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

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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

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Armillaria species and relative pathogenicity

NABS[a]	Species	Pathogenicity	Primary Host
I →	*A. ostoyae*	High	Conifers
VI	*A. mellea*	High	Hardwoods
	A. tabescens	High?	Hardwoods
II	*A. gemina*	Moderate?	Hardwoods
IX	*A. nabsnona*	Moderate?	Hardwoods
III	*A. calvescens*	Low?	Mixed
V	*A. sinapina*	Low?	Mixed
VII	*A. gallica*	Low?	Mixed
XI	*A. cepistipes*	Low?	Mixed
X	Unnamed	Rarely pathogenic	Mixed

[a]NABS = North American Biological Species

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

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Armillaria ostoyae: Primary cause of Armillaria disease in western conifers

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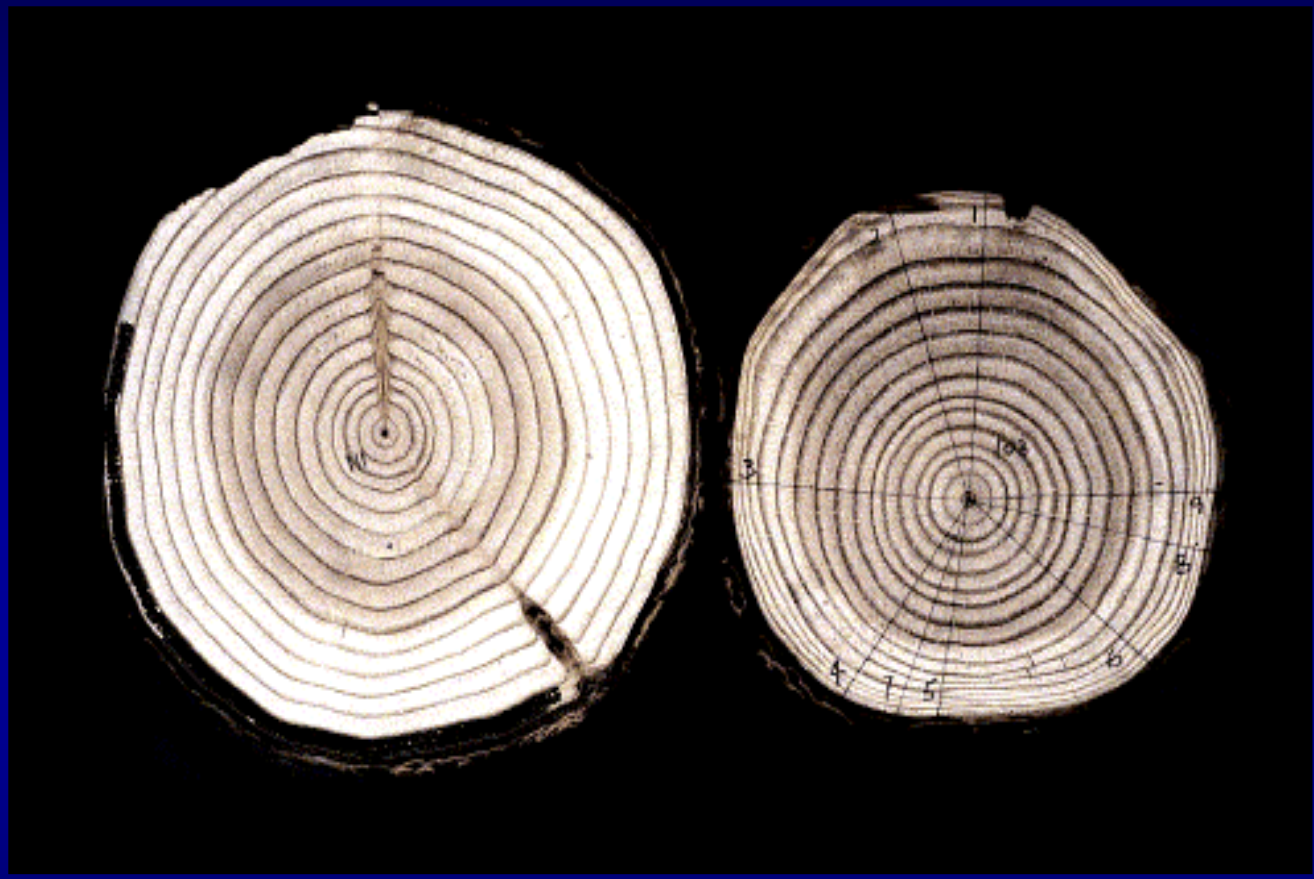
USDA Forest Service, Missoula Archives

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).

- Genetic variability within *A. ostoyae* has been demonstrated among genets (Anderson and Stasovski 1992, Piercey-Normore et al. 1997, Schulze et al. 1997, Chillali et al. 1998, Terashima et al. 1998, White et al. 1998, Sicoli et al. 2003).

- 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

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Materials and Methods

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Summary of *Armillaria* species and genets used in phylogeographic analysis

Armillaria ostoyae

Origin	# isolates	Collector(s) or Reference
Chihuahua, Mexico	1	C.G. Shaw III
Eastern, Finland	1	Unknown
Idaho, USA	23	IFTNC[c], G.I. McDonald
Montana, USA	8	B.A. Ferguson et al. 2003
		G.I. McDonald
New Hampshire, USA	1	T.C. Harrington
New Mexico, USA	14	G.I. McDonald, Omdal et al. 1995
Oregon, USA	6	Ferguson et al. 2003,
		G.I. McDonald
Primorye, Russia	1	G.M. Filip
Utah, USA	3	G.I. McDonald
Washington, USA	19	J.F. Ammirati, IFTNC[c],
		M-S. Kim, G.I. McDonald

[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

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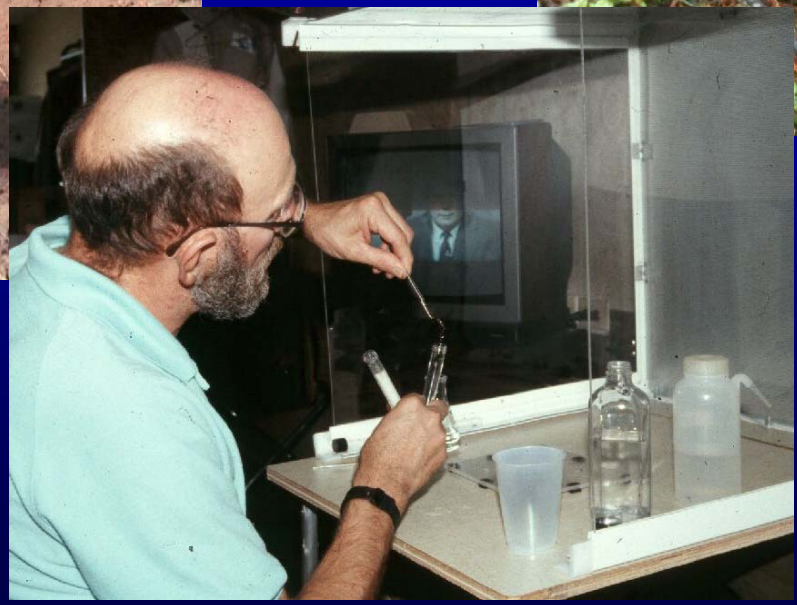
Armillaria inspections and collections



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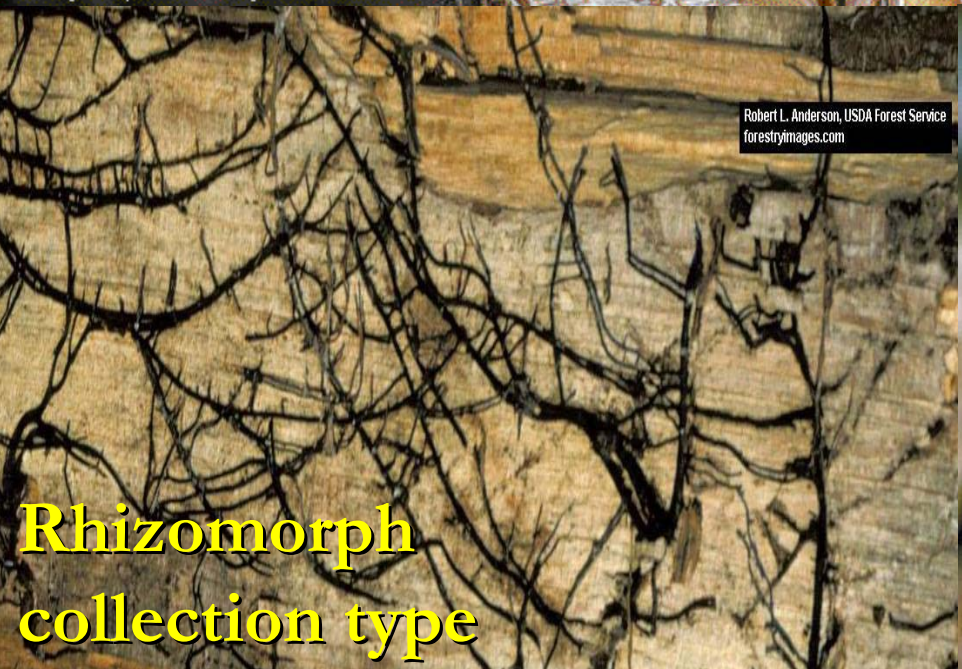
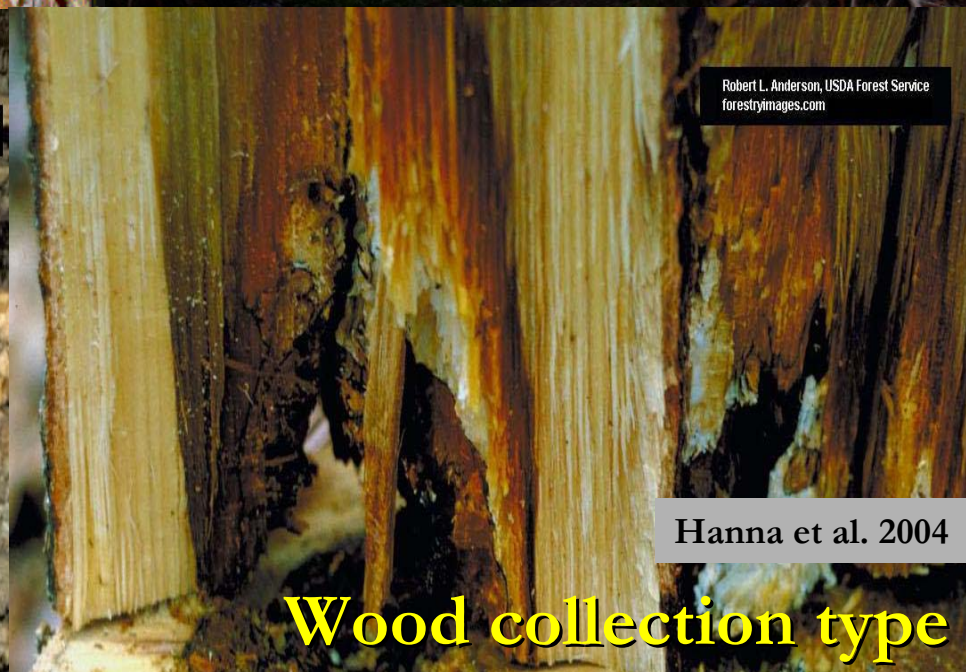
From G.I. McDonald


