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Leptographium bhutanense sp. nov., associated with the root collar weevil *Hylobitelus chenkupdorjii* on *Pinus wallichiana* in Bhutan

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Key words

Curculionidae
forest pests
Hylobitelus
Leptographium
ophiostomatoid fungi

Abstract *Leptographium* spp. are commonly associated with bark beetles and weevils (Coleoptera: Curculionidae), and some are important tree pathogens. In a recent survey of diseases and insect pests of conifer trees in Bhutan, the root collar weevil, *Hylobitelus chenkupdorjii* was found girdling young Himalayan blue pine (*Pinus wallichiana*) trees in Central Bhutan. Intensive wood staining and a *Leptographium* sp. were associated with damage by this insect. The fungus was also isolated from individuals of *H. chenkupdorjii*. It was tentatively identified based on morphology and then compared with other *Leptographium* spp. using DNA sequences for three gene regions. Morphological characteristics showed that the *Leptographium* sp. from *H. chenkupdorjii* is similar to, but distinct from *L. procerum* and *L. profanum*. DNA sequence comparisons revealed that the isolates from Bhutan resided in a distinct well-supported clade and confirmed that they represent an undescribed taxon for which the name *Leptographium bhutanense* sp. nov. is provided.

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INTRODUCTION

Bark beetles and weevils (Coleoptera: Curculionidae) are well-known vectors of *Leptographium* spp., some of which are important tree pathogens (Jacobs & Wingfield 2001, Kirisits 2004, Viiri 2004). At least four *Leptographium* species have been found associated with root and root collar weevils in the genus *Hylobius* (Jacobs & Wingfield 2001, Viiri 2004). *Leptographium procerum* is closely associated with the seriously damaging pine root collar weevil *Hylobius radicis* and other weevils with similar biology in North America (Wingfield 1983, Jacobs & Wingfield 2001). In Europe, this fungus is more loosely associated with the large pine weevil, *H. abietis* (Lévìeux et al. 1994, Viiri 2004), which is an important pest in conifer afforestations (Grégoire & Evans 2004). This weevil is also associated with *L. alethinum* in England and Scotland (Jacobs et al. 2001). In addition, *L. serpens* is found associated with *H. pales* in North America (Nevill & Alexander 1992), and *L. terebrantis* with *H. radicis* and *H. rhizophagus* in the USA (Wingfield 1983).

In a recent survey of diseases and pests of coniferous trees in Bhutan, a root and root collar weevil, *Hylobitelus chenkupdorjii,* was found girdling young Himalayan blue pine (*Pinus wallichiana*) trees in Central Bhutan. The wood surrounding weevil feeding was darkly stained and a *Leptographium* sp. was consistently present in and around the larval feeding galleries. The aim of this study was to identify the *Leptographium* sp. associated with *H. chenkupdorjii* based on morphology and comparisons of DNA sequences.

MATERIALS AND METHODS

Fungal isolates

Dying and recently killed *Pinus wallichiana* trees, approximately 6 to 10 years old (Fig. 1a) were commonly encountered in an afforestation area with natural and artificial regeneration of *P. wallichiana* and *Picea spinulosa* near the village of Dhur in the administrative district Bhumtang in Central Bhutan. Based on disease symptoms and signs, the trees were suspected of succumbing to Annosum root rot or Armillaria root disease. Isolation of a member of the *Heterobasidion annosum* species complex and an *Armillaria* sp. from the roots, butts or lower stems of a few saplings confirmed this view. Inspection of the bases of these trees also showed distinct feeding activity of an insect, which was identified as the root and root collar weevil, *Hylobitelus chenkupdorjii* (Fig. 1b) (Chhetri 1990). This weevil was either involved in killing the trees or it infested them during their decline due to root diseases. Wood surrounding the weevil feeding damage was stained with an intense black colour (Fig. 1c, d). Erect, long-stalked conidiophores typical of *Leptographium* spp. were common in and around the insect feeding galleries in the bark and on the wood surface.

