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Rediscovery of *Roesleria subterranea* from Japan with a discussion of its infraspecific relationships detected using molecular analysis

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Abstract

Roesleria subterranea, a distinctive hypogeous fungus, was collected from unidentified deciduous plant roots in red pine forests. The fungus had been documented several times in the past in Japan, but with no description. A description is given here based on specimens collected in Japan. The sequence of the D1-D2 region of the 28S rDNA obtained from the isolate was identical to those of the European and American specimens. Maximum parsimony analysis incorporating the present data and all other available ITS-5.8S sequences for *R. subterranea* showed that there are two infraspecific groups. One of them, composed of the isolates from *Vitis* spp. in Germany, Italy, and USA, was monophyletic. The other group, composed of isolates from deciduous trees in various countries, including Japan, was paraphyletic. The phylogenetic patterns indicate that the host may be more important than geographical distance for the genetic diversification of *R. subterranea*.

Key words

Geographic distribution, Helotiales, Hypogeous fungi, ITS-5.8S, Taxonomy

Introduction

Roesleria subterranea (Weinm.) Redhead is a distinctive hypogeous fungus currently placed in Roesleriaceae (Yao and Spooner 1999) or Helotiaceae Helotiales (Neuhauser et al. 2011). It is recognized as a facultative root parasite that causes damage to grape vines (Neuhauser et al. 2011). Ecologically, it occurs on the plant roots of various deciduous trees. Its morphology characterized by stalked apothecia with spores produced in evanescent asci in dried masses (mazaedia), shows a similarity to mazaediate lichens such as *Calicium* Pers. It is closely related to helotiaceous fungi, in particular *Hymenoscyphus* Gray and *Cudoniella* Sacc. (Kirchmair et al. 2008).

Roesleria subterranea is distributed primarily in Europe and North America, with rare reports from Asia. In Japan, three presumptive occurrences of *R. subterranea* have been reported. *Roesleria hypogaea* Thüm. & Pass. was first documented in Japan by Shirai (1894) as a possible agent of root rot in grapes. Hara (1930) later cited the fungus as of an uncertain occurrence in Japan. Togashi (1950) listed the name *Coniocybe pallida* (Pers.) Fr. as a causative agent of grape root rot and reported its occurrence in Hokkaido and Akita prefecture. This name was cited in fungi from the Tohoku area (North East of Japan) by Sawada (1952) as a synonym of *Roesleria pallida* (Pers.) Sacc., (Sawada 1952, p. 143). The fungus was thought to be a root rot agent of *Vitis vinifera* in Morioka. Currently, *Roesleria hypogaea* is treated as a synonym of *R. subterranea,* while *R. pallida* is recognized as *Sclerophora pallida* (Fr.) Yao & Spooner (Coniocybaceae based on *Calicium pallidum* Fr.), which was revealed to be a lichen name (Redhead 1984, Yao and Spooner 1999). Therefore, *R. subterranea* occurs in the northern part of Japan, but none of the authors provided a definite description of the species, nor are voucher specimens available.

A specimen of *R. subterranea* was recently collected from unknown deciduous tree roots in the red pine forests in Sugadaira heights, Nagano prefecture, Japan, from which an isolate was obtained. Because *R. subterranea* is a subterranean fungus with a unique phylogenetic position as a helotiaceous fungus, it is documented here with molecular data and reference to the specimens in Japan.

Materials and methods

Collection and isolation

A specimen was collected on roots of unknown trees beneath the log of *Pinus densiflora*, among the conifer forests of 30-year-old trees mixed with other deciduous trees such as *Betula platyphylla*, *Cerastrus orbiculatus*, *Ligustrum tschonoskii*, *Rhododendron japonicum*, *Swida controvers.*Although *Vitis coignetiae* is present in this forest, no individual was found nearby the collection site of the fungus, so it seems likely plausible that the host was not *Vitis*. The material was air-dried at 23°C for 24 hours. A small amount of spores were picked up by a fine needle and transferred to plates of malt extract agar (MEA; Nissui, Tokyo, Japan), cornmeal agar (CMA; Nissui), and half-strength malt extract-yeast extract agar (MEYE; malt extract 3 g; yeast extract 3 g, peptone 5 g, dextrose 10 g,

agar 15 g, DW 1 L). Germination was observed under a light microscope to obtain a pure culture. The specimen and isolate were deposited in the mycological herbarium of the National Museum of Nature and Science (TNS) and the National Institute of Technology and Evaluation, Biological Resource Center (NBRC). The color names and codes in the description followed the Pantone color bridge (Pantone Inc., Carlstadt, NJ, USA), adopting the CYMK color system. Additional specimens collected in Japan were investigated in the TNS fungal herbarium.

