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Target-specific PCR primers can detect and differentiate ophiostomatoid fungi from microbial communities associated with the mountain pine beetle *Dendroctonus ponderosae*

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ABSTRACT

The aim of this study was to develop DNA probes that could identify the major fungal species associated with mountain pine beetles (MPB). The beetles are closely associated with fungal species that include ophiostomatoid fungi that can be difficult to differentiate morphologically. The most frequently isolated associates are the pine pathogens *Grosmannia clavigera* and *Leptographium longiclavatum*, the less pathogenic *Ophiostoma montium*, and an undescribed *Ceratocystiopsis* species (*Cop.* sp.). Because growing, isolating and extracting DNA from fungi vectored by MPB can be time and labour intensive, we designed three rDNA primer sets that specifically amplify short rDNA amplicons from *O. montium*, *Cop.* sp. and the pine *Leptographium* clade. We also designed two primer sets on a gene of unknown function that can differentiate *G. clavigera* and *L. longiclavatum*. We tested the primers on 76 fungal isolates that included MPB associates. The primers reliably identified their targets from DNA obtained from pure fungal cultures, pulverized beetles, beetle galleries, and tree phloem inoculated with *G. clavigera*. The primers will facilitate large-scale work on the ecology of the MPB-fungal-lodgepole pine ecosystem, as well as phytosanitary/quarantine sample screening.

Introduction

Bark beetles, including the mountain pine beetle (*Dendroctonous ponderosae*, MPB), are associated with a diversity of microorganisms that the beetles vector when they colonize host trees. Some of these microorganisms are associated with specific beetle species, while others are associated with multiple vectors (Kirisits 2004; Six 2003b). The majority of filamentous fungi carried by bark beetles belong to a group of wood-colonizing fungi generally called ophiostomatoid fungi. For a few bark beetle ecosystems, these fungi have been isolated on artificial media and identified using morphological and molecular approaches (Harrington 1987, 1993; Six & Paine 1999). However, these methods are impractical for population and quarantine work when large numbers of samples need to be analyzed.

Most ophiostomatoid associates of MPB are specific to this beetle vector and are rarely isolated from other

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ecosystems (Lim et al. 2005). Ophiostomatoid fungi represent a diverse group of species that are present in various ecological niches (Wingfield et al. 1993). Among the fungi carried by MPB, those in the *Leptographium* clade (Lim et al. 2004), *Grosmannia clavigera* and *Leptographium longiclavatum* are known pine pathogens (Yamaoka et al. 1995; Lee et al. 2006a; Plattner et al. 2008) while *Ophiostoma montium* has been shown to be a weak pathogen of pine (Solheim & Krokene 1998). *Ceratocystiopsis* sp. from MPB has been reported as *Cop. manitobensis*-like or *O. minuta*. This species is mainly found on beetle bodies and in beetle galleries but does not colonize or stain pine sapwood (Kim et al. 2005; Lee et al. 2006b; Plattner et al. 2009). While the above fungal species have been isolated from the exoskeleton and gut of MPB, *G. clavigera* and *L. longiclavatum* have also been found in beetle mycangia (Six 2003a; Lee et al. 2006a). When MPBs attack healthy trees, the fungi may benefit the beetle by modifying toxic tree defence chemicals, reducing sapwood and phloem moisture, making the environment favourable to beetle reproduction and development and providing a source of nutrients to beetle progeny (Whitney 1982; Harrington 1993; Six 2003b). However, the specific roles of these fungal species in the MPB/lodgepole pine ecosystem and in the beetle life cycle remain uncertain. To clarify the relative abundance and the roles of the different MPB associates throughout the beetle life cycle in endemic and epidemic landscapes, requires identification methods that are specific, rapid and cost effective.

The diversity of fungi associated with MPB has been well established by culturing them on artificial media. Cultures are obtained by spreading washes of the beetle exoskeleton, gut and mycangia and by directly inoculating the medium by streaking the beetle across the media surface (Lee et al. 2006b; Six 2003a). The growing colonies need to be further purified and transferred to new media before morphological characteristics or DNA analyses can be conducted to identify each fungal species. All of these techniques are labour intensive, time consuming, and have other limitations (Harrington 1987; Jacobs et al. 2003; Lee et al. 2003; Lim et al. 2005). For example, isolation on artificial media can underestimate the presence or frequency of a fungal species. Yeasts are often present in high numbers and can prevent or mask the growth of filamentous fungi by competing for space and nutrients or by producing inhibitory metabolites (Luchi et al. 2005; Lee et al. 2006b, unpublished results).