Bark fan collection type
UC Statewide IPM Project
© Regents, University of California


Fruiting bodies


Robert L. Anderson, USDA Forest Service
forestryimages.com
Rhizomorph
collection type


Robert L. Anderson, USDA Forest Service
forestryimages.com
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Wood collection type

Armillaria spp. collections

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

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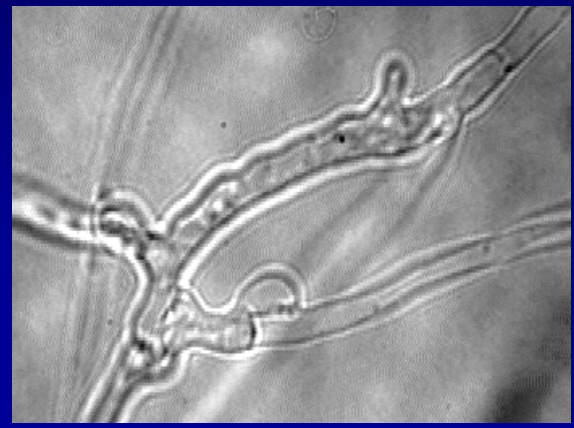
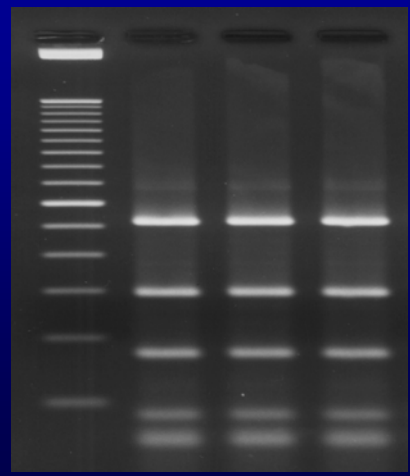
Armillaria species were identified

Somatic pairing

Mating tests



IGS RFLP



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Materials and Methods (Continued)

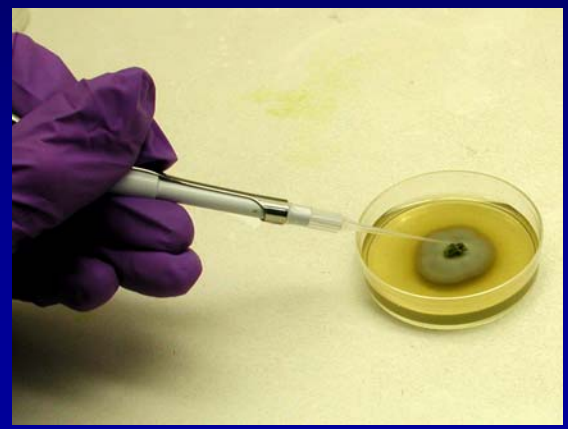
Using a direct-PCR method, sequences were obtained for nuclear ribosomal DNA

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).



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Primer sets used for amplification/sequencing

Region	Primer	Use	Reference
LSU:	5.8SR	Amplification	Vilgalys 2004
	LR7	Amplification	Vilgalys 2004
	LR0R	Sequencing	Vilgalys 2004
	LR5	Sequencing	Vilgalys 2004
	LR15	Sequencing	Vilgalys 2004
ITS:	ITS-1F	Amp & Seq	Gardes and Bruns 1993
	ITS4	Amp & Seq	White et al. 1990
IGS:	LR12R	Amp & Seq	Veldman et al. 1981
	O-1	Amp & Seq	Duchesne and Anderson 1990
	A5SR1	Amp & Seq	Hanna et al. 2004

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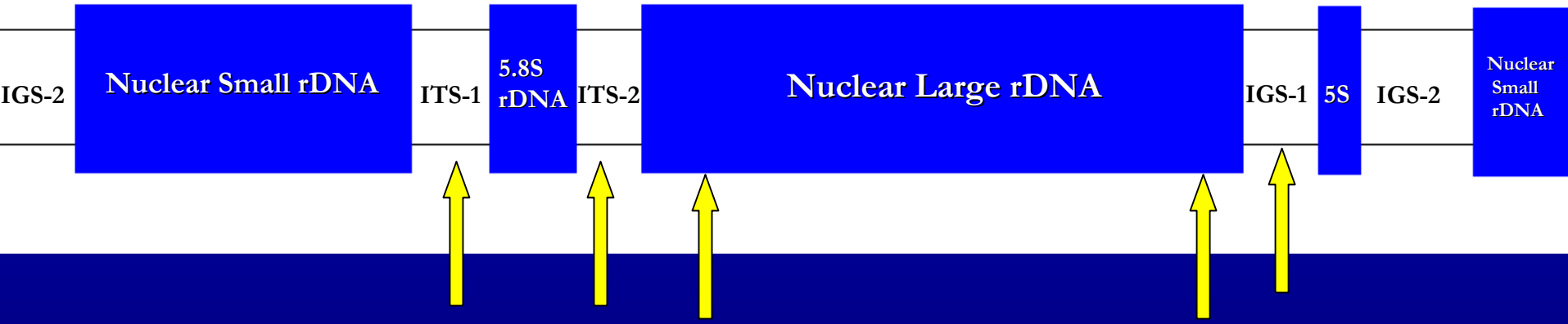
A direct PCR (polymerase chain reaction) method was used to amplify segments of nuclear ribosomal DNA.



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.

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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.

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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.

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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).

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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).

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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
…ATT**R**GCCA**Y**TTGCG**K**CCGTA**M**GGCA…
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.

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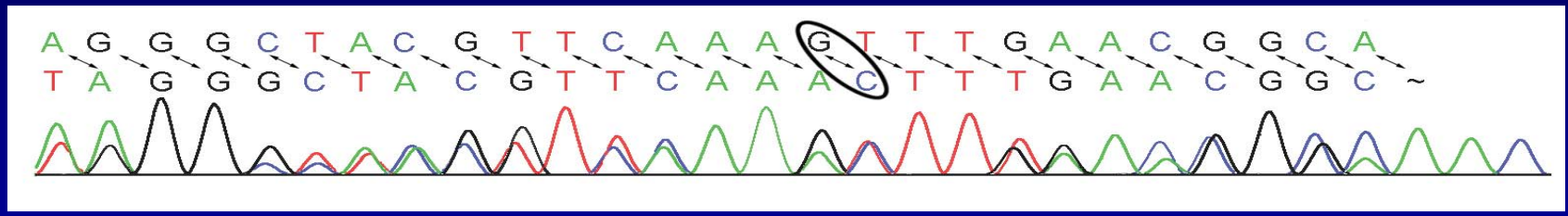
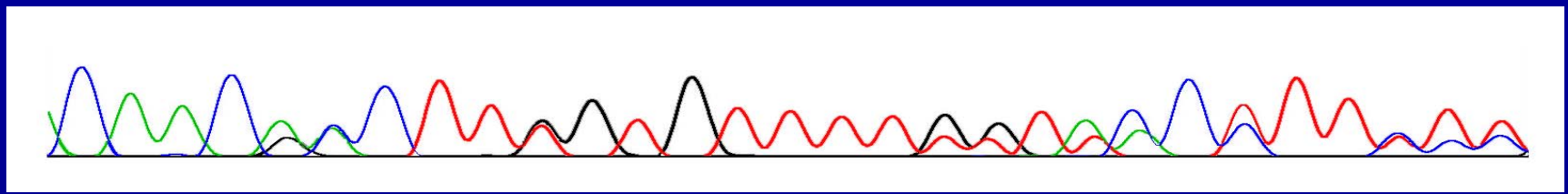
Three methods for editing chromatograms

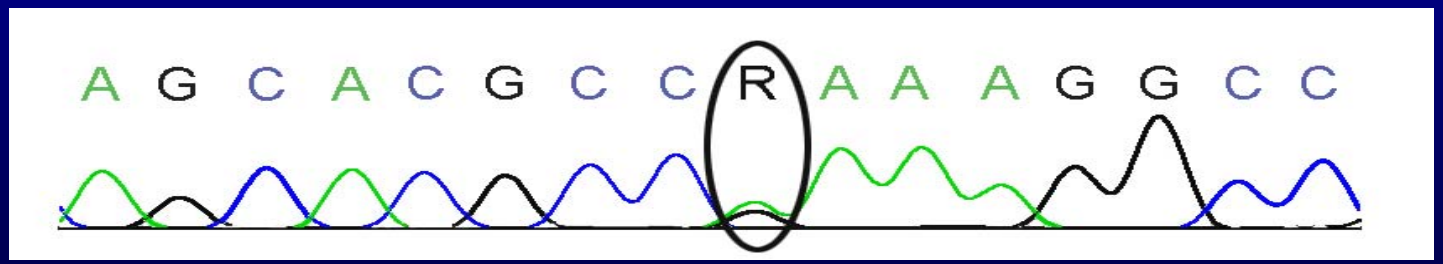
1a. Editing of a "frame-shift"



1b. Application of specific primers on heterogeneous PCR product



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



(R = A or G)

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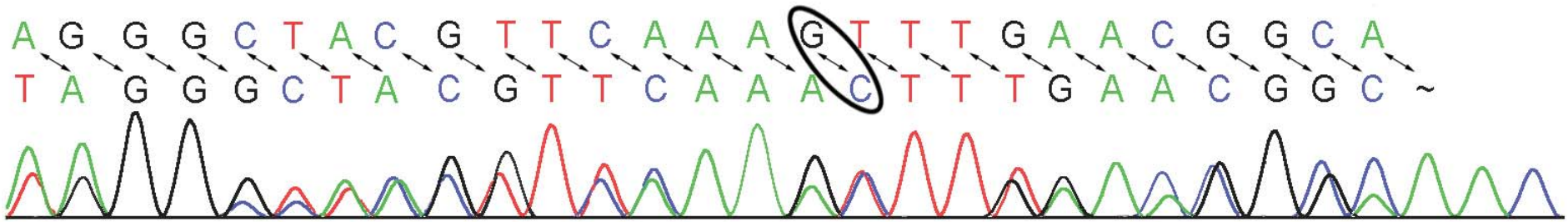
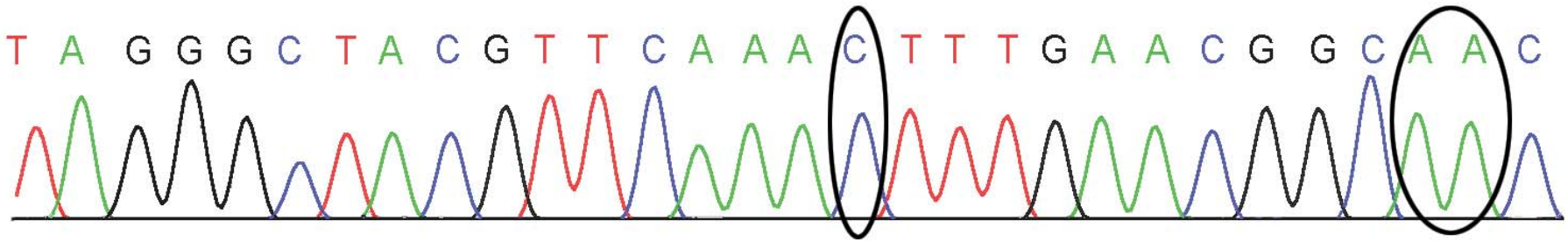
Editing of a "frame-shift"

