

## Discovery and description of a teleomorph for *Leptographium koreanum*

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**Abstract**—A *Leptographium* sp. unknown in Japan was isolated from Japanese red pine (*Pinus densiflora*) in this study. Pairing of different strains of the fungus gave rise to mature perithecia of an *Ophiostoma* sp. Characteristics of this teleomorph were similar to those of *Ophiostoma piceaperdum*, but the fungus had larger ascospores. The anamorph state had 2 to 3 primary branches, and was similar to *Leptographium truncatum*, *L. yunnanense*, *L. pini-densiflorae*, and particularly to *L. koreanum*. Comparisons of partial actin,  $\beta$ -tubulin, and rDNA sequences data showed that the Japanese fungus is *L. koreanum*. Pairing of strains from Japan with the ex-type culture of *L. koreanum* gave rise to mature perithecia confirming this identification. The teleomorph of *L. koreanum* is thus described here as *Ophiostoma koreanum*.

**Key words**—biological species, phylogenetic species, ophiostomatoid fungi, blue-stain fungi, DNA sequences

### Introduction

Species of *Leptographium* Lagerb. et Melin are best recognized as anamorphs of the ascomycete genus *Ophiostoma* Syd. et P. Syd. They include economically important agents of sap-stain as well as tree pathogens (Gibbs 1993, Seifert 1993). *Leptographium wingfieldii* M. Morelet and *L. wageneri* (W. B. Kendr.) M. J. Wingf. have relatively high levels of virulence, and the three varieties of the latter species are well-recognized root pathogens in the western United States (Cobb 1998, Solheim et al. 1993, Harrington 1988). These and other *Leptographium* spp., produce mitospores in slimy masses at the

apices of erect conidiophores that are specifically adapted to be carried by arthropods, particularly bark beetles (Coleoptera, Scolytinae, Jacobs and Wingfield 2001).

A recent monograph on *Leptographium* included 46 species (Jacobs & Wingfield 2001). The majority of these species occur in Europe and North America and it is well-recognized that this group of fungi has been poorly sampled in other parts of the world. This is particularly true for Asia where a large number of bark beetles are known to infest native conifers. This study represents part of an ongoing effort to catalogue the *Leptographium* spp. in Asia, and particularly in Japan.

Recent isolations from pine bark beetles and bark beetle-infested Japanese red pine (*Pinus densiflora* Siebold et Zucc.) and other pine species have yielded a *Leptographium* spp. that has previously not been collected in Japan. This fungus has 2-3 primary branches, relatively poorly developed rhizoids, and is morphologically similar to *L. truncatum* (M. J. Wingf. et Marasas) M. J. Wingf., *L. yunnanense* X. D. Zhou et al., *L. koreanum*, and *L. pini-densiflorae* Masuya et M. J. Wingf. (Jacobs & Wingfield 2001, Kim et al. 2005, Masuya et al. 2000, Zhou et al. 2000, Jacobs et al. 2005). However, unlike these fungi, isolates of the Japanese *Leptographium* sp. often produce protoperithecia in culture (Masuya et al. 1998). Masuya et al. (1998, 1999) have previously noted the fungus and reported it as an undescribed *Ophiostoma* sp. This fungus was isolated from 9 out of 13 investigated bark beetle species and appears to be widely distributed in Japan (Masuya et al. 2001). It was also found to be relatively virulent in inoculations on Japanese red pine where it produced longer lesions than various other blue-stain fungi (Masuya et al. 2003).

The unknown *Ophiostoma* sp. with a *Leptographium* asexual state from Japan is considered to be economically important for Japan thus an appropriate name for the fungus is required especially since its teleomorph is recognized. The aim of this study is to establish its identity.

## Materials and methods

### Fungal isolates

The fungal isolates used for morphological comparisons and mating experiments in this study are listed in Table 1. Five strains of the *Leptographium* sp. (MCC206, 214, 217, 364, 365) were used in the DNA sequence analyses. Comparisons of sequence data also included sequence data of other *Ophiostoma* and *Leptographium* species obtained from Genbank (Table 2).

### Morphology

A 5 mm-diam. plug of each isolate used in the morphological comparisons was placed in Petri dishes containing 2% malt extract agar (MEA, 20 g Difco malt extract, 15 g agar and 1000mL distilled water) and incubated at 20C in dark. After one month, two autoclaved pine twigs were placed on the surface of the medium to stimulate the development of fruiting structures. After an additional month of incubation, plates were inspected for the presence of perithecia. Where these structures were found, they were mounted on glass slides in 1% lacto-fuchsin for microscopic examination. In addition, perithecia were mounted after having been bleached with Sodium hypochlorite (1% available chlorine) for detailed observation of the cell arrangement of perithecial

Table 1. Isolates used for morphological comparisons and mating experiments in this study.

