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Rapid identification and detection of pine pathogenic fungi associated with mountain pine beetles by padlock probes

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ABSTRACT

Fifteen million hectares of pine forests in western Canada have been attacked by the mountain pine beetle (*Dendroctonus ponderosae*; MPB), leading to devastating economic losses. *Grosmannia clavigera* and *Leptographium longiclavatum*, are two fungi intimately associated with the beetles, and are crucial components of the epidemic. To detect and discriminate these two closely related pathogens, we utilized a method based on ligase-mediated nucleotide discrimination with padlock probe technology, and signal amplification by hyperbranched rolling circle amplification (HRCA). Two padlock probes were designed to target species-specific single nucleotide polymorphisms (SNPs) located at the inter-generic spacer 2 region and large subunit of the rRNA respectively, which allows discrimination between the two species. Thirty-four strains of *G. clavigera* and twenty-five strains of *L. longiclavatum* representing a broad geographic origin were tested with this assay. The HRCA results were largely in agreement with the conventional identification based on morphology or DNA-based methods. Both probes can also efficiently distinguish the two MPB-associated fungi from other fungi in the MPB, as well as other related fungi in the order Ophiostomatales. We also tested this diagnostic method for the direct detection of these fungi from the DNA of MPB. A nested PCR approach was used to enrich amplicons for signal detection. The results confirmed the presence of these two fungi in MPB. Thus, the padlock probe assay coupled with HRCA is a rapid, sensitive and reproducible method for the identification and detection of these ophiostomatoid fungi.

1. Introduction

The mountain pine beetle (*Dendroctonus ponderosae*; MPB) is the most destructive pest to lodgepole pine forests (Carroll et al., 2003). Currently, western Canada is in the midst of the largest epidemic in recorded history, and is suffering devastating economic and environmental losses, as over 15 million ha of lodgepole pine forests have been attacked (Kurz et al., 2008; Rice and Langor, 2009). The epidemic driven by the attack of MPB has been spreading northward in British Columbia and eastward into Alberta, Canada (Carroll et al., 2003; Kurz et al., 2008). MPB has a broad host range, attacking the native lodgepole, ponderosa, and western white pines as well as many non-native pines (Yamaoka et al., 1995; Carroll et al., 2003).

The fungal associates of MPB play a significant role in the epidemics because of their symbiotic relationships with the beetles (Six and Klepzig, 2004; Bleiker et al., 2009). They are found in the mycangia and on the exoskeleton of the beetles (Six, 2003; Six and Klepzig, 2004). They were suggested to help the beetle in overwhelming the host trees' defense (Raffa and Berryman, 1983), and provide nutrients throughout the beetles' life cycle (Six and Paine, 1998; Adams and Six, 2007). It is believed that the combined action of the beetles and fungi is responsible for rapid tree death (Yamaoka et al., 1995; Solheim and Krokene, 1998). Two of the most important fungal associates are pathogens belonging to the order Ophiostomatales (Ascomycetes) (Jacobs and Wingfield, 2001; Zipfel et al., 2006; DiGuistini et al., 2009). *Grosmannia clavigera* (formerly *Ophiostoma clavigerum*) is haploid throughout most of its life cycle, but is transiently diploid when it reproduces sexually (Lee et al., 2007). *G. clavigera* produces asexual and sexual spores in a slimy mass that is easily dispersed by beetles (Harrington, 1993; Six and Klepzig, 2004; Lee et al., 2007). It grows rapidly in the host tree phloem and through the sapwood where it produces melanin that discolors the wood

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(sapstain fungi) and blocks the host tree's water transport system (Yamaoka et al., 1995; Solheim and Krokene, 1998; Rice et al., 2007; Plattner et al., 2008). Another fungal associate, *Leptographium longiclavatum*, does not have a known sexual stage (Lee et al., 2005). It can also produce lesions on trees in inoculation experiments (Lee et al., 2006a; Rice et al., 2007). Both fungi have been frequently isolated from beetles and trees (Lee et al., 2006b; Rice and Langor, 2009).

These two fungi are morphologically similar in having hyaline, clavate conidia, even though they can be differentiated by the oblong to peanut-shaped conidia with a truncated end produced by *G. clavigera* (Harrington, 1993; Tsuneda and Hiratsuka, 1984; Lee et al., 2005), as well as longer conidiophores produced by *L. longiclavatum* (Lee et al., 2005). Also identification based solely on morphological features and growth rate is unreliable, and *L. longiclavatum* has previously been misidentified as *G. clavigera* (Lee et al., 2005). This poses difficulties for diagnosing diseases quickly, and accurate identification of these species requires the help of trained taxonomists (Jacobs et al., 2001).

Multi-gene phylogenies can differentiate these two fungi with strong statistical support, but they are very closely related in evolution with small variations in nucleotide sequences of ribosomal RNA (rRNA) (Lim et al., 2004; Lee et al., 2005). While routine sequencing of amplicons from the internal transcribed spacer (ITS) region, from the large subunit (LSU) regions of rRNA as well as homology comparison with sequence databases facilitated rapid species identification for most fungi (Nilsson et al., 2008), the sequencing of the ITS region in ophiostomatoid fungi presented a technical challenge due to high GC content (Mullineux and Hausner, 2009). Also, the highest scoring sequence from BLAST search may just happen to be the sequence most similar in length, given the small sequence polymorphisms in the ITS2 region and partial LSU among *G. clavigera*, *L. longiclavatum* and other closely related species such as *L. terebrantis* (Mullineux and Hausner, 2009). One of the most sensitive and common tests available for the molecular detection and separation of *G. clavigera* and *L. longiclavatum* involves the use of restriction fragment length polymorphism (RFLP) of beta-tubulin or ribosomal inter-generic spacer (IGS) region (Lee et al., 2003; Hausner et al., 2005). These assays may be time consuming and sensitive to contamination, which can lead to PCR inhibition, and are not amenable to detection directly from environmental samples. Alternative methods are needed to provide rapid and reliable detection and confirmation.

Understanding the ecology and population dynamics of these MPB-associated fungi is important for the surveillance and management of the beetle–fungal complex, and could improve prediction and modelling. The fungal communities on MPB or bark beetles have often been investigated by isolation from insect bodies using traditional culture-based morphological approaches (Jacobs and Wingfield, 2001; Lee et al., 2006b; Persson et al., 2009) or clone library construction from rRNA products amplified from total beetle DNA (Lim et al., 2005; Zhang et al., 2005; Persson et al., 2009). These methods are often time consuming, laborious, and only partly quantitative. Also methods of direct molecular analysis such as cloning may be insensitive to the target fungi (Persson et al., 2009).

