

Determining fungal diversity on *Dendroctonus ponderosae* and *Ips pini* affecting lodgepole pine using cultural and molecular methods

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and Colette Breuil**

**Mountain Pine Beetle Initiative
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Abstract

Several beetles (*Coleoptera: Scolytidae*) and their fungal associates cause severe damage to lodgepole pine in Western Canada and the northwestern United States. The fungus diversity from the surface of two bark beetle species, *Dendroctonus ponderosae* Hopkins (mountain pine beetle) and *Ips pini* Say (pine engraver), was surveyed using cultural and molecular methods. Nine fungal taxa were recognized by morphological characterizations. All nine taxa were isolated from the mountain pine beetle, whereas only seven of the nine taxa were isolated from the pine engraver. The identification was based on cultural morphology and high sequence similarities of the internal transcribed spacer (ITS) and large subunit ribosomal DNA (large subunit rDNA) region to sequences of known fungi. Fungal ITS regions were amplified from DNA directly extracted from the beetle surface. The PCR products were cloned and 250 clones were classified by their restriction pattern with *HaeIII* and *RsaI*. A total of 26 RFLP types were identified and subsequently sequenced. Among them, 15 RFLP (restriction fragment length polymorphism) types were identified as being present in mountain pine beetle and 14 were present in pine engraver. Sequence analysis of the RFLP types showed that 23 ascomycetes and 3 basidiomycetes were represented in the clone libraries, whereas the isolates from the cultural method represented 7 ascomycetes and 2 basidiomycetes. We found that yeast and non-staining filamentous euascomycetes fungi were detected efficiently using a molecular approach, while the major sap-staining fungi and decay fungi were best detected using cultural methods.

Key words: bark beetle; fungus diversity; ITS, lodgepole pine, mountain pine beetle.

Résumé

Plusieurs coléoptères (*Coleoptera: Scolytidae*) et leurs associés fongiques causent de sérieux dommages au pin tordu latifolié dans l'Ouest du Canada et le Nord-Ouest des États-Unis. La diversité des champignons à la surface de deux espèces de scolytes, *Dendroctonus ponderosae* Hopkins (dendroctone du pin ponderosa) et *Ips pini* Say (scolyte du pin), a été recensée à l'aide de méthodes de culture et de méthodes moléculaires. Neuf taxons fongiques ont été reconnus grâce à leurs caractères morphologiques. Ils ont tous été retrouvés sur le dendroctone du pin ponderosa, alors que seulement sept d'entre eux ont été retrouvés sur le scolyte du pin. L'identification s'est faite sur la base de la morphologie de la culture et des fortes similarités séquentielles entre l'espaceur intergénique (ITS) et une région de grande sous-unité d'ADN ribosomique (l'ADNr USL) des séquences des champignons connus. Les régions fongiques de l'ITS ont été amplifiées à partir de l'ADN extrait directement de la surface du coléoptère. Les produits de la PCR ont été clonés, et 250 clones ont été classifiés selon leur profil de restriction avec *HaeIII* et *RsaI*. En tout, on a identifié, puis séquencé, 26 types de polymorphismes de taille des fragments de restriction (RFLP). De ce nombre, on en a retrouvé 15 chez le dendroctone du pin ponderosa et 14 chez le scolyte du pin. Le séquençage des types de RFLP a montré que 23 ascomycètes et 3 basidiomycètes étaient représentés dans la banque de clones, alors que les isolats provenant de la méthode de culture représentaient 7 ascomycètes et 2 basidiomycètes. On a détecté efficacement la levure et les champignons ascomycètes unituniqués filamenteux qui ne tachent pas à l'aide de l'approche moléculaire, alors que les principaux champignons responsables de la tache colorée de l'aubier étaient plus facilement détectables à l'aide des méthodes de culture.

Mots clés : scolyte; diversité fongique; espaceur intergénique (ITS), pin tordu latifolié, dendroctone du pin ponderosa

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Introduction

Lodgepole pine (*Pinus contorta* Dougl. var. *latifolia* Engelm.) is an economically and ecologically important resource of western Canada and the northwestern United States. It is used in construction, for pulpwood, and for railway ties and poles. Several beetles (*Coleoptera: Scolytidae*) and their fungal associates cause severe damage to lodgepole pine in western Canada (Harrington and Cobb 1988). *Dendroctonus ponderosae*, known as the mountain pine beetle, is a serious threat for mature lodgepole and several other pine species (Safranyik et al. 1974). The mountain pine beetle possesses mycangia formed by invaginations of the exoskeleton that play a role in the dissemination of symbiotic fungi, *Ophiostoma clavigerum* (Rob.-Jeffer. & R.W. Davidson) T.C. Harr., *O. montium* (Rumbold) Arx and associated yeasts (Whitney and Farris 1970). The occurrence of ascomycete yeast with bark beetles was reported many years ago. *Pichia pini* (Holst) Phaff, *P. capsulata* (Wick.) Kurtzman, and *P. holstii* (Wick.) Kurtzman are closely associated with the mountain pine beetle throughout its life cycle in lodgepole pine (Rumbold 1941; Robinson 1962).

