Fungal associates of the lodgepole pine beetle, *Dendroctonus murrayanae*

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Abstract

Bark beetles are well known vectors of ophiostomatoid fungi including species of *Ophiostoma*, *Grosmannia* and *Ceratocystis*. In this study, the most common ophiostomatoid fungi associated with the lodgepole pine beetle, *Dendroctonus murrayanae*, were characterized. Pre-emergent and post-attack adult beetles were collected from lodgepole pines at four sites in British Columbia, Canada. Fungi were isolated from these beetles and identified using a combination of morphology and DNA sequence comparisons of five gene regions. In all four populations, *Grosmannia aurea* was the most common associate (74–100% of all beetles) followed closely by *Ophiostoma abietinum* (29–75%). Other fungi isolated, in order of their relative prevalence with individual beetles were an undescribed *Leptographium* sp. (0–13%), *Ophiostoma ips* (0–15%), *Ophiostoma piliferum* (0–11%), a *Pesotum* sp. (0–11%) and *Ophiostoma floccosum* (0–1%). Comparisons of the DNA sequences of *Leptographium* strains isolated in this study, with ex-type isolates of *G. aurea*, *Grosmannia robusta*, *Leptographium longiclavatum*, and *Leptographium terebrantis*, as well as with sequences from GenBank, revealed a novel lineage within the *Grosmannia clavigera* complex. This lineage included some of the *D. murrayane* isolates as well as several isolates from previous studies referred to as *L. terebrantis*. However, the monophyly of this lineage is not well supported and a more comprehensive study will be needed to resolve its taxonomic status as one or more novel taxa.

Introduction

Dendroctonus is one of the best studied genera of bark beetles and contains nineteen described species (Wood 1982). All of these species appear to be associated with fungi (Six 2003; Six and Klepzig 2004). These fungi are important agents of sapstain, many are pathogens although few are virulent to the host tree, and some are nutritional mutualists with their host beetles (Six and Wingfield 2011). Approximately one-third of *Dendroctonus* species are capable of eruptive population growth, resulting in either short- or long-term outbreaks that can cause extensive mortality of host trees, over large areas. These tree-killing beetles are considered among the most

important pests of conifers in North America (Furniss and Carolin 1977). The remaining species are less aggressive and are seldom considered for management. Of the less aggressive species, some also kill trees but are typically limited to dying, damaged or stressed hosts and do not develop extensive outbreaks. Several others are true parasites capable of producing brood in living trees and only kill trees when present in uncommonly high numbers.

The fungal associates of many of the economically important aggressive tree-killing *Dendroctonus* species have been relatively well characterized (reviewed in Six and Klepzig 2004). In contrast, the associates of most of the less aggressive, and especially the parasitic *Dendroctonus* species, are poorly known. This considerable gap in knowledge of the fungal associates of many *Dendroctonus* severely hampers our ability to develop and test appropriate hypotheses regarding the roles of the fungi with their beetle hosts.

One parasitic species of *Dendroctonus* for which fungal associates have not been described is the lodgepole pine beetle, *Dendroctonus murrayanae* Hopkins. This insect primarily colonizes *Pinus contorta* Dougl. ex Loud., although it has also been observed in *P. banksiana* Lamb. and *P. strobus* L. (Wood 1982; Furniss and Kegley 2008). Its geographic distribution extends from British Columbia to Ontario in Canada (Bright 1976) and south into Idaho, Montana, Utah, Wyoming, Colorado and Michigan in the United States (Wood 1982). *Dendroctonus murrayanae* is naturally rare and its numbers seldom reach levels that result in tree death (Safranyik *et al*. 1999, 2004). Usually only one pair, or less commonly, a few pairs of beetles, colonize an individual tree (Furniss and Kegley 2008).

Colonizing adult *D. murrayanae* mine the lower bole and root collars of mature healthy, injured, or weakened trees, and fresh stumps and windfall (Wood 1982; Safranyik *et al*. 1999). The female constructs an irregular vertical gallery under the outer bark and lays eggs in groups along the sides of the gallery. The larvae feed gregariously in a common excavation or brood chamber in the phloem layer between the outer bark and sapwood. Pupation and transformation to the adult stage takes place in the frass-filled brood chamber. One generation a year is apparently typical (Wood 1982; Furniss and Kegley 2008).

Phloem and sapwood surrounding successful galleries and brood chambers are usually stained dark blue or black. This is characteristic of colonization by a number of ophiostomatoid fungi (Seifert 1993) indicating that these beetles, like many other bark beetles, are likely to possess fungal symbionts. The objective of this study was to isolate and identify the most common and consistent fungal associates of *D. murrayanae*, and therefore, those most likely to be symbiotic.

Materials and methods

Collection of beetles and isolation of fungi

Adult *D. murrayane* were collected from *P. contorta* at four locations in British Columbia, Canada, in June 2004 (Table 1). At two sites, mature brood adults were collected just prior to emergence and dispersal. At the other two sites, collections were of adult beetles that had already emerged, dispersed, and colonized new trees (within 1 week of attack). Collections were made from greater than ten trees at each site except at Angstad Creek, where only two trees were located that contained live beetles. Brood adults were collected from the frass-filled communal brood/pupation chambers. Dispersed adults were collected from new gallery excavations under the bark. No more than two beetles were taken from any one gallery system. At Angstad Creek, all beetles were taken from different galleries. Live beetles were placed into individual vials containing small strips of moist paper towel and then placed onto ice and returned to the laboratory for isolation of fungi.

Table 1. The four sites in British Columbia and their location, where *D. murrayanae* beetles were collected from under bark of *P. contorta*.

Site	Dee Lake	Angstad Creek	West Lake	McCleod Lake	Total
Nearest landmark town	Winfield	Merritt	Prince George	Mackenzie	
Latitude	50°06′26″	49°50′23″	53°42′45″	54°54′08″	
Longitude	119°10′01″	120°45′57″	122°52′50″	122°55′18″	
Beetle stage	Brood adult	Brood adult	Dispersed adult	Dispersed adult	
Number of beetles	24	4	22	27	77
Number of beetles from which isolates came	24	4	21	27	76
G. aurea	24 (100)	3 (75)	20 (95.2)	20 (74.1)	67 (88.2)
O. abietinum	17 (70.8)	3 (75)	6 (28.6)	12 (44.4)	38 (50)
Leptographium sp. X	0 (0)	1 (25)	5 (23.8)	7 (25.9)	13 (17.1)
O. ips	0 (0)	0 (0)	1 (4.8)	4 (14.8)	5 (6.6)
Pesotum sp.	0 (0)	0 (0)	0 (0)	3 (11.1)	3 (4)
O. piliferum	0 (0)	0 (0)	0 (0)	3 (11.1)	3 (4)
O. flocossum	0 (0)	0 (0)	0 (0)	1 (3.7)	1 (1.3)

The numbers of isolates per fungal species obtained are listed, with in *parentheses* the percentage each number represents of the total number of isolates collected

Fungi were isolated by either streaking or squashing individual beetles onto the surface of 2% malt extract agar (MEA). Initial isolation cultures were incubated at approximately 22°C for at least 10 days. Sub-cultures were then made of each morphologically distinct fungus growing in each initial isolation plate.