We have developed an alternative method, based on ligase-mediated single nucleotide discrimination with padlock probe technology and signal amplification by hyperbranched rolling circle amplification (HRCA) (Nilsson, 2006; Kaocharoen et al., 2008) (Fig. 1). Padlock probes are long oligonucleotides of ~100 bases, and consist of two sequences complementary to the 5′ and 3′ terminals of the target sequence joined by genetic linker region. Upon hybridization to the target, the 5′ and 3′ terminals of the probe are juxtaposed and ligated to form a closed, circular molecule (Nilsson et al., 1994; Szemes et al., 2005; Nilsson, 2006; van Doorn et al., 2009). The intensity of the probe signal can be increased exponentially by HRCA (Fig. 1) (Lizardi et al., 1998; Faruqi et al., 2001). If there is a mis-match between the probe and the target at the 3′ terminal, ligation and amplification are prevented. This technology has been used for the differentiation of human pathogenic viruses (Wang et al., 2005; Stein et al., 2009), bacteria (Tong et al., 2007), and fungi (Kaocharoen et al., 2008; Zhou et al., 2008). In this study, we report the use of padlock probes in combination with HRCA for high-throughput rapid identification and differentiation of two major fungal associates from the MPB.

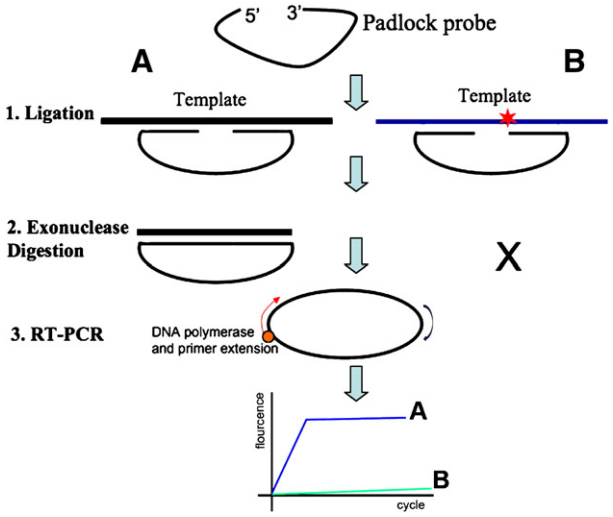

Fig. 1. Schematic representation of the different steps involved in the RCA method used. 1. Padlock probes containing the complementary sequences at the 5′ and 3′ ends hybridized to the target template. In the case of a perfect match, the probe is circularized by DNA ligase (A); while in the case of a mis-match, no ligation should occur (B). 2. Non-hybridized template is removed by the exonuclease. 3. Tandem repeat sequences are generated by the rolling circle amplification. 4. The amplification is monitored in real-time using SYBR green.

2. Materials and methods

2.1. Fungus and beetle samples

A total of 96 fungal strains, including 34 strains of *G. clavigera* and 25 strains of *L. longiclavatum*, 9 strains of *L. terebrantis* and 19 related species were obtained (Table 1). Most cultures are maintained at the Breuil culture collection of the University of British Columbia, Canada.

Thirty-one beetle samples were included in the study. Most were collected pre-flight in May–July 2006 from under the bark of attacked lodgepole pines in areas affected by the current outbreak (Houston, BC; Fort St James, BC; Banff, AB). Beetle samples were stored in 95% ethanol at 4 °C or −20 °C at the University of Northern British Columbia in Prince George, Canada, prior to DNA extraction.

2.2. Primer and padlock probe design for HRCA

DNA sequences spanning the fungal rRNA gene (18S, 5.8S and 28S) and the intervening ITS1 and ITS2 regions of major ophiostomatoid fungi and two target pathogens from GenBank were aligned using Clustal X (Thompson et al., 1997) and Se-Al v2 (Rambaut, 1999) (Treebase SN4966). New sequences (GU969297–GU969299) were also included in the alignment to identify informative SNPs between species (Lim et al., 2004) (Suppl. Fig. 1).

The padlock probes for *G. clavigera* (GC) and *L. longiclavatum* (LEP) were designed to recognize specific SNPs at positions 42 and 663 of the rRNA region, respectively (Suppl. Fig. 1; Table 2). The SNP at the ITS2 region had been previously reported in Lim et al. (2004). The probes were 96 nucleotides (nt) long, consisting of two adjacent target complementary sequences with a linker region (48 nt). To optimize binding to target sequences, the probes were designed with minimum secondary structure and with the *T*m of the 5′end probe-

Table 1
Strains used in this study and the results obtained by padlock probe (GC, LEP) and HRCA.