Ips pini, known as the pine engraver, occurs trans-continentially across North America (Lanier 1972) and colonizes most species of pine within its range (Wood 1982). The pine engraver frequently infests the trees being attacked by *Dendroctonus* species and thereby may accelerate the degradation of the tree. It does not possess glandular mycangia (Six 2003), but it does transport several fungal species (Furniss et al. 1995). Much is known about the mountain pine beetle; however, relatively little has been reported on fungi associated with the pine engraver.

The isolation of fungi from bark beetles has relied extensively on the dilution plating (Juzwik and French 1983; Klepzig et al. 1991) and the direct beetle streaking methods (Six and Bentz 2003). The failure of bacteria and fungi to grow under standard cultural conditions has been reported repeatedly; only 1% of bacteria and 5% to 10% of fungi have been described formally (Hawksworth 1991; Pace 1997). To circumvent the cultivation problem, an array of molecular techniques such as amplified rDNA restriction and ribosomal DNA (rDNA) sequencing analyses have been applied to elucidate microbial population structures in the environment (Smit et al. 1999; Allen et al. 2003). As cultivation-independent methods enable the detection of slowly growing or uncultivable fungi, it is likely that DNA extraction method gives a more complete view of fungal communities compared to traditional cultivation techniques. The wealth of sequence information that has been compiled in databases means that it is now possible to identify fungi at a far higher resolution using molecular techniques than can be achieved using cultural methods. Increased knowledge of the diversity surrounding the fungal community that beetles carry will further facilitate the understanding of beetle–fungal interactions.

The objective of this work was to compare the efficacy of the cultural and molecular methods to evaluate fungus diversity from the exoskeleton of two bark beetles. This is the first time that DNA extraction method has been applied to fungus diversity in mountain pine beetle and pine engraver.

Materials and methods

Beetle material

In early June 2003 at Manning Park (British Columbia, Canada), the mountain pine beetle and the pine engraver were collected from galleries at the bottom regions of five lodgepole pines attacked by mountain pine beetle the previous year. Two groups of five mountain pine beetles and two groups of five pine engravers were taken from each of five trees and were placed in separate microtubes. Half of these groups were used for culture and the other half were used for direct DNA extraction. Samples were kept in plastic bags on ice, transported to the University of British Columbia, and held at 4°C until the next day.

Fungal isolation

Tween-20 wash solution (0.01%) was added to each 1.5 ml microtube that included one bark beetle. The tubes were vortexed for 3 minutes at maximum speed, and the insect wash was diluted 50 times. Subsequently, 20 µL of the 50-fold diluted insect wash solution was spread on (i) plates of 1% Oxoid malt extract agar (MEA) with ampicillin for the general fungal flora, and (ii) plates of 1% Oxoid MEA with benomyl/ampicillin (BMEA) to select for decay basidiomycetes. All plates were checked daily to prevent overgrowth of a dominant isolate. As different forms of fungi were observed, they were transferred onto fresh plates to obtain pure cultures. The fungal isolates were grouped based on growth morphology on media and microscopic features. Species identification via classical methodology was achieved by macro- and micro-morphological analyses using taxonomic guides and standard procedures (Nobels 1965; Stalpers 1974; Kurtzman and Fell 1998; Jacobs and Wingfield 2001). This was complemented by identification of cultures using molecular methods.

DNA extraction and PCR amplification

Genomic DNA of each representative isolate was extracted from mycelia grown on media as described by Lecellier and Silar (1994). For direct DNA extraction from beetles surface, each tube consisting of five mountain pine beetles or five pine engravers was resuspended in 700 µL of extraction buffer [100 mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0), 100 mM NaCl and 2% SDS]. The beetles were removed from tubes after vortexing for 10 minutes. After 1 hour incubation at 75°C, glass beads (1/3 volume) were added to the tubes and vortexed again for 10 minutes. DNA was purified via a two-step phenol-chloroform extraction and precipitated with one volume of iso-propanol, then centrifuged immediately at 12,000 rpm at room temperature for 10 minutes. After removing the supernatant, the pellet was washed with 70% ethanol, allowed to air dry and resuspended in 40 µl of distilled water. The extracted DNA was stored at -20°C until further use.

Polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) from cultures was carried out using the ITS5 and ITS4 (White et al. 1990) primer sets. The large subunit (LSU) rDNA region was also amplified for four species of yeast, using the primer sets LR0R and LR3 (<http://www.biology.duke.edu/fungi/mycolab/primers.htm>). The LSU rDNA regions from only four yeast species were amplified for their identification. PCR amplification was performed as described by Lee et al. (2003). Amplified PCR products were directly sequenced after purification using Qiaquick PCR Purification Kit (Qiagen, Ontario).

For cloning, the ITS region was amplified from DNA extracted directly from the surface of the beetle bodies. All PCR reactions were performed in a PTC-100 thermal cycler (MJ research, USA) with the following cycling parameters: initial denaturation at 94°C for 4

minutes, then 30 cycles of denaturation at 94°C for 50 seconds, annealing at 52°C for 40 seconds, extension at 72°C for 50 seconds, with a final extension at 72°C for 10 minutes. From each PCR product, 3 µL was electrophoresed on 0.5% agarose gel containing EtBr in Tris-acetate EDTA (TAE) buffer. The PCR product sizes were determined by comparison to 1 kb DNA marker (GIBCO BRL, USA). To minimize PCR drift for cloning (Polz and Cavanaugh 1998), six replicate amplicons were pooled, then concentrated and purified with Qiaquick PCR Purification Kit.

Cloning and RFLP analysis

Purified PCR products were cloned using TOPO TA cloning kit (Invitrogen Inc. Burlington, ON, Canada) according to the manufacturers' instructions. Twenty-five white colonies from each library were selected and stored on agar plates. Selected clones were re-amplified using ITS 4 and ITS 5 primers. Amplified PCR products were digested with *Hae*III and *Rsa*I. Digestions were performed using 8 µL of PCR products, 1 µL of 10X reaction buffer, and 1 µL of each enzyme. Restriction fragment patterns were visualised on a 2.5% agarose gel (w/v).

Sequencing and phylogenetic analysis

PCR products of isolates and each representative RFLP type were further purified and then sequenced. Sequencing was performed on an ABI 3700 automated sequencer (Perkin-Elmer Inc. USA) at the DNA synthesis and sequencing facility, MACROGEN (Seoul, Korea). ITS and LSU region sequences of isolates were used for identification. For the phylogenetic analysis, ITS region sequences from isolates and different restriction fragment length polymorphism (RFLP) types were aligned with sequences obtained from BLAST searches (<http://www.ncbi.nlm.nih.gov/BLAST>). Sequences were first aligned using Clustal X algorithm (Thompson et al. 1997). Manual adjustment of alignments was done with the PHYDIT program version 3.2 (<http://plaza.snu.ac.kr/~jchun/phydit/>). Ambiguous regions of ITS regions in the alignments were excluded from further analyses. Parsimony analyses used the heuristic search option with simple addition sequences with MULPARS and TBR branch swapping. Gaps were treated as missing data. Branch stability was assessed by 1000 replicate parsimony bootstrap replications implemented with PAUP*4.0b10 (Swofford 2002).

Results

Cultural isolates and their identification

A total of 730 isolates were obtained from bark beetles, of which 418 isolates were from the mountain pine beetle and 312 from the pine engraver. We recognized nine different fungal taxa by morphological characterizations. All nine were isolated from the mountain pine beetle, but only seven were isolated from the pine engraver (Table 1). Two fungi isolated on BMEA had characteristics of basidiomycetous decay fungi with clamp-connections. Of all isolates, 1.7% and 1% consisted of decay fungi associated with the mountain pine beetle and the pine engraver, respectively. One of the decay fungi isolated from the mountain pine beetle was easily identified as *Heterobasidion annosum* (Fr.) Bref. due to its distinct morphology. It was characterized by the formation of diagnostic anamorph *Spiniger meinekellus* (Olson) Stalpers on media (Stalpers 1974). The identification of *H. annosum* was confirmed by ITS region sequence analysis. The other decay fungus isolated from both bark beetles exhibited fewer morphological features in culture; therefore, it was impossible to identify it to the species level. However, through the sequence BLAST search, the ITS sequence had 100% similarity with *Fomitopsis pinicola* (Swartz : Fries) P.Karsten.

Table 1. Fungal isolates recovered from mountain pine beetle and pine engraver from five lodgepole pine trees.

