Taxonomic Study of the *Grosmannia piceiperda* Complex based on Molecular Phylogeny and Morphology

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Contents

Chapter 1 Introduction

1.1. Taxonomic history of the genera *Grosmannia* and *Leptographium*

The genus *Grosmannia* Goid. is belonging to Ophiostomataceae Nannf. emend. Z.W. de Beer, Seifert & M.J. Wingf., Ophiostomatales Benny & Kimbr. emend. Z.W. de Beer, Seifert & M.J. Wingf., Sordariomycetes, Ascomycota (De Beer et al. 2013a, b). This genus is characterized by black perithecia with or without necks and with *Leptographium* Lagerb. & Melin asexual morph, which have dark pigmented erect conidiophores giving rise to series of branched conidiogenous apparatus produced conidia in slimy masses (Jacobs and Wingfield 2001; Zipfel et al. 2006). Currently, 84 species are recognized in these genera, 34 species in the *Grosmannia* and 50 species in the *Leptographium* (De Beer et al. 2013a). Most species in these genera cause sap stain of conifer timber and are vectored by bark beetles (Coleoptera: Curculionidae, Scolytinae) (Harrington and Cobb 1988; Wingfield et al. 1993; Jacobs and Wingfield 2001; Six and Wingfield 2011).

These genera have had complex taxonomic history intertwined with the genera *Ophiostoma* Syd. & P. Syd. and *Ceratocystis* Ellis & Halst., which are morphologically and ecologically similar. Lagerberg et al. (1927) described the genus *Leptographium* based on the single species *L. lundbergii* Lagerb. & Melin discovered in blue-stained timber of *Pinus silvestris* in Sweden. Then, Goidànich (1936) established the genus *Grosmannia* for species with *Leptographium* (as the genus *Scoplaria* Preuss at that time) asexual morph that had previously been placed in the genus *Ophiostoma*. The genus *Ophiostoma* was established by Sydow and Sydow (1919) based on the type species *O. piliferum* (Fr.) Syd. & P. Syd. and 11 other species. However, Goidànich (1936) did not recognize the genus *Leptographium*, and reduced it to a synonym of

Scoplaria. Unfortunately, the name *Scoplaria* was a homonym of a scientific name already in used for a plant, and the name *Leptographium* was reinstated by Shaw and Hubert (1952). However, Siemaszko (1939) reduced the genus *Grosmannia* to a synonym of *Ophiostoma*, because *Grosmannia ips* (Rumbold) Goid., one of the species transferred into *Grosmannia*, had a *Graphium* Corda asexual morph together with the *Leptographium* asexual morph.

Bakshi (1951) conducted a taxonomic examination of the sap-staining fungi and related genera (*Ceratostomella* Sacc., *Ceratocystis, Endoconidiophora* Münch, *Grosmannia, Linostoma* Höhn., *Ophiostoma,* and *Rostrella* Zimm). The genus *Ceratostomella* was established by Saccardo (1878). Then, Halsted (1890) described the genus *Ceratocystis* based on the species *C. fimbriata* Ellis & Halst. However, Saccardo (1892) reduced the genus *Ceratocystis* to a synonym of the coelomycete genus *Sphaeronaema* Fr., because he misidentified the ascospores that are produced from evanescent asci as conidia. Münch (1907) established the genus *Endoconidiophora* based on the type species *E. coerulescens* Münch with phialidic conidia, although its sexual morph was morphologically similar to the genus *Ceratostomella*. Von Höhnel (1918) redefined the genus *Ceratostomella* as having persistent asci and no ostiolar hyphae, and established the genus *Linostoma* for species forming evanescent asci and ostiolar hyphae. However, *Linostoma* was placed within the genus *Ophiostoma* by Sydow and Sydow (1919), because this genus was a homonym for a genus of plant. With this taxonomic background, Bakshi (1951) revealed that the genera producing evanescent asci should be treated as synonyms of *Ceratocystis*, with the exception of *Ceratostomella*. Moreau (1952) accepted Bakshi's conclusion and transferred 31 species to the genus *Ceratocystis*. Von Arx (1952) and von Arx and Müller (1954) called for conservation of the genus *Ophiostoma*. However, most subsequent studies (Hunt 1956;

Griffin 1968; Olchowecki and Reid 1974; Upadhyay 1981) accepted the proposal made by Bakshi (1951).

On the other hand, von Arx (1974) proposed separation of *Ceratocystis* and *Ophiostoma* based on asexual morph structures, with the former genus having a *Chalara* (Corda) Rabenh. asexual morph and the latter genus having other asexual morphs. This classification was also supported by biochemical characters. *Ceratocystis* does not have cellulose in its cell wall, while *Ophiostoma* does (Rosinski and Campana 1964; Smith et al. 1967; Jewell 1974). In terms of tolerance to cycloheximide, an antibiotic that disrupts protein synthesis, Harrington (1981) revealed that species of *Ophiostoma* could grow in its presence, while species of *Ceratocystis* were clearly inhibited. Based on these results, some subsequent researchers treated *Ceratocystis* and *Ophiostoma* as distinct genera (De Hoog and Scheffer 1984; Von Arx and van der Walt 1987). At that time, *Grosmannia* was treated as a synonym of *Ophiostoma*.

In the 1990s, molecular phylogenetic studies began being carried out. Hausner et al. (1993) and Spatafora and Blackwell (1994) conducted phylogenetic analysis using small subunit (SSU) or large subunit (LSU) of nuclear ribosomal DNA gene region (rDAN) sequences, and revealed that *Ophiostoma* and *Ceratocystis* were distantly related. The genus *Ophiostoma* was placed in Ophiostomatales, while *Ceratocystis* was in Microascales. Although these genera were found to be phylogenetically distinct at the order-level, they commonly co-occur in niches associated with insects. Therefore, the term “ophiostomatoid fungi” was created to group them for convenience (Wingfield et al. 1993). Moreover, Hausner et al. (1993) reduced *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr. to a synonym of *Ophiostoma*, because *Ceratocystiopsis* fell into the same clade as species of *Ophiostoma* in a phylogenetic analysis using SSU and LSU rDNA. Wingfield (1993) also proposed that *Ceratocystiopsis* and *Ophiostoma* should be treated

as one homogeneous group.

Zipfel et al. (2006) conducted a phylogenetic analysis of ophiostomatoid fungi using LSU rDNA and partial β-tubulin gene sequences. They revealed that *Grosmannia* and *Ceratocystiopsis*, which had been treated as synonyms of *Ophiostoma*, were separate genera, and could be distinguished based on sexual and asexual morph structures. Then, Six et al. (2011) carried out a phylogenetic analysis of *Grosmannia* and *Leptographium* species using ITS2 and LSU rDNA, and showed that seven species complexes exist in this genus (*G. clavigera* complex, *G. olivacea* complex, *G. penicillata* complex, *G. piceiperda* complex, *G. wageneri* complex, *L. lundbergii* complex, and *L. procerum* complex). Within genera, the term "species complex" is used to group species that are phylogenetically very closely related. Linnakoski et al. (2012a) recognized two more species complexes in this genus (*G. galeiformis* complex and *G. serpens* complex) based on ITS2 and LSU analysis.

In the most recent phylogenetic analysis of *Grosmannia* and *Leptographium* species, de Beer and Wingfield (2013) showed that monophyly of *Grosmannia* and *Leptographium* were not supported. They also revealed that one more species complex (*Raffaelea sulphurea* complex) and one genus (*Esteya* J.Y. Liou, J.Y. Shih & Tzean) that are morphologically distinct genera included between the genera *Grosmannia* and *Leptographium*. In light of these results, de Beer and Wingfield (2013) proposed that these genera should be collectively referred to as *Leptographium* sensu lato (s.l.).

1.2. Taxonomic history of the *Grosmannia piceiperda* complex

The *Grosmannia piceiperda* complex is one species complex within *Leptographium* s.l. The four species currently recognized in this complex are *G. aenigmatica* (K. Jacobs, M.J. Wingf. & Yamaoka) Zipfel, Z.W. de Beer & M.J. Wingf., *G. europhioides* (E.F. Wright & Cain) Zipfel, Z.W. de Beer & M.J. Wingf., *G. laricis* (Van der Westh., Yamaoka & M.J. Wingf.) Zipfel, Z.W. de Beer & M.J. Wingf., and *G. piceiperda* (Rumbold) Goid. (De Beer and Wingfield 2013). This complex is characterized by cucullate ascospores and a typical leptographium-like asexual morph (Linnakoski et al. 2012a; De Beer and Wingfield 2013).