Target species	Number of strain/strain code	Isolation date	Geographic origin	Host origin	GC	LEP
Grosmannia clavigera	ATCC18086	1965	Cache Creek, BC, Canada	*P. pondersosa* attacked by MPB	+	−
	CBS102.78	1976	Colorado, USA	–	+	−
	PY2-3b	2007	Kamloops, BC, Canada	–	+	−
	UAMH4585	1982	Riske Creek, BC, Canada	*P. contorta* attacked by MPB		
	KW1407	2001	Kamloops, BC, Canada	–	+	−
	4 (H18, H17, H51, H55)	2003	Houston, BC, Canada	–	+	−
	6 (STJ15, STJ3-26, STJ3-85, STJ16, STJ3-412, STJ3-89)	2003	Fort St James, BC, Canada	–	+	−
	5 (W3, W12, W4, W13, W14)	2003	Williams Lake, BC, Canada	–	+	−
	4 (B101, B5, BW28, B10)	2003	Banff, AB, Canada	–	+	−
	M6	2003	Manning Park, BC, Canada	–	+	−
	DPCHEBC10	2007	Cypress Hill, AB, Canada	–	+	−
	2 (DPLKDL3, DPLKDW4)	2007	Kelowna, BC, Canada	–	+	−
	NOF2991	2006	Graham Fire Base, AB, Canada	hybrid of *P. banksiana* and *P. contorta* attacked by MPB	+	−
	2 (HR14, HR39)	2003	Hell Roaring, Idaho USA	MPB *D. ponderosa*	+	−
	3 (HV4, HV33, HV34)	2003	Hidden Valley, Montana, USA	–	+	−
Leptographium longiclavatum	UAMH4876	1983	Blairmore, AB, Canada	*P. contorta* attacked by MPB	−	+
	2 (SL-W001, SL-Ww402)	2001	Kamloops, BC, Canada	–	−	+
	WG57EG-1	2003	Riske Creek, BC, Canada	–	−	+
	2 (868 AW 1-1, 868 AW1-2)	2004	Mount Robson National Park	–	−	+
	951 EW 2-1	2004	Prince George, BC, Canada	–	−	+
	2 (S5R140E2-2, S5R140A1-1)	2004	Little Fort, BC, Canada	–	−	+
	3 (924 A G-2, 925 E G-2, 922 A G-2)	2004	Sterns Lake, Canada	–	−	+
	5 (898 A EG1, 896 AW 2-1, 871 AW 1-2, 893 AW 1-1, 885 AW 1-1)	2004	Kamloops, BC, Canada	–	−	+
	888 AW 1-2	2004	Kamloops, BC, Canada	–	+	−
	889 AW 1-1	2004	Kamloops, BC, Canada	–	+	−
	2 (953 AW 2-1, 953 AW 2-2)	2004	Prince George, BC, Canada	–	−	+
	SL-KW1436	2004	Williams Lake, BC, Canada	–	−	+
	866 A EG1	2004	Northern BC, Canada	–	−	+
	DPLKG + T2B	2007	Kelowna, BC, Canada	–	−	+
	985 AMPBPC-2	2004	Quesnel, BC, Canada	MPB *D. ponderosa*	−	+
Related species						
Ambrosiella ips	CBS435.34	1934	Minnesota, USA	*Pinus* sp. sapwood	−	−
Ambrosiella sp.	WR43EW1-2	2003	Riske Creek, BC, Canada	*P. contorta* attacked by MPB	−	−
Ceratocystiopsis sp.	WY21BX12	2003	Williams Lake, BC, Canada	*P. contorta* attacked by MPB	−	−
	S3-89EW1-1	2003	Radium, BC, Canada	–	−	−
	954 AW HR1	2004	Prince George, BC, Canada	–	−	−
Entomocorticium sp.	WY47EW1-1	2003	Riske Creek, BC, Canada	*P. contorta* attacked by MPB	−	−
Leptographium lundbergii	DAOM64746	1961	Ontario, Canada	*P. strobus*	−	−
	UM1434	2004	NA	NA	−	−
Leptographium pyrinum	CMW3889	NA	California	*P. jeffrii*	−	−
Leptographium terebrantis	UAMH9722	1987	Victoria, BC, Canada	*P. contorta*	−	−
	AU123-239	1997	Big River, Saskatchewan, Canada	hybrid of *P. banksiana* and *P. contorta*	−	−
	WY42EW1-2	2003	Riske Creek, BC, Canada	*P. contorta*	−	−
	S3-78AW1-2	2003	Radium, BC, Canada	NA	−	−
	3 (MG5AGI-2, MY23 AW3-2, MG5AGI-1)	2003	Manning Park, BC, Canada	*P. contorta*	−	−
	986 EG-1	2004	Quesnel, BC, Canada	NA	−	−
	960 AW 2-2	2004	Prince George, BC, Canada	*P. contorta*	−	−
Leptographium wingfieldii	CMW2095	2004	NA	*P. strobus*	−	−
	CMW2096	NA	NA	*P. sylvestris*	−	−
	ATCC16936	NA	BC	*P. contorta*	−	−
Grosmannia aurea	AU98-Pr2-169	NA	Princeton, BC, Canada	*P. contorta*	−	−
Grosmannia huntii	CBS3987.77	1963	Park Forest, NY, USA	*P. monticola*	−	−
	CMW185	2001	South Africa	NA	−	−
Grosmannia robusta	CMW668	2001	South Africa	*Picea abies*	−	−
Ophiostoma abietinum	CMW1468	1986	BC, Canada	MPB *D. ponderosae*	−	−
Ophiostoma adjuncti	CMW135	NA	NA	NA	−	−
Ophiostoma bicolor	CBS492.77	1977	Colorado, USA	Ips gallery	−	−
Ophiostoma ips	CBS151.54	1952	Dalarna, Sweden	*P. sylvestris* gallery	−	−
	ATCC24285	NA	Canada	*P. contorta*	−	−
Ophiostoma minus	UM888A	NA	NA	NA	−	−
Ophiostoma montium	CBS151.78	1978	NA	*P. pondersa*	−	−
	SL P4	2001	Princeton, BC, Canada	MPB *D. ponderosa*	−	−
Ophiostoma nigrocarpum	CBS637.66	1962	Idaho, USA	Abies log infected with *Scolytus* sp.	−	−
Ophiostoma novo-ulmi	H327	1979	Brezno-Nizke, Slovakia	NA	−	−
Ophiostoma picea	DAOM229575	NA	Point Pleasant Park	*P. rubens* wood	−	−
	DAOM229578	NA	Point Pleasant Park	*P. rubens* wood	−	−

(–: same as above; NA: information not available; P.: Pinus; D.: Dendroctonus; MPB: mountain pine beetle).

Table 2
Padlock probes and padlock probe-specific primers used. The 5′ and 3′ ends of the primers, that are complementary to the target sequences, are underlined. The regions where the two padlock probe-specific primers (P1 and P2) bind for real-time amplification are in bold.

Probes	Sequence
GC	5′ CTG GGC GCA GGC CTG GCC GGA TC**A TGC TTC TTC GGT GCC CAT** TAC GAG GTG CGG ATA GCT AC**C GCG CAG ACA CGA TA**G TCT ACT GCG GCG GCC TGC A 3′
LEP	5′ GGA GTG CAC CGG CGC TGA GGA TCA TGC TTC TTC GGT GCC CAT TAC GAG GTG CGG ATA GCT AC**C GCG CAG ACA CGA TA**G TCT AGG CCT GCG CCC CGA 3′
P1	5′ ATGGGCACCGAAGAAGCA 3′
P2	5′ CGCGCAGACACGATA 3′

binding arm above the temperature used for probe ligation (62 °C). To increase the specificity, the 3′end binding arm was designed to have a *T*m (51–56 °C) below the ligation temperature. In addition, the linker region for each taxon-specific probe was carefully designed to (i) minimize similarity to other related ophiostomatoid fungi, and (ii) allow primer binding during RCA and amplification of the probe-specific signal. The sequences of the two primers (P1 and P2, *T*m 55 °C) that bind specifically to the linker region of the probes during HRCA are shown in Table 2.

2.3. DNA extraction and PCR amplification

DNA extraction for fungi was performed using the protocol previously described by Lee et al. (2005, 2007). Total DNA was extracted from adult beetles collected prior to emergence using a standard proteinase K and phenol/chloroform procedure (Sambrook and Russell, 2001). The quality of extracted DNA was evaluated through electrophoresis on 1% agarose gels using ethidium bromide staining and the quantity determined using a NanoDrop ND-1000 UV–Vis Spectrophotometer. DNA concentrations were normalized to 100 ng/ml in Tris–EDTA buffer (pH 8.0).

