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Phylogenetics and Adaptations of Basidiomycetous Fungi Fed upon by Bark Beetles (Coleoptera: Scolytidae)

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Abstract

Relatively few species of phloem-inhabiting bark beetles feed heavily on fungi. Basidiomycetous fungi appear to be more commonly fed upon than ascomycetous fungi, but the basidiomycete associates are poorly known because they generally do not produce sexual fruiting structures in culture. Phylogenetic analysis of mitochondrial and nuclear rDNA placed most isolates of basidiomycetes from bark beetles into two distinct clades. One of the basidiomycetes was identified as *Phlebiopsis gigantea*, a fungus that produces windborne basidiospores from fruiting bodies on the outside of logs and stumps, typical for wood decay fungi. However, *P. gigantea* also produces asexual spores (arthroconidia) in the pupal chambers of bark beetles, which provide food for the beetles and a mechanism for dispersal by their insect vectors. The other clade consisted of a monophyletic group of nine species nested within the large genus *Peniophora*, which are wood decay fungi with windborne basidiospores. One of these *Peniophora*-like species from bark beetles was described earlier as *Entomocorticium dendroctoni*, and examination of the basidia of this species and two other species in this clade revealed short, flattened sterigmata and an apparent lack of forcible discharge of basidiospores. The data suggest that *Entomocorticium* is derived from the paraphyletic genus *Peniophora*. The

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irreversible loss of forcible discharge of basidiospores has apparently allowed for more effective grazing by bark beetles and adherence of the basidiospores to the beetle exoskeleton for dispersal.

Keywords: Mycophagy, mycangia, bark beetles, Scolytidae, *Entomocorticium*, *Peniophora*, *Phlebiopsis*

1. Introduction

Many insects that feed on highly-lignified plant tissues cultivate and feed on fungi that colonize the plant tissue. For instance, most xylophagus (xylem-feeding) ambrosia beetles (Coleoptera: Platypodidae and Scolytidae) are mycophagus, that is they graze on ascomycetous fungi (ambrosia) for at least part of their life cycle (Batra, 1967; Beaver, 1989). However, mycophagus associations between phloephagus (phloem-feeding) bark beetles (Coleoptera: Scolytidae) and fungal symbionts appear to be relatively uncommon, presumably because the inner bark tissues of trees are relatively nutrient rich. Adults of many ambrosia beetles have special spore-carrying sacs called mycangia for transporting their symbiotic fungi, but only a few bark beetles are known to have well-developed mycangia (Batra, 1963; Beaver, 1989; Bright, 1993). The most studied and most common fungal associates of bark beetles are ascomycetous fungi (Francke-Grosmann, 1967; Harrington, 1993a, b; Whitney, 1982), which may aid the bark beetles in killing the tree host (Paine et al., 1997). Basidiomycetes have been reported from a few species of bark beetles, and some of these fungi may be important sources of nutrients for late instar larvae or young adults (Barras and Perry, 1972; Furniss et al., 1987; Happ et al., 1976a, b; Harrington, 1993a; Harrington et al., 1981; Klepzig et al., 2001; Tsuneda et al., 1993; Whitney and Cobb, 1972; Whitney et al., 1987). These basidiomycetes may be common wood decay fungi, such as those that produce windborne basidiospores from mushrooms or polyporous conks (Harrington et al., 1981), but lack of fruiting structures has precluded accurate taxonomic placement of the basidiomycetes intimately associated with bark beetles. It is not known if these beetle-associated basidiomycetes are polyphyletic or monophyletic, arising from a single evolutionary event that led to adaptation to bark beetle dispersal and ambrosial growth form. It is possible that these basidiomycetes are completely dependent on the bark beetles for dispersal, having lost their ability to produce windborne basidiospores, or they may have a dual life history, one with insect dispersal of asexual spores and another as free-living, wood decay fungi that produce windborne basidiospores, as has been found with some of the *Amylostereum* species associated with horntails (Hymenoptera: Siricidae) (Tabata et al., 2000).

In order to clarify the relationships among the basidiomycetes associated with bark beetles and their wood decay relatives, we isolated fungi from adult beetles and from pupal chambers of beetle species thought to feed on fungi. We emphasized two clades of *Dendroctonus* (Kelley and Farrell, 1998) for which mycophagy had been noted and mycangia had been described. Well-developed prothoracic mycangia were reported in female adults of *Dendroctonus brevicomis* LeConte, *D. frontalis* Zimmermann, and *D. adjunctus* (Francke-Grosmann, 1966, 1967; Barras and Perry, 1971, 1972; Six and Paine, 1996; Whitney and Cobb, 1972). Similar prominent bulges in the pronota of female *D. vitei* Wood, *D. mexicanus* Hopkins, and *D. approximatus* Dietz (Lanier et al., 1988; Wood, 1982) suggest that these species have functional prothoracic mycangia similar to those of their close relatives (*D. brevicomis*, *D. frontalis* and *D. adjunctus*).

Adults of two sister species of *Dendroctonus* (*D. ponderosae* Hopkins and *D. jeffreyi* Hopkins) in a second clade have mandibular pouches (maxillary mycangia) that frequently contain fungal spores. Young adults of these species are known to feed on fungi growing in pupal chambers before they leave the tree to breed and lay eggs in other trees (Six and Paine, 1997, 1998; Whitney and Farris, 1970; Whitney et al., 1987).