Fungal isolates	GenBank accession no. MPB ITS (LSU rDNA)	MPB					Pine engraver				
		T1 ¹	T2	T3	T4	T5	T1	T2	T3	T4	T5
Hemiascomycetes (yeast)											
<i>Pichia capsulata</i>	AY761153 (AY761149)	1	2	3	2	4	0	0	0	0	0
<i>Pichia holstii</i>	AY761154 (AY761150)	28	22	17	22	19	18	25	19	17	27
<i>Pichia scolyti</i>	AY761155 (AY761151)	45	42	28	37	33	32	38	35	34	29
Unidentified yeast	AY761156 (AY761152)	0	0	3	2	1	0	1	1	0	0
Filamentous Euascomycetes											
<i>Ceratocystiopsis</i> sp.	AY761157	2	0	2	3	0	0	3	0	3	0
<i>Ophiostoma clavigerum</i>	AY761158	3	2	4	6	0	0	1	0	0	0
<i>Ophiostoma montium</i>	AY761159	15	19	18	18	8	5	1	6	8	6
Basidiomycota											
<i>Fomitopsis pinicola</i>	AY761160	1	2	1	0	1	0	2	0	0	1
<i>Heterobasidion annosum</i>	AY761161	0	2	0	0	0	0	0	0	0	0
Total isolates		95	91	76	90	66	55	71	61	62	63

¹Denotes the sample trees where the beetles collected from.

Three sap-staining fungi were isolated and they represented 23.9% and 10.6% of the total isolates from the mountain pine beetle and the pine engraver, respectively (Table 1). The most common species was *Ophiostoma montium*. The other sap-staining fungus matched the characteristic of *O. clavigerum*, which had long clavate spores (12.5-85 µm) and long synnematosus conidiophores (500-1150 µm) with broom-shaped tips (Upadhyay 1981). Fifteen isolates of *O. clavigerum* were detected from the mountain pine beetle, but only one isolate was detected from the pine engraver. The identification of *O. montium* and *O. clavigerum* was confirmed by ITS sequences analyses. The third sap-staining fungus, isolated less frequently, had morphological features of *Ceratocystiopsis*. However, due to the scarcity of ITS region sequences of this genus in GenBank, the ITS 2 sequence matched the sequence of *Ceratocystiopsis ranaculosa* Perry & Bridges with 95% similarity.

Five hundred eighty-seven yeast isolates were divided into four different groups based on morphological features such as colony colour, colony shape, spore shape, and spore size. Due to limited morphological characteristics and similarity with other yeast species, we were unable to identify them. The nucleotide D1/D2 domains of the LSU rDNA region have been sequenced for all currently recognized ascomycetous yeasts. Most, but not all, ascomycetous yeast can be identified from their partial LSU rDNA region (Kurtzman and Robnett 1998). Therefore, we chose to identify the yeasts in our work based on their LSU rDNA sequence similarity to the yeasts of the GenBank. The LSU sequences derived from four representative yeast isolates matched those of *Pichia* species with more than 99% sequence similarity. Among them, three yeasts were identified as *P. capsulata*, *P. holstii*, and *P. scolyti*. They constituted 74.4% and 88.5% of total isolates from the mountain pine beetle and the pine engraver. *Pichia holstii* and *P. scolyti* were the most commonly isolated yeasts from both bark beetles. *Pichia capsulata* was found only in mountain pine beetle. The LSU sequences of less common unidentified yeast matched that of *Candida* sp. (AY242329) with 99.13% similarity. This yeast was isolated from both beetles (Table 1).

Molecular results and phylogeny

Genomic DNA was successfully isolated from the surfaces of two bark beetle species. The amplified PCR products of the ITS region were size fractionated by agarose gel electrophoresis. Their sizes ranged from 400 to 800 bp. From clone libraries, a total of 250 ITS clones (125 from the mountain pine beetle and 125 from the pine engraver) were analyzed. RFLP analysis using *Hae*III and *Rsa*I divided these clones into 26 RFLP types. Each RFLP type was represented by 1 to 64 ITS clones (Table 2). Among them, 15 RFLP types were identified as being present in the mountain pine beetle and 14 were present in the pine engraver (Table 2). Similarly, there was no difference among fungi from the pine engraver taken from the five different trees. However, there were differences between the mountain pine beetle and the pine engraver fungi across all five trees. Of the 26 RFLP types, only three types were found in both beetles (Table 2). BLAST search results and phylogenetic analysis revealed that the 18 RFLP types (69.2%) had closest match to Saccharomycetaceous yeasts. The most predominant type, BAF 1, was found in both bark beetle clone libraries. The next most common types, BAF 9 and BAF22, were present in mountain pine beetle, while BAF6, BAF9, and BAF12 were present in pine engraver. Other RFLP types were less frequent; five types (19.2%) clustered with the sequences of filamentous *Euascomycetes* and three types (11.5%) with those of *Basidiomycota*. Only five RFLP types matched to the isolates (Fig. 1): BAF1 matched with *P. scolyti*, BAF3 with *P. capsulata*, BAF9 with *P. holstii*, BAF10 with unidentified yeast, and BAF17 with *Ceratocystiopsis* sp.