To resolve these limitations and to address high throughput identification that is necessary for ecological and other biological studies, as well as for phytosanitary issues, our objectives were to establish rapid protocols for extracting DNA from fungal cultures and microbial communities from MPB bodies and tree phloem, develop primer sets for identifying the ophiostomatoid fungi associated with MPB and its galleries and to compare the efficacy of the DNA detection method with the traditional technique of isolation and identification. We anticipate that direct detection methods could be used to resolve the diversity of MPB fungal associates in various ecological niches and to rapidly confirm the presence or absence of the fungi in forest products destined for export.

Materials and methods

Study design and sampling

We compared the traditional isolation and culture technique to direct DNA extraction and PCR amplification for detecting the presence or absence of specific fungi on MPB bodies, beetle galleries and inoculated pine phloem. To assess the robustness of our methodology, the samples were obtained from different locations and were harvested at different times during the year. We also tested the methodology on a more controlled system: lodgepole pine phloem inoculated with a single pathogen, *Grosmannia clavigera*.

Twenty MPBs were collected from mature lodgepole pine trees from Bear Service Road near Merritt, BC that had been baited with Mountain Pine Beetle Tree Bait (Contech Enterprises, Delta BC) and attacked by MPB in the summer of 2009. The trees were harvested in Sep. 2009 and logs were incubated at room temperature in plastic bags for 3 m until adult beetles emerged. New adults were removed from the incubated logs and used for fungal isolation and direct DNA extraction.

Trees from the same site (Bear Service Road near Merritt, BC) attacked by MPB in the summer of 2009 were harvested during Nov. 2009 and kept at 4 °C for two weeks before MPB galleries were sampled. Two adjacent disks of phloem containing MPB galleries were removed with a 7.0 mm diameter cork borer; one disk was used to isolate the fungi on 1 % Oxoid malt extract agar (OMEA) while the other was used to extract the DNA directly. In total, there were 10 sample locations. At each location one disk was for isolation while the other was for direct DNA extraction.

Additional MPB galleries were collected during Oct. 2009 from trees attacked by an endemic population of MPB, in Prosser Creek, California. Samples were stored at 4 °C for four weeks prior to fungal isolation and DNA extraction. Sampling and isolation were carried out as described above. Thirteen sample locations were tested.

Finally, lodgepole pine trees were inoculated at the end of Aug. 2009 with *G. clavigera*, following the method of Lee et al. (2006b). For this test, the inoculation was performed at a density of eight inoculation points per tree, which does not cause tree death. Two months after inoculation with *G. clavigera*, bark pieces including phloem that contained fungal lesions of ~10 cm and the surrounding tissues, were collected. To confirm the presence or absence of *G. clavigera*, isolations were carried out with ~1–2 mm phloem samples located either inside the lesion or at 1–2 cm outside the lesion edge. From the same bark/phloem samples, additional phloem disks were removed with a 7.0 mm diameter cork borer from both inside and outside the lesions, to extract and amplify DNA with *G. clavigera*-specific primers (see below). Ten sample locations from different lodgepole pines inoculated with *G. clavigera*, were tested.

Fungal isolation

For MPB gallery isolation, one of the two gallery disks was cut into four pieces, placed on 1 % OMEA plates and incubated.

Growth of yeasts and filamentous fungi was monitored. Fungi were re-isolated and identified through morphology and DNA characterization, using the specific primers developed in this study. All the fungal isolations and cultures were incubated at ~22 °C in the dark.

For microbial isolation from MPBs, two beetles were placed in a 1.5 ml microfuge tube with 500 µl of 0.01 % Tween 20. The beetles were crushed with a pipette tip and vortexed for 10 min at medium speed. Then 50 µl of the supernatant were serially diluted 10, 100 and 1000 times. 50 µl of each dilution were spread onto 1 % OMEA. Colonies of yeast/bacteria and fungi were monitored. Fungal colonies were transferred into new media as soon as hyphal growth occurred. The initial inoculated media plates continued to be monitored for one or two more weeks to allow the growth of the slow growing species; these species were also transferred to new media. The fungi were identified as described above.

Primer design, testing and PCR conditions

Target-specific primers were designed from rDNA sequences of target species and close relatives that were downloaded from GenBank and aligned using the MUSCLE algorithm in CLC Main Workbench 5 (Edgar 2004; CLC bio, Cambridge, MA). Potential primer sites were selected from the alignment and were tested to minimize self-complementarities using Primer 3 v. 0.4.0 (Rozen & Skaletsky 2000).

