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

Frank Martin

USDA-ARS, Salinas, CA

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

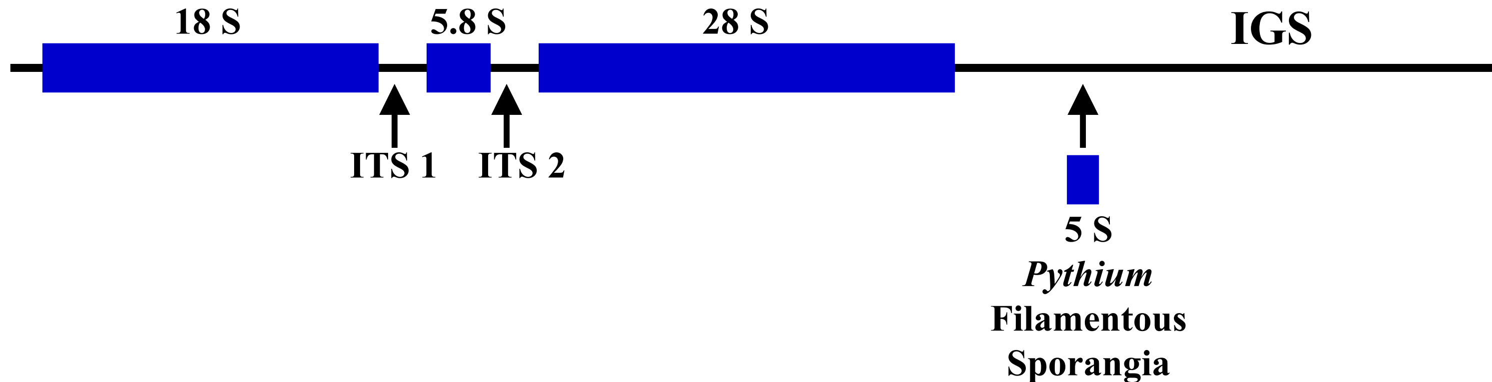
Molecular Loci Used for Species Identification

- Nuclear
  - rDNA
  - β-tubulin
  - Elicitin, cellulose binding elicitor lectin
  - Translation elongation factor 1 α
  - *Ypt1* gene
  - Elicitin gene *par1*, putative storage protein *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
    - ß -tubulin – phylogeny and molecular diagnostics
      - Kroon et al. 2004, Blair et al. 2008, Bilodeau et al. 2007
    - 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



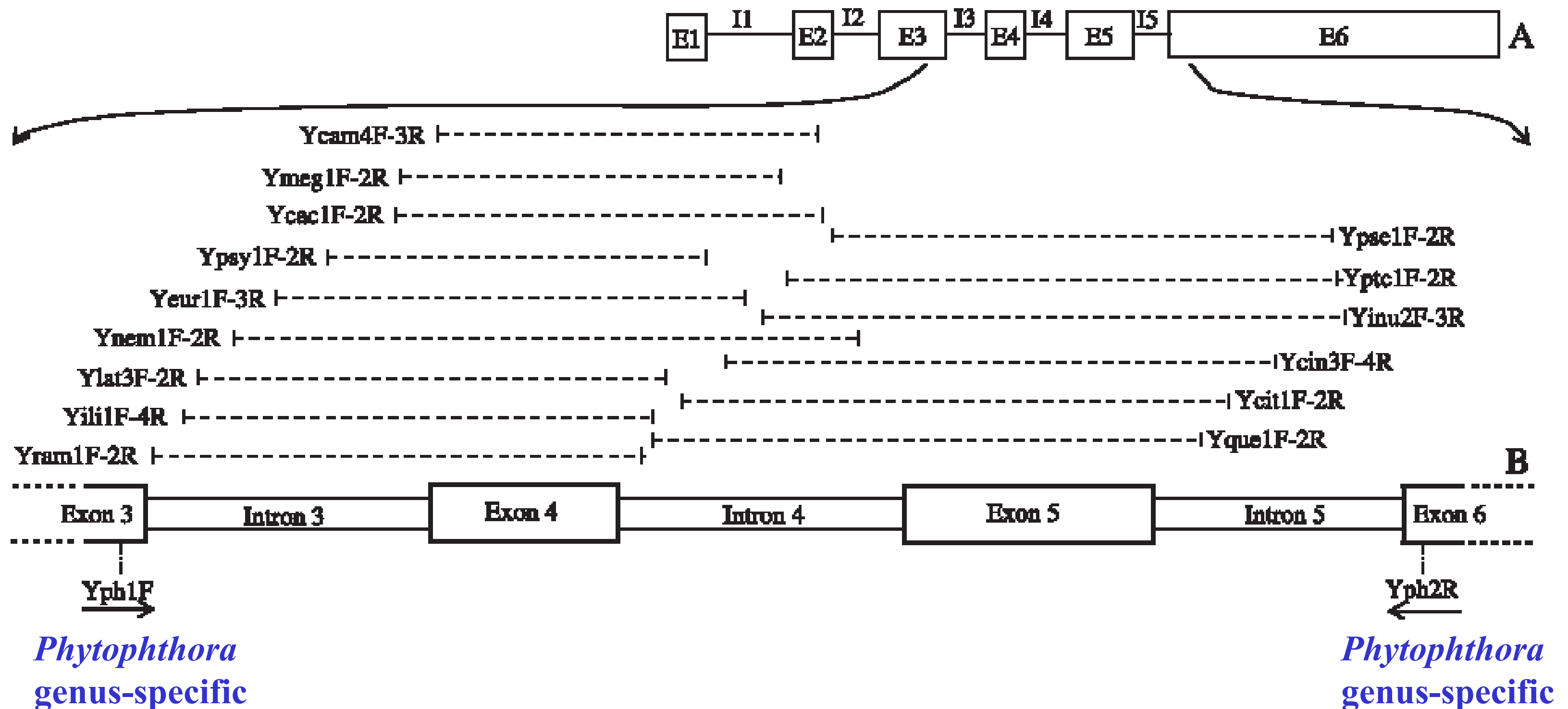
For *Pythium* species with spherical sporangia/hyphal swellings the 5 S rDNA is dispersed as an array in other regions of the genome

