

Phylogeny and genetic diversity of *Bridgeoporus nobilissimus* inferred using mitochondrial and nuclear rDNA sequences

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Abstract: The genetic diversity and phylogeny of *Bridgeoporus nobilissimus* have been analyzed. DNA was extracted from spores collected from individual fruiting bodies representing six geographically distinct populations in Oregon and Washington. Spore samples collected contained low levels of bacteria, yeast and a filamentous fungal species. Using taxon-specific PCR primers, it was possible to discriminate among rDNA from bacteria, yeast, a filamentous ascomycete and *B. nobilissimus*. Nuclear rDNA internal transcribed spacer (ITS) region sequences of *B. nobilissimus* were compared among individuals representing six populations and were found to have less than 2% variation. These sequences also were used to design dual and nested PCR primers for *B. nobilissimus*-specific amplification. Mitochondrial small-subunit rDNA sequences were used in a phylogenetic analysis that placed *B. nobilissimus* in the hymenochaetoid clade, where it was associated with *Oxyporus* and *Schizopora*.

Key words: *Abies procera*, Basidiomycete, conk, fungi, Fuzzy Sandoze, hymenochaetoid clade, molecular systematics, Noble fir, Noble polypore, old-growth forest, *Oxyporus*, Polyporaceae

INTRODUCTION

The fungus *Bridgeoporus* (= *Oxyporus*) *nobilissimus* (W.B. Cooke) Volk, Burdsall & Ammirati is a peren-

nial polypore that produces large basidiomata, or fruiting bodies, on the root crowns and trunks of living trees, snags and stumps. This species has been reported most commonly on large-diameter (at least 1 m) *Abies procera* (Noble fir), infrequently on *Abies amabilis* (Pacific silver fir) and possibly on *Tsuga heterophylla* (western hemlock), typically fruiting within 1 m of the ground. Unlike many polypores, *Bridgeoporus* has not been observed on logs, with the exception of one blow-down with intact roots, where the basidioma developed from the upturned root system/crown (Ammirati, pers comm, 1997). Endemic to Oregon and Washington, populations of *B. nobilissimus* have been identified in the Cascade, Coast and Olympic ranges at elevations of 1000–4000 feet (Hibler and O'Dell 1997, Castellano et al 1999).

The fruiting bodies of *B. nobilissimus* demonstrate three general shapes, depending largely on their location on the host. Hoof-shaped and shelf-like conks are found on the sides of hosts. Short, oblong-topped conks with tapering pore surfaces are found growing on the main roots of the host and centrally substipitate conks are located on the tops of stumps (Hibler and O'Dell 1997).

A description of macro- and microscopic morphological characteristics by Burdsall et al (1996) includes: The basidioma is characterized by a fibrous pileal surface with cracks and crevices from which vascular plants (e.g., *Oxalis* sp. and pteridophytes) and bryophytes often grow epiphytically. The pileal surface is covered with a dense mat of white mycelial fibers in young basidiomata, often somewhat agglutinated at the tips, which become darker with age, reaching several mm in length. These fibers often are green, due to epimycotic associations with several species of algae, including *Coccomyxa* sp. and *Charicium* species. *B. nobilissimus* lacks clamp connections at the septa and has a monomitic hyphal system with pseudocystidia arising from the trama. The spores are 5.5–6.5 × 3.5–4.5 μm, broadly ovoid, hyaline, smooth, thin-walled and inamyloid. The round pores are stratified with a layer of sterile tissue 2–3 mm thick between successive annual pore layers.

This polypore initially was named *Oxyporus nobilissimus* by William Bridge Cooke in 1949. A study by Burdsall et al (1996) identified characteristics of *O. nobilissimus* that were incompatible with the genus

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TABLE I. DNA isolates used in this study. Accession numbers AF508328–AF509233 designate ITS1/ITS2 rDNA sequences while Accession numbers AF509234–37 designate mitochondrial ssu rDNA sequences. DNAs may be obtained from Rusty Rodriguez at the Western Fisheries Research Center, Biological Resources Division, USGS, 6505 N.E. 65th St, Seattle, WA 98115

Sample	Accession numbers	Source of DNA	Location of conk
ac-1	AF508328	spores	Asahel Curtis, King Co., WA
ac-2	AF508329		
rn-a	AF508337	spores	Mt. Rainier National Park,
rn-b	AF508338, AF509235		Pierce Co., WA
gm-a, gm-b, gm-d, gm-f,	AF508330–3	spores	Goat Marsh, Mt. St. Helens,
gm-g	AF508334, AF509234		Cowlitz Co. WA
lm-a	AF508335	spores	Larch Mt., Multnomah Co.,
lm-g	AF508336		OR
wm ^a		spores	Wildcat Mt. Clackamas Co.,
			OR
sp-(b1-3), sp-d, sp-e, sp-i,	AF508339–43	spores	Snow Peak, Salem BLM Dist,
sp-k, sp-g, sp-m ^a	AF508345–7		Linn Co., OR
sp-h	AF508344, AF509236		
Bacterial and yeast iso-		Bacterial and yeast cultures	All locations listed above
lates ^a		from all spore collections	
gm-fy	AF509231	Culture from gm-f spore col-	Goat Marsh, Mt. St. Helens,
		lection	Cowlitz Co. WA
st-2	AF509232, AF509237	Mycelia cultured from spores	Mt. Rainier National Park,
tc-a ^a		and tissue of conks rn-a and	Pierce Co. WA
tc-b	AF509233	rn-b by Paul Stamets	

^a Accession number not available.