Species	Isolate No.*	Other No. *	Source	Collector	Origin
<i>Leptographium</i> sp.	MCC206	DAOM234395, JCM11853, MAFF410963	<i>Tomicus piniperda</i> on <i>Pinus densiflora</i>	H. Masuya	Japan
	MCC211	JCM11857, MAFF410964	<i>T. piniperda</i> on <i>P. densiflora</i>	H. Masuya	Japan
	MCC213	JCM11855, MAFF410965	<i>T. piniperda</i> on <i>P. densiflora</i>	H. Masuya	Japan
	MCC214	DAOM234396, JCM11854, MAFF410966	<i>T. piniperda</i> on <i>P. densiflora</i>	H. Masuya	Japan
	MCC215	JCM11859, MAFF410967	<i>T. piniperda</i> on <i>P. densiflora</i>	H. Masuya	Japan
	MCC217	JCM11860, MAFF410968	<i>T. piniperda</i> on <i>P. densiflora</i>	H. Masuya	Japan
	MCC364	JCM11858, MAFF410961	<i>Hylurgops interstitialis</i> in <i>P. parviflora</i> var. <i>pentaphylla</i>	H. Masuya	Japan
	MCC365	JCM11856, MAFF410962	<i>H. interstitialis</i> in <i>P. parviflora</i> var. <i>pentaphylla</i>	H. Masuya	Japan
	<i>L. lundbergii</i>	CBS352.29	CMW217, PREM50548	<i>P. sylvestris</i>	T. Lagerberg/E. Melin
<i>L. truncatum</i>	CMW21	PREM45896	Trunk of <i>P. radiata</i>	M. J. Wingfield	New Zealand
	CMW28	ATCC58099	Root of <i>P. taeda</i>	M. J. Wingfield	South Africa
	CMW30	PREM45699	Trunk of <i>P. strobus</i>	M. J. Wingfield	New Zealand
<i>L. koreanum</i>	KUC2072	DAOM234393	<i>P. densiflora</i> infested with <i>T. piniperda</i>	J. -J. Kim & G. -H. Kim	Korea
	KUC2102	DAOM234392	<i>P. koraiensis</i> infested with <i>T. piniperda</i>	J. -J. Kim & G. -H. Kim	Korea
<i>L. pini-densiflorae</i>	MCC071	JCM10479, MAFF410861, CMW5157	<i>P. densiflora</i> infested with <i>T. piniperda</i>	H. Masuya	Japan
	MCC194	JCM10480, MAFF410865, CMW5158	<i>P. densiflora</i> infested with <i>T. piniperda</i>	H. Masuya	Japan
<i>L. yunnanense</i>	CMW5304		<i>T. piniperda</i> on <i>P. yunnanensis</i>	XD Zhou	China

\* Culture collection source: ATCC, American Type Culture Collection, U.S.A; CBS, the culture collection of Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; CMW, culture collection of the Tree Pathology Co-operative Programme, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; DAOM, National Mycological Herbarium, Canada; JCM, Japan Collection of Microorganisms, RIKEN BioResource Center, Japan; KUC, the Korea University Culture Collection, Korean University, Korea; MAFF, Genetic Resources Center, Culture Collection of National Institute of Agrobiological Resources, Japan; MCC, culture collection of the senior author.

Table 2. Isolates used for molecular comparison.

Species	Isolate No.* <sup>1</sup>	Origin	GenBank No.		
			ITS2 & LSU	Actin	$\beta$ -tubulin
<i>Leptographium</i> sp.	MCC206	Japan	AB222065* <sup>2</sup>	AB222061* <sup>2</sup>	AB222063* <sup>2</sup>
	MCC214	Japan	AB222066* <sup>2</sup>	AB222062* <sup>2</sup>	AB222064* <sup>2</sup>
	MCC217	Japan			
	MCC364	Japan			
	MCC365	Japan			
<i>L. koreanum</i>	KUC2072	Korea	AY707196	AY707174	AY707183
	KUC2102	Korea	AY707197	AY707175	AY707184
<i>L. lundbergii</i>	CBS352.29	Sweden	AY707198	AY707176	AY707185
<i>L. pini-densiflorae</i>	MCC071	Japan	AY707199		AY707186
	MCC194	Japan	AY707200		AY707187
<i>L. pyrinum</i>	DLS879	USA	AY544604	AY544586	AY263185
<i>L. terebrantis</i>	C418	USA	AY544607	AY544589	AY263191
<i>L. truncatum</i>	CMW21	New Zealand	DQ062056		DQ061990
	CMW28	South Africa	DQ062052		DQ061986
	CMW30	New Zealand	DQ062054		DQ061988
<i>L. wingfieldii</i>	CMW2095	France	AY707204	AY707177	AY707190
<i>L. yunnanense</i>	CMW5304	China	AY707206	AY707179	AY707192
<i>Ophiostoma aenigmaticum</i>	CMW2199	Japan	AY553389		
	CMW2310	Japan	AY553390		
<i>O. aureum</i>	ATCC16936	Canada	AY544610	AY544592	AY263187
<i>O. clavigerum</i>	ATCC18086	Canada	AY544613	AY544595	AY263194
<i>O. huntii</i>	UAMH4997	Canada	AY544617	AY544599	AY349023
<i>O. laricis</i>	CMW1980	Japan	AF343691		
<i>O. piceaperdum</i>	C274	USA	AY707209	AY707182	AY707195
<i>O. robustum</i>	CMW668	USA	AY544619	AY544601	AY263185

\*<sup>1</sup> Culture collection source: ATCC, American Type Culture Collection, U.S.A.; C, Collection of T. C. Harrington, Iowa State University; CBS, the culture collection of Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; CMW, culture collection of the Tree Pathology Co-operative Programme, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; DLS, culture collection of D. L. Six; KUC, the Korea University Culture Collection, Korean University, Korea; MCC, culture collection of the senior author; UAMH, University of Alberta Microfungus Collection and Herbarium, Devonian Botanic Garden, Edmonton.

