

**Evaluation of molecular markers  
for *P. ramorum* detection and  
identification; testing for  
specificity using a standardized  
library of  
DNAs**

Project Coordinators:

Frank Martin (USDA-ARS)

Mike Coffey (UC Riverside)

# Project Objectives

- Evaluate a range of molecular diagnostic markers using a standardized library of DNAs sent blind to the cooperators.
  - Culture extracted DNA
  - DNA from field samples

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- Evaluate a range of molecular diagnostic markers using a standardized library of DNAs sent blind to the cooperators.
  - Culture extracted DNA
    - 317 isolates representing 60 described species and 11 taxonomically unclassified isolates
      - The species identity of the isolates used was confirmed by sequence analysis of the *Phytophthora* genus-specific amplicon spanning the *cox 2* gene and the ITS region
    - Sent out in 6 batches for total of 457 samples
      - Some samples sent out multiple times, when this was done the tubes were recoded.
    - 48 geographically diverse isolates of *P. ramorum* were sent out in 67 samples.
    - All DNA extractions done at UC Riverside in Mike Coffey's lab
      - DNA concentrations were sent at 10 ng/μl, but some labs diluted this

# Caveat for interpreting data from culture extracted DNA

- From all but one diagnostic assay the DNA concentration was between 6 and 10 ng
  - This is higher than would be found in nature
    - 13fg to 2 pg (Tooley et al. 2006)
    - 10-100 pg in 15-25 mg tissue (Tomlinson et al. 2005)
    - Mean *P. ramorum* DNA concentration 50 pg/ $\mu$ l, rarely above 2 ng or below 100 fg (Zeller, USDA-APHIS, unpublished)

# Project Objectives

- Evaluate a range of molecular diagnostic markers using a standardized library of DNAs sent blind to the cooperators.
  - Culture extracted DNA
  - DNA from field samples
    - 60 samples representing 16 plant species (forest and ornamental species) and 11 *Phytophthora* spp.
      - 9 samples were confirmed to have *P. ramorum* present
    - DNAs extracted using standard USDA-APHIS procedure by Cheryl Blomquist at CDFA
      - ID of pathogen based on recovery or sequencing of the ITS region
    - ID of pathogens present in the sample confirmed by sequence analysis of the *Phytophthora* genus-specific amplicon spanning the *cox 1* and 2 spacer region and of the ITS region (amplicon for the SSCP analysis)

# Diagnostic Markers Evaluated

| Diagnostic marker | Target locus           | Type of assay                                        | DNA concentration | Lab assay performed in       |
|-------------------|------------------------|------------------------------------------------------|-------------------|------------------------------|
| Mitochondrial     | <i>cox</i> spacer      | Conventional nested PCR                              | 10 ng             | Martin, USDA-ARS             |
|                   | <i>cox</i> spacer      | TaqMan real time PCR                                 | 10 ng             | Tooley, USDA-ARS             |
| Nuclear           | elicitin (NRC)         | TaqMan real time PCR                                 | 10 ng             | Hamelin, NRC-CFS, Quebec     |
|                   | $\beta$ -tubulin (NRC) | TaqMan real time PCR                                 | 10 ng             | Hamelin, NRC-CFS, Quebec     |
|                   | ITS (NRC)              | TaqMan real time PCR                                 | 10 ng             | Hamelin, NRC-CFS, Quebec     |
|                   | ITS (CSL)              | TaqMan real time PCR                                 | 10 ng             | Hughes, CSL, York, UK        |
|                   | ITS (UCB)              | TaqMan nested real time PCR                          | 62.5 fg           | Garbelotto, UC Berkeley      |
|                   | ITS (APHIS)            | Conventional nested PCR                              | 6 ng              | Zeller, USDA-APHIS-PPQ-CPHST |
|                   | ITS (APHIS)            | Conventional multiplexed PCR for <i>P. lateralis</i> | 6 ng              | Zeller, USDA-APHIS-PPQ-CPHST |
|                   | ITS (APHIS)            | Modified CSL Real time PCR                           | 6 ng              | Zeller, USDA-APHIS-PPQ-CPHST |
|                   | ITS (SSCP)             | Single Strand Conformational Polymorphism            | 10 ng             | Kubisiak, USDA-FS            |

