[Molecular Identification and Characterization of *Ceratocystiopsis* spp. Associated with the Mountain Pine Beetle (*Dendroctonus ponderosa*)]

[Graduating Essay – FRST 497]

[Ting Pu]

April 18, 2011

ABSTRACT

The mountain pine beetle (*Dendroctonus ponderosa*) attack has been the most devastating disturbance in the logdepole pine (*Pinus contorta*) forests in interior British Columbia, Canada. Previous studies have found several fungal species associated MPB: *Ophiostoma clavigerum, Ophiostoma monitum, Leptographium longiclavatum, Grosmannia clavigera* and some *Ceratocystiopsis* (*Cop.*) species. The aim of the study was to identify and characterize the *Ceratocystiopsis* species associated with MPB using molecular methods. In total, 72 samples were isolated from the beetles from 12 collection sites in BC and Alberta. They were distinguished as Cop. – like species based on morphological features. 61 of them were investigated in the phylogenetic analyses, using sequences of internal transcribed spacer regions (ITS), and portion of the large ribosomal subunit (LSU) of RNA, and β-tubulin gene. Surprisingly, the results showed that most of the isolates belonged to *Sporothrix*, which had rarely been reported as a symbiont with beetles in Canada. Results from β-tubulin gene showed that there were many variations under this genus. Future study will be needed to resolve the species-level taxonomy.

KEYWORDS

Ascomycetes, Ophiostoma, *Sporothrix*, phylogeny, taxonomy

Table of Contents

Index of Figures

Index of Tables

1.0 Introduction

In British Columbia, the mountain pine beetle (*Dendroctonus ponderosa*, MPB) (Coleoptera: Scolytidae) has already killed a total of 726 million cubic meters of timber cumulatively in the last two decades, most of which belong to *Pinus contorta* (Lodgepole pine) (The Government of British Columbia, 2011). This epidemic has devastating effects to the economy and forestry industry of the province. The MPB epidemic has been expanding beyond its native range into northern BC and eastward into Alberta, possibly due to global warming (The Government of British Columbia, 2011). While the beetles are colonizing the host trees, building tunnels underneath the bark, they also introduce the fungi to the hosts (Six, 2003). These fungi were carried in the mycangia and on the exoskeleton of the MPB (Six, 2003). They may benefit the attack by reducing the trees' defense to the beetles, dehydrating sapwood and phloem, and making the environment more favourable to beetle reproduction (Six and Klepzig, 2004). Therefore, it is of great importance in determining the ecological role and population biology of these fungal associates in the MPB/lodgepole pine system, and whether the fungi are pathogenic to the pine (Six and Klepzig, 2004).

The fungal associates with MPB are diverse, and the common filamentous fungi are *Grosmannia clavigera, Leptographium longiclavatum*, and *Ophiostoma montium* (Lee et al., 2006). There has been much research on the taxonomy, ecology, population structure and pathogenicity of these fungi (Lee et al., 2007, Roe et al., 2010). *Grosmannia clavigera* and *Leptographium longiclavatum* are found to be pathogenic (Solheim and Krokene, 1998, Rice et al., 2007, Plattner et al., 2009); while *Ophiostoma montium* has been suggested to be a weak pathogen (Solheim and Krokene, 1998, Khadempour, et al., 2010). Apart from these three fungi, *Ceratocystiopsis* (*Cop*.) species has also been consistently isolated

from the MPB (Lee et al., 2006). Although *Ceratocystiopsis* spp. may be nonpathogenic, its specific role in beetle associations and its effect on trees are not well established yet (Plattner et al., 2009). The taxonomy of the genus and species is also not well studied. Based on morphological characters, the *Ceratocystiopsis* strains from MPB resembled *Ceratocystiopsis minuta (*Plattner, et al., 2009). After extensive phylogenetic analysis, these strains were shown to be distinct in evolution from *Cop. minuta* as well as other described *Ceratocystiopsis* species from Europe and North America.

During the latest sampling of MPB, multiple fungal isolates that resemble *Ceratocystiopsis* species have been isolated based on colony characteristics. However they do not produce fruiting bodies readily in cultures and have little diagnostic characters. The purpose of the current investigation is to characterize the identity of these fungi using their DNA sequence data, and to study their genetic diversity using various molecular methods, and to examine their phylogenetic relationships to other described species in *Ophiostoma*.

2.0 Materials and Methods

2.1 Fungal Isolation and Culturing

Mountain pine beetles were sampled in different locations in BC and Alberta in 2010 (Figure 1). The isolation was done between June and July of 2010. The fungal associates were isolated from MPB in three different ways. Firstly, the beetles were washed in 1 ml sterile distilled water and the wash water (10 times dilution) was plated onto 2% malt extract agar (MEA) plates. Alternatively, each beetle was streaked across a plate of MEA. Thirdly for every beetle, a 5 mm (in diameter) sample of phloem was

flame sterilized for 3-5 seconds and placed on MEA.

Figure 1 Location map showing 12 sampling sites in Canada (Cranbrook, Kimberly, Cascades, Terrace, Mackenzie, Tumbler Ridge in BC; Cypress Hill, Canmore, Valemount, Fox Creek, Grande Prairies, PeaceArea in Alberta)

After incubation for one to two weeks, individual fungal culture was purified from these 'MEA plates' prepared as above. They were sorted based on morphotypes in terms of hyphal morphology, colony characteristics, and color. In total 72 cultures resembling *Ceratocystiopsis* species were isolated and used in this investigation, of which 60 were studied in molecular analyses (Figure 2).

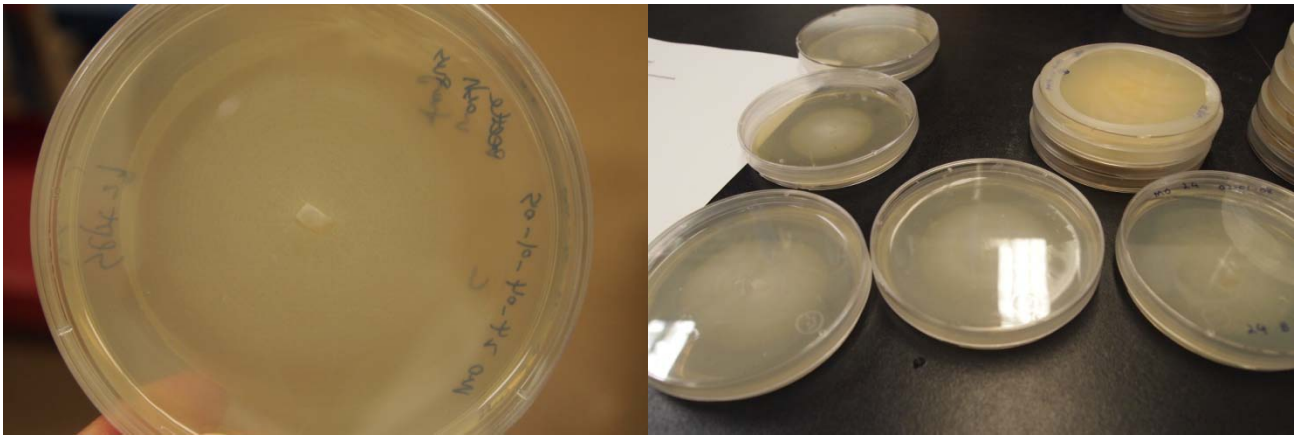

Figure 2 *Ceratocystiopsis*-liked species are maintained on MEA

2.2 DNA Extraction

After growing under room temperature for two to three weeks, the mycelium of the fungi was scraped from the surface of the agar plate into Eppendorf tubes with a ceramic bead for DNA extraction (Plattner et al., 2009). The mycelia were frozen at -20°C before they were used for DNA extraction. The original fungal colonies also were transferred into new MEA plates for backup. Genomic DNA was extracted according to a modified protocol of (Khadempour et al., 2010) - briefly 500 µL of TES buffer was added to each Eppendorf tube and then mixed in a shaker for five minutes with the addition of 1 µL lysing enzyme (*Trichoderma harzianum* SIGMA). The mixture was vortexed for a few seconds and incubated at 40 °C for 15 minutes. Then 250 µL sodium acetate was added, and the solution was well mixed and frozen at -80 °C for overnight. The next day, the extract was melted and centrifuged at 14, 000 rpm for 15 minutes. Then the supernatant is transferred to a new tube. RNA was digested with 2 µL of RNase A (Roche Applied Science, Laval, Quebec) and incubated at 37 °C for 20 minutes. The mixture was treated with 700 µL of Phenol: Chloroform: isoamyl alcohol (25: 24: 1), and gently rotated for 15 minutes for mixing, followed by centrifuge for 15 minutes at 14, 000 rpm to collect top aqueous layer. Then the top layer was transferred to a new tube, and added with 540 µL cold isopropanol and 60 µL sodium acetate to precipitate the DNA. After a few minutes of hand mixing and centrifuge, the

DNA pellet became visible and was washed with 1000 µL of 70% filtered ethanol. The tube was air dried for two hours after removing the ethanol. Then the DNA pellet was dissolved in 50 µL water and incubated overnight at 4 °C (Lee, R C Hamelin, Six, & Breuil, 2007).

