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

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Key words
Cucuilionidae 
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 chenkudpordorji 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. chenkudpordorji. 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. chenkudpordorji is similar to, but distinct from L. procerus 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, Víri 2004). At least four Leptographium species have been found associated with root and root collar weevils in the genus Hylobius (Jacobs & Wingfield 2001, Víri 2004). Leptographium procerus is closely associated with the seriously damaging pine root collar weevil Hylobius radicus 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 (Lévieux et al. 1994, Víri 2004), which is an important pest in conifer afforestation (G régoire & 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. radicus 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 chenkudpordorji, 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. chenkudpordorji 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 chenkudpordorji (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. chenkudpordorji 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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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 *Hylotibulus chenkupdorjii* (Table 2) by inoculating four OA plates per isolate and incubating these at temperatures ranging from 5 to 35 °C.
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 MgCl₂, 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 % agarose 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 INASE 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. | ITS2   | β–tubulin | EF1-α     |
|--------------------------|-------------|--------|-----------|-----------|
| Grosmannia aenigmatica   | CMW2199     | AY553389 | AY534937 | AY536183  |
| G. americana             | CMW2310     | AY553390 | AY534938 | AY536184  |
| G. aurea                 | CMW709      | AY553413 | AY534961 | AY536207  |
| G. huntii                | CMW714      | AY552071 | AY536205 | AY536206  |
| G. lariis                | CMW2868     | AY553394 | DQ354933 | DQ354938  |
| G. robusta               | CMW668      | AY553397 | AY534945 | AY536191  |
| G. serpens               | CMW2805     | AY553396 | AY534944 | AY536190  |
| Leptographium abietinum  | CMW2817     | DQ362080 | DQ62014  | DQ62047   |
| L. bhutanense            | CMW3083     | DQ362081 | DQ62015  | DQ62048   |
| L. douglasii             | CMW2078     | AY553381 | AY534929 | AY536175  |
| L. lundbergii            | CMW217      | AY553380 | AY534928 | AY536174  |
| L. neomexicanum          | CMW2079     | AY553382 | AY534930 | AY536176  |
| L. pineti                | CMW3831     | DQ620207 | DQ62010  | DQ62043   |
| L. pinidensiflorae       | CMW3837     | DQ620207 | DQ62011  | DQ62044   |
| L. procerum              | CMW1515     | DQ620202 | DQ62016  | DQ62049   |
| L. pyrnum                | CMW169      | DQ620207 | DQ62006  | DQ62039   |
| L. reconditum            | CMW15       | AY553383 | AY534931 | AY536177  |
| L. terebrantis           | CMW9        | AY553384 | EU452698 | EU52700   |
| L. truncatum             | CMW2402     | DQ620201 | DQ61985  | DQ62018   |
| L. wingfieldii           | CMW2096     | AY553398 | AY534946 | AY536192  |
| L. yunnanense            | CMW5152     | DQ620207 | DQ62007  | DQ62040   |

1 Isolates sequenced in this study are presented in bold. 2 ITS2 and partial 28S rRNA gene. 3 Partial β–tubulin 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. chenkupodorji* 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. Stipes 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 x 1–2 µm.

**Coloniae** atro­olivaceae (19”f), ad 45 mm diametro in 8 diebus in OA ad 25 °C crescent; ad 5 °C, 30 °C et 35 °C non crescent.

*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, 6–14 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 x 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.

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**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.
Discussions

Results of this study have shown that the Leptographium species associated with Hyllobitelus chenkupdorji 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, Grossmannia, 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 Grossmannia, 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. chenkupdorji 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. radicus and other Hyllobius species in North America, as well as with H. abietis in Europe (Wingfield 1993, Léviex et al. 1994, Jacobs & Wingfield 2001, Vieri 2004). Although the sample was relatively small, L. bhutanense was present on every tree found infested with H. chenkupdorji, 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. chenkudpordjor*, which is presumed to be its primary vector. It is unknown whether the fungus contributes to killing of *P. wallichiana* saplings, and whether *H. chenkudpordjor* 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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