A PCR-Based Identification Method for Species of Armillaria
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A PCR-based identification method for species of Armillaria

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Abstract: A portion of the Intergenic Spacer (IGS) of
the ribosomal RNA operon of 74 isolates of 11 Armillaria species from Europe and North America was amplified
using the polymerase chain reaction. Amplifications
were made from scrapes of living mycelium without DNA extraction. Alu I digests of the amplified
product were electrophoresed in agarose and stained
with ethidium bromide. With few exceptions, each taxon
had a unique combination of restriction fragments.
Most taxa had a single Alu I pattern, but two restriction
patterns were seen among isolates of A. borealis, A.
cepistipes, A. gallica, A. tabescens, and A. mellea. Armillaria ostoyae, A. gemina, one of the A. borealis types,
and one of the A. cepistipes types had identical sizes of
Alu I fragments, but each of these taxa could be distin-
guished by their polymorphisms after restriction
with the enzymes Nde I, Bsm I, or Hind II. European
isolates of A. gallica had a distinct Alu I restriction
pattern, but North American isolates of this species
had a restriction pattern identical to A. calvescens. IGS
amplification patterns were obtained from 8-year-old
spore prints and dried basidiomes, as well as fresh
wood decay without DNA extraction. The technique
allows for identification from decayed wood, basidio-
imes or mycelia of these Armillaria species in a single
day.

Key Words: Armillaria, identification, Intergenic
Spacer, rDNA

INTRODUCTION

Species of Armillaria (Fr.: Fr.) Staude (Agaricales, Tri-
cholomataceae) are among the most important root
disease pathogens of trees, but the confused taxonomy
of this genus has precluded a clear understanding of
the biology of the major species. Until the 1970s, most
plant pathologists referred to all Northern Hemi-
sphere species of Armillaria as A. mellea (Vahl: Fr.)
Kummer. Separating the common species and defining
their biology has been seriously impeded by difficul-
ties in species identification (Guillaumin et al.,
1991; Watling et al., 1991; Harrington et al., 1992;
Guillaumin et al., 1993). Characteristics of mycelial fans
and rhizomorphs vary among the species only to a
limited extent, and basidiomes are seasonal and rare
to uncommon in many regions. The fungus may be
difficult to isolate from some substrata, it grows slowly
in culture, there is little variation among Armillaria
species in cultural characteristics, and production of
basidiomes in culture is unreliable and time-consum-
ing.

Critical identifications of cultures of Armillaria rely
on pairings with haploid tester strains (Korhonen,
1978). Single basidiomorph stains are haploid and are
generally fluffy and unpigmented on malt extract agar.
Isolates from decay, fans or rhizomorphs are diploid
(Hintikka, 1973), generally produce relatively little aero-
ial mycelium, and the mycelium often has a reddish-
brown crust. Pairing of single basidiomorph strains of
the same species will usually result in diploidization,
and the fluffy mycelium tends to flatten. Transient
clamp connections may also be visible in compatible
pairings (Larsen et al., 1992). A haploid tester can be
diploidized upon pairing with a diploid isolate of the
same species. Subculturing from the pairing plates is
recommended (Harrington et al., 1992), but still, the
results are often ambiguous, particularly for identifi-
cation of diploid isolates (Siepmann, 1987; Shaw and
Loopstra, 1988; Rizzo and Harrington, 1992). These
pairing tests require up to 2 months for identification
after isolation in pure culture.

Other techniques have been explored for differenti-
ating species of Armillaria. Electrophoresis of iso-
zymes (Morrison et al., 1985), restriction fragments of
ribosomal or mitochondrial DNA (Anderson et al.,
1987; Anderson et al., 1989; Jahnke et al., 1987; Smith
and Anderson, 1989), or DNA-DNA hybridization
(Jahnke et al., 1987) can distinguish some of these
species. However, none of these techniques have been
widely tested or proven to be feasible for routine spe-
cies identifications.

Anderson and Stasowski (1992) published partial DNA
sequences for the IGS (Intergenic Spacer) region of the ribosomal RNA (rRNA) operon for most of the Northern Hemisphere species of Armillaria. Sequence variation among the limited number of isolates sampled suggested that restriction enzyme digests of this region may discriminate among the species. Using the polymerase chain reaction (PCR) and the primers of Anderson and Stasovski (1992), we amplified the IGS region and screened the PCR products using a number of restriction enzymes for unique restriction fragment length polymorphisms (RFLP) among the European and North American species of Armillaria. A 1-day procedure was developed that can identify the 11 taxa examined.