The quality of the DNA extracted was checked through electrophoresis on 0.8 % agarose gels, stained with ethidium bromide (Tsui et al., 2010). Only the high-quality DNA was used in Polymerase Chain Reaction. As Figure 3 shows, the ones with a bright band indicated that their DNA was of good quality.

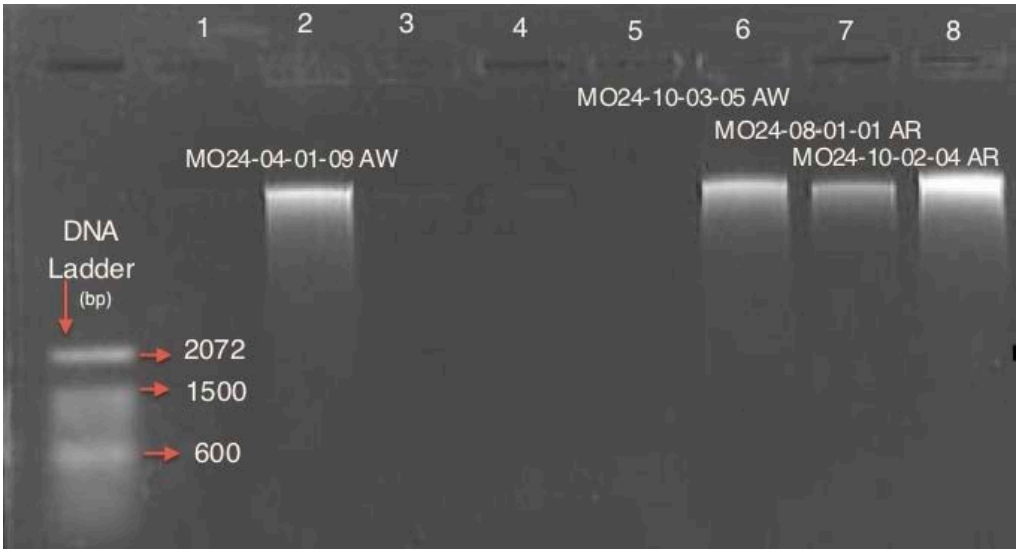

Figure 3 DNA quality check on 0.8 % agarose gel, stained with ethidium bromide; names of samples shown above the bands, the first column on the left is DNA ladder.

2.3 Polymerase Chain Reaction (PCR)

Partial large subunit ribosomal (LSU) rRNA was amplified with primers CTB6 and TW13, and internal transcribed spacer (ITS) region was amplified with primers ITS1F and ITS4 (Bruns Lab, 2002). A fragment of β-tubulin gene was also amplified with primers T1 and BT 12 (Plattner et al., 2009).

The total volume of each PCR reaction mixture was about 24 µL, which contained: 4 µL of 10x diluted DNA, 16.05 µL SIGMA water (Sigma-Aldrich Co., St. Louis, MO), 2.5 µL 10x PCR buffer, 0.75 µL of 50 mM $MgCl_2$, 0.5 µL of 10 mM deoxynucleo - tide triphosphates (dNTPs), 1 µL of each primer (25 µM), 0.2 µL of ampliTaq polymerase (Applied Biosystems, Foster City, CA) and possibly 0.2 µL dimethylsulfoxide (DMSO) (Gorton, 2004).

The amplification was carried out in 2720 thermocycler and GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, California), with the following condition: 3 minutes at 94 °C for denaturing, 34 cycles of 30 seconds at 94 °for denaturing, 30 seconds at 55 °C for annealing, and 1 minute at 72 °C for extension, a final elongation and extension 72 °C for 10 minutes. The final PCR products were kept at 4°C (Tsui et al., 2010).

The quality of the PCR products was assessed using the same method used for DNA quality check, but on 2% agarose gels. The ones showed bright bands on the gel were purified and sent for sequencing later on (Figure 4).

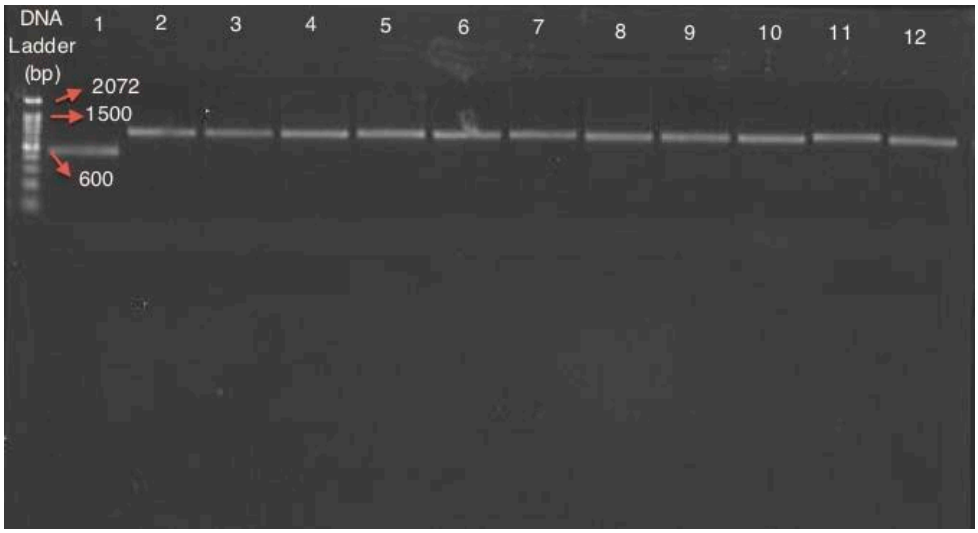

Figure 4 PCR products quality check (with ITS1F and ITS4) on 2% agarose gel after electrophoresis, stained with ethidium bromide; first column is DNA ladder

2.4 Sequencing

Purified PCR products were sent to Centre de Recherche du CHUQ (CHUL) in Quebec, Canada for sequencing, along with 5 µL of primers (1.6 µM) for each reaction. ITS region was sequenced with primers ITS5 and ITS4 (White et al., 1990). The sequences were determined by the latest platform from Applied Biosystems, the ABI 3730xl. Fragments were assembled using the software Geneious v. 5.3.4 (Drummond AJ et al., 2011), with some adjustments done manually. Some ambiguous regions were excluded for future analysis. The sequences were submitted to BLAST (Basic Local Alignment Search Tool) Search in the GenBank database to verify identity. The accession numbers were recorded.

2.5 Data Analysis

Sequences were aligned with Geneious v. 5.3.4 (Drummond AJ et al., 2011), and manually adjusted by Se-Al 2.0 (Rambaut, 1999). The alignment was analyzed with MEGA 4 (Tamara et al., 2007). The quality of sequences was checked, and multiple sequence alignment was generated with Clustal X (Thompson et al., 1997) using default settings implemented in Geneious (Drummond AJ et al., 2011), and then adjusted with MEGA 4 (Tamara et al., 2007). Phylogenetic analysis was performed using MEGA. Related sequences were downloaded from GenBank for comparison (Figure 8-10). Phylogenetic trees were developed using the neighbor joining method (Saitou and Nei, 1987), which is based on the distance matrix theory. The confidence level of the tree branches was assessed by bootstrap analysis (500 replicates) (de Meyer et al., 2008).

