Phylogeographic analyses of genetic variation within *Armillaria ostoyae* from the western United States

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A shift in tree species composition has occurred in many forests of the western USA

Hanna et al. 2004
Changing forest composition

Forest composition has shifted away from seral species (e.g., pine, larch) toward climax species (e.g., true fir, Douglas-fir)

As a result, Armillaria disease has become more prevalent

Hanna et al. 2004
## Armillaria species and relative pathogenicity

<table>
<thead>
<tr>
<th>NABS&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Species</th>
<th>Pathogenicity</th>
<th>Primary Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td><em>A. ostoyae</em></td>
<td>High</td>
<td>Conifers</td>
</tr>
<tr>
<td>VI</td>
<td><em>A. mellea</em></td>
<td>High</td>
<td>Hardwoods</td>
</tr>
<tr>
<td></td>
<td><em>A. tabescens</em></td>
<td>High?</td>
<td>Hardwoods</td>
</tr>
<tr>
<td>II</td>
<td><em>A. gemina</em></td>
<td>Moderate?</td>
<td>Hardwoods</td>
</tr>
<tr>
<td>IX</td>
<td><em>A. nabsnona</em></td>
<td>Moderate?</td>
<td>Hardwoods</td>
</tr>
<tr>
<td>III</td>
<td><em>A. calvescens</em></td>
<td>Low?</td>
<td>Mixed</td>
</tr>
<tr>
<td>V</td>
<td><em>A. sinapina</em></td>
<td>Low?</td>
<td>Mixed</td>
</tr>
<tr>
<td>VII</td>
<td><em>A. gallica</em></td>
<td>Low?</td>
<td>Mixed</td>
</tr>
<tr>
<td>XI</td>
<td><em>A. cepistipes</em></td>
<td>Low?</td>
<td>Mixed</td>
</tr>
<tr>
<td>X</td>
<td>Unnamed</td>
<td>Rarely pathogenic</td>
<td>Mixed</td>
</tr>
</tbody>
</table>

<sup>a</sup>NABS = North American Biological Species

Korhonen, 1978, Anderson and Ullich, 1979, Berube and Dessureault, 1988, Berube and Dessureault 1989, Volk et al., 1996

Hanna et al. 2004
Armillaria ostoyae: Primary cause of Armillaria disease in western conifers

Hanna et al. 2004

USDA Forest Service, Missoula Archives
Impact of Armillaria root disease on tree growth

Volume loss has been shown as high as 40% over 4-8 years in eighteen-year-old Douglas-fir.

Cruickshank et al., 2000
Previous studies on *A. ostoyae*

- Distinct differences in *A. ostoyae* epidemiology have been noted among coastal and interior regions of western North America (McDonald 1990, Goheen and Otrosina 1998, Morrison and Pellow 2002).

- Genets of *A. ostoyae* can show varying levels of pathogenicity and virulence (Omdal et al. 1995, Morrison and Pellow 2002).


- Little is known about intraspecific genetic variation within *A. ostoyae* and its relationship to phylogeny and ecological behavior.
Objectives

Assess genetic diversity among genets of *A. ostoyae* to examine intra- and interspecific phylogeographic relationships. Investigation of genetic diversity may be important to understand:

1) varying levels of pathogenicity and virulence within *A. ostoyae*

2) phylogeographic relationships among *A. ostoyae* genets and genets of other *Armillaria* species, and

3) adaptation to diverse environmental factors

Hanna et al. 2004
Materials and Methods

Hanna et al. 2004
## Summary of *Armillaria* species and genets used in phylogeographic analysis

