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Three new species and a new combination of *Triblidium*

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Abstract

Triblidiaceae (Rhytismatales) currently consists of two genera: *Triblidium* and *Huangshania*. *Triblidium* is the type genus and is characterised by melanized apothecia that occur scattered or in small clusters on the substratum, cleistohymenial (opening in the mesohymenial phase), inamyloid thin-walled asci and hyaline muriform ascospores. Before this study, only the type species, *Triblidium caliciiforme*, had DNA sequences in the NCBI GenBank. In this study, six specimens of *Triblidium* were collected from China and France and new ITS, mtSSU, LSU and RPB2 sequences were generated. Our molecular phylogenetic analysis and morphological study demonstrated three new species of *Triblidium*, which are formally described here: *T. hubeiense*, *T. rostriforme* and *T. yunnanense*. Additionally, our results indicated that *Huangshania* that was considered to be distinct from *Triblidium* because of its elongated, transversely-septate ascospores, is congeneric with *Triblidium*. Therefore, we have placed *Huangshania* in synonymy under *Triblidium*, rendering Triblidiaceae a monotypic family.

Keywords

Huangshania, phylogenetic, taxonomy, Triblidiaceae, three new taxa, muriform ascospores

Introduction

Triblidium Rebent.: Fr. is the type genus of Triblidiaceae Rehm (Rehm 1888–1896, 1912), which includes presumed saprobes on the bark of Pinaceae, Ericaceae and Fagaceae (Magnes 1997). In his monograph of the family, Magnes (1997) speculated that some species may exist in an endophytic state. Species of *Triblidium* are well documented in Europe, but they are poorly understood in Asia and America (Magnes

1997). Magnes (1997) revised *Triblidium* and accepted amongst the many included species only four species and one subspecies.

A history of Triblidiaceae is given in Karakehian et al. (2019). In brief, Magnes (1997) placed Triblidiaceae in Rhytismatales and treated Triblidiales as a synonym of Rhytismatales. Recent five-locus (Prieto et al. 2019) and 15-locus (Johnston et al. 2019) phylogeny analyses found high support for *Pseudographis* (Triblidiaceae) within Rhytismatales. The results of a three-gene phylogenetic analysis with expanded sampling by Karakehian et al. (2019) supported Magnes classification and the authors emended Triblidiaceae to include *Triblidium* and *Huangshania.*

We conducted a morphological analysis of a specimen of *T. caliciiforme* Rebent.: Fr., the type species of *Triblidium* and additional collections of Triblidiaceae. Phylogenetic relationships were inferred based on internal transcribed spacer (ITS), nuclear large subunit ribosomal DNA (LSU), mitochondrial small subunit ribosomal DNA (mtSSU) and the second largest subunit of RNA polymerase II (RPB2) gene.

Materials and methods

Morphological studies and isolation

A specimen of *Triblidium caliciiforme* was collected in France in June 2012 on *Quercus* sp. Other specimens were collected in China between 2006 and 2018. Mature dried ascomata were selected for morphological observation. All observations were made from dead herbarium material. Gross morphology was observed and photographed with a dissecting microscope (Nikon SMZ-1000). Standardised colour values matching the colour of the hymenium were taken from https://www.colorhexa.com/. Microscopic preparations were observed in distilled water, Lugol's solution (IKI), 5% potassium hydroxide (KOH) and lactophenol solution. Methods for morphological analysis follow Hou et al. (2009). Measurements of asci and ascospores were made in distilled water in 2019. For each structure, at least 25 measurements were recorded. Microphotographs were obtained using an Olympus BX51 compound microscope. Specimens are deposited in the Herbarium of the College of Life Science, Capital Normal University, Beijing, China (**BJTC**). Fresh specimens were used to obtain cultures directly from single ascoma, after washing and surface sterilisation, as follows: 75% ethanol for 10 s, 10% sodium hypochlorite for 3 min, washing in sterile water three times. The single ascoma was dried in sterilised tissue paper, placed on potato dextrose agar (PDA) with 50 mg/l chloramphenicol and incubated at room temperature (25 °C ± 3 °C). We were unable to obtain cultures from ascomata after a month.

DNA extraction and PCR amplification

Genomic DNA was extracted from ascomata using NuClean Plant Genomic DNA Kit (CWBIO, China), following the manufacturer's instructions and stored at -20 °C. Se-

quences of ITS, LSU, mtSSU and RPB2 were obtained. PCR amplifications were undertaken using primers ITS1F/ITS4 for ITS, mrSSU1/mrSSU3R for mtSSU, LR0R/LR5 for LSU and 5F/7CR for RPB2 (Vigalys and Hester 1990, White et al. 1990, Gardes and Bruns 1993, Rehner and Samuels 1994, Liu et al. 1999, Zoller et al. 1999). ITS, mtSSU and LSU PCR procedures in 25 μl reactions were carried out as outlined by Hou et al. (2009). PCR amplification of the RPB2 region was undertaken with an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 60 s, annealing at 55 °C for 60 s and elongation at 72 °C for 2 min and a final elongation at 72 °C for 10 min (Liu et al. 1999). The PCR products were purified, sequenced and edited by ZhongKe Xilin Biotechnology Co., Ltd. (Beijing, China). The new sequences were submitted to the NCBI GenBank database. Their accession numbers, as well as those for other ITS, LSU, mtSSU and RPB2 sequences downloaded from GenBank, are given in Table 1.

Table 1. Species and GenBank accession numbers of the sequences analysed in this study. "–" indicates data unavailable. Sequences generated for this study are in boldface.

Species	Voucher and strain	ITS	LSU	mtSSU	RPB2
Bisporella citrina	AFTOL-ID 1301	–	FJ176871	FJ190632	FJ238354
Coccomyces dentatus	AFTOL-ID 147	DQ491499	AY544657	AY544736	DQ247789
Coccomyces lauraceus	ICMP:18319	–	HM140504	HM143781	–
Coccomyces tumidus	Lantz 396 (UPS)	–	HM140510	HM143787	–
Colpoma quercinum	Lantz 368 (UPS)	–	HM140513	HM143789	–
Cryptomyces maximus	Lantz & Minter 424 (UPS)	–	HM140514	HM143790	–
Cudonia circinans	Lantz & Widen 402 (UPS)	–	HM140515	HM143791	–
Huangshania verrucosa	UME-29336a	MK751793	MK751802	MK751716	–
Hypoderma rubi	ICMP:17339	JF683419	HM140526	HM143801	–
Hypohelion scirpinum	Lantz 394 (UPS)	–	HM140531	HM143806	–
Lirula macrospora	Isolate 13	HQ902159	HQ902152	–	–
Lophodermium eucalypti	ICMP:16796	EF191235	HM140541	HM143817	–
Neofabraea malicorticis	AFTOL-ID 149	–	AY544662	AY544751	–
Pseudographis elatina	GJO-0090016	MK751794	MK751803	MK751717	–
Pseudographis pinicola	FH-18061706	MK751795	MK751804	MK751718	–
	FH-NB842	MK751796	MK751805	MK751719	–
Sporomega degenerans	Lantz 367 (UPS)	–	HM140567	HM143839	–
Spathularia flavida	KUS-F52331	JN033405	JN086708	JN086781	JN086859
Therrya abieticola	HOU447A	KP322574	KP322579	KP322587	–
Triblidium caliciiforme	FH-15071105	MK751797	MK751806	MK751720	–
	CUP-18080101	MK751798	MK751807	MK751721	–
	E-00012551	MK751799	MK751808	MK751722	–
	E-00012552	MK751800	MK751809	MK751723	–
	GJO-0088904	MK751801	MK751810	MK751724	–
Triblidium caliciiforme	**HOU1053**	**MN519485**	**MN540636**	**MN538985**	**MN547962**
Triblidium hubeiense	**HOU1350A**	**MN541813**	**MN541811**	**MN541828**	**MN565260**
Triblidium rostriforme	**HOU851A**	**MN541815**	**MN541820**	**MN541821**	**MN565263**
	HOU889	**MN541822**	**MN541817**	**MN541839**	**MN565262**
Triblidium yunnanense	**HOU470A**	**MN541818**	**MN541819**	**MN541810**	**MN565259**
	HOU1179	**MN541814**	**MN541809**	**MN541816**	**MN565261**
	HOU875A	**MN541840**	**MN541828**	**MN541812**	**MN551099**
Tryblidiopsis pinastri	AFTOL-ID 1319	–	DQ470983	–	DQ470935

Phylogenetic analysis

The sequences, used in this study, included 22 taxa for the ITS matrix, 32 taxa for the LSU matrix, 30 taxa for the mtSSU, and 11 taxa of RPB2. *Bisporella citrina* (Batsch) Korf & S.E. Carp. (Helotiales, Helotiaceae) and *Neofabraea malicorticis* (Cordley) H.S. Jacks. (Helotiales, Dermateaceae) were selected as outgroups. Maximum parsimony (MP) and Bayesian Inference (BI) analyses were performed on the concatenated ITS–LSU–mtSSU–RPB2 dataset. Each dataset was first aligned with Clustal X and then manually adjusted to allow maximum sequence similarity in Se-Al v.2.03a (Thompson et al. 1997; Rambaut 2000). Ambiguously aligned regions were excluded from the analysis by hand. Alignments were submitted to TreeBASE under accession number S25247. A partition homogeneity test was performed to determine the congruence of ITS, LSU, mtSSU and RPB2 (Farris et al. 1995; Huelsenbeck et al. 1996). After a positive outcome, the datasets were analysed together. The datasets were prepared and analysed with the maximum parsimony (MP) method using PAUP* 4.0b10 (Swofford 1998). The phylogenetic analysis was conducted using heuristic searches with 1000 replicates of random-addition sequence, tree bisection reconnection (TBR) branch swapping and no maxtree limit. All characters were equally weighted and unordered. Gaps were treated as missing data to minimise homology assumptions. A bootstrap analysis was performed with 1000 replicates, each with 100 random taxon addition sequences. Maxtrees were set to 1000 and TBR branch swapping was employed. For the Bayesian analysis, MrModeltest 2.3 with the Akaike Information Criterion (AIC) was used to choose the best-fit substitution models for the concatenated dataset: GTR+I+G for both ITS and LSU, HKY+I+G for mtSSU and SYM+G for RPB2. The Bayesian analysis was performed with MrBayes 3.1.2 (Huelsenbeck et al. 2011, Ronquist and Huelsenbeck 2003) with two sets of four chains (one cold and three heated) and the Stoprule option in effect, halting the analyses at an average standard deviation of split frequencies of 0.01. The sample frequency was set to 100 and the first 25% of trees were removed as burn-in and the remaining trees were kept and combined into one 50% majority-rule consensus tree. Bayesian Posterior Probabilities (PP) were obtained from the 50% majority consensus of the remaining trees. Clades receiving both bootstrap values of maximum parsimony (BP) ≥ 70% and PP ≥ 0.95 were considered to be significantly supported.

Results

Molecular phylogeny

The phylogenetic analyses, based on the concatenated four-locus (ITS, LSU, mtSSU, RPB2) DNA matrix, included 32 taxa and 3472 characters, of which 843 were parsimony-informative. The maximum parsimony analysis resulted in one most parsimonious tree with a length (TL) of 2991 steps, consistency index (CI) of 0.697, retention

index (RI) of 0.754 and homoplasy index (HI) of 0.303. Except for the two outgroup species, *B. citrina* and *N. malicorticis*, all the other taxa formed one highly supported clade. *Lirula macrospora* (R. Hartig) Darker resolved as sister to all the remaining taxa (Rhytismatales). This result is similar to the topology of Lantz et al. (2011). The Triblidiaceae samples formed a well-supported clade (BP = 100%, PP = 0.99; Fig. 1). The type species of *Huangshania*, *H. verrucosa* O.E. Erikss., is nested in the *Triblidium* clade (Fig. 1).

Taxonomy

Triblidium hubeiense **T. Lv & C. L. Hou, sp. nov.**
MycoBank No: 832358
Figs 2, 3

Diagnosis. Similar to *Triblidium sherwoodiae* but different by apically not swollen and unbranched paraphyses and homolateral curved ascospores, with a smaller L/W ratio of 1.4–2.3 (average ratio of 1.83) (average ratio of 2.52 for *T. sherwoodiae*).

Holotype. On dead twigs of *Rhododendron* sp., CHINA, Hubei Province, Shennongjia National Nature Reserve, 31.4360 N; 110.3014 E, alt. ca. 2900 m, 23 July 2018, C.-L. HOU1350A (BJTC 201908).

Description. Ascomata erumpent from the bark, circular or rectangular in outline, 1.3–2.0 mm diam., solitary or occasionally confluent, with a black (#211414) outer surface that is sculptured with polygonal areolae, opening by irregular splits to expose a yellow (#ffc14f) hymenium. In median vertical section, ascomata 500–600 µm thick. Covering stroma 270–300 µm thick near the central part of ascomata, decreasing to 65–110 µm at the edge, consisting of an outer layer of highly melanized hyphae with a few remnants of host tissue embedded in the surface and an inner layer of hyaline hyphae. Basal layer 65–160 µm thick, composed of highly melanized hyphae with hyaline hyphae towards the internal matrix of stroma that is 75–125 µm thick, composed of textura intricata. Subhymenium 45–75 µm thick consisting of small, irregular textura angularis. Excipulum absent. Paraphyses 200–230 × ca. 1 µm, filiform, multi-guttulate, guttulae visible in water and IKI but disappearing in both lactophenol solution and 5% KOH, not swollen and branched at the apex, extending past mature asci. Asci ripening sequentially, 160–200 × 15–24 µm, cylindrical, thin-walled, without circumapical thickening, rounded at the apex, 6–8-spored. Ascospores 20–30 × 12–18 µm, L/W ratio of 1.4–2.3 (average ratio of 1.83), ellipsoidal, often curved homolateral, hyaline, at first aseptate, becoming muriform at maturity, with 6–8 transverse septa and a few longitudinal and oblique septa, without a gelatinous sheath, inamyloid in IKI.

Conidiomata and zone lines not seen.

Known distribution. Known from a single collection from Shennongjia National Nature Reserve, Hubei Province, China.

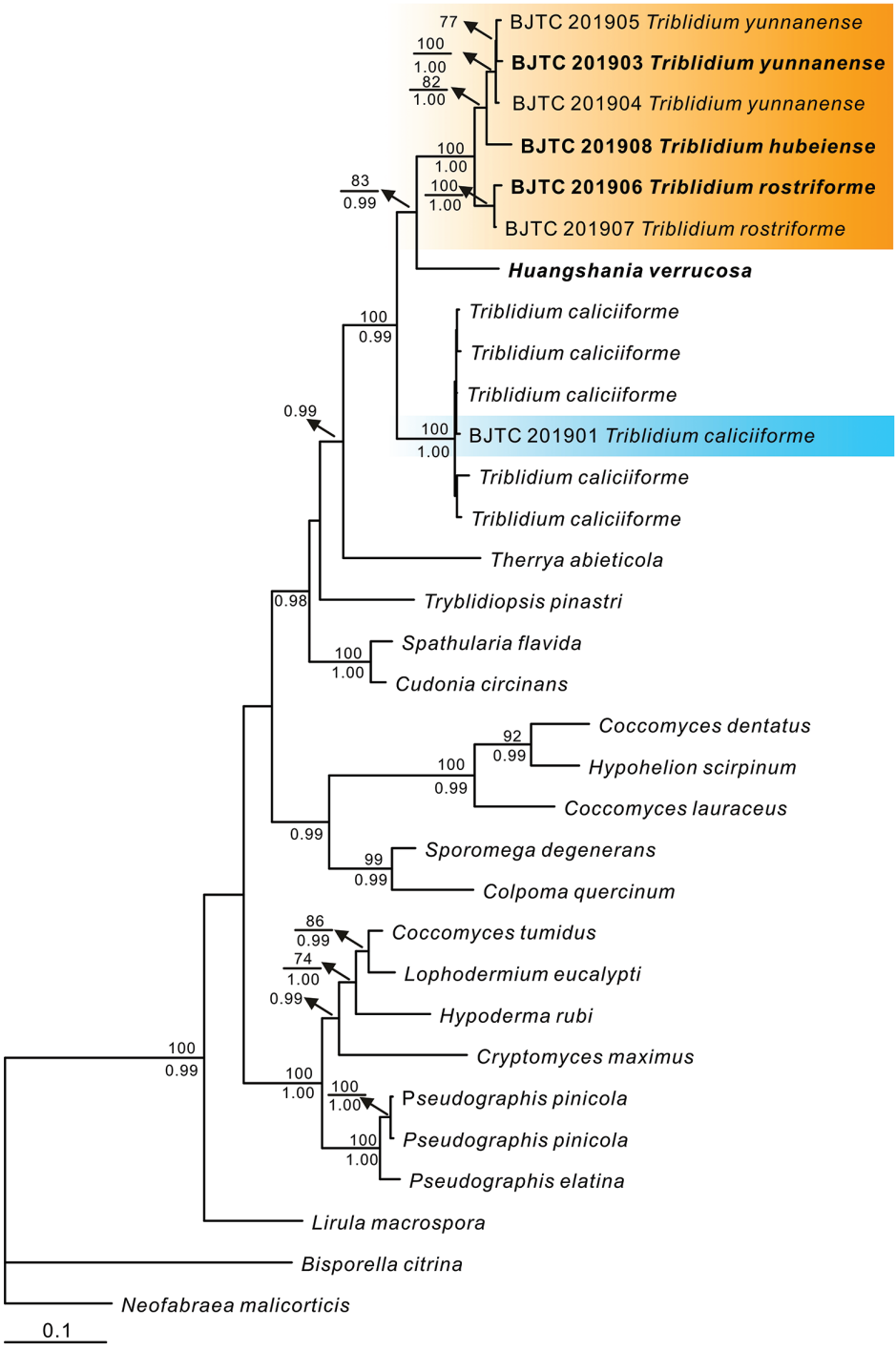

Figure 1. A phylogenetic tree generated by maximum parsimony and Bayesian analysis of the combined ITS, LSU, mtSSU and RPB2 sequences, using *B. citrina* and *N. malicorticis* as outgroups. Bootstrap values of maximum parsimony ≥ 70% are shown above the respective branches. Bayesian posterior probabilities ≥ 0.95 are marked below the branches. Sequences in bold indicate that the sequences are from the holotypes.

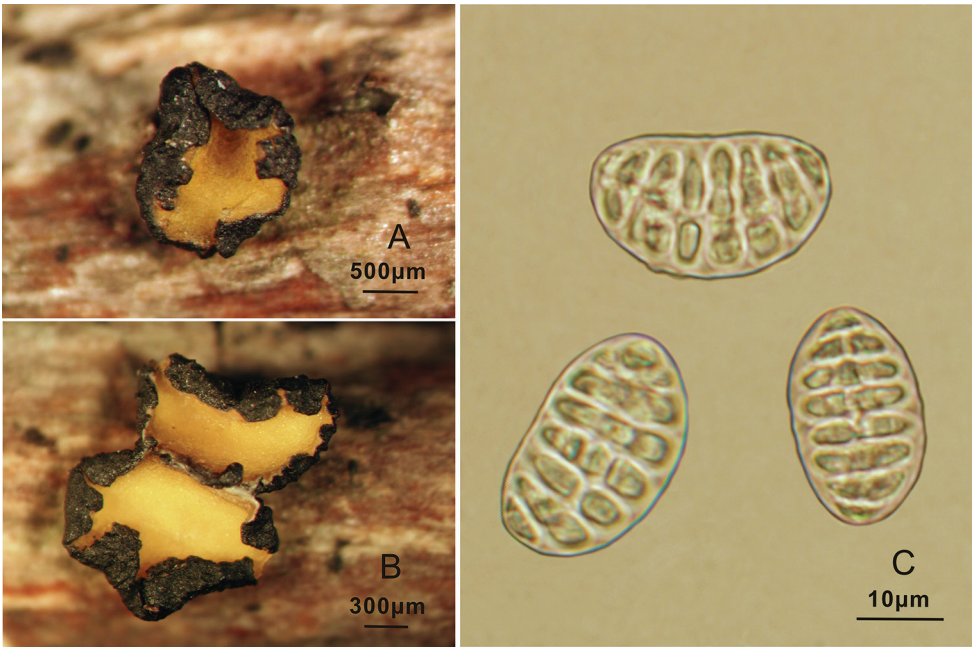

Figure 2. *Triblidium hubeiense* (Holotype, BJTC 201908) on *Rhododendron* sp. twig **A, B** mature dried ascomata observed under dissecting microscope **C** dead ascospores in water.

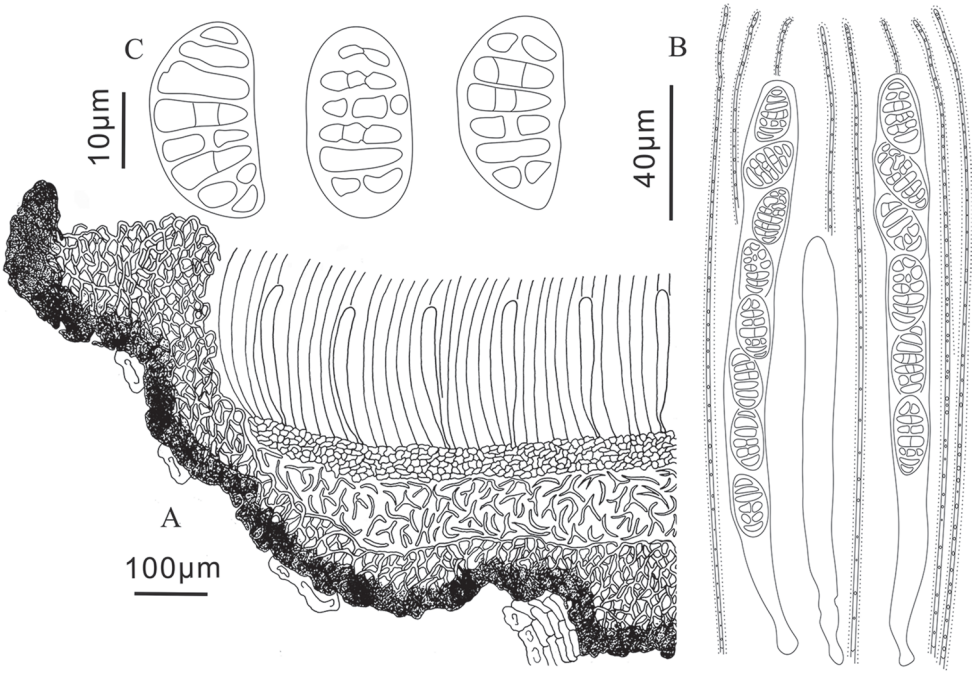

Figure 3. *Triblidium hubeiense* (Holotype, BJTC 201908) **A** ascoma in median vertical section **B** paraphyses, mature asci with ascospores and immature ascus **C** dead ascospores in water.

Etymology. Referring to the Hubei Province where the specimen was collected.

Comments. *Triblidium hubeiense* is similar to *T. sherwoodiae* Magnes and *T. carestiae* (De Not.) Rehm, but *T. sherwoodiae* has paraphyses with swollen terminal cell, straight ascospores and is only found on *Pinus ponderosa*; *T. carestiae* commonly has 3–8 ascospores per ascus, ascospores usually with beak-like structure at poles, 7–14 transverse septa and apically branched paraphyses.

Triblidium rostriforme **T. Lv & C. L. Hou, sp. nov.**
MycoBank No: 832359
Figs 4, 5

Diagnosis. Different from most *Triblidium* species by producing longer ascospores that have rostriform structures at the poles.

Holotype. On dead twigs of *Rhododendron* sp., CHINA, Yunnan Province, Lijiang, Laojunshan, 26.6831 N; 99.6997 E, alt. ca. 4056 m, 25 June 2011, C.-L. HOU 889 (BJTC 201906).

Description. Ascomata erumpent from bark, elliptical in outline, 0.85–1.7 mm diam., solitary, with a black (#211414) outer surface that is sculptured with polygonal areolae, opening by irregular splits to expose the hymenium. In median vertical section, ascomata 350–550 μm thick. Covering stroma 45–70 μm thick, consisting of an outer layer of highly melanized hyphae with some host tissues incorporated into the surface and an inner layer of hyaline hyphae. Basal layer 40–80 μm thick, composed of a lower, highly melanized layer with hyaline hyphae towards the internal matrix of the stroma which is 40–98 μm thick, composed of textura intricata. Subhymenium 25–45 μm thick, consisting of hyaline textura angularis. Excipulum moderately developed, formed by marginal paraphyses. Paraphyses 180–240 × ca. 1 μm, filiform, occasionally branched, sparsely guttulate, guttulae visible in water and IKI but disappearing in both lactophenol solution and 5% KOH. Asci ripening sequentially, 160–220 × 15–25 μm, cylindrical, thin-walled, without circumapical thickening, rounded at the apex, 8-spored. Ascospores 35–50 × 12–20 μm, L/W ratio of 2.0–3.8 (average ratio of 2.55), elliptical, with rostriform structures at the poles, hyaline, at first aseptate, becoming muriform at maturity, with usually 6 transverse septa and a few longitudinal and oblique septa, without gelatinous sheath, inamyloid in IKI.

Conidiomata and zone lines not seen.

Etymology. From Latin, *rostriforme*, referring to the beak-like protrusions observed at the ascospore poles.

Additional specimen examined. On dead twigs of *Rhododendron* sp., CHINA, Yunnan Province, Lijiang, Laojunshan, 26.6702 N; 99.7002 E, alt. ca. 4110 m, 24 June 2011, C.-L. HOU 851A (BJTC 201907).

Comments. *Triblidium rostriforme* is similar to *T. carestiae* (De Not.) Rehm but *T. carestiae* commonly has asci with 3–8 ascospores, ascospores with usually 7–14 transverse septa and ramose, multi-guttulate paraphyses.

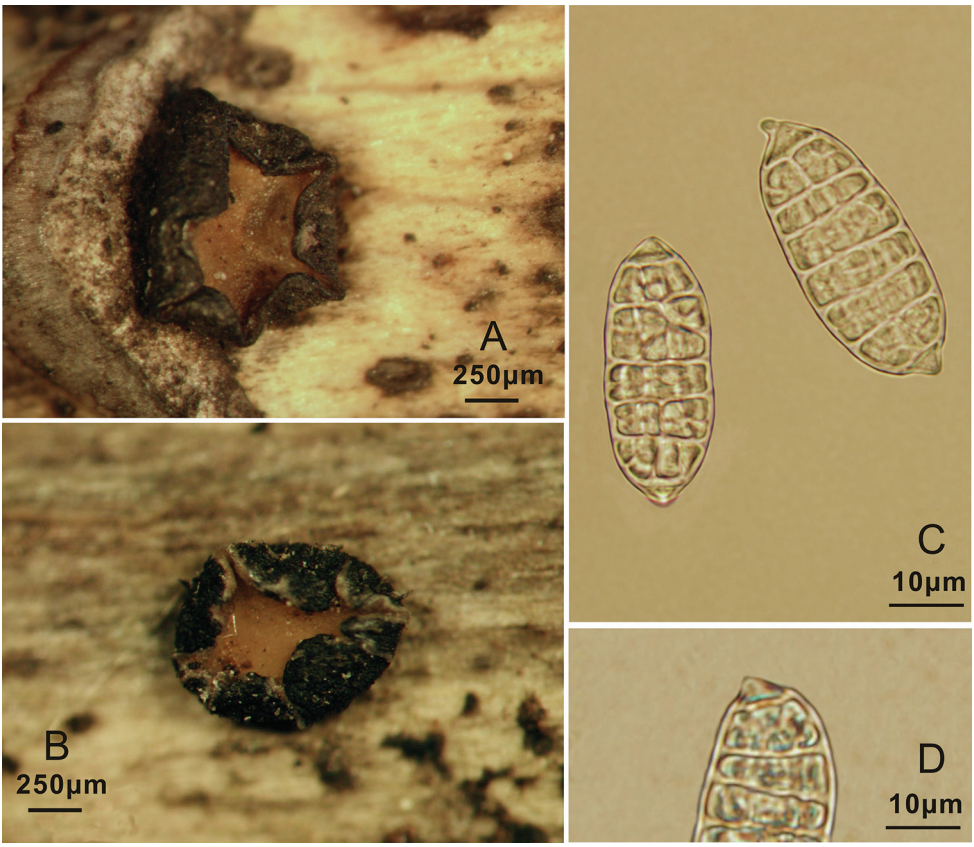

Figure 4. *Triblidium rostriforme* (Holotype, BJTC 201906) on *Rhododendron* sp. twig **A, B** mature dried ascomata observed under a dissecting microscope **C** dead ascospores in water **D** rostriform structure of ascospores.

***Triblidium yunnanense* T. Lv & C. L. Hou, sp. nov.**
MycoBank No: 832360
Figs 6, 7

Diagnosis. Different from *T. hafellneri* by its ascospores with 6–8 transverse septa, narrow asci and geographical range. Different from its phylogenetically closest relatives (*T. hubeiense* and *T. rostriforme*) by the size and the shape of ascomata and ascospores.

Holotype. On twigs of *Rhododendron* sp., CHINA, Yunnan Province, Lijiang, Laojunshan, 26.6571 N; 99.6944 E, alt. ca. 4070 m, 25 June 2011, C.-L. HOU 875A (BJTC 201903).

Description. Ascomata erumpent from bark, circular or slightly irregular in outline, 0.5–0.8 mm diam., solitary, with a black (#211414) outer surface that is sculptured with polygonal areolae, opening by irregular splits to expose the hymenium. In median vertical section, ascomata 300–400 μm thick. Covering stroma 45–75 μm, consisting of an outer layer of highly melanized hyphae with remnants of host tis-

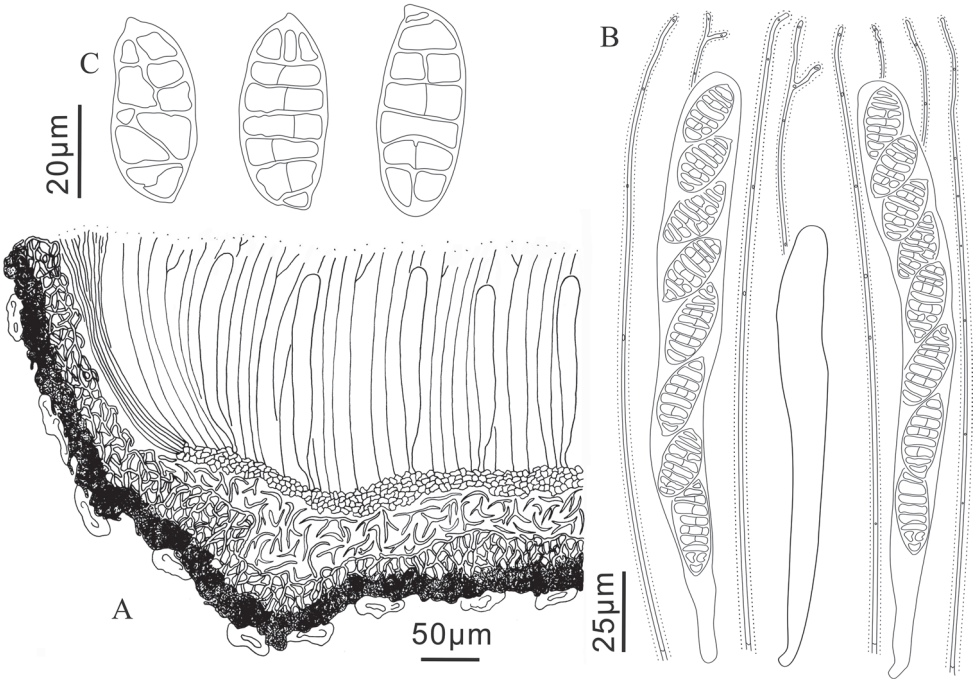

Figure 5. *Triblidium rostriforme* (Holotype, BJTC 201906) **A** ascoma in median vertical section **B** paraphyses, mature asci with ascospores and immature ascus **C** dead ascospores in water.

sue incorporated into the outer surface and an inner layer of hyaline hyphae. Basal layer 45–88 μm thick, composed of an outer layer of highly melanized hyphae and short, thick, hyaline hyphae towards the internal matrix of stroma that is 60–85 μm thick, composed of thick hyphae. Subhymenium 35–59 μm thick, consisting of hyaline textura angularis. Excipulum 25–35 μm thick, formed by marginal paraphyses. Paraphyses 180–230 × 1–1.2 μm, filiform, often branched, multi-guttulate, guttulae visible in water and IKI but disappearing in both lactophenol solution and 5% KOH. Asci ripening sequentially, 150–200 × 13–18 μm, cylindrical, thin-walled, without circumapical thickening, rounded at the apex, 6–8-spored. Ascospores 20–30 × 10–15 μm, L/W ratio of 1.7–2.5 (average ratio of 1.99), ellipsoid, hyaline, at first aseptate, becoming muriform at maturity, with usually 6–8 transverse septa and a few longitudinal and oblique septa, without gelatinous sheath, inamyloid in IKI.

Conidiomata and zone lines not seen.

Etymology. Referring to the Yunnan Province where the holotype specimens were collected.

Additional specimens examined. On twigs of *Rhododendron* sp., CHINA, Yunnan Province, Lijiang, Laojunshan, 26.6741 N; 99.6930 E, alt. ca. 4040 m, 11 July 2007, C.-L. HOU 470A (BJTC 201904). On dead twigs of *Rhododendron* sp., CHINA, Sichuan Province, Mt. Emeishan, 29.5185 N; 103.3329 E, alt. ca. 3010 m, 12 July 2014, C.-L. HOU 1179 (BJTC 201905).

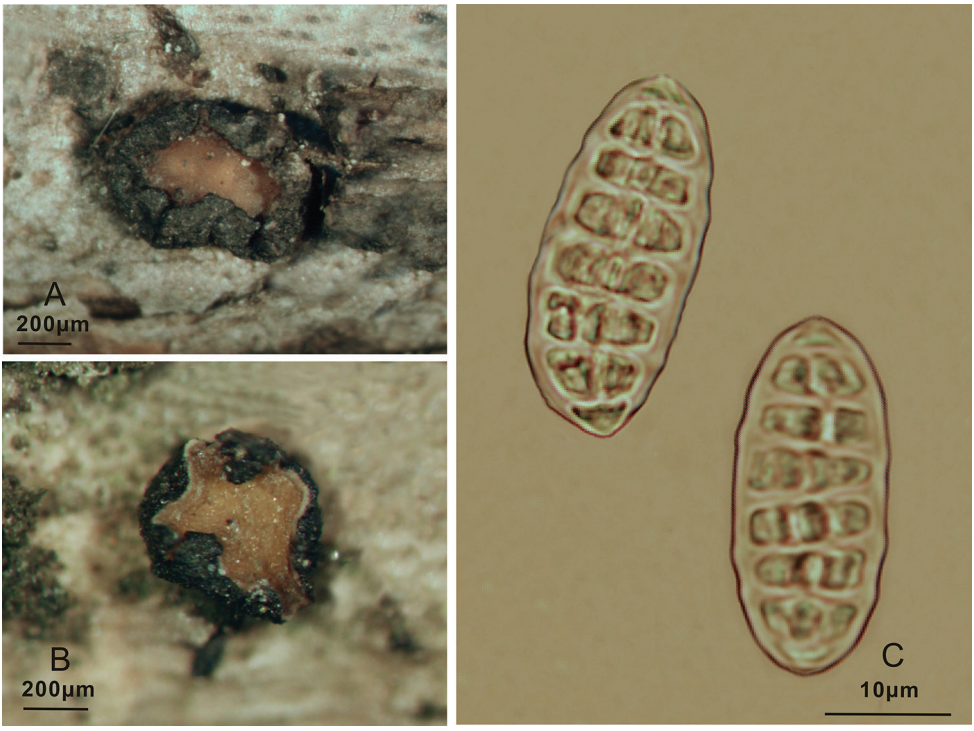

Figure 6. *Triblidium yunnanense* (Holotype, BJTC 201903) on *Rhododendron* sp. twig **A, B** mature dried ascomata observed under a dissecting microscope **C** dead ascospores in water.

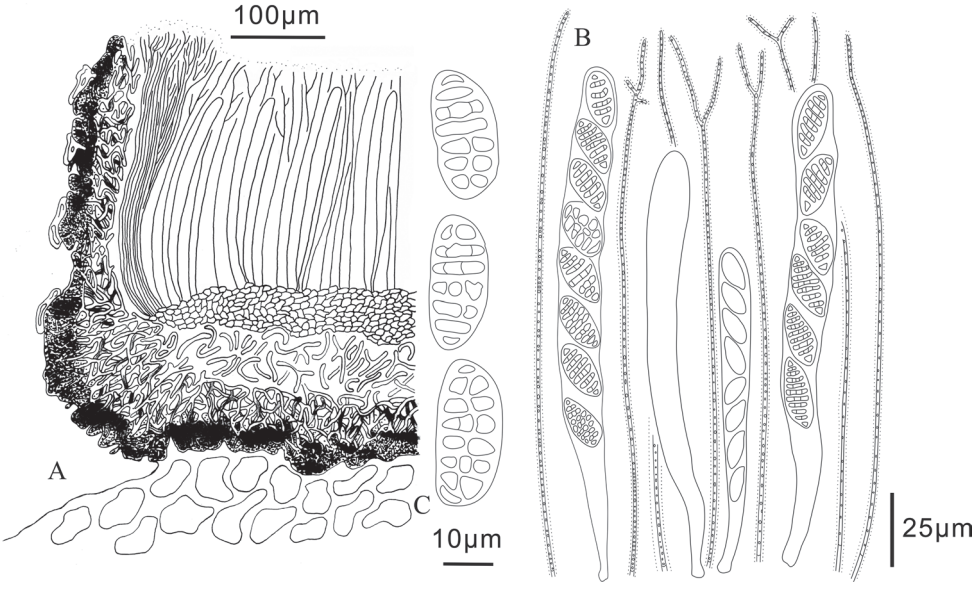

Figure 7. *Triblidium yunnanense* (Holotype, BJTC 201903) **A** ascoma in median vertical section **B** paraphyses, mature asci with ascospores and immature ascus **C** dead ascospores in water.

Comments. *Triblidium yunnanense* is similar to *T. hafellneri* Magnes, but the latter has asci 20–25 µm wide, ascospores with 7 transverse septa and occurs on *Vaccinum ovatum*, *Calluna vuglaris*, *Salix* spp., and *Nothofagus antarctica* in Europe and the Americas. *Triblidium yunnanense* has a close relationship to the two other new species in this study, but *T. rostriforme* has larger ascomata, ascospores with special beak-like structures and *T. hubeiense* has larger ascomata, unbranched paraphyses, a moderately developed excipulum, a thicker covering stroma, basal layer and subhymenium.

Triblidium verrucosum **(O.E. Erikss.) T. Lv, C. L. Hou & P. R. Johnst., comb. nov.**
MycoBank No: 832361

≡ *Huangshania verrucosa* O.E. Erikss., Systema Ascomycetum 11: 2, 1992.

Notes. The placement of this species in *Triblidium* is demonstrated by the phylogeny presented in Fig. 1. Eriksson (1992) discussed the similarities between *Huangshania* and *Triblidium* in macro-morphology and in the morphology of hamathecial tissues and asci. The two genera differed only in ascospore morphology (elongate-phragmosporous vs. ellipsoidal-muriform). Karakehian et al. (2019) reviewed that ascospore morphology is a poor predictor of relatedness amongst these fungi. *Huangshania verrucosa* is the type species of the genus, therefore, *Huangshania*, is synonymized here under *Triblidium*.

Discussion

The morphological characteristics of the species described here are typical of *Triblidium* (Magnes 1997): ascomata on twigs of *Rhododendron* spp., muriform, inamyloid ascospores, and highly melanized ascomata with roughened outer surfaces. Based on our molecular phylogenetic analyses (Fig. 1), the three newly described species form a highly supported clade, sister to *T. verrucosum*. *Triblidium yunnanense* and *T. hubeiense* form a well-supported subclade sister to *T. rostriforme*. The similarity of ITS amongst these three new species is 90–95%. The sequences generated from the specimens of *T. caliciiforme* collected from France on bark of *Quercus* sp., clustered well with other sequences accepted as *T. caliciiforme* by Karakehian et al. (2019).

The strongly supported phylogenetic relationship justifying the synonymy of *Huangshania* with *Triblidium* was not detected by Karakehian et al. (2019) because only the type species of *Triblidium* had DNA sequences available. If *Huangshania* is not placed in synonymy, the addition of the new Chinese *Triblidium* species described here would result in *Triblidium* being paraphyletic. The alternative solution, to erect a new genus for the Chinese species, has no morphological support, since these species are very similar to *T. caliciiforme* in both morphology and ecology. In 1992, Eriksson erected *Huangshania* as a genus only according to the morphology of the spores. Karakehian et al. (2019) examined the ascospores of *H. verrucosa* and noted

that ascospore morphology appears to be a poor predictor of phylogenetic relationships amongst these fungi. It is worth noting that the rostrum of the ascospores in *T. rostriforme* and *T. carestiae* bear some similarity to the plug-like appendages of *H. verrucosa*. Furthermore, we did not transfer *H. novae-fundlandiae* (Rehm) Magnes, another species in *Huangshania*, to *Triblidium* since sequences were lacking.

In conclusion, three new *Triblidium* species from China were described in detail by both morphological and phylogenetic analyses. The new species, discovered in China, illustrate that these fungi are more widespread than previously known. Sequences from these new collections have expanded the representation of this genus in NCBI GenBank and helped our understanding of the family Triblidiaceae. *Huangshania* is placed in synonymy with *Triblidium* in order to maintain its monophyly, further demonstrating that ascospore morphology alone may be a poor predictor of evolutionary relationships.

Key to species of *Triblidium*

1 Ascospores phragmosporous ..... ***T. verrucosum***
– Ascospores muriform **2**
2 Ascospores ellipsoid, without rostriform beaks at the poles **3**
– Ascospores ellipsoid with rostriform beaks at the poles..... **4**
3 Ascomata ≥1mm diam **5**
– Ascomata <1mm diam **6**
4 Paraphyses multi-guttulate, often branched at the apex; thick-walled asci with 3–8 ascospores; ascospores with 7–14 transverse septa ..... ***T. carestiae***
– Paraphyses sparsely guttulate, occasionally branched at the apex; asci thin-walled with 8-ascospores; ascospores with 6 transverse septa.... ***T. rostriforme***
5 Asci 20–25 µm wide; ascospores with 7 transverse septa; occurring on *Vaccinum ovatum*, *Calluna vuglaris*, *Salix* spp. *and Nothofagus antarctica* .... ***T. hafellneri***
– Asci 13–18 µm wide; ascospores with 6–8 transverse septa; only found on *Rhododendron* sp. ..... ***T. yunnanense***
6 Occurring mainly on *Fagaceae* spp. and *Pinus* spp..... **7**
– Occurring mainly on *Rhododendron* spp., asci 160–200 × 15–24 µm, ascospores 20–30 × 12–18 µm..... ***T. hubeiense***
7 Asci 230–280 × 25–30 µm, ascospores 30–48 × 12–20 µm.... ***T. caliciiforme***
– Asci 150–190 × 13–23 µm, ascospores 28–35 × 11–14 µm.... ***T. sherwoodiae***

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Taxonomy of two synnematal fungal species from *Rhus chinensis*, with *Flavignomonia* gen. nov. described

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Abstract

Rhus chinensis represents a commercially and ecologically important tree species in China, but suffers from canker diseases in Jiangxi Province. Synnemata, pycnidia and ascomata were discovered on cankered tissues. Strains were obtained from single ascospore or conidium within the fruiting bodies and identified based on morphological comparison and the phylogenetic analyses of partial ITS, LSU, *tef1* and *rpb2* gene sequences. As a result, two species were confirmed to represent two kinds of synnemata. One of these species is described herein as *Flavignomonia rhoigena* **gen. et sp. nov.**; and *Synnemasporella aculeans* is illustrated showing ascomata, pycnidia and synnemata. *Flavignomonia* is distinguished from *Synnemasporella* by the colour of the synnematal tips. Additionally, *Flavignomonia* can be distinguished from the other gnomoniaceous genera by the formation of synnemata.

Keywords

Diaporthales, Gnomoniaceae, systematics, taxonomy

Introduction

Many Diaporthales species are important branch canker pathogens, forming acervuli or pycnidia on diseased tissues (Rossman et al. 2007, Senanayake et al. 2017, Jiang et al. 2018, 2019, Wijayawardene et al. 2018, Yang et al. 2018, Voglmayr et al. 2019).