Isolations were made from conidiophores in the galleries of *H. chenkupdorjii* from five trees by lifting conidial masses directly from the conidiogenous apparatuses and transferring these to 2 % malt extract agar (MEA: 20 g Biolab malt extract, 20 g Biolab agar and 1 L deionised water). Isolations were also

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Fig. 1 Symptoms and damage caused by *H. chenkupdorjii* and its fungal associate on young *P. wallichiana*. a. A recently killed tree; b. young weevil adult in a pupal chamber in a pine root; c. cross-section through the stem of a tree infested by *H. chenkupdorjii* showing intensive black discolouration; d. roots of a young tree with larval feeding galleries of the weevil and intensive stain associated with the insect's infestation. — Ruler for d indicates centimetres and millimetres.

made from four young adult weevils, collected from the galleries, by crushing them onto the surface of 2 % MEA supplemented with 0.05 % cycloheximide.

All cultures used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. A relevant sub-set of isolates has also been deposited at the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Additional fungal cultures from Dhur and from a second locality, Tangsibi, also in the administrative district Bumthang, are stored in the culture collection of the Institute of Forest Entomology, Forest Pathology and Forest Protection, University of Natural Resources and Applied Life Sciences, Vienna (BOKU), Austria.

Morphological comparisons

Four isolates (CMW18649, CMW18650, CMW18651 and CMW18652) of the *Leptographium* sp. from Bhutan were grown on oatmeal agar (OA) (Gams et al. 2007) in incident light for 1 wk at 25 °C. Conidiophores were then mounted in 85 % lactic acid on glass slides, examined microscopically, and compared with other *Leptographium* species, especially those associated with weevils (Table 1). Thirty measurements were made for each morphological character used to define *Leptographium* spp. (Jacobs & Wingfield 2001) and averages determined.

Optimum temperatures for growth were established for the four isolates of the *Leptographium* sp. associated with *Hylobitelus chenkupdorjii* (Table 2) by inoculating four OA plates per isolate and incubating these at temperatures ranging from 5 to 35 °C

Table 1 Comparison of *Leptographium bhutanense* with other *Leptographium* species associated with root collar weevils.

Species	Host	Conidiophore length (µm)	Primary branch type	Rhizoids	Teleomorph	Conidium shape	Conidium size (µm)	Associated weevils	Reference
L. alethinum	*Abies* spp.	560–1270	B	Absent	Absent	Obovoid with truncate bases	4–9 × 2–3	*Hylobius abietis*	Jacobs & Wingfield (2001)
L. procerum	*Abies* spp., *Picea abies*, *Pinus* spp. and *Pseudotsuga menziesii*	150–760	B	Present	Absent	Obovoid to broadly ellipsoid	3–5 × 1–3	*H. abietis*, *H. pales*, *H. radicis* and *H. rhizophagus*	Jacobs & Wingfield (2001)
Grosmannia serpens	*Pinus* spp. and *Pseudotsuga menziesii*	250–1270	C	Present	Present	Oblong with truncate bases and rounded apices	3–5 × 1–2	*H. pales*	Jacobs & Wingfield (2001)
L. terebrantis	*Pinus* spp. and *Pseudotsuga menziesii*	142–508	B	Absent	Absent	Obovoid with truncate bases and rounded apices	4–10 × 2–3	*H. radicis* and *H. rhizophagus*	Jacobs & Wingfield (2001)
L. bhutanense	*P. wallichiana*	400–2300	B	Absent	Absent	Oblong to obovoid	3–5 × 1–2	*Hylobitelus chenkupdorjii*	

at 5 °C intervals. Colony diameters were measured after 8 d, and an average was calculated from the resultant 16 diameter readings. Colony colours were assessed according to Rayner (1970).

DNA sequencing and phylogenetic analyses

DNA was extracted from single hyphal tip cultures of the four isolates chosen for detailed study (Table 2) using PrepMan Ultra Sample reagent (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The concentration of isolated DNA was determined using a ND-1000 Nanodrop spectrophotometer (NanoDrop Technologies Inc. Wilmington, DE 19810, USA).