MATERIALS AND METHODS

Isolates.—At least four haploid or diploid isolates of each of the nine described and two nondescribed species of Armillaria in Europe and North America were included in the study (Table I). These isolates were identified by various investigators using pairing tests. All the isolates were grown on MYEA plates (2% malt extract, 0.2% yeast extract, 1.5% agar) at room temperature prior to amplification.

Template DNA.—DNA was isolated from a limited number of the cultures using the method of Lee and Taylor (1990). However, most of the results presented here were from amplifications done directly from Armillaria mycelium on MYEA. Neither the amplifications nor the results from the restriction digests were influenced by the origins of the DNA template.

For direct amplification from mycelium, a pipette tip was scraped approximately 1 cm across the actively growing mycelium at the edge of the colony. The tip was then dipped in the PCR reaction vessel containing the reaction mix and the mineral oil, and the mixture was vigorously stirred with the tip. In this manner fungal material attached to the tip was suspended in the reaction mix.

Polymerase chain reaction (PCR).—The Intergenic Spacer region (IGS) between the 3’ end of the large subunit ribosomal (LSU) RNA (rRNA) gene and the 5’ end of the 5S rRNA gene was amplified using PCR. The primers used were those recommended by Anderson and Stasovski (1992): LR12R, 5’CTGAACGCCTCTAGTCAGA3’ (Veldman et al., 1988) and 5’/AGTCCTATGCGCGTGGAT3’ (Duchesne and Anderson, 1990). The PCR reaction mixture included 2.5 units Taq polymerase (Promega, Madison, Wisconsin) per reaction, the buffer supplied with the enzyme, 4 mM MgCl₂, 200 μM dNTPs, and 0.5 μM of each primer. DNA (10 ng) or fungal mycelium were added as template for the reaction. Mineral oil was overlaid to prevent evaporation of the 100-μl final reaction volume. The thermocycler (MJ Research, Inc., Watertown, Massachusetts) conditions were an initial denaturation at 95 °C for 95 sec, followed by 35 cycles of 60 °C for 40 sec (annealing), 72 °C for 2 min (elongation) and 90 °C for 30 sec (denaturation). A final elongation was allowed for 10 min at 72 °C to ensure a double-stranded amplification product.

DNA restriction.—A number of restriction enzymes were tested for polymorphisms among a select group of Armillaria isolates. The enzyme Alu I gave the greatest polymorphisms, although some species had identical RFLP with this enzyme. DNA sequence of the IGS region from the end of the LSU to the 5S gene of the rRNA operon had been published for all the tested Armillaria species except A. mellea and A. tabescens (Scop.: Fr.) Emel. (Anderson and Stasovski, 1992), and these sequences were used to identify further diagnostic restriction enzymes. The five enzymes utilized were Alu I, Nde I or Hind II (Promega, Madison, Wisconsin), Bsm I (Stratagene, La Jolla, California), and Tha I (Gibco BRL, Life Science Technologies, Gaithersburg, Maryland).

The amplified DNA was not purified before restriction enzyme digestion. Alu I, Nde I or Hind II (2–4 units per reaction) was added directly to the PCR reaction mix (20 μl) after amplification and the digestion allowed to proceed for 1–16 h at 37 °C. The Bsm I and Tha I (2–4 units per reaction) digestions were performed at 65 °C for 1–16 h. NaCl was added to a final concentration of 50 mM for both the Nde I and Tha I digestions and to a final concentration of 100 mM for Bsm I digestions.

Electrophoresis.—Both the amplified DNA and the restriction enzyme fragments of these products were electrophoresed in agarose gels in a TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA (pH 8)) buffer system to determine the size of the amplification and restriction products. For routine analysis, 2% agarose gels were run at 100 V for 2 h, but more definite determinations of fragment sizes were based on larger gels of 3% MetaPhor agarose (FMC BioProducts, Rockland, Maine) run at 200 V for 3 h at 10 C. The gels were stained with ethidium bromide and visualized using UV light.

RESULTS

Good amplification of the IGS region was obtained with all isolates using direct amplification from mycelium. At least two amplifications were made of each of the isolates listed in Table I. The amplified product from all isolates of A. mellea was 875 base pairs (bp), and each isolate of A. tabescens yielded a product of
<table>
<thead>
<tr>
<th>Species (RFLP group)</th>
<th>Isolate number</th>
<th>Determined by, other isolate number</th>
<th>State, province, or country of origin</th>
<th>Fragment sizes (bp)</th>
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<td><strong>A. sinapina</strong></td>
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<td>B586 Mallett, 985</td>
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<td>B172 Worrall, A-G7Pa</td>
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<td>Germany</td>
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<td>B521 Guillaumin, T2.1</td>
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<td>B218 Gregory, DN1</td>
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<td>B519 Guillaumin, KB2</td>
<td>France</td>
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<td>B174 Worrall, AbG9</td>
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<td><strong>A. gallica (American)</strong></td>
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<td>B500 Anderson, 137-1</td>
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</table>
845 bp. All other isolates yielded a product of 920 bp.

