Rubroshiraia gen. nov., a second hypocrellin-producing genus in Shiraiaceae (Pleosporales)

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Abstract

Shiraiaceae is an important family in Pleosporales (Dothideomycetes), which includes medical fungi and plant pathogens. Two hypocrellin-producing taxa, Shiraia bambusicola and a novel genus Rubroshiraia gen. nov., typified by Rubroshiraia bambusae are treated in this article. Maximum likelihood analysis, generated via RAxML (GTR+G model), using a combined SSU, LSU, TEF1 and RPB2 sequence dataset, shows that Rubroshiraia is close to Shiraia and belongs to the family Shiraiaceae. Descriptions, illustrations and a taxonomic key are provided for the genera in Shiraiaceae. Rubroshiraia morphologically differs from Shiraia in having small and dark ascostromata and filiform ascospores. Production of the ascostromatal metabolites, hypocrellin A and B, were examined by HPLC and spectrophotometer. The content of hypocrellin A and B of specimen HKAS 102255 (R. bambusae) is twice that produced by HKAS 102253 (S. bambusicola). To clarify the relationship between R. bambusae and Hypocrella bambusae, type material of the latter was examined and provided the illustration.

Keywords

HPLC, metabolite, new genus, phylogeny, taxonomy

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Introduction

Liu et al. (2013) introduced the family Shiraiaceae Y.X. Liu, Zi Y. Liu & K.D. Hyde which is typified by *Shiraia* Henn. and placed the family in Pleosporales Luttr. ex M.E. Barr. Ariyawansa et al. (2013) accommodated *Grandigallia* M.E. Barr, Hanlin, Cedeño, Parra & R. Hern. in Shiraiaceae since it morphologically resembles *Shiraia*. Subsequent publications by Wijayawardene et al. (2014, 2017, 2018) agreed with this placement and, thus, the family currently comprises two genera.

*Shiraia* is typified by *S. bambusicola* Henn. (Hennings 1900), which is parasitic on living bamboo culms and has conspicuous large, pinkish, fleshy ascostromata with multi-locules located near the periphery, fissitunicate asci and hyaline, muri-form ascospores (Liu et al. 2013). *S. bambusicola* has been reported from temperate regions of Asia, such as China and Japan (Table 1) (Hino 1961; Li et al. 2009; Liu et al. 2013).

*Shiraia* has previously been placed in several families, depending on the opinions of authors. Hennings (1900) considered *Shiraia* to have unitunicate asci and treated as a member in the family Nectriaceae Tul. & C. Tul. (Hypocreales, Sordari-omycetes) when he established the genus. Based on its large and fleshy fruiting bodies, *Shiraia* was transferred to Hypocreaceae De Not by Saccardo (1902). Amano (1980) re-examined the type specimen and regarded *Shiraia* as having bitunicate asci and, hence, placed the genus in Pleosporaceae Nitschke (Pleosporales, Dothideomycetes). However, it was subsequently transferred to Dothideales, genera *incertae sedis* by Kirk et al. (2001).

Earlier classifications of *Shiraia* were based on morphological characters. The first attempt of DNA-based taxonomy (Cheng et al. 2004) confirmed that *Shiraia* belongs in Pleosporales and was phylogenetically close to species of Phaeosphaeriaceae M.E. Barr. Thus, Cheng et al. (2004) considered *Shiraia* as a member in Phaeosphaeriaceae. Liu et al. (2013) carried out significant studies on *Shiraia* taxonomy by re-examining the holotype and carrying out phylogenetic analysis, based on LSU sequence data. Liu et al. (2013) also designated an epitype of both sexual and asexual morphs and introduced Shiraiaceae in the Pleosporales.

*Shiraia bambusicola* has been reported as a pathogen on various bamboo species (Table 2) or as endophyte of bamboo culms (Morakotkarn et al. 2007, 2008). The bamboo genus *Brachystachyum* Keng is significantly affected by *S. bambusicola* (Table 2; Lai and Fu 2000). The holotype of *S. bambusicola* was recorded from a *Bambusa* sp. (Liu et al. 2013). *Shiraia bambusicola* has also been recorded on several common bamboo genera, including *Fargesia* Franch., *Phyllostachys* Sieb. et Zucc., *Pleioblastus* Nakai and *Indosasa* Mcclure (Lai and Fu 2000; Li et al. 2009). However, these hosts need to be further verified.

*Shiraia bambusicola* produces hypocrellins. Four hypocrellins have been extracted from the fungal stromata (Wan and Chen 1981; Kishi et al. 1991; Chen and Chen 2009). Endophytes, named as *Shiraia* spp., were also shown to produce hypocrellins on media (Lu et al. 2004; Morakotkarn et al. 2008; Liang et al. 2009; Zhang et al. 2014; Tong et al. 2017). The fruiting body of “Zhuhongjun” also contains hypocrellins
Rubroshiraia gen. nov., a second hypocrellin-producing genus in Shiraiaceae (Pleosporales)

A Chinese medical fungus named “Zhuhongjun” in Chinese, was identified as Hypocrella bambusae (Berk. & Broome) Sacc. by Liu (1978), based on its conspicuous and fleshy fruiting body. However, according to our knowledge, Zhuhongjun is similar to S. bambusicola and unrelated to Hypocrella. Therefore, the taxonomic status of this taxon needs to be clarified.

The monotypic genus Grandigallia, collected on Polylepis sericea Wedd. (Rosaceae), was introduced by Barr et al. (1987) with G. dictyospora M.E. Barr et al. as the type species. Grandigallia dictyospora was reported from Venezuela in a locality above 3,400 m and the fungus was found to produce large ascostromata (3–14 cm in diam.), with bitunicate asci and dictyospores (Barr et al. 1987).

In this study, ten specimens of S. bambusicola and a hypocrellin producing taxon (“Zhuhongjun” in Chinese) were collected from Yunnan Province in China. Morphological and phylogenetic studies were carried out to determine the taxonomic status of these taxa. Sequences from endophytic strains, named as Shiraia spp., were also downloaded from GenBank and included in the phylogenetic analyses. The metabolite content of hypocrellin extracted from the specimens was determined by HPLC (Chem 2012). Based on the morphology and phylogenetic analyses, the hypocrellin producing taxon “Zhuhongjun” is treated as a new genus in Shiraiaceae.

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### Table 1. Distribution of Shiraia bambusicola.

<table>
<thead>
<tr>
<th>Country</th>
<th>Province</th>
<th>References</th>
</tr>
</thead>
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<td>China</td>
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<td>Henan</td>
<td>Li et al. (2009)</td>
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<td>Li et al. (2009)</td>
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<td>Japan</td>
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<td>Osaka</td>
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### Table 2. List of bamboo hosts of Shiraia bambusicola.

<table>
<thead>
<tr>
<th>Bamboo host</th>
<th>References</th>
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<td>Brachystachyum densiflorum (Rendle) Keng</td>
<td>Lai and Fu (2000)</td>
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<td>Brachystachyum albostriatum G.H. Lai</td>
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<td>Phyllostachys nidularia Munro</td>
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<td>Phyllostachys praecox f. prevernalis S.Y. Chen &amp; C.Y. Yao</td>
<td>GenBank</td>
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<td>Pleioblastus amarus (Keng) Keng f.</td>
<td>GenBank</td>
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</table>

(Hudson et al. 1994; Huang et al. 2001). Hypocrellin seems to be an important feature when clarifying the taxa of Shiraiaceae.
Material and methods

Collecting and examination of specimens

Bamboo culms with large, reddish to pale yellow ascostromata were collected from Yunnan, China and brought to the laboratory in 2017. Samples were examined following the methods described in Dai et al. (2017). Micro-morphological characters were examined and photographed by differential interference contrast (DIC), using a Leica DM2500 compound microscope with a Leica DMC4500 camera. Fruiting bodies were observed by stereomicroscopy using a Leica S8AP0 and photographed by HDMI 200C. Measurements were made using Tarosoft (R) Image Frame Work software. Specimens have been deposited at the herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (KUN) and Herbarium Mycologicum, Academiae Sinicae (HMAS) in Beijing. Facesoffungi (Jayasiri et al. 2015) and Index Fungorum (Index Fungorum 2019) numbers were provided for new taxa. Type material of *H. bambusae* was loaned and examined from the Royal Botanic Gardens, Kew.

DNA extraction, PCR amplification and sequencing

The surface of fungal fruiting bodies was sterilised by 75% alcohol and rinsed three times in sterile water. The internal tissue with locules was cut into pieces and ground in a mortar into powder with liquid nitrogen. The powder was used to directly extract DNA with an OMEGA E.Z.N.A. Forensic DNA Kit, following the manufacturer’s instructions.

ITS5 and ITS4, NS1 and NS4 (White et al. 1990) and LROR and LR5 (Vilgalys and Hester 1990) primers were used for the amplification of internal transcribed spacers (ITS), small subunit rDNA (SSU) and large subunit rDNA (LSU), respectively. Translation elongation factor 1-α gene region (TEF 1-alpha) and RNA polymerase II second largest subunit (RPB2) genes were amplified by using EF1-983F and EF1-2218R (Rehner 2001), fRPB2-5f and fRPB2-7cr primers (Liu et al. 1999), respectively.

The final volume of the polymerase chain reaction (PCR) was prepared following Dai et al. (2017). The PCR thermal cycle programme of ITS, SSU, LSU, RPB2 and TEF 1-alpha genes amplifications were run under the same conditions as described in Dai et al. (2017). The quality of PCR products was checked by 1% Biowest agarose gel electrophoresis. Amplified PCR fragments were sequenced at Shanghai Majorbio Bio-Pharm Technology Co., Ltd. and BGI Tech Solutions Co., Ltd. (BGI-Tech), P.R. China. Generated new sequences of ITS, LSU, SSU, Rpb2 and TEF1 regions are deposited in GenBank (Table 4).
Table 3. HPLC condition used in this study.

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<td>Flow rate (ml/min)</td>
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<td>Mobile phase (%)</td>
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Table 4. List of newly generated sequences with their culture collection numbers and GenBank accession numbers.

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The holotype specimen is highlighted in bold. Abbreviations: HKAS: herbarium of Kunming Institute of Botany, Chinese Academy of Sciences.

Phylogenetic analysis

The BLAST searches in GenBank, using LSU and ITS sequence data were carried out to obtain the close strains. Additional sequences were downloaded from GenBank based on recent publications (Liu et al. 2017).

Single gene sequence alignments were carried out with MAFFT v. 7.215 (Katoh and Standley 2013, http://mafft.cbrc.jp/alignment/server/index.html) and edited manually when necessary in BioEdit v. 7.0 (Hall 2004). The alignments of LSU, SSU, Rpb2 and TEF1 regions were combined in MEGA6 version 6.0 (Tamura et al. 2013).

Maximum-likelihood (ML) analyses, including 1000 bootstrap replicates, were run using RAxMLGUI v.1.0. (Stamatakis 2006; Silvestro and Michalak 2011). Align-
ments in PHYLIP format were exchanged and loaded from the website (http://sing.ei.uvigo.es/ALTER/). The online tool Findmodel (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html) was used to determine the best nucleotide substitution model for each partition data.

Maximum-parsimony (MP) analyses were carried out in PAUP v. 4.0b10 (Swofford 2002) with 1000 replications. Maxtrees were set to 1000, branches of zero length were collapsed and all multiple equally most parsimonious trees were saved. The robustness of the most parsimonious trees was evaluated from 1000 bootstrap replications (Phillips et al. 2013).

Bayesian analyses were performed using MrBayes v. 3.0b4 (Ronquist and Huelsenbeck 2003). The model of evolution was performed using MrModeltest v. 2.2 (Nylander 2004). Posterior Probabilities (PP) (Rannala and Yang 1996; Zhaxybayeva and Gogarten 2002) were determined by Markov Chain Monte Carlo sampling (MCMC) in MrBayes v. 3.0b4 (Huelsenbeck and Ronquist 2001). Six simultaneous Markov chains were run for 1,000,000 generations and trees were sampled every 100th generation. The burn-in was set to 0.25 and the run was automatically stopped when the average standard deviation of split frequencies reached below 0.01 (Maharachchikumbura et al. 2015).

Trees were visualised with TreeView (Page 1996) or FigTree v. 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/) and, additionally, layouts were done with Adobe Illustrator CS v. 5. Maximum-likelihood bootstrap values (MLBP) and Maximum-parsimony bootstrap values (MPBP) equal to or greater than 50% are given for each tree. Bayesian posterior probabilities (BYPP) > 0.90 are indicated as thickened lines. The sequences used in this study are listed in Table 1. The combined alignment and phylogenetic tree were submitted at TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S24345).

HPLC profiling

Standards of hypocrellin A and hypocrellin B were purchased from Shanghai Tauto Biotech CO., Ltd. (http://www.tautobiotech.com) and used as received. Their purity is ≥ 98% (HPLC) and their structures are redrawn based on references (Wan and Chen 1981; Morakotkarn et al. 2008) and shown in Figure 1. The dry powder of ascostromata of S. bambusicola (HKAS102266) and “Zhuhongjun” (HKAS102270) was extracted followed the methods described by Stadler et al. (2001) and accurately weighed to 0.5 g and added to 25 ml of methanol and sonicated for 30 min. Semi-preparative HPLC was performed on an Agilent 1260 apparatus equipped with a UV detector and a CAPCELL PAK C18 (Agilent, 4.6 mm × 25 cm, 5 µm) column, with 38% solvent A: H₂O + 0.5% formic acid; 62% solvent B: acetonitrile, isocratic elution, UV/Vis the detection in the range of 265 nm (Table 3). The UV-Vis spectra were recorded at room temperature on a Perkin-Elmer Lambda 900 spectrophotometer (Fig. 5).
Rubroshiraia gen. nov., a second hypocrellin-producing genus in Shiraiaceae (Pleosporales)

Results

Phylogeny

To clarify the family placement of newly established taxa, maximum likelihood phylogenetic analysis was generated from RAxML (GTR+G model), based on combined SSU, LSU, TEF1 and RPB2 sequences data (Fig. 2). The combined alignment comprised 4025 characters including gaps for 127 ingroup taxa and one outgroup taxon *Dothidea insculpta* (CBS 189.58). Based on the phylogenetic tree in Fig. 2, the new collections cluster within family Shiraiaceae with high bootstrap support (96/1.00 MLBS/BSPP) and emerge as two groups, which are *S. bambusicola* lineage and a new clade named as *R. bambusae* in this paper. *Shiraia* and *Rubroshiraia* have more or less similar ascostromata and both of them can produce the metabolite hypocrellins. However, they can be phylogenetically distinguished with high bootstrap support (100/1.00 MLBS/BSPP) (Fig. 2). *Grandigallia* has not been included in phylogenetic analysis as it is lacking sequences in the GenBank. However, the new taxa can be morphologically distinguished from it. Shiraiaceae is phylogenetically close with family Phaeosphaeriaceae in Pleosporales and this has been confirmed by Liu et al. (2013).

To clarify the relationship between endophytic strains named as shiraia-like (*Shiraia* spp.) and Shiraiaceae, a phylogenetic tree was constructed (RAxML (GTR+G model), based on combined LSU and ITS sequences data and compared. The combined alignment comprises 1442 characters including gaps for 57 ingroup taxa and one outgroup taxon *Pleospora herbarum* (CBS 191.86). Of the 1442 characters of the combined matrix, 1116 were constant and 220 were parsimony informative. The endophytic strains separated into two lineages (Group A and group B) forming at the base clade of Shiraiaceae (Fig. 3). Several strains in group A ca. JP7, JP93, JP232, JP256, SUPER-H168, A8 and ML-2004, isolated from bamboo tissue can produce hypocrellins in media (Lu et al. 2004; Morakotkarn et al. 2008; Liang et al. 2009; Cai et al. 2011; Zhang et al. 2014). However, no hypocrellins were detected from Group B, which included three Japanese strains viz. JP119, JP151 and JP185 (Morakotkarn et al. 2008).
Figure 2. Maximum likelihood phylogenetic tree generated from RAxML (GTR+G model), based on combined LSU, SSU, TEF1 and RPB2 sequences data. ML values (MLBP) (> 50%), resulting from 1000 bootstrap replicates and Bayesian posterior probabilities (BYPP) greater than 0.90, are given at the nodes. The original isolate numbers' codes are noted after the species names. The tree is rooted to Dothidea insculpta (CBS 189.58). Ex-type or ex-epitype strains are in bold. Newly generated strains are in red and the new genus is in yellow background.
Rubroshiraia gen. nov., a second hypocrellin-producing genus in Shiraiaceae (Pleosporales)

Figure 3. Maximum likelihood phylogenetic tree generated from RAxML (GTR+G model), based on combined LSU and ITS sequences data. ML and MP values (MLBP/MPBP) (> 50%), resulting from 1000 bootstrap replicates, are given at the nodes. The original isolate numbers’ codes are noted after the species names. The tree is rooted to Pleospora herbarum (CBS 191.86). Ex-type or ex-epitype strains are in bold. Newly generated strains are in red.
Metabolites production

Stromatal extracts from specimens of *S. bambusicola* (HKAS102266) and *R. bambusae* (HKAS102270) contained high quantities of hypocrellin A (304.03 ng/ul and 790.86 ng/ul, respectively). Stromatal extracts from specimens of *S. bambusicola* contained 42.55 ng/ul hypocrellin B, whereas *R. bambusae* produces a higher quantity (204.60 ng/ul). The HPLC profiles of *S. bambusicola* and *R. bambusae* are depicted in Figure 4. The UV spectrum of the standards and of hypocrellin A and B from the samples (*S. bambusicola* HKAS 102253 and *R. bambusae* HKAS 102255) were recorded in alcohol and shown in Figure 5.

Taxonomy


Index Fungorum number: IF803884
Facesoffungi number: FoF 06202

**Notes.** The family Shiraiaceae was introduced by Liu et al. (2013) with a single genus and later *Grandigallia* was added to this family by Ariyawansa et al. (2013). In previous studies, Shiraiaceae was closely related with Phaeosphaeriaceae and their distinction was questionable (Cheng et al. 2004, Liu et al. 2013). However, our multi-gene analyses (Fig. 2) clearly indicate that Shiraiaceae and Phaeosphaeriaceae are distinct. Evidence is also borne out by the fact the Phaeosphaeriaceae have single ascostromata (Phookamsak et al. 2014), while in Shiraiaceae, ascostromata have multiple ascomata. Moreover, Shiraiaceae produces a high quantity of hypocrellins and no such metabolites, secreted by Phaeosphaeriaceae, were reported as far as we know (Phookamsak et al. 2014). In this study, the third genus (i.e. *Rubroshiraia*) is introduced to the family and produces hypocrellins. The endophytic strains in the phylogenetic tree in Figure (2) probably can be named as new genera, once the types are selected. Thus, currently three genera are placed in Shiraiaceae.

**Type genus.** *Shiraia* Henn., Bot. Jb. 28(3): 274 (1900).

**Type species.** *S. bambusicola* Henn., Bot. Jb. 28(3): 274 (1900).

**Shiraia bambusicola** Henn., Bot. Jb. 28(3): 274 (1900)

Fig. 6
Index Fungorum number: IF158454
Facesoffungi number: FoF 06203

**Description.** Parasitic on living branches of bamboo. **Sexual morph:** Ascostromata 1–6 cm long × 1–4 cm wide, solitary, superficial, subglobose, long ellipsoid to irregular, tuberculate, fleshy, white to pinkish, with locules lining the periphery,
Figure 4. Hypocrellin A and hypocrellin B HPLC-UV profiles (265 nm) of standards and stromatal HPLC-UV profiles (265 nm) of specimens of *Shiraia bambusicola* (HKAS 102253) (II) and *Rubroshiraia bambusae* (HKAS 102255) (III) and DAD spectra of major metabolites.
with dark ostiolate points appearing on surface. **Ascostromatic tissue** thick, pinkish, composed of wide, woven hyphae of textura intricata. **Locules** in vertical section 370–700 µm high × 370–700 µm diam. (\( \bar{x} = 541 \times 513 \) µm, n = 20), globose to subglobose, immersed in the peripheral layer of ascostromata, with 100–200 µm wide ostioles. **Peridium** 20–45 µm thick, composed of several layers of hyaline to light brown, small cells of textura angularis to textura intricata. **Hamathecium** composed of interthecal, hyaline septate, branched pseudoparaphyses, 1–2.5 µm wide. **Asci** 200–370 × 20–35 µm (\( \bar{x} = 291.6 \times 26.6 \) µm, n = 20), 4–6-spored, thick-walled, bitunicate, fissitunicate, cylindrical, short-pedicellate, with an ocular chamber. **Ascospores** 50–77 × 15–24 µm (\( \bar{x} = 62.3 \times 18.1 \) µm, n = 20), 1-seriate, overlapped, fusiform, muriform, hyaline, with 7 transverse septa, constricted at the septum, smooth-walled. **Asexual morph**: **Conidiomata** 200–500 µm high, 300–400 µm wide, loculate, forming within ascostromata, globose to subglobose or irregular. **Wall of locules** 20–40 µm thick, composed of several layers of hyaline to light brown, small cells of textura intricata. **Conidiophores** reduced to conidiogenous cells. **Conidiogenous cells** 3–6 × 2–3 µm (\( \bar{x} = 4.7 \times 2.1 \) µm, n = 10), blastic, cylindrical, hyaline, smooth-walled. **Conidia** 60–80 × 19–25 µm (\( \bar{x} = 75.4 \times 23.1 \) µm, n = 20), fusiform, muriform, hyaline, with irregularly transverse and longitudinal septa, straight to curved, smooth-walled.

**Culture characters.** Colonies growing slowly, attaining 30 mm diam. after 2 weeks at 27 °C under dark, circular, with even margin, floccose at the centre, drift white at margin, light greenish at centre, dark from below.

**Material examined.** CHINA, Yunnan province, Lijiang, on living branches of *Brachystachyum densiflorum* (Rendle) Keng, 3 May 2017, Dong-Qin Dai, DDQ00409.
Rubroshiraia gen. nov., a second hypocrellin-producing genus in Shiraiaceae (Pleosporales)

Figure 6. *Shiraia bambusicola* A–J sexual morph A fruiting bodies (HKAS102253, HKAS102254, HKAS102257, HKAS102261, HKAS102262) B–J photographs from material HKAS102253 B Surface of ascostromata showing the dark openings of ostiole C vertical section of ascostromata D vertical section of locule E pseudoparaphyses F, G asci (G Showing the fissitunicate asci) H–J ascospores K–M asexual morph K vertical section of asexual locules L–M conidia. Scale bars: 2 cm (A), 5 mm (B), 1 mm (C), 100 µm (D, K), 50 µm (F, G), 20 µm (H–J, L, M).

**Notes.** *Shiraia bambusicola* was erected by Hennings (1900), based on a collection from Japan. Liu et al. (2013) re-examined the holotype with 1–2.5 cm wide ascostromata, which is smaller than the new collections (1–4 cm wide in ascostromata) in China. The holotype has large ascospores compared with the new specimens in this study (75–125 × 23–47 µm vs. 50–77 × 15–24 µm). The epitype designated by Liu et al. (2013) which has similar-sized (50–77 × 15–24 µm) ascospores and similar ITS sequence, as in our new collections.

**Other genera included**


Index Fungorum number: IF12090
Facesoffungi number: FoF 06204

**Description.** See Ariyawansa et al. (2013).

**Type species.** *Grandigallia dictyospora* M.E. Barr et al., Mycotaxon 29: 196 (1987)

**Notes.** The monotypic genus *Grandigallia* was introduced by Barr (1987) and is typified by *G. dictyospora*. The fungus infects branches of *Polylepis sericea* Wedd. (*Rosaceae*) and produces conspicuous (3–14 cm in diam.) and black ascostromata. *Grandigallia* closely resembles *Shiraia* in having muriform ascospores, however, it differs by its black and larger ascostromata. Kirk et al. (2008) and Lumbsch and Huhndorf (2010) placed *Grandigallia* in Dothideomycetes, genera *incertae sedis*. Ariyawansa et al. (2013) re-examined the type material and transferred it to Shiraiaceae in Pleosporales. Wijayawardene et al. (2014, 2017, 2018) accepted this placement.

*Rubroshiraia* D.Q. Dai & K.D. Hyde, gen. nov.