# Results from culture extracted DNAs

| Diagnostic marker                                 | Type of assay          | <i>P. ramorum</i> Positive |           | <i>P. ramorum</i> Negative |          | Retest (E) |
|---------------------------------------------------|------------------------|----------------------------|-----------|----------------------------|----------|------------|
|                                                   |                        | True (A)                   | False (B) | False (C)                  | True (D) |            |
| Nested cox spacer                                 | Conventional (Martin)  | 66                         | 2         | 1                          | 388      | 0          |
| cox spacer                                        | Real time (Tooley)     | 67                         | 12        | 0                          | 378      | 0          |
| Elicitin (NRC)                                    | Real time (Hamelin)    | 67                         | 3         | 0                          | 387      | 0          |
| β-tubulin (NRC)                                   | Real time (Hamelin)    | 67                         | 4         | 0                          | 386      | 0          |
| ITS (NRC)                                         | Real time (Hamelin)    | 67                         | 5         | 0                          | 385      | 0          |
| ITS (CSL)                                         | Real time (Hughes)     | 66                         | 0         | 1                          | 379      | 11         |
| ITS (UCB)                                         | Real time (Garbelotto) | 64                         | 0         | 3                          | 390      | 0          |
| ITS nested (USDA-APHIS) <sup>a</sup>              | Conventional (Zeller)  | 67                         | 81        | 0                          | 309      | 0          |
| <i>P. lateralis</i> ITS (USDA-APHIS) <sup>a</sup> | Conventional (Zeller)  | 67                         | 17        | 0                          | 373      | 0          |
| ITS (USDA-APHIS) <sup>a</sup>                     | Real time (Zeller)     | 67                         | 35        | 0                          | 311      | 44         |
| ITS SSCP                                          | SSCP (Kubisiak)        | 67                         | 0         | 0                          | 390      | 0          |

<sup>a</sup> these assays were the USDA-APHIS approved diagnostic assays

# *P. ramorum* false negatives

- Conventional nested cox spacer region
  - 1 false negative – isolate was negative one out of three times it was tested
- CSL ITS TaqMan real time PCR
  - 1 false negative – isolate only tested once
- UCB nested ITS TaqMan real time PCR
  - 3 false negatives –
    - 1 isolate isolate was negative one out of three times it was tested (same isolate as the cox spacer false negative)
    - 2 isolates were only tested once (one was the same isolate that was a false negative in the CSL ITS TaqMan real time PCR)

## ***P. ramorum* false positive**

- For the APHIS approved conventional multiplex *P. lateralis* assay, primarily phylogenetically closely related species
  - *P. foliorum*
  - *P. hibernalis*
  - *P. lateralis*

# Analysis of accuracy of results

- Diagnostic sensitivity – proportion of true positives correctly identified
- Diagnostic specificity – proportion of true negatives correctly identified
- Positive Predictive Value (PPV) – proportion of samples with positive results correctly identified
- Negative Predictive Value (NPV) – proportion of samples with negative results correctly identified

As per Kox et al., (2007) with modification for diagnostic specificity and NPV

# Results from culture extracted DNAs

| Diagnostic marker                    | Type of assay          | <i>P. ramorum</i> Positive |           | <i>P. ramorum</i> Negative |          | Retest (E) |
|--------------------------------------|------------------------|----------------------------|-----------|----------------------------|----------|------------|
|                                      |                        | True (A)                   | False (B) | False (C)                  | True (D) |            |
| Nested cox spacer                    | Conventional (Martin)  | 66                         | 2         | 1                          | 388      | 0          |
| cox spacer                           | Real time (Tooley)     | 67                         | 12        | 0                          | 378      | 0          |
| Elicitin (NRC)                       | Real time (Hamelin)    | 67                         | 3         | 0                          | 387      | 0          |
| β-tubulin (NRC)                      | Real time (Hamelin)    | 67                         | 4         | 0                          | 386      | 0          |
| ITS (NRC)                            | Real time (Hamelin)    | 67                         | 5         | 0                          | 385      | 0          |
| ITS (CSL)                            | Real time (Hughes)     | 66                         | 0         | 1                          | 379      | 11         |
| ITS (UCB)                            | Real time (Garbelotto) | 64                         | 0         | 3                          | 390      | 0          |
| ITS nested (USDA-APHIS)              | Conventional (Zeller)  | 67                         | 81        | 0                          | 309      | 0          |
| <i>P. lateralis</i> ITS (USDA-APHIS) | Conventional (Zeller)  | 67                         | 17        | 0                          | 373      | 0          |
| ITS (USDA-APHIS)                     | Real time (Zeller)     | 67                         | 35        | 0                          | 311      | 44         |
| ITS SSCP                             | SSCP (Kubisiak)        | 67                         | 0         | 0                          | 390      | 0          |

