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ORIGINAL PAPER

Taxonomy and phylogeny of the *Leptographium procerum* complex, including *Leptographium sinense* sp. nov. and *Leptographium longiconidiophorum* sp. nov.

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Abstract *Leptographium procerum* (Ophiostomatales, Ascomycota) is a well-known fungal associate of pine root-infesting bark beetles and weevils, occurring in several countries of the world. The fungus is not a primary pathogen but has been associated with white pine root decline in the USA and with serious damage caused by the introduced red turpentine beetle (RTB) *Dendroctonus valens* in China. Several species closely related to *L. procerum* have been described during the past decade. The aim of this study was to reevaluate species boundaries in the *L. procerum* complex using multigene phylogenetic analyses and morphological comparisons. Phylogenetic analyses of seven gene regions (ITS2-LSU, actin, β-tubulin, calmodulin, translation elongation factor 1-α, and the mating type genes *MAT1-1-3* and *MAT1-2-1*) distinguished between nine species in the complex. These included *L. procerum*, *L. bhutanense*, *L. gracile*, *L. profanum*, *L. pini-densiflorae*, *L. sibiricum*, *L. sinoprocerum*, as well as two new species described here as *Leptographium sinense* sp. nov. from *Hylobitelus xiaoi* on *Pinus elliottii* in China, and *Leptographium longiconidiophorum* sp. nov. from *Pinus densiflora* in Japan. *Leptographium latens* is reduced to synonymy with *L. gracile*, and an epitype is designated for *L. procerum,* because a living culture associated with the holotype of *L. procerum* did not exist. Amplification patterns of the mating type genes suggest that all known species in the *L. procerum* complex are heterothallic, although sexual states have not been observed for any of the species. The results also suggest that Eastern Asia is most probably the centre of species diversity for the *L. procerum* complex.

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Introduction

The genus *Leptographium* Lagerb. & Melin was first described in 1927, with *L. lundbergii*, the causal agent of sapstain on pine timber, as the type species (Lagerberg et al. 1927). *Leptographium* spp. are characterized by mononematous, darkly pigmented conidiophores giving rise to brush-like conidiogenous structures that produce conidia in slimy droplets, facilitating insect dispersal (Jacobs and Wingfield 2001). Under the dual nomenclature system, *Leptographium* has been considered one of several anamorph genera in the Ophiostomatales (Wingfield 1993), and the sexual states of *Leptographium* spp. were treated under the genus *Grosmannia* Goid. (Zipfel et al. 2006). In 2011, dual nomenclature was abandoned (Hawksworth 2011) and the newly proposed one fungus one name principles require that the oldest genus name, in this case *Leptographium* (Lagerberg et al. 1927), should take preference over the younger name, *Grosmannia* (Goidànich 1936), irrespective of morph (De Beer and Wingfield 2013).

One of the *Leptographium* species that has gained most attention in terms of research during recent years is *Leptographium procerum,* which was first described from *Pinus banksiana*, *P. resinosa*, and *P. strobus* in 1962 in North America (Kendrick 1962). The fungus was associated with a disease known as white pine root decline (WPRD) that resulted in significant economic losses in the Christmas tree industry in North Central and Eastern America (Lackner and Alexander 1982; Alexander et al. 1988). However, Wingfield (1986) and Wingfield et al. (1988) argued strongly that the fungus was unlikely to play a primary role in tree death and that it was most likely a resinophillic fungus carried by insects that feed on stressed pine roots. Symptoms similar to those of WPRD were reported by Shaw and Dick (1979) in New Zealand and *L. procerum* was considered as possibly contributing to a root disease complex. More recently, *L. procerum* has been reported in association with root-infesting bark beetles from declining pines in the southeastern USA, although inoculation studies suggest that the fungus is not a serious pathogen in this area (Matusick et al. 2012). In all the above-mentioned studies, the identification of *L. procerum* was based on morphological characters of the fungus, and identities have yet to be confirmed based on DNA sequence data. In Europe, several recent studies showed that *L. procerum* is a common associate of several bark beetle and weevil species, occurring together with other ophiostomatoid fungi. The fungus was isolated from the roots of *Pinus sylvestris* and soil environments in Poland and identified based on ITS and β-tubulin sequences (Jankowiak et al. 2012; Jankowiak and Bilański 2013a, b, c).

In 2008, *L. procerum* was reported for the first time from China as an associate of the red turpentine beetle *Dendroctonus valens* (Lu et al. 2008), which is thought to have been introduced into that country from North America (Cognato et al. 2005). In northern China this beetle-fungus combination has contributed to the mortality of more than a half million hectares of native pine forests (Miao et al. 2001; Sun et al. 2013). Pathogenicity trials conducted in China on *P. tabuliformis* suggested that the fungus might be more pathogenic to pines in that country than in its native range in North America (Lu et al. 2010; Sun et al. 2013). The identity of *L. procerum* in China has been confirmed using DNA sequence analyses, which also confirmed its relatedness to several other species in the genus (Lu et al. 2008; 2009a, b; Taerum et al. 2013). In another study, Duong et al. (2013) characterized the mating type genes of *L. procerum* and its close relative, *L. profanum*, showing that both species are heterothallic.

De Beer and Wingfield (2013) defined ten species complexes in *Leptographium* sensu lato. In one of these complexes, *L. procerum* was the oldest known species and thus became the name-bearing species of the complex. At present, the *L. procerum* complex includes eight species (Linnakoski et al. 2012). Interestingly, apart from *L. procerum* (Kendrick 1962) and *L. profanum* (Jacobs et al. 2006) described from USA, and *L. sibiricum* from Russia (Jacobs et al. 2000), all other species in the complex have been described from East Asia (Linnakoski et al. 2012). *Leptographium pinidensiflorae* was described from Japan (Masuya et al. 2000), *L. bhutanense* from Bhutan (Zhou et al. 2008), and *L. sinoprocerum* (Lu et al. 2008), *L. gracile* and *L. latens* (Paciura et al. 2010) from China.

The three gene regions, the internal transcribed spacer region 2 and partial large subunit of the

ribosomal DNA (ITS2-LSU), β-tubulin (βT) and translation elongation factor-1 alpha (TEF-1α), that have typically been used for species delineation in *Leptographium* during the past decade, have also been used to identify *L. procerum* from China (Lu et al. 2008; 2009a, b; Taerum et al. 2013) as well as some of the new species in the complex. However, Paciura et al. (2010) and Linnakoski et al. (2012) showed that the ITS2-LSU and βT gene regions have limitations in distinguishing closely related species in the *L. procerum* complex. Duong et al. (2012) thus sequenced an additional two gene regions, calmodulin (CAL) and actin (ACT) and with sequences for five gene regions, were able to successfully delineate morphologically similar but cryptic species in the neighboring *G. serpens* complex in *Leptographium s. l.*

The aims of this study were to reevaluate the phylogenetic relationships and delineation of all species previously reported in the *L. procerum* complex, applying the five gene regions used by Duong et al. (2012). In addition, we used the recently developed diagnostic markers for the mating type genes *MAT1-1-3* and *MAT1-2-1* to identify the mating types for all isolates included in this study.

Materials and methods

Fungal isolates

All isolates used in the study are listed in Table 1. These were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Ex-type isolates of new species described in this study were deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands, while type specimens were deposited in the National Collection of Fungi (PREM), Pretoria, South Africa. Taxonomic novelties and typification events (Robert et al. 2013) were registered in MycoBank.

DNA extraction, PCR and sequencing

DNA extractions were made using fresh mycelium (ca. 100 mg) from isolates that had been incubated in 2 % liquid malt extract medium (20 g malt extract, Biolab, South Africa and 1,000 ml deionized water) for 4–5 days at 25 °C in the dark. PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, California, USA) was used for DNA extraction, following the manufacturer's protocols.

Seven gene regions were amplified for sequencing and phylogenetic analyses, including ITS2-LSU, ACT, βT, CAL, TEF-1α, *MAT1-1-3* and *MAT1-2-1*. The following primers were used: ITS3 & LR3 (White et al. 1990) for ITS2-LSU, Lepact-F & Lepact-R (Lim et al. 2004) for ACT, T10 (O'Donnell and Cigelnik 1997) & Bt2b (Glass and Donaldson 1995) for βT, CL2F & CL2R (Duong et al. 2012) for CAL, EF1-F & EF2-R (Jacobs et al. 2004) for TEF-1α, Oph-MAT1F1 & Oph-MAT1R2 (Duong et al. 2014) for *MAT1-1-3*, and Oph-HMG1 (5′- CGYAAGGAYMAYCACAAGGC -3′) & Oph-HMG2 (5′- GGRTGAAGMMKCTCAACCTG -3′) (Duong et al. unpublished) for *MAT1-2-1*.