Cultures of basidiomycetes from the bark beetle *Ips typographus* (Linnaeus) (Siemaszko, 1939) and two ambrosia beetles, *Xyleborus dispar* (Fabricius) and *Pityoborus comatus* (Zimmermann), were also available from culture collections and were compared to the *Dendroctonus* associates. Mycophagy by *X. dispar* (Batra, 1967; Happ et al., 1976b) and *P. comatus* (Furniss et al., 1987) had been previously known, but their basidiomycetous symbionts had not been well studied. Aside from these reports of basidiomycetes from *X. dispar* and *P. comatus*, only ascomycetous fungi and their asexual relatives have been found associated with ambrosia beetles.

We sequenced the mitochondrial small subunit (mt-ssu) region of the rDNA of these basidiomycetes and compared the sequences to those of other basidiomycetes in 14 families of Aphyllophorales and Agaricales (Hibbett and Donoghue, 1995). More detailed relationships among the basidiomycetes were studied using sequences of the internal transcribed spacer 1 and spacer 2 (ITS-1, ITS-2 and the 5.8s rDNA gene) and sequences of the first intergenic spacer region (IGS-1) of the rDNA operon.

2. Materials and Methods

Cultures of basidiomycetes were obtained from reference culture collections or were isolated by the authors (Table 1). Basidiomycetes associated with the prothoracic mycangia of *Dendroctonus approximatus*, *D. brevicomis* and *D.*

frontalis were isolated following surface sterilization of an excised prothorax, which contains the mycangium (Hsiau and Harrington, 1997). Propagules of other basidiomycetes were obtained by scraping white fungal growth from pupal chambers or larval galleries and streaking these propagules onto a selective medium for basidiomycetes. This medium, BSMA, contains 1% malt extract, 1.5% agar, 2 ppm benomyl powder and 100 ppm streptomycin sulfate (Harrington et al., 1981). Alternatively, these scrapings were serially diluted in sterile water and plated onto BSMA.

Template DNA for PCR (polymerase chain reaction) was obtained directly from mycelial scrapes (Harrington and Wingfield, 1995) for most of the isolates tested. No cultures of *Entomocorticium dendroctoni* Whitney et al. were available, so DNA was extracted (Taylor and Swann, 1994) from basidiospores and basidiome tissue of a dried specimen collected by H.S. Whitney (Nature Resources Canada, Pacific Forestry Center, Victoria, BC, Canada). The PCR products were generated utilizing primers MS1 and MS2 (White et al., 1990) for the mt-ssu-rDNA region; primers ITS5 and ITS4 (White et al., 1990) for the ITS region; and a newly-designed primer (P1, 5'-TTGCAGACGACTTGAATGG-3') and primer 5SRNA (5'-ATCAGACGGGATGCGGT-3', R. Vilgalys, Duke University) for the intergenic short spacer (IGS-1).

Sequences of mt-ssu-rDNA were generated using automatic sequencing (ABI PRISM 377 DNA sequencer and ABI PRISM 310 genetic analyzer, Perkin-Elmer) at the DNA Synthesis and Sequencing Facility at Iowa State University. Manual sequencing for ITS and IGS-1 used two-stage PCR amplification (Baldwin, 1992) to generate single-stranded PCR products as sequencing templates, and sequencing reactions were done following Wendel et al. (1995). Sequences were aligned using CLUSTAL V (Higgins et al., 1992), followed by manual adjustments. Representative sequences were deposited in GenBank (Table 1).

Mt-ssu-rDNA sequences of 62 species of the Aphyllophorales and Agaricales were kindly provided by David Hibbett (Hibbett and Donoghue, 1995). Because their data set was large, only a select number of taxa were included for the final analysis. The criterion for selecting taxa was based on sequence similarities to the sequences of the basidiomycetous fungi from *D. frontalis*, *D. approximatus*, *X. dispar*, and *I. typographus*. We then expanded this data set by generating sequences from cultures of basidiomycetes that had been previously associated with forest insects or that were suspected to be closely related to bark beetle associates (Table 1). In the final analysis, sequences of 16 taxa besides the bark beetle associates were used to generate a mt-ssu-rDNA tree.

Many of the bark beetle associates had mt-ssu-rDNA sequences similar to species of *Peniophora*, a large genus of wood decay fungi. Sequences of the ITS region of *Peniophora* species were kindly provided by Nils Hallenberg

(University of Göteborg, Sweden). We also sequenced the ITS region of isolates of other *Peniophora* species and of *Phlebiopsis gigantea* obtained from the Forest Products Laboratory of the U.S. Forest Service in Madison, Wisconsin (Table 1). Lastly, we generated IGS-1 sequences for select *Peniophora* species and related bark beetle associates (Table 1).

Sequence data sets were analyzed in PAUP (Swofford, 1993) using HEURISTIC searches with TBR branch swapping and MULPAR on. Ambiguously aligned characters were commonly found in all three datasets due to large insertions and deletions (indels), and these regions were excluded from the dataset before analysis. All remaining characters were given equal weight. All alignable gaps were treated as missing data. Branch supports of individual clades were estimated using bootstrapping, and decay indices were determined with the program AUTODECAY 3.0.3 (Eriksson and Wikstrom, 1996) in conjunction with PAUP.

