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Armillaria species of the Olympic Peninsula of Washington state, including confirmation of North American biological species XI

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Abstract: Forty-eight collections of *Armillaria* from the Olympic Peninsula of Washington State were identified to species using the conventional pairing protocol and/or a polymerase chain reaction-based (PCR) technique utilizing restriction fragment polymorphisms (RFLPs) of the intergenic spacer region (IGS) of the ribosomal DNA. The PCR-based technique yielded results comparable to those obtained by the conventional pairings. Several restriction patterns not previously reported in North American isolates were found using the enzyme *Alu* I. Collections from eight different host species were identified as *A. ostoyae, A. sinapina, A. nabsnona, A. gallica,* or North American biological species (NABS) XI. *Armillaria ostoyae, A. sinapina,* and *A. nabsnona* were the most often collected, with the first two occurring predominantly on gymnosperms and the latter mostly on angiosperms. *Armillaria gallica* and NABS XI were collected rarely and only from angiosperms. This is only the second report of the occurrence of NABS XI.

Key Words: distribution, intergenic spacer, intersterility groups, polymerase chain reaction

INTRODUCTION

The Olympic Peninsula of Washington contains the largest tract of temperate rainforest in the world. This unique area consists of a circle of mountains, the tallest being the centrally-located Mt. Olympus at about 2400 m. On average, the area receives about 350 cm of rainfall per year on the western side of the mountains, which faces the Pacific Ocean, while about 90 cm of rain falls each year in the "rainshadows" on the eastern side. The wet coastal areas support large expanses of coniferous trees, primarily *Picea sitchensis* (Bong.) Carr. and *Tsuga heterophylla* (Rafn.) Sarg. Inland from these areas the forests are dominated by *Pseudotsuga menzesii* (Mirb.) Franco, *T. heterophylla,* and *Thuja plicata* Donn. Drier areas of the peninsula contain large tracts of *P. menzesii, Abies* spp., and *Pinus contorta* Dougl. Riparian areas along rivers and streams and canopy gaps in the coniferous forest support several species of angiospermous trees, such as *Alnus rubra* Bong., *Acer macrophyllum* Pursh, and *Acer circinatum* Pursh (Franklin and Dyrness, 1973). Many areas of old growth temperate rainforest are preserved in the Olympic National Park and Olympic National Forests.

Armillaria species are major components of the mycota around the world. Few studies have examined the occurrence of these important root pathogens in the Pacific Northwest of the United States. There are many ecological similarities between the Olympic Peninsula and Vancouver Island in Canada, as well as much of the Canadian west coast north to the coastal areas of southeastern Alaska. Shaw and Loopstra (1988) identified North American biological species (NABS) V (= *A. sinapina* Bérubé & Dessureault) and NABS IX (= *A. nabsnona* Volk & Burdsall) in coastal southeastern Alaska. Morrison et al. (1985) identified these two species from the coastal areas of British Columbia as well as *A. gallica* Marxmüller &· Romagn. and *A. ostoyae* (Romagn.) Herink. They also reported finding NABS X and XI in inland areas. Theirs was the first and only report of the collection of NABS XI, which they determined to be partially compatible with the European species *A. cepistipes* Velenovský We have already described *A. nabsnona* from western North America (Volk et al., 1996).

To date, almost all identifications of *Armillaria* species are based on the haploid mating method developed by Hintikka (1973). This technique enabled Anderson and Ullrich (1979) to determine the biological species of *Armillaria* occurring in North America. Modifications of the technique, including haploid-diploid pairings, have been used extensively for species identification (Banik et al., 1995, Blodgett and Worrall, 1992, Barrington and Rizzo, 1993, Morrison et al., 1985). This technique has several drawbacks, e.g., the extensive time required to establish

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TABLE I. Substrates and number of basidiomata collections of the *Armillaria* species occurring on the Olympic Peninsula

Substrate	*Armillaria* species				NABS
	ostoyae	*sinapina*	*gallica*	*nabsnona*	*XI*
Acer circinatum Pursh	0	0	0	1	0
A. macrophyllum Pursh	0	0	0	2	0
Alnus rubra Bong.	0	0	0	3	4
Rubus spectabilis Pursh	1	0	0	0	0
Salix scouleriana Barratt ex Hook.	1	0	1	0	0
Picea sitchensis (Bong.)Carr.	2	3	0	0	0
Pseudotsuga menziesii (Mirb.)Franco	3	0	0	0	0
Tsuga heterophylla (Rafn.)Sarg.	10	4	0	0	0
Unidentified conifer	1	1	0	0	0
Unidentified	2	5	0	3	0
Total	20	13	1	9	4

and incubate the pairings, and the sometimes ambiguous results, especially when identifying diploid isolates (Shaw and Loopstra, 1988).