- 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



Phytophthora genus-specific

Phytophthora genus-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
  - Martin et al. 2000, 2003a, b, Hudspeth et al. 2000, Kageyama et al. 2005, Villa et al. 2006
- *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 β tubulin
      - Bilodeau et al. 2007, unpublished
- Consistency for rDNA copy number
  - In *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

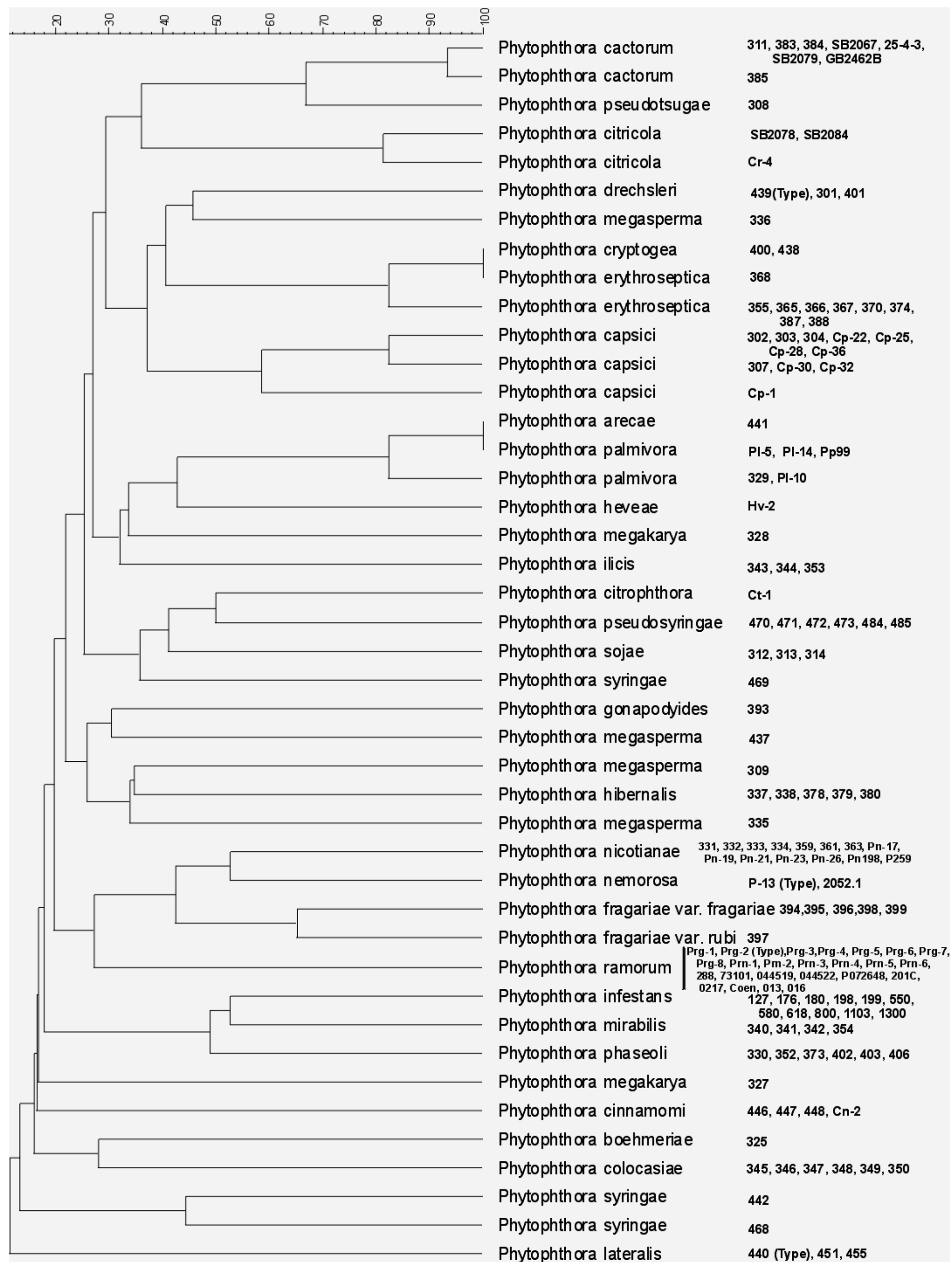
- Micro/macro arrays
  - Identification of isolates to species level
    - Reverse dot blot – Levesque et al. 1998
    - Reviewed in Lievens and Thomma 2005
  - Use single nucleotide polymorphisms (SNPs) on array to identify subpopulations

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)