Grosmannia piceiperda was first described by Rumbold (1936) as *Ceratostomella piceaperdum*, a blue-stain fungus on *Picea glauca* infested with the bark beetle *Dendroctonus rufipennis* (as *D. piceaperda*) in Canada. However, Rumbold (1936) did not designate the holotype. After that, BPI240-TRC (= BPI 595981) was designated as the lectotype of this species by Hunt (1956). Wright and Cain (1961) described *G. europhioides* as *Ceratocystis europhioides* based on isolates from *Picea* spp. and *Pinus* spp. in Canada (holotype: TRTC33700; *Picea mariana*, York Co., Ontario, Canada, May 10, 1958). In the original description, *G. europhioides* was morphologically similar to *G. piceiperda*. However, Wright and Cain (1961) did not compare *G. piceiperda* and *G. europhioides*.

Upadhyay (1981) conducted taxonomic examinations of *G. piceiperda* and *G. europhioides* using type materials of both species, and concluded that *G. europhioides* was a synonym of *G. piceiperda*. His conclusion was accepted in later studies such as Hutchison and Reid (1988) and Jacobs et al. (2000). On the other hand, several studies (Solheim 1986; Harrington and Cobb 1988; Yamaoka et al. 1997, 2004a, 2009) reported *G. europhioides* as a distinct species from *G. piceiperda*. Without the taxonomic

problem being solved, both species have been reported in association with conifers (mainly *Picea* spp. and *Pinus* spp.) infested by various species of bark beetles in North America (Solheim and Krokene 1998; Alamouti 2007, 2013), Europe (Solheim 1992; Kirisits 2004; Viiri and Lieutier 2004; Jankowiak 2006; Linnakoski et al. 2012a, b), New Zealand (Hutchison and Reid 1988), and Japan (Ohtaka et al. 2002b; Yamaoka et al. 2004a, 2009).

One of the remaining two species in the *G. piceiperda* complex, *G. laricis* was described by Van der Westhuizen et al. (1995) from samples in which *Ips subelongatus* (as *I. cembrae*) infested *Larix kaempferi* in Japan. This species is characterized by curved ascospores instead of cucullate ascospores. *Grosmannia aenigmatica* was described by Jacobs et al. (1998) based on cultures isolated in a previous study by Yamaoka et al. (1997) from *Ips typographus japonicus* infestation in *Picea jezoensis* var. *jezoensis* in Japan and originally identified as *Ophiostoma europhioides*. This species is characterized by perithecia with short necks. *Grosmannia laricis* and *G. aenigmatica* have been reported only from Japan and are considered distinct species (Linnakoski et al. 2012a; De Beer et al. 2013a).

A recent phylogenetic study based on β-tubulin and translation elongation factor-1 alpha (EF-1α) genes showed that the *G. piceiperda* complex could be separated into seven lineages (Linnakoski et al. 2012a). Two of these lineages are *G. laricis* and *G. aenigmatica*. Isolates identified as *G. piceiperda* or *G. europhioides* were included in the remaining five lineages. These lineages were composed of three North American lineages, one Russian lineage, and one European lineage. However, Linnakoski et al. (2012a) could not clarify the taxonomic treatment of *G. piceiperda* and *G. europhioides*, because they did not examine type specimens or authentic isolates of these species.

Grosmannia piceiperda and *G. europhioides* have been recognized as forest

pathogens, especially in Europe, because they have been consistently isolated from primary infestations of the bark beetle *Ips typographus*. For example, Jankowiak and Kolařík (2010) revealed that the *G. piceiperda* isolated from *Tetropium* spp. (cerambycid beetle) infesting *Picea abies* in Poland was pathogenic and killed two-year-old seedlings in an inoculation test. As such, these species have been recognized as potential pathogens. Polyphyly of *G. piceiperda* has been suggested in which each lineage has a different potential for pathogenicity to conifers. Therefore, taxonomic examination of the *G. piceiperda* complex is necessary for understanding forest pathology.

Some isolates that appeared to belong to the *G. piceiperda* complex were collected in previous studies from bark beetles infesting conifers in Japan (Yamaoka et al. 1998, 2004a, 2009). Yamaoka et al. (1998) examined ophiostomatoid fungi associated with *Ips subelongatus* (as *I. cembrae*) infesting *Larix kaempferi* in Japan, and isolated unidentified fungi that were morphologically similar to *G. europhioides* (as *Ophiostoma* sp.). Yamaoka et al. (2004a) reported isolates that were morphologically similar to *G. europhioides* (as *Ophiostoma* sp. D) associated with bark beetles infesting *Abies* spp. in Japan. Yamaoka et al. (2009) also examined ophiostomatoid fungi in association with *Ips subelongatus* infesting *L. kaempferi*, and reported species morphologically similar to *G. europhioides* (as *Grosmannia* sp. L2). As mentioned above, these Japanese isolates were morphologically similar and seemed to belong to the *G. piceiperda* complex. However, the phylogenetic relationship of these isolates remains unknown and their taxonomic positions are also unresolved. Consequently, examination of the Japanese isolates is necessary in order to clarify the taxonomy of the *G. piceiperda* complex.

1.3. Objective of this study

The purpose of this study was to clarify the phylogenetic relationships and taxonomic positions of species in the *Grosmannia piceiperda* complex based on molecular phylogenetic analysis and morphological observation.

Fifty-three Japanese isolates resembling species in the *G. piceiperda* complex that were isolated from several species of bark beetles infesting conifers in previous studies were used in this study. An additional thirty-eight isolates in the *G. piceiperda* complex isolated in North America, Europe, Russia and New Zealand were obtained from fungal culture collections.

The herbarium specimens of *G. piceiperda*, including the lectotype (BPI595981) and two related specimens (BPI595980 and BPI595982), and an ex-type isolate (NoF555) considered to be related to type specimen of *G. europhioides*, were obtained from the U.S. National Fungus Collections and the Fungus Culture Collection of the Northern Forestry Centre, respectively.

Chapter 2 Materials and Methods

2.1. Fungal isolate

A total of 91 isolates resembling species of the *G. piceiperda* complex were used in this study (Table 2.1). Fifty-three isolates among them are Japanese isolates that were obtained from 15 different bark beetle species (in seven genera) infesting 11 species of conifers (four genera) collected between 1989 and 2008 in Hokkaido, Iwate, Tochigi, Yamanashi, and Nagano prefectures in Japan. Thirty-eight of remaining isolates are non-Japanese isolates including six North American isolates, two Russian isolates, 23 European isolates, and five New Zealander isolates. One of six North American isolate, NoF555 (isolated from *Picea mariana*, York Co., Ontario, Canada, May 10, 1958, by Wright and Cain) that was deposited in the fungus culture collection of the Northern Forestry Centre, Edmonton, Alberta, Canada, was considered as an ex-type isolate of *G. europhioides*, because there was a note that the isolate was obtained from type material. And also, two isolates (CMW2428 and CMW2868) of *Grosmannia huntii* (R.C. Rob.) Zipfel et al. were used for outgroup in phylogenetic analyses.

Three specimens deposited in U.S. National Fungus Collections as *Ceratostomella piceaperda* were loaned. One of the specimens, BPI595981 which was collected on *Picea glauca*, Nova Scotia, St. Peters, Cape Breton, Canada, June 1930 by Rumbold who is author of *G. piceiperda*, was designated as lectotype by Hunt (1956). The other two specimens, i.e. BPI595980: isolated from *Dendroctonus rufipennis* in *Picea glauca*, Nova Scotia, St. Peters, Cape Breton, Canada, June 1930, by Blach R. E.; BPI595982: isolated from *Dendroctonus rufipennis* in *Picea glauca*, Nova Scotia, St. Peters, Cape Breton, Canada, June 1930, by Blach R. E., were not collected by Rumbold, but locality and date of collection and host were the same as lectotype.

2.2. Molecular phylogenetic analysis

2.2.1. DNA extraction, PCR amplification, and sequencing

All the 93 isolates and three dried specimens (BPI595980, 595981, and 595982) were used for molecular phylogenetic analyses. Isolates were cultured on 2% malt extract Ebios agar [2% MEBA; 20 g Difco malt extract, 1 g Ebios (Brewer's yeast preparation; Asahi food and healthcare Co., Tokyo, Japan), 15 g agar, 1000 ml distilled water] for two weeks. DNA was extracted from mycelium colony grown on MEBA or a few perithecia on dried specimens of *G. piceiperda*. Mycelium or perithecium was suspended in 50 µl of DNA extraction buffer [10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% sodium dodecyl sulfate (SDS), 0.01% Proteinase K]. The mixture was incubated at 37 °C for 60 min., then at 95 °C for 10 min. After centrifuged at 15,000 rpm for 2 min., the aqueous phase was pipetted out and transferred to a new tube to collect total DNA.