Identification of fungi

Morphology

Isolates were tentatively identified using morphological characters (Upadhyay 1981; Grylls and Seifert 1993; Jacobs and Wingfield 2001). For each morphological group, characteristic isolates were selected for DNA sequencing to confirm identifications. Representative isolates collected in this study have been deposited in the culture collections of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (CMW), the senior author at The University of Montana, Missoula, Montana, USA (DLS), and at the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands (Table 2).

Reference isolates

The morphology of the majority of isolates obtained in the present study resembled species in the *Grosmannia clavigera* complex. Fresh cultures of the ex-type and authentic isolates of *Grosmannia aurea*, *Leptographium terebrantis* and *L. longiclavatum* were obtained for references purposes from the CBS, the American Type Culture Collection (ATCC), Manassas, Virginia, USA, and the Mycothèque de l'Université catholique de Louvain (MUCL), Louvain-la-Neuve, Belgium (Online resources 1).

DNA extraction, PCR, and sequencing

To verify identifications based on morphology, DNA sequences were determined for representative isolates from each morphological group (Table 2), as well as the reference strains (Table 3). DNA extractions were performed from single spore isolates as described by Six *et al*. (2009). A fragment of the β-tubulin (βT) gene was amplified and sequenced for all selected isolates. In addition to the βT gene, four more gene regions were sequenced for the *Grosmannia* and *Leptographium* isolates.

These included the internal transcribed spacer 2 (ITS2) and partial large subunit (LSU) of the ribosomal RNA operon, the partial elongation factor 1α (EF-1α), partial actin, and an anonymous nuclear locus (UFM) used successfully by Roe *et al*. (2010) to distinguish between species in the *G. clavigera* complex.

Table 2. Culture collection and GenBank accession numbers for strains of fungi isolated from *D. murrayanae* that were sequenced in this study.

Species	Culture collection[a]			GenBank accession number									
	CMW	DLS	CBS	ITS2–LSU	HT[b]	βT	HT	EF-1α	HT	Actin	HT	Anon. locus	HT
Grosmannia aurea	15471					=DQ865286	AB1						
	15472	1205	121086			DQ865286	AB1						
	15474					=DQ865286	AB1						
	15475					=DQ865286	AB1						
	15478					=DQ865286	AB1						
	15482					=DQ865286	AB1						
	15483					=JF798455	AB4						
	15487					=DQ865286	AB1						
	15489					=DQ865286	AB1						
	15494					=DQ865286	AB1						
	15495					=DQ865286	AB1						
	15496			JF798474	AI1	=DQ865286	AB1	=JF798462	AE1			=JF798487	AU2
	15501			JF798475	AI2	JF798455	AB4	JF798461	AE2	JF798480	AA1	JF798486	AU1
	15504					=DQ865286	AB1	=JF798462	AE1	=JF798480	AA1	=JF798487	AU2
	15809			=JF798474	AI1	=DQ865286	AB1	JF798462	AE1	=JF798480	AA1	JF798487	AU2
	15811			=JF798475	AI2	=DQ865286	AB1	=JF798461	AE2	=JF798480	AA1	=JF798487	AU2
	15813			=JF798475	AI2	=DQ865286	AB1	=JF798461	AE2	=JF798480	AA1	=JF798487	AU2
	15818					=DQ865286	AB1	=JF798461	AE2	=JF798480	AA1	=JF798487	AU2
	15901					=JF798455	AB4	=JF798461	AE2	=JF798480	AA1	=JF798487	AU2
Leptographium sp. X	15457	1190		=JF798478	TI2	=DQ865285	TB1	=JF798472	TE2	=JF798482	TA2	=JF798489	TU1
	15470	1203	121089	JF798478	TI2	DQ865285	TB1	JF798472	TE2	JF798481	TA8	JF798488	TU2
	15493	1226		=JF798478	TI2	JF798456	TB4	=JF798472	TE2	JF798482	TA2	JF798489	TU1
	15502	1235		=JF798478	TI2	=JF798456	TB4	=JF798472	TE2	=JF798482	TA2	=JF798489	TU1
O. abietinum	15500	1233				=DQ865287							
	23436	1340	121088			DQ865287							
O. floccosum	23437	1341				DQ865289							
O. ips		1339	121087			DQ865284							
O. piliferum	15464	1197	121091			DQ865288							

[a] *CMW* culture collection of the FABI at the University of Pretoria, South Africa, *DLS* culture collection of D. Six at the University of Montana, USA, *CBS* Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands

[b] Each unique haplotype (HT) was assigned a number following the system of Roe et al. (2010). E.g. *AI1 G. aurea* ITS haplotype 1, and *AB4 G. aurea* βT haplotype 4, etc

Table 3. Data pertaining to DNA sequence data and phylogenetic analyses representing different loci.

Data set	Number of taxa	Number of char	Outgroup	MP		ML				MrBayes
				PIC	Number of trees	Subst. model	Pinvar	G	Nst	Burn-in
Grosmannia/Leptographium										
ITS2–LSU	99	363	*Ophiostoma* spp.	171	160	TrN+G	–	0.23	6	150
Combined 5 gene regions	62	2573	Midpoint rooted	78	28	TrN+I+G	0.31	0.01	6	100
Ophiostoma										
βT (*O. piliferum* group)	63	310	Midpoint rooted	104	45	GTR+G	–	0.174	6	150
βT (*O. stenoceras* group)	57	271	Midpoint rooted	121	24	HKY+G	–	0.187	4	350

Char characters, *PIC* number of parsimony informative characters, *Subst. model* best fit substitution model, *Pinvar* proportion of invariable sites, *G* gamma shape parameter, *Nst* number of substitution rate categories

The following primer combinations were used for amplification of the respective gene regions: T10 (O'Donnell and Cigelnik 1997) or Bt2a in combination with Bt2b (Glass and Donaldson 1995) to amplify the βT gene, ITS3 (White et al. 1990) and LR5 (Vilgalys and Hester 1990) for the ITS2–LSU fragment, EF1-728F (Carbone and Kohn 1999) or EF1F together with EF2R (Jacobs *et al*. 2004) for the EF-1α gene, Lepact F and Lepact R (Lim *et al*. 2004) for the actin gene, and UFM1_F

