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Leptographium sinoprocerum sp. nov., an undescribed species associated with *Pinus tabuliformis-Dendroctonus valens* in northern China

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Abstract: During a study of ophiostomatoid fungi associated with the invasive pest *Dendroctonus valens* in the *Pinus tabuliformis* ecosystem in northern China, a multigenic (ITS2-LSU, β-tubulin and EF1-α) phylogenetic analysis and examination of morphological features revealed in addition to *Leptographium procerum* the occurrence of an undescribed species. The new species, *Leptographium sinoprocerum*, belongs to the *L. procerum-L. profanum* clade. Both *L. procerum* and *L. sinoprocerum* are similar to each other and occur sympatrically in the ecosystem studied. Nevertheless *L. sinoprocerum* can be distinguished from *L. procerum* by shorter conidiophore stipes arising from both submerged and aerial hyphae, slightly more oblong conidia, a granular ornamentation on the submerged hyphae and dark olivaceous colonies on MEA.

Key words: elongation factor 1-α, ITS2-LSU, *Leptographium procerum*, ophiostomatoid fungi, phylogeny, β-tubulin

INTRODUCTION

Pinus tabuliformis Carr. is an indigenous tree of northern China that plays an important ecological role by protecting soil against erosion and contributing to regional socio-economic development (Wu and Feng 1994). Since 1998 the *P. tabuliformis* forests in the northern province of Shanxi have suffered from high mortality, with the death of more than 3 000 000 trees in about 3 y (Miao et al 2001). The invasive attacks during this time by the bark beetle *Dendroctonus valens* LeConte (Coleoptera, Scolytidae), which infested about 200 000 ha of pine forests in the province, were identified as the probable cause of these deaths. *Dendroctonus valens* is now spreading into the neighboring provinces of Hebei, Henan and Shaanxi. Commonly known as the red turpentine beetle (RTB), *D. valens* originating from the pine forests of North and Central America (Pajares and Lanier 1990) was introduced into China in about 1980, probably with the importation of unprocessed *Pinus ponderosa* lumber from the Pacific Northwest, USA (Sun et al 2003, Congnato et al 2005). Its subsequent naturalization in China was accompanied by several changes in its autecology (Wu et al 2002, Sun et al 2004, Yan et al 2005).

Although some information is available on its synecology in its original habitats, there is none about any potential association with ophiostomatoid fungi in the northern China *P. tabuliformis* forests. In North America several ophiostomatoid fungi have been recorded in association with RTB. *Leptographium terebrantis* S.J. Barras & T.J. Perry and *L. procerum* (W.B. Kendr.) M.J. Wingf. are the most commonly reported species (Harrington and Cobb 1983, Wingfield 1983, Owen et al 1987, Harrington 1988, Parmeter et al 1989, Klepzig et al 1991, Perry 1991, Six 2003). Other less commonly reported species include *L. wageneri* (W.B. Kendr.) M.J. Wingf. (Goheen and Cobb 1978, Harrington 1988, Perry 1991, Owen et al 2005, Schweigkofler et al 2005), *L. wingfieldii* M. Morelet (Jacobs et al 2004), *Grosmannia clavigerum* (R.C. Rob.-Jeffr. & R.W. Davidson) Zipfel et al (Six 2003), *O. ips* (Rumbold) Nannf. (Owen et al 1987, Parmeter et al 1989, Klepzig et al 1991, Perry 1991), *G. piceaperda* (Rumbold) Goid. (Perry 1991), *O. piliferum* (Fr.) Syd. & P. Syd. (Perry 1991), *Graphium* sp. (Owen et al 1987) and *Ceratosystiopsis collifera* Marmolejo & Butin (Marmolejo and Butin 1990).

Case studies of introductions of (invasive) alien gymnosperm-associated bark beetles in nonnative ecosystems showed that the resulting ecological consequences could be complex (Jacobs et al 2003, 2004; Hausner et al 2005, Zhou et al 2007).

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Introductions of their associated fungi, also representing taxa (or genotypes) that are alien to the newly colonized ecosystem (Jacobs et al 2003, 2004; Hausner et al 2005), often occur concomitantly. These simultaneous introductions have varying effects on the equilibrium of the native ecosystem; for example they might lead to a new beetle-fungi singular combination (Jacobs et al 2003, 2004) that might have a more serious impact than individual organisms (e.g. Kirisits 2004).

Extensive surveys of ophiostomatoid communities associated with RTB in *P. tabuliformis* forests therefore were carried out in the four infested provinces in northern China to identify the species present and their origin.

During this survey the most frequently isolated species initially was identified as *L. procerum*, based on morphological criteria (Kendrick 1962, Jacobs and Wingfield 2001). Subsequent phylogenetic inferences based on a set of three genomic markers however revealed two clades within the Chinese *L. procerum*, one of which corresponded to *L. procerum* s.s., but the second remained unnamed. After in-depth morphological characterization and comparisons in the literature this clade ultimately could not be linked to any described species and therefore was taken to be an undescribed species. The description is given below.

MATERIALS AND METHODS

Collection of samples and fungus isolations.—*Leptographium* strains were isolated from *D. valens* breeding galleries from phloem and sapwood near and beneath the galleries, from *Pinus tabuliformis* and in some cases from *P. bungeana*. Isolations from timber were carried out as described by Seifert et al (1993) on the surface of 2% water agar (agar: B&V S.R.L., Italy) in 9 cm Petri dishes. All isolated strains were purified by single conidial isolations, using the procedure described by Jacobs and Wingfield (2001) and routinely grown on 2% MEA (malt extract: Oxoid, England) at 20 C under alternating 12 h light/dark (Ritchie 2002). The strains were deposited in the BCCM/MUCL culture collection and the culture collection of Chinese Academy of Forestry (CXY).

After an initial analysis of macro- and microscopical characteristics, representative strains of each morphotype were selected for further in-depth morphological, physiological and molecular studies. *Leptographium* spp. were compared with reference strains, including ex-holotype or authentic strains, gathered from MUCL, CBS and CMW (TABLE I).

Cultural and morphological studies.—Using a sterile cork borer, agar disks of 5 mm diam were cut from the margin of the actively growing colonies of each strain on 2% MEA and placed on the center of plates of the same medium, ensuring the aerial mycelium in contact with media. Three replicate plates prepared for each strain were incubated in the dark at 5–35 C at 5 C intervals. Colony diameters on each dish were measured along two perpendicular lines after incubation for 2, 3, 4 and 7 d. The cycloheximide tolerance of the strains was assessed by measuring growth at 25 C on 2% MEA amended with cycloheximide 0.05, 0.1 and 0.5 g/L.

All microscopic measurements were done in lactic acid cotton blue from about 3 wk old cultures incubated at 25 C. Fifty measurements were made of each relevant structure, and the ranges were computed. In presenting the size range of the microscopic elements, 5% of the measurements have been excluded from each end; these are given in parentheses, where relevant.

DNA extraction, amplification and nucleotide sequencing.—DNA was extracted from freshly collected mycelium grown in liquid malt at 25 C in the dark for 7 d. DNA extractions and purification were carried out with an Invisorb Spin Plant Mini Kit (Invitek, Berlin), following the manufacturer's instructions. The primer pairs ITS3/LR3 (White et al 1990), Bt2a/Bt2b (Glass and Donaldson 1995) and EF1F/EF2R (Jacobs et al 2004) were used respectively for amplification of ITS2-LSU, β-tubulin gene, and elongation factor 1-α (EF 1-α) gene. PCR reactions were carried out in 50 μL volumes containing 100 ng DNA template, 2.5 U Taq DNA polymerase (Invitrogen), 1× buffer (20 mM Tris-Hcl, 50 mM KCl), 0.2 mM of each dNTP, 1.5 mM $MgCl_2$ and 0.2 μM of each primer. Successful PCR reactions resulted in a single band observed on a 1% agarose gel. PCR products were cleaned with an MSB Spin PCRapace Kit (250) (Invitek, Berlin), following the manufacturer's instructions.