The internal transcribed spacer (ITS) and large subunit (LSU) ribosomal RNA gene regions (rDNA) and portions of the actin (ACT), β-tubulin, and translation elongation factor 1-alpha (EF-1α) genes were amplified by polymerase chain reaction (PCR) using the primers ITS5 and ITS4 (White et al. 1990) for ITS; ITS3 (White et al. 1990) and LR3 (Vilgalys and Hester 1990) for ITS2-LSU; NL1 and NL4 (O'Donnell 1993) for LSU; Lepact F and Lepact R (Lim et al. 2004) for ACT; T10 (O'Donnell and Cigelnik 1997) and BT12 (Kim et al. 2003) or Bt2b (Glass and Donaldson 1995) for β-tubulin; and EF-1F and EF-2R (Jacobs et al. 2004) for EF-1α (Table 2.2). PCR reactions were performed using 25 µl reaction volumes each containing: 1 µl genomic DNA, 0.125 µl (0.25 unit) of ExTaq DNA polymerase (TaKaRa, Tokyo, Japan), 2 µl deoxynucleoside triphosphate (dNTP) mixture containing 2.5 mM of each dNTP, 2.5 µl ExTaq reaction

buffer containing 2 mM Mg^{2+}, 2.5 μl (0.2 μM) of each primer, and adding 14.375 μl distilled water to get 25 μl reaction volumes. Amplification was performed in a PCR System 9700 (Applied Biosystems, Foster City, CA, USA) according to the following protocol; initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing temperature and time of each primer sets were shown in Table 2.2, extension at 72 °C for 1 min, and a final extension at 72 °C for 8 min.

PCR products were electrophoresed in 1% agarose gels stained with ethidium bromide and visualized under UV light. PCR products were purified using Wizard® SV Gel and PCR Clean-Up Kit (Promega, Madison, WI, USA) following the manufacturer's instructions. Sequencing was performed using the BigDyeTM Terminator Cycle Sequencing Kit v.3.1 (Applied Biosystems) following the manufacturer's instructions using both forward and reverse primers and analyzed on an ABI 3500xL Genetic Analyzer (Applied Biosystems). Sequences were assembled and edited with BioEdit ver. 7.1.9 (Hall 1999), and were deposited in GenBank (Table 2.1).

2.2.2. Phylogenetic analyses

Sequences were aligned using the online version MAFFT 7 (http://mafft.cbrc.jp/alignment/server/) (Katoh and Standly 2013) with the G-INS-i option for ITS2–LSU rDNA, ACT, β-tubulin, and EF-1α genes. Sequences were manually edited when necessary using BioEdit ver. 7.1.9 (Hall 1999).

Phylogenetic trees were inferred with maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) analyses. All characters were equally weighted and sites including gap were deleted. The most appropriate substitution models for BI analyses were determined by comparing different evolutionary models via the Bayesian information criterion (BIC; Schwarz 1978) with Kakusan 4 nucleotide

substitution model selection program (Tanabe 2007, 2011). The combined dataset was tested for incongruence with the partition homogeneity test (PHT) as implemented in PAUP* version 4.0b10 (Swofford 2003).

The MP analyses were performed using PAUP* version 4.0b10 (Swofford 2003). Bootstrap analyses were performed with 1000 random addition replicates with tree bisection-reconnection branch swapping. The best tree topology of MP trees was conducted using the Kishino–Hasegawa likelihood test (Kishino and Hasegawa 1989) on PAUP*. The ML analyses were performed in RAxML 8.0.2 software (Stamatakis 2014) using the GTR + Gamma model of evolution and 1000 bootstrap replicates. The BI analyses were performed in MrBayes5D v.3.1.2.2012.12.13 (Tanabe 2008) that is modified version of MrBayes v.3.1.2 (Ronquist and Huelsenbeck 2003) to estimate the posterior probabilities of tree topologies with Markov Chain Monte Carlo (MCMC) searches. Each analysis was performed for 10,000,000 generations and trees were sampled every 100 generations. Convergence of the MCMC procedure was assessed by calculating the effective sampling size using Tracer 1.6 (Rambaut et al. 2014). The support of nodes was tested based on posterior probabilities obtained from a 50% majority rule consensus after deleting the trees in the burn-in period. Outgroups used were *Leptographium leptographioides* (R.W. Davidson) Zipfel et al. for ITS2 and LSU analysis, and *Leptographium huntii* for β-tubulin, EF-1α and combined dataset analyses. Reference sequences were obtained from GenBank (Table 2.3).

2.3. Morphological and growth studies

For morphological comparisons, representative isolates (see Table 2.1) were selected and incubated on 2% MEBA plates at 16 °C in darkness. After two-weeks of incubation, two pieces (about 1 cm × 3 cm × 5 mm) of autoclaved bark from *Larix kaempferi* were placed on the surface of the fungal colony to stimulate the development of asexual and sexual morphs. The asexual structures on the bark were examined after two weeks. After two more months, sexual structures on the bark were examined. These structures were mounted on glass slides with Shear's fluid (Chupp 1940) for conidiophores, polyvinyl alcohol (Omar et al. 1979) for ascomata, or 1% lacto-fuchsin for ascospores and conidia. The slides were observed under a differential interference contrast microscope (Leica DMLB: Leica microsystems, Wetzlar, Germany).

Dimensions of structures were measured and averages and ranges were calculated. Thirty measurements for each morphologically relevant structure were made. Tukey's HSD (honest significant different) test was performed to test for morphological differences between lineages in the *G. piceiperda* complex using the software package SPSS (SPSS Japan, Tokyo, Japan).

In order to determine range and optimal temperatures for growth in culture, two isolates for each lineage were selected. Disks of agar were cut from the actively growing margins of colonies on 2% MEBA for one week with a sterile cork borer (5 mm diam.) and transferred to the centers of 90 mm plates of 2% MEBA. Incubation was carried out under the darkness at 5−35 °C (5 °C intervals). Three replicates were conducted for each isolate. Average diameters of each culture were measured at 4, 6, 10 and 14 days of incubation until the mycelial growth reached the edges of the plates.

Table 2.1 – Isolates used in this study.

Species / lineage	Isolate no. [a]	Substrate		Locality	Date of Collection	GenBank accession no.			
		Bark beetle	Host			ITS2–LSU	β-tubulin	EF-1α	ACT
Grosmannia aenigmatica	YCC-72 (= NBRC111485) [b, c]	*Ips typographus japonicus*	*Picea jezoensis* var. *jezoensis*	Japan, Hokkaido	Aug 1989	○	○	○	○
	YCC-111 (= JCM9360) [b, c]	*I. typographus japonicus*	*P. jezoensis* var. *jezoensis*	Japan, Hokkaido	Jun 1990	○	○	○	○
G. huntii	CMW2824		*Pinus* sp.	USA	–	○	○	○	○
	CMW2868		*Pin. strobus*	USA	Jan 1980	○	○	○	○
	UAMH9784 [e]		*Pin. radiata*	New Zealand	1952	–	○	○	–
	UAMH9787 [e]		*Pin. nigra*	New Zealand	Jun 1982	–	○	○	–
	UAMH9788 [e]		*Pin. radiata*	New Zealand	May 1982	–	○	○	–
	UAMH9809 [e]		*Pin. radiata*	New Zealand	Feb 1988	–	○	○	–
	UAMH9964		*Pin. radiata*	New Zealand	Feb 1988	–	○	–	–
G. laricis	YCC-277 [b, c]	*I. subelongatus*	*Larix kaempferi*	Japan, Nagano	Jul 1989	○	○	○	○
	YCC-349 [b]	*I. subelongatus*	*L. kaempferi*	Japan, Nagano	Jun 1999	○	○	○	○
	YCC-389	*I. subelongatus*	*L. kaempferi*	Japan, Iwate	Jul 1999	○	○	○	○
	YCC-440 (= NBRC111486) [c]	*Dryocoetes hectographus*	*L. kaempferi*	Japan, Tochigi	Jul 2000	○	○	○	○
	YCC-441	*D. hectographus*	*L. kaempferi*	Japan, Tochigi	Jul 2000	○	○	○	○
	YCC-488 [b, c]	*I. subelongatus*	*L. kaempferi*	Japan, Tochigi	Jul 2001	○	○	○	○
	YCC-590 (= NBRC104091)	*D. pini*	*L. kaempferi*	Japan, Nagano	Jul 2006	○	○	○	○
G. piceiperda B	CMW448 (= CBS444.69)		*Pic. glauca*	USA	Jul 1965	○	○	○	○
	CMW452 (= CBS275.65) [b, c]	*Dryocoetes* sp.	*Pseudotsuga menziesii*	USA	Jul 1962	○	○	○	○

Table 2.1 – (continued)