PCR amplifications of DNA from pure cultures of fungi were carried out in 25 μl using a PTC-100 thermocycler (MJ Research Inc., Watertown, MA, USA). Reaction mixtures contained 20–40 ng DNA, 1× PCR buffer, 200 μM each dNTP, 1.5 μM of each primer ITS3 and TW13 (Eurofins MWG Operon, Huntsville, AL, USA; http://plantbio.berkeley.edu/~bruns/tour/primers.html), and 1U of Paq polymerase (Stratagene, Integrated Sciences). The PCR amplifications were performed for 3 min at 94 °C, followed by 30 cycles of 35 s at 94 °C, 35 s at 55 °C and 35 s at 72 °C, and a final extension at 72 °C for 7 min.

The DNA from 31 MPB was used as a template for direct amplification of rRNA genes from fungi carried in or on the beetles (Table 3). We used two approaches: first, total DNA from the beetles was amplified with the primer pairs ITS1F and TW13 (http://plantbio.berkeley.edu/~bruns/tour/primers.html) at 55 °C for 40 cycles (Wright et al., 2009). Second, we used a nested PCR strategy with the first round of PCR using ITS1F and TW13 at 55 °C for 40 cycles, followed by specific primer 171F<GCGGCCAGGCCTGCGCCCA>, which was designed in this study targeting the ITS2 region of rRNA for most ophiostomatoid fungi of the genera *Grosmannia*, and TW13 at 56 °C for 35 cycles. The amplicons were then purified with a Millipore Multiscreen PCR micro 96 well plate (Millipore, Billerica, MA, USA).

2.4. Preparation of templates for testing the sensitivity of padlock probes

The PCR amplicons of *G. clavigera* ATCC18086 and *L. longiclavatum* 922 AG2 were cloned into pGEM-T easy vector system (Promega, Madison, WI, USA) according to the manufacturer's protocol. The positive clones were selected and the linear templates were further generated by PCR amplification of plasmid DNA followed by quantification with a spectrophotometer. The copy numbers were estimated using a DNA calculator (http://www.uri.edu/research/gsc/resources/cndna.html).

2.5. Ligation of padlock probe

Ligation was performed in 10 μl reaction mixture containing 6.5 μl of PCR product, 2 U of *pfu* DNA ligase (Stratagene, Integrated Sciences) and 0.2 μM padlock probe in 20 mM Tris–HCl (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$, 0.1% Igepal, 0.01 mM rATP, and 1 mM DTT. The ligation reaction for amplicons from pure culture was conducted with one cycle of 3 min at 94 °C followed by 5 min ligation at 65 °C for GC and LEP probes. To test the PCR amplicons generated from the DNA of beetle samples, we used 15 cycles of ligation cycles.

For sensitivity testing, the GC probe was tested with 5×10^{10} copies of clone-derived templates in a serial dilution as well as 3.5×10^{10} of *L. longiclavatum* products as negative control in 5 cycles of ligation, while the LEP probe was ligated with 3.5×10^{10} of templates in a serial dilution as well as standard templates of *G. clavigera* as negative control, also in 5 cycles.

2.6. Exonucleolysis

Exonucleolysis was performed to remove non-ligated padlock probe and template PCR product, thus reducing subsequent ligation-independent amplification events. It was performed in 20 μl volumes by adding 10 U each of exonuclease I and exonuclease III (New England Biolabs, Ipswich, MA, USA) to the ligation mixtures, and incubated at 37 °C for 15 min and at 90 °C for 3 min.

2.7. Hyperbranched rolling circle amplification reaction

After exonucleolysis of the now circularized probes, HRCA reactions were performed in a 50 μl volume containing 8 U of *Bst* DNA polymerase (New England Biolabs), 400 μM deoxynucleoside triphosphate mix, 10 pmol of internal primers P1 and P2 (Table 2), 5% of dimethyl sulfoxide (v/v), and 10× SYBR Green I (Sigma-Aldrich). Probe signal were amplified by incubation at 65 °C for 30 min and the accumulation of dsDNA products was monitored using a Corbett RotorGene™ 3000 real-time PCR machine (Corbett Research, Mortlake, Australia). The assay (from ligation to HRCA reactions) with all the PCR amplicons have been repeated 3–4 times.

3. Results

3.1. Sensitivity

The sensitivity of the probes was assessed using standard templates in various concentrations. Probe GC was tested with 5×10^{10} copies, with 50%, 25%, 12.5%, 6.25%, 3.13%, 1.56%, 0.78% dilutions, and 3.5×10^{10} copies of *L. longiclavatum* templates as negative control. Similarly, probe LEP was tested with 3.5×10^{10} copies with serial dilutions and templates of *G. clavigera* as negative control in five ligation cycles (Fig. 2). The measurement of HRCA amplified probe signals indicated that a specific probe signal was detected with as low as 0.7% of the respective fungal DNA template-containing mixture (ca. 0.18 ng) from probe LEP (Fig. 2), whereas no signal was detected when there was no target template. HRCA is an isothermal reaction and also has potential for use in the quantification of DNA concentrations. The threshold cycle (Ct value) increased with the dilutions in the copies of DNA template.

3.2. Specificity

Padlock probes in combination with HRCA amplification in real-time PCR were applied to differentiate two closely related MPB fungal associates in pure cultures (Table 1). A fluorescent signal generated from the respective probe demonstrated a positive result, while no

Table 3
Results of padlock probes (GC and LEP) and HRCA testing on beetle DNA samples amplified with different primers pairs and amplification strategies.

Samples	ITS1F + TW13	ITS1F + TW13 followed by 171F + TW13	
	Both GC and LEP	GC	LEP
HO1	−	−	−
HO2	−	+	−
HO3	−	+	−
HO4	−	+	−
HO6	−	+	−
HO7	−	+	+
HO8	−	+	−
HO9	−	+	−
HO10	−	+	+
HO11	−	+	+
JP11	−	+	+
JP12	−	+	+
JP13	−	+	−
JP14	−	+	−
JP15	−	+	−
JP16	−	+	+
JP17	−	+	−
JP18	−	+	−
JP19	−	+	+
JP20	−	+	+
TM12	−	−	−
TM13	−	+	+
TM14	−	+	−
TM15	−	+	−
TM16	−	+	−
TM17	−	+	−
TM18	−	+	−
TM19	−	+	−
TM20	−	+	−
TM21	−	+	−
4A	−	+	−

HO: Houston, BC, Canada (Jun 2006); JP, Fort St. James, BC, Canada (Jul, 2005); TM: Banff, AB, Canada, (Jun 2006); 4A: Loon Lake, AB, Canada (Jul 2003).