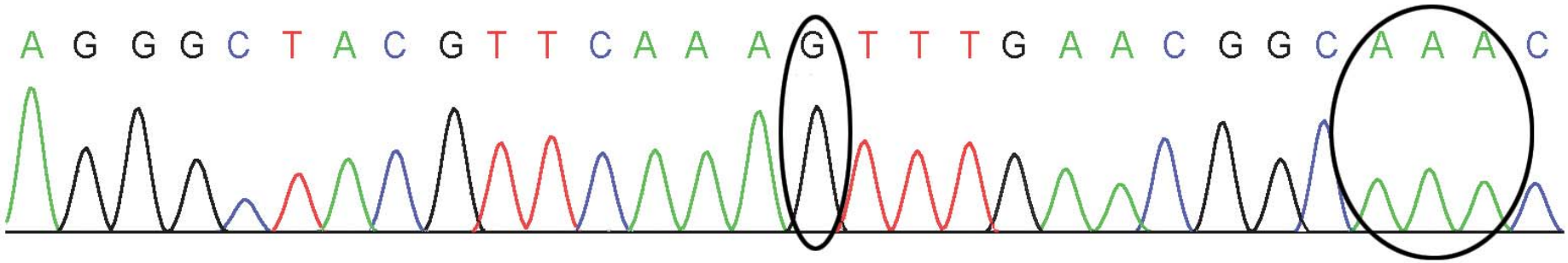
Downstream (toward 5S) IGS-1 rDNA section of isolate PC514



Edited (predicted) sequences and representative chromatograms



Primer site CTTTGAACGGCAAC



Primer site GTTTGAACGGCAAAC

Application of specific primers on heterogeneous PCR product

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

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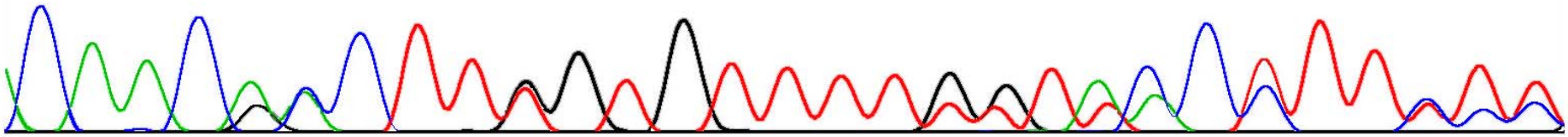
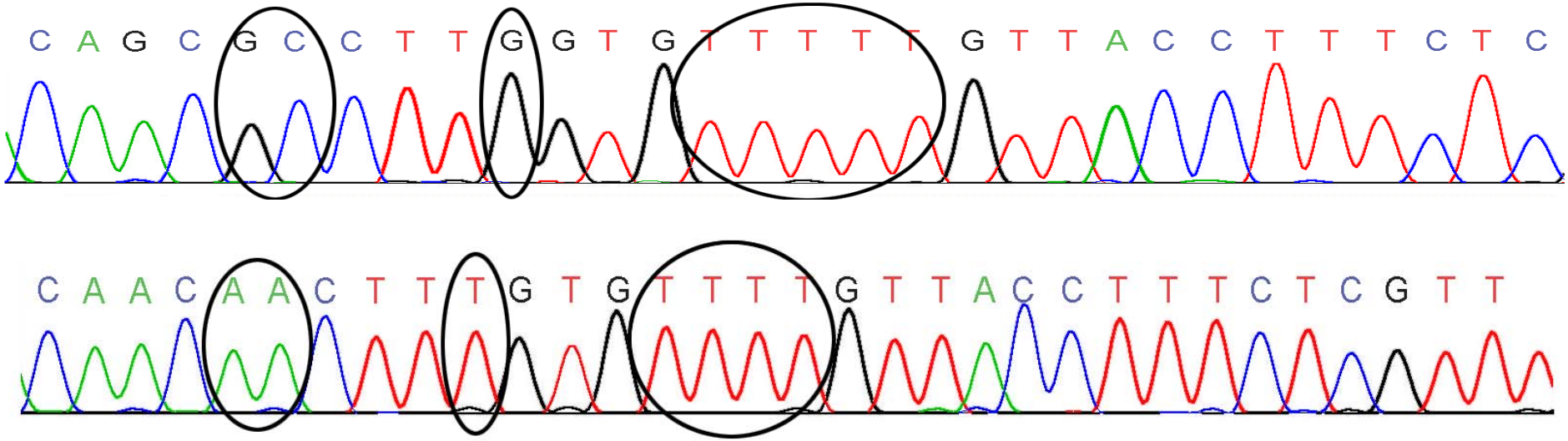
CTTTGAACGGCAAC and GTTTGAACGGCAAAC (Note: actual primers are reverse compliment)

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

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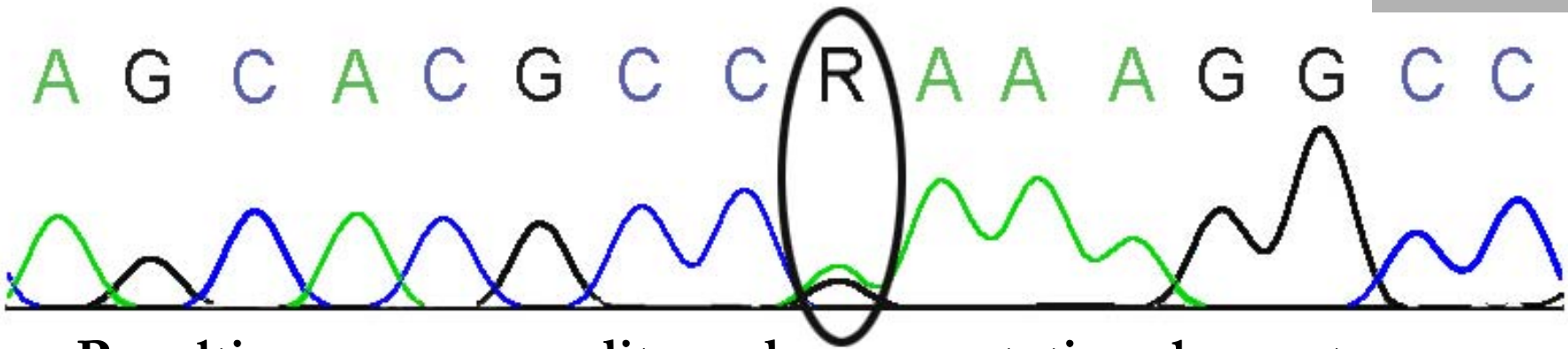
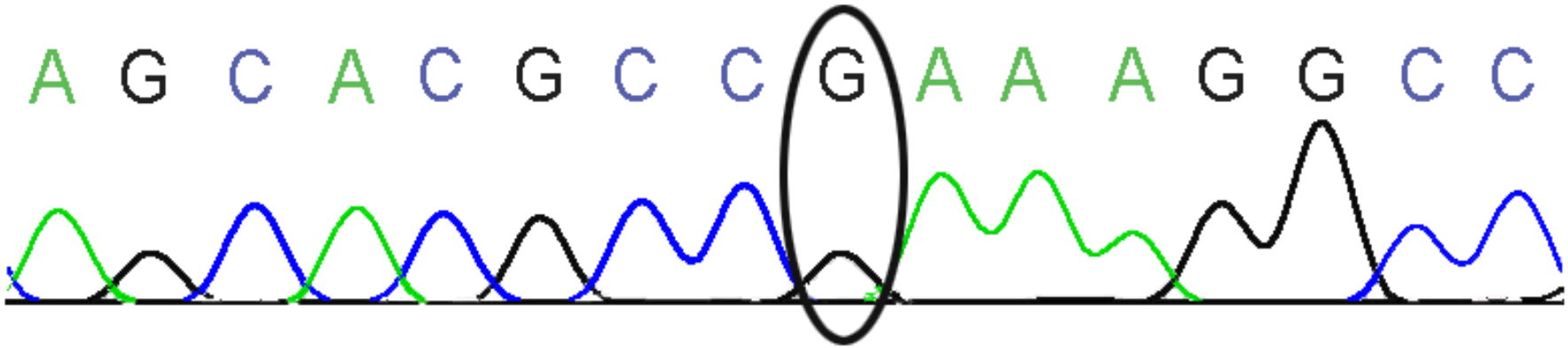
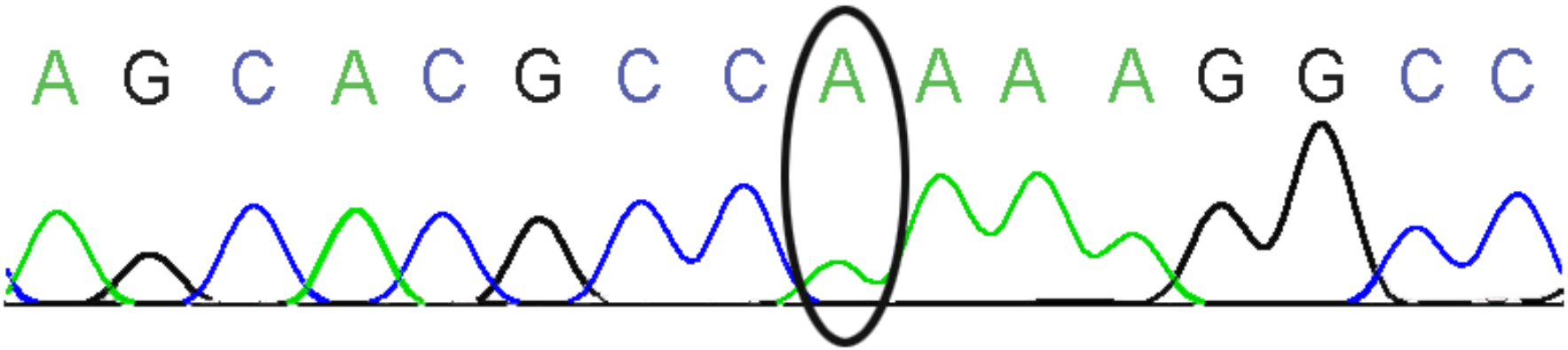