DNA extraction, polymerase chain reaction (PCR), and sequencing

The isolates were incubated in 2% malt extract broth for 2 weeks, and the mycelium was harvested. Approximately 50 mg of mycelium was mechanically lysed by a Qiagen Tissue Lyser Kit (Qiagen Inc., Mississauga, ON, Canada), using ceramic beads following the manufacturer's instructions. The DNA was extracted using a DNeasy Plant Mini Kit (Qiagen Inc.) following the manufacturer's instructions. To amplify the internal transcribed spacer (ITS1 and ITS2) and 5.8S rDNA regions (ITS-5.8S), the primer pair ITS1F and ITS4 (White et al. 1990) was used. To amplify the D1-D2 region of 28S rDNA (nLSU rDNA; D1-D2), the primer pair NL1 and NL4 (O'Donnell 1993) was used. The DNA was amplified using 40-µl PCR reactions containing 0.2 µM of each primer, 1 unit of TaKaRa Ex Taq DNA polymerase (Takara Bio, Otsu, Japan), and a deoxynucleoside triphosphate (dNTP) mixture containing 2.5 mM of each dNTP and ExTaq buffer containing 2 mM $MgCl_2$. PCR was carried out using a Gene Amp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). The DNA was denatured for 3 min at 95 °C, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 2 min, followed by final extension at 72 °C for 10 min. Residual primers, and unincorporated dNTPs were removed to prevent the inhibition of the following sequencing reaction using a ExoSAP-IT purification kit (USB Corp., Cleveland, OH, USA). Total DNA samples extracted for the present study were deposited in the Center for Molecular Biodiversity Research in National Museum of Nature and Science (ED16207). The sequencing reaction was carried out using a Big-Dye terminator cycle sequencing kit (Applied Biosystems) following the manufacturer's instruction using the same primers as those used for DNA amplification. Sequencing was conducted using an ABI 3130x Genetic Analyzer (Applied Biosystems).

Molecular phylogenetic analysis

The available sequences for *Roesleria subterranea* were obtained from GenBank (Table 1). The obtained sequence data for the ITS-5.8S region were aligned by Clustal W (Thompson et al. 1994) and edited manually when necessary using BioEdit v.7.0.5.2 (Hall 1999). Phylogenetic analysis was conducted by a maximum parsimony (MP) method. An MP heuristic search was carried out using the PAUP* version 4b10 (Swofford 2002) with 1,000 replications, each with the option of random sequence additions. Branch

Table 1. Sequences used in the present study. New sequences generated for this analysis are in bold.

Name	ID of the isolate	ITS-5.8S	28S rDNA*	Origin	Country
Roesleria subterranea	**NBRC108276**	**AB628057**	**AB628056**	**on roots of deciduous plant**	**Japan**
Roesleria subterranea	CBS 271.82	EF060309		on roots of *Populus* sp.	Netherlands
Roesleria subterranea	CBS 339.96	EF060308	EF608074	buried twig of deciduous shrub	Austria
Roesleria subterranea	CBS 320.33	EF060307		on roots of *Malus sylvestris*	Netherlands
Roesleria subterranea	CBS 201.25	EF060306		on roots of *Vitis vinifera*	USA
Roesleria subterranea	IB 2005/504	EF060305		"*V. berlandieri* × *V. viparia*"	Germany
Roesleria subterranea	IB 2005/511	EF060304		on roots of *V. berlandieri* × *V. viparia*	Germany
Roesleria subterranea	IB 2005/510	EF060303		on roots of *V. berlandieri* × *V. viparia*	Germany
Roesleria subterranea	IB 2005/507	EF060302		"*V. berlandieri* × *V. viparia*"	Germany
Roesleria subterranea	IB 2005/505	EF060301		on roots of *V. berlandieri* × *V. viparia*	Germany
Roesleria subterranea	IB 2005/506	EF060300	EF608075		Germany
Roesleria subterranea	IB 2005/508	EF060299		on roos of *V. rupestris* × *V. riparia*	Germany
Roesleria subterranea	CBS 407.51	EF060298	EF608073		Italy
Hymenoscyphus epiphyllus	isolate 1489	AY348580			
Hymenoscyphus immutabilis	isolate 71809	AY348584			
Hymenoscyphus scutula	MBH29259	AY789432			