To further differentiate *Grosmannia clavigera* from *Leptographium longiclavatum*, which are close relatives, a more specific set of primers was designed within a gene of unknown function (GenBank Accession number EE729832) that we selected from EST libraries (DiGuistini *et al.* 2007). This gene is currently being used in work to characterize *G. clavigera* populations (Massoumi Alamouti *et al.*, unpubl. data). We found that *G. clavigera* has a deletion of 21 bp in this gene whereas *L. longiclavatum* does not. Primer 3 was used to design specific primers for *L. longiclavatum*; however, while Primer 3 did not provide satisfactory primers for *G. clavigera*, Visual OMP (DNA Software, Ann Arbor, MI; SantaLucia 2007) generated an appropriate and specific primer set.

Each primer set was tested on ten isolates of the target species, including the holotype, as well as on one or two isolates of other ophiostomatoid fungi and other fungi that were occasionally found in the MPB microbial community (Hausner *et al.* 2003; Six *et al.* 2003; Kim *et al.* 2005; Zipfel *et al.* 2006; Lee *et al.* 2006a; Massoumi Alamouti *et al.* 2009). In total 76 isolates were tested in our screening analysis (Table 1). Identities of the isolates were confirmed morphologically and through DNA sequencing.

PCR reactions were carried out following standard methods (Kim *et al.* 2004). Table 2 shows the annealing temperatures of the primers. All reactions included general primer positive controls, LROR (Bunyard *et al.* 1994) and LR3 (Vilgalys & Hester 1990) and no template negative controls in order to ensure that negative bands were due to specificity of the primers and not to poor DNA quality. All reactions were repeated to ensure consistency. In order to ensure that the specific primers amplify their targets, we sequenced the amplicons for two representatives of each species from pure culture as well as from DNA extracted from MPBs. Purified PCR products were sent to Macrogen Inc. in Seoul, South Korea for sequencing (using the BigDyeTM terminator kit and run on ABI 3730XL). A specific primer set was considered satisfactory if it produced a PCR amplicon only from all the isolates of its respective species.

The minimum DNA concentration that each primer set could detect was determined using serially diluted target fungal DNA in the PCR reaction. Initial DNA concentration was determined with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

DNA extraction from pure cultures, MPBs, MPB galleries and fungal inoculated lodgepole pine phloem

We extracted the DNA of fungal species (Table 1) grown on 1 % OMEA. Cultures were harvested with a blunt scalpel when mycelia had covered approximately one quarter of a standard 100 × 15 mm Petri dish. DNA was extracted by adding the mycelia to a lysing matrix A tube (MP Biomedicals Solon, OH) with 500 µl of TES buffer (100 mM Tris, 100 mM EDTA and 2 % SDS). The tubes were shaken in a Mixer/Mill 8000 (SPEX CertiPrep, Metuchen, NJ) for 7 min. Then 0.2 % lysing enzyme (*Trichoderma harzianum*, SIGMA, St. Louis, MO) was added to each of the tubes and incubated at 40 °C for 15 min, with intermittent mixing. 250 µl of 3 M sodium acetate was added to the tubes that were then vortexed and placed in a −80 °C freezer for at least 15 min. The tubes were thawed, vortexed and centrifuged for 15 min at 14 000 rpm. The lysate was removed and the precipitate was washed twice with phenol:chloroform: isoamyl alcohol (25:24:1). Finally, DNA was precipitated in isopropanol (90 %) and sodium acetate (10 %), washed with 70 % ethanol and then resuspended in distilled water.

DNA was extracted from the same beetles from which we isolated the fungi. We added 50 µl of 10× concentrated TES buffer to the crushed beetles in Tween 20 and transferred the whole mixture into a lysing matrix A tube. The tubes were shaken for 30 s in a Mixer/Mill 8000 chamber that had been cooled to −20 °C. The remainder of the procedure was identical to that of DNA extraction from pure cultures.

The same procedure was used to extract DNA directly from MPB galleries and inoculated phloem. The samples were maintained at −20 °C until they were processed. The frozen samples were freeze-dried over-night in microfuge tubes with punctured lids. They were transferred to lysing matrix A tubes and shaken for 5 min in a Mixer/Mill 8000 chamber that had been cooled to −20 °C. Once removed, 500 µl of TES buffer and 0.2 % lysing enzyme were added to the tubes, which were then incubated at 40 °C for 15 min. 143 µl of 5 M NaCl and 100 µl of 10 % CTAB were added and the tubes were incubated for 10 min at 65 °C. The remainder of the procedure was identical to that of the DNA extraction from pure cultures.