Resulting sequence edits and representative chromatograms





Results

- Neighbor-joining, Parsimony, and Bayesian methods showed congruent results

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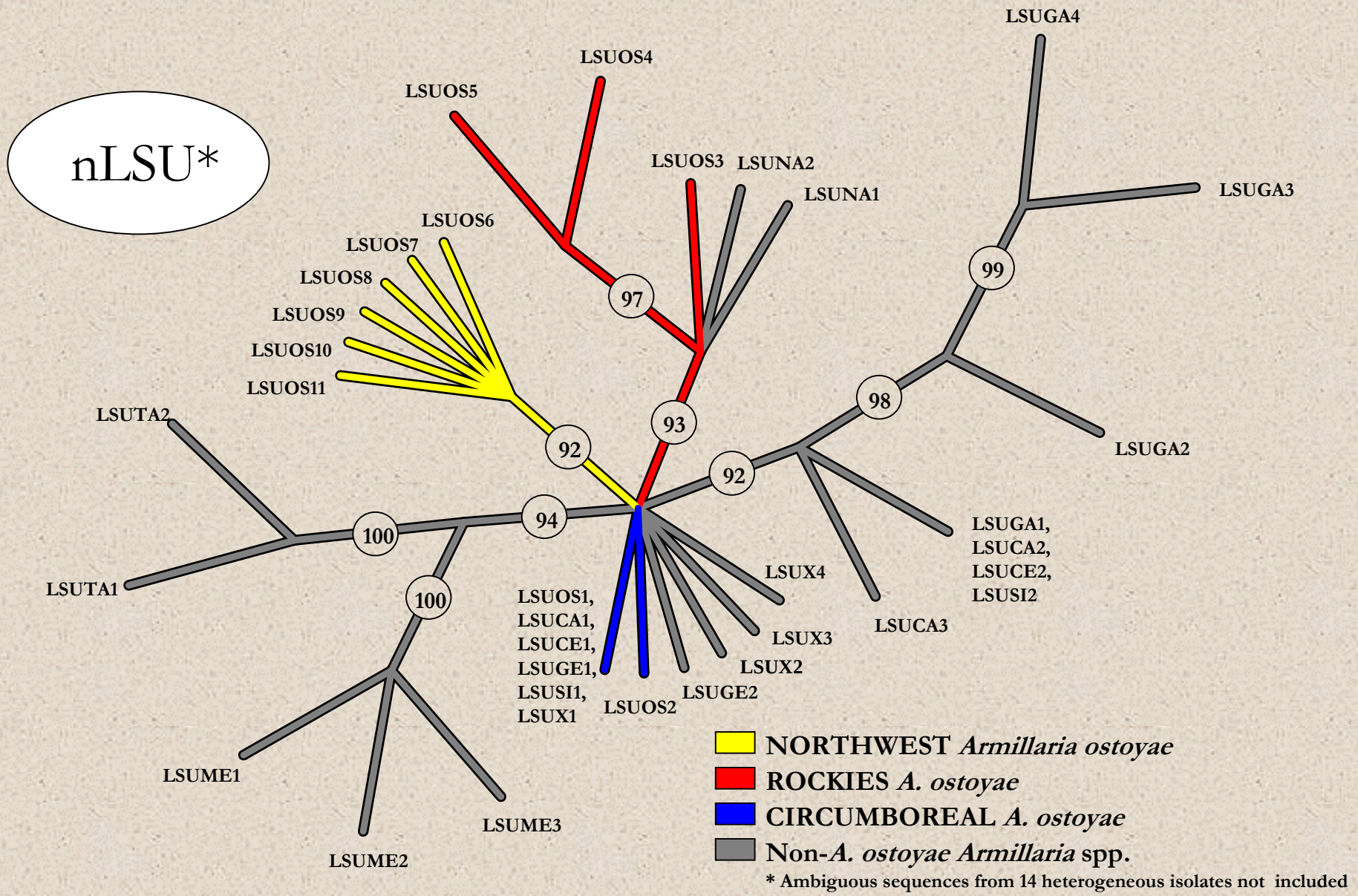

Radial 90% majority rule consensus tree of *Armillaria* spp. based on 24,001 trees from Bayesian inference analysis of the nuclear large ribosomal subunit (nLSU). Numbers between clades indicate estimated posterior probability.

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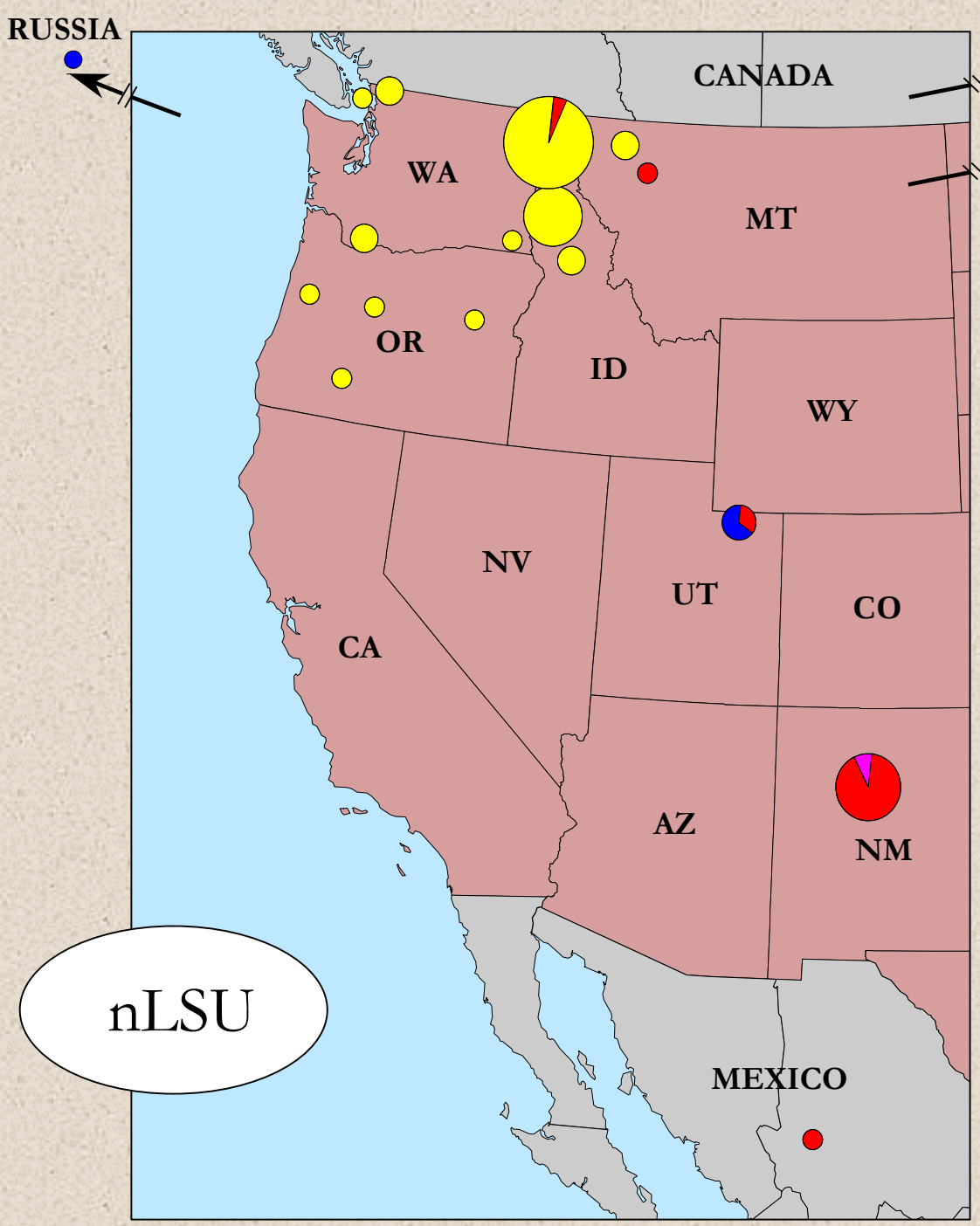

NORTHWEST
ROCKIES
ROCKIES x CIRCUMBOREAL
CIRCUMBOREAL

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.

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Circumboreal Group Distribution

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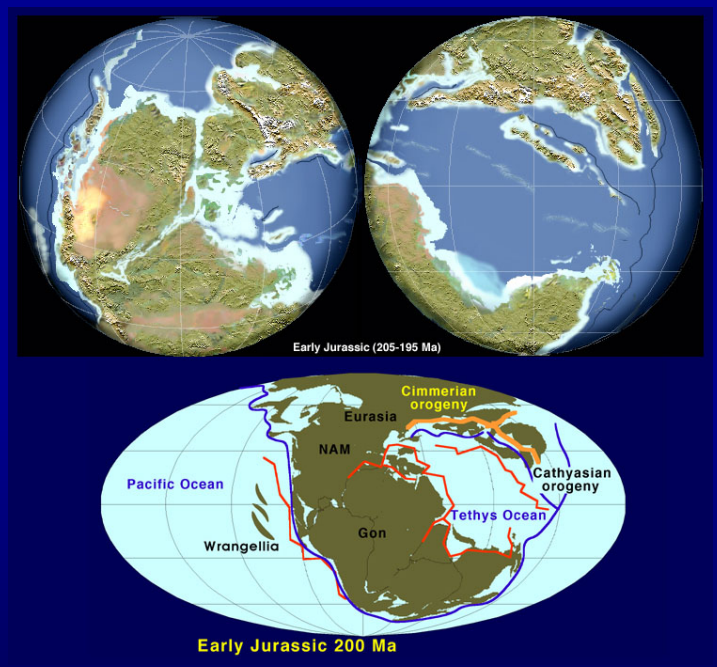
Observed trends in LSU sequence analyses raise the possibility that *A. ostoyae* distribution is related to paleogeographic events



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

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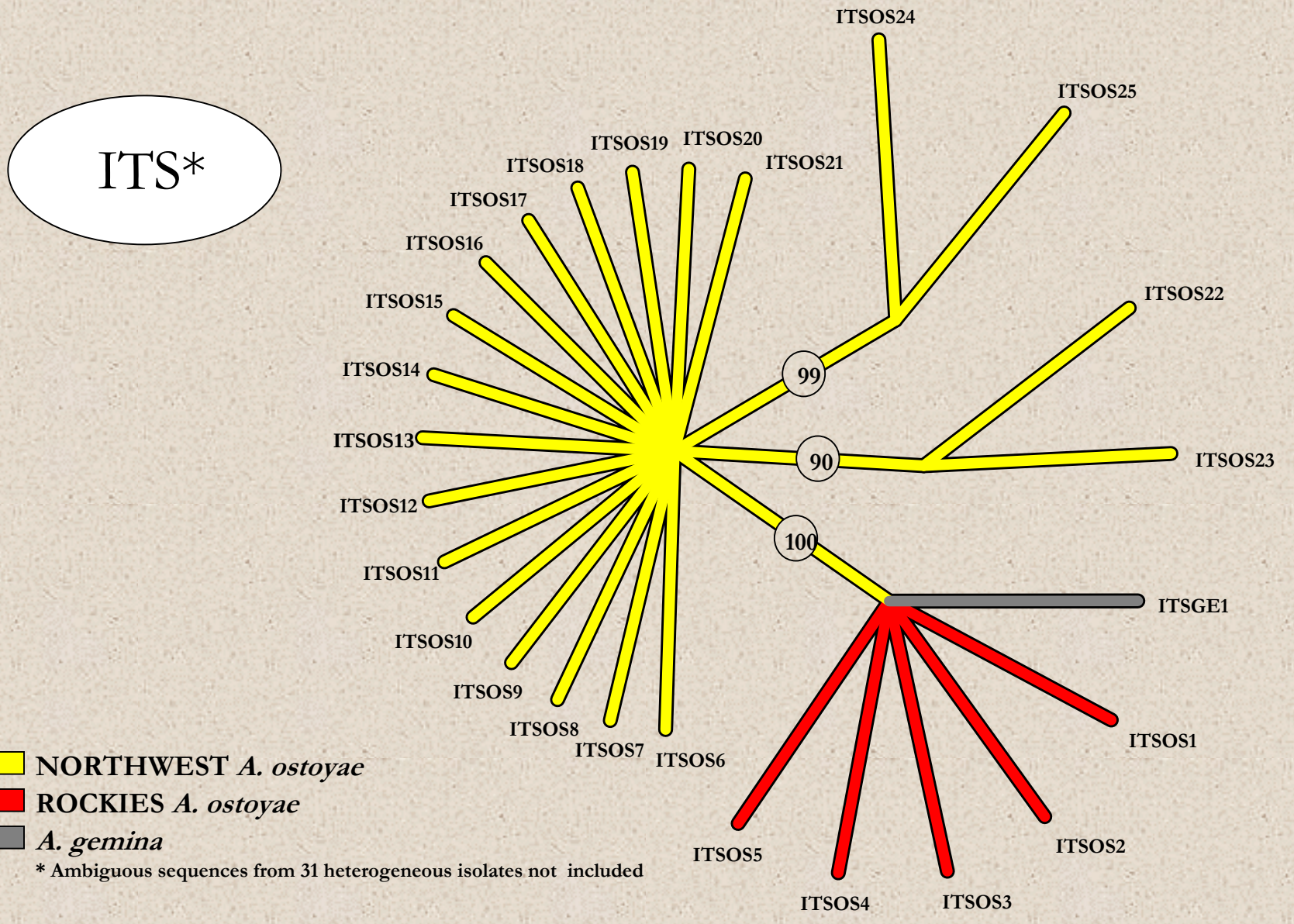