Barrington and Wingfield (1995) developed a PCR-based technique with potential as a quick, reliable method for identifying *Armillaria* species from cultures, basidiomata, rhizomorphs, and colonized wood. This method relies on restriction fragment length polymorphisms (RFLPs) of the amplified intergenic spacer region (IGS) of the nuclear ribosomal DNA. By using several restriction enzymes they were able to distinguish all North American *Armillaria* species, except *A. gallica* from *A. calvescens.* However, the number of isolates used for several of the species was small.

The objectives of this study were to determine the species of *Armillaria* occurring on the Olympic Peninsula in the old growth temperate rain forest and to assess the potential for using the PCR-based identification technique described by Harrington and Wingfield (1995) as a means of identifying collections to species.

MATERIALS AND METHODS

Basidiomata collection and isolate identification using known haploids. —During 1992 and 1993, basidiomata collections were made from the old growth forests in and around Olympic National Park and Olympic National Forest on the Olympic Peninsula of Washington State. Single spore isolates were obtained from many of these collections and two single spores from each collection were identified by pairing with known haploid tester isolates, following previously published protocol (Banik et al., 1995). Information on tester isolates used is available from the authors upon request.

Tissue isolates were obtained from most of the collections by aseptically removing a small piece of stipe or cap tissue and placing it on 1.5% malt extract, 2.0% agar (MEA). Tissue isolates that had no associated single spore isolates were identified using the dip-hap pairing protocol of Rizzo and Barrington (1992). All isolates collected are on deposit in the Center for Forest Mycology Research (CFMR) culture collection maintained at the USDA Forest Products Laboratory in Madison, WI.

Isolate identification using PCR-based protocol. —The IGS region of one tissue isolate from each collection was amplified and digested with the restriction enzyme *Alu* I using the technique of Barrington and Wingfield (1995) as modified by Volk et al. (1996). The restriction patterns obtained for tissue isolates that originated from hap-hap identified collections were collated by species and used as standards for identifying isolates lacking associated haploids, via comparison of their restriction patterns. This PCR-based identification was compared to the dip-hap identification of these isolates. Any discrepancies were checked by repeating the dip-hap pairings and the PCR and restriction digests.

Basidiomata lacking associated cultures were also identified by comparing their IGS restriction patterns to those of the standards. Template DNA for each basidioma was obtained by grinding a small piece of dried gill in 500 µL TE (10mM Tris, pH 8, 1mM EDTA) and following the protocol used for mycelia from cultures (Volk et al. 1996).

RESULTS

Five *Armillaria* species, *A. ostoyae, A. sinapina, A. nabsnona, A. gallica,* and NABS XI were identified from 47 specimens collected from seven different substrates (TABLE I). Amplification of the IGS region

TABLE II. *Alu* I restriction fragment sizes of the IGS region of tissue isolates of the *Armillaria* species occurring on the Olympic Peninsula.

Species	Number of isolates	Fragment sizes (bp)[a]
A. ostoyae	15	314(309–319), 207(203–211), 141(137–145)
A. sinapina	12	401(391–410), 237(229–245), 184(177–191)
A. gallica	1	400, 235, 175
A. nabsnona (Pattern 'a')	3	553(490–615), 210
A. nabsnona (Pattern 'c')	6	556(513–598), 314(302–327), 233(221–246), 203(191–216)
NABS XI	4	413(389–436), 203(198–207), 185

[a] Fragment sizes in base pairs were interpolated from DNA size standards, using a semilogarithmic scale. Values were obtained by averaging the sizes obtained from several different gels of each fragment from each isolate. Ninety-five percent confidence intervals are shown in parentheses. Confidence intervals were not calculated for fragments with small sample sizes (one isolate of a pattern type), or for which all determinations were the same.

of 45 of the collections yielded products similar in size to those reported by Barrington and Wingfield (1995) of 920 bp. Twenty-seven of these collections were identified using pairings of haploid mycelia and formed the basis for the PCR-based identification technique (TABLE II, FIG. 1). Fourteen collections identified from dip-hap pairings were checked using the PCR technique. Two discrepancies occurred between the pairing and PCR techniques. Repeating both methods indicated the original pairings had been incorrectly interpreted. Four basidiomata collections were identified using solely the PCR-based technique.