swapping by tree bisection-reconnection (TBR) and MulTrees were in effect. Support for the individual nodes was tested with bootstrap analysis under the equally weighted parsimony criterion. The bootstrap analysis was based on 1000 bootstrap replicates using the heuristic search option (TBR and MulTrees options, on) of ten replicates with random addition sequences. Based on the previous analysis (Kirchmair et al. 2008), *Hymenoscyphus epiphyllus* (Pers.) Rehm ex Kauffman (AY348580), *H. immutabilis* (Fuckel) Dennis (AY348584), and *H. scutula* (Pers.) W. Phillips (AY789432) were used as outgroups. The trees were visualized using the Treeview program, version 1.6.6 (Page 1996). The alignments were deposited into TreeBASE as TB2:S11877.

Results and discussion

Description and taxonomy

***Roesleria subterranea* (Weinm.) Redhead, Can. J. Bot. 62: 2516, 1984.**
Figs 1, 2

≡ *Pilacre subterranea* Weinm., Flora 15 (Beiblatt, Bd. 1), p. 458, 1832.

Note. For the detailed synonymy, see Redhead (1984).

Discription. Apothecia capitate, long-stalked, 0.5–1.5 mm high, hypogeous, on plant root; head spherical to irregular, 0.4 mm diam., composed of dried spore mass (mazaedia), powdery, grayish green (C16M0Y36K0); stalk 0.1 mm diam., base of apothecium with elongated cells (7–13 × 2.5–4.5 µm), pale-colored; ectal excipulum not observed due to maturation of apothecia. Asci 40–55 × 6.5–7.0 µm, cylindrical-clavate, thin walled, arising from croziers, eight-spored, evanescent, releasing ascospores by degradation of ascal wall; apex rounded, Meltzer reaction– with or without KOH pretreatment. Ascospores 5–6.4 × 4.5–5.5 µm, broadly elliptical, almost hyaline, pale-colored to dark-colored due to maturation, aseptate when young, becoming almost spherical to lenticular, transversely one-septate. Germination tubes usually occur from each cell of ascospores. Paraphyses cylindrical, simple, straight to waving, enlarged toward apex up to 6-µm wide, multiseptate, often long, extending beyond asci. Ascospores germinated on CMA, MEA, and MEYE to produce mycelium. On PDA, colonies of 65 mm diam in 30 days at 23 C, surface floccose; aerial mycelium well-developed at center, white; substratal mycelium dark green (C24M5Y98K35), becoming paler (C10M0Y54K0) toward the margin. Colonies were dark green (C24M0Y98K8) from the reverse, producing stronger colored patches, paler (C6M0Y54K0) toward margin. On MEA, colonies 65 mm diam in 30 days at 23 C, surface floccose; aerial mycelium well developed, light green (C10M0Y72K0) at center, becoming sparse and paler (C6M0Y54K0) toward margin; substratal mycelium obscured. Colonies dark green (C20M4K100Y32) at center from reverse, becoming paler (C6M0Y54K0) toward margin. On CMA, colonies 60 mm diam in 30 days at 23 C, mycelium sparse, with almost no coloration. Asexual state not observed in culture.

Specimens examined. Japan. Nagano Prefecture: Pinus densiflora forests approximately 30 years old, Sugadaira Montane Research Center, University of Tsukuba, Ueda (36°52.12'N; 138°34.97'E(DDM)), ca 1300–1360 m, 19 Nov 2010, Y. Degawa (TNS-F-38701), on unknown deciduous plant roots (not *Vitis*), multi-ascospore isolate FC-2678 (NBRC108276). Hokkaido isl., Sapporo: 20 Aug 1922, K. Togashi (TNS-F-185301) as *Calicium pallidum*, on *Vitis vinifera*. Iwate Prefecture: Morioka, Oct 1935, "College Orchard", "Murata" (TNS-F-185302) as *Calicium pallidum*, on *Vitis vinifera* (The words with double quotation indicate the data written on the original specimen label. The precise information is unclear, as this specimen was donated to TNS by K. Togashi.).

The specimen collected at Sugadaira showed similar morphological agreement with previous reports (Redhead 1984, Yao and Spooner 1999, Kirchmair et al. 2008). The taxonomy of *R. subterranea*, however, has been under debate. It was suggested to belong to Caliciaceae, Caliciales (Redhead 1984). Yao and Spooner (1999) proposed Roesleriaceae, but its placement was not mentioned by Kirk et al. (2008). Based on the molecular phylogeny on rDNA, it was classified as Helotiaceae, but Roesleriaceae as a distinct family has not been ruled out (Kirchmair et al. 2008, Neuhauser et al. 2011).

