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A new *Leptographium* species associated with *Tomicus piniperda* in south-western China

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***Tomicus* species (Coleoptera: Scolytidae) are serious pests of pines with a wide distribution in Europe, Asia and America. In Yunnan, south-western China, *T. piniperda* has destroyed more than 0.5 million ha of *Pinus yunnanensis* in the past 15 years. A blue stain fungus belonging to the genus *Leptographium* is associated with both the shoot-feeding and trunk-attacking stages of the beetle's life cycle. The fungus is morphologically similar to the anamorph of *Ophiostoma crassivaginatum* and to *L. pyrinum*, which are both characterised by short robust conidiophores and hyphae covered by a granular layer. Both these species have been isolated from conifers and are associated with insects. After comparing the fungus from *T. piniperda* with similar *Leptographium* species, using light and scanning electron microscopy, we concluded that it represents a new taxon, which is described here as *L. yunnanense* sp. nov.**

Key Words——China; *Leptographium*; morphology; taxonomy; *Tomicus piniperda*.

Tomicus piniperda L. (Coleoptera: Scolytidae), one of the world's major pine forest pests, is widely distributed throughout Europe, North America and Asia (Bakke, 1968; Haack and Kucera, 1993; Yin et al., 1984). In Europe, it is the principal bark beetle that attacks Scots pine (*Pinus sylvestris* L.) and is responsible for severe growth losses (Långström and Hellqvist, 1990). Recently, the beetle has been reported in North America, where it has contributed to a decline in quality of Christmas trees in the Great Lakes region (Haack and Lawrence, 1997). In China, it is regarded as one of five major pests of pines. In the past 15 years, it has destroyed more than 0.5 million ha of Yunnan pine (*P. yunnanensis* Franchet) (Ye, 1991), representing 52% of the total 5 million ha of forests.

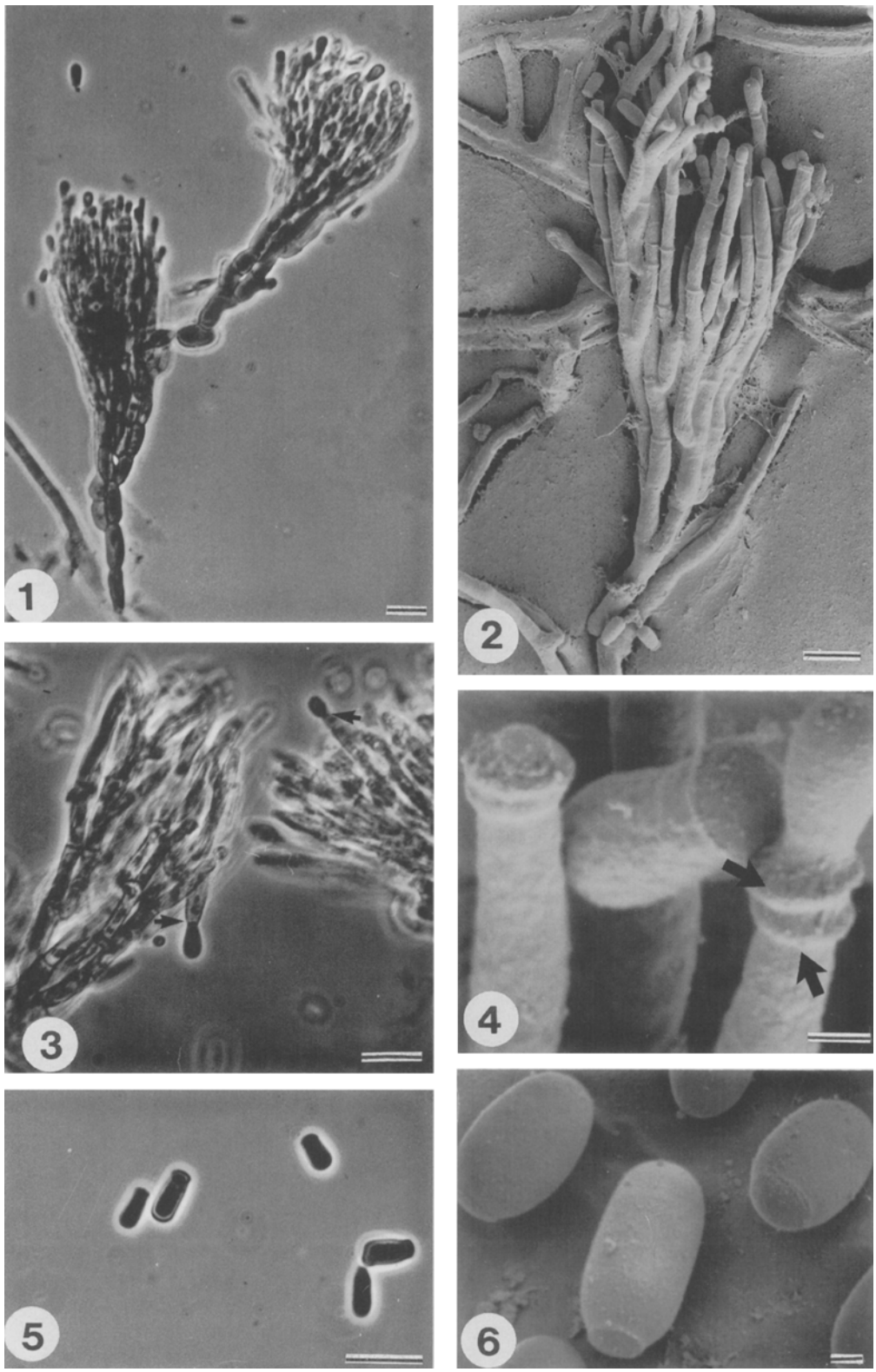
Bark beetles that infest conifers are commonly known to carry *Leptographium* spp. (Kendrick, 1962; Harrington, 1988; Wingfield, 1993; Wingfield and Gibbs, 1991). In some cases this association can lead to considerable losses to forestry (Solheim, 1992). The insects appear to be vectors of the fungi, while the fungi might serve as a source of food for the insect and contribute to the death of trees through mycelial penetration and toxin release (Paine et al., 1997). The nature of this association is, however, still actively being debated (Wingfield et al., 1995).