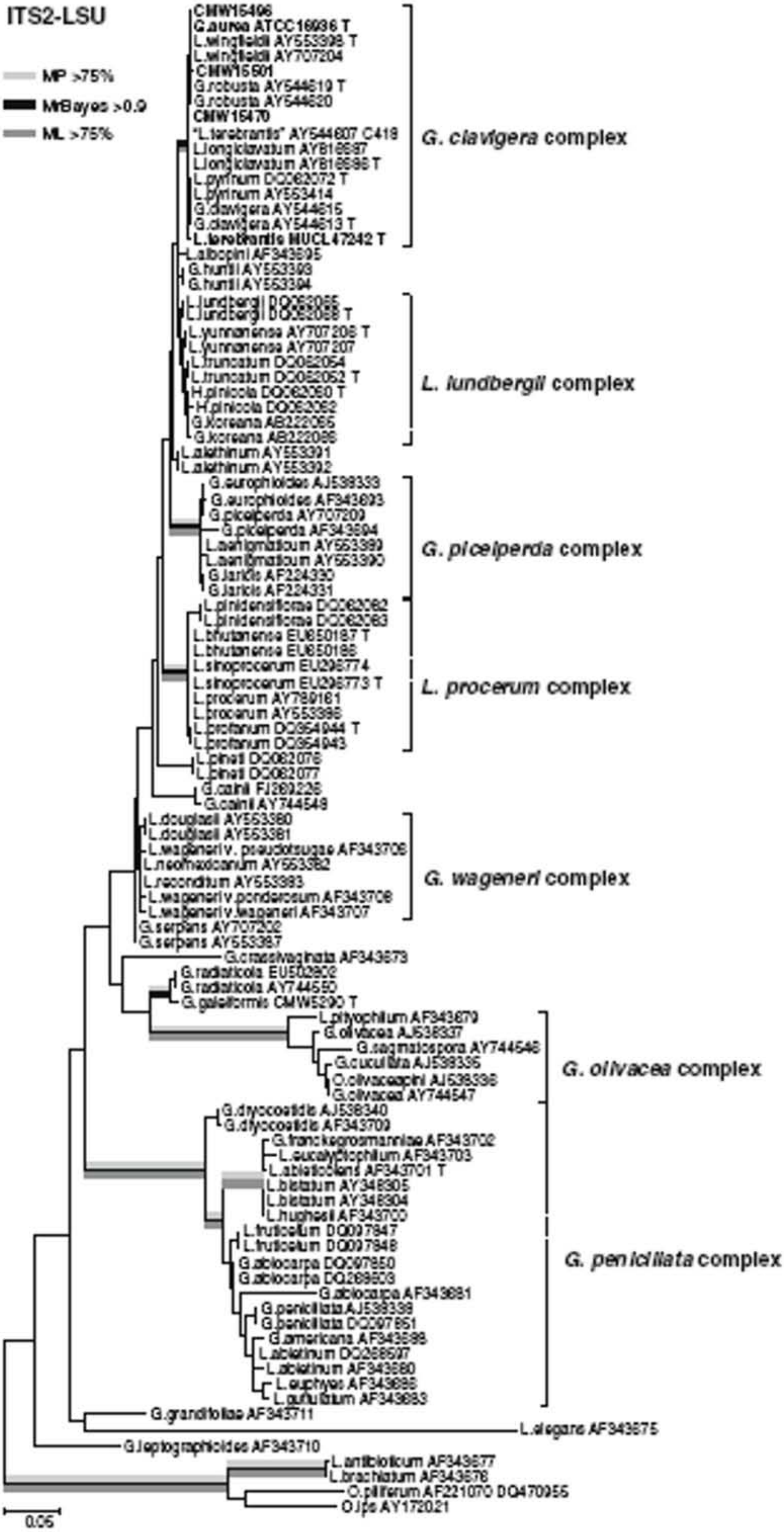
and UFM1_R (Roe *et al*. 2010) for the anonymous locus. Each PCR reaction mixture (25 µl total volume) consisted of 16.3 µl ultra-pure water (Adcock Ingram, Johannesburg, South Africa), 2.5 µl 10× buffer (Roche, Basel, Switzerland), 0.5 µl $MgCl_2$ (25 mM) (Roche), 2.5 µl dNTPs (2 mM each) (Fermentas, Burlington, Canada), 0.5 µl of each primer (10 µM), 2 µl of the DNA extract and 0.2 Faststart *Taq* polymerase (Roche). The ITS2–LSU region of some *Leptographium* isolates was GC-rich and 5 µl of the water in the reaction mixture was replaced with 5 µl 5 × GC solution (Roche). PCR conditions were: one cycle of denaturation at 96°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 52–58°C for 30 s, and extension at 72°C for 1 min, and one final cycle of extension at 72°C for 8 min.

PCR products were purified using a High Pure PCR Product Purification Kit (Roche). The purified PCR fragments were sequenced using the primers noted above and the Big Dye™ Terminator v.3.1 cycle sequencing premix kit (Applied Biosystems, Carlsbad, USA). Sequencing was performed on an ABI 3130X2 automated sequencer (Applied Biosystems). Consensus sequences were assembled from forward and reverse sequences using ContigExpress (a component of Vector NTI Advance™ 11, Invitrogen Corporation, Carlsbad, USA). All sequences obtained in this study were deposited in GenBank (Tables 2, 3).

Phylogenetic analyses

DNA sequence data sets were compiled using MEGA 4.0.2 (Tamura *et al*. 2007). An ITS2–LSU data set was compiled including one representative sequence (where possible that of the ex-type isolate) for each *Grosmannia* and *Leptographium* species (Fig. 1), to determine to which species complex the *Grosmannia*/*Leptographium*-like isolates obtained in this study, belonged. Based on these results, five separate data sets for the five gene regions were compiled for closely related species in the *G. clavigera* complex. These included all sequences for each gene region listed in Tables 2 and 3 and the data sets were analyzed separately (data not shown). The resulting data sets and phylogenetic trees were inspected carefully and anomalies were noted. For each locus different haplotypes were identified and labelled following the system used by Roe et al. (2010) (Tables 2, 3). The first letter of each three-digit label represented the species name (A for *aurea*, C for *clavigera*, etc.), the second letter the locus/gene region (B for βT, A for actin, etc.), and the third digit was the number assigned to each unique haplotype for that locus. E.g. actin sequences obtained from *G. clavigera* isolates belonged to one of three haplotypes, labeled as CA1, CA2 and CA3. A combined data set consisting of all five gene regions were compiled including only isolates for which data for all gene regions were available. The βT sequence data of *Ophiostoma*, *Pesotum* and *Sporothrix* isolates were separated into two data sets based on the presence or absence of βT introns.

Data sets were aligned separately from each other in the online version of MAFFT 6 using the E-INS-i strategy (Katoh and Toh 2008). Each of the data sets, including the combined data set, was subjected to three different analyses. Maximum parsimony (MP) analyses were done using MEGA 4.0.2 (Tamura *et al*. 2007). Maximum likelihood (ML) were conducted using PhyML 3.0 (Guindon and Gascuel 2003) and Bayesian inference (BI) in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003), employing the most appropriate substitution model for each data set selected with jModelTest 0.1 (Posada 2008). Node support for ML and MP trees was determined using 1,000 bootstrap replicates. For BI MCMC (Markov Chain Monte Carlo) chains were run for 5 million generations. Burn-in values were calculated using Tracer 1.4 (http://beast.bio.ed.ac.uk/Tracer). Phylogenetic trees were viewed and edited in MEGA 4.0.2 (Tamura *et al*. 2007).