\*<sup>2</sup> Accession numbers of the sequences obtained in this study.

necks and outer layers of the peridium. Morphologically characteristic structures were measured and averages and ranges computed. Fifty measurements for each structure were made.

### Mating experiments

Isolates of the unknown *Leptographium* sp. were paired in all possible combinations. In addition, four isolates originating from single ascospores derived from a single perithecium from a cross between isolates MCC206 and MCC214, were paired with each other in all possible combinations. We use MCC206 and MCC214 as tester isolates and try to pair each one with other species listed in Table 1. Negative control mating experiments also were done.

Plugs from 2-wk-old cultures on 2% MEA were excised with cork borer (5mm diam.) and placed on 2% MEA. Plates were incubated at 15 °C in the dark for 2 weeks. Two autoclaved pine twigs or sapwood blocks were then placed on the agar surface and the plates were incubated for an additional 2 weeks. Donor cultures were flooded with 600ml sterile and deionized water and conidial suspensions were prepared. This suspension was poured on the twigs in the each recipient culture. Plates were then incubated at 15 C and these were regularly inspected over a period of two months for the mature perithecia.

### DNA sequence comparisons

Cultures for DNA sequence comparisons were incubated on 2% MEA plates for four weeks. DNA was extracted using the methods described by Kim et al. (2005). Oligonucleotide primers used for both amplification and DNA sequencing of the internal transcribed spacer (ITS) 2 and partial large subunit (LSU) regions of the ribosomal DNA operon, portions of the actin and  $\beta$ -tubulin genes were the same as those used by Kim et al. (2005). Methods for amplification and sequencing of each gene or gene region were also as described by Kim et al. (2005). Both strands of fragments were sequenced and sequences have been deposited in GenBank (Table 2, Fig. 1).

Obtained sequences were analyzed together with previously published sequences to provide a sufficiently broad taxon sampling (Table. 2). Overall, the ITS2 and LSU rDNA—D1 sequence data set included 23 sequences including those derived in this study. The actin and  $\beta$ -tubulin gene sequence data set was comprised of 16 and 22 sequences including those obtained in this study.

Sequences were aligned using Clustal X version 1.81 (Thompson et al. 1997). Alignments were manually adjusted using the program BioEdit version 5.0.9 (Hall 1999). The aligned data set was analyzed using the program PAUP\*4.0 beta10 (Swofford 2002). A parsimony analysis was carried out using the heuristic search with simple stepwise addition, MAXTREE option set to 1000, and tree-bisection reconnection (TBR) option of the program. Gaps were treated as missing data and all characters were equally weighted. Bootstrap and jackknife values (each 1000 replicates) were also calculated.

## Results

### Morphology

The unknown *Leptographium* sp. from Japan was characterized by having mainly two primary branches and conidia with truncated bases. These characteristics are commonly found in various species of *Leptographium*. In particular, *L. truncatum*, *L. pini-densiflorae*, *L. koreanum*, and the *Leptographium* anamorphs of *Ophiostoma* spp. including *L. laricis* Van der Westh. et al., *L. aenigmaticum* K. Jacobs et al., *L. huntii* M. J. Wingf. and *L. piceaperdum* K. Jacobs & M. J. Wingf. have similar characters to those of the unknown *Leptographium* sp. from Japan. However, the stipe lengths of the unknown *Leptographium* sp. were longer than those of *L. piceaperdum*, *L. laricis* and *L. aenigmaticum*. Hyphal characteristics of the Japanese fungus were also different from those of *L. huntii*, which are typically serpentine, but could not be distinguished from all other species considered in this study. Primary branches of the Japanese *Leptographium* sp. were smaller than those of *L. truncatum*. Conidia of the *Leptographium* sp. were the

Table 3. Teleomorphic characters of the *Leptographium* species and morphologically similar species.

Character	<i>Leptographium</i> sp.	<i>O. piceaperdum</i> <sup>a</sup>	<i>O. laricis</i> <sup>b</sup>	<i>O. aenigmaticum</i> <sup>c</sup>	<i>O. huntii</i> <sup>d</sup>
Perithecial diam.	240–310	(170–) 199–312 (–370)	210–310	142–254	280–448
Perithecial neck length	520–1000	(280–) 503–603 (–850)	400–1320	115–310	140–720
Perithecial neck width at the base	52–75	(30–) 32–60	50–70	35–100	40–70
Perithecial neck width near the tip	25–33	20–30	20–50	20–45	21–42
Ascospore shape	Hat-shaped to cucullate	Hat-shaped	oblong to ellipsoid	Hat-shaped with elongated brims	Hat-shaped
Ascospore size	5.5–10.5 × 4.5–7.5	(3–) 4–5 × 2–3	6–11 × 2–4	4–5 × 1.8–3.5	3–4 × 1.5–2
Shape of perithecial outer wall composed cell	polygonal to irregularly shaped	–	–	–	elongate to oval
Size of perithecial outer wall composed cell	10–22 × 8–21	–	–	–	ca. 19.6 × 9.7