Radial 90% majority rule consensus tree of *Armillaria* spp. based on 24,001 trees from Bayesian inference analysis of the internal transcribed spacer and 5.8S rDNA (ITS). Numbers between clades indicate estimated posterior probability.

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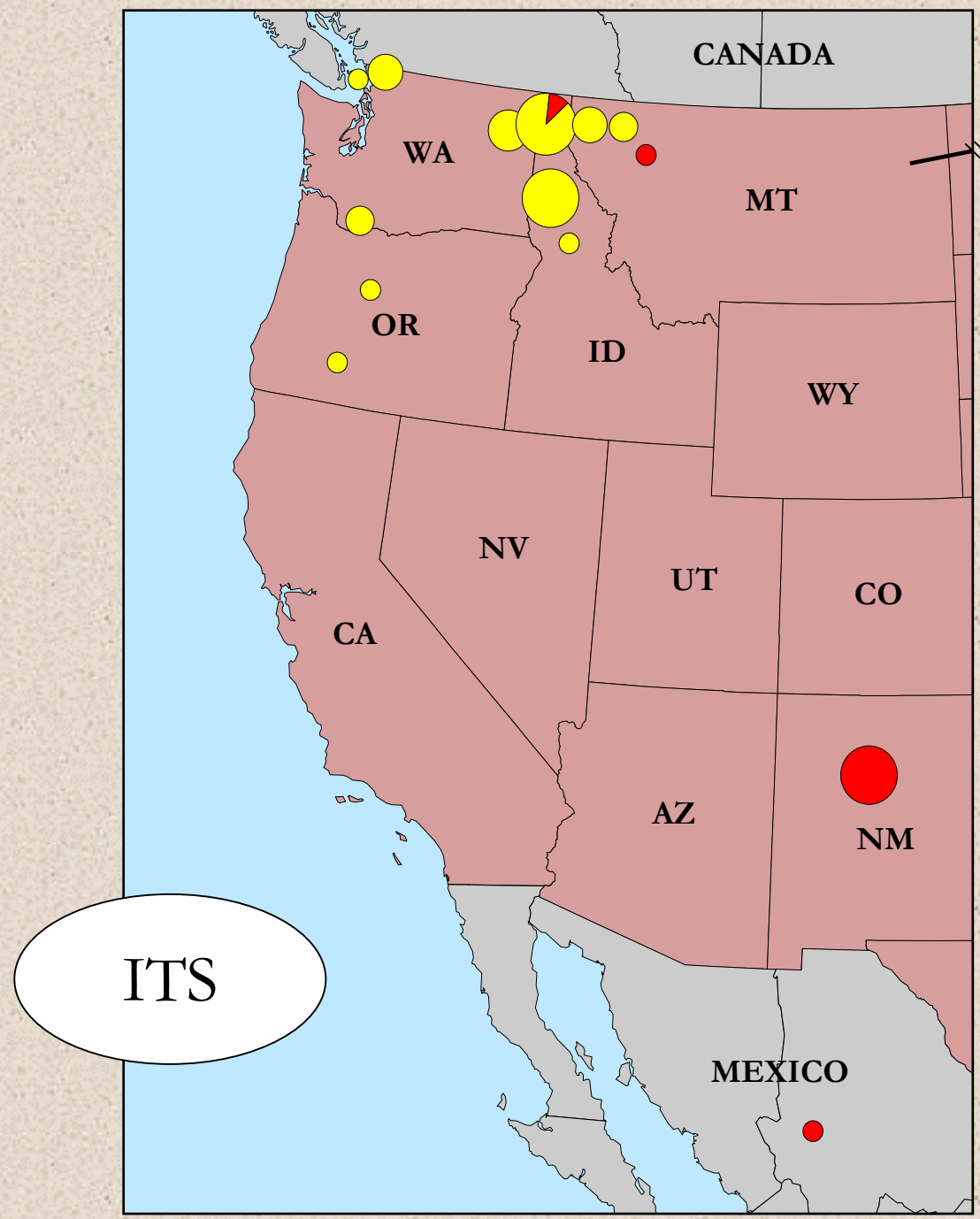

NEW HAMPSHIRE

NORTHWEST
ROCKIES & NEW HAMPSHIRE

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.

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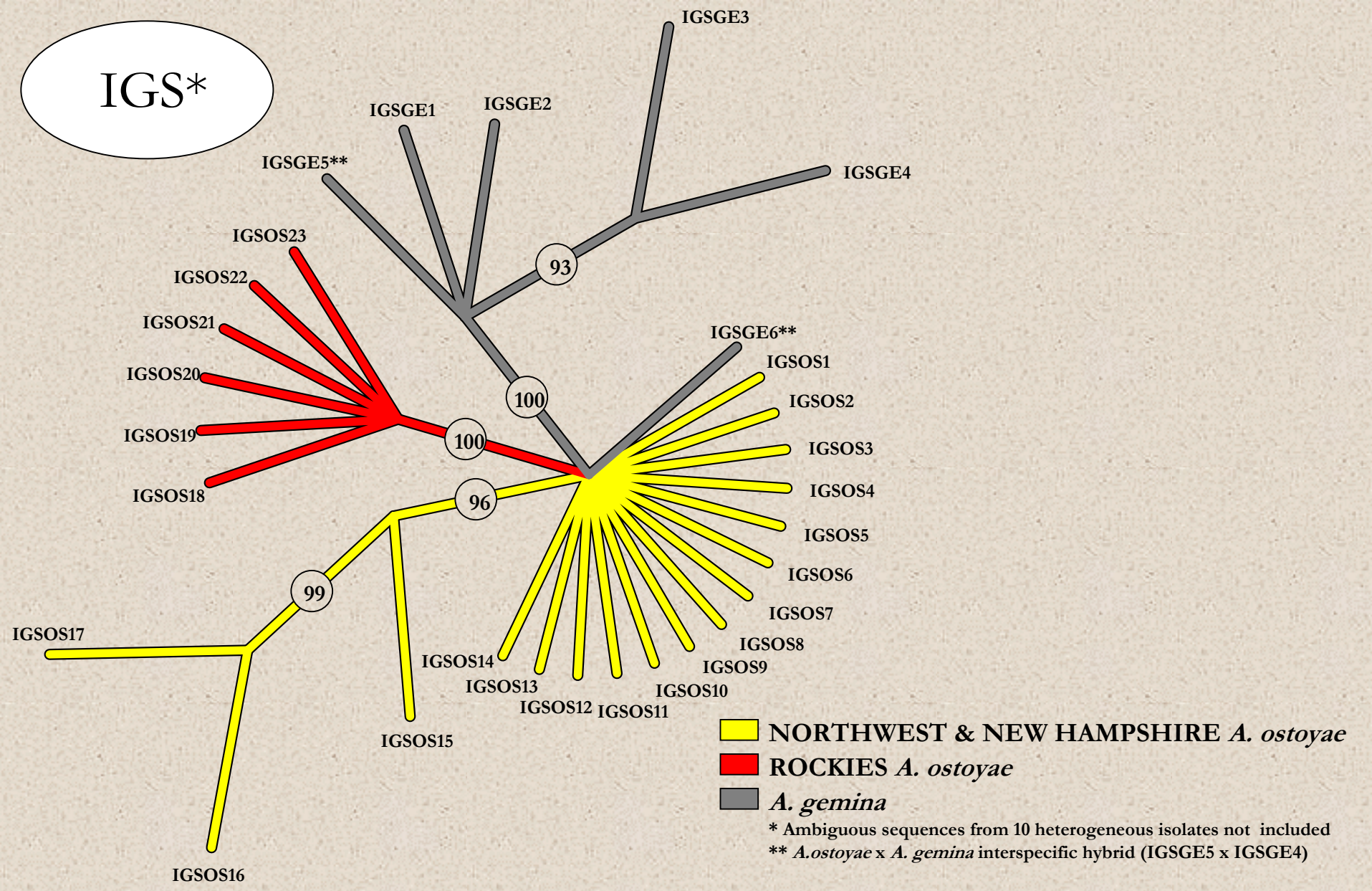

Radial 90% majority rule consensus tree of *Armillaria* spp. based on 24,001 trees from Bayesian inference analysis of the intergenic spacer one (IGS). Numbers between clades indicate estimated posterior probability.