3.0 Results

3.1 The identity of ITS and LSU sequences based on BLAST search.

In total, 161 sequences have been generated from 61 cultures and they were used in various phylogenetic analyses. For the ITS gene, 419 – 681 base pairs of DNA fragments were amplified and analyzed. Table 1 summarized some results revealed from BLAST search with all three genes. In the ITS gene, by comparing with the sequences in the Genbank database, the results indicated that most of the samples were similar to *Sporothrix spp.*, almost all producing 99% max identity. However there were a few exceptions, one *Ophiostoma sp.* and a few yeast species were also reported. Surprisingly only one of the cultures was similar to *Ceratocystiopsis* species. MPB1, MPB3 and UM 237, obtained from Dr. Breuil's lab as reference strains, were confirmed to be *Ceratocystiopsis* species.

DNA fragments of the LSU gene contained 564 – 587 base pairs. The variations among the sequences were small. The BLAST search results were congruent to that of ITS and showed that most of them of the samples were either *Ophiostoma nigrocarpum or O. stenoceras,* with 99% max identity.

With the β-tubulin gene, 844 – 1182 base pairs of DNA fragments were amplified and analyzed. Most samples seemed to be related to *Ophiostoma nigrocarpum* with 98% - 99% max identity. One species was found to be most similar to *Ophiostoma breviusculum,* while another is similar to *Fusarium ciliatum.* MPB1, MPB3 and UM237 were identified as *Ceratocystiopsis spp.* in all three genes.

Table 1 Results from BLAST search based on ITS, LSU and β-tubulin genes

SAMPLE NUMBER	GENE	ACCESSION NUMBER	SPECIES (GENBANK)	MAX IDENTITY
MO24-02-01-0712B	ITS	AY546720.1	*Sporothrix sp*. 1-CMW9488	99%
MO24-04-01-09AW	ITS	AY546722.1	*Sporothrix sp*. 1-CMW9492	99%
MPB1	ITS	AY761157.1	*Ceratocystiopsis sp.* M272	99%
UM237	ITS	EU913714.1	*Ceratocystiopsis manitobensis* strain UM237	97%
MO24-02-01-0712B	LSU	EF506941.1	*Ophiostoma nigrocarpum* 28S	99%
MO24-03-05-02CPW	LSU	DQ836904.1	*Ophiostoma stenoceras* isolate AFTOL-ID 1038 28S	99%
MO24-03-05-03CR	LSU	DQ836904.1	*Ophiostoma stenoceras* isolate AFTOL-ID 1038 28S	99%
MPB1	LSU	EU913677.1	*Ceratocystiopsis sp.* SWT3 28S	99%
UM237	LSU	EU913677.1	*Ceratocystiopsis sp.* SWT3 28S	99%
MO24-06-03-01AR	β-tubulin	AY789149.1	*Ophiostoma nigrocarpum* strain C201	98%
MO24-09-01-10BP	β-tubulin	EU913760.1	*Ophiostoma nigrocarpum* strain C201	98%
MO24-10-02-06AR	β-tubulin	AB200428.1	*Ophiostoma breviusculum*	97%
MO24-06-02-06AW	β-tubulin	EU860030.1	*Fusarium ciliatum* strain F-202, 821	97%
MPB1	β-tubulin	EU913748.1	*Ceratocystiopsis sp.* WY21TX2-2	99%
UM237	β-tubulin	EU913754.1	*Ceratocystiopsis manitobensis* strain UM214	100%

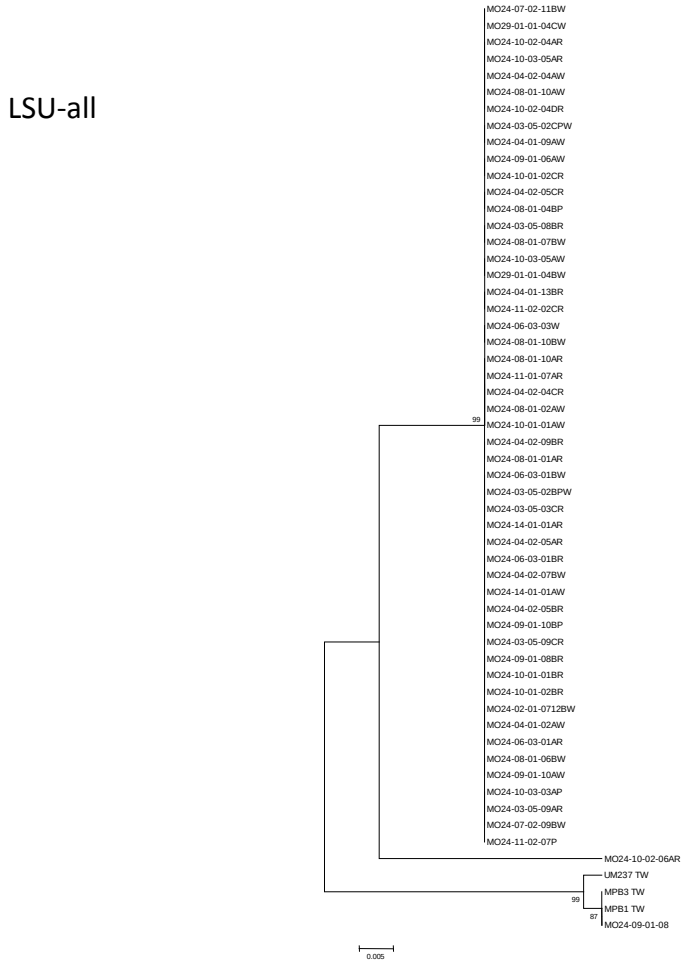

Figure 5 Phylogenetic tree generated by neighbor joining in MEGA using large subunit (LSU) ribosomal gene. 56 strains belonging to Ophiostomataceae were included. The tree was rooted in mid point with bootstrap values (%) above 40% indicated above the branches.

Based on the LSU gene, 56 strains were included in the phylogenetic analysis. As shown in the tree, the results were generally very consistent, which corresponded with the results from BLAST search well. There were only two main groups that both received 99% bootstrap supports. Within the first main group, 51 strains were similar to *Ophiostoma stenoceras*, while one strain was similar to *O. piceae*. The other main group contained four *Ceratocystiopsis* species, subdivided to two subgroups. UM237 was the most similar to *Ceratocystiopsis sp.* SWT3 28S (EU913677.1). The other three under fell under a subgroup of *Ceratocystiopsis sp.* SWT1 28S (EU913676.1), with a bootstrap value of 87%. My sampled strain (MO24-09-01-08) clustered with MPB1 and MPB3, the reference strains of

Ceratocystiopsis.

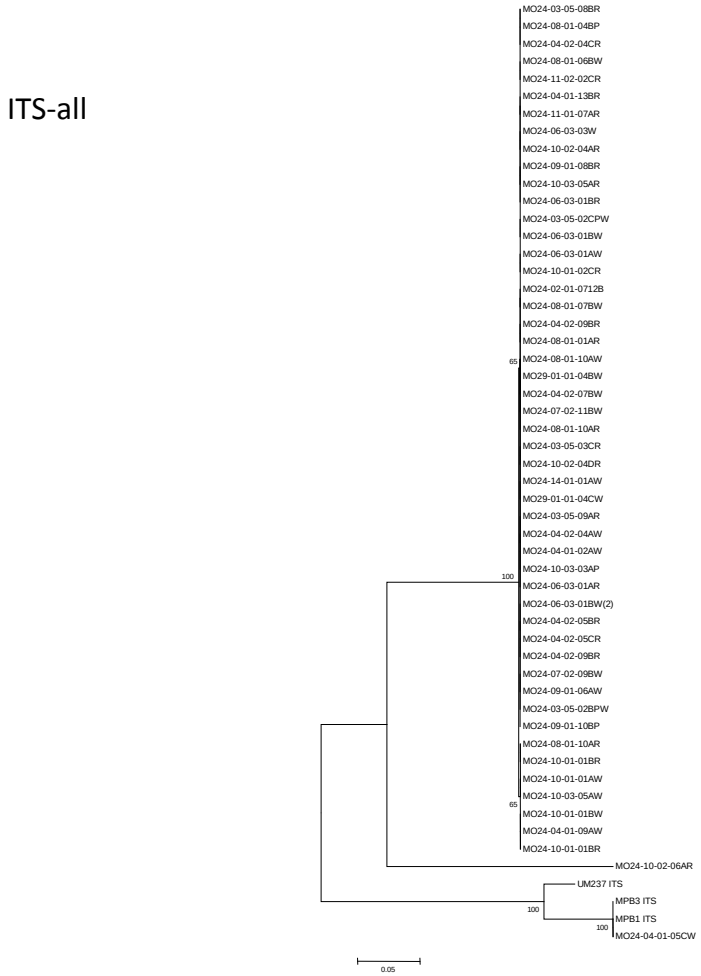

Figure 6 Phylogenetic tree generated with most samples (some divergent ones excluded, e.g. yeasts etc.) used in molecular analysis with ITS gene, bootstrap values (%) above 40% are indicated above the branches.