ITS2-LSU
MP >75%
MrBayes >0.9
ML >75%
CMW15496
G.aurea ATCC16936 T
L.wingfieldii AY553398 T
L.wingfieldii AY707204
CMW15501
G.robusta AY544619 T
G.robusta AY544620
CMW15470
"L.terebrantis" AY544607 C418
L.longiclavatum AY816687
L.longiclavatum AY816686 T
L.pyrinum DQ062072 T
L.pyrinum AY553414
G.clavigera AY544615
G.clavigera AY544613 T
L.terebrantis MUCL47242 T
G. clavigera complex
L.albopini AF343695
G.huntii AY553393
G.huntii AY553394
L.lundbergii DQ062065
L.lundbergii DQ062068 T
L.yunnanense AY707206 T
L.yunnanense AY707207
L.truncatum DQ062054
L.truncatum DQ062052 T
H.pinicola DQ062060 T
H.pinicola DQ062062
G.koreana AB222065
G.koreana AB222066
L. lundbergii complex
L.aletinum AY553391
L.aletinum AY553392
G.europhioides AJ538333
G.europhioides AF343693
G.piceiperda AY707200
G.piceiperda AF343694
L.aenigmaticum AY553389
L.aenigmaticum AY553390
G.laricis AF224330
G.laricis AF224331
G. piceiperda complex
L.pinidensiflorae DQ062082
L.pinidensiflorae DQ062083
L.bhutanense EU650187 T
L.bhutanense EU650188
L.sinoprocerum EU296774
L.sinoprocerum EU296773 T
L.procerum AY789161
L.procerum AY553396
L.profanum DQ354944 T
L.profanum DQ354943
L. procerum complex
L.pineti DQ062076
L.pineti DQ062077
G.cainii FJ269226
G.cainii AY744549
L.douglasii AY553380
L.douglasii AY553381
L.wageneri v. pseudotsugae AF343706
L.neomexicanum AY553382
L.reconditum AY553383
L.wageneri v. ponderosum AF343708
L.wageneri v. wageneri AF343707
G. wageneri complex
G.serpens AY707202
G.serpens AY553387
G.crassivaginata AF343673
G.radiaticola EU502802
G.radiaticola AY744550
G.galeiformis CMW5290 T
L.pityophilum AF343679
G.olivacea AJ538337
G.sagmatospora AY744546
G.cucullata AJ538335
O.olivaceapini AJ538336
G.olivacea AY744547
G. olivacea complex
G.dryocoetidis AJ538340
G.dryocoetidis AF343709
G.francke-grosmanniae AF343702
L.eucalyptophilum AF343703
L.abietocolens AF343701 T
L.bistatum AY348305
L.bistatum AY348304
L.hughesii AF343700
L.fruticetum DQ097847
L.fruticetum DQ097848
G.abiocarpa DQ097850
G.abiocarpa DQ268603
G.abiocarpa AF343681
G.penicillata AJ538339
G.penicillata DQ097851
G.americana AF343686
L.abietinum DQ268597
L.abietinum AF343680
L.euphyes AF343686
L.guttulatum AF343683
G. penicillata complex
G.grandifoliae AF343711
L.elegans AF343675
G.leptographioides AF343710
L.antibioticum AF343677
L.brachiatum AF343676
O.piliferum AF221070 DQ470956
O.ips AY172021
0.05

Figure 1. Phylogram resulting from a ML analysis of the partial ITS2 and LSU sequences of selected *Grosmannia* and *Leptographium* species. Bootstrap values obtained from MP and posterior probabilities of BI are presented as indicated. Isolate numbers of sequences produced in the present study are printed in bold type. T sequence of ex-type isolates

Results

Collection of beetles and isolation of fungi

A total number of 77 adult *D. murrayane* beetles were collected from the four sites, and from 76 of these beetles fungi were successfully isolated (Table 1). A total number of 130 ophiostomatoid isolates were obtained. Ubiquitous saprophytic fungi, including *Penicillium* spp., were also isolated from some beetles or were present as contaminants in the cultures. These were not common and were not considered further.

Identification of fungi

Morphology

The ophiostomatoid isolates could be separated into seven morphological groups, of which representatives were selected for DNA sequencing. Two of the species presented *Leptographium* anamorphs in culture, typical of those associated with *Grosmannia* spp. The other cultures all formed anamorph structures typically associated with *Ophiostoma* spp. Two of these groups formed *Sporothrix* anamorphs and one group sparse *Hyalorhinocladiella*-like structures in culture, while a single isolate produced *Pesotum*-like synnemata.

Selection of reference isolates and sequences

For reference purposes, published sequences obtained from GenBank had to be included in the phylogenetic analyses. Genbank sequences for the *Ophiostoma* species did not present problems. However, for the species in the *G. clavigera* complex there were several ambiguities between GenBank sequences of the same isolates determined in different studies, some resulting from misidentified isolates, culture or DNA contaminants, and others probably from errors in the sequencing process. We carefully compared all of these sequences within large phylogenies of all five gene regions (results not shown) including all available sequence data for the known species in the complex. Included were also the sequences we determined in the present study for the authentic isolates of *G. aurea*, *G. robusta*, *L. terebrantis* and *L. longiclavatum* (Online resources 1). Based on these data sets only the most reliable reference sequences were selected to be included in phylogenetic analyses with our isolates from *D. murrayane*. The selection of sequences was done as explained in Online Resource 1.

DNA sequencing

DNA sequence comparisons confirmed that the six morphological groups of isolates from *D. murrayanae* represented distinct taxa in the Ophiostomatales. We could not successfully amplify DNA for the seventh morphological group with the *Pesotum* anamorph, and thus did not obtain sequence data for that species. Two of the most dominant species grouped within the genera *Grosmannia* and *Leptographium*, while the remainder of the species grouped within *Ophiostoma* sensu (Zipfel *et al*. 2006).

Phylogenetic analyses of the ITS2–LSU sequences for the *Grosmannia* and *Leptographium* species showed that isolates of both taxa obtained in the present study grouped in the G. clavigera species

complex (Fig. 1). Results of the MP, ML and BI inferences analyses are given in Table 3. The topologies of the resulting trees were congruent and reflected by the ML tree (Fig. 1).

Analyses of the combined data set that included ITS–LSU, βT, EF-1α, actin and the anonymous locus (from Roe *et al*. 2010), revealed that the majority of isolates obtained in the present study, grouped in a strongly supported clade (Fig. 2) that included the ex-type of *G. aurea*. Other known species that were represented by strongly supported clades were *G. clavigera*, *L. wingfieldii*, *G. robusta*, and *L. longiclavatum* (Fig. 2). The ex-type isolate of *L. terebrantis*, and one other isolate of this species formed a well-supported clades very distinct from all the other species (Fig. 2). The ex-type isolate of *L. terebrantis* obtained from CBS and MUCL had identical sequences for all gene regions (Online resources 1) and thus only the data for the CBS isolate were included to represent this species in the analyses.