Species / lineage	Isolate no. [a]	Substrate		Locality	Date of	GenBank accession no.			
		Bark beetle	Host		Collection	ITS2–LSU	β-tubulin	EF-1α	ACT
G. piceiperda B	CMW2811 [b, c]		*Pic. rubens*	USA	Aug 1987	○	○	○	○
	NoF555		*Pic. mariana*	Canada	May 1958	○	○	○	○
G. piceiperda C	CMW446 (= CBS229.83)	*I. typographus*	*Pic. abies*	Norway	Jul 1980	○	○	○	○
	CMW3312 [b]	*I. typographus*	*Pic. abies*	Austria	1992	○	○	○	○
	CMW3313	*I. typographus*	*Pic. abies*	Austria	1993	○	○	○	○
	CMW3316 [b, c]	*Hylurgops glabratus*	*Pic. abies*	Austria	1993	○	○	○	○
	CMW3321 [b, c]		*Pic. abies*	Austria	1993	○	○	○	○
	CMW3322 [b, c]		*Pic. abies*	Norway	1992	○	○	○	○
	CMW3324		*Pic. abies*	Austria	1993	○	○	○	○
	RL618	*I. typographus*	*Pic. abies*	Finland	–	○	○	○	○
	RL678 [b, c]	*I. typographus*	*Pic. abies*	Finland	–	○	○	○	○
	RL679 [b, c]	*I. typographus*	*Pic. abies*	Finland	–	○	○	○	○
	1980-67/22 [d]	*I. typographus*	*Pic. abies*	Norway	1980	○	○	○	○
	1991-67/3/2 [d]	*Pityogenes chalcographus*	*Pic. abies*	Norway	1991	○	○	○	○
	1993-280/13a [d]	*Polygraphus poligraphus*	*Pic. abies*	Norway	1993	○	○	–	–
	1994-194/4 [d]		*Pic. abies*	Norway	1994	○	○	○	○
	1995-593/101 [d]	*Hylastes cunicularius*		Norway	1995	○	○	○	○
	1995-593/136 [d]	*D. autographus*		Norway	1995	○	○	–	–
	1995-593/81 [d]	*Hyla. cunicularius*		Norway	1995	○	○	–	–

Table 2.1 – (continued)

Species / lineage	Isolate no. [a]	Substrate		Locality	Date of Collection	GenBank accession no.			
		Bark beetle	Host			ITS2–LSU	β-tubulin	EF-1α	ACT
G. piceiperda C	2004-50/6 [d]	*Orthotomicus laricis*		Norway	2004	○	○	–	–
	2004-53/1 [d]	*O. laricis*		Norway	2004	○	○	–	–
	2004-96/3 [d]	*D. autographus*		Norway	2004	○	○	○	○
	2004-187/2 [d]	*D. autographus*		Sweden	2004	○	○	–	–
	2006-60/1 [d]	*I. amitinus*		Finland	2006	○	○	○	○
	2004-233/1 [d]	*Dryocoetes* sp.		Montenegro	2004	○	○	–	–
	2004-256/11/1 [d]	*Orthotomicus* sp.		Montenegro	2004	○	○	–	–
	2004-559/1 [d]	*D. autographus*		Montenegro	2004	○	○	○	○
G. piceiperda D	YCC-242 (= JCM9361)	*I. typographus japonicus*	*Pic. jezoensis* var. *jezoensis*	Japan, Hokkaido	Jul 1991	○	○	○	○
	YCC-549 (= NBRC111503) [b,	*Hylu. transbaicalicus*	*Pin. strobus*	Japan, Nagano	Jul 2005	○	○	○	○
	YCC-593 [b, c]	*D. hectographus*	*L. kaempferi*	Japan, Nagano	Jul 2006	○	○	○	○
	YCC-625 [b, c]	*Cryphalus* sp.	*Pic. koyamae*	Japan, Nagano	Jul 2006	○	○	○	○
	YCC-626 [b]	*Polygraphus* sp.	*Pic. koyamae*	Japan, Nagano	Jul 2006	○	○	○	○
	YCC-631 [b, c]	*Tomicus piniperda*	*Pin. banksiana*	Japan, Nagano	Jul 2006	○	○	○	○
	YCC-694 [b]	*Hylu. transbaicalicus*	*Pin. densiflora*	Japan, Nagano	Jun 2008	○	○	○	○
	YCC-695 [b, c]	*Hylu. transbaicalicus*	*Pin. densiflora*	Japan, Nagano	Jun 2008	○	○	○	○
	YCC-710 [b]	*D. autographus*	*Pic. koyamae*	Japan, Nagano	Jun 2008	○	○	○	○
	YCC-711	*Hylu. transbaicalicus*	*Pic. koyamae*	Japan, Nagano	Jun 2008	○	○	○	○

Table 2.1 – (continued)

Species / lineage	Isolate no. [a]	Substrate		Locality	Date of Collection	GenBank accession no.			
		Bark beetle	Host			ITS2–LSU	β-tubulin	EF-1α	ACT
G. piceiperda D	YCC-724 [b, c]	*Hylu. transbaicalicus*	*Pin. parviflora* var. *pentaphylla* f. *laevis*	Japan, Nagano	Jun 2008	○	○	○	○
	YCC-733 [b]	*Hylu. transbaicalicus*	*Pic. glauca*	Japan, Nagano	Jun 2008	○	○	○	○
	YCC-734 (= NBRC111504) [b]	*C. fulvus*	*Pic. glauca*	Japan, Nagano	Jun 2008	○	○	○	○
	YCC-735 [b, c]	*Hylu. interstitialis*	*Pic. glauca*	Japan, Nagano	Jun 2008	○	○	○	○
	YCC-736 [b]	*C. fulvus*	*Pic. glauca*	Japan, Nagano	Jun 2008	○	○	○	○
	CMW36626 [b]	*I. typographus*	*Pic. abies*	Russia	Jul 2004	○	○	○	○
	CMW36627 [b]	*I. typographus*	*Pic. abies*	Russia	Jul 2004	○	○	○	○
G. piceiperda F	UAMH10656 [b, c]	*I. perturbatus*	*Pic. glauca*	Canada	May 2003	○	○	○	○
	UAMH10657 [b, c]	*D. affaber*	*Pic. engelmannii* × *Pic. glauca*	Canada	May 2003	○	○	○	○
J-1	YCC-348 (= NBRC111487) [b, c]	*I. subelongatus*	*L. kaempferi*	Japan, Nagano	Jun 1999	○	○	○	○
	YCC-614 [c]	*D. autographus*	*Abies veitchii*	Japan, Nagano	Jul 2006	○	○	○	○
	YCC-705 [b, c]	*Hylu. transbaicalicus*	*Pic. koyamae*	Japan, Nagano	Jun 2008	○	○	○	○
	YCC-723 (= NBRC111488) [b, c]	*Hylu. transbaicalicus*	*Pin. parviflora* var. *pentaphylla* f. *laevis*	Japan, Nagano	Jun 2008	○	○	○	○
J-2	YCC-312 (= NBRC111489) [b, c]	*D. baikalicus*	*L. kaempferi*	Japann Nagano	Oct 1998	○	○	○	○
	YCC-314 (= NBRC111490) [b, c]	*D. baikalicus*	*L. kaempferi*	Japan, Nagano	Oct 1998	○	○	○	○
J-3	YCC-495 (= NBRC111491) [b, c]	*I. subelongatus*	*L. kaempferi*	Japan, Tochigi	Jul 2001	○	○	○	○

Table 2.1 – (continued)

Species / lineage	Isolate no. [a]	Substrate		Locality	Date of	GenBank accession no.			
		Bark beetle	Host		Collection	ITS2–LSU	β-tubulin	EF-1α	ACT
J-3	YCC-496 [b, c]	*D. baikalicus*	*L. kaempferi*	Japan, Tochigi	Jul 2001	○	○	○	○
	YCC-497 (= NBRC111492) [b, c]	*D. autographus*	*L. kaempferi*	Japan, Tochigi	Jul 2001	○	○	○	○
J-4	YCC-318 (= NBRC111493) [b, c]	*I. subelongatus*	*L. kaempferi*	Japan, Nagano	May 1999	○	○	○	○
	YCC-399 [c]	*O. laricis*	*L. kaempferi*	Japan, Nagano	May 1999	○	○	○	○
	YCC-591 (= NBRC111494) [b]	*D. pini*	*L. kaempferi*	Japan, Nagano	Jul 2006	○	○	○	○
J-5	YCC-300 (= JCM9814) [b, c]	*I. subelongatus*	*L. kaempferi*	Japan, Yamanashi	Jul 1992	○	○	○	○
	YCC-416 [b, c]	*C. montanus*	*A. mariesii*	Japan, Tochigi	Aug 2000	○	○	○	○
	YCC-417 (= JCM11721) [b, c]	*D. hectographus*	*A. mariesii*	Japan, Tochigi	Aug 2000	○	○	○	○
	YCC-468 (= NBRC111495) [c]	*D. autographus*	*A. mariesii*	Japan, Tochigi	Jul 2001	○	○	○	○
	YCC-469 [b, c]	*D. autographus*	*A. mariesii*	Japan, Tochigi	Jul 2001	○	○	○	○
	YCC-615 [b, c]	*Pol. proximus*	*A. veitchii*	Japan, Nagano	Jul 2006	○	○	○	○
	YCC-679 (= NBRC111496) [b, c]	*Pol. proximus*	*A. veitchii*	Japan, Nagano	Jun 2008	○	○	○	○
	YCC-681	*Pol. proximus*	*A. veitchii*	Japan, Nagano	Jun 2008	○	○	○	○
J-6	YCC-432 [b]	*D. hectographus*	*A. mariesii*	Japan, Tochigi	Aug 2000	○	○	○	○
	YCC-433 (= NBRC111497) [b]	*D. hectographus*	*A. mariesii*	Japan, Tochigi	Aug 2000	○	○	○	○
	YCC-470 (= NBRC111498) [b]	*C. montanus*	*A. mariesii*	Japan, Tochigi	Jul 2001	○	○	○	○
J-7	YCC-452 (= NBRC111499) [b]	*D. hectographus*	*A. mariesii*	Japan, Tochigi	Aug 2000	○	○	○	○
	YCC-453	*Pol. proximus*	*A. mariesii*	Japan, Tochigi	Aug 2000	○	○	○	○
	YCC-455 (= NBRC111500) [b]	*Pol. proximus*	*A. mariesii*	Japan, Tochigi	Jul 2000	○	○	○	○