Index Fungorum number: IF556564
Facesoffungi number: FoF 06205

**Etymology.** The epithet “Rubro” means red colour referring to reddish ascostromata similar to the genus *Shiraia*.

**Description.** Parasitic on living branches of bamboo. **Sexual morph:** *Ascostromata* solitary, superficial, globose to subglobose, fleshy, reddish, with locules lining the periphery, with dark ostiolate tips appearing on surface. **Ascostromatic tissue** thick,
Rubroshiraia gen. nov., a second hypocrellin-producing genus in Shiraiaceae (Pleosporales) 15

pinkish, composed of wider woven hyphae of textura intricata. **Locules** globose to subglobose, immersed in the peripheral layer of ascostromata, with narrow ostiolate openings. **Peridium** composed of several layers of hyaline to dark brown, small cells of textura angularis to textura intricata. **Hamathecium** of interthecial, hyaline, septate, branched pseudoparaphyses above asci. **Asci** 8-spored, thick-walled, bitunicate, fissitunicate, cylindrical, short-pedicellate, with an ocular chamber. **Ascospores** spirally arranged in ascii, filiform, hyaline, with transverse septa, smooth-walled. **Asexual morph:** Undetermined.

**Type species.** *R. bambusae* D.Q. Dai & K.D. Hyde.

**Notes.** The hypocrellin-producing fungus *R. bambusae* is a well-known taxon used in Chinese traditional medicine which is called “Zhuhongjun” or “Zhuxiaorouzhuojun” in Chinese. However, without molecular data, it was wrongly named as *H. bambusae* (Liu 1978).

*Hypocrella bambusae* was combined by Saccardo (1878), based on its linear asci and filiform ascospores. Index Fungorum (2019) lists its basionym as *Hypocrea bambusae* Berk. & Broome, which was collected on the inflorescences of bamboo in Sir Lanka and had linear asci and filiform ascospores (Berkeley and Broome 1875). Liu (1978) recorded a well-known Chinese medicinal ascomycete, producing 0.7–1.5 mm diam., hemispheric and reddish stromata with multi-locules, cylindrical asci and filiform ascospores which are spirally arranged and more than 250 µm long on bamboo culms. Liu (1978) identified this fungus as *H. bambusae*, probably based on its cylindrical asci and filiform ascospores. In addition, species of *Hypocrella* usually produce perithecial ascomata (Saccardo 1878). To our knowledge, no fungal records or herbal medicine like that described in Liu (1978) occur in Sir Lanka. Moreover, based on the examination of type material of *Hypocrea bambusae*, it has smaller (0.1 cm vs. 0.7–1.5 mm in diam.) and black stromata, unitunicate asci and ascospores are in a single fascicle but not significantly helically coiled (Fig. 7). Hence, we conclude that Liu (1978) made a wrong identification.

New collections of “Zhuhongjun” were collected and sequenced. The phylogenetic analyses showed it belongs to Shiraiaceae and is separate from *Shiraia* with high bootstrap support (100/1.00 MLBS/BSPP) (Fig. 2). *Grandigallia* has not been included in the phylogenetic tree as it is lacking gene sequences in the GenBank (retrieved date: 13 May 2019). However, *Grandigallia* can be morphologically distinguished from the new taxon in having black ascostromata and muriform ascospores (Barr 1987; Ariyawansa et al. 2013). Thus, this fungus is introduced as *R. bambusae* gen. et sp. nov in this study.

*Rubroshiraia bambusae* is often confused with *S. bambusicola* by Chinese traditional folk residents, probably because of the similarity of their ascostromata, parasitism on bamboo host and similar efficacy of medical treatment. However, it differs from *S. bambusicola* by its smaller sized ascostromata (0.7–1.2 cm long × 0.7–1 cm wide vs. 1–6 cm long × 1–4 cm wide) and distinct ascospores (filiform ascospores vs. fusiform and muriform ones). Both of the above species can produce the metabolites hypocrellin A and B, whereas *R. bambusae* contains almost double the content compared to *S. bambusicola* (Fig. 4).
**Rubroshiraia bambusae** D.Q. Dai & K.D. Hyde, sp. nov.

Fig. 7

Index Fungorum number: IF556564

Facesoffungi number: FoF 06206

**Etymology.** Refers the bamboo host.

**Holotype.** HKAS102255.

**Description.** Parasitic on living branches of bamboo. **Sexual morph:** Ascostromata 0.7–1.5 cm long × 0.7–1.3 cm wide, solitary, superficial, globose to subglobose, fleshy, reddish, with locules lining the periphery, with dark ostiolate points appearing on the surface. Ascostromatic tissue thick, pinkish, composed of wider woven hyphae of textura intricata. **Locules** in vertical section 800–1800 µm high × 1000–2000 µm diam. (\(\bar{x} = 1289.4 \times 1368.8 \, \mu m, \, n = 20\), globose to subglobose, immersed in the periphery layer of ascostromata, with 250–500 µm wide × 450–550 µm high ostioles. **Peridium** 20–35 µm thick, composed of several layers of hyaline to dark brown, small cells of textura angularis to textura intricata. **Hamathecium** of interthecal, hyaline septate, branched pseudoparaphyses, 1–3 µm wide. **Asci** 660–800 × 45–55 µm (\(\bar{x} = 751.6 \times 49.5 \, \mu m, \, n = 20\), 8-spored, thick-walled, bitunicate, fissitunicate, cylindric, short-pedicellate, with an ocular chamber. **Ascospores** 600–750 × 5.5–11 µm (\(\bar{x} = 728.8 \times 9.1 \, \mu m, \, n = 20\), spirally arranged in asci, filiform, hyaline, with 15–18 transverse septa, smooth-walled. **Asexual morph:** Undetermined.

**Material examined.** CHINA, Yunnan, Dali, on living branches of Fargesia spathacea Franch, 13 May 2017, Dong-Qin Dai, DDQ00411 (HKAS102255, holotype), Ibid. (HMAS 290447, isotype), Ibid. DDQ00412 (HKAS102256), Ibid. DDQ00416 (HKAS102260), Ibid. 20 June 2017, Dong-Qin Dai, DDQ00425 (HKAS102268), Ibid. DDQ00426 (HKAS102269), Ibid. DDQ00427 (HKAS102270), Ibid. DDQ00428 (HKAS102271), Ibid. DDQ00429 (HKAS102272), Ibid. DDQ00430 (HKAS102273), Ibid. DDQ00431 (HKAS102274).

**Key for distinguishing genera in Shiraiaceae**

1. Parasitising bamboo branches, ascostromata are white to reddish .............. 2
   – Parasitising Rosaceae branches, ascostromata are black ..................**Grandigallia**

2. Ascospores muriform .................................................................**Shiraia**
   – Ascospores filiform ...............................................................**Rubroshiraia**

Since the familial placement of *H. bambusae* is controversial in different studies (Berkeley and Broome 1875, Saccardo 1878, Liu 1978), we re-studied the isotype. Based on morphology, we conclude that it has unitunicate asci thus related to Sordariomycetes.
Rubroshiraia gen. nov., a second hypocrellin-producing genus in Shiraiaceae (Pleosporales)

Figure 7. Rubroshiraia bambusae (HKAS102255, holotype) A fruiting bodies B, C surface of ascostromata showing the openings of ostiole D vertical section of ascostromata E, F vertical section of locule G peridium of locule H asci and pseudoparaphyses I asci and asci ocular chamber J ascospores K, L immature asci. Scale bars: 1 cm (A), 25 mm (B), 2 mm (C, D), 500 µm (E, F), 200 µm (G), 50 µm (H–L).
**Hypocrella bambusae** (Berk. & Broome) Sacc. 1878

Fig. 8
Index Fungorum number: IF160297

**Basionym.** *Hypocrea bambusae* Berk. & Broome, 1873

**Description.** Parasitic on living inflorescence of bamboo. **Sexual morph:** Stromata around 0.14 cm diam., 0.06 cm high, solitary, superficial, subglobose, fleshy to coriaceous, black, with around 20 perithecia lining the periphery, with ostioles slightly raised above stroma surface. **Stromatic tissue** thick, brown to dark brown. **Perithecia** in vertical section around 100 µm diam., 200 µm high, pyriform, immersed in the periphery layer of stromata. **Asci** more than 220 µm long, 5–6 µm diam., 8-spored, unitunicate, cylindrical, with a glassy refractive cap around 3 µm from apex to base. **Ascospores** around 180 µm long, 1–1.5 µm diam., in a single fascicle but not significantly helically coiled, filiform, hyaline, with 9–10 transverse septa, with rounded ends, smooth-walled. **Asexual morph:** Undetermined.

**Material examined.** SRI LANKA, on inflorescence of bamboo, January 1855, G.H.K. Thwaites s.n. (ex herb. M.J. Berkeley), K(M)52469, isotype.

**Notes.** This taxon has typical morphology of the Clavicipitaceae, which is pyriform perithecia with a gradually tapering upper part and cylindrical asci with a glassy refractive cap. New collections are required and need to be sequenced to clarify its placement.

**Discussion**

Members of the family Shiraiaceae are distributed from Asia to South America but so far reported only from three countries, viz. China, Japan and Venezuela (Barr et al. 1987; Liu et al. 2013). The family comprises three genera, i.e. *Grandigallia*, *Rubroshiraia* and *Shiraia* wherein the former genus is lacking DNA sequences and, thus in here, we did not include it in the molecular analyses (Figs 2 and 3). These genera show the typical characters of Shiraiaceae, viz. conspicuous large, tuberculate, fleshy and multi-loculate ascostromata producing bitunicate asci. *Shiraia bambusicola* has various types of ascostromata, such as subglobose to tuberculate with white to pinkish colours (Fig. 6). However, the phylogenetic analysis shows these specimens with different types of ascostromata belong to same species (Figs 2 and 3). Thus, we assume that the different shapes of ascostromata are because of the host and different environmental conditions.

Stromatal methanol extracts of *Rubroshiraia* and *Shiraia* contain Hypocrellins (Fig. 4). However, so far no extracts have been reported from *Grandigallia*. Fresh material of *Grandigallia* is essential to determine the metabolites. *Rubroshiraia* has darker reddish ascostromata compared with *Shiraia*, probably because its stromatal methanol extracts contain larger quantity of hypocrellins. Some endophytes, named as Shiraia-like fungi, are known to produce hypocrellins on media. They were isolated from different parts of bamboo, such as seeds, nods and internodes (Lu et al. 2004; Mora-
Rubroshiraia gen. nov., a second hypocrellin-producing genus in Shiraiaceae (Pleosporales)

Other Shiraiia-like endophytes, isolated from the rhizome of Gastrodia, leaves of Huperzia serrata and from Triticum aestivum, phylogenetically cluster within the former group (Fig. 3). However, no hypocrellins were produced from their mycelium (Zhu et al. 2010; Wang et al. 2011, 2016). The bamboo tissue may be providing the needful substances for fungi to produce hypocrellins. The endophytic Shiraiia-like taxa (Fig. 3) appear as a distinct genus in Shiraiaceae. The nomination will be made once the type material is available.

Figure 8. Hypocrella bambusae (K(M)52469, isotype, images are accredited to the Royal Botanic Gardens, Kew) A, C fruiting bodies on inflorescence of bamboo B vertical section of stromata showing the perithecia locating D herbarium envelope E filiform ascospores F asci with caps (Staining by cotton blue). Scale bars: 5 mm (A), 200 µm (B), 2 mm (C), 20 µm (E, F).
Shiraia bambusicola has been used as a Chinese traditional folk-medicine, in curing rheumatoid arthritis, infantile convulsion and pertussis etc. for more than 400 years, because of its stromatal metabolites (Huang et al. 2001; Shen et al. 2002). Japanese scientists first obtained three perylenequinones from air-dried ascostromata of S. bambusicola and named them as hypocrellin A, B and C (Kishi et al. 1991). However, hypocrellin A was originally discovered by Wan and Chen (1981) from a different fungus on bamboo which was called as “Zhuhongjun” in Chinese and was erroneously identified as H. bambusae (Liu 1978). Later the fourth hypocrellin analogue (hypocrellin D) was named by Fang et al. (2006). Therefore, in total, four hypocrellins have so far been named. Hypocrellins are types of biologically active compounds and naturally occurring perylenequinones with photodynamic activity (Wan and Chen 1981; Kishi et al. 1991; Chowdhury et al. 2002; Liang et al. 2009; Liu et al. 2013). These secondary metabolites have gained much attention owing to their light-induced anti-tumour, anti-fungal and anti-viral activities (Wan and Chen 1981; Liang et al. 2009; Li et al. 2000a, b). In clinical trials, hypocrellin shows promising treatment for various skin diseases, such as skin cancer and white lesions of the vulva (Wan and Chen 1981; Li et al. 2000b). In China, a costly medicinal unguent named Bamboo Parasitic Fungus Ointment is made of hypocrellin B (Dai et al. 2018). Interestingly, it was proved that hypocrellin has bactericidal activities which inhibit various bacteria, such as Bacillus subtilis Ehrenberg and Micrococcus luteus Schroeter (Chen et al. 2010). In addition, hypocrellin A has an antiviral activity against human immunodeficiency virus (HIV-1) (Hudson et al. 1994) and is promising as a new-fashioned photoelectric conversion material (Li et al. 2000a).

Hypocrellin has wide application prospects, but it was earlier only found existing in ascostromata of S. bambusicola and “Zhuhongjun” (R. bambusae in this paper) (Wan and Chen 1981; Kishi et al. 1991). For gaining a high yield of Hypocrellin, scientists devoted themselves to looking for strains that can produce hypocrellin through fermentation production (Liang et al. 2009). Numerous endophytes, isolated from bamboo tissue such as culms, leaves, nodes and seeds, were published (Lu et al. 2004; Morakotkarn et al. 2007, 2008; Liang et al. 2009; Cai et al. 2011; Shen et al. 2012, 2014; Zhang et al. 2014), several of which had the potential for hypocrellin production (Lu et al. 2004; Morakotkarn et al. 2008; Liang et al. 2009; Zhang et al. 2014; Tong et al. 2017). However, the strains with promising industrial fermentation were identified as Shiraia sp. based on the blast search in GenBank by ITS sequences. More endophytes producing biologically active compounds, such as huperzine, isolated from the plant Huperzia serrata (Thunb. ex Murray) Trev., were also named as Shiraia sp. (Wang et al. 2011, 2016; Zhu et al. 2010). These strains usually have around 80%–90% ITS similarity with S. bambusicola, which also shows that they are phylogenetically close with members of Shiraiaceae. In this study, these endophytes are placed in Shiraiaceae, based on the phylogenetic analyses (Fig. 3).

According to Deng et al. (2017), polyketide synthase (SbaPKS) is involved in hypocrellin biosynthesis, based on the methods of CRISPR/Cas9 genome editing. It provides evidence for decoding the hypocrellin pathway (Deng et al. 2017). This pathway has the potential for producing high quality hypocrellins.
Rubroshiraia gen. nov., a second hypocrellin-producing genus in Shiraiaceae (Pleosporales)

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Rubroshiraia gen. nov., a second hypocrellin-producing genus in Shiraiaceae (Pleosporales)


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Rubroshiraia gen. nov., a second hypocrellin-producing genus in Shiraiaceae (Pleosporales) 25


Microsatellite based genetic diversity of the widespread epiphytic lichen Usnea subfloridana (Parmeliaceae, Ascomycota) in Estonia: comparison of populations from the mainland and an island

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Abstract
Understanding the distribution of genetic patterns and structure is an essential target in population genetics and, thereby, important for conservation genetics. The main aim of our study was to investigate the population genetics of Usnea subfloridana, a widespread lichenised fungus, focusing on a comparison of genetic variation of its populations amongst three geographically remote and disconnected regions, in order to determine relationships amongst environmental data, variation in lichen secondary chemistry and microsatellite data in genotyped populations. In all, 928 Usnea thalli from 17 populations were genotyped using seven specific fungal microsatellite markers. Different measures of genetic diversity (allelic richness, private allelic richness, Nei’s unbiased genetic diversity and clonal diversity) were calculated and compared between lichen populations. Our results revealed a low genetic differentiation of U. subfloridana populations amongst three distant areas in Estonia and also a high level of gene flow. The results support suggestion of the long-range vegetative dispersal of subpendulous Usnea subfloridana via symbiotic propagules (soralia, isidia or fragments of thalli). Our study has also provided evidence that environmental variables, including mean annual temperature and geographical longitude, shape the genetic structure of Usnea subfloridana populations in Estonia. Additionally, a weak but statistically significant correlation between lichen chemotypes and microsatellite allele distribution was found in genotyped specimens.

Keywords
Chemotypes, genetic diversity, environmental factors, lichenised fungi, microsatellites
**Introduction**

The disentangling processes which shape genetic patterns and structure of natural populations is of great importance in understanding basic questions concerning evolution, ecology and conservation biology of species. The distribution of genetic diversity, which is a significant part of overall biodiversity, could indicate patterns of gene flow, genetic drift and potential for local adaptation (Frankham et al. 2010). The vast majority of previous studies about microsatellite diversity of lichenised fungi have used threatened, regionally rare or narrowly distributed lichens (e.g. Nadyeina et al. 2014; Jones et al. 2015; Prieto et al. 2015). However, the genetic diversity of common taxa could also be of particular interest since common species could be similarly susceptible to genetic consequences of habitat fragmentation as rare species (Honnay and Jacquemyn 2007). To date, only a few investigations have studied the genetic diversity of common and widespread lichenised fungi and genetic differentiation of their populations using microsatellite markers (e.g. Mansournia et al. 2012; Degtjarenko et al. 2018).

The epiphytic fruticose lichen *Usnea subfloridana* Stirt. has a wide distribution across Eurasia, Macaronesia and North America (Nash et al. 2007; Randlane et al. 2009; Smith et al. 2009). This is one of the commonest *Usnea* species in Estonia (Northern Europe) being frequently found on Norway spruce (*Picea abies*), Scots pine (*Pinus sylvestris*) and Silver birch (*Betula pendula*), as well as other deciduous trees and lignum (Törra and Randlane 2007; Randlane et al. 2011). Recent microsatellite studies of *U. subfloridana* populations indicated that unconstrained gene flow and exchange of multilocus genotypes existed between two geographically remote regions (the maximum distance between the two regions was 184 km) within the mainland of Estonia or had occurred at least in the past (Degtjarenko et al. 2018). Moreover, the natural habitat characteristics, such as stand age and mean circumference of the host tree, did not reveal any significant influence on measures of genetic diversity of *U. subfloridana* populations (Degtjarenko et al. 2016, 2018). However, some negative impact caused by alkaline dust pollution has been recorded on the genetic variation of this species (Degtjarenko et al. 2016).

Microsatellites or simple sequence repeats (SSR) are highly variable DNA sequences of short tandem repeats of 1–6 bp with co-dominant inheritance and appear as widely used markers for studying genetic variation and structure of natural populations (Goldstein and Schlötterer 1999; Ellegren 2004). The microsatellites are highly polymorphic and species-specific markers, considered as a most promising tool for investigating genetic diversity of highly clonal and complex organisms such as lichens (Werth 2010). The microsatellites were usually assumed to be neutral markers, occurring mainly in non-coding DNA (Ellegren 2004). Recent studies, however, have questioned this assumption, since microsatellites are also found in coding regions (e.g. Gemayel et al. 2012; Gao et al. 2013), playing a role in species adaptation and phenotypic plasticity within and across generations (Vieira et al. 2016).
Lichens produce a great number of extracellular secondary metabolites; these are synthesised by the mycobiont, although the carbon which is necessary for these substances is provided by the photobiont and subsequently transported to the fungus (Elix and Stocker-Wörgötter 2008). The production of polyketides, the most studied class of secondary metabolites in lichens, is regulated by polyketide synthases (PKS), the genes for which have been found in clusters (Shen 2003). Secondary metabolites of lichen-forming fungi are considered to have a distinct function, such as protecting the thalli against herbivores, pathogens or UV-radiation (Molnár and Farkas 2010). The presence or absence of specific secondary substances or their replacements by another substance has played an important role in identification and classification of these organisms when correlated with morphological or geographical differences (Elix and Stocker-Wörgötter 2008). Hence, variation of secondary compounds in lichens is probably not selectively neutral (Werth 2010). In *U. subfloridana*, three chemotypes have been reported (Halonen et al. 1998, 1999) while, in Estonia, two of them are known: (i) with thamnolic acid and (ii) with squamatic acid as the main substance in the medulla (Törra and Randlane 2007).

In the present research, we studied the population genetics of *U. subfloridana*, a widespread lichenised fungus, concentrating on a comparison of genetic variation of populations amongst three geographically remote and disconnected (by sea) regions. The main aims of our research were: (i) to study the genetic differentiation of *U. subfloridana* populations, growing in the south-eastern and northern regions of mainland and on a western island in Estonia, Northern Europe; (ii) to compare the measures of genetic diversity of *U. subfloridana* populations amongst the three study areas; (iii) to find whether allele frequencies in studied populations correlate with environmental variables; and (iv) to check if there were correlations between lichen chemotypes and microsatellite allele distribution in genotyped data.

**Material and methods**

**Study area**

The study area is located in Northern Europe, in three geographically separate parts of Estonia: Lääne-Viru County, the northern region of mainland (hereafter N), Põlva County, the south-eastern region of mainland (hereafter SE) and Hiiumaa County, the second largest western island (hereafter W) of Estonia, located in the Baltic Sea (Fig. 1). According to climate norms from 1981 to 2010, N has a mean annual temperature of 5.7 °C, a mean annual precipitation of 587 mm, a mean wind speed of 3.9 m/s and a mean relative humidity of 80%, W has a mean annual temperature of 6.8 °C, a mean annual precipitation of 639 mm, a mean wind speed of 3.9 m/s and a mean relative humidity of 82% and SE has a mean annual temperature of 5.8 °C, a mean annual precipitation is 680 mm, a mean wind speed of 3.2 m/s and a
mean relative humidity of 80% (Estonian Weather Service 2019). The vegetation of Estonia belongs to the hemiboreal forest zone, lying in the transitional area, where the southern taiga forest subzone changes into the spruce-hardwood subzone (Ahti et al. 1968; Laasimer and Masing 1995). The study sites from both geographical regions were situated in *Pinus sylvestris*-dominated boreal forests, being classified as the Oxalis-*Vaccinium myrtillus*, the *Vaccinium myrtillus*, the Calamagrostio-Pinetum and the *Vaccinium vitis-idaea* forest site types. These forest types are also widely distributed in other Baltic states (Kairiūkštis 1966; Bušs 1997), in Fennoscandia (Dierßen 1996) and in northwest Russia (Fedorchuk et al. 2005).

**Data collection**

Fieldwork was carried out during the summer of 2011 (in SE), the autumn of 2014 (in N) and the autumn of 2016 (in W). The potential study sites for sampling were selected from forest survey maps using comparable forest characteristics (stand age and site type) from their forest survey (Forest Public Registry 2017). *Usnea subfloridana* populations, sampled from 17 study sites, three in N, eight in SE and six in W (Fig. 1; Table 1), were defined according to the boundaries of forest sites sharing the same values of forest survey data (forest site type, age of trees and proportion of trees in forest stand), according to the Forest Public Registry (2017). In each lichen population, an average of three *Usnea* thalli were collected from each Norway spruce tree, up to 6 m from the ground using a tree pruner. In total, 10–21 trees were surveyed and 30–66 thalli were randomly collected from each lichen population (Table 1). The tree circumference (BHC) was recorded for each sampled tree at breast height (1.3 m). The stand age was taken from the Forest Public Registry (2017).
Table 1. Characteristics of the studied *Usnea subfloridana* populations from the northern region (1–3), the southeastern region (4–11) and Hiiumaa island (12–17) of Estonia: sample size, geographical coordinates, tree variables, and measurements of genetic variation. Populations, the number of population; Specimens, the number of collected thalli per population; Trees, the number of host trees from which thalli were collected in each population; Latitude, latitudinal coordinates of the centre of forest site; Longitude, longitudinal coordinates of the centre of forest site; Age, the stand age (based on the oldest trees in the stands); BHC, mean circumference (cm) of the host tree per population (measured from each sampled tree at breast height 1.3 m); Squamous, the number of collected thalli containing squamous acid; Thamnolic acid, the number of collected thalli containing thamnolic acid; H, Nei's unbiased genetic diversity per population; A, standardized allelic richness per population; G, the number of multilocus genotypes per population; M, clonal diversity per population; P, private allelic richness per population.