Oxyporus and could find no other described genus appropriate for this species. Although the stratification of tube layers and context is a unique characteristic shared by *O. populinus* and *O. nobilissimus* (Cooke 1949, Gilbertson and Ryvarden 1987 p 499), all species of *Oxyporus* have true cystidia arising from the subhymenium, whereas *O. nobilissimus* has pseudocystidia of tramal origin. Burdsall et al (1996) considered the genus *Rigidoporus* the “closest placement” for *O. nobilissimus* because of these shared characteristics: pseudocystidia of tramal origin, thin- and thick-walled hyphae and similar basidium and basidiospore shape and size. Both *Rigidoporus* and *Oxyporus* cause a white rot in which lignin as well as other wood components are decomposed, whereas *O. nobilissimus* consistently has been associated with brown rot (Burdsall et al 1996), in which lignin is not appreciably degraded (Boddy and Watkinson 1995). However, the true rot characteristics of this species remain unresolved because efforts to culture *O. nobilissimus* have been unsuccessful to date (Cooke 1949; Burdsall et al 1996; Mueller G., pers comm, 1997). Because of these incompatibilities, the genus *Bridgeoporus* was created to accommodate *O. nobilissimus* (Burdsall et al 1996).

Bridgeoporus nobilissimus was placed on the Oregon Natural Heritage program's endangered species list in 1995 (as *Oxyporus nobilissimus*), making it

one of the first fungi to be so listed by any private or public agency in the United States (Lizon 1995). As of 2000, 81 sporocarps of *B. nobilissimus*, many of them in various states of decline, had been located at 12 sites (Hibler and O'Dell 1997; Castellano et al 1999; Cowden, pers comm, 2002). Genetic studies of *B. nobilissimus* have not been reported nor have the spores been germinated successfully under laboratory conditions to date.

The purpose of this study was to conduct a genetic analysis of spores from 22 individual *B. nobilissimus* sporocarps in six populations in the Pacific Northwest (TABLE I) and to develop a diagnostic system for detecting *B. nobilissimus* in wood or soil substrates. Nuclear rDNA was sequenced for genetic analysis to develop *Bridgeoporus*-specific polymerase chain reaction (PCR) primers and to examine relationships between *B. nobilissimus* spore DNA and the DNA of a putative filamentous fungus cultured from *B. nobilissimus* tissue and spore drops.

To clarify the relationship between *B. nobilissimus* and fungi from several other genera, the mitochondrial small-subunit rDNA was sequenced and this data was subjected to parsimony analysis.

METHODS

Sample collection and culture.—Spores were collected between 20 Aug 1997 and 28 Oct 1997 from 22 fruiting bodies

of *B. nobilissimus* in Washington and Oregon (TABLE I) by covering the exposed hymenium with aluminum foil for 24–48 h. In the laboratory the spores were washed off the foil into centrifuge tubes with sterile de-ionized distilled water. The spores then were centrifuged at $14\,000\times g$ for 5 min, and much of the supernatant was removed. After re-suspending, a sterile cotton tip was used to culture any contaminants on potato dextrose and Modified Mathur's Medium (M_3S) (Tu 1985) agar plates that were incubated at 25 C. The isolated colonies (referred to as spore-drop cultures) were cultured further in Luria Broth, M_3S and SOC broth.

Control samples of *Fomitopsis pinicola*, *Ganoderma applanatum* and *G. oregonense* were collected from conks in the South Fork of the Hoh River watershed in Olympic National Park. Tissue samples (approximately 0.5 cm^3) from the growing hymenium of each species were placed in 1.5 mL microcentrifuge tubes containing 0.5 mL of a DNA preservative (150 mM EDTA, 50 mM Tris pH 8.0, 2.0% n-lauroylsarcosine), and the DNA was extracted as below. Paul Stamets (Fungi Perfecti, P.O. Box 7634, Olympia, WA) provided three mycelial cultures derived from spores and tissue of *B. nobilissimus* for sequencing (TABLE I).

DNA extraction.—DNA was extracted from the mycelial cultures using Rodriguez's (1993) method. DNA was extracted from the spore samples using Bio 101 DNA FastPrep kit and a modification of Rodriguez's (1993) method. Approximately 100 μL of an aqueous spore suspension were placed in each Bio 101 homogenization tube containing 250 mg of 0.8 mm zircon beads with 2.0 mm spheres. Eight hundred μL of CLS-Y buffer were added and the tubes placed in the FastPrep cell disrupter for three cycles at speed 5.0 for 40 s/cycle. Tissue samples from the hymenium of control fungi were pulverized with a small pestle and incubated at 65 C for 30 min, then processed along with spore samples. Tubes were centrifuged for 5 min at $14\,000\times g$ and the supernatant transferred into 1.5 mL microcentrifuge tubes. 0.7 volumes of PEG/NaCl (20% polyethylene glycol mw 8000, and 2.5 N sodium chloride) solution were added, mixed thoroughly and iced 5–20 min to precipitate nucleic acids, proteins and polyphosphates. Tubes were centrifuged for 5 min at $14\,000\times g$, decanted, and pellets resuspended in 0.5 mL TE buffer (10 mM Tris pH 8.0, 1.0 mM EDTA). Any scum present on the surface of the supernatant was retained with the pellet. After the pellets dissolved, 0.5 volumes 7.5 N ammonium acetate were added and tubes were incubated in ice 5–20 min. Protein, RNA and polyphosphates, etc. were pelleted at $14\,000\times g$ for 5 min. The supernatant was transferred, 0.6 volumes of isopropyl alcohol added, and samples gently mixed and incubated in ice for 5–30 min to precipitate DNA. DNA was pelleted for 5 min at $14\,000\times g$ and resuspended in 0.5 mL TE Buffer. DNA was reprecipitated by the addition of NaCl to 0.1 M and two volumes of chilled 95% ethanol. Samples were incubated in ice for 5–30 min, DNA pelleted at $14\,000\times g$ for 5 min and resuspended in 0.5 mL TE Buffer. The size of the DNA was assessed by electrophoresis in a 0.7% agarose gel. The gels were stained with ethidium bromide and DNA visualized with 305 nm ultraviolet light (Sambrook et al 1989).