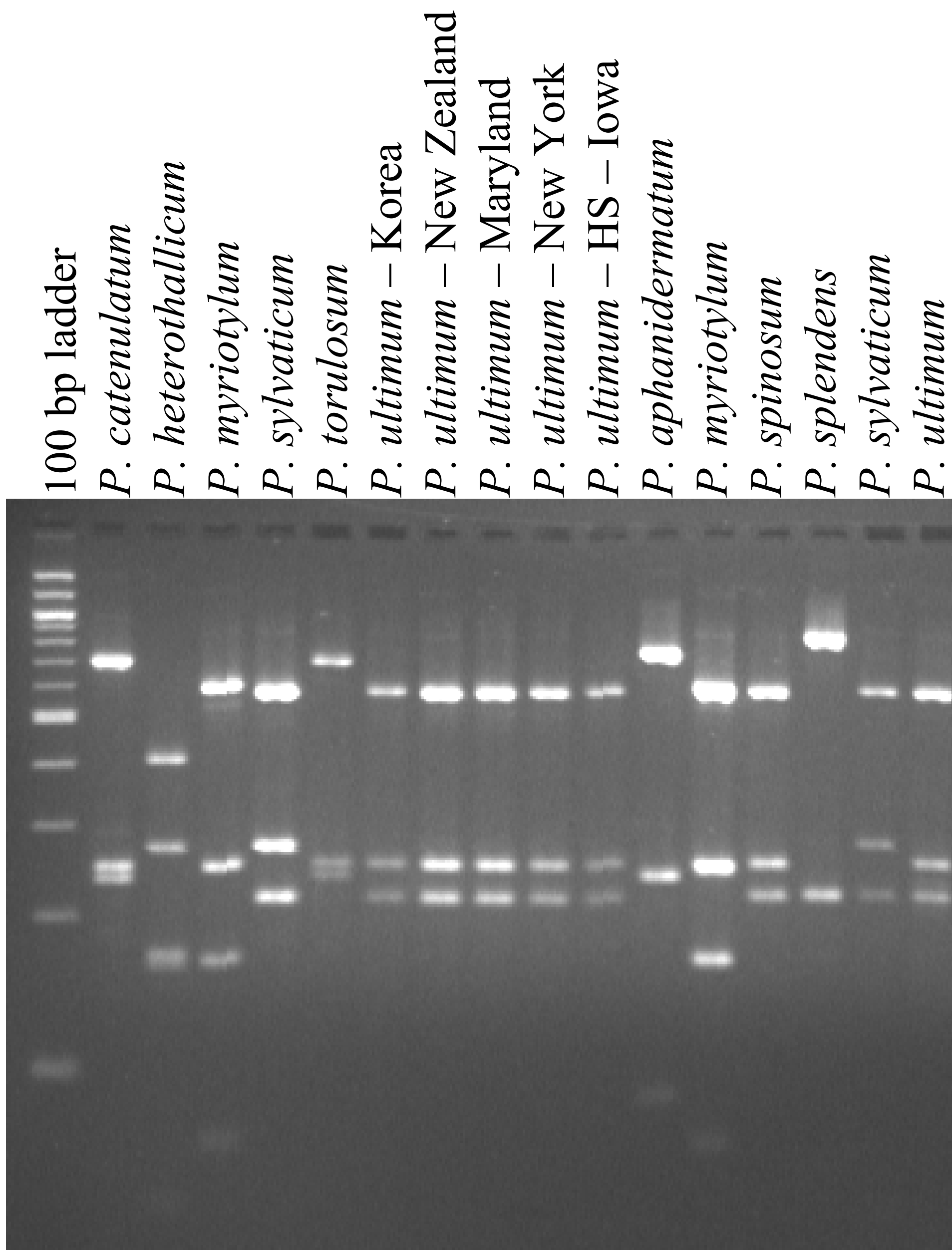


Martin and Tooley
Phytopathology
2004

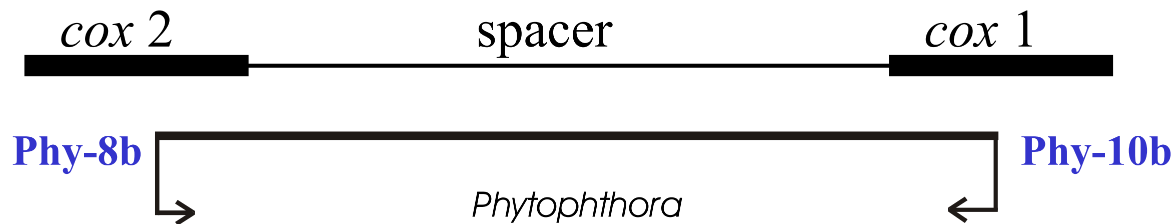
RFLP Analysis for ID of *Pythium* spp.

- 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

*Alu*1



Phytophthora genus-specific Amplification

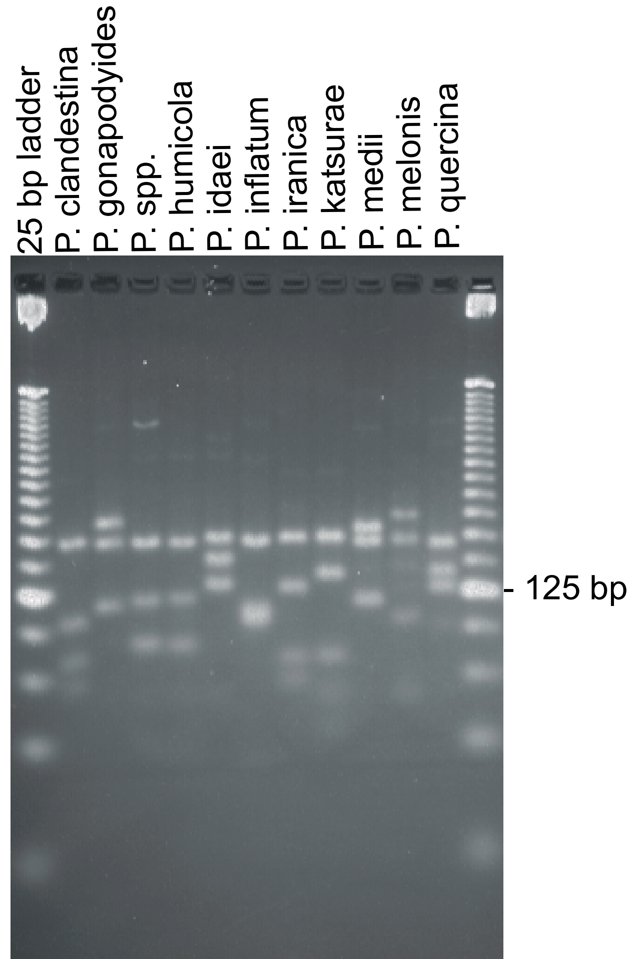


Approximately 450-500 bp

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*
    - Prospero et al. 2004, Ivors et al. 2006, Lees et al. 2006, Dobrowolski et al. 2002
  - *Pythium*
    - Lee and Moorman 2007
- Micro/macro arrays to identify SNPs
- Mitochondrial haplotypes
  - *Phytophthora infestans*

