Advances in systems for identification and diagnosis of *Phytophthora*, *Pythium* and related genera

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Identification of Isolates

- Challenges of morphological identification
  - Level of expertise needed
  - Not all isolates produce necessary structures
  - Overlap of morphological features
  - Convergent evolution
  - Time necessary
Molecular Identification

- Generally takes less time
- Less subjective for identification
- Can sometimes differentiate isolates below the species level.
Desired Marker Characteristics

• Look for a single region that is conserved within a species but variable between species.
• Have conserved sequences flanking variable region
• Amplicon size suitable for real-time PCR
• High copy number
Molecular Loci Used for Species Identification

- Nuclear
  - rDNA
  - β-tubulin
  - Elicitin, cellulose binding elicitor lectin
  - Translation elongation factor 1 α
  - Ypt1 gene
  - Elicitin gene \textit{par1}, putative storage protein \textit{Lpv}
  - 60S Ribosomal protein L10, enolase, heat shock protein 90, TigA gene fusion protein
Molecular Loci Used for Species Identification - Nuclear

• Nuclear
  – Multiple copy
    • rDNA – ITS region most commonly used for
      – sequence based ID (good representation in GenBank)
      – As source of sequences for designing species-specific markers
  – “Single” copy
    • Translation elongation factor 1 alpha – phylogeny
      – Kroon et al. 2004, Blair et al. 2008
    • β-tubulin – phylogeny and molecular diagnostics
    • Elicitin, cellulose binding elicitor lectin – molecular diagnostics
      – Bilodeau et al. 2007a, b
    • Ypt1 gene – molecular diagnostics
      – Schena et al. 2006, 2007
    • Elicitin gene par1, putative storage protein Lpv
      – Kong et al. 2003a, b
    • 60S Ribosomal protein L10, enolase, heat shock protein 90, TigA gene fusion protein – phylogeny
      – Blair et al. 2008
rDNA Organization

For Pythium species with spherical sporangia/hyphal swellings the 5 S rDNA is dispersed as an array in other regions of the genome
•Spacer regions between copies useful for species-specific markers

Cistron present in multiple copies in head to tail array
Ypt1 Gene Species-Specific Diagnostic Markers
Genus-specific primers and 15 species-specific

From Schena et al. 2007
Molecular Loci Used for Species Identification - Mitochondrial

Mitochondrial – multiple copy

- *cox1* – phylogeny and molecular diagnostics
  - Kroon et al. 2004a, b, Levesque et al. (bar code, personal comm.)

- *cox2* – phylogeny

- *cox1* and *cox2* spacer – molecular diagnostics
  - Martin et al. 2004, Tooley et al. 2006

- *nad1* – phylogeny
  - Kroon et al. 2004

- *nad5* – phylogeny
  - Ivors et al. 2004
Nuclear vs Mitochondrial Markers

• Mitochondria are uniparentally inherited from maternal parent
• Copy number may change depending on physiological status of the pathogen, so may not be best for quantification
Copy Number vs Sensitivity

• Multiple copy vs “single” copy
  – Similar \( C_t \) in real-time PCR for \( P. \) ramorum using ITS and elicitin markers,
    • The \( C_t \) for both these loci averaged 3.7 lower than \( \beta \) tubulin
      – Bilodeau et al. 2007, unpublished

• Consistency for rDNA copy number
  – In \textit{Pythium}, rDNA hybridizes to different number of chromosomal bands in PFGE
    • different hybridization intensity relative to other “single” copy probes as well.
  – Different real-time PCR \( C_t \) observed for various isolates of \( P. \) infestans when normalized to \( C_t \) of “single” copy loci (Z. Atallah, personal comm.)
Techniques Used for Molecular Identification

• Techniques used are dependent on the type of analysis that is needed
  – Identification of isolates to species level that have been cultured
  – Identification of isolates from field samples
  – Identification of a particular species of regulatory importance from field samples
  – Identification of subpopulations within a species
Molecular Techniques for Isolate Identification

- **DNA sequencing**
  - Specific genes for ID and phylogenetic analysis
  - *Pythium*
    - Nuclear – ITS, large ribosomal subunit, β tubulin,
    - Mitochondrial – *cox1*, *cox 2*
  - *Phytophthora*
    - Nuclear – ITS, β tubulin, translation elongation factor 1 α, elicitin, 60S Ribosomal protein L10, enolase, heat shock protein 90, TigA gene fusion protein, Ypt1
    - Mitochondrial – *cox1*, *cox2*, *nad1*, *nad5*
  - Molecular tool box for identification and characterization of *Phytophthora* spp.
    - 4 mtDNA intergenic regions, a portion of the rDNA-IGS, a portion of *Ypt1* (a ras related protein).
    - Schena and Cooke 2006
Molecular Techniques for Isolate Identification