PCR reactions were conducted in 25 μL reaction mixtures containing 5 μL of Mytaq buffer (including $MgCl_2$, dNTPs and reaction buffer), 0.5 μL of Mytaq polymerase (Bioline, USA), 0.5 μL of each primer (10 μM), 2 μL of DNA, and 16.5 μL of PCR grade water. Amplification for ITS2-LSU, ACT, βT, CAL, *MAT1-1-3* and *MAT1-2-1* gene regions was as follows: an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 55–52 °C for 30 s and 72 °C for 40 s, and a final chain elongation at 72 °C for 8 min. The TEF-1α gene region was amplified using an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 55–52 °C for 45 s and 72 °C for 1 min, and a final elongation at 72 °C for 8 min.

For DNA Sequencing, PCR products were purified using the High Pure PCR Product Purification Kit (Roche Applied Science, Mannheim, Germany) following the manufacturer's protocols. Sequencing PCRs were carried out using the same primer pairs that were used for PCR, together with the Big Dye Terminator 3.1 cycle sequencing premix kit (Applied Biosystems, Foster City, California, USA). The analyses of sequencing PCR products were done on ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). Forward and reverse sequences of each isolate were assembled with CLC Main Workbench 6.0 (CLC Bio, Aarhus, Denmark) to produce a consensus sequence.

Phylogenetic analyses

A total of seven different datasets were prepared for phylogenetic analyses. The ITS2-LSU sequences of

Table 1 Isolates used in present study

Species	Isolate no.[1,2]		Country	Host	Insect	GenBank accession no.[3]						
	CMW	Other				ITS2-LSU	ACT	βT	CAL	TEF-1α	*MAT1-1-3*	*MAT1-2-1*
Leptographium bhutanense	18649[H]	CBS 122076	Bhutan	*Pinus wallichiana*	*Hylobitelus chenkupdorjii*	EU650184	**KM491319**	**KM491352**	**KM491385**	**KM491461**	**KM491449**	–
	18650	CBS 122077	Bhutan	*P. wallichiana*	*H. chenkupdorjii*	EU650185	**KM491320**	**KM491353**	**KM491386**	**KM491462**	**KM491450**	–
	18651	CBS 122078	Bhutan	*P. wallichiana*	*H. chenkupdorjii*	EU650186	**KM491321**	**KM491354**	**KM491387**	**KM491463**	**KM491451**	–
	18652	–	Bhutan	*P. wallichiana*	*H. chenkupdorjii*	EU650187	**KM491322**	**KM491355**	**KM491388**	**KM491464**	–	**KM491428**
L. gracile	12316	CBS 123625	China	*P. armandii*	*Pissodes sp.*	HQ406842	**KM491323**	**KM491356**	**KM491389**	**KM491465**	–	**KM491429**
	12396	CBS 123624	China	*P. armandii*	*Pissodes sp.*	HQ406841	**KM491324**	**KM491357**	**KM491390**	**KM491466**	–	**KM491430**
	12398[H]	CBS 123623	China	*P. armandii*	*Pissodes sp.*	HQ406840	**KM491325**	**KM491358**	**KM491391**	**KM491467**	–	**KM491431**
L. latens	12310	CBS 123615	China	*P. armandii*	*Pissodes sp.*	HQ406843	**KM491330**	**KM491363**	**KM491396**	**KM491472**	–	**KM491435**
	12319	CBS 123616	China	*P. armandii*	*Pissodes sp.*	HQ406844	**KM491331**	**KM491364**	**KM491397**	**KM491473**	–	**KM491436**
	12438[H]	CBS 124023	China	*Picea koraiensis*	*Ips typographus*	HQ406845	**KM491332**	**KM491365**	**KM491398**	**KM491474**	–	**KM491437**
L. longiconidiophorum sp. nov.	2004[H]	CBS 135624	Japan	*P. densiflora*	–	**KM491421**	**KM491329**	**KM491362**	**KM491395**	**KM491471**	**KM491452**	–
L. pini-densiflorae	5157[H]	CBS 115261	Japan	*P. densiflora*	*Tomicus piniperda*	AY707199	**KM491333**	**KM491366**	**KM491399**	**KM491475**	**KM491453**	–
	5158	CBS 120508	Japan	*P. densiflora*	*T. piniperda*	DQ062082	**KM491334**	**KM491367**	**KM491400**	**KM491476**	**KM491454**	–
	5162	CBS120195	Japan	*P. densiflora*	*T. piniperda*	DQ062083	**KM491335**	**KM491368**	**KM491401**	**KM491477**		**KM491438**
L. procerum	661	CBS 516.63	USA	*P. resinosa*	–	**KM491422**	**KM491336**	**KM491369**	**KM491402**	**KM491478**	**KM491455**	–
	10217	CBS 120196	USA	*P. strobus*	*Dendroctonus valens*	AY553386	**KM491337**	**KM491370**	**KM491403**	**KM491479**	**KM491456**	–
	23285	CBS 128844	Russia	*P. sylvestris*	*Hylurgops palliatus*	JF279978	**KM491338**	**KM491371**	**KM491404**	**KM491480**	**KM491457**	–
	29993	MUCL 46323	China	*P. tabuliformis*	*D. valens*	EU296775	**KM491339**	**KM491372**	**KM491405**	**KM491481**	–	**KM491439**
	29994	MUCL 46361	China	*P. tabuliformis*	*D. valens*	EU296776	**KM491340**	**KM491373**	**KM491406**	**KM491482**	**KM491458**	–
	34542[E]	CBS 138288	USA	*P. resinosa*	*D. valens*	**KM491423**	**KM491341**	**KM491374**	**KM491407**	**KM491483**	–	**KM491440**
L. profanum	10550	–	USA	*Carya sp.*	–	DQ354943	**KM491342**	**KM491375**	**KM491408**	**KM491484**	–	**KM491441**
	10552[H]	CBS 120307	USA	*Nyssa sylvatica*	–	DQ354944	**KM491343**	**KM491376**	**KM491409**	**KM491485**	–	**KM491442**
	10554	CBS 120226	USA	*Cornus florida*	–	DQ354942	**KM491344**	**KM491377**	**KM491410**	**KM491486**	**KM491459**	–
L. sibiricum	4481[H]	CBS 115260	Russia	*Abies sibirica*	*Monochamus urussoni*	**KM491424**	**KM491345**	**KM491378**	**KM491411**	**KM491487**	–	**KM491443**
	4482	–	Russia	*A. sibirica*	*M. urussoni*	**KM491425**	**KM491346**	**KM491379**	**KM491412**	**KM491488**	–	**KM491444**
	4484	–	Russia	*A. sibirica*	*M. urussoni*	**KM491426**	**KM491347**	**KM491380**	**KM491413**	**KM491489**	–	**KM491445**
L. sinense sp. nov.	38171	CBS 316515	China	*P. elliottii*	*Hylobitelus xiaoi*	**KM491418**	**KM491326**	**KM491359**	**KM491392**	**KM491468**	–	**KM491432**
	38172[H]	CBS 135625	China	*P. elliottii*	*H. xiaoi*	**KM491419**	**KM491327**	**KM491360**	**KM491393**	**KM491469**	–	**KM491433**
	38173	CBS 316516	China	*P. elliottii*	*H. xiaoi*	**KM491420**	**KM491328**	**KM491361**	**KM491394**	**KM491470**	–	**KM491434**
L. sinoprocerum	26230	MUCL 46328	China	*P. tabuliformis*	*D. valens*	**KM491427**	**KM491348**	**KM491381**	**KM491414**	**KM491490**	**KM491460**	–
	29988	MUCL 47246	China	*P. bungeana*	*D. valens*	EU296774	**KM491349**	**KM491382**	**KM491415**	**KM491491**	–	**KM491446**
	29990[H]	MUCL 46352	China	*P. tabuliformis*	*D. valens*	EU296773	**KM491350**	**KM491383**	**KM491416**	**KM491492**	–	**KM491447**
	29992	MUCL 46331	China	*P. tabuliformis*	*D. valens*	EU296772	**KM491351**	**KM491384**	**KM491417**	**KM491493**	–	**KM491448**

