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Leptographium pini-densiflorae sp. nov. from Japanese red pine*

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A *Leptographium* species was isolated from dead *Pinus densiflora* at six sites in Japan. The fungus is morphologically most similar to *L. lundbergii* but could be distinguished from that species by its short stipes, primary branches of conidiophores, and conidia with a rounded to sub-truncate base. In addition, the colony morphology, growth rate and tolerance to the antibiotic cycloheximide of the *Leptographium* species and *L. lundbergii* differed markedly. Here we describe the fungus as a new species, *Leptographium pini-densiflorae*.

Key Words—blue-stain fungi; *Leptographium pini-densiflorae*; *Pinus densiflora*.

The genus *Leptographium* was established by Lagerberg and Melin (Lagerberg et al., 1927) to accommodate the type species *L. lundbergii* Lagerb. & Melin, which commonly occurs in Europe on blue-stained pine lumber. Species of *Leptographium* are characterized by having robust dematiaceous conidiophores that terminate in a conidiogenous apparatus comprised of a series of branches. These branches give rise to conidiogenous cells from which hyaline, single-celled conidia are produced in slimy masses (Lagerberg et al., 1927; Kendrick, 1962). The erect conidiophores above the substrate surface and the slimy conidial masses make *Leptographium* species ideally suited for transmission by insects.

Leptographium has a complex taxonomic history, which was summarised in two recent treatments (Harrington, 1988; Wingfield, 1993). Many species currently treated in *Leptographium* were formerly accommodated in *Verticicladiella* S. Hughes, a genus that had been segregated from *Leptographium* based on sympodial as opposed to percurrent conidial development (Hughes, 1953; Kendrick, 1962). Later, Wingfield (1985) showed that species of *Leptographium* and *Verticicladiella* have identical patterns of conidial development, and *Verticicladiella* was consequently reduced to synonymy with *Leptographium*.

Leptographium species are common anamorphs of *Ophiostoma* species. These fungi are best known as associates of bark beetles (Coleoptera: Scolytidae), the majority of which infest *Pinus* species. (Harrington, 1988). *Ophiostoma* species and their *Leptographium* anamorphs are unusual amongst the Ascomycetes having cellulose and rhamnose in their cell walls (Rosinski and Campana, 1964; Smith et al., 1967; Jewell, 1974; Weijman and de Hoog, 1975). Correlated with this characteristic is the fact that they are also able to tolerate very high concentrations of the antibiotic cycloheximide in artificial media (Harrington, 1981).

The majority of *Leptographium* species have been described from Europe and North America, and few species are known from Asia. Wingfield (1993) thus suggested that the lack of collections from Asia was a serious encumbrance to our knowledge of this group of fungi. Masuya et al. (1998) undertook a study of blue-stain fungi on Japanese red pine (*Pinus densiflora* Sieb. & Zucc.) infested with the pine shoot beetle *Tomicus piniperda* L. This study led to the collection of an unidentified *Leptographium* species. This fungus has subsequently been collected from *P. densiflora* at several sites in Japan. The aim of this study was to fully characterize the unidentified *Leptographium* species and to provide an appropriate name for it.

Materials and Methods

Isolations were made from the bark or sapwood of dead *P. densiflora* trees at six sites in Japan from 1995 to 1997. Two small pieces of bark or sapwood were placed in a Petri dish containing 1% malt agar (malt extract, 10 g; agar, 15 g; distilled water, 1000 mL) without surface-sterilization and kept at 15°C in the dark. After 2

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Table 1. List of the cultures used in this study, their locality and hosts.

Culture No.	Identity	Locality	Host
CMW28 (PREM45698)	*Leptographium lundbergii*	Wilgeboom, Eastern Transvaaal, South Africa	*Pinus taeda*
CMW30 (PREM45699)	*L. lundbergii*	Gwavas, North Island, New Zealand	*P. strobus*
CMW2398	*L. lundbergii*	Toronto, Ontario, Canada	*P. resinosa*
CMW2408			
(MAFF410548)	*L. lundbergii*	Morioka, Iwate, Japan	*P. densiflora*
MAFF410861 (MCC071)	*Leptographium* sp.	Tsukuba, Ibaraki, Japan	*P. densiflora*
MAFF410862 (MCC190)	*Leptographium* sp.	Nakajima, Ishikawa, Japan	*P. densiflora*
MAFF410863 (MCC192)	*Leptographium* sp.	Himeji, Hyogo, Japan	*P. densiflora*
MAFF410864 (MCC193)	*Leptographium* sp.	Ichinoseki, Iwate, Japan	*P. densiflora*
MAFF410865 (MCC194)	*Leptographium* sp.	Masuho, Yamanashi, Japan	*P. densiflora*
MAFF410866 (MCC205)	*Leptographium* sp.	Motegi, Saitama, Japan	*P. densiflora*

