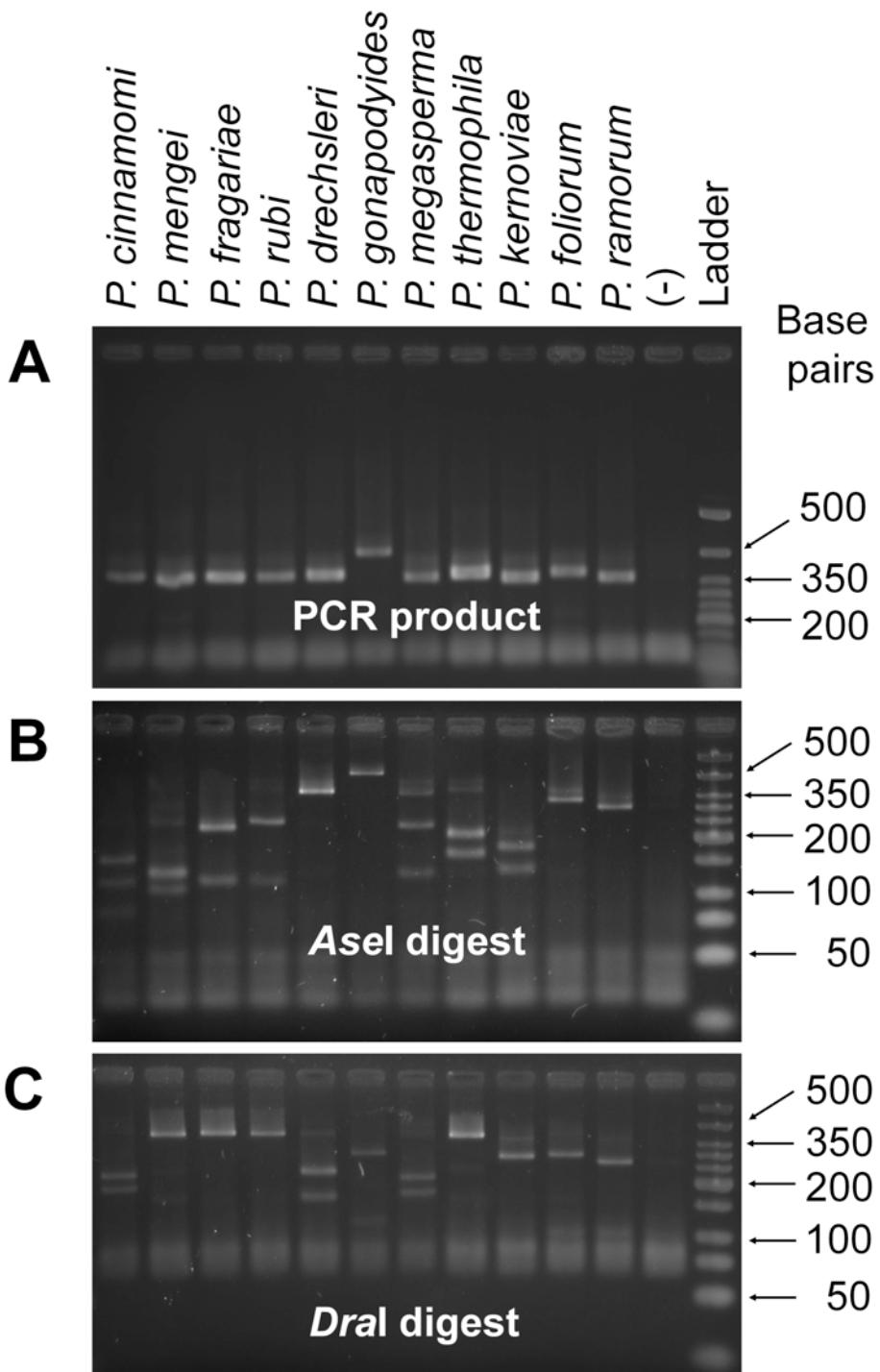


Bilodeau, G. J., Martin, F. N., Coffey, M. D., and Blomquist, C. L. 2014. Development of a multiplex assay for genus- and species-specific detection of *Phytophthora* based on differences in mitochondrial gene order. *Phytopathology* 104:733-748.