Table 1 continued

Species	Isolate no.[1,2]		Country	Host	Insect	GenBank accession no.[3]						
	CMW	Other				ITS2-LSU	ACT	βT	CAL	TEF-1α	*MAT1-1-3*	*MAT1-2-1*
Grosmannia alacris	621[H*]	CBS 128830	Portugal	*P. pinaster*	–	–	JN135318	JN135327	JN135296	JN135305	–	**KP171183**
	623[H*]	CBS 118621	Portugal	*P. pinaster*	–	–	JN135321	JN135327	JN135295	JN135306	**KP171181**	–
	2844[H*]	CBS 591.79	South Africa	*P. pinaster*	–	JN135313	JN135320	JN135329	JN135296	JN135304	–	**KP171184**
G. serpens	304[H]	CBS 141.36	Italy	*P. sylvestris*	–	JN135314	JN135325	JN135334	JN135300	JN135307	**KP171182**	–

Genbank accession numbers of sequences obtained in the present study are printed in bold type

[1] *CBS* Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; *CMW* Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; *MUCL* part of Belgian Coordinated Collections of Microorganisms, BCCM

[2,E] ex-epitype isolate; [H] ex-holotype isolate; [H*] in the case of *G. alacris* two isolates (CMW621 and CMW623) were crossed to produce sexual states that was deposited as the holotype. CMW 2844 is the ex-holotype isolate of the asexual form of the fungus, originally described as *Verticicladiella alacris*

[3] *ITS2* the internal transcribed spacer 2 region of the nuclear ribosomal DNA gene; *LSU* the 28S large subunit of the nrDNA gene; *ACT* Actin; *βT* Beta-tubulin; *CAL* Calmodulin; *TEF-1α* Translation elongation factor 1-alpha

the ex-type isolate of each species in the *L. procerum* complex (Table 1) were compared with those of 58 other species in *Leptographium s. l.* obtained from Genbank to show the placement of the complex within the genus. Sequences of *Fragosphaeria purpurea* and *F. reniformis* were selected as outgroup taxa. A smaller dataset of ITS2-LSU sequences consisting 35 sequences for the species in the *L. procerum* complex (Table 1), was also compiled for use in the combined analyses.

Four protein coding gene regions (ACT, βT, CAL and TEF-1α) in 35 isolates (Table 1) were sequenced for the delineation of closely related species in the *L. procerum* complex. These four data sets were included together with the ITS2-LSU data sub-set in the combined analyses. In the single gene and the combined datasets, sequences of *G. alacris* (CMW 2844, ex-type) and *G. serpens* (CMW 304, ex-type) from the study of Duong et al. (2012) were used as outgroup taxa.

The datasets for the two mating type gene regions (*MAT1-1-3* and *MAT1-2-1*) consisted of varying numbers of sequences, depending on the mating type of the respective isolates (Table 1). Fourteen isolates were included in the *MAT1-1-3* dataset, with sequences of *G. alacris* (CMW 623) and *G. serpens* (CMW 304) as outgroups (Duong et al. 2012). In the *MAT1-2-1* dataset, sequences of 23 isolates were included (Table 1). In this case two isolates of *G. alacris* (CMW 621 and CMW 2844) were used as outgroup taxa, since no *MAT1-2-1* sequences were available for *G. serpens*.

Because intron and exon composition of some protein coding genes vary between species and species complexes in *Leptographium s. l.* (De Beer and Wingfield 2013), it was important to consider the presence of introns in all sequences when alignments were made. To aid with the identification and appropriate alignment of introns, complete maps of the coding regions and introns of the four protein coding genes (Online Resources 1–4) were compiled based on the whole genome sequence of *Grosmannia clavigera* (kw1407; National Center for Biotechnology Information (NCBI), Genome PID: 39837) (DiGuistini et al. 2011). Alignments of the respective datasets were conducted using the online version of MAFFT 6.0 (Katoh et al. 2002). Alignments were checked manually in MEGA 5.1 (Tamura et al. 2011) and compared with the *G. clavigera* gene maps to ensure

introns and exons were aligned appropriately. In this process, amino acid sequences of the exons were also considered using MEGA 5.1. No manual modifications were necessary.

Three methods of phylogenetic analyses were applied to all datasets. These included maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). Before the analyses of the combined dataset, a partition homogeneity test (PHT) was conducted using PAUP* 4.0b10 (Swofford 2003) to examine the congruence of five gene regions.

MP analyses were executed in PAUP* 4.0b10 (Swofford 2003) with heuristic searches of 1,000 replicates, tree bisection and reconnection (TBR) branch swapping options and excluding gaps. To determine the confidence of branch nodes, 1,000 repetitions of bootstrap were conducted. Tree length (TL), consistency index (CI), retention index (RI) and homoplasy index (HI) and rescaled consistency index (RC) were recorded after generating the trees.

ML analyses were performed using the online version of PhyML 3.0 (Guindon et al. 2010). For these analyses the most appropriate substitution models were selected using jModelTest 2.1.1 (Posada 2008), and Nearest-Neighbor-Interchange (NNI) branch swapping were used. Confidence for nodes was determined by using 1,000 bootstrap replicates. Gaps were excluded.

BI analyses were conducted in MrBayes 3.2 (Ronquist et al. 2012) utilizing a Markov chain Monte Carlo (MCMC) method. The most appropriate evolutionary models were determined with jModelTest 2.1.1 as for ML analyses. Four independent Monte Carlo Markov chains were simultaneously run from a random starting tree for 5 million generations. Trees were sampled every 100 generations. Burn-in values were determined in Tracer 1.4 (Rambaut and Drummond 2007). Trees sampled in the burn-in phase were discarded and posterior probabilities were calculated from all the remaining trees.

Morphology, growth and mating studies

For microscope examination, isolates were inoculated on 2 % water agar (WA, 20 g Difco agar and 1,000 ml deionized water) adjacent to sterilized pine twigs on the agar surface, and incubated at 25 °C for 3–4 weeks (Duong et al. 2012). Fruiting structures on pine twigs were transferred with a needle to microscope slides and mounted in water. Culture characteristics were studied on Oatmeal agar (OA, 30 g oatmeal, 20 g Difco Bacto™ malt extract, from Becton, Dickinson & Company, and 1,000 ml deionized water), after incubation at 25 °C for 10–14 days. Descriptions of morphology and classifications were based on criteria recommended by Jacobs and Wingfield (2001), and colours were based on the charts of Rayner (1970).

In order to determine optimal temperatures for growth in culture, two isolates per species, including ex-type isolates, were selected. Disks of agar were cut from the actively growing margins of 5-day-old colonies of isolates with a sterile 5 mm cork borer and transferred to the centers of 90 mm plates of MEA. Three replicates were prepared for each isolate and incubated in the dark at 5–35 °C at 5 °C intervals. Average diameters of each colony were measured once every 2 days until the mycelial growth reached the edges of the plates.

The mating type of each isolate was determined based on the results of the mating type PCR reactions described above. Isolates of opposite mating type of the same species were paired with each other in all possible combinations. These pairings were done on water agar with sterilized pieces of pine wood using the technique described by Grobbelaar et al. (2010), and incubated for 3–4 months at 25 °C. Interspecific crosses were also performed using isolates of four morphologically indistinguishable species (*L. bhutanense*, *L. sinoprocerum*, *L. gracile* and *L. latens*). The crosses were inspected once a week for the presence of ascomata.