Primers ITS3 and LR3 (White et al. 1990) were used to amplify the internal transcribed spacer ITS2 and part of the large subunit (28S gene) of the rDNA operon. For the partial β-tubulin gene region, primers Bt2a and Bt2b (Glass & Donaldson 1995) were used. The primers EF1F and EF2R (Jacobs et al. 2004) were used to amplify a portion of the translation elongation factor 1-α gene region. Each PCR reaction (50 µL) included 100–200 ng DNA, 1× PCR reaction buffer, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.2 µM primer, and 2.5 U Super-Therm DNA Polymerase mixture (Hoffmann-La-Roche, US). PCR amplifications reactions were performed using an Eppendorf Mastercycler® Personal (Perkin-Elmer, Germany) with conditions similar to those described previously (Zhou et al. 2004), except that the annealing temperature was adjusted between 52 °C to 56 °C. PCR products were visualised under UV illumination on a 1 % agrose gel and purified using the High Pure PCR Product Purification Kit (Boehringer, Mannheim, Germany). PCR products were sequenced with the same primers, and conditions for sequencing were the same as those used by Zhou et al. (2004).

Sequence contigs were assembled using Vector NTI10, the edited sequences aligned in ClustalX (Thompson et al. 1997) and the alignments manually adjusted in Se-Al (Rambaut 2007). A partition homogeneity test (Farris et al. 1995) was performed to determine whether the three datasets could be combined. Phylogenetic relationships for the taxa were inferred using distance analysis in PAUP v.4.0b10 (Swofford 2003). In all datasets, the characters were treated as unweighted and gaps as missing data. A single tree for each dataset was obtained using neighbour-joining analysis with an uncorrected P-distance and rooted to midpoint. A bootstrap analysis (1 000 replicates using the neighbour-joining option) was performed to determine the confidence levels of the nodes.

For all the datasets, ambiguously aligned regions were coded and step matrices, used to assign different weights to these codes, were computed using INAASE 2.3b (Lutzoni et al. 2000). These weighted codes were used in the analysis to replace the ambiguous aligned regions.

RESULTS

Isolations and morphological characteristics

Isolations from *Leptographium* conidiophores in the galleries of *H. chenkupdorjii* consistently yielded cultures appearing to be a single *Leptographium* sp. The same fungus was also isolated from all four young adults of the insect.

Table 2 GenBank accession numbers for species and isolates included in DNA sequence comparisons.

Species	Isolate No.[1]	ITS[2]	β–tubulin[3]	EF1-α[4]
Grosmannia aenigmatica	CMW2199	AY553389	AY534937	AY536183
	CMW2310	AY553390	AY534938	AY536184
G. americana	CMW2929	DQ062078	DQ062012	DQ062045
	CMW495	DQ062079	DQ062013	DQ062046
G. aurea	CMW709	AY553413	AY534961	AY536207
	CMW714	DQ062071	DQ062005	DQ062038
G. huntii	CMW2868	AY553394	DQ354933	DQ354938
	CMW2824	AY553393	DQ354932	DQ354937
G. laricis	CMW1980	DQ062074	DQ062008	DQ062041
	CMW2014	DQ062075	DQ062009	DQ062042
G. robusta	CMW668	AY553397	AY534945	AY536191
	CMW2805	AY553396	AY534944	AY536190
G. serpens	CMW193	AY553387	AY534935	AY536181
	CMW60	AY553388	AY534936	AY536182
Leptographium abietinum	CMW2817	DQ062080	DQ062014	DQ062047
	CMW3083	DQ062081	DQ062015	DQ062048
L. bhutanense	**CMW18649; CBS 122076**	EU650184	EU650188	EU650192
	CMW18650; CBS 122077	EU650185	EU650189	EU650193
	CMW18651; CBS 122078	EU650186	EU650190	EU650194
	CMW18652	EU650187	EU650191	EU650195
L. douglasii	CMW2078	AY553381	AY534929	AY536175
	CMW725	AY553380	AY534928	AY536174
L. lundbergii	CMW217	DQ062065	DQ061999	DQ062032
	CMW2190	DQ062066	DQ062000	DQ062033
	CMW17264	DQ062068	DQ062002	DQ062035
L. neomexicanum	CMW2079	AY553382	AY534930	AY536176
L. pineti	CMW3831	DQ062076	DQ062010	DQ062043
	CMW3837	DQ062077	DQ062011	DQ062044
L. pinidensiflorae	CMW5158	DQ062082	DQ062016	DQ062049
	CMW5162	DQ062083	DQ062017	DQ062050
L. procerum	CMW12	EU244638	EU244640	EU244642
	CMW261	EU244639	EU244641	EU244643
L. profanum	CMW10550	DQ354943	DQ354935	DQ354940
	CMW10554	DQ354942	DQ354934	DQ354939
	CMW10552	DQ354944	DQ354936	DQ354941
L. pyrunum	CMW169	DQ062072	DQ062006	DQ062039
	CMW509	AY553414	AY534962	AY536208
L. reconditum	CMW15	AY553383	AY534931	AY536177
L. terebrantis	CMW9	AY553384	EU652698	EU652700
	CMW9A	EU652697	EU652699	EU652701
L. truncatum	CMW2402	DQ062051	DQ061985	DQ062018
	CMW28	DQ062052	DQ061986	DQ062019
L. wingfieldii	CMW2096	AY553398	AY534946	AY536192
	CMW2095	AY553400	AY534948	AY536194
	CMW2019	AY553399	AY534947	AY536193
L. yunnanense	CMW5152	DQ062073	DQ062007	DQ062040
	CMW5304	AY553415	AY534963	AY536209