Miles, T.D., Robideau, G., Martin, F.N., Bilodeau, G., Coffey, M.D. Validation of a TaqMan diagnostic assay for the systematic development of *Phytophthora* species-specific markers. (in preparation)

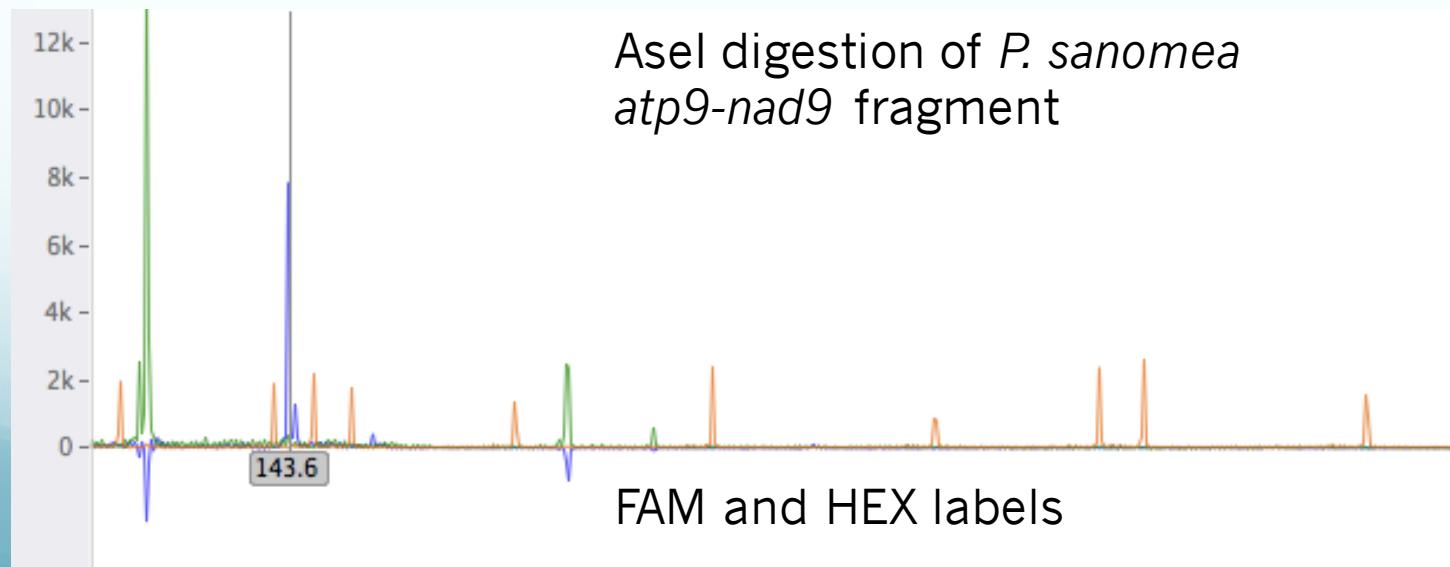
PCR-RFLP

- Amplification of the *atp9-nad9* locus and digestion of the fragment allows for species-specific detection
- Approximately 45 *Phytophthora* species can be detected using *AseI* and *DraI* when a size difference on bands 100 bp or larger differ in size by at least 10 bp or have different banding patterns using both enzymes
- Might be useful if multiple species are present



Fragment analysis following PCR-TRFLP

- Terminal dual labeling followed by fragment analysis allows further resolution down to 1-2 base pairs
- Preliminary data shows that this allows for higher resolution and should be particularly useful in samples with many *Phytophthora* species, particularly clade 6 species



Tools and alternative approaches to detect and study *Phytophthora* communities

- Using the TaqMan probe approach to investigate up to 5 *Phytophthora* species
- Restriction digest techniques to differentiate many species
- Metagenomic analysis using next generation sequencing
- Challenges:
 - All of these techniques require a DNA extraction and fairly clean DNA
 - Generally require access to a laboratory to perform extractions and amplification
 - A technique that was fast can work with a crude sample could be beneficial to study communities
 - Chose to investigate isothermal amplification techniques