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However, two diaporthalean species with synnemata were reported to cause cankers, namely *Synnemasporella aculeans* (syn. *Cryptodiaporthe aculeans*) and *S. toxicodendri* (Fan et al. 2018). These two species differ from the other diaporthalean taxa in conidiomata and form a distinct clade phylogenetically, which was named Synnemasporellaceae and distinguished by Fan et al. (2018).

Gnomoniaceae was initially introduced with *Gnomonia* as the type (Winter 1886). Species in Gnomoniaceae formed upright perithecia, with or without long or short necks and presence or absence of stromatic tissues (Barr 1978, Sogonov et al. 2008, Walker et al. 2012). In the recent monograph of Diaporthales, 34 genera were accepted in the family Gnomoniaceae (Senanayake et al. 2018). Subsequently, *Neognomoniopsis* and *Tenuignomonia* were added based on both molecular and morphological evidence (Crous et al. 2019, Minoshima et al. 2019).

Chinese gall (*Rhus chinensis* Mill.) has a range of uses as source of medicine, dye and oil, and has a wide distribution in China (Wang et al. 2014). However, cankers were found to be associated with different ascomata during our fungal collection trips in Jiangxi Province, China. The objectives of the present study were to identify these fungi based on morphological and phylogenetic evidence.

Materials and methods

Sample collections and isolation

We conducted our fungal collection surveys from April to October in China, and found *Rhus chinensis* to be one of the major tree species in Jiangxi Province. Twigs, branches and stems were carefully checked, and diseased tissues were cut into small pieces and packed in paper bags. Isolates were obtained by transferring the ascospores or conidial masses from ascomata to sterile PDA plates, incubating at 25 °C until spores germinated. Single germinating spores were transferred onto new PDA plates, which were kept at 25 °C in darkness. Specimens were deposited in the Museum of the Beijing Forestry University (**BJFC**) and axenic cultures maintained in the China Forestry Culture Collection Centre (**CFCC**).

Morphological analysis

Recognition and identification of the fungal species on *Rhus chinensis* was based on fruiting bodies formed on the bark. Ascomata and conidiomata were sectioned by hand using a double-edged blade, and microscopic structures were observed under a dissecting microscope. At least 10 conidiomata/ascomata, 10 asci, and 50 conidia/ascospores were measured to calculate mean and standard deviation. Measurements are reported as maxima and minima in parentheses and the range representing the mean plus and minus the standard deviation of the number of measurements given in parentheses (Voglmayr et al. 2017). Microscopy photographs were captured with a Nikon Eclipse

80i compound microscope equipped with a Nikon digital sight DS-Ri2 high definition colour camera, using differential interference contrast illumination. Nomenclatural novelties and descriptions were deposited in MycoBank (Crous et al. 2004).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from colonies grown on cellophane-covered PDA plates using a modified CTAB method (Doyle and Doyle 1990). PCR amplifications were performed in a DNA Engine Peltier Thermal Cycler (PTC-200; Bio-Rad Laboratories, Hercules, CA, USA). The primer sets ITS1/ITS4 (White et al. 1990) were used to amplify the ITS region. The primer pair LR0R/LR5 (Vilgalys and Hester 1990) was used to amplify the LSU region. The primer pairs EF1-688F/EF1-986R or EF1-728F/TEF1-LLErev (Carbone and Kohn 1999; Jaklitsch et al. 2005; Alves et al. 2008) were used to amplify *tef1* gene. The primer pair dRPB2-5f/dRPB2-7r (Voglmayr et al. 2016) was used to amplify the *rpb2* gene. The polymerase chain reaction (PCR) assay was conducted as described by Fan et al. (2018). PCR amplification products were assayed via electrophoresis in 2 % agarose gels. DNA sequencing was performed using an ABI PRISM 3730XL DNA Analyzer with a BigDye Terminater Kit v.3.1 (Invitrogen, USA) at the Shanghai Invitrogen Biological Technology Company Limited (Beijing, China).

Phylogenetic analyses

The preliminary identities of the isolates sequenced in this study were obtained by conducting a standard nucleotide BLAST search using the sequences generated from the above primers of the different genomic regions (ITS, LSU, *tef1* and *rpb2*). The BLAST results showed that three isolates were grouped in the family Gnomoniaceae, and five isolates in the genus *Synnemasporella*. The phylogenetic analyses for the three gnomoniaceous isolates were conducted based on Senanayake et al. (2018), supplemented by sequences of *Tenuignomonia styracis* and *Neognomoniopsis quercina* from Crous et al. (2019) and Minoshima et al. (2019). *Melanconis marginalis* (CBS 109744) in Melanconidaceae was selected as the out-group taxon. All sequences were aligned using MAFFT v. 6 (Katoh and Toh 2010) and edited manually using MEGA v. 6 (Tamura et al. 2013). Phylogenetic analyses were performed using PAUP v. 4.0b10 for maximum parsimony (MP) analysis (Swofford 2003), and PhyML v. 3.0 for Maximum Likelihood (ML) analysis (Guindon et al. 2010).

MP analysis was run using a heuristic search option of 1000 search replicates with random additions of sequences with a tree bisection and reconnection algorithm. Other calculated parsimony scores were tree length (TL), consistency index (CI), retention index (RI), and rescaled consistency (RC). ML analysis was performed using a GTR site substitution model including a gamma-distributed rate heterogeneity and a proportion of invariant sites (Guindon et al. 2010). The branch support was evaluated using a bootstrapping method of 1000 replicates (Hillis and Bull 1993). The matrix

was partitioned for the different gene regions. Phylograms were shown using FigTree v. 1.4.3 (Rambaut 2016). Novel sequences generated in the current study were deposited in GenBank (Table 1) and the aligned matrices used for phylogenetic analyses in TreeBASE (accession number: S25047).

Results

Phylogenetic analyses

The alignment based on the combined sequence dataset (ITS, LSU, *tef1*, and *rpb2*) included 42 in-group taxa and one out-group taxon, comprising 3368 characters in the aligned matrix. Of these, 2201 characters were constant, 224 variable characters were parsimony-uninformative and 943 characters were parsimony informative (282 from the ITS-LSU, 280 from *tef1*, 381 from *rpb2*). The MP analysis resulted in nine equally most parsimonious trees with identical tree backbone. The best ML tree (lnL = –20604.0384) was compatible with the MP strict consensus tree, except for unsupported clades in Fig. 1. As the trees obtained from different analytical methods were similar, only the ML tree was present in Fig. 1. The phylogram based on the four gene sequence matrix indicated that the three strains from the present study represent a novel genus in Gnomoniaceae.

Taxonomy

Flavignomonia **C.M. Tian, Q. Yang & N. Jiang, gen. nov.**
MycoBank No: 829530

Diagnosis. *Flavignomonia* is distinguished from *Synnemasporella* by the orange tips of its synnemata.

Type species. *Flavignomonia rhoigena* C.M. Tian & Q. Yang

Etymology. The generic name is derived from the colour of synnemata (flavus = yellow) and the genus name *Gnomonia*.

Description. Sexual morph: not observed. Asexual morph: Conidiomata synnematal. Synnemata long and determinate, growing from host tissue, with brown base and orange tip, straight to curved, parallel, with flat to slightly concave and dark zone of conidiogenous cells and host tissue at their bases. Conidiophores reduced to conidiogenous cells. Conidiogenous cells phialidic, aggregated, hyaline, straight to curved, cylindrical, arranged adjacent to one another at the end of the synnema, producing a single conidium. Conidia cylindrical to oblong, smooth, multiguttulate, hyaline.

Notes. *Flavignomonia* is included in Gnomoniaceae based on DNA sequences data. *Flavignomonia* is morphologically similar to *Synnemasporella* in forming synne-

Table 1. Strains used in the phylogenetic tree and their culture accession and GenBank numbers. Strains from this study are in bold.

Species	Strains	GenBank numbers			
		ITS	LSU	*tef1*	*rpb2*
Alnecium auctum	CBS 124263	KF570154	KF570154	KF570200	KF570170
Ambarignomonia petiolorum	CBS 116866	EU199193	AY818963	NA	EU199151
	CBS 121227	EU254748	EU255070	EU221898	EU219307
Amphiporthe tiliae	CBS 119289	EU199178	EU199122	NA	EU199137
Anisogramma anomala	529478	EU683064	EU683066	NA	NA
Anisogramma virgultorum	529479	EU683062	EU683065	NA	NA
Apiognomonia errabunda	AR 2813	DQ313525	NG027592	DQ313565	DQ862014
Apiognomonia veneta	MFLUCC 16-1193	MF190114	MF190056	NA	NA
Apioplagiostoma populi	858501	KP637024	NA	NA	NA
Asteroma alneum	CBS 109840	EU167609	EU167609	NA	NA
Asteroma sp.	Masuya 8Ah9-1	NA	AB669035	NA	NA
Cryptosporella hypodermia	CBS 116866	EU199181	AF408346	NA	EU199140
Discula destructiva	MD 254	AF429741	AF429721	AF429732	NA
Ditopella biseptata	MFLU 15-2661	MF190147	MF190091	NA	MF377616
Ditopella ditopa	CBS 109748	DQ323526	EU199126	NA	EU199145
Ditopellopsis sp.	CBS 121471	EU254763	EU255088	EU221936	EU219254
Flavignomonia rhoigena	**CFCC 53118**	**MK432674**	**MK429917**	NA	**MK578102**
	CFCC 53119	**MK432675**	**MK429918**	NA	**MK578103**
	CFCC 53120	**MK432676**	**MK429919**	NA	**MK578104**
Gnomonia gnomon	CBS 199.53	DQ491518	AF408361	EU221885	EU219295
	CBS 829.79	AY818957	AY818964	EU221905	NA
Gnomoniopsis alderdunensis	CBS 125680	GU320825	NA	NA	NA
Gnomoniopsis chamaemori	CBS 803.79	EU254808	EU255107	NA	NA
Gnomoniopsis racemula	AR 3892	EU254841	EU255122	EU221889	EU219241
Mamianiella coryli	BPI 877578	EU254862	NA	NA	NA
Marsupiomyces quercina	MFLUCC 13-0664	MF190116	MF190061	NA	NA
Marsupiomyces epidermoidea	MFLU 15-2921	NA	MF190058	NA	NA
Melanconis marginalis	CBS 109744	EU199197	AF408373	EU221991	EU219301
Neognomoniopsis quercina	CBS 145575	MK876399	MK876440	NA	NA
Occultocarpon ailaoshanense	LCM 524.01	JF779849	JF779853	NA	JF779856
	LCM 522.01	JF779848	JF779852	JF779862	JF779857
Ophiognomonia melanostyla	LCM 389.01	JF779850	JF779854	NA	JF779858
Ophiognomonia vasiljevae	AR 4298	EU254977	EU255162	EU221999	EU219331
Plagiostoma aesculi	AR 3640	EU254994	EU255164	NA	EU219269
Linospora capreae	CBS 372.69	NA	AF277143	NA	NA
Pleuroceras oregonense	AR 4333	EU255060	EU255196	EU221931	EU219313
Pleuroceras pleurostylum	CBS 906.79	EU255061	EU255197	EU221962	EU219311
Phragmoporthe conformis	AR 3632	NA	AF408377	NA	NA
Valsalnicola oxystoma	AR 5137	JX519561	NA	NA	NA
	AR 4833	JX519559	JX519563	NA	NA
Sirococcus tsugae	AR 4010	EF512478	EU255207	EU221928	EU219289
	CBS 119626	EU199203	EU199136	EF512534	EU199159
Synnemasporella aculeans	CFCC 52094	MG682086	MG682026	MG682066	MG682046
	CFCC 53123	**MK432679**	**MK429920**	**MK578148**	**MK578105**
	CFCC 53124	**MK432680**	**MK429921**	**MK578149**	**MK578106**
Synnemasporella aculeans	**CFCC 53125**	**MK432681**	**MK429922**	**MK578150**	**MK578107**
	CFCC 53126	**MK432682**	**MK429923**	**MK578151**	**MK578108**
	CFCC 53127	**MK432683**	**MK429924**	**MK578152**	**MK578109**
Synnemasporella toxicodendri	CFCC 52097	MG682089	MG682029	MG682069	MG682049
Tenuignomonia styracis	BPI 89278	NA	LC379288	LC379282	LC379294

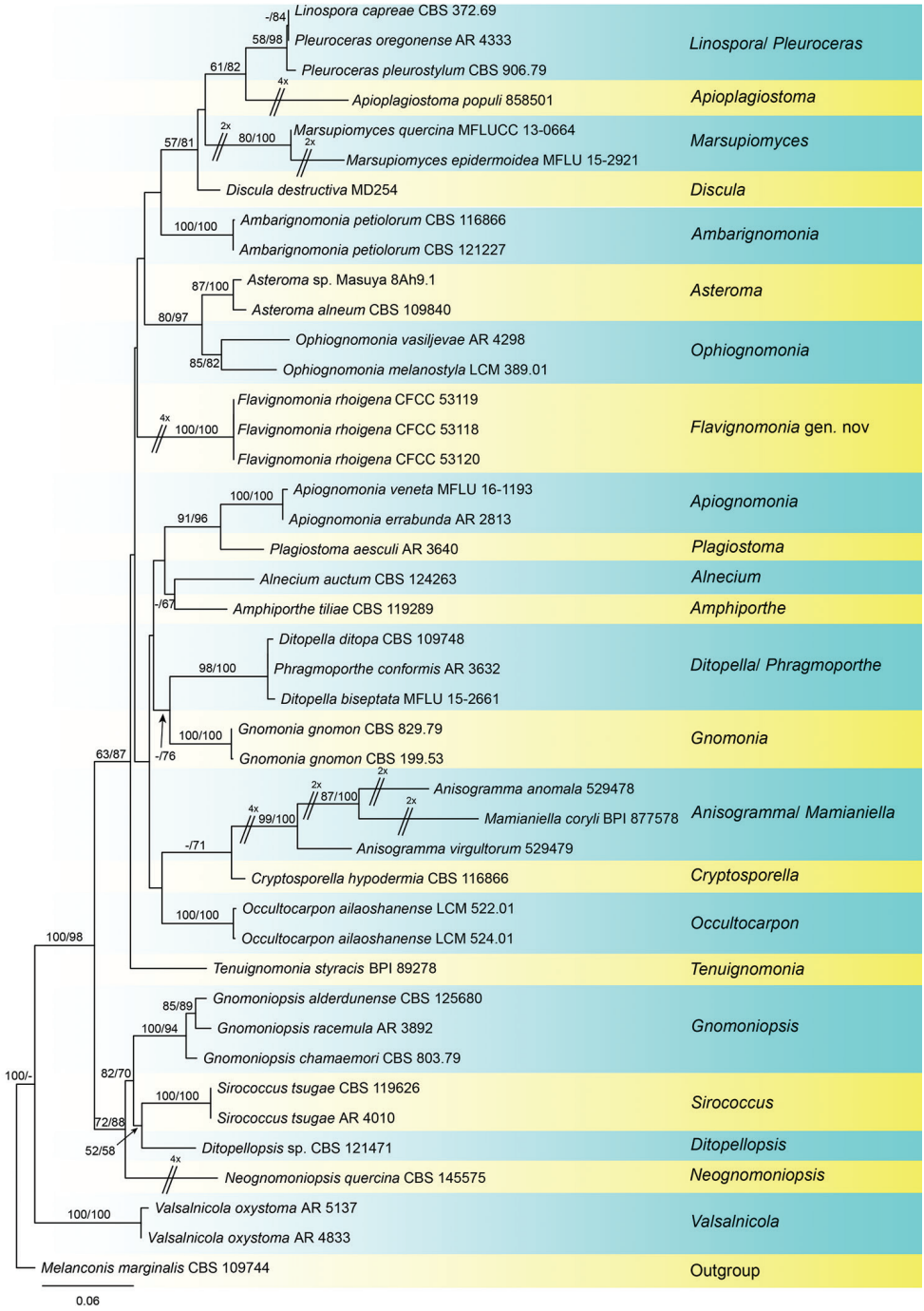

Figure 1. Phylogenetic tree based on an ML analysis of a combined DNA dataset of ITS, LSU, *tef1* and *rpb2* gene sequences for all genera with DNA data and some species of Gnomoniaceae. Bootstrap values ≥ 50 % for MP and ML analyses are presented at the branches. The scale bar represents the number of changes per site.

mata (Wehmeyer 1933, Fan et al. 2018). However, *Flavignomonia*, typified with *Flavignomonia rhoigena*, is distinguished from *Synnemasporella* species by its orange synnematal tips and hyaline conidia (Fan et al. 2018).

***Flavignomonia rhoigena* C.M. Tian & Q. Yang, sp. nov.**
Figure 2
MycoBank No: 829531

Diagnosis. *Flavignomonia rhoigena* can be distinguished from other gnomoniaceous species by the formation of synnemata.

Etymology. Named after the host genus, *Rhus*.

Description. Sexual morph: not observed. Asexual morph: Conidiomata synnematal. Synnemata (650–)750–1100 µm high, 150–300 µm diam, determinate, growing from host tissue, with brown base and orange tip, straight to curved, parallel, with flat to

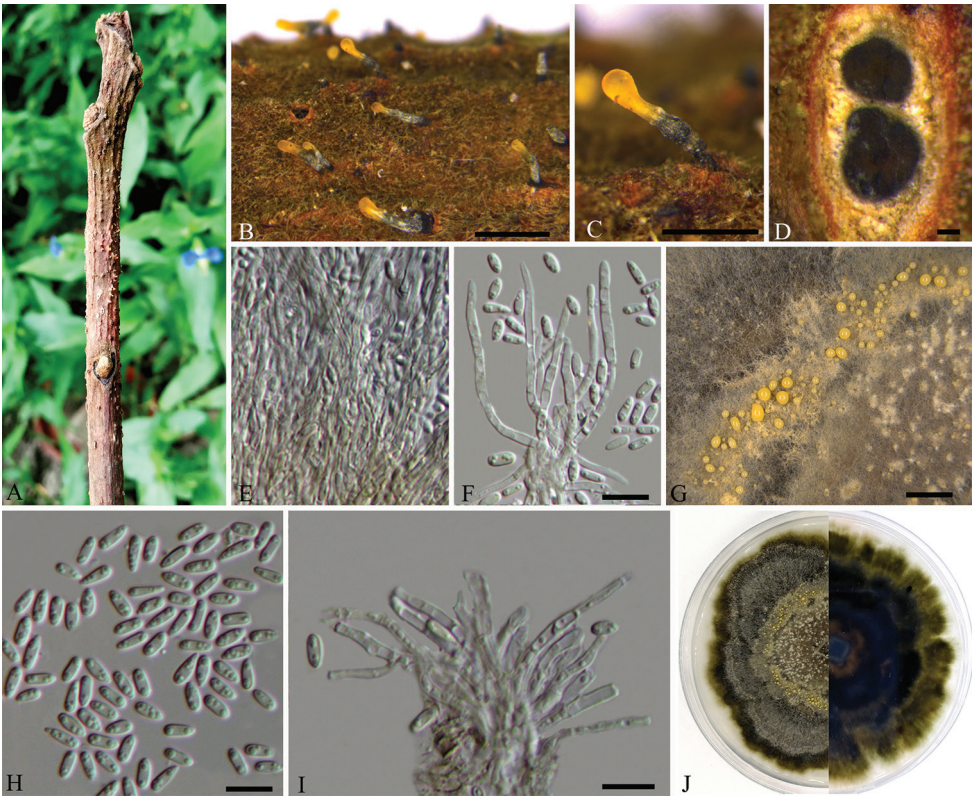

Figure 2. *Flavignomonia rhoigena* on *Rhus chinensis* (BJFC-S1766, holotype) **A–C** habit of conidiomata on twigs **D** transverse section through synnema **E** longitudinal section through synnema **F, I** conidiogenous cells attached with conidia **G** conidiomata on PDA **H** condia **J** the colony on PDA. Scale bars: 1 mm (**B**); 500 µm (**C**); 100 µm (**D**); 10 µm (**F, H–I**); 200 µm (**G**).

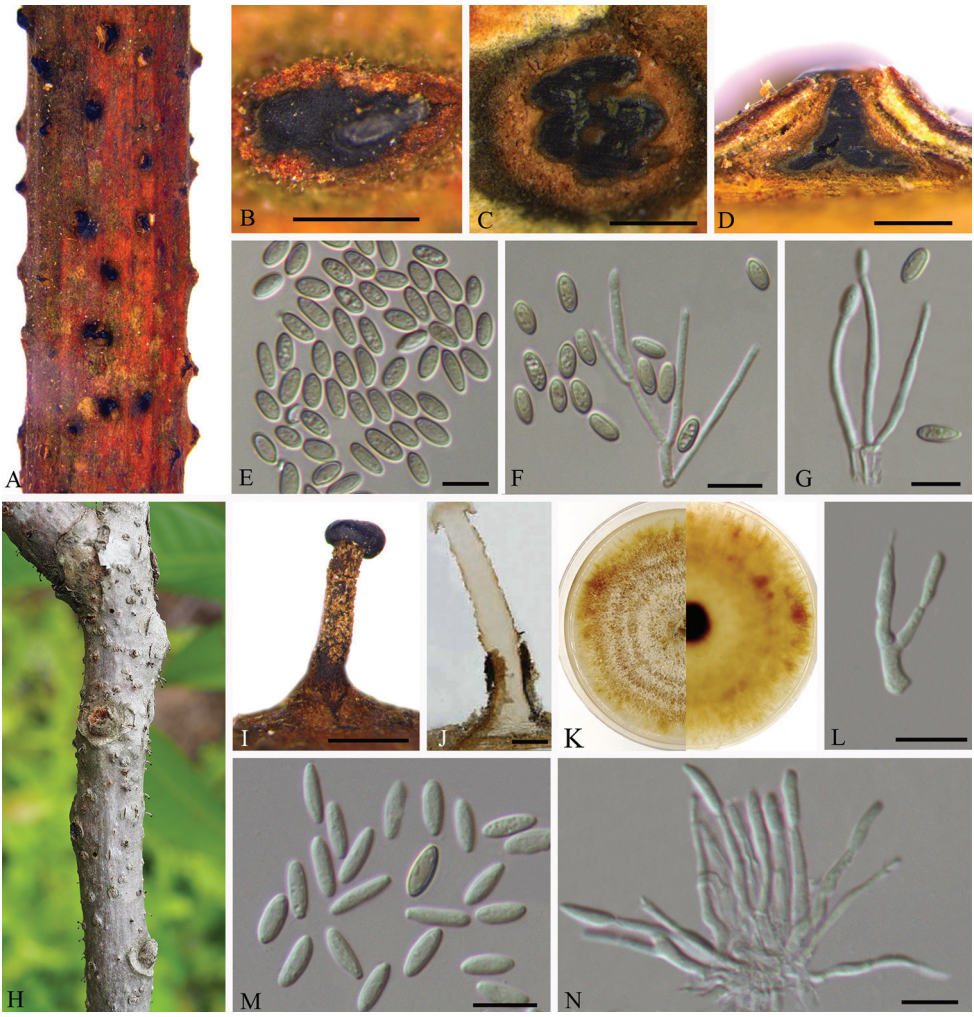

Figure 3. Asexual morphology of *Synnemasporella aculeans* on *Rhus chinensis* (BJFC-S1740) **A, B** habit of pycnidia on twigs **C** transverse section of pycnidium **D** longitudinal section through pycnidium **E** conidia **F, G** conidiogenous cells and conidia **H, I** habit of synnemata on twigs **J** longitudinal section through synnema **K** the colony on PDA **L, N** conidiogenous cells bearing conidia **M** conidia. Scale bars: 500 μm (**B–D, I, J**); 10 μm (**E–G, L–N**).

slightly concave and dark zone of conidiogenous cells and host tissue at their bases. Conidiophores reduced to conidiogenous cells. Conidiogenous cells (12.5–)16–22(–25) × 2 μm, phialidic, aggregated, hyaline, straight to curved, cylindrical, arranged adjacent to one another at the end of the synnema, producing a single conidium. Conidia cylindrical to oblong, smooth, multiguttulate, hyaline, (5–)5.5–7(–8) × 1.5–2 μm.

Culture characters. On PDA at 25 °C in darkness, initially white, becoming olivegreen to black after 3 wk, zonate with 3–4 well defined zones. Conidiomata distributed concentrically over agar surface.

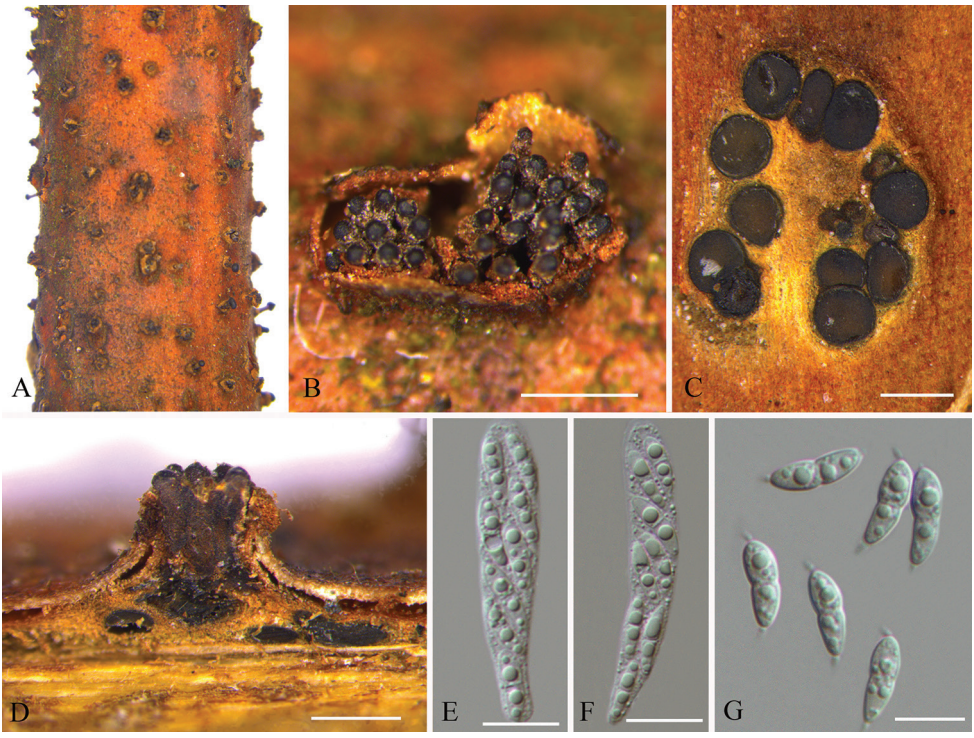

Figure 4. Sexual morphology of *Synnemasporella aculeans* on *Rhus chinensis* (BJFC-S1745) **A, B** habit of ascomata on twigs **C** transverse section of ascomata **D** longitudinal section through ascomata **E, F** asci **G** ascospores. Scale bars: 500 μm (**B–D**); 10 μm (**E–G**).

Specimen examined. CHINA, Jiangxi Province, Ganzhou City, Xunwu County, 24°52'31.34"N, 115°35'39.53"E, on branches of *Rhus chinensis*, 14 May 2018, Q. Yang, Y. Liu & Y.M. Liang (holotype BJFC-S1766, ex-type living cultures CFCC 53118, CFCC 53119 and CFCC 53120).

Notes. *Flavignomonia rhoigena* is the type species of *Flavignomonia* in the family Gnomoniaceae. It can be easily distinguished from the other gnomoniaceous genera by its unique conidiomata (Walker et al. 2004, Senanayake et al. 2018, Crous et al. 2019, Minoshima et al. 2019).

***Synnemasporella aculeans* (Schwein.) X.L. Fan & J.D.P. Bezerra, Persoonia 40: 130. 2018.**

Figure 3, 4

Description. Sexual morph: See Wehmeyer (1933) and Fan et al. (2018). Asexual morph: See Fan et al. (2018).

Specimens examined. CHINA, Jiangxi Province, Ganzhou City, Xunwu County, 24°52'31.34"N, 115°35'39.53"E, on branches of *Rhus chinensis*, 14 May 2018, Q. Yang, Y.

Liu & Y.M. Liang (BJFC-S1740, living culture CFCC 53123); Ganzhou City, Fengshan forest park, 25°44'32.14"N, 114°59'25.54"E, on branches of *Rhus chinensis*, 15 May 2018, Q. Yang, Y. Liu & Y.M. Liang (BJFC-S1753, living culture CFCC 53124 and CFCC 53125). 24°38'38.18"N, 115°33'58.45"E, on branches of *Rhus chinensis*, 16 May 2018, Q. Yang, Y. Liu & Y.M. Liang (BJFC-S1745, living culture CFCC 53126 and CFCC 53127).

Notes. *Synnemasporella aculeans* was proposed as a new combination in the new genus *Synnemasporella* based on the description of *Cryptodiaporthe aculeans* (Fan et al. 2018), which was introduced producing perithecial ascomata, and an asexual morph producing sporodochial and/or pycnidial conidiomata (Wehmeyer 1933). In the present study, five isolates from canker tissues on *Rhus chinensis* were congruent with *S. aculeans* based on morphology and DNA sequences data. This was the first time that the sexual morph of *Synnemasporella aculeans* in China had been collected.

Discussion

In this study, two diaporthalean species forming synnemata on *Rhus chinensis* were identified based on morphology and ITS, LSU, *tef1*, and *rpb2* sequence datasets. As a result, *Flavignomonia* typified with *F. rhoigena* is proposed as a new genus in Gnomoniaceae for its distinct phylogenic position and distinctive asexual fruiting body, Also, *Synnemasporella aculeans* strains were successfully isolated from perithecia, pycnidia and synnemata, which was confirmed by molecular data.

Nineteen fungal species have been recorded from the commercially and ecologically important tree species in China, including *Cladosporium cladosporioides*, *Cronartium quercuum*, *Mycosphaerella fushinoki*, *Pestalotiopsis diospyri*, *P. guepinii*, *P. mangiferae*, *P. sorbi*, *Phaeoramularia rhois*, *Phyllactinia corylea*, *Ph. rhoina*, *Pileolaria klugkistiana*, *Pi. shiraiana*, *Pseudocercospora rhoina*, *Ps. toxicodendri*, *Septoria* sp., *Tubercularia phyllophila*, *Uncinula verniciferae*, and two synnematal species from branch cankers in this study (Farr and Rossman 2019). *Flavignomonia rhoigena* and *Synnemasporella aculeans*, described and illustrated in the present study can be easily recognized by the asexual fruiting bodies, and they differ from each other in the colour of the synnematal tips.

Gnomoniaceae is a globally distributed fungal family on diverse plant hosts (Mejía et al. 2008, 2011a, 2011b, 2012, Sogonov et al. 2008, Walker et al. 2012, Senanayake et al. 2017, 2018). Host specificity of this family has been confirmed to be important in the evolution (Walker et al. 2014). Our newly discovered genus *Flavignomonia* was only found on *Rhus chinensis*, and more *Flavignomonia* species might be collected from the plant family Anacardiaceae in the future.

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RESEARCH ARTICLE

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Neptunomyces aureus gen. et sp. nov. (Didymosphaeriaceae, Pleosporales) isolated from algae in Ria de Aveiro, Portugal

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Abstract

A collection of fungi was isolated from macroalgae of the genera *Gracilaria*, *Enteromorpha* and *Ulva* in the estuary Ria de Aveiro in Portugal. These isolates were characterized through a multilocus phylogeny based on ITS region of the ribosomal DNA, beta-tubulin (*tub2*) and translation elongation factor 1 alpha (*tef1-a*) sequences, in conjunction with morphological and physiological data. These analyses showed that the isolates represented an unknown fungus for which a new genus, *Neptunomyces* **gen. nov.** and a new species, *Neptunomyces aureus* **sp. nov.** are proposed. Phylogenetic analyses supported the affiliation of this new taxon to the family Didymosphaeriaceae.

Keywords

Didymosphaeriaceae, marine fungi, phylogeny, taxonomy

Introduction

The family Didymosphaeriaceae is an important family in the order Pleosporales introduced by Munk (1953) and typified by the genus *Didymosphaeria* Fuckel with *D. epidermidis* as the type species. Members of this family are characterized by having brown 1-septate ascospores and trabeculate pseudoparaphyses that anastomose

mostly above the asci (Aptroot 1995, Hyde et al. 2013, Ariyawansa et al. 2014a, b). Species of Didymosphaeriaceae are saprobes, endophytes or pathogens of a wide variety of plant species worldwide (Ariyawansa et al. 2014a, Liu et al. 2015, Wanasinghe et al. 2016).

Accurate species' identification in genera of the family Didymosphaeriaceae was discussed in detail by Ariyawansa et al. (2014a). Phylogenetic analyses based on regions such as the internal transcribed spacer (ITS) region of the ribosomal DNA, beta-tubulin (*tub2*) and translation elongation factor 1 alpha (*tef1-a*) proved to be useful in delimiting taxa (Tennakoon et al. 2016, Ariyawansa et al. 2014b). Several studies have been conducted to resolve the boundaries of this family. First, Ariyawansa et al. (2014a) showed that *Montagnulaceae* and Didymosphaeriaceae were synonyms and thus, Ariyawansa et al. (2014b) synonymized *Montagnulaceae* under Didymosphaeriaceae and rearranged the family into 16 genera: *Alloconiothyrium*, *Barria*, *Bimuria*, *Deniquelata*, *Didymocrea*, *Didymosphaeria*, *Julella*, *Kalmusia*, *Karstenula*, *Letendraea*, *Montagnula*, *Neokalmusia*, *Paraconiothyrium*, *Paraphaeosphaeria*, *Phaeodothis* and *Tremateia*. Subsequently, in the last years, additional genera were added, namely *Paracamarosporium* and *Pseudocamarosporium* (Wijayawardene et al. 2014), *Spegazzinia* (Tanaka et al. 2015), *Xenocamarosporium* (Crous et al. 2015), *Austropleospora* and *Pseudopithomyces* (Ariyawansa et al. 2015) and *Laburnicola* and *Paramassariosphaeria* (Wanasinghe et al. 2016). More recently, Jayasiri et al. (2019) introduced *Cylindroaseptospora* and Gonçalves et al. (2019) reassigned the genus *Verrucoconiothyrium* previously included in the family Didymosphaeriaceae to the family *Didymelaceae*. Thus, the family Didymosphaeriaceae currently comprises 25 genera.

During an extensive survey of the fungal diversity from macroalgae species in the salt marsh of Ria de Aveiro in Portugal, we gathered a collection of fungal isolates. Here we report the morphological, cultural and phylogenetic characterization of these fungal isolates and introduce a novel genus and species to accommodate them.

Material and methods

Collection and isolation

Macroalgae (*Gracilaria gracilis*, *Enteromorpha intestinalis*, and other macroalgae species identified at genus-level only) were collected from various sites in the estuary Ria de Aveiro in Portugal (Table 1). Samples were placed in sterile plastic containers and maintained at 4 °C until fungal isolation. Algae samples were washed with autoclaved filtered saline water, cut into small pieces and placed on Potato Dextrose Agar (PDA) enriched with 3 % (w/v) sea salts (Sigma-Aldrich). Streptomycin and tetracycline, at final concentrations of 100 mg/L, were added to PDA to inhibit the growth of bacteria. From each sample (algae) 20 pieces of tissue were plated on PDA medium. The plates were incubated at 25 °C for 5 days and examined daily to observe the growth of fungal hyphae. Distinct fungal colonies were then transferred to new PDA plates for further isolation and purification.

Table 1. Sampling sites.

Locality name	GPS coordinates	Sampling date	Algae species collected
Ria de Aveiro	40°37'45"N, 8°43'27"W	26/09/18	*Ulva* sp.
	40°39'33"N, 8°43'27"W		*Enteromorpha* sp.
	40°40'38"N, 8°42'20"W		*Gracilaria gracilis*, *Ulva* sp.
	40°43'00"N, 8°42'04"W		*Enteromorpha intestinalis*, *Ulva* sp.

DNA isolation, amplification and analyses

Genomic DNA was extracted from fresh mycelium of cultures growing on PDA according to Möller et al. (1992). The primers ITS1 and ITS4 (White et al. 1990) were used for amplification and sequencing of the ITS region of the ribosomal DNA was as described by Alves et al. (2004). Beta-tubulin (*tub2*) gene was amplified and sequenced using T1 and Bt2b primers (Glass and Donaldson 1995, O'Donnell and Cigelnik 1997) with the cycling conditions previously described by Lopes et al. (2017). Translation elongation factor 1 alpha (*tef1-a*) gene was amplified and sequenced using EF1-688F and EF1-2218R primers (Rehner 2001, Alves et al. 2008). The amplified PCR fragments were purified with the NZYGelpure kit (NZYTech, Portugal) before sequencing at GATC Biotech (Cologne, Germany). The nucleotide sequences were analyzed with FinchTV v.1.4.0 (Geospiza Inc. www.geospiza.com/finchtv). A BLASTn search against the nucleotide collection (nr/nt) database using the ITS, *tub2* and *tef1-a* sequences was carried out to determine the closest matching sequences, which were added to the sequence alignment. Sequences were aligned with ClustalX v. 2.1 (Thompson et al. 1997), using the following parameters: pairwise alignment parameters (gap opening = 10, gap extension = 0.1) and multiple alignment parameters (gap opening = 10, gap extension = 0.2, transition weight = 0.5, delay divergent sequences = 25 %). Alignments were checked and edited with BioEdit Alignment Editor v.7.2.5 (Hall 1999). Phylogenetic analyses were done with MEGA7 v.7.0 (Kumar et al. 2016). All gaps were included in the analyses. MEGA7 v.7.0 was also used to determine the best substitution model to be used to build the Maximum Likelihood (ML) tree. ML analysis was performed on a Neighbour-Joining (NJ) starting tree automatically generated by the software. Nearest-Neighbour-Interchange (NNI) was used as the heuristic method for tree inference with 1,000 bootstrap replicates. The sequences generated in this study were deposited in GenBank and taxonomic novelties in MycoBank. Alignment and tree were deposited in TreeBase (TB2:S24556).

Morphology and growth studies

Observations of morphological characters were made with a SMZ1500 stereoscopic microscope (Nikon, Japan) and a Nikon Eclipse 80i microscope (Nikon, Japan) equipped with differential interference contrast. Fungal structures were mounted in 100% lactic acid. Photographs and measurements were taken with a Nikon DSRi1 camera (Nikon, Japan) and the NIS-Elements D program (Nikon, Japan). Colony characters and pigment production were registered after 2 weeks of growth on PDA, Malt Extract Agar (MEA) and

Oatmeal Agar (OA) incubated at 25 °C. Colony colors (obverse and reverse) were assessed according to the color charts of Rayner (1970). Morphological descriptions were based on cultures sporulating on PDA and pine needles, after 1-month incubation at 25 °C.

Temperature growth studies were performed for the new species described. A 5-mm diameter plug was taken from the margin of an actively growing colony (14-day-old) and placed in the center of PDA, MEA and OA plates. Three replicate plates per isolate were incubated at 10, 15, 20, 25, 30 and 35 °C in the dark. Colony diameter was measured after 1 and 2 weeks.

To evaluate the growth requirements for sea salts, the new species was cultured in PDA with 3% (w/m) sea salts. Three replicate plates per isolate were incubated at 25 °C for 2 weeks in the dark. After incubation the diameter of the colonies was measured and compared.

Results

Phenotype

Regarding conidial morphology, the fungal isolates studied were characterized by being aseptate and subcylindrical with rounded apices golden yellow conidia. For all media tested, the minimum, maximum and optimal growth temperatures were 10, 30 and 25 °C, respectively. No differences were observed in terms of colony diameter when grown in PDA with and without the addition of 3% sea salts, indicating that this fungus does not require salt for growth.

Phylogenetic analysis

BLASTn searches against the NCBI nucleotide database using the ITS, *tub2* and *tef1-a* sequences of the isolates retrieved various hits, of which those with the highest sequence similarity belonged to members of the family Didymosphaeriaceae. Based on a megablast search using the ITS sequence, the closest matches for MUM 19.38 = CMG 10A in GenBank were *Dothideomycetes* sp. (GenBank accession: HQ631008; Identities 549/564 (97%), no gaps) and *Letendraea* sp. (GenBank accession: LT796897; Identities 548/564 (97%), no gaps). The closest hits using the *tub2* sequence were *Letendraea* sp. (GenBank accession: LT796988; Identities 457/516 (89%), 5 gaps). Closest hits using *tef1-a* sequence also had highest similarity to *Letendraea* sp. (GenBank accession: LT797101; Identities 935/957 (98%), no gaps).

To confirm the phylogenetic placement of the fungal isolates within the family Didymosphaeriaceae, sequences of ITS, ITS + *tub2* and ITS + *tef1-a* were aligned against those of several genera/species belonging to Didymosphaeriaceae (Suppl. material 1: Table S1). The alignment of the ITS, ITS + *tub2* and ITS + *tef1-a* contained 60, 20 and 20 sequences (including the outgroup), and there was a total of 1010, 1352 and 1836 positions in the final dataset, respectively. In all ML phylogenetic trees

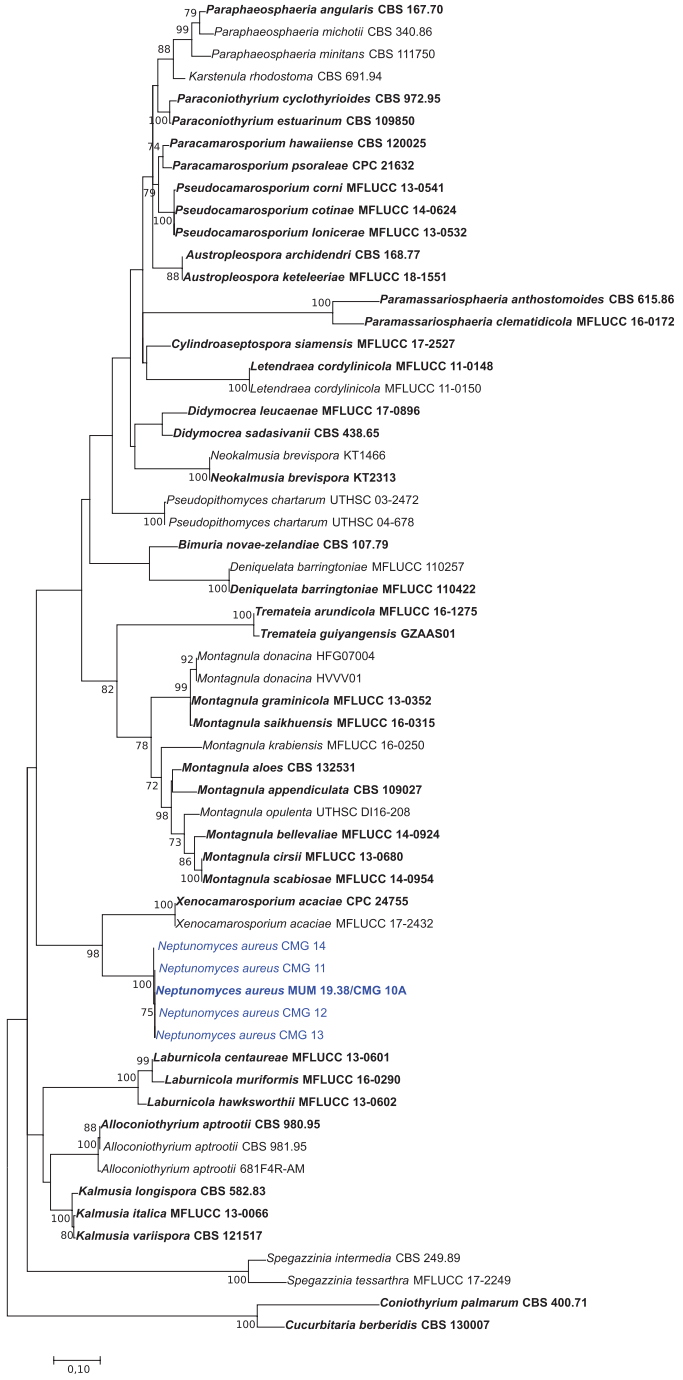

Figure 1. Phylogenetic relationships of Didymosphaeriaceae species based on ITS sequence data and inferred using the Maximum Likelihood method under the Kimura 2-parameter model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and rooted to *Cucurbitaria berberidis* (CBS 130007) and *Coniothyrium palmarum* (CBS 400.71). Bootstrap values (> 70%) are shown at the nodes. Ex-type strains are in bold and the isolates from the current study are in blue.