Bacterial and fungal DNA from sporedrop cultures were extracted in a manner similar to that of the spores. This exception applied: Instead of using the cell disrupter, the cultures were centrifuged and the pellets treated with 300 μL lysis buffer (EDTA 150 mM, Tris pH 8.0 at 50 mM, Sarkosyl 2.0%, Protease, and water to volume) vortexed for 1–5 s, and heated at 65 C. After 15–30 min the cultures were centrifuged at $14\,000\times g$ for 5 min and the supernatant transferred to a new 1.5 mL tube and the DNA purified as described above.

PCR amplification.—The ITS regions of nuclear rDNA of all samples were PCR amplified with primer sets ITS1/ITS4 (White et al 1990), ITS1-F/ITS4-B (Gardes and Bruns 1993) and Eubac27F/Eubac492R (DeLong 1992) to distinguish between ascomycete, basidiomycete and eubacterial DNA. PCR reactions were performed in 20 μL reactions containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM MgCl_2 , 0.2% Triton X-100, 200 μM each of dATP, dCTP, dGTP, dTTP (Pharmacia), 0.2 units *Taq* DNA polymerase, 250 ng of each oligonucleotide primer, 20–200 ng of fungal genomic DNA.

Amplifications involved 35 cycles of this temperature regime: Denaturing at 93 C for 15 s, annealing at 64 C for 1.5 min and synthesis at 72 C for 1.5 min. No ramp times were imposed from denaturing to annealing temperatures or from annealing to synthesis temperatures. The cycles were preceded by 2 min of denaturation at 93 C.

PCR primers MS1 and MS2 (White et al 1990) were used to amplify the mitochondrial small-subunit rDNA from *B. nobilissimus* spore samples and from mycelia of st-2 samples. The protocol was the same as described above, except that the annealing temperature was 60 C.

Electrophoresis of PCR-amplified products was performed in 2.0% agarose gels for 1.5 h at 7.0 V/cm^2 (Sambrook et al 1989). PCR products were stained with ethidium bromide and visualized with 305 nm ultraviolet light.

Sequence analysis and design of diagnostic primers.—The QIAEX II Agarose Gel Extraction Protocol (Qiagen Inc., 28159 Avenue Stanford, Valencia, CA 91355) was used to purify the ITS1-F/ITS4-B and MS1/MS2 PCR products that then were quantified by fluorescence and sequenced by the chain termination method (Sanger et al 1977). ITS sequences were aligned using Sequencher[®] to generate consensus sequences. The consensus sequences for *B. nobilissimus* and the mycelial cultures (st-2, tc-a and tc-b) were aligned and primers were designed from regions of the sequences showing the greatest differences between these organisms. The *B. nobilissimus* primers Bn215-118 (ATG TGCTCGTTGCCCTTGAC) and Bn217-190 (AGTTGTCCG AAGACGATTAG) were located 118 and 190 bp from primers ITS1-F and ITS4-B, respectively. The primers for the mycelial cultures, St215-184 (GGTCTTAATCGACTCTGCTC) and St217-35 (GGACGATTGGAAGCCGAATA) were located 184 and 35 bp from ITS1-F and ITS4-B, respectively. Sequences of *B. nobilissimus* can be obtained from GenBank with the access numbers shown in TABLE I.

Nested primer PCR.—To perform nested primer PCR (npPCR), dual primer reaction products amplified with

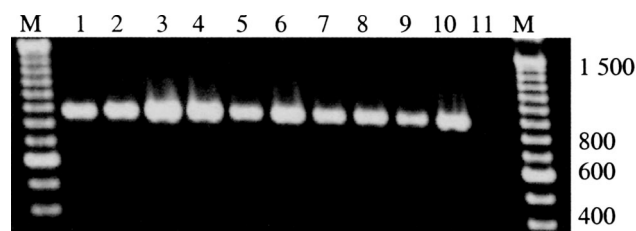


FIG. 1. PCR amplification of *Bridgeoporus nobilissimus* isolates with ITS1-F/ITS4-B rDNA primers. Lanes 1–10 contain the following individual samples from four populations in Oregon and Washington: ac-1, wm, lm-a, sp-b1, sp-b3, sp-d, sp-e, sp-g, sp-i, sp-k. Lane 11 is a no DNA control and lanes M contain 100 bp DNA ladders with some sizes in bp shown at right.

ITS1-F/ITS4-B primers were diluted from 100× to 10 000× with 10 mM Tris pH 8.5 buffer and 2 µL transferred to a 0.5 mL microcentrifuge tube containing 18 µL fresh PCR reaction buffer and primers Bn215-118/Bn217-190 or St215-184/St217-35. The thermocyclers were programmed similarly to the dpPCR reactions, except the annealing temperatures were 68 C.

Phylogenetic analysis.—To evaluate the phylogenetic relationships of *B. nobilissimus*, mt-ssu rDNA sequences obtained from three spore preparations (gm-g, rn-b, sp-h) and one putative mycelial isolate (st-2) were added to a reference dataset containing 85 species of homobasidiomycetes, emphasizing wood-decaying taxa. The heterobasidiomycetes *Auricularia auricula-judae* and *Dacrymyces chrysospermus* were included for rooting purposes. All species other than *B. nobilissimus* were represented by sequences of nuclear small-subunit rDNA (ca 1.8 kb) as well as the mt-ssu rDNA. Sequences in the reference dataset were used in a study on the evolution of wood-rotting homobasidiomycetes (Hibbett and Donoghue 2001), which should be consulted for strain information and accession numbers.