Sequencing reactions were performed with CEQ DTCS Quick Start Kit® (Beckman Coulter), following the manufacturer's instructions, with the same PCR primers as above. Nucleotide sequences were determined with a CEQ 2000 XL capillary automated sequencer (Beckman Coulter).

Phylogenetic analysis.—Preliminary indications of the relationships among the strains were obtained with the BLAST option (Altschul et al 1997) at GenBank. Sequences for the taxa other than those derived from this study were downloaded from GenBank. The nucleotide sequences were aligned automatically with Clustal W 1.83 (Pearson and Lipman 1988) and manually adjusted where necessary.

Phylogenetic analysis of the aligned sequences was performed with the maximum parsimony method of PAUP* version 4.0b10 (Swofford 2001), with gaps treated as fifth base. Most parsimonious trees were identified using heuristic searches with random addition sequence (1000), MAXTREES set to 200 and further evaluated by bootstrap analysis, retaining clades compatible with the 50% majority rule in the bootstrap consensus tree. The analysis conditions were tree bisection reconnection branch swapping (TBR); starting tree obtained via stepwise addition; steepest descent not in effect; MULTREES effective. Because there was no significant conflict between the various phylogenies based on individual sequences the three individual datasets were combined for joint analysis. *Leptographium yunnanense* (CMW 5304) was chosen as outgroup in these analyses.

TABLE I. *Leptographium* spp. strains used for morphological, physiological and molecular studies and GenBank accession numbers for the sequences

Species	Strains[a]	Source and associated insect	Origin	GenBank accession number[b]			Date	Collector
				ITS2-LSU	β-tubulin	EF 1-α		
L. sinoprocerum	MUCL 46331	Phloem adjacent to *D. valens* gallery in *P. tabuliformis*	China, Shanxi	EU296772	EU296778	EU296785	Jan 2005	Authors
	MUCL 46352 ex-holotype	*D. valens* gallery in *P. tabuliformis*	China, Hebei	EU296773	EU296779	EU296786	Aug 2004	Authors
	MUCL 47246	*D. valens* gallery in *P. bungeana*	China, Shanxi	EU296774	EU296780	EU296787	May 2005	Authors
L. procerum	MUCL 46323	Sapwood underneath *D. valens* gallery in *P. tabuliformis*	China, Shanxi	EU296775	EU296781	EU296788	Dec 2004	Authors
	MUCL 46361	*D. valens* gallery in *P. tabuliformis*	China, Shaanxi	EU296776	EU296782	EU296789	Jul 2004	Authors
	MUCL 8094 authentic = CBS 516.63	Interior of roots with resinous lesions in *P. resinosa*	USA, New York	—	EU296783	EU296790	1959	D.S. Welch
L. albopini	MUCL 47429 ex-holotype = CMW 2065	Insect-infested roots of *P. strobus*	USA, Virginia	(AF343696)	—	—	1979	A. Lackner
L. terebrantis	MUCL 47242 ex-holotype = CBS 337.70	Pupal chamber of *D. terebrans* in *P. taeda*	USA, Louisiana	EU296777	EU296784	EU296791	1966	S.J. Barras
L. yunnanense	MUCL 47426 ex-holotype = CMW 5304	*T. piniperda* infesting *P. yunnanensis*	China, Yunnan	(AY553415)	(AY534963)	(AY536209)	Dec 1996	X.D. Zhou

[a] CBS, Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; CMW, Fungal genetic resource collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa.

[b] Accession numbers in parentheses were downloaded from GenBank. —, The sequence missed because of unsuccessful gene amplification.

RESULTS

Sixty-five *Leptographium* strains were isolated, mainly from RTB breeding galleries and the surrounding discolored phloem and sapwood. The morphospecies *L. procerum* was the dominant species, representing about 66% of the strains.

Amplification of the ITS2-LSU region produced an amplicon of approximately 900 base pairs (bp). The final ITS2-LSU dataset comprised 25 strains (14 species) and 582 characters, including gaps after the alignment and exclusion of 19 positions in two ambiguous regions; 526 characters were constant, 18 parsimony uninformative and 38 parsimony informative. Amplification of the β-tubulin gene produced an amplicon of approximately 430 bp. The final β-tubulin gene dataset comprised 21 strains (12 species) and 319 characters, including gaps after the alignment and exclusion of 131 positions in four ambiguous regions; 217 characters were constant, 15 parsimony uninformative and 87 parsimony informative. Amplification of the EF 1-α gene produced an amplicon of about 900 bp. The final EF 1-α gene dataset comprised 21 strains (12 species) and 629 characters, including gaps after the alignment and exclusion of 221 positions in five ambiguous regions; 351 characters were constant, 29 parsimony uninformative and 249 parsimony informative.

The concatenated dataset (ITS2-LSU + β-tubulin gene + EF 1-α gene) comprised 20 strains (12 species) and 1530 characters after the exclusion of 371 positions in 11 ambiguous regions; 1105 were constant, 51 parsimony uninformative and 374 parsimony informative.

Two analyses were carried out, considering the gaps either as missing data or as fifth base. The topologies of the trees regarding the recovery and relative position of the clades were concordant in the analyses, based either on single sequence data or concatenated datasets (FIGS. 1–4). The different treatment of gaps did not change the topologies of the phylogenetic trees (data not shown) but for the ITS2-LSU dataset in which morphospecies *L. procerum* appeared as single clade. In all other analyses the Chinese strains tentatively identified as *L. procerum* were distributed over two distinct clades; they either clustered with an authentic strain of *L. procerum* (MUCL 8094) to form an *L. procerum* s.s. clade or formed a second clade (*L. procerum*-China clade) with good bootstrap support in all analyses (99–100%), except in the ITS2-LSU-based inference (FIGS. 1–4). Furthermore in all analyses the *L. procerum* s.s. clade was found to be more closely related to *L. profanum* K. Jacobs et al, a species described from angiosperm in the southern Appalachian Mountains in southeastern USA, than to the *L. procerum*-China clade. These three clades formed a well supported larger clade, together with *L. pini-densiflorae* H. Masuya & M.J. Wingf. and *L. pineti* K. Jacobs & M.J. Wingf. (FIGS. 1–4).

Subsequent morphological re-examination of the *L. procerum* s.s. and *L. procerum*-China strains segregated them into two homogeneous groups. The Chinese strains of the *L. procerum* s.s. clade agreed globally with the species description in the literature (Kendrick 1962, Jacobs and Wingfield 2001) and with MUCL 8094. They deviated slightly by producing longer conidiophore stipes, commonly reaching 3900 µm long. MUCL 8094 rarely produced conidiophore stipes longer than 2500 µm under our experimental conditions. Kendrick (1962) originally reported stipes up to 1250 µm long, while Jacobs and Wingfield (2001) noted an even smaller size of about 200–500 µm, occasionally reaching 700 µm. Hausner et al (2005) noted lengths of up to 2200 µm in Canadian strains.

