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

Diana L. Six · Z. Wilhelm de Beer · Tuan A. Duong · Allan L. Carroll · Michael J. Wingfield

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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.

Keywords Bark beetle · Symbiosis · *Leptographium* · *Ophiostoma* · *Grosmannia aurea*

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D. L. Six (✉)
Department of Ecosystem and Conservation Sciences, College of Forestry and Conservation, The University of Montana, Missoula, MT 59812, USA
e-mail: diana.six@cfc.umt.edu

Z. W. de Beer · M. J. Wingfield
Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria 0002, South Africa

T. A. Duong
Department of Genetics, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria 0002, South Africa

A. L. Carroll
Department of Forest Sciences, University of British Columbia, Vancouver, BC V6T 1Z4, Canada

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). *D. 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.

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.

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

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.

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

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

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.

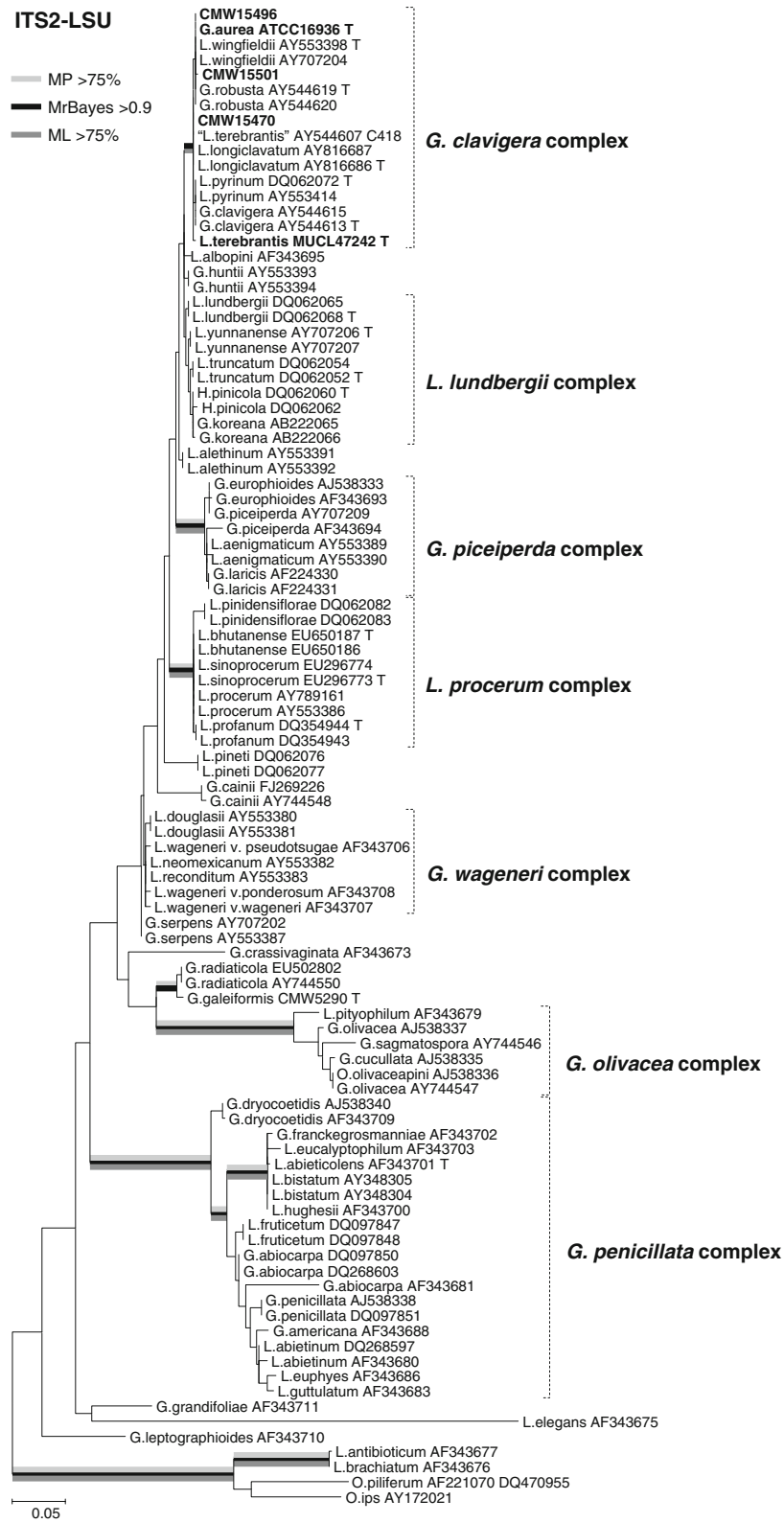

Fig. 1 Phylogram resulting from a ML analysis of the partial ITS2 and LSU sequences of selected *Grosmannia* and *Leptographium* species. Bootstrap values obtained from MP and posterior probabilities of BI are presented as indicated. Isolate numbers of sequences produced in the present study are printed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

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).