Results

DNA sequencing and phylogenetic analyses

A summary of the most important parameters applied in, as well as outcomes of, the phylogenetic analyses are presented in Table 2. The trees resulting from ML, MP and BI analyses of the *Leptographium s. l.* data, resulted in the species in the *L. procerum* complex always grouping together, although without significant statistical support (Online Resource 5). The complex grouped between the *Grosmannia olivacea* and *Grosmannia serpens* complexes. Tree topologies obtained from ITS2-LSU analyses were not able to separate all the species within the complex from each other. In all the ITS2-LSU trees *L. procerum, L. sinoprocerum, L. bhutanense*, *L. gracile*, *L. latens*,

Table 2 Parameters used and outcomes of all phylogenetic analyses in the present study

		ITS2-LSU	ACT	βT	CAL	TEF-1α	Combined	*MAT1-1-3*	*MAT1-2-1*
Alignments	Exons(introns)	Not applicable	5(5)6	2(2)3(3) 4(4)5(−)6	3(3)4(4) 5(−)6	3(3)4(4)5	–	1, 2/1	2, 3/2
	Number of taxa	70	35	35	35	35	35	14	23
	Total	612	814	484	537	792	3,232	379	205
	Constant	419	707	363	379	506	2,532	257	147
	Uninformative	62	1	5	3	8	20	6	5
	Informative	133	106	116	155	278	680	116	53
MP	Tree number	1,000	1	1	2	1	1	2	1
	Tree length	398	121	143	186	368	847	128	62
	CI	0.585	0.967	0.944	0.919	0.894	0.920	0.984	0.919
	RI	0.891	0.990	0.981	0.974	0.962	0.973	0.988	0.959
	RC	0.521	0.958	0.926	0.895	0.860	0.895	0.973	0.881
	HI	0.415	0.033	0.056	0.081	0.106	0.080	0.016	0.081
Model tests	Subst. models	GTR+I+G	GTR+G	GTR+G	GTR+G	GTR+G	GTR+G	K80	HKY+I
ML	P-inv	0.511	–	–	–	–	–	–	0.341
	Gamma	0.686	0.257	0.550	0.508	0.683	0.341	–	–
BI	Burn-in	50	40	30	30	30	30	20	20

MP maximum parsimony, *ML* maximum likelihood, *BI* Bayesian inference, *Uninformative* Number of parsimony-uninformative characters, *Informative* Number of parsimony-informative characters, *CI* consistency index, *RI* retention index, *RC* rescaled consistency index, *HI* homoplasy index, *Subst. model* substitution models in phylogeny, *P-inv* proportion of invariable sites, *Gamma* Gamma distribution shape parameter

grouped with the four unidentified isolates from China and Japan. *Leptographium profanum* grouped within the main lineage containing the latter seven species, but differed by 1 bp from the other species in the lineage (Online Resource 5). The only two species that were distinct, were *L. pini-densiflorae* and *L. sibiricum* that formed an unsupported sub-lineage within the complex.

The intron/exon composition in the amplified ACT gene region of all species in the complex (Table 2) corresponded with that of *G. clavigera* (Online Resource 1). Sequences for the ACT dataset distinguished between all the species in the complex, apart from *L. sinoprocerum*, *L. gracile* and *L. latens* for which all isolates had identical sequences (Online Resource 6). The single isolate from Japan and the isolates obtained from China in the present study (Table 1), formed a well-supported monophyletic lineage distinct from all the other species in the complex, but differed in 1 bp from each other in exon 5 (Online Resource 7). They were thus respectively labeled as Taxon 1 and Taxon 2.

In the alignment of the βT gene region, intron 5, which is not present in *G. clavigera* (Online Resource 2), but is found in some other *Leptographium* spp. (De Beer and Wingfield 2013), was lacking in all species of the *L. procerum* complex (Table 2). Analyses of the βT region (Online Resource 6) distinguished between most species in the complex, apart from *L. sinoprocerum*, *L. gracile* and *L. latens* that had identical sequences. Sequences for this gene region also failed to distinguish between Taxa 1 and 2 from China and Japan.

Within the sequence data for the CAL region, the intron/exon composition (Table 2) corresponded with that of *G. clavigera* (Online Resource 3) with intron 5 being absent while it is found in some other species complexes in the Ophiostomatales (De Beer, unpublished). The phylogenies obtained from this data set showed differences between most species in the complex, including Taxa 1 and 2, but in this case *L. bhutanense* and *L. sinoprocerum* had identical sequences, as did *L. gracile* and *L. latens* (Online Resource 6).

The intron/exon composition of the TEF-1α gene of species in the *L. procerum* complex (Table 2) did not correspond with that of *G. clavigera*, which lacks intron 4 (Online Resource 4). Phylogenetic analyses of this gene resolved all the species in the complex, apart

from *L. gracile* and *L. latens* which had identical sequences (Online Resource 6).

The partition homogeneity test (PHT) of the combined dataset (ITS2-LSU, ACT, βT, CAL and TEF-1α) gave a *P* value of 0.648, indicating that data of the five gene regions could be combined. The MP, ML and BI analyses of the combined dataset provided trees with similar topologies and no conflicts (Fig. 1). Six of the known species (*L. bhutanense, L. procerum*, *L. profanum*, *L. pini-densiflorae*, *L. sibiricum* and *L. sinoprocerum*) in the *L. procerum* complex were well-defined. Similarly, the isolate from Japan (Taxon 1) and those from China (Taxon 2) formed distinct lineages. Two of the previously known species, *L. gracile* and *L. latens,* had identical sequences in all the gene regions (Online Resources 5 and 6). The ten taxa grouped in three well-supported major lineages (Fig. 1), labeled as groups A, B, and C. Group A included *L. bhutanense, L. sinoprocerum, L. gracile, L. latens,* Taxon 1 and Taxon 2. Group B included *L. procerum* and *L. profanum*, and Group C *L. pini-densiflorae* and *L. sibiricum*.

The *MAT1-1-3* data set included 14 isolates (Taxon 1, *L. bhutanense*, *L. procerum*, *L. profanum*, *L. pini-densiflorae* and *L. sinoprocerum*), representing six species of the *L. procerum* complex. In the resulting phylogeny (Fig. 2), four of the six species could be distinguished, but *L. bhutanense* and *L. sinoprocerum* had identical sequences.

The phylogenetic analyses of aligned data set of the *MAT1-2-1* gene (Fig. 2) could only distinguish between *L. procerum*, *L. profanum*, *L. pini-densiflorae* and *L. sibiricum*, while *L. bhutanense*, *L. sinoprocerum, L. gracile*, *L. latens* and Taxon 2 all had identical sequences.

Morphology, growth in culture and mating studies

All five groups representing the Chinese isolates and the Japanese isolate formed olivaceous leptographium-like asexual states similar to *L. bhutanense* and *L. sinoprocerum*. Moreover, *L. pini-densiflorae* and *L. sibiricum* could be distinguished from the other species with colorless mycelium on both OA and MEA. Morphological differences between Taxa 1 and 2 and the other species are discussed in the *Notes* provided for the new species descriptions in the "Taxonomy" section.

The optimal growth temperature for most isolates was 25 °C, with the only exception being *L. bhutanense* that grew best at 20 °C and *L. pini-densiflorae* and *L. sibiricum* which grew best at 30 °C. Four species (*L. bhutanense*, *L. latens*, *L. gracile* and *L. sinoprocerum*) were extremely slow growing or had no growth at 5 or 30 °C. None of the isolates in the study grew below 5 °C, or at 35 °C. None of the crosses between isolates in the *L. procerum* complex gave rise to ascomata.

Taxonomy

Multilocus phylogenetic analyses of 33 isolates revealed nine well-supported lineages in the *L. procerum* complex. Six of these lineages represented previously described species. These included *L. procerum*, for which an epitype is designated below. One isolate from Japan, previously identified as *L. procerum* based on morphology, formed a distinct lineage representing a new species (Taxon 1), as did the isolates collected from *H. xiaoi* in China (Taxon 2).

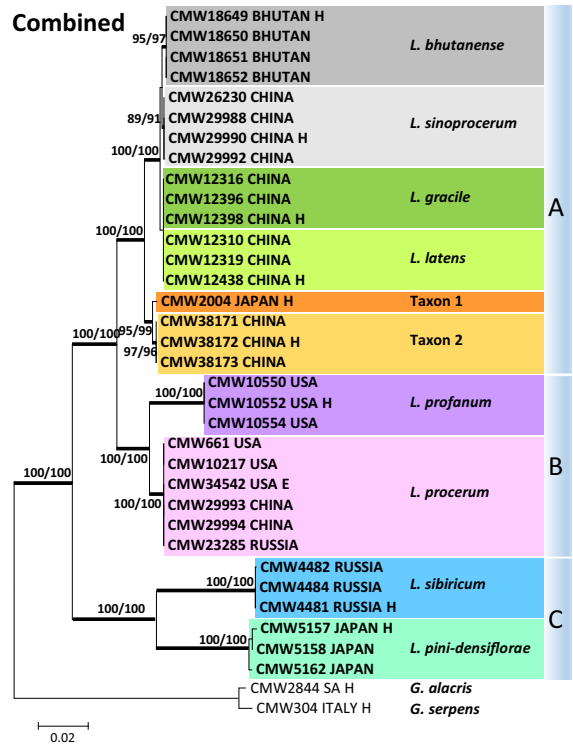

Fig. 1 ML trees of the *Leptographium procerum* complex generated from the DNA sequences of the ribosomal ITS2-LSU regions, combined with those of four protein-coding gene regions, including ACT, βT, CAL and TEF 1-α. *Bold* branches indicate posterior probabilities values ≥ 0.95. Bootstrap values ≥ 75 % are recorded at nodes as ML/MP. H ex-holotype isolates, E ex-epitype isolate

These two taxa are described here as novel species. The ninth lineage consisted of isolates of both *L. gracile* and *L. latens,* including those linked to the holotypes of the two species. Isolates of these two species had identical sequences in all six gene regions and they are reduced to synonymy. Based on the recommendations of De Beer and Wingfield (2013), all species in the complex are treated in the genus *Leptographium sensu lato*.