Isothermal amplification tools

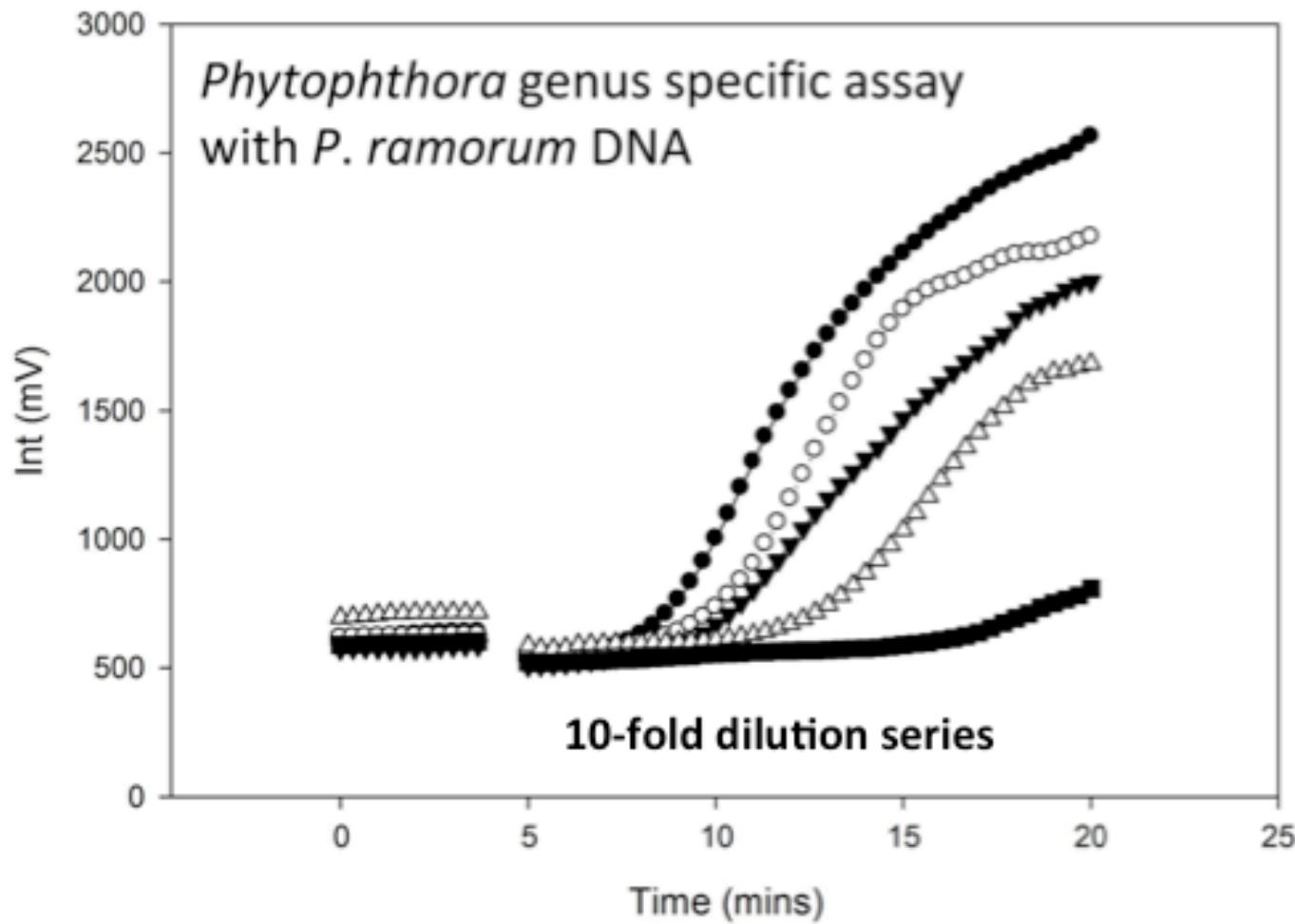
- New recombinase polymerase amplification tools developed using the *trnM-trnP-trnM* and *atp9-nad9* loci
 - Advantages:
 - Similar to TaqMan, so easier to transfer existing technology
 - Uses crude DNA extraction tools and does **not** require traditional DNA extraction
 - A nested PCR technique has been developed so there is no need to do additional DNA extractions
 - This method could be performed in the laboratory or in the field
 - Plant tissue only has a minor affect on amplification