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

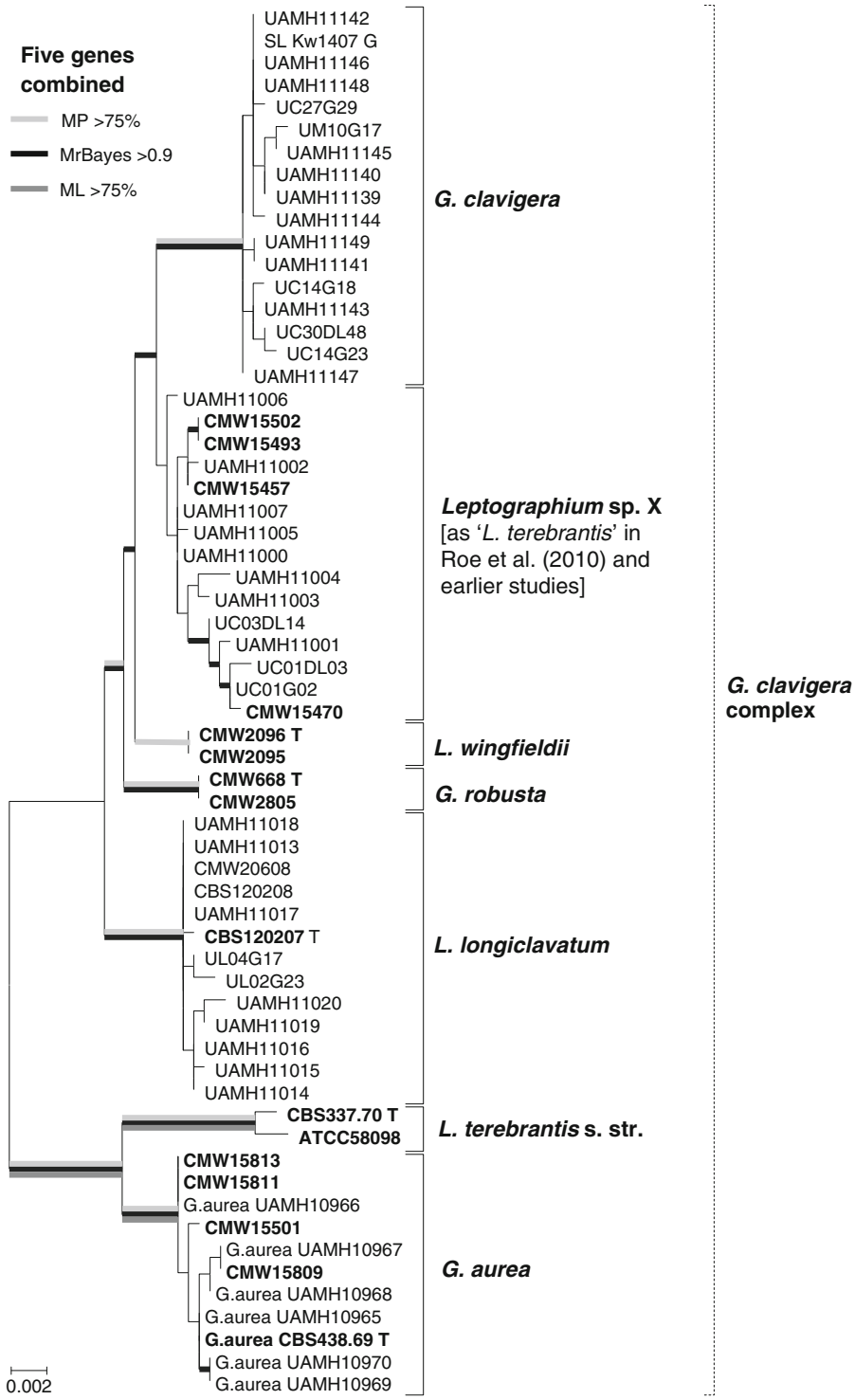

Fig. 2 Phylogram resulting from a ML analysis of a combined data set consisting of sequences of five gene regions for species in the *G. clavigera* species complex. The gene regions included were: ITS2–LSU (566 bp), βT (357 bp), EF-1α (516 bp), actin (741 bp), and an anonymous locus (393 bp). Bootstrap 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

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.

Discussion

Results of this study revealed that some relatively well-known *Ophiostoma* and *Grosmannia* species are associated with *D. murrayanae*. *G. 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*. *H. 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

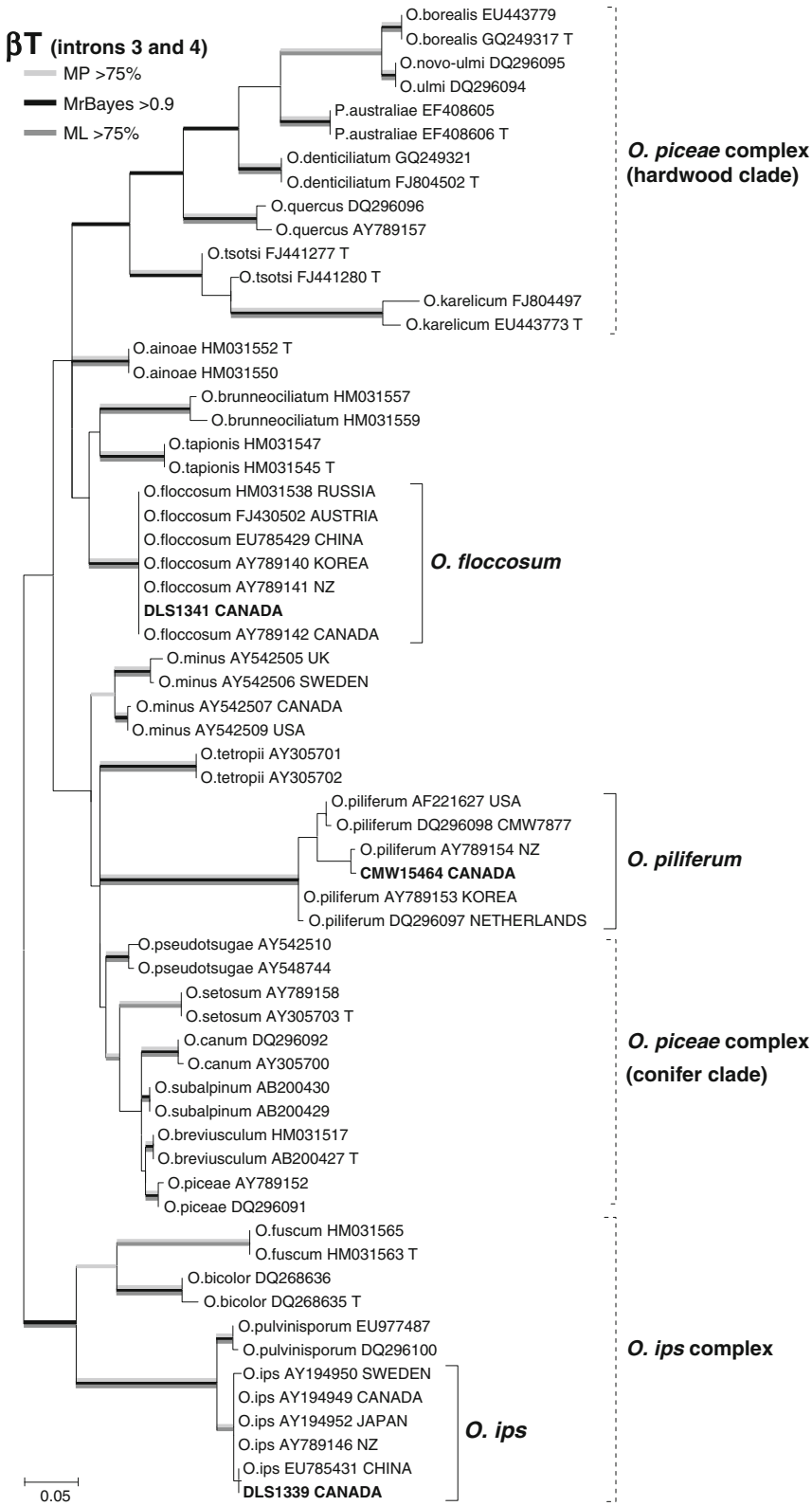

Fig. 3 Phylogram resulting from a ML analysis of the βT sequences of selected *Ophiostoma* species. Bootstrap values obtained from MP and posterior probabilities of BI are presented as indicated. Isolate numbers of sequences produced in the present study are printed in *bold* type. *T* sequence of ex-type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