Leptographium procerum (W.B. Kendr.) M.J. Wingf., Trans. Br. Mycol. Soc. 85: 92 (1985). MB 105454

≡ *Verticicladiella procera* W.B. Kendr., Can. J. Bot. 40: 783 (1962). (Basionym) MB 340902

Sexual state not observed. *Asexual state*, *conidiophores* occurring singly or in groups of up to three, macronematous, mononematous, erect, arising directly from the mycelium, (150–) 245–570 (–760) µm long. *Rhizoids* present. *Stipes* olivaceous, 3–10 septa, not constricted at septa, (125–) 206–496 (–690) µm long. Apical cells not swollen at apex, 3–15 µm wide. Basal cells not swollen at apex. *Conidiogenous apparatus* (25–) 39.4–75 (–90) µm long, excluding the conidial mass, with 2 to 5 series of cylindrical branches. Primary branches light olivaceous, smooth, cylindrical, aseptate, arrangement of primary branches Type B-more than two branches, (11–) 16–22 (–34) × (3–) 4–5 (–7) µm. Secondary branches light olivaceous, aseptate, (8–) 11–12 (–15) × (2–) 3–4 (–7) µm. Tertiary branches hyaline, aseptate, (7–) 7–13 (–14) × 2–6 µm. Quaternary branches aseptate, (7–) 8–12 (–13) × 2–5 µm. *Conidiogenous cells* discrete, 2–4 per branch, cylindrical, tapering slightly at apex, (11–) 15–18 (–22) × 1–2 µm. *Conidia* hyaline, aseptate, obovoid to broadly ellipsoid with truncate bases and rounded apices, 3–5 × 1–3 µm. *Colonies* on 3 % OA flat, hyaline at the beginning, then becoming light olivaceous to dark olivaceous. Hyphae submerged in agar with no aerial mycelium, concentric rings observed. Colony margin smooth or slightly effuse. Conidiophores forms abundantly in clusters on OA. *Colonies* on 2 % MEA flat, with optimal growth at 25 °C, reaching 25 mm in diam. in 7 days. No growth below 10 °C nor above 30 °C.

Mating system Heterothallic based on presence of *MAT* genes, but no sexual states were obtained in crosses.

Type material **Holotype** CANADA, Quebec, Baie St. Paul, from *Pinus banksiana* stump, Sep 1959, coll. W. B. Kendrick, DAOM 63700. **Paratypes** USA, New York state, Montgomery Co., from interior of roots with resinous lesions of *Pinus resinosa*, Feb 1959, coll. D. S. Welch, DAOM 62093; Newfield, DAOM 62094; CANADA, Columbia Co., DAOM 62095; Stockton, Chatauqua Co., DAOM 62096; Ontario, Sudbury, from *Pinus strobus* heart rot, Sep 1952, S. N. Linzon, DAOM 33940. SWEDEN, Södermanland, Järna, from galleries of *Pissodes pini* on *Pinus* sp., Aug 1959, coll. A. Mathiesen-Käärik, DAOM 63686.

Epitype (designated here): USA, Maine, Massabesic Experimental Forest, from *Dendroctonus valens* gallery on *Pinus resinosa,* Nov 2009, coll. M. J. Wingfield. PREM 61058 (herbarium specimen of dried culture); CMW 34542 = CBS 138288 (ex-epitype culture), MBT 198257.

Additional isolates examined USA, Vermont, from *Pinus strobus*, 2000, coll. K. Jacobs, CMW 10217.

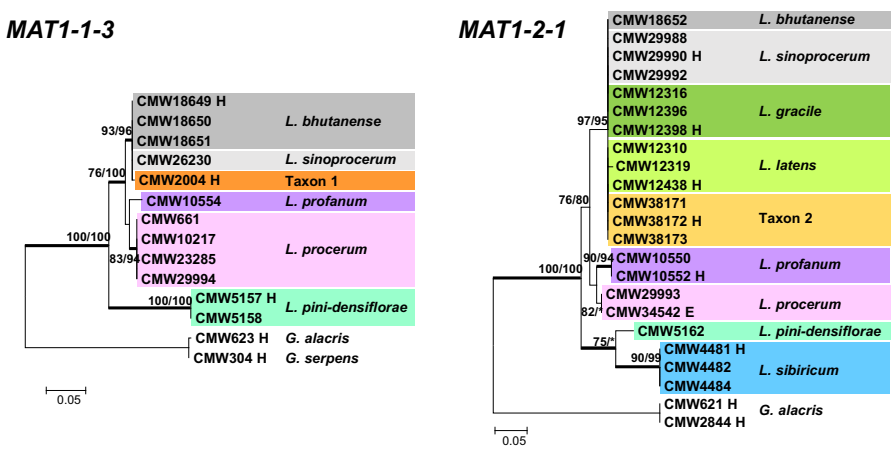

Fig. 2 ML trees of the *Leptographium procerum* complex generated from DNA sequences of the *MAT1-1-3* and *MAT1-2-1* gene regions. *Bold* branches indicate posterior probabilities values ≥ 0.95. * bootstrap values < 75 %. H ex-holotype isolates, E ex-epitype isolate

RUSSIA, Lisino-Corpus, from *Pinus sylvestris*, associated with *Hylurgops palliatus*, 2012, coll. R. Linnakoski, CMW 23285 = CBS 128844. CHINA, from *Pinus tabuliformis*, sapwood underneath gallery of *Dendroctonus valens*, 2004, coll. Q. Lu., CMW 29993 = MUCL 46323; CMW 29994 = MUCL 46361.

Hosts/Substrate Abies fraseri, *A. grandis*, *Picea abies**, *Pinus banksiana*, *P. bungeana*, *P. clausa*, *P. contorta*, *P. densiflora*, *P. echinata*, *P. elliottii*, *P. monticola*, *P. nigra*, *P. ponderosa*, *P. radiata**, *P. resinosa**, *P. strobus**, *P. sylvestris**, *P. tabuliformis**, *P. taeda*, *P. virginiana*, *Pseudotsuga menziesii*.

Insect vectors Dendroctonus frontalis, *D. terebrans*, *D. valens**, *Hylastes ater**, *H. opacus**, *Hylobius abietis**, *H. pales*, *H. radicis*, *Hylurgops palliatus**, *Hylurgus ligniperda*, *Ips typographus*, *I. sexdentatus**, *Pachylobius picivorus*, *Pissodes approximatus*, *Pissodes castaneus**, *P. nemorensis*, *P. pini**, *P. piniphilus**, *Pityogenes* sp., *Tomicus piniperda*, *Tetropium fuscum**, *Xyleborus* sp.

Known distribution North America (Canada and USA*), Europe (Sweden, Poland*, UK, France and Russia*), Asia (Japan and China*), South Africa, and New Zealand*.

* Hosts, vectors and origin from studies where the identity of *L. procerum* was confirmed based on DNA sequences (Jacobs et al. 2004; Jankowiak 2012; Jankowiak and Bilański 2013a, b, c; Jankowiak and Kolařík 2010; Kim et al. 2005; Lu et al. 2008, 2009a, b; Linnakoski et al. 2012; and from the present study). All unmarked hosts and vectors were listed by Jacobs and Wingfield (2001), but identifications were based on morphology only.