<sup>a</sup> Diagnostic sensitivity (%) =  $100 \times A / (A + C)$

<sup>b</sup> Diagnostic specificity (%) =  $100 \times D / (D + B + E)$

<sup>c</sup> PPV = positive predictive value (%) =  $100 \times A / (A + B)$

<sup>d</sup> NVP = negative predictive value (%) =  $100 \times D / (D + C + E)$

# Results from culture extracted DNAs

| Diagnostic marker                    | Type of assay          | Diagnostic sensitivity | Diagnostic specificity | PPV  | NVP               |
|--------------------------------------|------------------------|------------------------|------------------------|------|-------------------|
| Nested cox spacer                    | Conventional (Martin)  | 98.5                   | 99.5                   | 97.1 | 99.7              |
| cox spacer                           | Real time (Tooley)     | 100                    | 96.9                   | 84.8 | 100               |
| Elicitin (NRC)                       | Real time (Hamelin)    | 100                    | 99.2                   | 95.7 | 100               |
| β-tubulin (NRC)                      | Real time (Hamelin)    | 100                    | 99.0                   | 94.4 | 100               |
| ITS (NRC)                            | Real time (Hamelin)    | 100                    | 98.7                   | 93.1 | 100               |
| ITS (CSL)                            | Real time (Hughes)     | 98.5                   | 97.2 <sup>a</sup>      | 100  | 96.9 <sup>a</sup> |
| ITS (UCB)                            | Real time (Garbelotto) | 95.5                   | 100                    | 100  | 99.2              |
| ITS nested (USDA-APHIS)              | Conventional (Zeller)  | 100                    | 79.2                   | 45.3 | 100               |
| <i>P. lateralis</i> ITS (USDA-APHIS) | Conventional (Zeller)  | 100                    | 95.6                   | 79.8 | 100               |
| ITS (USDA-APHIS)                     | Real time (Zeller)     | 100                    | 79.7 <sup>a</sup>      | 65.7 | 87.6 <sup>a</sup> |
| ITS SSCP                             | SSCP (Kubisiak)        | 100                    | 100                    | 100  | 100               |

<sup>a</sup> Calculations for these samples included the samples requiring a retest due to the C<sub>t</sub> of the amplification being between 36 and 40

# Results from field samples

| Diagnostic marker                                 | Type of assay          | <i>P. ramorum</i> Positive |           | <i>P. ramorum</i> Negative |          | Retest (E) |
|---------------------------------------------------|------------------------|----------------------------|-----------|----------------------------|----------|------------|
|                                                   |                        | True (A)                   | False (B) | False (C)                  | True (D) |            |
| Nested cox spacer                                 | Conventional (Martin)  | 9                          | 0         | 0                          | 51       | 0          |
| cox spacer                                        | Real time (Tooley)     | 9                          | 0         | 0                          | 51       | 0          |
| Elicitin (NRC)                                    | Real time (Hamelin)    | 9                          | 0         | 0                          | 51       | 0          |
| $\beta$ -tubulin (NRC)                            | Real time (Hamelin)    | 9                          | 1         | 0                          | 50       | 0          |
| ITS (NRC)                                         | Real time (Hamelin)    | 9                          | 0         | 0                          | 51       | 0          |
| ITS (CSL)                                         | Real time (Hughes)     | 9                          | 0         | 0                          | 51       | 0          |
| ITS (UCB)                                         | Real time (Garbelotto) | 6                          | 1         | 3                          | 50       | 0          |
| ITS nested (USDA-APHIS) <sup>a</sup>              | Conventional (Zeller)  | 9                          | 9         | 0                          | 42       | 0          |
| <i>P. lateralis</i> ITS (USDA-APHIS) <sup>a</sup> | Conventional (Zeller)  | 9                          | 11        | 0                          | 40       | 0          |
| ITS (USDA-APHIS) <sup>a</sup>                     | Real time (Zeller)     | 9                          | 1         | 0                          | 37       | 13         |
| ITS SSCP                                          | SSCP (Kubisiak)        | 9                          | 0         | 0                          | 47       | 4          |