The product of at least two amplifications of each isolate was digested with Alu I and electrophoresed separately with markers to determine the sizes of the restriction fragments (Fig. 1). Only fragments larger than 100 bp were scored because fragments smaller than this were difficult to see clearly and tended to be obscured by the prominent “primer dimer” band produced during amplification. The number and sizes of fragments from the two or more digestions were consistent for each isolate.

One or two Alu I digestion patterns were found in each of the 11 taxa tested (Table I, Fig. 1). Twelve different patterns were found among the isolates tested. Based on the published sequences of Anderson and Stasovski (1992), it was possible to create a restriction map (Fig. 2) for eight of these patterns, which includes all of the tested species of Armillaria except A. mellea and A. tabescens. Two patterns were seen in A. borealis Marxmüller & Korhonen, A. cepistipes Velenovsky, and A. gallica Marxmüller & Romagnesi. The restriction maps show that the two patterns within each of these species is due to a difference in one or two restriction sites. Two restriction patterns were also seen in A. mellea and A. tabescens, but the lack of published sequence data for these two species prevented unequivocal determination of the variation in restriction sites.

The IGS amplification products of isolates of A. ostoyae (Romagnesi) Herink, A. gemina Bérubé & Dessureault, A. borealis type A, and A. cepistipes type B had the same Alu I restriction sites. However, examination of the IGS DNA sequences revealed other diagnostic restriction enzymes for these species. Amplified products of all isolates listed in Table I were digested with Nde I, but only the isolates of A. borealis and A. ostoyae were cleaved, yielding products of 550 and 370 bp (Fig. 3). Amplified products of the listed isolates of A. gemina and A. borealis did not digest with Bsm I, but the products of all of the listed A. ostoyae isolates except B177 restricted, yielding fragments of 620 and 300 bp (Fig. 3). The amplified product of another A. ostoyae isolate, B747, gave inconsistent digestion with Bsm I. As predicted from the sequence data, Hind II digested A. cepistipes isolates, giving fragment sizes of 580 and 340 bp, but did not cleave the IGS amplification products of A. ostoyae, A. gemina or A. borealis.

North American and European isolates of A. gallica differed in their Alu I restriction patterns (Table I, Fig. 2). The North American isolates had an Alu I pattern identical to that of A. calvescens Bérubé & Dessureault isolates. Examination of the IGS DNA sequences from North American A. gallica and A. cal-
vescens (Anderson and Stasovski, 1992) indicated that the two taxa differ only at a single nucleotide/base pair, and this difference should result in differential restriction by the enzyme Tha I. However, we found that the amplified DNA from only some isolates of each taxon were cleaved at this site with Tha I, and inconsistencies were also found between two amplified products from the same isolate. Therefore, we were not able to distinguish the amplified DNA from the North American isolates of A. gallica and A. calvscens using this or any other enzyme.

We attempted amplifications from dried samples and decay without extraction of DNA by scraping the tissues with a pipette tip as described for the amplification from fresh mycelia. We successfully amplified the IGS region from small amounts of 6- to 8-yr-old spore prints of A. gallica, gill tissue of a 7-yr-old dried basidiome of A. mellea, two 8-yr-old dried basidiomes of A. ostoyae, and wood from two oak trees that were decayed by A. gallica. Generally, small (barely visible) amounts of material worked better than larger amounts for amplifications. Small pieces of wood were likely present in the scrapings from decay. We also attempted amplifications from the core of dried rhizomorph tissue, but without success; fresh rhizomorph tissue might prove more amenable to the technique. The amplified products from the other tissues were the correct length for the respective species. The Alu I restriction patterns were as found in other North American isolates of A. gallica, A. mellea (type A), and A. ostoyae (Table 1).

DISCUSSION

Digestions of DNA from the IGS region with the restriction enzyme Alu I proved reliable for unambiguous identification of pure cultures of most of the Armillaria species known to Europe and North America. Because DNA extraction from pure cultures, dried samples or decay was unnecessary, diagnostic restriction patterns could be obtained in a single day. The IGS region was amplified from 8-yr-old spore prints and basidiome tissue, which should allow for nondestructive study of herbarium samples critical to the taxonomy and nomenclature of this difficult genus. Further, the IGS region was amplified directly from decayed wood, which will greatly facilitate rapid and unambiguous identification of fresh field materials for ecological work.