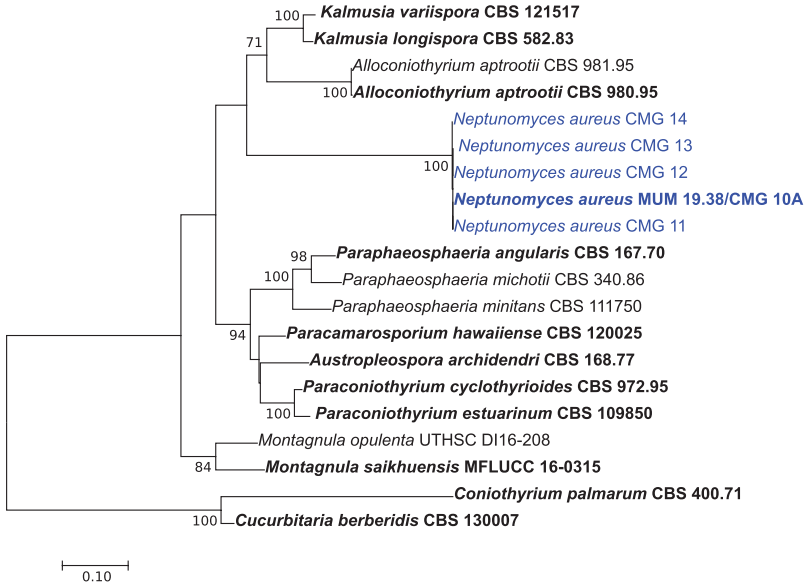

Figure 2. Phylogenetic relationships of Didymosphaeriaceae species based on ITS and *tub2* sequence data and inferred using the Maximum Likelihood method under the Kimura 2-parameter model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and rooted to *Cucurbitaria berberidis* (CBS 130007) and *Coniothyrium palmarum* (CBS 400.71). Bootstrap values (> 70%) are shown at the nodes. Ex-type strains are in bold and the isolates from the current study are in blue.

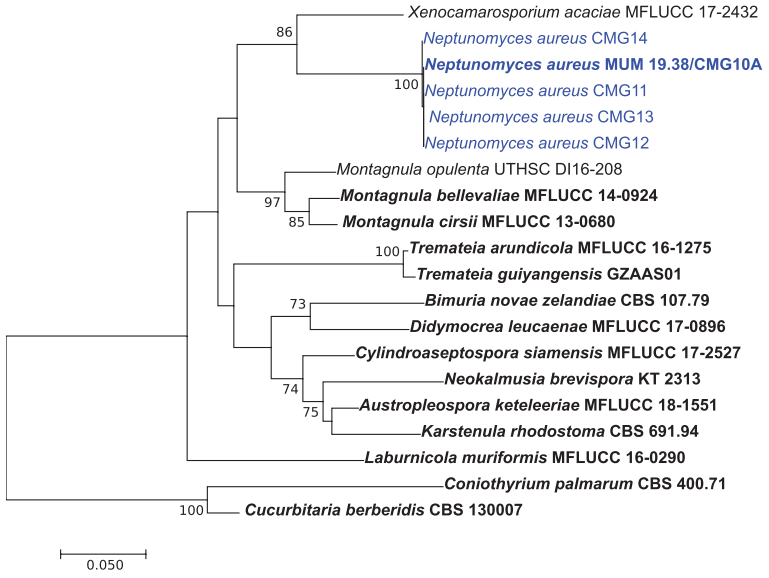

Figure 3. Phylogenetic relationships of Didymosphaeriaceae species based on ITS and *tef1-a* sequence data and inferred using the Maximum Likelihood method under the Kimura 2-parameter model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and rooted to *Cucurbitaria berberidis* (CBS 130007) and *Coniothyrium palmarum* (CBS 400.71). Bootstrap values (> 70%) are shown at the nodes. Ex-type strains are in bold and the isolates from the current study are in blue.

(Figs 1–3), all novel isolates clustered in a monophyletic clade that received high (100 %) bootstrap support within the family Didymosphaeriaceae with a close relationship with the genera *Alloconiothyrium* and *Kalmusia* (ITS + *tub2*, Fig. 2) and *Xenocamarosporium* (ITS + *tef1-a*, Fig. 3). Thus, this novel lineage is phylogenetically well delimited, and it is clearly distinct from the other genera of Didymosphaeriaceae described so far and therefore it is proposed here as a new genus and a new species.

Taxonomy

Neptunomyces **M. Gonçalves, T. Vicente & A. Alves. Portugal, gen. nov.**
MycoBank No: 831436

Description. Asexual morph: mycelium consisting of septate, smooth hyphae, thick-walled, hyaline and rarely with nucleus. Conidia aseptate, golden yellow, smooth, subcylindrical with rounded apices. Chlamydospores not observed. Sexual morph unknown.

Etymology. Referring to Neptune (Latin: *Neptūnus*) the god of the seas in Roman mythology.

Type species. *Neptunomyces aureus* M. Gonçalves, T. Vicente & A. Alves. Portugal

Neptunomyces aureus **M. Gonçalves, T. Vicente & A. Alves. Portugal, sp. nov.**
MycoBank No: 831437
Fig. 4

Type. Portugal, Ria de Aveiro (40°40'38"N, 8°42'21"W), isolated from *Gracilaria gracilis*, 26th September 2018, M. Gonçalves, (holotype: a dried culture sporulating on pine needles AVE-F-1; ex-type living culture, MUM 19.38 = CMG 10A).

Etymology. Referring to the golden yellow conidia.

Diagnosis. Phylogenetic analysis based on the ITS, ITS and *tub2* and ITS and *tef1-a* dataset considered in the present study clustered the retrieved strains in a monophyletic lineage in the family Didymosphaeriaceae. Therefore, a new genus *Neptunomyces* gen. nov., and a new species *Neptunomyces aureus* sp. nov. are here proposed.

Description. Mycelium smooth, white, 2–3 µm wide hyphae. Hyphae thick-walled, smooth, hyaline and rarely with nucleus. Conidiomata aggregated or solitary, globose to subglobose, dark brown, immersed or rarely superficial. Conidiomata wall pseudoparenchymatous. Conidiophores reduced to ampulliform to subcylindrical, hyaline, smooth conidiogenous cells (mean ± S.D. = 5.2 ± 0.3 × 2.0 ± 0.6 µm, n = 20). Conidia solitary, subcylindrical with rounded apices, aseptate, initially hyaline, smooth, becoming golden yellow (mean ± S.D. = 7.0 ± 0.6 × 2.7 ± 0.2 µm, n = 100). Sexual morph unknown.

Culture characteristics. On 2 weeks old PDA and OA plates, at 25 °C, colonies growing to 50 mm in diameter, regular and above and a little immersed into agar. PDA obverse white near the center getting flesh orange towards the borders; reverse buff orange in the center and lighter in periphery. OA obverse skimmed milk white;

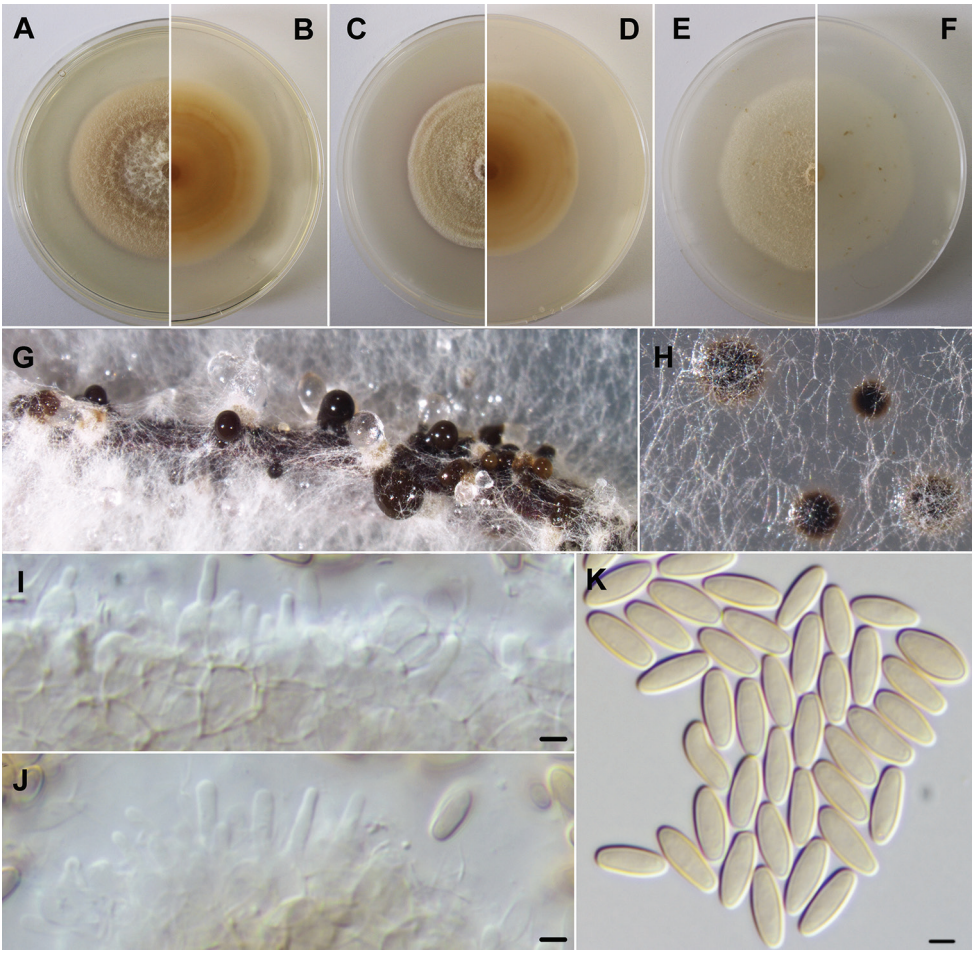

Figure 4. *Neptunomyces aureus* (MUM 19.38). **A, B** Colony after 2 weeks at 25 °C on PDA (obverse and reverse) **C, D** colony after 2 weeks at 25 °C on MEA (obverse and reverse) **E, F** colony after 2 weeks at 25 °C on OA (obverse and reverse) **G, H** conidiomata after 1 month at 25 °C on pine needles and PDA. **I, J** conidiogenous cells **K** conidia. Scale bars: 2.5 µm.

reverse snow white. On 2 weeks old MEA plates, at 25 °C, colonies growing to 44 mm in diameter, regular and above and a little immersed into agar. Obverse orange-colored white; reverse reddish orange in the center and ochre yellow in periphery. At 35 °C, there was no growth in any media tested.

Distribution. Estuary Ria de Aveiro, Portugal

Additional specimens examined. Portugal, Ria de Aveiro (Table 1), isolated from *Ulva* sp., *Enteromorpha intestinalis* and *Enteromorpha* sp. (Supp. material 1: Table S1). M. Gonçalves, living cultures CMG 11, CMG 12, CMG 13 and CMG 14.

Notes. *Neptunomyces aureus* clustered in a distinct lineage in the family Didymosphaeriaceae with high p-distances (= 0.07) of nucleotide sites among the two-loci se-

Table 2. Comparison of *Neptunomyces aureus* and *Xenocamarosporium acaciae*.

Species		*Neptunomyces aureus*	*Xenocamarosporium acaciae*
Strain		MUM 19.38	CBS 139895
Nucleotide differences	ITS	65	
	tef1-a	58	
(p-distance)	ITS + *tef1-a*	0.07	
Conidia	Size (µm)	7.0 ± 0.6 × 2.7 ± 0.2	(11–)12–14(–15) × (3.5–)4(–5)
	Morphology	Subcylindrical	Ellipsoidal to subcylindrical
	Apex and base	Rounded	Obtuse and rounded to truncate base
	Color	Hyaline becoming golden yellow	Hyaline becoming golden brown
	Septation	Aseptate	(1–)3-septate
Conidiogenous cells	Size (µm)	5.2 ± 0.3 × 2.0 ± 0.6	7–12 × 5–7
	Morphology	Ampulliform	Ampulliform
	Color	Hyaline	Hyaline
References		Present study	Crous et al. 2015

quences (ITS and *tef1-a*) with closest genus *Xenocamarosporium*. Although the morphology of conidiomata, conidiomata wall and conidiogenous cells can be very similar in the genera of this family, conidial morphology distinguishes *Neptunomyces* from *Xenocamarosporium* (Table 2).

Discussion

This study adds to the family Didymosphaeriaceae a new genus/species, namely *Neptunomyces aureus* isolated from macroalgae in the estuary of Ria de Aveiro in Portugal. The family Didymosphaeriaceae contains now 26 genera described.

The majority of the genera in the Didymosphaeriaceae remain under studied, which makes the family still poorly understood and not well resolved (Wanasinghe et al. 2016). In fact, there was no β-tubulin and *tef1-a* sequence data available for many species and therefore the phylogenetic analyses presented did not encompass all known species of the family. For example, phylogenetic analyses based on ITS + *tub2* revealed that *N. aureus* is closely related to the genera *Alloconiothyrium* and *Kalmusia*, while on ITS + *tef1-a* it is related to the genus *Xenocamarosporium*, since there is no *tef1-a*/*tub2* for *Alloconiothyrium*, *Kalmusia* and *Xenocamarosporium*, respectively. However, this family contains several well supported clades, most of which correspond to monotypic genera (*e.g. Alloconiothyrium*, *Bimuria*, *Karstenula*, *Xenocamarosporium*), or genera with only two species (*e.g. Cylindroaseptospora*, *Deniquelata*, *Didymocrea*).

Comparison of the ITS and *tef1-a* sequences from *N. aureus* and the closest genus/species *X. acaciae* revealed 65 and 58 base pair differences, respectively, with high p-distances (= 0.07) supporting the establishment of *Neptunomyces* as a distinct genus. Although the morphology of conidiomata, conidiomata wall and conidiogenous cells are similar, the conidiogenous cells of *N. aureus* are smaller than those of *X. acaciae*. Also, both can be easily discriminated by their conidia morphology, color and size. The

conidia of *N. aureus* are aseptate, subcylindrical with rounded apices and initially hyaline and soon become golden yellow, while conidia of *X. acaciae* are mostly tri-septate, ellipsoidal to subcylindrical, sometimes with truncate base and golden brown. Moreover, conidia of *N. aureus* are considerably smaller than those of *X. acaciae*.

Neptunomyces aureus was isolated from healthy tissues of the macroalgae analyzed, where it may occur as endophyte or epiphyte. Further investigations are essential for clarifying its biology, ecology, physiological characteristics and host-specificity. Moreover, we did not obtain any sexual morph for this new species and there is no molecular support to link possible sexual taxa.

So far, species of Didymosphaeriaceae seem to be cosmopolitan in distribution: they have been recorded from both temperate and tropical regions. Also, Didymosphaeriaceae have been found on various hosts and substrates, including plants, humans and soil, being regarded as saprobes, endophytes or pathogens of a wide variety of plant substrates worldwide (Ariyawansa et al. 2014a, Liu et al. 2015, Wanasinghe et al. 2016). However, most Didymosphaeriaceous genera occur on plants of more than 20 host families, the majority of them being monocotyledons and herbaceous plants, such as *Anacardiaceae*, *Asparagaceae*, *Asteraceae*, *Caprifoliaceae*, *Euphorbiaceae*, *Fagaceae*, *Lecythidaceae* and *Poaceae*. Reports of Didymosphaeriaceous species in marine/estuarine environments are almost non-existent. So far, this new genus/species has been found only in association with macroalgae species. Garzoli et al. (2018) reported, for the first time, some species within this family in *Padina pavonica*, a brown alga collected in the Mediterranean Sea: *Paraconiothyrium variabile*, *Paraphaeosphaeria neglecta* and another eight unidentified Didymosphaeriaceous species. Also, *Paraconiothyrium estuarinum* was isolated from sediments of an estuarine environment (Verkley et al. 2004) and *Paraphaeosphaeria michotii* from *Phragmites australis*, also typically found in estuaries (Eriksson 1967).

Physiological tests allowed us to characterize the retrieved isolates as a slight halophile as they grow equally well in the presence and absence of 3% sea salts. Information regarding NaCl tolerance is still poorly described in *Didymosphaeriaceous* species, but future studies related to tolerance to salinity in these organisms (especially in this new species) may provide physiological unique characteristics which may have some biotechnological potential.

Acknowledgements

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Supplementary material 1

Table S1. List of isolates used in this study

Authors: Micael F.M. Gonçalves, Tânia F.L. Vicente, Ana C. Esteves, Artur Alves

Data type: species data

Link: https://doi.org/10.3897/mycokeys.60.37931.suppl1

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RESEARCH ARTICLE

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A four-locus phylogeny of rib-stiped cupulate species of *Helvella* (Helvellaceae, Pezizales) with discovery of three new species

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Abstract

Helvella species are ascomycetous macrofungi with saddle-shaped or cupulate apothecia. They are distributed worldwide and play an important ecological role as ectomycorrhizal symbionts. A recent multi-locus phylogenetic study of the genus suggested that the cupulate group of *Helvella* was in need of comprehensive revision. In this study, all the specimens of cupulate *Helvella* sensu lato with ribbed stipes deposited in HMAS were examined morphologically and molecularly. A four-locus phylogeny was reconstructed using partial sequences of the heat shock protein 90, nuclear rDNA internal transcribed spacer region 2, nuclear large subunit ribosomal DNA and translation elongation factor 1-α genes. Three clades were revealed in *Helvella* sensu stricto. Twenty species were included in the analysis, of which 13 are distributed in China. Three new species, *H. acetabuloides*, *H. sichuanensis* and *H. tianshanensis*, are described and illustrated in detail. A neotype was designated for *H. taiyuanensis*. *Helvella calycina* is a new record for China, while *Dissingia leucomelaena* should be excluded from Chinese mycota. Hsp90 and ITS2 are recommended as useful supplementary barcodes for species identifications of the genus.

Keywords

Ascomycota, DNA barcode, phylogeny, taxonomy, typification

Introduction

The genus *Helvella* L. contains a group of ascomycetous macrofungi with saddle-shaped or cupulate apothecia. *Helvella* species are distributed worldwide, especially in temperate regions (Dissing 1966, Abbott and Currah 1997). Some of them are edible, for example, *H. bachu* Q. Zhao, Zhu L. Yang & K.D. Hyde (Zhao et al. 2016a) and *H. taiyuanensis* B. Liu, Du & J.Z. Cao (Liu et al. 1985), and some are medicinal, for example, *H. lacunosa* Afzel. (Shameem et al. 2016). They are also important as ectomycorrhizal symbionts (Tedersoo et al. 2006, Healy et al. 2013, Hwang et al. 2015).

Helvella was established in 1753 and more than 400 names attributable to the genus have been recorded in the databases of Index Fungorum and MycoBank. Several taxonomic treatments were proposed, based on morphological characters (Table 1). Seven sections were established by Dissing (1966): sections *Acetabulum*, *Crispae*, *Elasticae*, *Ephippium*, *Lacunosae*, *Leucomelaenae* and *Macropodes*. Amongst them, the sections *Acetabulum* and *Leucomelaenae* included the species having cup-shaped apothecia with ribbed stipes. Similarly, six to eight infrageneric groups (sections or subgenus) were recognised by different authors (Weber 1972, Häffner 1987, Abbott and Currah 1997). Meanwhile, many additional species were added to the genus (Weber 1975, Harmaja 1976, 1977a, b, 1978, 1979, Abbott and Currah 1988). A checklist of cupulate *Helvella* species having ribbed stipes and their infrageneric positions are summarised in Table 2. Recently, *Helvella* sensu stricto was found to be associated with *Balsamia* Vittad., *Dissingia* K. Hansen, X.H. Wang & T. Schumach., *Midotis* Fr., *Pindara* Velen. and *Underwoodia* Peck in Helvellaceae (Hansen and Pfister 2006; Hansen et al. 2019). Amongst them, *Dissingia* was proposed to accommodate the species formerly placed in *Helvella* section *Leucomelaenae* (Hansen et al. 2019).

With the development of molecular phylogenetics, the taxonomy of *Helvella* has been re-evaluated. Sequences of nuclear large and small subunit ribosomal DNA (LSU and SSU) were adopted for phylogenetic inference of *Helvella* sensu lato and its allied genera (Hansen and Pfister 2006, Tedersoo et al. 2006, Laessoe and Hansen 2007). Protein-coding genes, RNA polymerase II the largest subunit (RPB1), the second largest subunit (RPB2) and translation elongation factor 1-α (TEF1) were also applied (Bonito et al. 2013, Hansen et al. 2013). Nguyen et al. (2013) explored *Helvella* phylogeny using large-scale sequence analysis of LSU and the nuclear rDNA internal transcribed spacer region (ITS) and reported two new species from North America based on molecular and morphological evidence. On the basis of examinations of the type specimens and LSU sequence analysis, Landeros et al. (2012, 2015) concluded that the sections *Elasticae*, *Helvella*, *Lacunosae* and *Leucomelaenae* were monophyletic. Skrede et al. (2017) studied molecular characteristics of 55 European species, described seven new species based on the sequence divergences of LSU, RPB2, TEF1 and heat shock protein 90 gene (Hsp90), and designated neotypes and epitypes for 30 of them. Five clades and 18 lineages were distinguished according to the phylogeny inferred from the combined Hsp90 and RPB2 datasets. The above work provides background information for understanding the species concept of *Helvella*. In their updated study, Hansen et al. (2019) defined *Helvella* s. s., treated the cupulate *H. leucomelaena* (Pers.)

Table 1. Comparison of the taxonomic systems established in *Helvella*.

Dissing (1966)	Weber (1972)	Häffner (1987)	Abbott and Currah (1997)	Hansen et al. (2019)
Section *Leucomelaenae* Dissing	Section *Leucomelaenae* Dissing	Section *Leucomelaenae* Dissing	Subgenus *Leucomelaenae* (Dissing) S.P. Abbott	*Dissingia* K. Hansen, X.H. Wang & T. Schumach.
		Section *Solitariae* Häffner		*Helvella* L.
Section *Acetabulum* Dissing	Section *Acetabulum* Dissing	Section *Acetabulum* Dissing		
Section *Crispae* Dissing	Section *Helvella* L.	Section *Helvella* L.	Subgenus *Helvella* L.	
Section *Lacunosae* Dissing	Section *Lacunosae* Dissing	Section *Lacunosae* Dissing		
Section *Elasticae* Dissing	Section *Elasticae* Dissing	Section *Elasticae* Dissing	Subgenus *Elasticae* (Dissing) S.P. Abbott	
Section *Ephippium* Dissing	Section *Ephippium* Dissing	Section *Ephippium* Dissing		
Section *Macropodes* Dissing	Section *Macropodes* Dissing	Section *Macropodes* Dissing	Subgenus *Macropodes* (Dissing) S.P. Abbott	
			Subgenus *Cupuliformes* S.P. Abbott	
			Subgenus *Silvicolae* (S.P. Abbott) S.P. Abbott	*Midotis* Fr.

Nannf. lacking crozier at the ascus base as a separate genus *Dissingia*, retrieved the generic name *Pindara*, and transferred *H. aestivalis* (R. Heim & L. Rémy) Dissing & Raitv. to *Balsamia*. Brief comparisons amongst different taxonomic treatments are shown in Table 1.

In China, Teng (1963) recorded 11 species of *Helvella* and Tai (1979) listed 15 taxa. Liu, Cao and their collaborators (Liu et al. 1985, Liu and Cao 1988, Cao and Liu 1990, Cao et al. 1990) published nine species, new to the genus. With the additional investigations, our knowledge of the group accumulated (Zhuang 1989, 1995, 1996, 1997, 1998, Zhuang and Wang 1998a, 1998b, Yu et al. 2000, Wang and Chen 2002, Xu 2002, Zhuang 2004, Zhuang and Yang 2008). Zhuang et al. (2018) provided a checklist of 37 *Helvella* species occurring in China up to 2013. Recently, Zhao and his collaborators (Ariyawansa et al. 2015, Zhao et al. 2015, Hyde et al. 2016, Wang et al. 2016, Zhao et al. 2016a, 2016b, Tibpromma et al. 2017) described 12 new species with two bearing cupulate apothecia (Table 2), as well as two new Chinese records, *H. subglabra* N.S. Weber and *H. ulvinenii* Harmaja. There are about 51 species currently known from the country.

The present study is aimed at exploring species diversity of the cupulate *Helvella* species with ribbed stipes.

Materials and methods

Fungal materials and morphological observations

Collections of the cupulate *Helvella* species with ribbed stipes, deposited in the Herbarium Mycologicum Academiae Sinicae (**HMAS**), were re-examined, including those originally deposited in the Mycological Herbarium of Shanxi University (**MHSU**). Specimens recently collected from Beijing, Inner Mongolia, Hubei and Sichuan prov-

Table 2. A checklist of cupulate *Helvella* species sensu lato with ribbed stipes.

Species	Section *Acetabulum*	Section *Leucomelaenae*	Section *Solitariae*	Section *Macropodes*	Subgenus *Leucomelaenae*	Remark
Acetabula calyx Sacc., 1873	–	Syn. of *H. solitaria* (Dissing 1966); Syn. of *H. leucomelaena* (Harmaja 1977a)	–	–	Syn. of *H. leucomelaena* (Abbott and Currah 1997)	Syn. of *H. leucomelaena* (Landeros et al. 2015)
Balsamia aestivalis (R. Heim & L. Rémy) K. Hansen, Skrede & T. Schumach, 2019	–	Häffner 1987	–	–	Abbott and Currah 1997	as *Helvella aestivalis*
Dissingia crassitunicata (N.S. Weber) T. Schumach & Skrede, 2019	–	Weber 1975, Häffner 1987	–	–	Abbott and Currah 1997	as *Helvella crassitunicata*
Dissingia confusa (Harmaja) K. Hansen & X.H. Wang, 2019	–	Harmaja 1977a, Häffner 1987	–	–	Syn. of *H. leucomelaena* (Abbott and Currah 1997)	as *Helvella confusa*
Dissingia leucomelaena (Pers.) K. Hansen & X.H. Wang, 2019	–	Dissing 1966, Weber 1975, Häffner 1987	–	–	Abbott and Currah 1997	as *Helvella leucomelaena*
Dissingia oblongispora (Harmaja) T. Schumachand Skrede, 2019	–	Harmaja 1978, Häffner 1987	–	–	Abbott and Currah 1997	as *Helvella oblongispora*
Helvella acetabulum (L.) Quél, 1874	Dissing 1966, Weber 1972, Häffner 1987	–	–	–	Abbott and Currah 1997	Valid species
Helvella arctoalpina Harmaja, 1977	Harmaja 1977b, Häffner 1987	–	–	–	Syn. of *H. verruculosa* (Abbott and Currah 1997)	Valid species
Helvella calycina Skrede, T.A. Carlsen & T. Schumach, 2017	–	–	–	–	–	Valid species
Helvella costata Schwein, 1822	–	–	–	–	Syn. of *H. acetabulum* (Abbott and Currah 1997)	Valid species
Helvella costifera Nannf, 1953	Dissing 1966, Häffner 1987	–	–	–	Abbott and Currah 1997	Valid species
Helvella dryadophila Harmaja, 1977	Harmaja 1977b, Häffner 1987	–	–	–	Syn. of *H. verruculosa* (Abbott and Currah 1997)	Valid species
Helvella floriforma Q. Zhao & K.D. Hyde, 2016'	–	–	–	–	–	Valid species
Helvella griseoalba N.S. Weber, 1972	Weber 1972, Häffner 1987	–	–	–	Syn. of *H. costifera* (Abbott and Currah 1997)	Valid species
Helvella helvellula (Durieu) Dissing, 1966	–	Dissing 1966	–	–	–	Member of lasunosa clade (Skrede et al. 2017)
Helvella hyperborea Harmaja, 1978	Harmaja 1978, Häffner 1987	–	–	–	Abbott and Currah 1997	Valid species

Species	Section *Acetabulum*	Section *Leucomelaenae*	Section *Solitariae*	Section *Macropodes*	Subgenus *Leucomelaenae*	Remark
Helvella jiaohensis J.Z. Cao, L. Fan & B. Liu, 1990*	–	–	–	–	–	Holotype lost
Helvella jilinensis J.Z. Cao, L. Fan & B. Liu, 1990*	–	–	–	–	–	Holotype lost
Helvella pedunculata Harmaja, 1978	–	Harmaja 1978, Häffner 1987	–	–	Syn. of *H. leucomelaena* (Abbott and Currah 1997)	?Syn. of *H. costifera* (Skrede et al. 2017)
Helvella pocillum Harmaja, 1976	Häffner 1987	Harmaja 1976	–	–	–	Syn. of *B. aestivalis* (Hansen et al. 2019)
Helvella queletii Bres, 1882	–	Syn. of *H. solitaria* (Harmaja 1977a, Häffner 1987)	–	Dissing 1966, Weber 1972	Syn. of *H. solitaria* (Abbott and Currah 1997)	Syn. of *H. solitaria* (Landeros et al. 2012)
Helvella robusta S.P. Abbott, 1988	Abbott and Currah 1988	–	–	–	Abbott and Currah 1997	Valid species
Helvella solitaria P. Karst, 1871	–	Dissing 1966	Häffner 1987	–	Abbott and Currah 1997	Valid species
Helvella taiyuanensis B. Liu, Du & J.Z. Cao, 1985*	–	–	–	–	–	Neotypification here
Helvella tinta Q. Zhao, B. Feng & K.D. Hyde, 2016*	–	–	–	–	–	Valid species
Helvella ulvinenii Harmaja, 1979	Harmaja 1979	–	Häffner 1987	–	Abbott and Currah 1997	Syn. of *H. solitaria* (Landeros et al. 2015)
Helvella unicolor (Boud.) Dissing, 1966	Dissing 1966, Häffner 1987	–	–	–	Abbott and Currah 1997	In need of reassessment (Skrede et al. 2017)
Helvella verruculosa (Sacc.) Harmaja, 1978	–	–	–	–	Abbott and Currah 1997	In need of reassessment (Skrede et al. 2017)

Syn.: synonym; * indicates the species originally described from China.

inces were identified (Table 3). Morphological observations were conducted following Wang and Zhuang (2019). In measurements, Q refers to length/width ratio of ascospores for which the medians are given.

DNA extraction, PCR amplification and sequencing

Well-preserved specimens were selected for DNA extraction using a Plant Genomic DNA Kit (DP305, TIANGEN Biotech, Beijing, China). Partial Hsp90, ITS2, LSU and TEF1 were amplified by PCR using primers H_hspf and H_hspr (Skrede et al. 2017), ITS3 and ITS4 (White et al. 1990), LROR and LR5 (Vilgalys and Hester 1990) and EF1-983F and EF1-1567R (Rehner and Buckley 2005). Products were sequenced on an ABI 3730 DNA Sequencer (Applied Biosystems).

Table 3. Fungal species and sequences used in phylogenetic analyses.

Species	Voucher	Locality	*HSP90*	ITS	LSU	*TEF1*	Label	Reference
Balsamia aestivalis (R. Heim & L. Rémy) K. Hansen, Skrede & T. Schumach.	KH.10.133	Sweden	–	–	MK100250	MK113869	*Balsamia aestivalis*	Hansen et al. 2019
	O-253217	Norway	–	–	MK100251	MK113870	*Balsamia aestivalis*	Hansen et al. 2019
Balsamia platyspora Berk.	TUR206101	Finland	–	–	MK100252	MK113871	*Balsamia platyspora*	Hansen et al. 2019
Dissingia confusa (Harmaja) K. Hansen & X.H. Wang	H437*	Norway	KY784529	–	KY773164	–	*Helvella confusa*	Skrede et al. 2017
	HMAS 27728*	Qinghai, China	**MK652180**	**MK592119**	–	–	*Helvella confusa*	This study
	HMAS 38328*	Xinjiang, China	**MK652181**	**MK592120**	–	–	*Acetabula leucomelas*	This study
Dissingia crassitunicata (N.S. Weber) T. Schumach. & Skrede	H222*	Canada	KY784342	–	KY773053	–	*Helvella crassitunicata*	Skrede et al. 2017
Dissingia leucomelaena (Pers.) K. Hansen & X.H. Wang	H404, epitype	Sweden	KY784500	–	–	–	*Helvella leucomelaena*	Skrede et al. 2017
	H115*	USA	KY784253	–	KY772970	–	*Helvella leucomelaena*	Skrede et al. 2017
	KH.06.01 = H115	USA	–	–	KC012682	KC109207	*Helvella leucomelaena*	Hansen et al. 2013
	He273	Australia	–	–	JX993075	–	*Helvella leucomelaena*	Landeros et al. 2015
	He286, isotype	Italy	–	–	JX993051	–	*Acetabula calyx*	Landeros et al. 2015
	HMAS 61351	Denmark	**MK652201**	–	–	–	*Helvella leucomelaena*	This study
	HMAS 61356*	Sweden	**MK652202**	**MK592137**	–	–	*Helvella leucomelaena*	This study
Dissingia oblongispora (Harmaja) T. Schumach. & Skrede	H132*	Norway	KY784265	–	KY772983	–	*Helvella oblongispora*	Skrede et al. 2017
	HMAS 38329*	Xinjiang, China	**MK652203**	**MK592138**	–	–	*Helvella acetabulum*	This study
	HMAS 74657*	Gansu, China	**MK652204**	**MK592139**	–	–	*Helvella leucomelaena*	This study
	HMAS 75147*	Sichuan, China	**MK652205**	**MK592140**	–	**MK652162**	*Helvella leucomelaena*	This study
	HMAS 75151	Sichuan, China	**MK652206**	**MK592141**	–	–	*Helvella leucomelaena*	This study
	HMAS 75183	Sichuan, China	**MK652207**	**MK592142**	–	–	*Helvella leucomelaena*	This study
	HMAS 75960	Sichuan, China	**MK652208**	**MK592143**	–	–	*Helvella cupuliformis*	This study
	HMAS 86050	Xinjiang, China	–	**MK592144**	–	–	*Helvella acetabulum*	This study
	HMAS 86051	Xinjiang, China	–	**MK592145**	–	**MK652163**	*Helvella acetabulum*	This study
	HMAS 86160	Shanxi, China	–	**MK592146**	–	–	*Helvella leucomelaena*	This study
Helvella acetabuloides X.C. Wang & W.Y. Zhuang	HMAS 279703*, CFSZ 2044, holotype	Inner Mongolia, China	**MK652219**	**MK592155**	–	**MK652168**	*Helvella acetabulum*	This study
	HMAS 23842*	Shaanxi, China	**MK652220**	–	–	–	*Acetabula vulgaris*	This study
Helvella acetabulum (L.) Quél.	H410, epitype	Sweden	KY784506	–	KY773154	–	*Helvella acetabulum*	Skrede et al. 2017
	H133*	Norway	KY784266	–	KY772984	KY772875	*Helvella acetabulum*	Skrede et al. 2017
	HMAS 7046*	Czech	**MK652177**	**MK592116**	–	–	*Acetabula vulgaris*	This study
	HMAS 61353	Denmark	**MK652176**	–	–	–	*Helvella acetabulum*	This study
	HMAS 243823*	UK	**MK652174**	**MK592114**	**MK592099**	–	*Helvella acetabulum*	This study
	HMAS 23839	Qinghai, China	**MK652171**	**MK592112**	–	–	*Helvella acetabulum*	This study
	HMAS 23841	Beijing, China	**MK652172**	**MK592113**	–	–	*Helvella acetabulum*	This study
	HMAS 23843	Qinghai, China	**MK652173**	–	–	–	*Acetabula vulgaris*	This study
	HMAS 38129	Xinjiang, China	**MK652175**	**MK592115**	–	–	*Helvella acetabulum*	This study

Species	Voucher	Locality	*HSP90*	ITS	LSU	*TEF1*	Label	Reference
Helvella acetabulum (L.) Quél.	HMAS 75176*	Sichuan, China	**MK652178**	**MK592117**	–	**MK652156**	*Helvella acetabulum*	This study
Helvella arctoalpina Harmaja	H293, holotype	Norway	KY784406	–	–	–	*Helvella arctoalpina*	Skrede et al. 2017
	H033*	Norway	KY784207	–	KY772924	KY772841	*Helvella arctoalpina*	Skrede et al. 2017
Helvella calycina Skrede, T.A. Carlsen & T. Schumach.	H022*, epitype	Norway	KY784198	–	KY772915	KY772833	*Helvella calycina*	Skrede et al. 2017
	HMAS 279704*, CFSZ 2658	Inner Mongolia, China	**MK652179**	**MK592118**	**MK592100**	**MK652157**	*Helvella acetabulum*	This study
Helvella costata Schwein.	H100*	USA	KY784244	–	KY772962	–	*Helvella costata*	Skrede et al. 2017
Helvella costifera Nannf.	H298, epitype	Sweden	KY784409	–	–	–	*Helvella costifera*	Skrede et al. 2017
	H131*	Norway	KY784264	–	KY772982	KY772874	*Helvella costifera*	Skrede et al. 2017
	HMAS 61361	Shanxi, China	**MK652185**	–	–	–	*Helvella acetabulum*	This study
	HMAS 71778	Beijing, China	**MK652186**	**MK592124**	–	–	*Helvella costifera*	This study
	HMAS 83510	Xinjiang, China	**MK652187**	**MK592125**	–	–	*Helvella costifera*	This study
	HMAS 88497	Shanxi, China	**MK652188**	**MK592126**	–	–	*Helvella acetabulum*	This study
	HMAS 139024*	Shaanxi, China	**MK652182**	**MK592121**	**MK592101**	–	*Helvella* sp.	This study
	HMAS 187120*	Beijing, China	**MK652183**	**MK592122**	**MK592102**	**MK652158**	*Helvella* sp.	This study
	HMAS 280301*	Yunnan, China	**MK652184**	**MK592123**	**MK592103**	**MK652159**	*Helvella* sp.	This study
Helvella dryadophila Harmaja	H302, holotype	Norway	KY784412	–	–	–	*Helvella dryadophila*	Skrede et al. 2017
	H180*	Norway	KY784309	–	KY773024	KY772883	*Helvella dryadophila*	Skrede et al. 2017
Helvella floriforma Q. Zhao & K.D. Hyde	HKAS 90224, Holotype	Yunnan, China	–	–	KX239771	–	*Helvella floriforma*	Hyde et al. 2016
Helvella griseoalba N.S. Weber	He164, holotype	USA	–	–	JX993066	–	*Helvella griseoalba*	Landeros et al. 2015
	H306*	USA	KY784416	–	–	–	*Helvella griseoalba*	Skrede et al. 2017
Helvella hyperborea Harmaja	H491*	Finland	KY784569	–	–	–	*Helvella hyperborea*	Skrede et al. 2017
	HMAS 23840	Gansu, China	**MK652189**	–	–	–	*Helvella acetabulum*	This study
	HMAS 38331	Xinjiang, China	**MK652190**	–	–	–	*Helvella costifera*	This study
	HMAS 83506	Xinjiang, China	**MK652191**	**MK592127**	–	–	*Helvella costifera*	This study
	HMAS 83507	Xinjiang, China	**MK652192**	**MK592128**	–	–	*Helvella costifera*	This study
	HMAS 83508	Xinjiang, China	**MK652193**	**MK592129**	–	–	*Helvella costifera*	This study
	HMAS 83509	Xinjiang, China	**MK652194**	**MK592130**	–	–	*Helvella costifera*	This study
	HMAS 83511	Xinjiang, China	**MK652195**	**MK592131**	–	**MK652160**	*Helvella costifera*	This study
Helvella hyperborea Harmaja	HMAS 83512	Xinjiang, China	**MK652196**	**MK592132**	–	–	*Helvella costifera*	This study
	HMAS 85476	Xinjiang, China	**MK652197**	**MK592133**	–	–	*Helvella acetabulum*	This study
	HMAS 85591*	Shanxi, China	**MK652198**	**MK592134**	–	–	*Helvella leucomelaena*	This study
	HMAS 85673*	Shanxi, China	**MK652199**	**MK592135**	–	–	*Helvella solitaria*	This study
	HMAS 86043*	Xinjiang, China	**MK652200**	**MK592136**	–	**MK652161**	*Helvella costifera*	This study
Helvella robusta S.P. Abbott	He163, holotype	Canada	–	–	JX993079	–	*Helvella robusta*	Landeros et al. 2015
Helvella sichuanensis X.C. Wang & W.Y. Zhuang	10706*, HMAS 254610, holotype	Sichuan, China	**MK652221**	**MK592156**	**MK592107**	**MK652169**		This study

Species	Voucher	Locality	*HSP90*	ITS	LSU	*TEF1*	Label	Reference
Helvella solitaria P. Karst.	H370, epitype	Sweden	KY784470	–	–	–	*Helvella solitaria*	Skrede et al. 2017
	H004*	Norway	KY784184	–	KY772902	KY772819	*Helvella solitaria*	Skrede et al. 2017
	He248, holotype	Finland	–	–	JX993085	–	*Helvella ulvinenii*	Landeros et al. 2015
	HMAS 41140*	Netherlands	**MK652211**	**MK592148**	–	–	*Helvella queletii*	This study
	HMAS 58371	Czech	**MK652212**	–	–	–	*Helvella queletii*	This study
	HMAS 27727*	Qinghai, China	**MK652209**	**MK592147**	–	–	*Helvella confusa*	This study
	HMAS 27951	Jilin, China	**MK652210**	–	–	–	*Helvella confusa*	This study
	HMAS 73509	Sichuan, China	**MK652213**	**MK592149**	–	–	*Helvella acetabulum*	This study
	HMAS 75175*	Sichuan, China	**MK652214**	**MK592150**	–	**MK652164**	*Helvella leucomelaena*	This study
Helvella taiyuanensis B. Liu, Du & J.Z. Cao	HMAS 85689*, neotype	Shanxi, China	**MK652217**	**MK592153**	–	–	*Helvella taiyuanensis*	This study
	HMAS 277500*	Yunnan, China	**MK652216**	**MK592152**	**MK592105**	**MK652166**	*Helvella* sp.	This study
	11925*, HMAS 254611	Beijing, China	**MK652215**	**MK592151**	**MK592104**	**MK652165**		This study
	MCCNNU 6499*, HMAS 279702	Hubei, China	**MK652218**	**MK592154**	**MK592106**	**MK652167**	*Helvella solitaria*	This study
Helvella tianshanensis X.C. Wang & W.Y. Zhuang	HMAS 86040*, holotype	Xinjiang, China	**MK652222**	**MK592157**	**MK592108**	**MK652170**	*Helvella costifera*	This study
	HMAS 88611*	Xinjiang, China	**MK652223**	**MK592158**	–	–	*Helvella acetabulum*	This study
Helvella tinta Q. Zhao, B. Feng & K.D. Hyde	HKAS 82560, holotype	Sichuan, China	–	KX239842	KX239772	–	*Helvella tinta*	Hyde et al. 2016
Helvella crispa (Scop.) Fr.	H408*, epitype	Sweden	KY784504	–	–	–	*Helvella crispa*	Skrede et al. 2017
	H135	Norway	KY784268	–	KY772986	–	*Helvella crispa*	Skrede et al. 2017
	HKAS 75434	Germany	–	JX462572	KR493479	KT254487	*Helvella crispa*	Zhao et al. 2015
Helvella elastica Bull.	H066*	Sweden	KY784230	–	KY772950	KY772858	*Helvella elastica*	Skrede et al. 2017
Helvella lacunosa Afzel.	H407, epitype	Sweden	KY784503	–	KY773152	–	*Helvella lacunosa*	Skrede et al. 2017
	H039*	Norway	KY784213	–	KY772930	KY772845	*Helvella lacunosa*	Skrede et al. 2017
Helvella macropus (Pers.) P. Karst.	H412, epitype	Sweden	KY784507	–	–	–	*Helvella macropus*	Skrede et al. 2017
	H073*	Norway	KY784233	–	KY772954	KY772863	*Helvella macropus*	Skrede et al. 2017
Midotis lingua Fr.	H283*	Switzerland	KY784397	–	KY773093	–	*Wynnella silvicola*	Skrede et al. 2017
	HMAS 67962*	Germany	**MK652224**	**MK592159**	**MK592109**	–	*Wynnella auricula*	This study
	HMAS 71896*	Shanxi, China	**MK652225**	**MK592160**	**MK592110**	–	*Wynnella silvicola*	This study
	HMAS 74656	Gansu, China	**MK652226**	**MK592161**	**MK592111**	–	*Helvella silvicola*	This study
	HMAS 83548	Xinjiang, China	**MK652227**	**MK592162**	–	–	*Wynnella auricula*	This study
Pindara terrestris Velen.	KH.12.67	Sweden	–	–	MK100279	MK113889	*Pindara terrestris*	Hansen et al. 2019
	S-F327988	Sweden	–	–	MK100280	MK113896	*Pindara terrestris*	Hansen et al. 2019
	T. Kekki 168	Finland	–	–	MK100281	MK113897	*Pindara terrestris*	Hansen et al. 2019
Underwoodia columnaris Peck	Kanouse 1951	USA	–	–	U42685	–	*Underwoodia columnaris*	O'Donnell et al. 1997

* Taxa included in the four-locus sequence analysis; Note: GenBank accession numbers in bold indicating the newly generated sequences.