Table 2.1 – (continued)

Species / lineage	Isolate no. [a]	Substrate		Locality	Date of Collection	GenBank accession no.			
		Bark beetle	Host			ITS2–LSU	β-tubulin	EF-1α	ACT
J-7	YCC-501 [b]	*C. montanus*	*A. mariesii*	Japan, Tochigi	Jul 2001	○	○	○	○
J-8	YCC-507 (= NBRC111501) [b]	*D. hectographus*	*Tsuga diversifolia*	Japan, Tochigi	Jul 2001	○	○	○	○
	YCC-508 (= NBRC111502) [b]	*D. hectographus*	*T. diversifolia*	Japan, Tochigi	Jul 2001	○	○	○	○

[a] **CBS**: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. **CMW**: Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. **JCM**: Japan Collection of Microorganisms, RIKEN BioResource Center, Tsukuba, Japan. NBRC: NITE Biological Resource Center, Kisarazu, Japan. **RL**: Cultures of Riikka Linnakoski, Department of Forest Sciences, University of Helsinki, Finland. **UAMH**: Microfungus Collection and Herbarium, Devonian Botanic Garden, University of Alberta, Edmonton, Canada. **YCC**: Cultures of Yuichi Yamaoka, Culture collection of the Laboratory of Plant parasitic Mycology, Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Japan.

[b] Isolates used for morphological study of sexual morph.

[c] Isolates used for morphological study of asexual morph.

[d] Culture of Halvor Solheim, Norwegian Forest Research Institute, Norway.

[e] Isolates had been identified as the *G. piceiperda* at the time of acceptance.

Table 2.2 – Primers used in this study.

Primer name	Alignment (5’ – 3’)	Annealing		Reference
		Temperature	Time (second)	
ITS5	GGAAGTAAAAGTCGTAACAAGG	54	30	White et al. 1990
ITS4	TCCTCCGCTTATTGATATGC			White et al. 1990
ITS3	GCATCGATGAAGAACGCAGC	58	45	White et al. 1990
LR3	CCGTGTTTCAAGACGGG			Vilgalys and Hester 1990
NL1	GCATATCAATAAGCGGAGGAAAAG	57	30	O’Donnell 1993
NL4	GGTCCGTGTTTCAAGACGG			O’Donnell 1993
T10	ACGATAGGTTCACCTCCAGAC	55	60	O’Donnell and Cigelnik 1997
BT12	GTTGTCAATGCAGAAGGTCTCG			Kim et al. 2003
Bt2b [a]	ACCCTCAGTGTAGTGACCCTTGGC	60	30	Glass and Donaldson 1995
Lepact F	TACGTCGGTGACGAGGC	59	60	Lim et al. 2004
Lepact R	CAATGATCTTGACCTTCAT			Lim et al. 2004
EF-1F	TGCGGTGGTATCGACAAGCGT	60	50	Jacobs et al. 2004
EF-2R	AGCATGTTGTCGCCGTTGAAG			Jacobs et al. 2004

[a] The annealing temperature and time of Bt2b were shown the setting when were used in conjunction with the primer T10.

Table 2.3 – Sequence data obtained from GenBank used in this study.

Species / lineage	Isolate no. [a, b]	Substrate		Country	GenBank accession no.		
		Bark beetle	Host		ITS2-LSU	β-tubulin	EF-1α
Grosmannia abiocarpa	MUCL18351 [T]		*Picea engelmannii*	USA	AJ538339	–	–
G. aenigmatica	CMW2199 [T]	*Ips typographus japonicus*	*P. jezoensis*	Japan	AY553389	AY534937	–
G. aenigmatica	CMW2310	*I. typographus japonicus*	*P. jezoensis*	Japan	–	AY534938	AY536184
G. aenigmatica	CBS501.96	*I. typographus japonicus*	*P. jezoensis*	Japan	–	KF779131	–
G. alacris	CMW2844 [T]		*Pinus pinaster*	South Africa	JN135313	–	–
G. aoshimae	YCC607	*Polygraphus proximus*	*Abies veitchii*	Japan	GU134162 + GU134178	–	–
G. aurea	CMW714		*Pin. contorta* var. *latifolia*	Canada	AF343699	–	–
G. cainii	WIN(M)71-13 [T]		*Pic. mariana*	Canada	AY744548	–	–
G. clavigera	ATCC18086 [T]	*Dendroctonus* sp.	*Pin. ponderosa*	Canada	AY544613	–	–
G. cucullata	CBS218.83 [T]	*I. typographus*	*Pic. abies*	Norway	AJ538335	–	–
G. dryocoetidis	CBS376.66 [T]		*A. lasiocarpa*	Canada	AJ538340	–	–
G. francke-grosmanniae	CMW445 [T]		*Quercus* sp.	Germany	AF343702	–	–
G. galeiformis	CMW5290 [T]	*Tomicus piniperda*	*Pin. sylvestris*	Scotland	AY744552	–	–
G. huntii	CMW2824		*Pinus* sp.	USA	–	DQ354932	DQ354937
G. huntii	CMW2868			USA	–	DQ354933	DQ354938
G. koreana	MAFF4140963 [T]	*T. piniperda*	*Pin. densiflora*	Japan	AB222065	–	–
G. laricis	CMW1980 [T]	*I. subelongatus*	*Larix kaempferi*	Japan	DQ062074	DQ062008	DQ062041
G. laricis	CMW2014	*I. subelongatus*	*L. kaempferi*	Japan	–	DQ062009	DQ062042

Table 2.3 – (continued)

Species / lineage	Isolate no. [a, b]	Substrate		Country	GenBank accession no.		
		Bark beetle	Host		ITS2-LSU	β-tubulin	EF-1α
G. laricis	CMW1913	*I. subelongatus*	*L. kaempferi*	Japan	–	DQ296113	–
G. leptographioides	CMW2803		*Q. alba*	USA	AF343710	–	–
G. olivacea	CBS138.51 [T]		*Pin. sylvestris*	Sweden	AJ538337	–	–
G. olivaceapini	MUCL18368 [T]	*Dendroctonus* sp.	*Pin. ponderosa*	USA	AJ538336	–	–
G. penicillata	CBS140.36 [T]		*Pic. abies*	Germany	DQ097851	–	–
G. piceiperda [c]	MUCL18355			USA	AJ538333	–	–
G. piceaperda [c]	3PG4A-Op	*Dryocoetes affaber*	*Pic. glauca*	Canada	DQ268613	–	–
G. piceiperda B	CMW448		*Pic. glauca*	USA	JF279973	JF280025	JF280079
G. piceiperda B	CMW452		*Pseudotsuga menziesii*	USA	–	JF280033	JF280078
G. piceiperda B	CMW2811		*Pic. rubens*	USA	AY707209	AY707195	JF280077
G. piceiperda B	CO3-081A			Canada	DQ268614	DQ268644	–
G. piceiperda C	CMW446	*I. typographus*	*Pic. abies*	Norway	JF279971	JF280032	JF280076
G. piceiperda C	CMW660		*Pic. abies*	Finland	AF343694	DQ296112	–
G. piceiperda C	CMW3312	*I. typographus*	*Pic. abies*	Austria	JF279970	JF280026	JF280074
G. piceiperda C	CMW3313	*I. typographus*	*Pic. abies*	Austria	JF279972	JF280030	JF280073
G. piceiperda C	CMW3314	*I. typographus*	*Pic. abies*	Austria	–	JF280031	JF280075
G. piceiperda C	CMW36628	*Pityogenes chalcographus*	*Pic. abies*	Finland	JF279969	–	JF280072
G. piceiperda C	87RbPRJ		*Pin. sylvestris*	Poland	–	KU319080	KU319136
G. piceiperda D	CMW36626	*I. typographus*	*Pic. abies*	Russia	JF279968	JF280024	JF280070