### *Armillaria ostoyae*

<table>
<thead>
<tr>
<th>Origin</th>
<th># isolates</th>
<th>Collector(s) or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chihuahua, Mexico</td>
<td>1</td>
<td>C.G. Shaw III</td>
</tr>
<tr>
<td>Eastern, Finland</td>
<td>1</td>
<td>Unknown</td>
</tr>
<tr>
<td>Idaho, USA</td>
<td>23</td>
<td>IFTNC&lt;sup&gt;c&lt;/sup&gt;, G.I. McDonald</td>
</tr>
<tr>
<td>Montana, USA</td>
<td>8</td>
<td>B.A. Ferguson et al. 2003, G.I. McDonald</td>
</tr>
<tr>
<td>New Hampshire, USA</td>
<td>1</td>
<td>T.C. Harrington</td>
</tr>
<tr>
<td>New Mexico, USA</td>
<td>14</td>
<td>G.I. McDonald, Omdal et al. 1995</td>
</tr>
<tr>
<td>Oregon, USA</td>
<td>6</td>
<td>Ferguson et al. 2003, G.I. McDonald</td>
</tr>
<tr>
<td>Primorye, Russia</td>
<td>1</td>
<td>G.M. Filip</td>
</tr>
<tr>
<td>Utah, USA</td>
<td>3</td>
<td>G.I. McDonald</td>
</tr>
<tr>
<td>Washington, USA</td>
<td>19</td>
<td>J.F. Ammirati, IFTNC&lt;sup&gt;c&lt;/sup&gt;, M-S. Kim, G.I. McDonald</td>
</tr>
</tbody>
</table>

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*a* Each isolate represents a distinct genet  
*b* NABS = North American Biological Species  
*c* Intermountain Forest Tree Nutrition Cooperative, Department of Forest Resources, University of Idaho, Moscow, ID 83844  
Hanna et al. 2004
Armillaria inspections and collections

From G.I. McDonald

Hanna et al. 2004

From G.I. McDonald
**Armillaria** spp. collections

- *Armillaria* spp. isolates were reduced to unique genets by somatic pairing

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Armillaria species were identified

Somatic pairing

Mating tests

IGS RFLP

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Materials and Methods (Continued)

Using a direct-PCR method, sequences were obtained for nuclear ribosomal DNA Large subunit (LSU),

Internal transcribed spacer and 5.8S (ITS), and

Intergenic spacer one (IGS)

from 77 genets of Armillaria ostoyae from western North America (and 3 individuals each from 10 North American Armillaria species).

Hanna et al. 2004
<table>
<thead>
<tr>
<th>Region</th>
<th>Primer</th>
<th>Use</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSU:</td>
<td>5.8SR</td>
<td>Amplification</td>
<td>Vilgalys 2004</td>
</tr>
<tr>
<td></td>
<td>LR7</td>
<td>Amplification</td>
<td>Vilgalys 2004</td>
</tr>
<tr>
<td></td>
<td>LR0R</td>
<td>Sequencing</td>
<td>Vilgalys 2004</td>
</tr>
<tr>
<td></td>
<td>LR5</td>
<td>Sequencing</td>
<td>Vilgalys 2004</td>
</tr>
<tr>
<td></td>
<td>LR15</td>
<td>Sequencing</td>
<td>Vilgalys 2004</td>
</tr>
<tr>
<td>ITS:</td>
<td>ITS-1F</td>
<td>Amp &amp; Seq</td>
<td>Gardes and Bruns 1993</td>
</tr>
<tr>
<td></td>
<td>ITS4</td>
<td>Amp &amp; Seq</td>
<td>White et al. 1990</td>
</tr>
<tr>
<td></td>
<td>O-1</td>
<td>Amp &amp; Seq</td>
<td>Duchesne and Anderson 1990</td>
</tr>
<tr>
<td></td>
<td>A5SR1</td>
<td>Amp &amp; Seq</td>
<td>Hanna et al. 2004</td>
</tr>
</tbody>
</table>
A direct PCR (polymerase chain reaction) method was used to amplify segments of nuclear ribosomal DNA.

PCR product was then sequenced and nuclear ribosomal DNA regions investigated for phylogenetic signal. These segments of DNA repeat several hundred times within the genome of an individual.

Hanna et al. 2004
Phylogenetic analysis

• Careful attention was given to both sequence editing and alignment.

• Only sequences having an non-ambiguous alignment were compared.

For example: *A. mellea* and *A. tabescens* were only compared to other North American *Armillaria* species in the LSU region. ITS and IGS regions contain numerous indels making alignment impractical.