Crude suspect root sample

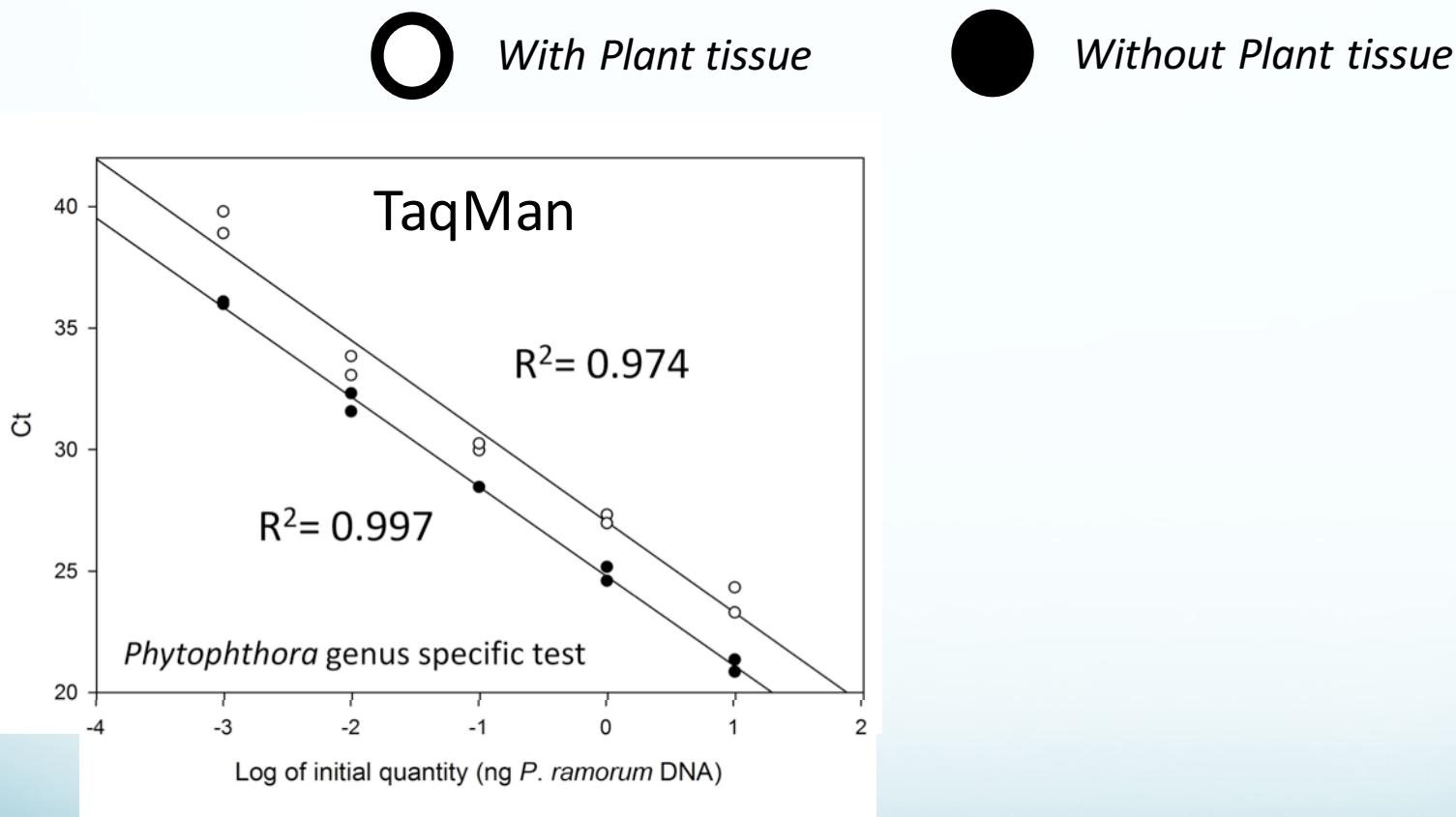
What does RPA detection look like?