Table 2.3 – (continued)

Species / lineage	Isolate no. [a, b]	Substrate		Country	GenBank accession no.		
		Bark beetle	Host		ITS2-LSU	β-tubulin	EF-1α
G. piceiperda D	CMW36627	*I. typographus*	*Pic. abies*	Russia	–	JF280023	JF280071
G. piceiperda E	3YT2P-Op	*I. perturbatus*	*Pic. glauca*	Canada	DQ268611	DQ268642	–
G. piceaperda E	3PG4P-Op	*I. perturbatus*	*Pic. engelmannii* × *P. glauca*	Canada	DQ268612	DQ268643	–
G. piceaperda F	FAE2D-19-16-Gp	*Dry. affaber*	*Pic. glauca*	Canada	–	FJ269189	–
G. piceaperda F	RAE6D-3-21-Gp	*Dry. affaber*	*Pic. glauca*	Canada	FJ269221	FJ269188	–
G. radiaticola	KUC2036 [T]		*Pin. radiata*	New Zealand	AY744551	–	–
G. robusta	CMW668 [T]	*Dendroctonus* sp.	*Pin. ponderosa*	USA	AY544619	–	–
G. serpens	CMW304 [T]		*Pin. sylvestris*	Italy	JN135314	–	–
G. yunnanensis	CMW5304 [T]	*T. piniperda*	*Pin. yunnanensis*	China	AY553415	–	–
Leptographium abieticolens	CMW2865 [T]		*A. balsamea*	USA	AF343701	–	–
L. abietinum	CMW759				AF343680	–	–
L. alethinum	CMW3766 [T]	*Hylobius abietis*		England	AF343685	–	–
L. altius	CMW12471 [T]		*Pic. koraiensis*	China	HQ406851	–	–
L. americanum	CMW495 [T]		*L. decidua*	USA	DQ062079	–	–
L. bistatum	GYH2799 [T]		*Pin. radiata*	New Zealand	AY348305	–	–
L. bhutanense	CMW18649 [T]	*Hylobitelus chenkupdorjii*	*Pin. wallichiana*	Bhutan	EU650187	–	–
L. castellanum	CMW2321 [T]		*Pin. occidentalis*	Dominican Republic	JN135317	–	–
L. celere	CMW12422 [T]		*Pin. kesiya*	China	HQ406834	–	–

Table 2.3 – (continued)

Species / lineage	Isolate no. [a, b]	Substrate		Country	GenBank accession no.		
		Bark beetle	Host		ITS2-LSU	β-tubulin	EF-1α
L. chlamydatum	CMW11592 [T]		*Pic. abies*	Norway	EU979333	–	–
L. conjunctum	CMW12473 [T]	*Hylurgops major*	*Pin. yunnanensis*	China	HQ406831	–	–
L. curviconidium	CMW12425 [T]	*I. typographus*	*Pic. koraiensis*	China	HQ406850	–	–
L. curvisporum	CMW17260 [T]		*Pic. abies*	Norway	EU979328	–	–
L. douglasii	CMW2078		*Pse. menziesii*	USA	AY553381	–	–
L. eucalyptophilum	CMW4848 [T]		*Eucalyptus urophylla* × *E. pellita*	Democratic Republic of the Congo	AF343703	–	–
L. euphyes	CMW301		*Pin. strobus*	New Zealand	AF343686	–	–
L. fruticetum	DAOM234389 [T]	*I. perturbatus*	*Pic. engelmannii* × *P. glauca*	Canada	DQ097847	–	–
L. gibbsii	CMW1376 [T]	*Hylastes ater*		UK	JN135316	–	–
L. gracile	CMW12398 [T]	*Pissodes* sp.	*Pin. armandii*	China	HQ406840	–	–
L. guttulatum	CMW742 [T]	*T. piniperda*	*Pin. sylvestris*	France	AF343683	–	–
L. hughesii	CMW4052		*Aquilaria* sp.	Vietnam	AF343700	–	–
L. latens	CMW12438 [T]	*I. typographus*	*Pic. koraiensis*	China	HQ406845	–	–
L. longiclavatum	SL-Kw1436 [T]	*Den. ponderosae*	*Pin. contorta* var. *latifolia*	Canada	AY816686	–	–
L. longiconidiophorum	CMW2004 [T]		*Pin. densiflora*	Japan	KM491421	–	–
L. lundbergii	CMW17264 [T]		*Pin. sylvestris*	Sweden	DQ062068	–	–
L. manifestum	CMW12436 [T]	*I. subelongatus*	*L. olgensis*	China	HQ406839	–	–
L. neomexicanum	CMW2079		*Pin. ponderosa*	USA	AY553382	–	–

Table 2.3 – (continued)

Species / lineage	Isolate no. [a, b]	Substrate		Country	GenBank accession no.		
		Bark beetle	Host		ITS2-LSU	β-tubulin	EF-1α
L. pinicolum	CMW2398 [T]		*Pin. resinosa*	Canada	DQ062060	–	–
L. pini-densiflorae	CMW5157 [T]	*T. piniperda*	*Pin. densiflora*	Japan	AY707199	–	–
L. pistaciae	CMW12499 [T]		*Pistacia chinensis*	China	HQ406846	–	–
L. pityophilum	CMW2840 [T]		*Pin. merbusii*	Italy	AF343679	–	–
L. procerum	CMW34542 [T]	*Den. valens*	*Pin. resinosa*	USA	KM491423	–	–
L. profanum	CMW10552 [T]		*Carya* sp.	USA	DQ354944	–	–
L. pyrinum	CMW169 [T]	*Den. adjunctus*	*Pin. ponderosa*	USA	DQ062072	–	–
L. reconditum	CMW15		*Zea mays*	South Africa	AF343690	–	–
L. sinense	CMW38172 [T]	*Hylobitelus xiaoi*	*Pin. elliottii*	China	KM491419	–	–
L. sinoprocerum	MUCL46352 [T]	*Den. valens*	*Pin. tabuliformis*	China	EU296773	–	–
L. taigense	CMW36630 [T]	*Hylurgops palliatus*	*Pin. sylvestris*	Russia	JF279980	–	–
L. terebrantis	CBS337.70 [T]	*Den. terebrans*	*Pin. taeda*	USA	EU296777	–	–
L. truncatum	CMW28 [T]		*Pin. taeda*	South Africa	DQ062052	–	–
L. wageneri var. *ponderosum*	CMW2812		*Pinus* sp.	USA	AF343708	–	–
L. wageneri var. *pseudotsugae*	CMW154		*Pse. menziesii*	USA	AF343706	–	–
L. wageneri var. *wageneri*	CMW402		*Pinus* sp.	USA	AF343707	–	–

Table 2.3 – (continued)

Species / lineage	Isolate no. [a, b]	Substrate		Country	GenBank accession no.		
		Bark beetle	Host		ITS2-LSU	β-tubulin	EF-1α
L. wingfieldii	CMW2096		*Pin. sylvestris*	France	AY553398	–	–
L. yamaokae	CMW 4726 [T]		*Pin. densiflora*	Japan	JN135315	–	–

[a] ATCC: American Type Culture Collection, Manassas, VA, USA. CBS: Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre, Utrecht, The Netherlands. CMW: Cultures of Michael J. Wingfield, Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. DAOM: Canadian National Mycological Herbarium, Agriculture and Agri-Food Canada, Ottawa, Canada. MAFF: Genetic Resources Center, National Institute of Agrobiological Sciences, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan. MUCL: Mycothèque de l´Université Catholique de Louvain, Louvain-la-Neuve, Belgium.

[b] T: Ex-type isolate.

[c] The lineage in the *G. piceiperda* complex could not be determined.

Chapter 3 Results

3.1. Molecular phylogenetic analysis

3.1.1. Molecular phylogenetic relationship using ITS2–LSU rDNA

The ITS2 and LSU rDNA dataset were consisted of 89 sequences obtained in this study (Table 2.1) and 81 reference sequences obtained from GenBank (Table 2.3). *Grosmannia* and *Leptographium* species were separated into nine major lineages (*G. clavigera* complex, *G. galeiformis* complex, *G. olivacea* complex, *G. penicillata* complex, *G. piceiperda* complex, *G. serpens* complex, *G. wageneri* complex, *L. lundbergii* complex, and *L. procerum* complex). The 87 isolates used in this study were included in the *Grosmannia piceiperda* complex, forming a clade with high support values [83% of maximum parsimony (MP) bootstrap (BS), 94% of maximum likelihood (ML) BS, and 1 of Bayesian inference (BI) posterior probabilities (PP)]. On the other hand, five of New Zealand isolates were not revealed phylogenetic relationship in the *Grosmannia* and *Leptographium* because sequences of ITS2-LSU rDNA could not be obtained.