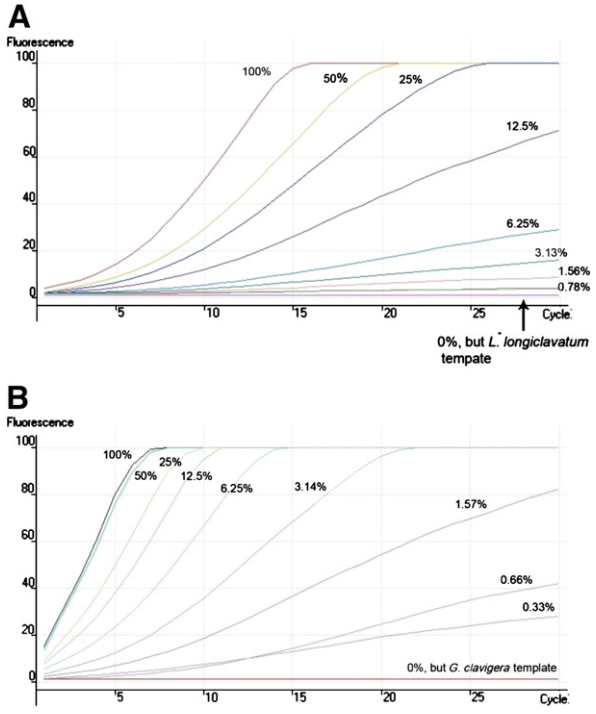

Fig. 2. Sensitivity testing of padlock probes to the serial dilutions of different copies of artificial templates. (A) Probe OP was tested against *G. clavigera* (5×10^{10} and dilutions, as well as *L. longiclavatum* as negative control) and (B) Probe LEP was tested on *L. longiclavatum* (3.5×10^{10} and dilutions, as well as *G. clavigera* as negative control).

signal indicated a negative result (Table 1, Suppl. Fig. 2). All strains identified by morphology or DNA RFLP as *G. clavigera* generated fluorescent signals after hybridization with the GC probe (Table 1). Most *L. longiclavatum* strains identified based on morphological features were also positive to the LEP probe with the exception of two strains: 888 AW1-2 and 889 AW1-1 (Table 1). These two strains were positive to probe GC and negative to probe LEP. We therefore sequenced the ITS and partial LSU sequences of both strains, which revealed that they shared identical sequences to *G. clavigera* (data not shown). We also tested the specificity of the designed padlock probes against 17 related species, including six isolates of the most closely related species, *L. terebrantis*, and they all showed negative results to both probes (Table 1).

3.3. Detection of fungi from beetle DNA

An experiment was also performed to determine if the developed method/technology was effective for fungal detection directly from beetles. PCR products were amplified from a single primer pair and combined with a group-specific primer 171F in a nested PCR strategy (Table 3). In order to obtain the required sensitivity, we increased the number of ligation cycles to 15 but kept other ligation and HRCA conditions the same. Using the fungal-specific primer pair ITS1F and TW13, which has been widely used in fungus identification PCR, the current protocol did not detect either of the two fungi in the beetle samples (Table 3). However, the nested PCR approach enhanced the level of pathogen detection. *G. clavigera* was detected in 29/31 (>90%) beetles and *L. longiclavatum* in 9/31 beetles (29%). Nine beetle samples (three from Houston, five from Fort St. James, and one from Banff) were positive for the two fungi, and two (HO1 and TM12) were negative for both fungi (Table 3). The no-template control was negative.

4. Discussion

4.1. Molecular diagnostics of fungi

Padlock probes offer advantages over other techniques such as conventional real-time PCR for detection of SNPs (Nilsson, 2006), and the ligation reaction is sensitive to mis-match between the probes and the target (Wang et al., 2005; Kaocharoen et al., 2008; Zhou et al., 2008; Stein et al., 2009). In this study, the padlock probes developed in association with HRCA were highly specific as demonstrated by the absence of cross-reactions in the fungal taxa of related species. *Grosmannia aurea*, *G. robusta*, *L. lundbergii*, and *L. pyrinum* are evolutionarily closely related based on multi-gene phylogeny (Lim et al., 2004) and isozyme analyses (Zambino and Harrington, 1992; Lee et al., 2003). Traditionally, these fungi have been differentiated based on morphological characters (Tsuneda and Hiratsuka, 1984; Jacobs and Wingfield, 2001), which is sometimes confusing because of overlapped characteristics and degeneration of characters due to repeated cultures and long-term storage (Lee et al., 2003).

More importantly, the probes are specific and they differentiated all the strains of *G. clavigera* and *L. longiclavatum* from 9 strains of *L. terebrantis* (Table 1), which has also been isolated from many species of pine trees, bark beetles, and occasionally in the galleries of lodgepole pines attacked by the MPB (Jacobs and Wingfield, 2001; Six et al., 2003). *Leptographium terebrantis* shares some morphological similarities to the anamorphs of *G. clavigera*, and is considered as a basal to *G. clavigera* and *L. longiclavatum* based on molecular data (Lim et al., 2004). The possible genetic relationships of *L. terebrantis* with other ophiostomatoid fungi have been studied using a variety of isolates and molecular markers, but were not clearly resolved (Six et al., 2003).

Ophiostoma montium and *Ceratocystiopsis* sp. are two other common fungi associated with MPB (Six, 2003; Lee et al., 2006b; Rice and Langor, 2009), and *O. montium* appears to be pathogenic (Yamaoka et al., 1995; Rice et al., 2007). Since they are only distantly

related to *G. clavigera* and *L. longiclavatum*, it was not surprising that the signal from both probes was negative. However, since those species are intimately associated with MPB, this result is important for assays using environmental samples.

Routine sequencing of ITS regions of ribosomal RNA genes followed by homology-based searches on databases such as GenBank is common for fungal species identification and will become more important and useful with the development of barcoding databases (Nilsson et al., 2008). However, this approach is only applicable when pure cultures are available and is not easily amenable to high-throughput processing. We are currently working on assessing the distribution of fungal associates of MPB at the landscape level and, therefore, a high-throughput method of fungal identification is required.

The proposed diagnostic protocol using padlock probes and HRCA is rapid, sensitive and reproducible, thus reducing the chance of false positives. Because of its high level of specificity, we were able to target the ribosomal genes that are well characterized in the database, and abundant in genomes but that comprise only few SNPs among species. Our method requires only a PCR reaction, the addition of buffers, primers and enzymes, followed by warming for 60 min at 65 °C, which is then followed by real-time PCR assay. Subsequently, the hands-on time of the whole experiment is less than 5 h, which is significantly shorter than the time required for PCR fingerprinting, RFLP analysis, or sequencing (Lee et al., 2003; Lim et al., 2004). In addition, interpretation of the results is straightforward and is based on a simple positive or negative result obtained during HRCA, eliminating subjective criteria as needed during the analysis of such complex banding patterns as those obtained by PCR fingerprinting.