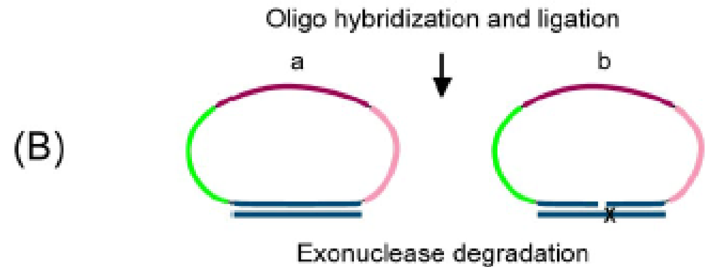
Species-Specific PCR for Pathogen Detection

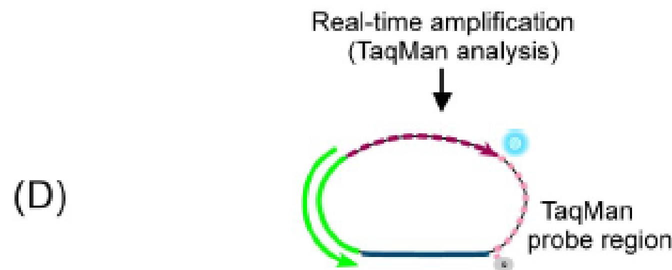
- Conventional vs real-time PCR
  - Due to less sensitivity and the time necessary for running the sample conventional PCR less common in diagnostic setting
- Important to have multiplexed
  - Plant marker as internal control for DNA extraction
  - Genus-specific marker is desirable
- Different chemistries 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

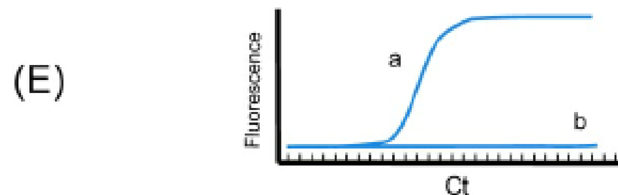
Approaches to Enhance Specificity

- 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

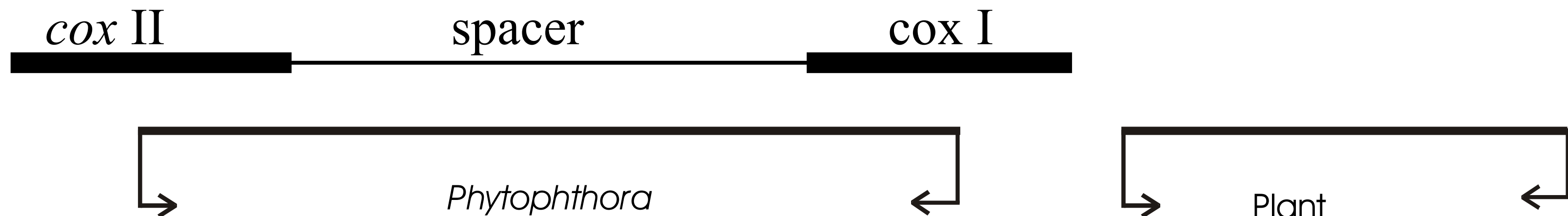
- Reported as diagnostic for *Phytophthora ramorum*
  - Tomlinson et al. 2007
- Does not require a thermal cycler (just a temperature controlled block)
- Can visualize results
  - On a gel by electrophoresis
  - Intercalation of a dye
  - Increased turbidity (production of Mg pyrophosphatase)
  - Real-time 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

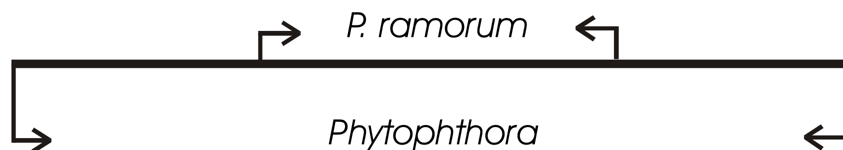
- See more sequence variation than in many nuclear regions
- Target has high copy number
- Want to identify region where variable sequences are flanked by conserved sequences to simplify marker development for additional species
- Use in conjunction with plant and *Phytophthora* genus specific markers