Phylogenetic analyses

Sequences obtained from this study and those retrieved from GenBank are listed in Table 3. Four single gene datasets and two combined datasets were compiled. Sequences were aligned using MAFFT 7.221 (Katoh and Standley 2013) and subsequently processed with BioEdit 7.1.10 (Hall 1999). A Maximum-Likelihood (ML) tree for each single gene data was generated using MEGA 6.0.6 (Tamura et al. 2013) with the most suitable nucleotide substitution model and 1,000 replicates of bootstrap (BP) tests. For the combined four-gene dataset, the ML tree was determined using RAxML-HPC2 on XSEDE 8.2.12 on CIPRES Science Gateway (Miller et al. 2010) with the default GTRCAT model. Bayesian Inference (BI) analysis was performed with MrBayes 3.2.6 (Ronquist et al. 2012) using a Markov Chain Monte Carlo (MCMC) algorithm. Appropriate nucleotide substitution models and parameters were determined via MrModelTest 3.7 (Posada and Crandall 1998). The first 25% of the trees were excluded as the burn-in phase and posterior probability (PP) values were estimated with the remaining 75% of trees. *Helvella crispa* (Scop.) Fr., *H. elastica* Bull., *H. lacunosa* Afzel. and *H. macropus* (Pers.) P. Karst. are the representatives of the formerly recognised sections *Crispae*, *Elasticae*, *Lacunosae* and *Macropodes*, respectively. *Midotis lingua* Fr. served as the outgroup taxon of the four-gene phylogeny and *Underwoodia columnaris* Peck worked for the two-gene analysis.

Results

Fifty-one specimens of the rib-stiped cupulate species of *Helvella* s. l. deposited in HMAS and five recent collections were examined. A total of 125 sequences of the *Helvella* and *Dissingia* samples and 11 of the outgroup taxa were submitted to GenBank (Table 3).

The combined four-locus dataset included 48 taxa of *Helvella* s. s. and *Dissingia* in an alignment of 1788 bp, including 236 bp of Hsp90, 348 bp of ITS2, 690 bp of LSU and 514 bp of TEF1. Kimura 2-parameter (K2) with gamma distribution (+G) was determined as the most suitable model for ML analysis. Tamura-Nei with gamma distribution and invariant sites (TrN+I+G) was selected by Akaike Information Criterion as the best fit for the BI analysis. As shown in Figure 1, three clades and some independent lineages were recognised amongst the cupulate taxa of *Helvella* s. s. Clade 1 consisted of *H. calycina*, *H. costifera* and *H. tianshanensis*; Clade 2 included *H. solitaria* and *H. taiyuanensis*; and Clade 3 contained *H. acetabuloides*, *H. acetabulum*, *H. arctoalpina*, *H. costata* and *H. sichuanensis*. *Helvella dryadophila*, as an independent lineage, was sister to Clade 3, which was not supported by two of the single gene analyses (Suppl. material 1: Figures S1 and S4). *Helvella griseoalba* and *H. hyperborea* were situated outside the clades in all analyses.

The combined LSU and TEF1 dataset was comprised of 38 taxa of *Balsamia*, *Dissingia*, *Helvella*, *Midotis*, *Pindara* and *Underwoodia*. The alignment is of 1239 bp, including 711 bp of LSU and 528 bp of TEF1. Tamura-Nei with gamma distribution (TN93+G) was determined as the most suitable model for ML analysis.

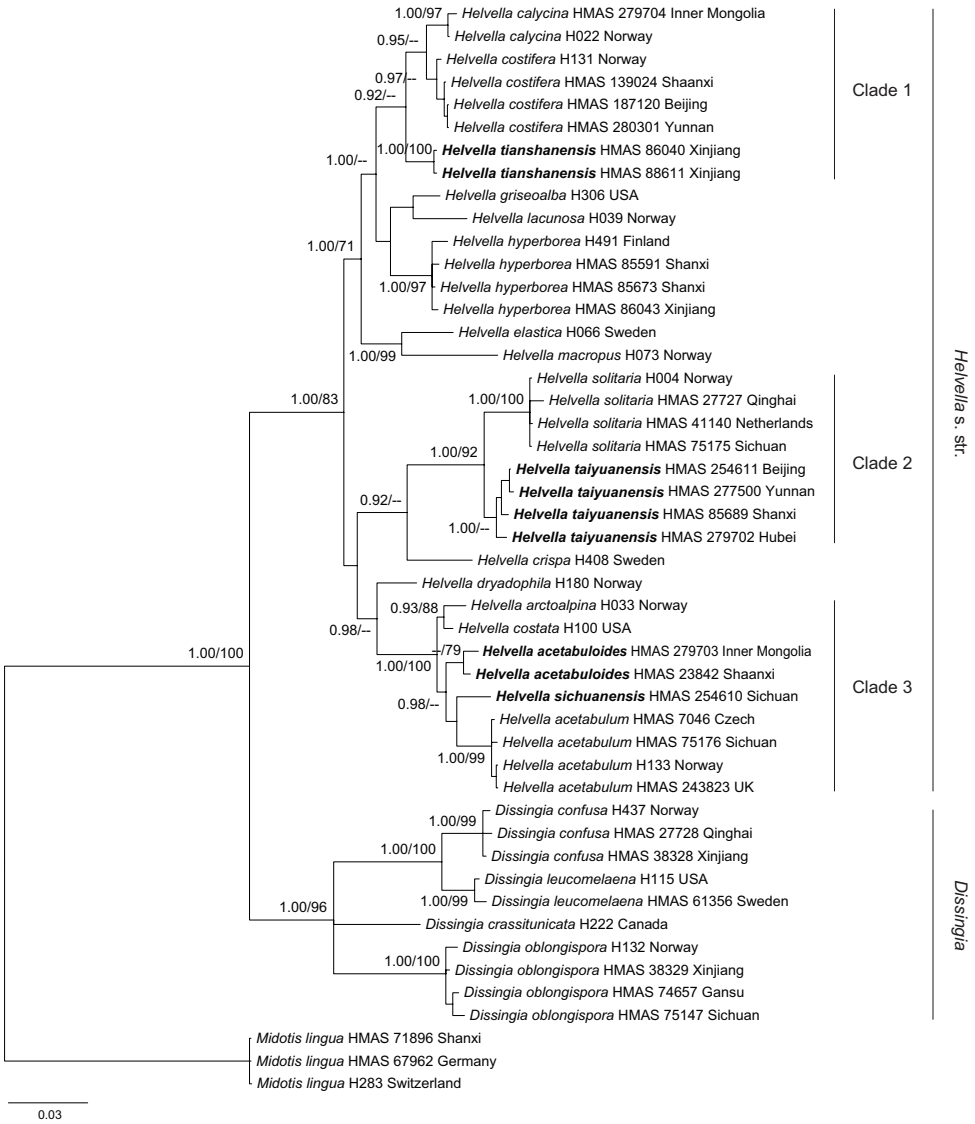

Figure 1. Bayesian phylogenetic tree of *Helvella* and *Dissingia* inferred from combined Hsp90, ITS2, LSU and TEF1 dataset. Posterior probability values ≥ 0.90 (left) and bootstrap values ≥ 70% (right) are indicated at nodes.

Clades 1–3 were supported and *H. dryadophila* was outside Clade 3 (Figure 2), which are congruent with the four-gene analysis (Figure 1).

The Hsp90 dataset consisted of 84 sequences of *Helvella* and *Dissingia*. K2+G was determined as the most suitable model for ML analysis. Clades 2 and 3 were monophyletic, but Clade 1 was poorly supported (Suppl. material 1: Figure S1). The positions of the three undescribed species were consistent with that of the four-locus phylogeny.

Figure 2. Maximum likelihood phylogeny of Helvellaceae inferred from combined LSU and TEF1 dataset. Bootstrap values ≥ 50% are indicated at nodes.

The ITS2 dataset possessed 53 taxa of *Helvella* and *Dissingia*. Tamura 3-parameter with gamma distribution (T92+G) was determined as the most suitable model for ML analysis. Clades 1–3 were strongly supported. *Helvella tinta*, excluded from these clades, appeared to be sister of *H. hyperborea* (Suppl. material 1: Figure S2).

The LSU dataset comprised 40 sequences of *Helvella* and *Dissingia*. TN93+G was determined as the most suitable model for ML analysis. Clades 1–3 of *Helvella* were monophyletic, in which *H. floriforma* and *H. robusta*, absent in other trees, were located. *Dissingia* seemed to be not monophyletic (Suppl. material 1: Figure S3).

The TEF1 dataset consisted of 26 taxa of *Helvella* and *Dissingia*. K2+G was determined as the most suitable model for ML analysis. Clades 1–3 of *Helvella* were strongly supported (Suppl. material 1: Figure S4) and the phylogenetic positions of the three undescribed species recalled that of the multigene phylogeny (Figure 1).

Taxonomy

New species

Helvella acetabuloides **X.C. Wang & W.Y. Zhuang, sp. nov.**
Fungal Names: FN 570634
Figure 3a–d

Holotype. CHINA. Inner Mongolia Autonomous Region, Chifeng City, Harqin Banner, Shijia Town, Toudaoyingzi Village, 41°53'20"N, 119°1'1"E, on the ground under *Ostryopsis davidiana* Decne., 8 Aug 2002, T.Z. Liu & T.H. Liu, HMAS 279703 (= CFSZ 2044).

Etymology. The species epithet refers to its similarity to *H. acetabulum*.

Description. Apothecia stipitate to subsessile, cupulate, margin undulate, involute or revolute, 2.2–4.8 cm high and 2.5–4 cm diam. when dry; hymenium dull brown to reddish-brown when dry, receptacle surface light brown to brown when dry, glabrous; stipe terete or flattened, buff, light yellowish-brown to brown, surface ribbed, 0.5–3 × 0.4–1.3 cm, typically fluted with sharp-edged or rarely blunt ribs, ribs branching at the upper half of receptacle surface, reaching to the edge or ending 1–2 mm from the edge. Ectal excipulum of *textura angularis*, 75–100 µm thick, cells hyaline, outer cells arranged in chains, 16–21.5 × 7–8 µm. Medullary excipulum of *textura intricata*, 180–220 µm thick, hyphae hyaline. Asci subcylindrical, tapering and with crozier at base, 8-spored, 235–280 × 15–20 µm. Paraphyses filiform with apical portion very slightly enlarged, septate, hyaline, 4.5–5.5 µm wide at apex and 4–4.5 µm below. Ascospores ellipsoidal, hyaline, smooth, uniguttulate, 14–20 × 10–14.5 µm, median 16.2 × 12.3 µm, Q = 1.2–1.55, median 1.375, n = 50.

Additional specimen examined. CHINA. Shaanxi Province, Baoji City, Taibai County, Mt. Taibai, 34°1'53"N, 107°25'33"E, alt. 2270 m, on the ground in broadleaf forest, 26 Jun 1958, J.H. Yu 106, HMAS 23842.

Notes. *Helvella acetabuloides* is nested with *H. acetabulum*, *H. arctoalpina*, *H. costata* and *H. sichuanensis* in Clade 3 (Figure 1). Its hymenium is reddish-brown when dry and different from that of *H. acetabulum* (brown when dry) and those of *H. arctoalpina* and *H. sichuanensis* (black when dry, Harmaja 1977b). The two specimens cited are identical in sequences of *Hsp90*. *Helvella acetabuloides* differs from *H. acetabulum* in 6 bp for Hsp90 (H410, epitype), 14 bp for ITS2 (HMAS 243823) and 17 bp for TEF1 (H133). It is distinguished from *H. arctoalpina* in 2 bp of Hsp90 (H293, holotype) and 11 bp of TEF1 (H033), from *H. costata* in 3 bp of Hsp90. It differs from *H. sichuanensis* in 1 bp of Hsp90, 20 bp of ITS2 and 11 bp of TEF1. PCR amplification of LSU failed.

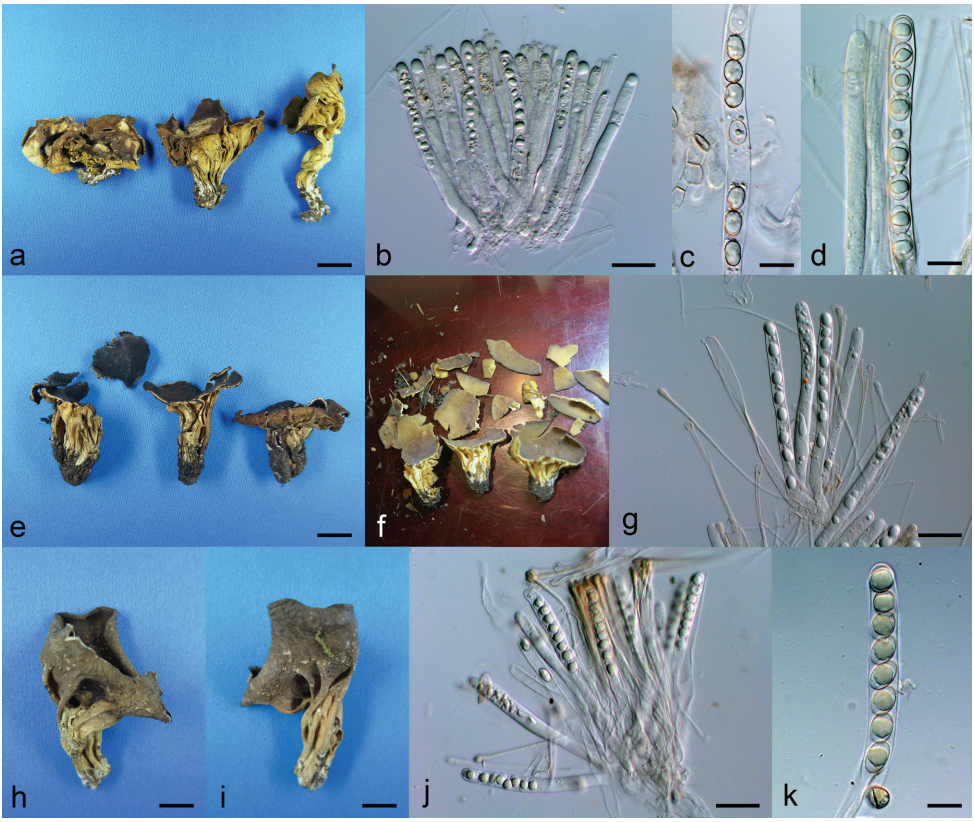

Figure 3. a–d *Helvella acetabuloides:* **a** mature apothecia when dry (CFSZ 2044) **b** asci (HMAS 23842) **c, d** ascospores in ascus (c: CFSZ 2044, d: HMAS 23842) **e–g** *Helvella sichuanensis* (HMAS 254610): **e** mature apothecia when dry **f** mature apothecia when fresh **g** ascospores in asci **h–k** *Helvella tianshanensis* (HMAS 86040): **h, i** Mature apothecium when dry **j** asci **k** ascospores in ascus. Scale bars: 1 cm (**a, e**); 0.75 cm (**h, i**); 50 µm (**b, g, j**); 20 µm (**c, d, k**).

***Helvella sichuanensis* X.C. Wang & W.Y. Zhuang, sp. nov.**
Fungal Names: FN 570635
Figure 3e–g

Holotype. CHINA. Sichuan Province, Garzê Tibetan Autonomous Prefecture, Daocheng County, Yading National Nature Reserve, 28°25'6"N, 100°21'26"E, alt. 3900 m, on the ground of mixed forest, 18 Aug 2016, J.P. Wang & X.C. Wang 10706, HMAS 254610.

Etymology. The species epithet refers to the type locality of the fungus.

Description. Apothecia stipitate, shallow-cupulate, margin entire and flattened when fresh, undulate, involute or revolute when dry, 5–6 cm diam. when fresh and 2.5–3.5 cm high when dry; hymenium yellowish-brown when fresh, nearly black when dry, receptacle surface buff to light brown when fresh, light brown to dark brown when dry, glabrous; stipe terete or flattened, buff to light brown, surface ribbed, 2.5–3 × 1.5–3 cm when fresh, 2–2.5 × 0.5–1.5 cm when dry, typically fluted with sharp-edged or rarely

blunt ribs, ribs branching at the upper half of receptacle surface, reaching to the edge or ending 3–5 mm from the edge. Ectal excipulum of *textura angularis*, 100–180 µm thick, cells hyaline to light brown, outer cells 15–45 × 9–35 µm. Medullary excipulum of *textura intricata*, 300–500 µm thick, hyphae hyaline. Asci subcylindrical, tapering and with crozier at base, 8-spored, 225–325 × 13–18.5 µm. Paraphyses filiform with apical portion obviously swollen, septate, hyaline to light brown, 7–10.5 µm wide at apex and 3–4.5 µm below. Ascospores ellipsoidal, hyaline, smooth, uniguttulate, 15.5–18.5 × 10–12.5 µm, median 16.9 × 11.2 µm, Q = 1.3–1.7, median 1.48, n = 40.

Notes. *Helvella sichuanensis* belongs to Clade 3 (Figure 1). Its hymenium is nearly black when dry, which is similar to that of *H. arctoalpina*, but different from those in *H. acetabulum* (brown when dry) and *H. acetabuloides* (reddish-brown when dry). When fresh, the hymenium is yellowish-brown, while that of *H. arctoalpina* is brown. Molecularly, it differs from *H. acetabulum* in 7 bp of Hsp90 (H410, epitype), 14 bp of ITS2 (HMAS 243823), 17 bp of LSU (H133) and 15 bp of TEF1 (H133); from *H. arctoalpina* in 1 bp of Hsp90 (H293, holotype), 25 bp of LSU (H033) and 11 bp of TEF1 (H033); and from *H. costata* in 2 bp of Hsp90 and 13 bp of LSU. The sequence divergences between *H. sichuanensis* and *H. acetabuloides* are 1 bp of Hsp90, 20 bp of ITS2 and 12 bp of TEF1.

***Helvella tianshanensis* X.C. Wang & W.Y. Zhuang, sp. nov.**
Fungal Names: FN 570636
Figure 3h–k

Holotype. CHINA. Xinjiang Uygur Autonomous Region, Changji Hui Autonomous Prefecture, Jimsar County, 43°59'44"N, 89°10'31"E, alt. 1700 m, on the ground, 31 Jul 2003, W.Y. Zhuang & Y. Nong 4661, HMAS 86040.

Etymology. The species epithet refers to the type locality of the fungus.

Description. Apothecia stipitate, cupulate, margin undulate, involute, 2.5–3.5 cm high and 2–3 cm diam. when dry; hymenium greyish-brown, brown to dark brown, receptacle surface yellowish-brown to brown; stipe terete or flattened, buff, yellowish-brown, orange brown to brown, surface ribbed, 2–2.5 × 0.5–1.3 cm, typically fluted with rarely blunt ribs, ribs branching at the upper half of receptacle surface, reaching to the edge or ending 3–12 mm from the edge. Ectal excipulum of *textura angularis*, 120–150 µm thick, hyphae hyaline, outer cells 35–40 × 20–40 µm. Medullary excipulum of *textura intricata*, 350–600 µm thick, hyphae hyaline. Asci subcylindrical, tapering and with crozier at base, 8-spored, 240–275 × 12–24 µm. Paraphyses filiform, slightly enlarged at apical portion, septate, hyaline to light brown, 6–7.5 µm wide at apex and 3–4.5 µm below. Ascospores ellipsoidal, hyaline, smooth, uniguttulate, 17–21 × 11.5–13.5 µm, median 18.8 × 12.3 µm, Q = 1.35–1.7, median 1.51, n = 30.

Additional specimen examined. CHINA. Xinjiang Uygur Autonomous Region, Urumqi City, Urumqi County, 43°28'47"N, 87°27'27"E, 12 Aug 1985, L. Fan & K. Tao 161, HMAS 88611.

Notes. *Helvella tianshanensis* nested with *H. calycina* and *H. costifera* in Clade 1 (Figure 1). These three species are hardly separated by gross morphology and anatomic structures. *Helvella tianshanensis* differs from *H. calycina* in 4 bp of Hsp90 (H022, epitype), 16 bp of ITS2 (HMAS 279704), 9 bp of LSU (H022) and 15 bp of TEF1 (H022); and it is different from *H. costifera* in 3 bp of Hsp90 (H298, epitype), 12 bp of ITS2 (HMAS 187120), 11 bp of LSU (H131) and 13 bp of TEF1 (H131). The two specimens of the new species are identical in Hsp90 and ITS2.

New Chinese record

***Helvella calycina* Skrede, T.A. Carlsen & T. Schumach., Persoonia 39: 221, 2017**

Specimen examined. CHINA. Inner Mongolia Autonomous Region, Xilingol League, Zhenglan Banner, Yihehaierhan Sumu, 42°23'8"N, 116°10'17"E, 21 August 2005, on the ground, T.Z. Liu & X.L. Bai, HMAS 279704 (= CFSZ 2658).

Notes. *Helvella calycina* is a new record for China. It was known only from Norway and Denmark. The Chinese collection extends its distribution to Asia. The Chinese collection is identical with the epitype in TEF1 but with 2 bp differences for Hsp90 and 1 bp for LSU.

Neotypification

***Helvella taiyuanensis* B. Liu, Du & J.Z. Cao, Acta Mycol. Sin. 4(4): 211, 1985**
Fungal Names: FN 570637
Figure 4

Neotype is designated here. CHINA. Shanxi Province, Lvliang City, Jiaocheng County, Guandishan National Forest Park, 37°54'25"N, 111°35'40"E, on the ground in mixed forest, 16 Jul 1987, Y.M. Li, HMAS 85689 (= MHSU 758).

Additional specimens examined. CHINA. Beijing City, Mentougou District, Xiaolongmen National Forest Park, 39°58'2"N, 115°26'43"E, alt. 1100 m, on the ground in mixed forest, 4 Aug 2018, X.C. Wang et al. 11925, HMAS 254611. Hubei Province, Yichang City, Xingshan County, Longmenhe National Forest Park, 31°21'12"N, 110°30'40"E, on the ground, 23 Jul 2017, R. Wang & X. Zhang 420526MF0679, MCCNNU 6499, HMAS 279702. Yunnan Province, Diqing Tibetan Autonomous Prefecture, Dêqên County, Yunling Town, Meili Snow Mountain, 28°23'23"N, 98°47'49"E, alt. 3150 m, on the ground, 12 Aug 2016, Y. Li 920, HMAS 277500.

Notes. This species was originally described, based on a single specimen collected by Y.M. Li from Taiyuan City, Shanxi Province in 1983 (Holotype: HBSU 2449, Liu et al. 1985). Unfortunately, the type specimen was destroyed by a fire in MHSU

Figure 4. *Helvella taiyuanensis* **a** specimen sheet (HMAS 85689) **b** mature apothecia when dry (HMAS 85689) **c** mature apothecia when fresh (HMAS 254611) **d** mature apothecia when fresh (HMAS 277500) **e** mature apothecium when fresh (HMAS 279702) **f–h** ascospores in ascus (**f, g**: HMAS 85689, **h**: HMAS 254611). Scale bars: 0.8 cm (**b, d**); 2 cm (**c**); 20 μm (**f**), applies to **g, h**.

in 1984 (Cao 1988, Cao et al. 1990). To protect fungal collections after the fire, the remaining specimens, deposited in MHSU, were moved to HMAS. The neotype specimen HMAS 85689 was collected by the same collector as the type specimen of *H. taiyuanensis* and identified by one of the original authors J.Z. Cao (Cao 1988). Its detailed morphological characteristics are in accordance with the original description. We thus treat it as authentic material. As other specimens were neither cited in the protologue nor filed under this name, we thus designate HMAS 85689 as the neotype specimen of *H. taiyuanensis*.

Helvella taiyuanensis was once treated as a synonym of *H. solitaria* sensu Dissing (1966), based on morphological features (Cao 1988), but the molecular differences between them are clear in the multigene analysis (Figure 1). It should be a tenable species. The four specimens of the fungus examined are variable in colour of the hymenium and receptacle surface when dry or fresh, but stable in cupulate to saddle-shaped apothecia (Figure 4). Phylogenetic analyses indicate that they belong to the same species (Figures 1, 2 and Suppl. material 1: S1–S4) although minor sequence divergences exist amongst collections. The maximum sequence divergences amongst collections are 1 bp in Hsp90, 6 bp in ITS2, 3 bp in LSU and 7 bp in TEF1.

Discussion

A total of about 28 rib-stiped cupulate species of *Helvella* and *Dissingia* have been reported in the world (Table 2) and 17 of them were investigated in this study. With the discovery of the three new species and one new record, 13 species were confirmed to be distributed in China. Amongst them, six are known only from China, five

(*D. oblongispora*, *H. acetabulum*, *H. calycina*, *H. costifera* and *H. hyperborea*) are found in Europe and China and *D. confusa* and *H. solitaria* are widespread in Europe, Asia and North America. Amongst the Chinese helvellas, *H. acetabulum*, *H. costifera* and *H. taiyuanensis* show a relatively wide distribution range and occur in at least four provinces. However, *H. calycina*, *H. floriforma*, *H. sichuanensis*, *H. tianshanensis* and *H. tinta* were known only from a single locality. Eight species are in northwest China (Gansu, Qinghai, Shaanxi and Xinjiang), eight in the southwest (Sichuan and Yunnan) and seven in the north (Beijing, Inner Mongolia and Shanxi). However, the Chinese record of *H. leucomelaena* (≡ *D. leucomelaena*) (Teng 1963, Tai 1979, Zhuang 1998) is questionable since many specimens in HMAS, filed under that name, were based on misidentifications (Table 3).

As shown in the multigene phylogeny (Figure 1), three clades were formed amongst the investigated species. The cupulate *Helvella* taxa are clustered or mixed with the saddle-shaped ones. This gives the hint that the apothecial shape changed several times during the evolution. Clade 2, Clade 3 and *H. dryadophila* belong to the acetabulum-solitaria lineage (Skrede et al. 2017); however, this lineage was not herein supported due to joining of the non-cupulate species *H. crispa*. Clade 1 is in accordance with the costifera lineage (Skrede et al. 2017) with the addition of *H. tianshanensis*. Our results clearly support the separation of *Dissingia* from *Helvella* s. l. (Hansen et al. 2019).

Supplementary DNA barcodes are essential for delimitation of *Helvella* species. LSU is the most commonly used region for *Helvella* species identification (Nguyen et al. 2013, Landeros et al. 2015, Skrede et al. 2017). LSU is capable of distinguishing cupulate *Helvella* species (Suppl. material 1: Figure S3); whereas, its PCR amplification success rate is low (10/56), especially for specimens subject to long storage. A similar situation is witnessed in TEF1, which was suggested as a secondary barcode for fungi (Stielow et al. 2015). Although the primers for this region were reported working well on DNAs extracted from fresh materials, the amplifications from dried *Helvella* specimens were not easy (Skrede et al. 2017). The amplification success rate of TEF1 in our study was again low (15/56). Hsp90 was first applied to *Helvella* by Skrede et al. (2017) and is recommended due to its short sequence length, high amplification success rate, usefulness in species delimitation and its reasonable phylogenetic informative properties. It was successfully amplified from 53 of the 56 specimens studied and is able to distinguish all the involved species (Suppl. material 1: Figure S1). RPB2 was also applied in the recent studies (Skrede et al. 2017, Hansen et al. 2019), but did not work well since the amplicons of the newly designed primers, H_rpb2r2 and H_rpb2f, had a lower species resolution than that of Hsp90. The fragment is also too short to align with the existing sequences in GenBank.

ITS is recommended as the universal barcode for fungi (Schoch et al. 2012), which is applied widely to elucidate species diversity of the pezizalean ectomycorrhizae (Tedersoo et al. 2006, Healy et al. 2013, Hwang et al. 2015). However, very limited ITS sequences of cupulate *Helvella* species were available in GenBank. The trials of obtaining ITS amplicons, using the universal primers for many *Helvella* species, usually failed owing to primer mismatch (Skrede et al. 2017). The success rate of ITS amplification in our work was extremely low (2/56) upon using the primer pairs ITS5

and ITS4. Functional *Helvella*-specific ITS primers are expected to be developed. Our amplifications of the ITS2 region by the primers ITS3 and ITS4 reached a relative high success rate (47/56) with the tested species well separated (Suppl. material 1: Figure S2). We thus propose to use Hsp90 and ITS2 as supplementary DNA barcodes for rib-stiped cupulate species of *Helvella*.

Acknowledgements

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Supplementary material 1

Figures S1–S4

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Data type: phylogenetic data

Explanation note: **Figure S1.** Maximum-likelihood phylogenetic tree of *Helvella* and its allies inferred from Hsp90 dataset. Bootstrap values ≥ 50% are indicated at nodes. **Figure S2.** Maximum-likelihood phylogenetic tree of *Helvella* and its allies inferred from ITS2 dataset. Bootstrap values ≥ 50% are indicated at nodes. **Figure S3.** Maximum-likelihood phylogenetic tree of *Helvella* and its allies inferred from LSU dataset. Bootstrap values ≥ 50% are indicated at nodes. **Figure S4.** Maximum-likelihood phylogenetic tree of *Helvella* and its allies inferred from TEF1 dataset. Bootstrap values ≥ 50% are indicated at nodes.

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The genus *Simplicillium*

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Abstract

Simplicillium species have a wide host range and an extensive distribution. Some species are associated with rusts, as well as other plant pathogenic fungi and play an important role in biological control. In this study, two specimens of *Simplicillium* were collected from Chiang Mai Province, Thailand. *Simplicillium formicae* sp. nov. was isolated from an infected ant and *S. lanosoniveum* from *Ophiocordyceps unilateralis* which is a new host record. Species were initially identified using ITS gene sequences and confirmed using morphology coupled with phylogenetic analyses of a combined nrLSU, nrSSU, TEF and RPB1 dataset. *Simplicillium formicae* differs from other species in the genus by the presence of flask-shaped synnemata and phialides with intercalary nodes. *Simplicillium lanosoniveum* resembles other collections of the species by its completely solitary, tapering phialides and globose to ellipsoidal conidia which adhere in a slimly head. A key to species of *Simplicillium* is also provided.

Keywords

new species, Thailand, ant fungi, taxonomy, phylogeny

Introduction

Zare and Gams (2001) introduced *Simplicillium* to accommodate four taxa including the type species *S. lanosoniveum* and three other species, *S. lamellicola*, *S. obclavatum* and *S. wallacei*. *Simplicillium* species were historically placed in *Verticillium* sect. *Prostrata* which was described by Gams (1971) for prostrate conidiophore-producing species. Later, most of the species of *Verticillium* sect. *Prostrata* were reported as members in Clavicipitaceae, based on molecular data (including SSU, LSU and ITS sequences), whereas *Simplicillium* species consistently formed a monophyletic group apart from the other described taxa in this family (Zare et al. 2000; Gams and Zare 2001; Sung et al. 2001; Zare and Gams 2001). Recently, Clavicipitaceae was divided into three families, based on multi-gene phylogenetic analyses and *Simplicillium* was assigned to Cordycipitaceae (Hypocreales, Hypocreomycetidae, Sordariomycetes) (Sung et al. 2007; Maharachchikumbura et al. 2016; Wijayawardene et al. 2018). Zare and Gams (2008) excluded *Simplicillium wallacei* from *Simplicillium* and transferred it to *Lecanicillium* due to the basal position being closer to the latter genus than to the former genus in the cladogram of ITS data. Subsequently, ten species viz. *Simplicillium chinense* (Liu and Cai 2012), *S. aogashimaense*, *S. cylindrosporum*, *S. minatense*, *S. subtropicum*, *S. sympodiophorum* (Nonaka et al. 2013), *S. lanosoniveum* var. *tianjinensis* (Dong et al. 2014), *S. calcicola* (Zhang et al. 2017), *S. coffeanum* (Gomes et al. 2018) and *S. filiforme* (Crous et al. 2018) were restricted to *Simplicillium*, based on the phylogenetic analyses of ITS sequence data and strong morphological evidence. Its sexual-asexual connection has been established with *S. lanosoniveum* linked to a *Torrubiella* sp. (Zare and Gams 2001).

Simplicillium species have a wide distribution and are considered as mammal and plant-parasitic, symbiotic, entomopathogenic, fungicolous and nematophagous fungi, as they have a broad spectrum of hosts and substrates, such as insects, plants, rusts, nematodes, human nails, canine tissues and mushrooms, *Chroococcus* sp., soil, freshwater, marine and terrene environments (Zare and Gams 2001; Guo et al. 2012; Liu and Cai 2012; Dong et al. 2014; Liang et al. 2016; Sun et al. 2019). Several studies have been shown that *Simplicillium* species have a high ecological and economical value for biocontrol and bioactive compounds (Takata et al. 2013; Yan et al. 2015; Hyde et al. 2019). For example, *Simplicillium lanosoniveum* can be a phytopathogen, causing brown spots and lesions on flowers (Chen et al. 2008) or a mycoparasite on soybean rust (Ward et al. 2012; Gauthier et al. 2014) or a pathogen on aphids and other phytopathogens (Chen et al. 2017) or an anti-*Trichomonas vaginalis* agent (Scopel et al. 2013). *Simplicillium chinense* can be a biological control agent against plant parasitic nematodes (Zhao et al. 2013; Luyen 2017). *Simplicillium lamellicola* can suppress plant bacterial diseases and grey mould diseases of tomato (*Solanum lycopersicum*) and ginseng (*Panax ginseng*) (Dang et al. 2014; Shin et al. 2017). *Simplicillium obclavatum* has the ability to produce multiple xylanases and endoglucanases that have the potential to be used in biofuels, animal feed and food industry applications (Roy et al. 2013). Bioactive compounds with anti-fungal and anti-bacterial profiles and pharma-

ceutical exopolysaccharides have been isolated from *S. lanosoniveum* (Yu et al. 2013; Fukuda et al. 2014; Xing et al. 2016; Dong et al. 2018). Linear and cyclic peptides with anti-fungal and anti-viral properties have also been discovered from the secondary metabolites of *S. obclavatum* (Liang et al. 2016, 2017).

Recent studies have shown that Thailand supports an amazing fungal diversity with numerous new species that have the potential for biotechnological application (Hyde et al. 2018, 2019). In this study, we introduce a novel species, *Simplicillium formicae* from northern Thailand and a new record of *S. lanosoniveum* with evidence from a combination of molecular analyses and morphological characteristics to reserve a natural resource for future studies regarding biocontrol in the forestry, agricultural and pharmaceutical industries.

Material and methods

Sample collection and isolation

The Mushroom Research Centre (MRC) is a disturbed rainforest located in Chiang Mai Province, Thailand (Aung et al. 2008). The forest consists of various tall tree and lower shrubs. The climate of Chiang Mai is controlled by tropical monsoons and the weather is typically hot and humid with temperatures often close to or above 30 °C. Frequent rain and thunder showers usually last from June to late October (Chiang Mai Buddy website: https://chiangmaibuddy.com/welcome-to-chiang-mai/weather-and-climate/, accessed 26.8.2019). Two ant fungi were found anchored to the underside of two different shrubby leaves in the forest at the Mushroom Research Centre. These two fresh specimens; HKAS 102459 and HKAS 102447 were collected and placed in plastic containers and transported to the laboratory for subsequent study. Interestingly, the ant fungus HKAS 102447 was already dead and was colonised by a saprobic fungus. The isolate MFLUCC 18–1385 was separated from this saprobe which occurred on the surface of specimen HKAS 102447 via single spore isolation. The isolate MFLUCC 18–1379 was separated from specimen HKAS 102459 by directly cultivating the hyphae which covered the surface of the ant host. These two isolates were cultured with potato dextrose agar (PDA, 1% w/v peptone) and incubated at room temperature (25 °C).

Morphological studies

For long-term deposit, these two specimens were dried with allochroic silica gel to protect them from contamination of opportunistic fungi and to retain the informative taxonomic characters. The macro-morphological characters were observed with a stereoscope (Olympus SZ61) and the micro-morphological features were examined with a compound microscope (Nikon ECLIPSE Ni). Important characteristics such as myce-

lium, phialides and conidia were captured with a digital camera (Canon EOS 600D). Measurements of perithecia, synnemata, phialides and conidia were taken using the Tarosoft (R) Image Frame Work programme and the images used were processed with Adobe Photoshop CS3 Extended v. 10.0 (Adobe, San Jose, CA).

DNA extraction, PCR amplification and sequencing

DNA was extracted from fresh mycelium of isolates MFLUCC 18–1379 and MFLUCC 18–1385 and from stromal tissue of ant fungus HKAS 102447 (the host of isolate MFLUCC 18–1385) using a DNA extraction kit (Biospin Fungus Genomic DNA Extraction Kit, BioFlux, China), following the instructions of the manufacturer. Extracted DNA was stored at 4 °C for use in regular work and duplicated at –20 °C for long-term storage. The internal transcribed spacer (ITS1-5.8S-ITS2, ITS) was amplified with primer ITS4 and ITS5 (White et al. 1990) and was used for individual gene phylogenetic analyses. The large subunit (LSU), small subunit rDNA (SSU), translation elongation factor 1-alpha gene (TEF1-α) and RNA polymerase II largest subunit 1 (RPB1) were also amplified as described in Wei et al. (2018) and used for multi-gene phylogenetic analyses. The PCR products were sent to Sangon Company, Kunming City, Yunnan Province, China for sequencing using the above primers. Newly generated sequences, used in the study, were submitted to GenBank to be assigned their accession numbers.

Sequence alignments and phylogenetic analyses

The raw sequences were verified with Finch TV version 1.4.0 (Mccredden 2016) and assembled with BioEdit v. 7.0.9.1 (Hall 1999). Sequence data were downloaded from GenBank based on BLAST searches of ITS sequences and with reference to the recent publications (Table 1). Most *Simplicillium* species are lacking protein-coding genes, but ITS sequences are available for all the species that are useful in understanding the intraspecific relationships within *Simplicillium* (Liu and Cai 2012, Nonaka et al. 2013, Dong et al. 2014 and Crous et al. 2018). Therefore, phylogenetic analyses, based on ITS regions, were generated throughout *Simplicillium* for the primary identification. Multi-gene phylogenetic analysis of the combined SSU, LSU, TEF and RPB1 sequences from representative species in Hypocreales was afterwards performed to confirm the taxonomic placements of our isolates.

The generated sequences of each gene region were aligned separately with representative sequences using MAFFT v. 7 web server (http://mafft.cbrc.jp/alignment/server) (Kuraku et al. 2013; Katoh et al. 2017). The uninformative gaps and ambiguous regions were manually removed and different gene regions were concatenated using BioEdit v. 7.0.9.1 (Hall 1999). The maximum Likelihood (ML) analyses was performed using RAxML-HPC2 on XSEDE (8.2.10) at CIPRES Science Gateway V. 3.3 (https://www.phylo.org/portal2/home.action), with default setting, except the boot-

Table 1. Strains and GenBank accession numbers from related references used in multi-gene tree.

Taxon	Voucher no.	Host/substrate	SSU rRNA	LSU rRNA	tef1	rpb1	Reference
Akanthomyces tuberculata	OSC 111002	Lepidoptera	DQ522553	DQ518767	DQ522338	DQ522384	Spatafora et al. (2007)
Aschersonia badia	BCC 8105	Scale insect	DQ522537	DQ518752	DQ522317	DQ522363	Spatafora et al. (2007)
Aschersonia placenta	BCC 7957	Scale insect	DQ522538	DQ518753	DQ522318	DQ522364	Spatafora et al. (2007)
Balansia henningsiana	GAM 16112=AEG96-27a	*Panicum* sp.	AY545723	AY545727	AY489610	AY489643	Spatafora et al. (2007)
Balansia pilulaeformis	AEG 94-2	Poaceae	AF543764	AF543788	DQ522319	DQ522365	Spatafora et al. (2007)
Claviceps fusiformis	ATCC 26019	Poaceae	DQ522539	U17402	DQ522320	DQ522366	Spatafora et al. (2007)
Claviceps paspali	ATCC 13892	Poaceae	U32401	U47826	DQ522321	DQ522367	Spatafora et al. (2007)
Claviceps purpurea	GAM 12885	*Dactylis glomerata*	AF543765	AF543789	AF543778	AY489648	Spatafora et al. (2007)
Cordyceps farinosa	OSC 111005	Lepidoptera pupa	DQ522558	DQ518773	DQ522348	DQ522394	Spatafora et al. (2007)
Cordyceps heteropoda	OSC 106404	Cicada	AY489690	AY489722	AY489617	AY489651	Spatafora et al. (2007)
Cordyceps militaris	OSC 93623	Lepidoptera	AY184977	AY184966	DQ522332	DQ522377	Spatafora et al. (2007)
Cordyceps ophioglossoides	OSC 106405	*Elaphomyces* sp.	AY489691	AY489723	AY489618	AY489652	Spatafora et al. (2007)
Cordyceps pruinosa	ARSEF 5413	*Iragoides fasciata*	AY184979	AY184968	DQ522351	DQ522397	Spatafora et al. (2007)
Cordyceps scarabaeicola	ARSEF 5689	Scarabaeidae pupa	AF339574	AF339524	DQ522335	DQ522380	Spatafora et al. (2007)
Cordyceps tenuipes	OSC 111007	Lepidoptera pupa	DQ522559	DQ518774	DQ522349	DQ522395	Spatafora et al. (2007)
Drechmeria balanoides	CBS 250.82	Nematoda	AF339588	AF339539	DQ522342	DQ522388	Spatafora et al. (2007)
Drechmeria gunnii	OSC 76404	Lepidoptera larva	AF339572	AF339522	AY489616	AY489650	Spatafora et al. (2007)
Drechmeria sinensis	CBS 567.95	Nematoda	AF339594	AF339545	DQ522343	DQ522389	Spatafora et al. (2007)
Engyodontium aranearum	CBS 309.85	Spider	AF339576	AF339526	DQ522341	DQ522387	Spatafora et al. (2007)
Epichloë typhina	ATCC 56429	*Festuca rubra*	U32405	U17396	AF543777	AY489653	Spatafora et al. (2007)
Hypocrella nectrioides	GJS 89-104	Scale insect	U32409	U47832	DQ522347	DQ522393	Spatafora et al. (2007)
Hypocrella schizostachyi	BCC 14123	Scale insect	DQ522557	DQ518771	DQ522346	DQ522392	Spatafora et al. (2007)
Lecanicillium antillanum	CBS 350.85	Agaric	AF339585	AF339536	DQ522350	DQ522396	Spatafora et al. (2007)
Lecanicillium lecanii	CBS 101247=IMI 304807	*Coccus viridis*	AF339604	AF339555	DQ522359	DQ522407	Spatafora et al. (2007)
Lecanicillium wallacei	CBS 101237=IMI 331549	Lepidoptera		AY184967	EF469073	EF469102	Zare and Gams (2008); Kouvelis et al. (2008)
Metacordyceps chlamydosporia	CBS 101244	Mollusca	DQ522544	DQ518758	DQ522327	DQ522372	Spatafora et al. (2007)
Metacordyceps taii	ARSEF 5714	Lepidoptera	AF543763	AF543787	AF543775	DQ522383	Spatafora et al. (2007)
Metapochonia goniodes	CBS 891.72	Nematoda	AF339599	AF339550	DQ522354	DQ522401	Spatafora et al. (2007)
Metarhizium album	ARSEF 2082	*Cofana spectra*	DQ522560	DQ518775	DQ522352	DQ522398	Spatafora et al. (2007)
Metarhizium flavoviride	ARSEF 2037	*Nilaparvata lugens*	AF339580	AF339531	DQ522353	DQ522400	Spatafora et al. (2007)
Metarhizium majus	ARSEF 3145	*Oryctes rhinoceros*	AF339579	AF339530	AF543774	DQ522399	Spatafora et al. (2007)

Taxon	Voucher no.	Host/substrate	SSU rRNA	LSU rRNA	tef1	rpb1	Reference
Myriogenospora atramentosa	AEG 96-32	*Andropogon virginicus*	AY489701	AY489733	AY489628	AY489665	Spatafora et al. (2007)
Ophiocordyceps acicularis	OSC 128580	Coleoptera	DQ522543	DQ518757	DQ522326	DQ522371	Araújo et al. (2018)
Ophiocordyceps aphodii	ARSEF 5498	Coleoptera	DQ522541	DQ518755	DQ522323		Araújo et al. (2018)
Ophiocordyceps brunneipunctata	OSC 128576	Coleoptera	DQ522542	DQ518756	DQ522324	DQ522369	Araújo et al. (2018)
Ophiocordyceps irangiensis	OSC 128577	Ant	DQ522546	DQ518760	DQ522329	DQ522374	Araújo et al. (2018)
Ophiocordyceps irangiensis	OSC 128578	Ant	DQ522556	DQ518770	DQ522345	DQ522391	Araújo et al. (2018)
Ophiocordyceps melolonthae	OSC 110993	Scarabaeidae larva	DQ522548	DQ518762	DQ522331	DQ522376	Araújo et al. (2018)
Ophiocordyceps nutans	OSC 110994	Stink bug	DQ522549	DQ518763	DQ522333	DQ522378	Araújo et al. (2018)
Ophiocordyceps ravenelii	OSC 110995	*Phyllophaga* sp.	DQ522550	DQ518764	DQ522334	DQ522379	Araújo et al. (2018)
Ophiocordyceps sphecocephala	OSC 110998	Wasp	DQ522551	DQ518765	DQ522336	DQ522381	Araújo et al. (2018)
Ophiocordyceps stylophora	OSC 111000	Elateridae grub	DQ522552	DQ518766	DQ522337	DQ522382	Araújo et al. (2018)
Ophiocordyceps unilateralis	OSC 128574	Ant	DQ522554	DQ518768	DQ522339	DQ522385	Araújo et al. (2018)
Ophiocordyceps variabilis	ARSEF 5365	Diptera larva	DQ522555	DQ518769	DQ522340	DQ522386	Spatafora et al. (2007)
Rotiferophthora angustispora	CBS 101437	Rotifera	AF339584	AF339535	AF543776	DQ522402	Spatafora et al. (2007)
Simplicillium calcicola	LC5586 = CGMCC3.17943	Calcaire	KY883301	KU746752	KX855252		Spatafora et al. (2007)
Simplicillium lamellicola	CBS 116.25	*Agaricus bisporus*	AF339601	AF339552	DQ522356	DQ522404	Spatafora et al. (2007)
Simplicillium lanosoniveum	CBS 704.86	*Hemileia vastatrix*	AF339602	AF339553	DQ522358	DQ522406	Spatafora et al. (2007)
Simplicillium obclavatum	CBS 311.74	Air above sugarcane filed	AF339567	AF339517	EF468798		Spatafora et al. (2007)
Tolypocladium fractum	OSC 110990	*Elaphomyces* sp.	DQ522545	DQ518759	DQ522328	DQ522373	Spatafora et al. (2007)
Tolypocladium japonicum	OSC 110991	*Elaphomyces* sp.	DQ522547	DQ518761	DQ522330	DQ522375	Spatafora et al. (2007)
Torrubiella ratticaudata	ARSEF 1915	*Euophrys* sp.	DQ522562	DQ518777	DQ522360	DQ522408	Spatafora et al. (2007)
Verticillium epiphytum	CBS 384.81	*Hemileia vastatrix*	AF339596	AF339547	DQ522361	DQ522409	Spatafora et al. (2007)

strap iterations were set to 1,000 and the substitution model set to GTRGAMMA + I (Miller and Blair 2009). Maximum Parsimony analysis (MP) was performed by PAUP v. 4.0b10 (Swofford 2002) with the heuristic search option and Tree-Bisection-Reconnection (TBR) branch-swapping algorithm for 1000 random replicates. Branches that have a minimum branch length of zero were collapsed. Gaps were treated as "missing" and starting tree(s) were generated via stepwise addition (Hillis and Bull 1993). Tree Length [TL], Consistency Index [CI], Retention Index [RI], Rescaled Consistency Index [RC] and Homoplasy Index [HI]) were calculated for all parsimonious trees. For Bayesian analysis, the best models of each gene were selected under Akaike Information Criterion (AIC) employing MrModeltest v. 2.3 (Nylander et al. 2008) and

PAUP v. 4.0b10 (Ronquist and Huelsenbeck 2003). Bayesian analysis was performed using MrBayes v. 3.1.2 (Rannala and Yang 1996; Zhaxybayeva and Gogarten 2002) to evaluate posterior probabilities (BYPP) with the Markov Chain Monte Carlo sampling (MCMC) method. Trees were sampled and printed to output at every 1000 generations. The first 25% of sampled trees were discarded as part of a burn-in procedure, the rest of the trees were used to create the consensus tree and the average standard deviation of split frequencies was set as 0.01.