The second group of *Leptographium*-like isolates from *D. murrayane* grouped in all analyses of the separate βT, EF-1α and Actin gene regions (data not shown) among several isolates labeled as 'L. terebrantis' in several previous studies (Six *et al*. 2003; Lee *et al*. 2003, 2005; Lim *et al*. 2004; Kim *et al*. 2005; Roe *et al*. 2010). In the combined analyses, our isolates also grouped among the so-called '*L. terebrantis*' isolates from Roe *et al*. (2010). Sequences for all four genes of these '*L. terebrantis*' isolates were however distinctly different from those of the ex-type of *L. terebrantis*. Furthermore, these isolates showed a high level of variability. Although they grouped in a seemingly monophyletic lineage in the combined tree (Fig. 2), this lineage did not have any statistical support in the separate (data not shown) nor combined analyses (Fig. 2). We labeled this taxon as *Leptographium* sp. X (Table 2; Fig. 2).

βT sequences of three of the *Ophiostoma* species obtained in the present study all contained introns 3 and 4, but no intron 5. This is characteristic of the lineage containing the type species for the genus, *O. piliferum* and other well-known species such as *O. piceae* (Zipfel *et al*. 2006). The one group produced DNA fragments of 289 bp in size, and fragments of both the other groups were 277 bp. After alignment with similar species the data set consisted of 310 characters (Table 3). Based on our analyses the isolates from *D. murrayane* grouped respectively, with isolates of *O. piliferum*, *O. floccosum* and *O. ips* (Fig. 3).

The group of isolates producing *Sporothrix* anamorphs in culture, presented βT sequences (231 bp in length) that included introns 3 and 5, but no intron 4. This is characteristic of the lineage within *Ophiostoma* containing *S. schenckii* and *O. stenoceras* (Zipfel *et al*. 2006). The isolate from *D. murrayane* (DLS1340) grouped with isolates closely resembling the ex-type of *Ophiostoma abietinum* (Fig. 4) that came from previous studies in Canada and the USA (Aghayeva *et al*. 2004; Kim *et al*. 2005).

Isolation frequencies

At all four sites where *D. murrayanae* was sampled, *G. aurea* was the most commonly isolated fungus (74–100%) (Table 1). The next most commonly isolated species was *O. abietinum* (29–75%). Other associates in order of their relative prevalence were the unkown *Leptographium* sp. X (0–13%), *O. ips* (0–15%), *O. piliferum* (0–11%), the unknown *Pesotum* sp. (0–11%), and *O. floccosum* (0–1%). More than one fungus was isolated from 41% of beetles collected at West Lake, 71% at Dee Lake, 78% at McCleod Lake, and 100% of beetles collected at Angstad Creek. The total number of species isolated within a site ranged from seven (McCleod Lake) to two (Dee Lake) (Table 1). Only *G. aurea* and *O. abietinum* were isolated from beetles at all sites. No statistical comparisons were made comparing fungal prevalence between dispersed and brood adults because of unequal samples sizes and because of the potential for confounding site effects.