Phylogenetic analysis was carried out using only ITS region sequences. The alignment included sequences from 9 isolates and 26 RFLP types, along with other sequences from GenBank. Alignment was accomplished by inserting gaps, which resulted in a total of 989 characters. Three hundred sixteen ambiguous alignment characters were excluded from the data set. Six hundred seventy-three characters were used in the final analysis, which included 220 parsimony-uninformative characters and 453 informative characters. Due to limited ITS sequence data within the database, the closest matching of many RFLP types could not be established. The level of sequence divergence between the closest clone sequences was below 97% sequence similarity. Parsimony analysis yielded four most parsimonious trees (MPTs: length 2131; consistency index [CI] 0.4894; retention index [RI] 0.7724). One of those trees is

shown in Fig. 1. The phylogenetic analysis placed the fungi in three major groups representing two groups of *Ascomycota* (*Euascomycetes* and *Hemiascomycetes*) and one of *Basidiomycota*. These groups were separated by strongly supported branches (Bootstrap value, 94%) (Fig. 1). The majority of RFLP types and yeast isolates were confirmed to be Saccharomycetaceous yeasts falling in the Hemiascomycete group with a 94% parsimony bootstrap support. The yeast group consisted of a total 18 RFLP types with high similarity to ITS sequences of *Pichia* species in the database. Five RFLP types were placed in filamentous *Euascomycetes*. The filamentous fungi, including sap-staining ones, appeared to be associated with the pine engraver. The *Basidiomycota* clade included two isolates and three RFLP types. *Fomitopsis pinicola*, *H. annosum*, and BAF23 were grouped into the *Homobasidiomycetes* with high bootstrapping support (94%). BAF23 matched *Entomocorticium dendroctoni* Whitney with 99.65%. BAF26 was most closely related to a member of *Tremellales* in *Heterobasidiomycetes* and BAF25 was matched to *Rhodotorula lamellibrachiae*, which is classified among the *Urediniomycetes*.

Table 2. Number of clones of each RFLP type obtained from both bark beetles and closest matches from FASTA searches.

RFLP type	Acc. No	Clone No and source ¹				ITS size (bp)	RFLP pattern		Closest match in BLAST ²	Identity [%] ³
		MPB	Source	PE	Source		<i>Hae</i> III	<i>Rsa</i> I		
Hemiascomycetes (yeast)										
BAF1	AY761155	64	All	30	All	651	13,79,149,411	651	<i>Pichia scolyti</i> AB054111	552/566 (97%)
BAF2	AY761162	5	T2,T5			615	13,149,454	515	<i>Pichia scolyti</i> AB054111	355/369 (96%)
BAF3	AY761153	2	T2			645	13,149,484	645	<i>Pichia scolyti</i> AB054111	369/390 (94%)
BAF4	AY761163	3	T1			649	13,79,150,408	214,436	<i>Candida</i> sp. AY559447	543/600 (90%)
BAF5	AY761164			2	T2	653	13,79,150,412	213,441	<i>Candida atlantica</i> AJ539368	362/382 (94%)
BAF6	AY761165			40	T1,T2,T3	657	79,161,418	10,155,493	<i>Pichia mexicana</i> AB054110	560/574 (97%)
BAF7	AY761166	1	T4			580	79,502	580	<i>Pichia scolyti</i> AB054111	357/361 (98%)
BAF8	AY761167	1	T2			521	79,443	521	<i>Pichia scolyti</i> AB054111	345/359 (96%)
BAF9	AY761154	24	All	11	T1,T2,T4,T5	661	82,575	661	<i>Candida ernobii</i> AY585212	540/545 (99%)
BAF10	AY761156	2	T3	1	T3	681	84,598	681	<i>Candida ernobii</i> AY585212	407/448 (90%)
BAF11	AY761168	1	T5			658	79,580	658	<i>Candida ernobii</i> AY585212	379/395 (95%)
BAF12	AY761169			23	T1,T3,T4,T5	658	178,481	658	<i>Lacazia loboi</i> AF035675	179/187 (95%)
BAF13	AY761170			1	T4	658	173,486	658	<i>Pichia guilliermondii</i> AF455495	170/175 (97%)
BAF14	AY761171	3	T5			791	791	791	<i>Kluyveromyces thermotolerans</i> AY046207	172/177 (97%)
BAF15	AY761172			5	T1	644	644	644	<i>Saccharomyces crataegensis</i> AF411061	452/546 (83%)
BAF16	AY761173			2	T4,T5	767	248,520	767	<i>Pichia guilliermondii</i> AF455495	179/183 (97%)
BAF22	AY761174	12	T2,T3,T4			404	66, 66,272	404	<i>Clavispora lusitaniae</i> AY321475	320/374 (86%)
BAF24	AY761175	1	T2			456	456	456	<i>Candida petrohuensis</i> AY585213	111/119 (93%)
Filamentous Eusascomycetes										
BAF17	AY761157	2	T1,T4			692	12,42,57,104, 144,333	692	<i>Ceratocystiopsis ranaculosa</i> AY542504	396/416 (95%)
BAF18	AY761176			2	T2,T3	575	575	575	<i>Cladosporium tenuissimum</i> AF393724	542/543 (99%)
BAF19	AY761177			3	T3	613	89,102,124,298	613	<i>Sepedonium boletiphagum</i> AF054867	511/572 (89%)
BAF20	AY761178			1	T4	604	6,65,93,93,349	604	<i>Penicillium brevicompactum</i> AY373898	558/560 (99%)
BAF21	AY761179	1	T5			582	31,122,431	210,382	Leaf litter ascomycete AF502745	444/458 (96%)
Basidiomycota										
BAF23	AY761180			2	T4	660	41,146,473	80,145,436	<i>Entomocorticium dendroctoni</i> AF119506	544/548 (99%)
BAF25	AY761181	3	T1			611	611	611	<i>Rhodotorula lamellibrachiae</i> AB025999	548/574 (95%)
BAF26	AY761182			2	T4,T5	578	104,474	578	<i>Tsuchiyaea wingfieldii</i> AF444327	525/537 (97%)