Results

Primer design and testing

We designed three sets of target-specific primers from ribosomal DNA (Fig 1) for each of the three groups of ophiostomatoid fungi that occupy different ecological niches in the MPB system. The set of Lepto primers amplify species in the

Table 1 – List of isolates.

Species	Isolate	Host/substrate	Location	Identified by
MPB-associated ophiostomatoid fungi				
Ceratocystiopsis sp. 1	WY21BX12	*P. contorta* MPB gallery	BC	J.-J. Kim
	954 AW HR1	*P. contorta* MPB gallery	BC	J.-J. Kim
	WY13TX13	*P. contorta* MPB gallery	BC	J.-J. Kim
	WG51EW1-2	*P. contorta* MPB gallery	Riske Creek, BC	J.-J. Kim
	S389EW1-1	*P. contorta* MPB gallery	Radium, BC	J.-J. Kim
	S5R133A2-1	*P. contorta* MPB gallery	Little Fort, BC	J.-J. Kim
	MR14EW3-2	*P. contorta* MPB gallery	Manning Park, BC	J.-J. Kim
	MR16AW3-2	*P. contorta* MPB gallery	Manning Park, BC	J.-J. Kim
	987 AW 2-1	*P. contorta* MPB gallery	Doig River at Quesnel, BC	J.-J. Kim
	S5R135A2-2	*P. contorta* MPB gallery	Little Fort, BC	J.-J. Kim
Grosmannia clavigera	ATCC18086	*P. ponderosae*	BC	RC Robinson-Jeffrey
	SL-Kw1407	*P. contorta* sapwood	Kamloops, BC	S. Lee
	H55	*P. contorta* sapwood	Houston, BC	S. Lee
	B8	*P. contorta* sapwood	Banff, AB	S. Lee
	B10	*P. contorta* sapwood	Banff, AB	S. Lee
	BW26	*P. contorta* sapwood	Banff, AB	S. Lee
	BW28	*P. contorta* sapwood	Banff, AB	S. Lee
	M4	*P. contorta* sapwood	Manning Park, BC	S. Lee
	M16	*P. contorta* sapwood	Manning Park, BC	S. Lee
	DPLKGT6A	*P. contorta* sapwood	Kelowna, BC	S. Massoumi Alamouti
Leptographium longiclavatum	SL-W001	*P. contorta* sapwood	Kamloops, BC	S. Lee
	DPLKG	*P. contorta* sapwood	Kelowna, BC	S. Massoumi Alamouti
	Pa-3	*P. albicaulis*	Unknown	K. Bleiker
	PY8GA	*P. ponderosae* sapwood	Kamloops, BC	S. Massoumi Alamouti
	PY8-1	*P. ponderosae* sapwood	Kamloops, BC	S. Massoumi Alamouti
	927 A G-2	*P. contorta* sapwood	Northern BC	J.-J. Kim
	DPSMWC2	*Picea* sp.	Kamloops, BC	C. Breuil
	DPSIWC1	*Picea* sp.	Kamloops, BC	C. Breuil
	SLWw402	*P. contorta* sapwood	BC	S. Lee
	866 A EG1-L23	*P. contorta* sapwood	Northern BC	J.-J. Kim
Ophiostoma montium	CBS151.78-2	*P. ponderosa*	Unknown	R.W. Davidson
	92-628/55/4	*P. contorta*	Sunday Creek, New Princeton, BC	Solheim
	MG3DG1	*P. contorta* MPB gallery	Manning Park, BC	J.-J. Kim
	MG4EWI1-1	*P. contorta* sapwood	Manning Park, BC	J.-J. Kim
	MR12AW2-2	*P. contorta* sapwood	Manning Park, BC	J.-J. Kim
	MG6DGI-1	*P. contorta* Ips gallery	Manning Park, BC	J.-J. Kim
	WG56AW2-1	*P. contorta* sapwood	Riske Creek, BC	J.-J. Kim
	WG57AGF-1	*P. contorta* MPB gallery	Riske Creek, BC	J.-J. Kim
	S3G90E	*P. contorta* sapwood	Radium, BC	J.-J. Kim
	S4G116AW1-2	*P. contorta* sapwood	Cranbrook, BC	J.-J. Kim
Other ophiostomatoid fungi				
Ambrosiella sp.	WY42EW3-1	*P. contorta* sapwood	Riske Creek, BC	J.-J. Kim
	WR43EW1-2	*P. contorta* sapwood	Riske Creek, BC	J.-J. Kim
Ceratocystiopsis brevicomi	UM 1452	*D. brevicomis*	CA, USA	T. Harrington
Ceratocystiopsis manitobensis	UM214	*P. resinosa*	MB, Canada	J. Reid
	UM237	*P. resinosa*	MB, Canada	J. Reid
Ceratocystiopsis minima	UM 235	*P. banksiana*	MB, Canada	J. Reid
	UM 85	*P. resinosa*	MB, Canada	J. Reid
Ceratocystiopsis minuta	CBS145.59	Unknown	USA	New York Bot. Garden
	CBS463.77	*P. engelmannii*	USA, New Mexico, Irez Ritos	R.W. Davidson
	UM 1532	Unknown	Krynki For. District Poland	Dr. Jankowiak
	YCC 294	Gallery of I. cembrae	Japan	Y. Yamaoka
Ceratocystiopsis minuta-bicolor	UM 480	*P. contorta*	AB, Canada	J. Reid
	UAMH 9551	*P. contorta*	AB, Canada	J. Reid
Ceratocystiopsis pallidobrunnea	UM 51	*Populus tremuloides*	MB, Canada	J. Reid
Ceratocystiopsis ranaculosa	CBS 216.88	*P. taeda*	LA, USA	J.R. Bridges
Grosmannia aurea	ATCC16936	*P. contorta* sapwood	BC	RC Robinson-Jeffrey
	854 AW1-1	*P. contorta* sapwood	Robson National Park, BC	J.-J. Kim