Two other genera are known for their occurrence on plant roots. One is *Moserella* Pöder & Scheuer, known as a root endophyte of *Picea*, and the other is *Roeslerina* Redhead, obtained from coniferous roots (Redhead 1984, Yao and Spooner 1999). Because these two genera and *Roesleria* have mazaedia, mazaedia formation may be suggested to be a common convergent character in hypogeous apothecial fungi.

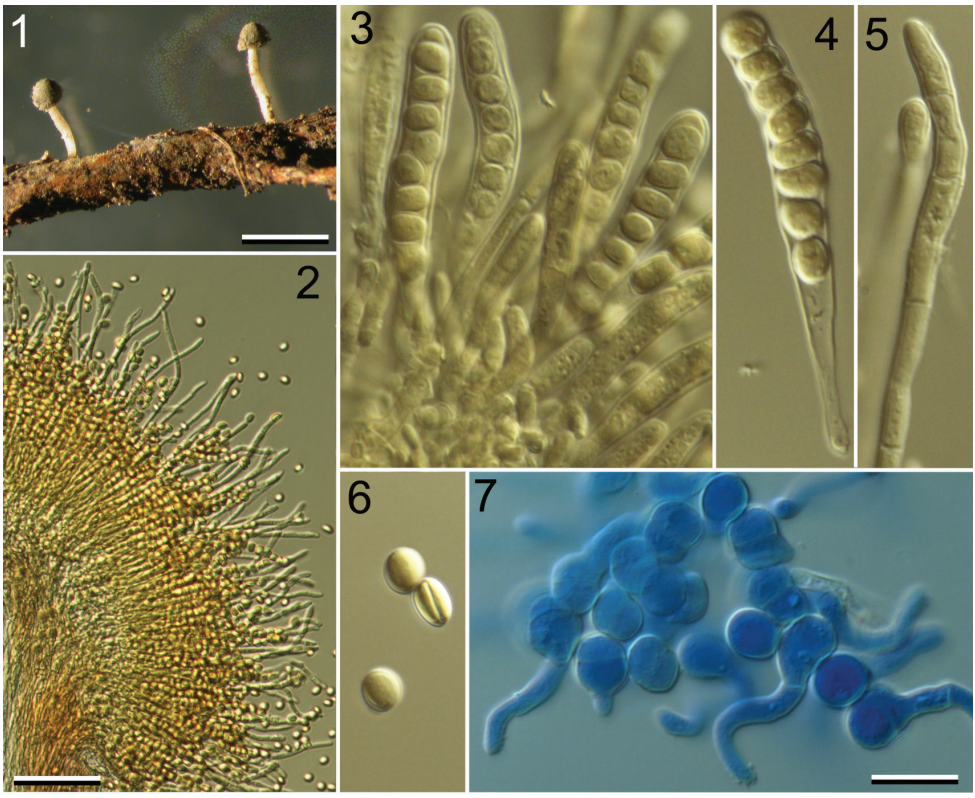

Figure 1. *Roesleria subterranea* (TNS-F-38701). **1** Growing habits of two mature mazaedia on the root tip of an unidentified tree **2** Hymenium of mazaedium, showing protruding paraphyses **3** Enlarged hymenium showing developmental stages of asci and paraphyses **4** Mature ascus containing eight ascospores **5** Paraphysis **6** Discharged matured ascospores; on the right, a transversely one-septate spore is shown in side view **7** Germination of ascospores on MEYE, showing that germination tubes from one of the two-celled spores **2–6** Mounted in Meltzer's solution **7** Mounted in lacto-aniline blue. *Bars*: **1** = 2 mm; **2** = 50 µm; **3–7** = 10 µm.

We found two specimens (TNS-F-185301 and 185302) of *R. subterranea* deposited in TNS as *Calicium pallidum*, which were reported by Togashi (1950). Although *Calicium pallidum* is a lichen name, it is also a misapplied name for *R. subterranea*. We did not attempt DNA extract analysis of TNS-F-185301 and TNS-F-185302 because the specimens lacked sufficient material to guarantee a DNA, and in our experience, such DNA is damaged due to repeated fumigation.