<table>
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<th>Variables</th>
<th>Region</th>
<th>Population</th>
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<th>Southeastern (SE)</th>
<th>Hiiumaa (W)</th>
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<tr>
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Chemical and molecular analyses

All collected *Usnea* thalli were air dried, cleaned to remove other lichen specimens and examined under a stereomicroscope. Thin layer chromatography (TLC) with solvent A (Orange et al. 2001) was used to confirm the identification of collected *Usnea* species. Then, 50 mg of each specimen was maintained in 1.5 ml microtubes at –20 °C until molecular analyses. The total genomic DNA was extracted using PowerPlant Pro DNA Isolation Kit and DNeasy Plant Mini Kit (MO BIO Laboratories, Inc., Qiagen, USA), according to the manufacturer’s protocol. Seven fungal microsatellite loci (*Us02, Us03, Us04, Us05, Us06, Us08 and Us09*) were amplified in two multiplex PCR using QIAGEN Multiplex PCR Kit, following the instructions described in Törra et al. (2014) and Degtjarenko et al. (2016). Fragment lengths of PCR products were determined on a 3730xl DNA Analyzer (Applied Biosystems) with LIZ-500 as the internal size standard. The alleles were sized and genotyped using GeneMapper Software ver 5 (Applied Biosystems).

Statistical analyses

The basic measurements of population genetics (the total number of alleles, mean number of alleles per locus, Nei’s unbiased genetic diversity (H) and allelic richness (A)) for *U. subfloridana* populations were calculated in the Microsatellite Analyzer ver 2.65 (MSA) (Dieringer and Schlötterer 2003). The measures of A were standardised using the rarefaction procedure implemented in the software MSA (Dieringer and Schlötterer 2003). The allelic richness of private alleles (P) per population was calculated using software HP-Rare (Kalinowski 2005). The number of multilocus genotypes (G), the percentage of multilocus genotypes, i.e. clonal diversity or genotypic diversity (M; the proportion of different genotypes in the population, G/N) and total number of multilocus genotypes from all populations were calculated in the software R (R Core Team 2013), using the R script by Werth et al. (2006). One-way analysis (ANOVA, type III) in the TIBCO Statistica ver 13.3 (TIBCO Software Inc.) was used to compare the different measurements of genetic diversity (A, H, P and M) amongst the three regions, N, SE and W.

The number of shared multilocus genotypes between populations was calculated in the software ARLEQUIN ver 3.5 (Excoffier and Lischer 2010). Clone correction of the genotyped dataset was performed in the software R (R Core Team 2013) using the R package ‘poppr’ (Kamvar et al. 2014; 2015). Hierarchical analyses of molecular variance (AMOVA) with 1023 permutations to estimate genetic differentiation were performed using ARLEQUIN ver 3.5 (Excoffier and Lischer 2010). The first, second and third AMOVA were performed at the tree level; genotyped individuals (364 multilocus genotypes and 124 trees) from populations of W, genotyped individuals (112 multilocus genotypes and 32 trees) from populations of N and genotypes individuals (452 multilocus genotypes and 166 trees) from populations of SE were analysed separately
where each tree was treated as a distinct population. The fourth AMOVA was undertaken using all genotyped individuals (928 multilocus genotypes) and the fifth without identical multilocus genotypes or clone corrected dataset (403 multilocus genotypes). The rate of gene flow (Nm) across seven loci amongst 17 populations was estimated using GenAlex ver 6.5 (Peakall and Smouse 2012). Index of Association (Ia) was calculated to measure the extent of linkage equilibrium within a dataset by quantifying the amount of recombination amongst a set of sequences and observing association between alleles at different loci (Smith et al. 1993). The Ia was measured in the software R (R Core Team 2013) using the R package ‘poppr‘ (Kamvar et al. 2014; 2015).

To assess the variation in data of *U. subfloridana* multilocus genotypes, the principal component analysis (PCA) was performed, implemented with the programme package Canoco 5.0 (Šmilauer and Lepš 2014). The data matrix of alleles in seven loci from 928 sample specimens was used. Variable ‘Lichen substance’ (squamatic or thamnolic acid) was used to group the samples. Subsequently, the redundancy analysis (RDA) (Šmilauer and Lepš 2014) was performed and nine explanatory variables were used to assess the correlation with accounted multilocus genotypes. The explanatory variables used in RDA were ‘Lichen substance’ and environmental variables as Latitude, Longitude, Stand Age, a mean BHC of sample trees per population, a mean annual temperature, a mean annual precipitation, a mean wind speed and a mean relative humidity for populations in the region.

To assess the significance of the associations between allele frequency in the populations and environmental variables, the second RDA was implemented in the same programme package. The data matrix of frequency of 62 alleles in 17 populations was used. The number of records of each allele in each population was counted and log-transformed for data analyses. The same environmental variables as in the first RDA were used as the explanatory variables. In both RDA models, the interactive forward selection procedure with randomisation tests was employed to select the most important environmental variables influencing variation in response data, retaining variables with an independent significant contribution at the p < 0.05 level. Subsequently, variation partitioning analysis (VPA) in the same programme was employed. The unique effects of statistically significant explanatory variables and the shared proportion of variation, explaining the distribution of multilocus genotypes (the first RDA) or allele frequency in populations (the second RDA), was calculated. The statistically significant contribution of variables was tested by the permutation test (Monte-Carlo permutation test, 4999 unrestricted permutations).

**Results**

In total, 62 alleles at seven microsatellite loci, all polymorphic (Table 1), in 928 specimens from 17 *U. subfloridana* populations were recorded. The minimum number of alleles was four in locus *Us04* and the maximum was 15 in locus *Us03* and, on average, 2.8–9.1 were detected per locus across 17 populations. The mean number of alleles per
population varied from 5.3 to 6.1 in populations on the western island in Estonia (W), from 5.3 to 6.2 in populations in the south-eastern region of mainland (SE) and from 4.9 to 5.4 in populations in the northern region of the mainland (N). There were 403 different multilocus genotypes across 928 genotyped specimens in 17 lichen populations. Allelic richness (A) varied from 4.86 to 5.94 across all lichen populations and Nei’s unbiased genetic diversity (H) ranged from 0.58 to 0.67 (Table 1). Other detailed measurements of genetic diversity per population are given in Table 1. The mean gene flow (Nm) for all populations across seven loci was 7.29.

The results of ANOVA showed that Nei’s unbiased genetic diversity (H) depended significantly on the region ($F_{(2, 12)} = 10.74, p = 0.001$); H was higher in populations from W and lower in populations from N. The clonal diversity (M) also depended significantly on the region ($F_{(2, 14)} = 5.62, p = 0.02$); M was higher in populations from N and lower in populations from W. The allelic richness (A; $F_{(2, 14)} = 2.83, p = 0.09$) and private allelic richness (P; $F_{(2, 14)} = 0.18, p = 0.83$ did not differ amongst the three regions (N, SE and W).

The analyses for checking shared haplotypes amongst populations in the software ARLEQUIN ver 3.5 indicated that all *Usnea* populations shared the identical multilocus genotypes with other populations, as well as amongst three regions, N, W and SE (Fig. 2; Suppl. material 1). The Index of Association (Ia) differs significantly from zero which means that *Usnea* multilocus genotypes are likely undergoing clonal reproduction in study populations (Kamvar et al. 2014; 2015). The first, second and third AMOVA results showed that most of the total genetic variation (96.1% in W, 97.5% in SE and 95.1% in N) was due to the differences amongst individuals within study tree and 0.5%, 0.3% and 0.7% of genetic variation (for W, SE and N, respectively) was found amongst populations (Table 2). The fourth AMOVA results (928 genotypes) revealed that most of the total genetic variation (97.7%) was due to the differences amongst individuals within studied *Usnea* populations; a low proportion (1.8%) of genetic variation was attributed to regional differences (Table 2). The results from the fifth AMOVA (clone corrected, 403 genotypes) showed that most of the total genetic variation (98.7%) was also due to differences amongst individuals within studied *Usnea* populations and 1.2% of genetic variation was found between the regions (Table 2).

In the PCA ordination of multilocus genotypes of *U. subfloridana*, the first ordination axis accounted for 34.5% and the second axis for 20.5% of variation in the sample data. The sampled specimens constituted a rather homogenous cluster in the PCA ordination plot and only a minor distinction, according to the presence of thamnolic or squamatic acid, was visible (Fig. 3). In the RDA analyses with nine explanatory variables, the model accounted for 5.7% of variation in the response data. According to the results of the interactive forward selection of explanatory variables, the variables ‘Lichen substance’ and ‘Temperature’ contributed significantly to the explanation of variation in the response data (Fig. 4). The other environmental variables did not make a significant contribution to the model and were left out during the interactive forward selection of variables ($p \geq 0.05$). The results of variation partitioning analysis (VPA) showed that two variables, ‘Lichen substance’ and ‘Temperature’, represent 5.5% of
Figure 2. Total number of shared haplotypes between populations of *Usnea subfloridana* in the southeastern (SE), the western (W) and northern (N) regions of Estonia; the thickness of lines reflects the number of shared haplotypes between populations.

Figure 3. *Usnea subfloridana* multilocus genotypes in the principal component analysis (PCA) ordination plot of the first and second axes. Samples are grouped according to the presence of lichen substance: samples containing thamnolic (square) or squamatic acid (circle).
the variation of *U. subfloridana* multilocus genotypes (adjusted variation), the former being 4.1% and the latter 1.3% of the total variation, while the co-effect of both variables was less than 0.1% \((p = 0.0005)\).

The model of the second RDA with allele frequency data in studied populations accounted for 36.1% of variation in the response data. According to the results of the interactive forward selection of explanatory variables, the variables ‘Temperature’ and ‘Longitude’ contributed significantly to the explanation of variation in the response data (Fig. 5), the other explanatory variables not making a significant contribution to the model and were left out during the interactive forward selection of variables \((p \geq 0.05)\). The results of variation partitioning analysis (VPA) showed that the two explanatory variables, ‘Temperature’ and ‘Longitude’, represent 27.0% of the variation of allele frequency data (adjusted variation), the former being 8.2% and the latter 7.6% of the total variation, while the co-effect of both variables was 11.2% \((p = 0.0005)\). Alleles, occurring only (e.g. 326 and 346 \((Us05)\), 201\((Us08)\), coded as 8201)) or being more frequent (e.g. 322 and 330 \((Us05)\), 195\((Us08)\), 345 \((Us09)\)) in populations of western Estonia with an average higher air temperature, are located in the positive side of the

<table>
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<th>Source of variation</th>
<th>d.f.</th>
<th>Sum of squares</th>
<th>Variance</th>
<th>Percentage %</th>
<th>P</th>
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Microsatellite based genetic diversity of the widespread epiphytic lichen *Usnea subfloridana*

**Figure 4.** *Usnea subfloridana* multilocus genotypes (Us02, Us03, Us04, Us05, Us06, Us08, Us09) and explanatory variables mean annual air temperature (‘Temp’) and the presence of thamnolic acid (‘Tham’) in a lichen sample in the bi-plot of the redundancy analysis (RDA) of the first and second axes.

**Figure 5.** Sample populations of *Usnea subfloridana* and explanatory variables mean annual air temperature (‘Temp’) and geographical longitude of populations (‘Long’) in the bi-plot of the redundancy analysis (RDA) of the first and second axes. The shape of symbols indicates the geographical location of studied populations (square – south-eastern region of mainland, circle - western island and diamond – north-eastern region) and the size of symbols indicates the number of different alleles found in the studied populations.
Discussion

Lichen-forming fungi, reproducing purely sexually, are assumed to have a longer dispersal distance and exhibit less genetic structure than clonally reproducing species via isidia/soredia or fragments of thalli (Werth 2010; Singh et al. 2015; Alors et al. 2017). However, our study showed that Usnea subfloridana, which usually reproduces asexually by symbiotic propagules, exhibited a very long dispersal range and a negligible level of genetic differentiation in populations from three geographically remote areas, namely the south-eastern (SE) and northern (N) regions of the Estonian mainland and Hiiumaa (W), an island in the Baltic Sea (Fig. 1; Table 2). Small-scale AMOVA also exhibited similar results; a negligible level of genetic differentiation was found amongst populations in three study regions (Table 2). Using highly variable microsatellite markers, our study also showed high levels of gene flow (Nm = 7.29) or genetic similarity amongst all studied U. subfloridana populations. Moreover, our results demonstrated that Usnea population shared common identical multilocus genotypes amongst all studied populations and also amongst the three regions (Fig. 2; Suppl. material 1). Our previous study also recorded a very low genetic differentiation of U. subfloridana populations between two distant areas in Estonia, suggesting spatially unrestricted
Microsatellite based genetic diversity of the widespread epiphytic lichen *Usnea subfloridana*  

dispersal of individuals and unconstrained gene flow in *U. subfloridana* populations (Degtjarenko et al. 2018). However, the genetic variation, attributed to regional differences, was slightly higher in the current study (1.8% and 1.2%; Table 2) than in the previous microsatellite study (0.5%) concerning *U. subfloridana* populations in Estonia (Degtjarenko et al. 2018). A possible explanation for this is that the maximum distance amongst the populations was longer (viz. the maximum distance was 295 km in the current and 184 km in the previous study) and, in addition, one region (W) with six populations was relatively isolated, i.e. an island. Similar levels of genetic differentiation at local scales have been indicated in previous studies; for example, for populations of *Lobaria pulmonaria* L., a lichen-forming fungus that also reproduces predominantly asexually (e.g. Walser 2004; Scheidegger et al. 2012). According to the test of Index of Association (Ia), our study found that multilocus genotypes of *U. subfloridana* in our localities most likely originated from asexual reproduction (Suppl. material 2). Therefore, it appears that the long-range dispersal of subpendulous *U. subfloridana* occurred via symbiotic propagules (soralia, isidia or fragments of thalli) that had to travel several kilometres over the sea.

The genetic diversity of natural populations is shaped by cumulative synergy of historical and present-day processes (Hewitt 2000; Frankham et al. 2010). Previous studies have shown that the climatic and habitat heterogeneity could be important in explaining the levels of genetic diversity of lichen populations; for example, annual precipitation had an effect on genetic diversity of *Lobaria pulmonaria* populations in the Iberian Peninsula (Otálora et al. 2015). Nadyeina et al. (2014) demonstrated that the microclimatic factors (air humidity and temperature) influenced the distribution of gene pools of *L. pulmonaria* in the Carpathian Mountains. The genetic diversity of *L. pindarensis* Räsanen was significantly influenced by altitude, revealing higher levels of genetic diversity at a high elevation in the Himalayas (Devkota et al. submitted). Belinchón et al. (2018) showed that *Nephroma parile* (Ach.) Ach. and *N. laevigatum* Ach. were also related to measured environmental and habitat variables, indicating micro-evolutionary responses to the environment. Our studies showed that genetic diversity (H) of *Usnea subfloridana* populations was higher on Hiiumaa island while clonal diversity (M) was higher in the northern region (N) of Estonia. This is contrary to our *a priori* assumption that relatively isolated populations, occurring on islands, will exhibit a lower genetic diversity and higher levels of clonal diversity. Moreover, the allele distribution in studied populations correlated with a mean annual temperature and geographical longitude (Fig. 5). These findings suggest (in our study – average annual temperature and longitude) are important factors for shaping the genetic structure and patterns of *U. subfloridana* populations in Estonia and indicating local adaptation to landscape conditions. The results of the redundancy analysis (RDA) indicated that, for example, several alleles occurring only or being more frequent are found in the populations of western Estonia with a higher average air temperature while other alleles, occurring only or being more frequent in populations of south-eastern Estonia, are related to a lower average air temperature (Fig. 6). Habitat quality measured as age of stand or host tree could be important in explaining the distribution of genetic
diversity of lichen populations (Jüriado et al. 2011; Otálora et al. 2011; Scheidegger et al. 2012). However, RDA showed that the age of forest stands and circumference of host trees did not have a significant effect on the distribution of allele frequencies in the studied populations. This also accords with our earlier observations, which showed that stand age or tree circumference were not of great importance in explaining the genetic patterns of *U. subfloridana* populations (Degtjarenko et al. 2016, 2018). Overall, our results reveal that, despite extensive gene flow and low genetic differentiation amongst the distant areas, geographical longitude and temperature heterogeneity might promote the current levels of genetic diversity of *U. subfloridana* populations amongst these three remote regions in Estonia.

Recent studies highlighted that microsatellites could be found in coding regions and be linked with adaptation and phenotypic consequences (e.g. Gemayel et al. 2012; Gao et al. 2013). In our study, for the first time any probable correlations between the chemotypes of a lichen-forming fungus, *U. subfloridana* and its microsatellite allele distribution in genotyped data were checked. Both chemotypes of *U. subfloridana* are common in Estonia (Tõrra and Randlane 2007) and nearly evenly represented in study regions (Table 1). The results of this study showed that only a minute difference, according to the presence of thamnolic or squamatic acid, was visible (Fig. 3) in sampled specimens, representing a rather homogenous cluster in the PCA ordination plot (Fig. 3). Recently, Lagostina et al. (2018) showed that chemotypes were not correlated with any of the genetic clusters of the two morphotypes, *U. antarctica* Du Rietz and *U. auranticoatra* (Jacq.) Bory, in the study delimiting species within the genus *Usnea* subgenus *Neuropogon*. However, the chemical variation across these species was not randomly distributed between the two morphotypes nor amongst the genetic clusters (Lagostina et al. 2018). The presence of particular secondary compounds is also known to be specific to a certain habitat or ecoregion and to respond to the environmental gradients (e.g. Nash and Zavada 1977; Culberson et al. 1977; Zhou et al. 2006). We speculate that the correlation between particular microsatellite alleles and secondary metabolite could be relevant to the adaptation of populations to a certain geographical region and contribute thus to the minute isolation by geographical distance.

**Conclusion**

We studied the population genetics of *U. subfloridana*, a widespread lichenised fungus, concentrating on a comparison of genetic variation of populations amongst three geographically remote and disconnected (by sea) regions in Estonia. We recorded a very low genetic differentiation of *U. subfloridana* populations amongst three distant areas, suggesting spatially unrestricted dispersal of individuals and unconstrained gene flow in *U. subfloridana* populations. Furthermore, geographical longitude and the mean annual temperature might play an important role in forming genetic variation in *U. subfloridana* populations in Estonia. This work contributes to the existing knowledge of population genetics of highly clonal and complex organisms, such as lichens.
Acknowledgements

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References


Microsatellite based genetic diversity of the widespread epiphytic lichen *Usnea subfloridana*


**Supplementary material 1**

Authors: Polina Degtjarenko, Inga Jüriado, Tiina Mandel, Tiiu Törra, Andres Saag, Christoph Scheidegger, Tiina Randlane

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Link: https://doi.org/10.3897/mycokeys.58.36557.suppl1

**Supplementary material 2**

Authors: Polina Degtjarenko, Inga Jüriado, Tiina Mandel, Tiiu Törra, Andres Saag, Christoph Scheidegger, Tiina Randlane

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Phylogeny and species delimitations in the entomopathogenic genus *Beauveria* (Hypocreales, Ascomycota), including the description of *B. peruviensis* sp. nov.

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Abstract

The genus *Beauveria* is considered a cosmopolitan anamorphic and teleomorphic genus of soilborne necrotrophic arthropod-pathogenic fungi that includes ecologically and economically important species. Species identification in *Beauveria* is difficult because of its structural simplicity and the lack of distinctive phenotypic variation. Therefore, the use of multi-locus sequence data is essential to establish robust species boundaries in addition to DNA-based species delimitation methods using genetic distance, coalescent, and genealogical concordance approaches (polyphasic approaches). In this regard, our study used multilocus phylogeny and five DNA-based methods to delimit species in *Beauveria* using three molecular makers. These polyphasic analyses allowed for the delimitation of 20–28 species in *Beauveria*, confirming cryptic diversity in five species (i.e. *B. amorpha*, *B. bassiana*, *B. diapheromeriphila*, and *B. pseudobassiana*) and supporting the description of *B. peruviensis* as a new taxon from northeastern Peru. The other five species were not evaluated as they did not have enough data (i.e. *B. araneola*, *B. gryllotalpidicola*, *B. loeensis*, *B. medogensis*, and *B. rudraprayagi*). Our results demonstrate that the congruence among different methods in a polyphasic approach (e.g. genetic distance and coalescence methods) is more likely to show reliably supported species boundaries. Among the methods applied in this study, genetic distance, coalescent approaches, and multilocus phylogeny are crucial when establishing species boundaries in *Beauveria*.
Keywords
Beauveria, fungal diversity, multi-locus phylogeny, Peru, polyphasic approaches, species delimitation

Introduction

Around 1800, a silkworm disease called “calcine”, “real del segno” or “muscardine” was causing great trouble in Italy and France (Redaelli and Visocchi 1940). Experiments developed by Agostino Bassi in Mariago, Italy showed that a parasitic fungus produced this disease (Redaelli and Visocchi 1940). Balsamo (1835) confirmed this discovery and concluded that the incrustation and white efflorescence, which covered the body of a dead silkworm, were a fungus of the genus Botrytis. He first named this species Botrytis paradoxa Balsamo and later Botrytis bassiana Balsamo (Balsamo 1835). Then, this species was transferred to its own genus and Beauveria Vuillemin was established on the basis of B. bassiana Vuillemin as the type species (Vuillemin 1912).

The genus Beauveria is considered a cosmopolitan genus of soilborne necrotrophic arthropod-pathogenic fungi that includes ecologically and economically important species (Rehner et al. 2011, Kepler et al. 2017, Chen et al. 2018). Morphologically, Beauveria genus have been characterized asexually by having conidiogenous cells arising from short, often one-celled, more or less swollen stalk cells, often in dense clusters, or scattered or in whorls from undifferentiated hyphae; they consist of a globose to fusiform basal part, and a geniculate, denticulate rachis. Conidia one-celled, hyaline, smooth, thin-walled, globose to ellipsoidal (de Hoog 1972). The sexual morphs form stromata solitary, paired or gregarious, unbranched, fleshy texture, fertile area apical, cylindrical to clavate, yellowish to orange; perithecia partially immersed, in longitudinal section oval to ovoid; and asci hyaline with cylindrical and filiform ascospores (Kepler et al. 2017).

Based on the end of dual nomenclature for different morphs of the same fungus in 2011 (McNeill et al. 2012), Kepler et al. (2017) phylogenetically established the genetic boundaries in Cordycipitaceae regardless of life-stage or the associated morphological differences. One of the most significant changes was the recognition of Beauveria as a genus separate from Cordyceps. Although direct links between species of Beauveria and cordyceps-like sexual morphs have been demonstrated from molecular data and culture-based experiments (Shimazu et al. 1988, Li et al. 2001, Huang et al. 2002, Shrestha et al. 2014), their respective type species are not congeneric (Kepler et al. 2017). Thereby, the clade composed of Beauveria currently includes the traditional species known from asexual morphs, as well as several taxa previously described for sexual morphs in Cordyceps (Sanjuan et al. 2014, Kepler et al. 2017).

Initially, Beauveria was delimited based on diagnostic features, and three species were recognized, i.e., B. bassiana, B. brongniartii and B. alba (Limber) Saccas (de Hoog, 1972). New additions were included by de Hoog and Rao (1975), Samson and Evans (1982), Bissett and Widden (1986) and Rehner et al. (2006). Molecular analyses confirmed the monophyly and placement of seven species of Beauveria within Cordycipitaceae (Rehner and Buckley 2005, Sung et al. 2007). More recent molecular studies based on multilocus phylogenetic analysis that included the Bloc nuclear intergenic region, internal
transcribed spacer (ITS), translation elongation factor-1α (TEF), and RNA polymerase II largest subunit (RPB1) and second largest subunit (RPB2) demonstrated that Beauveria is composed of 26 species (Rehner et al. 2011, Sanjuan et al. 2014, Kepler et al. 2017, Chen et al. 2018). These phylogenetic studies also revealed that the most commonly reported species, namely, B. bassiana and B. brongniartii, encompass cryptic lineages with worldwide distributions (Rehner et al. 2006, 2011, Ghikas et al. 2010). Although morphologically distinctive as a genus, species identification in Beauveria, especially in the conidiogenic state, is difficult because of its structural simplicity and lack of distinctive phenotypic variation. Thus, numerous registered mycoinsecticide formulations based on B. bassiana and B. brongniartii that are extensively used for the control of insect pests worldwide (Faria and Wraight 2007) are not likely based on these species (Rehner et al. 2006).

In the Amazonian region, a total of five species have been reported (Rehner et al. 2011, Sanjuan et al. 2014). Two of these species B. acridophila (T. Sanjuan & Franco-Mol.) T. Sanjuan, B. Shrestha, Kepler & Spatafora and B. diapheromeriphila (T. Sanjuan & S. Restrepo) T. Sanjuan, B. Shrestha, Kepler & Spatafora, and a lectotype, namely, B. locustiphila (Henn.) B. Shrestha, Kepler & Spatafora were recently described on the basis of molecular data and their sexual stages were characterized (Sanjuan et al. 2014). Additionally, two species of Beauveria were reported from Peru: B. amorpha Samson & Evans and B. bassiana, but only the former has been confirmed by molecular analysis while the latter is extensively used in coffee rust programs to control the expansion of the coffee borer (Rehner et al. 2011).