<sup>a</sup> Jacobs & Wingfield (2001), <sup>b</sup> Van der Westhuizen et al. (1995), <sup>c</sup> Jacobs et al. (1998), <sup>d</sup> Robinson-Jeffrey & Grinchenko (1964)

Table 4. Result of mating experiment of the *Leptographium* sp.

	donor	recipient																
		MCC206	MCC211	MCC213	MCC214	MCC215	MCC217	MCC364	MCC365	KUC2102	KUC2072	CBS352.29	CMW21	CMW28	CMW30	MCC071	MCC194	CMW5304
<i>Leptographium</i> sp.	MCC206	-	-	-	++	-	++	-	-	+	+	-	-	-	-	-	-	-
	MCC211	-	-	+	-	-	-	-	-	NT	NT	-	-	-	-	-	-	NT
	MCC213	-	+	-	++	-	-	+	-	NT	NT	-	-	-	-	-	-	NT
	MCC214	++	-	+	-	-	-	-	++	-	-	-	-	-	-	-	-	-
	MCC215	-	-	-	+	-	-	-	-	NT	NT	-	-	-	-	-	-	NT
	MCC217	-	-	+	-	-	-	-	+	NT	NT	-	-	-	-	-	-	NT
	MCC364	-	-	++	-	-	-	-	++	-	-	-	-	-	-	-	-	-
	MCC365	-	-	-	++	-	+	-	-	-	-	-	-	-	-	-	-	-
	KUC2102	+	NT	NT	-	NT	NT	-	-	NT	NT	NT	NT	NT	NT	NT	NT	NT
KUC2072	+	NT	NT	-	NT	NT	-	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	
<i>L. lundbergii</i>	CBS352.29	-	-	-	-	-	-	-	-	NT	NT	-	-	-	-	-	-	NT
<i>L. truncatum</i>	CMW21	-	-	-	-	-	-	-	-	NT	NT	-	-	-	-	-	-	NT
	CMW28	-	-	-	-	-	-	-	-	NT	NT	-	-	-	-	-	-	NT
	CMW30	-	-	-	-	-	-	-	-	NT	NT	-	-	-	-	-	-	NT
<i>L. pini-densiflorae</i>	MCC071	-	-	-	-	-	-	-	-	NT	NT	-	-	-	-	-	-	NT
	MCC194	-	-	-	-	-	-	-	-	NT	NT	-	-	-	-	-	-	NT
<i>L. yunnanense</i>	CMW5304	-	NT	NT	-	NT	NT	-	-	NT	NT	NT	NT	NT	NT	NT	NT	NT

\*- no perithecia produced, +: perithecia (n <50) produced, ++: perithecia (n >50) produced, NT: not tested.

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same as those of *L. koreanum*, which are oblong to ovoid and relatively straight and thus, different to those of other species.

The morphology of the teleomorph structures produced as a result of the pairing of various strains was similar to those of *Ophiostoma piceaperdum* (Rumbold) Arx, *O. aenigmaticum* K. Jacobs et al., and *O. huntii* (Rob.-Jeffer.) de Hoog & R. J. Scheff. where ascospores were hat-shaped and perithecial necks had no ostiolar hyphae. Perithecia sizes and neck lengths overlapped between species and could not be clearly separated. However, the ascospores of the unidentified *Ophiostoma* sp. were larger than those of the other species with which it might be confused (Table 3).

### Mating experiments

Two isolates (MCC206 and MCC214) of the unidentified *Leptographium* sp. from Japan produced protoperithecia in culture, but none of them produced mature perithecia unless they were paired with other isolates. Pairing of isolates with themselves did not result in perithecia. The results of the pairing of isolates in all possible combinations showed that some isolates were able to produce perithecia (Table 4). In addition, an isolate of the Japanese *Leptographium* sp. produced perithecia, when it was crossed with the ex-type strain of *L. koreanum* (KUC2102), and not when it was paired with other fungal species such as *L. truncatum* and *L. pini-densiflorae*. The result of crosses between single ascospore isolates showed clearly that the mating behavior of this fungus was heterothallic with two mating types.

### DNA sequence comparisons

Phylogenetic analyses of sequences for the ITS2 and partial LSU of rDNA gene regions showed that isolates of the unknown *Leptographium* sp. from Japan is clearly distinct from *O. piceaperdum*, *O. laricis*, *O. aenigmaticum* and *L. pini-densiflorae* but that it reside a monophyletic group with isolates of *L. koreanum*, *L. truncatum*, *L. yunnanense* and other *Ophiostoma* spp. and *Leptographium* spp. (Fig. 1a). This result was supported by strong bootstrap/Jackknife values (83/78). From a total of 610 characters, 572 characters were constant, 13 variable characters were parsimony uninformative and 25 were informative. The heuristic search gave rise to 390 most parsimonious trees, of which one was chosen for presentation (Fig. 1a). The tree had a length of 44 steps with a Consistency Index (CI) of 0.9091, a Homoplasy Index (HI) of 0.0909, and a Retention Index (RI) of 0.9565.