¹ MPB, mountain pine beetle and PE, pine engraver.² The entire sequence information comprising ITS 1 region, the 5.8S rDNA, the ITS 2 region and the 5' end of the 28S rDNA was used for BLAST searches. The names listed were taken from the respective database accessions and do not necessarily reflect current use.³ Identity [%] was represented by matched nucleotide / compared nucleotide. Accessions and identity in bold type are used in tree construction.

Discussion

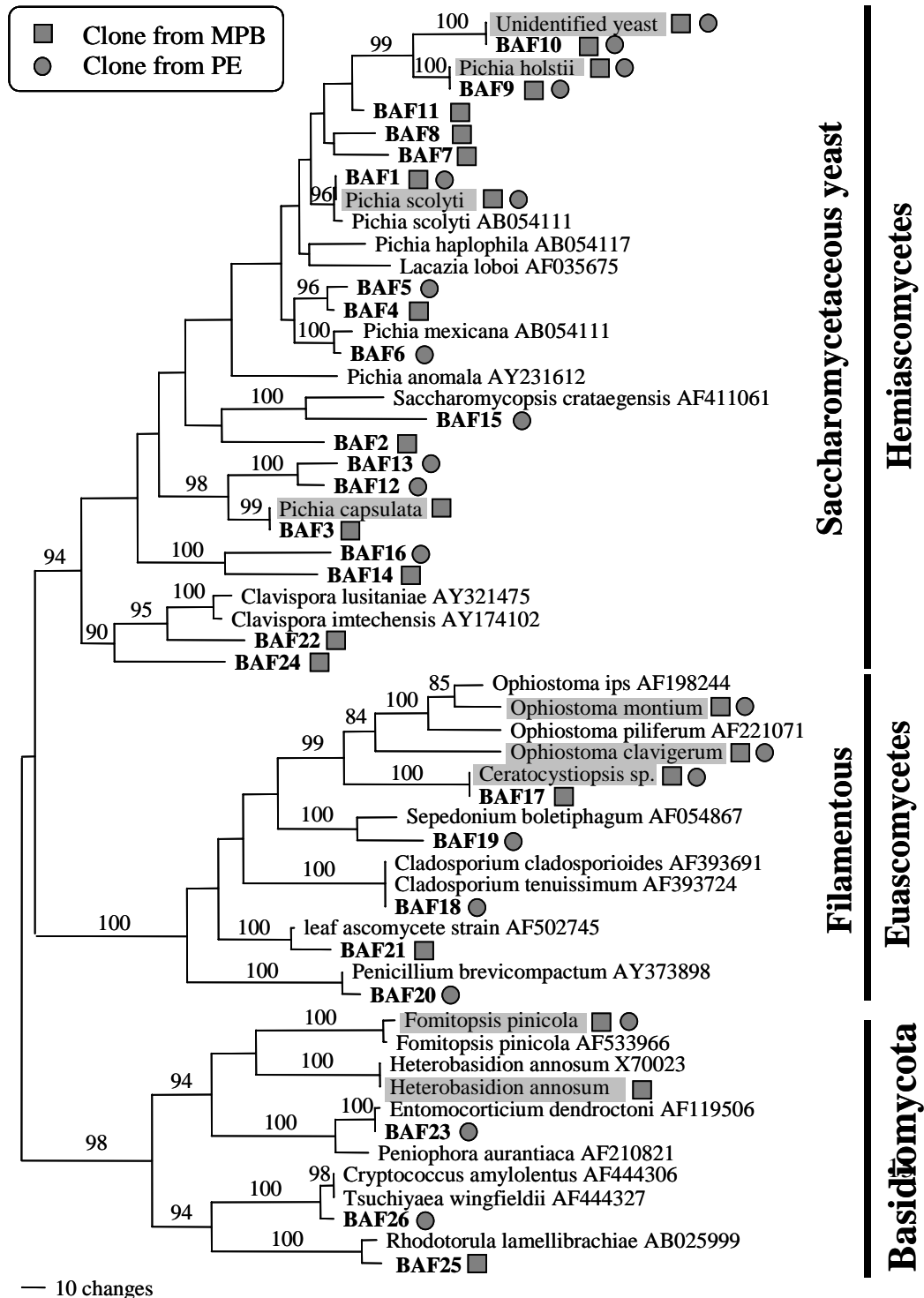
The culture and DNA methodology used in this work have complementary strengths. Yeasts and non-staining filamentous *Euscomycetes* were, most efficiently, detected by the molecular methods, while the major sap-staining fungi and decay fungi were more frequently detected using the culture methods. Similar fungal communities were found on mountain pine beetle and pine engraver using the cultural method, while different fungal species were detected with the DNA approach.