3. Results

Fungal isolations

Many of the pupal chambers of *Dendroctonus* species were found to have luxuriant fungal growth. Microscopic examination of this material sometimes revealed basidia and basidiospores or tightly packed chains of globose spores, as in the pupal chambers of *D. fronatlis* and *D. brevicomis*. Arthroconidia (cylindrical, asexual spores in chains) typical of some basidiomycetes were observed in some pupal chambers of *D. ponderosae*. The ascomycetous fungus *Ophiostoma clavigerum* (Robins.-Jeff. & Davids.) Harrington was also common in pupal chambers of *D. ponderosae* and *D. jeffreyi* (Six and Paine, 1997), *Ceratocystiopsis ranaculosus* Perry & Bridges was common in the chambers of *D. frontalis* (Harrington and Zambino, 1990), and *C. brevicomi* Hsiau & Harrington was common in the chambers of *D. brevicomis* (Hsiau and Harrington, 1997).

White fungal growth in pupal chambers was scraped with a sterile needle and placed on BSMA medium for isolation. Many of these isolation attempts yielded basidiomycetous fungi, identified based on their tolerance to the fungicide benomyl (in BSMA) and by the presence of clamp connections on their hyphae. Some of the examined cultures produced basidia and basidiospores in culture, though most did not. Other basidiomycete cultures were obtained by isolations from prothoracic mycangia of *D. frontalis*, *D. brevicomis*, and *D. approximatus*. Our isolates from beetles and pupal chambers, as well as other beetle isolates from referenced culture collections, were tentatively grouped into species based on pigmentation and texture of mycelium and growth rate.

Table 1. Isolates and GenBank accession numbers for rDNA sequences used in phylogenetic analyses. Sequences were of the mitochondrial small subunit (mt-ssu), internal transcribed spacer region (ITS), or the first intergenic spacer region (IGS-1) of the nuclear rDNA.

Species	Isolates	mt-ssu	ITS	IGS
Entomocorticium dendroctoni	DAVFP #23165		AF119506	AF119526
Entomocorticium species A	B17, B248, B319, B322, B348, B408, B419, B709-712, B717, B721-722, B1032	AF119547	AF119509	AF119530
Entomocorticium species B	B317, B884, B887-B889, B890, B892-895, B1035-1036, B1038	AF119546	AF119508	AF119528
Entomocorticium species C	B896 (MMF-4485)	AF119548	AF119510	AF119531
Entomocorticium species D	B1039-1041	AF119550	AF119503	AF119524
Entomocorticium species E	B1043, B1060-1061	AF119551-119552	AF119504-119505	AF119525
Entomocorticium species F	B1048, B1050-1051	AF119553	AF119507	AF119527
Entomocorticium species G	B1067, B1069	AF119549	AF119511	AF119523
Entomocorticium species H	B1063	AF119545	AF119512	AF119529
Species I, *Phlebiopsis gigantea*	B1073	AF119539		
Species J, near *Antrodia* sp.	B267 (CBS 101.07)	AF119536		
Species K, *Phlebiopsis gigantea*	B1078 (UAMH 4919)	AF119538		
Phlebiopsis gigantea	B115	AF119537		
Amylostereum chailletii	B29	AF119542		
Dendrophora albobadia	B1029 (FP-101822-Sp)	AF119543	AF119522	AF119535
Gloeocystidium ipidophilum	B1076	AF119540		
Hyphoderma praetermissum	B76	AF119541		
Peniophora aurantiaca	B1018		AF119517	
Peniophora cinerea	B1020		AF119518	
Peniophora duplex	B1022		AF119519	AF119533
Peniophora nuda	B1006		AF119513	AF119534
Peniophora picea	B1010		AF119515	AF119532
Peniophora pithya	B1013 (HHB-9125-Sp)		AF119521	
Peniophora pithya	B1012 (FCUG 2226)	AF119544	AF119520	
Peniophora pseudo-pini	B1007		AF119514	
Peniophora rufa	B1014		AF119516	

Table 2. Beetle associates, tree hosts, location of origin, growth rate and morphology of the bark beetle associates studied.

Species	Associated beetle	Tree host	Geographic origin of cultures	Colony diameter (mm) after 8 days[a]	Mycelial morphology on malt agar and known spores
Gloeocystidium ipidophilum	*Ips typographus*	*Picea abies*	Norway	ND[b]	Conidial state?
Entomocorticium dendroctoni	*D. ponderosae*	*P. contorta* var. *latifolia*	Canada	ND	Smooth, light tan; basidiospores 7–12 × 4–6 μm[c]
Entomocorticium species A	*D. frontalis*	*Pinus* spp.	South Carolina, Mississippi, Louisiana, Texas	17–26	Velvety, brown
Entomocorticium species B	*D. brevicomis*	*Pinus* spp.	California	15–17	Velvety and crustose, radially striate, brown to light gray-brown
Entomocorticium species C	*Pityoborus comatus*	*P. elliottii*	Florida	26–27	Downy, creamy yellow
Entomocorticium species D	*D. ponderosae*	*P. lambertiana*	California	62–70	Downy, creamy white and striate when young, becoming light brown with age
Entomocorticium species E	*D. ponderosae* and *D. jeffreyi*	*P. contorta* var. *latifolia* and *P. jeffreyi*	California	62–72	Velvety to farinaceous, creamy yellow to reddish-brown, not striate
Entomocorticium species F	*D. ponderosae*	*P. contorta* var. *latifolia*	California	21–36	Velvety, with patches of aerial mycelium, azonate to zonate, brown

Table 2. Continued.