Phytophthora ramorum Multiplex Amplification

First Round Amplification



Second Round Amplification



Additional details: 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

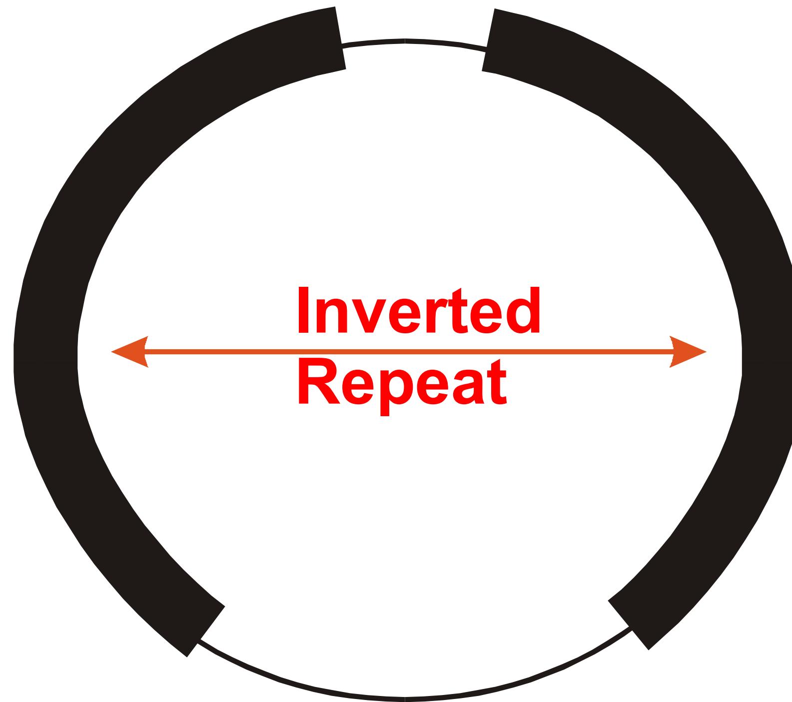
Mitochondrial Genome Sequencing

- *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*

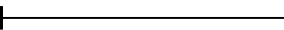
Pythium mtDNA



Single Copy
Region

IR

IR

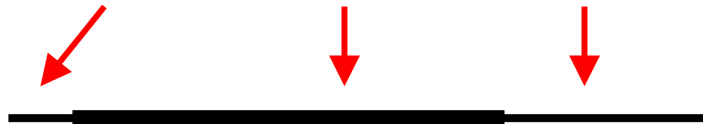


Linear Mitochondrial Genomes of *Pythium* spp.

- 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

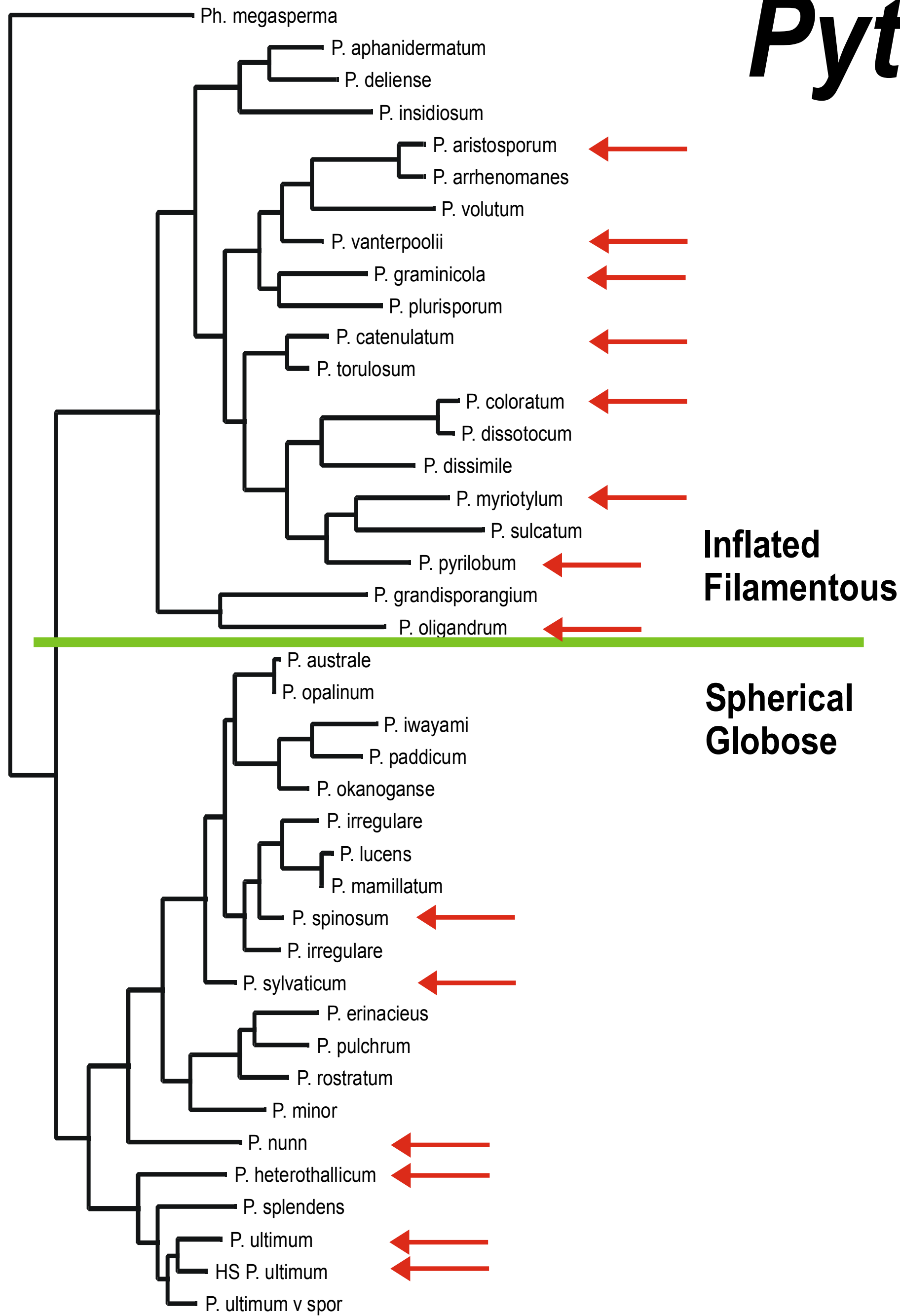
Genome Sizes for *Pythium* spp.