DISCUSSION

Of the collections identified, *A. ostoyae* was the most common, comprising 43% of the collections. *A. ostoyae* was found almost exclusively on coniferous hosts, especially *Tsuga heterophylla* (TABLE I). The association of *A. ostoyae* with conifers is similar to the findings of Morrison et al. (1985) in British Columbia and for studies in other areas of North America (Blodgett and Worrall, 1992, Barrington and Rizzo, 1993, Mallett, 1990). The fragment sizes of the IGS region digested with *Alu* I of the *A. ostoyae* isolates approximate those reported by Barrington and Wingfield (1995) of 310,200, and 135 base pairs (bp) (TABLE II, FIG. 1).

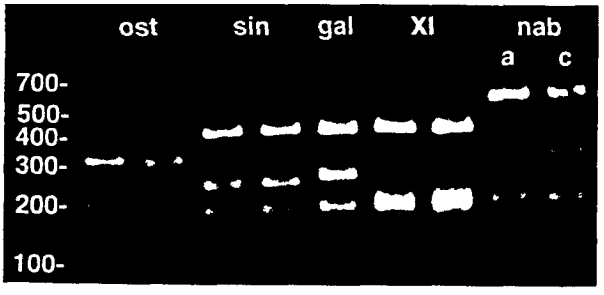

FIG. 1. Representative patterns of *Alu* I digestion products of the IGS region of *Armillaria ostoyae* (ost), *A. sinapina* (sin), *A. gallica* (gal), NABS XI, and *A. nabsnona* (nab, Patterns "a" and "c") occurring on the Olympic Peninsula. Size markers in base pairs are shown on the far left. The digital image shown is from a photograph of a single gel.

Armillaria sinapina was the second most often collected species, comprising 28% of the collections. It was collected only from *Picea sitchensis* and *Tsuga heterophylla* (TABLE I), which is in contrast to the results of Morrison et al. (1985) in British Columbia, where they collected this species predominantly from deciduous substrates. *Armillaria sinapina* in other parts of North America has also been associated more often with deciduous substrates (Dumas, 1988, Barrington and Rizzo, 1993, Mallett, 1990). This may represent a distinct ecological difference between isolates of this species from the Olympic Peninsula and those from other locations. The restriction pattern for *A. sinapina* was different than the 399, 200, and 135 bp fragment sizes reported by Barrington and Wingfield (1995) (TABLE II, FIG. 1). However, they did not test isolates from the Pacific Northwest. The restriction pattern was also different from that hypothesized by Barrington and Wingfield (1995) for an *A. sinapina* isolate from British Columbia based on sequence data reported by Anderson and Stasovski (1992). According to the sequence data this isolate should exhibit *Alu* I banding pattern of 399, 200, and 183 bp, the same as *A. cepistipes* type A reported by Harrington and Wingfield (1995).

Armillaria nabsnona was the third most common species, comprising 19% of the collections. This species was found primarily on deciduous substrates especially *Alnus rubra* (TABLE I). This is similar to what Morrison et al. (1985) and Volk et al. (1996) report-

ed for this species. Two different banding patterns were found in *A. nabsnona* tissue isolates (TABLE II, FIG. 1). One of the fragment patterns observed (Pattern-a) matched the 534, 200 bp pattern reported by Barrington and Wingfield (1995) and the other (Pattern-c) was previously reported by Volk et al. (1996) in their description of the species. A third pattern, pattern-b, reported by Volk et al. (1996) was found only in single spore isolates. See Volk et al. (1996) for an in depth discussion of the *Alu* I restriction patterns present in *A. nabsnona*.