Culture collections are as follows: PREM, the National Collection of Fungi, Pretoria, South Africa; CMW, Culture Collection of the Tree Pathology Co-operative Programme, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; MAFF, Genetic Resources Center, Culture Collection of National Institute of Agrobiological Resources, Japan; MCC, Culture Collection of H. Masuya.

mo, fungi that had grown on the plates were isolated by picking up hyphae or conidial masses using a sterilized tungsten needle and transferring these structures to Petri dishes containing 2% malt agar (malt extract, 20 g; agar, 15 g; distilled water, 1000 mL). Petri dishes were incubated at 15°C in dark for 2 wk.

All isolates used in this study (Table 1) were incubated at 15°C in the dark, and after 2 wk, small pieces of sterilized pine twig or bark were added to the plates to stimulate sporulation. Conidiophores and conidia that were produced in cultures were mounted on glass slides in 1% lacto-fuchsin and observed and measured using a light microscope.

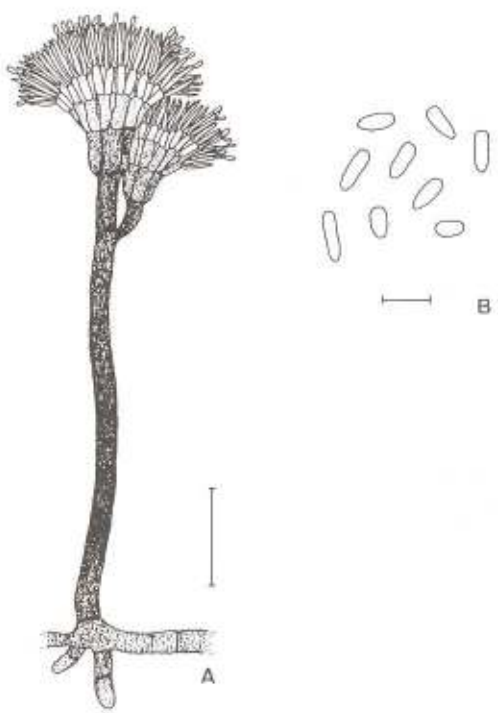


Fig. 1. *Leptographium pini-densiflorae*.
A. Conidiophore. B. Conidia. Scale bars = 50 μm in Fig. 1A; 5 μm in Fig. 1B.