Although morphologically similar to *L. procerum* s.s., strains from the *L. procerum*-China clade deviate at both the macro- and microscopic levels. They produce darker colonies overall (dark olivaceous versus light olivaceous in *L. procerum* s.s.) that were especially obvious in early development (FIG. 5) (5–15 d). Microscopically the conidiophores in the strains of the *L. procerum*-China clade rise from both submerged and aerial hyphae whereas in *L. procerum*, which lacks aerial mycelium, the conidiophores rise only from submerged mycelium. The conidiophores born on submerged hyphae bear a rhizoid-like base in strains from both clades, a feature prominent in *L. procerum* s.s. (FIGS. 8, 10, 11; Jacobs and Wingfield 2001, TABLE II), whereas those born on aerial mycelium in strains of the *L. procerum*-China clade consistently lack this feature (FIG. 9). The conidiophore stipes of strains from the *L. procerum*-China clade are distinctly shorter than those of the Chinese strains of *L. procerum* s.s., their size being (34–)62–1020(–1266) µm ($\bar{x}$ = 314 µm) and (38–)93–3812 (–3984) µm, ($\bar{x}$ = 810 µm), respectively. However as emphasized above the size of the conidiophore stipes in *L. procerum* s.s. appears to be variable and therefore should be treated with caution. Conidia in strains from the *L. procerum*-China clade are slightly more oblong than those in *L. procerum* s.s. (FIG. 16, TABLE II). In addition strains from the *L. procerum*-China clade have a granular ornamentation on the submerged hyphae (FIG. 17), a characteristic not reported or observed in *L. procerum* strains.

Other characters are subtler and variable/unstable and might be strains or environment dependant. For example strains from both clades form concentric rings. Although reported as typical of *L. procerum* s.s. (Kendrick 1962, Jacobs and Wingfield 2001) it seems

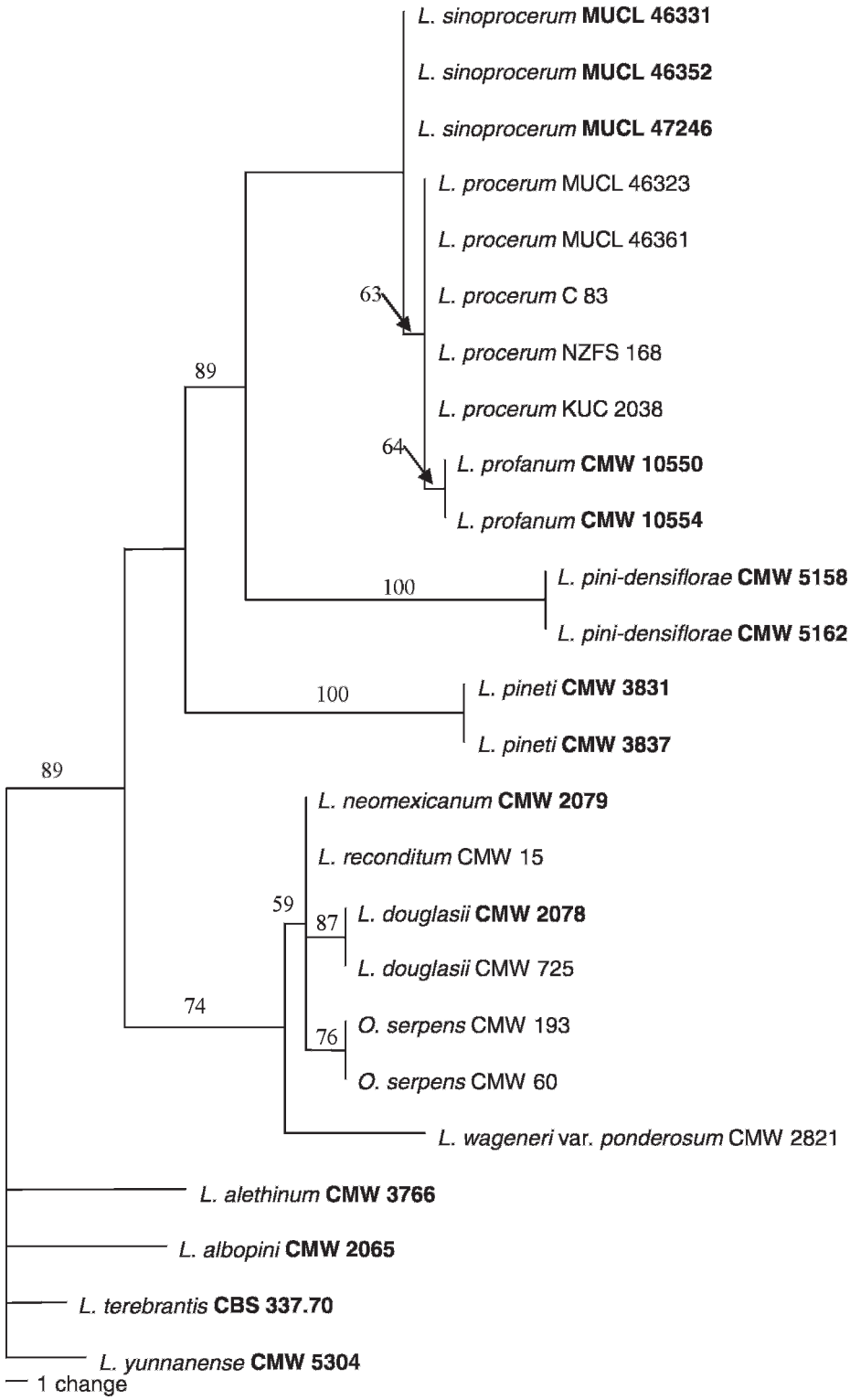


FIG. 1. One of the nine most parsimonious trees based on ITS2-LSU sequences using a heuristic search (76 step in length, CI = 0.829, RI = 0.916). Bootstrap values >50% are indicated above branch nodes. Boldface represents the authentic or ex-type strains of the species.