Phylogenetic trees were visualised with FigTree v1.4.0 (Rambaut 2006) and edited in Microsoft PowerPoint, then saved as a PDF format and finally altered to JPG format using Adobe Illustrator CS6 (Adobe Systems Inc., United States). The finalised alignments and trees were submitted in TreeBASE (http://www.treebase.org/), with submission ID 24238 (ITS) and 24240 (multi-gene).

Results and discussion

Phylogenic analysis

The combined four gene dataset comprised 60 taxa from three families (Cordycipitaceae, Ophiocordycipitaceae and Clavicipitaceae) (Table 1) with *Cosmospora coccinea*, *Nectria cinnabarina*, *Ophionectria trichospora* and *Viridispora diparietispor*a as the outgroup taxa. The RAxML analysis of the combined dataset yielded a best scoring tree (Figure 1) with a final ML optimisation likelihood value of –39792.595439. The alignment comprised 3469 total characters including gaps, of which 2077 were constant, 338 variable characters parsimony-uninformative and 1054 characters parsimony-informative. The Kishino-Hasegawa (KH) test showed CI = 0.281, RI = 0.527, RC = 0.148 and HI = 0.719. The matrix had 1655 distinct alignment patterns, with 6.42% undetermined characters or gaps. Estimated base frequencies were as follows: A = 0.241091, C = 0.260362, G = 0.272837, T = 0.225710; substitution rates AC = 0.985172, AG = 2.843760, AT = 0.887714, CG = 0.898140, CT = 6.284116, GT = 1.000000; gamma distribution shape parameter α = 0.585080. MrModeltest v. 2.3 imply that GTR+I+G is the best-fit model for LSU and RPB1, SYM+I+G for SSU and TEF sequences.

The ITS dataset comprised 49 taxa from all *Simplicillium* species that are currently available in GenBank (Figure 2) with *Cordyceps militaris* (CBS178.59) (Cordycipitaceae, Hypocreales) as the outgroup taxon. The RAxML analysis of the ITS dataset yielded a best scoring tree (Figure 2) with a final ML optimisation likelihood value of –3155.597177. The alignment comprised 570 total characters including gaps, of which 346 were constant, 43 variable characters parsimony-uninformative and 181 characters parsimony-informative. The Kishino-Hasegawa (KH) test showed CI = 0.681, RI = 0.856, RC = 0.583 and HI = 0.319. The matrix had 283 distinct alignment patterns, with 6.45% undetermined characters or gaps. Estimated base frequencies were as follows: A = 0.232003, C = 0.283823, G = 0.254774, T = 0.229400; substitution rates

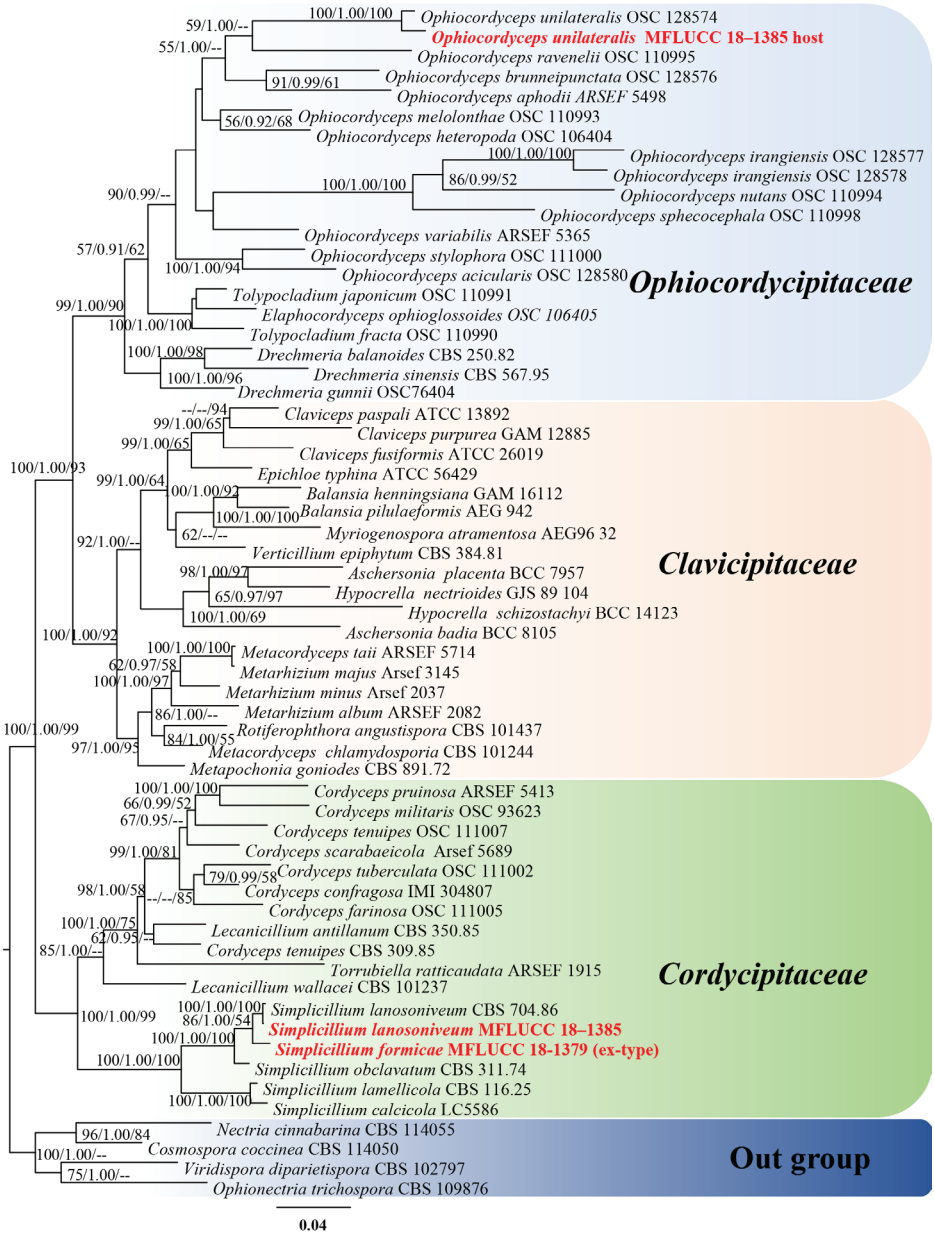

Figure 1. Phylogram generated from maximum likelihood analysis based on combined SSU, LSU, TEF and RPB1 sequence data. Bootstrap values for maximum likelihood (ML, left) and maximum parsimony (MP, right) equal to or greater than 50% and Bayesian posterior probabilities (BYPP, middle) equal to or greater than 0.90 are placed nearby the note. The newly generated sequences are indicated in red bold.

AC = 2.623562, AG = 2.645665, AT = 2.248749, CG = 1.653083, CT = 5.842034, GT = 1.000000; gamma distribution shape parameter α = 0.980038. MrModeltest v. 2.3 imply that GTR+I+G is the best-fit model for ITS sequences.

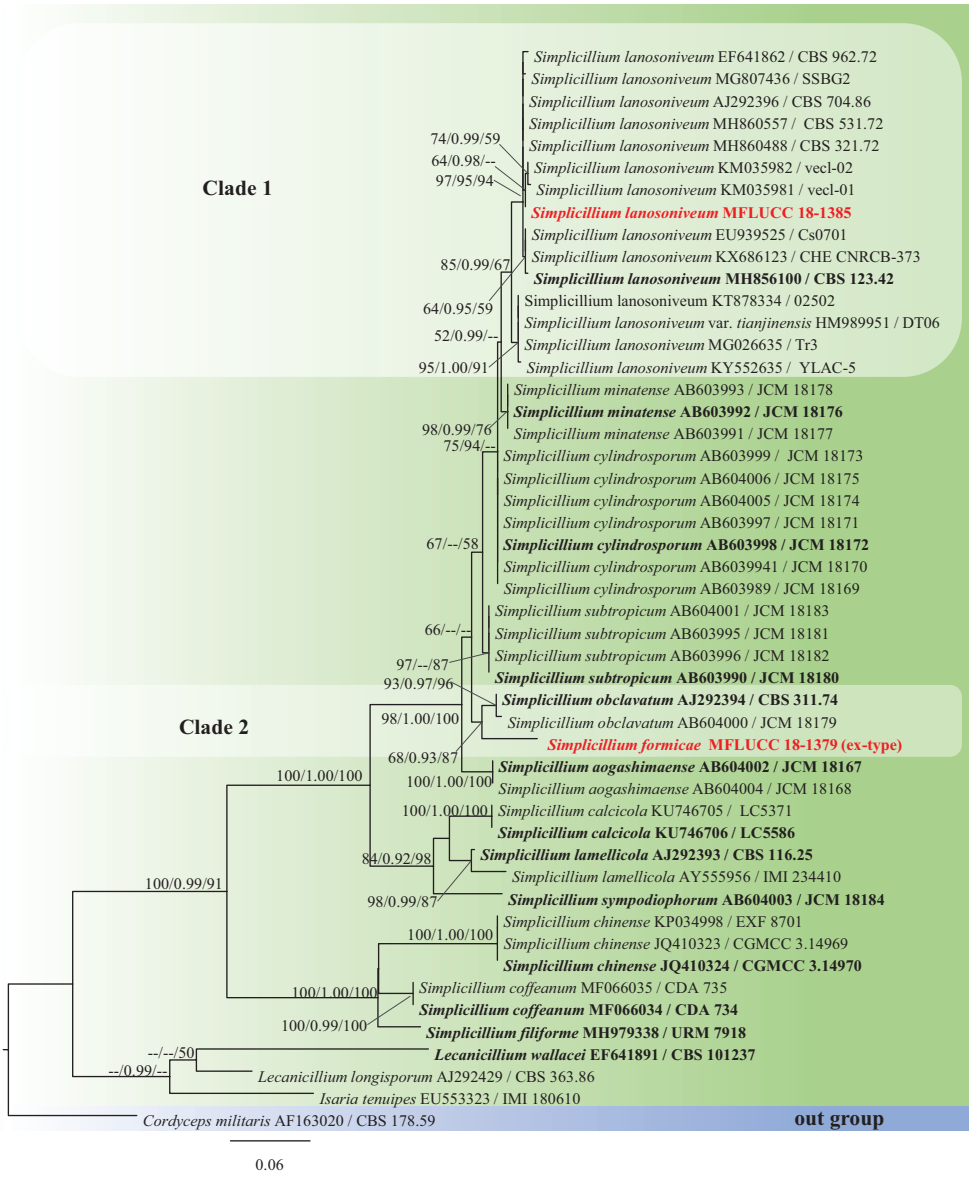

Figure 2. Phylogram generated from maximum likelihood analysis based on ITS sequence data. Bootstrap values for maximum likelihood (ML, left) and maximum parsimony (MP, right) equal to or greater than 50% and Bayesian posterior probabilities (BYPP, middle) equal to or greater than 0.90 are placed nearby the branches, respectively. The newly generated sequences are indicated in red bold and the type species are highlighted in black bold.

The multi-gene phylogenetic analyses showed that our isolates MFLUCC 18-1379 and MFLUCC 18–1385 grouped with the remaining *Simplicillium* species with strong support (100% ML, 1.00 BYPP, 100% MP, Figure 1) in Cordycipitaceae. The host of isolate MFLUCC 18–1385 grouped with *Ophiocordyceps unilateralis* (OSC

128574) in Ophiocordycipitaceae with a significant statistical support (100% ML, 1.00 BYPP, 100% MP, Figure 1). In the individual ITS-based phylogenetic tree, the isolate MFLUCC 18-1379 constituted a close affiliation to *Simplicillium obclavatum* with moderate bootstrap support (68% ML, 0.93 BYPP, 87% MP, Figure 2, clade 2). The fungal isolate MFLUCC 18-1385 grouped with the remaining *Simplicillium lanosoniveum* strains with 85% ML, 0.99 BYPP and 67% MP support (Figure 2, clade 1).

Taxonomy

Simplicillium W. Gams & Zare, Nova Hedwigia 73(1-2): 38 (2001)

Hyperparasitic on rusts or parasitic on nematodes or occurring in soil. ***Asexual morph***: *Mycelium* thin, hyaline, septate, branched, smooth-walled. *Phialides* arising from prostrate aerial hyphae or rope-like and flask-shaped synnemata, typically solitary, rarely in whorls of 2–3, gradually tapering towards the apex, elongate, slender, smooth-walled, phialidic. *Conidia* hyaline, oval, spindle-shaped, cylindrical, subglobose to ellipsoidal, fusoid to filiform, straight to curved, smooth-walled. Conidia commonly form in small globose heads, sometimes in branched, unbranched, zigzag or imbricate chains, occasionally in sympodial proliferation with cylindrical conidium-bearing denticles. Colonies of species in this genus are usually fast growing, reaching 10–38 mm within 10 days on PDA, white, reverse brownish-cream to pale yellow, margin entire, cottony, fluffy or floccose. Some species produce yellow or orange pigment. Crystals are commonly present in the agar. ***Sexual morph***: *Torrubiella* (Zare and Gams 2001; Liu and Cai 2012; Nonaka et al. 2013; Dong et al. 2014; Gomes et al. 2018; Zhang et al. 2017).

In this study, we introduce a new species and a new host species as described below.

Simplicillium formicae **D.P. Wei & K.D. Hyde, sp. nov.**
Index Fungorum number: IF556432
Facesoffungi number: FoF 05813
Figure 3, 4

Etymology. the epithet refers to its host–ant.

Holotype. HKAS 102459; living culture: MFLUCC 18–1379.

Description. Parasitic on ant (Formicidae). ***Asexual morph:*** Hyphomycetous. *Mycelium* rarely septate, hyaline, smooth-walled, covering the whole body of the ant host. *Synnemata* 250–350 × 65–100 ($\bar{x}$ = 300 × 86, n = 10) μm, forming at the head region of ant host in circular arrangement, flask-shaped, hyaline to yellowish, composed of dense hyphae, somehow curved. *Phialides* 25–100 × 0.5–1.5 ($\bar{x}$ = 49 × 1.1, n = 20) μm, arising from procumbent hyphae or synnemata, blastic, enteroblastic, phialidic, monophialidic, discrete, terminal, unbranched, solitary, aseptate, hyaline, smooth-walled, slender, occasionally a swollen node present. *Conidia* 2–3.5 × 1.5–2.5 ($\bar{x}$ = 2.6 × 2,

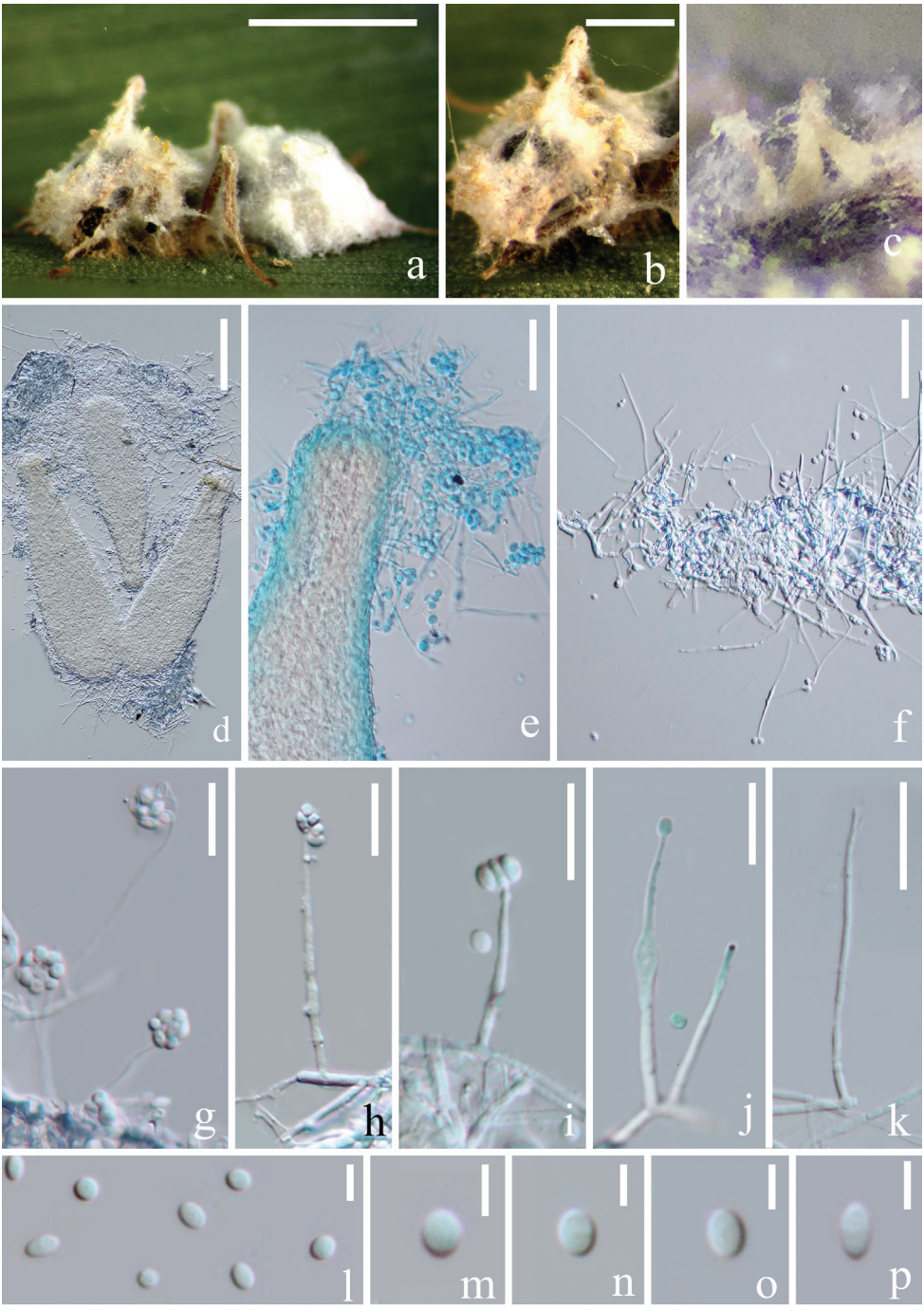

Figure 3. *Simplicillium formicae* (from HKAS 102459, holotype) **a** superficial hyphae associated with the ant host **b–e** flask-shaped synnemata **f–k** phialides bearing conidia **l–p** conidia. Scale bars: 1000 µm (**a**); 500 µm (**b**); 100 µm (**d**); 30 µm (**e, f**); 15 µm (**j, k**); 10 µm (**l–p**) (**e** stained with cotton blue solution).

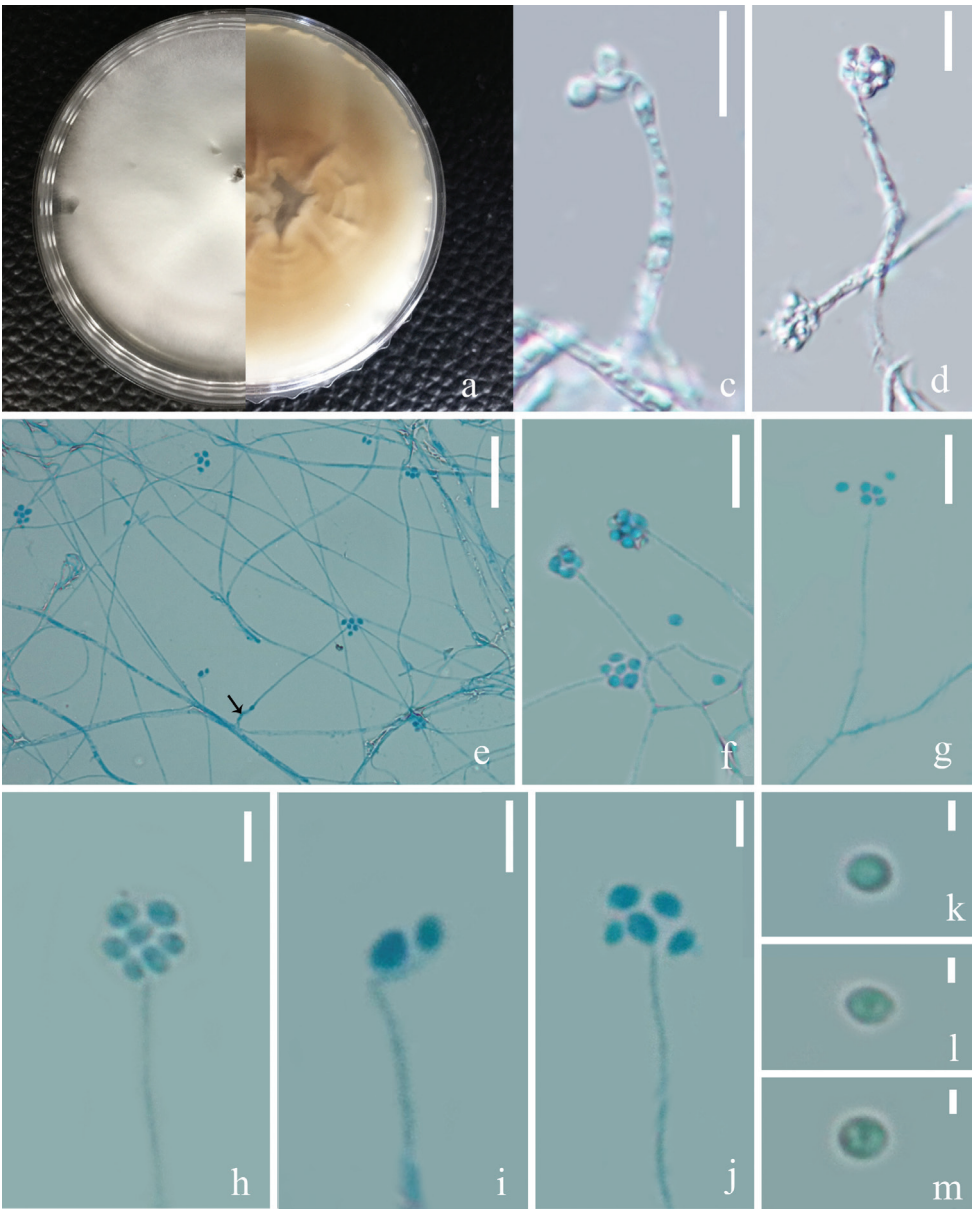

Figure 4. *Simplicillium formicae* (MFLUCC 18–1379, ex-type living culture) **a** upper and reverse view of cultures on PDA after 30 days **e–g** phialides indicated with black arrow **c, d, h–j** conidial mass on the tip of phialides **k–m** conidia. Scale bars: 10 μm (**c, d, f, g**); 20 μm (**e**); 3 μm (**h–j**); 1 μm (**k–m**) (**e–j** stained with cotton blue solution).

n = 30) μm, globose to ellipsoidal, hyaline, one-celled, smooth-walled, round at both ends, adhering in slimy head on the tip of phialides. ***Sexual morph:*** Undetermined.

Culture characteristics. The colonies were rapid-growing on PDA medium, reaching a diameter of 2.5–3 ($\bar{x}$ = 2.6, n = 9) cm, in 13 days at 22 °C, entire margin, circu-

lar, velvety and white from above, with radial crack and primrose-yellow on reverse. *In vitro*, *Synnemata* absent. *Phialides* 25–75 × 0.4–0.6 ($\bar{x}$ = 50 × 0.55, n = 10) µm, arising from procumbent hyphae, blastic, enteroblastic, phialidic, discrete, terminal, unbranched, solitary, aseptate, hyaline, smooth-walled, relatively slender and long. *Conidia* 1.5–3 × 1.5–2.5 ($\bar{x}$ = 2.3 × 1.7, n = 100) µm, hyaline, globose to ellipsoidal, aseptate, smooth-walled, slightly guttulate, adhering in slimy head on the tip of phialides.

Material examined. THAILAND, Chiang Mai Province, Mushroom Research Centre, on an adult ant, 1 April 2018, *Deping Wei*, MRC18040102 (***holotype:*** HKAS 102459; ***ex-type living culture:*** MFLUCC 18–1379). Sequences generated from this strain have been deposited in GenBank with accession numbers: SSU = MK765046, LSU = MK766512, ITS = MK766511, TEF = MK926451, RPB1 = MK882623.

Note. Isolate MFLUCC 18–1379 has a close phylogenetic relationship with *Simplicillium obclavatum*, based on ITS sequence analysis. The new isolate is similar to *Simplicillium obclavatum* in terms of shape and dimensions of the conidia with slender phialides tapering towards the apex. However, they have a different conidial arrangement, by *Simplicillium obclavatum* having short-imbricate chains, whereas the new fungus has subglobose to globose head. There are numerous synnemata in a circular arrangement which can be observed in our isolate and those are absent in *Simplicillium obclavatum*. The comparisons of ITS sequences between our isolate MFLUCC 18–1379 and ex-type strain of *Simplicillium obclavatum* (CBS 311.74) show 23 bp differences within 550 bp (4.2%). Thereby, we identify our isolates as a new species according to Jeewon and Hyde (2016).

Simplicillium lanosoniveum **(J.F.H. Beyma) Zare & W. Gams, Nova Hedwigia 73(1–2): 39 (2001)**

Facesoffungi number: FoF 05814
Index Fungorum number: 532459
Figure 5

Cephalosporium lanosoniveum J.F.H. Beyma, Antonie van Leeuwenhoek 8: 121 (1942) (Basionym)

Ex-type. Netherlands, on hair of *Cibotium schiedei* in greenhouse, 1942, F.H. van Beyma, CBS123.42.

Description. Saprophytic on *Ophiocordyceps unilateralis*. ***Asexual morph:*** Hyphomycetous. *Mycelium* aseptate, hyaline, smooth-walled. *Phialides* 20–40 × 1.1–2 ($\bar{x}$ = 30 × 1.6, n = 20) µm, arising from the prostrate mycelium, blastic, enteroblastic, phialidic, monophialidic, discrete, terminal, aseptate, hyaline, smooth-walled, solitary, tapering toward the apex. *Conidia* 2–4.5 × 1–3 ($\bar{x}$ = 3 × 1.8, n = 60) µm, hyaline, amerospores, globose to ellipsoidal, smooth-walled, adhering in globose to ellipsoidal head at the apex of phialides. ***Sexual morph:*** Undetermined.

Culture characters. The colonies on PDA medium were rapid-growing, reaching a diam. of 5.5 cm in 30 days at 22 °C, white, entire margin, velvety, with radial cracks and primrose-yellow on the reverse.

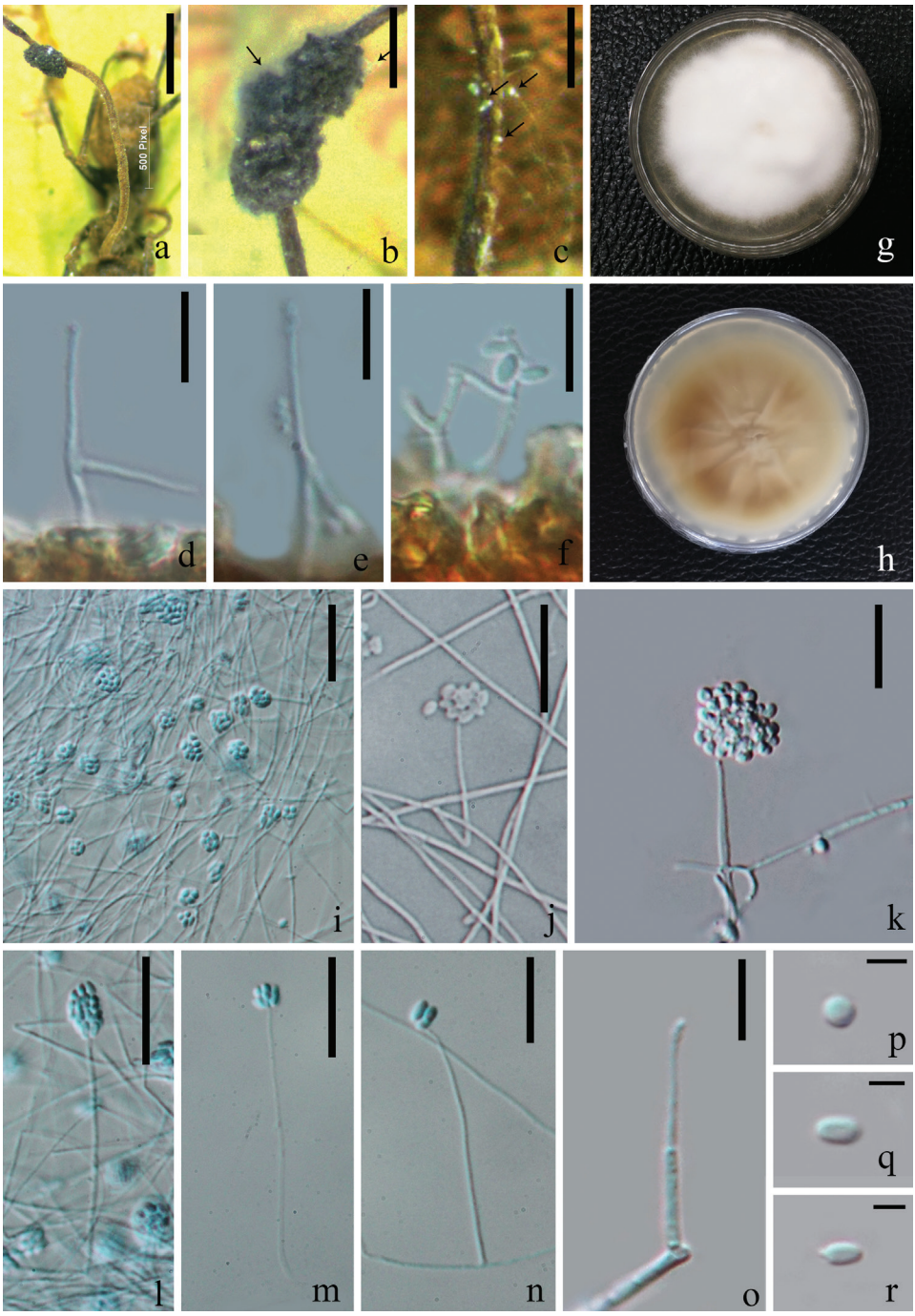

Figure 5. *Simplicillium lanosoniveum* (**a–f** from HKAS 102447, **g–r** from MFLUCC 18–1385) **a** host (*Ophiocordyceps unilateralis*); **b, c** hyphae associated with host indicated with black arrows **g, h** upper and reverse view of cultures on PDA after 40 days incubation **i–l** conidial mass on the tip of phialides **m–o** phialides bearing conidia **p–r** conidia. Scale bars: 15 μm (**i–m**); 10 μm (**d–f, n, o**); 3 μm (**p–r**) (**i, l–n** stained with cotton blue solution).

Host and distribution: Saprophytic on fungi, endophytic or symbiotic or pathogenic on plant, parasitic on rust, nematode and insect, occurring on soil, animal hair or human bronchoalveolar lavage fluid, with a cosmopolitan distribution (see Table 2).

Material examined. THAILAND, Chiang Mai Province, Mushroom Research Centre, on *Ophiocordyceps unilateralis*, 19 February 2018, *Deping Wei*, MRC18021901 (HKAS 102447; living culture: MFLUCC 18–1385). Sequences generated from this strain have been deposited in GenBank with accession numbers: SSU = MK752791, LSU = MK752849, ITS = MK752683, TEF = MK926450, RPB1 = MK882622.

Note. Our isolate MFLUCC 18–1385 colonised on a decayed *Ophiocordyceps unilateralis* with white hyphae. In a thorough examination of the *Ophiocordyceps unilateralis* host, we found the phialides and conidia of our isolate grown on the surface of the host (Figure 5). Phylogenetically, our isolate grouped with the strains of *Simplicillium lanosoniveum* with high bootstrap support (85% ML, 0.99 BYPP, 67% MP, Figure 2). The nucleotides comparison between our isolate and the type strain of *Simplicillium lanosoniveum* (CBS123.42) showed only 5 bp differences out of 539 in the ITS region. This evidence proves that our isolate is a strain of *S. lanosoniveum*, according to Jeewon and Hyde (2016). Morphologically, it resembles *S. lanosoniveum* with solitary phialides without verticillate branches and conidia adhering on a slimy head. Most of the previous descriptions of this species were given in hand-drawings and scanning electron microscopy (SEM) patterns (Zare and Gams 2001; Ward et al. 2012; Gauthier et al. 2014). *Simplicillium lanosoniveum* has been reported from *Enhalus acoroides* (seagrass) in Trang Province, Thailand. In this study, we introduce our isolate MFLUCC 18–1385 as a new host record of *Simplicillium lanosoniveum* from *Ophiocordyceps unilateralis* and provide the updated morphological features for a better understanding of this species. *Simplicillium lanosoniveum* has been frequently reported as a hyperparasite of rust and plant pathogenic fungi. Therefore, this species has a high potential of being a natural source of microbial agents against microbiological diseases in commercial agriculture (Baiswar et al. 2014; Berlanga-Padilla et al. 2018). At first, we included all available sequences of *S. lanosoniveum* from GenBank in the individual gene tree. Some strains did not group with other strains but distributed throughout the genus in primary analyses (data not shown), so we excluded those strains from the final phylogenetic analysis. Most of the reported strains of *S. lanosoniveum*, including the invalid strains, are listed in Table 2 to show their distribution and host range, as well as the sequence data availability.

***Ophiocordyceps unilateralis* (Tul. & C. Tul.) Petch, Trans. Br. mycol. Soc. 16(1): 74 (1931)**

Index Fungorum number: 281145;
Facesoffungi number: FoF 05815
Figure 6

Description. Parasitic on ants (*Formicidae*). ***Sexual morph:*** *Stromata* up to 14 mm in length, 0.5 mm wide in the broadest part, cylindrical, brown, slightly tapering towards the apex, single, piercing through the dorsal neck region of the ant host.

Table 2. Distribution, host and available sequence data of *Simplicillium lanosoniveum* strains.

Species	Strain no.	Host and habitat	Origin	Available gene region	Morphological description	Reference
S. lanosoniveum	CBS123.42	Hair of *Cibotium schiedei* (Plant)	Netherland	ITS, LSU		GenBank; Zare and Gams (2001)
	Cs0701	*Salvinia molesta* (Plant)	Taiwan	ITS	√	Chen et al. (2008)
	PSU-ES104	***Enhalus acoroides* (Plant)**	**Trang Province, Thailand**	**ITS**		**Supaphon et al. (2014)**
	CBS 531.72	*Salvinia rotundifolia* (Plant)	USA	ITS		Zare and Gams (2001)
	Tr3	*Salvia miltiorrhiza* (Plant)	China	ITS		GenBank
	YLAC-5	Endophytic on *Inula aconitum* (Plant)	China	ITS		GenBank
		Endophytic on seaweed (Plant)	India	SSU		GenBank
	E1, E3, E5	Endophytes of *Sophora alopecuroides* (Plant)	Ningxia, China	SSU, ITS	√	Yu et al. (2013)
	GA-B1	*Grewia asiatica* (Plant)	Shivalik region, Jammu, India	SSU		GenBank
	IMI 303103b	*Hemileia vastatrix* (Rust)	Colombia	ITS, SSU		Zare and Gams (2001), Kouvelis et al. (2008)
	AMH 9654	Rust pustules on leaves of *Elaeagnus* sp.	India	LSU, ITS	√	Baiswar et al. (2014)
	D082307-2A	Soybean rust	Louisiana	ITS	√	Ward et al. (2011)
	vecl-02	Rust of *Eleagnus latifolia*	India	ITS		GenBank
	vecl-01	Rust of *Eleagnus latifolia*	India	ITS		GenBank
	CBS 704.86	*Hemileia vastatrix* (Rust)	Venezuela	ITS, SSU, LSU, TEF, RPB1, RPB2, ATP	√	GenBank; Zare et al. (2000)
	S-599	*Coleosporium plumeriae* (Rust)	Campos dos Goytacazes, GJ, Brazil	ITS		Berlanga-Padilla et al. (2018)
	D082307-2A-GFP15	*Phakopsora pachyrhizi* (Rust)	Florida, USA		√	Gauthier et al. (2014)
	HKAS 102447	***Ophiocordyceps unilateralis* (Fungi)**	**Chiang Mai, Thailand**	**SSU, LSU, ITS, TEF, RPB1**	**√**	**This study**
	TYL001	*Pseudaulacaspis pentagona* (Insect)	Shanxi Province, China	ITS, SSU	√	Wang et al. (2016)
	SSBG2	*Coccus hesperidum* (Insect)	The South-Siberian Botanical Garden, Russia	ITS	√	Skaptsov et al. (2017)
	TAMA 173	*Aphidoidea* sp. (Insect)	Ibaraki, Japan	ITS		Fukuda et al. (2014)
	CHE-CNRCB 373	*Diaphorina citri* (Insect)	Colima, USA	ITS		Berlanga-Padilla et al. (2018)
	ARSEF 8822	Culicid (Insect)	Tanzania			Hubner-Campos et al. (2013)
	ARSEF7550	Coccoidea (Insect)	Brazil	TEF		GenBank
	1T9BA	Tick (Insect)	New York, USA	ITS		Greengarten et al. (2011)
	Btab03	*Bemisia tabaci* (Insect)	South Korea	ITS		GenBank
	113-8	Mosquitoes (Insect)	Japan	ITS		Ishii et al. (2015)

Species	Strain no.	Host and habitat	Origin	Available gene region	Morphological description	Reference
S. lanosoniveum	7S	*Heterodera schachtii* (Nematode)	Iran	ITS		GenBank
		Hair of giant panda (Animal)	China	ITS		GenBank
	2502	Bronchoalveolar lavage fluid (Human)	China	ITS		GenBank
	41559-3	Cave and mine	New York State, USA	ITS, LSU		GenBank
	CBS 321.72		Malaysia	SSU, LSU, ITS		Genbank; Summerbell et al. (2011)
	CBS 322.72		Malaysia	ITS		GenBank

Note: '√' means related data are available. The strains collected from Thailand are indicated with **black bold**.

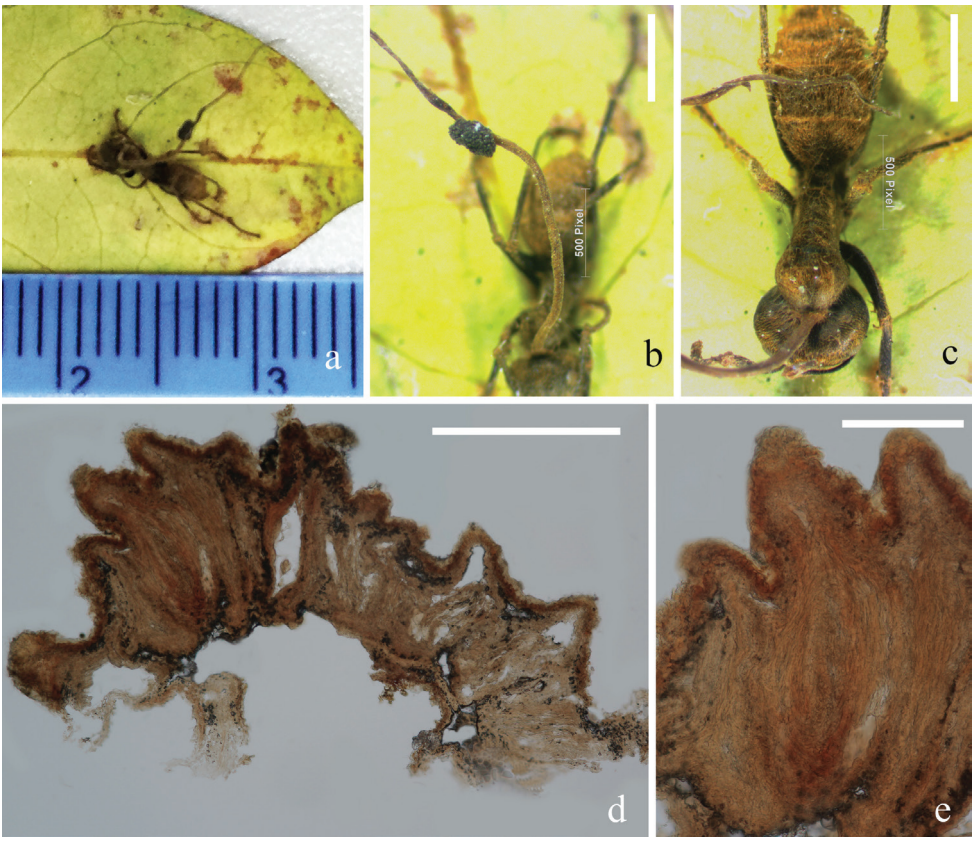

Figure 6. *Ophiocordyceps unilateralis* (from HKAS 102447) **a** stroma emerging from host **b** ascomata on stroma **c** host (Formicidae) **d, e** decayed perithecia. Scale bars: 500 μm (**b, c**); 300 μm (**d**); 100μm (**e**).