Hanna et al. 2004
Phylogenetic analysis (continued)

- Repetitive sequences were eliminated from the sequence alignments so that only unique genotypes would be compared.

- Neighbor-joining, Parsimony, and Bayesian methods of phylogenetic inference were used for phylogenetic inference.

Hanna et al. 2004
Direct PCR reveals heterogeneous sequences

Direct PCR has been previously shown to detect 90% of the heterogeneous rDNA products in an individual and the relative peak height seems to reflect relative copy number (Rauscher et al. 2002).

Hanna et al. 2004
Direct PCR and heterogeneous sequences

• Sequence chromatograms from direct PCR showed that heterogeneity was common within Armillaria individuals for all rDNA regions analyzed.

• Heterogeneity within rDNA regions show evidence of intra-individual variation (intra- or interspecific hybridization).

Hanna et al. 2004
Direct PCR and heterogeneous rDNA

- Phylogenetic signal is reduced when heterogeneous rDNA is characterized by ambiguous characters at polymorphic nucleotide sites.

For example, sequences with 4 dimorphic sites

\[ \ldots \text{ATT} \text{RGCCAYTTGC} \text{GKCCGTAMGGC} \ldots \]

may represent 16 possible genotypes.

- When possible, chromatograms showing heterogeneous PCR product were split into homogenous sequence representations before phylogenetic analysis with three different methods.

- Only individuals with well-defined rDNA sequences were used for phylogenetic analyses.

Hanna et al. 2004
Three methods for editing chromatograms

1a. Editing of a “frame-shift”

1b. Application of specific primers on heterogeneous PCR product

1c. Splitting a single SNP (single nucleotide polymorphism)

(R = A or G)

Hanna et al. 2004
Editing of a “frame-shift”
Downstream (toward 5S) IGS-1 rDNA section of isolate PC514

Edited (predicted) sequences and representative chromatograms

Primer site CTTTGAACGGGCAAAC

Primer site GTTTGAACGGGCAAAC
Application of specific primers on heterogeneous PCR product

Primers designed from the predicted sequences from previous slide are applied to the product

**CTTTGAACGGCAAC** and **GTTTGAACGGGCAAAAC** (Note: actual primers are reverse compliment)

Upstream (toward LSU) IGS-1 rDNA section of isolate PC514

Resulting sequences and chromatograms from reverse direction sequencing “unzipping”
Splitting a single SNP (single nucleotide polymorphism)
nLSU rDNA section of isolate SSF4

Hanna et al. 2004

Resulting sequence edits and representative chromatograms
Results

• Neighbor-joining, Parsimony, and Bayesian methods showed congruent results
Radial 90% majority rule consensus tree of *Armillaria* spp. based on 24,001 trees from Bayesian inference analysis of the nuclear large ribosomal subunit (nLSU). Numbers between clades indicate estimated posterior probability.

* Ambiguous sequences from 14 heterogeneous isolates not included

Hanna et al. 2004
Phylogeographic distribution of *Armillaria ostoyae* genets based on major clades from a 90% majority rule consensus tree produced from sequences of the nuclear large ribosomal subunit (nLSU) using Bayesian inference analysis.

Hanna et al. 2004
Circumboreal Group Distribution

Utah

New Hampshire

Finland

Russian Far East

Hanna et al. 2004
Observed trends in LSU sequence analyses raise the possibility that *A. ostoyae* distribution is related to paleogeographic events.

Coetzee et al. (2003) postulate that relationships among Southern Hemisphere Armillaria species is related to continental drift and the breakup of Gonwanaland.

Dr. Ron Blakey – Northern Arizona University
Radial 90% majority rule consensus tree of Armillaria spp. based on 24,001 trees from Bayesian inference analysis of the internal transcribed spacer and 5.8S rDNA (ITS). Numbers between clades indicate estimated posterior probability.

Hanna et al. 2004
Phylogeographic distribution of *Armillaria ostoyae* genets based on major clades from a 90% majority rule consensus tree produced from sequences of the internal transcribed spacer and 5.8S rDNA (ITS) using Bayesian inference analysis.

