Comparative Mitochondrial Genomics and the Development of a Genus and Species Specific Diagnostic TaqMan Assay for *Phytophthora*

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Desired Characteristics for Diagnostic Assays for *Phytophthora* Detection

• High copy number for greatest sensitivity
• Amplicon size suitable for real-time PCR
• High level of specificity
• Potential for systematically developing species-specific markers for all/most species in the genus
• Be capable of detection at a genus specific level
Phytophthora Multiplex Detection

Phytophthora amplicon

gene

gene
**Phytophthora** Multiplex Detection

**Phytophthora** amplicon

![Diagram of Phytophthora amplicon with gene and TaqMan Probe]
Using the MtDNA for Development of Diagnostic Markers

• Rather than looking at individual sequences one at a time, will approach this by comparison of mtDNA genomic sequences
  – Look for gene order differences with related genera and plants to enhance specificity of the markers
Mitochondrial Genome Sequencing

- *Pythium* spp.
  - 15 species
  - 18 genomes
    - 2 isolates for 3 species to evaluate intraspecific variation

- *Phytophthora* spp.
  - 21 species
  - 22 genomes
    - 2 isolates of 1 species to evaluate intraspecific variation
Phytophthora ramorum

Length: 39,314 bp
37 genes
26 tRNAs for 19 AA
7 ORFs, 1 unique

Inverted Repeat
-1,150 bp in length
-Includes 528 bp ORF
<table>
<thead>
<tr>
<th>Species</th>
<th>Genome Size (kb)</th>
</tr>
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<tbody>
<tr>
<td>P. cactorum</td>
<td>38,068</td>
</tr>
<tr>
<td>P. nemorosa</td>
<td>38,663</td>
</tr>
<tr>
<td>P. capsici</td>
<td>38,427</td>
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<tr>
<td>P. nicotianae</td>
<td>37,743</td>
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<tr>
<td>P. cinnamomii</td>
<td>39,274</td>
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<tr>
<td>P. palmivora</td>
<td>38,761</td>
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<td>P. citricola</td>
<td>38,529</td>
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<td>P. pseudosyringae</td>
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<td>P. foliorum</td>
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<td>P. heveae</td>
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<td>P. ramorum</td>
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<td>P. hibernalis</td>
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<td>P. infestans</td>
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<tr>
<td>P. sojae</td>
<td>42,977</td>
</tr>
</tbody>
</table>

-39,870

Genome sizes similar to *Pythium* if one arm of the inverted repeat in *Pythium* is removed
Is gene order related to phylogenetic relationships in *Phytophthora*?

- Gene order is fairly well conserved
  - Inversions and translocation observed but these found at only 6 junctions of genes
- No consistent correlation between gene order and phylogenetic relationship
Mitochondrial Gene Order Differences

Phytophthora vs Pythium & Plants

- Three conserved gene order differences have been found in Phytophthora compared to Pythium and plants
- These regions were sequenced for virtually all Phytophthora species
  - Represent the core of isolates used in the multigene phylogeny of Phytophthora by Blair et al. 2008
  - Additional “species” newly described or in the process of being describes included as well
  - Many DNAs obtained from Mike Coffey, UC Riverside
Background of Genus-Specific Amplicon

• Approximately 340 bp amplicon
  – Have sequence data for approximately 700 isolates representing nearly all described *Phytophthora* spp.
  – Potential for species-specific probe in spacer region
    • Yes – 73+ species (80+ if locked nucleic acids used)
    • No - 14 species
      – Sequence too AT rich or interspecific variability not suitable for development of species-specific probes
      – Does not amplify *P. bisheria, P. alticola* and *P. frigida*

Guillaume Bilodeau – post doc working on this project
Evaluation of Marker System