Armillaria gallica was collected once from *Salix scouleriana* (TABLE I). The *Alu* I pattern for this isolate was significantly different from the 582, 240 bp fragment sizes reported by Harrington and Wingfield (1995) for North American *A. gallica* (TABLE II, FIG. 1). However it did approximate the 399, 240, 183 bp pattern they reported for European *A. gallica.* The restriction pattern of the Olympic Peninsula *A. sinapina* isolates was statistically inseparable from that of the *A. gallica* isolate, although visually the patterns were slightly different. Barrington (pers. comm.) did not detect differences in restriction patterns between isolates of these two species from the west. Attempts to further distinguish *A. gallica* and *A. sinapina* by restricting IGS PCR products with *Hinc* II, *Hae* III, *Pst I, Msp I, Rsa I, Sau3A I, Bsm I, Tha I* , *Nde* and *Bgl II* were unsuccessful.

Single spore isolates from four collections were incompatible with testers of any of the eight NABS identified by Anderson (1986). These collections were found to be compatible with one another and to possess different mating type alleles, indicating they originated from different genets. Pairings of 4 testers of these collections (one single spore from each collection) with 4 single spore isolates of Morrison's group XI isolate 82–14, resulted in 9 out of 16 compatible pairs, 3 of 16 questionable, and 4 of 16 negative. Therefore, these four collections were identified as NABS XI. Because of NABS XI's reported compatibility with European *A. cepistipes* (Morrison et al., 1985), the 4 new NABS XI testers were paired with 5 *A. cepistipes* testers (provided by J. J. Guilluamin). This resulted in 12 of 20 compatible pairs, 4 of 20 questionable, and 4 of 20 negative pairings. The four negative pairings are somewhat suspect since they all involved the same *A. cepistipes* isolate, which also failed to demonstrate compatibility with any of the other *A. cepistipes* isolates. Thus, the report by Morrison et al. (1985) of NABS XI's compatibility with *A. cepistipes* is substantiated.

Anderson et al. (1980) reported compatibility between *A. cepistipes* and NABS X. In routine screening of isolates, 14 single spore isolates of NABS XI, some from each of the four Olympic Peninsula collections, were paired with 10 single spore isolates of NABS X from 7 different collections resulting in 48 unique pairings. In these pairings one was rated positive, two questionable, and the remainder negative. Based on these pairings, the assertion of Morisson et al. (1985) that NABS XI is incompatible with NABS X is sup ported. The relationships between NABS X, XI, and *A. cepistipes* are currently being examined more thoroughly using molecular biological techniques.

Our four NABS XI collections were all from *Alnus ruin-a,* which is in agreement with the collection of this species on deciduous hosts by Morrison et al. (1985) (TABLE I). The fragment sizes obtained from the *Alu* I digest of the IGS region for NABS XI approximate the 399, 200, 183 bp sizes reported for *A. cepistipes* type A by Harrington and Wingfield (1995) (TABLE II, FIG. 1).

The *Armillaria* species found on the Olympic Peninsula are the same as those reported from British Columbia (Morrison at al. 1985), except that NABS X was not found. The existence of NABS XI is corroborated and additional isolates of this species are now available for study.

The PCR based identification method proposed by Barrington and Wingfield (1995) is definitely useful for identifying isolates from a limited geographical area, once a baseline using known isolates has been established. The technique is much faster than using pairings for identification and the results are less ambiguous, especially compared to dip-hap pairings. To separate the species of *Armillaria* occurring on the Olympic Peninsula, digestion of the amplified IGS region with only one enzyme, *Alu* I, was necessary. For each species, a single banding pattern was exhibited in the amplified IGS region when restricted with *Alu*- I, except for *A. nabsnona,* which had two different patterns (TABLE II, FIG. 1). The patterns for *A. ostoyae, A. nabsnona,* and NABS XI were easily distinguished from each other and from those of *A. gallica* and *A. sinapina,* which were very similar. The finding of restriction patterns other than those reported by Barrington and Wingfield (1995) for several of the North American species suggests that more work is needed before the technique can be applied reliably to the identification of *Armillaria* isolates from widely distributed geographical areas. This work has contributed significantly to the database required to make such identifications possible.

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