Phylogenetic analysis of the  $\beta$ -tubulin gene sequences resulted in the most parsimonious tree shown in Fig. 1b. This analysis also showed that the unknown *Leptographium* grouped with high bootstrap/Jackknife support (92/82) in the clade containing *L. koreanum*, and not with *L. truncatum*, *L. yunnanense*, *L. lundbergii*, *O. piceaperdum*, and *O. aenigmaticum*. From a total of 360 characters, 235 characters were constant, 19 variable characters were parsimony-uninformative and 106 were informative. The heuristic search found 87 most parsimonious trees of which one was chosen for presentation (Fig. 1b). The tree had a length of 221, a Consistency Index (CI) of 0.8190, a Homoplasy Index (HI) of 0.1810, and a Retention Index (RI) of 0.8987.

The phylogenetic analysis of the actin gene sequences showed that the unknown *Leptographium* sp. from Japan resided in a monophyletic group with *L. koreanum* (Fig. 1c) with high bootstrap/Jackknife support (99/98). *Leptographium yunnanense*

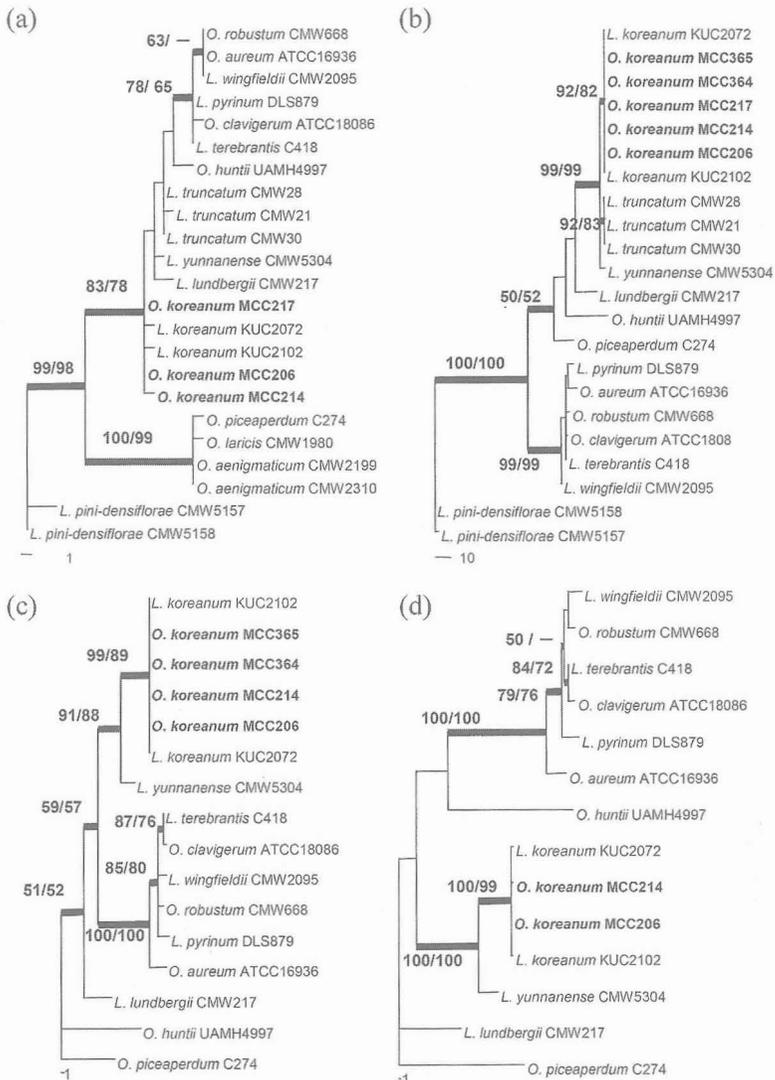


Fig. 1. The most parsimonious trees for each of the three nuclear gene datasets and the combined datasets: (a) rDNA, (b)  $\beta$ -tubulin, (c) actin, and (d) combined. The tree is unrooted. Bootstrap and Jackknife values > 50% with 1000 replications are indicated at the left of the branches (bootstrap value/Jackknife value).

appeared in a sister clade to *L. koreanum*. From a total of 763 characters, 668 characters were constant, 51 variable characters were parsimony-uninformative and 44 were informative. The heuristic search gave rise to two most parsimonious trees of which one (Fig. 1c) is presented. The tree length was 132 with a Consistency Index (CI) of 0.8788, a Homoplasy Index (HI) of 0.1212, and a Retention Index (RI) of 0.9171.