Similarly, 54 ITS sequences were included in the phylogenetic analysis. The neighbor joining tree demonstrated a similar pattern to the one of LSU. But there were more subgroups within the main group. The first clade, where the majority of the sequences fell under, including two sub-clades, represented *Sporothrix sp.* 1-CMW9485 (AY546718.1) and *Sporothrix sp.* 1- CMW9492 (AY546722.1) respectively, both with weak bootstrap supports of 65%. The second clade, which contained only 1 strain, represented *Ophiostoma rachisporum* (HM031491.1). The other main group, which was supported by a bootstrap value of 100%, had four strains. It was further divided into two subgroups,

with a 100% bootstrap value. UM237 represented *Ceratocystiopsis manitobensis* (DQ268610.1) closely, while the other three were more similar to Ceratocystiopsis sp. WY21TX2-2 (EU913709.1).

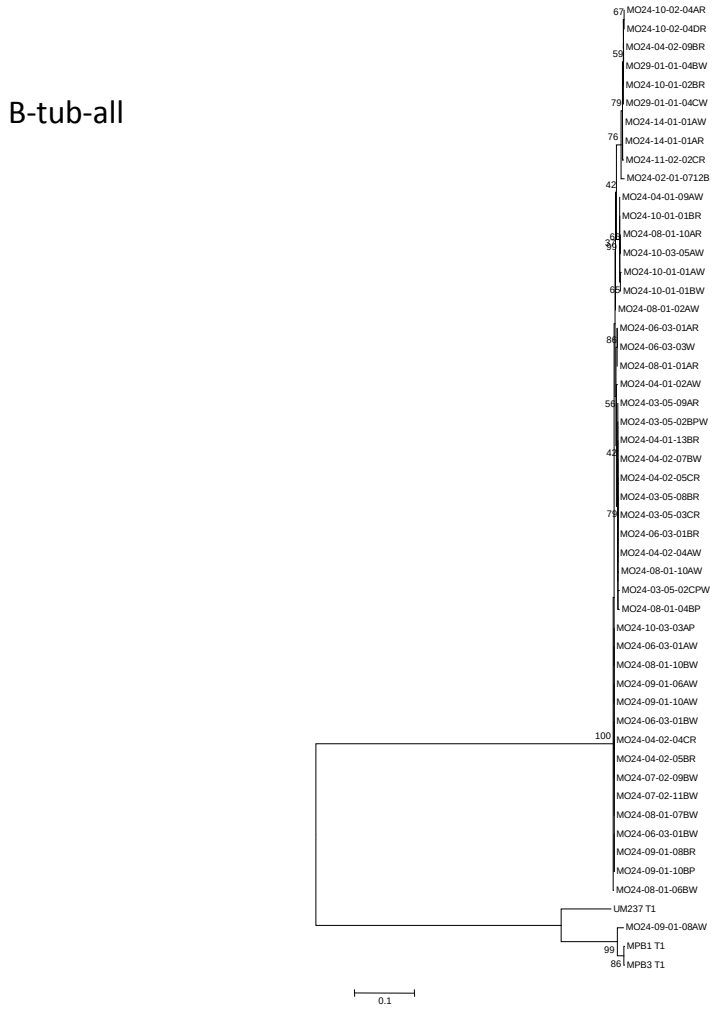

Figure 7 Phylogenetic tree generated with most samples (some divergent ones excluded, e.g. yeasts etc) used in molecular analysis with β-tubulin gene, bootstrap values (%) are indicated above the branches.

With the β-tubulin gene, 52 strains were included in the phylogenetic analysis. This tree was the most divergent one compared to the other two shown above. The general pattern was similar, but there were way more subgroups within the first main group. Even though most of the strains in the first clade belonged to *Sporothrix sp.*, they were more variable in species level, which will be shown in more details in Figure 10. The second main group still represented *Ceratocystiopsis sp.*.

3.2 Comparison of species to those in the GenBank.

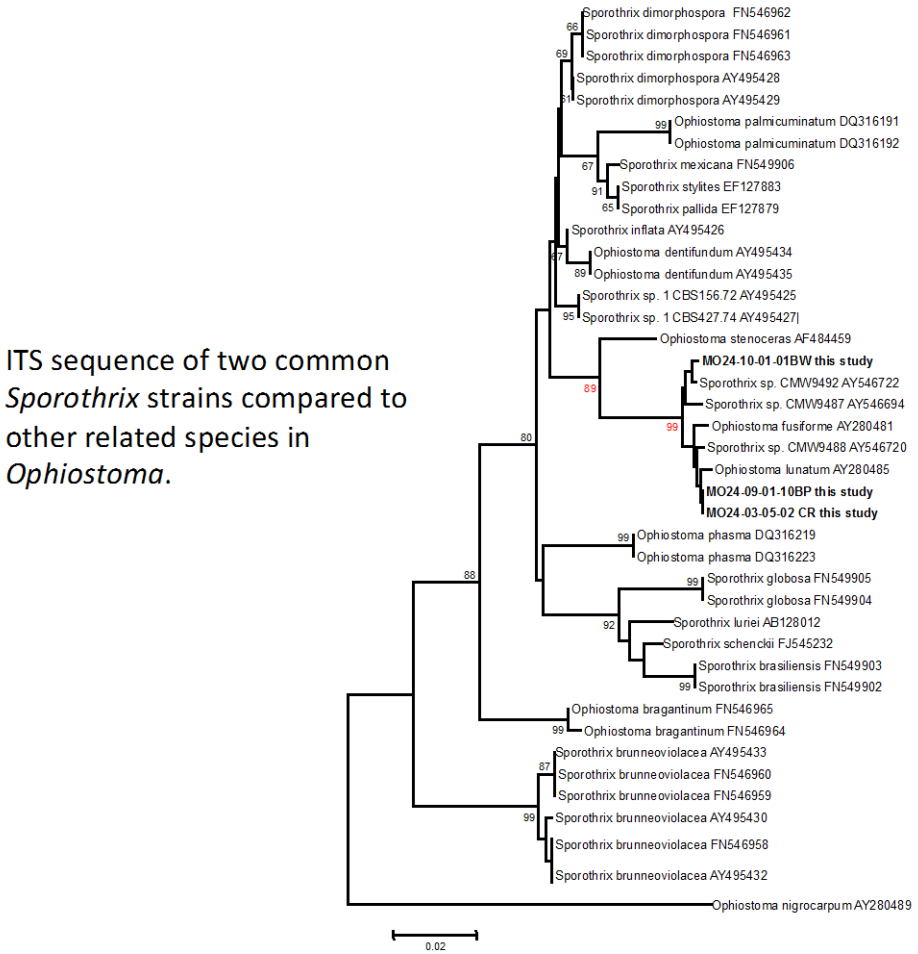

Figure 8 Phylogenetic tree generated with three *Sporothrix* strains based on ITS region from the study (printed in bold) and other related *Ophiostoma* species downloaded from the Genbank; *Ophiostoma nigrocarpum* was used as outgroup.