Table 1 – (continued)

Species	Isolate	Host/substrate	Location	Identified by
Grosmannia robusta	CMW668	*Picea abies*	S. Africa	
Leptographium pyrinum	CMW3889	*P. jeffryi*	Unknown	Unknown
Leptographium terebrantis	CBS337.70	*P. taeda* gallery of *D. terebrans*	Elisabeth, Louisiana, USA	S. Barras
	AU189-6-T6	*P. contorta* sapwood	Blaimore, AB	A. Uzunovic
	DPCHWC11	*P. contorta* sapwood	Cypress Hill, SK	S. Massoumi Alamouti
	DPCHWC12	*P. contorta* sapwood	Cypress Hill, SK	S. Massoumi Alamouti
Ophiostoma abietinum	CMW1468	Unknown	Unknown	Unknown
Ophiostoma adjuncti	CMW135	*P. ponderosae*	Unknown	R.W. Davidson
Ophiostoma bicolor	CBS492.77	Picea sp. Ips gallery	Colorado, USA	R.W. Davidson
Ophiostoma ips	CBS137.36	*Ips integer* gallery	USA, Oregon	C.T. Rumbold
	ATCC24285	*Pinus contorta* sapwood	Canada	H.S. Whitney
Ophiostoma minus	S4-105E1-1	*P. contorta* sapwood	Cranbrook, BC	J.-J. Kim
	S3-74E1-1	*P. contorta* sapwood	Radium, BC	J.-J. Kim
Ophiostoma nigrocarpum	CBS 637.66	*Abies* sp.	Idaho, USA	R.W. Davidson
Ophiostoma piceae	DAOM229575	Unknown	Unknown	K. Seifert
	DAOM229576	Unknown	Unknown	K. Seifert
Basidiomycetes				
Entomocorticium sp.	WR42AW3-1	*P. contorta* sapwood	Riske Creek, BC	J.-J. Kim
	WY47EW1-1	*P. contorta* sapwood	Riske Creek, BC	J.-J. Kim
Trichaptum abietinum	S4-99A	*P. contorta* sapwood	Cranbrook, BC	J.-J. Kim

Cultures were from American Type Culture Collection (ATCC), Centraalbureau voor Schimmelcultures (CBS, Netherlands), Reid's culture collection at University of Manitoba (UM), University of Alberta Microfungus Herbarium (UAMH), Yamaoka's culture collection, Japan (YCC) and Breuil at University of British Columbia.

Leptographium clade, including the pathogens *Grosmannia clavigera* and *Leptographium longiclavatum*, as well as *Grosmannia aurea*, *Leptographium pyrinum*, *Grosmannia robusta* and *Leptographium terebrantis* that are usually not associated with MPB. The Omon primers amplify *Ophiostoma montium*. The CopMPB primers amplify *Ceratocystiopsis* sp. (Fig 2).

Since we could not differentiate *L. longiclavatum* and *G. clavigera* with rDNA primers, we designed specific primer sets to differentiate the two species using a gene of unknown function. These two specific primer sets, Llongi and Gclavi differentially amplify *L. longiclavatum* and *G. clavigera*, respectively. The primer sequences and the conditions for the amplification are shown Table 2. The Gclavi primer set detected only the *G. clavigera* isolates and none of the other closely related species. The Llongi primer set reacted with *L. longiclavatum* but not with *G. clavigera* or most species from the *Leptographium* clade; the one exception was *L. terebrantis*, which is rarely associated with the early MPB colonization of the trees.