A combined sequence data set was also produced including 14 sequences representing the ITS2 and LSUrDNA—D1 domain, and parts of the actin, and  $\beta$ -tubulin genes. As was true for the analyses of the sequence data for the individual genes or gene regions, the phylogenetic analyses (Fig. 1d) showed that the unknown *Leptographium* sp. from Japan resided in a monophyletic group with *L. koreanum*, with a high bootstrap/Jackknife values (100/99). In this tree (Fig. 1d), *L. yunnanense* resided in a sister group to *L. koreanum*. From a total of 1728 characters, 1510 characters were constant, 110 variable characters were parsimony-uninformative and 108 were informative. The heuristic search found two most parsimonious trees of which one was chosen for presentation. The tree length was 305 with a Consistency Index (CI) of 0.8659, a Homoplasy Index (HI) of 0.1344, and a Retention Index (RI) of 0.9014.

### Taxonomy

Results of the DNA sequence and morphological comparisons show clearly that the *Leptographium* anamorph of unknown *Ophiostoma* species from Japan is conspecific with *L. koreanum*. Furthermore, crosses between isolates of this fungus and those of *L. koreanum* have given rise to its *Ophiostoma* teleomorph. Teleomorph characteristics are also distinct from other *Ophiostoma* spp. with *Leptographium* anamorphs. Thus, on the basis of the mating behavior, morphological characteristics and DNA sequence comparisons, we described the teleomorph of *L. koreanum* as follows:

***Ophiostoma koreanum*** Masuya, J.-J. Kim & M. J. Wingf. sp. nov. Figs. 2-7.

Anamorph: *Leptographium koreanum* J.-J. Kim & G.-H. Kim, Mycol. Res. 109(3): p. 275, 2005.

*Perithecia* basi nigra, globosa vel subglobosa, 240–310  $\mu\text{m}$  diam, Collum cylindraceum, curvatum vel rectum, 560–1000  $\mu\text{m}$  longum, ad basim 52–75  $\mu\text{m}$  latum. ad apicem 25–33  $\mu\text{m}$  latum, apice obtusum vel truncatum, hyphis ostioli non praeditum. Asci evanescenti. Ascospores hyalinae, aseptatae, aspectu laterali cucullatae, aspectu frontali triangulatae, vagina hyalina circumdantes, 5.5–10.5  $\times$  4.5–7.5  $\mu\text{m}$ .

*Etymology*: Derived from the name of anamorph, *Leptographium koreanum*.

*Perithecia* superficial or partly embedded in the substratum and medium. Basal part black, globose to subglobose, 240–310 (mean 290)  $\mu\text{m}$  diam. without hyphal ornamentation, outer layer of the peridium composed of thick-walled, more or less isodiametric, polygonal or irregularly shaped cells, 10–22  $\times$  8–21 (mean 18  $\times$  12)  $\mu\text{m}$ . Necks dark brown to black, broad at the base, becoming cylindrical or slightly tapered at the tip, straight or curved, 560–1000 (mean 680)  $\mu\text{m}$  long, 52–75 (mean 68)  $\mu\text{m}$  wide at base, 25–33 (mean 30)  $\mu\text{m}$  wide near the tip, composed of dark, thick-walled, squamous cells, 4–10  $\times$  2–4  $\mu\text{m}$ , terminating in an obtuse to truncate apex. Ostiolar hyphae absent. Asci evanescent, clavate when young, subglobose when mature, up to 25  $\times$  20  $\mu\text{m}$ . Ascospores, hyaline, one-celled, hat-shaped or cucullate in side view, triangular