• Micro/macro arrays
  – Identification of isolates to species level
    • Reverse dot blot – Levesque et al. 1998
    • Reviewed in Lievens and Thomma 2005
  – Use single nucleotide polymorphisms (SNPs) on array to identify subpopulations
Molecular Techniques for Isolate Identification

• Single Strand Conformational Polymorphism
  – SSCP of ITS sequences - Both *Pythium* and *Phytophthora* spp.
    • C. Hong’s lab at VPI (2003 – 2005)
    • Automated sequencer for *Phytophthora* ID
      – Tom Kubisiak, USDA Forest Service, MS (unpublished)
  – SSCP with *cox* spacer region for *Phytophthora* spp.
    • E. Hansen (unpublished)
PCR-RFLP for Isolate Identification

• RFLP analysis of PCR amplified fragments
  – ITS region of the rDNA
    • *Phytophthora* – David Cooke (PhytID)
    • *Pythium* – Chen et al. 1992, Wang and White 1997
  – MtDNA
    • *cox* 1 and 2 gene cluster
      – *Phytophthora* - Martin and Tooley 2004
      – *Pythium* – Martin (unpublished)
    • Spacer between *cox* 1 and 2 genes
      – *Phytophthora* - Martin (unpublished)
RFLP Analysis for ID of *Pythium* spp.

- Similar in approach to *Phytophthora* RFLP analysis
  - Different primers used
  - Amplicon a little more than half the size of the *Phytophthora* amplicon

- Tested on over 160 isolates representing 40+ species
  - Clearly delineated species
  - Limited intraspecific variation
|---------------|---------------|-------------------|---------------|--------------|-------------|-------------------|-------------------------|-----------------------|------------------------|------------------|----------|---------|-----------|----------|

*Alu1*
**Phytophthora genus-specific Amplification**

*cox 2*  
**spacer**  
*cox 1*

**Phy-8b**  
**Phytophthora**  
**Phy-10b**

Approximately 450-500 bp

Primers amplify *Phytophthora*, but not the *Pythium* and plant species tested

• Analysis can be done directly on amplifications from infected tissue
RFLP Analysis of * Phytophthora * Genus-specific Amplicon for Species ID
Molecular Techniques for Identification of Subpopulations

- RAPDs
- AFLPs
  - *Phytophthora*
    - Lamour and Hausbeck 2001, Ivors et al. 2004
  - *Pythium*
    - Garzon et al. 2005a, b
- Inter simple sequence repeats (ISRR)
  - *Pythium*
    - Vasseur et al. 2005
- Microsatellites
  - *Phytophthora*
  - *Pythium*
    - Lee and Moorman 2007
- Micro/macro arrays to identify SNPs
- Mitochondrial haplotypes
  - *Phytophthora infestans*
Species-Specific PCR for Pathogen Detection

- Conventional vs real-time PCR
  - Due to less sensitivity and the time necessary for running the sample conventional PCR less common in diagnostic setting
- Important to have multiplexed
  - Plant marker as internal control for DNA extraction
  - Genus-specific marker is desirable
- Different chemistries for real-time PCR
  - TaqMan – perhaps most common
  - Scorpion – need less time to run cycle than TaqMan, so need less time to complete assay
  - Molecular beacons
Approaches to Enhance Specificity

• Nested amplification
  – Advantage that in also increases sensitivity
  – Disadvantage that it adds a few steps and has more opportunities for errors

• Locked nucleic acids
  – Allows higher annealing temperatures to be used

• Padlocked probes
  – Szemes et al. 2005

• Analysis of hybridization melt kinetics
  – Anderson et al. 2006
Padlock Probes to Improve Specificity

T1, T2 – species-specific sequences

P1, P2 – forward and reverse primers

Zip – sequences generated to be species-specific for TaqMan probe

Szemes et al. 2005
Considerations when starting to use PCR markers reported in the literature

• At least initially try using exact procedures reported
• Validate technique in your lab
  – Amplification conditions
  – Block uniformity
Loop Mediated Isothermal Amplification

- Reported as diagnostic for *Phytophthora ramorum*
  - Tomlinson et al. 2007
- Does not require a thermal cycler (just a temperature controlled block)
- Can visualize results
  - On a gel by electrophoresis
  - Intercalation of a dye
  - Increased turbidity (production of Mg pyrophosphatase)
  - Real-time PCR
- Some limitations
  - Less sensitive than TaqMan assay (10 pg vs 250 fg)
  - Commonly used dye has to be added at the end of the reaction as it inhibits the reaction
Using Mitochondrial Sequences for a Systematic Approach for Marker Development

• See more sequence variation than in many nuclear regions
• Target has high copy number
• Want to identify region where variable sequences are flanked by conserved sequences to simplify marker development for additional species
• Use in conjunction with plant and *Phytophthora* genus specific markers
**Phytophthora ramorum**

**Multiplex Amplification**

**First Round Amplification**

\[ cox \text{ II} \quad \text{spacer} \quad cox \text{ I} \]

[Diagram showing the first round amplification process with Phytophthora and Plant at the ends and an intermediate spacer.]