<sup>a</sup> these assays were the USDA-APHIS approved diagnostic assays

# Results from field samples

| Diagnostic marker                    | Type of assay          | <i>P. ramorum</i> Positive |           | <i>P. ramorum</i> Negative |          | Retest (E) |
|--------------------------------------|------------------------|----------------------------|-----------|----------------------------|----------|------------|
|                                      |                        | True (A)                   | False (B) | False (C)                  | True (D) |            |
| Nested cox spacer                    | Conventional (Martin)  | 9                          | 0         | 0                          | 51       | 0          |
| cox spacer                           | Real time (Tooley)     | 9                          | 0         | 0                          | 51       | 0          |
| Elicitin (NRC)                       | Real time (Hamelin)    | 9                          | 0         | 0                          | 51       | 0          |
| β-tubulin (NRC)                      | Real time (Hamelin)    | 9                          | 1         | 0                          | 50       | 0          |
| ITS (NRC)                            | Real time (Hamelin)    | 9                          | 0         | 0                          | 51       | 0          |
| ITS (CSL)                            | Real time (Hughes)     | 9                          | 0         | 0                          | 51       | 0          |
| ITS (UCB)                            | Real time (Garbelotto) | 6                          | 1         | 3                          | 50       | 0          |
| ITS nested (USDA-APHIS)              | Conventional (Zeller)  | 9                          | 9         | 0                          | 42       | 0          |
| <i>P. lateralis</i> ITS (USDA-APHIS) | Conventional (Zeller)  | 9                          | 11        | 0                          | 40       | 0          |
| ITS (USDA-APHIS)                     | Real time (Zeller)     | 9                          | 1         | 0                          | 37       | 13         |
| ITS SSCP                             | SSCP (Kubisiak)        | 9                          | 0         | 0                          | 47       | 4          |

<sup>a</sup> Diagnostic sensitivity (%) =  $100 \times A / (A + C)$

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<sup>c</sup> PPV = positive predictive value (%) =  $100 \times A / (A + B)$

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# Analysis of accuracy of results

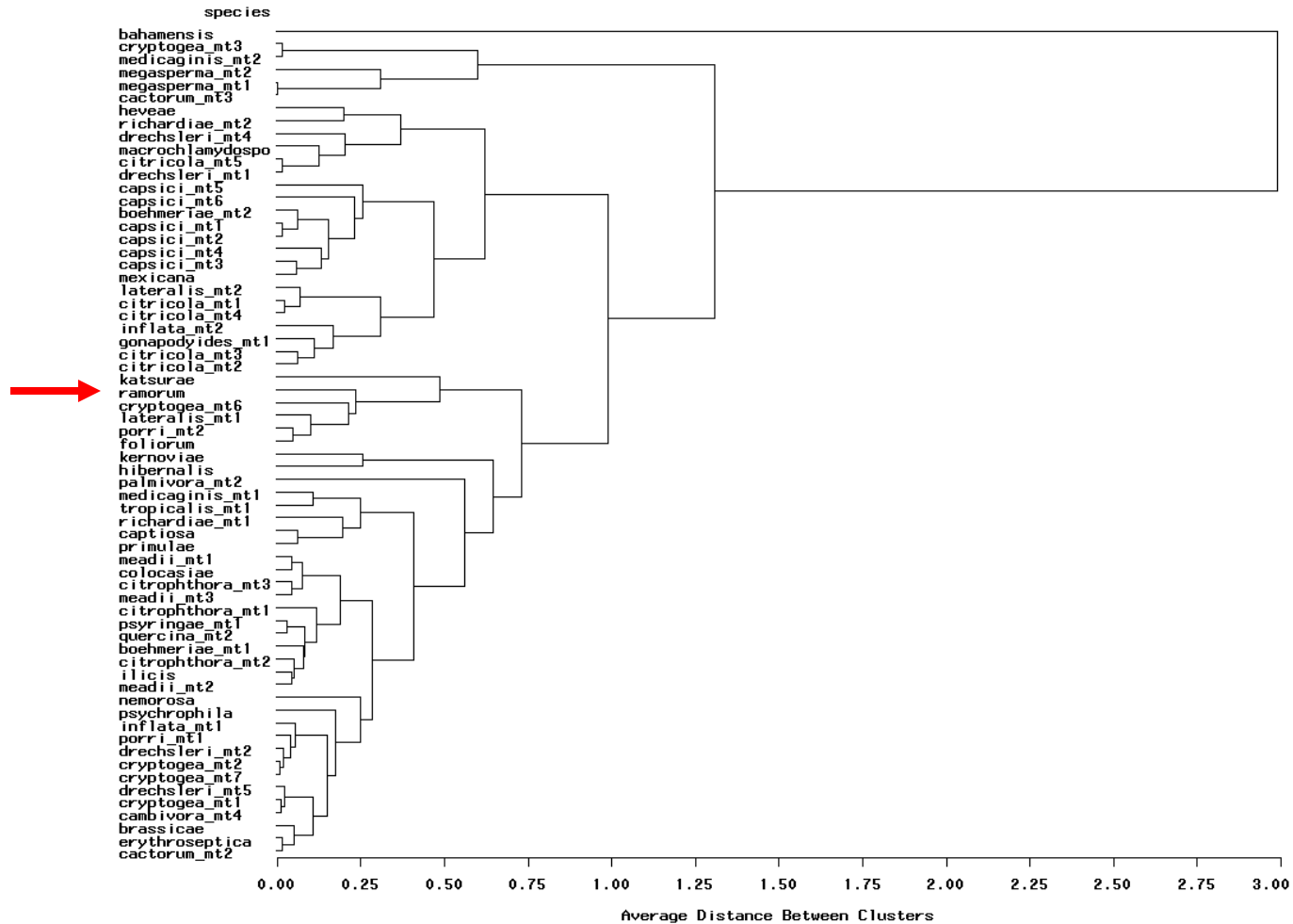
- Diagnostic sensitivity – proportion of true positives correctly identified
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# Results from field samples