Sequences were manually aligned to the reference dataset, and divergent regions were excluded from analysis. Phylogenetic analyses in PAUP*4.0 (Swofford 1999) used equally weighted parsimony. An initial search with 1000 heuristic searches was performed, with random taxon addition sequences and TBR branch swapping, keeping up to 10 trees per replicate. Trees found in the initial search were used as input trees for TBR branch swapping with MAXTREES set to autoincrease. Bootstrap analysis used 1000 replicates, with one heuristic search per replicate. Each bootstrap replicate used a single random taxon addition sequence with TBR swapping, keeping up to 10 trees.

RESULTS

Microscopic composition of spore samples.—Microscopic observations of the spore samples revealed at least four different cell morphologies indicating contamination by other organisms (data not shown). Spores appearing very similar in shape and size to the description of *Fomitopsis pinicola* spores, as detailed by

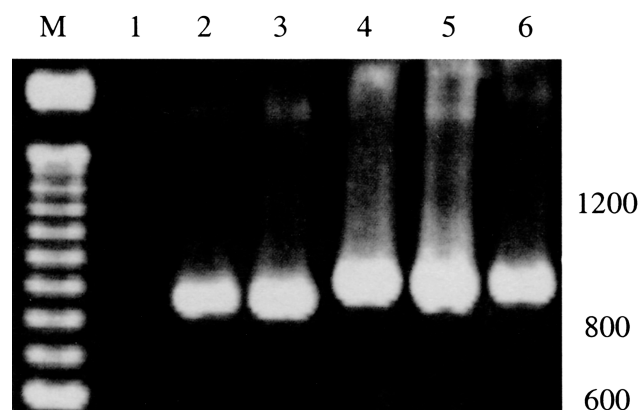


FIG. 2. ITS1-F/ITS4-B PCR amplification of *Fomitopsis* sp. cultures isolates tc-a and tc-b (lanes 2 and 3) and *Bridgeoporus nobilissimus* isolates gm-b, rn-b and sp-b2 (lanes 4–6). Lanes M and 1 are a 100 bp DNA ladder and no DNA control, respectively.

Florance and Shaw (1988), were noted frequently. Culture analysis indicated that each of the spore samples contained low levels of bacteria and yeast (data not shown). Although these cultures were not defined taxonomically, they were used to extract genomic DNA for PCR analysis of rDNA (see below). In addition, only one filamentous fungus (st-2, tc-a, tc-b) was cultured from the spores and tissue of *B. nobilissimus* (Paul Stamets unpubl) and it was characterized by rDNA sequence analysis (described below).

Nuclear rDNA sequence analysis.—Microscopic analysis indicated that either the physical separation of spores from contaminating organisms or the germination of *B. nobilissimus* spores would be required for the extraction of pure *B. nobilissimus* DNA. However, efforts to accomplish either were unsuccessful. Therefore, DNA from spore samples and from the spore-drop cultures was PCR amplified with primers specific to the rDNA of ascomycetes (ITS1/ITS4, White et al 1990), basidiomycetes (ITS1-F/ITS4-B, Gardes and Bruns 1993) and eubacteria (Eubac27F/Eubac492R, DeLong 1992) for comparative analysis.

An 850 bp product was amplified from all 22 *B. nobilissimus* samples from six populations with the primers ITS1-F/ITS4-B (FIG. 1). The mycelial cultures (st-2, tc-b) also amplified with ITS1-F/ITS4-B producing an 800 bp product (FIG. 2). When amplified with the primer set ITS1/ITS4 (FIG. 3A, B) and with the primer set Eubac27F/492R (FIG. 4), *B. nobilissimus* spore DNA produced products that were similar in size to those produced by the yeast and bacterial spore-drop cultures. However, with one exception (gm-fy), the yeast and bacterial cultures did not amplify with the Basidiomycete primers ITS1-F/ITS4-B. Sequence analysis of this anomalous 850 bp