Some care must be taken in interpreting the restric-
A. ostoyae

| 310 | 89| 135 | 200 |

A. gemina

| 310 | 89| 135 | 200 |

A. borealis (type A)

| 310 | 89| 135 | 200 |

A. borealis (type B)

| 310 | 89| 104 | 200 |

A. sinapina

| 399 | 135 | 200 |

A. cepistipes (type A)

| 399 | 183 | 200 |

A. cepistipes (type B)

| 310 | 89| 135 | 200 |

NABS IX

| 534 | 200 |

NABS X

| 399 | 183 | 142 |

A. gallica (European)

| 399 | 183 | 240 |

A. gallica (N. American)

| 582 | 240 |

A. calvescens

| 582 | 240 |

Fig. 3. A 2% agarose gel stained with ethidium bromide showing the Nde I and Bsm I digestion products of the IGS region of representative isolates of Armillaria ostoyae (OST), A. gemina (GEM) and A. borealis (BOR). The occurrence of digested and undigested DNA fragments in the same lane is interpreted as incomplete digestion. Markers (M) are 100-bp ladders with the lowest band being 100 bp in size.

Fig. 2. IGS restriction maps for the enzyme Alu I based on the sequences published by Anderson and Stasovski (1992) and restriction patterns seen in the isolates listed in Table I and illustrated in Fig. 1. Numbers designate the approximate length (bp) of diagnostic fragments. Restriction maps for A. tabescens and A. mellea were not developed.
use of the recommended restriction buffer, use of higher concentrations of the restriction enzyme, and longer digestion periods would give more complete digestions, but these steps would add some expense and considerable time to the technique.

Among the restriction enzymes that we screened, Alu I proved the most informative for separating the taxa, but A. ostoyae, A. gemina and some of the A. borealis isolates had the same restriction pattern. The IGS sequences for these taxa are similar (Anderson and Stasovski, 1992). The first two species are morphologically similar (Bérubé and Dessureault, 1989) but much different in biology (Rizzo and Harrington, 1993). In northeastern North America, the amplified products from A. ostoyae, but not A. gemina, should be cleaved by Bsm I or Nde I. In Europe, where both A. ostoyae and A. borealis are known, digestions with Bsm I could distinguish the two species in most cases. Although the IGS sequence for A. cepistipes differs substantially from the above three species (Anderson and Stasovski, 1992), the A. cepistipes type B restriction pattern with Alu I was the same as the A. ostoyae pattern. However, the IGS product of A. cepistipes can be distinguished from the other species tested by the presence of a Hind II restriction site.

Both A. borealis isolates sequenced by Anderson and Stasovski (1992) had the predicted A. borealis type B Alu I digestion pattern and not the type A pattern. The type A A. borealis pattern produced by Alu I digestion is identical to that of A. ostoyae, but, unlike A. ostoyae, all isolates of A. borealis lack a Bsm I restriction site in this region. However, the amplified DNA from our isolate B177 (Germany) of A. ostoyae failed to digest with Bsm I, and A. ostoyae isolate 337 (Germany) of Anderson and Stasovski (1992) had an IGS sequence lacking the Bsm I site. Thus, our A. ostoyae isolate B177 and their A. ostoyae isolate 337 would be identified as A. borealis type A using our technique. Comparisons of the IGS sequences (Anderson and Stasovski, 1992) show that the sequence of isolate 337 is similar to both A. borealis and A. ostoyae. Thus, it could be speculated that isolates B177 and 337, if accurately identified by pairings, represent evolutionary intermediates between the closely related A. borealis and A. ostoyae.

The IGS region for A. cepistipes type A and type B differed at two Alu I restriction sites. Our type A isolates had a restriction pattern consistent with published sequences of isolates 311 (Finland) and 316 (France) of Anderson and Stasovski (1992). Their isolate 304 (Finland), however, had an IGS sequence much different from that of the other two isolates of A. cepistipes they studied and had a predicted Alu I restriction pattern different from any that we tested. None of the isolates studied by Anderson and Stasovski (1992) had an IGS sequence that would result in the type B A. cepistipes pattern with Alu I digestion. There are, therefore, at least two, and possibly three, different A. cepistipes IGS types.

