

P. ramorum diagnostics - update



USDA APHIS PPQ CPHST
March, 2006

YOU HAVE VERMIN



The Times (UK)
11/17/05

Provisional Approval Process



- ✓ Protocol still evolving and improving
 - ✓ > 20 labs in system
 - ✓ 7 approved: CDFA, ODA, Oregon State Univ., WSDA, U Tenn., Univ. FL, USDA-AMS (surge lab)
- ✓ serves as template for future plans for lab accreditation and process certification:
(NPPLAP - National Plant Protection Laboratory Accreditation Program)

Current PA protocol:



1. Documentation on lab layout, equipment, scientific background of personnel
2. Inspection of facility – infrastructure, expertise, record keeping, follow through of process
 - scout for Real-Time capabilities in anticipation of new protocol
3. Proficiency Panel – currently tests only part of process (nested PCR)
 - Research under way by UC Berkeley to assist in development of start to finish PT panels; main thrust is to determine the range of *P. ramorum* DNA found in nature, and design panel to target average amount of DNA and average of the lowest 10% tail
 - Current PA labs need only to complete a PT for real-time PCR
4. Memorandum of Agreement

Experiences of Inspection Team



VARIETY!!!

- ✓ state dep't of ag labs, NPDN labs, Univ. Labs, other USDA labs
- ✓ everything from traditional diagnostic labs retrofitted for molecular diagnostics to 1-2 year old labs specifically designed for molecular diagnostics
- ✓ expertise range from traditional mycology to biochemistry and molecular biology

NPGBL



- ✓ PPQ is currently caught up on samples
- ✓ characterization of 'Azalea' Phytophthora that occasionally triggers false positive in nested PCR
 - ✓ Work from NPGBL, CDFA
- ✓ Real-Time PCR validated
 - ✓ Multi-lab 'Ring test' - 6 labs (USDA-AMS, USDA-CPHST, CDFA, CFIA, U Tenn., CSL)
 - ✓ Afforded us opportunity to 'validate' ABI platforms (7000, 7900)
 - ✓ Indicated problem with OmniMix beads
 - ✓ doesn't trigger false positive with 'Azalea' Phytophthora depending on assay conditions

Evaluation of *P. ramorum* Diagnostics



Project Coordinators:

Frank Martin (USDA-ARS)

Mike Coffey (UC Riverside)

Goal: To identify the (next) best diagnostic test for Pr that is:

- 1) relatively simple and rapid,
- 2) sensitive,
- 3) highly selective,
- 4) target is a genetic locus distinct from ITS

Objective:



The objective of this project is to assemble a library of DNA from isolates at the World *Phytophthora* Collection at UC Riverside representing the phylogenetic groupings and geographic diversity of the genus.

These will be sent blind to cooperating labs to evaluate a variety of molecular diagnostic or analytical methods for *P. ramorum*.

Experimental Approach



✓ Species examined

- ✓ > 400 isolates collected from different regions of the world representing all or nearly all described species included in the trials

✓ DNA Extraction

- ✓ DNA extracted at UC Riverside and adjusted to 10 ng/μl
 - ✓ Sequence analysis of the ITS region will be done to confirm isolate identification
- ✓ Sub-samples sent to cooperators in numbered tubes for evaluation
- ✓ After cooperators forward results to Mike Coffey they are told the species classification of the samples

Diagnostic Tests Evaluated

- ✓ Approved Methods (Garbelotto ITS method):
 - ✓ Conventional
 - ✓ Matteo Garbelotto, UC Berkeley
 - ✓ USDA-APHIS-CHPST (nested and multiplex)
 - ✓ Real-time PCR (CSL ITS method)
 - ✓ Kelvin Hughes, CSL
 - ✓ USDA-APHIS-CHPST
- ✓ Real-time
 - ✓ Matteo Garbelotto, UC Berkeley
- ✓ Real-time PCR (ITS, β tubulin, elicitin)
 - ✓ Richard Hamelin, NRC Canada Forest Service
- ✓ Mitochondrial marker (cox 1 and 2 region)
 - ✓ Conventional – Frank Martin, USDA-ARS
 - ✓ Real-time – Paul Tooley, USDA-ARS

Diagnostics for *Phytophthora* spp.