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



[a]Sizes in bp

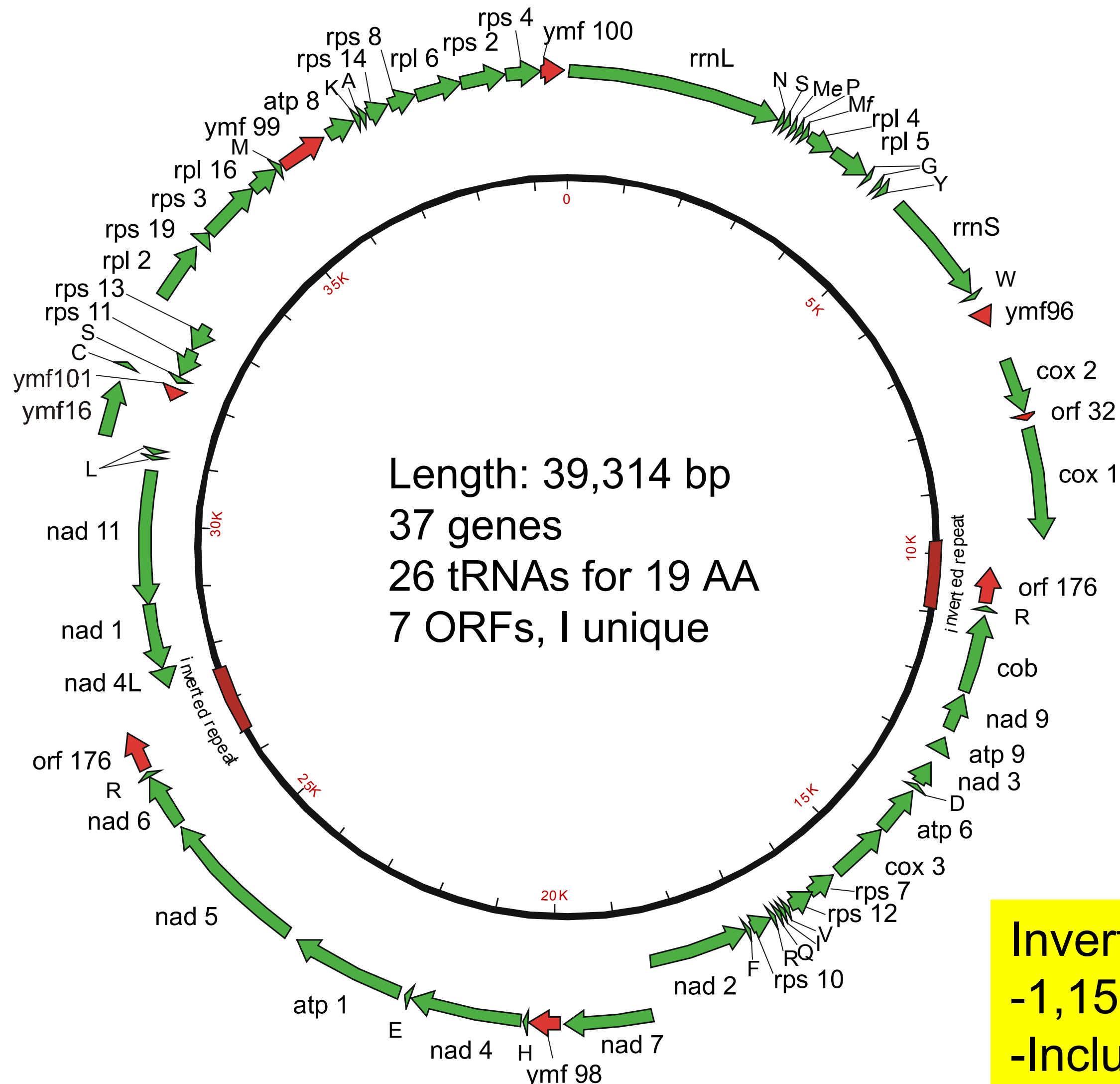
Pythium spp.