Species	Associated beetle	Tree host	Geographic origin of cultures	Colony diameter (mm) after 8 days[a]	Mycelial morphology on malt agar and known spores
Entomocorticium species G	*D. ponderosae*	*P. ponderosa*	Colorado	42–50	Downy, later becoming chamois-like, pinkish cream to brown; basidiospores 6–9 × 4–8 µm
Entomocorticium species H	*D. ponderosae*	*P. ponderosa*	Colorado	71	Felty, creamy white; basidiospores 8–14 × 4–6 µm
Species I, *Phlebiopsis gigantea*	*Dendroctonus approximatus*	*Pinus ponderosa*	Colorado	ND	Arthroconidia
Species J, near *Antrodia* sp.	*Xyleborus dispar*	Unknown	Unknown	ND	Chlamydospores
Species K, *Phlebiopsis gigantea*	*D. ponderosae*	*P. contorta* var. *latifolia*	Canada	ND	Arthroconidia

[a]Colony diameters measured after 8 days of growth on MYEA plates at room temperature. [b]ND = not determined. [c]The description is based on Whitney et al., 1987.

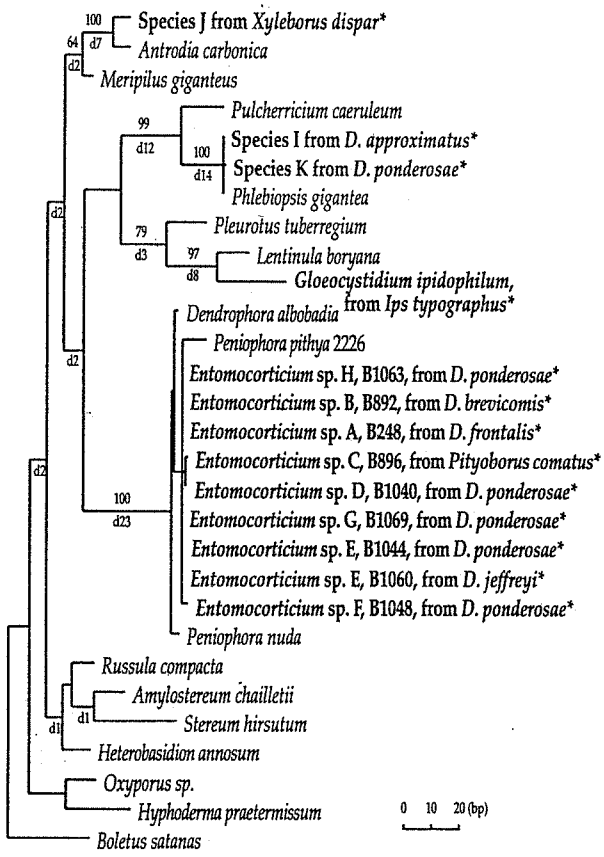

Figure 1. One of 19 most parsimonious trees generated from mt-ssu-rDNA sequences of bark and ambrosia beetle associates and other Holobasidiomycetes (tree length = 409 steps, CI = 0.501, RI = 0.744). Bootstrap values greater than 50% are shown above branches, and decay indices are shown in d values below branches. Taxa closely associated with bark or ambrosia beetles are indicated by an asterisk.

These putative species were arbitrarily assigned letter designations A through K (Table 2).

Mitochondrial small subunit sequences

Three hypervariable regions (Hibbett and Donoghue, 1995) were excluded from analyses due to ambiguous alignment of sequences among taxa. Phylogenetic reconstruction of the remaining dataset of 459 characters resulted in 19 most parsimonious trees. One of the shortest trees is shown in Fig 1. Most

of the variation among the 19 trees was due to minor rearrangement within the *Peniophora/Dendrophora/Entomocorticium* clade. This branch was found in all 19 trees and was strongly supported by bootstrap and decay indices. Included in this clade were the *Peniophora* species, the closely related *Dendrophora albobadia*, the mycangial fungi from *D. brevicomis*, *D. frontalis* and *Pityoborus comatus*, and several unnamed fungi isolated from the pupal chambers of *D. ponderosae* or *D. jeffreyi*.

Another well-supported clade (d12) contained *Phlebiopsis gigantea* (Fr.) Jülich, *Pulcherricium caeruleum* (Lamarck:Fr.) Parm., and two unidentified isolates from bark beetles. Placement of the mycangial fungus from *D. approximatus* (fungal species I) and fungal species K from the pupal chambers of *D. ponderosae* in the *Phlebiopsis gigantea* subclade was very strongly supported (d14). The mt-ssu-rDNA sequences of these three isolates were identical except for three one-base indels.