4.2. Detection from beetle samples

The two fungi for which padlock probes were developed were detected when assays were conducted directly from MPB DNA, confirming the close relationship revealed from cultures (Six, 2003; Six and Klepzig, 2004; Adams and Six, 2007). However, detection rates varied for the two species studied. *G. clavigera* was detected much more frequently than *L. longiclavatum*. This was largely congruent with relative abundance data based on culture studies (Lee et al., 2005, 2006b; Lim et al., 2005; Rice and Langor, 2009). *G. clavigera* has been more commonly isolated from beetle larvae and tree galleries than *L. longiclavatum* (Lee et al., 2006b; Rice and Langor, 2009). The lower occurrence of *L. longiclavatum* may be related to its rare presence on beetle body surface (Lee et al., 2005). Recently *L. longiclavatum* was suggested to be more prevalent in northwestern Alberta with greater tolerance to colder temperatures (Rice and Langor, 2009). The occurrence and abundance of fungi also varied in beetles at different stages of flight and after colonizing trees (Adams and Six, 2007). The probes developed here will be useful in testing hypotheses regarding the patterns of fungal species abundance.

Molecular detection of ophiostomatoid fungi directly from beetle samples has not been well developed. A species-specific primer with real-time PCR detected *L. wagneri* on 37% of the bark beetle samples collected, in which one beetle sample contained one to five beetle specimens (Schweigkofler et al., 2005). Our results show that *G. clavigera* and *L. longiclavatum* were found in over 90% and 20% of the MPB, respectively, which suggests that the method developed is very efficient in detecting these fungi. Mountain pine beetles carry fungi in their mycangia and guts, and on the surface of their exoskeleton (Six and Klepzig, 2004; Bleiker et al., 2009). Using the culture-based morphological approach, *G. clavigera* was isolated in less than 30% of MPB in biodiversity surveys (Six, 2003; Lee et al., 2006b), and around 60% in a MPB developmental study (Adams and Six, 2007). *L. longiclavatum* was also isolated from 1 out of 68 (1.5%) beetles (Lee et al., 2006b). The culture-based fungal detection approach suffers from an excess of false negatives because of the presence of contaminants or aggressive non-ophiostomatoid fungal associates that can overgrow the targeted species. The molecular approach of generating fungus-ITS clones from MPB DNA followed by RFLP type classifications, did not detect either *G. clavigera* or *L. longiclavatum* from 250 clones (Lim et al., 2005). A nested PCR approach combined with cloning increased the detection rate of ophiostomatoid pathogens to 0.9–11% (Persson et al., 2009). The low detection value was possibly due to preferential PCR reaction, high GC content and low frequency of target DNA (Lim et al., 2005).

Fungal DNA is often at low levels and is difficult to detect with regular PCR assays in environmental sample. Therefore primer specificity play a vital role in the molecular assessment of fungi from the environment (Martin and Rygiewicz, 2005; Zhang et al., 2005). Persson et al. (2009) used a nested PCR approach to avoid chimeric sequences from beetle DNA with the fungal-specific primers NLC2 and NSA3 (cited in Martin and Rygiewicz (2005)) in the first reaction, followed by primers ITS1F and ITS4. The specificity of primer pairs and PCR strategies are also important in allowing selective or enriching amplification of fungal rRNA genes from MPB.

Several factors may have contributed to the negative data obtained from primers ITS1F and TW13 from beetle DNA and the 29 positive data of *G. clavigera* from subsequent nested PCR approach (Table 3). Target competition could result in ITS1F and TW13 not binding to their fungal targets in the presence of many copies of rRNA genes of beetles, thus producing a low yield of fungal amplicons. The biodiversity of microbes associated with MPB is high and contains bacteria, yeasts and other filamentous fungi (Lim et al., 2005; Zhang et al., 2005; Lee et al., 2006b; Adams et al., 2008; Vasanthakumar et al., 2008; Persson et al., 2009). Without highly specific primers, there has been preferential amplification of non-target microbes such as yeasts (up to 77% of the sequenced clones) (Lim et al., 2005; Persson et al., 2009). Since the two species-specific SNPs are located in both the ITS and LSU rRNA gene regions, we had to amplify an amplicon that span at least 1 kb. Such large-sized amplicons are not often desirable from DNA of low yield.

The low level of DNA from fungal associates within the beetle's DNA may present a problem to normal quantitative real-time PCR, the use of amplicons will ensure template quantity for accurate identification of target pathogens from ligation-based diagnostics technology such as padlock probes (van Doorn et al., 2007, 2009; Stein et al., 2009). The nested PCR strategy on DNA obtained directly from beetles is useful to generate or enrich amplicons for padlock probe and HRCA. This makes the technique useful enough in avoiding the growth of pure fungal cultures before any molecular test can be carried out. Upon modification and optimization of enzymatic digestion of DNA, such as *Hin*dIII and *Bam*HI (Szemes et al., 2005; van Doorn et al., 2009), the padlock probe based ligation assay can identify and detect target organisms in as little as 0.01% of environmental source DNA (van Doorn et al., 2009). Also, padlock probes containing a tag sequence or a ZipCode sequence (unique sequence identifier) or biotin-labeled, in conjunction with qPCR and Luminex™ technology or microarray technology, can be used for multiplex pathogen detection and quantification (Szemes et al., 2005; van Doorn et al., 2007, 2009; Eriksson et al., 2009).

5. Conclusion

Species-specific padlock probes used in combination with hyper-branched rolling circle PCR differentiate two closely-related fungi associated with MPB within 5 h. This clearly can be an efficient alternative to existing detection and identification methods such as morphological identification following isolation and culturing, or sequencing target genes for identification. This high-throughput and reliable screening tool allows identification of a large number of pathogens. This study also shows the possibility of detecting *G. clavigera* and *L. longiclavatum* in total DNA extracted from MPB for distribution monitoring. The technology may be applied to a wide

range of beetle pests for fungal detection without the need for fungal isolation and cultivation. Therefore, it represents a promising tool to better understand pathogen population dynamics and to provide alternatives in quarantine control facilities.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mimet.2010.07.016.

References

Adams, A.S., Six, D.L., 2007. Temporal variation in mycophagy and prevalence of fungi associated with developmental stages of *Dendroctonus ponderosae* (Coleoptera: Curculionidae). Environ. Entomol. 36, 64–72.

Adams, A.S., Six, D.L., Adams, S.M., Holben, W.E., 2008. *In vitro* interactions between yeasts and bacteria and fungal symbionts of the mountain pine beetle (*Dendroctonus ponderosae*). Microb. Ecol. 56, 460–466.