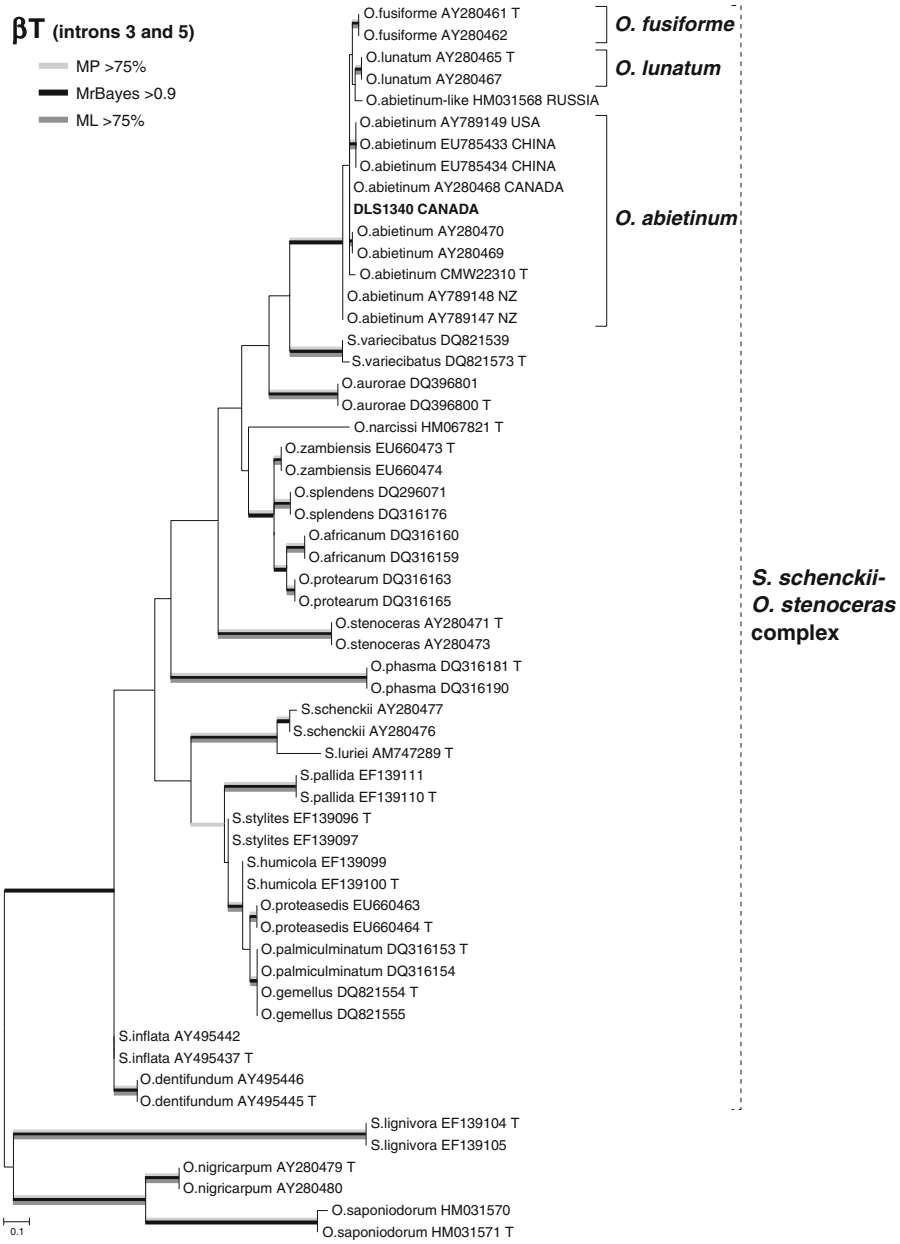

Fig. 4 Phylogram resulting from a ML analysis of the *βT* sequences of species in the *S. schenckii–O. stenoceras* complex. Bootstrap values obtained from MP and posterior probabilities of BI are presented as indicated. Isolate numbers of sequences produced in the present study are printed in *bold*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

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). *D. 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). *O. 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* 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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Online Resource 1:

Comparison and selection of reference sequences for species of the *Grosmannia aurea* complex:

For several species in the *Grosmannia clavigera*-complex there are ambiguous sequences obtained from the same isolates present in GenBank. These sequences were determined in different studies from ex-type isolates ([T]) maintained in and sourced from different culture collections. For example, the ex-type isolate of *G. aurea* (Table A) was obtained from ATCC for the study of Lee et al. (2003). The same isolate, but obtained from CBS, was used by Hausner et al. (2005). This isolate is furthermore maintained in the CMW collection under three different numbers. One of these cultures (CMW667) was used by Zipfel et al. (2006), the second (CMW709) by Jacobs et al. (2004, 2005), and the third (CMW714) by Jacobs et al. (2001, 2005). Similar situations existed for *G. robusta*, *L. pyrinum*, and *L. terebrantis* (references to these are listed in the table). To show and clarify inconsistencies between some of these sequences, all sequences of the same isolate produced in different studies were included in our initial analyses (data not shown). The same isolates are thus presented in the table more than once, in some cases with different culture collection numbers, showing the accession numbers as used in the various publications. Accession numbers for sequences obtained in the present study are printed in bold type. The sequences that we recommend for use in future phylogenetic studies are highlighted in grey.

Species	**Isolate no**	**GenBank Acc. no.**										**[2]Reference**
		ITS2-LSU	**[1]HT**	**β-tubulin**	**HT**	**EF-1α**	**HT**	**Actin**	**HT**	**Anonymous locus**	**HT**	
G. aurea: **ex-type isolate**	[T]ATCC16936	AY544610	AI1	AY263187	AB1	AY544633	AE2	AY544592	AA2		AU2	Lee et al. 2003, 2005; Lim et al. 2004
	[T]ATCC16936 (=CMW30732)	**=JF798473**	AI1	**=JF798454**	AB1	**=JF798463**	AE2	**=JF798479**	AA2	**=JF798485**	AU2	present study
	[T]CBS438.69	#AY935606	-1									Hausner et al. 2005
	[T]CBS438.69 (=CMW29869)	**JF798473**	AI1	**JF798454**	AB1	**JF798463**	AE2	**JF798479**	AA2	**JF798485**	AU2	present study
	[T]CMW667	*DQ294389	AI1	DQ296109	AB1							Zipfel et al. 2006
	[T]CMW709	AY553413	AI1	AY534961	AB1	AY536207	-2					Jacobs et al. 2004, 2005
	[T]CMW714	DQ062071	AI1	DQ062005	AB1	DQ062038	AE2					Jacobs et al. 2005
	[T]CMW714	AF343699	-2									Jacobs et al. 2001
	[T]CMW714	**=JF798473**	AI1	**=JF798454**	AB1	**=JF798463**	AE2	**=JF798479**	AA2	**=JF798485**	AU2	present study
	[T]MUCL19069 (=CMW29989)	**=JF798473**	AI1	**=JF798454**	AB1	**JF798464**	AE4	**=JF798479**	AA2	**=JF798485**	AU2	present study
G. aurea: **other isolates**	AU98Pr2-128	AY544611	AI1			AY544634	AE2	AY544593	AA1			Lim et al. 2004
	AU98Pr2-141			AY263186	AB1							Lee et al. 2003
	AU98Pr2-169	AY544612	AI1	AY263188	AB4	AY544635	AE2	AY544594	AA1			Lee et al. 2003; Lim et al. 2004; Roe et al. 2010
	UAMH10965	GU370267	AI1	GU370181	AB2	GU370224	AE2	GU370138	AA1	GU370310	AU1	Roe et al. 2010
	UAMH10966	GU370271	AI2	GU370185	AB1	GU370228	AE2	GU370142	AA1	GU370314	AU2	Roe et al. 2010
	UAMH10967	GU370265	AI1	GU370179	AB1	GU370222	AE1	GU370136	AA1	GU370308	AU2	Roe et al. 2010
	UAMH10968	GU370291	AI1	GU370205	AB2	GU370248	AE1	GU370162	AA1	GU370334	AU1	Roe et al. 2010
	UAMH10969	GU370293	AI1	GU370207	AB2	GU370250	AE3	GU370164	AA1	GU370336	AU1	Roe et al. 2010
	UAMH10970	GU370260	AI1	GU370174	AB3	GU370217	AE3	GU370131	AA1	GU370303	AU1	Roe et al. 2010