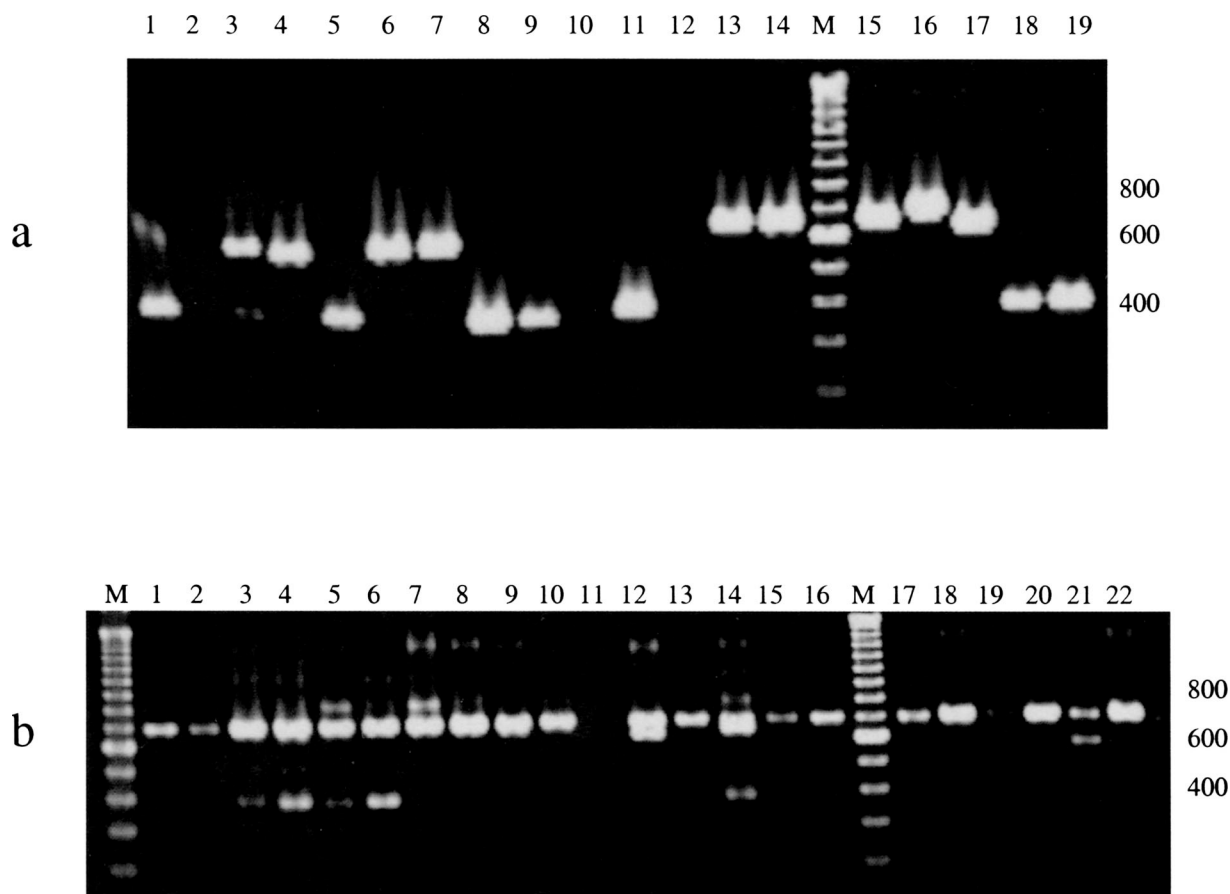


FIG. 3. Yeast isolates cultured from *Bridgeoporus nobilissimus* spore samples produced a product ranging in size from 350 to 675 bp when amplified with the primers ITS1/ITS4 (photo a; lanes 1–19). *Bridgeoporus nobilissimus* isolates (photo b; lanes 1–22: ac-1, ac-2, rn-a, rn-b, gm-a, gm-b, gm-d, gm-f, gm-g, lm-a, lm-g, wm, sp-b1, sp-b2, sp-b3, sp-d, sp-e, sp-g, sp-h, sp-i, sp-k, sp-m) amplified with ITS1/ITS4 primers produced a more consistent product that included bands mirroring the yeast bands. Lanes M are 100bp DNA standards.

yeast product (GenBank accession # AF509231) showed no similarity to the *B. nobilissimus* sequences.

Sequence analysis of the ITS1 regions revealed 98% identity between 20 *B. nobilissimus* isolates from all geographic areas. The poor quality of sequences

from isolates wm and sp-g prevented their inclusion in the ITS analysis. Specific sequence variation was observed in samples lm-a, rn-a, and ac-1 at bp 132 and in sample sp-m with bp 52, 53, 97 and 148 upstream of the 18S subunit.

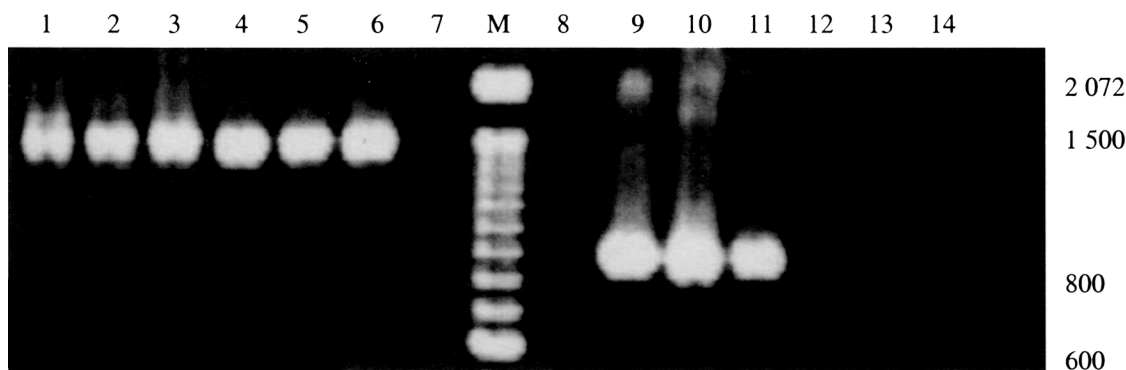


FIG. 4. Eubac27F/492R PCR amplification of *Bridgeoporus nobilissimus* (lanes 1–3:gm-b, rn-b, sp-b2) and three bacterial cultures (lanes 4–6) isolated from *B. nobilissimus* spore samples. In lanes 9–14 the above samples, in the same order, were amplified with ITS1-F/ITS4-B. Lanes 7 and 8 are no DNA controls and lane M is a 100 bp standard.

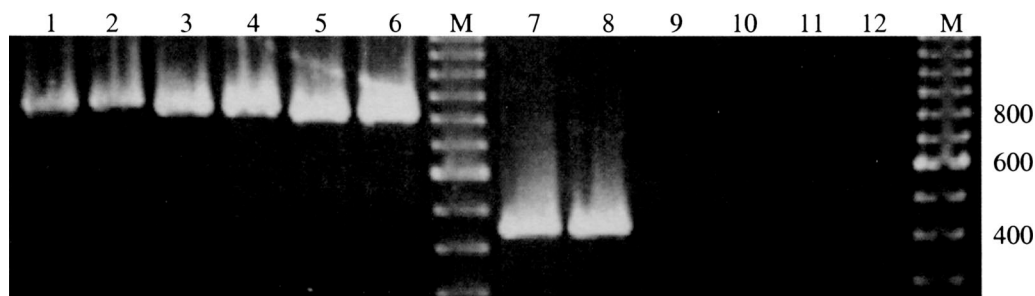


FIG. 5. PCR amplification with ITS1-F/ITS4-B (lanes 1–6) and Bn215-118/217-282 (lanes 7–12) rDNA primers demonstrating specificity of the *Bridgeoporus* primer. These isolates are represented: *B. nobilissimus* rn-b and gm-b (lanes 1, 2 and 7, 8), *Fomitopsis* sp. st-2, *F. pinicola*, *Ganoderma oregonense* and *G. applanatum* (lanes 3–6 and 9–12). Lanes marked M are 100 bp DNA standards.