Given the problems with species delimitation in fungi using morphology, molecular data are becoming the standard for delimiting species and testing their traditional boundaries (Rehner et al. 2011). The recognition of distinct clades in gene trees as species is likely to be misleading in understanding the evolutionary history of taxa (Lu et al. 2016). Therefore, the use of multi-locus sequence data is essential to establish robust species boundaries (Lumbsch and Leavitt 2011). Most researchers, however, did not carefully examine the species boundaries but simply recognized distinct clades in single-gene trees as separate species (Stewart et al. 2014). Estimating the species tree and species delimitation using genetic distance (e.g. automated barcode gap discovery algorithm, ABGD; and statistical parsimony, SPN), coalescent (e.g. generalized mixed Yule coalescent, GMYC; and Bayesian phylogenetics and phylogeography, BPP), and genealogical concordance (genealogical concordance phylogenetic species recognition, GCPSR) methods have proven very useful and have been used for a range of animal and plant taxa (Liu et al. 2016). These methods have otherwise not been used much in fungi, especially in studies of pathogenic fungi (Millanes et al. 2014, Liu et al. 2015). Therefore, the use of several methodologies and data sets to delimit species is recommended, and subsequently, the achievement of congruent results across the methods is likely to prove most useful for framing reliably supported species boundaries (Carstens et al. 2013).
ods. Three nuclear molecular markers (Bloc, rpb1, and tef1) were used to examine their phylogenetic relationships and to assess species boundaries within the genus Beauveria.

Materials and methods

Collection of specimens and isolation

Fungal strains were isolated from infected coffee borers (Hypothenemus hampei) obtained from infected coffee berries according to Gerónimo-Torres et al. (2016). They were collected during July and August 2017 from three districts in the province of Rodriguez de Mendoza, Amazonas, Peru (Fig. 1). Briefly, infected coffee berries were preserved at 5 °C until coffee borers were recovered from them. The coffee borers with signs of fungal infection were cleaned superficially in 0.5% sodium hypochlorite solution and rinsed with sterile distilled water. Then, insects were placed in a humid chamber (90% RH and 25 °C) for 8 days to allow the growth of the entomopathogenic fungus. Once visible mycelia appeared on the borers under observations with a stereo microscope (Nikon SMZ18, Tokyo, Japan), these were transferred to a Petri-dish containing potato dextrose agar (PDA; Merck, Darmstadt, Germany).

Identification of isolates

Fifty-five fungal strains were incubated as monosporic cultures on PDA at 25 °C for 15 days. Morphological characterization of the fungus was performed as described by Rehner and Buckley (2005). Microscope observations were made from fungal mycelia and other structures stained with methylene blue (0.1–0.5%). Photomicrographs were taken under an inverted microscope (IX83; Olympus, Tokyo, Japan) with an integrated camera (Nikon D810, Tokyo, Japan). Fungal strains were deposited as semisolid and dry material in the herbarium of Toribio Rodriguez de Mendoza National University (UT), Peru.

Molecular phylogenetic analyses

Genomic DNA was extracted from semisolid PDA cultures using the NucleoSpin Plant II Kit (Macherey-Nagel, Düren, Germany), following the manufacturer’s instructions. Three genes were sequenced, i.e., Bloc, rpb1, and tef1. Each gene was amplified using polymerase chain reaction (PCR) with MasterMix (Promega, Wisconsin, USA) in the following reaction mixture: 10 ng of DNA and 0.25–0.5 pmol of forward and reverse primers for a total volume of 10 µl. The PCR protocols and primer combinations for Bloc (B5.1F, B5.4F, B3.1R, B3.3R), rpb1(RPB1A, RPB1A_VH6R, RPB1B_VH6Fa, RPB1B_G2R), and tef1 (983F, 1567RintB) followed Rehner et al. (2011). The se-
Phylogeny and species delimitations of *Beauveria*

Figure 1. Collections of the 55 strains of *B. amazonensis* sp. nov. from the Rodriguez de Mendoza Province.

Sequences of the forward and reverse strands were determined commercially by Macrogen Inc. (Macrogen, Seoul, Korea). New *Bloc*, *rpb1*, and *tef1* sequences were deposited in GenBank (Table 1). These sequences and others obtained from GenBank were initially aligned with Muscle algorithms (Thompson et al. 1994) and were adjusted manually with MEGA6 software (Tamura et al. 2013).
## Table 1. List of species used in the molecular analyses.

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The phylogeny was based on concatenated data combining *Bloc*, *rpb1*, and *tef1* (101 sequences, Table 1). Selection of the best-fitting nucleotide substitution model was conducted using the program PartitionFinder (Lanfear et al. 2012) with three partitions (*Bloc*, *rpb1*, and *tef1*). The best partition strategy and model of sequence evolution were selected based on the Bayesian Information Criterion (BIC). The general time reversible nucleotide substitution model with a gamma distribution and a proportion of invariable sites (GTR + Γ + I) was selected for all partitions. Maximum likelihood (ML) analyses were conducted with the RAxML HPC-AVX program (Stamatakis 2014) implemented in the raxmlGUI 1.3.1 interface (Silvestro and Michalak 2012) using a GTRGAMMAI model with 1000 bootstrap replications. Bayesian inference (BI) was performed with MrBayes v. 3.2.6 software (Ronquist et al. 2012) using Metropolis-coupled MCMC and the GTR + Γ + I model. We conducted two runs each with four chains (three hot and one cold) for 10,000,000 generations, sampling trees every 1,000 generations. We plotted likelihood vs. generation using the Tracer Version v. 1.6 program (Rambaut et al. 2014) to reach a likelihood plateau and set the burn-in value.

DNA-based species delimitation

Although 26 species have been molecularly confirmed in *Beauveria* (Rehner et al. 2011, Kepler et al. 2017, Chen et al. 2018), only 21 of these species and *Beauveria* sp. from Peru were used in the DNA-based delimitation methods. *Beauveria araneola* W.H. Chen, Y.F. Han, Z.Q. Liang & D.C. Jin, *B. gryllotalpidicola* Luangsa-ard, Ridkaew & Tasanathi, *B. loeiensis* Luangsa-ard, Ridkaew & Tasanathi, *B. medogensis* Imoulan & Y.J. Yao, and *B. rudraprayagi* Y. Agrawal, P. Mual & B.D. Shenoy were not used due to abundant missing data and short sequences for the three markers (e.g. ~731 bp for *rpb1* and ~720 bp for *tef1*).

We explored five different DNA-based delimitation methods using *Bloc*, *rpb1*, and *tef1* data sets to assess species boundaries in *Beauveria*. Although *B. acridophila*, *B. blattidicola* M. Chen, Aime, T.W. Henkel & Spatafora, *B. diapheromeriphila*, *B. locustiphila*, and *B. staphylinidicola* (Kobayasi & Shimizu) B. Shrestha, Kepler & Spatafora lack *Bloc* sequences, these species were used in the analysis to evaluate its status in the new circumscribed *Beauveria*. Two of these DNA-based delimitation methods are based on genetic distance [statistical parsimony network analysis (SPN) (Hart and Sunday 2007) and automatic barcoding gap detection (ABGD) (Puillandre et al. 2012)], two in coalescence [generalized mixed Yule coalescent method (GMYC) (Pons et al. 2006) and Bayesian phylogenetics and phylogeography (BPP) (Rannala and Yang 2003)], and one in genealogical concordance [genealogical concordance phylogenetic species recognition (GCP-SR) (Quaedvlieg et al. 2014)]. For the SPN analyses of *Bloc*, *rpb1*, and *tef1*, data sets were generated in TCS 1.21 (Clement et al. 2000) with a maximum connection probability set at 95% statistical confidence. The ABGD method was tested via a web interface (ABGD web, http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html). Before analysis, the model criteria were set as follows: variability (P) between 0.001 (Pmin) and 0.1 (Pmax), minimum gap width (X) of 0.1, Kimura-2-parameters and 50 screening steps.
To perform the GMYC delimitation method, an ultrametric tree was constructed in BEAST v.2.0.2 (Drummond et al. 2012), relying on the uncorrelated lognormal relaxed clock, the GTR + Γ + I model, and a coalescent tree prior. Bayesian Markov chain Monte Carlo was run for 50 million generations, and trees and parameters were sampled every 1000 generations. Log files were visualized in Tracer v.1.6 (Rambaut et al. 2014) for assessing the stationary state of parameters on the basis of the value of estimate-effective sample size (ESS). After removing 25% of trees as burn-in, the remaining trees were used to generate a single summarized tree in TreeAnnotator v.2.0.2 (part of the BEAST v.2.0.2 package) as an input file for GMYC analyses. The GMYC analyses with a single threshold model were performed in R (R Development Core Team, http://www.R-project.org) under the ‘splits’ package using the ‘gmyc’ function (R-Forge, http://r-forge.r-project.org/projects/splits/).

To validate the outcomes of single locus species delimitation, a multilocus BPP was applied using the program BP&P v.2.0 (Rannala and Yang 2003, 2003, 2003 and Rannala 2010, Liu et al. 2015). The three-gene data (Bloc, rpb1, and tef1) were used as input for BPP under the A11 model (A11: species delimitation = 1, species tree = 1). Specimens were a priori assigned to species based only on the minimum number of species from the results of the phylogenetic analysis. The guide tree derived from the three-gene ML analysis was used. Five variables (ε1~ε5) were automatically fine-tuned following the instructions of BP&P (Rannala and Yang 2003, 2003 and Rannala 2010). The prior distribution of θ and τ could have influenced the posterior probabilities for different models (Yang and Rannala 2010). Analyses were run with three different prior combinations (Leaché and Fujita 2010). Each analysis was run three times to confirm consistency between runs. Two independent MCMC analyses were run for 100,000 generations with the ‘burn-in’ = 20,000.

GCPSR was implemented by identifying independent evolutionary lineages (IELs) and by exhaustive subdivision of strains into phylogenetic species. The criteria used to identify IELs and exhaustive subdivision were the same as those used by Brankovics et al. (2018). These were implemented using Perl scripts developed by Brankovics et al. (2018) and available at GitHub (https://github.com/b-brankovics/GCPSR).

Results
Molecular phylogeny

In the phylogeny of Beauveria species, the analyzed data matrix included 1592 base pairs (bp) for Bloc, 2890 bp rpb1, and 1181 bp for tef1 of 101 individuals. Phylogenetic trees obtained from ML and BI analyses confirmed the robustly supported monophyly of the genus Beauveria (Fig. 2). The tree topologies for the individual genes (tef1, Bloc, and rpb1) did not show congruence (Suppl. material 1: Figs S1–S3). These trees showed topological differences, especially in the clades composed of B. asiatica / B. majiangensis and by B. bassiana / B. staphylinidicola / Beauveria sp. from Peru. Although the individual gene trees did not show congruence with the combined data, the latter resolved these clades, suggesting conspecificity in the first clade and sister relationship
Figure 2. Phylogenetic tree based on maximum likelihood inference of combined Bloc, RPB1, Tef1 data. Value above branches = Maximum likelihood bootstrap values (BS) / Bayesian posterior probabilities. Grey bars represent species delimitation results from ABGD-, SPN-, GMYC- and BPP based algorithmic methods based on Bloc, RPB1, and Tef1 sequences. Scale bar indicates the number of nucleotide substitution per site. a: delimited as the same species. B. araneola, B. gryllotalpidicola, B. loeiensis, B. medogensis, and B. rudraprayagi were not delimited by any DNA-based algorithm due to abundant missing data in their sequences.

The genetic divergence comparisons showed that the minimum threshold (p-distance) to distinguish genetic species in Beauveria was 1.3%, 0.4%, and 0.2% for Bloc, rpb1, and tef1, respectively, as occurred between B. australis and B. asiatica. (Table 2).
Phylogeny and species delimitations of *Beauveria*

**Table 2.** Genetic distance (p-distances) in percentage for species of *Beauveria* for three markers.

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**Table 3.** Species number in *Beauveria* identified under DNA-based species-delimitations methods and phylogeny.

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<tr>
<td><em>B. diapheromeriphi</em></td>
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<td>1</td>
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<td><em>B. hoplocheli</em></td>
<td>1 1 1 8 1 1 8 1 1 1 1 1 x 1 1 1 1 1</td>
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<td><em>B. kipukae</em></td>
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<tr>
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<tr>
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<tr>
<td><em>B. malawiensis</em></td>
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<tr>
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<tr>
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<td><em>B. sinensis</em></td>
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<tr>
<td><em>B. staphylinidicola</em></td>
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<tr>
<td><em>B. varroae</em></td>
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<tr>
<td><em>B. vermiconia</em></td>
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<tr>
<td><em>B. peruvianis</em></td>
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</tr>
<tr>
<td><strong>Total</strong></td>
<td>20 22 28 35 16 3 63 28*** 26*** 16* 1 22</td>
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</tbody>
</table>

x = non recognized as species, - = not evaluated, * = posterior probabilities higher or equal than 0.53, *** = highly significant

Species delimitation

The species-delimitation methods based on genetic distance (ABGD, SPN), coalescence (GMYC, BPP), and genealogical concordance (GCPSR) showed incongruent results for the three genes (Fig. 2, Table 3). Among these methods, the highest number of species was delimited in the GMYC analysis for the *Bloc* gene, whereas conservative results were observed in BPP. The species delimitations by SPN and GCPSR have inadequate and contradictory results. The genetic distance method based on the barcode gap (ABGD) found similar species numbers for *Bloc*, *rpb1*, and *tef1*, differing
only in the species recognized in the clades *B. asiatica* / *B. majiangensis* and *B. bassiana* / *B. staphylinidicola* / Beauveria sp. from Peru. In the former clade, there were 3, 1 and 2 species for *Bloc*, *rpb1*, and *tef1*; whereas in the latter clade, there were 7, 1, and 5 species for *Bloc*, *rpb1*, and *tef1*. The GMYC identified relatively conserved results in *RPB1* (28) and *tef1* (26) and plenty of species in the *Bloc* data set (63). This high number of species for the *Bloc* data set is a consequence of the splitting of the main clades into different species but lacking significance (Suppl. material 1: Table S1, Fig. S4). Regarding the multi-locus coalescent species validation (BPP), the highest posterior probabilities for *Bloc*, *rpb1*, and *tef1* were found by recognizing 16 species based on the results from the phylogenetic analysis and single species delimitation methods (Suppl. material 1: Table S2). Conversely, the BPP analyses with the maximum number of species (39 and 62) were not used based on the inadequate results from SPN and GCPSR. Although there were incongruent results among different methods, the conservative results from species delimitation methods (ABGD and GMYC) and phylogenetic analysis suggest that *Beauveria* is composed of 20–28 and 26 species, respectively. These results also suggest that the clade composed of *B. asiatica* / *B. majiangensis*, *B. diapheromeriphila*, and *B. bassiana* / *B. staphylinidicola* / Beauveria sp. from Peru were genetically composed of more than one species. Our analysis also revealed that *Beauveria* sp. from Peru was supported as a distinct species by ABGD (*Bloc* gene), GMYC, BPP, and phylogeny. Thereby, the description of *Beauveria* sp. as a new species is proposed.

Morphological observations

*Beauveria peruviensis* D.E.Bustamante, M.S.Calderon, M.Oliva, S.Leiva, sp. nov.
MycoBank No: 829032

Fig. 3

**Diagnosis.** Species very similar morphologically to *Beauveria bassiana*, but differing in the sister phylogenetic relationship with this species (Fig. 2). The sequence divergence between *B. peruviensis* and *B. bassiana* is 3.5–4.1% for *Bloc*, 0.3–0.5% for *rpb1*, and 0.2–0.4% for *tef1*. *B. peruviensis* is occurring in coffee plantations located in the middle altitudes of the Amazon region of Peru.


**Description.** Colony growth on PDA, 15–38 mm diam. after 15 d at 25 C, 1.4–1.9 daily rate of radial growth, velutinous and closely appressed to agar surface, up to 3.5 mm thick, white, changing to yellowish white in older sections of the colony. Conidia aggregated as ca. 0.1 mm spherical clusters and white in mass. Colony reverse colorless or yellowish white to grayish white. Odor indistinct. Vegetative hyphae septate, branched, hyaline, smooth-walled, 1–1.5 µm wide. Conidiogenous cells, phialidic, solitary or occurring in dense lateral clusters, base subsphaerical, 3–6
Figure 3. Morphology of *Beauveria amazonensis*. A, B Colony growth on PDA showing the habit C–F conidiogenous cells and conidia.

μm wide, sympodially branched neck tapering into a long slender denticulate rachis, geniculate or irregularly bent, 2.0–3.5 × 1.5–2.5 μm. Conidia, 2–3 × 1–3 μm, Q = 1.0–1.8 (Lₘ = 2.5 μm, Wₘ = 2.2 μm, Qₘ = 1.6), mainly globose, slightly ellipsoid, oblong or cylindrical, hyaline, aseptate, walls smooth and thin. Mycelium on the host is granular-pulverulent, sometimes funiculose or rarely producing synnemata, white, rarely yellowish. Hyphae of the aerial mycelium bearing a conidial apparatus as described above. Basal parts of the conidiogenous cells globose, subglobose or somewhat flask-shaped.

**Distribution.** This species is widely spread on coffee plantations in the middle altitudes of the Amazon region in northeastern Peru.

**Ecology.** *B. peruviensis* was isolated from coffee borers (*Hypothenemus hampei*) obtained from coffee grains. Only the asexual stage was found.

**Etymology.** The specific epithet ‘*peruviensis*’ is derived from the country where the samples were collected.

Notes. Beauveria peruviensis is practically indistinguishable in morphology to other Beauveria species. The shape and size of the conidia and the colony color of B. peruviensis among other morphological features have been observed in B. bassiana, B. kipukae, B. pseudobassiana, and B. varroae (Rehner et al. 2011). The lack of diagnostic morphological features to distinguish Beauveria peruviensis was overcome by delimiting this species with DNA-based methodologies.

Discussion

Accurate species identification within the entomopathogenic fungi Beauveria is crucial for disease control and prevention (Lu et al. 2016). This genus has recently been circumscribed, and its taxonomy has been updated with new combinations and the description of new species based mainly on multi-locus phylogenies in the absence of diagnostic features that delimit species (Sanjuan et al. 2014, Shrestha et al. 2014, Kepler et al. 2017, Chen et al. 2017, 2018). In addition to phylogenies, other methodologies and data sets to delimit species are recommended to establish well-supported boundaries among species (Carstens et al. 2013) because most researchers simply recognize distinct clades in either single- or multi-locus trees as species (Stewart et al. 2014). In this regard, our study used phylogeny and five DNA-based methods to delimit species in Beauveria using three molecular makers. Although incongruence among some of these methods was observed in our analyses, a genetic distance (ABGD), a coalescence method (BPP), and the multilocus phylogeny strongly supported 20–28 different species, including the new species B. peruviensis from Peru.
The use of multi-locus sequence data is essential to establish robust species boundaries (Lumbsch and Levitt 2011), and our results for *Beauveria* showed well-supported clades, although it resulted in incongruence to the single locus phylogenies (Suppl. material 1: Figs S1–S3). This conflict can be a result of incomplete lineage sorting, horizontal gene transfer, gene duplication and loss, hybridization, or recombination (Degnan and Rosenberg 2009). This study cannot determine which of these scenarios are occurring in *Beauveria*; nevertheless, it serves as a baseline for investigating causes of gene tree discordance that can be identified by further analyses at the genomic level (Patterson et al. 2006, Lu et al. 2016). According to our multilocus phylogeny, 22 of the 26 molecularly confirmed species in *Beauveria* were recognized. Previous studies have delimited *B. araneola*, *B. gryllotalpidicola*, *B. loeiensis*, *B. medogensis*, and *B. rudraprayagi* as valid species on the basis of their phylogenies (Agrawal et al. 2014, Imoulan et al. 2016, Chen et al. 2017); however, our study did not include these sequences because they have abundant missing data, and thus, their status was not evaluated. These species would require further revision to be recognized as supported lineage within the genus *Beauveria*.

Regarding the genetic distance methods, the ABGD showed similar results when delimiting *Beauveria* species to those from the multilocus phylogeny. The additional putative species in ABGD is mainly due to the split of *B. bassiana*. This confirms that *B. bassiana* encompasses cryptic lineages as proposed initially by Rehner et al. (2011). Therefore, the original *B. bassiana* should be the clade that includes the specimen from the type locality, namely, Italy (Vuillemin 1912). Additionally, these results delimited *B. majiangensis* and *B. asiatica* as different lineages, although the multilocus analysis showed low support. *B. majiangensis* needs further analysis with additional and longer sequences to confirm its status because one or only a few individuals often fail to represent the species as a whole (Davis and Nixon 1992, Walsh 2000). On the other hand, the SPN method showed conflicting results among the *Bloc*, *RPB1*, and *tef1* loci, leading to incorrect inferences. The number of species inferred by SPN greatly matched the number of Linnaean species in mitochondrial markers (e.g., COI) (Hart and Sunday 2007). Therefore, our nuclear markers due to indels might generate many reticulations that allow inadequate species delimitation in our data (Paradis 2018).

In the coalescence methods, although 6 species were not included in the BPP analysis due to the lack of their *Bloc* sequences, this method supports the conservative results obtained from the multilocus phylogeny. BPP supported the status of 16 species (posterior probabilities higher than 0.52), which are not high supportive, but these probabilities are not supportive at all when splitting or merging species in the BPP analysis (Suppl. material 1: Table S2). Zhang et al. (2011) found that the correct species model was inferred with a high posterior probability with only one or two loci when 5 or 10 sequences were sampled from each population or with 50 loci when only one sequence was sampled, and they also demonstrated that the migration rate might affect these results. This suggests that further analysis might need to increase the number of sequences per locus among different populations of species of *Beauveria* and assess their migration rate to obtain supportive delimitations. Moreover, the highly
significant results obtained from the GMYC method for the \textit{tef1} and \textit{rpb1} loci partially support the ABGD and multilocus analyses. The additional number of putative species in the GMYC analyses, as occurred with the ABGD, is due to the presence of more than one lineage in \textit{B. amorpha}, \textit{B. bassiana}, \textit{B. diapheromeriphila}, and \textit{B. pseudobassiana} confirming cryptic diversity (Rehner et al. 2011). The performance in empirical studies of the ABGD and GMYC tends to under- and oversplit species, respectively (Luo et al. 2018). However, our results suggest that GMYC and ABGD are appropriate for determining cryptic diversity in \textit{Beauveria} by splitting well-supported clades from the multi-locus phylogeny.

Regarding \textit{B. peruviensis}, ABGD (Bloc), SPN (Bloc), GMYC, BPP, and the phylogenetic analyses support this species as a different lineage from \textit{B. bassiana} and \textit{B. staphylinidicola}. Additionally, the genetic divergence between \textit{B. peruviensis} and these species is higher than the minimum threshold observed in species of \textit{Beauveria} (Table 2). In our study, \textit{Beauveria peruviensis} showed morphological indistinctiveness to other \textit{Beauveria} species that produce globose/subglobose/ellipsoid conidia. Additionally, \textit{B. peruviensis} conidia is also similar in size to other \textit{Beauveria}, especially \textit{B. bassiana}. Previously, Rehner et al. (2011) noted that \textit{B. bassiana} is hardly distinguishable from other species of \textit{Beauveria}. The lack of diagnostic morphological features to delimit species in \textit{Beauveria} was overcome by the application of molecular methods in fungal taxonomy. The segregation of \textit{B. peruviensis} from \textit{B. bassiana} and \textit{B. staphylinidicola} confirmed that phylogenetic diversity and DNA-species delimitation methods discover taxa within morphologically defined species (Goldstein et al. 2000, Lu et al. 2016). Ecologically, the segregation of \textit{B. peruviensis} from \textit{B. bassiana} and \textit{B. staphylinidicola} is supported by the specificity of \textit{B. peruviensis} to the coffee borer from Amazon and the well-supported lineage in the phylogenetic analysis that might indicate the presence of a barrier in gene flow in nature (Van Valen 1976, Lu et al. 2016).

Recently, polyphasic approaches have been used to reflect the natural classification of species within many important fungal genera (Aveskamp et al. 2010, Milic et al. 2012, Lu et al. 2016). These approaches frequently incorporate morphological and phylogenetic analyses and metabolomics, but few of them use genetic distance and coalescent methods (Lu et al. 2016). The use of polyphasic analysis, including DNA-based delimitation methods, allowed the establishment of boundaries among species of morphologically conserved genera such as \textit{Beauveria} and thus provided support for the description of new taxa (e.g., \textit{B. peruviensis}) or validated the taxonomic uncertain of others (e.g., \textit{B. majiangensis}). Although more recent methods avoid arbitrary cut-offs (Knowles and Carstens 2007), our results demonstrate that the congruence among this method and other methods used in a polyphasic approach (e.g., genetic distance, coalescence methods) are more likely to prove reliably supported species boundaries (Carstens et al. 2013). Among the methods applied in this study, ABGD, GMYC, BPP, and multilocus phylogeny are crucial when establishing species boundaries in \textit{Beauveria}. 