● 10ng/ μ l ○ 1ng/ μ l ▲ 0.1ng/ μ l △ 0.01ng/ μ l ■ 0.001ng/ μ l



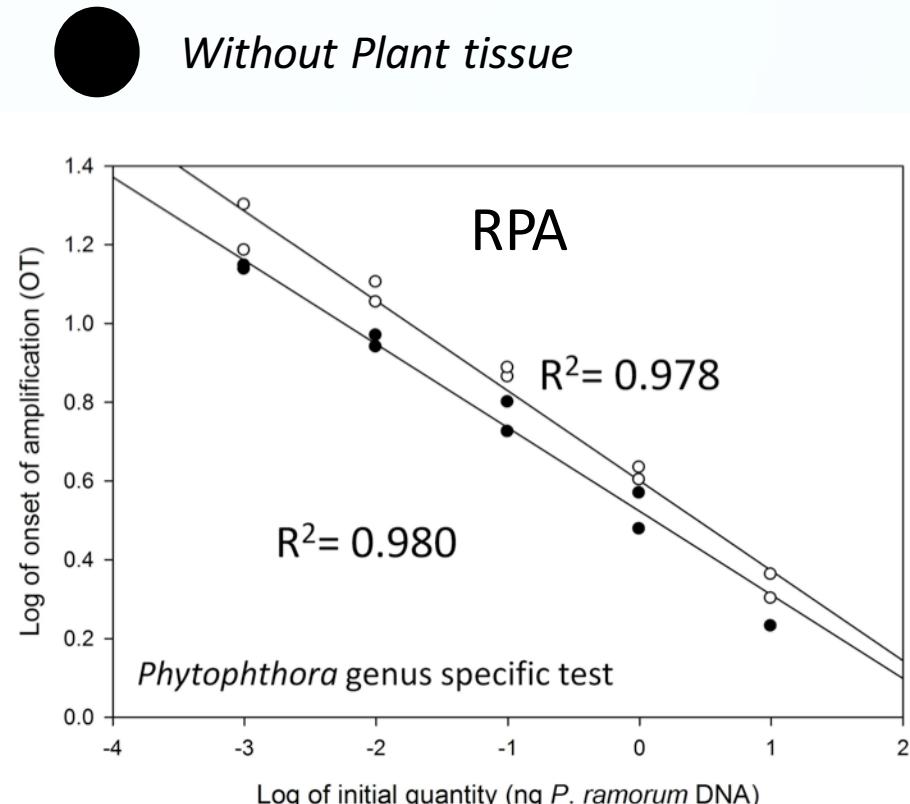
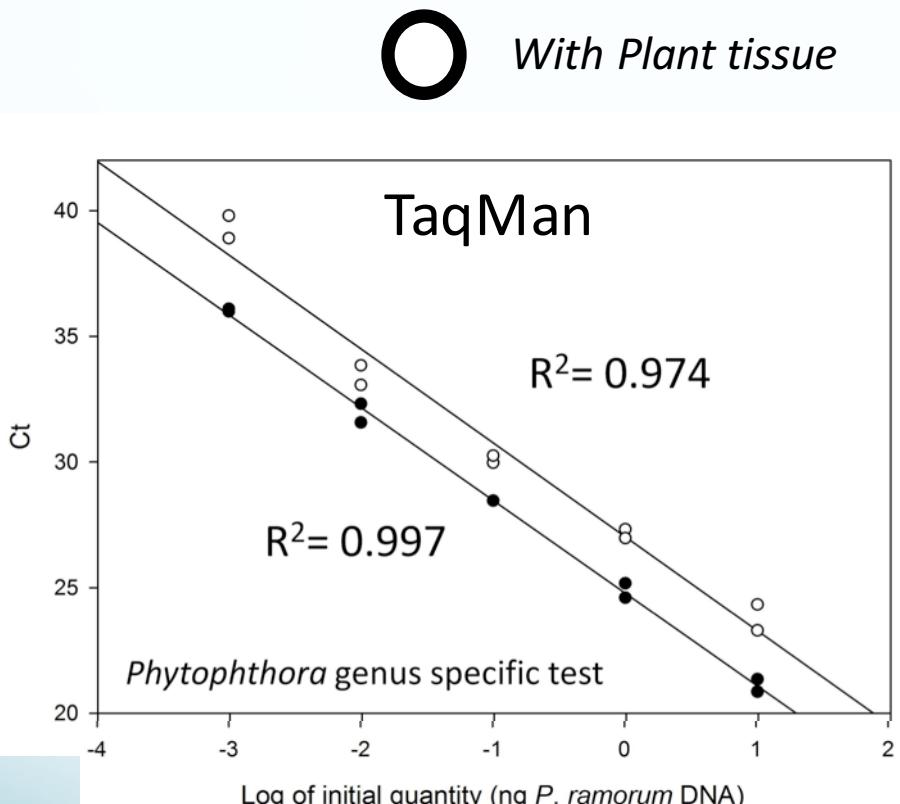
Miles, T. D., Martin, F. N., and Coffey, M. D. 2015. Development of rapid isothermal amplification assays for detection of *Phytophthora* spp. in plant tissue. *Phytopathology* 105:265-278.