Leptographium species can generally be recognised by their long mononematous conidiophores with dark stipes and complex conidiogenous apparatuses, consisting of a series of branches. These branches terminate in conidiogenous cells that produce numerous conidia through annellidic conidium development (Kendrick, 1962). In most cases the conidiogenous cells are characterised by delayed secession of the conidia, which gives them the appearance of sympodial conidium development (Van Wyk et al., 1988). The hyaline and aseptate conidia of *Leptographium* species are accumulated in slimy masses at the apices of the conidiophores, making these fungi ideal for insect dispersal.

Limited research has been done on the taxonomy of fungi associated with bark beetles in Asia (Kaneko and Harrington, 1990; Yamaoka et al., 1997, 1998; Masuya et al., 1998, 1999) and no previous study has been conducted in China. During the course of a study to isolate and identify the blue stain fungi associated with *T. piniperda* attacking *P. yunnanensis* in south-western China, a *Leptographium* species was isolated in association with *T. piniperda*. The aim of this study was to identify and name this fungus.

Materials and Methods

Sampling and fungal isolation In excess of 1200 adult *T. piniperda* beetles were collected during the beetle's shoot-feeding and trunk-attacking stages in four different localities of Yunnan, south-western China, approximately 250 km apart. Collections were made during one season of the beetle's life cycle. Each infested shoot, including a beetle within it, and each gallery including a pair of beetles within the gallery, were placed in a separate



Figs. 1–6. *Leptographium yunnanense* (CMW 5304) on MEA. 1. Conidiophores with dark olivaceous stipes and complex conidiogenous apparatuses (Bar = 10 μm). 2. Complex conidiogenous apparatus (Bar = 20 μm). 3, 4. Conidiogenous cells showing false sympodial, and annellidic conidiogenesis indicated by arrows (Bar = 10 μm in Fig. 3, 1 μm in Fig. 4). 5, 6. Conidia (Bar = 10 μm in Fig. 5, 1 μm in Fig. 6).