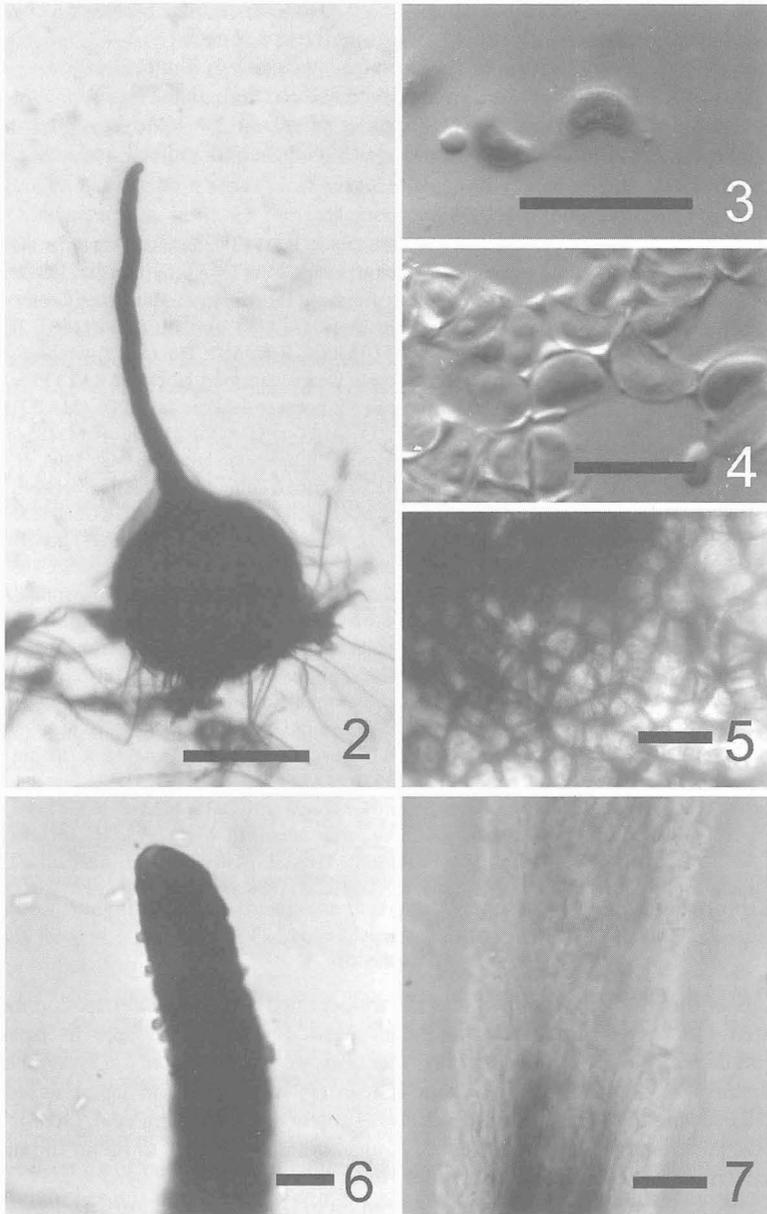


Fig. 2—7. *Ophiostoma koreanum*. Fig. 2. Perithecium. Fig. 3. Ascospore. Fig. 4. Ascospores. Fig. 5. Outer layer of the peridium composed of thick-walled, more or less isodiametric, polygonal or irregularly shaped cells. Fig. 6. Tip of perithecial neck. Fig. 7. Perithecial neck, composed of dark, thick-walled, squamous cells. Bars: Fig. 2 = 100 $\mu$ m, Figs. 3—5, 7 = 10 $\mu$ m, Fig. 6 = 5 $\mu$ m.

in front view, 5.5—10.5 × 4.5—7.5 (mean 7.5 × 5.5) µm, surrounded by a distinct hyaline wall, sometimes accumulating in a white mass at the tips of neck.

Morphology of anamorph is as described for *L. koreanum* (Kim et al. 2005).

Hosts: *Pinus densiflora*, *P. koraiensis* Siebold & Zucc. and other *Pinus* spp.

Isolated from following insects: *Tomicus piniperda* L., *Hylurgops interstitialis* (Chapuis), *Hylastes parallelus* Chapuis, *Hylastes plumbeus* Blandford and other bark beetle species.

Known distribution: Korea, Japan.

CULTURES AND SPECIMENS EXAMINED—HOLOTYPE: DAOM234414, dried culture derived from the pairing of the cultures KUC2102 (MAT-1) (DAOM234392) (KOREA. YEOJU: Sawmill, Central Forest Products Processing & Marketing Center, *P. koraiensis* log, 10 Aug 2000, J.-J. Kim & G.-H. Kim) and MCC206 (MAT-2) (DAOM234395, JCM11853, MAFF410963) ( JAPAN. IBARAKI: Tsukuba, *T. piniperda* adult, 4 Apr 1995, H. Masuya) on *P. contorta* Dougl. sapwood block. PARATYPES: FPH (= TFM) 7605, dried culture from pairing between isolates MCC206 (MAT-2) (DAOM234395, MAFF410963) and MCC214 (MAT-1) (DAOM 234396, JCM11854, MAFF410966) ( JAPAN. YAMANASHI: Masuho, *T. piniperda* adult, 15 May 1996, H. Masuya) on *P. densiflora* twigs, FPH (TFM) 7606, dried culture from pairing between isolates MCC213 (MAT-2) (JCM11855, MAFF410965) (JAPAN. FUKUSHIMA: Amasakae, *T. piniperda* adult, 24 Jun 1996, H. Masuya) and MCC214 (MAT-1) on *P. densiflora* twigs, FPH (TFM) 7607, dried culture from pairing between isolates MCC365 (MAT-2) (JCM11856, MAFF410962) (JAPAN. IWATE: Matsukawa, *H. interstitialis* adult, 1 Jun 2000, H. Masuya) and MCC214 (MAT-1) on *P. densiflora* twigs. Additional cultures examined. KOREA. YEOJU: Sawmill, Central Forest Products Processing & Marketing Center, *P. koraiensis* log, 10 Aug 2000, J.-J. Kim & G.-H. Kim (KUC 2072, MAT-1, DAOM 234393, CMW14199). Bongwha: Sawmill, National Forestry Cooperatives Federation, *P. densiflora* log, 9 Aug 2000, J.-J. Kim & G.-H. Kim (KUC 2078, MAT-1, CMW 14201, PREM 58261). JAPAN. IWATE: Ichinoseki, *T. piniperda* adult, 19 Jun 1996, H. Masuya (MCC211, MAT-1, JCM11857, MAFF410964), HOKKAIDO: Yamabe, bark of *P. sylvestris* dead tree, 7 Nov 1996, H. Masuya (MCC215, MAT-2, JCM11859, MAFF410967), IBARAKI: Tsukuba, *T. piniperda* adult, 18 May 1995, H. Masuya (MCC217, MAT-1, JCM11860, MAFF410968), IWATE: Matsukawa, *H. interstitialis* adult, 1 Jun 2000, H. Masuya (MCC364, MAT-1, JCM11858, MAFF410961).