Species	Isolate no	GenBank Acc. no.										[2]Reference
		ITS2-LSU	[1]HT	β-tubulin	HT	EF-1α	HT	Actin	HT	Anonymous locus	HT	
***G. clavigera*: ex-type isolate**	[T]ATCC18086 (=CBS438.69)	AY544613	CI1	AY263194	CB1	AY544636	CE3	AY544595	CA4			Lee et al. 2003; Lim et al. 2004
***G. clavigera*: other isolates**	C843	AY544614	CI1	AY263196	CB2	AY544637	CE3	AY544596	CA4			Lee et al. 2003; Lim et al. 2004
	[G]SL-Kw1407	AY544615	CI1	AY263195	CB1	AY544638	CE1	AY544597	CA1	ACYC01001508	CU1	Lee et al. 2003; Lim et al. 2004; Roe et al. 2010
	AU98Pr3-18	AY544616	CI3	AY544624	CB1	AY544639	CE1	AY544598	CA1			Lim et al. 2004
	MO5	#AY761158	CI1									Lim et al. 2005
	SL-St.J11	AY816691	CI1	AY263201	CB1			AY816684	CA1			Lee et al. 2005
	SL-Wg602	AY816692	CI1	AY263205	CB1			AY816685	CA1			Lee et al. 2005
	UAMH11139	GU370273	CI1	GU370187	CB1	GU370230	CE2	GU370144	CA1	GU370316	CU1	Roe et al. 2010
	UAMH11140	GU370288	CI1	GU370202	CB1	GU370245	CE2	GU370159	CA1	GU370331	CU1	Roe et al. 2010
	UAMH11141	GU370290	CI1	GU370204	CB1	GU370247	CE1	GU370161	CA2	GU370333	CU2	Roe et al. 2010
	UAMH11142	GU370289	CI1	GU370203	CB1	GU370246	CE1	GU370160	CA1	GU370332	CU1	Roe et al. 2010
	UAMH11143	GU370274	CI1	GU370188	CB1	GU370231	CE1	GU370145	CA1	GU370317	CU3	Roe et al. 2010
	UAMH11144	GU370278	CI1	GU370192	CB1	GU370235	CE1	GU370149	CA2	GU370321	CU1	Roe et al. 2010
	UAMH11145	GU370286	CI1	GU370200	CB1	GU370243	CE2	GU370157	CA2	GU370329	CU1	Roe et al. 2010
	UAMH11146	GU370287	CI1	GU370201	CB1	GU370244	CE1	GU370158	CA1	GU370330	CU1	Roe et al. 2010
	UAMH11147	GU370298	CI1	GU370212	CB1	GU370255	CE1	GU370169	CA1	GU370341	CU2	Roe et al. 2010
	UAMH11148	GU370301	CI1	GU370215	CB1	GU370258	CE1	GU370172	CA1	GU370344	CU1	Roe et al. 2010
	UAMH11149	GU370296	CI1	GU370210	CB1	GU370253	CE1	GU370167	CA2	GU370339	CU2	Roe et al. 2010
	UC30DL48	GU370259	CI1	GU370173	CB1	GU370216	CE1	GU370130	CA3	GU370302	CU3	Roe et al. 2010
	UC27G29	GU370261	CI1	GU370175	CB1	GU370218	CE1	GU370132	CA3	GU370304	CU1	Roe et al. 2010
	UM10G17	GU370280	CI2	GU370194	CB1	GU370237	CE2	GU370151	CA2	GU370323	CU1	Roe et al. 2010
	UC14G18	GU370264	CI1	GU370178	CB1	GU370221	CE1	GU370135	CA2	GU370307	CU3	Roe et al. 2010
	UC14G23	GU370266	CI1	GU370180	CB1	GU370223	CE2	GU370137	CA3	GU370309	CU3	Roe et al. 2010
***G. robusta*: c**	[T]CMW668	AY544619	-1	AY263190	-2	AY544642	RE1	AY544601	RA1			Lee et al. 2003; Lim et al. 2004; Roe et al. 2010
	[T]CMW668	AY553397	RI1	AY534945	RB1	AY536191	RE1					Jacobs et al 2004
	[T]CMW668			**JF798458**	RB1	**JF798465**	RE1			**JF798491**		present study
***G. robusta*: other isolate**	CMW2805			**JF798457**	RB1	**JF798466**	RE1			**JF798490**		present study
	CMW2805	AF343705	Lg									Jacobs et al. 2001
	CMW2805	AY544620	-1	AY263189	-2	AY544643	RE1	AY544602	RA1			Lee et al. 2003; Lim et al. 2004
	CMW2805	AY553396	RI1	AY534944	RB1	AY536190	-2					Jacobs et al. 2004
	CMW2805	#DQ294398	-5	DQ296118	Gp							Zipfel et al. 2006
***L. longiclavatum*: ex-type isolate**	[T]CBS120207 (=CMW20607 =SL-Kw1436)	AY816686	LI1	AY288934	LB1	**JF798467**	LE2	AY816679	LA5	**JF798492**	LU1	Lee et al. 2005; present study
***L. longiclavatum*: other isolates**	CMW20608 (=SL-Kp11)	AY816687	LI1	AY816712	LB1	**JF798468**	LE2	AY816680	LA1	**JF798493**	LU1	Lee et al. 2005; present study