Diagnostic tests for identification of isolates to a species level

- ✓ SSCP
 - ✓ Tom Kubisiak, USDA Forest Service
- ✓ Cox 1 and 2 gene cluster RFLP
 - ✓ Frank Martin, USDA-ARS

Current Status of Evaluations



- ✓ 358 samples have been sent in 5 groups to the cooperators
- ✓ There were 216 samples in first four groups
 - ✓ 44 described species, 9 unidentified spp.
 - ✓ 140 different isolates/species
 - ✓ DNA from some isolates have been sent out on multiple groups, especially if there are any false positive or negative results reported
- ✓ Currently working on 142 additional samples in group 5
- ✓ Three additional groups of isolates to finish the tests will be sent out shortly
- ✓ Follow-up tests (e.g., relative sensitivity, environmental samples)

Other activities associated with Pr diagnostics:

✓ The Monrovia Experiment

✓ Results indicate that:

- ✓ 42% culture +ve
- ✓ Nested PCR appears to be more sensitive 75.6% +ve vs 54.6% +ve by Real time
- ✓ No +ve cultures that were both nested or Real time -ve
- ✓ 2 cultures Real time -ve, but none that were Nested -ve
- ✓ 34.7% Nested +ve's were culture -ve
- ✓ 1.0% of culture +ve were nested -ve
- ✓ ELISA is a relatively effective pre-screen

Cooperative Agreement with UC Berkeley



- ✓ Direct, systematic comparison of diagnostic methods:
 1. Nested PCR
 2. CSL TaqMan
 3. V8 PARP
 4. Corn meal PARP
 5. ELISA

Cooperative Agreement with UC Berkeley - Preliminary Results



- ✓ Experimental approach includes intensive sampling of a limited number of sites across the entire natural ecological and geographic range of *P. ramorum* in California
- ✓ Symptomatic plant material collected in two different seasons (wet vs. dry) and processed by two independent labs:
- ✓ 289 samples from 24 spp., thus far...

Cooperative Agreement with UC Berkeley - Preliminary Results

1. Results are highly host-specific; there is a strong effect of plant species on efficacy of diagnostic method
2. For one or two species CSL is superior to Nested
3. CSL real time is less effective than nested PCR; 15% diagnosed by nested and not by CSL were confirmed by isolation
4. Very low rate of false positives if no cultures were obtained. For example, when both PCR and ELISA +ve, the ID is authentic, 97-99%
5. Both isolation media are comparable, but V8-based PARP is better
6. ELISA sensitive, but will of course detect other *Phytophthora* spp. Data analysis still ongoing, but suggest that it is appropriate to trust ELISA negative results as negative.

(These data were obtained with spring sampling when the plant tissue is still in good condition. More information about ELISA will be obtained when fall samples are analyzed.)

PRELIMINARY

Potentially Actionable Suspect Samples (PASS) System



- ✓ Purpose: To clearly define when a sample requires Federal confirmatory testing
 - ✓ Usually, the first presumptive identification of a pest or pathogen of Federal regulatory concern.
 - ✓ Subsequent samples from a pre-defined area around the first sample would not require confirmatory testing, but new finds outside of the area would be treated as new PAS samples. (i.e., we would accept results of external lab.)
 - ✓ PASS samples would also encompass any sample that involves unusual or unexpected circumstances, such as a new host, new location, etc.
 - ✓ Policy is specific to any pest or pathogen, based on what is known on the biology and epidemiology of the organism.

PASS and *P. ramorum*

Sample Routing:

If the sample is an Initial Presumptive Positive from a:	Then the sample is a:
National survey site	PASS Sample and must be sent to an APHIS Reference Laboratory for confirmation.
Compliance Inspection site	
Cleanliness Inspection site	
Certification Inspection site	
TF plant at a TF site that ships interstate	
TF plant at a TF site in a state other than the source site	
(TB) site	
Any unusual or unexpected detection or one not otherwise covered above	
If the sample is a subsequent Presumptive Positive from:	Then the sample is:
National survey site	Not a PASS sample if the sample is covered by previous confirmation of the PASS sample.
Compliance Inspection site	
Cleanliness Inspection site	
Certification Inspection site	
TF plant at a TF site that does not ship interstate and is in the same state as the source site	
TB site (originating source)	
If the sample is an Initial Presumptive Positive or a Subsequent Presumptive Positive from:	Then the sample is a:
Any TF site where the sample is from any plant not part of the TF shipments(s)	PASS Sample and must be sent to an APHIS Reference Laboratory for confirmation.
Any sample that will require Federal regulatory action	
Any previously undescribed or unknown host(s)	
Any host(s) new to the US	
Any Environmental location, including home owner yards, natural landscape or forest location(s) whether or not associated with a positive nursery	