## Discussion

Results of this study have shown that the unidentified *Leptographium* sp. commonly isolated from pine bark beetles and bark beetle-infested *Pinus* spp. in Japan is morphologically and phylogenetically identical to *L. koreanum*. *Leptographium koreanum* was originally isolated from Korean (*P. koraiensis*) and Japanese red (*P. densiflora*) pine in Korea and was described as a new species by Kim et al. (2005). This fungus was frequently isolated from *T. piniperda* and it appears to be an important causal agent of blue-stain of conifer timber in Korea.

In this study, we have shown that *L. koreanum* in Japan is able to form a teleomorph when sexually compatible isolates are crossed. We have thus described the teleomorph of the fungus as *O. koreanum*. This fungus has been known in Japan for more than a decade where it was reported as an undescribed *Ophiostoma* sp. by Masuya et al (1998,

1999), frequently associated with the pine shoot beetle, *T. piniperda*. The Japanese *Leptographium* strains could be also paired with the ex-type strain of *L. koreanum* confirming that these two are biological heterothallic species. The distribution and ecology of *L. koreanum* and *O. koreanum* thus appear to be similar in Korea and Japan.

The teleomorph of *L. koreanum* described in this study is characterized by hat-shaped ascospores and long necks without ostiolar hyphae. In this regard it is similar to that of *O. piceaperdum* sensu Jacobs et al. (2000) and related species. Taxonomic status of *O. piceaperdum*, however, remains questionable. Morphological comparisons of the dried type specimens led Jacobs et al. (2000) to treat *O. europhioides* (E. F. Wright & Cain) H. Solheim and *O. pseudoeurophioides* (Olchow. & J. Reid) Georg Hausner et al. as synonyms of *O. piceaperdum*. However, recent DNA based comparisons by Hausner et al. (2000) showed that *O. europhioides*, *O. pseudoeurophioides* and *O. piceaperdum* reside in different clades. This suggests that the species concept of *O. piceaperdum* sensu Jacobs et al (2000) deserves reconsideration. Indeed, Olchowecki & Reid (1974) showed that the *Leptographium* anamorph of *O. pseudoeurophioides* has curved conidia, unlike *O. piceaperdum* illustrated by Jacobs et al. (2000). Because the ex-type cultures of *O. pseudoeurophioides* and *O. piceaperdum* are no longer available, it is not possible to consider their morphological characteristics or to make DNA based phylogenetic comparisons. These studies are required but must await further collections.

*Leptographium koreanum* does not have curved conidia and its LSU rDNA sequences do not correspond with those of *O. pseudoeurophioides* (GenBank accession No. AF155678). These species are, therefore, clearly different. In addition, our DNA sequence comparisons have shown that *O. koreanum* is not related to well-defined culture of *O. piceaperdum*. We are thus confident that the new species does not represent *O. piceaperdum*.

*Ophiostoma aenigmaticum* was described by Jacobs et al. (1998) from Japan. This species had previously been treated as *O. europhioides* by Yamaoka et al. (1997) but was differentiated from *O. europhioides* based on the characteristic of elongated brims of the ascospores (Jacobs et al. 1998). Because *O. koreanum* does not have ascospores with elongated brims and because *O. aenigmaticum* is homothallic, as opposed to the heterothallic *O. koreanum*, these species can easily be distinguished from each other. In addition, they also differ in their hosts and insect vectors. DNA based comparisons in this study have also shown that *O. aenigmaticum* is more closely related to *O. piceaperdum* than to *L. koreanum*.

The heterothallic mating behavior appears to be relatively uncommon in *Ophiostoma* with *Leptographium* anamorphs. While heterothallism has been noted for many ascomycetes including those from the genus *Ophiostoma* (Brasier 1993), *O. huntii* is the only *Ophiostoma* with a *Leptographium* anamorph that has previously been shown to display this mating behaviour (Jacobs et al. 1998). This is in contrast to species such as *O. piceaperdum* sensu Jacobs et al. (2000), *O. europhioides*, and *O. aenigmaticum* that have been reported to be homothallic. The fact that *O. koreanum* is heterothallic clearly distinguishes it from related *Ophiostoma* species with *Leptographium* anamorphs.

A relatively small number of *Leptographium* spp. have known teleomorphs and the majority of these appear to have hat-shaped ascospores (Jacobs & Wingfield 2001). In some cases such as that of *Leptographium wagneri* var. *ponderosae*, perithecia thought to represent an *Ophiostoma* state have been found in galleries of beetle vectors but these

have never been found in culture. This makes it difficult to confirm the anamorph/teleomorph connection. It is not uncommon to discover teleomorph states in fungi thought to exist only in the asexual form (Kuhls et al. 1996, Hodge et al. 1996, Chaverri et al. 2001). The discovery of the teleomorph of *L. koreanum* arising from crosses between different isolates suggests that some other *Leptographium* species might also be heterothallic and might have the ability to produce perithecia in crosses of sexually compatible isolates under suitable environmental conditions. This is clearly an area of research worth pursuing.

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