Phylogeny and species delimitations of \textit{Beauveria} 63
Acknowledgements

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Phylogeny and species delimitations of *Beauveria*


Supplementary material I

Tables S1, S2, Figures S1–S4
Authors: Danilo E. Bustamante, Manuel Oliva, Santos Leiva, Jani E. Mendoza, Leidy Bobadilla, Geysen Angulo, Martha S. Calderon
Data type: molecular data
Explanation note: Table S1. Results of the Generalized Mixed Yule-Coalescent (GMYC) analyses under the single threshold model. Table S2. Highest posterior probabilities of the three-gene Bayesian species delimitation analysis (BPP) by jointing species delimitation and species tree inference. Figure S1. Phylogenetic tree based on maximum likelihood inference of combined *Bloc* data. Figure S2. Phylogenetic tree based on maximum likelihood inference of combined *RPB1* data. Figure S3. Phylogenetic tree based on maximum likelihood inference of combined *Tef1* data. Figure S4. Bayesian inference ultrametric gene tree.
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Link: https://doi.org/10.3897/mycokeys.58.35764.suppl1


**Clitopilus lampangensis (Agaricales, Entolomataceae), a new species from northern Thailand**

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**Abstract**

A new species of agaricomycetes, *Clitopilus lampangensis*, is described based on collections from northern Thailand. This species was distinguished from previously described *Clitopilus* species by its pale yellow to grayish yellow pileus with the presence of wider caulocystidia. Molecular phylogenetic analyses, based on the data of the internal transcribed spacers (ITS) and the large subunit (LSU) of the nuclear ribosomal DNA, and the second largest subunit of RNA polymerase II (*rpb2*) genes, also support the finding that *C. lampangensis* is distinct from other species within the genus *Clitopilus*. A full description, color photographs, illustrations and a phylogenetic tree showing the position of *C. lampangensis* are provided.

**Keywords**

Agaricomycetes, gill mushroom, morphology, phylogeny, tropics

**Introduction**

The genus *Clitopilus* was proposed by Kummer (1987) with *C. prunulus* (Scop.) P. Kummer as the type species. It belongs to the family Entolomataceae of the order Agaricales. This genus is saprotrophic and is widely distributed, especially in northern temperate areas (Singer 1986; Baroni and Watling 1999; Moncalvo et al. 2002; Kirk et al.)
2008; Hartley et al. 2009; Crous et al. 2012; Raj and Manimohan 2018). *Clitopilus* is characterized by basidiocarps that are clitocyboid, omphalinoid or pleurotoid, mostly whitish or occasionally grayish or brownish in color, with pink or pinkish brown spore prints, ellipsoid basidiospores with longitudinal ridges that appear angular in a polar view, and hyphae lack clamp connections (Singer 1986; Noordeloos 1988). There are 30 species of *Clitopilus* worldwide (Kirk et al. 2008), although there are 201 species names recorded in the Index Fungorum (http://www.indexfungorum.org/Names/Names.asp). The taxa list in the Index Fungorum includes synonyms and misidentifications, as well as some species that are not well documented. Formerly, the genus *Clitopilus* included *Rhodocybe* (Moncalvo et al. 2002; Co-David et al. 2009; Vizzini et al. 2011a). However, molecular phylogenetic analyses have provided powerful tools for the identification of *Clitopilus*, leading to the separation of *Clitopilus* from *Rhodocybe* as well as the related genera (*Clitocella* and *Clitopilopsis*) (Cooper 2014; Kluting et al. 2014; Raj and Manimohan 2018).

Only six species, *Clitopilus apalus* (Berk. & Br.) Petch, *C. crispus* Pat. *C. doimaesalongensis* Jatwong, Karun. & K.D. Hyde, *C. chalybescens* T.J. Baroni & Desjardin, *C. peri* (Berk. & Br.) Petch and *C. prunulus*, have been reported in Thailand (Baroni et al. 2001; Chandrasrikul et al. 2011; Kluting et al. 2014; Jatwong et al. 2017). During an investigation of macrofungi in northern Thailand, we found a population of *Clitopilus* which we describe here as a new species based on the morphological and molecular characteristics. To confirm its taxonomic status, the phylogenetic relationship of the new species was determined by the ITS and LSU of the rDNA, and the rbp2 genes.

**Materials and methods**

**Sample collection**

Basidiocarps were collected in Mae Moh District, Lampang Province, northern Thailand in 2018. Basidiocarps were wrapped in aluminum foil and kept in plastic specimen boxes to be transported to the laboratory. Notes on the macromorphological features and photographs were obtained within 24 h of collection. The specimens were dried at 40–45 °C and deposited at the Herbarium of the Sustainable Development of Biological Resources Laboratory, Faculty of Science, Chiang Mai University (SDBR-CMU), and BIOTEC Bangkok Herbarium (BBH), Pathumthani, Thailand.

**Morphological studies**

Macromorphological data were recorded from fresh specimens. The recording of color names and codes followed Kornerup and Wanscher (1978). Micromorphological data were recorded from dry specimens rehydrated in 95% ethanol followed by distilled water, 3% KOH or Melzer’s reagent. Anatomical features were based on at least 50
measurements of each structure as seen under a light microscope (Olympus CX51, Japan). For spore statistics, \( Q \) is the ratio of spore length divided by spore width and \( \bar{Q} \) is the average \( Q \) of all specimens ± standard deviation.

**Molecular phylogenetic studies**

Genomic DNA of dry specimens (1–10 mg) was extracted using a Genomic DNA Extraction Mini-Kit (FAVORGEN, Taiwan). The ITS region of DNA was amplified by polymerase chain reactions (PCR) using ITS4 and ITS5 primers (White et al. 1990), the LSU of rDNA gene were amplified with LROR and LRO5 primers (Vilgalys and Hester 1990), and \( rpb2 \) gene was amplified with the bRBP2-6F and bRBP2-7.1R primers (Matheny 2005). The amplification program for these three domains was performed in separated PCR reaction and consisted of an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s (ITS), 52 °C for 45 s (LSU), and 54 °C for 1 min (\( rpb2 \)), and extension at 72 °C for 1 min on a peqSTAR thermal cycler (PEQLAB Ltd., UK). PCR products were checked on 1 % agarose gels stained with ethidium bromide under UV light. PCR products were purified using a PCR clean up Gel Extraction NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Germany) following the manufacturer's protocol. The purified PCR products were directly sequenced. Sequencing reactions were performed and the sequences were automatically determined in the genetic analyzer at 1st Base company (Kembangan, Malaysia) using the PCR primers mentioned above. Sequences were used to query GenBank via BLAST (http://blast.ddbj.nig.ac.jp/top-e.html).

For phylogenetic analyses, the sequences from this study, previous studies and the GenBank database were used and provided in Table 1. The multiple sequence alignment was carried out using MUSCLE (Edgar 2004), and the combined ITS and LSU alignment, and \( rpb2 \) alignment were deposited in TreeBASE under the study ID 24373 and 24374, respectively. Phylogenetic trees were constructed using maximum likelihood (ML) and Bayesian inference (BI) algorithms, implemented by RAxML v7.0.3 (Stamatakis 2006) and MrBayes v3.2.6 (Ronquist et al. 2012), respectively. *Rhodocybe griseoaurantia* and *R. pallidogrisea* were used as outgroup. The best-fit substitution model for BI and ML analyses were estimated by jModeltest 2.1.10 (Darriba et al. 2012) using Akaike information criterion (AIC). For ML analysis, the bootstrap (BS) replicates were set as 1000 and used to test phylogeny (Felsenstein 1985). Clades with bootstrap values (BS) of ≥ 70% were considered significantly supported (Hillis and Bull 1993). For the BI analysis, the Markov chains were run for one million generations, with six chains and random starting trees. The chains were sampled every 100 generations. Among these, the first 2,000 trees were discarded as burn-in, while the postburn-in trees were used to construct the 50% majority-rule consensus phylogram with calculated Bayesian posterior probabilities. Bayesian posterior probabilities (PP) ≥ 0.95 were considered significant support (Alfaro et al. 2003).
### Table 1. Sequences used for phylogenetic analysis. The newly generated sequences are in bold.

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Results

Phylogenetic analyses

The topology of each single-gene of ITS and LSU, and the combined ITS and LSU phylagrams were found to be similar. However, differences were observed in the topology of the \textit{rbp2} gene. Therefore, we present only the combined ITS and LSU gene phylagram (Fig. 1), and the single \textit{rbp2} gene phylagram (Fig. 2). The combined ITS and LSU sequence dataset consisted of 34 taxa and were comprised of 1774 characters including gaps (ITS: 1–779, LSU: 780–1774). The sequence dataset of \textit{rbp2} consisted of 27 taxa and the aligned dataset was comprised of 620 characters that included gaps. The GTR model with gamma rate heterogeneity and invariant sites (GTR+G+I) was the best-fit model used for both ML and BI analyses that were selected by AIC. The average standard deviation of the split frequencies fell to 0.011364 and 0.009837 in the BI analysis of the combined ITS and LSU, and \textit{rbp2} sequences, respectively after one million generations. This was observed after the 50\% majority consensus phylogram was constructed. The ML analysis of the combined ITS and LSU sequences was based on the parameters estimated under the GTR+I+G model, and the proportion of the invariable sites and the gamma shape parameters were 0.0250 and 0.9320, respectively. Additionally, the tree with log likelihood (-8211.7515) was built after 1000 bootstrapping replications. In the ML analysis of the \textit{rbp2} sequence that was based on the GTR+I+G model, the proportion of the invariable sites and the gamma shape parameters were 0.5400 and 1.7960, respectively, while the tree with log likelihood (-3640.1616) was built after 1000 bootstrapping replications.

Both the combined ITS and LSU, and the \textit{rbp2} phylograms indicated that the sequences were of a new species, \textit{C. lampangensis}, that had formed a monophyletic clade with high BS (100 \%) and PP (1.0) support (Figs 1, 2). A combined ITS and LSU phylogram revealed that the new species was a sister taxon to \textit{C. chalybescens}. In addition, the \textit{rbp2} phylogram indicated that the new species was a sister taxon to \textit{C. chalybescens} and \textit{C. peri}.

Taxonomy

\textit{Clitopilus lampangensis} J. Kumla, N. Suwannarach & S. Lumyong, sp. nov.
MycoBank No.: 830890
Fig. 3

\textbf{Diagnosis.} Distinguished from other \textit{Clitopilus} species by its pale yellow to grayish yellow pileus with the presence of caulocystidia, and from \textit{C. chalybescens} by its wider caulocystidia, longer basidiospores, and lack of grayish blue color change on the pileus and stipe when bruised.

\textbf{Etymology.} ‘lampangensis’, referring to Lampang Province, where the holotype was found.
Figure 1. Phylogram derived from maximum likelihood analysis of the combined ITS and LSU region of nuclear rDNA of 34 sequences. *Rhodocybe griseoaurantia* and *R. pallidogrisea* were used as outgroup. The numbers above branches represent maximum likelihood bootstrap percentages (left) and Bayesian posterior probabilities (right). Only bootstrap values ≥ 50 % are shown, and the scale bar represents ten substitutions per nucleotide position. The fungal species obtained in this study are in bold.
A new *Clitopilus* species from Thailand

**Figure 2.** Phylogram derived from maximum likelihood analysis of *rpb2* gene of 27 sequences. *Rhodocybe griseoaurantia* and *R. pallidogrisea* were used as outgroup. The numbers above branches represent maximum likelihood bootstrap percentages (left) and Bayesian posterior probabilities (right). Only bootstrap values ≥ 50% are shown, and the scale bar represents ten substitutions per nucleotide position. The fungal species obtained in this study are in bold.

**Holotype.** THAILAND, Lampang Province, Mae Moh District, (18°24'21"N, 99°42'26"E, elevation 380 m), on ground in a tropical deciduous forest, May, 2018, J. Kumla & N. Suwannarach, SDBR-CMUJK 0147 and BBH 43590 (isotype).

**Gene sequence (from holotype).** MK764933 (ITS), MK764935 (LSU) and MK784127 (*rpb2*).
Basidiocarps small, clitocyboid. Pileus 35–50 mm diam., initially convex or somewhat plano-convex with or without a central depression, becoming deeply umbilicate with age; surface pale yellow (4A3) to greyish yellow (4B5), somewhat velutinous, finely pruinose all over; margin incurved to slightly inrolled, entire or slightly wavy. Lamellae subdecurrent to decurrent, white (1A1), crowded, up to 2.5 mm wide, with lamellulae of 1–3 lengths; edge entire or slightly wavy, concolorous with the sides. Stipe 20–25 × 5–8 mm, central, solid; surface white (1A1) to yellowish white (4A2),
A new Clitopilus species from Thailand

Finely pruinose all over, densely so towards the apex; base with white cottony mycelium. Odor strong farinaceous. A pale pinkish spore print.

Basidiospores 7.0–9.0 × 3.0–5.0 µm, Q = 1.40–2.33, Q = 1.82 ± 0.27, ellipsoid in polar view, amygdaliform to limoniform in side view, with 6–8 prominent longitudinal ridges, colorless, thin-walled. Basidia 17.0–25.0 × 4.0–8.0 µm, clavate, colorless, thin-walled, 2- and 4-spored; sterigmata up to 4 µm long. Lamella-edge fertile. Pleurocystidia and cheilocystidia absent. Lamellar trama subregular; hyphae 2.5–4.0 µm wide, hyaline, thin-walled. Pileus trama compact, hyaline, cylindrical hyphae 5–10 µm wide. Pileipellis a cutis of loosely interwoven hyphae; 3–5 µm wide, hyaline, thin-walled, and terminal cells; subcylindric or narrowly clavate, 4–8 µm wide. Stipitipellis at stipe apex a layer of repent, hyaline, cylindrical hyphae 4–8 µm wide, thin-walled. Caulocystidia 25.5–42.5 × 8.0–15.0 µm, single or clustered, erect or repent, varying in shape from cylindrical to clavate, hyaline, slightly thick-walled. Clamp connections absent in all tissues.

Ecology and distribution. Fruiting solitary or gregarious on soil in a tropical deciduous forest. Known only from northern Thailand

Specimens examined. THAILAND, Lampang Province, Mae Moh District, (18°24’20”N, 99°42’3”E, elevation 375 m), on ground in a tropical deciduous forest, May, 2018, N. Suwannarach & J. Kumla, SDBR-CMUNK 0047, GenBank sequence MK764934 (ITS), MK773856 (LSU) and MK784128 (rpb2).

Discussion

The present study has identified a new species of Clitopilus acquired from northern Thailand based on both morphological characteristics and phylogenetic analyses. Clitopilus lampangensis is characterized by its clitocyboid, pale yellow to grayish yellow basidiocarps, pinkish spore-print, ellipsoid basidiospores with longitudinal ridges and hyphae lacking clamp connections. Thus, these morphological characteristics support its placement into the genus Clitopilus (Singer 1986; Noordeloos 1988). Based on the morphology, the pale yellow to grayish yellow pileus of C. lampangensis distinguishes it from the white and grayish pileus of Clitopilus species, with the exceptions of C. catalonicus, C. djellouliae, C. fasciculatus, C. gallaecicus, C. giovanellae, C. incrustatus, C. luteocinnamomeus and C. prunulus, (Kummer 1871; Singer 1942; Noordeloos 1984; Baroni and Halling 2000; Moreno et al. 2007; Ovrebo and Baroni 2007; Vila et al. 2008; Contu et al. 2011; Desjardins et al. 2015). The characteristics of the basidiocarps and size of the basidia, caulocystidia and basidiospores of C. lampangensis were compared with related Clitopilus species (Table 2). The presence of caulocystidia in C. lampangensis clearly distinguishes it from these related species. Moreover, the pileus of C. lampangensis (35–50 mm in diameter) are larger than C. djellouliae (6–18 mm in diameter; Contu et al. (2011)), C. giovanellae (5–15 mm in diameter; Singer (1942) and Moreno et al. (2007)) and C. catalonicus (up to 15 mm in diameter; Vila et al. (2008)). Prior to this study, C. apalus, C. crispus, C. doimaesalongensis, C. chalybescens, C. peri and C. prunulus had been found in Thailand.
Table 2. Comparison of *Clitopilus lampangensis* with the closely related species.

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Origin</th>
<th>Pileus</th>
<th>Basidia</th>
<th>Caulocystidia</th>
<th>Basidiospores</th>
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<td><em>C. lampangensis</em></td>
<td>Thailand</td>
<td>35–50 mm in diameter,</td>
<td>17.0–25.0 × 4.0–8.0 µm, 2–4 streigmata</td>
<td>25.5–42.5 × 8.0–15.0 µm</td>
<td>Ellipsoid, 7.0–9.0 × 3.0–5.0 µm, 6–8 longitudinal ridges</td>
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<tr>
<td></td>
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<td>pale yellow to greyish yellow</td>
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<tr>
<td><em>C. chalybescens</em> a</td>
<td>Thailand</td>
<td>15–90 mm in diameter, white, yellowish white to greyish blue</td>
<td>15.0–21.0 × 5.1–8.0 µm, 4 streigmata</td>
<td>16.0–32.0 × 5.0–7.0 µm</td>
<td>Ellipsoid, 5.3–7.5 × 3.6–5.0 µm, 8–10 longitudinal ridges</td>
</tr>
<tr>
<td><em>C. peri</em> b, c</td>
<td>India, Sri Lanka, Thailand</td>
<td>8–22 mm in diameter, white</td>
<td>16.0–18.0 × 5.0–7.0 µm, 4 streigmata</td>
<td>Absent</td>
<td>Ellipsoid, 6.7–8.5 × 3.0–4.0 µm, 6–9 longitudinal ridges</td>
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<tr>
<td><em>C. prunulus</em> c</td>
<td>Thailand, United State</td>
<td>25–80 mm in diameter, white, yellowish white to greyish or yellow cream</td>
<td>25.0–47.0 × 7.0–12.0 µm, 4 streigmata</td>
<td>Absent</td>
<td>Ellipsoid, 9.0–14.0 × 4.5–8.0 µm, 6–8 longitudinal ridges</td>
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<td><em>C. fasciculatus</em> d</td>
<td>Netherlands</td>
<td>20–70 mm in diameter, pale brown</td>
<td>Sizes were not reported, 4 streigmata</td>
<td>Absent</td>
<td>Ellipsoid, 4.5–6.3 × 3.0–4.0 µm, 3–6 longitudinal ridges</td>
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<tr>
<td><em>C. gallaecicus</em> e</td>
<td>Spain</td>
<td>80–90 mm in diameter, creamy, ochre to ochre-brown</td>
<td>20.0–35.0 × 8.5–10.5 µm, 4 streigmata</td>
<td>Absent</td>
<td>Ellipsoid, 8.0–14.5 × 4.5–7.5 µm, 3–6 longitudinal ridges</td>
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<tr>
<td><em>C. incrutatus</em> f</td>
<td>Costa Rica, United State</td>
<td>80–90 mm in diameter, greyish brown</td>
<td>16.0–24.0 × 7.0–8.0 µm, 4 streigmata</td>
<td>Absent</td>
<td>Ellipsoid, 5.0–6.5 × 3.0–4.0 µm, 3–6 longitudinal ridges</td>
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<td><em>C. djellouliae</em> g</td>
<td>France</td>
<td>6–18 mm in diameter, light yellowish brown</td>
<td>22.0–32.0 × 7.5–8.5 µm, 4 streigmata</td>
<td>Absent</td>
<td>Ellipsoid, 6.0–9.0 × 4.0–6.0 µm</td>
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<tr>
<td><em>C. giovanellae</em> h</td>
<td>Italy, Spain</td>
<td>5–15 mm in diameter, greyish to light brown</td>
<td>14.0–22.0 × 6.5–9.5 µm, 4 streigmata</td>
<td>Absent</td>
<td>Ellipsoid, 5.0–8.0 × 3.0–4.0 µm</td>
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<tr>
<td><em>C. latocinnamomeus</em> i</td>
<td>Panama</td>
<td>15–45 mm in diameter, light cinnamon-brown</td>
<td>19.0–27.0 × 6.0–7.0 µm, 4 streigmata</td>
<td>Absent</td>
<td>Subglobose to ellipsoid, 4.5–6.0 × 3.5–5.0 µm</td>
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<tr>
<td><em>C. catalonicus</em> j</td>
<td>Panama</td>
<td>Up to 15 mm in diameter, light yellowish brown</td>
<td>32.0–40.0 × 6.4–8.0 µm, 4 streigmata</td>
<td>Absent</td>
<td>Ellipsoid, 5.3–7.5 × 3.7–4.5 µm</td>
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</table>

aThis study, bBaroni et al. (2001), cJatuwong et al. (2017), dPegler (1986), eKlutting et al. (2014), fKummer (1871), gDesjardin et al. (2015), hNoordeloos (1984), iBlanco-Dios (2013), jBaroni and Halling (2000), kContu et al. (2011), lSinger (1942), mMoreno et al. (2007), nOvrebo and Baroni (2007) and oVila et al. (2008).

However, *C. apalus*, *C. crispus*, *C. peri* and *C. doimaesalongensis* differ from *C. lampangensis* by their white to chalk-white pileus and a lack of caulocystidia (Pegler 1986; Yang 2000; Jatuwong et al. 2017). The larger basidia and basidiospores, and the absence of caulocystidia in *C. prunulus* clearly differentiate it from *C. lampangensis* (Kummer 1871; Desjardin et al. 2015) (Table 2). Both *C. lampangensis* and *C. chalybescens* have caulocystidia (Baroni et al. 2001; Jatuwong et al. 2017). However, the width of the caulocystidia and the length of the basidiospores of *C. chalybescens* are narrower and shorter than in *C. lampangensis* (Table 2) (Baroni et al. 2001; Jatuwong et al. 2017).

The phylogenetic analyses of the combined ITS and LSU, and *rpb2* sequences confirmed that *C. lampangensis* formed a monophyletic clade which clearly separated it from the other *Clitopilus* species. *Clitopilus lampangensis* forms a sister taxon to *C. chalybescens* and *C. peri*. *Clitopilus perii* differs from *C. lampangensis* by its smaller white basidiocarps (8–22 mm in diameter) and the absence of caulocystidia (Pegler 1986).
A new Clitopilus species from Thailand

Additionally, the different morphological characteristics that exist between *C. lampangensis* and *C. chalybecens* have been mentioned above.

Therefore, a combination of the morphological characteristics and the molecular analyses strongly support recognition of a new fungus species. This discovery is considered important in terms of stimulating a deeper investigation of macrofungi in Thailand, and will help researchers to better understand the distribution and ecology of *Clitopilus*.

**Key to *Clitopilus* species known from Thailand**

1. Pileus white to chalk-white colors ...............................................................2
   
   – Pileus white or with other colors .............................................................5
2. Stipe ≥ 3 mm thick .................................................................................3
   
   – Stipe < 3 mm thick ................................................................................C. peri
3. Basidia < 8 µm wide ...............................................................................4
   
   – Basidia ≥ 8 µm wide, basidiospores 6.8–9.2 × 4.1–5.5 µm .....................

   ..................................................................................................................C. doimaesalongensis
4. Basidia up to 25 µm, basidiospores 6–8.5 × 4.5–5.5 µm ......................C. apalus
   
   – Basidia up to 30 µm, basidiospores 5.5–9 × 4–6 µm .............................C. cripus
5. Pileus white to pale grayish or yellowish cream colors ........................6
   
   – Pileus pale yellow to greyish yellow colors, caulocystidia present, basidiospores 7.0–9.0 × 3.0–5.0 µm ..............................................C. lampangensis
6. Basidia ≥ 25 µm long, caulocystidia absent, basidiospores 8.0–12.0 × 4.0–6.5 µm ..............................................................................C. prunulus
   
   – Basidia < 25 µm long, caulocystidia present, basidiospores 5.3–7.5 × 3.6–5.0 µm .................................................................C. chalybecens

**Acknowledgements**

This work was supported by grants from Chiang Mai University and Center of Excellence on Biodiversity (BDC), Office of Higher Education Commission (BDC-PG3-161005), Thailand. We are grateful to staff of Mae Moh Forestry Industry Organization for their excellent field assistance, and Dr. Eric H.C. McKenzie for English proof reading.