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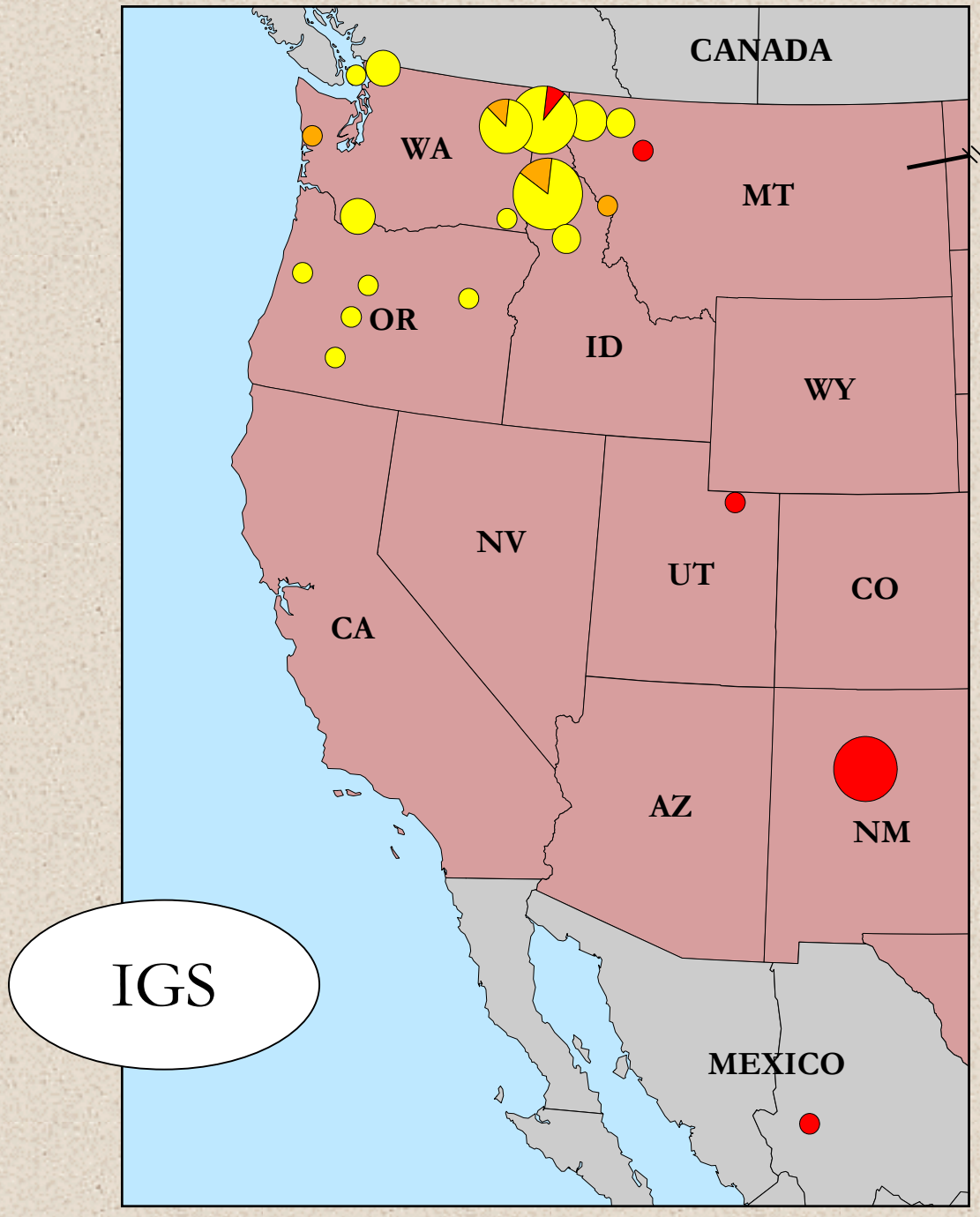

NEW HAMPSHIRE

NORTHWEST & NEW HAMPSHIRE
ROCKIES
NORTHWEST x ROCKIES

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.

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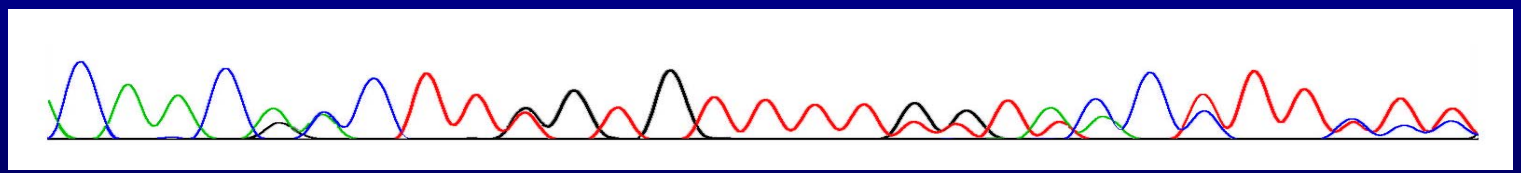
Results (continued)

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

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Armillaria ostoyae intraspecific hybridization - IGS-1 rDNA

C G A A C G G G T A A G C T A A C A A C G C C T T G G T G T T T T T G T T A C C

NABS I – Northwest Group

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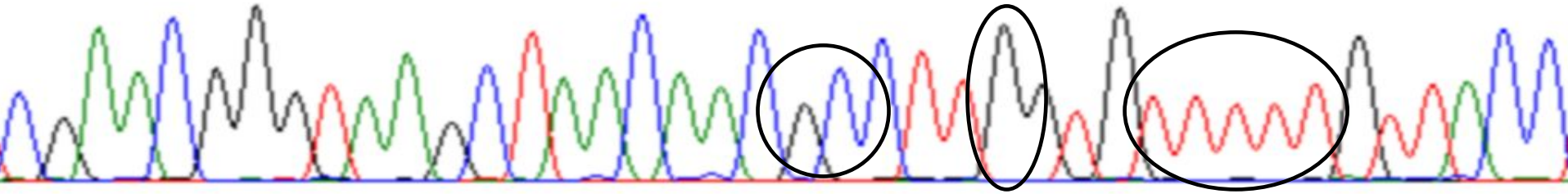
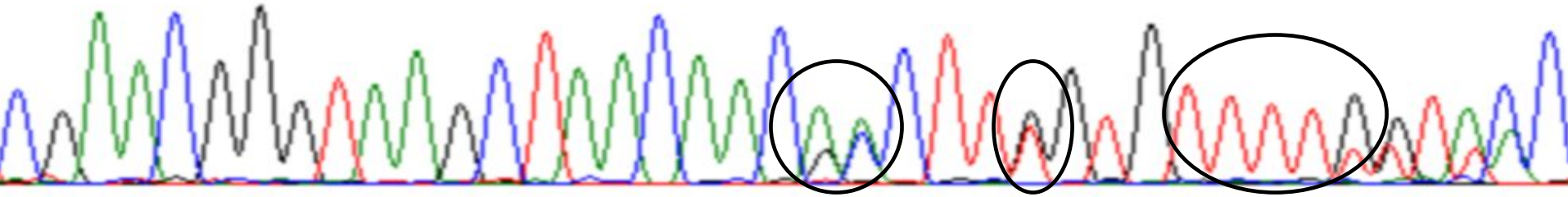


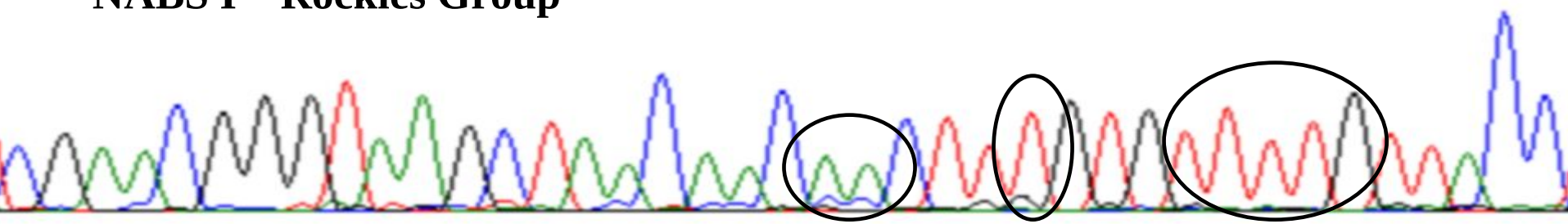
C G A A C G G G T A A G C T A A C A A C A N C T T N G T G T T T T G G T A C C

NABS I - Northwest x Rockies Hybrid



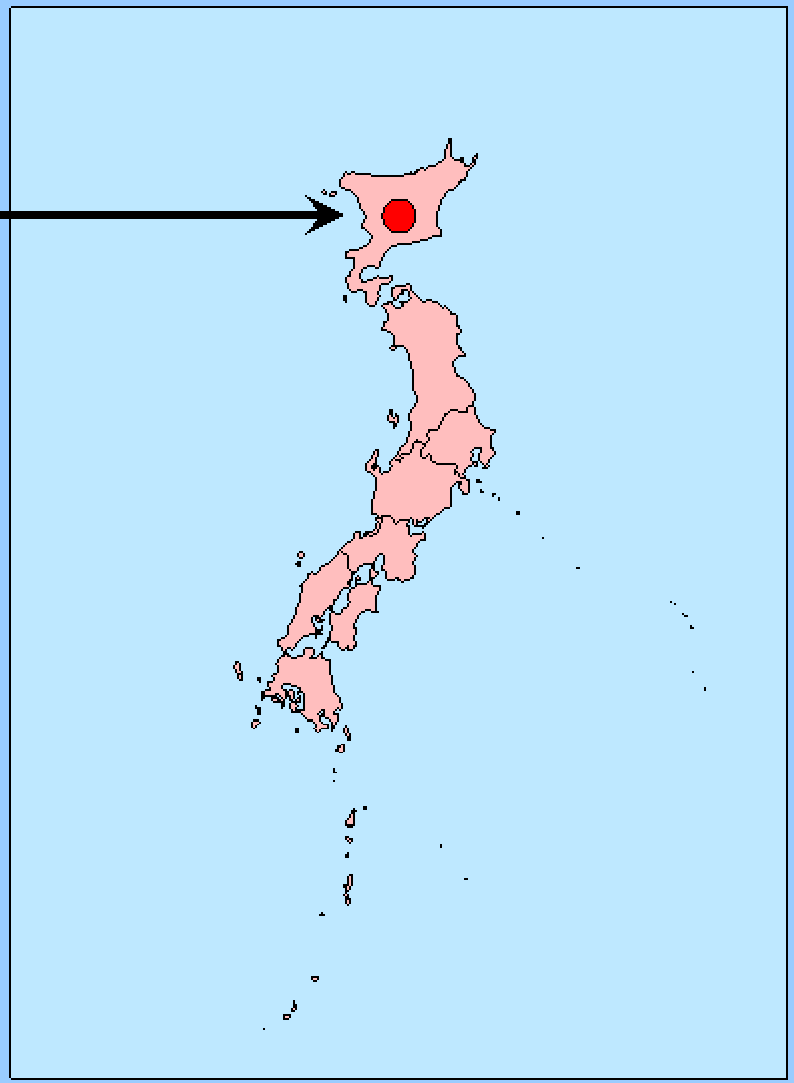
C G A A C G G G T A A G C T A A C A A C A A C T T T G T G T T T T G T T A C C

NABS I – Rockies Group



Asahikawa, Japan
Terashima et al. 1998

Single clone from *Armillaria ostoyae* rDNA IGS-1 region (collected from orchid)