The distinction between A. sinapina, A. cepistipes and NABS XI remains clouded (Anderson et al., 1987; Bérubé and Dessureault, 1988; Guillaumin et al., 1989). Armillaria sinapina is known from northern North America (Bérubé and Dessureault, 1988; Shaw and Loopstra, 1988; Mallett, 1990; Blodgett and Worrall, 1992a; Harrington and Rizzo, 1993). Armillaria cepistipes is reported from Europe (Guillaumin et al., 1993), but NABS XI (group F sensu Morrison) from northwestern North America is at least partially interfertile in pairing studies with A. cepistipes and may be the same species (Morrison et al., 1985). We found only one Alu I restriction pattern among our A. sinapina isolates, and this pattern was predicted by the sequence for A. sinapina isolate 48 (New York) of Anderson and Stasovski (1992). The sequence of their A. sinapina isolate 205 (British Columbia), however, would give an Alu I restriction pattern identical to A. cepistipes type A. Unfortunately, we did not have isolates of NABS XI available for testing, nor did Anderson and Stasovski (1992) sequence the IGS region of NABS XI isolates.

The other two undescribed North American taxa of Armillaria (NABS IX and X) have unique IGS sequences (Anderson and Stasovski, 1992) and Alu I restriction patterns. It should be pointed out, however, that only a limited number of isolates of NABS IX and X were used in these respective studies.

In most cases where two Alu I restriction patterns were seen within an Armillaria species, a difference at a single restriction site would explain the discrepancy. The geographic origin of the isolates did not appear to correspond with the within-species variation, except in A. gallica. The North American A. gallica pattern is consistent with the sequence published for the North American isolates 434 (Michigan), 137 (Michigan) and 90 (Vermont) studied by Anderson and Stasovski (1992). Their sequence for the European isolate 332 (France), however, differs substantially from the North American A. gallica isolates, and the predicted Alu I restriction pattern for this isolate is consistent with the pattern obtained with our European A. gallica isolates.

The sequence of the IGS region of the North American A. gallica isolates is more similar to the North American species A. calvescens than to the sequence in the European A. gallica isolate (Anderson and Stasovski, 1992). In fact, the only difference found in the sequence of these two North American species is at a single Tha I site, but we found digestion of the IGS region with this restriction enzyme to be unreliable. Basidiomes of the two species are morphologically distinct (Bérubé and Dessureault, 1989), but these species are similar in other respects, including their weak pathogenicity and large, monopodially branched rhi-
zomorphs (Bérubé and Desjardins, 1989; Blodgett and Worrall, 1992a, b; Harrington and Rizzo, 1993; Rizzo and Harrington, 1993). In northeastern North America, where A. calvus is known to occur, A. calvus may have recently evolved from A. gallica and is better adapted to the more northern hardwood forests dominated by maple, whereas A. gallica remains more prevalent in forests dominated by oak and at locations typically south of the known distribution of A. calvus (Bérubé and Desjardins, 1989; Blodgett and Worrall, 1992a, b; Harrington and Rizzo, 1993; Rizzo and Harrington, 1993).

Only the A. tabescens isolates had amplified products of 845 bp. One isolate appeared to have an extra Alu I restriction site, but otherwise, the six isolates had the same restriction pattern. Two isolates of A. tabescens from Korea also had this pattern (data not shown). The A. tabescens isolates from Europe and North America also had the same restriction pattern with Mse I (data not shown). This near uniformity in restriction patterns suggests that A. tabescens from Europe and North America is a single species (Darmok et al., 1993), in spite of the suggestion of intersterility between isolates from the two continents (Guillaumia et al., 1993). Each of the A. mellea isolates tested had an amplified product of 875 bp and gave one of two unique Alu I restriction patterns: the pattern shown by five collections from northeastern North America and the pattern shown by the isolates from Britain and California. An isolate from Japan had the Alu I pattern of the latter group (data not shown). All A. mellea isolates tested had the same pattern when digested with Mse I (data not shown). When digested with Cfo I, the two British isolates had a pattern that differed from the other A. mellea isolates (data not shown). Thus, there was some variability in A. mellea in the IGS restriction patterns that may correspond with the geographic origin of the isolates, but the data are very limited.

More sampling would likely reveal new restriction patterns among the Armillaria species of Europe and North America, but the most common Alu I patterns were likely revealed by this study. With our limited data, it appears that a combination of Alu I digests and digestions with other select enzymes can distinguish all the recognized Armillaria species of these two continents except the North American isolates of A. gallica and A. calvus. This exception and other data point to questions concerning the evolution, biogeography and taxonomy of this important genus of tree pathogens.

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