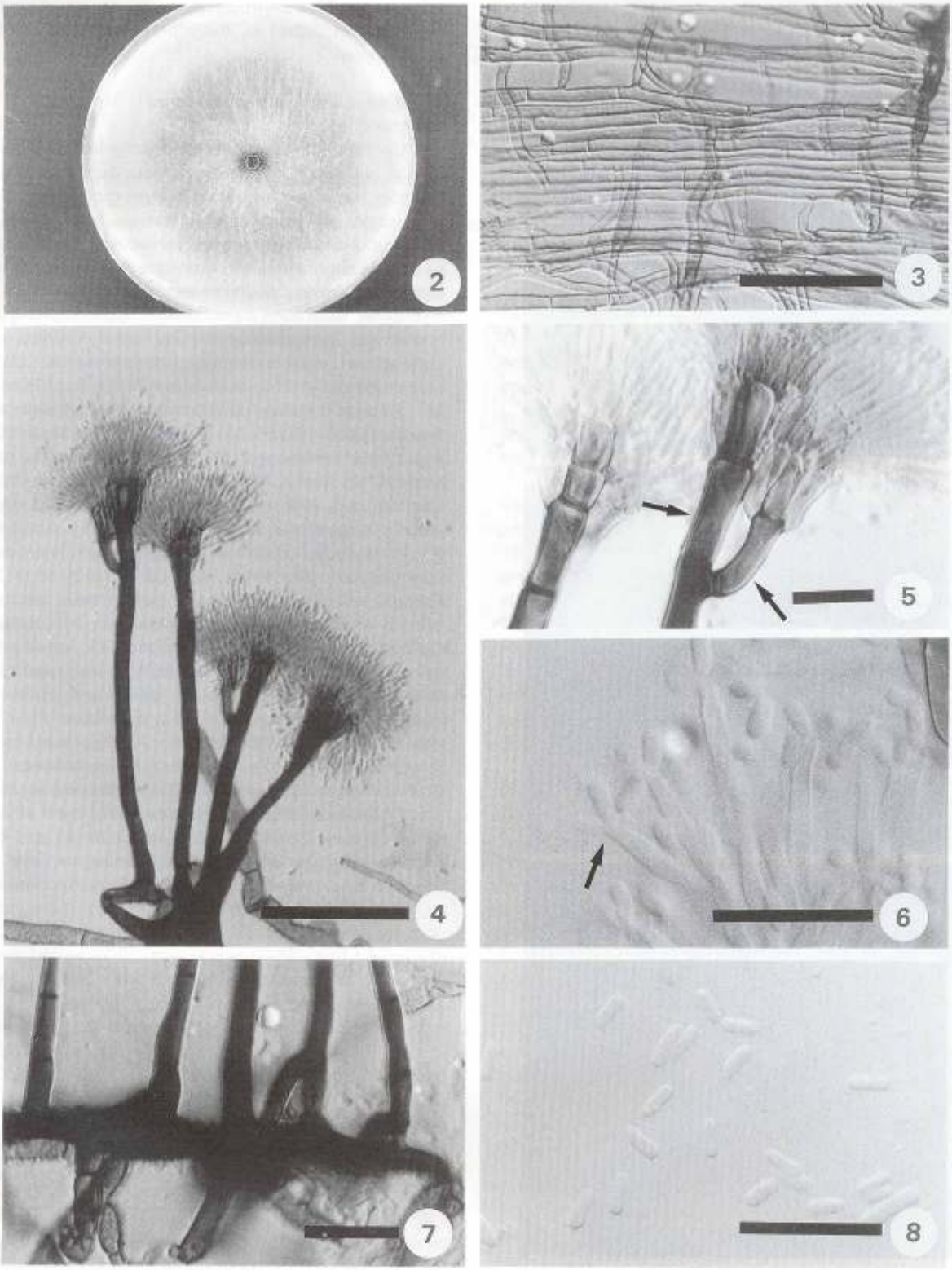
For scanning electron microscopy (SEM), agar disks 8 mm in diam were cut from the colonies and fixed in 3% glutaraldehyde overnight. They were then dehydrated in a graded ethanol series, passed through ethanol-isoamylacetate, and dried with a Hitachi critical point drier. The specimens were examined using a Hitachi S-4200 scanning electron microscope.

The growth rates of isolates were determined at 4, 10, 15, 20, 25, and 30°C. Agar disks 5 mm in diam were cut from actively growing colonies of each isolate and placed at the center of plates containing 2% malt extract agar. Three replicate plates were prepared for each isolate. In addition, cycloheximide tolerance of isolates was also examined at different concentrations (0, 0.05, 0.1, 0.5, 1.0, 2.5, and 5.0 g/L). Colony diam on each plate was measured after 1 wk of incubation at 20°C, and growth rates were calculated as mm/d.