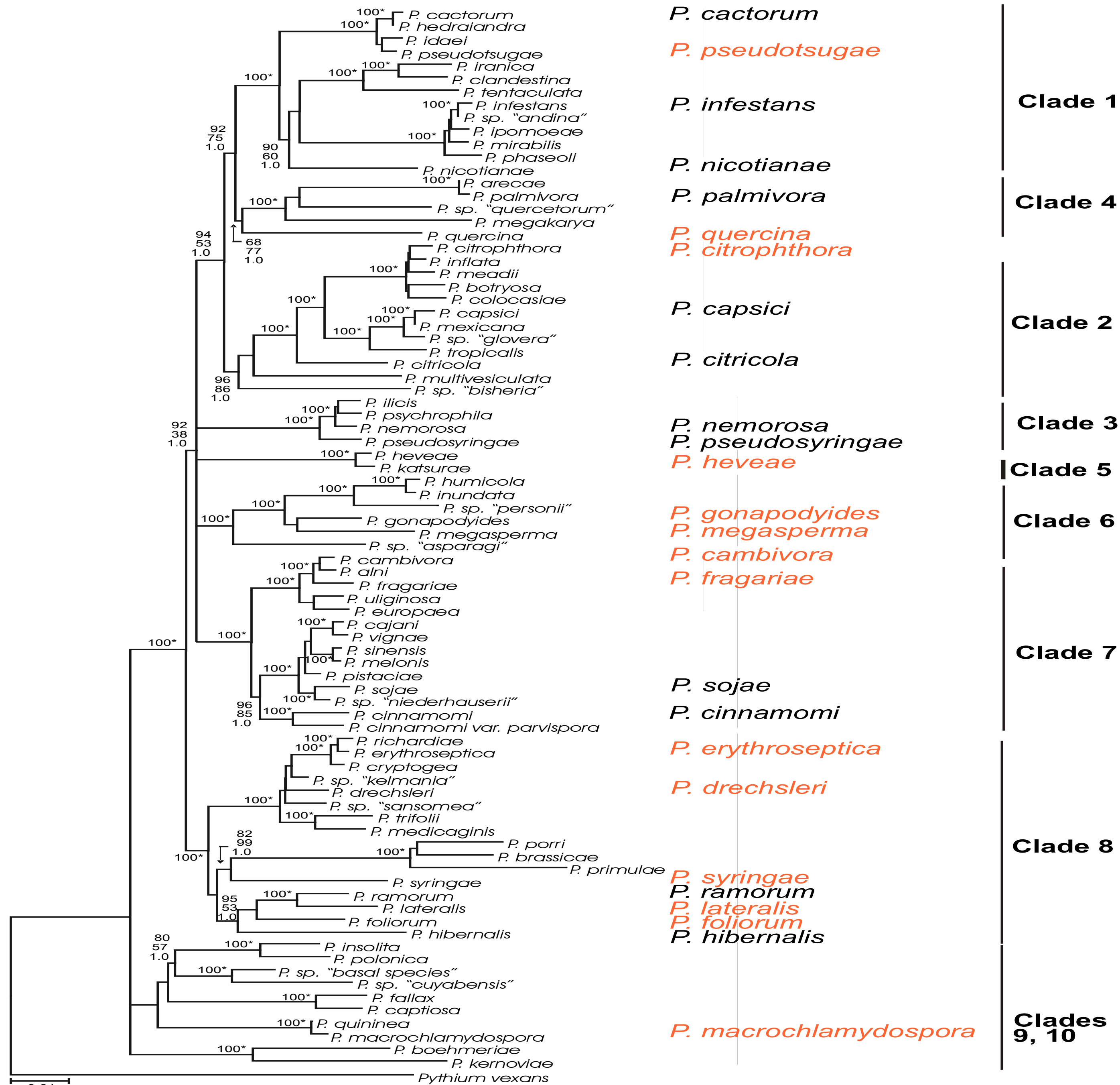
Phytophthora Mitochondrial Genome Organization

- 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



Inverted Repeat
-1,150 bp in length
-Includes 528 bp ORF



Multilocus phylogeny of Blair et al. (2008)

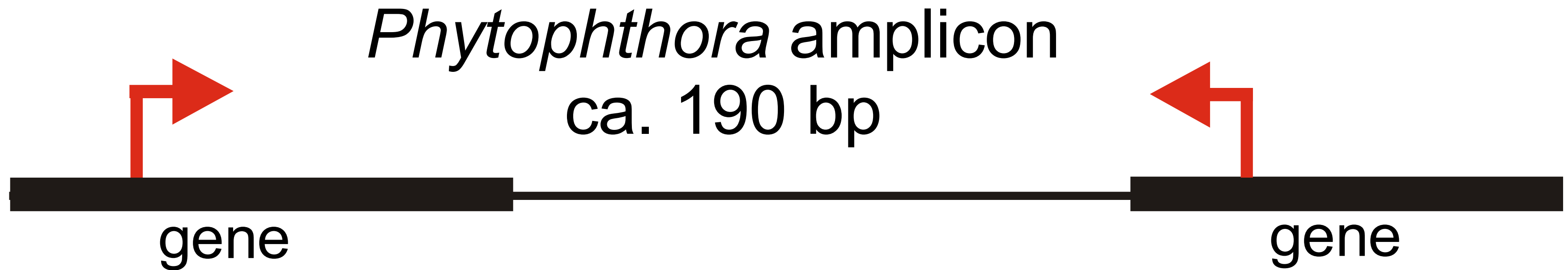
Is gene order related to phylogenetic relationships in *Phytophthora*?

- 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*

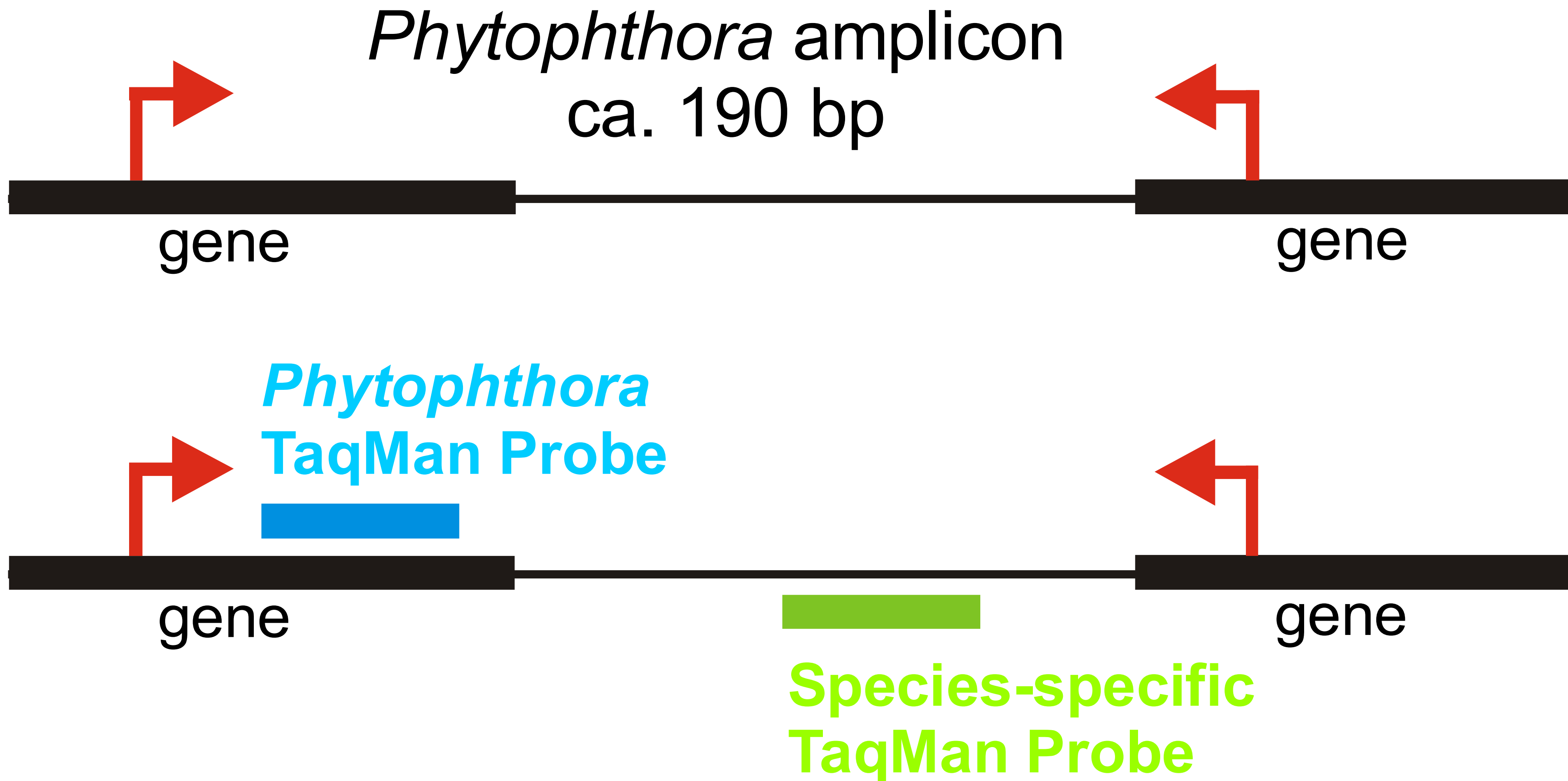
- Two conserved differences in gene order compared to *Pythium* have been identified
- Both regions have been sequenced in 90+ isolates representing 60+ species to assess intra- and interspecific variation.
- One region has been selected for further study based on the sequence data
  - Interspecific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