Gloeocystidium ipidophilum Siem. from *Ips typographus* and *Lentinula boryana* (Berkeley & Montagne) Pegler formed a clade that was sister to *Pleurotus tuberregium* (Fr.) Singer (d3), an oyster mushroom (Tricholomataceae). Another clade (d7) consisted of *Antrodia carbonica* (Overholts) Ryvarden & Gilbertson and a fungus (sp. J) isolated from the galleries of the ambrosia beetle *X. dispar*, and these two species grouped weakly (d2) with *Meripilus giganteus* (Persoon) P. Karsten. The fungus from *X. dispar* had negative reactions on gallic and tannic acid media, a physiological test indicative of the brown rot mechanism of wood decay (Davidson et al., 1938). *Antrodia carbonica* and *M. giganteus* are also brown rot fungi. The placement of this brown rot clade in relation to the other Holobasidiomycetes was not clear.

Peniophora and Entomocorticium ITS sequences

Phylogenetic analysis of ITS sequences of the *Peniophora* clade yielded 133 most parsimonious trees with a branch length of 188 steps. One of the most parsimonious trees is shown in Fig. 2. *Dendrophora albobadia* (Schweinitz) Chamuris was chosen as the outgroup taxon because it is morphologically similar to *Peniophora*, other than the presence of dendrohyphidia (Chamuris, 1987).

The most significant feature of the ITS tree was a strongly supported clade (decay index of 3, bootstrap value of 93%) that included the beetle-associated species, including *E. dendroctoni*, and *Peniophora pithya* (Persoon) J. Eriksson (FCUG2226). All 133 trees had this branch. The other *P. pithya* isolate (B1012) was placed sister to *P. rufa* (Persoon) Boidin, so it appears that one of the two *P. pithya* isolates may be misidentified. With no fruiting structures and only

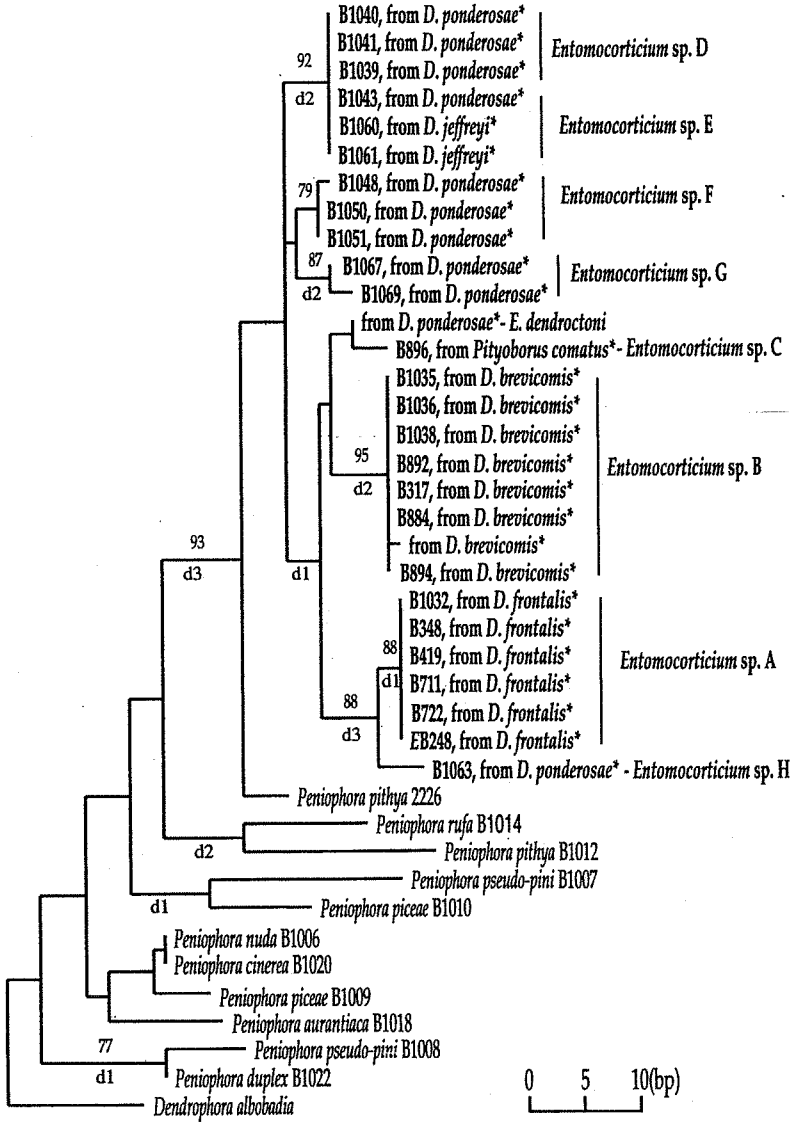

Figure 2. One of 133 most parsimonious trees from the internal transcribed spacer and 5.8S rDNA sequences of *Peniophora* and *Entomocorticium* species (tree length = 188 steps, CF = 0.484, RI = 0.773). Bootstrap values greater than 50% are shown above branches, and decay indices (d value) are shown below branches.

sterile mycelium, we were not able to determine which, if either, of these cultures is truly *P. pithya*. *Peniophora piceae* (Schleicher) Boidin and *P.*