clean plastic bag. All beetle samples were inoculated into 1 m long (15–20 cm diam) freshly cut and uninfected Yunnan pine logs waxed at both ends using the technique described by Furniss et al. (1990), and maintained at room temperature. After 4 wk, fungi were isolated from the lesions that developed in the phloem and transferred to potato dextrose agar (PDA, 150 g potatoes, 15 g dextrose, 20 g Biolab agar in 1000 ml distilled water) amended with 0.04% streptomycin. Purified colonies were transferred to 2% malt extract agar (MEA, 20 g Biolab malt extract, 20 g Biolab agar and 1000 ml distilled water) plates and incubated at 25°C until the onset of sporulation. Fungal structures for microscopic examination were mounted on glass slides in lactophenol. Fifty measurements of each relevant morphological structure were made. Colours were determined with the aid of colour charts (Rayner, 1970). All cultures used in this study are maintained in the Culture Collection of Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, Republic of South Africa (CMW) and representative material including dried and living cultures has been deposited in the National Collection of Fungi (PREM, PPRI), South Africa.

Scanning electron microscopy For scanning electron microscopy, small blocks of agar cut from sporulating colonies were fixed in 3% glutaraldehyde and 0.5% osmium tetroxide in a 0.1 M phosphate buffer, dehydrated in a graded acetone series and critical-point dried.