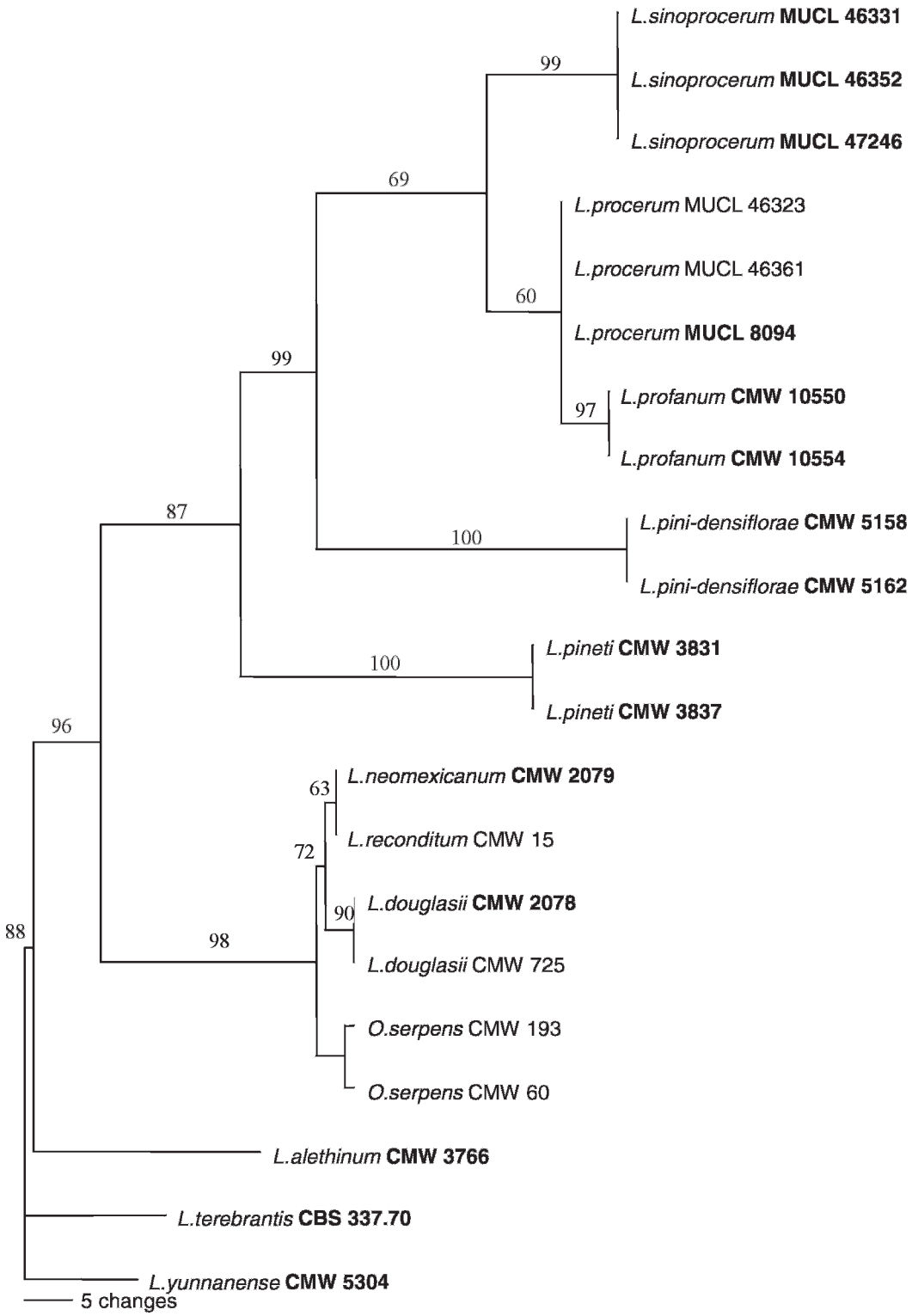


FIG. 2. One of the three most parsimonious trees based on β-tubulin gene sequences using a heuristic search (167 step in length, CI = 0.820, RI = 0.921). Bootstrap values >50% are indicated above branch nodes. Boldface represents the authentic or ex-type strains of the species.

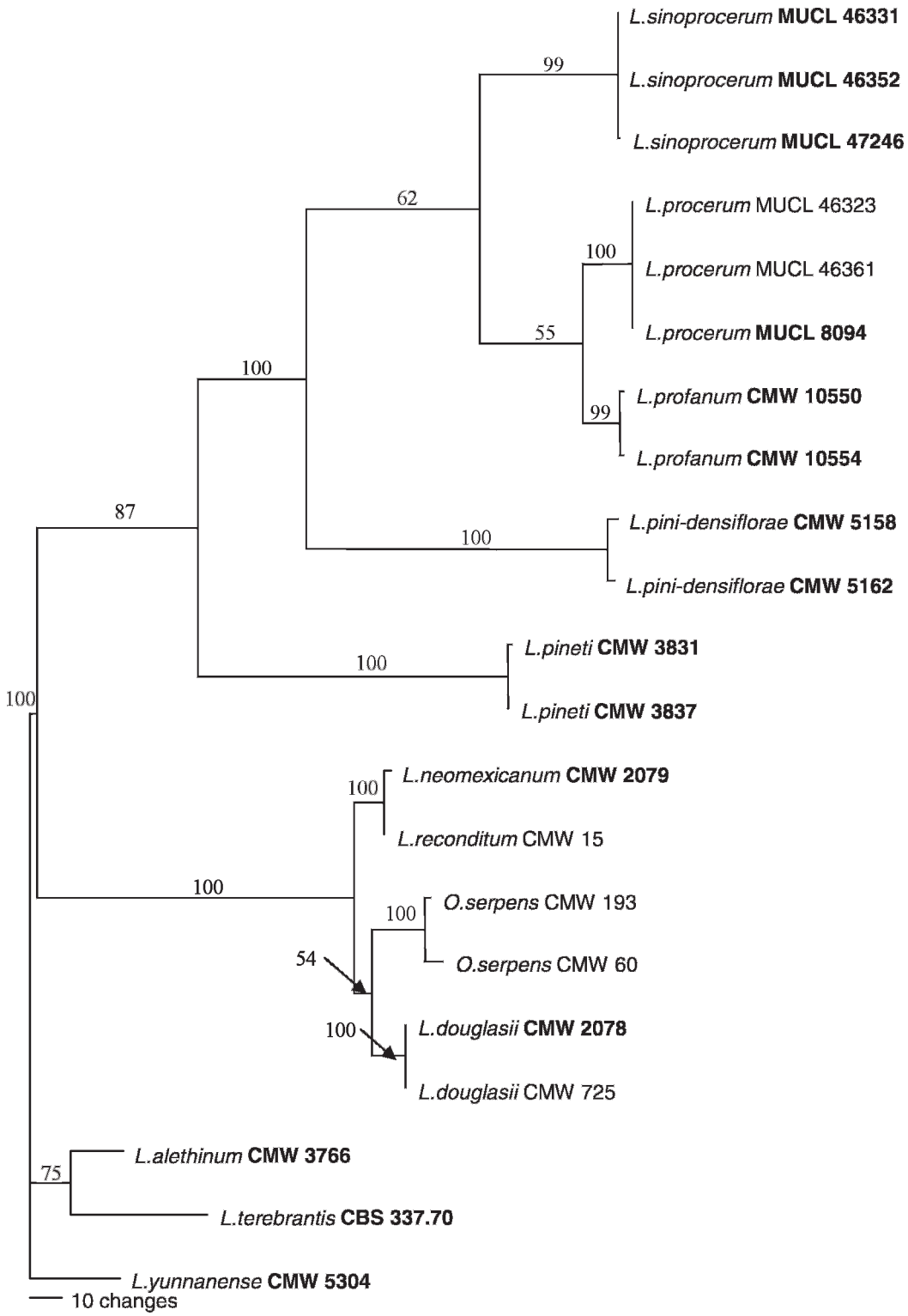


FIG. 3. Single most parsimonious trees based on EF 1-α gene sequences using a heuristic search (540 step in length, CI = 0.815, RI = 0.918). Bootstrap values >50% are indicated above branch nodes. Boldface represents the authentic or ex-type strains of the species.