Species	Isolate no	GenBank Acc. no.										[2]Reference
		ITS2-LSU	[1]HT	β-tubulin	HT	EF-1α	HT	Actin	HT	Anonymous locus	HT	
***L. longiclavatum*: other isolates**	CBS120208 (=CMW20609 =SL-Pw5)	AY816689	LI1	AY288935	LB1	**JF798469**	LE2	AY816682	LA1	**JF798494**	LU1	Lee et al. 2005; Roe et al. 2010
	SL-W001	AY816688	LI1					AY816681	LA1			
	C187	AY816690	LI1					AY816683	LA4			
	UAMH 11013	GU370276	LI1	GU370190	LB1	GU370233	LE2	GU370147	LA1	GU370319	LU1	Roe et al. 2010
	UAMH 11014	GU370282	LI1	GU370196	LB1	GU370239	LE1	GU370153	LA1	GU370325	LU1	Roe et al. 2010
	UAMH 11015	GU370275	LI1	GU370189	LB1	GU370232	LE1	GU370146	LA1	GU370318	LU1	Roe et al. 2010
	UAMH 11016	GU370277	LI1	GU370191	LB1	GU370234	LE1	GU370148	LA1	GU370320	LU1	Roe et al. 2010
	UAMH 11017	GU370279	LI1	GU370193	LB1	GU370236	LE2	GU370150	LA1	GU370322	LU1	Roe et al. 2010
	UAMH 11018	GU370297	LI1	GU370211	LB1	GU370254	LE2	GU370168	LA1	GU370340	LU1	Roe et al. 2010
	UAMH 11019	GU370299	LI1	GU370213	LB1	GU370256	LE1	GU370170	LA1	GU370342	LU1	Roe et al. 2010
	UAMH 11020	GU370300	LI1	GU370214	LB1	GU370257	LE2	GU370171	LA2	GU370343	LU1	Roe et al. 2010
	UL02G23	GU370262	LI1	GU370176	LB1	GU370219	LE3	GU370133	LA3	GU370305	LU1	Roe et al. 2010
	UL04G17	GU370263	LI1	GU370177	LB1	GU370220	LE3	GU370134	LA1	GU370306	LU1	Roe et al. 2010
***L. pyrinum*: ex-type isolate**	[T]CMW169 (=ATCC34943 =CBS 119897)	AF343689	La									Jacobs et al. 2001
	[T]CMW169 (=ATCC34943 =CBS119897)	DQ062072	PI1	DQ062006	PB1	DQ062039	PE1					Zhou et al. 2008
	[T]CMW509 (=ATCC34943 =CBS120181)	AY553414	PI1	AY534962	PB1	AY536208	-3					Jacobs et al. 2004; Zhou et al. 2008
[2]***L. pyrinum* A**	DLS879	AY544604	-2	AY263185	-7	AY544627	-7	AY544586				Lee et al. 2003; Lim et al. 2004
	CMW3889 (=DLS879)	AY544605	-2	AY544621	-7	AY544628	PE1	AY544587				Lim et al. 2004
***L. terebrantis:* ex-type isolate**	[T]CBS337.70 (=CMW29841)	**JF798477**	SI1	**JF798459**	SB1	**JF798470**	SE1	**JF798483**	SA1	**JF798495**	SU1	present study
	[T]CMW9	AF343698	Lp									Jacobs et al. 2001
	[T]CMW9	AY553384	Lp	AY534932	Lp	AY536178	Lp					Jacobs et al. 2004
	[T]CMW9			EU652698	-2	EU652700	-10					Zhou et al. 2008
	[T]CMW9a	EU652697	Lp	EU652699	-2	EU652701	-10					Zhou et al. 2008
	[T]CMW663	EU785383	-2	EU785349	-7	EU785412	-18					Lu et al. 2009a
	[T]CMW663	Contaminated										present study
	[T]MUCL47242	EU296777	SI1	EU296784	SB1	EU296791	-2					Lu et al. 2008, 2009b
	[T]MUCL47242 (=CMW29991)	**=JF798477**	SI1	**=JF798459**	SB1	**=JF798470**	SE1	**=JF798483**	SA1	**=JF798495**	SU1	present study

Species	Isolate no	GenBank Acc. no.										[2]Reference
		ITS2-LSU	[1]HT	β-tubulin	HT	EF-1α	HT	Actin	HT	Anonymous locus	HT	
***L. terebrantis*: other isolates**	ATCC58098 (=CMW30731)	**JF798476**	SI2	**JF798460**	SB1	**JF798471**	SE2	**JF798484**	SA1	**JF798496**	SU2	present study
	CMW2814 (=CBS115209)	EU785385	-1	EU785354	-6	EU785406	-17					Lu et al. 2009a
	CMW11 (=CBS298.85)	EU785386	-1	EU785348	-6	EU785403	-17					Lu et al. 2009a
***L. wingfieldii:* ex-type isolate**	[T]CMW2096	AF343684	La									Jacobs et al. 2001
	[T]CMW2096	AY553398	WI1	AY534946	WB1	AY536192	WE1			**JF798498**	WU1	Jacobs et al. 2004; present study
	[T]CMW2096	AY707205	WI1	AY707191	WB1			AY707178	WA1			Kim et al. 2005
***L. wingfieldii:* other isolates**	CMW2095	AY553400	WI1	AY534948	WB1	AY536194	WE1			**JF798497**	WU1	Jacobs et al. 2004; present study
	CMW2095	AY707204	WI1	AY707190	WB1			AY707177	WA1			Kim et al. 2005
	CMW2019	AY553399	WI1	AY534947	WB1	AY536193	WE2					Jacobs et al. 2004
	CMW10224	AY553401	WI1	AY534949	WB1	AY536195	WE1					Jacobs et al. 2004
***Leptographium* sp. X** (as *L. terebrantis* in previous publications)	AU156-12-13	AY544609	TI2	AY544623	TB5	AY544632	TE6	AY544591	TA7			Lim et al. 2004
	AU98Pr2-155	AY544608	TI2	AY544622	TB1	AY544631	TE1	AY544590	TA1			Lim et al. 2004; Lee et al. 2005;
	C418	AY544607	TI2	AY263191	TB1	AY544630	TE1	AY544589	TA2			Six et al. 2003; Lee et al. 2003, 2005; Lim et al. 2004; Kim et al. 2005; Roe et al. 2010
	LPWYLT-1			AY267826	TB1							Lee et al. 2003
	MY23AW3			AY672911	TB1							Kim et al. 2005
	SL-A57			DQ118421	TB1							Lee et al. unpubl.
	UAMH9722	AY544606	TI2	AY263192	TB1	AY544629	TE1	AY544588	TA1			Lee et al. 2003, 2005; Lim et al. 2004
	UAMH 11000	GU370272	TI1	GU370186	TB1	GU370229	TE1	GU370143	TA2	GU370315	TU1	Roe et al. 2010
	UAMH 11001	GU370292	TI1	GU370206	TB1	GU370249	TE3	GU370163	TA1	GU370335	TU2	Roe et al. 2010
	UAMH 11002	GU370283	TI2	GU370197	TB2	GU370240	TE4	GU370154	TA2	GU370326	TU1	Roe et al. 2010
	UAMH 11003	GU370284	TI1	GU370198	TB1	GU370241	TE5	GU370155	TA4	GU370327	TU1	Roe et al. 2010
	UAMH 11004	GU370281	TI2	GU370195	TB3	GU370238	TE1	GU370152	TA6	GU370324	TU1	Roe et al. 2010
	UAMH 11005	GU370285	TI1	GU370199	TB2	GU370242	TE1	GU370156	TA2	GU370328	TU2	Roe et al. 2010
	UAMH 11006	GU370294	TI1	GU370208	TB1	GU370251	TE3	GU370165	TA3	GU370337	TU1	Roe et al. 2010
	UAMH 11007	GU370295	TI1	GU370209	TB1	GU370252	TE1	GU370166	TA5	GU370338	TU1	Roe et al. 2010
	UC03DL14	GU370268	TI1	GU370182	TB1	GU370225	TE1	GU370139	TA1	GU370311	TU1	Roe et al. 2010
	UC01G02	GU370269	TI2	GU370183	TB2	GU370226	TE1	GU370140	TA1	GU370312	TU2	Roe et al. 2010
	UC01DL03	GU370270	TI2	GU370184	TB2	GU370227	TE2	GU370141	TA1	GU370313	TU2	Roe et al. 2010