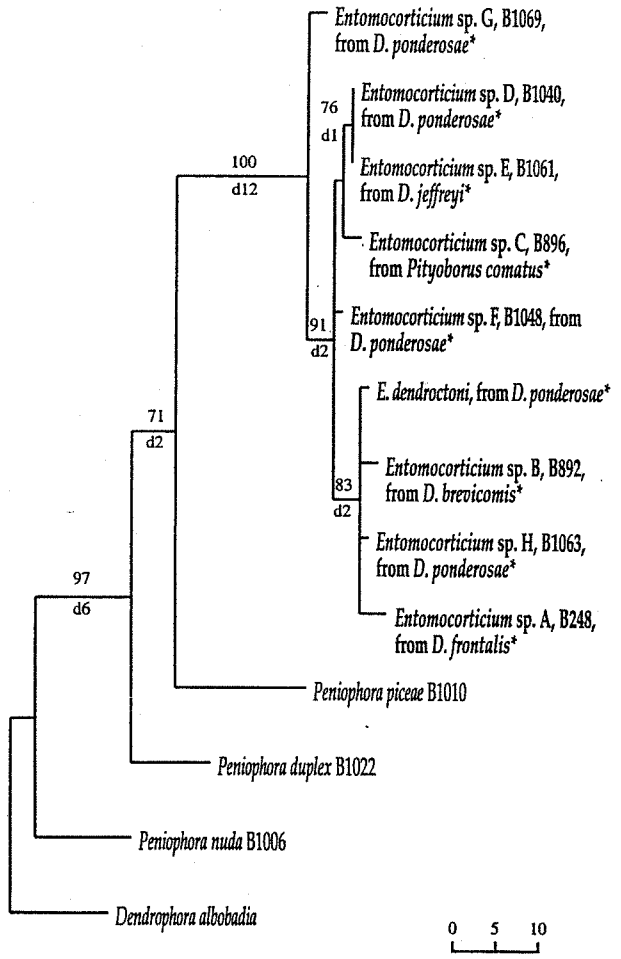

Figure 3. One of six most parsimonious trees from sequences of the intergenic short spacer (IGS-1) of *Peniophora* and *Entomocorticium* species (tree length = 100 steps, CI = 0.850, RI = 0.769). Bootstrap values greater than 50% are shown above branches, and decay indices (d value) are shown below branches.

pseudo-pini Weresub & Gibson were placed as sister taxa, while *P. nuda* (Fries) Bresadola and *P. cinerea* (Persoon) Cooke were another species pair. Generally, the clades within *Peniophora* were not strongly supported, except for the *Entomocorticium/P. pithya* FCUG2226 clade, and there was some support for grouping *Entomocorticium* A with H (d3). *Entomocorticium* species D and E had identical ITS sequences.

Peniophora and Entomocorticium IGS-1 sequences

Phylogenetic analyses of IGS-1 sequences found six most parsimonious trees of 100 steps using 177 alignable characters. One of the six most parsimonious trees is shown in Fig. 3. The incongruence among the six trees was mainly in the placement of *E. dendroctoni* and *Entomocorticium* sp. B.

The *Entomocorticium* clade was very strongly supported (d12) by the IGS-1 data. The IGS-1 sequence of species G appeared to be basal to the *Entomocorticium* clade (Fig. 3). As in the ITS sequences, the IGS-1 sequences of species D and E were identical. The mycangial fungi from *D. frontalis* and *D. brevicomis* (species A and B, respectively) grouped weakly (d2) with *E. dendroctoni* and species H.

Phlebiopsis gigantea ITS sequences

The ITS sequences of three isolates of *Phlebiopsis gigantea* from basidiomes on decayed wood (Table 1) were compared to the ITS sequences of species I and species K from bark beetles. The length of aligned ITS sequence was 591 bp. The ITS sequences of isolates from *D. approximatus* (B1073 and B1075) and from *D. ponderosae* (B1078) were identical to each other and to those from *P. gigantea* isolates B1079, B1103, and B1104.

4. Discussion

Phylogenetic analyses and morphological comparisons suggest that at least two groups of basidiomycetes have adapted to associations with phloem-feeding bark beetles. The relatives of the bark beetle associates we studied are wood decay fungi, which typically have wind-disseminated basidiospores forcibly discharged from distinctive sterigmata on the top of basidia. *Phlebiopsis gigantea* forms arthroconidia as well as airborne basidiospores, and the arthroconidia would appear to be suitable for grazing by insects and for adherence to insect exoskeletons for dispersal.

In *Peniophora*, however, adaptation to insect dispersal appears to have resulted in a more drastic, probably irreversible adaptation, that is, loss of forcible discharge of basidiospores. The rDNA analyses suggest that *Entomocorticium* is a unique monophyletic lineage dependent on bark beetles for dispersal. In return, it appears that the *Phlebiobsis* and *Entomocorticium* species provide nutritional benefit to their beetle vectors (Bridges, 1983; Coppedge et al., 1995; Klepzig et al., 2001; Tsuneda et al., 1993; Whitney et al., 1987).

Phlebiopsis gigantea

The mycangial fungus of *D. approximatus* was isolated from the prothoracic segments of two out of five adult beetles, a fairly high percentage considering that the beetles were frozen before isolation and the surface sterilization was severe. No fungi were isolated from the other three adults. In addition, we attempted direct amplification from DNA extracted from whole adult *D. approximatus* (from beetles collected in southwestern Colorado, DNA extracted by S. Kelley, then at the University of Colorado) using basidiomycete-specific primers for the ITS region (ITS1F and ITS4B, Gardes and Bruns, 1993). The sequence of this PCR product was identical to that of the other two isolates from *D. approximatus*. This suggests that the fungus is intimately associated with *D. approximatus*, and this is the first indication that this beetle has a mycangial fungus.