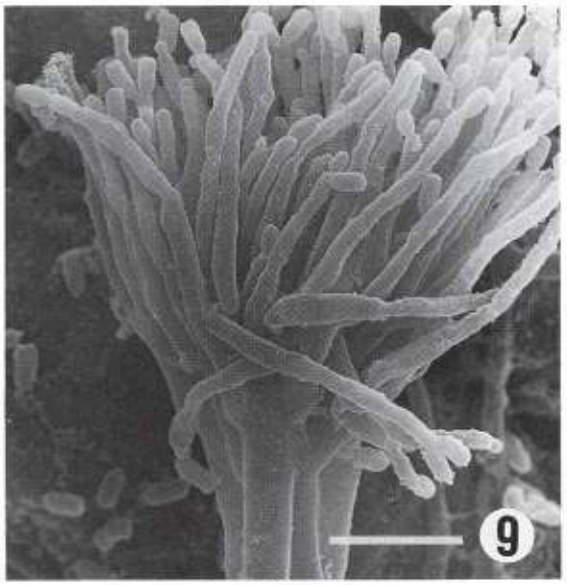
Taxonomy

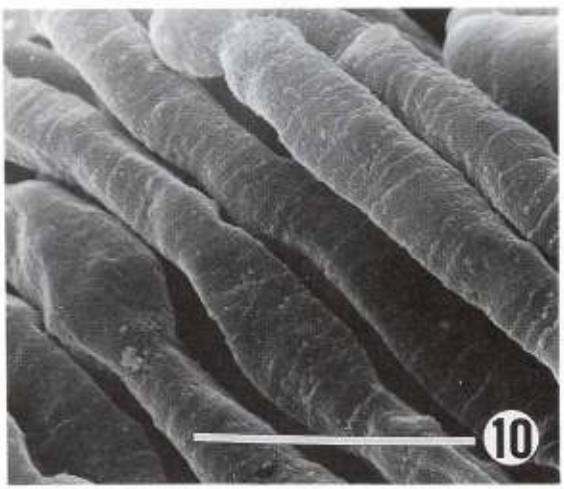
The unidentified *Leptographium* species was compared with all accepted *Leptographium* species and found to be most similar to *L. truncatum* (M. J. Wingf. & Marasas) M. J. Wingf. (Wingfield and Marasas, 1983). *Leptographium truncatum* has been collected from Japanese red pine killed by the pine wood nematode *Bursaphelenchus xylophilus* (Steiner & Buhrer) in Japan (Kaneko and Harrington, 1990). Recently, Strydom et al. (1997) neotypified *L. lundbergii* and proposed that *L. truncatum* is a synonym of *L. lundbergii* based on rDNA-ITS2 sequence data. In this study, we follow the taxonomy of Strydom et al. (1997).

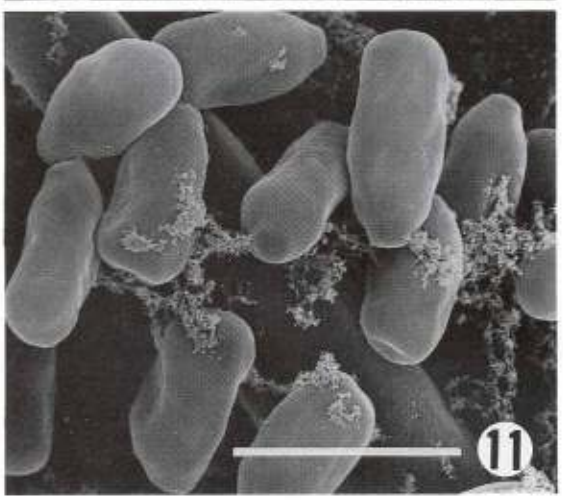
The unidentified *Leptographium* species was compared with four cultures of *L. lundbergii* (Table 1). We were thus able to distinguish the unidentified *Leptographium* species from *L. lundbergii* based on morpho-



Figs. 2–8. Morphological characteristics of *Leptographium pini-densiflorae*.
2. Colonies on MA grown for 2 wk at 20°C. 3. Mycelial strands. 4. Conidiophores in group. 5. Primary branches of conidiophore (arrows). 6. Conidiogenous cells showing the appearance of sympodial development (arrow). 7. Rhizoids at the bases of conidiophores. 8. Hyaline, oblong to ellipsoid, sometimes clavate conidia with rounded apices and rounded to subtruncate bases. Scale bars = 50 μm in Figs. 3, 5; 5 μm in Figs. 4, 6–8.







Figs. 9–11. Scanning electron micrographs of conidiogenous cells and conidia of *L. pini-densiflorae*.
9. Conidiogenous apparatus. 10. Conidiogenous cells showing percurrent proliferation. 11. Conidia with subtruncate bases. Scale bars=10 μm in Fig. 9; 5 μm in Figs. 10, 11.

logical characteristics, growth rate, and cycloheximide tolerance. This *Leptographim* species isolated from *P. densiflora* in Japan is, therefore, described below as a new species.