- 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

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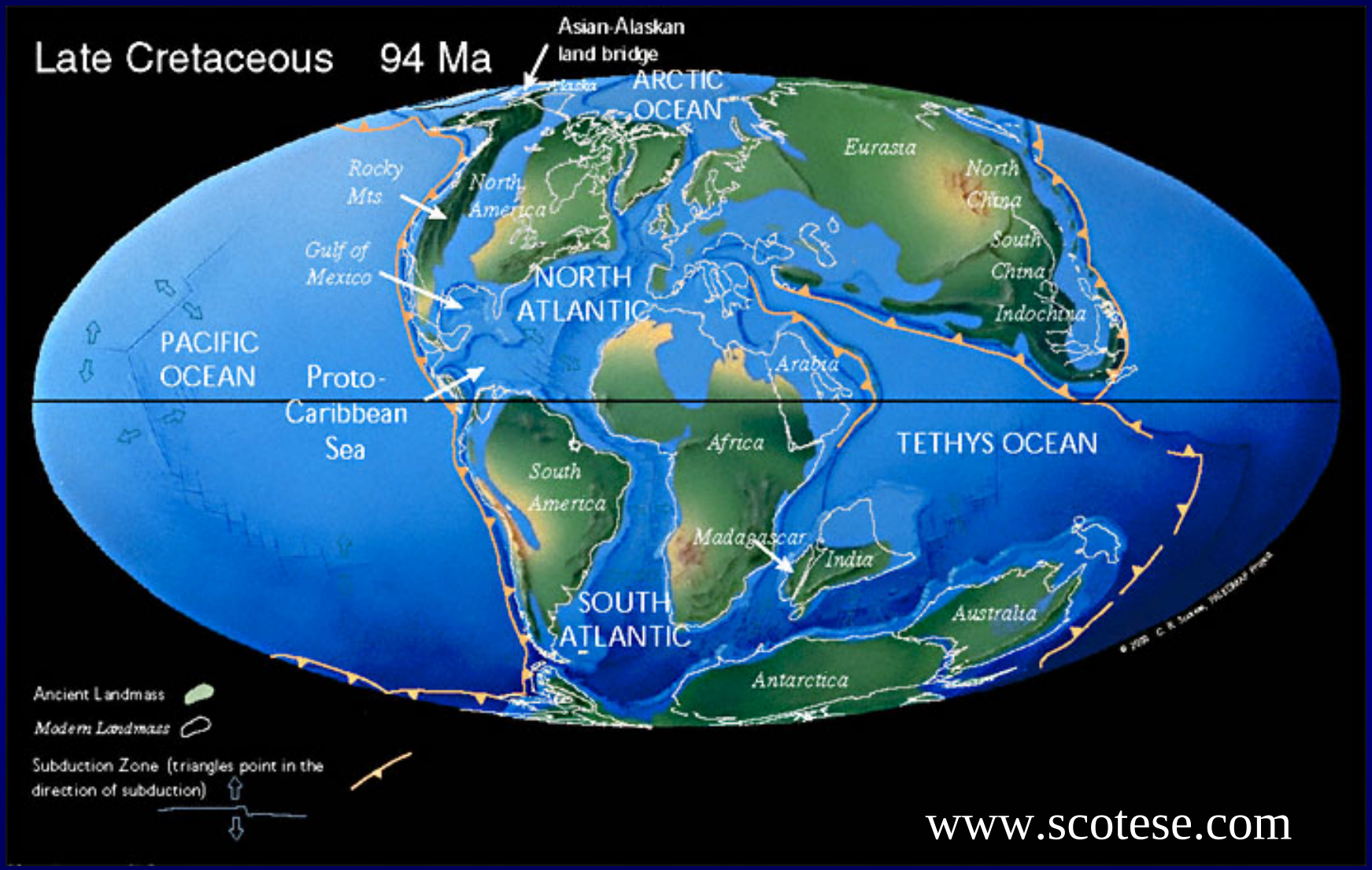

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Factors associated with genetic races and intraspecific hybridization within *A. ostoyae*

- 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

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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.**

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Acknowledgements

Intermountain Forest Tree Nutrition Co-op

University of Idaho

BOISE Corp.

USDA Forest Service

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**

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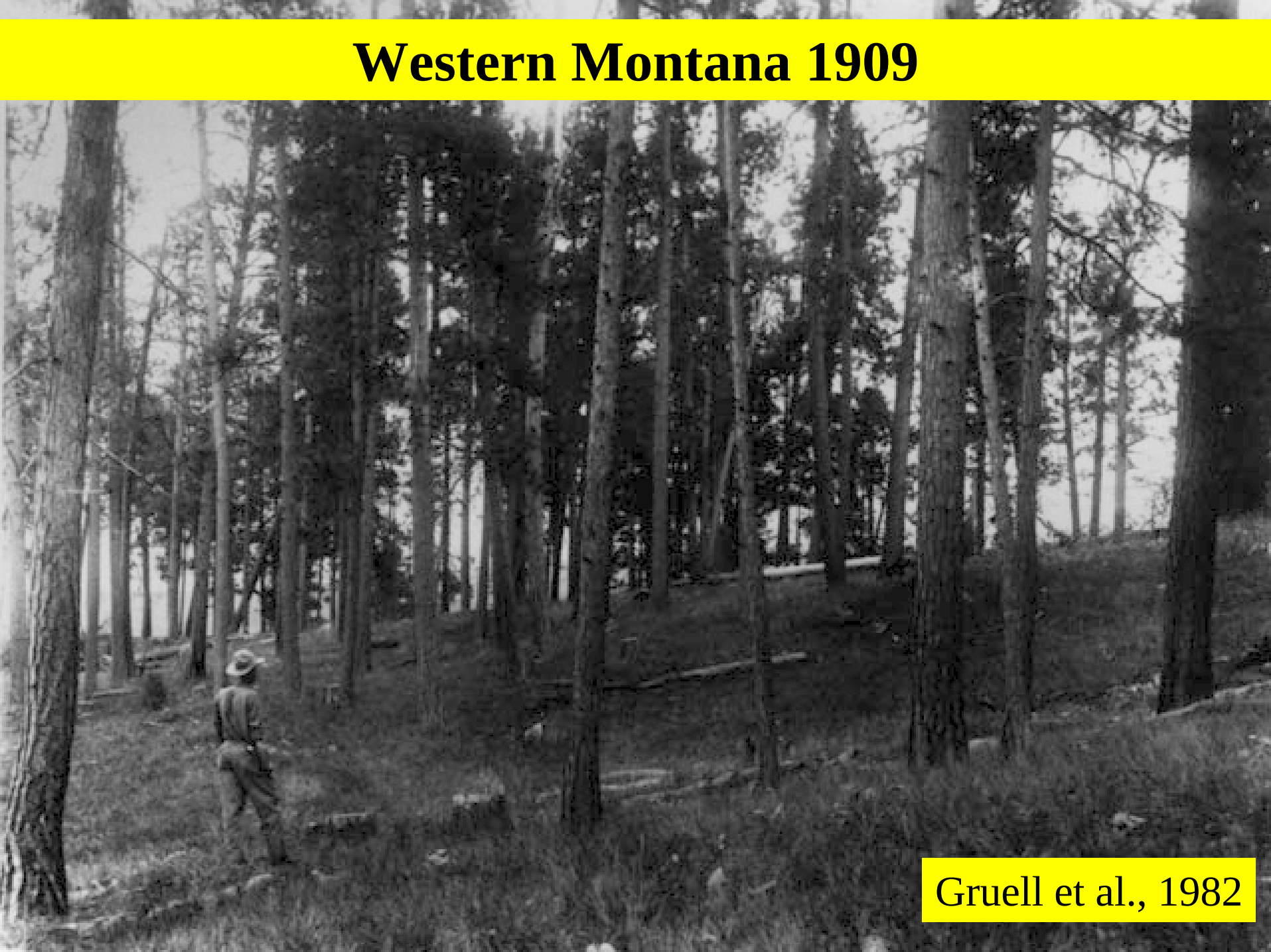
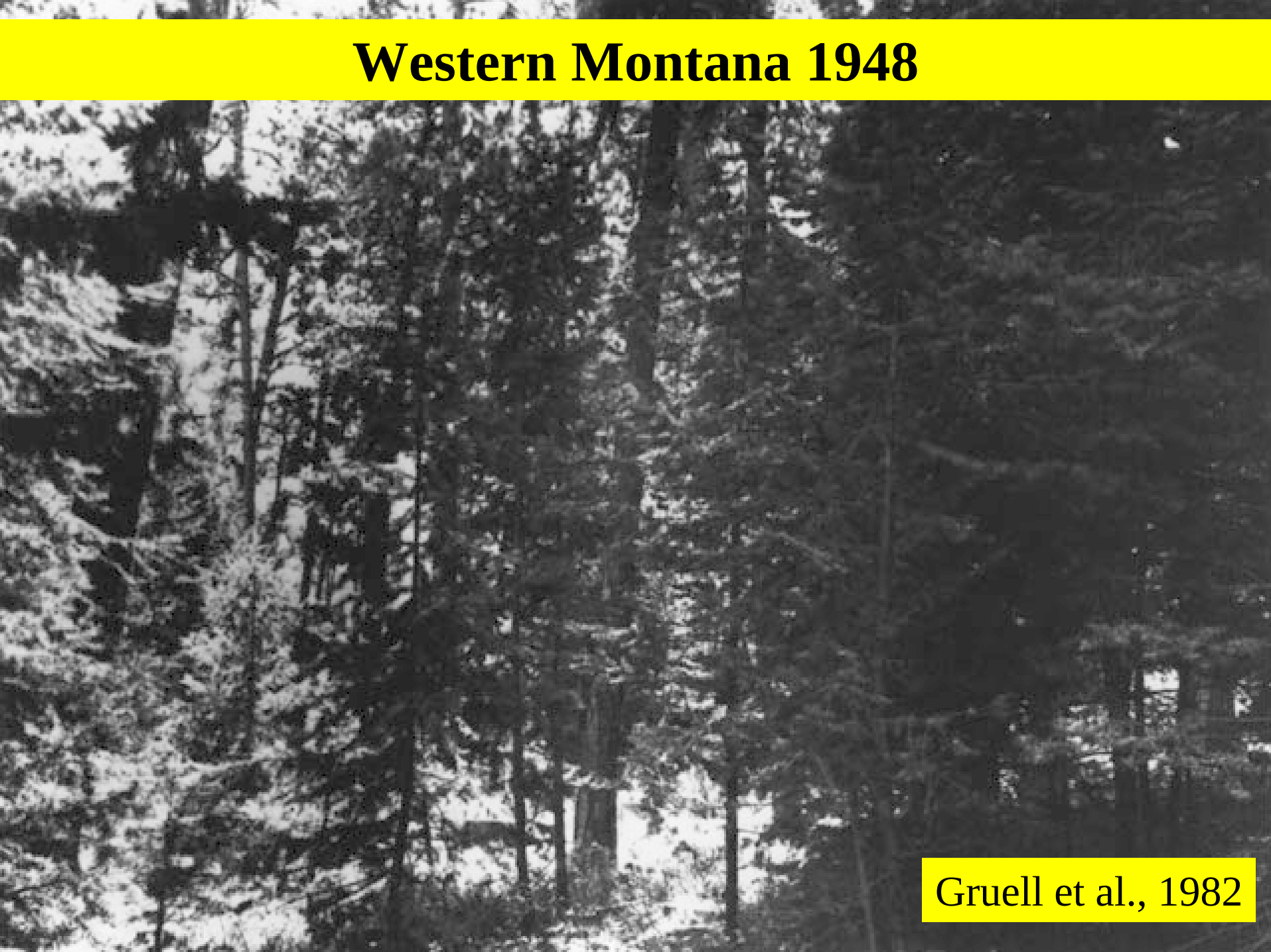
Western Montana 1909



Gruell et al., 1982

Western Montana 1948



Gruell et al., 1982

Hanna et al. 2004

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.