Phytophthora Multiplex Amplification



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

Phytophthora Multiplex Amplification



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 *cox*1 gene
- If so, can they be used as a marker to help monitor populations of the pathogen?

Martin (2008) Current Genetics 54:23-34

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

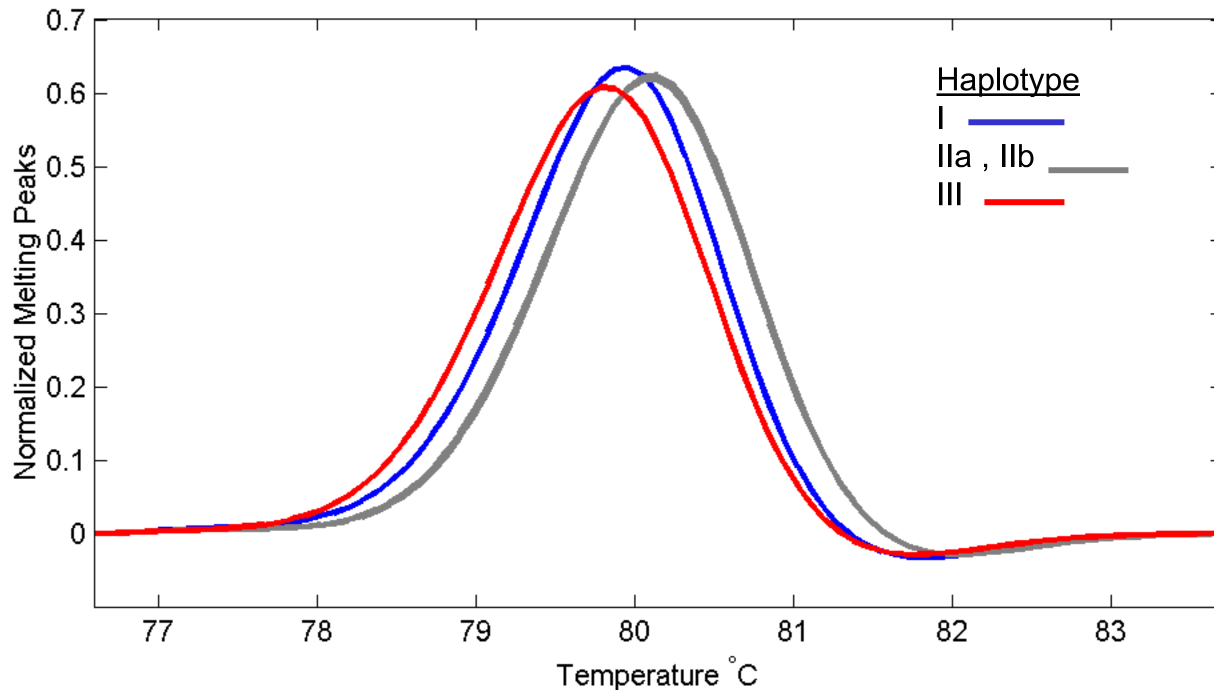
P. ramorum Mitochondrial Haplotypes

Marker	# Variable Bases	mtDNA Haplotypes
Prv-9	1	I – EU II - US , III – Washington Nursery
ymf-16	2	I – EU , III – Washington Nursery II - US
*cox*2 + spacer	3	III – Washington Nursery I = II
Prv-1	2	III – Washington Nursery I = II
Prv-8	2	I – EU II - US III – Washington Nursery
Prv-11	2	I – EU II - US III – Washington Nursery
Prv-13	8	I – EU II – US III – Washington Nursery
*cox*1	4	I – EU II – US III – Washington Nursery
Prv-14	4	I – EU IIa – US IIb – Oregon forest III – Washington Nursery

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



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
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