**Second Round Amplification**

\[ P. \text{ ramorum} \]

[Diagram showing the second round amplification process with Phytophthora and P. ramorum at the ends.]

**Additional details:** [http://www.ars.usda.gov/Research/docs.htm?docid=8728](http://www.ars.usda.gov/Research/docs.htm?docid=8728)
Genomic Sequencing of the MtDNA for Marker Development

- Rather than looking at individual sequences one at a time, will approach this by looking at genomic sequences of the mitochondrial DNA
  - Identify conserved/variable regions to focus on
  - 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.
  - 12 species
  - 13 genomes
    - 2 isolates of 1 species to evaluate intraspecific variation
Mitochondrial Genome Organization

- Circular orientation
  - Some *Pythium* spp. have linear genomes
- Inverted repeats?
  - Yes – *Pythium*, *Saprolegnia*, *Achlya*, *Aplanopsis*, *Leptolegnia*, *Saparomyces*
  - No – *Phytophthora*
    - Small inverted repeat (< 1.5 kb) present in *P. ramorum* and *P. hibernalis*
Pythium mtDNA

Inverted Repeat

Single Copy Region
Linear Mitochondrial Genomes of *Pythium* spp.

• Occur as concatamers
• Found in all species examined
  – For most species linear arrangements are present in very low amounts
• Termini correspond to the small unique region
• Termini have hairpin loop
## Genome Sizes for *Pythium* spp.

<table>
<thead>
<tr>
<th>Species</th>
<th>Small Unique(^a)</th>
<th>Inverted Repeat(^a)</th>
<th>Large Unique(^a)</th>
<th>Genome Size One arm IR(^a)</th>
<th>Genome Size Total(^a)</th>
<th>% Genome IR</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. catenulatum</em></td>
<td>2,704</td>
<td>24,964</td>
<td>10,253</td>
<td>37,921</td>
<td>62,885</td>
<td>79.4</td>
</tr>
<tr>
<td><em>P. graminicola</em></td>
<td>7,280</td>
<td>27,611</td>
<td>9,915</td>
<td>44,806</td>
<td>72,417</td>
<td>76.3</td>
</tr>
<tr>
<td><em>P. heterothallicum</em></td>
<td>3,368</td>
<td>21,269</td>
<td>13,066</td>
<td>37,703</td>
<td>58,972</td>
<td>72.1</td>
</tr>
<tr>
<td><em>P. myriotylum</em></td>
<td>3,900</td>
<td>28,342</td>
<td>12,148</td>
<td>44,390</td>
<td>72,732</td>
<td>77.9</td>
</tr>
<tr>
<td><em>P. nunn</em></td>
<td>3,304</td>
<td>22,346</td>
<td>13,103</td>
<td>38,754</td>
<td>61,100</td>
<td>73.1</td>
</tr>
<tr>
<td><em>P. oligandrum</em></td>
<td>1,372</td>
<td>30,911</td>
<td>10,291</td>
<td>42,574</td>
<td>73,485</td>
<td>84.1</td>
</tr>
<tr>
<td><em>P. sylvaticum</em></td>
<td>3,395</td>
<td>20,599</td>
<td>13,102</td>
<td>37,096</td>
<td>57,695</td>
<td>71.4</td>
</tr>
<tr>
<td><em>P. ultimum</em></td>
<td>2,711</td>
<td>21,954</td>
<td>13,068</td>
<td>37,733</td>
<td>59,687</td>
<td>73.6</td>
</tr>
</tbody>
</table>

\(^a\)Sizes in bp
Phytophthora Mitochondrial Genome Organization

• Lack an inverted repeat
  – Exceptions
    • *P. megasperma*, less than 0.9 kb based on Southern analysis (Schumard-Hudspeth and Hudspeth 1990)
    • *P. ramorum*, 1,150 bp (Martin et al. 2007)
    • *P. hibernalis*, ca. 1,500 bp
• Has the same genes found in *Pythium*
  – Some differences in ORFs
• Differences in gene order
**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
Is gene order related to phylogenetic relationships in *Phytophthora*?