Based on Figure 6, there were two subgroups of *Sporothrix* strains within the main group. In this analysis, one strain from the first subgroup and two strains from the other subgroup were selected and compared with other related *Ophiostoma* species downloaded from the Genbank. Those related species were referred from (Madrid, Gené, Cano, Silvera, & Guarro, 2010), (de Meyer et al., 2008) and (Zhou, 2004). Figure 8 showed that all my three samples (MO24-10-01-01BW, MO24-09-01-10BP, and Ting1-its) were clustered closely with five related species: *Sporothrix sp.* 1-CMW9492, *Sporothrix sp.* 2-CWM9487, *Ophiostoma fusiforme*, *Sporothrix sp.* 1-CMW9488 *and Ophiostoma lunatic*, supported by

a 99% bootstrap value. This group as a whole was grouped together with another species - *Ophiostoma stenoceras*, with 89% bootstrap support (shown in red on Figure 8).

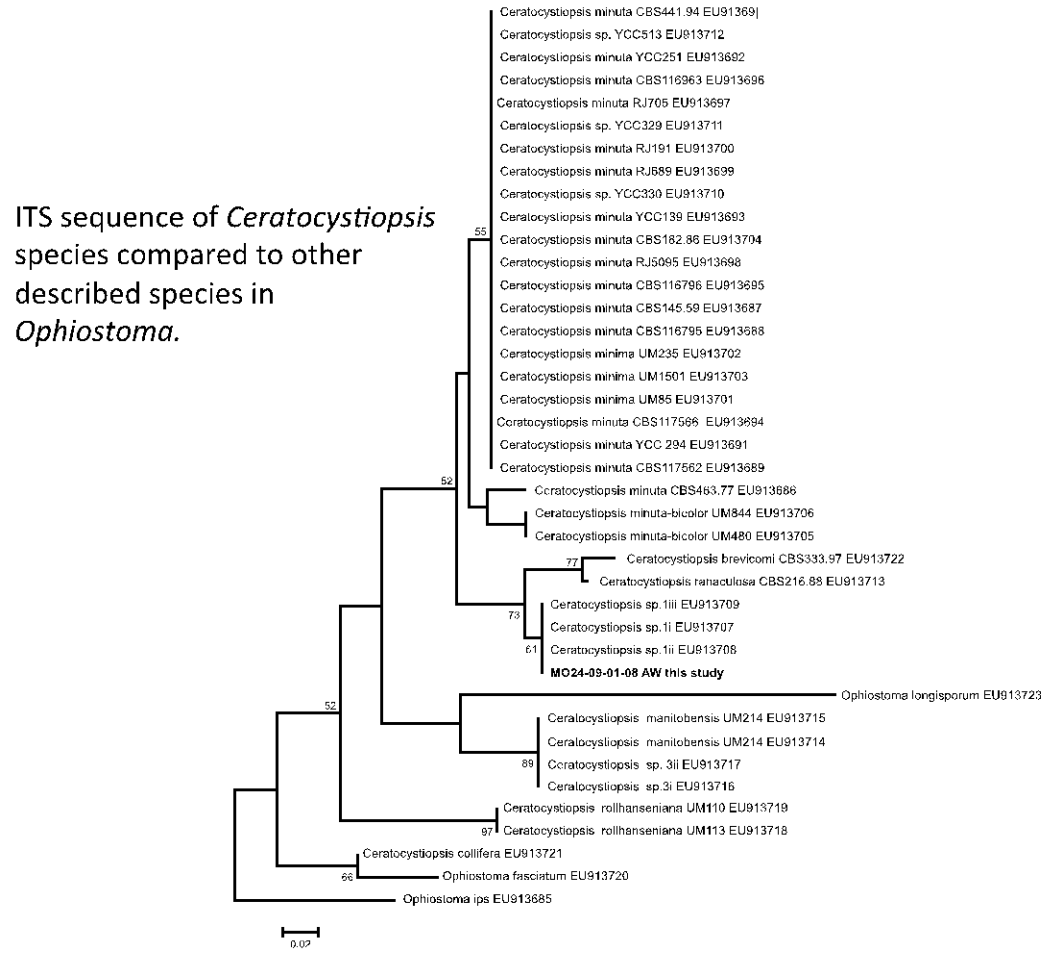

Figure 9 Phylogenetic tree generated with one *Ceratocystiopsis* strain based on ITS region from the study and other related *Ophiostoma* species downloaded from the Genbank; *Ophiostoma ips* EU913685 was used as outgroup.

Based on the results from ITS, two *Ceratocystiopsis* strains were reported, MO24-04-05-01CW and MO24-09-01-08AW (but only MO24-04-05-01CW was included in Figure 6). In order to better understand the relationship between theses strains and other previously reported *Ceratocystiopsis* species, MO24-09-01-08AW was compared with other related sequences downloaded from the Genbank (Plattner et al., 2009). Figure 9 indicated that my sample was clustered weakly (64% bootstrap value) with three undefined *Ceratocystiopsis* species: *Ceratocystiopsis* sp. WY21TX2-2, *C.* sp. WY21TS1-2, and *C.* sp. WY13TX1-3. This whole group was grouped with another group

containing two species: *C. brevicomis and C. ranaculosa* with 73% bootstrap support.

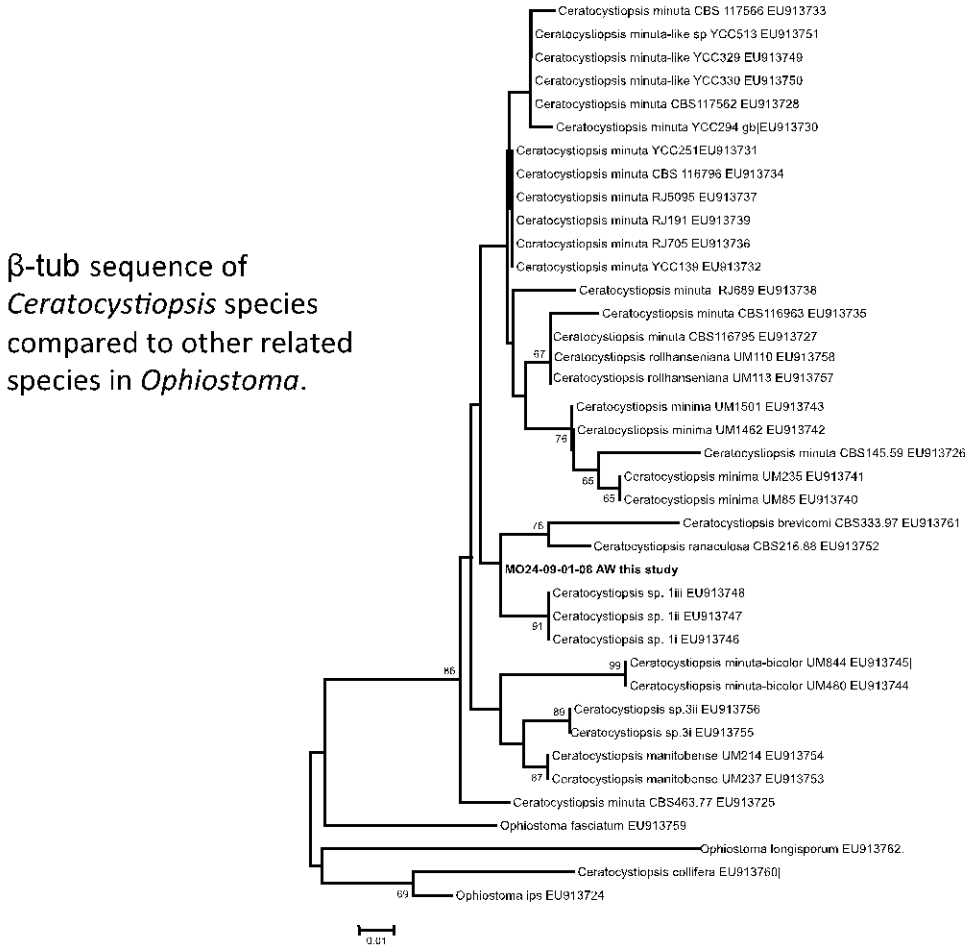

Figure 10 Phylogenetic tree generated with one *Ceratocystiopsis* strain based on β-tubulin region from the study (printed in bold) and other related *Ophiostoma* species downloaded from the Genbank.

