P. ramorum AMPLIFICATION PROCEDURE (3/26/04)

Martin, F.N, Tooley, P.W. and Blomquist, C. (2004) Molecular detection of *Phytophthora ramorum,* the causal agent of sudden oak death in California, and two additional species commonly recovered from diseased plant material. Phytopathology 94: 621-631

Amplification Conditions

- Tubes: 500 µl thin walled tubes
- Reaction vol: 25 µl
- Taq Polymerase: AmpliTaq (N8080172)
- dNTP mix was Promega
- Primers: from Invitrogen, working concentration of 12.5 µM, stock solutions stored at –80 C
- Glycerol: use a 50% stock solution – make with Roche Glycerol (cat. # 100 647) using a syringe to measure the glycerol. Store in the freezer in 0.5 ml aliquots.
- Water: Sigma (cat. # W4502)
- Thermalcycler: Eppendorf Mastercycler Gradient, 3 C/sec ramp used

Primers

- FMPl-2b – plant primer (dGCGTGGACCTGGAATGACTA)
- FMPl-3b – plant primer (dAGGTTGTATTAAAGTTTCGATCG)
- FMPhy-8b – *Phytophthora* genus-specific primer (dAAAAGAGAAGGTGTTTTTTATGGA)
- FMPhy-10b - *Phytophthora* genus-specific primer (dGCAAAAGCACTAAAAATTAAATATAA)
- FMPr-1a – *P. ramorum* specific primer (dGTATTTAAAATCATAGGTGTAATTTG)
- FMPr-7 – *P. ramorum* specific primer (dTGGTTTTTTTAATTTATATTATCAATG)

First Amplification Master Mix

2.5 µl	Buffer
3.0 µl	$MgCl_2$ (3 mM)
0.5 µl	dNTP mix
2.0 µl	FMPhy-8b (12.5 µM)
2.0 µl	FMPhy-10b (12.5 µM)
0.2 µl	FMPl-2b (12.5 µM)
0.2 µl	FMPl3b (12.5 µM)
1.0 µl	Glycerol (50%)

water is added to bring the final vol. to 19 µl for most amplifications

- Mix the master mix well (finger vortex a 1.5 ml tube, then quick spin in centrifuge; using a 0.5 ml tube or just pipetting back and forth does not always mix well enough and inconsistent results were obtained)
- Aliquot 19 µl into a 500 µl thin walled tube
- Add 1 µl of the purified DNA and place tube on ice.
    - For the DNA samples from California Department of Food and Agriculture (field samples extracted with the FastDNA kit of Qbiogene) 2 µl are used so the water in the master mix is adjusted to give a vol. of 18 µl/tube.
- Once all the tubes have the DNA added and are placed on ice, the *Taq* is diluted at a rate of 0.2 µl AmpliTaq + 4.8 µl H_2O per amplification.
- Add the *Taq* and mix well (pipette back and forth several times) and return the tube to ice.
- Start the amplification program on the thermalcycler and put on "pause"
    - Once the lid temperature and block temperature are reached, the tubes are placed in the wells, the lid closed and the program restarted.
- After amplification, 12 µl of the reaction mix is run on a 1.5% gel (a 3% NuSieve 3:1 gel is used if sharp bands are desired) to visualize the bands.

- If the water blank has a band or the positive control does not amplify, the entire amplification is discarded and the samples rerun.
- Occasionally this single amplification will provide a *Phytophthora* specific fragment that will be indicative of the presence of a *Phytophthora* spp., but generally the pathogen DNA concentration is pretty low in forest samples relative to the rest of the plant DNA in the sample. So even if a *Phytophthora* specific band is not seen, continue with the second round of amplification (while this is an issue with forest samples, it may not with samples form ornamental plants).
- A 100 bp ladder from New England BioLabs is used as a size marker.

Amplification program

1. 95 C for 3 minutes
2. 95C for 1 minute
3. 66 C for 1 minute
4. 72 for 1 minute
5. go to 2 and repeat 39 more times
6. 72 C for 5 minutes
7. 10 C, hold

Notes on amplification

- The plant primers are present at $1/10^{th}$ the concentration of the *Phytophthora* genus specific primers due to the greater amount of plant DNA that is present in the sample. If a greater amount of the plant primers are used they compete with the genus specific primers for amplification and will provide a fainter *Phytophthora* genus specific band (the plant primers amplify a smaller fragment and are therefore more efficient in amplification).
- In each amplification I include a known sample (*P. ramorum* DNA) as well as a blank tube with no DNA
- The presence of glycerol enhances the specificity of the amplification, without it a band may be amplified by some, but not all, plant species.
- Adding BSA to enhance the level of template amplification has been tried
  - BSA: from New England BioLabs, dilute a 100 mg/ml stock solution 1:20 and add at a rate of 1µl per tube (make in 100 µl aliquots and store in the freezer; after repeated freeze-thaw cycles or if it is more than a few weeks old discard and remake)
  - BSA is not essential for the amplification, it just tends to give more amplified product
  - I tend not to use BSA anymore as nonspecific background bands were sometimes encountered.