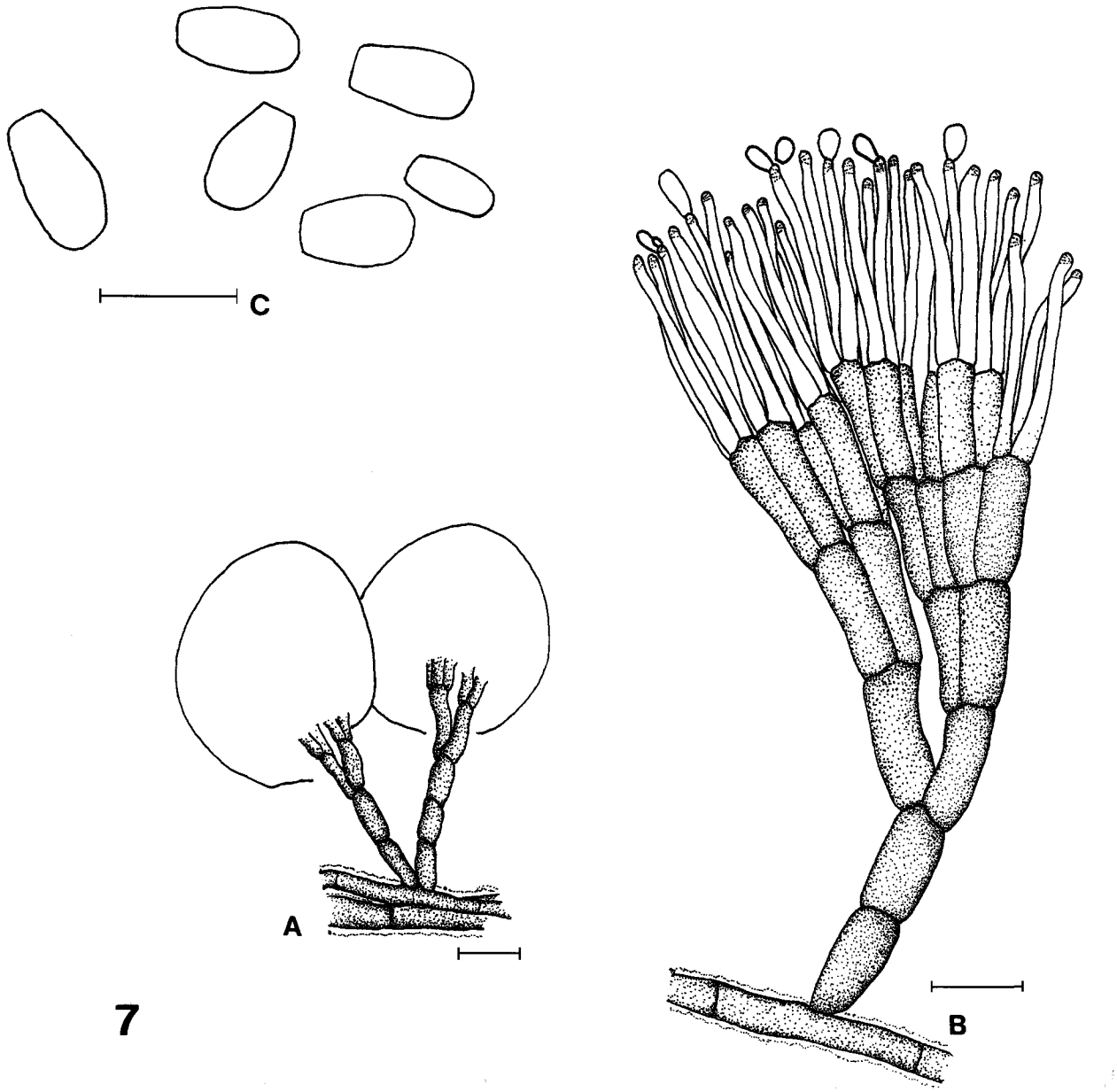


Fig. 7. *Leptographium yunnanense* (CMW 5304) on MEA. A. Habit sketch of the conidiophores (Bar=50 μm). B. Conidiogenous apparatus (Bar=10 μm). C. Conidia (Bar=10 μm).

Specimens were mounted and coated with gold palladium alloy and examined using a JEOL JSM 840 scanning electron microscope.

Optimal temperature for growth The optimal growth temperature for the isolates (CMW 5152, CMW 5153, CMW 5304 and CMW 5305) was determined by inoculating eight MEA plates for each temperature with a 6.0 mm diam agar disk taken from the actively growing margin of a fresh isolate. The plates were incubated at temperatures ranging from 5 to 35°C at 5°C intervals. Colony diameters were measured on the fourth and the eighth day after commencing the experiment, and an average was calculated from eight random readings.

Cycloheximide sensitivity Cycloheximide tolerance of isolates (CMW 5152, CMW 5153, CMW 5304 and CMW 5305) was determined by growing them on 2% MEA amended with 0.5 g/l cycloheximide. Dishes were incubated in the dark at 25°C for 7 d, and two colony diameters were measured at right angles. Five replicate plates were used and the growth rate (mm/day) was determined based on the average of ten diameter readings.

Results

A *Leptographium* species was isolated from 12.3% of adult *T. piniperda* that had been collected from both shoot-feeding and trunk-attacking stages. Isolates of the *Leptographium* species are characterised by small robust conidiophores and an optimal growth temperature of 25°C. This species also proved to be tolerant to cycloheximide, with no reduction in growth when grown on 0.5 g/l of the antibiotic. Isolates of this species produced a considerable number of conidia. In older cultures, the spore masses become hardened, making the observation of the sporulating structures difficult. The hyphae of the *Leptographium* species were characterised by granular surfaces. Comparison with known species of *Leptographium* led us to conclude that this is a previously undescribed *Leptographium* species, and it is described herein.

Leptographium yunnanense X. D. Zhou, K. Jacobs, M. J. Wingfield & M. Morelet, sp. nov. Figs. 1–7