Genetic variations and geological distribution

The sequence of the D1-D2 region of 28S rDNA was almost identical to the available sequences from European specimens. The present sequence differed from EF608073,

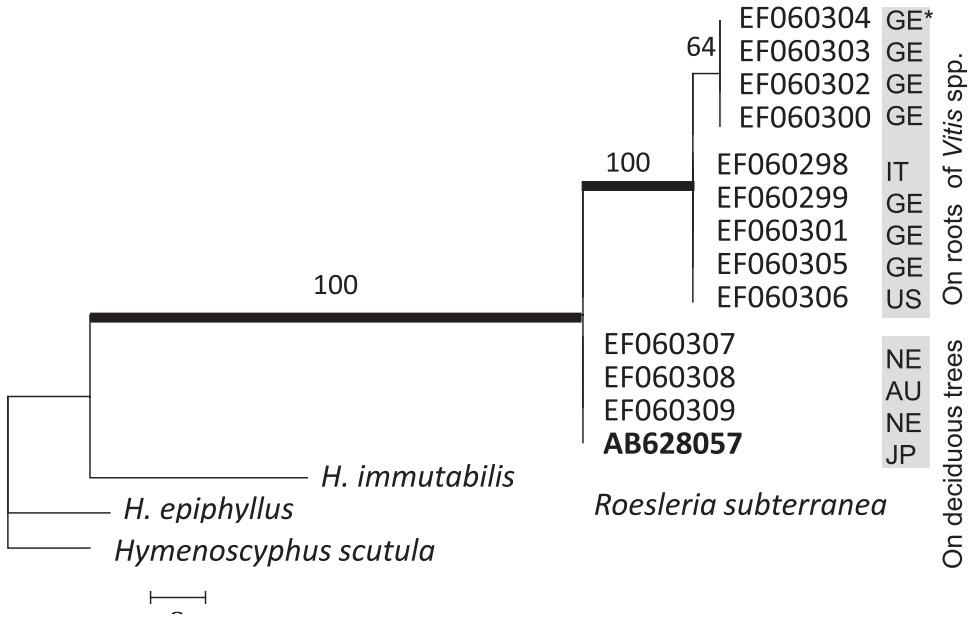

Figure 2. A single most parsimonious tree of *Roesleria subterranea* (ITS-5.8S sequences). Tree length = 31, CI = 0.8065, HI = 0.1935, RI = 0.8723, RC = 0.7035. The tree was constructed from a dataset of all available ITS-5.8S sequences of *R. subterranea* from various hosts and localities. The numbers on the branches indicate the bootstrap values (BP) of 1000 replications in maximum parsimony analysis when the BP exceeds 50%. The scale bar indicates the number of substitutions. Abbreviations indicate the localities of the isolates: AU, Austria; GE, German; IT, Italy; NE, Netherlands; US, USA; JP, Japan.

EF608074, and EF608075 at three, one, and four sites, respectively, among the 554 sites that were compared. The ITS-5.8S sequence was almost identical to the available sequences, and the identification based on morphology was confirmed.

In the MP analysis, we incorporated the available ITS-5.8S sequences of *R. subterranea,* using the three species of *Hymenoscyphus* used in Kirchmair et al. (2008) as outgroups. The aligned dataset included 466 characters, of which 28 characters were excluded from the analyses due to ambiguous alignment. The remaining 438 characters were used for the analysis, including a total of 23 parsimony-informative characters.

A single most parsimonious tree was obtained (Fig. 2). Within the tree, a monophyly of isolates from *Vitis* spp. from Germany, Italy and USA was strongly supported. The remaining group of this clade were found to be paraphyletic and composed of the isolates from various deciduous trees. The sequence obtained from the Japanese isolate (NBRC 108276) was included in this group (Fig. 2). Although the sequence of the latter group seemed to be identical in Fig. 2, the original sequence obtained from the Japanese isolate (AB628057) was not identical with several nucleotide differences to the other three. However, these differences were present only in the ambiguously aligned region, which was excluded from the analysis.

Although the tree topology depends on the choice of outgroup (data not shown), the monophyly of the species as a whole and the monophyly of the clade of isolates from *Vitis* ("*Vitis*-clade") were highly stable (BS values were 100 in the present data and also in Kirchmair et al. (2008)). However, a group of isolates from other hosts besides *Vitis* ("non-*Vitis* group") was paraphyletic. This result suggests that the host specificity was more important than the geographical distance as a factor for infraspecific genetic diversification.