[1] Isolates sequenced in this study are presented in **bold**.
[2] ITS2 and partial 28S rRNA gene.
[3] Partial β-tublin gene.
[4] Partial translation elongation factor 1-α gene.

Morphological characteristics of the *Leptographium* sp. were similar to, but different from those of previously described *Leptographium* spp., including those associated with root and root collar weevils (Table 1). The species was most similar to *L. procerum* and *L. profanum*. *Leptographium procerum* typically forms concentric rings of growth on the agar medium (Wingfield 1985, Jacobs & Wingfield 2001), a characteristic not found in the *Leptographium* sp. from Bhutan. Comparisons showed that the strains associated with *H. chenkupdorjii* in Bhutan had substantially longer conidiophores than *L. profanum*.

DNA sequence comparisons

Results of the partition homogeneity test showed that the three datasets could be combined. The aligned set of the combined data from the ITS2, 28S, β-tubulin and translation elongation factor 1-α gene regions consisted of 1941 characters. Fourteen ambiguous regions were identified and coded. A total of 797 ambiguous characters were excluded from the analysis and replaced with the weighted codes (TreeBASE: SN3867). DNA sequence comparisons showed that the isolates from

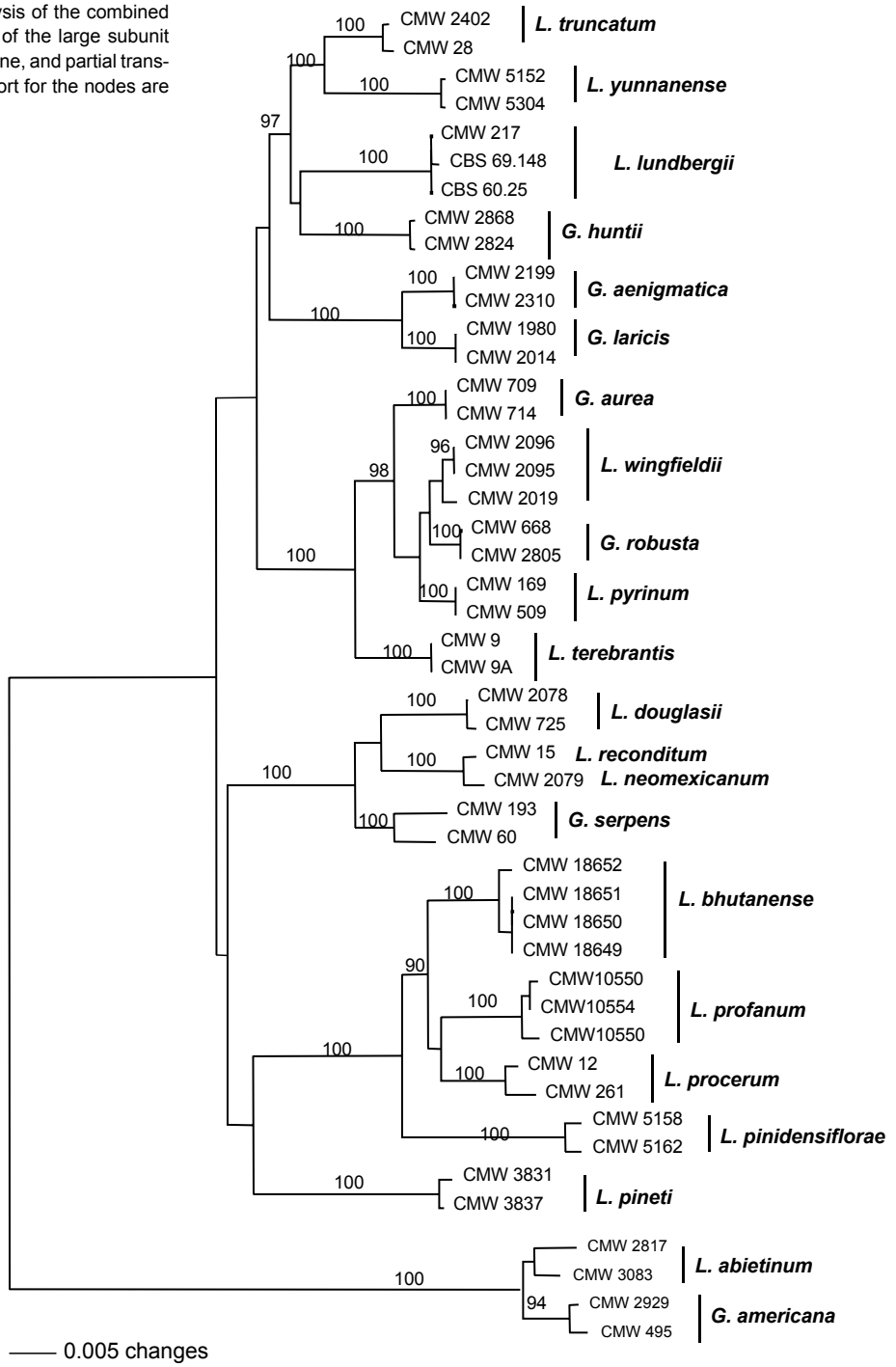

Fig. 2 Neighbour-joining tree derived from analysis of the combined dataset of DNA sequences of the ITS2 and part of the large subunit (28S gene) of the rDNA operon, partial β-tubulin gene, and partial translation elongation factor 1-α gene. Bootstrap support for the nodes are indicated above branches.

Bhutan reside in a distinct clade (Fig. 2) close to *L. profanum* and *L. procerum*. This group had a bootstrap support value of 100 %, confirming the *Leptographium* sp. associated with *H. chenkupdorjii* to be distinct from others known in the genus.

Taxonomy

Morphological characteristics of the *Leptographium* sp. associated with *H. chenkupdorjii* on *P. wallichiana* in Bhutan were similar to, but different from those of *L. procerum* and *L. profanum*. DNA sequence comparisons also showed clearly that this fungus was distinct from all described species of *Leptographium* for which sequences are available. The following description is thus provided:

Leptographium bhutanense X.D. Zhou, K. Jacobs & M.J. Wingf., *sp. nov.* — MycoBank MB511811; Fig. 3, 4

Conidiophorae (380–)800–1490(–2300) µm longae, sine structuris rhizoidiformibus. Stipae hyalinae vel olivaceae, cylindricae, 3–8-septatae, (190–)700–1260(–2000) µm longae. Apparatus conidiogenus (80–)100–120(–150) µm longus massa conidiorum exclusa, ramis cylindricis in 2–4 seriebus. Conidia hyalina, non septata, oblonga vel obovoidea, 3–5 × 1–2 µm. Coloniae atro-olivaceae (19''f), ad 45 mm diametro in 8 diebus in OA ad 25 °C crescunt; ad 5 °C, 30 °C et 35 °C non crescunt.

Etymology. Name refers to the country Bhutan, where the fungus was found.