Previously, fungus surveys from bark beetles have focused mainly on staining fungi because they cause important economic losses to the forest product industry (Uzunovic et al. 1999; Lee et al. 2003; Six and Bentz 2003). Other fungi that also are typically associated with mountain pine beetle and pine engraver and do not discolour sapwood have been systematically overlooked. The finding that 76.9% of cloned DNAs was yeast indicated that yeast species are commonly associated with mountain pine beetle and pine engraver, and also suggested that yeasts play an important ecological role.

The sequence similarities of the ITS and LSU rDNA regions are broadly used and are considered to be an excellent basis for studying fungus diversity in ecological investigations (Smit et al. 1999; Horton and Bruns 2001; Buchan et al. 2002; Allen et al. 2003). A major impediment in such work is a lack of reference ITS sequence information in the public databases. Due to the scarcity of Saccharomycetaceous yeast ITS region sequences, many of the clones sequenced in the current work clustered with other ascomycetous sequences and matched database sequences across a very small length of the sequence or with low similarity.

Our phylogenetic analysis showed that at least five different genera of yeasts were associated with bark beetles. Three genera, *Pichia*, *Clavispora* and *Saccharomycopsis*, are included in Saccharomycetaceous yeasts, and two genera, *Rhodotorula* and *Cryptococcus*, are classified with basidiomycetous yeasts. *Pichia*, *Clavispora* and *Rhodotorula* were detected on mountain pine beetle, while *Pichia*, *Saccharomycopsis* and *Cryptococcus* were found on pine engraver. *Pichia* species are commonly associated with eggs, larvae, pupae, and adult bark beetles, and are also frequently isolated from the walls of beetle galleries and pupal chambers, as well as from xylem tissues (Robinson 1962). *Pichia pini*, *P. capsulata* and *P. holstii* have been reported from mountain pine beetle (Grosmann 1930; Robinson 1962; Kurtzman and Fell 1998). We detected 18 RFLP types of *Pichia* even though only two restriction enzymes were used on the ITS PCR products. In contrast to previous reports, we did not find *P. pini* (Rumbold 1941; Robinson 1962); instead, *P. scolyti* was commonly detected among the 12 *Pichia* species from mountain pine beetle and the eight *Pichia* species from the pine engraver. Other common isolates, found only on the mountain pine beetle, clustered with *Clavispora* species. This group is basal to the other Saccharomycetaceous yeasts. Although species of *Clavispora* have been reported from flies and moths, they have not been reported on mountain pine beetle and pine engraver (Kurtzman and Fell 1998).