How does RPA compare to TaqMan technologies?



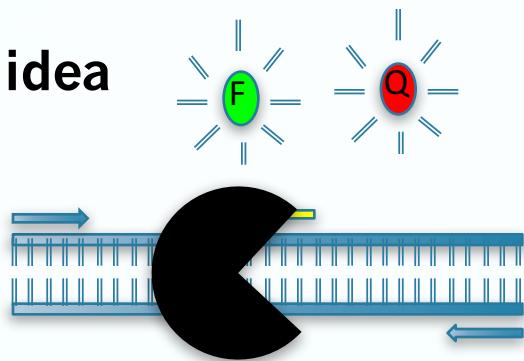
Miles, T. D., Martin, F. N., and Coffey, M. D. 2015. Development of rapid isothermal amplification assays for detection of *Phytophthora* spp. in plant tissue. *Phytopathology* 105:265-278.

How does RPA compare to TaqMan technologies?

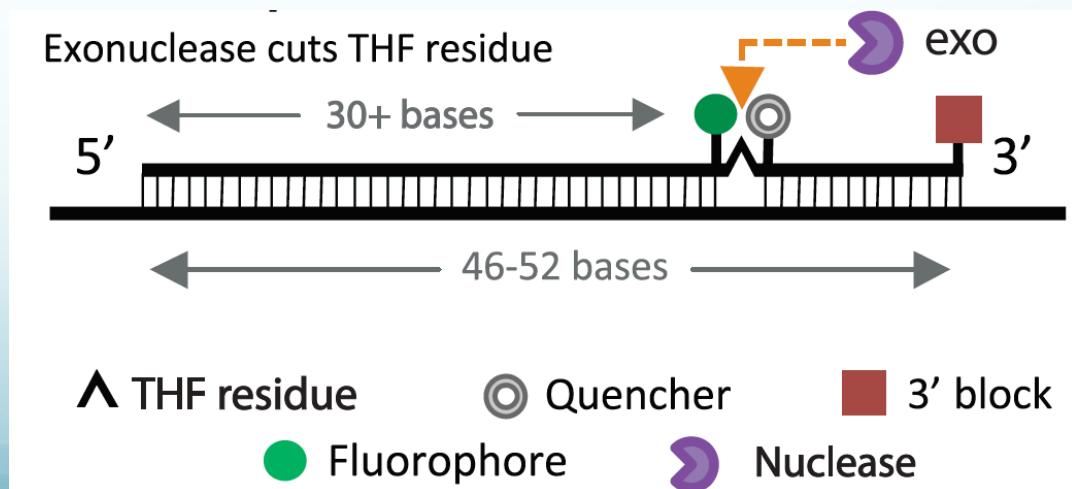
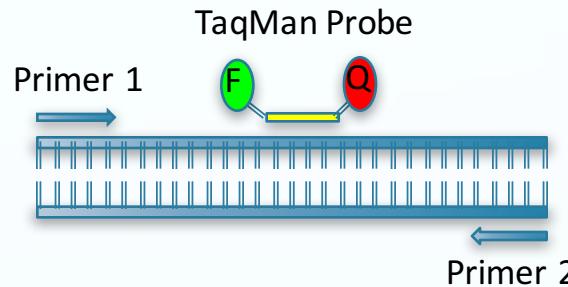


RPA versus TaqMan: Primer and probe design

General idea



- TaqMan
 - Primers: 18-25 base pairs
 - Probe: 25-35 base pairs
- RPA (TwistAmp exo)
 - Primers- 25-35 base pairs
 - Probe: 46-52 base pairs, with several modifications



How does plant tissue affect amplification?