Primers were designed to amplify regions of approximately 200 bases. The annealing temperatures were selected between 60 and 65 °C (Table 2).

Comparing fungal isolation on media with DNA detection techniques

We optimized the DNA extraction protocols for pure cultures and microbial communities associated with MPB bodies and galleries. Extraction from pure cultures and beetles were

Table 2 – Sequences and summary information for each target-specific primer set.

Primer name	Sequence	Annealing temperature (°C)	Amplicon length (bp)	Min. DNA detection (ng/ml)
OmonF	CCTGTCCGAGCGTCATTTCC	60	224	4×10^{-5}
OmonR	CCAAGAGAAGAATCCTGGACTGCT			
LeptoF	GACGCGAGTCTGCCTCCTT	63	195	4×10^{-5}
LeptoR	CGCCAGAAGCATCCTCTCCA			
CopMPBF	AGTCTTAACGAGCGTCTGAGTAGGA	64	227	4×10^{-6}
CopMPBR	AACACCAGCGCTAGGCGCACTG			
LlongiF	TCGAGGCTGAGAAGGTCCTGGTCA	65	263	4×10^{-5}
LlongiR	GCAAGGACGCCGAGCAGTTTCT			
GclaviF	CGCTCTCCCCTAGTTCCTGCTCT	65	174	4×10^{-5}
GclaviR	CGGGCGCCGAGGGTTACTAGGA			

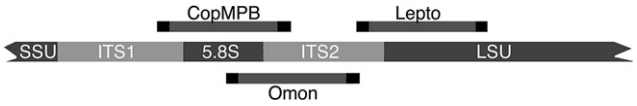

Fig 1 – Schematic diagram indicating the relative position of the target-specific primers on the rDNA.

completed in less than 4 h, while for MPB galleries and fungal inoculated phloem extractions took 6 h after the samples had been freeze-dried. In contrast, the fungal isolation technique required on average two to three weeks before a final identification could be confirmed using either DNA sequencing or the specific DNA primer sets developed in this work.

In inoculated phloem, *Grosmannia clavigera* was detected within and outside the edge of the lesions using the Gclavi primer set. Similar data were obtained with fungal isolation on 1 % OMEA; *G. clavigera* was present inside and outside the lesion edge. Both methods gave consistent results.

Overall, *Cop.* sp. and yeasts were abundant in our adult beetle samples. We isolated *Cop.* sp. three times more frequently than the other filamentous fungi. As well, the beetle body isolation produced eight to ten times more yeast colonies than filamentous fungi. Yeasts were also isolated from every beetle gallery sample. We only sampled a single point from each gallery, which is not representative of the whole gallery and does not allow for quantification.

On MPB bodies, *Cop* sp. was detected on all samples with both methods. For the other fungi associated with MPB we observed some variation in detecting *G. clavigera*, *Leptographium longiclavatum* and *Ophiostoma montium* between the two methods. From the BC MPB galleries, we were successful at detecting *Cop.* sp. with the DNA detection method but not with the isolation method. With the isolation method, the other fungal species were more frequently detected. Finally, for the MPB gallery samples from California, while the two methods gave overall similar results, species varied somewhat between samples from adjacent galleries. We detected neither *Cop* sp. nor *L. longiclavatum*, in the California samples using either method, however, these two species have not been reported in MPB galleries sampled in the US (Table 3).

Discussion

In this work we successfully designed and tested three sets of rDNA primers that we used to differentiate fungal species grown in artificial media and directly from bodies of MPBs. While our rDNA primers were able to distinguish species from genera *Ceratocystiopsis*, *Ophiostoma* and *Grosmannia*, the *Leptographium* primers cross-reacted with different species closely related to the two MPB-associated fungal pathogens: *Grosmannia clavigera* and *Leptographium longiclavatum*. We selected the rDNA region for the design of primers because rDNA genes are present in high copy numbers and are easily amplified in fungi. As well, they have been the most commonly targeted genes for clarifying ophiostomatoid systematics and phylogeny (Hausner *et al.* 1993; Jacobs & Wingfield 2001; Zipfel *et al.* 2006). While they provide valuable information at the genus and sometimes at the species levels, they do not effectively distinguish closely related species like *G. clavigera*, *L. longiclavatum* and *Leptographium terebrantis* (Six *et al.* 2003; Lim *et al.* 2004).