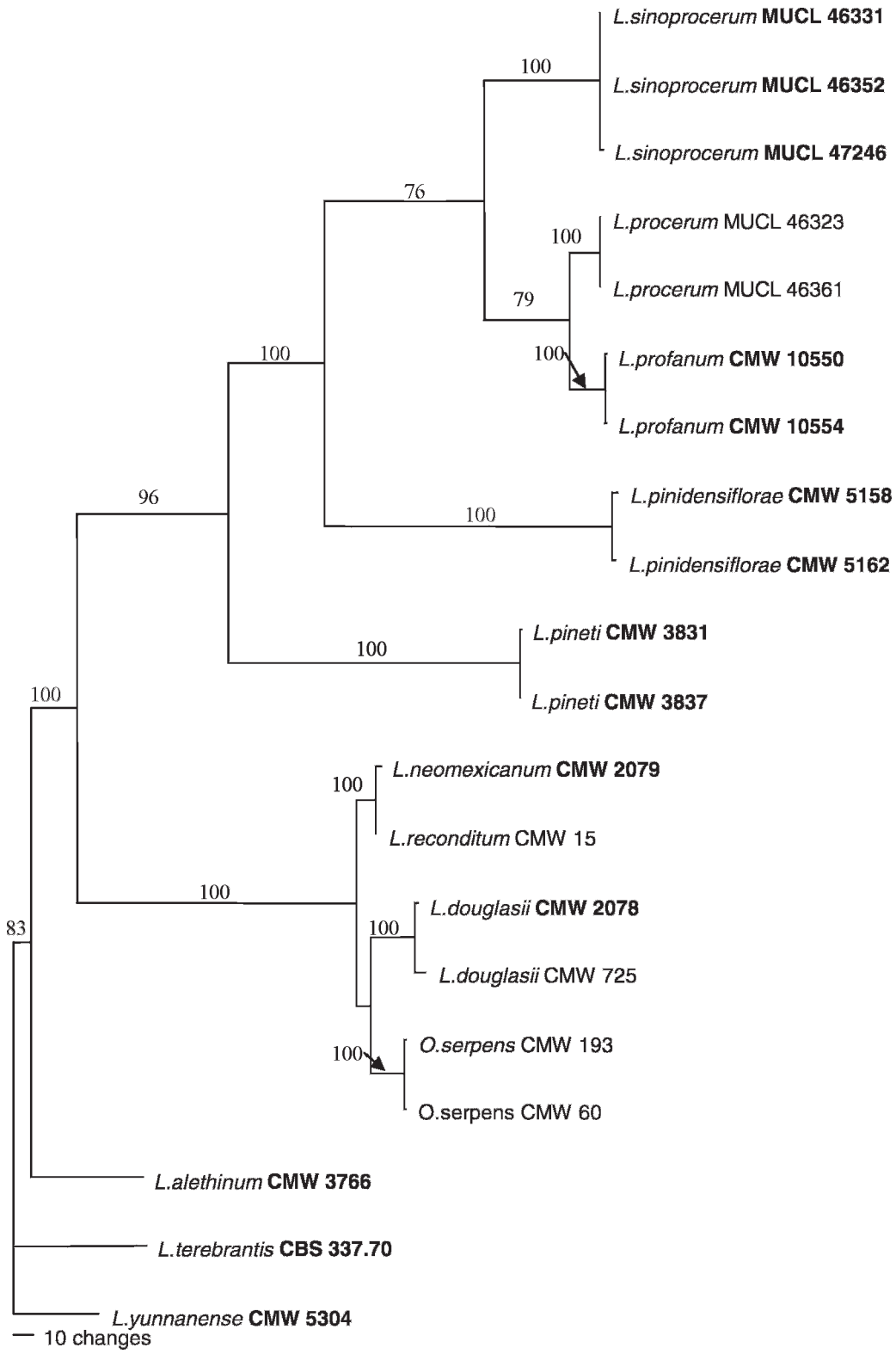
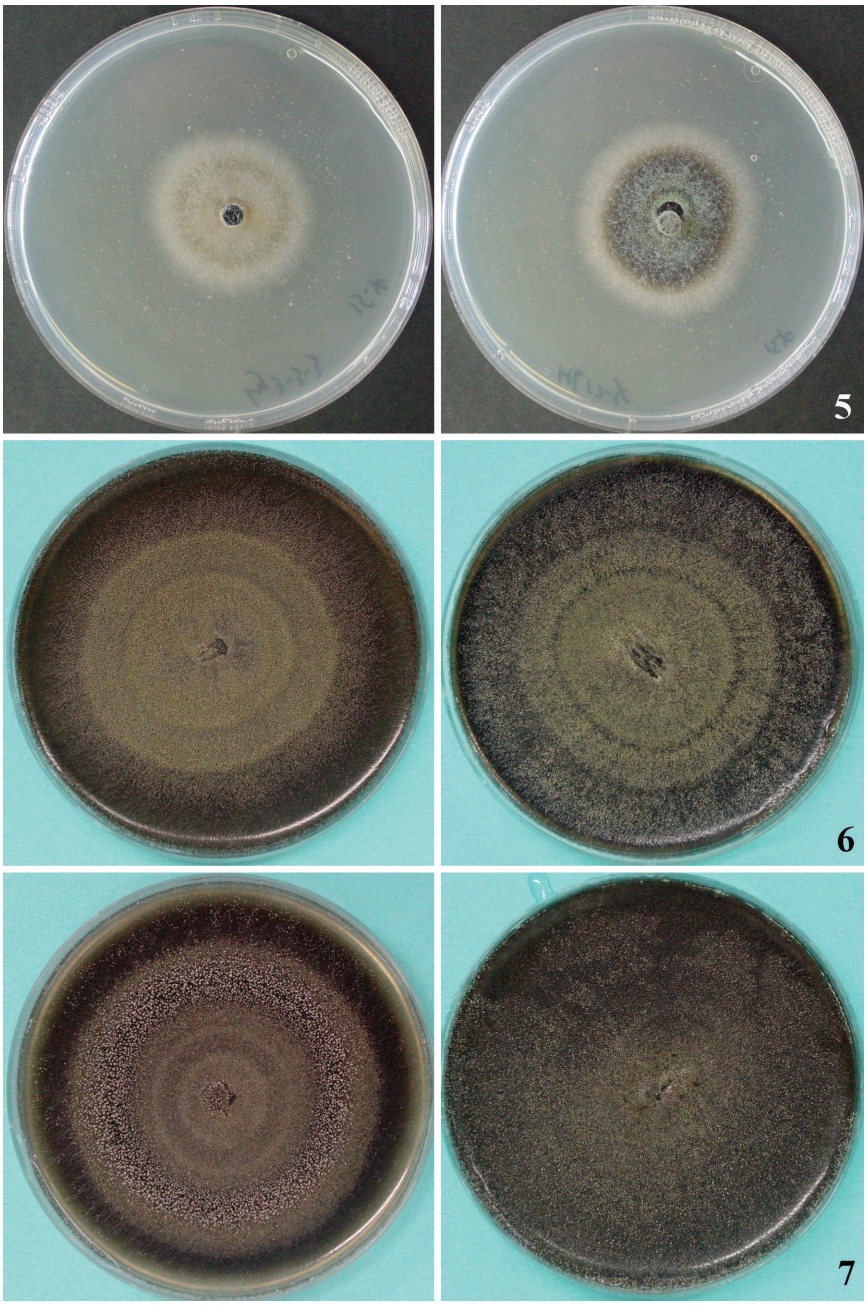


FIG. 4. One of the two most parsimonious trees based on concatenated dataset of ITS2-LSU, β-tubulin gene and EF 1-α gene sequences using a heuristic search (773 step in length, CI = 0.812, RI = 0.912). Bootstrap values >50% are indicated above branch nodes. Boldface represents the authentic or ex-type strains of the species.



FIGS. 5–7. Colony comparisons on 2% MEA at 25 C in darkness of representative strains of *Leptographium procerum* (MUCL 46323, 46361) and *L. sinoprocerum* (MUCL 46352, 46331). 5. MUCL 46323 (left) and 46352 at 6 d old showing a darker colony of *L. sinoprocerum* than *L. procerum*. 6. MUCL 46323 (left) and 46352 at 30 d old showing concentric rings in both species. 7. MUCL 46361 (left) and 46331 at 30 d old showing variable culture colonies with extra long conidiophores common in *L. procerum* and concentric rings absent in *L. sinoprocerum*.

to be variable (Hausner et al 2005 pers obs). It is also less obvious (FIG. 6) and even absent (FIG. 7) in strains of the *L. procerum*-China clade.