As can be seen from Figure 7, one of my samples, MO24-09-01-08AW was identified as *Ceratocystiopsis* species based on β-tubulin gene. Using similar approach, this strain was compared with other related species downloaded from the Genbank (Plattner et al., 2009). Figure 10 demonstrated that my sample was related to five *Ceratocystiopsis* species: *Ceratocystiopsis* sp. WY21TX2-2, *C.* sp. WY21TS1-2, *C.* sp. WY13TX1-3, *C. brevicomis and C. ranaculosa.* But this relationship was also very weak as it only had a bootstrap value of 49%.

3.3 Genetic diversity of *Sporothrix* based on β-tubulin gene

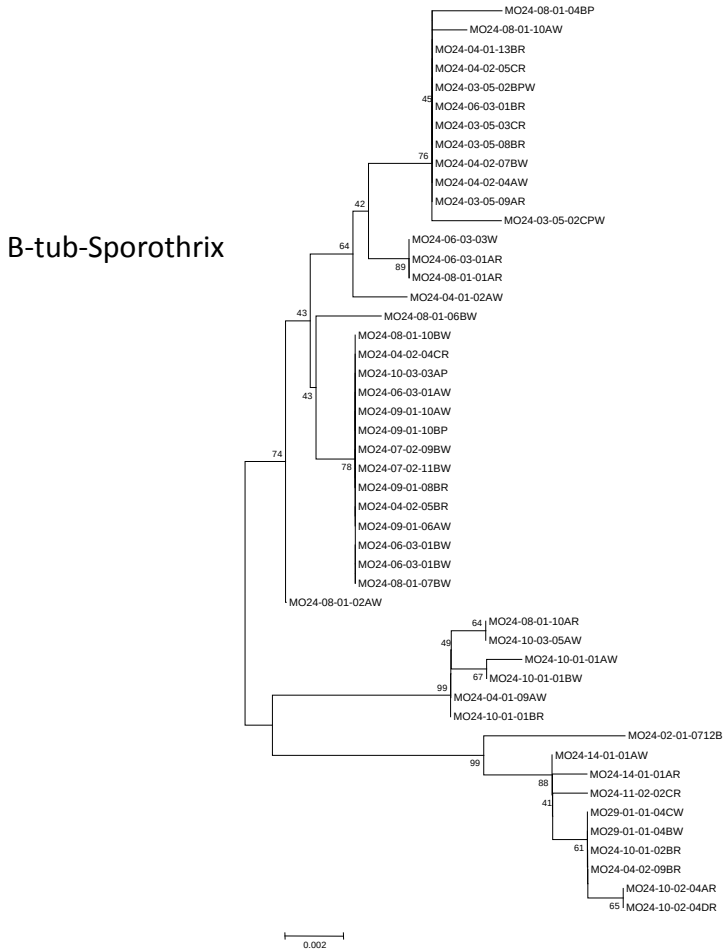

Figure 11 Phylogenetic tree generated with all *Sporothrix* strains based on β-tubulin region from this study

As Figure 7 suggested, there were many variations among the samples under the main *Sporothrix* group, as there were many subgroups. A phylogenetic tree with only *Sporothrix* species based on the β-tubulin gene was generated (Figure 11). The results showed that my samples were mainly divided into two groups with 74% bootstrap support. Within the first clade, there were several small clades but not strongly supported by their bootstrap values. However, MO24-08-01-02AW was identified separately from the other samples within the first clade. Under the second main clade, there were two sub-clades (with 99% bootstrap support). They contained some small groups too, but also with weak bootstrap values.

4.0 Discussion

The Ophiostomatoid fungi contain a group of ascomycetes habiting in wood or associated with insects, with a broad distribution throughout the world (Aghayeva, Michael J Wingfield, Z Wilhelm de Beer, & Kirisits, 2004). *Ophiostoma*, and its related genera *Ceratocystiopsis* and *Grosmannia*, are well known for their association with bark beetles, among which some are forest pathogens (Aghayeva et al., 2004). They were also found to have caused sapstain in freshly cut wood (de Meyer et al., 2008).

To our surprise, most of the samples investigated in this study belong to *Sporothrix*, with only a few exceptions. *Sporothrix* has rarely been reported as a symbiont of Mountain Pine Beetle in Canada before. One study in 2004 found three *Sporothrix* species in Canada (Aghayeva et al., 2004). Morphologically, the genus *Sporothrix* was described by De Hoog (1974) as having conidiogenous cells arising laterally or terminally from any place on the hyphae, and producing nonseptate conidia on denticles developed sympodially. The three isolates found in Canada were characterized by having cylindrical conidia, which was different from other anamorphs of *Ophiostoma* species (Aghayeva et al., 2004). *Ophiostoma fusiforme* usually has guttuliform or fusiform conidia, while *O. lunatum* has clavate or crescent shaped conidia, and *lastly O. stenoceras* has broadly ellipsoidal ones (Aghayeva et al., 2004). Based on the results from ITS gene, the two common strains from this study formed a close relationship with *Sporothrix* CMW 9492, *S.* CMW 9487, *O. fusiforme*, *S.* CMW 9488, *O. lunatum* and *O. stenoceras*. Compared with the study done in 2004, it is possible that the two *Sporothrix* species found in this study are the similar to the three discovered before.

4.1 The discovery of *Sporothrix* sp. commonly associated with MPB

Sporothrix schenckii is well recognized as the human pathogen which causes the disease sporotrichosis (de Meyer et al., 2008). Many studies have been carried out due to its importance to human health, while other species of *Sporothrix* are not fully understood. In South Africa, two groups of *Sporothrix* species were isolated from wood utility poles. *Sporothrix stylites* was separated from pine poles and rosebush wood. It was found to have some phylogenetic relationship to *S. pallid* (de Meyer et al., 2008). *Sporothrix humicola* was isolated from soils, and it was formerly treated as "environmental" isolates of *S. schenckii*. Another species *S. lignivora* was isolated from eucalypt poles (de Meyer et al., 2008). *Sporothrix mexicana* is a newly discovered species from Mexico. It was determined as closely related to *S. stylites* and *S. humicola* from phylogenetic analyses (de Meyer et al., 2008). Some species in the *Ophiostoma stenoceras – S. schenckii* complex are from conifer-infesting bark beetles, such as *O. nigrocarpum* and *O. aurorae* (de Meyer et al., 2008).

Two undefined *Sporothrix* species were reported as associates with the bark beetle *Dendroctonus mexicanus* in Mexico, together with *Ceratocystiopsis minuta*, one *Ophiostoma galeformis* - like species, *O. pluriannulatum* and one *Hyalorhinocladiella* species. Another *Sporothrix* species was isolated from the galleries of *Ips calligraphus* (Zhou, 2004), summarized in Table 2.

Table 2 Fungi Isolates from bark beetles and their galleries in Mexico
Source: Adapted from (Zhou, 2004)

Species	Isolation no.[a]	GenBank accession no.	Host	Insect
Ceratocystiopsis minuta	CMW10771		*Pinus pseudostrobus*	*Dendroctonus mexicanus*
Ophiostoma galeiformis-like	CMW9490		*P. pseudostrobus*	*D. mexicanus*
Sporothrix sp. 1	**CMW9485**	AY546718	*P. pseudostrobus*	*D. mexicanus*
	CMW9486	AY546719		
	CMW9488	AY546720		
	CMW9491	AY546721		
	CMW9492	AY546722	*P. maximinoi*	*Ips calligraphus*
Sporothrix sp. 2	**CMW9487**	AY546694	*P. pseudostrobus*	*D. mexicanus*
	CMW9489	AY546695		
O. pluriannulatum	CMW10772		*P. pseudostrobus*	*D. mexicanus*
	CMW10773		*P. maximinoi*	*I. calligraphus*
O. pulvinisporum	**CMW9020**	AY546713	*P. pseudostrobus*	*D. mexicanus*
	CMW9022	AY546714		
	CMW9023			
	CMW9024		*P. maximinoi*	*I. calligraphus*
	CMW9026	AY546715		
	CMW9028			
	CMW9493	AY546716		

[a] Isolate numbers in bold type refer to isolates used for rDNA sequence analyses.