The ITS2 region of 19 isolates from all geographic areas displayed 100% identity. Sequences from wm, sp-m and gm-g were not included in the ITS2 analysis due to poor sequence quality. A BLAST analysis with the *B. nobilissimus* ITS sequences revealed no similarities with other taxa in GenBank.

The ITS2 region of the mycelial cultures (st-2 and tc-b) had 98.5% identity with *Fomitopsis pinicola*. The ITS1 region of *F. pinicola* was not found in the database, and no comparison could be made. As a result, the mycelia of st-2 and tc-b cultures were relabeled as a *Fomitopsis* sp.

Fidelity of taxon-specific primers.—Nested PCR reactions, with the primer sets ITS1-F/ITS4-B and Bn215-118/Bn 217-190, specifically amplified *B. nobilissimus* and did not amplify DNA from other polypores or the *Fomitopsis* sp. cultures (FIG. 5). In addition, npPCR allowed *B. nobilissimus* DNA to be detected at concentrations of 100 pg/ μ L (FIG. 6). The *Fomitopsis* sp.-specific primer set St215-184 and St217-35 detected *Fomitopsis* sp. at concentrations of 1 pg/ μ L (data not shown) and did not amplify *B. nobilissimus* or other polypore DNA, including *F. pinicola* (FIG. 7). Thus, although this culture (st-2) likely represents a species of *Fomitopsis*, it probably is not *Fomitopsis pinicola*.

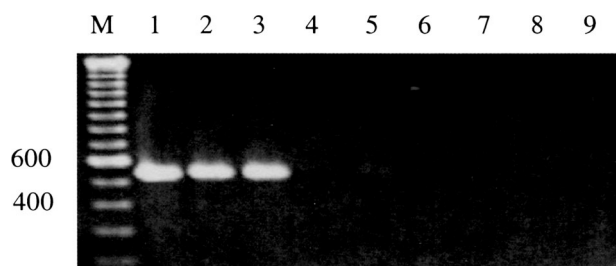


FIG. 6. Dilution series of *Bridgeoporus nobilissimus* DNA in PCR's demonstrating sensitivity of primer Bn215-118/217-190. Isolate gm-f is shown in lanes 1–8 with tenfold DNA dilutions ranging from 10ng/ μ L to 1fg/ μ L, respectively.

Phylogenetic analysis.—Equally weighted parsimony analysis resulted in 13 equally parsimonious trees (5011 steps, CI = 0.309, RI = 0.479; FIG. 8). The sequences from the three spore preparations (gm-g, rn-b, sp-h) were strongly supported as monophyletic (bootstrap = 100%; FIG. 8) and are nested in the hymenochaetoid clade, which is weakly supported (bootstrap = 63%). The closest relatives of the *B. nobilissimus* sequences were *Schizopora paradoxa* and *Oxyporus* sp. The sequence from the *Fomitopsis* sp. (st-2) was nested in the polyporeoid clade, where it is part of a strongly supported clade (bootstrap = 100%) that also included *Daedalea quercina* and *Fomitopsis pinicola*.

DISCUSSION

Bridgeoporus nobilissimus is a complex symbiotic organism. The perennial fruiting body occurs on living and dead trees, where the hirsute pileus is inhabited by vascular plants and bryophytes. The dense mycelial fibers on the pileal surface have epimycotic associations with several species of algae including *Coccomyxa* sp. and *Charicium* spp (Burdall et al 1996). Participants in the 1996 study also noted several variations on cell morphologies during microscopic analysis of *B. nobilissimus* spore samples (Volk, T., pers comm, 1997). Therefore, it is not surprising that the spore samples of the current study contained other fungi, yeast and bacteria. While there is significant evidence of bacteria initiating basidiome formation (Rainey 1991) and stimulating germination of spores (Mayo et al 1986, Ali and Jackson 1989) in other fungi it is not yet known what role these microorganisms play in the biology of *B. nobilissimus*.

Although the intermingling of organisms has made the study of this polypore challenging, the use of taxon-specific primers to differentially amplify DNA mixtures is effective (Camacho et al 1997) and enabled us to analyze genetically *B. nobilissimus*. The regions