All of the known isolates of the "*Vitis* clade" were obtained from cultivated grape samples. On the other hand, isolates of "non-*Vitis* grade" including our isolate were from the roots of *Malus sylvesris*, *Populus* sp. and other unknown wild deciduous trees. It is therefore hypothesized that isolates highly specific to *Vitis* are more widely distributed in the cultivated grape fields than is currently recognized. To clarify whether such a host specificity is present or not, inoculation experiments are indispensable. In addition, further isolates from various hosts and localities should be collected and examined to know the infraspecific relationships of the species.

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Buwchfawromyces eastonii gen. nov., sp. nov.: a new anaerobic fungus (Neocallimastigomycota) isolated from buffalo faeces

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Abstract

The novel anaerobic fungus *Buwchfawromyces eastonii* **gen. nov.**, **sp. nov.**, belonging to order Neocallimastigales (phylum Neocallimastigomycota) is described. Morphologically similar to *Piromyces* but genetically quite distinct, this fungus (isolate GE09) was first isolated from buffalo faeces in west Wales and then subsequently isolated from sheep, cattle and horse in the same area. Phylogenetic analysis of LSU and ITS sequence confirmed that *B. eastonii* isolates formed a distinct clade close to the polycentric *Anaeromyces* spp. The morphology of GE09 is monocentric with monoflagellate zoospores. However, the sporangial stalk (sporangiophore) is often distinctly swollen and the proximal regions of the rhizoidal system twisted in appearance.

Key words

Ruminant, symbiosis, fungal phylogenetics, buffalo, DNA barcoding

Introduction

At present six genera of anaerobic fungi are recognised, differentiated by thallus morphology and zoospore flagellation (Gruninger et al. 2014). However, the lack of other reliable morphological features, combined with difficulties in the cultivation of these fungi, exchange of cultures between different labs and their morphological variability in culture, has hindered more definitive study of their diversity. As the amount of DNA barcode data (mostly based on the internal transcribed spacer regions, ITS1 and ITS2), both for pure cultures and from environmental DNA sequencing projects has increased, it has become apparent that the six existing clades represent only a subset of the full diversity of the anaerobic fungi (Griffith et al. 2010). For instance, Liggenstoffer et al. (2010) generated 250,000 ITS1 sequences from a diverse range of host species and identified eight potentially novel clades. Furthermore, reconciliation of morphological features and DNA barcode data has led to the reassignment of some taxa (Fliegerová et al. 2012). Following revision of the taxonomy of kingdom Fungi, the anaerobic fungi are now assinged to phylum Neocallimastigomycota (Hibbett et al. 2007). However, the status of the anaerobic fungi as a distinct phylum as opposed to a class within phylum Chytridiomycota remains a matter of debate (Frey 2012; Powell and Letcher 2014).

The genus *Anaeromyces* was first discovered by Breton et al. (1990), describing *Anaeromyces mucronatus*. A near-simultaneous publication by Ho et al. (1990) named a morphologically similar fungus as *Ruminomyces elegans* but in recognition of the rules of priority, Ho and Barr (1993a) later renamed this species as *A. elegans*. Neither viable cultures nor DNA from these original isolates remains (Prof. Yin Wan Ho and Dr. Brigitte Gaillard-Martinie, pers. comms.), though the >80 ITS sequences submitted to GenBank under this genus name suggests that cultures conforming to the morphological description of *Anaeromyces* (uniflagellate zoospores, polycentric mycelium) are commonly isolated from the digestive tracts of different types of herbivorous mammals.

The taxonomic status of one isolate (GE09) is addressed, for which data were previously submitted to GenBank (five ITS cloned from this single isolate; EU414755–EU414759) under the generic name *Anaeromyces* (Edwards et al. 2008). More detailed genetic analysis has been conducted to clarify its taxonomic position, along with its morphological characterisation. Microscopic examination and phylogenetic reconstruction, using both the ITS1 and LSU regions of the rRNA locus, showed that GE09 and three other similar isolates (isolated from different host species) were clearly distinct from the main *Anaeromyces* clade. We therefore conclude that these four isolates represent a new genus, which we name *Buwchfawromyces* gen. nov.