Second Round Amplification

- Samples from the first round amplification are diluted 1:100
  - There are several reasons for this:
    - It significantly dilutes the plant DNA so the second round of amplification with the genus specific primers (which is done in the absence of glycerol and at a lower annealing temp so the species specific fragments amplify) will not amplify plant sequences.
    - The plant primers currently in use generate a fragment that is close in size to the *P. ramorum* diagnostic fragment, so diluting the samples this much will eliminate this as a potential source of false positives.
      - Dilutions of 1:25. 1:50 work as well, but I like to use the 1:100 just to make sure. There does not appear to be any difference in the final level of the amplified diagnostic fragment between any of the dilutions.
      - New plant primers have been constructed that will not interfere with this assay. I have not finished testing them on all plant species yet so I have kept the prior primers (please see the website for details).

- To ensure that the *Phytophthora* genus-specific fragment is amplified to diagnose the presence of *Phytophthora* spp. other than *P. ramorum* a low level of the genus specific primers are included in the second amplification mixture.
    - As noted above, the pathogen DNA is often present in very low concentrations relative to the plant DNA, which means that often times it will not amplify a genus specific fragment with just a single round. Unfortunately adding more DNA to the reaction mix may inhibit amplification due to the presence of PCR inhibitors, so the approach I have chosen is do a second round of amplification with the genus-specific primers.
        - The primer concentration this amplification is lower than the first round so it does not out compete the species-specific primers for amplification.
        - This multiplex amplification will not give single bands for each primer pair when *P. ramorum* is present.
            - The species-specific primers are internal to the genus specific primers, so bands at about 290 bp are generated by one of the species specific primers + one of the genus-specific primers.
        - It is important that the 134 bp species-specific fragment is amplified to give a positive assessment. The reason for this is that while the species-specific primers are specific for *P. ramorum* (they should not anneal to any of the other species we have evaluated), it is possible that one of them may anneal to some other species and generate a band 293-296 bp in size along with one of the genus-specific primers (from review of the sequence data I would think that it would be unlikely that both primers will anneal, so the 134 bp fragment diagnostic of *P. ramorum* will not be amplified). To prevent false positives, if there is any question I have repeated the second round amplification with just the species-specific primers in the second round amplification. Conversely, if it is not important to know if there is another *Phytophthora* spp. present, the genus specific primers can be omitted from the second round amplification in the first place.

<u>Second Amplification Master Mix</u>

2.5 µl	Buffer
3.0 µl	$MgCl_2$ (3 mM)
0.5 µl	dNTP mix
0.2 µl	FMPhy-8b (12.5 µM)
0.2 µl	FMPhy-10b (12.5 µM)
2.0 µl	FMPr-1a (12.5 µM)
2.0 µl	FMPr-7 (12.5 µM)

- Bring vol to 19 µl/reaction with water, add 1µl of diluted DNA and place on ice.
- Add 5 µl of diluted AmpliTaq (0.2 µl AmpliTaq + 4.8 µl H_2O per amplification.)
- Start the amplification program on the thermalcycler and put on "pause"
    - Once the lid temperature and block temperature are reached, the tubes are placed in the wells, the lid closed and the program restarted.
- After amplification, 12 µl of the reaction mix is run on a 3% NuSieve 3:1 gel to visualize the bands.
    - If the water blank has a band or the known tube does not amplify, the entire amplification is discarded and the samples rerun.

<u>Amplification program</u>

1. 95 C for 3 minutes
2. 95C for 30 sec
3. 64 C for 30 sec
4. go to 2 and repeat 34 more times
5. 72 C for 5 minutes
6. 10 C, hold

GENERAL NOTES

- One of the first things that will need to be tested when this procedure is use done on a different PCR machine is to evaluate the effect of annealing temp on amplification. The annealing temperature is very important to for the specificity of the genus–specific primers and differences have been encountered when using different thermalcyclers.
    - At 65 annealing temp with glycerol the primers do not amplify plant DNA from the species that have been tested using the Eppendorf thermalcycler (most of the hosts from which *P. ramorum* has been isolated, lettuce, citrus, pine, watermelon, cucumber, tomato, lettuce, cherry, juniper, redwood, sugar beet, strawberry, ..). However, using an Applied Biosystems 9700 thermalcycler in Paul Tooley's lab in Ft. Detrick low background amplification was observed *Kalmia latifolia* 'Madeline' (approximately 0.3 kb), *U. californica* (approximately 0.8 kb), *Glycine max* (approximately 0.4 kb) and *Solanum tuberosum* (approximately 0.3 kb).
    - Removing the glycerol form the amplification mixture and using a 65 C annealing temp will allow background amplification of cherry, citrus, live oak, Juniper, and strawberry.
    - With a 66 C annealing temp without addition of glycerol no amplification of plant DNA is observed.
    - The annealing temperatures that we used with our thermalcycler (Eppendorf) for the first and second round amplification was 66 C and 64 C, respectively. However, when we used a MJ unit the temperatures had to be reduced by 1 C for the marker system to work. I believe this is due to differences in block calibration between machines (possibly differences in ramp rates as well) and highlights the importance of validating your machine before getting started with using the diagnostic assay. Several suggestions:

        - use purified *P. ramorum* DNA and healthy plant DNA from the hosts you will be evaluating to check the annealing temps for specificity. If the annealing temp is too low for the first round amplification some plant DNA (citrus) may give a band the same size as the *Phytophthora* genus-specific amplicon (with the plants we have looked at these bands will not be amplified by the *P. ramorum* species-specific primers in the second round nested amplification). Dilute these amplifications 1:100 and then test the annealing temp for the *P. ramorum* specific primers.

            - After the optimum annealing temperatures have been determined the plant and *P. ramorum* DNA are combined in the master mix and 12 tubes are placed in a uniform pattern across the thermalcycler block to test for block uniformity. Both the first and second amplification cycles are tested this way and the separate tubes are compared for intensity of amplification and lack of background bands. **To ensure our thermalcycler is functioning properly we repeat this test on a regular basis.**

        - If the annealing temp for the first round amplification with the *Phytophthora* genus-specific primer pair is to high there will be low level of genus-specific amplicon generated, which will reduce the sensitivity of detection using the species specific primer pair.

        - It might be useful to run a several concentrations of *P. ramorum* DNA in the first genus-specific amplification to evaluate the limit of detection with the *P. ramorum* species-specific primers using the annealing temperatures that have been chosen. In my lab we are able to get detection down to 2 fg total DNA.

- Genus specific primers (Phy-8b + 10b)
    - Under the conditions indicated above Phy-8b + 10b primer pair amplifies all of the *Phytophthora* species with the exception of low amplification of *P. lateralis* and *P. sojae*.

- Both these species are amplified by the earlier version of this primer pair, Phy 8 + 10.
    - While this primer pair will amplify all *Phytophthora* spp. and none of the plant material tested, it also amplifies *Pythium* spp. as well. For this reason it cannot be used for assaying any plant part that comes into contact with the soil.

- This primer pair did not amplify any of the *Pythium* spp. tested.

- *P. ramorum* species-specific primers
    - When total DNA is used for amplification very faint bands larger than the *P. ramorum* diagnostic amplicon can sometime be seen for *P. erythroseptica, P. palmivora, P. citrophthora*, and *P. drechsleri*.
    - When the first amplification is done with the genus-specific primers, there is no background amplification observed for any plant or other *Phytophthora* spp.
    - These primers did not amplify DNA from total DNA of the plant species tested.

- Evaluation of other thermalcycler
    - Many of these amplifications were tested on a MJ PT-100 machine (1.2 C/sec ramp time). They worked well, but at annealing temps 1 C below what was reported above. This highlights the importance of validating the thermalcycler for annealing temperature and block uniformity before using this marker system.

TROUBLE SHOOTING

- Bands in the 450-500 bp range when plant DNA only is in the amplification mixture – the stringency needs to be increased. Make sure the glycerol is included in the master mix, increase the annealing temperature by 1 C,
- Faint *P. ramorum* diagnostic band – the annealing temperature may be too high for the first round amplification with the genus-specific primer pair or for the second round amplification with the *P. ramorum*-specific primers. Run samples with *P. ramorum* and plant DNA individually in separate tubes to optimize the annealing temperatures to prevent background amplification of plant DNA by the genus-specific primers. With the two round nested amplification procedure as little as 2 fg total *P. ramorum* DNA should be detected.
- Inconsistent amplifications – if there is inconsistent amplification of positive controls or amplification of negative controls the components of the master mix are discarded and fresh tubes opened. The uniformity of temperature control of the block is also tested.
    - BLOCK UNIFORMITY – to evaluate the uniformity in temperature control in the thermalcycler block the initial validation of the thermalcycler with the individual tubes of *P. ramorum* and plant DNA is done with the tubes placed in the same wells each time a run is done. After the optimum annealing temperatures have been determined the plant and *P. ramorum* DNA are combined in the master mix and 12 tubes are placed in a uniform pattern across the thermalcycler block. Both the first and second amplification cycles are tested this way and the separate tubes are compared for intensity of amplification and lack of background bands. **To ensure our thermalcycler is functioning properly we repeat this test on a regular basis.**