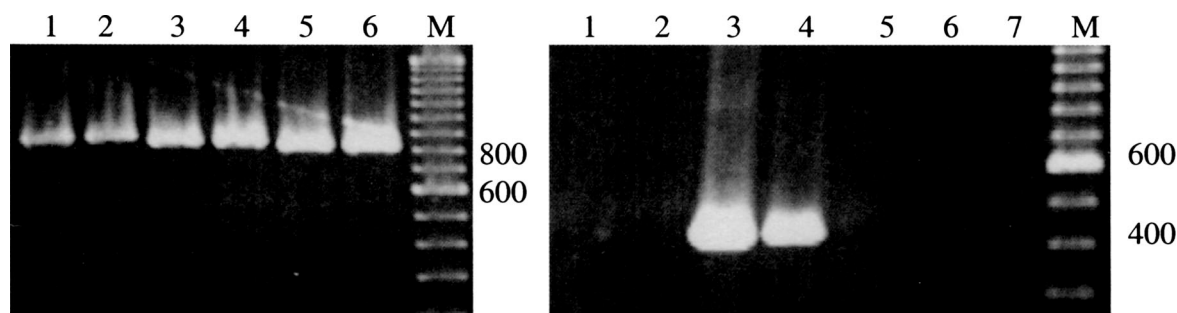


FIG. 7. PCR amplification with ITS1-F/ITS4-B (left photo) and St215-184/217-35 (right photo) rDNA primers demonstrating specificity of the *Fomitopsis* sp. primer. In the photo on the left, these isolates are represented in lanes 1–6: *Bridgeoporus nobilissimus* rn-b and gm-b, *Fomitopsis* sp. st-2, *F. pinicola*, *Ganoderma oregonense* and *G. applanatum*. The right photo contains these samples: *B. nobilissimus* isolates gm-f, ac-2 (lanes 1, 2), *Fomitopsis* sp. isolates st-2, tc-b (lanes 3, 4) and *F. pinicola*, *Ganoderma applanatum* (lanes 5, 6). Lane 7 is a no DNA control and lanes M are 100 bp DNA standards.

of the nuclear genome coding for the ribosomal subunits have evolved at different rates. While the areas coding for the large and small subunits have been highly conserved among eukaryotes, the spacer regions between the subunits are highly variable among morphologically distinct fungal species. And yet, in these same spacer regions, intraspecific variation is low (Hillis and Dixon 1991, Gardes and Bruns 1993, Redecker et al 1997). Distinguishing among taxa was made possible by using primers embedded in the highly conserved regions. These same primers amplified the highly variable spacer regions that we were able to sequence and thus detect genetic differences among individual sporocarps.

Sequence analysis of both the ITS regions and the mitochondrial small rDNA region indicated that all of the fruiting bodies from which spore samples were obtained are of the same species. Because *B. nobilissimus* is an endangered species, we did not harvest any tissue samples and thus no comparison was made between spore and tissue DNA. However, the likelihood of the same contaminant being present on all 22 widely separated conks is remote. Morphological variation of the fruiting bodies might be due to differences in the location of the sporocarp on the substrate, nutritional inconsistencies or variation in types of colonizing organisms. It also is clear from the ITS sequencing that the three mycelial cultures (st-2, tc-a, tc-b) derived from tissues and spore-drops of *B. nobilissimus* are probably a species of *Fomitopsis* (FIG. 8).

The Forest Ecosystem Management Assessment Team (FEMAT) Report 1993 (p. IV 86–89) stated the need for information on old-growth associated fungi of the Pacific Northwest. It specifically noted that a long-term study of *Oxyporus nobilissimus* should be initiated and that it should include distance and effectiveness of spore dispersal. Such a study would re-

quire a molecular diagnostic system to detect *B. nobilissimus* in its vegetative stage.

The vegetative stage of a polypore may grow for a considerable span of time as it accumulates resources to form its large fruiting bodies (Gilbertson and Ryvarden 1986, p 5). In the case of *B. nobilissimus*, which is found only on ancient trees, this stage could span centuries, perhaps the entire life of the tree. During the vegetative stage it is not identifiable without the use of molecular techniques. A useful diagnostic system to determine the location of *B. nobilissimus* mycelium before it develops the sporocarp is the *B. nobilissimus*-specific PCR primer set. This primer set will enable future studies to be conducted on spore dispersal patterns and colonization patterns of the substrate by amplifying DNA extracted from soil and wood samples near existing conks.

Some questions this system could address regarding *B. nobilissimus* are: *i.* What are the specific habitat requirements (age and diameter range of host trees)? *ii.* How long does *B. nobilissimus* inhabit a substrate before fruiting? *iii.* What is its relationship with the associated yeast, bacteria and the *Fomitopsis* sp.? *iv.* Is it saprobic, pathogenic or possibly a commensal or mutualistic endophyte to *Abies procera*?

The taxonomic placement of *B. nobilissimus* has been problematical (Burdall et al 1996). Phylogenetic analysis of four mt-ssu rDNA sequences in the context of a broad dataset of lignicolous homobasidiomycetes placed three of the sequences in the hymenochaetoid clade (Hibbett and Thorn 2001) and the misidentified sequence in the polyporoid clade (FIG. 8). Taxa in the hymenochaetoid clade include members of the Hymenochaetaceae and other white-rotting, lignicolous species that traditionally have been placed in the Corticiaceae s. lat. and Polyporaceae s. lat. (Hibbett and Thorn 2001). The latter includes *Oxyporus*, which is represented in the refer-