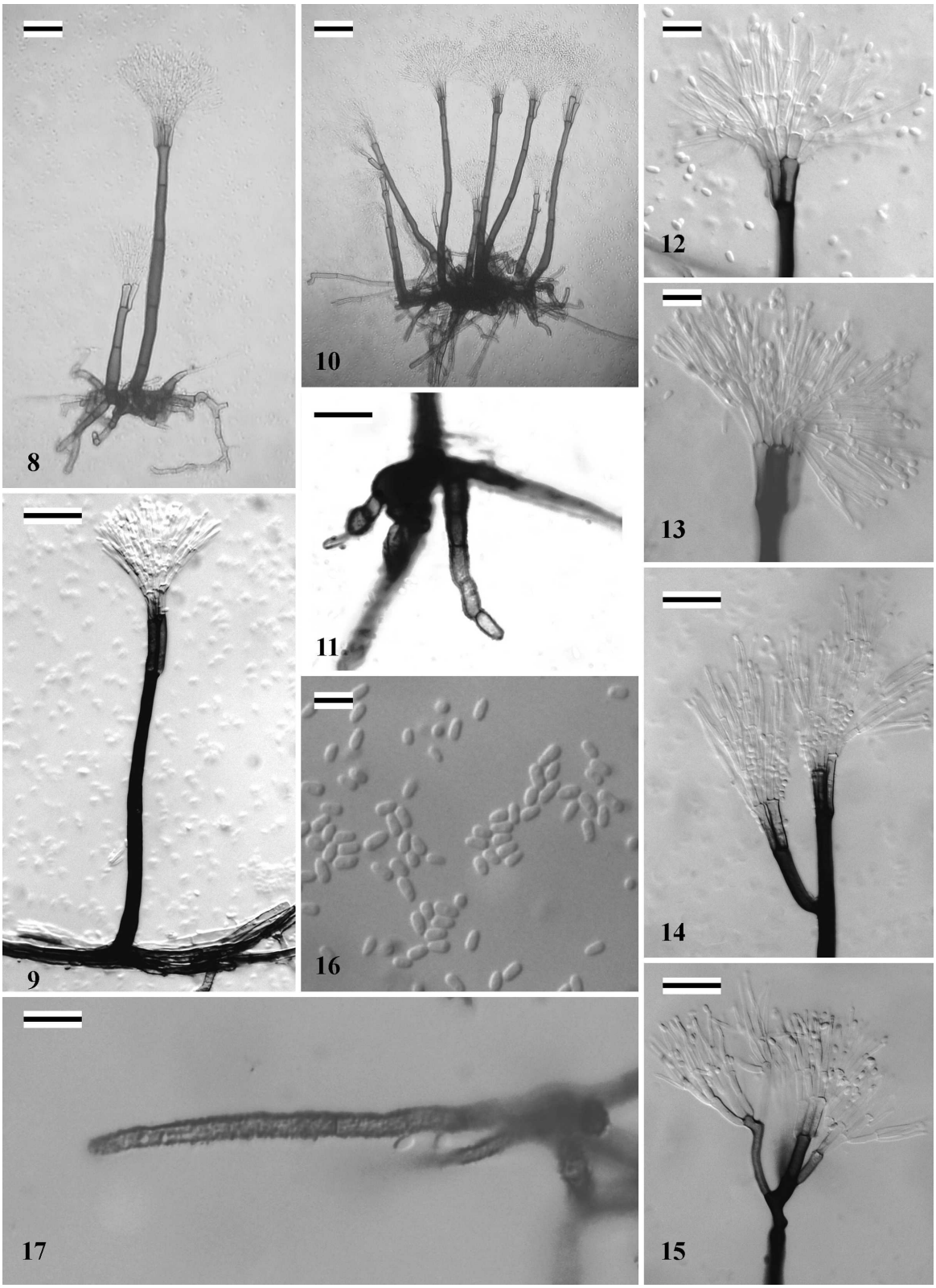
The congruent results of both morphological and molecular studies support the fact that the strains of the *L. procerum*-China clade represent an undescribed species in the *L. procerum*-*L. profanum* clade. It is described below as *Leptographium sinoprocerum*.

TAXONOMY

Leptographium sinoprocerum Lu, Decock, et Maraite sp. nov. FIGS. 8–17

Etymology. *sino* refers to the type locality, China; *procerum* refers to the similarity to *L. procerum*

Crescit optime ad 25 C. Non crescit infra 5 C vel supra 35 C. In 2% MEA cum alio 0.05, 0.1 et 0.5 g/l cycloheximide, crescentiae diminutive post septem dies ad 25 C in



FIGS. 8–17. *Leptographium sinoprocerum* sp. nov. (MUCL 46352). 8–9. Mononematous, macronematous conidiophores with and without rhizoid-like structures. Bar = 30 μm. 10. Conidiophores grouped. Bar = 50 μm. 11. Rhizoid-like structures of conidiophores. Bar = 10 μm. 12–15. Conidiogenous apparatus with various arrangements of primary branches, the branching pattern shown on 12 and 13 were the most common while those on 14 and 15 were rare. Bar = 30 μm. 16. Oblong to obovoid conidia. Bar = 10 μm. 17. Granular hyphae. Bar = 10 μm.

obscuro erat 6.0, 14.5 et 19% respectu. Coloniae in 2% MEA 59 mm diam in 7 dies attingentes, primo atroolivaceae deinde ad marginem atrobrunneae; odore laeve foetido; margine laeve effuso. Hyphae proparte maxima in aerio mycelio submersae, hyalinae ad laeve olivaceae, hyphae submersae granulatae, rectae sed interdum curvatae, rare ad septa constrictae, 2–4(–8) µm latae. Conidiophorae singulae vel ad riginta aggregatae, mononematosae, macronematosae, (78–)122–1140(–1352) µm longi, rhizoideis munitae. Stipites olivacei cylindracei, simplices, 1–15-septati, (34–)62–1020(–1266) µm longi. Apparatus conidiogenus (40–)42–128(–140) µm longi, massa conidiali exclusa, 2–5 seriebus ramorum cylindricorum munitus. Rami primarii 2–3 metutae primariae sed plerumque duo, ramo centrale distincto nullo, laeve olivacei, cylindrici, aseptati, (11.7–)11.7–30.7(–36.7) × (2.0–)2.3–7.9(–8.6) µm. Rami secundarii laeve olivacei, aseptati, (9.4–)11.4–20.6(–22.6) × (1. 6–)1.9–5.6(–6.2) µm. Rami tertiani hyalini, aseptati, (7.8–)7.8–16.4(–16.4) × (1.6–)1.6–3.9(–3.9) µm. Rami quartani hyalini, aseptati, (7.0–)7.0–15.4(–20.3) × (1.6–)1.6–2.4(–2.4) µm. Cellulae conidiogenae discretae, hyalinae, sursum attenuatae, (8.6–)10.4–23.8(–24.2) × (1.2–)1.2–2.0(–2.0) µm. Conidia hyalina, aseptata, oblonga ad subclavata, 2.3–5.5 × 1.2–2.3 µm.