Conidiophores (Fig. 3a, 4c) occurring singly or in groups of 2–11 arising directly from the medium, erect, macronematous, mononematous, (380–)800–1490(–2300) µm in length; rhizoid-like structures absent. *Stipes* hyaline to olivaceous, cylindrical, simple, 3–8-septate, (190–)700–1260(–2000) µm long, 7–8.5 µm wide below primary branches, apical cell not swollen, 38–45 µm wide at slightly swollen base. *Conidiogenous apparatus* (Fig. 3b, 4a) (80–)100–120(–150) µm long, excluding the conidial mass, with 2–4 series of cylindrical branches. *Primary branches* 2–3, dark-olivaceous (19''f), smooth, cylindrical, aseptate, 18–30 µm long and arrangement of the primary branches on the stipe follows type B (more than two branches sensu Jacobs & Wingfield 2001); *secondary branches* pale olivaceous (21''k), aseptate, 15–16 µm long; *tertiary branches* hyaline, aseptate, 8–14 µm long; *quaternary branches* hyaline, aseptate. *Conidiogenous cells* (Fig. 3c, 4a), discrete, 2–4 per branch, cylindrical, 9–13 µm long and 1–2 µm wide. *Conidia* (Fig. 3d, 4b) hyaline, aseptate, oblong to obovoid, 3–5 × 1–2 µm, belonging to the conidial shape category A (oblong to obovoid conidia) and the small size category (C, 3–5 µm, as defined by Jacobs & Wingfield 2001).

Cultural characteristics — Colonies reaching 45 mm diam after 8 d at 25 °C on OA; no growth observed at 5, 30 and 35 °C. *Hyphae* superficial or submerged; aerial mycelium present,