Notes Leptographium procerum was first described as *Verticicladiella procera*. The genus *Verticicladiella* was subsequently reduced to synonymy with *Leptographium* based on similarities of conidial development as revealed by scanning electron microscopy, and a new combination for this species in *Leptographium* was thus provided (Wingfield 1985). The holotype of *L. procerum* originates from *P. resinosa* in Quebec, Canada, while four of the paratype specimens are from *Pinus resinosa* from various locations in New York, USA. The remaining two paratypes originated from *Pinus strobus* in Ontario and pine in Sweden respectively (Kendrick 1962). There are no living cultures of the holotype and paratypes that could be used for DNA sequencing. During recent surveys of fungal associates of *Dendroctonus valens* infesting *P. resinosa* in the Northeastern USA (Taerum et al. 2013), many fresh isolates of *L. procerum* were obtained. One of these, CMW 34542 from *P. strobus* in Maine, corresponds with the original descriptions based on morphology and with other *L. procerum* isolates based on DNA sequences and it is consequently designated here as the epitype for the species.

Taxon 1

Leptographium longiconidiophorum M.L. Yin, Z.W. de Beer & M.J. Wingf., sp. nov. Fig. 3

Mycobank MB 805970

Etymology Name reflects the exceptionally long conidiophores that distinguish it from all other species in the *L. procerum* complex.

Sexual state not observed. *Asexual state*, *conidiophores* occurring singly or in groups of up to 10, macronematous, mononematous, erect, arising directly from the mycelium, (1,030–) 1,580–2,150 (–2,460) µm long. *Rhizoids* present. *Stipes* dark olivaceous, 2–14 septa, not constricted at septa, (979–) 1,403–1,980 (–2,320) µm long. Apical cells occasionally swollen at apex, (6–) 9–14 (–16) µm wide. Basal cells occasionally swollen at apex, (9–) 11–17 (–20) µm wide. *Conidiogenous apparatus* (60–) 110–125 (–142) µm long, excluding the conidial mass, with multiple series of cylindrical branches. Primary branches olivaceous, smooth, cylindrical, not swollen at apex, aseptate, arrangement of primary branches was Type A—only two branches, (25–) 30–35 (–42) × (4.9–) 5.8–6.9 (–7.2) µm. Secondary branches light olivaceous, frequently swollen at apex, aseptate, (12–) 16–20 (–27) × (3.9–) 4.3–4.9 (–5.6) µm. Tertiary branches light olivaceous, aseptate, (9–) 13–17 (–20) × (3.5–) 4.1–4.7 (–5.0) µm. Quaternary branches light olivaceous to hyaline, aseptate, (5–) 9–15 (–17) × (1.6–) 2.1–2.7 (–3.2) µm. *Conidiogenous cells* discrete, hyaline, 2–3 per branch, aseptate, cylindrical, tapering slightly at the apex, (16–) 19–22 (–25) × (1.3–) 1.6–1.9 (–2.1) µm. *Conidia* hyaline, aseptate, elliptical, (2.7–) 3.5–4.8 (–5.1) × (1.6–) 1.9–2.2 (–2.4) µm. *Colonies* on 3 % OA flat, hyaline at the beginning, then becoming light olivaceous to dark olivaceous. Hyphae superficial on agar with olivaceous aerial mycelium, no concentric rings observed. Colony margin smooth. Conidiophores forms abundantly in clusters on OA. Colonies on 2 % MEA flat, with

optimal growth at 25 °C, reaching 30.5 mm in diam. in 7 days. No growth below 10 °C and at 35 °C or above.

Mating system Heterothallic based on the presence of *MAT* genes, but no sexual state was found in laboratory crosses.

Type material **Holotype** JAPAN, Kofu, from *Pinus densiflora*, 2002, coll. M. J. Wingfield, PREM 60872 (herbarium specimen of dried culture); CMW 2004 = CBS 135624 (ex-holotype culture).

Hosts/Substrate Pinus densiflora.

Known distribution Japan.

Notes Leptographium longiconidiophorum is phylogenetically closely related to but clearly distinct from *L. sinense* (Fig. 1 and Online Resource 6), differing from that species in 1 bp in ACT, 3 bp in CAL, and 5 bp in TEF-1α (Online Resource 7). This fungus can be distinguished from all other species in the complex, including *L. sinense,* by its conidiophores that are much longer (almost double the length) than those of the other species. Furthermore, its conidiophores are often produced in clusters distributed over the medium and do not form concentric rings on OA such as those of *L. sinense*. Despite the fact that only one isolate of this species was available for study, we describe it here based on the clear phylogenetic and morphological differences with other species in the complex.

Taxon 2

Leptographium sinense M.L. Yin, Z.W. de Beer & M.J. Wingf., sp. nov. Fig. 4

Mycobank MB 805971

Etymology Name refers to China where it was first collected.

Sexual state not observed. *Asexual state*, *conidiophores* occurring singly, macronematous, mononematous, erect, arising directly from the mycelium, (608–) 753–893 (–1,039) µm long. *Rhizoids* present. *Stipes* dark brown, 3–9 septa, not constricted at the septa, (544–) 608–770 (–936) µm long. Apical cells occasionally swollen at the apex, (7–) 10–13 (–15) µm wide. Basal cells occasionally swollen at apex, (9–) 11–17 (–20) µm wide. *Conidiogenous apparatus* (120–) 144–175 (–196) µm long, excluding the conidial mass, with multiple series of cylindrical branches. Primary branches brown, smooth, cylindrical, not swollen at apex, aseptate, arrangement of primary

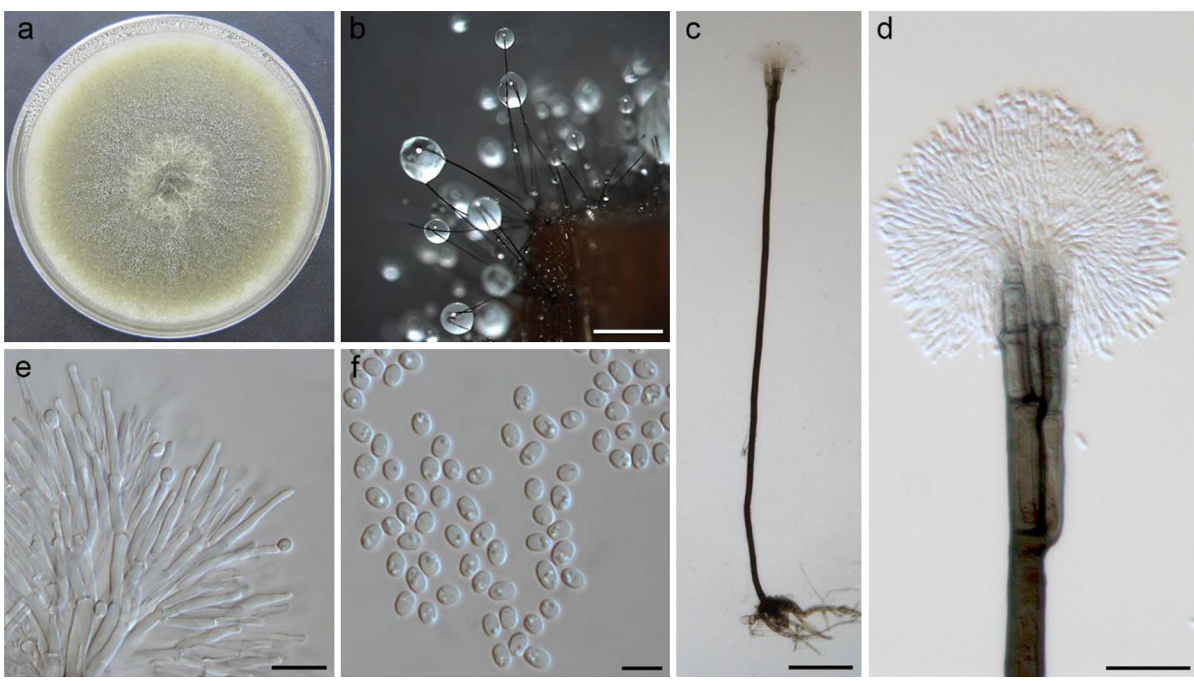

Fig. 3 Morphological characters of *Leptographium longiconidiophorum* (CMW2004) **a** Fourteen days old culture on 90 mm OA; **b** mononematous asexual morph on wood tissue on WA; **c** conidiophore; **d** conidiogenous apparatus; **e** conidiogenous cells; **f** conidia. *Scale bars* b = 500 µm, c = 100 µm, d = 20 µm, e = 10 µm, f = 5 µm