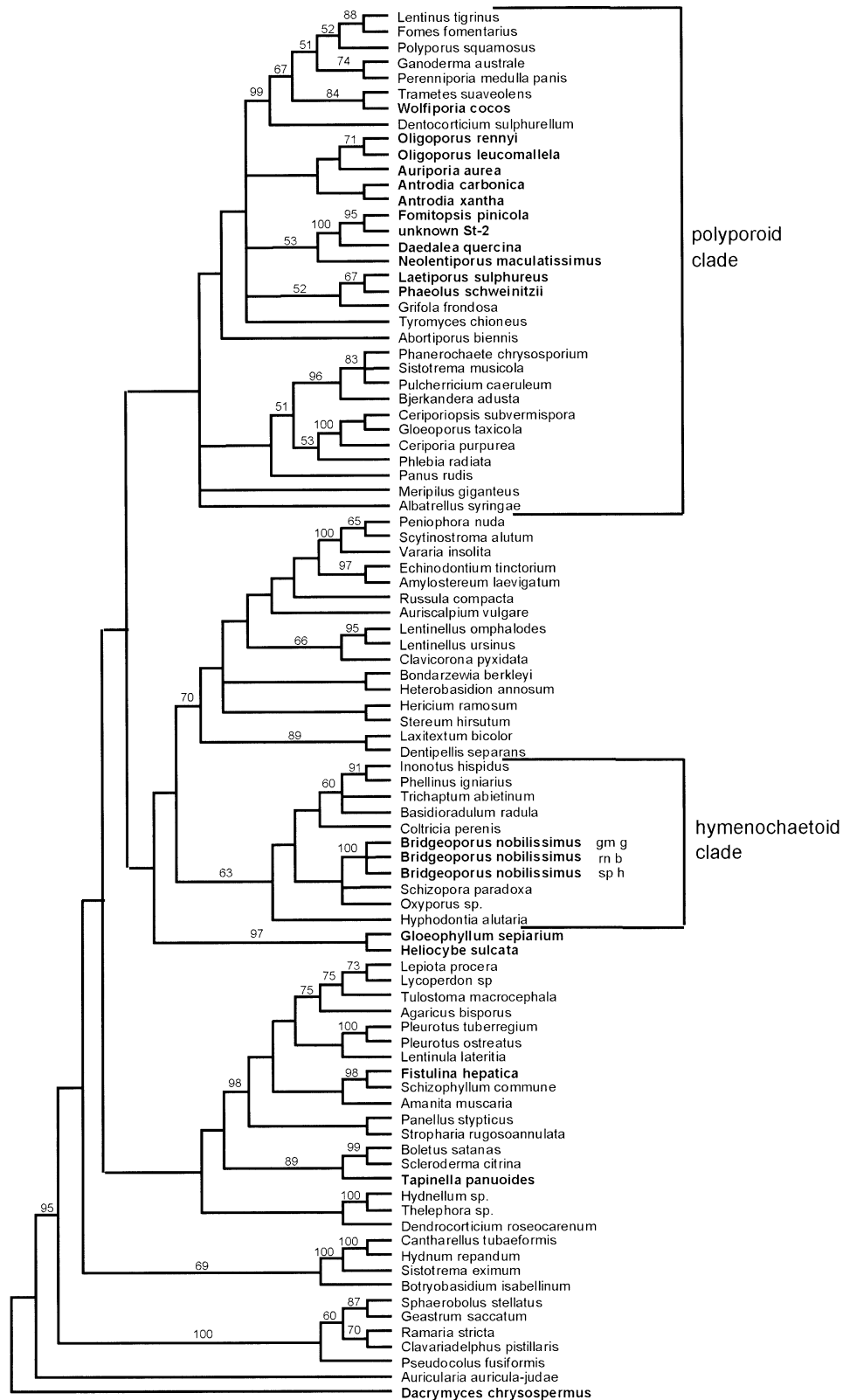


FIG. 8. Phylogenetic relationships of *Bridgeoporus nobilissimus* inferred from mt-ssu and nuc-ssu rDNA sequences. Strict consensus of 13 equally parsimonious trees (5011 steps, CI = 0.309, RI = 0.479). Bootstrap values above 50% are shown above internodes. Names of brown rot taxa are in boldface.

ence dataset by one sequence identified as *Oxyporus* sp. (probably *O. populinus*, D. Hibbett pers obs). Burdsall et al (1996) segregated *Bridgeoporus* from *Oxyporus*, based on anatomical features and rot type. Nevertheless, results of the molecular phylogenetic analysis place *Bridgeoporus* in the white-rotting hymenochaetoid clade (Hibbett and Donoghue 1995) and in a weakly supported clade containing *Bridgeoporus*, *Oxyporus* and *Schizopora* (FIG. 8). If this is correct, then *Bridgeoporus* might represent another independent origin of the brown-rot mode of wood decay in the homobasidiomycetes (Hibbett and Donoghue 2001). On the other hand, *B. nobilissimus* actually might cause a white rot and the brown rot observed in the association is due to another species, possibly *Fomitopsis*. Gilbertson and Ryvarden (1986) noted that *O. populinus* resembles *B. nobilissimus*, which they classified in *Oxyporus*, in having a “perennial sessile basidiocarp with the tube layers separated by thin layers of context.” In addition, *B. nobilissimus* and *O. populinus* both occur on living trees (hardwoods, in the case of *O. populinus*) and the pilei typically support mosses and other epimycota. Although the tramal origin of the cystidia makes this unlikely, *B. nobilissimus* indeed might be an *Oxyporus*, as described by Cooke (1949). However, the weak support for many nodes in the hymenochaetoid clade and limited sampling of *Oxyporus* spp. limit the conclusions that can be made at this time. The inclusion of *Bridgeoporus* and other nonxanthochroic taxa (e.g., *Trichaptum*, *Schizopora*) in the hymenochaetoid clade is at odds with the traditional delimitation of the Hymenochaetales. Nevertheless, members of the hymenochaetoid clade that have been studied at the ultrastructural level are united by possession of a nonperforated parenthesome (Hibbett and Thorn 2001). It would be valuable to determine whether *Bridgeoporus* has a nonperforated parenthesome, which would support its placement in the hymenochaetoid clade. Future analyses also should include additional isolates of *Oxyporus* spp., as well as *Rigidoporus* spp., and to seek genes that resolve relationships within the hymenochaetoid clade.

While rotting characteristics are considered important indicators at the genus level, they are not necessarily so at the family level. The characteristic of producing brown rot appears to have evolved repeatedly. Past phylogenetic studies of Polyporaceae have implicated a history of extensive convergence and parallelism in macro-morphological features, as well as considerable homoplasy in such physiological features as rotting characteristics (Hibbett and Donoghue 1995, 2001). Therefore it is not uncommon for fungi within the same family to have different rotting characteristics. Because *B. nobilissimus* has not been

cultured successfully and its life history is poorly understood, its association with brown rot can be classified only as correlation rather than causal at this time.

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