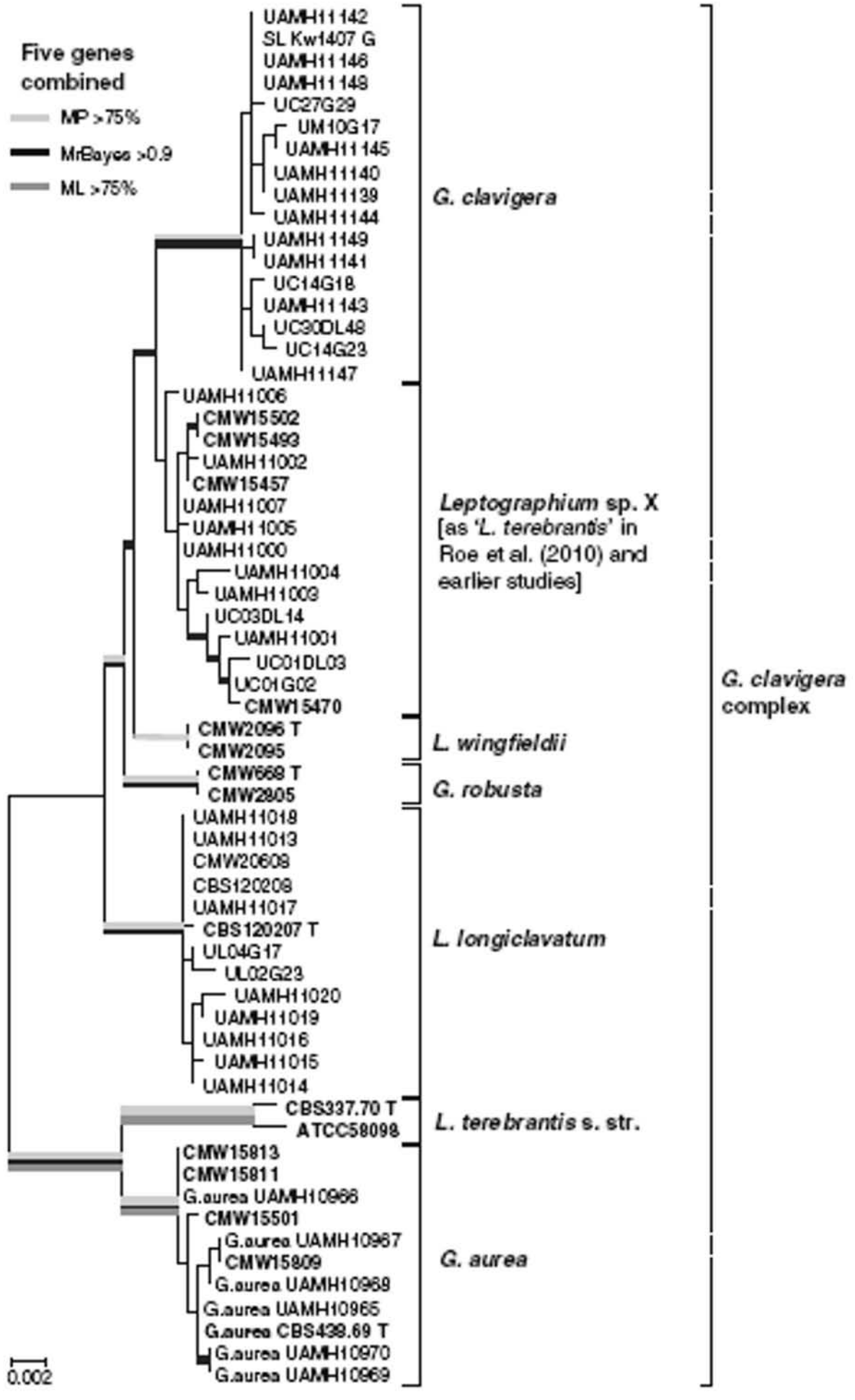

Five genes combined
MP >75%
MrBayes >0.9
ML >75%
UAMH11142
SL Kw1407 G
UAMH11146
UAMH11148
UC27G29
UM10G17
UAMH11145
UAMH11140
UAMH11139
UAMH11144
UAMH11149
UAMH11141
UC14G18
UAMH11143
UC30DL48
UC14G23
UAMH11147
G. clavigera
UAMH11006
CMW15502
CMW15493
UAMH11002
CMW15457
UAMH11007
UAMH11005
UAMH11000
UAMH11004
UAMH11003
UC03DL14
UAMH11001
UC01DL03
UC01G02
CMW15470
Leptographium sp. X [as 'L. terebrantis' in Roe et al. (2010) and earlier studies]
CMW2096 T
CMW2095
L. wingfieldii
CMW668 T
CMW2805
G. robusta
UAMH11018
UAMH11013
CMW20608
CBS120208
UAMH11017
CBS120207 T
UL04G17
UL02G23
UAMH11020
UAMH11019
UAMH11016
UAMH11015
UAMH11014
L. longiclavatum
CBS337.70 T
ATCC58098
L. terebrantis s. str.
CMW15813
CMW15811
G.aurea UAMH10966
CMW15501
G.aurea UAMH10967
CMW15809
G.aurea UAMH10968
G.aurea UAMH10965
G.aurea CBS438.69 T
G.aurea UAMH10970
G.aurea UAMH10969
G. aurea
G. clavigera complex
0.002

Figure 2. Phylogram resulting from a ML analysis of a combined data set consisting of sequences of five gene regions for species in the *G. clavigera* species complex. The gene regions included were: ITS2–LSU (566 bp), βT (357 bp), EF-1α (516 bp), actin (741 bp), and an anonymous locus (393 bp). Bootstrap values obtained from MP and posterior probabilities of BI are presented as indicated. Isolate numbers of sequences produced in the present study are printed in bold type. T sequence of ex-type isolates

Discussion

Results of this study revealed that some relatively well-known *Ophiostoma* and *Grosmannia* species are associated with *D. murrayanae*. *Grosmannia aurea* was the fungus most commonly associated with this bark beetle. This fungus was originally described as *Europhium aureum* R.C. Rob. and R.W. Davidson from two isolates taken from bark beetle-infested trees. One of these was from *P. contorta* attacked by *Dendroctonus* in British Columbia, and another the other was from an unidentified pine containing unidentified beetles in Wyoming (Robinson-Jeffrey and Davidson 1968). Recently, the fungus was placed in the genus *Grosmannia* based on phylogeny and the presence of a *Leptographium* anamorph (Zipfel *et al*. 2006).

There have been few contemporary reports of *G. aurea* and little is known of its association with insects. Harrington (1988) reported isolations of this fungus from *Hylurgops porosus* (LeConte). However, our results suggest that *G. aurea* is symbiotic with *D. murrayanae*. *Hylurgops porosus* and *D. murrayanae* commonly occur together in the same tree, often with galleries constructed in close proximity to one another (DLS Pers. Observation, Furniss and Kegley 2008). Adult *H. porosus* have even been observed in *D. murrayane* brood/pupal chambers (D.L. Six and A.L. Carroll, pers. observ.). However, we consistently isolated *G. aurea* from *D. murrayanae* that were not adjacent to *H. porosus* galleries, indicating that this fungus is associated with *D. murrayanae*, regardless of whether *H. porosus* is present. Systematic isolations of fungi from *H. porosus* would help to reveal whether both beetles are commonly associated with this fungus, or if *H. porosus* is only incidentally associated with *G. aurea* when its galleries abut or overlap *D. murrayanae* galleries. While common with *D. murrayanae*, G. aurea has also been found with *D. ponderosae* (Roe *et al*. 2010). *Dendroctonus murrayanae* and *D. ponderosae* occasionally cohabit the same tree but it is unknown if the isolation of *G. aurea* from *D. ponderosae* was due to such co-occurrences of the two beetle species or if *G. aurea* has a broader distribution of insect hosts than *D. murrayanae*.

The second most prevalent species found with *D. murrayane* has βT sequences that matched a sequence in GenBank for an undescribed *Sporothrix* sp. (CMW 1468) collected in Canada from *D. ponderosae* Hopkins (Fig. 4). This sequence came from a study by Aghayeva *et al*. (2004). The ITS sequence of this isolate grouped in an earlier study, close to that of the ex-type isolate of *O. abietinum* (De Beer et al. 2003), but in that study both isolates were erroneously labeled as *O. nigrocarpum* (Davidson) de Hoog. Authentic isolates of *O. nigrocarpum*, including the ex-type, are only distantly related to these isolates (Fig. 2). In this study, we determined the βT sequence for the ex-type isolate of *O. abietinum*, and although slight variations exist between the sequence of this isolate and similar ones from previous studies (Aghayeva *et al*. 2004; Kim *et al*. 2005), we believe that treating them all as *O. abietinum* is most appropriate for the time being (Fig. 2). *Ophiostoma abietinum* was the second most commonly isolated fungus from *D. murrayanae* in this study, being isolated from approximately 50% of beetles overall. This fungus, along with *G. aurea*, is clearly symbiotic with the beetle. However, *O. abietinum* has been isolated from wood (*Pinus* and *Abies*) and various conifer-infesting bark beetles in Canada, the USA, Mexico, New Zealand, Korea and South Africa (reported as *O. abietinum* in Marmolejo and Butin 1990, and Zhou *et al*. 2006; as *O. nigrocarpum* in De Beer *et al*. 2003, and Kim et al. 2005; as '*Sporothrix* sp.' in Aghayeva *et al*. 2004). It is thus not restricted to association with *D. murrayanae*, which occurs only in Canada and the northern USA and infests mainly *P. contorta* and to a lesser degree, a couple of other *Pinus*