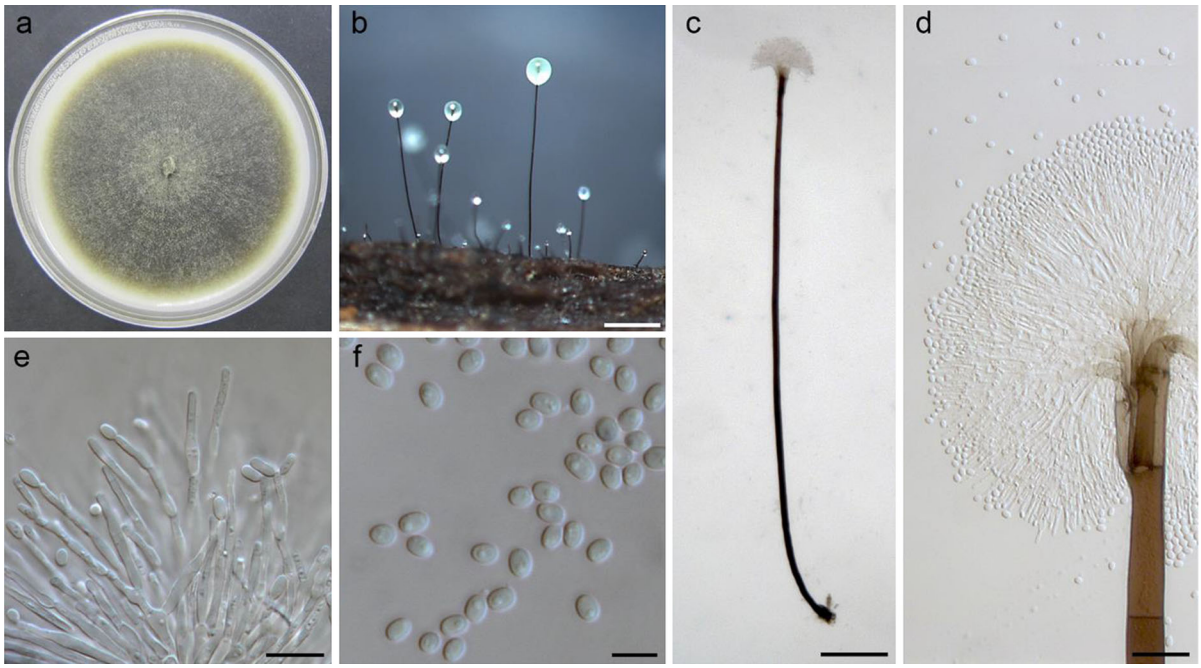

Fig. 4 Morphological characters of *Leptographium sinense* (CMW38172) **a** Fourteen days old culture on 90 mm OA; **b** mononematous asexual morph on wood tissue on WA; **c** conidiophore; **d** conidiogenous apparatus; **e** conidiogenous cells; **f** conidia. *Scale bars* b = 300 µm, c = 100 µm, d = 20 µm, e = 10 µm, f = 5 µm

branches was Type A - only two branches, (19–) 21–28 (–36) × (4.5–) 5.5–8.9 (–9.7) µm. Secondary branches light brown, frequently swollen at apex, aseptate, (10–) 15–24 (–30) × (2.9–) 4.3–5.4 (–6.6) µm. Tertiary branches light brown to hyaline, aseptate, (10–) 12–18 (–21) × (2.9–) 3.4–4.1 (–4.9) µm. Quaternary branches hyaline, aseptate, (5–) 8–12 (–16) × (1.7–) 2.0–2.5 (–3.3) µm. *Conidiogenous cells* discrete, hyaline, 2–3 per branch, aseptate, cylindrical, tapering slightly at the apex, (10–) 12–16 (–20) × (1.2–) 1.6–2.2 (–2.5) µm. *Conidia* hyaline, aseptate, elliptical to round, (2.9–) 3.4–4.1 (–4.9) × (1.9–) 2.4–2.7 (–3.0) µm. *Colonies* on 3 % OA flat, hyaline at the beginning, then becoming light olivaceous to dark olivaceous. Hyphae superficial on the agar with olivaceous aerial mycelium, multiple concentric rings observed. Colony margin smooth. Conidiophores forms abundantly in clusters on OA. Colonies on 2 % MEA flat, with optimal growth at 25 °C. No growth below 10 °C and at 35 °C or above.

Mating system Heterothallic based on the presence of *MAT* genes but a sexual state was not produced in crosses.

Type material **Holotype** CHINA, Jiangxi province, from *Pinus elliottii*, 2010, coll. X.D. Zhou, PREM 60873 (herbarium specimen of dried culture), CMW 38172 = CBS 135625 (ex-holotype culture).

Additional isolates examined CHINA, Jiangxi province, from *Pinus elliottii*, 2010, coll. M. Yin, R. Chang & X.D. Zhou, CMW 38171 = CBS 316515; CMW 38173 = CBS 316516.

Hosts/Substrate Pinus elliottii.

Insect vectors Hylobitelus xiaoi

Known distribution Jiangxi, China.

Notes Leptographium sinense grouped close to, but distinct from *L. longiconidiophorum* in phylogenetic analyses (Fig. 1 and Online Resource 6), and can clearly be separated from that species based on sequences in three gene regions (Online Resource 7). Morphologically, it differs from *L. longiconidiophorum* with its shorter and darker conidiophores occurring singly and in multiple concentric rings on OA.

Leptographium gracile D. Paciura, Z.W. de Beer & M.J. Wingf., Persoonia 25:103 (2010). MB 516736

= *Leptographium latens* D. Paciura, Z.W. de Beer & M.J. Wingf., Persoonia 25:104 (2010). MB 516737

Type material For *L. gracile* CHINA, Yunnan Province, Midu, from *Pinus armandii* infested by

Pissodes sp., July 2001, coll. X.D. Zhou & Z.W. de Beer, **holotype** PREM 59995 (herbarium specimen of dried culture), CMW 12398 = CBS 123623 (ex-holotype culture); **paratype** PREM 59996 (herbarium specimen of dried culture), CMW 12396 = CBS 123624 (ex-paratype culture); Yunnan Province, Lijiang, from *Pinus armandii*, infested by *Pissodes* sp., July 2001, coll. X.D. Zhou & Z.W. de Beer, **paratype** PREM 59997 (herbarium specimen of dried culture), CMW 12316 = CBS 123625 (ex-paratype culture). For *L. latens* CHINA, Yunnan Province, Midu, from *Picea koraiensis* infested by *Ips typographus*, July 2001, coll. X.D. Zhou & Z.W. de Beer, **holotype** PREM 60007 (herbarium specimen of dried culture), CMW 12438 = CBS 124023 (ex-holotype culture); Yunnan Province, Lijiang, from *Pinus armandii* infested by *Pissodes* sp., July 2001, coll. X.D. Zhou & Z.W. de Beer, **paratype** PREM 60008 (herbarium specimen of dried culture), CMW 12310 = CBS 123615 (ex-paratype culture); Yunnan Province, Midu, from *Pinus armandii* infested by *Pissodes* sp., July 2001, coll. X.D. Zhou & Z.W. de Beer, **paratype** PREM 60009 (herbarium specimen of dried culture), CMW 12319 = CBS 123616 (ex-paratype culture).

Notes Paciura et al. (2010) described *L. gracile* and *L. latens*, comparing the species based on sequences of three gene regions (ITS2-LSU, β-tubulin and TEF-1α). They distinguished between the two species based on differences in the latter two gene regions and slightly longer conidia of *L. latens*. In the present study, seven gene regions were used for phylogenetic analyses and all the genes previously used to distinguish *L. gracile* and *L. latens* were resequenced together with the two closely related species, *L. bhutanense* and *L. sinoprocerum.* The results showed that *L. gracile* and *L. latens* had identical sequences in all gene regions (Fig. 1 and Online Resources 6 and 7), while *L. bhutanense* and *L. sinoprocerum* could be distinguished from them and from each other. We thus consider *L. gracile* to be a synonym of *L. latens.*

Discussion

In this study, the taxonomy of all species in the *L. procerum* complex was revised based on phylogenetic analyses of sequences for seven gene regions. Representative isolates of eight known species were included, as well as a collection of unidentified isolates from China, and an isolate from Japan previously thought to represent *L. procerum*. The isolates from the latter two countries were shown to represent novel taxa that were described as *L. sinense* and *L. longiconidiophorum.* Furthermore, an epitype was designated for *L. procerum*, and two species previously described from China (*L. gracile* and *L. latens*) were shown to be conspecific. The *L. procerum* complex now includes nine well-defined species (*L. bhutanense*, *L. gracile*, *L. longiconidiophorum, L. pini-densiflorae*, *L. procerum, L. profanum*, *L. sibiricum, L. sinense,* and *L. sinoprocerum*) primarily occurring in association with bark beetles infesting pine trees. Sequences obtained for the mating type genes of these species suggest that they are all heterothallic.