Ascomatal cushion hemisphere, up to 1.2 mm in diam., laterally attaching to the erect stroma stalk, dark brown, with ostioles protruding from the cushions. *Perithecia* 200–400 × 50–120 ($\bar{x}$ = 294 × 81, n = 10) μm, sub-immersed, flask-shaped. *Asci* and ascospores were too old to observe their features. ***Asexual morph*:** Undetermined.

Note. This collection was already decayed and was colonised by other fungi which we introduced as a new host record of *Simplicillium lanosoniveum* from Thailand. The outline of this specimen was intact, while its asci and ascospores were too old to analyse. We retrieved DNA through direct sequencing from the stromal tissue.

Sequences generated from this specimen have been deposited in GenBank with accession numbers: SSU = MK752759, LSU = MK752812, ITS = MK752874. The herbarium material is deposited at KUN herbarium, Yunnan Province, China. In the multi-gene phylogenetic tree, this collection groups with *Ophiocordyceps unilateralis* (OSC 128574) with a strongly supported bootstrap value (100% ML, 1.00 BYPP, 100% MP, Figure 1). Therefore, we identify this collection as *O. unilateralis*, based on its morphologic features and molecular evidence.

Key to accepted species of *Simplicillium*

1a	Conidia formed in sympodia	***S. sympodiophorum***
1b	Conidia solitary, borne on the tip of phialides	***S. calcicole***
1c	Conidia aggregate in chains	**2**
1d	Conidia aggregate in subglobose to ellipsoidal heads	**3**
1e	Conidia aggregate in globose heads	**4**
2a	Conidia 2.5–3.5 × 1–2 µm, obclavate to ellipsoidal, formed in short imbricate chains	***S. obclavatum***
2b	Conidia 3.5–5.0 × 1.0–1.5 µm, oval, ellipsoidal or cylindrical, formed in vertical chains	***S. chinense***
2c	Conidia 7.2–12.5 × 1 µm, long, fusiform to short filiform, hyaline, straight to curved, formed in vertical chains	***S. filiforme***
3a	Phialides 15–50 × 0.7–1.0 µm, colonies light to dark-brown reverse on PDA, usually with yellow pigment diffusing into the agar	***S. lamellicola***
3b	Phialides 11–44 (–70) × 1.0–2.4 µm, colonies cream-coloured reverse on PDA, no diffused pigment	***S. coffeanum***
4a	Present flask-shaped synnemata	***S. formicae***
4b	Synnemata absent	**5**
5a	Conidia cylindrical	**6**
5b	Conidia globose to subglobose or ellipsoidal	7
6a	Phialides 23–53 × 1.2–2.0 µm, long	***S. cylindrosporum***
6b	Phialides 17–32 × 1.2–2.0(–2.5) µm, short	***S. aogashima***
7a	Phialides 35–75 × 1.2–3.0 µm, conidia 4.5–6.0 × 2.5–3.5 µm, colonies light yellow to deep tawny in reverse view on PDA	***S. lanosoniveum* var. *tianjinensis***
7b	Phialides 15–39 × 0.7–1.9 µm, conidia 1.5–3 × 0.7–1.3 µm, colonies brownish-cream to pale yellow reverse on PDA	***S. lanosoniveum***
7c	Phialides 11–31(–47) × 1.0–1.7 µm, conidia 2.0–3.5 × 1.8–2.5(–2.8) µm, colonies brown reverse on PDA	***S. minatense***
7d	Phialides (15–)20–42(–50) × 1.0–2.3 µm; conidia 2.3–4.0(–4.5) × 1.5–3.3 µm, colonies brownish-orange to brown reverse on PDA	***S. subtropicum***

Conclusion

A new species *Simplicillium formicae* and a new host record species *Simplicillium lanosoniveum* from *Ophiocordyceps unilateralis* were introduced, based on phylogenetic analyses and morphological evidence. The host and distribution of *S. lanosoniveum* was summarised and a key to *Simplicillium* was provided.

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Taxonomy and phylogeny of the *Leptographium olivaceum* complex (Ophiostomatales, Ascomycota), including descriptions of six new species from China and Europe

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Abstract

The *Leptographium olivacea* complex encompasses species in the broadly defined genus *Leptographium* (Ophiostomatales, Ascomycota) that are generally characterized by synnematous conidiophores. Most species of the complex are associates of conifer-infesting bark beetles in Europe and North America. The aims of this study were to reconsider the delineation of known species, and to confirm the identity of several additional isolates resembling *L. olivacea* that have emerged from recent surveys in China, Finland, Poland, Russia, and Spain. Phylogenetic analyses of sequence data for five loci (ACT, TUB, CAL, ITS2-LSU, and TEF-1α) distinguished 14 species within the complex. These included eight known species (*L. cucullatum*, *L. davidsonii*, *L. erubescens*, *L. francke-grosmanniae*, *L. olivaceum*, *L. olivaceapini*, *L. sagmatosporum*, and *L. vescum*) and six new species (herein described as *L. breviuscapum*, *L. conplurium*, *L. pseudoalbum*, *L. rhizoidum*, *L. sylvestris*, and *L. xiningense*). New combinations are provided for *L. cucullatum*, *L. davidsonii*, *L. erubescens*, *L. olivaceum*, *L. olivaceapini*, *L. sagmatosporum* and *L. vescum*. New Typifications: Lectotypes are designated for *L. olivaceum*, *L. erubescens* and *L. sagmatosporum*. Epitypes were designated for *L. olivaceapini* and *L. sagmatosporum*. In addition to phylogenetic separation, the synnematous asexual states and ascomata with almost cylindrical necks and prominent ostiolar hyphae, distinguish the *L. olivaceum* complex from others in *Leptographium*.

Keywords
bark beetle, *Leptographium*, integrative taxonomy, new species, *Ophiostomatales*, phylogeny

Introduction

Species of *Leptographium* are commonly associated with bark beetles and weevils, and are responsible for causing sapstain on a wide range of primarily coniferous trees (Jacobs and Wingfield 2001). The genus also includes some important tree pathogens such as species in the *Leptographium wageneri* complex that cause black stain root disease (Goheen and Hansen 1978). In their monograph of *Leptographium*, Jacobs and Wingfield (2001) treated the asexual states of 46 species in the genus, all characterized by mononematous conidiophores branched at their apices. Conidia aggregate in slimy droplets at the apices of these structures, which make these species well-adapted for arthropod dispersal.

Following the "one fungus one name" principles adopted in the Melbourne Code (Hawksworth 2011), De Beer and Wingfield (2013) re-evaluated the taxonomy of *Leptographium*, considering available DNA sequence data for all species. Ninety-four species were included and ten species complexes were defined within a broadly defined concept for *Leptographium sensu lato*, based on phylogenies resulting from ribosomal internal transcribed spacer (**ITS**) and partial LSU sequences.

One of the species complexes recognized in *Leptographium* s.l. by De Beer and Wingfield (2013) was the *L. olivaceum* complex. Earlier, Zipfel et al. (2006) had shown that *L. olivaceum* produces synnematous asexual states, which is unlike mononematous conidiophores traditionally defining *Leptographium*. In extended phylogenies, Massoumi Alamouti et al. (2007), Six et al. (2011), and Linnakoski et al. (2012) showed that additional species with synnematous asexual states grouped in a monophyletic lineage with *L. olivaceum.* Six et al. (2011) referred to this lineage as the *L. olivaceum* species complex for the first time and they included *L. olivaceum* (Mathiesen-Käärik, 1951), *L. sagmatosporum* (Wright & Cain, 1961), *L. olivaceapini* (Davidson, 1971), and *L. cucullatum* (Solheim, 1986) in their phylogeny. Subsequently, *L. davidsonii* (Olchowecki & Reid, 1974) and *L. vescum* (Davidson, 1958) were shown to also belong to this complex (Linnakoski et al. 2012, De Beer and Wingfield 2013).

The six species currently residing in the *L. olivaceum* complex have morphologically similar sexual and asexual states. They produce globose ascomata with long, nearly cylindrical necks, terminating in prominent ostiolar hyphae on which sticky droplets are formed that contain orange-section shaped ascospores with cucullate gelatinous sheaths (Mathiesen-Käärik 1951, Davidson 1958, Wright and Cain1961, Davidson 1971, Olchowecki and Reid 1974, Solheim 1986). This study includes isolates representing all species in the *L. olivaceum* complex as well as morphologically similar isolates from recent surveys of fungi in China, Europe, and Russia. The aims of the study were to reconsider and redefine the species boundaries in the *L. olivaceum* complex based on phylogenetic analyses of multilocus regions, to provide neotypes for species where type specimens have been lost or are inadequate, and to describe new species in this complex.

Methods

Isolates

All isolates included in this study are listed in Table 1. Reference isolates were obtained from the culture collection (**CMW**) of the Forestry and Agricultural Biotechnology Institute (**FABI**), University of Pretoria, South Africa. Ex-type isolates of newly described species were deposited in the Westerdijk Fungal Biodiversity Institute (**CBS**), Utrecht, in the Netherlands. Type specimens of new species were deposited in the National Collection of Fungi (**PREM**), Pretoria, South Africa. Taxonomic novelties and new typification events for known taxa were registered in MycoBank (Robert et al. 2013).

DNA extraction, PCR and sequencing

DNA extractions were done as described by Yin et al. (2015). For sequencing and phylogenetic analyses, five loci were amplified: internal transcribed spacer 2 and large subunit (ITS2-LSU), actin (ACT), beta tubulin (TUB), calmodulin (CAL) and translation elongation factor-1 alpha (TEF-1α). Primers used were: ITS3 and LR3 (White et al. 1990) for ITS2-LSU, Lepact-F and Lepact-R (Lim et al. 2004) for ACT, T10 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995) for TUB, CL2F and CL2R (Duong et al. 2012) for CAL, EF2-F (Marincowitz et al. 2015) and EF2-R (Jacobs et al. 2004) for TEF-1α.

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), and 16.5 µL of PCR grade water. PCR conditions for these five gene regions followed the protocols described by Yin et al. (2015). PCR products were purified with Sephadex G-50 columns (6%).

PCR products were sequenced with the same primers used for PCR, together with the Big Dye Terminator 3.1 cycle sequencing premix kit (Applied Biosystems, Foster City, California, USA). BigDye PCRs were conducted in 12 µL: sequencing Buffer 4.0 µL, Big Dye 1.0 µL, PCR Grade Water 4.0 µL, primer 1.0 µL, PCR product 2.0 µL; PCR conditions were: 1 min at 96 °C; 25 cycles of 10 sec at 96 °C, 5 sec at 50 °C, and 1min at 60 °C; and finally held at 12 °C. BigDye PCR products were also cleaned up with Sephadex. Sequence analyses were done on the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). Consensus sequences were generated from forward and reverse sequences in the CLC Main Workbench 6.0 (CLC Bio, Aarhus, Denmark).

Phylogenetic analyses

Five sequence datasets were analyzed. The ITS2-LSU sequences of the ex-type isolate of every species in the *L. olivaceum* complex (Table 1) were compared with sequences of other known species in *Leptographium* obtained from GenBank to show the placement

Table 1. Isolates used in the present study.

Species[1]	Isolate no.[2]		Country	Host	Insect	GenBank accession no. [3]				
	CMW no.	CBS no.				ITS2-LSU	ACT	TUB	CAL	TEF-1α
Leptographium breviscapum	38888 [H]	136507	China	*Picea crassifolia*	*Polygraphus poligraphus*	**MN516697**	**MN517641**	**MN517672**	**MN517707**	**MN517742**
	38889 [P]	136508	China	*Picea crassifolia*	*Polygraphus poligraphus*	**MN516698**	**MN517642**	**MN517673**	**MN517708**	**MN517743**
	38890		China	*Picea crassifolia*	*Polygraphus poligraphus*	**MN516699**	**MN517643**	**MN517674**	**MN517709**	**MN517744**
L. conplurium	23289 [P]	128834	Finland	*Picea abies*	*Dryocoetus autographus*	**MN516701**	**MN517644**	JF279994	**MN517710**	**MN517745**
	23295		Finland	*Picea abies*	*Dryocoetus autographus*	**MN516702**	**MN517645**	JF279993	**MN517711**	JF280036
	23315 [H]	128923	Finland	*Picea abies*	*Dryocoetus autographus*	**MN516700**	**MN517646**	JF279989	**MN517712**	**MN517746**
	23316		Finland	*Picea abies*	*Hylastes brunneus*	**MN516703**	**MN517647**	JF279990	**MN517713**	**MN517747**
L. cucullatum	1140=1141 [H]	218.83	Norway	*Picea abies*	*Ips typographus*	AJ538335	**MN517619**	JF280000	**MN517685**	**MN517724**
	1871		Japan	*Pinus jezoensis*	*Ips typographus*	**MN516704**	**MN517620**	JF280001	**MN517686**	**MN517725**
	5022		Austria	*Picea abies*	*Ips typographus*	**MN516705**	**MN517621**	JF280002	**MN517687**	**MN517726**
	23123	128299	Russia	*Picea abies*	*Ips typographus*	**MN516706**	**MN517622**	JF280003	**MN517688**	JF280042
	23190		Russia	*Pinus sylvestris*	*Ips typographus*	**MN516707**	**MN517623**	JF280005	**MN517689**	JF280043
	27983		Russia	*Picea abies*	*Dryocoetus autographus*	**MN516708**	**MN517624**	**MN517658**	**MN517690**	**MN517727**
	27984		Russia	*Picea abies*	*Dryocoetus autographus*	**MN516709**	**MN517625**	**MN517659**	**MN517691**	**MN517728**
	36623		Russia	*Picea abies*	*Ips typographus*	**MN516710**	**MN517626**	**MN517660**	**MN517692**	**MN517729**
L. davidsonii	790 [H]		Canada	*Pseudotsuga menziesii*	–	**MN516711**	**MN517627**	**MN517661**	**MN517693**	**MN517730**
	3094		Canada	*Picea* sp.	unknown bark beetle	**MN516712**	**MN517628**	**MN517662**	**MN517694**	**MN517731**
	3095		Canada	*Picea* sp.	unknown bark beetle	**MN516713**	**MN517629**	**MN517663**	**MN517695**	**MN517732**
L. erubescens	40672 [H]	278.54	Sweden	*Pinus sylvestris*	–	**MN516714**	**MN517656**	**MN517683**	**MN517722**	**MN517756**
L. francke-grosmanniae	445 [H]	356.77	Germany	*Quercus* sp.	*Hylecoetus dermestoides*	**MN516715**	**MN517618**	**MN517657**	**MN517684**	**MN517723**
L. olivaceum	23348	128836	Finland	*Picea abies*	*Ips typographus*	**MN516717**	**MN517630**	**MN517664**	**MN517696**	JF280049
	23350	128837	Finland	*Picea abies*	*Ips typographus*	**MN516718**	**MN517631**	**MN517665**	**MN517697**	JF280050
	28090		Russia	*Pinus sylvestris*	*Ips typographus*	**MN516719**	**MN517632**	**MN517666**	**MN517698**	**MN517733**
	31059 [H]	138.51	Sweden	*Pinus sylvestris*	–	**MN516716**	**MN517633**	JF279997	**MN517699**	**MN517734**
	31060	152.54	Sweden	–	–	**MN516720**	**MN517634**	JF279998	**MN517700**	**MN517735**
L. olivaceapini	63	503.86	USA	–	–	**MN516721**	**MN517635**	**MN517667**	**MN517701**	**MN517736**
	116 [E]	504.86	USA	–	–	**MN516722**	**MN517636**	**MN517668**	**MN517702**	**MN517737**
L. pseudoalbum	40671 [H]	276.54	Sweden	*Pinus sylvestris*	*Tomicus piniperda*	**MN516723**	**MN517655**	**MN517682**	**MN517721**	**MN517755**
L. rhizoidum	22809 [H]	136512	Spain	*Pinus radiata*	*Hylastes ater*	**MN516724**	**MN517648**	**MN517675**	**MN517714**	**MN517748**
	22810 [P]	136513	Spain	*Pinus radiata*	*Hylastes attenuatus*	**MN516725**	**MN517649**	**MN517676**	**MN517715**	**MN517749**
	22811		Spain	*Pinus radiata*	*Ips sexdentatus*	**MN516726**	**MN517650**	**MN517677**	**MN517716**	**MN517750**
	22812		Spain	*Pinus radiata*	*Hylurgops palliatus*	**MN516727**	**MN517651**	**MN517678**	**MN517717**	**MN517751**
L. sagmatosporum	34135 [E]	113452	Canada	*Pinus strobus*	–	**MN516728**	**MN517637**	**MN517669**	**MN517703**	**MN517738**
L. sylvestris	23300 [P]	128833	Finland	*Picea abies*	*Ips typographus*	**MN516729**	**MN517639**	JF279996	**MN517705**	**MN517740**
	34140 [T]	136511	Poland	*Pinus sylvestris*	–	**MN516730**	**MN517640**	**MN517671**	**MN517706**	**MN517741**
L. vescum	34186 [H]	800.73	USA	*Picea engelmannii*	*Ips pilifrons, Dendroctonus engelmanni*	**MN516731**	**MN517638**	**MN517670**	**MN517704**	**MN517739**
L. xiningense	38891 [H]	136509	China	*Picea crassifolia*	*Polygraphus poligraphus*	**MN516732**	**MN517652**	**MN517679**	**MN517718**	**MN517752**
	39237 [P]	136510	China	*Picea crassifolia*	*Polygraphus poligraphus*	**MN516733**	**MN517653**	**MN517680**	**MN517719**	**MN517753**
	39238		China	*Picea crassifolia*	*Polygraphus poligraphus*	**MN516734**	**MN517654**	**MN517681**	**MN517720**	**MN517754**

[1] **Bold** type = new species in the present study.

[2] CMW = Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; CBS = Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands. [H] = ex-holotype; [E] = ex-epitype; [P] = ex-paratype

[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; TUB = Beta-tubulin; CAL= Calmodulin; TEF-1α = Translation elongation factor 1-alpha; **Bold** type = Genbank accession numbers of sequences obtained in the present study.

of the complex within the genus. Sequences of *Fragosphaeria purpurea* and *F. reniformis* were used to represent the outgroup taxa. Four protein coding gene regions (ACT, TUB, CAL, and TEF-1α) were sequenced (Table 1) for 39 isolates (Table 1) in order to delineate closely related species in the *L. olivaceum* complex. Sequences for *L. procerum* and *L. profanum* from the study of Yin et al. (2015) were selected to represent the outgroup taxa for the four protein-coding gene regions as well as in the combined dataset.

Alignments of loci were conducted in MAFFT 7.0 online (Katoh and Standley 2013), then checked manually in MEGA X (Kumar et al. 2018) and compared with the gene maps (Yin et al. 2015) to ensure that introns and exons were aligned appropriately. Three methods were used for phylogenetic analyses including Maximum parsimony (MP), Maximum Likelihood (ML), and Bayesian Inference (BI). A partition homogeneity test was conducted in PAUP* 4.0b10 (Swofford 2002) to consider the congruence of the four protein-coding gene regions before analyses of the combined dataset. The most important parameters used in phylogenetic analyses and statistical values related to all datasets analyzed are presented in Table 2.

MP analyses were executed in PAUP* 4.0b10 (Swofford 2002) with heuristic searches of 1000 replicates and tree bisection and reconnection (TBR) branch swapping options. Gaps were treated as the fifth base. Bootstrap analysis (1000 pseudo replicates) was performed to determine the confidence levels of the branch nodes. Tree length (TL), consistency Index (CI), retention Index (RI), Homoplasy Index (HI), and Rescaled Consistency Index (RC) were recorded after generating the trees.

The best substitution models (Table 2) for the two likelihood methods (ML and BI analyses) were selected congruously in jModelTest 2.1.1 (Pasoda 2008). MEGA X (Kumar et al. 2018) was used for ML analyses with Nearest-Neighbor-Interchange (NNI) branch swapping option. Node support values were determined using analysis of 1000 bootstrap pseudo replicates.

For BI analyses, the Markov Chain Monte Carlo (MCMC) method was used in MrBayes 3.2 (Ronquist et al. 2012). Four MCMC chains were simultaneously run from a random starting tree for five million generations. Trees were sampled every 100 generations. Burn-in values were determined in Tracer v1.7 (Rambaut et al. 2018). Trees sampled in the burn-in phase were discarded and posterior probabilities were calculated from all the remaining trees.

Morphology and growth studies

In order to describe their morphology, isolates of new species were inoculated on to 2% water agar (WA, 20 g Difco agar and 1000 ml deionized water) amended with sterilized pine twigs (*Pinus pinaster*) and examined microscopically as described by Yin et al. (2015). Culture characteristics were recorded on Oatmeal agar (OA, 30 g oatmeal, 20 g Difco Bacto malt extract, from Becton, Dickinson and Company, and 1000ml deionized water) incubated at 25 °C for 10–14 days. Color descriptions were defined using the charts of Rayner (1970). Growth studies were conducted on 2 % Malt extract agar (MEA) following the procedure described by Yin et al. (2015).

Table 2. Parameters used and statistical values related to all phylogenetic analyses in the present study.

		ITS2-LSU	ACT	βT	CAL	TEF-1α	Combined
Alignments	Number of taxa	59	41	41	41	41	41
	Total	603	809	288	579	781	2457
	Constant	456	622	209	435	479	1785
	Uninformative	46	20	8	22	45	95
	Informative	101	127	71	122	257	577
MP	Number of trees	396	13	4	15	10	12
	Tree length	289	276	154	404	619	1486
	CI	0.740	0.812	0.786	0.884	0.837	0.821
	RI	0.934	0.935	0.933	0.956	0.941	0.935
	RC	0.691	0.759	0.733	0.845	0.787	0.767
	HI	0.259	0.188	0.214	0.116	0.163	0.179
Model tests	Selected Models	GTR+I+G	HKY+I+G	HKY+G	HKY+I	HKY+G	HKY+I +G
ML	P-inv	0.378	0.527	–	0.623	–	0.441
	Gamma	0.257	0.287	0.179	–	0.618	0.712
BI	Burn-in	100	300	300	300	300	300

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 used in phylogenetic analyses, P-inv = proportion of invariable sites, Gamma = Gamma distribution shape parameter.

Results

Phylogenetic analyses

The phylogenetic trees arising from the analyses of the ITS2-LSU data for *Leptographium* s.l. showed the *L. olivaceum* complex grouping between the *L. galeiformis* and *L. procerum* complexes with strong statistical support (Fig. 1). Within the complex, the ITS2-LSU sequences could not distinguish between some of the species, e.g. between *L. rhizoidum* and *L. sagmatosporum*; *L. davidsonii* and *L. vescum*; *L. conplurium*, *L. pseudoalbum* and *L. erubescens*. *Leptographium francke-grosmanniae* grouped peripheral to other species in the complex, but remained part of a strongly supported lineage including all the species under consideration.

The ACT data matrix included part of exon 5 (sites 1–678), intron 5 (sites 679–785) and part of exon 6 (sites 786–809). The intron/exon composition of this gene region was congruent with that of the *L. procerum* complexes (Yin et al. 2015). Analyses of this gene region (Fig. 2) separated all known species and revealed six new taxa in the complex.

The TUB dataset included part of exon 4 (sites 1–41), intron 4 (sites 42–113), exon 5 (114–168) and part of exon 6 (sites 169–288). Intron 5 was lacking in the *L. olivaceum* complex, corresponding with most other species complexes in *Leptographium* s.l. (De Beer and Wingfield 2013). In the resulting phylogenies (Fig. 2), most known species and all new taxa could be separated, apart from the *L. davidsonii* and *L. vescum* isolates that formed a single clade.

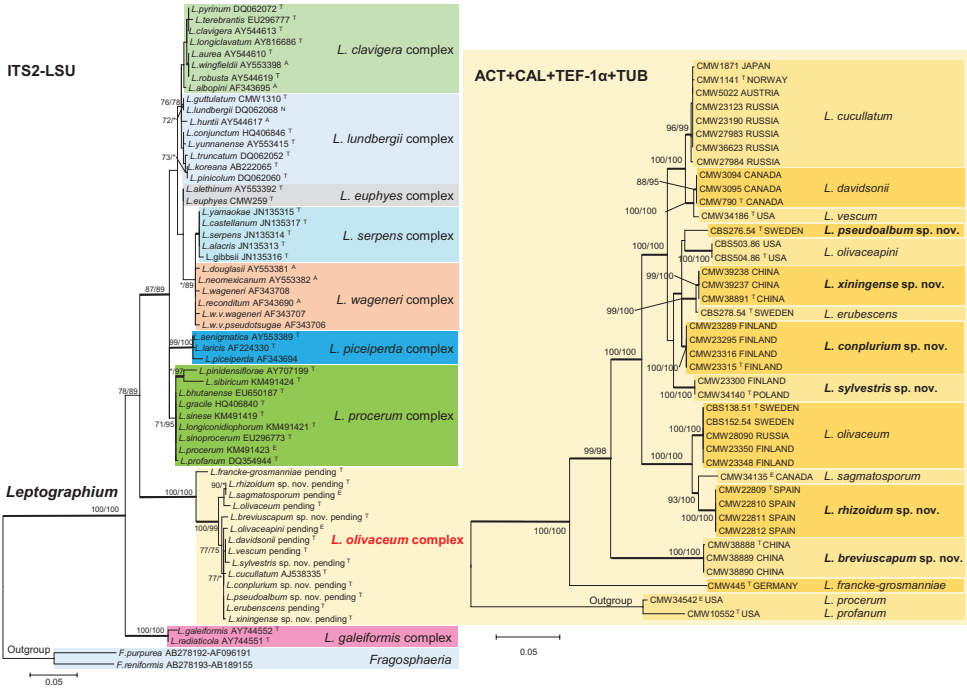

Figure 1. Left side: ML tree of the genus *Leptographium* generated from the ITS2-LSU DNA sequence data. Sequences generated from this study are printed in bold type. Bold branches indicate posterior probabilities values ≥0.95. Bootstrap values ≥75% are recorded at nodes as ML/MP. * Bootstrap values <75%. Scale bar represents 5 nucleotide substitutions per 100 nucleotides. Right side: ML trees of the *L. olivaceum* complex generated from the DNA sequences of combined four protein-coding gene regions, including ACT, CAL, TEF-1α, and TUB. Bold branches indicate posterior probabilities values ≥0.95. Bootstrap values ≥75% are recorded at nodes as ML/MP. * Bootstrap values <75%. Scale bar represents 5 nucleotide substitutions per 100 nucleotides.

The aligned DNA sequences for the CAL gene region included exon 3 (sites 1–16), intron 3 (sites 17–165), exon 4 (sites 166–291), intron 4 (sites 292–451), exon 5 (452–526), and part of exon 6 (sites 527–579). The intron/exon arrangement corresponded with that of the *L. clavigerum* and *L. procerum* complexes (Yin et al. 2015), with intron 5 lacking in this complex. Phylogenetic analyses of the CAL dataset (Fig. 2) recovered all currently accepted species in the complex.

The TEF-1α gene region used in phylogenetic analyses, included part of exon 3 (sites 1–9), intron 3 (sites 10–461), exon 4 (462–599), intron 4 (600–686), and part of exon 5 (687–781). Intron 4 of the TEF-1α gene was present in the *L. olivaceum* complex as is also true for the *L. procerum*, *L. galeiformis*, *L. wageneri* and *L. serpens* complexes, while it is absent in several other species complexes in *Leptographium* s. l. (De Beer and Wingfield 2013, Yin et al. 2015). Analysis of the TEF-1α dataset (Fig. 2) made it possible to separate all species in the complex.

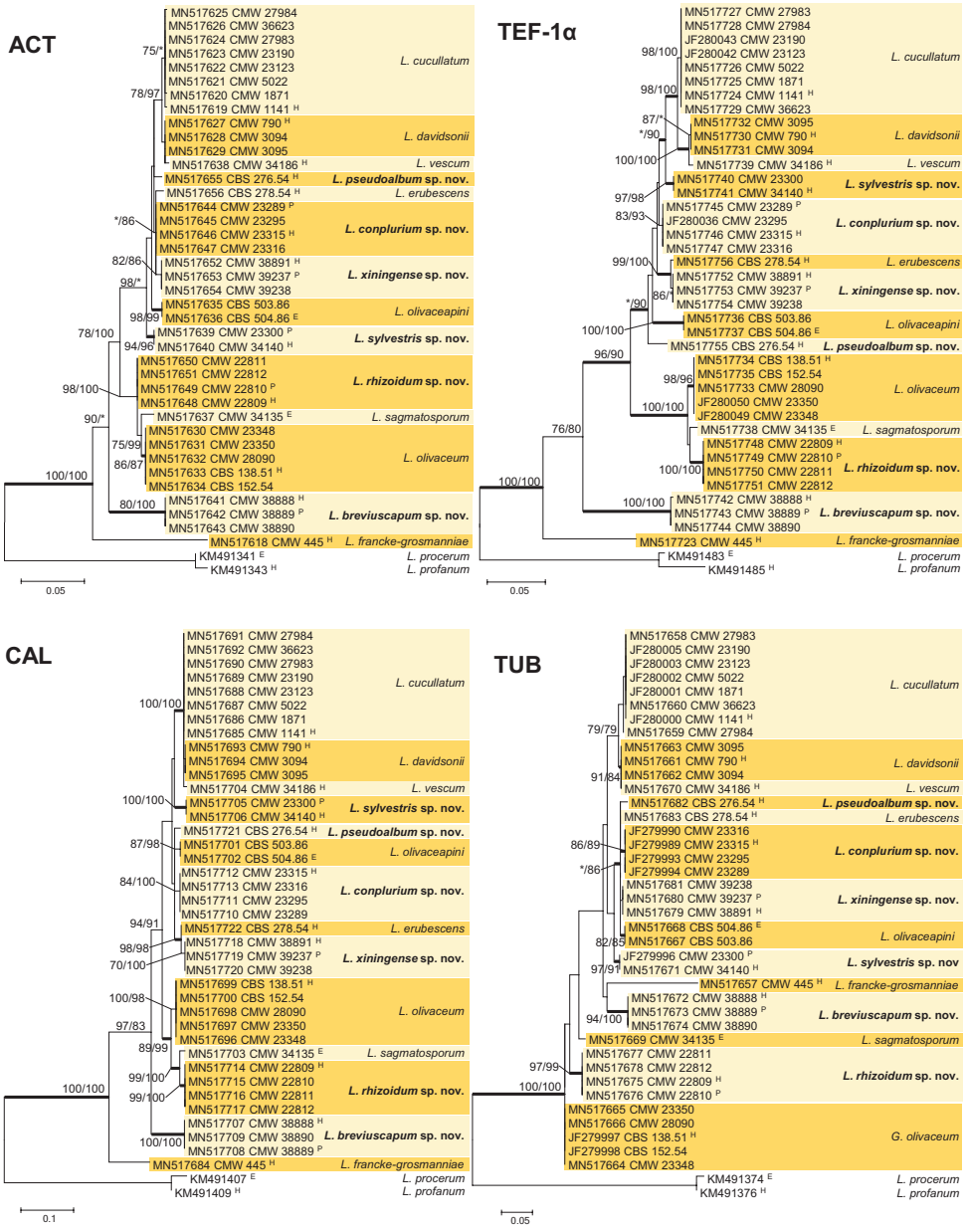

Figure 2. ML trees of the *L. olivaceum* complex generated from DNA sequences of four protein-coding gene regions. Bold branches indicate posterior probabilities values ≥0.95. Bootstrap values ≥75% are recorded at nodes as ML/MP. * Bootstrap values <75%. Scale bar represents nucleotide substitutions.

The partition homogeneity test conducted on the combined data set for the four protein coding genes (ACT, TUB, CAL and TEF-1α) resulted in a P-value of 0.081, indicating that these regions could be combined. The MP, ML, and BI analyses gener-

ated were consistent with each other. Fourteen species with significant statistical support were defined in the *L. olivaceum* complex (Fig. 1), including eight known species (*L. cucullatum*, *L. davidsonii*, *L. vescum*, *L. olivaceapini*, *L. erubescens*, *L. olivaceum*, *L. sagmatosporum*, and *L. francke-grosmanniae*) and six new species from Europe and China.

Morphology and growth studies

Isolates of the six new species emerging from this study were similar in growth in culture, with colors initially hyaline, later turning pale yellowish or pale olivaceous. Mononematous synnemata were common in the cultures and hyphae were superficial on the agar. The droplets containing conidia were initially hyaline, becoming yellowish with age. Morphological differences among all these new species are discussed in the *Notes* sections provided with the new species descriptions in the Taxonomy section. A sexual state was induced only in isolates of *L. sylvestris* after incubation at 25 °C for three weeks.

Other than *L. sylvestris* that grew fastest at 30 °C, the optimal growth temperature for all isolates of the new species was 25 °C. None of the isolates of the new species grew at 5 °C or 35 °C, only *L. rhizoidum* was able to grow (2.5 mm/d) at 35 °C.

Taxonomy

Sequence data for 39 isolates included in the present study revealed 14 taxa in the *L. olivaceum* complex. One of these species, *L. erubescens*, was previously treated as a synonym of *L. cucullatum* but our data distinguished clearly between the two species. A new combination is thus provided for *L. erubescens*. Lectotypes and epitypes are designated here for *L. olivaceum*, *L. sagmatosporum* and *L. erubescens*. The remaining six taxa in the complex represented novel species and descriptions are provided for them.

Leptographium breviuscapum **M.L. Yin, Z.W. de Beer & M.J. Wingf., sp. nov.**
MycoBank No: 823576
Fig. 3

Etymology. The epithet (brevius-, short, and -scapum, branch) refers to very short conidiophores.

Type. CHINA, Qinghai province, from *Picea crassifolia* infested with *Polygraphus poligraphus*, Aug. 2010, *M.L. Yin* & *X.D. Zhou*, (PREM 60914 **holotype**, ex-holotype cultures CBS 136507 = CMW 38888); Qinghai province, from *Picea crassifolia* infested with *P. poligraphus*, Aug. 2010, *M.L. Yin & X.D. Zhou*, (PREM 60915 **paratype**, ex-paratype cultures: CBS 136508 = CMW 38889).

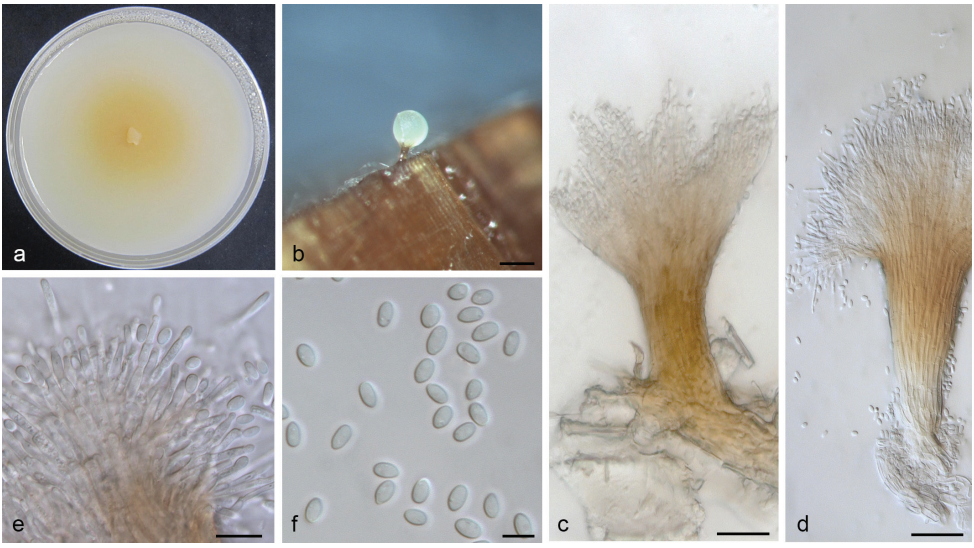

Figure 3. *Leptographium breviuscapum* sp. nov. (CMW 38888) **a** fourteen-days old culture on OA with black background **b** synnematous asexual state on wood tissue on WA **c–d** conidiophore **e** conidiogenous cells **f** conidia. Scale bars: 100 μm (**b**), 25 μm (**c**), 25 μm (**d**), 10 μm (**e**), 5 μm (**f**).

Description. *Sexual state* not observed. *Conidiophores* occasionally observed on wood of WA, macronematous, synnematous, short, wide at the stipe, light brown to yellowish, expanding branches at the apex, 150–230 μm in length including conidiogenous apparatus, 20–25 μm wide at base, 40–45 μm wide at apex, 100–150 μm wide at conidiogenous apparatus. *Conidiogenous cells* discrete, hyaline, cylindrical, percurrent proliferation, (8–)9–13(–15) × 1.8–2.5 μm. *Conidia* hyaline, one-celled, smooth, ellipsoidal, (3.7–)4–4.5(–5) × 2.5–3 μm. *Culture characteristics*: Colonies on OA, hyaline at first, later becoming light yellowish in the center, mycelium superficial on agar. Mostly mycelium observed in culture, synnemata sparse. Optimal temperature for growth 25 °C, growth reduced at 10 °C and 30 °C, no growth at 35 °C.

Host tree. *Picea crassifolia*.

Insect vector. *Polygraphus poligraphus*.

Distribution. Qinghai, China.

Note: The asexual state of *L. breviuscapum* has very short conidiophores making it very easy to distinguish from that of other species in the complex.

Additional material examined. Qinghai province, from *Picea crassifolia* infested with *Polygraphus poligraphus*, Aug. 2010, *M.L. Yin* & *X.D. Zhou*, (culture: CMW 38890). Yunnan province, from *Pinus yunnanensis* infested with *Tomicus yunnanense*, Sep. 2017, *M.L. Yin*, (culture: SCAU-475). Yunnan province, from *Pinus yunnanensis* infested with *Tomicus yunnanense*, Sep. 2017, *M.L. Yin*, (culture: SCAU-478).

Leptographium conplurium **M.L. Yin, Z.W. de Beer & M.J. Wingf., sp. nov.**
MycoBank No: 823572
Fig. 4

Etymology. The epithet refers to synnemata produced abundantly in culture.

Type. FINLAND, Ilomantsi, from *Picea abies* infested with *Dryocoetes autographus*, Aug. 2005, *Z.W. de Beer*, (PREM 60918-**holotype**, ex-holotype cultures: CBS 128923 = CMW 23315); Ilomantsi, from *P. abies* infested with *D. autographus*, Aug. 2005, *Z.W. de Beer*, (PREM 60919-**paratype**, ex-paratype cultures: CBS 128834 = CMW 23289).

Description. *Sexual state* not observed. *Conidiophores* macronematous, synnematous, 300–700 µm including conidiogenous apparatus, synnemata occasionally swollen at the base, frequently swollen at the stipe, brown to black, expanding branches at the apex, (25–)40–50(–80) µm in width, abundantly produced in culture. *Conidiogenous cells* discrete, terminal, hyaline, cylindrical, (8–)12–17(–20) × 1.5–2.3 µm. *Conidia* hyaline, one-celled, ellipsoidal to cylindrical, (3.9–)4.3–4.9(–6.3) × 1.9–2.5 µm. *Culture characteristics*: colonies on OA, hyaline at first, later becoming light yellowish in the center, concentric rings present, hyphae hyaline, appressed and immersed. Optimal growth temperature is 25 °C with radial growth rate 2.5 (± 0.5) mm/d, growth reduced at 10 °C and 30 °C, no growth at 35 °C.

Host tree. *Picea abies*.

Insect vectors. *Dryocoetes autographus*, *Hylastes brunneus*.

Distribution. Finland.

Notes. All isolates of this species were initially recognized as a cryptic species closely related to *L. cucullatum* and *L. olivaceapini* by Linnakoski et al. (2012). Our results confirmed that they represent an undescribed taxon.

Additional material examined. FINLAND, Ilomantsi, from *Picea abies* infested with *Dryocoetes autographus*, Aug. 2005, *Z.W. de Beer*, (culture: CMW 23295); Ilomantsi, from *P. abies* infested with *Hylastes brunneus*, Aug. 2005, *Z.W. de Beer*, (culture: CMW 23316).

Leptographium cucullatum **(H. Solheim) M.L. Yin, Z.W. de Beer & M.J. Wingf., comb. nov.**
MycoBank No: 831546

≡ *Ophiostoma cucullatum* H. Solheim, Nord. J. Bot. 6: 202 (1986). (Basionym)
≡ *Grosmannia cucullata* (H. Solheim) Zipfel, Z.W. de Beer & M.J. Wingf., Zipfel et al. (2006) Stud. Mycol. 55: 90.

Type. NORWAY, Vestfold, Lardal, from *Ips typographus* caught when leaving a log of *Picea abies*, 20 Aug 1981, *H. Solheim*, (CBS H-15306 and CBS H-3560-**holotype**, ex-holotype cultures: CMW 1140 = CBS 218.83 = 81-83/16).

Descriptions. Solheim (1986, pp 202–203, fig. 2); Wingfield et al. (1989, pp 92–95, figs 1–10); Yamaoka et al. (1997, pp 1220–1221 figs 22–26); Harrington et al. (2001, pp 128–129, figs 41, 44).

Host trees. *Picea abies*, *Picea jezoensis*, *Pinus sylvestris*.

Insect vectors. *Dryocoetus autographus*, *Ips typographus*, *Ips typographus japonicus*.

Distributions. Europe (Austria, Norway, Poland, Russia), Japan

Notes. Harrington et al. (2001) suggested that *Phialographium erubescens* represented the asexual state of *L. cucullatum*. Comprehensive data from the present study distinguish between the two species. See details under *L. erubescens*.

Additional material examined. AUSTRIA, Tyrol, Ehrwald, from *I. typographus* in *Picea abies*, July 1997, *T. Kirisits*, CMW 5022; JAPAN, Hokkaido, Furano, from an adult of *Ips typographus japonicus* in *Picea jezoensis*, 31 July 1991, *Y. Yamaoka*, CMW 1871 = JCM 8816; RUSSIA, Ohtama, from *I. typographus* in *P. abies*, June 2004, *J. Ahtiainen*, CMW 23123 = CBS 128299;RUSSIA, Lisino-Corpus,from *I. typographus* in *Pinus sylvestris*, *R. Linnakoski*, CMW 23190; RUSSIA, Kivennapa, Lintula, from *Dryocoetus autographus* in *P. abies*, Oct 2007, *R. Linnakoski*, CMW 27983, CMW 27984; RUSSIA, Karelia, from *I. typographus* in *P. abies*, *H. Roininen*, CMW 36623.

Leptographium davidsonii **(Olchow. & J. Reid) M.L. Yin, Z.W. de Beer & M.J. Wingf., comb. nov.**

MycoBank No: 831547

≡ *Ceratocystis davidsonii* (Olchow. & J. Reid), Can. J. Bot. 52: 1698 (1974). (Basionym)
≡ *Ophiostoma davidsonii* (Olchow. & J. Reid) H. Solheim, Nord. J. Bot. 6: 203 (1986).
≡ *Grosmannia davidsonii* (Olchow. & J. Reid) Zipfel, Z.W. de Beer & M.J. Wingf., Zipfel et al., Stud. Mycol. 55: 90 (2006).

Type. CANADA, British Columbia, Seymour Arm, from *Pseudotsuga menziesii*, 1971, *J. Reid*, (WIN (M) 71-30-**holotype**, ex-holotype cultures: CMW 790 = IMI 176524 = JCM 7867).

Descriptions. Olchowecki & Reid (1974, pp 1698–1699, figs 230–238); Upadhyay (1981, pp 42–43, figs 58–62); Mouton et al. (1993, pp 376–377, figs 15–18); Ohtaka et al. (2002, pp 154–156, figs 6–10).