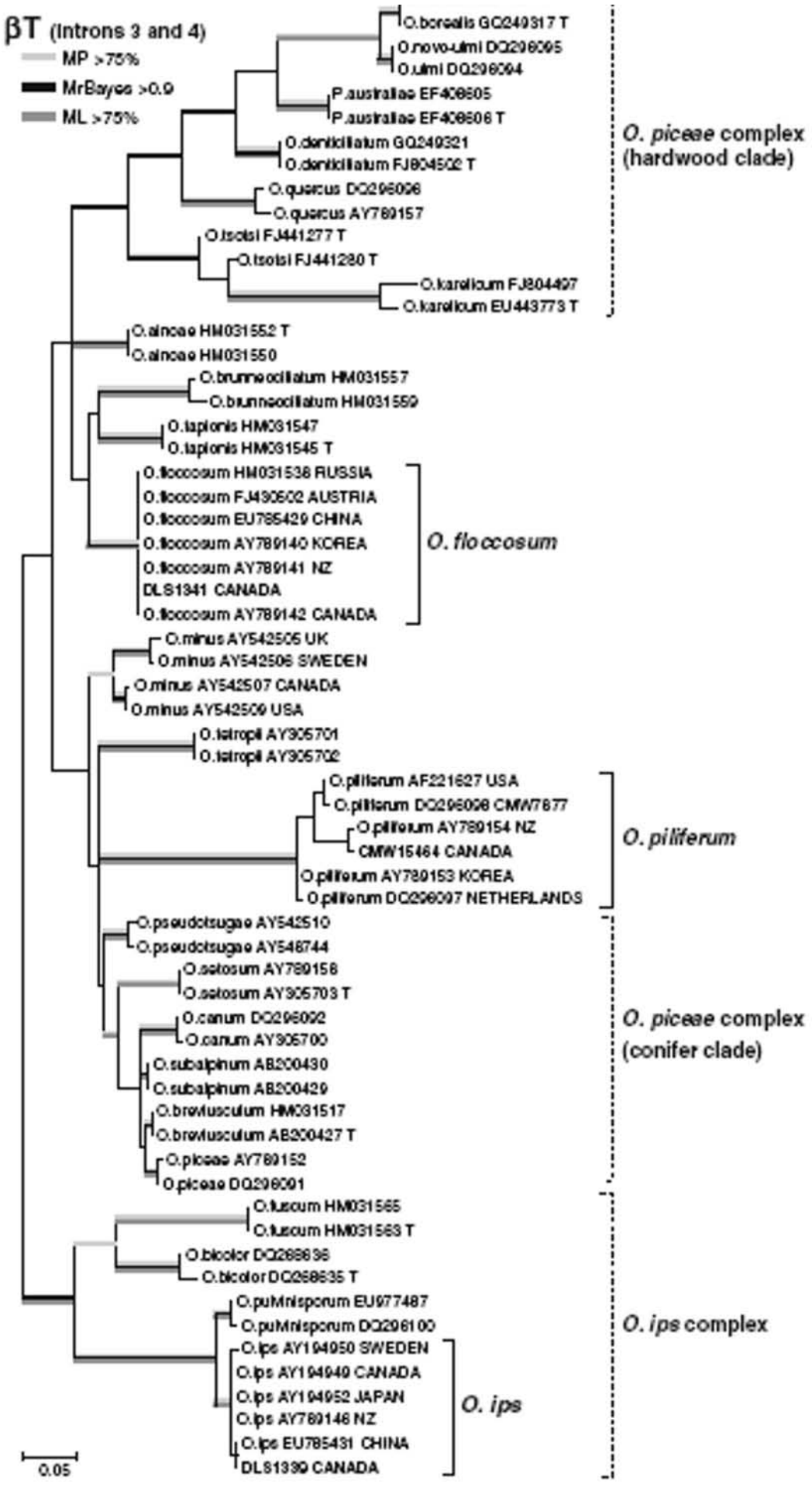

βT (Introns 3 and 4)
MP >75%
MrBayes >0.9
ML >75%
O.borealis GQ249317 T
O.novo-ulmi DQ296095
O.ulmi DQ296094
P.australiae EF408605
P.australiae EF408606 T
O.denticillatum GQ249321
O.denticillatum FJ804502 T
O.quercus DQ296096
O.quercus AY789157
O.tsotsi FJ441277 T
O.tsotsi FJ441280 T
O.karelicum FJ804497
O.karelicum EU443773 T
O. piceae complex (hardwood clade)
O.aincae HM031552 T
O.aincae HM031550
O.brunneociliatum HM031557
O.brunneociliatum HM031559
O.tapionis HM031547
O.tapionis HM031545 T
O.floccosum HM031538 RUSSIA
O.floccosum FJ490602 AUSTRIA
O.floccosum EU785429 CHINA
O.floccosum AY789140 KOREA
O.floccosum AY789141 NZ
DLS1341 CANADA
O.floccosum AY789142 CANADA
O. floccosum
O.minus AY542505 UK
O.minus AY542506 SWEDEN
O.minus AY542507 CANADA
O.minus AY542509 USA
O.istropil AY905701
O.istropil AY905702
O.piliferum AF221627 USA
O.piliferum DQ296098 CMW7677
O.piliferum AY789154 NZ
CMW15464 CANADA
O.piliferum AY789153 KOREA
O.piliferum DQ296097 NETHERLANDS
O. piliferum
O.pseudotsugae AY542510
O.pseudotsugae AY548744
O.setosum AY789158
O.setosum AY905703 T
O.canum DQ296092
O.canum AY905700
O.subalpinum AB200430
O.subalpinum AB200429
O.brevisiculum HM031517
O.brevisiculum AB200427 T
O.piceae AY789152
O.piceae DQ296091
O. piceae complex (conifer clade)
O.fuscum HM031565
O.fuscum HM031563 T
O.bicolor DQ288636
O.bicolor DQ288635 T
O.pulmisporum EU977487
O.pulmisporum DQ296100
O.ips AY194950 SWEDEN
O.ips AY194949 CANADA
O.ips AY194952 JAPAN
O.ips AY789146 NZ
O.ips EU785431 CHINA
DLS1339 CANADA
O. ips
O. ips complex
0.05

Figure 3. Phylogram resulting from a ML analysis of the βT sequences of selected *Ophiostoma* species. Bootstrap values obtained from MP and posterior probabilities of BI are presented as indicated. Isolate numbers of sequences produced in the present study are printed in bold type. T sequence of ex-type isolates.

species.

The third most prevalent species was an unknown *Leptographium* sp. (X) that roughly resembled *L. terebrantis* in culture. However, our sequences of the newly requested ex-type isolate from CBS and MUCL, showed that *L. terebrantis sensu stricto* groups distinct from all the other species in the *G. clavigera* complex. Our isolates from *D. murrayane* grouped consistently with a number of isolates previously reported as *L. terebrantis* (Kim *et al*. 2005; Lee *et al*. 2003, 2005; Lim *et al*. 2004, Roe *et al*. 2010; Six *et al*. 2003). Unfortunately, the lineages containing the sequences from our study and those from the previous studies did not have statistical support in any of the analyses we conducted, not in the separate data sets for the five different gene regions (data not shown), nor in the combined tree (Fig. 3). The latter data set included 11 isolates, each of which representing a different haplotype. Although our four isolates had identical sequences in some gene regions to some of the isolates of Roe *et al*. (2010), the haplotypes of the concatenated multilocus data of our isolates did not correspond to any of the 11 haplotypes designated by Roe *et al*. (2010). Based on this incongruence of sequence data, we are hesitant to describe a novel taxon based on our four sequenced isolates. We suggest a more comprehensive study where at least some of the isolates of previous studies can be included and compared with our isolates. A re-evaluation of '*L. terebrantis*' isolates included in the study of Lu *et al*. (2009), should also from part of the future work since none of the βT and EF-1α sequences match any of the sequences produced in the present study, including those of the ex-type isolate.