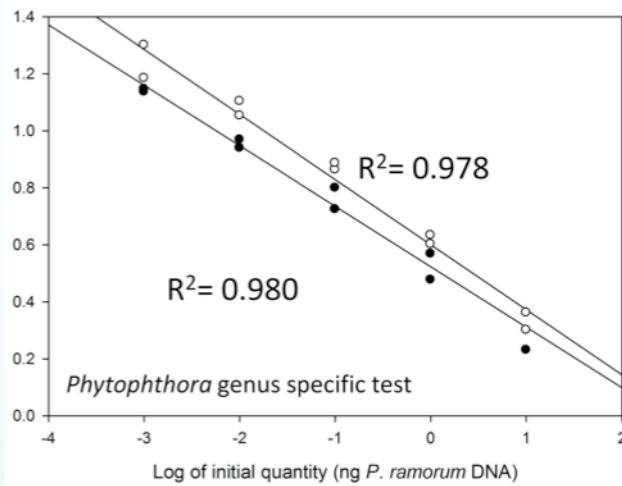


With Plant tissue

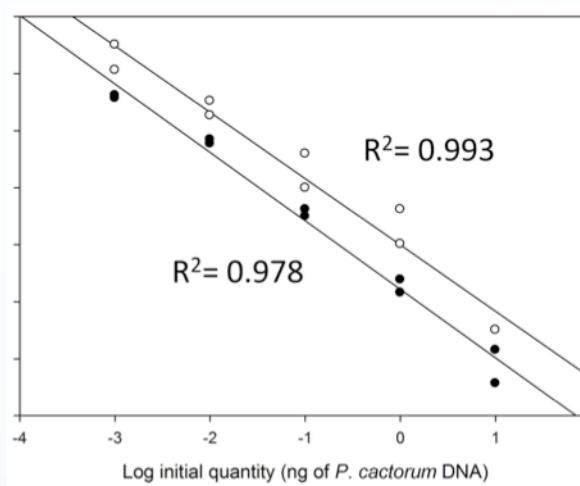


Without Plant tissue

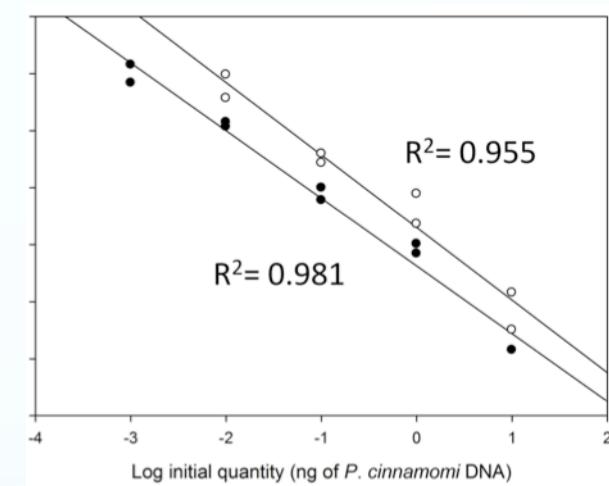
P. ramorum



P. cactorum



P. cinnamomi



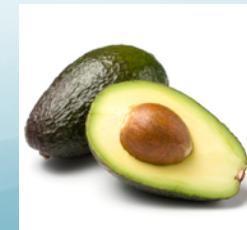
With and without
bay laurel leaf tissue



With and without
strawberry crown tissue



With and without
avocado root tissue



Multiple platforms to optically read RPA reactions

A



C



B



D



Other devices are available to read reactions immunologically

Future uses of these *Phytophthora* mitochondrial loci

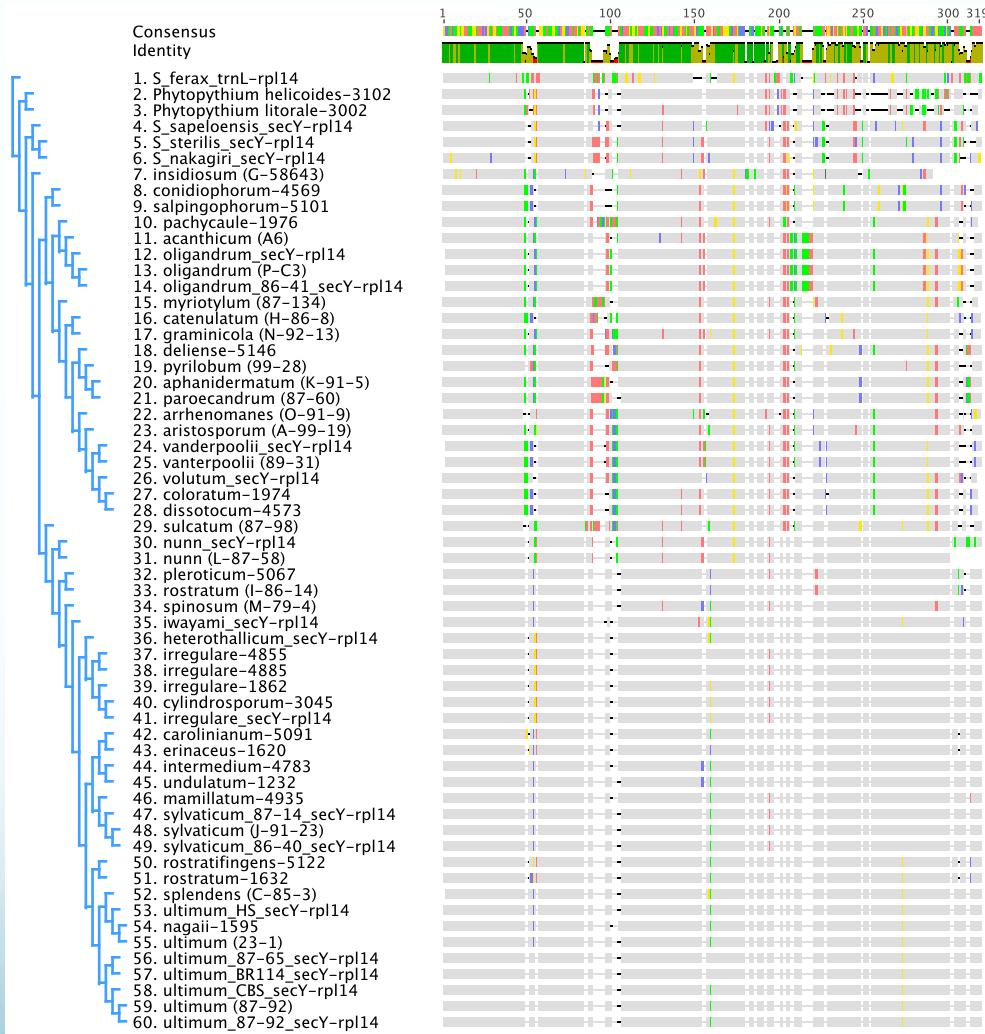
- Size of amplicon for *atp9-nad9* can be reduced for next generation sequencing
 - *trnM-trnP-trnM*- 225 bps
 - *atp9-nad9*-375-400 bps (could be reduced to 275-300)
- Large database for identification for both loci (larger for *atp9-nad9*)
- Validated on 130 *Phytophthora* taxa and both gene orders are present in all *Phytophthora* spp. (except *atp9-nad9* *P. bisheri* and *P. frigida*)

Gene order differences in *Pythium*

- Mitochondrial locus that appears to be well conserved in *Pythium* but not *Phytophthora*, *Phytopythium* or plants
- A sequence database of over 90 isolates representing 37 species has been collected across each clade
- Currently trying to collect more sequence data on this locus

TaqMan and isothermal diagnostics