The *D. approximatus* cultures produced arthroconidia in culture, which prompted us to compare it to *Phlebiopsis gigantea* and another unidentified, arthroconidia-producing basidiomycete (species K, UAMH 4919), which had been found in galleries of *D. ponderosae* (Tsuneda et al., 1993). The 491 bp mt-ssu-rDNA sequences for cultures of species I from mycangia of *D. approximatus*, species K from *D. ponderosae* galleries, and cultures from fruiting bodies of *P. gigantea* differed only by three one-base indels.

Further, the three collections of *P. gigantea* from basidiomes on decayed wood had identical ITS sequences to those of cultures of species I and K, thus supporting that these fungi are conspecific. Species I differs from the other isolates of *P. gigantea* in slower growth and having conidia and hyphae of wider diameter, perhaps indicative of an ambrosial growth habit. We also found that the mycangial fungi of *D. brevicomis* and *D. frontalis* grow slower than the non-mycangial beetle associates in the *Entomocorticium* clade.

Phlebiopsis gigantea is a typical wood decay fungus with adaptations for wind dispersal, that is, forcible discharge of basidiospores from basidia that form a tightly compacted hymenium on the surface of a basidiome on the outer surface of woody stems or logs.

In addition to the sexual state, *P. gigantea* can also form a dense layer of asexual spores (conidia or arthrospores), and these spores may be suitable for grazing by beetles and for dispersal by the insects. Thus, *P. gigantea* has apparently maintained the typical dispersal mechanism (wind) for basidiospores while adding insect dispersal of conidia by insects. Some species of *Amylostereum* similarly maintain the typical wind dispersal of basidiospores with the added adaptation of conidia or hyphal fragments for growth in the mycangia of horntails (Tabata et al., 2000).

Entomocorticium and Peniophora

Phylogenetic relationships inferred from mt-ssu-rDNA, ITS, and IGS-1 sequences resolved the placements of the *Dendroctonus frontalis* and *D. brevicomis* mycangial fungi with the mycangial fungus from *Pityoborus comatus* and several species from *D. jeffreyi* and *D. ponderosae*. One of these *D. ponderosae*-associated fungi was named *E. dendroctoni* (Whitney et al., 1987). The tentative identification (Furniss et al., 1987) of the mycangial fungus from *P. comatus* as *Holtermannia corniformis*, a member of the Tremellales, appears to be in error, as the species falls within the *Entomocorticium* clade.

The ITS data resolved some relationships among the *Peniophora* species but failed to resolve relationships among the *Entomocorticium* species, which showed little variation in ITS or IGS sequences. The relationships seen among the *Peniophora* species generally agree with the phylogenetic studies of Hallenberg et al. (1996), except for *P. rufa*. *Peniophora rufa* appeared to be basal to the other *Peniophora* species in the ITS2 tree of Hallenberg et al. (1996), but not in our trees. Two isolates of *P. pithya* had differing ITS sequences in our study. The sequence of isolate B1012 was close to *P. rufa*, which might be expected, but isolate 2226 was basal to the *Entomocorticium* species in both the mt-ssu-rDNA and ITS sequences, and in the ITS sequence analysis, grouping of the *Entomocorticium* species with *P. pithya* isolate 2226 was very strongly supported. The ITS data suggest that at least one of the two isolates is misidentified. Unfortunately, we were unable to obtain clear IGS-1 sequences for B1012 and 2226. It is possible that isolate 2226 is actually an *Entomocorticium* species, but we have no sexual state of this isolate to do the needed morphological comparisons. If isolate 2226 is correctly identified as *P. pithya*, perhaps this species is close to the ancestor of the *Entomocorticium* clade.

Both ITS and IGS-1 analyses support our contention that the *Entomocorticium* clade is monophyletic and relatively young, probably derived from a *Peniophora* species. The genus *Peniophora* seems to be paraphyletic based on our trees. Although such paraphyletic taxa are generally considered undesirable, there are several recognized subgenera in the genus *Peniophora* (Eriksson et al., 1978; Hallenberg et al., 1996), and it is likely that the genus *Peniophora* will be divided into several genera in the future.

Cultures of *Entomocorticium* species G and H formed basidia with short sterigmata identical to those described for *E. dendroctoni* (Whitney et al., 1987), and the basidiospores form a thick layer on top of the hymenium. The thick-walled and sticky basidiospores of *Entomocorticium* are not forcibly discharged and accumulate in a thick layer for grazing and for adherence to the exoskeleton of adult beetles. The spores likely accumulate in the maxillary mycangia of *D. ponderosae* and *D. jeffreyi* while the young adults are grazing.

These unique adaptations for insect dispersal are in contrast to their apparent close relative *Peniophora*, a large corticioid genus of wood decay fungi with wind-disseminated basidiospores that are discharged from well-developed sterigmata (Hallenberg et al., 1996). The *Peniophora* species are otherwise morphologically similar to the *Entomocorticium* species, including the sterile cells (cystidia) in the hymenium.