Hanna et al. 2004
Radial 90% majority rule consensus tree of *Armillaria* spp. based on 24,001 trees from Bayesian inference analysis of the intergenic spacer one (IGS). Numbers between clades indicate estimated posterior probability.

* Ambiguous sequences from 10 heterogeneous isolates not included
** *A. ostoyae* x *A. gemina* interspecific hybrid (IGSGE5 x IGSGE4)

Hanna et al. 2004
Phylogeographic distribution of Armillaria ostoyae genets based on major clades from a 90% majority rule consensus tree produced from sequences of the intergenic spacer one (IGS) using Bayesian inference analysis.

Hanna et al. 2004
Results (continued)

rDNA heterogeneity (intra-individual variation) was common within the 77 A. ostoyae individuals

LSU – 37 (48%)
ITS – 45 (58%)
IGS – 46 (60%)

Only 16% of individuals were homogenous in all three rDNA regions

Hanna et al. 2004
Armillaria ostoyae intraspecific hybridization - IGS-1 rDNA

NABS I – Northwest Group

Hanna et al. 2004

NABS I - Northwest x Rockies Hybrid

NABS I – Rockies Group
• The Rockies group appears to have evolutionary ties to *Armillaria ostoyae* in Japan.

• From this we can hypothesize movement of this group to or from Asia.

• 70-100 million years ago a land bridge may have been present to facilitate this movement.

• At the time Alaska and northern Asia had a temperate climate.

Hanna et al. 2004
Factors associated with genetic races and intraspecific hybridization within *A. ostoyae*

- Adaptation to a variety of environments
- May be responsible for differing levels of pathogenicity and virulence
- “Hybrid vigor” may contribute to enhanced growth, survival, and/or pathogenicity
- Hybrids may adapt better to environmental change

Hanna et al. 2004
Conclusions

- *Armillaria ostoyae* is genetically diverse.

- Phylogenetic results from the LSU region show three distinct groups (circumboreal, Rockies, and Northwest) of *A. ostoyae* present in western North America.

- Genetically diverse groups of *Armillaria ostoyae* show various evolutionary histories and spatial distributions.

- There is phylogeographic congruence of Rockies and Northwest groups among three different rDNA regions.

Hanna et al. 2004
Acknowledgements

Intermountain Forest Tree
Nutrition Co-op

University of Idaho

BOISE Corp.

USDA Forest Service

Hanna et al. 2004
During the last century many forests within the western United States have seen a shift in tree species composition due to:

• Fire Suppression
• White Pine Blister Rust
• Selective Harvesting and Planting Practices

Hanna et al. 2004
Western Montana 1909

Gruell et al., 1982
White Pine Blister Rust

• By 1940 *Cronartium ribicola* the fungus responsible for white pine blister rust had reached epidemic proportions on the remaining white pine forests.

• Today less than 10% of the original 5 million acres of white pine cover type exists.

Hanna et al. 2004

Fins et al., 2001
Selective Harvesting and Planting Practices

Very few stands of white pine remain. Those that do contain genes resistant to blister rust. With each generation natural selection produces trees that are more resistant. Unfortunately as much as 90% of the potential genetic resistance within white pine was lost due to selective logging of the best trees and later in blister rust “salvage” operations.

Fins et al., 2001
Symptoms of *Armillaria* Root Disease

Resinosis

Crown thinning and/or stress cones

Hanna et al. 2004
**Armillaria ostoyae (NABS I)**

- Serious plant pathogen found throughout the Northern Hemisphere that causes root and butt rot on diverse woody plant hosts.

- Adversely impacts commercial timber production by causing tree mortality and growth reduction.
Direct PCR and heterogeneous product

• When possible, chromatograms showing heterogeneous PCR product were split into homogenous sequence representations before phylogenetic analysis with three different methods

Hanna et al. 2004
Characteristics of *Armillaria*

- *Armillaria* root disease was first described by Robert Hartig in 1873
- Fungus / Basidiomycete
- Constructs rhizomorphs from vegetative hyphae
- Fruiting bodies are mushrooms
- Some *Armillaria* mycelium is luminescent

*Hanna et al. 2004*
Example of *Armillaria ostoyae* Mortality Centers

Hanna et al. 2004
As a result...