Coloniae in 2% MEA ad 25°C optime crescentes et post 7 dies 13 mm diam attingentes, margine integrae. Mycelia immersa vel emersa, hyphis aeriis sparsis emittentia, atro-olivacea vel hyalina, ad exterius granulares. Conidiophora singula, erecta, macronematosa, mononematosa, 74.0–233.0 μm longa; structura rhizoideiformis absens. Apparatus conidiogeni praeter massam conidii (40.0–)83.0–88.0(–127.0) μm longi, ex ramis cylindricis 2 vel 4-seriatis compositi; rami primarii 2 vel 3, pallide olivacei vel hyalini, cylindrici, laeves, 0-1-septati, (9.0–)12.0–15.0(–20.0) × 3.0–7.0 μm; rami secundarii pallide olivacei vel hyalini, aseptati, (9.0–)13.0 × 15.0(–20.0) × 3.0–6.0 μm; rami tertiarii aseptati, 7.0–19.0(–24.0) × 2.0–5.0 μm; rami quartii (11.0–)14.0–17.0(–20.0) μm. Cellulae conidiogenae discretae, 2 vel 3 per ramum, cylindricae, apicem versus leviter attenuatae, (18.0–)23.0–26.0(–32.0) μm longae, 2.0–4.0 (–6.0) μm latae. Conidia oblonga vel obovoidea, lasi truncata, ad apicem apparatus conidiogeni in massa guttulata mucilaginosa accumulata, (4.0–)7.0–8.0(–11.0) × 2.0–6.0 μm.

Colonies with optimal growth at 25°C on 2% MEA, reaching 13 mm in diam in 7 d; no growth below 10°C or above 30°C; able to withstand high concentrations of cycloheximide, with no reduction in growth on 0.5 g/l cycloheximide after 7 d at 25°C in the dark; on MEA dark olivaceous (19″f), with smooth margins; mycelia submerged or on top of agar with sparse aerial hyphae, dark olivaceous to hyaline, granular outer surface, not constricted at the septa, (2.0–)3.0–7.0(–9.0) μm in diam. Conidiophores occurring singly, arising directly from the mycelium, erect, macronematous, mononematous, 74.0–227.0(–233.0) μm in length; rhizoid-like structures

Table 1. Comparison of morphological characteristics of *Leptographium yunnanense, Ophiostoma crassivaginatum* anamorph and *L. pyrinum*.

	L. yunnanense	*O. crassivaginatum* anam.	*L. pyrinum*
Substrate	*Pinus yunnanensis* *P. densata* *P. kesiya*	*Picea mariana, P. glauca, Pinus resinosa, P. strobus, P. sylvestris, Populus grandidenta, P. tremuloides, Fraxinus nigra*[a]	*Pinus ponderosa*[c]
Insect association	*Tomicus piniperda*	*Trypodendron retusus*[b]	*Dendroctonus adjunctus*[d]
Conidiophore length	74.0–227.0(–233.0) μm	25.0–106.0(–118.0) μm	(117.5–)215.0–236.5(–392.5) μm
Primary branches	2–3	2–3	2–4
Rhizoids	absent	absent	present
Conidium shape	obovoid	oblong to obovoid	oblong, almost pear-shaped
Conidium length	(4.0–)7.0–8.0(–11.0) μm	(4.0–)4.5–5.5(–10.0) μm	5.0–12.0 μm
Conidium width	2.0–6.0 μm	1.0–2.5 μm	4.0–6.0 μm
Granular hyphae	present	present	present
Teleomorph	absent	present	absent

a) Griffin (1968), Olchowecki and Reid (1974); b) Harrington (1988); c) Davidson (1978); d) Davidson (1978), Harrington (1988), Perry (1991), Six and Paine (1996).

absent; stipe light olivaceous, cylindrical, simple, with 0–4 septa, occasionally constricted, 11.0–66.0 (–112.0) μm long (from first basal septum to below primary branches), 4.0–9.0 μm wide below primary branches; apical cells not swollen, (3.0–)5.0–6.0(–11.0) μm wide at base; basal cells not swollen (Figs. 1, 7a, b). Conidiogenous apparatus (40.0–)83.0–88.0(–127.0) μm long (excluding the conidial mass), with 2 to 4 series of cylindrical branches; primary branches light olivaceous to hyaline, smooth, cylindrical, with 0–1 septum, (9.0–) 12.0–15.0(–20.0) μm long, 3.0–7.0 μm wide; secondary branches light olivaceous to hyaline, aseptate, (9.0–) 13.0–15.0(–20.0) μm long, 3.0–6.0 μm wide; tertiary branches light olivaceous to hyaline, aseptate, 7.0–19.0(–24.0) μm long, 2.0–5.0 μm wide; quaternary branches (11.0–)14.0–17.0(–20.0) μm long, 2.0–5.0 μm wide (Figs. 2, 7b). Conidiogenous cells discrete, 2–3 per branch, cylindrical, tapering slightly toward the apex, (18.0–)23.0–26.0(–32.0) μm long, 2.0–4.0(–6.0) μm wide. Conidium development occurring through replacement wall building with holoblastic ontogeny, percurrent proliferation and delayed secession, giving the false impression of sympodial proliferation (Minter et al., 1982; Van Wyk et al., 1988) (Fig. 4). Conidia accumulating in slimy droplets at the apex of conidiogenous apparatus, oblong to obovoid, with a truncate base, (4.0–)7.0–8.0 (–11.0) × 2.0–6.0 μm (Figs. 5, 6, 7c).