**References**


A new Clitopilus species from Thailand


Three novel insect-associated species of *Simplicillium* (Cordycipitaceae, Hypocreales) from Southwest China

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Abstract

In this paper, we introduce three new species of *Simplicillium*, viz. *S. cicadellidae*, *S. formicidae* and *S. lepidopterorum*, which were isolated from an infected leafhopper, ant and carpenterworm, respectively. Morphological comparisons and phylogenetic analyses based on multigene datasets (LSU+RPB1+RPB2+TEF and ITS+LSU) support the establishment of the three new species. *Simplicillium cicadellidae* was distinguished from other species in morphological characteristics by having smaller phialides and ellipsoidal conidia, and lacking octahedral crystals. The reverse of colonies were yellowish (#FFBF00), especially in the middle, and radially sulcate. *Simplicillium formicidae* was morphologically distinguished from other by having longer phialides and filiform to fusoid conidia, and by lacking octahedral crystals. *Simplicillium lepidopterorum* was morphologically distinguished from other species by having smaller, ellipsoidal to fusiform conidia, and by lacking octahedral crystals. The reverse of the colony was pale white. The three new species are likely to be nourished by plant to animal (especially insect) nutrients based on the evolutionary pattern of the Hypocreales, and they are described herein as being clearly distinct from other species in *Simplicillium*.

Keywords

Commensal fungi, morphology, nutritional preference, phylogeny
Introduction

The genus *Simplicillium* W. Gams & Zare was introduced by Zare and Gams (2001) with *S. lanosoniveum* (J. F. H. Beyma) Zare & W. Gams as the type species. The genus is characterized with its complete lack of verticillate branching; mostly solitary phialides, which are discrete, aculeate and narrow and arise from aerial hyphae; conidia short-ellipsoidal to suglobose or obclavate, and adhering in globose heads or imbricate chains (Zare and Gams 2001). The members of *Simplicillium* are fungicolous and occur on various substrata (Zare and Gams 2001; Chen et al. 2008; Baiswar et al. 2014; Gauthier et al. 2014; Gomes et al. 2018). Furthermore, Zare and Gams (2001) introduced three additional species, viz., *S. lamellicola* (F. E. V. Sm.) Zare & W. Gams, *S. obclavatum* (W. Gams) Zare & W. Gams and *S. wallacei* H. C. Evans. The typical characteristics of *Simplicillium* include mostly solitary phialides, conidia adhering in globose, slimy heads or imbricate chains, and commonly present crystals in the agar (Zare and Gams 2001). Later, Zare and Gams (2008) transferred *S. wallacei* to *Lecanici-illum* W. Gams & Zare based on the phylogenetic analysis of internal transcribed spacer (ITS) region and this transfer was confirmed by Sung et al. (2007).


Kepler et al. (2017) re-evaluated the Cordycipitaceae based on the multigene dataset (SSU, LSU, TEF, RPB1 and RPB2), and indicated that *Simplicillium* species group in a clade and are the earliest diverging lineage in Cordycipitaceae. The nuclear ribosomal ITS and LSU were first used to identify cryptic diversification among *Simplicillium* species by Liu and Cai (2012) and then were widely applied in the identification of *Simplicillium* species by Nonaka et al. (2013), Zhang et al. (2017), Gomes et al. (2018) and Crous et al. (2018).

Zare and Gams (2001) noted that *Simplicillium* species were found on various substrata and fungi. Other substrata were found later, such as limstone and wood (Liu and Cai 2012; Zhang et al. 2017). Many bioactive compounds were discovered in *Simplicillium*, such as alkaloids (Fukuda et al. 2014), peptides (Liang et al. 2016; 2017; Dai et al. 2018), diketopiperazine (Yan et al. 2015), xylanases (Roy et al. 2013), anthraquinones (Huang et al. 2015), antibiotics (Takata et al. 2013; Dong et al. 2018), and especially Simpotentin, which is a new potentiator of amphotericin B activity against *Candida albicans* (C. P. Robin) Berkhout and has showed great potential ap-
Novel insect-associated Simplicillium species

Applications in medicine (Uchida et al. 2019). Furthermore, the antimicrobial activities and entomopathogenicity has meant that Simplicillium has potential applications in biocontrol (Ward et al. 2012; Zhao et al. 2013; Le Dang et al. 2014; Lim et al. 2014; Chen et al. 2017; Skaptsov et al. 2017). However, as far as we know, there are limited reports of Simplicillium species isolated from infected insects.

Three infected insect specimens were found during a survey of araneogenous fungi and allies from southwestern China. Some fungal strains were isolated and purified from the three specimens. Based on polyphasic approach (morphological, ecological characteristics along with a phylogenetic analysis), they were identified as three new species, Simplicillium cicadellidae sp. nov., S. formicidae sp. nov. and S. lepidopterorum sp. nov.

Materials and methods

Collection and isolation

Three infected insect specimens (DL1004, GY1101 and GY2913) were collected from Dali, Rongjiang Country (26°01'58.70"N, 108°24'48.06"E) and Tongmuling (26°23'25.92"N, 106°41'3.35"E), Huaxi District, Guizhou Province, on 1 October, 9 November and 31 July, 2018, respectively. The surface of the specimens were rinsed with sterile water, followed by surface sterilization with 75% ethanol for 3–5 s. A part of the insect body was cut off and used to inoculate a piece of tissue in haemocoel on potato dextrose agar (PDA) and improved potato dextrose agar (PDA, 1% w/v peptone) (Qu et al. 2018). The strain was isolated and cultured at 22 °C for 14 d under 12 h light/12 h dark conditions following protocols described by Zou et al. (2010). Strains DL10041, DL10042, GY11011, GY11012, GY29131 and GY29132 were obtained.

Culture and identification

The strains were incubated in PDA at 25 °C for 14 d. Macroscopic and microscopic morphological characteristics of the fungi were examined using classical mycological techniques, and the growth rates were determined. The fresh hyphae were observed with an optical microscope (OM, BX35, Olympus, Japan) following pretreatment with lactophenol cotton blue solution or normal saline. The ex-type cultures and dried culture as holotype specimens were deposited in GZAC, Guizhou University, Guiyang, China.

DNA extraction, PCR amplification and nucleotide sequencing

DNA extraction was carried out in accordance with Liang et al. (2009). The extracted DNA was stored at −20 °C. The amplification of large subunit ribosomal
RNA (LSU) genes was performed using NS1-1/AB28 primers (Curran et al. 1994). Translation elongation factor 1 alpha (TEF) and DNA-directed RNA polymerase II largest subunit 2 (RPB2) were amplified using 983F/2218R and RPB2-5F/RPB2-7Cr primers according to van den Brink et al. (2012). DNA-directed RNA polymerase II largest subunit 1 (RPB1) was amplified with the primer pair CRPB1 and RPB1-Cr (Castlebury et al. 2004). The internal transcribed spacer (ITS) region was amplified using ITS4/ITS5 primers by PCR following the procedures described by White et al. (1990). PCR products were purified using the UNIQ-10 column PCR products purification kit [no. SK1141; Sangon Biotech (Shanghai) Co., Shanghai, China] in accordance with the manufacturer’s protocol and sequenced at Sangon Biotech (Shanghai) Co. The resulting sequences were submitted to GenBank.

The new species *Simplicillium cicadellidae*, *S. formicidae* and *S. lepidopterorum* were registered in MycoBank with the numbers MB 831336, MB 831337 and MB 831335, respectively.

**Sequence alignment and phylogenetic analyses**

DNA sequences generated in this study were assembled and edited using DNASTAR Lasergene software (version 6.0). Sequences of ITS, LSU, RPB1, RPB2 and TEF were selected based on previously published data by Nonaka et al. (2013), Zhang et al. (2017), Gomes et al. (2018), Crous et al. (2018) and Mongkolsamrit et al. (2018). Multiple sequence alignments for ITS, LSU, RPB1, RPB2 and TEF were carried out using MAFFT v7.037b (Katoh and Standley 2013). Sequence editing was performed with MEGA6 (Tamura et al. 2013), and the resulting output was in Fasta file format. The concatenated LSU+RPB1+RPB2+TEF and ITS+LSU sequences were assembled by SequenceMatrix v.1.7.8 (Vaidya et al. 2011). Gene concordance was assessed with the ‘hompart’ command in PAUP4.0b10 (Swofford 2002).

Two different analyses have been carried out using Bayesian inference (BI) and maximum likelihood (ML) methods. Analysis 1: To check the relationship between *Simplicillium* species and its allies in Cordycipitaceae based on the combined dataset of (LSU+RPB1+RPB2+TEF). Analysis 2: To check the relationship among *Simplicillium* spp. based on the combined dataset of (ITS+LSU). For the BI analysis, two runs were executed simultaneously for 10,000,000 generations, saving trees every 500 generations, with the GTR+G nucleotide substitution model across all the partitions, in MrBayes 3.2 (Ronquist et al. 2012). After the analysis was finished, each run was examined with the program Tracer v1.5 (Drummond and Rambaut 2007) to determine burn-in and confirm that both runs had converged. For the ML analysis in RAxML (Stamatakis 2014), the GTRGAMMA model was used for all the partitions in accordance with recommendations in the RAxML manual against the use of invariant sites. The final alignment is available from TreeBASE under submission ID: 24549 (http://www.treebase.org)
Results

Phylogenetic analyses

A phylogenetic tree of *Simplicillium* in Cordycipitaceae was generated from the maximum-likelihood (ML) and Bayesian inference (BI) based on a combined data set of LSU, RPB1, RPB2 and TEF sequence data. Statistical support (≥ 50%/0.5) is shown at the nodes for ML bootstrap support/BI posterior probabilities (Fig. 1). The strain numbers are noted after each species’ name. The tree is rooted with *Purpureocillium lilacinum* (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson (CBS 284.36 and CBS 431.87). The concatenated sequences including 40 taxa and contained 2,205 characters with gaps (LSU: 447, RPB1: 518, RPB2: 560, and TEF: 680).

A phylogenetic tree of *Simplicillium* species level was generated from the maximum-likelihood (ML) and Bayesian inference (BI) analysis based on a combined data set of ITS and LSU sequence data set. Statistical support (≥ 50%/0.5) are shown at the nodes for ML bootstrap support/BI posterior probabilities. The strain numbers are noted after each species’ name. The tree is rooted with *Pochonia chlamydospora* (Goddard) Zare & W. Gams (CBS 103.65). The dataset includes 16 taxa and consists of 1,000 characters with gaps (ITS: 489 and LSU: 511).

Analysis 1: family Cordycipitaceae. The RAxML analysis of the combined dataset (LSU+RPB1+RPB2+TEF) yielded a best scoring tree (Fig. 1) with a final ML optimization likelihood value of –24,337.973328. Parameters for the GTR model of the concatenated data set was as follows: estimated base frequencies; A = 0.242689, C = 0.276532, G = 0.270879, T = 0.209901; substitution rates AC = 0.926706, AG = 2.728719, AT = 0.823168, CG = 0.803225, CT = 6.257555, GT = 1.000000; gamma distribution shape parameter α = 0.410435. The Bayesian analysis resulted in 20,001 trees after 10,000,000 generations. The first 4,000 trees, representing the burn-in phase of the analyses, were discarded, while the remaining 16,001 trees were used for calculating posterior probabilities in the majority rule consensus tree. In the phylogenetic tree (Fig. 1), *Simplicillium cicadellidae*, *S. formicidae* and *S. lepidopterorum* cluster with other *Simplicillium* species in a clade, and within the earliest diverging lineage in Cordycipitaceae.

Analysis 2: *Simplicillium* species. The RAxML analysis of the combined dataset (ITS+LSU) yielded a best scoring tree (Fig. 2) with a final ML optimization likelihood value of –4,849.039588. Parameters for the GTR model of the concatenated data set was as follows: Estimated base frequencies; A = 0.243952, C = 0.258870, G = 0.268223, T = 0.228956; substitution rates AC = 1.296760, AG = 2.678402, AT = 1.354112, CG = 1.488619, CT = 5.097242, GT = 1.000000; gamma distribution shape parameter α = 0.462419. The Bayesian analysis resulted in 20,001 trees after 10,000,000 generations. The first 4,000 trees, representing the burn-in phase of the analyses, were discarded, while the remaining 16,001 trees were used for calculating posterior probabilities in the majority rule consensus tree. In the phylogenetic tree
Figure 1. Phylogenetic relationships among the genus *Simplicillium* and its allies in Cordycipitaceae based on multigene dataset (LSU, RPB1, RPB2 and TEF). Statistical support values (≥0.5/50%) are shown at the nodes for ML bootstrap support/BI posterior probabilities. The tree is rooted with *Paraprocyclicium filicium* (CBS 284.36 and CBS 431.87). The new species are in bold face. T in the upper right corner indicates the type strains.
Figure 2. Phylogenetic relationships among the new taxa *S. cicadellidae*, *S. formicidae*, *S. lepidopterorum* and other *Simplicillium* species by ITS+LSU sequences. Statistical support values (≥ 0.5/50%) are shown at the nodes for ML bootstrap support/BI posterior probabilities. The tree is rooted with *Pochonia chlamydosporia* (CBS 103.65). The new species are in bold face. T in the upper right corner indicates the type strains.

(Fig. 2), *Simplicillium* species were resolved into four obvious clades. *S. cicadellidae*, *S. formicidae* and *S. lepidopterorum* were nested in a subclade and formed three independent branches, which received maximum statistical support (BI posterior probabilities 1, ML bootstrap 100%).
### Table 1. Taxa included in the phylogenetic analyses

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Novel insect-associated *Simplicillium* species

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*Pochonia chlamydosporia* CBS 103.65 MH858504

*Purpureocillium lilacinum* CBS 284.36 FR75484 EF468898 EF468941 EF468792

*Samoniella alboaurantianum* CBS 240.32 EF468844 EF468987 EF468940 EF468791

*S. aurantia* CBS 262.58 MG665232 JQ425685

*S. inthanonensis* TBRC 7271T MF140728 MF140791 MF140818 MF140846

*S. inthanonensis* TBRC 7272 MF140727 MF140817 MF140818 MF140845

*S. inthanonensis* TBRC 7273 MF140726 MF140816 MF140818 MF140844

*S. inthanonensis* TBRC 7915T MF140725 MF140790 MF140815 MF140849

*S. inthanonensis* TBRC 7916 MF140724 MF140789 MF140814 MF140848

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*Simplicillium* JCM 18167T AB604002

*Simplicillium* JCM 18168 AB604004

*S. calcicola* LC 5371 KU746705 KU74675

*S. calcicola* LC 5586T KU746706 KU746752

*S. chinense* LC 1342 JQ410323 JQ410321

*S. chinense* LC 1345 NR155782 JQ410322

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*S. coffeanum* COAD 2061 MF066035 MF066033

*S. cylindrosporum* JCM 18169T AB603989

*S. cylindrosporum* JCM 18170 AB603994

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*S. cylindrosporum* JCM 18174 AB604005

*S. cylindrosporum* JCM 18175 AB604006

*S. filiforme* URM 7918 MH979338 MH979399

*S. formicidae* DL10041T MN006241 MN006247 MN022269 MN022267

*S. formicidae* DL10042 MN006242 MN006248 MN022270 MN022268

*S. lamellicola* CBS 116.25T AJ292393 AF339552 DQ522404 DQ522462 DQ522356

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*S. lamellicola* UAMH 4785 AF108480

*S. lamellicola* KYK00006 AB378533

*S. lanosoniveum* CBS 704.86 AJ292396 AF339553 DQ524206 DQ522464 DQ522358

*S. lanosoniveum* CBS 101267 AJ292395 AF339554 DQ522405 DQ522463 DQ522357

*S. lepidopterorum* GY29131T MN006246 MN006251 MN022273 MN022265

*S. lepidopterorum* GY29132 MN006245 MN006252 MN022274 MN022266

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*S. minatense* JCM 18177 AB603991

*S. minatense* JCM 18178 AB603993

*S. obclavatum* CBS 311.74T AJ292394 AF339517 EF468798

*S. obclavatum* JCM 18179 AB604000

*S. subtropicum* JCM 18180T AB603990

*S. subtropicum* JCM 18181 AB603995

*S. subtropicum* JCM 18182 AB603996

*S. subtropicum* JCM 18183 AB604001

*S. sympodiothorum* JCM 18184T AB604003

*T = type strains, strain and sequences generated in this study are shown in bold.*
Taxonomy

*Simplicillium cicadellidae* W.H. Chen, C. Liu, Y.F. Han, J.D. Liang, Z.Q. Liang
sp. nov.
MycoBank: MB 831336
Figure 3

**Etymology.** The epithet *cicadellidae* refers to an insect host in family Cicadellidae.

**Diagnosis.** Characterized by phialides always solitary and rather long and narrow, 12.9–18.3 × 0.8–1.1 µm. Conidia adhering in globose slimy heads, mostly ellipsoidal,

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**Figure 3.** *Simplicillium cicadellidae* **A** infected leafhopper (Hemiptera) **B–C** culture plate, showing the front (**B**) and the reverse (**C**) of the colony, cultured on PDA medium **D–F** phialides solitary, conidia adhering ellipsoidal slimy head and conidia **G** conidia. Scale bars: 10 mm (**B, C**), 10µm (**D, E, F, G**).
Novel insect-associated Simplicillium species

1.8–2.8 × 1.4–1.8 µm. Octahedral crystals absent. Reverse of colony yellowish, especially in the middle, and radially sulcate.

**Type.** CHINA, Guizhou Province, Huaxi District (26°23’25.92”N, 106°41’3.35”E), 9 November 2018, Wanhao Chen, holotype GZAC GY1101, ex-type culture GZAC GY11011. Sequences from isolated strain GY11011 has been deposited in GenBank with accession numbers: ITS = MN006243, LSU = MN006249, RPB1 = MN022271 and TEF = MN022263.

**Description.** Colonies reaching 45–47 mm in diameter in 14 d on PDA; white; reverse yellowish, especially in the middle, and radially sulcate. Hyphae septate, hyaline, smooth-walled, 0.9–1.9 µm wide. Phialides arising from aerial hyphae, gradually tapering towards apex, without basal septa, always solitary and rather long and narrow, 12.9–18.3 × 0.8–1.1 µm. Conidia adhering in ellipsoidal slimy heads, mostly ellipsoidal, hyaline, smooth-walled, 1.8–2.8 × 1.4–1.8 µm. Octahedral crystals absent.

**Host.** Leafhopper (Hemiptera)

**Distribution.** Huaxi District, Guizhou Province, China

**Remarks.** Zare and Gams (2001) summarized the typical characteristics of Simplicillium as having mostly solitary phialides arising from aerial hyphae, conidia adhering in globose slimy heads or imbricate chains, crystals commonly present, fungicolous and on various other substrata. Simplicillium cicadellidae was easily identified as belonging to Simplicillium because of its solitary phialides, conidia adhering in ellipsoidal slimy heads, and lack of octahedral crystals. Comparing with the typical characteristics of 12 species (Table 2), it was easily distinguished from other species in having the phialides always solitary and rather long and narrow (12.9–18.3 × 0.8–1.1 µm), the conidia adhering in globose slimy heads, which are mostly ellipsoidal (1.8–2.8 × 1.4–1.8 µm), and the octahedral crystals absent. The reverse of colony was yellowish, especially in the middle, and radially sulcate. Based on ITS and LSU rDNA, S. cicadellidae is phylogenetically close to S. formicidae and S. lepidopterorum. However, S. cicadellidae has ellipsoidal conidia and shorter phialides (12.9–18.3 × 0.8–1.1 µm), and the reverse of colony was yellowish.

Simplicillium formicidae W.H. Chen, C. Liu, Y.F. Han, J.D. Liang, Z.Q. Liang, sp. nov. MycoBank: MB 831337

Figure 4

**Etymology.** The epithet formicidae refers to an insect host in family Formicidae.

**Diagnosis.** Characterized by phialides always being solitary and rather long and narrow, 51–70.1 × 0.7–0.9 µm. Conidia adhering in globose slimy heads, mostly filiform to fusoid, 3.9–7.9 × 0.8–1.3 µm. Octahedral crystals absent.

**Type.** CHINA, Guizhou Province, Rongjiang County (26°01’58.70”N, 108°24’48.06”E), 1 October 2018, Wanhao Chen, holotype GZAC DL1004, ex-type culture GZAC DL10041. Sequences from isolated strain DL10041 has been deposited in GenBank with accession numbers: ITS = MN006241, LSU = MN006247, RPB1 = MN022269 and RPB2 = MN022267.
Description. Colonies reaching 26–32 mm in diameter in 14 d on PDA; white; reverse pale brown to brown, and with brown secretions. Hyphae septate, hyaline, smooth-walled, 1.2–1.8 µm wide. Phialides arising from aerial hyphae, gradually tapering towards the apex, without basal septa, always solitary and rather long and narrow, 51–70.1 × 0.7–0.9 µm. Conidia adhering in globose slimy heads, mostly filiform to fusoid, hyaline, smooth-walled, 3.9–7.9 × 0.8–1.3 µm. Octahedral crystals absent.

Host. Ant (Hymenoptera)

Distribution. Rongjiang County, Guizhou Province, China

Remarks. Simplicillium formicidae was easily identified as belonging to Simplicillium because of its solitary phialides, conidia adhering in globose slimy heads, and lack of octahedral crystals. Compared with the typical characteristics of 12 species (Table 2), it was easily distinguished from those species by having the phialides always solitary and rather long and narrow (51–70.1 × 0.7–0.9 µm) and the conidia mostly filiform to fusoid (3.9–7.9 × 0.8–1.3 µm), and adhering in globose slimy heads, and in having octahedral crystals absent. Based on ITS and LSU rDNA, S. formicidae is phylogenetically close to S. cicadellidae and S. lepidopterorum. However, S. formicidae has larger filiform to fusoid conidia (3.9–7.9 × 0.8–1.3 µm).
Novel insect-associated *Simplicillium* species

*Simplicillium lepidopterorum* W.H. Chen, C. Liu, Y.F. Han, J.D. Liang & Z.Q. Liang, sp. nov.
MycoBank: MB 831335
Figure 5

**Etymology.** The epithet *lepidopterorum* refers to an insect host in order Lepidoptera.

**Diagnosis.** Characterized by phialides always being solitary and rather long and narrow, 15.3–26.2 × 0.7–1.4 µm, Conidia adhering in globose slimy heads, mostly ellipsoidal, 1.6–2.4 × 1.4–1.7 µm. Octahedral crystals absent. The reverse of colony was pale white.

**Type.** CHINA, Guizhou Province, Huaxi District (26°23’25.92”N, 106°41’3.35”E), 31 July 2018, Wanhao Chen, **holotype** GZAC GY2913, ex-type culture GZAC GY29131, sequences from isolated strain GY29131 has been deposited in GenBank with accession numbers: ITS = MN006246, LSU = MN006251, RPB1 = MN022273 and TEF = MN022265.

**Description.** Colonies reaching 48–51 mm in diameter in 14 d on PDA; white; reverse pale white. Hyphae septate, hyaline, smooth-walled, 1.1–2.2 µm wide. Phi-

![Figure 5. Simplicillium formicidae A isolated substrate an infected ant (Hymenoptera) B–C culture plate, showing the front (B) and the reverse (C) of the colony, cultured on PDA medium D, E phialides solitary, conidia adhering globose slimy head and conidia F conidia. Scale bars: 10 mm (B, C), 10µm (D, E, F).](image-url)
alides arising from aerial hyphae, gradually tapering towards the apex, without basal septa, always solitary and rather long and narrow, 15.3–26.2 × 0.7–1.4 µm. Conidia adhering in globose slimy heads, ellipsoidal to fusiform, hyaline, smooth-walled, 1.6–2.4 × 1.4–1.7 µm. Octahedral crystals absent.