Host trees. *Abies veitchii*, *Picea* sp, *Pseudotsuga menziesii*.

Insect vector. *Dryocoetes hectographus*.

Distribution. USA, Japan.

Notes. The orange section shaped to hemispherical ascospores makes this species distinct from others in the complex (Ohtaka et al. 2002). This fungus was also reported associated with *Dryocoetes hectographus* on *Abies veitchii* in Japan based on morphology (Ohtaka et al. 2002), but the identity of the Japanese isolates needs to be verified with DNA sequences.

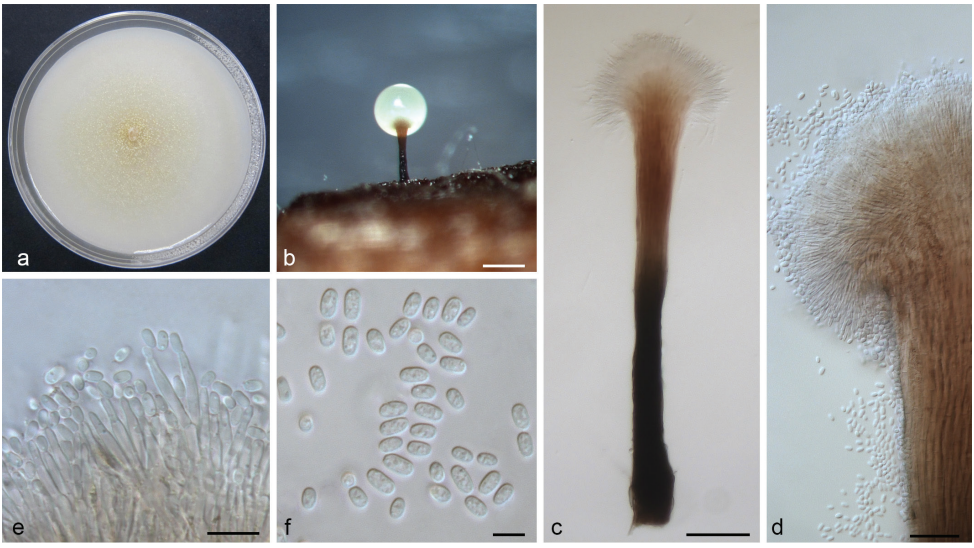

Figure 4. *Leptographium conplurium* sp. nov. (CMW 23315). **a** fourteen-days old culture on OA with black background; b. synnematous asexual state on wood tissue on WA **c** conidiophore **d** conidiogenous apparatus **e** conidiogenous cells **f** conidia. Scale bars: 200 µm (**b**), 50 µm (**c**), 20 µm (**d**), 10 µm (**e**), 5 µm (**f**).

Additional material examined. CANADA, British Columbia, Lake Louise, from small Scolytinae sp. in *Picea* sp. Aug 1994, *M. J. Wingfield*, (cultures: CMW 3094, CMW 3095).

***Leptographium erubescens* (Math.-Käärik) M.L. Yin, Z.W. de Beer & M.J. Wingf., comb. nov.**
MycoBank No: 823577

≡ *Graphium erubescens* Math.-Käärik, Medd. Skogs for skninginst. 43: 62 (1953). (Basionym)
≡ *Pesotum erubescens* (Math.-Käärik) G. Okada, Stud. Mycol. 45: 184 (2000).
≡ *Phialographium erubescens* (Math.-Käärik) T.C. Harr. & McNew, Mycologia 93: 129 (2001).

Type. SWEDEN, from pine poles and board, *A. Mathiesen-Käärik*, **lectotype** designated here, represented by line drawings (fig. 8b, p. 58; fig. 9d–f, p. 61) from Mathiesen-Käärik (1953), **MBT 379456**; Uppland, Skutskär, from piled timber of *Pinus sylvestris*, 1952, *A. Mathiesen-Käärik*, (Isotype CBS H-7193, CBS H-7194, ex-type cultures: CMW 40672 = CBS 278.54 = JCM 9747 = No. Sk 13-52).

Descriptions. Mathiesen-Käärik (1953, p.62, figs8b, 9f–d); Harrington et al. (2001, pp 128–129, figs 42, 43, 45).

Host tree. *Pinus sylvestris*.

Insect vector. unknown.

Distribution. Sweden.

Notes. This species was first described by Mathiesen-Käärik (1953) from pine timber in Sweden. No specimen numbers and very little detail (e.g. no host locality or collection dates) were provided in the protologue. Furthermore, no specimen number and little detail are listed under this species name in the herbarium of the Museum of Evolution, Uppsala, which incorporated Mathiesen-Käärik's collection. However, in 1954 she deposited an isolate (No. Sk 13-52) in the CBS labeled as *L. erubescens*. Two dried specimens (CBS H-7193, CBS H-7194) are linked to this isolate and these are labeled as isotypes. It is reasonable to assume that this isolate represents the original material, but there is no conclusive evidence that this is true. We have thus designated the line drawings from the protologue (Mathiesen-Käärik 1953) as the lectotype.

Harrington et al., (2001) suggested that *Graphium erubescens* (as *Phialographium erubescens*) represented the asexual state of *L. cucullatum* (as *O. cucullatum*) based on ITS sequences. However, based on sequences produced in the present study, the extype culture of *L. erubescens* differs from that of *L. cucullatum* in 1bp in ITS2-LSU, 17 bp in ACT, 17 bp in BT, 30 bp in CAL, and 48 bp in TEF-1α. We have thus treated these species as distinct and have provided a new combination for *L. erubescens*.

Leptographium francke-grosmanniae **(R.W. Davidson) K. Jacobs & M.J. Wingf., *Leptographium* species: p. 99 (2001)**

MycoBank No: MB375135

≡ *Ceratocystis francke-grosmanniae* R.W. Davidson, Mycologia 63: 6 (1971). (Basionym)

≡ *Ophiostoma francke-grosmanniae* (R.W. Davidson) De Hoog & R.J. Scheff., Mycologia 76: 297 (1984).

≡ *Grosmannia francke-grosmanniae* (R.W. Davidson) Zipfel, Z.W. de Beer & M.J. Wingf., Stud. Mycol. 55: 90 (2006).

Type. GERMANY, Reinbeck near Hamburg, from *Quercus* sp. associated with *Hylecoetus dermestoides*, May 1967, *H. Francke-Grosmann*, (**holotype** BPI 595654, exholotype cultures: RWD 828 = ATCC 22061 = CBS 356.77 = CMW 445).

Descriptions. Davidson (1971, pp 6–7, figs 1, 10, 11, 17); Upadhyay (1981, p. 45, figs 73–78); Mouton et al. (1992, figs 1–11); Wingfield (1993, p. 48, figs 6–7); Jacobs and Wingfield (2001, pp 99–102, figs 73–75).

Host tree. *Quercus* sp.

Insect vector. *Hylecoetus dermestoides*.

Distribution. Germany.

Notes. *Leptographium francke-grosmanniae* groups peripheral to other species in the *L. olivaceum* complex (Figs 1–3). Morphologically, the ascospores are almost cylindrical and its ascomatal necks correspond with other species in the complex. But *L. francke-grosmanniae* produces mononematous conidiophores, in contrast to the synne-

mata produced by the other species, which also explains why it is the only species in the complex previously treated in *Leptographium*. The mode of conidiogenesis of *L. francke-grosmanniae* (Mouton et al. 1992) appears similar to that of other species where the conidiogenous cells that appear phialidic under a light microscope arise from percurrent proliferation (Wingfield et al. 1989, Wingfield et al. 1991, Mouton et al. 1993). However, the apices of the apparent "phialides" are substantially more flared than those of other species in the complex and they could be more different than assumed by Mouton et al. (1993). *Leptographium francke-grosmanniae* is also unusual in the *L. olivaceum* complex in having an angiosperm host.

Leptographium francke-grosmanniae was originally described as *Ceratocystis francke-grosmanniae* from larval galleries of *Hylecoetus dermestoides* on *Quercus* sp. in Germany (Davidson 1971). De Beer and Wingfield (2013) showed that sequences for this species produced in different studies were inconsistent. Based on comparisons of the ITS2 region, the sequences of ex-holotype generated in the present study are consistent with those produced by Mullineux and Hausner (2009) for ATCC 22061 and Hamelin et al. (unpublished) for CBS 356.77, but differ substantially from sequences produced by Jacobs et al. (2001b). In the LSU gene region, our sequences are identical to those of Hausner et al. (2000), but they differed from that of Jacobs et al. (2001a, b) for CMW 445.In the β-tubulin gene region, the sequence of CMW 445 in the present study was consistent with that provided by Kim et al. (2004) for CMW 445 and Hamelin et al. (unpublished sequence in GenBank) for CBS 356.77. We thus suggest that the two sequences for *L. francke-grosmanniae* produced by Jacobs et al. (2001a, b) are incorrect. Sequences of another isolate from the USA (CMW 2975), previously identified as *L. francke-grosmanniae* (Zipfel et al. 2006), differ substantially from the ex-holotype culture. Thus, this isolate (CMW 2975) does not represent *L. francke-grosmanniae*, and its taxonomic placement needs reconsideration.

Leptographium olivaceum **(Math.-Käärik) M.L. Yin, Z.W. de Beer & M.J. Wingf., comb. nov.**
MycoBank No: 831548

≡ *Ophiostoma olivaceum* Math.-Käärik, Svensk. Bot. Tidskr. 45: 212 (1951). (Basionym)
≡ *Ceratocystis olivacea* (Math.-Käärik) J. Hunt, Lloydia 19: 29 (1956).
≡ *Grosmannia olivacea* (Math.-Käärik) Zipfel, Z.W. de Beer & M.J. Wingf., Zipfel et al., Stud. Mycol. 55: 91 (2006).

Type. SWEDEN, Hällnäs, Västerbotten, from the galleries of *Acanthocinus aedilis* in pine wood, *A. Mathiesen-Käärik*, **lectotype** designated here, represented by line drawings (fig. 2a–g, p. 213) from Mathiesen-Käärik (1951), **MBT 379459**; from dead wood of *Pinus sylvestris*, Jan 1949, *A. Mathiesen-Käärik*, (ex-type cultures: CMW 31059 = CBS 138.51, MBT 2063).

Descriptions. Mathiesen-Käärik (1950, p. 298); Mathiesen-Käärik (1951, pp 212–215, fig. 2); Hunt (1956, pp 29–30); Griffin (1968, pp 707–708, figs 49–52,

82); Olchowecki and Reid (1974, pp 1699–1700, Pl. XIII fig. 262); Upadhyay (1981, pp 52–54, figs 116–121); Mouton et al. (1993, pp 376–377, figs 19–22).

Host trees. *Betula papyrifera*, *Picea abies*, *Picea mariana*, *Pinus sylvestris*.

Insect vectors. *Acanthocinus aedilis*, *Dendroctonus rufipennis*, *Ips typographus*, *Polygraphus rufipennis*.

Distributions. Canada, Finland, Russia, Sweden, USA.

Notes. This species was first described invalidly (no Latin diagnosis) from *Pinus sylvestris* infested by a longhorn beetle *Acanthocinus aedilis* in Sweden (Mathiesen-Käärik 1950). Mathiesen-Käärik (1951) then validated the name with a more detailed description accompanied by a Latin diagnosis. In the original descriptions of *L. olivaceum* by Mathiesen-Käärik (1950, 1951), the host tree, beetle and location of the collection was noted, but no mention was made of a specimen. The herbarium specimens of Mathiesen-Käärik were initially curated in the herbarium of the Statens Skogsforsknings institut, Experimentalfältet, Sweden. The collection was later incorporated into the herbarium of the Museum of Evolution, Uppsala. Only one herbarium specimen (UPS:BOT:F-130986) of *L. olivaceum*, collected from the same host, beetle and location by T. Hedquist, is available from that collection. However, an isolate of *L. olivaceum* (No. 297-49 = CBS 138.51), collected in 1949, also from the original host and location, was deposited in the CBS by Mathiesen-Käärik in 1951. Although we were not able to confirm that this isolate was from the original collection, it was treated as the ex-type culture of the species in previous studies (Duong et al. 2012, Linnakoski et al. 2012, De Beer and Wingfield 2013). In view of the absence of concrete evidence that this isolate represents the original material, we have designated the line drawings from the protologue (Mathiesen-Käärik 1951) as lectotype.

More recently, it was reported from *Picea abies* and *Pinus sylvestris* infested by *Ips typographus* and *Dryocoetes autographus* in Finland and Russia, in a study where the identities were confirmed using DNA sequence analyses (Linnakoski et al. 2012). Griffin (1968) reduced *L. vescum* to synonymy with *L. olivaceum*, but data from the present study confirmed that these two species are phylogenetically distinct.

Additional material examined. FINLAND, Jouhteninen, from *Ips typographus* in *Picea abies*, July 2005, *Z.W. de Beer*, (cultures: CMW 23348 = CBS 128836, CMW 23350 = CBS 128837). RUSSIA, Uuksujärvi, from *I. typographus* in *Pinus sylvestris*, Oct 2007, *R. Linnakoski*, (culture CMW 28090). SWEDEN, Oct 1954, *A. Mathiesen-Käärik*, (cultures: CMW 31060 = CBS 152.54).

Leptographium olivaceapini **(R.W. Davidson) M.L. Yin, Z.W. de Beer & M.J. Wingf., comb. nov.**

MycoBank No: 831549

≡ *Ceratocystis olivaceapini* R.W. Davidson, Mycologia 63: 7 (1971). (Basionym)

≡ *Ophiostoma olivaceapini* (R.W. Davidson) K.A. Seifert & G. Okada, *In* Okada et al., Can. J. Bot. 76: 1504 (1998).

≡ *Grosmannia olivaceapini* (R.W. Davidson) Z.W. de Beer, R. Linnakoski & M.J. Wingf., *In* Linnakoski et al., Antonie van Leeuwenhoek 102: 375–399 (2012).

Type. USA, New Mexico, Santa Fe, from *Pinus ponderosa* tree infested *Dendroctonus* sp. and other bark beetles, 10 July 1964, *R.W. Davidson*, (**holotype** BPI 595910 = RWD 548D; BPI 595914 = RWD 548D isotype); USA, Arizona, Flagstaff, from *Pinus ponderosa* infested with *Dendroctonus* sp., 24 July 1964, *R.W. Davidson*, (BPI 596223= RWD 581-D isotype); Arizona, Flagstaff, from *P. ponderosa* infested with *Dendroctonus* sp., 3 Oct 1986, *T. Hinds*, (**epitype** PREM 61051, designated here, ex-epitype cultures CBS 504.86 = CMW 116 = COLO 479, **MBT 379458**).

Descriptions. Davidson (1971, pp 7–10, figs 2, 12, 18); Upadhyay (1981, p. 54, figs 122–129); Mouton et al. (1993, pp 372–373, figs 1–4).

Host trees. *Pinus ponderosa*.

Insect vectors. *Dendroctonus* sp.

Distribution. USA.

Notes. No living culture associated with the holotype (BPI 595910) or isotype (BPI 595914) of *L. olivaceapini* exists. However, T. Hinds, a collaborator of R.W. Davidson and later curator of the RWD culture collection, provided an isolate (COLO 479) labeled as *C. olivaceapini* to M.J. Wingfield, who later deposited this in the CBS (CBS 504.86). The species name and origin provided by Hinds with the isolate corresponds to a second specimen mentioned by Davidson (1971, p. 10) in the protologue (RWD 581-D = BPI 596223). In our opinion, the isolate (COLO 479) most probably originated from the specimen (RWD 581-D). We could not confirm with certainty that BPI 296223 originated from RWD 581-D and thus designated a dried culture of COLO 479 as the epitype for *L. olivaceapini*.

Additional Material examined: USA, Arizona, Flagstaff, from *P. ponderosa* infested with *Dendroctonus* sp., 3 Oct 1986, *T. Hinds*, (PREM 61051, cultures CBS 504.86 = CMW 116 = COLO 479). Minnesota, *Pinus resinosa*, Nov 1986, M.J. Wingfield, (cultures: CBS 503.86 = CMW 63).

Leptographium pseudoalbum **M.L. Yin, Z.W. de Beer and M.J. Wingf., sp. nov.**
MycoBank No: 823571
Fig. 5

Etymology. The epithet refers to the previous, incorrect identification of the ex-holotype isolate of this species as *Graphium album*.

Type. SWEDEN, from *Pinus sylvestris* infested by *Tomicus piniperda*, 1953, *Mathiesen-Käärik*, (PREM 61050-**holotype**, ex-holotype cultures: CBS 276.54 = CMW 40671 = JCMW 9774 = C 1225).

Description. *Sexual state* not observed. *Conidiophores* macronematous, synnematous, 120–270 µm including conidiogenous apparatus, synnemata frequently swollen at base, frequently wider at stipe, expanding branches at apex, brown to hyaline, (11–)25–34(–40) µm in width. *Conidiogenous cells* discrete, terminal, percurrent and phialidic proliferation, hyaline, cylindrical, (9–)10–14(–18) × 1.8–2.8 µm. *Conidia* hyaline, one-celled, ellipsoidal to cylindrical, (3.5–)4.3–5.2(–6.5) × 2.4–3.3 µm. *Cultural characteristics*: Colonies on OA, hyaline at first, later becoming white and gray in

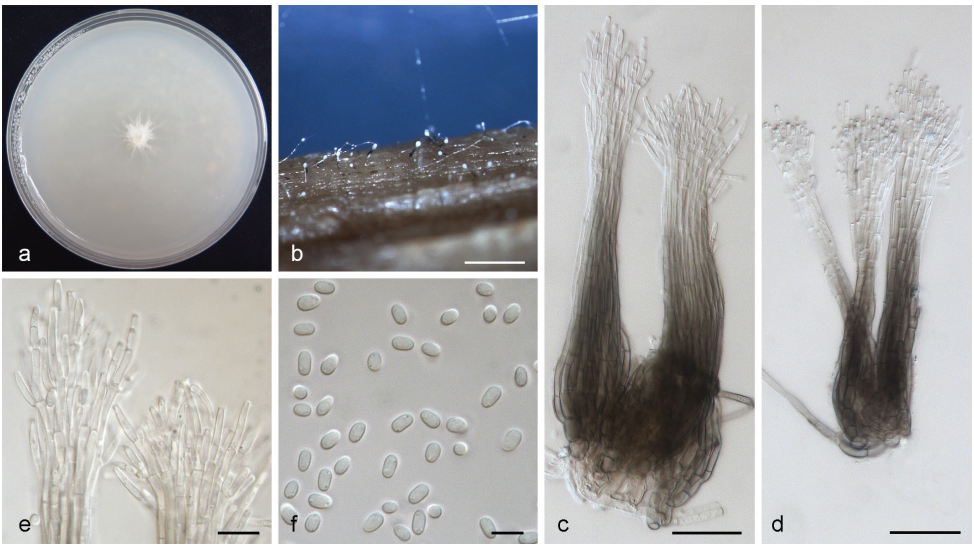

Figure 5. *Leptographium pseudoalbum* sp. nov. (CBS 276.54) **a** fourteen-days old culture on OA with black background; b. synnematous asexual state on wood tissue on WA **c–d** conidiophore **e** conidiogenous cells **f** conidia. Scale bars: 200 µm (**b**), 25 µm (**c**), 25 µm (**d**), 10 µm (**e**), 5 µm (**f**).

the center, hyphae hyaline, appressed and immersed, aerial mycelium frequently present on wood tissue, phialographium-like asexual morph abundant. Optimal growth temperature on MEA:25 °C with radial growth rate 3.0 (± 0.5) mm/d, while growth slightly reduced at 10 °C and 30 °C, and no growth occurred at 35 °C.

Host. *Pinus sylvestris*.

Insect vector. *Tomicus piniperda*.

Distribution. Sweden.

Notes. This species was initially identified as *Graphium album* (Corda) Sacc. by Mathiesen-Käärik (1953). However, Okada et al. (2000) and Harrington et al. (2001) questioned the identification by Mathiesen-Käärik (1953) and showed that this isolate belonged in the Ophiostomatales and grouped close to *L. erubescens*. This study showed that Mathiesen-Käärik's isolate representing an undescribed species in the *L. olivaceum* complex, for which we have provided the name *L. pseudoalbum*.

Leptographium rhizoidum **M.L. Yin, Z.W. de Beer and M.J. Wingf., sp. nov.**
MycoBank No: 823575
Fig. 6

Etymology. The epithet refers to the rhizoid-like structures at the synnematal bases.

Type. SPAIN, Morga, from *Pinus radiata* infested by *Hylastes ater*, July. 2004, *P. Romon & X.D. Zhou*, (PREM 60922-**holotype**, ex-holotype cultures: CBS 136512 = CMW 22809); Morga, from *Pinus radiata* infested by *Hylastes attenuatus*, July. 2004,

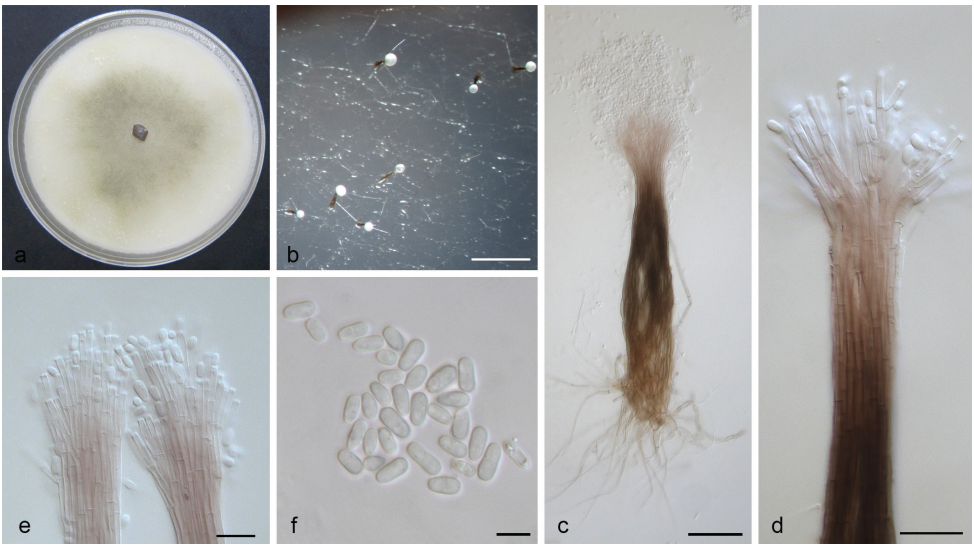

Figure 6. *Leptographium rhizoidum* sp. nov. (CMW 22809). **a** fourteen-days old culture on OA with black background **b** synnematous asexual state on wood tissue on WA **c** conidiophore **d** conidiogenous apparatus **e** conidiogenous cells **f** conidia. Scale bars: 200 µm (**b**), 50 µm (**c**), 20 µm (**d**), 10 µm (**e**), 5 µm (**f**).

P. Romon & X.D. Zhou, (PREM 60923-**paratype**, ex-paratype cultures: CBS 136513 = CMW 22810).

Description. *Sexual state* not observed. *Conidiophores* macronematous, synnematous, 200–350 µm including conidiogenous apparatus, synnemata frequently swollen at the base, frequently wider at the stipe, brown to light brown, expanding branches at the apex, (15–)35–45(–70) µm in width. *Conidiogenous cells* discrete, terminal, percurrent and phialidic proliferation, hyaline, cylindrical,(10–)14–17(–19) × 2–3 µm. *Conidia* hyaline, one-celled, cylindrical to obovoid, (5.1–)6.5–7.8(–10.5) × 2.1–3.5 µm. *Cultural characteristics*: Colonies on OA, hyaline at first, later becoming olivaceous in the center, hyphae hyaline, appressed and immersed, aerial mycelium frequently present on wood tissue, synnemata abundant in WA cultures, Optimal growth temperature on MEA is 25 °C with radial growth rate 6.0 (± 0.5) mm/d, growth slightly reduced at 10 °C and 35 °C.

Host tree. *Pinus radiata*.

Insect vectors. *Hylastes ater*, *H. attenuatus*, *Hylurgops palliatus*, *Ips sexdentatus*.

Distribution. Spain.

Note: Isolates of *L. rhizoidum* from pine-infesting bark beetles in Spain were initially identified as *L. olivaceum* based on ITS sequences by Romon et al. (2007). Our data showed them to be distinct from that species. This species produced more abundant and longer rhizoids than others in the complex.

Other Material examined: SPAIN, Morga, from *Pinus radiata* infested by *Ips sexdentatus*, July. 2004, *P. Romon & X.D. Zhou*, (culture: CMW 22811); Morga, from *P. radiata* infested by *Hylurgops palliatus*, July. 2004, *P. Romon & X.D. Zhou*, (culture: CMW 22812).

***Leptographium sagmatosporum* (E.F. Wright & Cain) M.L. Yin, Z.W. de Beer & M.J. Wingf., comb. nov.**
MycoBank No: 831550

≡ *Ceratocystis sagmatospora* E.F. Wright & Cain, Can. J. Bot. 39: 1226 (1961). (Basionym).
≡ *Phialographium sagmatosporae* H.P. Upadhyay and W.B. Kendr., Mycologia 66: 183 (1974).
≡ *Ophiostoma sagmatosporum* (E.F. Wright & Cain) H. Solheim, Nord. J. Bot. 6: 203 (1986).
≡ *Graphium sagmatosporae* (H.P. Upadhyay & W.B. Kendr.) M.J. Wingf. & W.B. Kendr., Mycol. Res. 95: 1332 (1991).
≡ *Pesotum sagmatosporum* (H.P. Upadhyay & W.B. Kendr.) G. Okada & K.A. Seifert, in Okada et al., Can. J. Bot. 76: 1504 (1998).
≡ *Grosmannia sagmatospora* (E.F. Wright & Cain) Zipfel, Z.W. de Beer & M.J. Wingf., *In* Zipfel et al. Stud. Mycol. 55: 91 (2006).

Type. CANADA, Ontario, Ontario, NE. of Mansfield, Dufferin Co., from *Pinus resinosa*, Nov. 8 1958, *E.F. Wright* &*R.F. Cain*, **lectotype** designated here, represented by line drawings (fig. 23, p. 1225, figs 24–33, p. 1228) from Wright and Cain (1961), **MBT 379455**; Ontario, Stittsville, 13 Lucas Lane, 4511.9 N 7558.8 W, from old bark beetle galleries in *Pinus strobus*, Sept. 2000, *K. Jacobs*, (**epitype** PREM 61054, designated here, ex-epitype cultures: CMW 34135 = CBS 113452, **MBT 379454**).

Descriptions. Wright and Cain (1961, pp 1226–1229, figs 23–33); Griffin (1968, pp 708, 712–713); Olchowecki and Reid (1974, p. 1701, Pl. XIII figs 254, 257); Upadhyay (1981, p. 60, figs 167–171).

Host trees. *Pinus strobus*, *Picea mariana*.

Insect vectors. unknown bark beetle species.

Distribution. Canada.

Notes. This species was originally described from bark beetle galleries and freshly cut surfaces of *Picea mariana*, *Pinus resinosa* and *Pinus strobus* in Canada (Wright and Cain 1961). The Royal Ontario Museum Fungarium (TRTC), Canada, informed the authors of this study that the holotype (TRTC 36427) of *L. sagmatosporum* was permanently lost. There is also no living culture available from the holotype. We have thus designated the line drawings in the protologue as the lectotype. An isolate (CMW 34135), also from pine in Ontario, identified as *L. sagmatosporum* based on morphology (K. Jacobs, unpublished) and used in previous studies to represent the species (Duong et al. 2012, Linnakoski et al. 2012, De Beer and Wingfield 2013), its dry specimen is designated here as the epitype.

Additional Material examined: CANADA, Ontario, NE. of Mansfield, Dufferin Co., from *Pinus resinosa*, Nov. 8 1958, *E.F. Wright* & *R.F. Cain*, TRTC 34600; NW. of Nobleton, York Co., from *Pinus strobus*, July 1 1957, *E.F. Wright* & *R.F. Cain*, TRTC 33034; Twp. West of 11 H, Challener Lake, Sudbury Dist., from *Pinus strobus*, June 20 1960, *E.F. Wright* & *R.F. Cain*, TRTC 36245, 36251, 36255, 36264, 36265;Twp.

5F, Aubinadong R., Algoma Dist, from *Pinus strobus*, June 17 1960, *E.F. Wright* & *R.F. Cain*, TRTC 36246; Twp. West of 11 H, Challener Lake, Sudbury Dist., from *Picea mariana*, June 20 1960, *E.F. Wright* & *R.F. Cain*, TRTC 36263.

Leptographium sylvestris **M.L. Yin, Z.W. de Beer and M.J. Wingf., sp. nov.**
MycoBank No: 823574
Fig. 7

Etymology. The epithet refers to the host species where the holotype was collected.

Type. POLAND, Chrosnica, from *Pinus sylvestris*, Jan. 2008, *R. Jankowiak*, (PREM 60920-**holotype**, ex-holotype cultures: CBS 136511 = CMW 34140). FINLAND, Jouhteninen, from *Picea abies* infested with *Ips typographus*, Aug. 2005, *Z.W. de Beer*, (PREM 60921-**paratype**, ex-paratype cultures: CBS 128833 = CMW 23300).

Description. *Sexual state* develop on wood on WA in 14–21 days. *Perithecia* superficial on wood and agar, base brown to black, globose, unornamented, 91–110 µm in diameter, necks dark brown, cylindrical, slightly curved, 200–480 µm long (including ostiolar hyphae), 26–32 µm wide at base, 15–21 µm wide at the tip. *Ostiolar hyphae* present, pale brown, straight, septate, numerous, divergent, tapering at the tip, up to 190 µm long. *Asci* not seen. *Ascospores* one-celled, hyaline, fusiform to orange section shaped in side view, ellipsoidal in face view, globose in end view, (4.0–)4.5–5.5(–5.8) × (2.5–)2.8–3.7(–3.9) µm including hyaline gelatinous sheath, 0.3–0.6 µm thick. *Conidiophores* macronematous, synnematous, swollen at the base, occasionally wider at the stipe, brown to light brown, expanding branches at the apex, 260–500 × 14–57 µm including conidiogenous apparatus. *Conidiogenous cells* discrete, hyaline, cylindrical, 2–3 per branch, percurrent proliferation, (10–)11–15(–18) × 1.5–2.5 µm. *Conidia* hyaline, obovate to clavate, (3.6–)4.5–4.9(–5.2) × (1.6–)1.7–1.9(–2.1) µm. *Cultural characteristics*: Colonies on OA, hyaline at first, later becoming dark yellowish in the center, mycelium appressed and immersed, Perithecia and *Pesotum*-like asexual morph co-occur in culture. Optimal growth temperature is 30 °C, radial growth rate 5.0 (± 0.5) mm/d, growth reduced at 10 °C, no growth at 35 °C.

Host trees. *Pinus sylvestris*, *Picea abies*.

Insect vector. *Ips typographus*.

Distributions. Poland, Finland.

Notes. The Finnish isolate (CMW 23300) was considered by Linnakoski et al. (2012) to be the same undescribed species as the isolates described above as *L. conplurium*. The addition of a newly obtained isolate from Poland in the present study, confirmed that the two isolates represented a distinct taxon, clearly separated from all other species in the complex. This is the only new species for which ascomata were obtained in culture. Single ascospore isolates of this species produced ascomata in culture, suggesting that the species is homothallic. The common characters of sexual states of species in this complex are having ascomata with sheath and ostiolar hyphae on the top of neck. This species differs from others by its fusiform to orange section shaped ascospores and slightly curved neck.

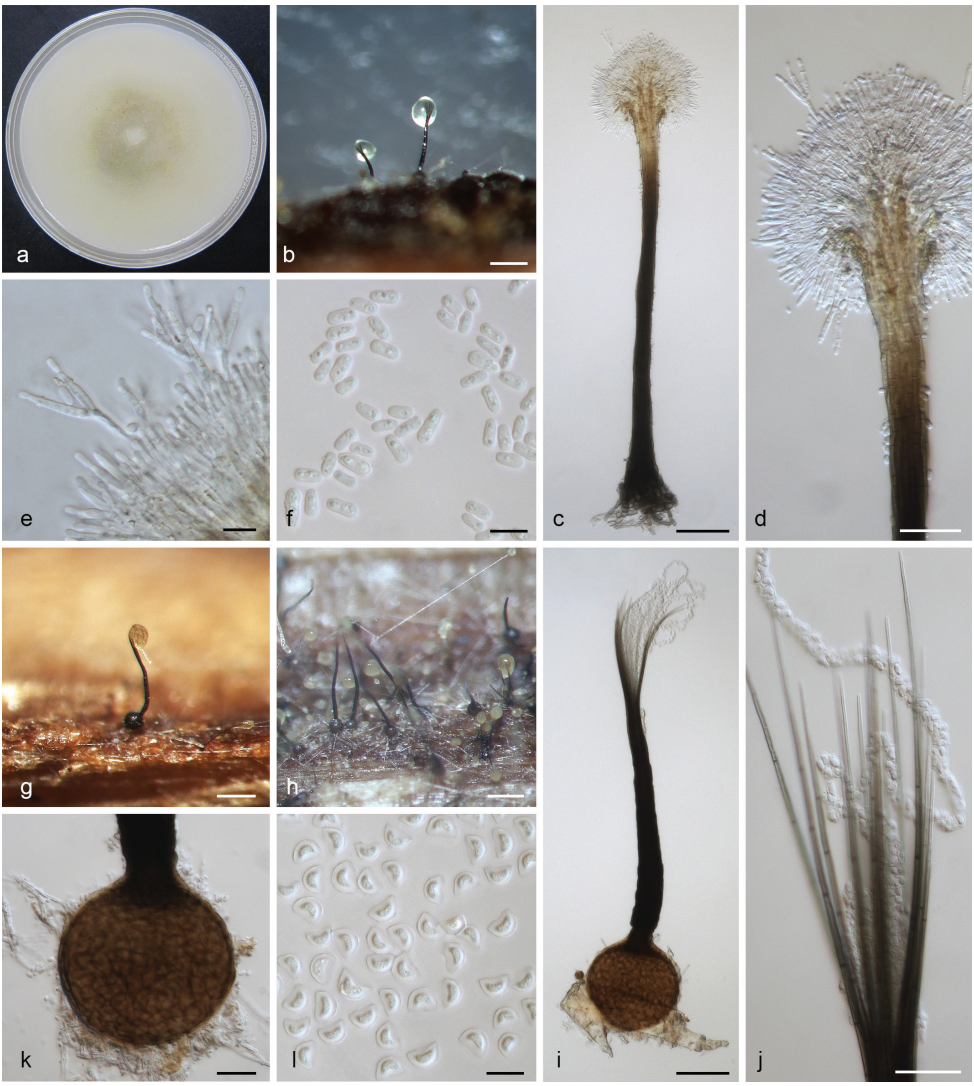

Figure 7. *Leptographium sylvestris* sp. nov. (CMW 34140) **a** fourteen-days old culture on OA with black background **b** synnematous asexual state on wood tissue on WA **c** conidiophore **d** conidiogenous apparatus **e** conidiogenous cells **f** conidia **g–h** the sexual state on wood tissue on WA **i** ascoma **j** ostiolar hyphae **k** ascomatal base **l** ascospores. Scale bars: 100 µm (**b**), 50 µm (**c**), 25 µm (**d**), 10 µm (**e**), 5 µm (**f**), 100 µm (**g**), 100 µm (**h**), 50 µm (**i**), 25 µm (**j**), 20 µm (**k**), 5 µm (**l**).

Leptographium vescum **(R.W. Davidson) M.L. Yin, Z.W. de Beer & M.J. Wingf., comb. nov.**
MycoBank No: 831551

≡ *Ceratocystis vesca* R.W. Davidson, Mycologia 50: 666. (1958) (Basionym)
≡ *Ophiostoma vescum* (R.W. Davidson) Hausner, J. Reid & Klassen. Can. J. Bot. 71: 1264. (1993)

≡ *Grosmannia vesca* (R.W. Davidson) Zipfel, Z.W. de Beer & M.J. Wingf., Zipfel et al., Stud. Mycol. 55: 92. (2006)

Type. USA, Colorado, Fort Collins, from *Ips pilifrons* and *Dendroctonus engelmanni* in *Picea engelmannii*, Jan. 31, 1956, *F.F. Lombard* & *R.W. Davidson*, (**holotype** BPI 595662 = FP 70807, ex-holotype cultures: ATCC 12968 = CBS 800.73 = CMW 34186).

Descriptions. Davidson (1958, p. 666); De Hoog and Scheffer (1984, p. 295, fig. 2); Samuels (1993, p. 16, fig. 1C–F).

Host tree. *Picea engelmannii*.

Insect vectors. *Ips pilifrons*, *Dendroctonus engelmanni*.

Distribution. USA.

Notes. The perithecia of *L. vescum* are smaller than in related species and ascospores are different in shape and size. This species was treated as a synonym of *L. olivaceum* by various authors (Griffin 1968, Olchowecki and Reid 1974, Upadhyay 1981). However, the sequences produced by Hausner et al. (1993, 2000), confirmed by our results, showed that the two species are distinct.

Leptographium xiningense **M.L. Yin, Z.W. de Beer and M.J. Wingf., sp. nov.**
MycoBank No: 823573
Fig. 8

Etymology. The epithet refers to the locality where the species was first collected.

Type. CHINA, Qinghai Province, from *Picea crassifolia* infested by *Polygraphus poligraphus*, Aug. 2010, *M.L. Yin* & *X.D. Zhou*, (PREM 60916-**holotype**, ex-holotype cultures CBS 136509 = CMW 38891); Qinghai Province, from *Picea crassifolia* infested by *Polygraphus poligraphus*, Aug. 2010, *M.L. Yin*, (PREM 60917-**paratype**, ex-paratype cultures CBS 136510 = CMW 39237).

Description. *Sexual state* not observed. *Conidiophores* macronematous, synnematous, 450–550 µm including conidiogenous apparatus, synnemata occasionally slightly swollen at the base, wider at the stipe, black to brown, expanding branches at the apex, light brown to hyaline, (25–)39–44(–50) µm in width. *Conidiogenous cells* discrete, terminal, percurrent and phialidic proliferation, hyaline, cylindrical, (11–)15–18(–19) × 2–3 µm. *Conidia* hyaline, one-celled, cylindrical to obovoid, (3.9–)4.2–4.5(–4.8) × 1.8–2.4 µm. *Cultural characteristics*: Colonies on OA, spore drops hyaline at first, later becoming light to dark yellowish in the center, hyphae hyaline, appressed and immersed, *synnemata* predominant, aerial mycelium occasionally present on wood tissue, Optimal growth temperature on MEA is 25 °C with radial growth rate 2.0 (± 0.5) mm/d, growth reduced at 10 °C, no growth at 30 °C.

Host tree. *Picea crassifolia*.

Insect vector. *Polygraphus poligraphus*.

Distribution. China.

Note. This species groups closely with *L. conplurium* and *L. erubescens*, but can be distinguished by its dark conidial droplets. In addition, the synnematous

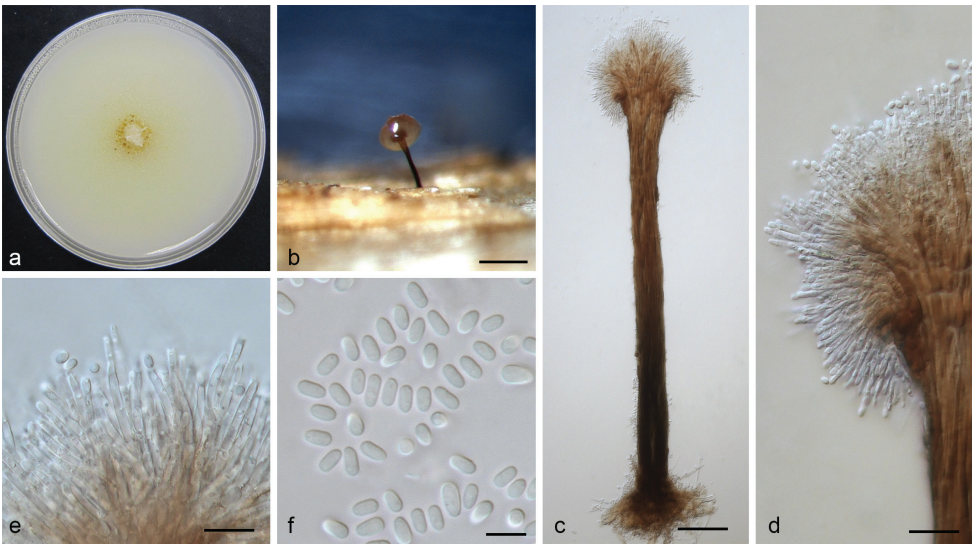

Figure 8. *Leptographium xiningense* sp. nov. (CMW 38891) **a** fourteen-days old culture on OA with black background **b** synnematous asexual state on wood tissue on WA **c** conidiophore **d** conidiogenous apparatus **e** conidiogenous cells **f** conidia. Scale bars: 300 μm (**b**), 50 μm (**c**), 20 μm (**d**), 10 μm (**e**), 5 μm (**f**).

conidiophores of this species were shorter, and its conidia were bigger than that of *L. erubescens*.

Additional material examined. CHINA, Qinghai Province, from *Picea crassifolia* infested by *Polygraphus poligraphus*, Aug. 2010, *M.L. Yin* & *X.D. Zhou*, (culture: CMW 39238). Chongqing, from *Pinus armandii* infested by *Dendroctonus armandi*, Nov. 2018, *M.L. Yin*, (culture: SCAU-530). Chongqing, from *Pinus armandii* infested by *Dendroctonus armandi*, Nov. 2018, *M.L. Yin*, (culture: SCAU-531).

Discussion

Among the five loci used in the phylogenetic analyses, ACT, CAL, and TEF-1α were able to distinguish among all species in the *L. olivaceum* complex. In contrast, TUB sequences could not distinguish between *L. davidsonii* and *L. vescum*. Although ITS2-LSU sequences provided reasonable resolution for species complexes at the genus level, this region could not be used to distinguish among closely related species. Of the five gene regions, TEF-1α had the most variable sites and this is consistent with the results of Yin et al. (2015) for the *L. procerum* complex. This also supports their suggestion that TEF-1α is suitable for use as a barcoding gene for accurate species identification in *Leptographium*.

In this study, we have clarified the previous confusion related to the ex-type isolate of *L. francke-grosmanniae*, and although our phylogenetic data placed it close to the complex, it grouped separated from all other species. This is consistent with its mon-

onematous morphology that distinguishes it from all other species in the complex that produce synnematous asexual states. Furthermore, it is unique in that it does not come from the galleries of a conifer-infesting scolytine bark beetle like the other species, but from the large timberworm beetle, *Hylecoetus dermestoides* (Coleoptera: Lymexylidae), infesting a *Quercus* sp. (Davidson 1971). Some beetles in the latter genus are known to vector ambrosial yeasts (Batra and Francke-Grosmann 1961), but the role and biology of *L. francke-grosmanniae* in these galleries on oak remains unknown. If these beetle ecosystems in hardwoods are explored further, it seems reasonable to expect that additional species related to *L. francke-grosmanniae* could be discovered. These would most likely emerge as a species complex distinct from the *L. olivaceum* complex.

All species in the *L. olivaceum* complex, with the exception of *L. francke-grosmanniae*, share various characteristics. Apart from similar sexual and asexual morphology (as discussed in the introduction), these species are all associated with scolytine bark beetles infesting primarily species of pine (*Pinus*) and spruce (*Picea*). Only *L. davidsonii* has been reported from another conifer genus, namely *Pseudotsuga* (Douglas-fir). However, there is no evidence for strong host or beetle specificity among these fungi. The European spruce bark beetle, *Ips typographus*, for example, infests various species of spruce and pine, and *L. cucullata*, *L. olivacea*, and *L. poloniae*, have been isolated from this beetle or its galleries. Nothing is known regarding the pathogenicity of any of the species in the complex, but Griffin (1968) and Davidson (1958) showed that some species were responsible for the blue-stain of the timber.

In terms of the distribution of species in the *L. olivaceum* complex, our data suggest that most of these taxa are geographically restricted to the continents from which they have been recorded. Four species have been reported only from North America, namely *L. davidsonii*, *L. olivaceapini*, *L. sagmatosporum*, and *L. vescum*, while *L. olivaceum*, *L. erubescens* and four of the new species have been found only in Europe and western Russia. Two of the new species originate from China. Only *L. cucullatum* has been found in Europe and East Asia, specifically Japan.

