A Practical Guide to the Molecular ID of AMF in Roots: Pitfalls and Promises

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What are the Arbuscular Mycorrhizal Fungi (AMF)?

“… 200 described morphospecies might be a strong underestimation of the true diversity of the Glomeromycota”
Redecker, *Mycologia* 2006
Why Do Molecular ID?

Historical AMF Ecology
- Spore Counts and Root Colonization

BUT
- Known problems with morphological distinction
- Ecological disconnect between root associates and spores

Spore counts “measure the sporulation activity of the fungi rather than a direct measure of diversity”

Clapp et al. 2002
This is Why

Molecular ID of AMF

- Identify AMF in root sample without need for spores
- More direct measure of diversity
- Better correlate with function/habitat/host specificity/environmental changes

NOT an old guy!

Kyle Garrone www.farwestfungi.com

www.udel.edu

Schechter and Bruns, 2008
Overview of Techniques

AMF are better than ECM!!

AMF are better than ECM!!

Schechter and Bruns, 2008

users.ugent.be/~avierstr/principles/pcr.html

www.udel.edu

commons.wikimedia.org
Sampling

- How many plants per site?
- How much root tissue to sample per plant?

- Depends on your goal
  - Survey of unknown?
  - Genetic diversity of focus taxa?
What You Need To Start

- **COLONIZED ROOTS!!**
  - 1 - 10% = Good Luck
  - 15 - 30% = Definitely with some work
  - 50% + = No Problem

- **Know your root tissue**
  - Pigmented and lignified roots = PCR inhibitors
    - PVP, DMSO, BSA (Ma and Michailides 2007)
DNA Extraction

- Detailed Protocol in Handout
- Highlights
  - Break up root tissue
  - Buffer with facilitator
  - Chloroform: iso-amyl alcohol extraction
  - Qiagen column purification
PCR of AMF from Roots

- Small target in a sea of plant DNA
- Lots of “other” fungi too

So, amplification method and molecular marker choice are important

Hunts Needle in a Haystack

How long does it take to find a needle in a haystack? Jim Moran, Washington, D.C., publicity man, recently dropped a needle into a convenient pile of hay, hopped in after it, and began an intensive search for (a) some publicity and (b) the needle. Having found the former, Moran abandoned the needle hunt.
Which Molecular Marker to Choose?

<table>
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<tr>
<th>Marker</th>
<th>Pro</th>
<th>Con</th>
<th>Amp method</th>
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| SSU (18S) | -Large database  
-Can align across all taxa | -Misses some  
-Picks up other fungi | Direct |
| LSU (28S) | -Some specific group primer sets  
-Can align across all taxa | -Very small database | Nested |
| ITS/5.8S | -Specific group primer sets  
-Can distinguish between closely related taxa | -Small database  
-Only aligns 5.8S  
-Lots of variation | Nested |
PCR Protocol

Detailed protocol in handout - SSU AM1/NS31

TIPS

- If low yields:
  - Use good polymerase
  - Try different DNA dilutions
  - Optimize PCR
    - Annealing temps - gradient PCR
    - Buffer - know what’s in yours

CUTTING COSTS

- smaller rxns, make own polymerase, optimize dNTP conc., make own PCR buffer

www.med.yale.edu/genetics/ward/tavi/p06.html
Cloning Protocol

Detailed protocol in handout - PCR-Script Amp (Stratagene)

TIPS - to increase # of clones and proportion of +’s

- Eliminate small stuff & concentrate PCR products
  - Gel Purify
  - MinElute or SpeedVac

- If it’s still not concentrated enough
  - In ligation rxn:
    - Add max amount of PCR product (eliminate water)
    - Lower the concentration of vector

CUTTING COSTS: 1/2 reactions, make own cells/vector, use small (1 cm) root segments
Got Clones, Now What?

How many to pick?

- Depends on your question
  - How much diversity do you expect?
  - Do you need to saturate diversity?
  - Only need +/- of specific taxa?
  - Only dominate taxa matter?

- Do a test
  - Pick 50+ from representative sample
  - Sequence all
  - Do virtual “cutter” test for RFLP clone screen
Screening Clones

- Restriction fragment length polymorphism (RFLP)

RFLP type 1 = Clone 1 & 2
RFLP type 2 = Clone 3
RFLP type 3 = Clone 4
Screening Clones

- Restriction fragment length polymorphism (RFLP)

So, check before you choose restriction enzymes to screen clones
Sequencing

Detailed protocol in handout - Big Dye v. 3.1 (Applied Biosystems)

TIPS -
  - Check Clean PCR Concentration - make sure it’s within range

CUTTING COSTS -
  - 1/8 reactions

www.laughparty.com
Got Sequences, Now What?

“There is no simple ‘one sequence, one species’ correlation in the Glomeromycota” - Redecker 2006

- Determine “operational taxonomic units”
  - OTUs, Phylotypes, Ribotypes, etc.
  - Compare seq. to each other and “known” taxa

- Must do phylogenetic analysis!
  - NJ, ML, MrBayes
  - BLAST can LIE!
    - Lots of non-AMF sequences with AMF names
  - Check for Chimeras
    - Bellerophon, Ribosomal Database Project (RDP)
Example

1200 Sequences
Combined at 98% sequence similarity = putative OTUs
Aligned representative sequences and built a tree with “known” taxa
98% similar groupings = Well-supported clades
Named OTUs based on Genus
Summary

- Choose appropriate sampling scheme
- Have good starting material
  - At least know the potential for problems
- Choose appropriate molecular marker
- Follow tips to improve success
- Decide on clone screening method
  - Yes/no, which restriction enzyme
- Determine “OTU”
  - DO PHYLOGENETIC ANALYSIS!!