Leptographium pini-densiflorae Masuya & M. J. Wingfield, sp. nov. Figs. 1–11

Coloniae in 2% agaro maltoso primo hyalinae, dein pallide olivaceae, hyphis aeriis abundantibus formantes. Mycelia recta vel curvata, in superficie medii radiatim divaricatae vel in medium immersa, hyalina vel pallide brunnea, interdum brunnea, verrucosa, 1.5–12 μm diam ex hyphis separatim vel plerumque 3–12 subparallele reptantibus composita. Hyphae aeriae hyalinae vel pallide brunneae, interdum verrucosae. Conidiophora macronematosa, mononematosa, e latere hyphorum singulariter vel saepe laxe aggregatim exorientia. Rhizoidea basi conidiophororum evolventia, sed in hyphis aeriis nulla. Stipites erectus, pallide brunnei vel atro-brunnei, 1–6-septati, 32–190 (–320) μm longi, ad basim 3–10.5 lati. Apparatus conidiogeni 22–80 μm longi massa conidica exclusi, ex ramis 3–5 (plerumque 3–4) seriatis constantes; rami primarii usque quatuor sed plerumque duo inter se adjacentes, ramus centralis distinctus nullus, 6–24×2–6.4 μm. Cellulae conidiogenae discretae, hyalinae, sursum attenuatae, 5.3–13 (–16.2)×1.1–2.6 μm. Conidiogenesis holoblastica, percurrens, per separationem retardatam ut in proliferatione sympodiali visa. Conidia hyalina, oblonga vel ellipsoidea, interdum clavata, apice rotundata, basi rotundata vel subtruncata, 2.6–8.3 (–13)×0.9–3.0 μm, circa apparatum conidiogenum in massa hyalina mucilaginosa accumulata.

HOLOTYPUS: FPH (TFM=) 7389; colonia exsiccata in cultura ex ligno Pini densiflorae, Tsukuba-shi, Ibaraki Pref, Japonia, 27 Maius, 1996, a H. Masuya isolata.

Colonies at first hyaline becoming light olivaceous, aerial hyphae abundant on 2% malt extract agar (Fig. 2). Mycelia straight or curved, spreading radially on the medium and immersed in the medium, hyaline to pale

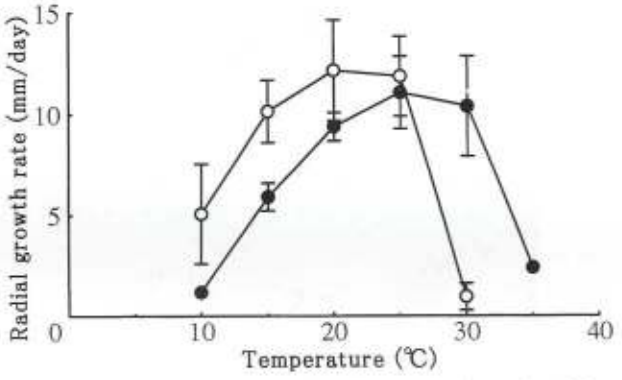


Fig. 12. Mean growth rate (mm/d±standard error) of *Leptographium pini-densiflorae* (MAFF410861, 410862, 410863, 410864, 410865, 410866) and *L. lundbergii* (CMW28, 30, 2398, 2408) on malt extract agar at various temperatures.
○: *L. lundbergii*. ●: *L. pini-densiflorae*.

Table 2. Growth rates of *Leptographium pini-densiflorae* and *L. lundbergii* on malt extract agar supplemented with various concentrations of cycloheximide.

Species	Concentrations of cycloheximide (g/L)						
	0	0.05	0.1	0.5	1	2.5	5
L. pini-densiflorae[a)]	6.4 (±0.1)[c)]	5.8 (±0.2)	5.7 (±0.2)	5.4 (±0.2)	5.2 (±0.2)	4.8 (±0.2)	4.2 (±0.2)
L. lundbergii[b)]	13.0 (±2.9)	12.4 (±2.3)	12.5 (±3.1)	11.5 (±2.6)	10.8 (±2.2)	8.9 (±2.2)	7.5 (±2.0)

a) MAFF410861, 410862, 410863, 410864, 410865, 410866.
b) CMW28, 30, 2398, 2408.
c) Growth rates represent the means of three measurements of each culture grown at 15°C, shown in mm/d (±standard error).