The *Ophiostoma montium* primer set is specific for this species. In previous work, we showed that *O. montium* and *Ophiostoma ips* were often misidentified, even for specimens from culture collections (Kim *et al.* 2003; Massoumi Alamouti *et al.* 2009). The two species are very similar morphologically and

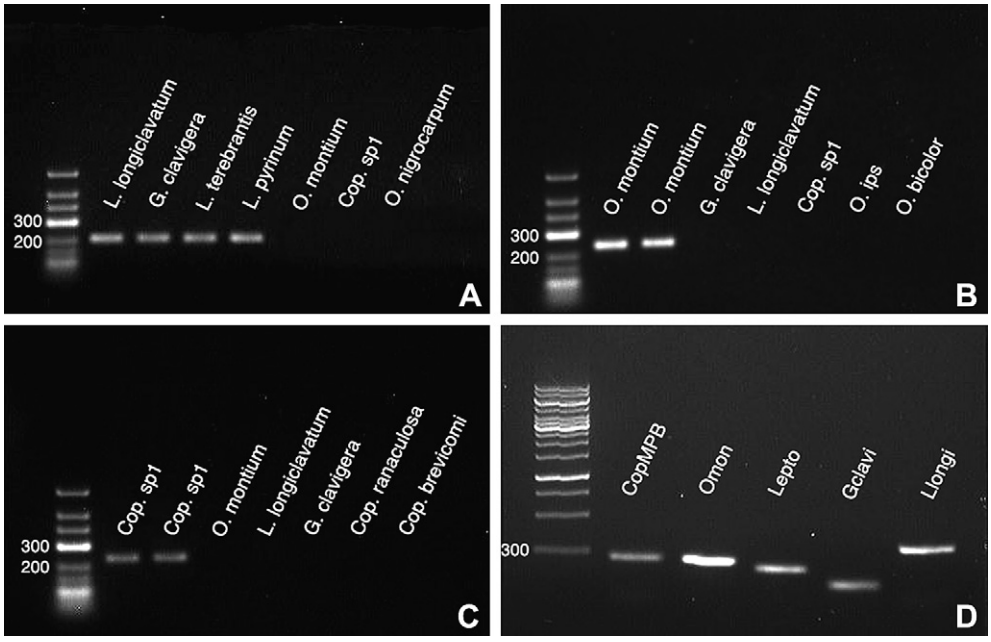

Fig 2 – Agarose gel images of PCR amplicons obtained with target-specific primers: A, B and C are results obtained with primer sets Lepto, Omon and CopMPB, respectively, on DNA extracted from pure culture. D shows the PCR amplicons obtained with target-specific primer sets on DNA extracted from two MPBs and their associated microbial communities.

Table 3 – Targeted fungi detected from MPBs and MPB galleries using either isolation on 1 % OMEA or PCR specific primers on DNA extracted from selected samples.

	BC MPB galleries		CA MPB galleries		MPB bodies	
	Isolation[a]	PCR[b]	Isolation[a]	PCR[b]	Isolation[a]	PCR[b]
O. montium	5	3	11	7	0	2
Cop. sp.	0	5	0	0	10	10
Leptographium	8	5	6	9	4	5
G. clavigera	3	3	6	5	1	0
L. longiclavatum	2	1	0	0	1	0
Total samples taken	10		13		10[c]	

MPB, mountain pine beetle; BC, British Columbia; CA, California.
Values represent the total number of samples for each substrate in which each fungus was detected.
a Microorganisms isolated on 1 % MEA and further identified by morphology and by PCR using specific primers.
b PCR method: DNA was extracted from selected samples and amplified with specific primers.
c Each sample included two adult beetles.

both are vectored by bark beetles and colonize pine trees. However, *O. montium* seems more specifically associated with MPB, while *O. ips* is distributed worldwide and does not appear specifically associated with a particular beetle (Zhou *et al.* 2007). In previous work we differentiated the two species by growing them at different temperatures and by amplifying their β-tubulin genes. *O. montium* had a single amplicon of 607 bp while *O. ips* had two amplicons of 776 and 876 bp (Kim *et al.* 2003). In the current work we designed the *O. montium* primers in the ITS region of the rDNA, since β-tubulin is sometimes difficult to amplify, especially when the DNA is of low concentration and of lower quality, which is often the case for ecological samples (Arbeli & Fuentes 2007).