As can be seen from Table 2, all the three related species found in this study - CMW 9492, CMW 9487, and CMW 9488 had been described in the research from Mexico. The results from the study done by Zhou in 2004 showed that the vector for CWM 9492 was *Ips calligraphus*, while the vector for the other two was *Dendroctonus mexicanus*, which is commonly known as Mexican Pine Beetle. The host tree for CMW 9492 was *Pinus maximinoi* (Thinleaf pine), and for the other two was *Pinus pseudostrobus* (smooth-barked Mexican pine) (Zhou, 2004). This was the first time that those *Sporothrix* isolates were reported, so it was reasonable to assume that these species originated from Mexico and spread up North to Canada and possibly to the US. But further study will be needed to confirm this hypothesis.

4.2 Genetic diversity of Sporothrix based on β-tubulin gene

As discussed earlier in the Results section, there were many variations in species level under the genus *Sporothrix* (Figure 11), based on the results from β-tubulin gene. Therefore, it was difficult to accurately determine which species each of them was. The fact that *Sporothrix* is not well studied makes the taxonomy of this genus more confusing. The *Ophiostoma stenoceras – Sporothrix schenckii*

complex contains many uncertain species (Madrid, Gené, Cano, Silvera, & Guarro, 2010). Traditionally, *S. schenckii* was believed to be the only human pathogenic species within the genus, however, recent phylogenetic and phenotypic studies revealed three relevant pathogenic species: *Sporothrix brasiliensis* Marimon, Gene, Cano & Guarro, *S. globosa* Marimon, Gene, Cano & Guarro and *S. luriei* (Ajello & Kaplan) Marimon, Gene, Cano & Guarro (Madrid, Gené, Cano, Silvera, & Guarro, 2010). Other species contained in this complex include: *Sporothrix inflata* de Hoog, *Sporothrix pallida* (Tubaki) Matsush., *Sporothrix stylites* de Meyer, Z.W. de Beer & M.J. Wingf., *Sporothrix mexicana* Marimon, Gene, Cano & Guarro (Madrid, Gené, Cano, Silvera, & Guarro, 2010). Figure 12 shows the diversity of the species under the *Ophiostoma stenoceras – Sporothrix schenckii* complex.

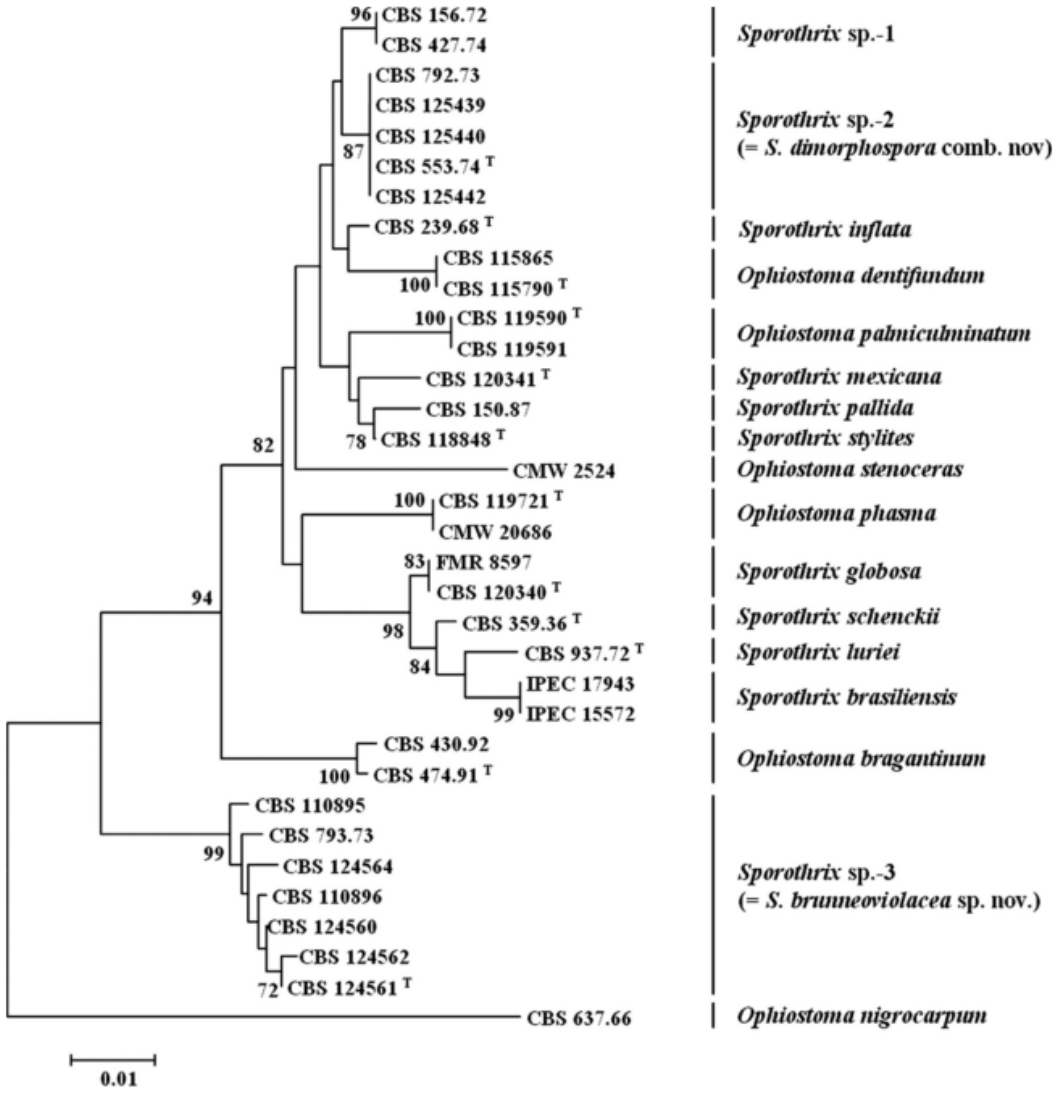

Figure 12 Diversity of *Sporothrix* species with the combined ITS and β-tubulin dataset. Source: Adapted from (Madrid, Gené, Cano, Silvera, & Guarro, 2010).

Even though the species were genetically divergent, they showed similarities in asexual reproductive structures, as they adapted to similar ecological habitats (Madrid, Gené, Cano, Silvera, & Guarro, 2010). It was reported that some morphologically similar species differed in physiological features (Madrid, Gené, Cano, Silvera, & Guarro, 2010). For example, the ability to digest raffinose distinguished *Sporothrix brunneoviolacea* and *S. dimorphospora* from *Sporothrix* sp.-1, and *S. inflata* (Madrid, Gené, Cano, Silvera, & Guarro, 2010). But more tests needed to be done to further distinguish *Sporothrix* sp.-1, from *S. inflata*. Madrid et al. concluded that the evolutionary relationships of many

Sporothrix species remained unresolved, and there were certain species that are genetically heterogeneous (Madrid, Gené, Cano, Silvera, & Guarro, 2010).

4.3 The relationship between *Ceratocystiopsis* and *Sporothrix*

The purpose of this study was to identify the fungal species isolated from MPB, with the expectation that most of them would be *Ceratocystiopsis* species based on morphological features. However, only two species were confirmed to be *Ceratocystiopsis* species after the phylogenetic analyses. They were the most closely related to *C. brevicomis*, *C. ranaculosa* and three undefined *Ceratocystiopsis* species: EU 913709, EU 913707, and EU 913708.