Basidiospores have not been observed in the three mycangial *Entomocorticium* species (A, B, and C), and it is not clear if the cells forming in the pupal chambers of *D. frontalis* are asexual spores or basidiospores. However, Klepzig et al. (2001) illustrated a basidiomycete from a pupal chamber of *D. frontalis* that had the characteristic basidiospores and cystidia of an *Entomocorticium* species, and that fungus may have been *Entomocorticium* sp. A. These mycangial species of *D. frontalis* and *D. brevicomis* (*Entomocorticium* sp. A and B) grow slower than the other *Entomocorticium* species, perhaps indicative of their more specialized habitat. The ambrosial-like growth in the pupal chambers of *D. frontalis* and *D. brevicomis*, whether due to the accumulation of basidiospores or conidia, likely serves as the propagules for entrance into the mycangium of the young adults. In the mycangium, *Entomocorticium* sp. A grows in a yeast-like phase (Happ et al., 1976a).

Another bark beetle, *Ips avulvus*, is thought to benefit from feeding on fungi (Yearian et al., 1972), and a basidiomycete was found in pupal chambers of this beetle by Brian Sullivan (personal communication, US Forest Service, Pineville, Louisiana). Inner bark material containing galleries and pupal chambers of *I. avulvus* was sent by B. Sullivan to the junior author, and the basidiospores, basidia and cystidia typical of an *Entomocorticium* species were found there. This fungus was isolated in pure culture, and ITS sequences of the *I. avulvus* associate were very similar to those of the other *Entomocorticium* species (Harrington, unpublished). Thus the *I. avulvus* symbiont appears to represent a tenth species in the genus *Entomocorticium*.

The Xyleborus dispar associate

Unlike the other beetle-associated basidiomycetes, the mycangial fungus from the ambrosia beetle *Xyleborus dispar* appears to be a brown rot fungus and may be related to the genus *Antrodia*. This isolate is characterized by fast growth and production of chlamydospores in culture. The chlamydospores tend to occur within dense columns of hyphae. Perhaps these chlamydospores serve as food for the ambrosia beetles. However, this beetle, like other ambrosia beetles, has an asexual ascomycete, *Ambrosiella hartigii* Batra, as its primary mycangial associate (Batra, 1967). Happ et al. (1976b) noted that an

unidentified basidiomycete was sometimes found in mycangia of *X. dispar*. We assume that our isolate is the same basidiomycete, but we are not certain that it is symbiotic with *X. dispar*. Nonetheless, it is not related to the basidiomycetes associated with bark beetles.

Gleocystidium ipidophilum and other insect associates

Gloeocystidium ipidophilum was thought to be related to *E. dendroctoni* by Whitney et al. (1987), but this speculation was not supported by our mt-ssu-rDNA sequence data. *Gloeocystidium ipidophilum* was placed in a weakly-supported clade with *Lentinula boryana* and *Pleurotus tuberregium*. Some members of *Gloeocystidium* have been transferred to *Hyphoderma* (Donk, 1962; Eriksson and Ryvarden, 1975), but the mt-ssu-rDNA sequence of *G. ipidophilum* was quite distinct from that of *H. praetermissum* (P. Karsten) J. Eriksson & Strid. Our cultures of *G. ipidophilum* were isolated by Solheim (1992) from sapwood of Norway spruce attacked by *Ips typographus*. The fungus was reported to sporulate in the galleries of this bark beetle (Siemaszko 1939), but no symbiotic association between the beetle and fungus has been noted. The morphology of the basidium is unique and difficult to distinguish from hyphae in culture, and these may actually be conidiophores. It is not clear if *I. typographus* has a symbiotic relationship with *G. ipidophilum*.

Two other wood decay fungi associated with insects were included in the analysis. *Heterobasidion annosum* was reported to be loosely associated with scolytid beetles (Bakshi, 1952; Himes and Skelly, 1972; Hunt and Cobb, 1982; Kadlec et al., 1992). *Amylostereum chailletii* is a mycangial fungus of *Urocerus gigas* L. and other horntails (Redfern, 1989; Tabata et al., 2000). The mt-ssu-rDNA sequences of *H. annosum* and *A. chailletii* suggest that they are not closely related to bark and ambrosia beetle associates.

5. Conclusions

According to our phylogenetic analyses, intimate associations with bark beetles have evolved at least twice in the basidiomycetes. We hypothesize that dependence on fungi for nutrition is a recently derived character in the bark beetles, found in at least two closely related clades of *Dendroctonus*, perhaps the two most advanced clades within the genus. This dependence on fungi for nutrition may have provided a new niche for fungal speciation, a niche fulfilled by several species of ascomycetes and basidiomycetes. Besides *Phlebiopsis gigantea*, which also exists in the absence of bark beetles, the other basidiomycetes that we discovered in bark beetle mycangia and pupal chambers comprise a unique genus of basidiomycetes that appears to have

recently evolved from a *Peniophora* ancestor. The loss of sterigmata and the forcible discharge of basidiospores would appear to be an irreversible adaptation that allows for the accumulation of basidiospores for grazing by the beetles. The sticky nature of these basidiospores may also be well suited for adhesion to the exoskeleton of the beetles and entrance into mycangia, with subsequent dispersal to new trees as the adult beetles seek fresh material for egg-laying and brood development.

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