3.1.2. Molecular phylogenetic relationship using partial of β-tubulin gene

The β-tubulin dataset was composed of 86 sequences obtained in this study (Table 2.1) and 25 reference sequences obtained from GenBank including sequences used by Linnakoski et al. (2012a) (Table 2.3). Results showed that the *G. piceiperda* complex consisted of nine lineages (BT-1 to BT-9), although all of these lineages excepting for the lineage BT-9 were not well supported (85% < MP BS and ML BS, and 1 < BI PP). The Japanese isolates used in this study were grouped in six of these lineages (BT-2, BT-3, BT-4, BT-6, BT-8, and BT-9). The non-Japanese isolates were included in five

lineages (BT-1, BT-4, BT-5, BT-6 and BT-7), that were previously recognized by Linnakoski et al. (2012a) as the lineages *G. piceiperda* C, *G. piceiperda* D, *G. piceiperda* B, *G. piceiperda* E and *G. piceiperda* F, respectively. Two lineages, BT-8 and BT-9, were found as new lineage in this study and that were composed of only Japanese isolates.

Two isolates (UAMH10656 and UAMH10657) were expected to belong to the lineage *G. piceiperda* E. However, sequence data of β-tubulin taken from these isolates in this study were coincided with the sequence data of the lineage *G. piceiperda* F (accession numbers: FJ269188, FJ269189) and different from the sequence data of the lineage *G. piceiperda* E (accession numbers: DQ268642, DQ268643). *G. piceiperda* F created monophyletic lineage in this study.

3.1.3. Molecular phylogenetic relationship using partial of EF-1α gene

The EF-1α dataset was composed of 83 sequences obtained in this study (Table 2.1) and 16 reference sequences from GenBank including sequencences used by Linnakoski et al. (2012a) (Table 2.3). Results showed that the *G. piceiperda* complex separated into 14 lineages (EF-1 to EF-14), although most of these lineages were not well supported (85 % < MP BS and ML BS, and 1 < BI PP).

The non-Japanese isolates were included in four lineages (EF-1, EF-2, EF-13 and EF-14). Three lineages (EF-1, EF-2 and EF-14) consistent with the lineages *G. piceiperda* D, *G. piceiperda* B and *G. piceiperda* C indicated by Linnakoski et al. (2012a), respectively. The lineage EF-13 was composed of isolates of *G. piceiperda* F and confirmed that this lineage create monophyletic lineage. The Japanese isolates used in this study were grouped in 11 of these lineages (EF-1, EF-3 to EF-12), and all lineages except for the lineage EF-1 were composed of only Japanese isolates. Four of

the 11 lineages (EF-1, EF-3, EF-7 and EF-12) contained the same isolates as lineages BT-4 (including *G. piceiperda* D, BT-2 (= *G. aenigmatica*), BT-9 and BT-8 detected in the β-tubulin analysis. Isolates of lineage BT-3 in the β-tubulin analysis were separated into two lineages (EF-10 and EF-11) in this analysis. Isolates in lineage BT-6 were divided into five lineages (EF-4 to EF-6, EF-8 and EF-9). The lineages EF-3 and EF-10 included isolates previously identifies as *G. aenigmatica* and *G. laricis*, respectively.

3.1.4. Molecular phylogenetic relationship using combined dataset

The result of partition homogeneity test for the combined dataset of four gene regions (ITS2–LSU rDNA, ACT, β-tubulin, and EF-1α) gave a *P* value of 0.3730, which was indicated that each dataset could be combined for phylogenetic analysis.

The combined dataset was composed of 80 sequences obtained in this study (Table 2.1). The results of combined dataset analyses showed that *Grosmannia piceiperda* complex was separated into 14 lineages. Although four lineages (i.e. J-1, J-2, J-5, and *G. piceiperda* D) were not well supported, remaining ten lineages were well supported [85% > MP BS and ML BS, and 1 > BI PP].

All of the 14 lineages (*G. aenigmatica*, *G. laricis*, *G. piceiperda* B, C, D, F, and J-1 to J-8) contained the same isolates as lineages EF-3, EF-10, EF-2, EF-14, EF-1, EF-13, EF-11, EF-6, EF-5, EF-4, EF-9, EF-8, EF-12, and EF-7 detected in the EF-1α analysis, respectively.

Two of these 14 lineages (*G. piceiperda* B and *G. piceiperda* F) were composed of North American isolates. One of other lineage (*G. piceiperda* C) was composed of European isolates. One of other lineage (*G. piceiperda* D) was composed of Russian and Japanese isolates. Ten of remaining lineages (*G. aenigmatica*, *G. laricis*, J-1 to J-8) were composed of only Japanese isolates.

In phylogenetic analyses, an ex-type isolate of *G. europhioides* (NoF555) was included in the lineage *G. piceiperda* B. On the other hand, phylogenetic position of *G. piceiperda* was not resolved, because any sequence data were not obtained from lectotype (BPI595981) and related specimens (BPI595980 and BPI595982) of *G. piceiperda*.

3.2. Morphological observation

Morphological characteristics of the Japanese and non-Japanese isolates belonging to the 14 lineages in the combined dataset analysis were compared. Four lineages had unique characteristics and were distinguishable from the other nine lineages. *Grosmannia laricis* produced curved ascospores, which distinguish this group from all other lineages in the complex, which have cucullate ascospores. The lineage J-6 was the only lineage characterized by multiple perithecial necks. Compared to other lineages, lineage J-7 produced smaller ascospores and shorter perithecial necks. The lineage J-8 was characterized by longer perithecial necks and larger ascospores. Furthermore, lineages J-6, J-7, and J-8 were characterized by the absence of an asexual morph.

The remaining ten lineages (*G. aenigmatica*, *G. piceiperda* B, C, D, and F, and J-1 to J-5) were morphologically similar, but distinguishable based on differences in size of ascospores, diameter of the perithecial base, length of perithecial neck, length of conidiophores, stipe, and conidiogenous apparatus, and size of conidia. The lineages *G. piceiperda* D, *G. piceiperda* C and *G. piceiperda* F produced shorter perithecial necks (less than 300 μm). However, they could be distinguished based on width of perithecial base and length of conidiophores. The *G. piceiperda* C formed wider width of perithecial base than the *G. piceiperda* D and the *G. piceiperda* F, and the *G. piceiperda* F produced shorter conidiophores than the *G. piceiperda* D. The lineage *G. piceiperda* B

was similar to *G. aenigmatica*, but former lineage produced longer stipe and shorter conidiogenous apparatus than the later species. Remaining five Japanese lineages (J-1 to J-5) also could be distinguished each other. The lineage J-5 produced longer perithecial necks than other four lineages. The lineage J-1 formed wider width of perithecial base than the lineages J-2 to J-4. The lineage J-3 produced longer length of conidiophore than the lineages J-2 and J-4. The lineage J-2 produced smaller size of conidia than the lineage J-4. Consequently, all the lineages of the *G. piceiperda* complex were morphologically distinguishable and regarded as distinct species.

The lectotype (BPI595981) and related specimens (BPI595980, BPI595982) of *G. piceiperda* produced narrow width of perithecial base and relatively longer perithecial necks, and size of ascospores were. Conidiophores and conidiogenous apparatus of these specimens were relatively shorter, and size of conidia were. Although asexual morph structures were similar to the lineage *G. piceiperda* B, sexual morph structures did not match with any lineages. Therefore, *G. piceiperda* did not match with any lineages.

Chapter 4 Discussion

Multigene phylogenetic analyses and examination of morphological characteristics carried out in this study suggested that the *G. piceiperda* complex includes some cryptic species. In previous studies, phylogenetic analysis for the *G. piceiperda* complex has been only conducted by Linnakoski et al. (2012a). Our results support the results opinion of their study and recognized several previously unrecognized lineages in the *G. piceiperda* complex. In the present study, 53 Japanese isolates and 33 non-Japanese isolates of the *G. piceiperda* complex were analyzed. Results of this study showed that this species complex could be separated into 14 lineages based on the combined multigene dataset (ITS2-LSU rDNA, ACT, β-tubulin, and EF-1α) analyses, these were also morphologically distinct.