**Host.** Carpenter worm (Lepidoptera)

**Distribution.** Huaxi District, Guizhou Province, China

**Remarks.** *Simplicillium lepidopterorum* was easily identified as belonging to *Simplicillium* because of its solitary phialides, conidia adhering in globose slimy heads, and lack of octahedral crystals. Comparing with the typical characteristics of 12 species (Table 2), *S. lepidopterorum* could easily distinguished from other species by having the phialides always solitary and rather long and narrow, 15.3–26.2 × 0.7–1.4 µm. Conidia ellipsoidal (1.6–2.4 × 1.4–1.7 µm), adhering in globose slimy heads, and in

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**Table 2.** Morphological comparison of three new species with other *Simplicillium* species

<table>
<thead>
<tr>
<th>Species</th>
<th>Morphological characteristics</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phialide (Conidiogenous cell) (µm)</td>
<td>Conidia (µm)</td>
</tr>
<tr>
<td><em>S. aogashimaense</em></td>
<td>(19–)23–53 × 1.2–2.0</td>
<td>cylindrical, 4.2–6.5 × 1.2–2.0</td>
</tr>
<tr>
<td><em>S. calcicola</em></td>
<td>14–38 × 1–2</td>
<td>micro-: globose, oval or ellipsoidal, 2.3–5.5 × 1.0–1.5 reconam: fusiform, 4.2–8 × 1-2</td>
</tr>
<tr>
<td><em>S. chinense</em></td>
<td>(6.0–)15–30–(68.0) × 1.5</td>
<td>oval, ellipsoidal or cylindrical 3.5–5.0 × 1.0–1.5</td>
</tr>
<tr>
<td><em>S. coffeanum</em></td>
<td>11–40(–70) × 1.0–2.4</td>
<td>micro-: spindle-shaped, 5.3–8.8 × 1.0–1.6 reconam: ellipsoidal to fusiform, 2.2–3.8 × 0.8–1.5</td>
</tr>
<tr>
<td><em>S. cylindrosporum</em></td>
<td>17–32 × 1.2–2.0(–2.5)</td>
<td>cylindrical, 3.0–4.5(–5.0) × 1.0–2.0</td>
</tr>
<tr>
<td><em>S. filiforme</em></td>
<td>9–18 × 1</td>
<td>fusoid to filiform, 7.2–12.5 × 1</td>
</tr>
<tr>
<td><em>S. lamellicola</em></td>
<td>15–50 × 0.7–1.0</td>
<td>micro-: spindle-shaped, 4.5–9.0 × 0.8–1.2 reconam: oval to ellipsoidal, 2.0–3.0 × 0.7–1.2</td>
</tr>
<tr>
<td><em>S. lanoonivorum</em></td>
<td>15–35 × 0.7–1.5</td>
<td>subglobose, oval, ellipsoidal 1.5–3 × 0.7–1.2</td>
</tr>
<tr>
<td><em>S. minatense</em></td>
<td>11–31(–47) × 1.0–1.7</td>
<td>globose to subglobose, sometimes ellipsoidal, 2.0–3.5 × 1.8–2.5(–2.8)</td>
</tr>
<tr>
<td><em>S. obclavatum</em></td>
<td>30–52 × 0.8–1.2</td>
<td>obclavate to ellipsoidal, 2.5–3.5 × 1–2</td>
</tr>
<tr>
<td><em>S. subtropicum</em></td>
<td>(15–)20–42(–50) × 1.0–2.3</td>
<td>subglobose to ellipsoidal, 2.3–4.0(–4.5) × 1.5–3.3</td>
</tr>
<tr>
<td><em>S. sympodiophorum</em></td>
<td>20–34(–47) × 0.5–1.3</td>
<td>oval to ellipsoidal, 2.2–3.5 × 1.0–2.0</td>
</tr>
<tr>
<td><em>S. cicadellidae</em></td>
<td>12.9–18.3 × 0.8–1.1</td>
<td>ellipsoidal, 1.8–2.8 × 1.4–1.8</td>
</tr>
<tr>
<td><em>S. formicidae</em></td>
<td>51–70.1 × 0.7–0.9</td>
<td>filiform to fusoid, 3.9–7.9 × 0.8–1.3</td>
</tr>
<tr>
<td><em>S. lepidopterorum</em></td>
<td>15.3–26.2 × 0.7–1.4</td>
<td>ellipsoidal, 1.6–2.4 × 1.4–1.7</td>
</tr>
</tbody>
</table>

a–f: data are derived from Zare and Gams (2001), Nonaka et al. (2013), Zhang et al. (2017), Liu and Cai 2012, Gomes et al. (2018) and Crous et al. (2018), respectively.
Novel insect-associated Simplicillium species

having the octahedral crystals absent. Based on ITS and LSU rDNA, S. lepidopterorum is phylogenetically close to S. cicadellidae and S. formicidae. However, S. lepidopterorum has ellipsoidal conidia, longer phialides (15.3–26.2 × 0.7–1.4 µm), and the reverse of colony was pale white.

Key

1  Conidia in globose or subglobose heads ............................................................... 2
   – Conidia in chains or solitary ........................................................................... 11
2  Macro- and microconidia present ................................................................. 3
   – Only one type of conidia present ............................................................. 4
3  Octahedral crystals present ........................................................................ 5
   – Octahedral crystals absent ........................................................................... 9
4  Octahedral crystals present .......................................................................... S. coffeae
   – Octahedral crystals absent ........................................................................ S. coffeae
5  Conidia cylindrical ................................................................................... 6
   – Conidia subglobose or ellipsoidal ............................................................... 7
6  Chlamydospores present, conidia 4.2–6.5 × 1.2–2.0 µm........... S. aogashimaense
   – Chlamydospores absent, conidia 3.0–4.5 (–5.0) × 1.0–2.0 µm .............. S. aogashimaense
   ...................................................................................................................................... S. cylindrosorum
7  Conidia subglobose to ellipsoidal ................................................................ 8
   – Conidia oval or ellipsoidal to subcylindrical, 1.5–3.0 × 0.7–1.3 µm .............. S. lanosoniveum
   .............................................................................................................................. S. subtropicum
8  Conidia subglobose to ellipsoidal, 2.3–4.0 (–4.5) × 1.5–3.3 µm ..................... S. subtropicum
   – Conidia globose to subglobose, sometimes ellipsoidal, 2.5–3.5 × 1.8–2.5
     (–2.8) µm .................................................................................................................. S. minatense
9  Conidia ellipsoidal ........................................................................................ 10
   – Conidia filiform to fusoid .............................................................................. S. formicidae
10 The reverse of colony pale white, phialide 12.9–18.3 × 0.8–1.1 µm ................... S. cicadellidae
    – The reverse of colony yellowish, phialide 15.3–26.2 × 0.7–1.4 µm ............ S. lepidopterorum
11 Denticles present in conidiogenous cell (phialide) ...................... S. sympodiphorum
   – Denticles absent in conidiogenous cell (phialide) ......................................... 12
12 Macro- and microconidia present ............................................................ S. calcicola
   – Only one type of conidia present ................................................................. 13
13 Conidia ellipsoidal ......................................................................................... 14
   – Conidia fusoid to filiform, form zigzag chains ........................................ S. filiforme
14 Conidia in branched or unbranched chains, 3.5–5.0 × 1.0–1.5 µm ...... S. chinense
   – Conidia in short imbricate chains, 2.5–3.5 × 1.0–2.0 µm .......... S. obclavatum
Discussion

Two types of the evolutionary correlation patterns between fungi and hosts are known, co-evolutionary patterns and the more frequent host jump events (Spatafora et al. 2007). The generation of host jumping is closely related to a common living environment (Vega et al. 2009). Nutritional sources are very important factors in determining whether a host has undergone a host jump. The nutritional model of Hypocreales fungi is from plants (including living plants and plant residues) to animals (especially insects), and finally to fungi. Plants and their residues were the initial sources of nutrition for the common ancestor of Hypocreaceae and Clavicipitaceae. The jumps from plants to animals and then to fungi indicate that the fungal nutrient requirements have changed with the environment (Spatafora et al. 2007). Prediction of the characteristics and evolutionary placement of any given member should be based on the correlation between molecular-phylogenetic genealogy and nutritional preferences (Spatafora et al. 2007; Vega et al. 2009). Additionally, host insect species are an important diagnostic feature in the identification of entomopathogenic fungi.

Among the 12 reported *Simplicillium* species, *S. aogashimaense* (soil), *S. calcicola* (calcareous rock), *S. chinense* (decaying wood), *S. cylindrosporum* (soil), *S. minatense* (soil), *S. obclavatum* (air), *S. subtropicum* (soil) and *S. sympodiophorum* (soil) were isolated from soil, marine water, rock, decaying wood and air (Zare and Gams 2001; Liu and Cai 2012; Nonaka et al. 2013; Liang et al. 2017). *Simplicillium filiforme* and *S. coffeanum* were isolated as endophytic fungi from plants (Crous et al. 2018; Gomes et al. 2018). *Simplicillium lamellicola* belongs to the hyperparasite fungi (Shin et al. 2017). *Simplicillium lanosoniveum* was reported as both an endophytic and hyperparasite fungi (Baiswar et al. 2014). It has been reported that *Simplicillium* is pathogenic to insects. Unfortunately, there are limited reports of insect-related *Simplicillium*.

The hosts of *Simplicillium cicadellidae* and *S. lepidopterorum* were larvae of Cicadidae and Lepidoptera, which feed through piercing-sucking and chewing. Moreover, *S. formicidae* was isolated from an infected ant. These three strains are likely to receive nutrients from plants (including living plants and plant residues) and animals (especially insects) based on the evolutionary pattern of Hypocreales. *Simplicillium cicadellidae, S. formicidae* and *S. lepidopterorum* represent three new species based on their nutritional preferences. To our knowledge, this is the first report of insect-associated *Simplicillium* species.

ITS and LSU have been widely used in the identification of *Simplicillium* (Liu and Cai 2012; Nonaka et al. 2013; Zhang et al. 2017; Sliva et al. 2018). In the present study, the combined dataset (ITS+LSU) was used to analysis of phylogenetic relationships among the new taxa and other *Simplicillium* species. Additionally, RPB1, RPB2 and TEF loci were added to analysis that the relationship among *Simplicillium* and its allies. The new species clustered with other *Simplicillium* species in a clade (Fig. 1), and this was consistent with morphological characteristics based identification. Six strains were clustered into three subclades (Fig. 2) and were distinctly different from other reported *Simplicillium* spp. Additionally, three species, *S. chinense, S. coffeanum* and...
S. filiforme were clustered in a subclade, and these species were associated with plants. This may be because of their nutritional preferences. Therefore, S. cicadellidae, S. formicidae and S. lepidopterorum are based on morphological characteristics, ecological characteristics and a phylogenetic analysis.

Acknowledgements

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References


100


Novel insect-associated Simplicillium species


Behind the veil – exploring the diversity in *Phallus indusiatus* s.l. (Phallomycetidae, Basidiomycota)

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Abstract

Studies have demonstrated that many cosmopolitan species actually consist of divergent clades that present high levels of morphological stasis throughout their evolutionary histories. *Phallus indusiatus* s.l. has been described as a circum-tropical species. However, this distribution may actually reflect the lack of taxonomic resolution due to the small number of diagnostic morphological characters, which leads to the identification of new records as populations of *P. indusiatus*. Here, we examine the diversity of *P. indusiat-us*-like species in Brazilian Amazonia. We show a clear congruence between detailed morphological data and ITS, nuc-LSU and atp6 based phylogenetic analyses and three new species are described within the Brazilian indusiate clade. These results highlight the importance of more detailed investigation, with the inclusion of molecular information, in Neotropical fungi.

Keywords

Amazonia, atp6, ITS, Neotropics, nuc-LSU, Phallales

Introduction

The worldwide distribution of fungal species hypotheses has been questioned by modern molecular analyses. Studies have demonstrated that many cosmopolitan species actually consist of divergent clades that present high levels of morphological stasis
throughout their evolutionary histories (Mueller et al. 2001, Bickford et al. 2007, Geml et al. 2008, Davis et al. 2014). *Phallus indusiatus* Vent. – also known as the “veiled lady” mushroom – has been described as a circum-tropical species, with records for South and Central America (Dennis 1960, Saénz and Nassar 1982, Leite et al. 2007, Cheype 2010), Mexico (Guzmán et al. 1990), Africa (Dissing and Lange 1962, Dring 1964, Demoulin and Dring 1975, Dring and Rose 1977, Desjardin and Perry 2015), Asia (Dennis 1953, Liu 1984, Hosaka 2010) and Australia (Smith 2005). For some groups of fungi, spore dispersal mechanisms may support the idea of transoceanic dispersal connecting geographically isolated populations (Halling et al. 2008, Hosaka et al. 2008). However, the current distribution of *P. indusiatus* may actually reflect the lack of taxonomic resolution due to the small number of diagnostic morphological characters, which leads to the identification of new records as populations of *P. indusiatus*. The insect-dependent mechanism of spore dispersal may also have played an important role in determining the current distribution of *P. indusiatus*.

As in phalloid fungi in general, few morphological characters are available to delimit species in *Phallus*. In addition, most of the widely used diagnostic characters – such as colour and sizes – show high plasticity, another factor that may lead to misidentifications and mask the real diversity within the genus (Kreisel 1996, Calonge 2005). As a consequence of these taxonomic uncertainties, a great number of synonyms are reported for several species of this clade. *Phallus indusiatus* is an emblematic example, where at least nineteen synonyms and several distinct forms have been described (Lloyd 1909, Liu 1984, Guzmán et al. 1990, Kreisel 1996, Calonge 2005, Das et al. 2007, Cheype 2010).

Due to lack of resolution when using morphological characters to identify *Phallus* species, we believe that several specimens that have been identified as *P. indusiatus* might actually consist of independently evolving entities. In fact, some new species with minimal, yet noticeable morphological differences from *P. indusiatus*, have been proposed. For instance, *P. serrata* H.L. Li, L. Ye, P.E. Mortimer, J.C. Xu & K.D. Hyde, described for China, differs by the meshes of the indusium with serrate edges (Li et al. 2014); *P. echinovolvatus* (M. Zang, D.R. Zheng and Z.X. Hu) Kreisel has a whitish volva with mycelioid projections on the surface (Zang et al. 1988); and *P. flavidus* Kreisel & Hausknecht, described for the Seychelles, has yellowish pigments on the receptacle and indusium (Kreisel and Hausknecht 2009). Some of these species were described with the support of molecular analyses, which reinforces the importance of this kind of analysis to resolve these taxonomic uncertainties. At least three species resembling *P. indusiatus* were described for Brazil: *Phallus moelleri* Lloyd, *Dictyophora callichroa* Möller and *Dictyophora phalloidea* Desv. In the original descriptions, they present some inherent characteristics that distinguish them from *P. indusiatus*, such as the above-ground development of the volva in *D. phalloidea* and the orange receptacle and pinkish receptacle apex in *D. callichroa* (Möller 1895). Lloyd (1909) described *P. moelleri* based on a Brazilian species and synonymised *D. callichroa* with it. All three species are now considered synonyms of *P. indusiatus* by some authors and Index Fungorum (Lloyd 1909, Fischer 1928, Saénz and Nassar 1982, Calonge 2005, Kreisel and Hausknecht 2009).

*Phallus indusiatus* was described by Étienne Pierre Ventenat in 1798, based on a specimen from Suriname. In 1809, Desvaux created a new genus, *Dictyophora* Desv.,
mainly characterised by the presence of an indusium, a skirt-like structure that expands from the receptacle towards the ground. Ventenat’s species was transferred to Dictyophora and named *D. indusiata* (Vent.) Desv. Kreisel (1996) considered that the importance of an indusium for the taxonomy of the genus was overestimated, hence he downgraded *Dictyophora* to a section of *Phallus*. More recently, with the introduction of molecular data to the systematics and taxonomy of fungi, studies have shown that the indusium is a recurrent character, which independently emerged several times during the evolution of the group (Hosaka et al. 2006, Cabral et al. 2012, Marincowitz et al. 2015, Trieveiler-Pereira et al. 2017). Today, *P. indusiatus* Vent. is the valid name for Ventenat’s species. *Phallus indusiatus* is widespread in Brazil, with records from four of the six Brazilian biomes (Magnago et al. 2013), but information concerning its diversity and distribution is still incomplete.

In this study, we examined the diversity of *P. indusiatus*-like species in Brazilian Amazonia. We show a clear congruence between detailed morphological data and DNA-based phylogenetic analyses and three new species are described within the Brazilian indusiate clade. These results highlight the importance of more detailed investigation, with the inclusion of molecular information, in Neotropical fungi.

**Material and methods**

**Morphological data**

Specimens of *Phallus* sp. with white indusium were collected during the rainy seasons of 2013 to 2015 in various areas of the Amazon Rainforest domain (Figure 1). We included in the analyses four additional specimens attributed to *P. indusiatus* borrowed from the Herbarium of the Instituto de Botânica (São Paulo) and the Universidade Federal de Rio Grande do Norte-Fungos, which were collected in various areas of the Atlantic Rainforest domain. Other *Phallus* species were included in the molecular analysis to increase taxon coverage, both from GenBank and newly sequenced specimens from the Palearctic-Oriental region (Suppl. material 1: Table S1). Species were morphologically described based on fresh and dried material. Macroscopic characters were described based on field notes and photographs, while microscopic details were obtained by mounting slides with fragments from different layers and structures of dried basidiome in 5% potassium hydroxide (KOH) and/or stained with Congo red dye. We followed the specific literature for species identification (Lloyd 1909, Kreisel 1996, Calonge 2005, Kreisel and Hausknecht 2009) and colours were described following Küppers (1979).

**DNA extraction, amplification and sequencing**

DNA extraction followed Hosaka (2009). The nuclear ribosomal ITS and nuc-LSU regions, as well as mitochondrial *atp6* region, were amplified using previously described
Figure 1. Currently known distributions of the Phallus species described in this study. Highlighted areas are the Brazilian Biomes (IBGE 2012): Amazonian Rainforest, Cerrado, Caatinga, Atlantic Rainforest, Pantanal and Pampa.

primers and protocols (Vilgalys and Hester 1990, White et al. 1990, Kretzer and Bruns 1999). DNA fragments were visualised in 1% agarose gel stained with GelRed™ (Bi- otium) under UV light. The fragments were purified using Ilustra ExoProStar (GE Healthcare) and then sequenced using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) with the same primer pairs. After sequencing, some ITS electropherograms presented double peaks; in order to resolve these, the ITS PCR fragments were cloned following Marincowitz et al. (2015). All ribotypes were included in the phylogenetic analyses.

Molecular phylogenetic analyses

We submitted each sequence to a BLAST search to identify the closest relatives and to check for possible contamination. The closest sequences resulting from the BLAST search and sequences with genus names of Phallus or Dictyophora were retrieved from GenBank and added to the dataset. All sequences were aligned and manually edited with Geneious R6.1 (Biomatters Ltd.). Two analyses were run, one for the ITS dataset (ITS) and the other with ITS, nuc-LSU and atp6 concatenated matrix (CONC). The ITS final aligned matrix contained 618 positions, while the concatenated matrix contained 1896 positions (571 for ITS, 794 for nuc-LSU and 529 for atp6). These two
matrices were analysed separately. Based on a previous phylogeny (Trierveiler-Pereira et al. 2014), species of the genus *Mutinus* were chosen as outgroups. Maximum Parsimony (MP) analyses were performed with PAUP* (Swofford 2003) using heuristic searches with the TBR branch-swapping algorithm; the initial tree was obtained by stepwise addition of random additional sequences repeated 100 times and 1000 replicates as bootstrap (bs) settings. For Bayesian analysis (BA), the substitution model of evolution was chosen with MrModelTest (Nylander 2004). The analyses were run in MrBayes 3.2.6, as follows: two parallel runs were executed with four incrementally-heated simultaneous MCMC simulations over 5 million generations, with trees sampled every 1000 generations. The consensus trees were reconstructed with the remaining trees after the burn-in stage, which was defined based on the average standard deviation of split frequency values. The confidence values were estimated with posterior probabilities (pp). Trees were visualised and edited in FigTree version 1.4.2. All data are available in TreeBASE under ID 21524.

**Results**

A total of 19 recently collected specimens of *Phallus* spp. with white indusium were studied, 15 of which were collected in Brazilian Amazonia, while four other specimens were collected from the Brazilian Atlantic Rainforest (SP and UFRN-Fungos herbaria) (Figure 1). Additionally, we obtained sequences from 21 *Phallus* specimens from Japan, Russia, Vietnam and Thailand. The collection localities, herbarium vouchers and GenBank accession numbers can be found in the Suppl. material 1: Table S1, as well as in species descriptions.

**Phylogenetic analyses**

We obtained 95 sequences, amongst which 54 were ITS, 19 were nuc-LSU and 22 were *atp6* (Suppl. material 1: Table S1). The ITS final aligned matrix contained 618 positions, while the concatenated matrix contained 1896 positions (571 for ITS, 795 for nuc-LSU and 530 for *atp6*). Maximum Parsimony and Bayesian analyses with both matrices (ITS and CONC) resulted in trees with the same intraspecific relationships, but with different topologies (Figures 2, 3; MP trees in Suppl. material 2: Figures S1, S2). For Maximum Parsimony analysis, of the 618 positions from the ITS matrix, 382 were informative and resulted in a most parsimonious tree with 2006 steps (CI = 0.458, RI = 0.859, RC = 0.394), while of the 1896 positions from the CONC matrix, 502 were informative and resulted in a most parsimonious tree with 1097 steps (CI = 0.547, RI = 0.709, RC = 0.388). In all of the phylogenetic trees obtained in this study, the Brazilian specimens of *Phallus* grouped together (ITS: pp = 1, bs = 94%; CONC: pp = 1, bs = 100%). This clade can be divided into six groups, which correspond to the four morphospecies identified and described here (coloured clades on Figures 2,
Figure 2. Phylogenetic tree obtained by Bayesian analysis with ITS. Brazilian clades corresponding to the new species and *P. indusiatus* are indicated (the holotype of each species is in bold). Posterior probabilities and bootstrap values are on the nodes (pp/bs), values of pp < 0.95 and bs < 90 are not shown. The black dots indicate specimens under *Phallus indusiatus* deposited in Genbank and downloaded for this study.
Exploring the diversity in *Phallus indusiatus* s.l.

*Phallus denigricans* T.S.Cabral, B.D.B.Silva & Baseia, sp. nov.
MycoBank No: 824632

**Figure 3.** Phylogenetic tree obtained by Bayesian analysis with concatenated data (ITS, nuc-LSU and atp6). Brazilian clades corresponding to the new species and *P. indusiatus* are indicated (the holotype of each species is in bold). Posterior probabilities and bootstrap values are on the nodes (pp/bs), values of pp < 0.95 and bs < 90 are not shown (except for *P. denigricans* clade).

*D. indusiata* retrieved from GenBank, all from Asia (China and Japan), as well as those collected by us in this study, form a paraphyletic clade with intercontinental disjunct distributions. Based on morphological similarities and the geographical proximity to the type locality (Suriname) of the Amazonian specimens collected and supported by the molecular data, one Brazilian clade (blue on Figures 2, 3) corresponds to *P. indusiatus* sensu stricto.

**Taxonomy**

*Phallus denigricans* T.S.Cabral, B.D.B.Silva & Baseia, sp. nov.
MycoBank No: 824632

**Figure 4**

**Diagnosis.** This species is characterised by the campanulate receptacle slightly constricted at the base, pale yellow, reticulated, with a prominent apical pore, epigeous development of basidiome, volva varying from white to dark brown and spores up to 4.6 × 2.5 μm.
Figure 4. Phallus denigricans UFRN-Fungos 2805, holotype. A Basidiome B blackish and smooth volva in detail C white volva with projections D receptacle with a prominent pore E spores F pseudoparenchymatous hyphae of pseudostipe G hyphae from rhizomorphs H hyphae from volva. Scale bars: 20 mm (A–D), 20 µm (E), 40 µm (F–H).

Holotype. BRAZIL. Amazonas: São Gabriel da Cachoeira, Itacoatiara Mirim Community (0.304167S, 66.8403W), 1 April 2013, Komura DL (INPA-Fungos 272383). GenBank accessions: MG678486 (ITS), MG678455 (nuc-LSU), MG678541 (atp6).
Exploring the diversity in *Phallus indusiatus* s.l.

Immature basidiomes not observed. Fresh expanded basidiome 98 mm high. Receptacle [25] 26 × 19 [25] mm, campanulate, but slightly constricted at the base, with a prominent apical pore, deeply reticulated surface. Pseudostipe [81] 54 × 10 [22] mm, cylindrical, spongy, white (N00 A00 M00); pseudoparenchymatous, composed of globose to elongate-ovoid cells, [20.5] 18.5–65.5 [60.8] × [17.5] 19–52.5 [51.2] µm, hyaline. Indusium poorly developed, extending to 2/3 of pseudostipe, white (N00 A00 M00), 53 mm in length, attached to the apex of the pseudostipe, polygonal to irregular meshes up to 13 × 8 mm. Volva epigeous, white (N00 A00 M00) in some specimens to dark brown (N00 A00 M50) in others, with smooth surface or sometimes with small hyphae projections on surface; formed by filamentous hyphae, septate, branched, hyaline, clamp connections present, [2.5] 1.8–5 [3.5] µm diameter, with inflated ends up to 15.5 µm diameter. Rhizomorphs composed of at least two types of hyphae: filamentous thin-walled hyphae, with clamp connections; and thicker hyphae (7–16 µm) that seem to communicate with each other by pores on the inflated tips. Crystals in globose cells were found distributed amongst the hyphae of volva and rhizomorphs of some of the white volva species, measuring 8.2–11.5 × 6.8–10.6 µm. Gleba olive brown (N99 A50 M10), mucilaginous. Basidiospores elongated, smooth, 3.6–4.6 × 2.2–2.5 µm, hyaline in 5% KOH.