brown, sometimes brown, verrucose, 1.5–12 μm diam, occurring singly or aggregated in strands of 3–12 hyphae (Fig. 3); aerial hyphae hyaline to pale brown, sometimes verrucose. Conidiophores macronematous, mononematous, arising laterally from hyphae, single but often also in loosely arranged groups on the hyphae (Figs. 1A , 4), with rhizoidal hyphae at the bases (Fig. 7), but not developed on aerial hyphae. Stipes erect, pale brown to dark brown, 1–6-septate, 32–190 (–324) ($\bar{x}$=86.3) μm long and 3–10.5 ($\bar{x}$=6.2) μm wide at base (Fig. 4). Conidiogenous apparatus 22–80 ($\bar{x}$=46.5) μm long (excluding conidial mass) consisting of three to five but mostly three or four series of branches (Figs. 5, 9). Up to four primary branches present but mostly two adjacent to each other without a distinct central branch, 6–24×2–6.4 ($\bar{x}$=13×4) μm. Conidiogenous cells discrete, hyaline, tapering from base to apex, 5.3–13 (–16.2)×1.1–2.6 ($\bar{x}$=10×1.6) μm (Fig. 6). Conidium development replacement wall building with holoblastic ontogeny and percurrent ontogeny, but with delayed separation giving a false appearance of sympodial proliferation (Figs. 6, 10). Conidia hyaline, oblong to ellipsoid, sometimes clavate, with rounded apices and rounded to subtruncate bases (Figs. 1B, 8, 11), 2.6–8.3 (–13)×0.9–3.0 ($\bar{x}$=4.9×1.8) μm, accumulating around the conidiogenous apparatus in a hyaline mucilaginous mass.

The growth rate of colonies on 2% malt extract agar 5.4–7.2 ($\bar{x}$=6.4) mm/d at 20°C. Growth reduced at temperatures below 20°C and above 30°C, and no growth occurred at 4°C. The fungus is tolerant to cycloheximide, with growth at 20°C reduced by approximately 65% on 2% malt extract agar containing 5.0 g/L cycloheximide (Table 2).

HOLOTYPE: FPH (=TFM) 7389, dried culture of MAFF410861 (JCM10479) from Tsukuba, Ibaraki Pref., Japan, on *Pinus densiflora* infested by *Tomicus piniperda*, isolated by H. Masuya on 27 May 1996, from conidial mass.

PARATYPE: FPH (TFM) 7390, dried culture of MAFF410865 (JCM10480) from Masuho, Yamanashi Pref., Japan, on *Pinus densiflora* infested by *Tomicus piniperda*, isolated by H. Masuya on 15 May 1996, from conidial mass.

ETYMOLOGY: pini-densiflorae=of *Pinus densiflora* in Latin.

Of all accepted *Leptographium* species, *L. pini-densiflorae* is morphologically most similar to *L. lundbergii*. It can, however, be distinguished from the latter species based on a number of morphological characteristics. The most obvious of these is the fact that *L. lundbergii* has conidia with distinctly truncate bases. This is in contrast to the rounded to sub-truncate conidial bases in *L. pini-densiflorae*. In addition, the mean length of the conidiophores of *L. pini-densiflorae* (110 μm) is less than that of *L. lundbergii* (180 μm).

The culture characteristics of *L. pini-densiflorae* are very distinct from those of *L. lundbergii*. Cultures of *L. pini-densiflorae* are light olivaceous, 19″k (Rayner, 1970), whereas those of *L. lundbergii* are dark mouse-grey, 15‴k (Rayner, 1970). Isolates of *L. lundbergii* also grow on 2% malt extract agar more rapidly than do those of *L. pini-densiflorae* (Table 2). In addition, the former fungus has an optimum temperature for growth between 20 and 25°C and is unable to grow at 35°C, whereas the latter fungus displays optimum growth between 25 and 30°C and is also able to grow at 35°C (Fig. 12).

Many species of *Leptographium* are known to cause blue-stain of the sapwood in lumber (Gibbs, 1993), and some cause root disease in standing trees (Harrington, 1988; Wingfield et al., 1988). In a preliminary inoculation test, *L. pini-densiflorae* showed no effect on the health of five-year-old *P. densiflora* seedlings (our unpublished data), and we thus presume that the fungus is not a primary pathogen. At this stage, we believe that the fungus causes sap stain only, which is typical of many *Leptographium* species.

Masuya et al. (1998) isolated *L. pini-densiflorae* from the pine shoot beetle *T. piniperda*, although the frequency of isolation was low. It is thus possible that this fungus is an accidental associate of *T. piniperda*, rather than being closely linked to the biology of the insect. Future studies are planned to consider the vector relationships of the fungus and its pathogenicity to healthy trees.

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