- A high percentage of forest stands within the Inland Northwest are no longer dominated by fire dependent seral tree species such as western larch, western white pine, and Ponderosa pine.

- Shade tolerant species such as grand fir and Douglas-fir now make up the majority of our forest stands. These stands are often young and dense.

- Remaining old growth stands are in danger due to the abundance of “ladder fuels”. This creates an increased risk of stand replacing crown fires.

Langston, 1995
• Douglas-fir and grand fir are highly susceptible to *Armillaria ostoyae*.

• Plantations of Ponderosa pine not locally adapted to a site can also be highly susceptible.

• Locally adapted seral species such as western larch, western white pine, and Ponderosa pine show the greatest resistance to *Armillaria* root rot.

Shaw and Kile, 1991
Armillaria Species and Genet Identification

Black line = different species
Colorless antagonisms = same species

Photo: Mee-Sook Kim
Microscopic observation of *Armillaria* mating for species identification

Clamp connections

Hanna et al. 2004

From G.I. McDonald
Inferring phylogenies from genes containing a small number of informative sites becomes highly ambiguous if hybrid individuals are polymorphic at those sites.

<table>
<thead>
<tr>
<th>Polymorphic site 1</th>
<th>R = G or A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymorphic site 2</td>
<td>Y = C or T</td>
</tr>
<tr>
<td>Polymorphic site 3</td>
<td>K = G or T</td>
</tr>
<tr>
<td>Polymorphic site 4</td>
<td>M = A or C</td>
</tr>
</tbody>
</table>

2 polymorphic sites = 4 possible genotypes
3 polymorphic sites = 8 possible genotypes
4 polymorphic sites = 16 possible genotypes

Hanna et al. 2004
A triplicate of reverse primers were created and applied to product shown to contain a SNP at base pair position 683 of the IGS-1 region.

<table>
<thead>
<tr>
<th>Region</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGS:</td>
<td>AOHR1T (5’-TGCCGTTCGGGAA3’)</td>
</tr>
<tr>
<td></td>
<td>AOHR1G (5’-TGCCGTTCGAAAAC3’)</td>
</tr>
<tr>
<td></td>
<td>AOHR1C (5’-TGCCGTTCGGGAA3’)</td>
</tr>
</tbody>
</table>
Phylogenetic approaches

• Techniques for reducing heterogeneous individuals into homogenous parental representation were not always possible.

• Remaining individuals containing two or more nucleotide sites showing intra-individual variation were excluded from phylogenetic analysis to eliminate ambiguity of phylogenetic signal.

Hanna et al. 2004
A. ostoyae individuals were sequenced in each of three regions:

Chromatograms showing heterogeneity (intra-individual variation):

<table>
<thead>
<tr>
<th>Region</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSU</td>
<td>37</td>
<td>48%</td>
</tr>
<tr>
<td>ITS</td>
<td>45</td>
<td>58%</td>
</tr>
<tr>
<td>IGS</td>
<td>46</td>
<td>60%</td>
</tr>
</tbody>
</table>

Reduced to two homogenous sequences with editing techniques:

<table>
<thead>
<tr>
<th>Region</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSU</td>
<td>23</td>
<td>30%</td>
</tr>
<tr>
<td>ITS</td>
<td>14</td>
<td>18%</td>
</tr>
<tr>
<td>IGS</td>
<td>-36</td>
<td>47%</td>
</tr>
</tbody>
</table>

Remaining heterogeneous individuals excluded from analysis:

<table>
<thead>
<tr>
<th>Region</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSU</td>
<td>14</td>
<td>18%</td>
</tr>
<tr>
<td>ITS</td>
<td>31</td>
<td>40%</td>
</tr>
<tr>
<td>IGS</td>
<td>10</td>
<td>13%</td>
</tr>
</tbody>
</table>

Only 16% of individuals were homogenous in all three regions.
Armillaria Mortality Centers

USDA Forest Service, Missoula Archives