**Habitat and distribution.** On soil, in a fragment of upland old-growth forest. So far restricted to the Brazilian Atlantic and Amazon forests, found in the municipalities of Barcelos, Parintins, Sáo Gabriel da Cachoeira and Maraã (State of Amazonas, Brazil); and Natal (State of Rio Grande do Norte).

**Etymology.** with reference to the volva becoming blackish.


**Notes.** *Phallus flavidus* Kreisel & Hauskn. could be comparable with *P. denigricans* by the conical receptacle and the indusium size; however, *P. flavidus* has smaller spores (up to 3.6 × 1.8 µm), the surface of the volva is light grey with an orange flush and the indusium is cream to yellow (Kreisel and Hausknecht 2009). *Phallus impudicus* var. *obliteratus* (Malençon) Kreisel has a reticulate white receptacle and a rudimentary white indusium; *Phallus denigricans* also has a poorly-developed indusium, but it is very different from *P. impudicus* var. *obliteratus*, where the indusium is hidden under the receptacle (Calonge 2005, Kreisel and Hausknecht 2009). *Phallus callichrous* (Møller) Lloyd is a species described from Brazil, with white indusium and differs from *P. denigricans* by having an orange to pink receptacle and reddish-violet rhizomorphs. Recently, another indusiate species was described for Brazil, *Phallus aureolatus*, but it differs from *P. denigricans* mainly by the strongly developed pore and the meruloid
surface of the receptacle (Trierveiler-Pereira et al. 2017), in addition to its different phylogenetic placement (Figures 2, 3). *Phallus echinovolvatus* (M. Zang, D.R. Zheng & Z.X. Hu) Kreisel is another white-indusiate species, characterised mainly by the volva covered with echinulate hyphae projections; in *P. denigricans*, hyphae projections on the volva surface can also be found in some specimens, but they are smaller than in *P. echinovolvatus* (Zang et al. 1988). In *P. indusiatus*, the receptacle is campanulate, the immature basidiome is hypogeous, so that the volva is buried under the ground when the basidiome is fully developed, the indusium is completely developed reaching the ground and the volva and rhizomorphs have pinkish pigments (Ventenat 1798). On the other hand, in *P. denigricans* the campanulate receptacle is constricted at the base, the basidiome has a completely epigeous development, the indusium is poorly-developed reaching only 2/3 of the basidiome and the rhizomorphs and volva are white to brownish.

It is not rare to find *Phallus* specimens with a blackish volva; recently, a new species was described, *P. fuscoechinovolvatus* (Song et al. 2018), but it is quite different from *P. denigricans* mainly by the strongly echinulated volva. *Phallus merulinus* (Berk.) Cooke and *P. atrovolvatus* Kreisel & Calonge are very similar, differing by the volva colour – that is black in *P. atrovolvatus* and white in *P. merulinus* – and the habitat (Calonge 2005). In our ITS phylogenetic analyses (Figure 3), specimens identified as *P. atrovolvatus* and *P. merulinus* grouped together in the same clade, indicating a possible identity between these two species. However, no type material was analysed here, which prevents a reliable determination of the species boundaries between *P. atrovolvatus* and *P. merulinus*. Similarly, in *P. denigricans*, we found specimens with white and pale white to brownish volva all grouping in the same clade in phylogenetic trees (Figures 2, 3). This suggests that the volva colour might change due to the soil properties or with the maturity of the basidiome. Therefore, this specific characteristic – pale or darker volva – should be carefully analysed before it can be used as a diagnostic character in *Phallus* species.

In both the Bayesian and Maximum Parsimony phylogenetic trees (Figures 2, 3 and Suppl. material 2: Figures S1, S2, specimens of *P. denigricans* grouped in a clade with high support values (ITS tree: pp = 1, bs = 100%), in concordance with morphological data.

*Phallus purpurascens* T.S.Cabral, B.D.B.Silva & Baseia, sp. nov.
MycoBank No: 824633

Figure 5

**Diagnosis.** This species is characterised by its large basidiome (up to 200 mm), the indusium reaching 2/3 of the basidiome, the purplish volva and rhizomorphs and the thimble-like and strongly reticulated receptacle.

Figure 5. Phallus purpurascens SINOP27, paratype. A Fresh basidiome B gregarious immature basidiome, with purplish pigments on surface C longitudinal section of an immature basidiome, showing the purplish volva and rhizomorphs. Phallus purpurascens UFRN-Fungos 2808, holotype. D Spores E rhizomorphs hyphae F pseudoparenchymatous hyphae from pseudostipe G hyphae from volva H crystals in globose cells found on volva. Scale bars: 20 mm (A–C), 20 µm (C–H).
Immature basidiomes whitish (N₆₀A₆₀M₅₀) with purplish pigments (A₁₀M₁₀C₁₀), globose to subglobose, up to 56 × 43 mm, growing gregariously. Fresh expanded basidiome up to 200 mm high. Receptacle up to 45 × 29 mm, thimble-like, flat at the apex with an apical pore; strongly reticulated surface, shallow reticulations up to 3.2 × 1.7 mm, white (N₆₀A₆₀M₆₀). Pseudostipe up to 122 × 21 mm, cylindrical, spongy, white (N₀₀A₀₀M₀₀); pseudoparenchymatous, composed of globose to elongate-ovoid cells, 37–65.5 × 22.5–48 µm, hyaline. Indusium well-developed, extending up to 2/3 of the pseudostipe, white (N₀₀A₀₀M₀₀), up to 100 mm in length, attached to the apex of the pseudostipe; polygonal meshes up to 10 × 5 mm. Volva semi-hypogeous, white (N₀₀A₀₀M₀₀) becoming purplish (A₁₀M₁₀C₁₀) when exposed, with a smooth surface; formed by filamentous hyphae, septate, branched, hyaline, clamp connections present, 3.1–6.6 µm diameter; with crystal deposits in globose cells widely distributed amongst the hyphae, 17.5–38 × 20.5–35.7 µm. Rhizomorphs composed of at least two types of hyphae: filamentous thin-walled hyphae, with clamp connections; and thicker hyphae (3–6.5 µm) that seem to communicate with each other by pores on the inflated tips. Gleba olive-brown (N₉₉A₅₀M₁₀), mucilaginous. Basidiospores cylindrical, smooth, 4.4–5 × 2.5–3.4 µm, hyaline in 5% KOH.

Habitat and distribution. on soil, in a fragment of upland secondary forest. It was found in the municipalities of Manaus (State of Amazonas, Brazil) and Sinop (State of Mato Grosso, Brazil).

Etymology. with reference to the volva becoming purple.

Other specimens examined (paratypes). Mato Grosso: Sinop, Parque Florestal de Sinop (11.8359S, 55.5008W), 7 November 2013, Cabral TS (SINOP26, SINOP27, SINOP28, SINOP30).

Notes. This species is the most distinctive amongst our collections, mainly due to its large basidiome, the purplish volva and rhizomorphs and the strongly reticulated receptacle. Phallus rubrovolvatus (M. Zang, D.G. Ji & X.X. Liu) Kreisel is one of the largest white-indusiate species (up to 330 mm); it differs from P. purpurascens by the deep red volva, the fragile indusium, by larger reticulations on the receptacle and smaller spores (3.7–4 × 2–2.5 µm) (Liu 1984, Calonge 2005). Additionally, in the phylogenetic analysis (Figures 2, 3), P. rubrovolvatus does not group with P. purpurascens, which confirms their separate identities. Phallus callichrous has an orange to pink receptacle, reddish-violet rhizomorphs and orange receptacle with pink margin (Möller 1895, Kreisel and Hausknecht 2009), which differ from the white receptacle, purplish volva and rhizomorphs of P. purpurascens; unfortunately, there is little information available for this Brazilian species (Calonge 2005). Phallus multicolor (Berk. & Broome) Cooke is similar to P. purpurascens in the purplish volva and rhizomorphs, but it differs by the cream to orange indusium and the light yellow pseudostipe (Lloyd 1909, Calonge 2005, Kreisel and Hausknecht 2009). Phallus indusiatus differs from P. purpurascens by the smaller basidiome, the hypogeous development of the immature basidiome and smaller spores (up to 4.1 × 2.2 µm), the well-developed indusium reaching the ground and the campanulate receptacle with wider reticulations (Ventenat 1798). The phylogenetic analyses show specimens of P. purpurascens grouping in a clade with high
support values (ITS tree: pp = 1, bs = 100%; CONC tree: pp = 1, bs = 98%), confirming its distinct identity.

*Phallus purpurascens* was found in a fragment of secondary forest, in an extremely threatened area of the Amazonian forest domain in the State of Mato Grosso, Brazil. This state was the second most deforested in Brazil in 2018 (INPE 2018), meaning that species in this area may be suffering the consequences of habitat fragmentation, which is one of the main causes of decline in fungal species (Courtecuisse 2008). Thus, this new species record shows the urgency of cataloguing fungal biodiversity of threatened areas, such as Neotropical forests.

**Phallus squamulosus** T.S.Cabral, B.D.B.Silva & Baseia, sp. nov.
MycoBank No: 824634
Figure 6

**Diagnosis.** This species is characterised by its immature basidiome and volva with a squamous surface, white receptacle with shallow reticulations and a wide pore.


Immature basidiomes whitish (N60A60M50), up to 39 × 34 mm, ovoid, with squamous surface. Fresh expanded basidiome up to 95 mm high. Receptacle 20 × 28 mm, campanulate to thimble-like, with a wide apical pore; and a strongly but shallow reticulated surface, reticulations 1.6–2 × 0.8–1.2 mm. Pseudostipe 60 × 12 mm, cylindrical, spongy, white (N00A00M00); pseudoparenchymatous, composed of globose to elongate-ovoid cells, 18–71 × 10.5–35 µm, hyaline. Indusium well-developed, extending to 2/3 of pseudostipe, white (N00A00M00), 44 mm in length, attached to the apex of the pseudostipe; polygonal to rounded meshes up to 6 × 3 mm. Volva epigeous, whitish (N60A60M50) to pale yellow (N00C00A30), with squamous surface; formed by filamentous hyphae, septate, branched, hyaline; clamp connections present, 2.5–4.5 µm diameter. Rhizomorphs whitish (N00A00M00), composed of filamentous thin-walled hyphae, with crystal deposits in globose cells distributed amongst the hyphae, 15–17.9 × 14–17 µm. Gleba olive-brown (N99A50M10), mucilaginous. Basidiospores elongated, smooth, 3.5–4.4 × 1.8–2.2 µm, hyaline in 5% KOH.

**Habitat and distribution.** found growing on sandy soil, in a fragment of ombrophilous forest in the Atlantic Rainforest domain.

**Etymology.** with reference to the volva covered with small scales.

**Notes.** Only one specimen of this species has been found to date in the northern Atlantic Rainforest domain, but it is quite distinct from other species found in this study. We could not find white-indusiate species records with squamous exoperidium in the available literature. However, *P. duplicatus*, described in Martín and Tabarés (1994), presents an immature basidiome with fine scales on the exoperidium, but this character is not found in other described *P. duplicatus* (Lloyd 1909, Liu 1984,
Figure 6. *Phallus squamulosus* UFRN-Fungos 2806, holotype. A Fresh basidiome B immature basidiome with squamous surface C spores D pseudoparenchymatous hyphae from pseudostipe E hyphae from volva F hyphae from rhizomorphs and crystals deposits on globose cells. Scale bars: 20 mm (A, B), 20 µm (C–F).
Kreisel and Hausknecht 2009, Kibby and McNeil 2012). Nevertheless, the material described by Martín and Tabarés (1994) differs from *P. squamulosus* mainly by having a conic-campanulate receptacle with crenulate disc on the apex. *Phallus denigricans* presents small hyphae projections on immature exoperidium surfaces of some specimens, but these projections are arranged differently in *P. squamulosus*, where they appear as scales. *Phallus indusiatus* is different from *P. squamulosus* by the campanulate receptacle with a smaller pore and deeper reticulations, the indusium extending to the ground and the immature basidiome that is hypogeous with a smooth surface and pinkish pigments.

Mycobank No: 245788

Figure 7

≡ *Dictyophora indusiata* (Vent.) Desv., J. Bot., Paris 2: 92 (1809)
≡ *Hymenophallus indusiatus* (Vent.) Nees, Syst. Pilze (Würzburg): 251 (1816)
≡ *Hymenophallus duplicatus* (Bosc) Nees, Syst. Pilze (Würzburg): 251 (1816)
≡ *Phallus duplicatus* Bosc, Mag. Gesell. naturf. Freunde, Berlin 5: 86 (1811)
≡ *Dictyophora duplicata* (Bosc) E. Fisch., in Berlese, De Toni & Fischer, Syll. fung. (Abellini) 7(1): 6 (1888)
≡ *Dictyophora rosea* (Ces.) E. Fisch., in Saccardo, Syll. fung. (Abellini) 7(1): 6 (1888)
≡ *Dictyophora phalloidea* var. *rosea* (Ces.) Lloyd, Synopsis of the known phalloids 7: 20 (1909)
≡ *Dictyophora phalloidea* var. *callichroa* (Möller) Lloyd, Synopsis of the known phalloids 7: 20 (1909)
≡ *Phallus callichrous* (Möller) Lloyd, Mycol. Writ. 7: 6 (1907)
≡ *Phallus indusiatus* var. *rochesterensis* (Lloyd) Lloyd, Synopsis of the known phalloids 7: 81 (1909)
≡ *Phallus rochesterensis* Lloyd, Synopsis of the known phalloids 7: 20 (1909)
≡ *Dictyophora phalloidea* var. *rochesterensis* (Lloyd) Sacc. & Trotter, Syll. fung. (Abellini) 21: 460 (1912)
≡ *Dictyophora indusiata* f. *aurantiaca* Kobayasi, Nov. fl. jap. 2: 83 (1938)

**Neotype.** (designated here): BRAZIL. Pará: Belterra, Floresta Nacional do Tapajós, Jamaraqua Community (2.812667S, 55.033083W), 25 March 2014, Cabral TS
Figure 7. Phallus indusiatus. Fresh basidiome of A INPA-Fungos 264931 (neotype), and B INPA-Fungos 264929, showing the volva with pinkish pigments C spores D pseudoparenchymatous hyphae from pseudostipe E hyphae from volva and crystals deposits on globose cells F hyphae from rhizomorphs. Scale bars: 20 mm (A, B); 10 µm (C); 40 µm (D); 20 µm (E, F).

(INPA-Fungos 264931). GenBank accessions: MG678500, MG678501, MG678502 (ITS); MG678463 (nuc-LSU); MG678550 (atp6).

Immature basidiomes not observed. Fresh expanded basidiome 120 mm high. Receptacle 25 × 25 mm, campanulate, with an apical pore, reticulated surface. Pseudostipe 67 × 12 mm, cylindrical, spongy, white (N00 A00 M00); pseudoparenchymatous, composed of globose to elongate-ovoid cells, 29.3–56.8 × 17.2–44 µm, hyaline. In-
dusium in full development extending to the ground, white (N 00 A 00 M 00 ), 74 mm in length, attached to the apex of the pseudostipe; polygonal to rounded meshes up to 7 × 4 mm, composed of pseudoparenchymatous cells, 31–53.8 × 23.8–41 µm. Volva hypogeous, white (N 00 A 00 M 00 ), with pinkish pigments (N 00 M 10 C 00 ); outer layer papery, composed of filamentous hyphae, 3.22–6.5 µm, yellowish, septate, with clamp connections; crystal deposits in globose cells distributed amongst the hyphae, 11.5–13.8 × 19.6–22.7 µm. Rhizomorphs composed of filamentous thin-walled hyphae, with clamp connections. Gleba olive-brown (N 99 A 50 M 10 ), mucilaginous. Basidiospores elongated, smooth, 3.6–4.1 × 1.5–2.2 µm, hyaline in 5% KOH.

Habitat and Distribution. found on sandy soil, in dense old-growth forest. It has a questionable circum-tropical distribution, with records for South and Central America, Mexico, Africa, Asia and Australia, but we believe that the distribution is restricted to South America.

Other specimens examined. BRAZIL. Pará: Belterra, Floresta Nacional do Tapajós, Jamarqua Community (2.812667S, 55.033083W), 25 March 2014, Cabral TS (INPA-Fungos 264929, INPA-Fungos 264930); São Paulo, Parque Estadual das Fontes do Ipiranga (23.54S, 46.63W), January 2011, Oliveira, J.J.S. (SP416389); March 2011, Ventura, P.O. (SP416393); Capelari, M. (SP416087).

Notes. According to Ventenat’s original description, *P. indusiatus* is characterised by the hypogeous volva, the campanulate and reticulated receptacle and by the indusium reaching the ground. The indusium is white, but it can become reddish as it matures. Ventenat does not give information on the colour of the volva and rhizomorphs, but some authors state that the volva can be light pinkish and rhizomorphs can be pinkish to violet (Calonge 2005, Kreisel and Hausknecht 2009). Our collection presents the same characteristics of the original description and those in the key for indusiate species presented by Kreisel and Hausknecht (2009a); in addition, some of the specimens are from the State of Pará, which is geographically close (about 970 km in a straight line) and with the same forest domain as the type locality (Suriname). Therefore, we believe that the specimens that are nested in the same clade in the phylogenetic trees (Figures 2, 3), all collected in the Brazilian Amazonian and Atlantic rainforests, correspond to *P. indusiatus* sensu stricto. Since Ventenat’s original description does not designate a type specimen and, consequentially, it is not possible to find the original material in herbarium for comparison, we designated here a neotype for *Phallus indusiatus*, in accordance with the provisions of the International Code of Nomenclature for algae, fungi and plants (ICN) (Article 9.8) (Turland et al. 2017).

Discussion

Molecular and morphological analyses, as well as geographical distributions, support the description of three new species within the *Phallus indusiatus*-like specimens from Brazil, with partially overlapping distributions. Our results suggest that a great number of species might be hidden within the circum-tropical *P. indusiatus* species concept, since the sequence data obtained from GenBank are clearly polyphyletic with different
relationships with other *Phallus* species (Figures 2, 3). In a similar way, several studies have unveiled cryptic fungal diversity hidden within species complexes, especially after the integration of phenotypic, single-DNA and next-generation sequencing (NGS) data (Geml et al. 2006, Jargeat et al. 2010, Kasuya et al. 2012, Kõljalg et al. 2013, Sousa et al. 2017, Accioly et al. 2019). For instance, Geml et al. (2008) revealed that at least eight phylogenetic species are found in the worldwide distribution of *Amanita muscaria* (L.) Lam, with strong intercontinental genetic disjunctions and intracan
tinental phylogeographic structure. Sousa et al. (2017) revealed four species within the pepper pot *Myriostoma* (Phallomycetidae, Basidiomycota), which has always been considered a monotypic worldwide genus. Long-distance dispersal and cosmopolitanism seems not to be the rule in fungal geographical distribution and, for this reason, there are few species with truly worldwide distributions (Salgado-Salazar et al. 2013).

Peay et al. (2016) affirm that climate, environment and dispersal play important roles in shaping fungal communities, where endemism is the most common result in continental and global-scale studies, instead of cosmopolitanism. This becomes clear when analysing the *P. indusiatus* s.l. distribution. As in all gasteroid fungi – basidiomycetes that produce spores inside the fruiting body – this species has a passive mechanism of spore dispersal (statismospory) (Wilson et al. 2011). Phalloid fungi have developed a peculiar spore dispersal mechanism that depends mainly on insects as vectors for dispersal and this factor, together with environmental conditions, should limit *P. in-
dusiatus* s.l. geographical distributions, generating the species mosaic observed here.

Regarding the Brazilian indusiate clade, we suggest that species within this group are, in fact, divergent entities that maintained the general ancestral phenotype (*P. indusiatus* s.l.) throughout their evolutionary history, due to high levels of morphological stasis. This would explain the high frequency of taxonomic uncertainties, which generates a great number of synonyms of *P. indusiatus*. The maintenance of a conserved morphology due to low rates of phenotypic variation has been widely discussed in evolution (Davis et al. 2014). Two main mechanisms have been proposed to explain the small levels of morphological change through time: genetic and developmental constraints may restrict the appearance of phenotypic variation; or there is strong stabilising selection for a phenotype (Lee and Frost 2002, Geml et al. 2008, Davis et al. 2014). In our hypothesis, because the different species in *P. indusiatus* occupy similar niches and, therefore, they are in similar environmental conditions, they are likely to experience similar selective pressures. A similar pattern was found by Mueller et al. (2001) in two disjunct and paraphyletic populations of *Suillus spraguei* (Berk. & M.A. Curtis) Kuntze, that presented no noticeable morphological differences, probably as a result of stabilising selection. For the future, this could be tested for other *Phallus indusiatus*-like species from other continents and alternative methodologies should be applied, such as ancestral state reconstruction.

When studying phalloid species, it is noticeable that macro-characters are more variable than micro-characters. For instance, spores are often cylindrical to bacillloid and smooth throughout the order (except for Gastrosporiaceae), probably as an adaptation for dispersal, since they are dispersed through the gut and do not adhere on the bodies of insects (Tuno 1998, Oliveira and Morato 2000). The presence of rounded crystals in
Exploring the diversity in Phallus indusiatus s.l.

Globose cells amongst hyphae of the volva and rhizomorphs was reported for Phallales species (Iofisidou and Agerer 2002), but it is not a commonly used character in species descriptions. Probably these crystals consist of calcium oxalate, as found in other Phallomycetidae species, such as Gastrosporium simplex Mattir. and Geastrum Pers. (Iofisidou and Agerer 2002, Zamora et al. 2013), but further studies about function and composition in Phallus are needed. These crystals are present in most of the species described here, although on different parts: only on the volva of Phallus purpurascens, only on rhizomorphs of Phallus squamulosus and on both volva and rhizomorphs in Phallus denigricans. Further studies are needed in order to evaluate the taxonomic value of the presence of crystals in phalloid fungi. For instance, the presence, shape and the arrangement of oxalate crystals were found to be important characters to delimit species in Geastrum (Zamora et al. 2013).

On the other hand, macro-characters, such as the shape, surface and colour of the main structures (receptacle, pseudostipe, indusium, volva and rhizomorphs), are important characters for infrageneric classification (Kreisel 1996). In this study, the phylogenetic clades of Phallus-like species were differentiated, based on these features (Table 1), confirming their importance as diagnostic characters. Given that these diagnostic characters are lost once phalloid specimens are dehydrated, it is extremely important that newly described species and new records should be well illustrated with coloured photographs of fresh material. In addition, we believe that molecular data are indispensable for delimiting and describing species in Phallus.

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Supplementary material 1

Table S1
Authors: Tiara S. Cabral, Bianca D.B. Silva, María P. Martín, Charles R. Clement, Kentaro Hosaka, Iuri G. Baseia
Data type: species data
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Link: https://doi.org/10.3897/mycokeys.58.35324.suppl1

Supplementary material 2

Figure S1
Authors: Tiara S. Cabral, Bianca D.B. Silva, María P. Martín, Charles R. Clement, Kentaro Hosaka, Iuri G. Baseia
Data type: phylogenetic tree
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Link: https://doi.org/10.3897/mycokeys.58.35324.suppl2
Supplementary material 3

Figure S2
Authors: Tiara S. Cabral, Bianca D.B. Silva, María P. Martín, Charles R. Clement, Kentaro Hosaka, Iuri G. Baseia
Data type: phylogenetic tree
Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.
Link: https://doi.org/10.3897/mycokeys.58.35324.suppl3

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Two morphological features of the new Psathyrella species, described in the paper, need to be emended: the presence of pleurocystidia and the spore length range.

Figure 3 (G, H and I) showed pyriform cells on the cap cuticle, not pleurocystidia. This mistake originated from a wrong labelling of the photo folder. Conversely to what is stated in Table 1 and on pages 93 and 100, pleurocystidia were not observed. Coherently with the DNA analysis, this correction addresses the discussion on page 99 to the certain inclusion of our fungus in the Section Spintrigerae (Fr.) Konr. & Maubl. and in the “candolleana” clade.

Due to a transcription error, the spore length range was 7.2–8.8 µm instead of 7.2–11.8 µm.

Missing reference (cited in the Introduction as Lansdown et al. 2018):