Materials examined: Cultures on 2% MEA, isolated from *T. piniperda* infesting *P. yunnanensis*, Yunnan, south-western China, collected by Xu Dong Zhou, Hui Ye and HuaSun Ding, March 1997, CMW 5304 (=PPRI 6907, living culture) (holotype: PREM 56579, dried culture); December 1996, CMW 5305 (=PPRI 6908, living culture) (paratype: PREM 56580, dried culture); March 1997, CMW 5153 (=PPRI 6923, living culture) (paratype: PREM 56578, dried culture); August, 1995, CMW5152 (=PPRI 6909, living culture), no PREM number for this isolate presently.

Discussion

Leptographium yunnanense can easily be recognised by its small conidiophores, which are abundantly produced on the surface of MEA. As with other species in *Leptographium* (Harrington, 1988), *L. yunnanense* can tolerate high concentrations of cycloheximide. *Leptographium yunnanense* is typical of the other members of this genus: i.e., numerous conidia produced through annellidic conidium development and accumulated in slimy masses on the apices of the conidiophores. In older cultures, spore masses flow from the conidiophores, become sticky and cover the entire structure. This makes the study of the conidiophore structure in older cultures extremely difficult.

Leptographium yunnanense is morphologically similar to the *Leptographium* anamorph of *O. crassivaginatum* (H. D. Griffin) T. C. Harr. and *L. pyrinum* R. W. Davidson. These species are all characterised by short robust conidiophores and hyphae that appear to have a granular surface. Furthermore, these species have all been isolated from conifers and are associated with insects (Griffin, 1968; Davidson, 1978).

Leptographium yunnanense, lacking the teleomorph, can be distinguished from the anamorph of *O. crassivaginatum* based on its slightly longer conidiophores (Table 1). *Leptographium yunnanense* and the anamorph of *O. crassivaginatum* have conidia of similar length, while those of *L. yunnanense* are almost twice as broad as those of *O. crassivaginatum* anamorph. This makes the conidia of *L. yunnanense* distinctly obovoid compared with the oblong conidia of *O. crassivaginatum* anamorph (Griffin, 1968).

Leptographium yunnanense can be distinguished from *L. pyrinum* based on the considerably longer conidiophores in the latter species (Table 1). *Leptographium yunnanense* and *L. pyrinum* have conidia of similar dimensions, but can be distinguished based on the pear-shaped conidia of *L. pyrinum*, compared with the obovoid conidia of *L. yunnanense*. *Leptographium pyrinum* is also characterised by conidiophores with rhizoids (Davidson, 1978), while these structures are absent in *L. yunnanense*. In addition, *L. yunnanense* is associated with *T. piniperda* in China, while *L. pyrinum* is associated with *Dendroctonus* species in the western USA (Davidson, 1978).

This is the first report on fungi associated with *T. piniperda* in China. The beetle has been considered to be a secondary pest, which usually colonizes weakened, stressed and recently killed trees (L°0angström and Hellqvist, 1993). However, in China, it can also attack healthy trees (Ye, 1991). *Leptographium yunnanense* occurs in China, not in Europe, where the beetles infest and feed on shoots as well as colonise trunks of living trees. The insect, therefore, appears to be a much more serious pest in China than in Europe.

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