Colonies on 2% MEA, reaching 59 mm diam in 7 d at 25 C, initially dark olivaceous then dark brown toward the edge, even or occasional with a vague concentric pattern of cream-colored rings; odor slightly fetid; margin slightly effuse. Hyphae mostly submerged with aerial mycelium, hyaline to light olivaceous, submerged hyphae granular, straight but sometimes curving, occasionally constricted at the septa, 2–4(–8) µm wide. Conidiophores occurring singly or in groups of up to 8, rising directly from the submerged mycelium and the aerial mycelium, erect, macronematous, mononematous, (78–)122–1140 (–1352) µm long ($\bar{x}$ = 389.5 µm), with rhizoid-like structures rising from the basal cells, from submerged mycelium. Stipes olivaceous, not constricted, cylindrical, simple, 1–15-septate, (34–)62–1020(–1266) µm long ($\bar{x}$ = 314.2 µm), 3–9 µm wide below primary branches, apical cell not swollen, 3–12 µm wide at base, basal cell not swollen. Below stipes knot-like structures present in medium. Conidiogenous apparatus (40–)42–128(–140) µm long ($\bar{x}$ = 75.3 µm), excluding the conidial mass, with series of 2–5 cylindrical branches. Primary branches, 2–3 without larger central branch, mostly 2, light olivaceous, smooth, cylindrical, aseptate, (11.7–)11.7–30.7 (–36.7) µm long and (2.0–)2.3–7.9(–8.6) µm wide ($\bar{x}$ = 21.2 × 5.1 µm); secondary branches light olivaceous, aseptate, (9.4–)11.4–20.6(–22.6) µm long and (1. 6–)1.9–5.6(–6.2) µm wide ($\bar{x}$ =15.8 × 3.8 µm); tertiary branches hyaline, aseptate, (7.8–)7.8–16.4 (–16.4) µm long and (1.6–)1.6–3.9(–3.9) µm wide ($\bar{x}$ =11.6 × 2.7 µm); quaternary branches hyaline, aseptate, (7.0–)7.0–15.4(–20.3) µm long and (1.6–)1.6–2.4(–2.4) µm wide ($\bar{x}$ = 10.4 × 2.0 µm). Conidiogenous cells hyaline, discrete, 2–4 per branch, cylindrical, tapering slightly at the apex, (8.6–)10.4–23.8(–24.2) µm long and (1.2–)1.2–2.0(–2.0) µm wide ($\bar{x}$ = 16.3 × 1.5 µm). Conidia hyaline, aseptate, oblong to subclavate, 2.3–5.5 × 1.2–2.3 µm, ($\bar{x}$ =3.8 × 1.7 µm, ratio length to width 1.5–3.1). Conidial droplet hyaline initially, becoming cream-colored, amber or yellow with age.

Optimal growth temperature: about 25 C; cardinal temperature >5 C and <35 C.

Cycloheximide tolerant: on 2% MEA amended with 0.05, 0.1, and 0.5 g/L cycloheximide, growth reduction after 7 d at 25 C in the dark were respectively 6.0, 14.5 and 19%.

Specimens examined. CHINA. HEBEI Province: Xinzhuang forest farm, *D. valens* gallery in *Pinus tabuliformis*, 22 Aug 2004, *Quan Lu*, *MUCL 46352 (ex-living culture MUCL 46352, CXY 943)* (HOLOTYPE, MUCL, CXY). HEBEI Province: Xinzhuang forest farm, *D. valens* gallery in *Pinus tabuliformis*, 22 Aug 2004, *Quan Lu*, *MUCL 46351 (CXY 942)*; SHANXI Province: Sandaochuan forest farm, *D. valens* gallery in *Pinus tabuliformis*, 8 Jul 2004 and 29 Dec 2004, *Quan Lu*, *MUCL 46328 (CXY 913)*, *MUCL 46330 (CXY 916)*, *MUCL 46331 (CXY 917)*, *MUCL 46332 (CXY 918)*; Wujinshan forest farm, *D. valens* gallery in *Pinus bungeana*, 9 Jun 2005, *Quan Lu*, *MUCL 47246 (CXY 1191)*; Liangma forest farm, *D. valens* gallery in *Pinus tabuliformis*, 11 Jun 2005, *Quan Lu*, *MUCL 47247 (CXY 1140)*; *Tomicus* sp. gallery in *Pinus tabuliformis*, 11 Jun 2005, *Quan Lu*, *MUCL 47248 (CXY 1145)*; HENAN Province: Huixian County, Shanglajiang village, sapwood beneath *D. valens* gallery in *Pinus tabuliformis*, 12 Jun 2005, *Quan Lu*, *MUCL 47249 (CXY 1192)*; SHAANXI Province: Daling forest farm, *D. valens* gallery in *Pinus tabuliformis*, 22 Jul 2004, *Quan Lu*, *MUCL 46360 (CXY 951)*.

DISCUSSION

The phylogenetic species recognition principle involving three DNA loci was applied to a set of Chinese *Leptographium* strains to unravel its genetic and species diversity. All phylogenetic analyses, based on either independent DNA loci or joint dataset (Genealogical Concordance PSR), revealed a clade that remained isolated and directly assignable to none of the known species, although being morphologically related to *L. procerum*. This clade was interpreted as representing an undescribed species, described as *L. sinoprocerum*.

The closest relatives of *L. sinoprocerum* are *L. profanum* and *L. procerum*, these three species forming a well supported, few divergent clade in all the phylogenetic inferences assessed (FIGS. 1–4). The three species also are related morphologically. *Leptographium pini-densiflorae* and *L. pineti* also are related to the *L. sinoprocerum*-*L. procerum*-*L. profa-*

num clade, although more distantly, and these five species form a well supported clade in all analyses (FIGS. 1–4). This agrees with the results of Massoumi Alamouti et al (2006) and Jacobs et al (2006), which related respectively *L. pini-densiflorae* to *L. procerum* and *L. profanum*.

Leptographium albopini M.J. Wingf. et al also is closely related morphologically to *L. sinoprocerum* but phylogenetically distant (FIG. 1). Critical examination of strains of *L. sinoprocerum*, *L. procerum* and *L. albopini* and comparison with the original description of *L. profanum* (Jacobs et al 2006) made it possible to identify a combination of consistently different morphological traits (TABLE II).

Leptographium sinoprocerum, *L. albopini* and *L. profanum* all have similar conidiophores, both in length and structure, and conidia (Wingfield et al 1994, Jacobs et al 2006). The conidiophores in *L. albopini* and *L. profanum* occur singly, while in *L. sinoprocerum* they are grouped (FIG. 10). The primary branches of *L. albopini* usually vary 2–4, whereas *L. sinoprocerum* has only 2–3 primary branches, never 4. Like *L. procerum*, *L. albopini* forms no aerial mycelium. The colony color of *L. albopini* is hyaline, becoming light olivaceous. The colony color of *L. sinoprocerum* however initially is dark olivaceous, becoming dark brown with age. Unlike most *Leptographium* species, *L. profanum* was isolated from hardwood. The obvious characters distinguishing *L. sinoprocerum* from *L. profanum* are the rhizoid-like basal ramification as well as rough-walled hyphae in the former species and smooth hyphae in the latter. In addition the growth rate of *L. sinoprocerum* is about twice fast as that of *L. profanum*.

Meanwhile *L. sinoprocerum* also bears similarities and differences to *L. euphyes* K. Jacobs & M.J. Wingf. (Jacobs et al 2001a) and *L. peucophilum* K. Jacobs & M.J. Wingf. (Jacobs et al 2000). Both species can be distinguished from *L. sinoprocerum* by a much slower growth rate (respectively 19 mm diam in 6 d and 10 mm diam in 10 d on 2% MEA, at each optimal temperature) and shorter conidiophores (respectively [204–]300[–315] and [230–]310–352[–520]). Both species also consistently lack concentric rings in culture but this feature is also variable in *L. sinoprocerum* (see above).