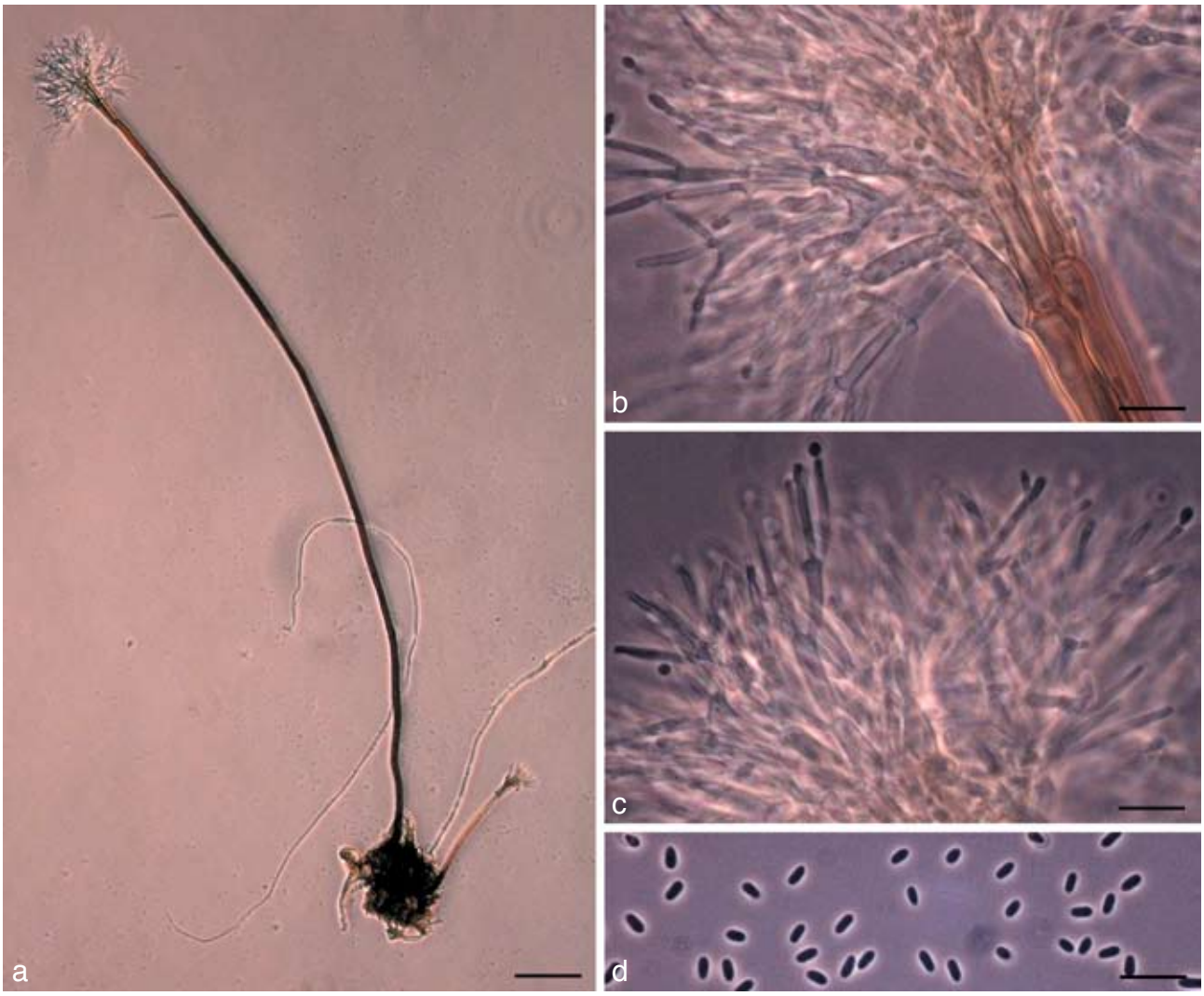

Fig. 3 *Leptographium bhutanense* sp. nov. a. Conidiophore indicating the arrangement of the primary branches on the stipe as Type A; b. conidiogenous apparatus with a complex series of branches; c. conidiogenous cells showing percurrent conidium development; d. oblong to obovoid conidia. — Scale bars: a = 30 µm; b, c = 3.5 µm; d = 6 µm.

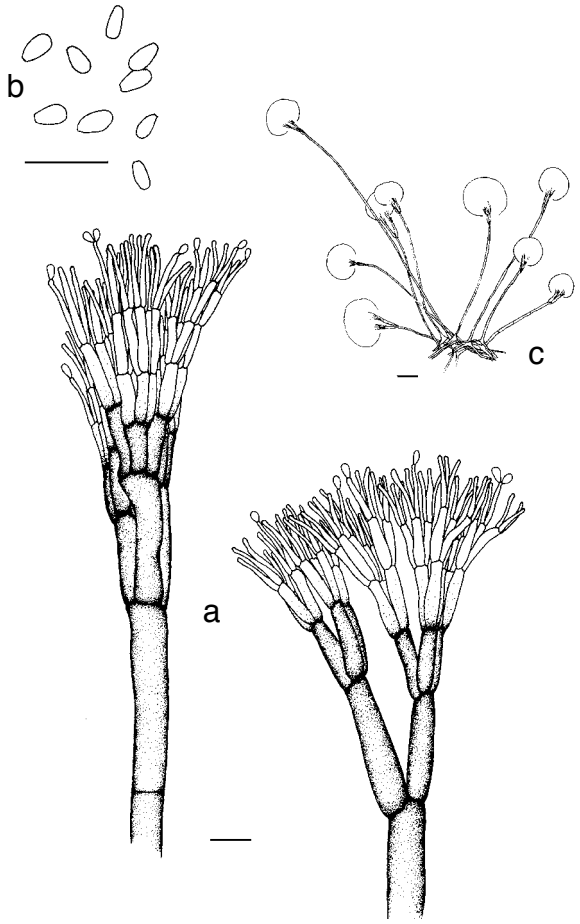

Fig. 4 Line drawings of morphological characters of *Leptographium bhutanense* sp. nov. a. Conidiophores; b. oblong to obovoid conidia; c. habit sketch. — Scale bars: a, b = 10 µm; c = 100 µm.

hyaline, smooth, effuse. Colonies dark-olivaceous (19''f). When old, cultures become white at the centre with numerous, often confluent spore masses and a pale olivaceous (21"k), effuse margin.

Specimens examined. Bhutan, Dhur, Bumthang, isolated from *Pinus wallichiana* infested by *Hylobitelus chenkupdorjii*, July 2005, *M.J. Wingfield, D.B. Chhetri* & *T. Kirisits*, PREM 59752 holotype, culture ex-type CMW 18649 = CBS 122076; PREM 59753 paratype, culture ex-paratype CMW 18650 = CBS 122077; PREM 59754 paratype, culture ex-paratype CMW 18651 = CBS 122078; PREM 59755 paratype, culture ex-paratype CMW 18652.