Bleiker, K.P., Potter, S.E., Lauzon, C.R., Six, D.L., 2009. Transport of fungal symbionts by mountain pine beetles. Canad. Entomol. 141, 503–514.

Carroll, A.L., Taylor, S.W., Regniere, J., Safranyik, L., 2003. Effects of climate change on range expansion by the mountain pine beetle in British Columbia. In: Shore, T.L., Brooks, J.E., Stone, J.E. (Eds.), Mountain Pine Beetle Symposium: Challenges and Solutions. 30 and 31 October 2003. Can. For. Serv. Pac. For. Cent. Inf. Rep. BC-X-399, Victoria, BC, Canada, pp. 223–232.

DiGuistini, S., Liao, N.Y., Platt, D., Robertson, G., Seidel, M., Chan, S.K., Docking, T.R., Birol, I., Holt, R.A., Hirst, M., Mardis, E., Marra, M.A., Hamelin, R.C., Bohlmann, J., Breuil, C., Jones, S.J.M., 2009. *De novo* genome sequence assembly of a filamentous fungus using Sanger, 454 and Illumina sequence data. Genome Biol. 10, R94.

Eriksson, R., Jobs, M., Ekstrand, C., Ullberg, M., Herrmann, B., Landegren, U., Nilsson, M., Blomberg, J., 2009. Multiplex and quantifiable detection of nucleic acid from pathogenic fungi using padlock probes, generic real time PCR and specific suspension array readout. J. Microbiol. Methods 78, 195–202.

Faruqi, A.F., Hosono, S., Driscoll, M.D., Dean, F.B., Alsmadi, O., Bandaru, R., Kumar, G., Grimwade, B., Zong, Q., Sun, Z., Du, Y., Kingsmore, S., Knott, T., Lasken, R.S., 2001. High-throughput genotyping of single nucleotide polymorphisms with rolling circle amplification. BMC Genomics 2, 4.

Harrington, T.C., 1993. Biology and taxonomy of fungi associated with bark beetles. In: Schowalter, T.D., Filip, G.M. (Eds.), Beetle–Pathogen Interactions in Conifer Forests. Academic Press, San Diego, USA, pp. 37–51.

Hausner, G., Iranpour, M., Kim, J.-J., Breuil, C., Davis, C.N., Gibb, E.A., Reid, J., Loewen, P.C., Hopkin, A.A., 2005. Fungi vectored by the introduced bark beetle *Tomicus piniperda* in Ontario, Canada, and comments on the taxonomy of *Leptographium lundbergii*, *Leptographium terebrantis*, *Leptographium truncatum*, and *Leptographium wingfieldii*. Can. J. Bot. 83, 1222–1237.

Jacobs, K., Wingfield, M.J., 2001. *Leptographium* Species: Tree Pathogens, Insect Associates, and Agents of Blue Stain. APS Press, St. Paul, MN, USA.

Jacobs, K., Wingfield, M.J., Wingfield, B.D., 2001. Phylogenetic relationships in *Leptographium* based on morphological and molecular characters. Can. J. Bot. 79, 719–732.

Kaocharoen, S., Wang, B., Tsui, K.M., Trilles, L., Kong, F., Meyer, W., 2008. Hyperbranched rolling circle amplification as a rapid and sensitive method for species identification within the *Cryptococcus* species complex. Electrophoresis 29, 3183–3191.

Kurz, W.A., Dymond, C.C., Stinson, G., Rampley, G.J., Neilson, E.T., Carroll, A.L., Ebata, T., Safranyik, L., 2008. Mountain pine beetle and forest carbon feedback to climate change. Nature 452, 987–990.

Lee, S., Kim, J.J., Fung, S., Breuil, C., 2003. A PCR-RFLP marker distinguishing *Ophiostoma clavigerum* from morphologically similar *Leptographium* species associated with bark beetles. Can. J. Bot. 81, 1104–1112.

Lee, S., Kim, J.-J., Breuil, C., 2005. *Leptographium longiclavatum* sp. nov., a new species associated with the mountain pine beetle, *Dendroctonus ponderosae*. Mycol. Res. 109, 1162–1170.

Lee, S., Kim, J.-J., Breuil, C., 2006a. Pathogenicity of *Leptographium longiclavatum* associated with *Dendroctonus ponderosae* to *Pinus contorta*. Can. J. For. Res. 36, 2864–2872.

Lee, S., Kim, J.-J., Breuil, C., 2006b. Diversity of fungi associated with the mountain pine beetle, *Dendroctonus ponderosae* and infested lodgepole pines in British Columbia. Fungal Divers. 22, 91–105.

Lee, S., Hamelin, R.C., Six, D.L., Breuil, C., 2007. Genetic diversity and the presence of two distinct groups in *Ophiostoma clavigerum* associated with *Dendroctonus ponderosae* in British Columbia and the northern Rocky Mountains. Phytopathology 97, 1177–1185.

Lim, Y.W., Massoumi Alamouti, S., Kim, J.-J., Lee, S., Breuil, C., 2004. Multigene phylogenies of *Ophiostoma clavigerum* and closely related species from bark beetle-attacked *Pinus* in North America. FEMS Microbiol. Lett. 237, 89–96.

Lim, Y.W., Kim, J.-J., Lu, M., Breuil, C., 2005. Determining fungal diversity on *Dendroctonus ponderosae* and *Ips pini* affecting lodgepole pine using cultural and molecular methods. Fungal Divers. 19, 79–94.

Lizardi, P.M., Huang, X.H., Zhu, Z.G., Bray-Ward, P., Thomas, D.C., Ward, D.C., 1998. Mutation detection and single-molecule counting using isothermal rolling-circle amplification. Nat. Genet. 19, 225–232.

Martin, K.J., Rygiewicz, P.T., 2005. Fungal specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. BMC Microbiol. 5, 28.

Mullineux, T., Hausner, G., 2009. Evolution of rDNA ITS1 and ITS2 sequences and RNA secondary structures within members of the fungal genera *Grosmannia* and *Leptographium*. Fungal Genet. Biol. 46, 855–867.

Nilsson, M., 2006. Lock and roll: single-molecule genotyping in situ using padlock probes and rolling-circle amplification. Histochem. Cell Biol. 126, 159–164.

Nilsson, M., Malmgren, H., Samiotaki, M., Kwiatkowski, M., Chowdhary, B.P., Landegren, U., 1994. Padlock probes: circularizing oligonucleotides for localized DNA detection. Science 265, 2085–2088.

Nilsson, R.H., Kristiansson, E., Ryberg, M., Hallenberg, N., Larsson, K.-H., 2008. Intraspecific *ITS* variability in the kingdom *Fungi* as expressed in the international sequences databases and its implications for molecular species identification. Evol. Bioinform. 4, 193–201.