- New mitochondrial *Pythium* locus
 - TaqMan diagnostics
 - Amplicon size:~225 bps
 - Genus specific detection
 - Sensitivity ~100 fg/ μ l
 - Specificity tested on over 130 *Phytophthora* taxa and 37 *Pythium* spp. and 3 *Phytophytium* species
 - Isothermal diagnostics
 - Genus specific detection capability so far
 - Sensitivity ~200-300 fg/ μ l



Utility of these tools for detection and investigating communities

- These tools should have utility when multiple species are present in a single biological sample
- Due to the fact that mitochondrial genes are generally high copy number these assays should be quite sensitive compared to many nuclear loci
- Their short size compared with previously developed markers (i.e. cox 1-2 spacer) could allow for future metagenomic studies using HiSeq/MiSeq and better understand *Pythium* and *Phytophthora* communities
- The development of both TaqMan and isothermal technologies allow for flexible data collection

Acknowledgements

- Guillaume Bilodeau- CFIA
- Mike Coffey – UC Riverside
- Funding:
 - California Avocado Commission
 - California USDA-Specialty Crop Block Grant Program
- Provided plant samples for validation:
 - Jim Adaskaveg, Mark Bolda, Cheryl Blomquist, Helga Forster, Steve Koike, Mansun Kong, Heather Scheck, Wolfgang Schweigkofler, Karen Suslow, Steve Tjosvold and Lela Walker