It is interesting to note that all the isolates of *Leptographium* sp. X from the previous studies (as '*L. terebrantis*') originated from pine in British Columbia and the USA. One of the isolates (C418) came from *D. brevicomis* in the USA (Six *et al*. 2003), while all the Canadian isolates came from *D. ponderosae* or stained wood from *D. ponderosae*-attacked trees (Kim *et al*. 2005; Lee *et al*. 2003, 2005; Lim *et al*. 2004; Roe *et al*. 2010).

The presence of these fungi with *D. murrayanae* may be, at least in part, due to the presence of other beetle species in trees from which our collections were made. In several instances, we observed galleries of *Pseudips mexicanus* (Hopkins), *Orthotomicus latidens* (LeConte), *H. porosus*, and *H. rugipennis* (Mannerheim) located near, or directly adjacent to, *D. murrayanae* galleries. *Pseudips* and *Ips* are known to vector *O. ips* (Furniss *et al*. 1995; Kirisits 2004; Harrington 2005), and *Hylurgops* spp. are known to vector L. terebrantis (Harrington 1982, 1988; Harrington and Cobb 1983). Because all five beetle species often develop in the same general area within a tree, co-mingling of their respective fungal associates is likely to be a common occurrence. Our observations and isolations indicate that *D. murrayanae* may often develop in the presence of a fungal community, rather than with a single fungus. However, other than *G. aurea* and *O. abietinum*, the composition of that community is likely to vary.

It is not known what effects the fungi associated with *D. murrayanae* may have on their host. However, the success of both the beetle and the fungi appears to be linked to where in the tree the beetles attack, and potentially, site conditions. Safranyik *et al*. (1999, 2004) observed that *D. murrayanae* tended to be more common at wetter sites. Our observations indirectly support this conclusion. We observed that most successful attacks occurred near soil level in deep crevices in the tree bole that contained very wet phloem and sapwood. These wet areas were stained a deep blue or black by the fungi. Most attacks that occurred on areas of the bole containing drier phloem (well above soil line, not constructed in crevices) were unstained, encrusted with resin and not successful.

Furniss and Kegley (2008) also observed that most successful galleries were located within 5 cm of the soil line and that none occurred above 20 cm. Overall, the majority of the galleries we observed were unsuccessful (no brood). Of those that were successful, brood production was typically low (approx. 2–12 brood per parental pair). None of the attacks that we observed resulted in the death of the tree.

In summary, our results indicate that *D. murrayanae* is symbiotically associated with *G. aurea* and *O. abietinum*. However, rather than developing in the presence of these fungi alone, it appears that the beetle may often develop in the presence of a larger community of fungi, some of which may be present as a result of the co-occurrence of other bark beetle vectors in the tree. Very few of the parasitic *Dendroctonus* spp. such as *D. murrayanae* have been carefully examined for their fungal associates. Studies on additional examples will contribute to an expanding understanding of the biology of these beetles and of the fungi that are associated with them.

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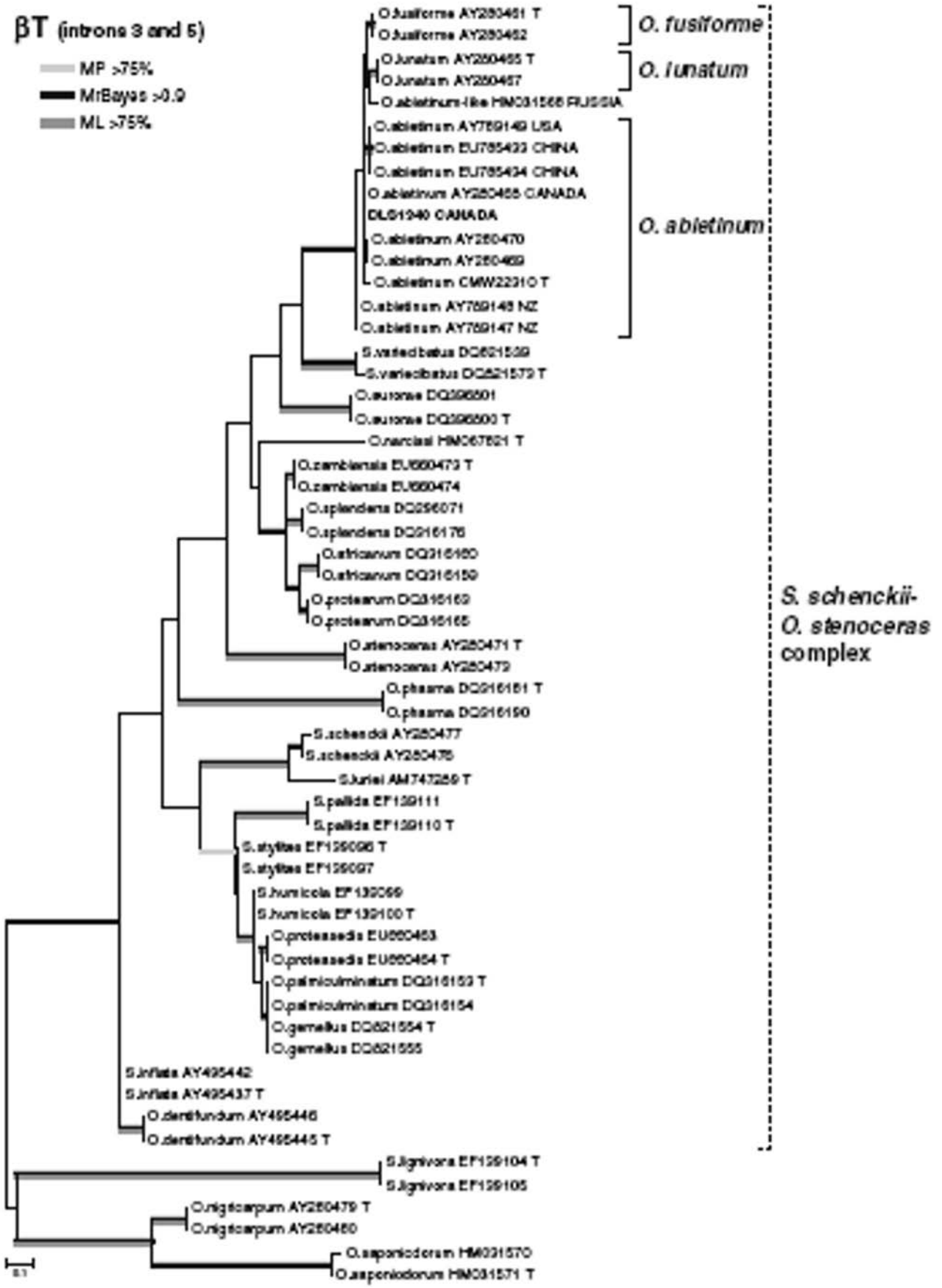

Figure 4. Phylogram resulting from a ML analysis of the βT sequences of species in the *S. schenckii–O. stenoceras* complex. Bootstrap values obtained from MP and posterior probabilities of BI are presented as indicated. Isolate numbers of sequences produced in the present study are printed in bold type. T sequence of ex-type isolates

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