| Diagnostic marker                                 | Type of assay          | Diagnostic sensitivity | Diagnostic specificity | PPV  | NVP               |
|---------------------------------------------------|------------------------|------------------------|------------------------|------|-------------------|
| Nested cox spacer                                 | Conventional (Martin)  | 100                    | 100                    | 100  | 100               |
| cox spacer                                        | Real time (Tooley)     | 100                    | 100                    | 100  | 100               |
| Elicitin (NRC)                                    | Real time (Hamelin)    | 100                    | 100                    | 100  | 100               |
| $\beta$ -tubulin (NRC)                            | Real time (Hamelin)    | 100                    | 98.0                   | 90   | 100               |
| ITS (NRC)                                         | Real time (Hamelin)    | 100                    | 100                    | 100  | 100               |
| ITS (CSL)                                         | Real time (Hughes)     | 100                    | 100                    | 100  | 100               |
| ITS (UCB)                                         | Real time (Garbelotto) | 66.6                   | 98.0                   | 85.7 | 94.3              |
| ITS nested (USDA-APHIS) <sup>f</sup>              | Conventional (Zeller)  | 100                    | 82.3                   | 50.0 | 100               |
| <i>P. lateralis</i> ITS (USDA-APHIS) <sup>f</sup> | Conventional (Zeller)  | 100                    | 78.4                   | 45.0 | 100               |
| ITS (USDA-APHIS) <sup>f</sup>                     | Real time (Zeller)     | 100                    | 72.5 <sup>a</sup>      | 90.0 | 74.0 <sup>a</sup> |
| ITS SSCP                                          | SSCP (Kubisiak)        | 100                    | 92.1 <sup>a</sup>      | 100  | 92.2 <sup>a</sup> |

<sup>a</sup> Calculations for these samples included the samples requiring a retest due to the C<sub>t</sub> of the amplification being between 36 and 40

# UPGMA analysis of SSCP results



# Conclusions

- The diagnostic assays accurately identified *P. ramorum*
  - Some false negatives were observed, but for some this may be a reflection of operator error
- Specificity for *P. ramorum* was good for most assays
  - Primarily phylogenetically closely related species
  - The nested conventional ITS diagnostic assay had the least specificity, but this assay is not stand alone, diagnosis is based on the combined results of 2 other assays
  - Important to keep in mind how the high DNA concentration relative to naturally infected tissue that was used and how this might increase the chances of a false positive (especially for real time PCR assays)
    - To get a better idea of specificity as it relates to the analysis of field samples a dilution series of DNA would need to be run
- Using a combination of diagnostic assays improved the accuracy of correct diagnosis for *P. ramorum*
- The SSCP procedure can identify *P. ramorum* and be helpful in identifying other species that are present.