Persson, Y., Vasaitis, R., Långström, B., Öhrn, P., Ihrmark, K., Stenlid, J., 2009. Fungi vectored by the bark beetle *Ips typographus* following hibernation under the bark of standing trees and in the forest litter. Microb. Ecol. 58, 651–659.

Plattner, A., Kim, J.-J., DiGuistini, S., Breuil, C., 2008. Variation in pathogenicity of a mountain pine beetle-associated blue-stain fungus, *Grosmannia clavigera*, on young lodgepole pine in British Columbia. Can. J. Plant Pathol. 30, 457–466.

Raffa, K.F., Berryman, A.A., 1983. Physiological aspects of lodgepole pine wound responses to a fungal symbiont of the mountain pine beetle, *Dendroctonus ponderosae* (Coleoptera: Scolytidae). Can. Entomol. 115, 723–734.

Rambaut, A., 1999. Se-Al: Sequence Alignment Editor. Available at <http://evolve.zoo.ox.ac.uk/>. Department of Zoology, University of Oxford, UK.

Rice, A.V., Langor, D.W., 2009. Mountain pine beetle-associated blue-stain fungi in lodgepole × jack pine hybrids near Grande Prairie, Alberta (Canada). For. Path. 39, 323–334.

Rice, A.V., Thormann, M.N., Langor, D.W., 2007. Virulence of, and interactions among, mountain pine beetle associated blue-stain fungi on two pine species and their hybrids in Alberta. Can. J. Bot. 85, 316–323.

Sambrook, J., Russell, D.W., 2001. Molecular Cloning: a Laboratory Manual, 3rd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schweigkofler, W., Otrosina, W.J., Smith, S.L., Cluck, D.R., Maeda, K., Peay, K.G., Garbelotto, M., 2005. Detection and quantification of *Leptographium wageneri*, the cause of black-stain root disease, from bark beetles (Coleoptera: Scolytidae) in Northern California using regular and real-time PCR. Can. J. For. Res. 35, 1798–1808.

Six, D.L., 2003. A comparison of mycangial and phoretic fungi of individual mountain pine beetles. Can. J. For. Res. 33, 1331–1334.

Six, D.L., Klepzig, K.D., 2004. *Dendroctonus* bark beetles as model systems for studies on symbiosis. Symbiosis 37, 207–232.

Six, D.L., Paine, T.D., 1998. Effects of mycangial fungi and host tree species on progeny survival and emergence of *Dendroctonus ponderosae* (Coleoptera: Scolytidae). Environ. Entomol. 27, 1393–1401.

Six, D.L., Harrington, T.C., Steimel, J., McNew, D., Paine, T.D., 2003. Genetic relationships among *Leptographium terebrantis* and the mycangial fungi of three western *Dendroctonus* bark beetles. Mycologia 95, 781–792.

Solheim, H., Krokene, P., 1998. Growth and virulence of mountain pine beetle associated blue-stain fungi, *Ophiostoma clavigerum* and *Ophiostoma montium*. Can. J. Bot. 76, 561–566.

Steain, M.C., Dwyer, D.E., Hurt, A.C., Kol, C., Saksena, N., Cunningham, A.L., Wang, B., 2009. Detection of influenza A H1N1 and H2N2 mutation conferring resistance to oseltamivir using rolling circle amplification. Antiviral Res. 84, 242–248.

Szemes, M., Bonants, P., de Weerdt, M., Baner, J., Landegren, U., Schoen, C.D., 2005. Diagnostic application of padlock probes — multiplex detection of plant pathogens using universal microarrays. Nucleic Acids Res. 33, e70.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.

Tong, Z., Kong, F., Wang, B., Zeng, X., Gilbert, G.L., 2007. A practical method for subtyping of *Streptococcus agalactiae* serotype III, of human origin, using rolling circle amplification. J. Microbiol. Methods 70, 39–44.

Tsuneda, A., Hiratsuka, Y., 1984. Sympodial and annellidic conidiation in *Ceratocystis clavigera*. Can. J. Bot. 62, 2618–2624.

van Doorn, R., Szemes, M., Bonants, P., Kowalchuk, G.A., Salles, J.F., Ortenberg, E., Schoen, C.D., 2007. Quantitative multiplex detection of plant pathogens using a novel ligation probe-based system coupled with universal, high-throughput real-time PCR on OpenArrays™. BMC Genomics 8, 276.

van Doorn, R., Slawiak, M., Szemes, M., Dullemans, A.M., Bonants, P., Kowalchuk, G.A., Schoen, C.D., 2009. Robust detection and identification of multiple oomycetes and fungi in environmental samples by using a novel cleavable padlock probe-based ligation detection assay. Appl. Environ. Microbiol. 75, 4185–4193.

Vasanthakumar, A., Handelsman, J., Schloss, P.D., Bauer, L.S., Raffa, K.F., 2008. Gut microbiota of an invasive subcortical beetle, *Agrilus planipennis* Fairmaire, across various life stages. Environ. Entomol. 37, 1344–1353.

Wang, B., Potter, S.J., Lin, Y., Cunningham, A.L., Dwyer, D.E., Su, Y., Ma, X., Hou, Y., Saksena, N.K., 2005. Rapid and sensitive detection of severe acute respiratory syndrome coronavirus by rolling circle amplification. J. Clin. Microbiol. 43, 2339–2444.

Wright, S.H.A., Berch, S.M., Berbee, M.L., 2009. The effect of fertilization on the below ground diversity and community composition of ectomycorrhizal fungi associated with western hemlock (*Tsuga heterophylla*). Mycorrhiza 19, 267–276.

Yamaoka, Y., Hiratsuka, Y., Maruyama, P.J., 1995. The ability of *Ophiostoma clavigerum* to kill mature lodgepole-pine trees. Eur. J. For. Pathol. 25, 401–404.

Zambino, P.J., Harrington, T.C., 1992. Correspondence of isozyme characterization with morphology in the asexual genus *Leptographium* and taxonomic implications. Mycologia 84, 12–25.

Zhang, N., Suh, S.O., Blackwell, M., 2005. Microorganisms in the gut of beetles: evidence from molecular cloning. J. Invertebr. Pathol. 84, 226–233.

Zhou, X., Kong, F., Sorrell, T.C., Wang, H., Duan, Y., Chen, S.C.A., 2008. Practical method for detection and identification of *Candida, Aspergillus,* and *Scedesporium* spp. by use of rolling circle amplification. J. Clin. Microbiol. 46, 2423–2427.

Zipfel, R.D., de Beer, Z.W., Jacobs, K., Wingfield, B.D., Wingfield, M.J., 2006. Multi-gene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct from *Ophiostoma*. Stud. Mycol. 55, 75–97.