- While some differences in gene order may be associated with phylogenetic relationships, many are not.
- Interspecific comparisons of genomes reveals some regions are more variable than others
  - Gene order in some regions highly conserved in genus
Development of New Marker System for *Phytophthora*

- Two conserved differences in gene order compared to *Pythium* have been identified.
- Both regions have been sequenced in 90+ isolates representing 60+ species to assess intra- and interspecific variation.
- One region has been selected for further study based on the sequence data
  - Interspecific polymorphisms
  - Intraspecific sequence conservation
  - %GC of sequences
Phytophthora Multiplex Amplification

Phytophthora amplicon
ca. 190 bp

Gene order differences between *Phytophthora* and *Pythium*
- also with plant mtDNA from GenBank search
Phytophthora Multiplex Amplification

Phytophthora amplicon
ca. 190 bp

Phytophthora TaqMan Probe

Species-specific TaqMan Probe
Mitochondrial Haplotype Determination

• Can intraspecific variation be used as haplotype markers to differentiate isolates?
  – *P. infestans* – Ia, Ib, IIa, IIb

• Are there specific places in the genome that are more prone to variation to simplify looking for haplotype markers from a wider number of species?
  – Genomic rearrangements leading to intraspecific differences in gene order tend to occur at specific places. Is this also a region more prone to intraspecific variation as well?
Phytophthora ramorum Mitochondrial Haplotypes

• Is there intraspecific variation in the sequences of the mitochondrial genome that can be used to assign haplotype?
  • Kroon et al. – SNP in cox1 gene

• If so, can they be used as a marker to help monitor populations of the pathogen?

**Phytophthora ramorum**

*Intraspecific Sequence Conservation*

- California vs European mtDNA genomic sequence
  - 13 single nucleotide polymorphisms
  - 1 insertion of 180 bp
- Additional polymorphisms when looking at 40 other isolates
  - 15 new SNPs
Evaluation of Mitochondrial Haplotypes

• Identification of SNPs
  – Designed primers to amplify and sequence regions that are variable in comparisons between the US and EU mt genomes.
  – Looked at other regions that were polymorphic in comparisons among other species.

• Determination of haplotypes
  – Total of 7,496 bp (or 19% of the genome) examined
  – Looked at 40 isolates from geographically diverse areas
# P. ramorum Mitochondrial Haplotype

<table>
<thead>
<tr>
<th>Marker</th>
<th># Variable Bases</th>
<th>mtDNA Haplotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prv-9</td>
<td>1</td>
<td>I – EU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II - US, III – Washington Nursery</td>
</tr>
<tr>
<td>ymf-16</td>
<td>2</td>
<td>I – EU, III – Washington Nursery</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II - US</td>
</tr>
<tr>
<td>cox2 + spacer</td>
<td>3</td>
<td>III – Washington Nursery</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I = II</td>
</tr>
<tr>
<td>Prv-1</td>
<td>2</td>
<td>III – Washington Nursery</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I = II</td>
</tr>
<tr>
<td>Prv-8</td>
<td>2</td>
<td>I – EU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II - US</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III – Washington Nursery</td>
</tr>
<tr>
<td>Prv-11</td>
<td>2</td>
<td>I – EU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II - US</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III – Washington Nursery</td>
</tr>
<tr>
<td>Prv-13</td>
<td>8</td>
<td>I – EU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II – US</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III – Washington Nursery</td>
</tr>
<tr>
<td>cox1</td>
<td>4</td>
<td>I – EU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II – US</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III – Washington Nursery</td>
</tr>
<tr>
<td>Prv-14</td>
<td>4</td>
<td>I – EU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IIa – US</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IIb – Oregon forest</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III – Washington Nursery</td>
</tr>
</tbody>
</table>
Non-Sequence Based Haplotype Determination

• Melt curve analysis of amplicons
  – Using the Idaho Technology Light Scanner
  – Redesigned the amplification primers so a smaller amplicon was generated (for the most part less than 200 bp)
*P. ramorum* Mitochondrial Haplotype Melt Curve Analysis

![Melt Curve Analysis Graph](image)
Non-Sequence Based Haplotype Determination

- Melt curve analysis of amplicons
  - Using the Idaho Technology Light Scanner
  - Redesigned the amplification primers so a smaller amplicon was generated (for the most part less than 200 bp)

- Has worked well for most regions for differentiating haplotypes
  - Can differentiate IIa from IIb
Acknowledgements

• MtDNA genomic sequencing
  – *P. ramorum* and *P. sojae* (Current Genetics 51:285-296)
    • J. Boore, D. Bensasson – JGI, Walnut Creek, CA
    • B. Tyler – VBI, VPI Blacksburg, VA
  – *Pythium* and other *Phytophthora* spp.
    • P. Richardson et al., JGI, Walnut Creek, CA

• Thanks to the USDA-CSREES-NRI Plant Biosecurity Grant Program for supporting this work