Among the seven gene regions that were used in the phylogenetic analyses, TEF-1α was the most variable and most informative region, distinguishing between all nine species in *L. procerum* complex as defined based on the combined analyses of five of the gene regions. The CAL gene region was also useful in delineating the species, but with slightly fewer variable sites than TEF-1α. In contrast, ITS2-LSU has the lowest number of variable sites and could distinguish only two species (*L. pini-densiflorae* and *L. sibiricum*) from all the other species that all had almost identical sequences for this region. The partial *MAT* genes were not useful in distinguishing between closely related species. Although the ITS region has been suggested as barcoding region for fungi (Schoch et al. 2012), our results suggest that the ITS2-LSU region would be useful only to place isolates in a particular complex in *Leptographium s. l.* and not to distinguish between species in that complex. We suggest that the TEF-1α gene region should be considered as an additional barcoding gene for accurate species identification in this genus.

All isolates in the *L. procerum* complex included in this study had either one of the two *MAT* genes, suggesting that all species in the complex are heterothallic. This is consistent with the findings of Duong et al. (2013) who showed that *L. procerum* and *L. profanum* are heterothallic. We were, however, not able to induce a sexual state for any of the species in laboratory crosses. The fact that the sexual state has not been observed in nature and despite some intensive searching (Wingfield, unpublished), could be due to a cryptic nature or the absence of long-necked perithecia

that are characteristic for the majority of *Leptographium* spp. for which sexual states are known (Jacobs and Wingfield 2001). This would be consistent with the fact that some species, e.g. *G. clavigera* (Robinson-Jeffrey and Davidson 1968) and *G. yunnanense* (Yamaoka et al. 2008), are known to produce cleistothecial ascomata that are embedded in the woody substrate and thus not visible during routine inspections of bark beetle galleries.

The three major lineages making up the *L. procerum* complex as defined in this study corresponded to the geographical origin of the isolates. Group B (Fig. 1) included two species, *L. procerum* and *L. profanum*, and could be referred to as the North American-European lineage. *Leptographium procerum* was initially known only from North America (Canada and USA) (Kendrick 1962), subsequently it was found in other parts of the world, including Europe [e.g. UK (Wingfield and Gibbs 1991) and Poland (Jacobs and Wingfield 2001; Jankowiak and Bilański 2013a, b, c)], and Asia [e.g. Japan (Masuya et al. 1999) and more recently China (Lu et al. 2008)]. The species has also been found in association with introduced bark beetles on exotic pines in New Zealand (Wingfield and Marasas 1983) and South Africa (Zhou et al. 2001). This fungus has been reported on many different *Pinus* spp. on which its various and generally non-host specific vectors feed. Several recent studies suggest that *L. procerum* is the dominant associate of *D. valens* (Taerum et al. 2013), but the fungus is certainly not carried specifically by that beetle, as it has been isolated from various other bark beetles and weevils (Alexander et al. 1988; Jacobs and Wingfield 2001; Jankowiak and Bilański 2013a, b, c), most notably those that infest roots or root collars of trees. This explains why the fungus has often been associated with root diseases of trees, even though its role as pathogen has been questioned (Wingfield 1986; Wingfield et al. 1988; Jacobs and Wingfield 2001; Jankowiak 2006). More recently the suggestion has been made that *L. procerum* might be a soil fungus in pine forests that infests roots through wounds caused by insects (Jankowiak et al. 2012).

Leptographium profanum, also residing in the North American–European lineage (Group B), is the only species in the *L. procerum* complex that has been isolated from hardwood trees. It is known only from central Alabama (USA) where it was isolated from roots of *Nyssa sylvatica*, *Cornus florida* and a *Carya* sp. (Jacobs et al. 2006). Unlike other species in the complex, there is no evidence that *L. profanum* is associated with bark beetles. It remains uncertain whether it is a soil-inhabiting fungus, or carried by mites, or whether it might be associated with some undiscovered root-infesting insect species.

All seven species belonging to Groups A and C (Fig. 1) of the *L. procerum* complex are from conifers in Asia. Group A is the largest group and includes five species, *L. bhutanense*, *L. gracile*, *L. sinoprocerum*, *L. sinense* and *L. longiconidiophorum*, while Group C includes two species, *L. pini-densiflorae* and *L. sibiricum*. Three of these (*L. gracile*, *L. sinoprocerum*, *L. sinense*) have been reported only from China (Lu et al. 2008; Paciura et al. 2010; and isolates from the present study). *Leptographium longiconidiophorum* and *L. pini-densiflorae* have been recorded only from Japan (Masuya et al. 2000; present study), while *L. bhutanense* is from Bhutan (Zhou et al. 2008), and *L. sibiricum* from Russia (Jacobs et al. 2000). Most of these species were isolated from pines, but *L. gracile* (in Group A) has also been collected on spruce (Paciura et al. 2010), and *L. sibiricum* (in Group C) was isolated from *Abies* (Jacobs et al. 2000). The insects associated with species in Groups A and C are mainly weevils and bark beetles, with *L. sibiricum* being the only exception and collected as an associate of a cerambycid beetle (Jacobs et al. 2000). Pathogenicity has been tested for only *L. sinoprocerum* residing in the Asian lineages. This species was thought to be less pathogenic than the other fungal associates of *D. valens* based on the lesions resulting from inoculations in the crowns of mature *P. tabuliformis* in China (Lu et al. 2009b).

The two new species described in the present study were both isolated from *Pinus* spp. Of these, *L. longiconidiophorum* was from *P. densiflora* in Japan adding to several species in *Leptographium s. l.* reported in association with various bark beetles from *P. densiflora* in Japan, including *L. procerum* (Masuya et al. 1999, 2009). All these identifications have been based on morphology, and although *L. longiconidiophorum* has longer conidiophores than *L. procerum*, it is possible that some of the isolates reported from Japan as the latter species, might have represented *L. longiconidiophorum*.

The other new species, *L. sinense,* was isolated from the pine weevil *Hylobitelus xiaoi* infesting *P. elliottii* in China. This tree is native to the southeastern

United States, and was introduced to China in the 1940s (Wen et al. 2004). During last 30 years, *P. elliottii* has become the dominant pine species in commercial plantations in southern China due to its fast growth rate and economic value (Wen et al. 2004). *Hylobitelus xiaoi,* which is native to China (Zhang 1997), has become known as a common pest in over 80,000 ha of pine forests, including *P. elliottii,* in this country (Wen et al. 2004). The weevil has contributed to the mortality of over 15,000 pine seedlings in Jiangxi Province alone (Wen et al. 2004). These weevils breed in healthy hosts, where the larvae invade the inner bark of the lower stem while the adults infest the inner bark of branches (Wen et al. 2004). The isolates in the present study originated from both the larvae and adults of *H. xiaoi*, as well as their galleries. As the fungal associates of *H. xiaoi* have not previously been studied, *L. sinense* is the first ophiostomatoid fungus reported in association with this insect. Although the insect is exotic to China, the phylogenetic placement of *L. sinense* in the Asian lineage of the *L. procerum* complex, suggests that the fungus might be native to China.

The availability of DNA sequencing techniques that distinguish between cryptic species, has led to the discovery of new species in the *L. procerum* complex. Of the nine species recognized in the complex, the majority were described for the first time from Asia, and all except *L. profanum* have been recorded from this continent. Current knowledge thus suggests that East Asia could be the center of species diversity in the *L. procerum* complex. Yet very little is known regarding the biology, ecology and population genetics of these fungi. It will be interesting to obtain more samples of species in this complex from Europe, America and other continents in the future, to understand more clearly their origins, diversity, host range, and pathways of movement.

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