- Primers tested on a range of *Phytophthora* species
  - Amplified all *Phytophthora* spp. (100+)
    - Only exception is *P. bisheria, P. alticola and P. frigida*
  - Did not amplify wide range of
    - *Pythium* species
    - Plant species
- Primers and TaqMan *Phytophthora* specific probe
  - Detected all *Phytophthora* spp. that were amplified
  - Did not detect any *Pythium* or plant species
- Multiplexed with internal positive control
  - Detects plant DNA (*cox1* gene)
Phytophthora Species-Specific Markers

Interspecific Comparisons

- Species specific TaqMan probe specificity confirmed with purified DNA

  - P. alni (3 sub spp.)
  - P. cactorum
  - P. cambivora
  - P. cinnamomii
  - P. citricola group
  - P. fragariae
  - P. kernoviae
  - P. nicotianae
  - P. palmivora
  - P. pseudosyringae
  - P. ramorum
  - P. rubi
  - P. syringae
  - P. gonapodyides
Phytophthora Species-Specific Markers
Intra- and Inter-Specific Comparisons

• Species specific TaqMan probes

  \(P.\) \textit{alni} (12 isolates + 37 of other spp.*)
  \(P.\) \textit{cactorum} (59 + 5 of other spp)
  \(P.\) \textit{cambivora} (17 + 32 of other spp.)
  \(P.\) \textit{cinnamomii} (60 + 14 of other spp.)
  \(P.\) \textit{citricola} group (79)
  \(P.\) \textit{fragariae} (20 + 26 of other spp)
  \(P.\) \textit{gonapodyides} (25 + 13 other spp.)
  \(P.\) \textit{kernoviae} (5 + 15 of other spp.)
  \(P.\) \textit{nicotianae} (43 + 12 of other spp.)
  \(P.\) \textit{palmivora} (60 + 20 of other spp.)
  \(P.\) \textit{pseudosyringae} (5 + 33 of other spp.)
  \(P.\) \textit{ramorum} (10 + 6 of other spp.)
  \(P.\) \textit{rubi} (14 + 32 of other spp.)
  \(P.\) \textit{syringae} (20 + 29 of other spp.)

* Other species refers to isolates of other phylogenetically related species
**Phytophthora** Species-Specific Markers

- Species-specific TaqMan probes under testing
  - *P. europaea*   *P. pinifolia*
  - *P. lateralis*  *P. quercina*
  - *P. nemorosa*
Field Validation of Markers

• DNA from environmental samples
• Identification confirmed by
  – Culturing pathogen when possible
  – Sequencing of genus-specific amplicon and BLAST analysis from sequence database
Field Validation of Markers

• Big Sur - *P. ramorum*, confirmed by culturing
• Raspberry roots – *P. rubi/P. fragariae* (Nik Grunwald, OSU; J. Gigot, WSU)
• UK – *P. kernoviae* (J. Tomlinson)
• California plants – 99 samples (Cheryl Blomquist, CDFA)
• Oregon stream baiting – 43 samples (Melodie Putnam, OSU)
• Pennsylvania plants – 265 samples from natural ecosystems (Seong Kim, Penn. Dept. of Agri.)
Second *Phytophthora* Genus-Specific TaqMan Assay

- Approximately 206 bp
  - Smaller size may be compatible with diagnostic markers for other pathogens
- Detects all *Phytophthora* spp.
  - Does not detect *Pythium* or plant species
- Can be used for developing species-specific markers
  - But only for about half the number of species compared to the other locus used for species-specific marker
Characteristics of Diagnostic Markers

- High copy number
- Capable of simultaneous detection at a
  - Genus-specific level – highly specific due to gene order differences
  - Species-specific level
- Sequence data suggest should be capable of designing species-specific TaqMan probes for approximately 80+ species
- Genus-specific amplicon can be sequenced and the extensive sequence database used to identify to a species level
Acknowledgements

• MtDNA genomic sequencing
  – *Pythium* and other *Phytophthora* spp.
    • P. Richardson et al., JGI, Walnut Creek, CA

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