Methods

Enrichment culture from faeces

Anaerobic fungal cultures were enriched using a basal medium (Davies et al. 1993; Orpin 1977) supplemented with milled wheat straw. The basal medium was made up as follows: 150 ml salts solution 1 (3 g.L^{-1} K_2HPO_4 in distilled water), 150 ml salts solution 2 (3 g KH_2PO_4, 6 g $(NH_4)_2SO_4$, 6 g NaCl, 0.6 g $MgSO_4.7H_2O$ and 0.5 g $CaCl_2$; dissolved in 1 L distilled water, in that order), yeast extract (3 g; Oxoid, Basingstoke, UK), tryptone (10 g; Fisher Scientific Ltd., Loughborough, UK), resazurin (2 ml of 0.1% solution) and hemin (2 ml of 0.05% solution dissolved in 1:1 ethanol / 50 mM NaOH) were added and the volume made up to 850 ml with distilled water. After boiling (until light red in colour) and cooling, 150 ml centrifuged (clarified) rumen fluid, 6 g $NaHCO_3$ and 1 g L-cysteine-HCl were added (final volume 1 L). The resultant solution was then deoxygenated by gassing with CO_2 for 1 h. The medium was dispensed anaerobically in 9 ml aliquots into 15 ml Hungate tubes containing milled wheat straw (5 mg.ml^{-1}; 0.5% w/v; dry-sieved through 2 mm mesh). Tubes were then capped with a polypropylene bung held in place by a screw cap, and autoclaved (121 °C/15 min).

Freshly voided faecal samples (ca. 20 g) were collected and transported to the laboratory within 1 h. Aliquots (ca.10 g fresh matter) of these samples were then homogenised for 2 min with 90 ml of pre-warmed (39 °C) basal medium (without wheat straw, yeast extract or tryptone) in a pre-sterilised Stomacher 400 Circulator Bag (polythene; 177 × 305 mm) using a Seward Stomacher 400 Circulator Paddle Blender (Seward Ltd., Worthing, W. Sussex, UK). A 10-fold serial dilution of this faecal homogenate was then prepared in pre-warmed basal medium (1 ml transferred to 9 ml basal medium in 15 ml Hungate tube). The 10^{-4}, 10^{-5} and 10^{-6} dilutions were used to inoculate (1 ml) the tubes of basal medium (9 ml) supplemented with wheat straw. A mixed solution of penicillin, ampicillin and streptomycin sulphate in 50% (v/v) ethanol (5 mg.ml^{-1} of each; 10 ml.L^{-1} added to give a final medium concentration of 50 μg.ml^{-1}) was also added to tubes before they were recapped, in order to inhibit bacterial growth. Tubes were incubated in the dark at (39 °C), and routine subculture was conducted at 3–5 d intervals. Exposure of the samples to oxygen was prevented by undertaking the manipulations in a box flushed with CO_2. Cultures were also grown on basal medium containing cellobiose (5 mg.ml^{-1}) instead of wheat straw. Purity of the isolates was ensured by three cycles of cultivation in roll tubes (Joblin 1981), with well-separated colonies being excised for subculture with a mycological spear.

For cryopreservation of cultures, a method based on the procedures suggested by Sakurada et al. (1995) and Yarlett et al. (1986) was devised. A 5× cryopreservation solution was prepared by mixing 49.7 g ethylene glycol with 155 ml clarified rumen, 200 μl of 0.1% resazurin, 0.2 g L-cysteine and 1.2 g $NaHCO_3$ under anaerobic conditions (total volume 200 ml; 3.2 M ethylene glycol). This solution was bubbled with CO_2 for ca. 3 h, prior to anaerobically dispensing 10 ml aliquots into Hungate tubes which were autoclaved and stored at -20 °C until use. The 5× cryopreservation solution was added

to 3–5 d old wheat straw cultures (2.5 ml per 10 ml culture) under anaerobic conditions in Hungate tubes. After mixing, tubes were chilled in ice water for 15 min and then anaerobically dispensed in 2 ml aliquots into sterile 2 ml cryovials. Cryovials were placed at -80 °C overnight, before being transferred to liquid nitrogen for longer-term storage. Storage of cryopreserved cultures at -80 °C is possible for up to a few months but for prolonged storage, liquid nitrogen was found to be much more reliable.

Microscopy

Microscopy was conducted on cultures, grown on either wheat straw or cellobiose, using an epifluorescence microscope (Olympus BX50) with images recorded using a Nikon Coolpix 995 digital camera. For visualisation of nuclei, DAPI (0.3 $mg.ml^{-1}$ in 50 mM Tris-HCl, pH 7.2) was added, and for enhanced definition of cell walls and septa, Calcofluor white M2R (100 μM; Day et al. 2002) was used (UV-W filters: 365 nm excitation/420 nm emission).