[1] 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 LA3 =*L. longiclavatum* actin haplotype 3, etc. The number of bp differences in which ambiguous sequences differ from reliable sequences of the same isolate are indicated with a – sign. In some cases sequences in GenBank represent other species that are abbreviated as follows: *La=Leptographium abietinum; Lp=L. procerum; Lg=L. guttulatum; Gp=Grosmannia piceaperda*

[2] References to studies in which these isolates were used in phylogenetic analyses.

[3] The isolate DLS879 most probably represents a species distinct from the true *L. pyrinum*.

[T] Ex type isolates.

[G] Isolate used for whole genome sequencing (DiGuistini et al. 2009).
Only ITS2 sequences were available, excluding the LSU fragment.
* Only LSU sequences were available, excluding the ITS fragment.

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Online Resource 1:

Comparison and selection of reference sequences for species of the *Grosmannia aurea* complex:

For several species in the *Grosmannia clavigera*-complex there are ambiguous sequences obtained from the same isolates present in GenBank. These sequences were determined in different studies from ex-type isolates ([T]) maintained in and sourced from different culture collections. For example, the ex-type isolate of *G. aurea* (Table A) was obtained from ATCC for the study of Lee et al. (2003). The same isolate, but obtained from CBS, was used by Hausner et al. (2005). This isolate is furthermore maintained in the CMW collection under three different numbers. One of these cultures (CMW667) was used by Zipfel et al. (2006), the second (CMW709) by Jacobs et al. (2004, 2005), and the third (CMW714) by Jacobs et al. (2001, 2005). Similar situations existed for *G. robusta*, *L. pyrinum*, and *L. terebrantis* (references to these are listed in the table). To show and clarify inconsistencies between some of these sequences, all sequences of the same isolate produced in different studies were included in our initial analyses (data not shown). The same isolates are thus presented in the table more than once, in some cases with different culture collection numbers, showing the accession numbers as used in the various publications. Accession numbers for sequences obtained in the present study are printed in bold type. The sequences that we recommend for use in future phylogenetic studies are highlighted in grey.

Species	**Isolate no**	**GenBank Acc. no.**										**[2]Reference**
		ITS2-LSU	**[1]HT**	**β-tubulin**	**HT**	**EF-1α**	**HT**	**Actin**	**HT**	**Anonymous locus**	**HT**	
G. aurea: **ex-type isolate**	[T]ATCC16936	AY544610	AI1	AY263187	AB1	AY544633	AE2	AY544592	AA2		AU2	Lee et al. 2003, 2005; Lim et al. 2004
	[T]ATCC16936 (=CMW30732)	**=JF798473**	AI1	**=JF798454**	AB1	**=JF798463**	AE2	**=JF798479**	AA2	**=JF798485**	AU2	present study
	[T]CBS438.69	#AY935606	-1									Hausner et al. 2005
	[T]CBS438.69 (=CMW29869)	**JF798473**	AI1	**JF798454**	AB1	**JF798463**	AE2	**JF798479**	AA2	**JF798485**	AU2	present study
	[T]CMW667	*DQ294389	AI1	DQ296109	AB1							Zipfel et al. 2006
	[T]CMW709	AY553413	AI1	AY534961	AB1	AY536207	-2					Jacobs et al. 2004, 2005
	[T]CMW714	DQ062071	AI1	DQ062005	AB1	DQ062038	AE2					Jacobs et al. 2005
	[T]CMW714	AF343699	-2									Jacobs et al. 2001
	[T]CMW714	**=JF798473**	AI1	**=JF798454**	AB1	**=JF798463**	AE2	**=JF798479**	AA2	**=JF798485**	AU2	present study
	[T]MUCL19069 (=CMW29989)	**=JF798473**	AI1	**=JF798454**	AB1	**JF798464**	AE4	**=JF798479**	AA2	**=JF798485**	AU2	present study
G. aurea: **other isolates**	AU98Pr2-128	AY544611	AI1			AY544634	AE2	AY544593	AA1			Lim et al. 2004
	AU98Pr2-141			AY263186	AB1							Lee et al. 2003
	AU98Pr2-169	AY544612	AI1	AY263188	AB4	AY544635	AE2	AY544594	AA1			Lee et al. 2003; Lim et al. 2004; Roe et al. 2010
	UAMH10965	GU370267	AI1	GU370181	AB2	GU370224	AE2	GU370138	AA1	GU370310	AU1	Roe et al. 2010
	UAMH10966	GU370271	AI2	GU370185	AB1	GU370228	AE2	GU370142	AA1	GU370314	AU2	Roe et al. 2010
	UAMH10967	GU370265	AI1	GU370179	AB1	GU370222	AE1	GU370136	AA1	GU370308	AU2	Roe et al. 2010
	UAMH10968	GU370291	AI1	GU370205	AB2	GU370248	AE1	GU370162	AA1	GU370334	AU1	Roe et al. 2010
	UAMH10969	GU370293	AI1	GU370207	AB2	GU370250	AE3	GU370164	AA1	GU370336	AU1	Roe et al. 2010
	UAMH10970	GU370260	AI1	GU370174	AB3	GU370217	AE3	GU370131	AA1	GU370303	AU1	Roe et al. 2010