Little is known about the biology or ecology of *L. sinoprocerum*. So far no teleomorph is known. All paired crosses proved to be unsuccessful in producing perithecia (unpubl data). To date the species is known from the *Pinus tabuliformis-P. bungeana* ecosystems in four northern provinces of China infested by RTB. In these forests it was isolated mainly in association with and from the RTB breeding galleries in *P. tabuliformis*; this suggests that RTB might act as a vector. *Leptographium* species are adapted to be carried by bark-infesting beetles or other insects that act as vectors (Harrington 1988, Wingfield et al 1993, Jacobs and Wingfield 2001), and in its native area of occurrence *D. valens* is associated frequently with *Leptographium* species (e.g. Wingfield 1983, Klepzig et al 1991).

Leptographium sinoprocerum also was isolated once from a *Tomicus* gallery. *Tomicus* spp. also have been shown to be efficient vectors of some *Leptographium* species in other areas (Solheim and Långström 1991, Zhou et al 2000, Masuya et al 1999, Kim JJ et al 2005, Sabbatini Peverieri et al 2006). However *Tomicus* spp. as well as other native bark beetles and their breeding galleries were not extensively sampled in the surveyed RTB-infested sites or in neighboring RTB-free areas and certainly not before the RTB invasion, preventing us from drawing any conclusions about its original aut- and synecology, particularly any association(s) with native insect vector(s) and its relative abundance.

The geographic origin and distribution range of *L. sinoprocerum* are partially known. Although the hypothesis of a simultaneous introduction of *L. sinoprocerum* with RTB cannot be dismissed—there are other examples of simultaneous introduction of *Leptographium* species with their vectors (e.g. Wingfield and Gibbs [1991] and Jacobs et al [2001a])—the hypothesis of a native origin is favored. A similar situation occurred in Korea with *L. bistatum* J.J. Kim & G.H. Kim, a species isolated from imported pine timber from New Zealand but strongly suspected to be a Korean native taxon (Kim JJ et al 2004). So far the species is known only from northern China. An extensive survey of currently undisturbed, RTB-free ecosystems would be necessary to learn more about the distribution and ecology of this species.

Leptographium procerum s.s. also was isolated frequently from the *P. tabuliformis*-RTB niche in northern China. This is the first record of this species in northern China. However it has been reported previously from Korea and Japan either on local trees (*Pinus koraiensis* Oh 1999, cited by Kim GH et al 2005), associated with naturally occurring *Tomicus piniperda* and *T. minor* in *P. densiflora* (Coleoptera, Scolytidae) (Masuya et al 1999), or on imported timber (*P. radiata* from New Zealand, Kim GH et al 2005). *Leptographium procerum* is distributed widely and reported from North America (USA, Canada) (e.g. Kendrick 1962, Harrington 1988, Hausner et al 2005), Europe (Kendrick 1962, Halambek 1981, Gibbs and Inman 1991, Wingfield and Gibbs 1991, Jacobs et al 2001a), New Zealand (Shaw and Dick 1980, Wingfield and Marasas 1983, Thwaites et al 2005) and South Africa (Jacobs et al 2001a, Zhou et al

TABLE II. Comparison of *Leptographium sinoprocerum* sp.nov. with most resembling species

	L. sinoprocerum	*L. procerum*	*L. profanum*	*L. albopini*	*L. euphyes*
Region	China	U.S.A, Canada, Europe, South Africa, New Zealand, Korea, Japan, China	U.S.A	U.S.A.	New Zealand
Host	*P. tabuliformis*, *P. bungeana*	Many conifers	*Carya* sp., *Nyssa sylvatica*, *Cornus florida*	*Pinus* spp.	*Pinus* spp.
Insect association	*D. valens*, *Tomicus* sp.	Many beetles including *D. valens*	None	*Hylastes* sp.	*Tomicus piniperda*
Conidiophore	Occurring single or in groups from submerged and aerial mycelium	Occurring singly or in groups from the mycelium directly	Occurring singly from medium	Occurring singly from the mycelium directly	Occurring singly from the mycelium directly
Rhizoids	Present, knot-like structure	Prominent present	Absent	Present, interwoven in a knot	Present
Stipe length (μm)	(34–)62–1020(–1266)	Up to 1250, reaching 2500[a]	(120–)284–644(–850)	70–960, mean 323	(143–)224(–354)
Conidiogenous apparatus length (μm)	(40–)42–128(–140)	36–130	(70–)84–129(–160)	40–92	(31–)73(–93)
Series of branches	2–5	3–5 usually	Multiple series	3–6	3–4
Number of primary branches	2–3	2–3 usually	2–3	2–4	2–3
Primary branches (μm)	11–37 × 2–9	13–45 × 5–9	8–37 × 3–7	—[b]	11–47 × 5–8
Conidium (μm)	Oblong to subclavate, 2–6 × 1–3	Obovoid, 2–7 × 1–3	Ellipsoid to obovoid with truncate bases and round apices, 3–6 × 2	Oblong, 3–8 × 1–4	Obovoid with truncated ends, occasionally oblong, 4–6 × 2–3
Colony on 2% MEA	Dark olivaceous first and later dark brown, sometime with faintly cream-colored concentric rings toward the edge	Dark mouse gray to olivaceous toward the edge with concentric rings	Dark olive	Hyaline first and later light olivaceous	Olivaceous
Hyphae	Loose aerial mycelium, roughened	Little or no aerial mycelium, smooth	Abundant aerial mycelium, smooth	No aerial mycelium, roughened	No aerial mycelia, smooth
Optimum growth temperature	25 C	25 C	25 C	25 C	25 C
Growth rate on 2% MEA					
- by authors (radius, mm/day)	3.6 ± 0.3	3.5 ± 0.4	—	3.8 ± 0.3	—
- from literature (diameter)MEA	—	About 75 mm in 15 d[c]	24.5 mm in 8 d	33 mm in 4 d	19 mm in 6 d
Reference	MUCL 46352	Kendrick 1962, Jacobs and Wingfield 2001	Jacobs et al 2006	Wingfield et al 1994	Jacobs et al 2001a

[a] Personal observation of authentic strain MUCL 8094.

[b] no records in original literatures or this study.

[c] Kendrick (1962) didn't indicate the temperature under which this growth was measured. However Jacobs and Wingfield (2001) measured the growth as reaching 23 mm diam in 9 d at 25 C on 2% MEA. The Chinese strains of *L. procerum* and MUCL 8094 have similar radial growth rates about 3.6 mm/d (56 mm diam in 7 d) under same condition.

2001). In North America *D. valens* acts as one of the vectors of *L. procerum* and thus this species might have entered China with RTB. However no data are available from the time that RTB was introduced (1980s). Further genetic analysis of *L. procerum* populations from various geographic areas would be necessary to ascertain their relationships and geographic origin.

Studies of *Leptographium* in eastern Asia, especially China, are still few and fragmentary. Three species have been described so far from the eastern temperate Asian mainland in addition to *L. sinoprocerum*, *L. bistatum* (Kim JJ et al 2004), *L. koreanum* J.J. Kim & G.H. Kim (Kim JJ et al 2005) and *L. yunnanense* X.D. Zhou et al (Zhou et al 2000). These species phylogenetically are related distantly to *L. sinoprocerum* (Kim JJ et al 2004, 2005; FIGS. 1–4). Intensive surveys of the numerous ecosystems in China that harbor a great diversity of trees will certainly lead to the discovery of more undescribed species and perhaps, using phylogenetic methods, new lineages within *Leptographium* and other ophiostomatoid fungi originating in eastern Asia, as demonstrated in two out of the 34 provinces in China by Zhou et al (2006).

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