Ceratocystiopsis brevicomis was firstly described by P.T.W. Hsiau and T.C. Harrington in 1997. They were originally isolated from *Pinus ponderosa* in California in 1990 and 1991. *Ceratocystiopsis brevicomis* is commonly associated with *Dendroctonus brevicomis* (western pine beetle), and it is similar to *C. ranaculosus* (Perry and Bridges), which is carried by *D. frontalis* (southern pine beetle) (Hsiau & Harrington, 1997). They have similar heterothallic mating system, and they only form perithecia when pairing happens between strains of opposite mating type (Hsiau & Harrington, 1997). *C. brevicomis* has larger perithecia and longer ascospores compared to *C. ranaculosus*.

Some other *Ceratocystiopsis* species has been reported as symbionts with bark beetles, such as *C. minuta* (Zhou, 2004). In Europe, *Ceratocystiopsis minuta* was found associated with *Ips typographus* to infest Norway spruce, and with *Tomicus* species to infest *Pinus* species (Zhou, 2004). In North America, it was commonly found with *Dendroctonus* and *Ips* species to infest different softwood trees (Zhou, 2004). It was also present in the study done in Mexico (Zhou, 2004).

One study in 1980 found that some *Ceratocystiopsis* species (which was formerly known as *Ceratocystis*) were related to *S. schenckii* in some respects (Travassos & Lloyd, 1980). First of all, the main surface antigens of *Sporothrix schenckii* – rhamnomannans were synthesized by many *Ceratocystiopsis* species, which were not often found in pathogenic fungi. Secondly, it concluded that some *Ceratocystiopsis* species had same conidial forms as *Sporothrix*, and some lived in the same habitats as *S. schenckii*, such as soils and plants (Travassos & Lloyd, 1980). Later on, with the development in DNA sequence – based on phylogenetic studies, it was confirmed that the two genera *Ophiostoma* and *Ceratocystis* shared polyphyletic origins (Zipfel et al., 2006).

5.0 Conclusion

This study discovered some new fungal isolates from mountain pine beetle in Canada, dominated by *Sporothrix* species with a few exceptions. Unexpectedly, only two out of the 61 samples investigated turned out to be *Ceratocystiopsis*. Phylogenetic analyses based on β-tubulin gene showed that there were much diversity among the *Sporothrix* species, but many of the species were undefined yet. It also suggested some phylogenetic relationship between the genus *Sporothrix* and *Ceratocystiopsis*. Further research will be needed to distinguish the isolates in species level. In addition, more investigations need to be carried out in order to understand the physiological characteristics of *Sporothrix*, the role it plays in the MPB association and its pathogenicity to host trees.

6.0 Literature Cited

Aghayeva, D. N., Wingfield, Michael J, Beer, Z Wilhelm de, & Kirisits, T. (2004). Two new Ophiostoma species with Sporothrix anamorphs from Austria and Azerbaijan. *Mycologia*, *96*(4), 866-78.
Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/21148906.

Bruns Lab. (2002). Primer Sequences. *University of California at Berkeley*. Retrieved April 17, 2011, from http://plantbio.berkeley.edu/~bruns/tour/primers.html.

Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, Duran C, Field M, Heled J, Kearse M, Markowitz S, Moir R, Stones-Havas S, Sturrock S, Thierer T, Wilson A. (2011). Geneious v5.4, Available from http://www.geneious.com/

Gorton, C. (2004). Phylogenetic analysis of the bluestain fungus Ophiostoma minus based on partial ITS rDNA and β-tubulin gene sequences. *Mycological Research*, *108*(7), 759-765. doi: 10.1017/S0953756204000012.

Hsiau, P. T.-W., & Harrington, T. C. (1997). *Ceratocystiopsis brevicomi* sp. nov., a Mycangial Fungus from Dendroctonus brevicomis (Coleoptera: Scolytidae). *Mycologia*, *89*(4), 661. doi: 10.2307/3761004.

Khadempour L, Massoumi Alamouti S, Hamelin R, Bohlmann J, Breuil C. (2010). Target-specific PCR primers can detect and differentiate ophiostomatoid fungi from microbial communities associated with the mountain pine beetle Dendroctonus ponderosae. Fungal Biology, 114, 825-833.

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*(9), 1177-85. doi: 10.1094/PHYTO-97-9-1177.

Lee S, Kim JJ, Breuil C. (2006). Diversity of fungi associated with the mountain pine beetle, Dendroctonus ponderosae and infested lodgepole pines in British Columbia. Fungal Diversity, 22, 91-105.

Madrid, H., Gené, J., Cano, J., Silvera, C., & Guarro, J. (2010). *Sporothrix brunneoviolacea* and *Sporothrix dimorphospora*, two new members of the Ophiostoma stenoceras-Sporothrix schenckii complex. *Mycologia*, *102*(5), 1193-203. doi: 10.3852/09-320.

Meyer, E. M. de, Beer, Z. W. de, Summerbell, R. C., Moharram, a M., Hoog, G. S. de, Vismer, H. F., et al. (2008). Taxonomy and phylogeny of new wood- and soil-inhabiting Sporothrix species in the Ophiostoma stenoceras-Sporothrix schenckii complex. *Mycologia*, *100*(4), 647-661. doi: 10.3852/07-157R.

Plattner, a, Kim, J.-J., Reid, J., Hausner, G., Lim, Y. W., Yamaoka, Y., et al. (2009). Resolving taxonomic and phylogenetic incongruence within species Ceratocystiopsis minuta. *Mycologia*, *101*(6), 878-887. doi: 10.3852/08-132.

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

Rice AV, Thormann MN, Langor DW. (2007). Virulence of, and interactions among, mountain pine beetle associated blue stained fungi on two pine species and their hybrids in Alberta. Canadian Journal of Botany, 85, 316-323.

Roe AD, Rice AV, Bromilow SE, Cooke J, Sperling F. (2010). Multilocus species identification and fungal DNA barcoding: insights from blue stain fungal symbionts of the mountain pine beetle. Molecular Ecology Resources doi: 10.1111/j.1755-0998.2010.02844.x

Saitou N, Nei M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406-425

Six DL. (2003). A comparison of mycangial and phoretic fungi of individual mountain pine beetles. Canadian Journal of Forest Research, 33, 1331-1334.

Six DL, Klepzig KD. (2004). Dendroctonus bark beetles as model systems for studies on symbiosis. Symbiosis, 37, 207-232.

Solheim H, Krokene P. (1998). Growth and virulence of mountain pine beetle associated blue-stain fungi, Ophiostoma clavigerum and Ophiostoma montium. Canadian Journal of Botany, 76, 561-566.

Tamura K, Dudley J, Nei M & Kumar S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596-1599.

The Government of British Columbia. (2011). Facts About B.C.' s Mountain Pine Beetle, (April), 1-2.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higguns DG. (1997). The CLUSTAL_X Windows Interface: Flexible stratgies for Multiple Sequene Alignment Aided by Quality Analysis Tools. Nucleic Acids Res 24: 4876-4882.

Travassos, L. R., & Lloyd, K. O. (1980). Sporothrix schenckii and related species of Ceratocystis. *Microbiological reviews*, *44*(4), 683-721. Retrieved from

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=373199&tool=pmcentrez&rendertype=abstract

Tsui, C. K. M., Wang, B., Khadempour, L., Alamouti, S. M., Bohlmann, J., Murray, B. W., et al. (2010). Rapid identification and detection of pine pathogenic fungi associated with mountain pine beetles by padlock probes. *Journal of microbiological methods*, *83*(1), 26-33. Elsevier B.V. doi: 10.1016/j.mimet.2010.07.016.

White TJ, Bruns T, Lee S, Taylor J, (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Shinsky JJ, White TJ, editors. *PCR Protocols: A Guide to Methods and Applications*, 315–322. Academic Press, San Diego.

Zhou, X. (2004). Characterisation of Ophiostoma species associated with pine bark beetles from Mexico, including O. pulvinisporum sp. nov. *Mycological Research*, *108*(6), 690-698. doi: 10.1017/S0953756204009918.

Zipfel, R. D., Beer, Z. W. D., Jacobs, K., Wingfield, B. D., Wingfield, Michael J, Luttrell, M., et al. (2006). Multi-gene phylogenies define Ceratocystiopsis and Grosmannia distinct from Ophiostoma. *DNA Sequence*, 75-97.