Species	Isolate no	GenBank Acc. no.										[2]Reference
		ITS2-LSU	[1]HT	β-tubulin	HT	EF-1α	HT	Actin	HT	Anonymous locus	HT	
***G. clavigera*: ex-type isolate**	[T]ATCC18086 (=CBS438.69)	AY544613	CI1	AY263194	CB1	AY544636	CE3	AY544595	CA4			Lee et al. 2003; Lim et al. 2004
***G. clavigera*: other isolates**	C843	AY544614	CI1	AY263196	CB2	AY544637	CE3	AY544596	CA4			Lee et al. 2003; Lim et al. 2004
	[G]SL-Kw1407	AY544615	CI1	AY263195	CB1	AY544638	CE1	AY544597	CA1	ACYC01001508	CU1	Lee et al. 2003; Lim et al. 2004; Roe et al. 2010
	AU98Pr3-18	AY544616	CI3	AY544624	CB1	AY544639	CE1	AY544598	CA1			Lim et al. 2004
	MO5	#AY761158	CI1									Lim et al. 2005
	SL-St.J11	AY816691	CI1	AY263201	CB1			AY816684	CA1			Lee et al. 2005
	SL-Wg602	AY816692	CI1	AY263205	CB1			AY816685	CA1			Lee et al. 2005
	UAMH11139	GU370273	CI1	GU370187	CB1	GU370230	CE2	GU370144	CA1	GU370316	CU1	Roe et al. 2010
	UAMH11140	GU370288	CI1	GU370202	CB1	GU370245	CE2	GU370159	CA1	GU370331	CU1	Roe et al. 2010
	UAMH11141	GU370290	CI1	GU370204	CB1	GU370247	CE1	GU370161	CA2	GU370333	CU2	Roe et al. 2010
	UAMH11142	GU370289	CI1	GU370203	CB1	GU370246	CE1	GU370160	CA1	GU370332	CU1	Roe et al. 2010
	UAMH11143	GU370274	CI1	GU370188	CB1	GU370231	CE1	GU370145	CA1	GU370317	CU3	Roe et al. 2010
	UAMH11144	GU370278	CI1	GU370192	CB1	GU370235	CE1	GU370149	CA2	GU370321	CU1	Roe et al. 2010
	UAMH11145	GU370286	CI1	GU370200	CB1	GU370243	CE2	GU370157	CA2	GU370329	CU1	Roe et al. 2010
	UAMH11146	GU370287	CI1	GU370201	CB1	GU370244	CE1	GU370158	CA1	GU370330	CU1	Roe et al. 2010
	UAMH11147	GU370298	CI1	GU370212	CB1	GU370255	CE1	GU370169	CA1	GU370341	CU2	Roe et al. 2010
	UAMH11148	GU370301	CI1	GU370215	CB1	GU370258	CE1	GU370172	CA1	GU370344	CU1	Roe et al. 2010
	UAMH11149	GU370296	CI1	GU370210	CB1	GU370253	CE1	GU370167	CA2	GU370339	CU2	Roe et al. 2010
	UC30DL48	GU370259	CI1	GU370173	CB1	GU370216	CE1	GU370130	CA3	GU370302	CU3	Roe et al. 2010
	UC27G29	GU370261	CI1	GU370175	CB1	GU370218	CE1	GU370132	CA3	GU370304	CU1	Roe et al. 2010
	UM10G17	GU370280	CI2	GU370194	CB1	GU370237	CE2	GU370151	CA2	GU370323	CU1	Roe et al. 2010
	UC14G18	GU370264	CI1	GU370178	CB1	GU370221	CE1	GU370135	CA2	GU370307	CU3	Roe et al. 2010
	UC14G23	GU370266	CI1	GU370180	CB1	GU370223	CE2	GU370137	CA3	GU370309	CU3	Roe et al. 2010
***G. robusta*: c**	[T]CMW668	AY544619	-1	AY263190	-2	AY544642	RE1	AY544601	RA1			Lee et al. 2003; Lim et al. 2004; Roe et al. 2010
	[T]CMW668	AY553397	RI1	AY534945	RB1	AY536191	RE1					Jacobs et al 2004
	[T]CMW668			**JF798458**	RB1	**JF798465**	RE1			**JF798491**		present study
***G. robusta*: other isolate**	CMW2805			**JF798457**	RB1	**JF798466**	RE1			**JF798490**		present study
	CMW2805	AF343705	Lg									Jacobs et al. 2001
	CMW2805	AY544620	-1	AY263189	-2	AY544643	RE1	AY544602	RA1			Lee et al. 2003; Lim et al. 2004
	CMW2805	AY553396	RI1	AY534944	RB1	AY536190	-2					Jacobs et al. 2004
	CMW2805	#DQ294398	-5	DQ296118	Gp							Zipfel et al. 2006
***L. longiclavatum*: ex-type isolate**	[T]CBS120207 (=CMW20607 =SL-Kw1436)	AY816686	LI1	AY288934	LB1	**JF798467**	LE2	AY816679	LA5	**JF798492**	LU1	Lee et al. 2005; present study
***L. longiclavatum*: other isolates**	CMW20608 (=SL-Kp11)	AY816687	LI1	AY816712	LB1	**JF798468**	LE2	AY816680	LA1	**JF798493**	LU1	Lee et al. 2005; present study

Species	Isolate no	GenBank Acc. no.										[2]Reference
		ITS2-LSU	[1]HT	β-tubulin	HT	EF-1α	HT	Actin	HT	Anonymous locus	HT	
***L. longiclavatum*: other isolates**	CBS120208 (=CMW20609 =SL-Pw5)	AY816689	LI1	AY288935	LB1	**JF798469**	LE2	AY816682	LA1	**JF798494**	LU1	Lee et al. 2005; Roe et al. 2010
	SL-W001	AY816688	LI1					AY816681	LA1			
	C187	AY816690	LI1					AY816683	LA4			
	UAMH 11013	GU370276	LI1	GU370190	LB1	GU370233	LE2	GU370147	LA1	GU370319	LU1	Roe et al. 2010
	UAMH 11014	GU370282	LI1	GU370196	LB1	GU370239	LE1	GU370153	LA1	GU370325	LU1	Roe et al. 2010
	UAMH 11015	GU370275	LI1	GU370189	LB1	GU370232	LE1	GU370146	LA1	GU370318	LU1	Roe et al. 2010
	UAMH 11016	GU370277	LI1	GU370191	LB1	GU370234	LE1	GU370148	LA1	GU370320	LU1	Roe et al. 2010
	UAMH 11017	GU370279	LI1	GU370193	LB1	GU370236	LE2	GU370150	LA1	GU370322	LU1	Roe et al. 2010
	UAMH 11018	GU370297	LI1	GU370211	LB1	GU370254	LE2	GU370168	LA1	GU370340	LU1	Roe et al. 2010
	UAMH 11019	GU370299	LI1	GU370213	LB1	GU370256	LE1	GU370170	LA1	GU370342	LU1	Roe et al. 2010
	UAMH 11020	GU370300	LI1	GU370214	LB1	GU370257	LE2	GU370171	LA2	GU370343	LU1	Roe et al. 2010
	UL02G23	GU370262	LI1	GU370176	LB1	GU370219	LE3	GU370133	LA3	GU370305	LU1	Roe et al. 2010
	UL04G17	GU370263	LI1	GU370177	LB1	GU370220	LE3	GU370134	LA1	GU370306	LU1	Roe et al. 2010
***L. pyrinum*: ex-type isolate**	[T]CMW169 (=ATCC34943 =CBS 119897)	AF343689	La									Jacobs et al. 2001
	[T]CMW169 (=ATCC34943 =CBS119897)	DQ062072	PI1	DQ062006	PB1	DQ062039	PE1					Zhou et al. 2008
	[T]CMW509 (=ATCC34943 =CBS120181)	AY553414	PI1	AY534962	PB1	AY536208	-3					Jacobs et al. 2004; Zhou et al. 2008
[2]***L. pyrinum* A**	DLS879	AY544604	-2	AY263185	-7	AY544627	-7	AY544586				Lee et al. 2003; Lim et al. 2004
	CMW3889 (=DLS879)	AY544605	-2	AY544621	-7	AY544628	PE1	AY544587				Lim et al. 2004
***L. terebrantis:* ex-type isolate**	[T]CBS337.70 (=CMW29841)	**JF798477**	SI1	**JF798459**	SB1	**JF798470**	SE1	**JF798483**	SA1	**JF798495**	SU1	present study
	[T]CMW9	AF343698	Lp									Jacobs et al. 2001
	[T]CMW9	AY553384	Lp	AY534932	Lp	AY536178	Lp					Jacobs et al. 2004
	[T]CMW9			EU652698	-2	EU652700	-10					Zhou et al. 2008
	[T]CMW9a	EU652697	Lp	EU652699	-2	EU652701	-10					Zhou et al. 2008
	[T]CMW663	EU785383	-2	EU785349	-7	EU785412	-18					Lu et al. 2009a
	[T]CMW663	Contaminated										present study
	[T]MUCL47242	EU296777	SI1	EU296784	SB1	EU296791	-2					Lu et al. 2008, 2009b
	[T]MUCL47242 (=CMW29991)	**=JF798477**	SI1	**=JF798459**	SB1	**=JF798470**	SE1	**=JF798483**	SA1	**=JF798495**	SU1	present study