Fig. 1. One of the most parsimonious trees comparing ITS sequences of the isolates and RFLP types from surfaces of mountain pine beetle (MPB) and pine engraver (PE) to reference sequences. Bootstrap support values are indicated (when greater than 70%) above corresponding branches. Isolates from cultural method are in shaded box. Taxa originating from the representative RFLP types are in bold. Square and circle indicate that fungi were found on mountain pine beetle and pine engraver, respectively.



Our results, from the cultural approach, were generally consistent with previous results. Only a few species of sap-staining fungi have been found on mountain pine beetle. *Ophiostoma clavigerum* and *O. montium* have been known to be closely associated with the mountain pine beetle throughout its life cycle in lodgepole pine (Rumbold 1941; Robinson 1962) and were the most prevalent isolates in this work. These two fungi are known to be the primary invaders of fresh sapwood (Whitney and Farris 1970; Solheim 1995). A *Ceratocystiopsis* sp. with similar morphological features to the genus *Ophiostoma* was present in lower numbers. However, its DNA sequence as well as its morphology could not be matched to known *Ceratocystiopsis* species (Zhou et al. 2004), suggesting that this isolate may be a new species. Because little is known about fungi associated with pine engraver, we were unable to compare our results with previous observations. In this work, however, the same sap-staining fungi (*Ceratocystiopsis* sp. and *O. montium*) and yeasts (*Pichia holstii*, *P. scolyti*, and unidentified yeast) were isolated from both the mountain pine beetle and the pine engraver infesting the same host tree.

Although most associations among bark beetles and fungi involve ascomycetes, a few associations with basidiomycetes have been characterized (Castello et al. 1976; Alexander et al. 1980; Whitney et al. 1987; Garcia and Morrell 1999). Three basidiomycetous fungi, *Entomocorticium dendroctoni*, *Fomitopsis pinicola* and *Heterobasidion annosum*, were detected in this work. *Entomocorticium* species appear to be the most common and widespread basidiomycetous associates of bark beetles (Tsuneda et al. 1993). Some evidence suggests that *E. dendroctoni* may contribute nutritionally to the mountain pine beetle (Whitney et al. 1987). The white pocket rot fungus, *H. annosum*, has been associated with bark and wood-boring beetles (Alexander et al. 1980; Garcia and Morrell 1999). The brown rot fungus, *F. pinicola*, has also been found in Douglas-fir beetles (*Dendroctonus pseudotsugae* Hopkins) that were trapped in flight or were removed from galleries in Douglas-fir (Castello et al. 1976). Our results support the hypothesis of Castello et al. (1976) that mycelium fragments of decay fungi can be trapped in the intersegmental areas of beetles' bodies. While there are also many examples of decay fungi dispersed by beetles (Nuss 1982; Thomsen and Koch 1999), the importance of mountain pine beetle and pine engraver in disseminating decay fungi needs to be determined.

Except for *Ceratocystiopsis* sp., the DNA method did not detect decay and sap-staining fungi. Our inability to detect certain species may have resulted from several factors including low proportion of associated fungi, DNA extraction bias, PCR drift and the GC ratio. The low density of decay fungi in the overall microflora may have inhibited their detection. In culture, the presence of benomyl favors decay fungi and reduces competitive microflora. Although the bead beating DNA extraction method has been used to study fungal communities, differences in cell wall composition between different fungus types (sap-staining fungi contain cellulose in addition to chitin) might affect cell lysis (Jewell 1974; Yeates and Gillings 1998; Kresk and Wellington 1999; Griffiths et al. 2000). To minimize PCR drift by decreasing PCR bias, we pooled six PCR amplicons (Polz and Cavanaugh 1998). The GC ratio of the amplified DNA region might also affect PCR amplification. The GC ratios from the

total ITS region of *O. clavigerum* and *O. montium* were high at 63.7% and 59.4%, respectively. *Ophiostoma clavigerum* and related *Leptographium* species are known to have high GC ratios, especially in their ITS 1 region, and so only the ITS2 region and part of the LSU region have been used in the molecular phylogeny of these fungi (Hausner et al. 2000; Jacobs et al. 2001; Lim et al. 2004). In contrast to the sap-staining fungi, the other fungi found in this work had low GC ratios. The GC ratio of Saccharomycetaceous yeasts was fairly low, ranging from 36.8 to 44.1%. This suggested that the high GC ratio of the sap-staining fungi might have a negative effect on PCR amplification, affecting their detection by the molecular method.

Despite the biases and limitations of the molecular method, a more diverse fungal community was found using DNA analyses than using cultures, and our results showed that the cultural approach alone is not sufficient for determining fungus diversity.

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