The results of this study incorporating data for morphology, ecology, and phylogenetic inference based on DNA sequences for five loci have confirmed that the *L. olivaceum* complex is a well-defined species complex in *Leptographium*. Moreover, this integrative approach has been recently employed to resolve lower-level taxonomy in several other groups of fungi such as the Ophiocordycipitaceae (Araújo et al. 2015), Pyronemataceae (Sochorová et al. 2019), Laboulbeniaceae (Haelewaters et al. 2018), Geastraceae (Sousa et al. 2017), and Helvellaceae (Skrede et al. 2017). The combination of multiple properties as independent lines of evidence (e.g., morphology, DNA, substratum, and/or geography) is the way to move forward in fungal taxonomy in general.

Conclusions

In the present study, DNA sequences for five loci were amplified and used to reconstruct phylogenies for species in the *L. olivaceum* complex. Multilocus phylogenies

distinguished clearly among the eight previously described species and also revealed six species: *L. breviuscapum*, *L. conplurium*, *L. pseudoalbum*, *L. rhizoidum*, *L. sylvestris*, and *L. xiningense* that are newly described. TEF-1α was recognized as the best candidate gene to distinguish all species in the complex. For several of the previously known species, problems relating to type specimens were identified, and to resolve these, seven new combinations, two epitypes and three lectotypes have been designated. Following the "one fungus one name" principles, this study provided a model solution to resolving interspecific relationships within the species complexes in the Ophiostomatales. More work should be done on other unresolved species complexes of *Leptographium* and other lineages in the Ophiostomatoid fungi in the future.

Acknowledgements

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Supplementary material 1

The sequence alignment of combined four protein-coding gene regions

Authors: Mingliang Yin, Michael J. Wingfield, Xudong Zhou, Riikka Linnakoski, Z. Wilhelm de Beer

Data type: phylogenetic data.

Explanation note: The alignment was generated from MAFFT V7 Online, and it contained sequences of four protein coding genes (actin, beta-tubulin, calmodulin, and translation elongation factor 1 alpha) of all the isolates in the Leptographium olivacerum complex.

Link: https://doi.org/10.3897/mycokeys.60.39069.suppl1

Supplementary material 2

The sequence alignment of ITS2-LSU gene region

Authors: Mingliang Yin, Michael J. Wingfield, Xudong Zhou, Riikka Linnakoski, Z. Wilhelm de Beer

Data type: phylogenetic data.

Explanation note: The alignment was generated from MAFFT V7 Online, and it contained sequences of Internal transcribed spacer 2 and large-subunit rRNA genes of all isolates used in this study.

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RESEARCH ARTICLE

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The genus *Massalongia* (lichenised ascomycetae) in the Southern Hemisphere

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Abstract

The species of *Massalongia* recorded and described from the Southern Hemisphere are revised and it is shown that only one is present; *M. patagonica* which is widespread, with populations in Australia and New Zealand that differ from the South American populations, but at present best regarded as part of the variation of that species. Records from this hemisphere of all other species placed in the genus are incorrect. The type species, *M. carnosa*, is restricted to the Northern Hemisphere. Two species, *M. antarctica* and *M. novozelandica* cannot be identified precisely due to lack of sufficient type material and with the types as the only collections known of these, but none belongs in *Massalongia* according to available data. *Massalongia griseolobata* (from Gough Isl.) is shown here to belong in the Pannariaceae and is part of the parmelielloid clade. *M. intricata* (from South Georgia) and *M. olechiana* (from South Shetland) have both recently been correctly transferred to the genus *Steinera* in the Arctomiaceae.

Keywords

Peltigerales, Massalongiaceae, phylogeny, taxonomy, South Hemisphere

Introduction

The genus *Massalongia* was described by Körber (1855), based on the species *Lichen carnosus* described by J. Dickson in 1790 on material collected in Scotland, but later often called *Pannaria muscorum* (Ach.) Duby, an illegitimate, superfluous name. This reflects the difficulties which the early lichenologists had in classifying the species. Mo-

lecular studies (Wedin et al. 2007; Muggia et al. 2011) have shown that *Massalongia* does not belong in the Pannariaceae, but is best placed in a family of its own, Massalongiaceae, in the Peltigerales. There is, however, only one major study of the species and their variation, made by Henssen (1963) covering North America. She accepts two species; the widespread, variable *M. carnosa* and the nearly crustose, microphylline, local Californian endemic *M. microphyllizans* (Hasse ex Nyl.) Henssen. Jørgensen (2000), whilst revising the Pannariaceae, had studied the type of *Massalongia fauriei* (Hue) Zahlbr. and found the poor type (the only material existing) to belong in *Fuscopannaria leucophaea* s.lat., now transferred to the genus *Vahliella* (Jørgensen 2008; Wedin et al. 2010). An additional Asian species has been recorded from the Philippines based on Rehm (1916). This is, however, based on a misunderstanding of *Massalongiella imperatae* Rehm., which is a non-lichenised ascomycete as originally described. In Europe, Harmand recognised a variety which Gyelnik (1940), in his notorious treatment of the Pannariaceae in Rabenhorst's Kryptogamenflora, raised to species rank as *Massalongia meizospora*, only representing a form with somewhat longer, 3-septate spores. In addition, he established a new *Massalongia rabenhorstiana*, the type of which has disappeared. It is most certainly only a synonym of *M. carnosa*, still the only species on the Northern Hemisphere in addition to *M. microphyllizans*, a species in need of a phylogenetic study.

The situation in the Southern Hemisphere is different, though it took a long time before any species in the genus was recognised. Zahlbruckner (1917) was the first when he recorded *Massalongia carnosa* from the Falkland Islands, followed by Lamb (1958) who recorded it from Patagonia. Later, it was mentioned from several regions in the Southern Hemisphere (Smith and Corner 1973; Lindsay 1974; Galloway 1985; Redon 1985; Jørgensen 1986; Jørgensen and Elix 1988; Øvstedal and Smith 2001). In addition, several new species were described from the Southern Hemisphere; *M. antarctica* Dodge (from the Antarctic Peninsula, Dodge 1971), *M. novozelandica* Dodge (from subantarctic New Zealand, Dodge 1971), *M. griseolobata* Øvstedal (from Gough Isl., Øvstedal and Gremmen 2010), *M. intricata* Øvstedal (from South Georgia, Øvstedal and Smith 2001) and *M. olechiana* Alstrup & Søchting (from South Shetland, Alstrup and Søchting 2011).

During fieldwork in Chile in 2015, one of the authors (A.E.) discovered a strange *Pannaria*-like lichen which, on closer inspection, proved to be a *Massalongia* with some differences from *M. carnosa*, as known by us from Norway. However, since this is a variable species, we felt that a more detailed study, including molecular screening, would be useful. This being done and the distinction of this material proven, we found it necessary to check on the surprisingly high number of species of *Massalongia* described from the Southern Hemisphere. This proved to be time-consuming and complicated, since it was difficult to get hold of suitable material and, when molecularly checked, often not giving clear results and involving quite unrelated lichen families. Fortunately, Ertz et al. (2017) solved some of our problems and, eventually, this new species was named by Kitaura and Lorenz in Liu et al. (2018). However, our project

contains more data than their work includes and we present these here in an attempt to give full clarification of the taxonomic situation for the genus in the Southern Hemisphere. Some additional phylogenetic data were also added on the genus in the Northern Hemisphere.

Material and methods

The specimens

Specimens of *Massalongia* were obtained from various herbaria for phylogenetic analyses, see Tables 1 and 2. In addition, we microscopically studied material from the following herbaria: BAA, BG, BM, C, CANB, CANL, CHR, F, FH, H, MSC, NY, TROM and UPS. A total of 130 ascospores from collections from both hemispheres were drawn in detail and measured for comparison.

DNA extraction, Amplification and Sequencing

Total genomic DNA was extracted using DNeasy Plant Mini Kit (Qiagen). Four DNA markers where amplified; the mitochondrial small subunit rDNA (mtSSU rDNA: primers mrSSU1 and mrSSU3R (Zoller et al. 1999)), the internal transcribed spacer (ITS) and the large subunit (LSU) regions of the nuclear ribosomal RNA gene (primers ITS1f (Gardes and Bruns 1993), ITS4 (White et al. 1990), LSU155 and LSU362 (Döring et al. 2000), LSU635/LR3 and LSU1125/LR6 (Vilgalys and Hester 1990)) and the gene coding for the largest subunit of RNA polymerase II (RPB1: primers PRB1-BCR (Wedin et al. 2009), gRPB1-A (Stiller and Hall 1997) and fRPB1-C (Matheny et al. 2002)).

PCR reactions consisted of 1× GeneAmp PCR Buffer II (Applied Biosystems), 2.5 μM $MgCl_2$ (Applied Biosystems), 20 μM dNTPs (Promega), 0.4 μM of each primer, 0.03 U AmpliTaq DNA Polymerase (Applied Biosystems), 2–5.0 μl of genomic DNA extract and distilled water to a total volume of 25 μl. PCR reactions were performed on a C1000 Touch thermal cycler (Bio-Rad Laboratories), with the following temperatures; initial denaturation at 94 °C for 4 min, followed by a 62–56 °C touchdown annealing for the first 6 cycles, ending with 30 cycles at 56 °C for 30 sec, polymerisation at 72 °C for 1 min 45 sec and a final elongation at 72 °C for 10 min.

Direct sequencing of PCR products was run with the PCR primers using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems) on an ABI Prism 3700XL DNA analyser (Applied Biosystems) at the DNA Sequencing Facility (UiB), Norway. Sequences were assembled and edited using Geneious v.11.0.2 (Kearse et al. 2012).

Newly generated sequences with GenBank accession numbers are listed in Tables 1, 2, together with sequences downloaded from GenBank.

Table 1. List of specimens used for phylogenetic analyses of the broad analysis of the *Massalongiaceae* and *Pannariaceae*, with vouchers and accession numbers from GenBank. Bold accession numbers are new in this study.

Species and ID	Voucher	mtSSU	RPB1
Austrella arachnoidea	Jørgensen 8200 (BG)	KC608054	KC608108
Collema furfuraceum	Wedin 6187 (BM)	AY340488	GQ259048
Collema nigrescens	Wedin 7046 (UPS)	GQ259020	GQ259049
Collema parvum	Nordin 5500 (UPS)	GQ259021	GQ259050
Degelia duplomarginata	Wedin 8023 (S)	KC608058	–
Degelia durietzii	Elvebakk 02-296 (TROM)	KC608059	–
Degelia gayana	Wedin 6112 (UPS)	AY652619	–
Degeliella rosulata	Galloway 840b (BG)	KC608063	–
Degeliella versicolor	Galloway 840a (BG)	KC608064	–
Erioderma pedicellatum	mrSSU: MacPitcher s.n. 2007 (BG-L85909), RPB1: MacPitcher s.n. 2007 (BG-L85911)	KC608065	KC608110
Erioderma verruculosum	AFTOL-ID 337	DQ972990	DQ973062
Fuscoderma applanatum	Tibell 19076 (BG)	KC608112	KC608066
Fuscopannaria pacifica	Tønsberg 29359 (BG)	KC608074	KC608118
Fuscopannaria praetermissa	mrSSU: Tønsberg 36838 (BG) RPB1: Wedin 7671 (UPS)	KC608075	GQ259056
Joergensenia cephalodina	Passo 269 (BCRU 4895)	EU885330	–
Lecidea fuscoatra	Wedin 6860 (UPS)	AY756401	AY756408
Leciophysma furfurascens	Nordin 5695 (UPS)	GQ259028	GQ259058
Leioderma erythrocarpum	Schumm and Frahm s.n. 2009 (BG, dupl of hb. Schumm 15583)	KC608078	–
Leioderma pycnophorum	Wedin 8013 (S)	GQ259031	GQ259059
Leptochidium albociliatum	Tønsberg 29087 (BG)	DQ900632	GQ259060
	Muggia TSB38886	JF938191	–
	Spribille 20997 (COLO)	JF938193	–
Lobaria pulmonaria	mrSSU: Wedin 6167 (UPS) RPB1: Wedin 5092(UPS)	AY340503	GQ259068
Lobaria scrobiculata	AFTOL-ID 128	AY584621	DQ883736
Massalongia carnosa	Tønsberg 44267 (BG)	**MN708314**	**MN714653**
	Tønsberg 45410 (BG)	**MN708315**	**MN714654**
	Johnsen L-86694 (BG)	**MN708316**	–
	Ezhkin 1289 (SAK)	**MN708317**	**MN714655**
	Spribille 22021 (COLO)	JF938205	–
	Haikonen 20961 (H)	EU558817	–
	Hermansson 8916 (UPS)	AY340509	GQ259071
	Spribille 21565 (COLO)	JF938204	–
Massalongia patagonica	Elvebakk 99:775 (TROM)	**MN708318**	**MN714656**
	Elvebakk 15:033 (SGO)	**MN708319**	**MN714657**
	Buck 60287 (NY)	**MN708320**	**MN714658**
	Gremmen K-789 (BG)	**MN708321**	**MN714659**
	Galloway 5616 (CHR)	**MN708322**	**MN714660**
	Galloway s.n. (CHR)	**MN708323**	**MN714661**
	Kitaura 4181 (CGMS)	MG243608	–
	Kitaura 4188 (CGMS)	MG243607	–
	Kitaura 4168 (CGMS)	MG243609	–
Massalongia griseolobata	Gremmen 2006-91 (BG)	**MN708324**	–
Nephroma parile	Wedin 6169 (UPS)	AY340512	GQ259072
Pannaria hookeri	Jørgensen s.n. (BG)	KC608083	KC608124
Pannaria immixta	Elvebakk 02-352b (BG)	KC608084	KC608125
Pannaria rubiginella	Thor 10050 (S)	GQ259037	GQ259074
Parmeliella appalachensis	Lendemer 578 and Smith (BG)	KC608090	–
Parmeliella miradorensis	Tønsberg 23053 (BG)	KC608094	KC608136
Parmeliella nigrocincta	Elvebakk 02-356 (BG)	KC608095	KC608137

Species and ID	Voucher	mtSSU	RPB1
Parmeliella pannosa	Ståhl s.n. 1999 (BG)	KC608096	–
Parmeliella triptophylla	Wedin 7037 (UPS)	AY652623	GQ259075
Pectenia atlantica	Lindblom and Blom L251 (BG)	KC608057	KC608109
Pectenia cyanoloma	Purvis, James and Smith 1995 (BM)	AY340491	GQ259052
Pectenia plumbea	AFTOL-ID 1046	DQ912300	DQ912374
Placynthium nigrum	Wedin 6778 (UPS)	AY340518	GQ259079
Polychidium muscicola	Obermayer 8547 (UPS)	DQ900634	GQ959080
	Spribille 26411 (KLGO)	JF938220	–
Pseudocyphellaria aurata	Purvis, James and Smith 7/5/1995 (BM)	AY340520	GQ259082
Psoroma hypnorum	Ihlen 453 (BG)	KC608100	KC608142
Santessoniella arctophila	Kristinsson s.n. (BG)	KC608104	KC608145
Sticta fuliginosa	Wedin 6078 (BM)	AY340529	GQ259089
Vahliella carnifornica	Tønsberg 26316 and Goward (BG)	HQ268594	HQ268593
Vahliella leucophaea	Wedin 6849 (UPS)	AY652621	GQ259090

Table 2. List of specimens used for phylogenetic analyses of the species delimitation in *Massalongia*, *M.* referring to *Massalongia*, *P.* to *Polychidium* and *L.* to *Leptochidium*, with vouchers and accession numbers from GenBank. Bold accession numbers are new in this study.

Species and ID	Voucher	Area	ITS	LSU	mtSSU	RPB1
M. carnosa	Tønsberg 44267 (BG)	USA: Alaska	**MN708327**	**MN708327**	**MN708314**	**MN714653**
	Tønsberg 45410 (BG)	USA: Alaska	**MN708328**	**MN708328**	**MN708315**	**MN714654**
	Johnsen L-86694 (BG)	Norway	**MN708329**	**MN708329**	**MN708316**	–
	Ezhkin 1289 (SAK)	East Russia	**MN708330**	**MN708330**	**MN708317**	**MN714655**
	Spribille 22021 (COLO)	USA: Montana	–	–	JF938205	–
	Hermansson 8916 (UPS)	Sweden	–	AY340554	AY340509	GQ259071
	Rui andTimdal 13267 (O)	Norway	MG243601	–	MG243611	–
	Hansen 1138 (COLO, H)	Greenland	MG243599	MG243615	MG243610	–
	Hansen 1057 (H)	Greenland	MG243603	MG243616	MG243612	–
	Türk 17280 (H)	Austria	MG243602	MG243614	MG243613	–
M. patagonica	Elvebakk 99:775 (TROM)	Chile	**MN708331**	**MN708331**	**MN708318**	**MN714656**
	Elvebakk 15:033 (SGO)	Chile	**MN708332**	**MN708332**	**MN708319**	**MN714657**
	Buck 60287 (NY)	Chile	**MN708333**	**MN708333**	**MN708320**	**MN714658**
	Gremmen K-789 (BG)	Australia	–	–	**MN708321**	**MN714659**
	Galloway 5616 (CHR)	New Zealand	**MN708334**	**MN708334**	**MN708322**	**MN714660**
	Galloway s.n. (CHR)	New Zealand	**MN708335**	**MN708335**	**MN708323**	**MN714661**
	Kitaura 4181 (CGMS)	Argentina	MG243604	MG243617	MG243608	–
	Kitaura 4188 (CGMS)	Argentina	MG243606	MG243619	MG243607	–
	Kitaura 4168 (CGMS)	Argentina	MG243605	MG243618	MG243609	–
P. muscicola	Obermayer 8547 (UPS)	Austria	–	DQ900647	DQ900634	GQ259080
L. albociliatum	Tønsberg 29087 (BG)	USA	–	DQ900644	DQ900632	GQ259060

Phylogenetic analyses

To align the sequences, MAFFT v7.309 (Katoh et al. 2002; Katoh and Standley 2013) implemented in Geneious v.11.0.2 (Kearse et al. 2012) was used with default settings, followed by manual adjustments. Suitable substitution models for the separate datasets were identified using MrAIC v.1.4.6 (Nylander 2004).

Two different datasets were analysed; one broad analysis of *Massalongiaceae* and *Pannariaceae* to test whether the included species is part of *Massalongia* (Table 1) and a

second analysis for species delimitation within *Massalongia* (Table 2). For the broader dataset, mtSSU and RPB1 were concatenated, using *Lecidea fuscoatra* as outgroup and for the species delimitation in *Massalongia*, mtSSU, LSU, ITS and RPB1 was concatenated using *Polychidium muscicola* as outgroup.

Separate analyses of all genes and concatenated datasets were run as Bayesian MCMC searches using MrBayes v.3.2.1 (Ronquist and Huelsenbeck 2003) with default options; substitution model GTR+G+I, 10 million generations starting with a random tree, four simultaneous chains and using the default temperature of 0.2. Every 1000th tree was saved. Phylogenetic trees were visualised using Geneious v. 11.0.2 (Kearse et al. 2012).

Results

Phylogeny

The two resulting concatenated datasets consisted for the broad analysis of *Massalongiaceae* and *Pannariaceae* of 63 taxa with 1435 characters, whereas for the species delimitation in *Massalongia*, of 21 taxa with 2983 characters (details in Table 3).

The resulting phylogenetic consensus tree from the broad analysis of *Massalongiaceae* and *Pannariaceae* are given in Fig. 1. Both *Massalongia carnosa* and *M. patagonica* are with high support a part of the *Massalongiaceae*, together with *Polychidium muscicola* and *Leptochidium albociliatum*. *M. griseolobata* is a part of the Pannariaceae, in the "Parmelielloid" clade 1 from Ekman et al. (2014) with high support. Within this clade, *M. griseolobata* is a part of a supported group with no internal resolution, including *Degeliella rosulata*, *Degeliella versicolor*, *Leioderma*, *Erioderma* and *Joergensenia*.

The resulting phylogenetic consensus tree from the species delimitation analysis of *Massalongia* is given in Fig. 2. *M. patagonica* from the Southern Hemisphere and *M. carnosa* from the Northern Hemisphere are nicely separated in two sister groups with high support.

The samples of *M. patagonica* from New Zealand are grouped in a separate subclade from the rest of the samples from Australia, Chile and Argentina. The phylogenetic tree indicates a high genetic variance within *M. patagonica* throughout the Southern Hemisphere, but further studies are necessary to evaluate these differences.

The samples from the Northern Hemisphere make a monophyletic clade with little variation between the samples and a sample from Sweden is practically identical to those analysed from Alaska and Greenland.

Table 3. List of numbers of characters, taxa and constant variables, from the two concatenated datasets.

Dataset	Numbers of characters	Numbers of taxa	Number of constant characters	Number of variable characters
Broad analysis of the *Massalongiaceae* and *Pannariaceae*	1435	63	724	711
Species delimitation in *Massalongia*	2983	32	2745	238

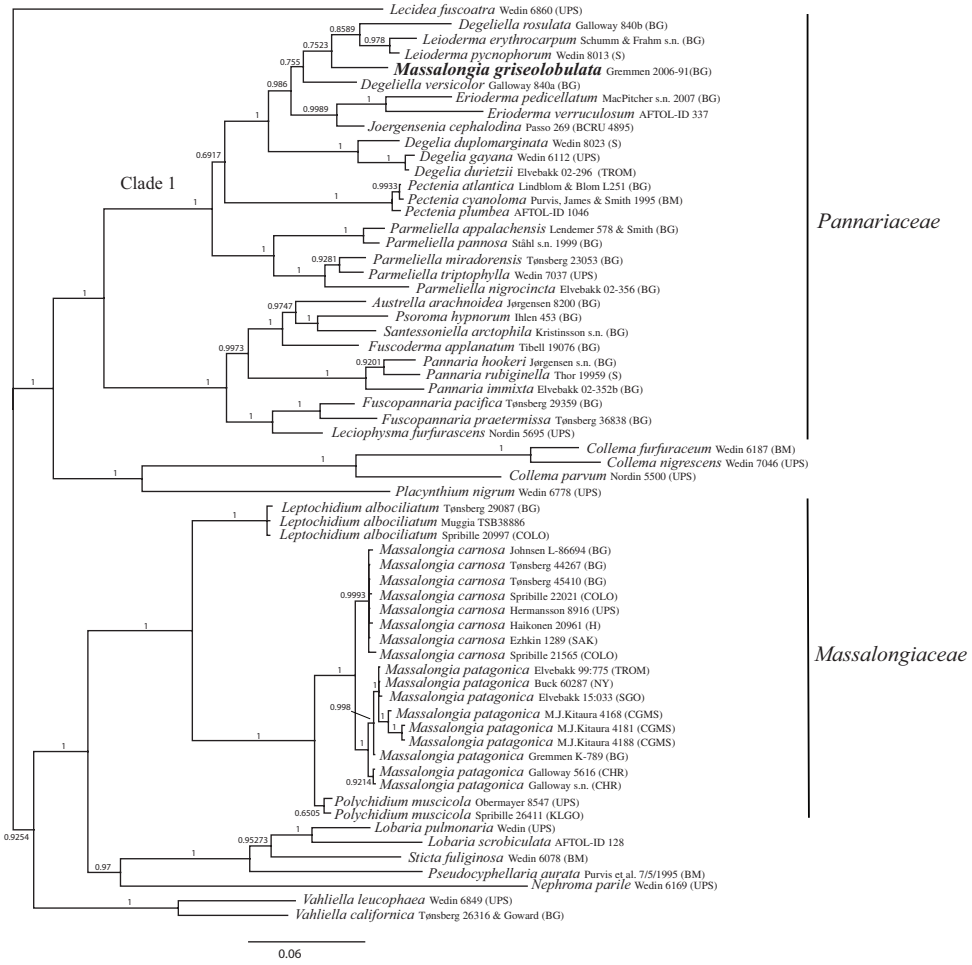

Figure 1. The phylogenetic tree of a concatenated broad dataset of the *Massalongiaceae* and *Pannariaceae*, resulting from Bayesian MCMC analyses.

Taxonomy

The only species from the Southern Hemisphere which, according to our data belongs in *Massalongia*, is *M. patagonica*, the details of which are as follows:

***Massalongia patagonica* Kitaura & Lorenz in Liu et al.**

MycoBank No: 824006

Massalongia patagonica Kitaura & Lorenz in Liu et al., Sydowia 70: 249–252 (2018) – Holotypus: Argentina, Ushuaia, National Park of Tierra del Fuego, Lapataia Bay, muscicolous on the rock, 54°50'41.42"S, 68°33'52.31"W, 6 m alt., 25 December 2016, *leg.* M.J.Kitaura, J.Bordin, A.A.Spielmann & D.Peralta 4188 (CGMS).

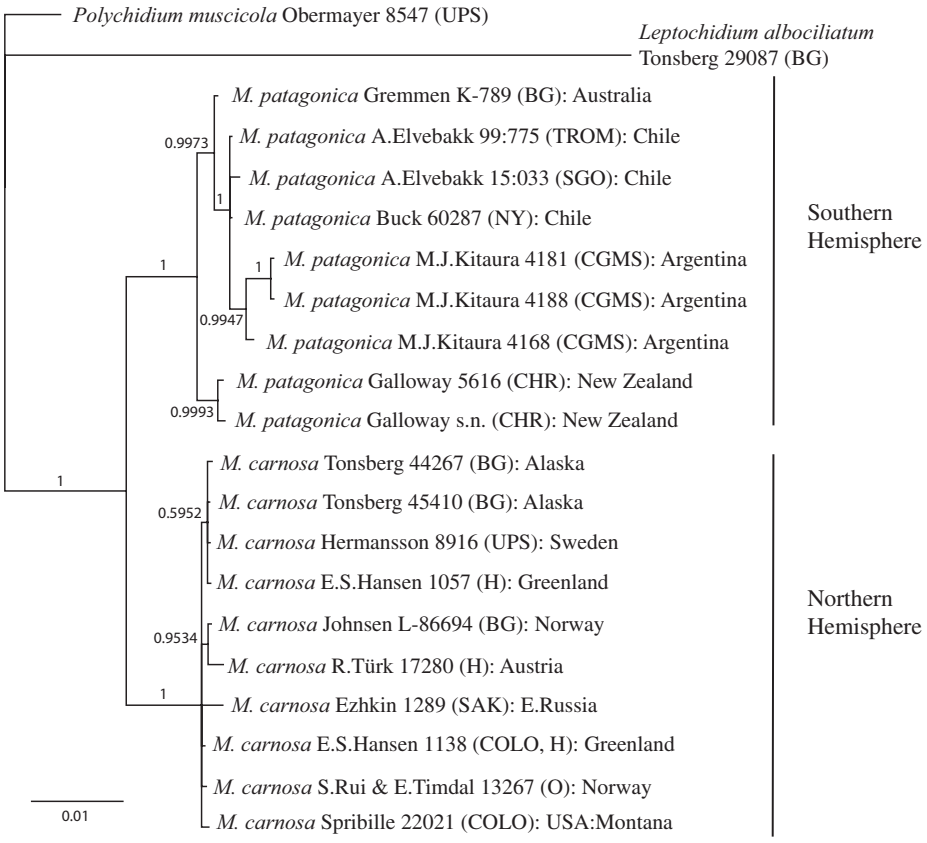

Figure 2. The phylogenetic tree of a concatenated dataset for species delimitation of *Massalongia*, resulting from Bayesian MCMC searches.

Description. *M. patagonica* (Fig. 3) is morphologically similar to *M. carnosa*. Generally, spore characters are the best distinguishing characters (Fig. 4). The spores of *M. carnosa* are longer and 92% of 72 measured spores were in the range 23–35 µm. By contrast, 70% of 58 measured spores of *M. patagonica* were in the range 15–22 µm. This means that there is an overlap in sizes between these two species. However, the spores of *M. patagonica* are often two-septate, sometimes three-septate, which is very rare in *M. carnosa*. Here follows a more detailed treatment of *Massalongia patagonica* Kitaura & Lorenz:

Thallus foliose, forming rosettes up to 3 cm, mostly muscicolous; ***lobes*** 0.5–1.5 mm broad, up to 1 cm long, irregularly and repeatedly divided with isidioid marginal outgrowths, simple to sparingly branched, sometimes developing into branched lobule systems, lobules, 0.1 mm wide. ***Upper surface*** brown, glabrous and glossy; ***upper cortex*** 20–30 µm thick, paraplectenchymatic, of thick-walled (ca. 1.5–2 µm wide) cells with 7–12 µm large lumina; ***photobiont layer*** 40–60 µm thick, often also developed in the subhymenium; ***cyanobiont Nostoc***, cells bluish-green, irregularly

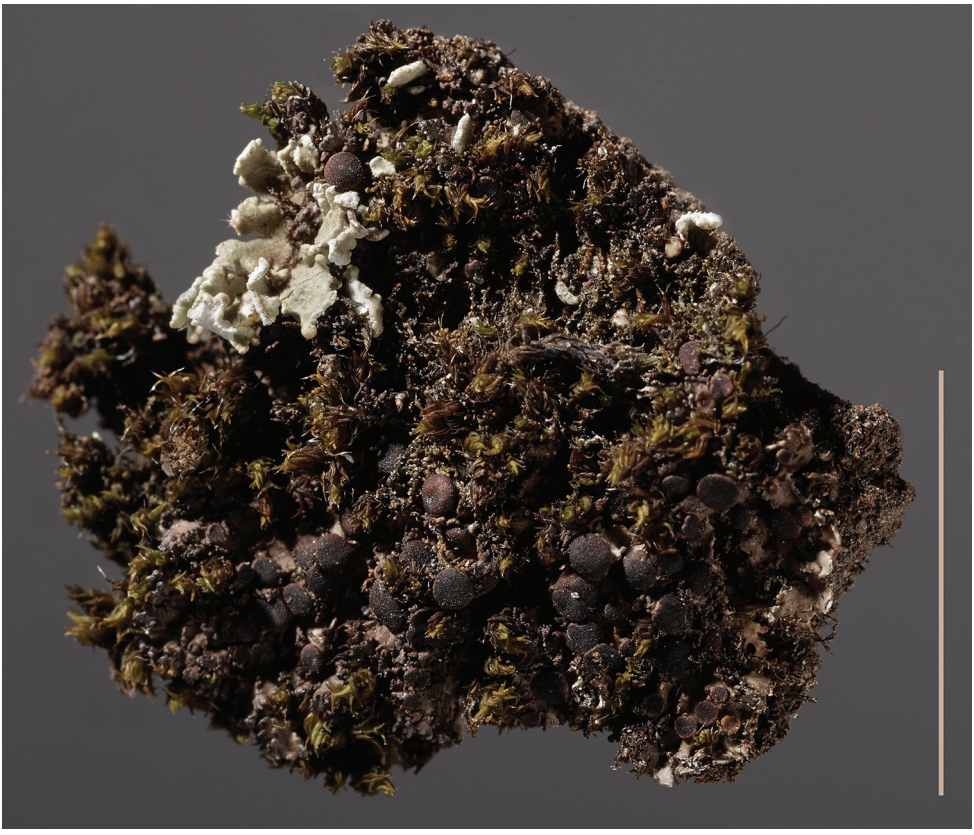

Figure 3. *Massalongia patagonica*, AE 15-033. Scale bar: 1 cm.

subglobose to ellipsoid, 5–9 × 6–11 µm in size, arranged within 20–40 µm large glomeruli without visible chain structures, chain structures visible in some liberated cells; ***medulla*** loose, 60–80 µm thick; ***lower cortex*** absent, with scattered rhizohyphae.

Apothecia common to scattered, substipitate, laminal, 1–2 mm wide; ***thalline excipulum*** lacking, true excipulum weakly prominent; ***epithecium*** 5–10 µm thick, of protruding brown and strongly swollen, pyriform paraphyse end cells, 4–6 µm wide, 7–10 µm long, paraphyses undivided to sparingly divided, 2–4 µm thick; ***hymenium*** ca. 60 µm thick, IKI + blue; ***asci*** clavate 50–70 × 10–15 µm, 8-spored, with distinct internal apical IKI + blue sheath-like structures, sometimes also with weak tube structures; ***ascospores*** narrowly ellipsoid, occasionally asymmetric, 1- to 2 (3)-septate, (13) 20–25 (28) × 5–7.5 µm. ***Hypothecium*** ca. 60 µm thick, weakly brownish, IKI negative. ***Conidiomata*** not seen.

Chemistry. All reactions negative, no lichen substances detected by TLC.

Habitat and distribution. This is a species of wet to dry rock surfaces or boulders, usually growing in between mosses or on plant remains. It has a widely scattered distribution in South America, ranging from the temperate forests of south-central Chile, including the Juan Fernandez Islands and Patagonia, with two widely separated

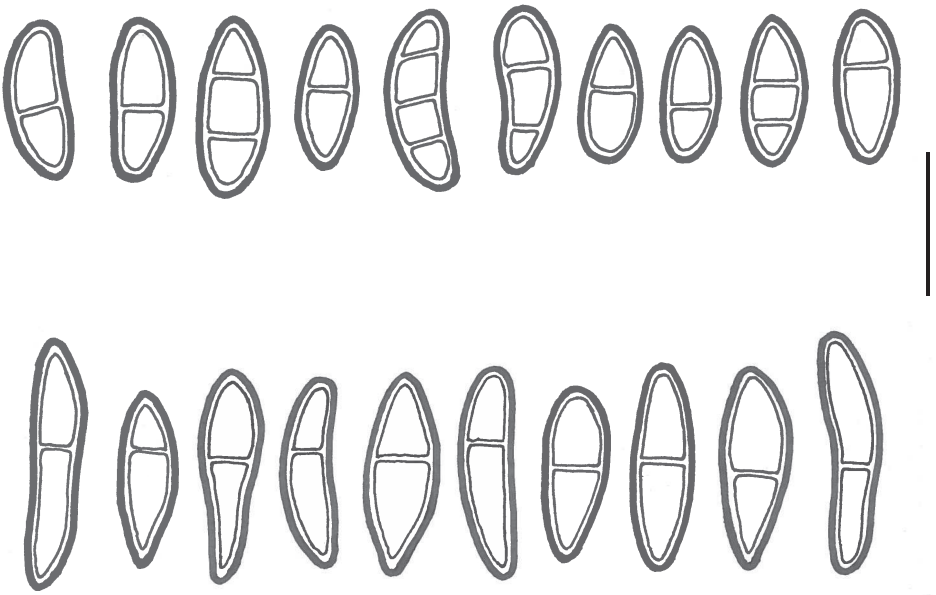

Figure 4. *Massalongia* ascospores, *patagonica* above, *carnosa* below. Scale bar: 20 µm.

collections from southernmost Chile and Argentinean Tierra del Fuego. In addition, it is known from the Falkland Islands, Antarctica, mountains of SE Australia, where it is rare and from several localities in New Zealand.

Specimens examined. Antarctica: South Shetland Islands, King George Isl., Admiralty Bay, creeping slopes above Paradise Cove, 26 Jan 1980, *R. Ochyra* 1224/80 (BG, H); Urbanek, Crag between Polar Committee Glacier and Ladies Icefall, in Ezcurra Inlet, 20 Feb 1980, *R. Ochyra* 2319/80 (BG, H).

Argentina: Patagonia: Chubut, Lago Verde, near Futalaufquen, 1 Feb 1950, *I. M. Lamb* 5877 (over mosses on a rock in open scrub, about 30 m above the lake), 5880 (over mosses on a rock in open forest about 15 metres above the lake) (CANL, UPS).

Australia: New South Wales, near the summit of Mt. Guthrie, Kosciusko National Park, on moss over granite rocks, 9 Feb 1978, *J.A. Elix* 4360 (CANB); Kosciusko National Park, near Digger's Creek, 21 Jan 1976, *J. A. Elix* 1722 (CANB); Île Australia near Kerguelen Isl., on moss cushions, 492823S, 695329E, 45 m alt., 31 Dec 2003, *NJM Gremmen*, K-789 (BG).

Chile: IX Región de la Araucanía: Reserva Nacional Malalcahuello, W bank of Río Colorado, 500 m W of the Entrance/CONAF building and 200 m S of the junction between the paths Sendero Coloradito and Sendero Sierra de Colorado; 38°25'45"S, 71°32'44"W, 1380 m alt., over *Grimmia* mosses on a S-facing rock outcrop in a *Nothofagus dombeyi-Araucaria araucana* forest, probably affected by river water during high flooding events, 9 Jan 2015, *A. Elvebakk* 15:033 (SGO, BG, UPS, BM, TROM); Archipiélago de Juan Fernandez: Isla Alejandro Selkirk (Mas Afuera), Los Innocentes, 4 Dec 1965, *H. Imshaug* (MSC); Valdivia, Corral, *R. Thaxter* (MSC); XII Región de

Magallanes y de la Antárctica Chilena, Provincia Magallanes, Morro Chico, 52°03'S, 71°28'W, 200 m alt., on acrocarpous mosses on a NW-facing rocky slope, 28 Nov 1999, *A. Elvebakk* 99:775 (TROM); Provincia Antártica Chilena, Comuna Cabo de Hornos, Isla Grande de la Tierra del Fuego, Bahía Yendegaia, NNE shore opposite Caleta Ferrari, 54°50'28"S, 68°47'52"W, 13 Jan 2013, *W.R. Buck* 60287 (NY 01886528).

Falkland Islands: W. Falkland, Chartres, Luxton NNR, 30 Jan 2015, *A. Fryday* 10999 (MSC).

New Zealand: Canterbury, Cass, between Sugar Loaf and Cass Hill, 761 m alt., 18 Feb 1991, *A. J. Fife* 9761 (CHR); Banks Peninsula, Mt. Sinclair, summit, 5 Feb 1970, *D. J. Galloway* (CHR); Mt. Cook National Park, *D. J. Galloway* (CHR); Otago, Deep Stream, above DCC water intake, 13 Feb 1998, *D. J. Galloway* 0170 (CHR); Otago, Old Man Range, N of Obelisk, 5 Feb 2009, *D. J. Galloway* 404009 (CHR); St. Mary's Range, Anakin's Skifield, 22 Feb 2006, *D. J. Galloway* (CHR); Lake Onslow near huts, amongst moss in drainage cracks of schist rock in grassland, 30 Jul 1998, *D. J. Galloway* 404012 (CHR); Otago, Pomahaka River- Hukarere, rock slabs above river, 13 Apr 1998, *D.J. Galloway* 404011 (CHR); North Rough Ridge, near "Great Tor", 12 Apr 1998, *D. J. Galloway* (CHR).

The other taxa originally described from the Southern Hemisphere as *Massalongia* species, are listed alphabetically, according to the epithet at the end of the discussion.

Discussion

The result of the phylogenetic analyses of *Massalongia* (Fig. 2) show that *Massalongia carnosa* and *M. patagonica* are located in different supported clades, as separate species as also described by Kitaura and Lorenz in Liu et al. (2018), *M. patagonica* being restricted to the Southern Hemisphere, whereas *M. carnosa* occurs only in the Northern Hemisphere. The clade with *M. carnosa* includes one circumarctic and circumboreal species, with low genetic diversity, whereas *M. patagonica* is more variable and shows a geographic pattern within this species. The material from New Zealand groups in a distinct branch within the *M. patagonica* clade and is superficially much more similar to the material of *M. carnosa*, but has extra short ascospores measured in two samples from New Zealand, all spores were shorter than 23 µm. This could be a result of the preference for moist, mossy habitats (Galloway 2007) as opposed to the drier, often exposed habitats in Chile. The material from Australia and New Zealand is, therefore, best classified as part of the *M. patagonica* complex.

That species is also found as far west as the Juan Fernandez Islands and is also possibly present on the Antarctic Peninsula and the Bouvet Island, but the material examined was sparse, sterile and too old for molecular studies.

Still, *M. patagonica* is not morphologically easily distinguished from *M. carnosa*; the two species have different spores, although there is an overlap zone in both length and degree of septation. Both species have a gross morphology showing high variation, probably due to habitat modifications, depending on light exposure, competition, moisture and water availability.

Chilean material of *M. patagonica* tends to have thicker, narrower and clearly radiating lobes than most material of *M. carnosa*. However, in cases where habitat information is available, they appear to be dry, but exposed to nutrient supplies by spring flooding (the Río Colorado collection), wind-transported saline lake dust (the Morro Chico collection) or seashore spray (the Tierra del Fuego collection). The New Zealand material, on the other hand, treated as *M. carnosa*, is cited as widespread and from moist habitats by Galloway (2007).

This detailed phylogenetic signal within *M. patagonica* is the result of a long history of evolution and isolation in austral areas, although shorter than the split-up between *M. carnosa* and *M. patagonica*. There is a record of *M. carnosa* from Mt. Kinabalu on Borneo (Sipman 1993) which could have indicated a migration route between a northern and a southern distribution area of the genus; however, a check of the material deposited at herbarium B revealed that it instead represents a sterile, richly squamulose specimen of a *Parmeliella* species. Future studies should investigate phylogeographic relationships between the three accepted species and the molecular distances between *M. patagonica* in New Zealand, Australia and South America/West Antarctica.

The examination of all relevant material from the Southern Hemisphere, shows the following, treated alphabetically according to the epithet:

Massalongia antarctica Dodge is a species only known from the type specimen from Lambda Island at the tip of the Antarctic Peninsula (Siple 380c-2, FH!). The type specimen is minute and sterile and consists of two different species, none of which belongs in *Massalongia*. The one fitting best with the description has a crustose, hemigelatinous thallus in accordance with species of the Arctomiaceae. There are no apothecia present in the collection and the description of the apothecia, given by Dodge (1968), is at variance with characters of *Massalongia*, indicating a species of the Arctomiaceae, most probably in *Arctomia*. There is, however, no known species with such a distinctly crustose thallus. More material is needed to identify this taxon more exactly. The sample also contains squamules with a trebouxioid photobiont and this is possibly *Pertusaria corallifera* Vain. as pointed out by Castello and Nimis (1995).

Massalongia griseolobata Øvstedal is a species only known from the type specimen (from Gough Isl., coll. Gremmen 2006-91, BG!). Even if only incipient apothecia were found, we do not hesitate to place this species in the Pannariaceae, based on morphology and the original description of the asci. They are recorded to have apically blue in tholus in iodine with a weak ring-structure. (*Massalongia* has sheet-like structures, Jørgensen 2007). The molecular study confirms this (Fig. 1). The species groups in the parmelielloid clade (Clade 1) in the tree by Ekman et al. (2014), with *Degeliella*, *Leiderma*, *Erioderma* and *Joergensiana.* This is an unresolved group of subantarctic taxa (Jørgensen and Andersen 2015) in need of further studies.

Massalongia intricata Øvstedal was correctly transferred to the genus *Steinera* by Ertz et al. (2017). *S. intricata* has a semi-gelatinous thallus producing apothecia on special lobules, just as species in the Arctomiaceae.

Massalongia novozelandica Dodge was recorded by Galloway (2007), but the holotype (the only material) has not been possible to obtain. However, the original descrip-

tion of the spores being brownish at maturity with disappearing septae (clearly pseudoseptae) is at variance with characters found in *Massalongia*. We agree with Galloway that this is probably a parasite growing on the thallus of a species in the Pannariceae.

Massalongia olechiana Alstrup and Søcht. was correctly transferred to the genus *Steinera* in the Arctomiaceae by Ertz et al. (2017).

Massalongia patagonica Kitaura and Lorenz, the recently described species (Fig. 3), belongs in the genus and, according to our phylogenetic tree (Figs 1, 2), prove to be distinct from *M. carnosa*, the latter being restricted to the Northern Hemisphere. The two species are morphologically variable due to the ecological conditions, but have different spores (Fig. 4), usually shorter than 25 µm in *M. patagonica*, but variable both in length and number of septae in both species. Chilean material of *M. patagonica* tends to have thicker, narrower and clearly radiating lobes than most material of *M. carnosa.*

Conclusion

From these facts, we conclude that there is only one, widespread species of *Massalongia* in the Southern Hemisphere, *M. patagonica*, though the populations in Australia and New Zealand differ somewhat molecularly, but more data is necessary to decide their taxonomic status. *M. patagonica* has a wider distribution than indicated in the original paper, also southwards and westwards. Previous records of several species in the Southern Hemisphere proved incorrect, most of them belonging in other genera.

The type species *M. carnosa* is restricted to the Northern Hemisphere, where it is widespread and variable, but without distinct molecular groupings requiring taxonomic recognition. There is also a local endemic, *M. microphyllizans* in California.

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