Species	Isolate no	GenBank Acc. no.										[2]Reference
		ITS2-LSU	[1]HT	β-tubulin	HT	EF-1α	HT	Actin	HT	Anonymous locus	HT	
***L. terebrantis*: other isolates**	ATCC58098 (=CMW30731)	**JF798476**	SI2	**JF798460**	SB1	**JF798471**	SE2	**JF798484**	SA1	**JF798496**	SU2	present study
	CMW2814 (=CBS115209)	EU785385	-1	EU785354	-6	EU785406	-17					Lu et al. 2009a
	CMW11 (=CBS298.85)	EU785386	-1	EU785348	-6	EU785403	-17					Lu et al. 2009a
***L. wingfieldii:* ex-type isolate**	[T]CMW2096	AF343684	La									Jacobs et al. 2001
	[T]CMW2096	AY553398	WI1	AY534946	WB1	AY536192	WE1			**JF798498**	WU1	Jacobs et al. 2004; present study
	[T]CMW2096	AY707205	WI1	AY707191	WB1			AY707178	WA1			Kim et al. 2005
***L. wingfieldii:* other isolates**	CMW2095	AY553400	WI1	AY534948	WB1	AY536194	WE1			**JF798497**	WU1	Jacobs et al. 2004; present study
	CMW2095	AY707204	WI1	AY707190	WB1			AY707177	WA1			Kim et al. 2005
	CMW2019	AY553399	WI1	AY534947	WB1	AY536193	WE2					Jacobs et al. 2004
	CMW10224	AY553401	WI1	AY534949	WB1	AY536195	WE1					Jacobs et al. 2004
***Leptographium* sp. X** (as *L. terebrantis* in previous publications)	AU156-12-13	AY544609	TI2	AY544623	TB5	AY544632	TE6	AY544591	TA7			Lim et al. 2004
	AU98Pr2-155	AY544608	TI2	AY544622	TB1	AY544631	TE1	AY544590	TA1			Lim et al. 2004; Lee et al. 2005;
	C418	AY544607	TI2	AY263191	TB1	AY544630	TE1	AY544589	TA2			Six et al. 2003; Lee et al. 2003, 2005; Lim et al. 2004; Kim et al. 2005; Roe et al. 2010
	LPWYLT-1			AY267826	TB1							Lee et al. 2003
	MY23AW3			AY672911	TB1							Kim et al. 2005
	SL-A57			DQ118421	TB1							Lee et al. unpubl.
	UAMH9722	AY544606	TI2	AY263192	TB1	AY544629	TE1	AY544588	TA1			Lee et al. 2003, 2005; Lim et al. 2004
	UAMH 11000	GU370272	TI1	GU370186	TB1	GU370229	TE1	GU370143	TA2	GU370315	TU1	Roe et al. 2010
	UAMH 11001	GU370292	TI1	GU370206	TB1	GU370249	TE3	GU370163	TA1	GU370335	TU2	Roe et al. 2010
	UAMH 11002	GU370283	TI2	GU370197	TB2	GU370240	TE4	GU370154	TA2	GU370326	TU1	Roe et al. 2010
	UAMH 11003	GU370284	TI1	GU370198	TB1	GU370241	TE5	GU370155	TA4	GU370327	TU1	Roe et al. 2010
	UAMH 11004	GU370281	TI2	GU370195	TB3	GU370238	TE1	GU370152	TA6	GU370324	TU1	Roe et al. 2010
	UAMH 11005	GU370285	TI1	GU370199	TB2	GU370242	TE1	GU370156	TA2	GU370328	TU2	Roe et al. 2010
	UAMH 11006	GU370294	TI1	GU370208	TB1	GU370251	TE3	GU370165	TA3	GU370337	TU1	Roe et al. 2010
	UAMH 11007	GU370295	TI1	GU370209	TB1	GU370252	TE1	GU370166	TA5	GU370338	TU1	Roe et al. 2010
	UC03DL14	GU370268	TI1	GU370182	TB1	GU370225	TE1	GU370139	TA1	GU370311	TU1	Roe et al. 2010
	UC01G02	GU370269	TI2	GU370183	TB2	GU370226	TE1	GU370140	TA1	GU370312	TU2	Roe et al. 2010
	UC01DL03	GU370270	TI2	GU370184	TB2	GU370227	TE2	GU370141	TA1	GU370313	TU2	Roe et al. 2010

[1] 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 LA3 =*L. longiclavatum* actin haplotype 3, etc. The number of bp differences in which ambiguous sequences differ from reliable sequences of the same isolate are indicated with a – sign. In some cases sequences in GenBank represent other species that are abbreviated as follows: *La=Leptographium abietinum; Lp=L. procerum; Lg=L. guttulatum; Gp=Grosmannia piceaperda*

[2] References to studies in which these isolates were used in phylogenetic analyses.

[3] The isolate DLS879 most probably represents a species distinct from the true *L. pyrinum*.

[T] Ex type isolates.

[G] Isolate used for whole genome sequencing (DiGuistini et al. 2009).
Only ITS2 sequences were available, excluding the LSU fragment.
* Only LSU sequences were available, excluding the ITS fragment.

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