The *Ceratocystiopsis* sp. primer set, CopMPB, was also very efficient in differentiating MPB-associated species from the closely related *Cop. ranaculosa* and *Cop. brevicomis* (Plattner *et al.* 2009). These three species have been isolated from bark beetles or trees infested by *Dendroctonous frontalis*, *Dendroctonous valens* and *Dendroctonous brevicomis*, respectively. The red turpentine beetle (*D. valens*) and the western pine beetle (*D. brevicomis*) inhabit *Pinus ponderosae* and have been found in BC. The southern pine beetle (*D. frontalis*) seems to inhabit mainly loblolly and short-leaf pines (Davidson & Prentice 1967). It is important to note that very little work has been done on the fungi associated with these beetles. The CopMPB primers differentiated the MPB species from the more distantly related *Cop. manitobensis* and *Cop. manitobensis*-like from *Ips perturbatus*, which were isolated in Canada (Massoumi Alamouti *et al.* 2007). All of the species found in Canada (MPB associates, *Cop. manitobensis* and *manitobensis*-like) grow slowly on 2 % OMEA and form white colonies. They also produce similar conidia from their *Hyalorhinocladiella* anamorphs (Plattner *et al.* 2009).

To differentiate the MPB-associated *Leptographium* species we developed two more specific primer sets Llongi and Gclavi from a protein-coding gene with an unknown function (DiGuistini *et al.* 2007). While the Gclavi primer set exclusively amplifies *G. clavigera*, the Llongi primer set is effective at differentiating *L. longiclavatum* from *G. clavigera* but not from *L. terebrantis*. It is important to note that we rarely isolated *L. terebrantis* from trees that have recently been attacked by MPB; rather, we occasionally found it in trees that have been infested for a year or more (Kim *et al.* 2005). Furthermore, from our own work and work by others it seems that the *L. terebrantis* species reported in the literature will need to be reassessed, as we recently found that some of the species assigned as *L. terebrantis* for MPB and a few other beetles were genetically different from the *L. terebrantis* holotype isolated from *D. terebrans* (Six & Massoumi Alamouti, unpubl. data). Since we have now sequenced the complete genome of *G. clavigera* (DiGuistini *et al.* 2009) and are comparing some of its genes with closely related species, it may be possible in the future to design primers to further differentiate *L. longiclavatum* from the *L. terebrantis* complex.

From our sampling from different hosts and sites we can conclude that PCR and isolation methods detect fungi in a range of ecological niches, and each method has strengths and weaknesses. The methods gave comparable results when a single species was dominant; either in the case of *Cop* sp. being naturally abundant on MPB bodies or *G. clavigera* that was the only fungus present as it was inoculated in trees. Both methods are efficient under these circumstances.

However, the methods performed differently when the fungi were less abundant or when other microorganisms (e.g. yeasts) dominated the sample. This was evident in the BC MPB galleries where we did not detect *Cop.* sp. using isolation but did with the DNA detection method. The effects of competition between the fungi on media hamper the isolation technique in its ability to detect slower-growing or less abundant species. This could explain why *Cop.* sp. was only recently reported as an MPB associate (Plattner *et al.* 2009). In contrast, on the MPB bodies, the PCR method detected *Leptographium* with the Lepto rDNA primer set, but neither *G. clavigera* nor *L. longiclavatum* with their species-specific primers. Because in earlier experiments, while we optimized our DNA extraction method, we were able to detect these species with the specific primers on a pair of beetles (data not shown), these two species were likely much less abundant on the MPBs used in this work, making them difficult to detect with primers that target single copy genes. Among other variables that might affect the DNA extraction method is the cell wall composition of the targeted fungi. Cell walls of staining fungi

that contain chitin, cellulose and melanin are difficult to disrupt, in contrast to spores and mycelium of non-staining species (e.g. *Cop*. sp.) (data not shown). Our results suggest two issues. First, the abundance and the cell wall composition of the targeted fungi are important variables in the direct DNA extraction; second, neither PCR nor isolation methods will always give a true representation of this ecosystem's microflora.

For the beetle galleries we used adjacent samples of phloem for isolation and PCR methods. Differences in the diversity and abundance of the fungi between the two locations may explain why results varied between the methods. Despite this uncertainty, both detection methods worked well for detecting the fungi associated with the MPB and the PCR method requires less time and expertise.

In conclusion, we showed that PCR was able to detect the targeted ophiostomatoid fungi from DNA extracted from pure cultures, MPBs and their associated microbial communities. This method can detect species without requiring that fungi be isolated from their substrates, and can be completed for a large number of samples in one day. The method can be used to detect fungi in imported and exported wood and we anticipate that it will be effective in field surveys that characterize fungal diversity at diverse geographic scales.

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