Fins et al., 2001

Selective Harvesting and Planting Practices

Photo: BC Ministry of Forests

Hanna et al. 2004

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

Photo: John Hanna

Photo: Raini Rippy

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.**

Photo: Raini Rippy

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*

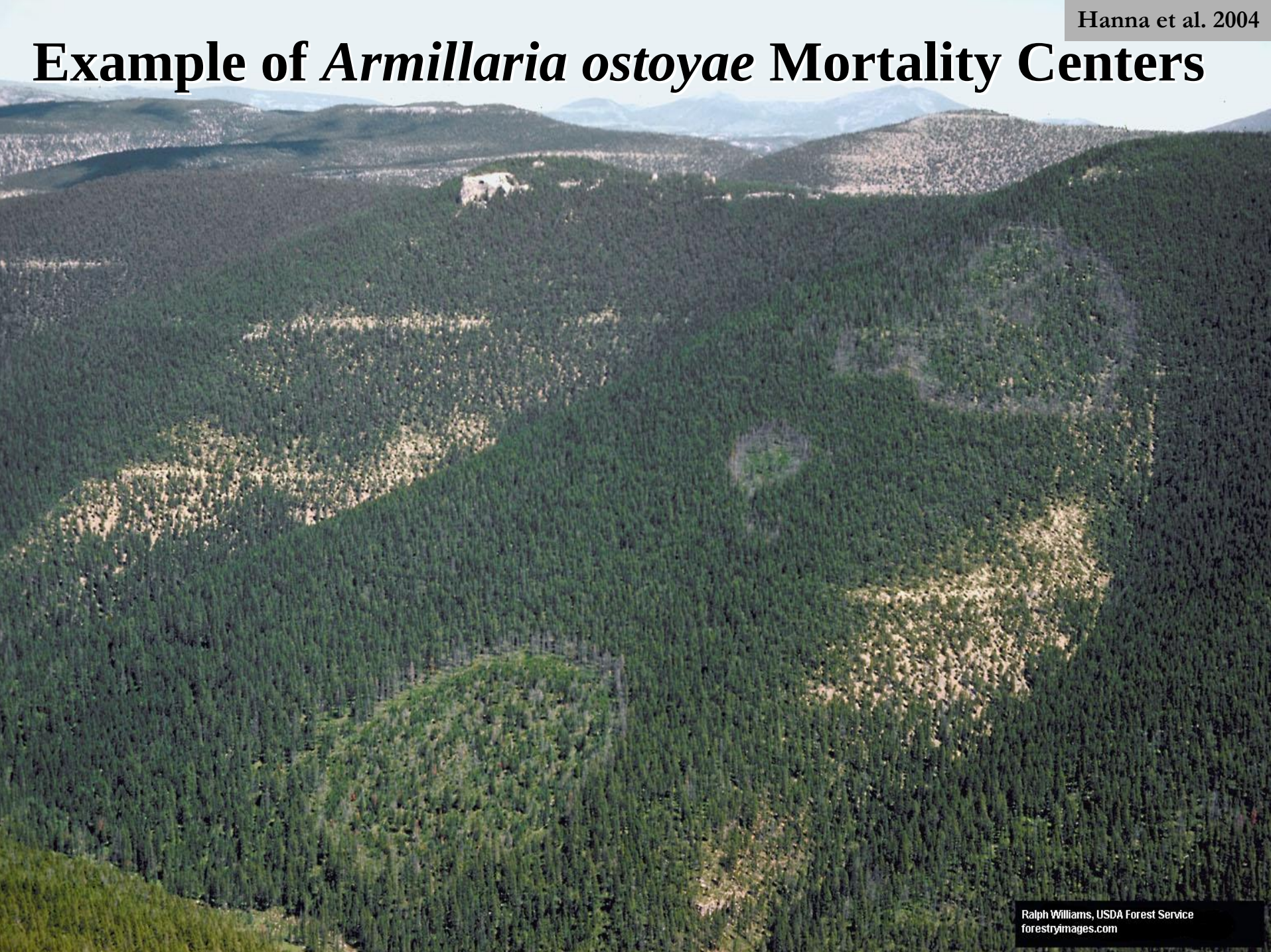
- Armillaria root disease was first described by Robert Hartig in 1873
- Fungus / Basidiomycete
- Constructs rhizomorphs from vegetative hyphae
- Fruiting bodies are mushrooms
- Some *Armillaria* mycelium is luminescent

Photo: Raini Rippy

Hanna et al. 2004

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


Photo: Moosefest.com

• 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

Hanna et al. 2004

Hanna et al. 2004

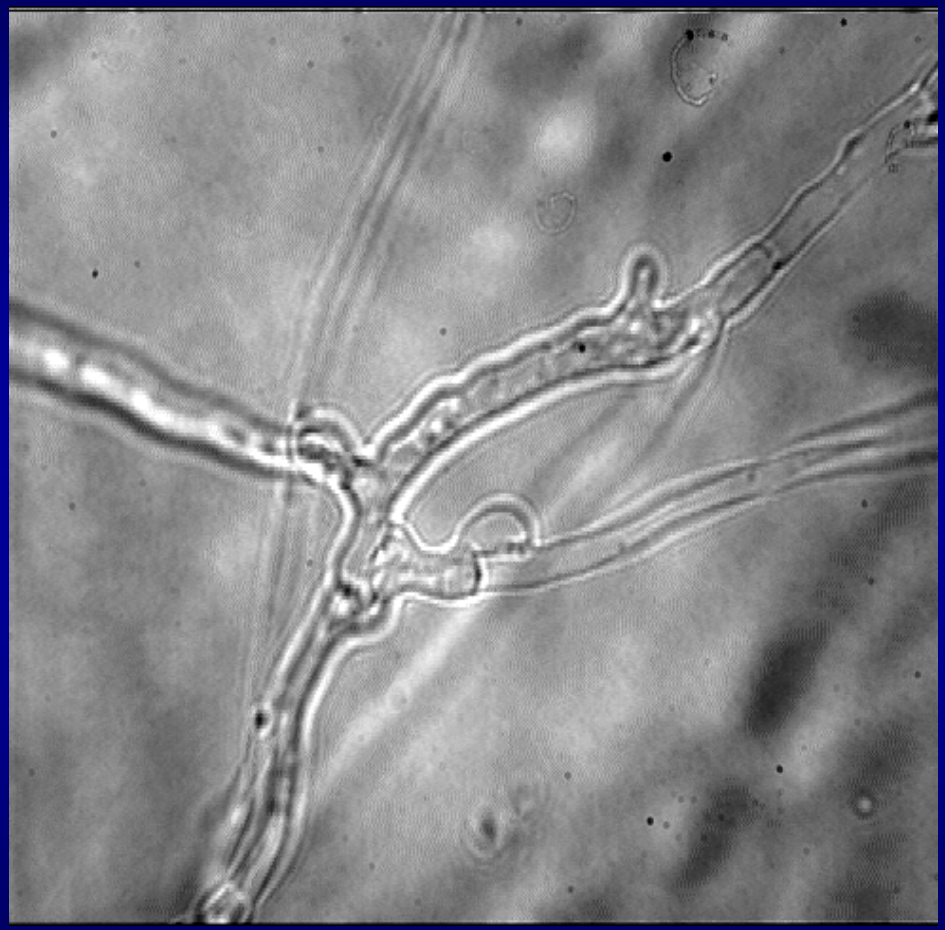
Armillaria Species and Genet Identification

Black line
= different species

Colorless antagonisms
= same species

Photo: Mee-Sook Kim

Microscopic observation of *Armillaria* mating for species identification

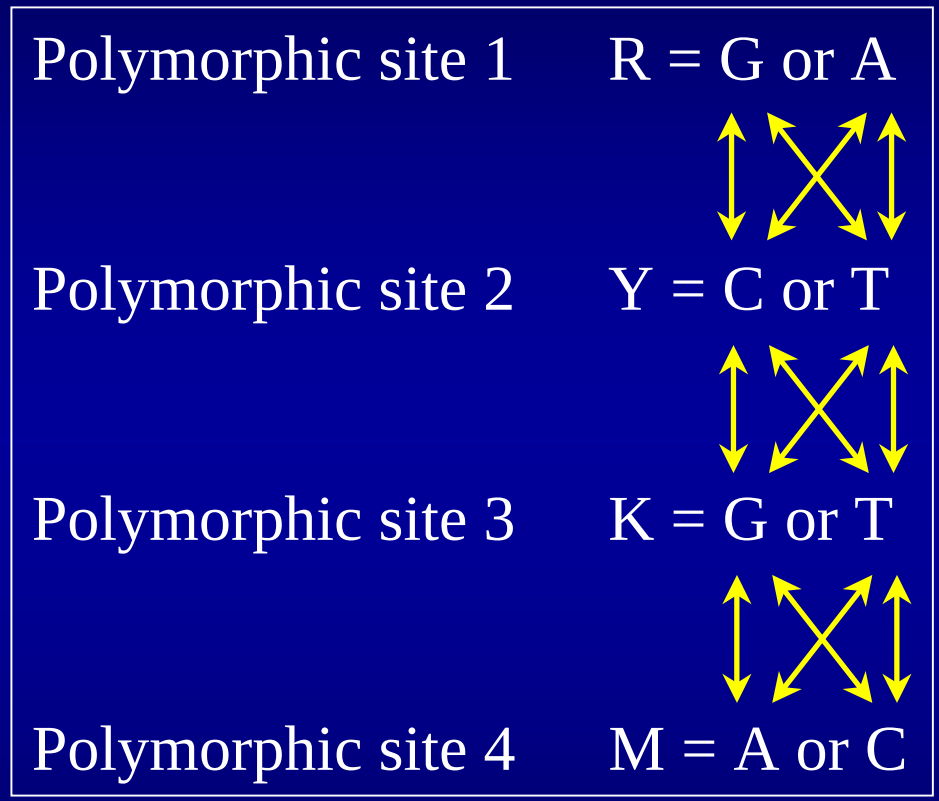


Clamp connections

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



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

Hanna et al. 2004

Primer sets applied to heterogeneous PCR product

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

Region	Primer	
IGS:	AOHR1T	(5'-TGCCGTTCAAAA-3')
	AOHR1G	(5'-TGCCGTTCAAAC-3')
	AOHR1C	(5'-TGCCGTTCAAAG-3')

Hanna et al. 2004

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 phylogenic signal.

Hanna et al. 2004

77 *A. ostoyae* individuals were sequenced in each of three regions

Chromatograms showing heterogeneity (intra-individual variation)

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

Reduced to two homogenous sequences with editing techniques

LSU – 23 (30%) ITS – 14 (18%) IGS -36 (47%)

Remaining heterogeneous individuals excluded from analysis

LSU – 14 (18%) ITS – 31 (40%) IGS – 10 (13%)

Only 16% of individuals were homogenous in all three regions

Hanna et al. 2004

Hanna et al. 2004

Armillaria Mortality Centers

USDA Forest Service, Missoula Archives