The ITS2 and LSU rDNA sequences have been frequently used to investigate the phylogeny of *Leptographium* s.l (Jacobs et al. 2001, 2004, 2005; Lim et al. 2004; Masuya et al. 2004, 2005; Kim et al. 2005a, b; Alamouti et al. 2006, 2007; Paciura et al. 2010; Six et al. 2011; Duong et al. 2012; Linnakoski et al. 2012a; Yin et al. 2015), and ITS rDNA has recently been recommended as a DNA barcoding region for fungi (Schoch et al. 2012). However, the ITS2 and LSU rDNA sequences would be useful only to place isolates in a particular complex within *Leptographium* s.l., but not to distinguish between species within that complex (Lim et al. 2004; Paciura et al. 2010; Six et al. 2011; Duong et al. 2012; Linnakoski et al. 2012a; Yin et al. 2015). The findings of this study agreed with previous studies, indicating that the ITS2 and LSU rDNA sequences were insufficient for understanding the phylogenetic relationship between species in the *G. piceiperda* complex.

The partial sequences of protein-coding genes, such as ACT, β-tubulin, EF-1α and

calmodulin were used to reveal the phylogenetic relationships among species in *Leptographium* s.l. (Kim et al. 2005a, b; Masuya et al. 2005; Alamouti et al. 2006, 2007; Paciura et al. 2010; Duong et al. 2012; Linnakoski et al. 2012a; Yin et al. 2015). These protein-coding genes were useful for making phylogenetic inferences of species-level relationships. Previously, phylogenetic analysis of protein-coding genes in the *G. piceiperda* complex had only been conducted by Linnakoski et al. (2012a). They separated the *G. piceiperda* complex into seven (*G. aenigmatica*, *G. laricis*, *G. piceiperda* B, C, D, E and F) and five (*G. aenigmatica*, *G. laricis*, *G. piceiperda* B, C and D) lineages using β-tubulin and EF-1α, respectively. The results of the present study showed that this species complex was separated into nine lineages (BT-1 to BT-9) based on the partial β-tubulin gene and into 14 lineages (EF-1 to EF-14) based on EF-1α. This study supported the results of an earlier study (Linnakoski et al. 2012a) and showed several previously unrecognized lineages among the Japanese isolates of the *G. piceiperda* complex.

Among these 14 lineages, 10 (*G. aenigmatica*, *G. laricis*, *G. piceiperda* B, C, F, J-3, J-4, and J-6 to J-8) were considered distinct species, because they were well supported in phylogenetic analyses and had unique morphological characteristics. The remaining four lineages (i.e., *G. piceiperda* D, J-1, J-2, and J-5) were not well supported in the phylogenetic analyses. However, the lineage *G. piceiperda* D could be distinguished based on morphological differences. This lineage produced short perithecial neck, narrow perithecial base, and moderate length conidiophores. The lineage J-1 was closely related to *G. laricis*. However, *G. laricis* was phylogenetically well supported and produced curved ascospores. Therefore, these lineages were considered distinct species. The lineage J-2 was closely related to lineages J-3 and J-4, although lineages J-3 and J-4 were well supported in phylogenetic analysis. These lineages could also be

distinguished by the size of their ascospores, length of conidiophores, and size of conidia. Lineage J-5 was also morphologically distinguishable based on its long perithecial necks and conidiophores. Therefore, these lineages were considered different species.

This study revealed that phylogenetic analysis using combined multigene dataset is necessary to infer reliably the phylogenetic relationships in the *G. piceiperda* complex. Moreover, this study indicated that incubation of cultures under the same conditions was indispensable to know the real status of species by morphological comparisons. Consequently, it was concluded that all of the 14 lineages distinguished by combined multigene combined dataset analysis and morphological comparisons are distinct species.

Summary

The genus *Grosmannia* is belonging to Ophiostomataceae, Ophiostomatales, Sordariomycetes, Ascomycota. This genus is characterized by black perithecia with or without necks and with *Leptographium* asexual morph, which have dark pigmented erect conidiophores giving rise to series of branched conidiogenous apparatus produced conidia in slimy masses. Most species in these genera cause sap stain of conifer timber and are vectored by bark beetles. The most recent phylogenetic analysis of comprehensive *Grosmannia* and *Leptographium* species showed that *Grosmannia* and *Leptographium* were not supported to monophyletic lineage. Therefore, it was proposed that these genera are together referred to *Leptographium* sensu lato (s.l.). Moreover, it was shown that 10 species complexes exist in the *Leptographium* s.l. The *Grosmannia piceiperda* complex is one of these species complexes.

The four species currently recognized in this complex are *G. aenigmatica*, *G. europhioides*, *G. laricis*, and *G. piceiperda*. This complex is characterized by cucullate ascospores and a typical leptographium-like asexual morph. *Grosmannia aenigmatica* and *G. laricis* were described based on the isolates from *Ips typographus japonicus* infesting *Picea jezoensis* var. *jezoensis* and *Ips subelongatus* infesting *Larix kaempferi* in Japan, respectively. Both species have been reported only from Japan. On the other hand, *G. piceiperda* and *G. europhioides* were described based on the isolates from *Picea glauca* infested with *Dendroctonus rufipennis* and *Picea* spp. and *Pinus* spp. in Canada, respectively. Then, *Grosmannia piceiperda* and *G. europhioides* have been reported from several species of bark beetles and conifers in several countries.

A recent phylogenetic study based on β-tubulin and translation elongation factor-1 alpha (EF-1α) genes showed that the isolates identified as *G. piceiperd* or *G.*

europhioides could be separated into five lineages. However, it could not clarify the taxonomic treatments of both species, because taxonomic study using type specimens or authentic isolate of *G. piceperda* and *G. europhioides* have not been conducted. Furthermore, some isolates that appeared to belong to the *G. piceiperda* complex were collected in previous studies from bark beetles infesting conifers in Japan. However, the phylogenetic relationship of these isolates remains unknown and their taxonomic positions also unresolved. Therefore, the purpose of this study was to clarify the taxonomic treatment of species in the *Grosmannia piceiperda* complex based on molecular phylogenetic analysis and morphological observation.

A total of 91 isolates resembling species of the *G. piceiperda* complex were used in this study. Fifty-three Japanese isolates resembling species in the *G. piceiperda* complex that were isolated from several species of bark beetles infesting conifers in previous studies were used in this study. An additional thirty-eight isolates in the *G. piceiperda* complex isolated in North America, Europe, Russia, or New Zealand were obtained from fungal culture collections. The herbarium specimens of *G. piceiperda* that were lectotype (BPI595981) and two related specimens (BPI595980 and BPI595982) and an ex-type isolate (NoF555) considered to be related to type specimen of *G. europhioides*, were obtained from the U.S. National Fungus Collections and the Fungus Culture Collection of the Northern Forestry Centre, respectively.

Molecular phylogenetic analysis were carried out using internal transcribed spacer 2 (ITS2) and large subunit (LSU) ribosomal RNA gene regions (rDNA) and portions of the actin (ACT), β-tubulin, and translation elongation factor 1-alpha (EF-1α) genes. Phylogenetic relationships were estimated by maximum patrimony, maximum likelihood, and Bayesian Phylogenetic trees were inferred with maximum parsimony, maximum likelihood, and Bayesian inference methods. For morphological comparisons,

representative isolates were selected and incubated on 2% MEBA plates. After two-weeks of incubation, two pieces of autoclaved bark from *Larix kaempferi* were placed on the surface of the fungal colony. The asexual structures on the bark were examined after two weeks. After two more months, sexual structures on the bark were examined.

The results of combined multigene dataset (ITS2-LSU, ACT, β-tubulin, and EF-1α) analysis showed that *Grosmannia piceiperda* complex was separated into 14 lineages. Two of these 14 lineages (*G. piceiperda* B and *G. piceiperda* F) were composed of North American isolates. One of other lineage (*G. piceiperda* C) was composed of European isolates. One of other lineage (*G. piceiperda* D) was composed of Russian and Japanese isolates. Ten of remaining lineages (*G. aenigmatica*, *G. laricis,* J-1 to J-8) were composed of only Japanese isolates. An ex-type isolate of *G. europhioides* (NoF555) was included in the lineage *G. piceiperda* B. On the other hand, phylogenetic position of *G. piceiperda* was not resolved, because any sequence data were not obtained from lectotype and related specimens of *G. piceiperda*.

In the results of morphological comparisons, the 14 lineages in the *Grosmannia piceiperda* complex could be morphologically distinguishable. Therefore, all the lineages were considered as distinct species. However, morphological characters of type specimens of *G. piceiperda* were not match with any lineages. From these results, 14 lineages detected in this study were different species from *G. piceiperda*, and it was concluded that 11 lineages (*G. piceiperda* C, D, F, and J-1 to J-8) should be treated as undescribed species.

In conclusion, this study revealed that phylogenetic analysis using combined multigene dataset is necessary to infer reliably the phylogenetic relationships in the *G. piceiperda* complex. Moreover, this study indicated that incubation of cultures under the

same conditions was indispensable to know the real status of species by morphological comparisons. The results in this study were considered to be applicable as the basic information for the further study of ecological, evolutional, and forest pathological studies.

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