DISCUSSION

Results of this study have shown that the *Leptographium* species associated with *Hylobitelus chenkupdorjii* infesting *Pinus wallichiana* in Bhutan represents an undescribed taxon for which the name *L. bhutanense* has been provided in this study. Very little is presently known about the occurrence, taxonomy and ecology of ophiostomatoid fungi in the Himalayas. To our best knowledge, *L. bhutanense* is the first *Leptographium* species from this part of Asia that has been determined to species level. Other precisely characterised ophiostomatoid fungi from the Himalayan region include *Ophiostoma himal-ulmi*, described from the Western Himalayas (Brasier & Mehrotra 1995), as well as *Ceratocystis bhutanensis* and *C. moniliformis* occurring in Bhutan (van Wyk et al. 2004). Furthermore, surveys in Bhutan, conducted in 2001 and 2005, have documented a diverse assemblage of ophiostomatoid fungi in this Eastern Himalayan country, that includes, besides *C. bhutanensis* and *C. moniliformis*, a number of species of *Ceratocystiopsis*, *Grosmannia*, *Ophiostoma*, *Leptographium* and *Pesotum* (Kirisits et al. 2002, 2008, van Wyk et al. 2004, Konrad 2006). Many of these fungi are suspected to represent hitherto unknown taxa and investigations on their taxonomic placement are continuing.

Leptographium bhutanense is most similar to *L. procerum* (Wingfield 1985, Jacobs & Wingfield 2001) and *L. profanum* (Jacobs et al. 2006). However, it can be distinguished from these species based on morphology and DNA sequence comparisons. According to our current knowledge, the species also has a unique geographical occurrence, host and insect associate, which should make it easy to distinguish from its closer relatives.

Morphologically, *L. bhutanense* and the two species most closely related to it have long conidiophores, most commonly with only two primary branches present. *Leptographium procerum* forms typical concentric rings in the cultures while these have not been observed in the cultures of *L. bhutanense*. *Leptographium bhutanense* is morphologically almost identical to *L. profanum*, and distinguishing between these species may be difficult. The conidiogenous cells of *L. profanum* are, however, longer than those of *L. bhutanense.* These characters results in the former species having an almost fan-like conidiogenous apparatus, while that of *L. bhutanense* has a brush-like appearance. DNA sequence comparison further showed that isolates of *L. bhutanense* reside in a clade close to but distinct from *L. procerum* and *L. profanum*.

No sign of a teleomorph was found for *L. bhutanense* despite searching for ascomata in cultures and in galleries of the insects. This is similar to *L. procerum,* where a sexual state has never been found, even though great effort has been made to detect one (Wingfield unpubl. observations). If a teleomorph were to be found, it would reside in the genus *Grosmannia*, which is a segregate of *Ophiostoma* s.l. and phylogenetically accommodates *Leptographium* spp. and their teleomorphs (Zipfel et al. 2006).

The ecology of *L. bhutanense* is different from that of *L. profanum*. The latter species was isolated from hardwood roots in the USA and it is not known to be associated with an insect vector (Jacobs et al. 2006). In contrast, *L. bhutanense* is associated with *H. chenkupdorjii* infesting the roots and root collar zone of young conifers in Bhutan. This ecological habit of *L. bhutanense* is remarkably similar to that of *L. procerum*. The latter fungus is casually associated with various bark beetle species (Jacobs & Wingfield 2001, Kirisits 2004) and is consistently found in association with the root collar weevil *H. radicis* and other *Hylobius* species in North America, as well as with *H. abietis* in Europe (Wingfield 1983, Lévìeux et al. 1994, Jacobs & Wingfield 2001, Viiri 2004). Although the sample was relatively small, *L. bhutanense* was present on every tree found infested with *H. chenkupdorjii,* and it was also isolated from adults of this insect. Thus, the intimacy of this association appears to be similar to that of *L. procerum* with root collar weevils.

Leptographium bhutanense is very closely associated with *H. chenkupdorjii,* which is presumed to be its primary vector. It is unknown whether the fungus contributes to killing of *P. wallichiana* saplings, and whether *H. chenkupdorjii* can kill trees in the absence of the fungus. Pathogenicity tests with *L. procerum*, which has an association with a very similar insect, have shown that the fungus is only mildly pathogenic, and only rarely can kill trees in the absence of the insect (Wingfield 1986). Nonetheless, these fungi might contribute to the tree-killing process or to some other feature of the ecology of their vectors. Studies to consider the pathogenicity of *L. bhutanense* to *P. wallichiana* in Bhutan would help to resolve such questions.

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