Phylogenetic analysis

DNA extraction was carried out using the CTAB (hexadecyltrimethylammonium bromide) method of Doyle and Doyle (1987), with modifications as described by Griffith and Shaw (1998). Cultures were harvested after 3–6 d incubation, and the biomass washed three times with sterile distilled water before being freeze–dried and ground to a powder. Ground biomass (ca. 50 mg) was used for DNA extraction, and the purified DNA resuspended in 50 μl TE buffer (pH 8.0) before being stored at -20 °C.

For genetic analysis, the D1/D2 domain of large-subunit (LSU) ribosomal DNA and internal transcribed spacer 1 (spanning ITS1 and ITS2) were amplified, using the primer pairs NL1 (GCATATCAATAAGCGGAGGAAAAG) / NL4 (GGTCCGTGTTTCAAGACGG) (Dagar et al. 2011; Fliegerová et al. 2006) and GM1 (TGTACACACCGCCCGTC) / MN106 (CGTTGTAAAACACTCAWAACC) (Edwards et al. 2008), respectively. Sequence management was conducted within the Geneious (v6.1.6) bioinformatics package (Drummond et al. 2011), using MAFFT (Katoh et al. 2002) for sequence alignment (default settings).

Phylogenetic reconstruction was conducted using TOPALi (v2.5) (Milne et al. 2004). For LSU analysis, maximum likelihood analysis was conducted using PhyML (Guindon et al. 2010) and the TrN+gamma substitution model recommended by TOPALi. For analysis of the ITS, only the ITS1 region was used since the great majority of available sequences cover only this region (and not ITS2). To establish the phylogenetic position of isolate GE09, ITS1 sequences derived from five clones were aligned with environmental and isolate sequences belonging to the four closely related monoflagellate genera. The resulting MAFFT alignment was trimmed to include only 15 bp of the flanking 18S and 5.8S regions, and duplicate sequences were removed

(retaining sequences relating to cultured fungi, if present). For ITS1 analysis, maximum likelihood analysis was conducted using PhyML (Guindon et al. 2010) and the GTR substitution model recommended by TOPALi. Both the ITS1 and LSU alignments have been submitted to TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S16672).

Results and discussion

Ecology

The isolate GE09 was originally isolated from the faeces of a domesticated Asian water buffalo (*Bubalus bubalis*) on 26th Feb 2004 at Panthwylog Farm, Llanon, Ceredigion, Wales (N52.279; W-4.169). The buffalo was part of a herd kept outdoors, maintained on grass pasture supplemented with grass silage. More recently, three additional pure cultures (each from a different host species have been isolated), all identical in morphology and LSU DNA barcode to GE09. These isolates were obtained as follows: from cow (*Bos taurus*) faeces (isolate HoCal4.C3.3; Nant yr Arian, Ponterwyd, Ceredigion; 6th Feb 2013; N52.416; W-3.891), sheep (*Ovis aries*) faeces (HoCal4.B3c, also Nant yr Arian, 6th Feb 2013) and horse (*Equus ferus caballus*) faeces (isolate HoCal4.D1.2; Aberystwyth University Lluest livery yard; 6th Feb 2013; N52.410; W-4.051).

Morphology

Thalli of isolate GE09, when grown on wheat straw or cellobiose as a carbon source, were consistently monocentric, with rhizoids radiating from a single developing sporangium. Mature sporangia were spherical to ovoid 30 to 80 μm long and 20 to 60 μm wide (Fig. 1). No apical projections, as found in *Anaeromyces mucronatus* (Breton et al. 1990) or *Piromyces mae* (Li et al. 1990) (referred to as a mucro or papilla respectively by these authors), were observed.

Zoospores (spherical; diameter 9–11 μm) were readily observed in 3–5 d old cultures grown on wheat straw and consistently bore a single flagellum (30–40 μm long; 3–4× longer than the length of the zoospore body). However, the process whereby zoospores were released from the sporangium was not observed. On a single occasion, a large (30 μm diameter) zoospore-like structure bearing numerous flagella, each emerging from a different point on the zoospore body, was detected (Suppl. material 1A). This is possibly the result of the agglomeration and fusion of numerous zoospores (hence the numerous flagella that are visible), similar to the structure previously observed by Orpin (1975) in *N. frontalis* (Orpin's Fig. 2; Suppl. material 1B).

The most distinctive feature of the thalli of GE09 were the swollen sporangiophores (40–80 μm long and 15–50 μm wide), occasionally comparable in volume to the sporangium they supported (Fig. 1E–H). Also visible was a septum at the point

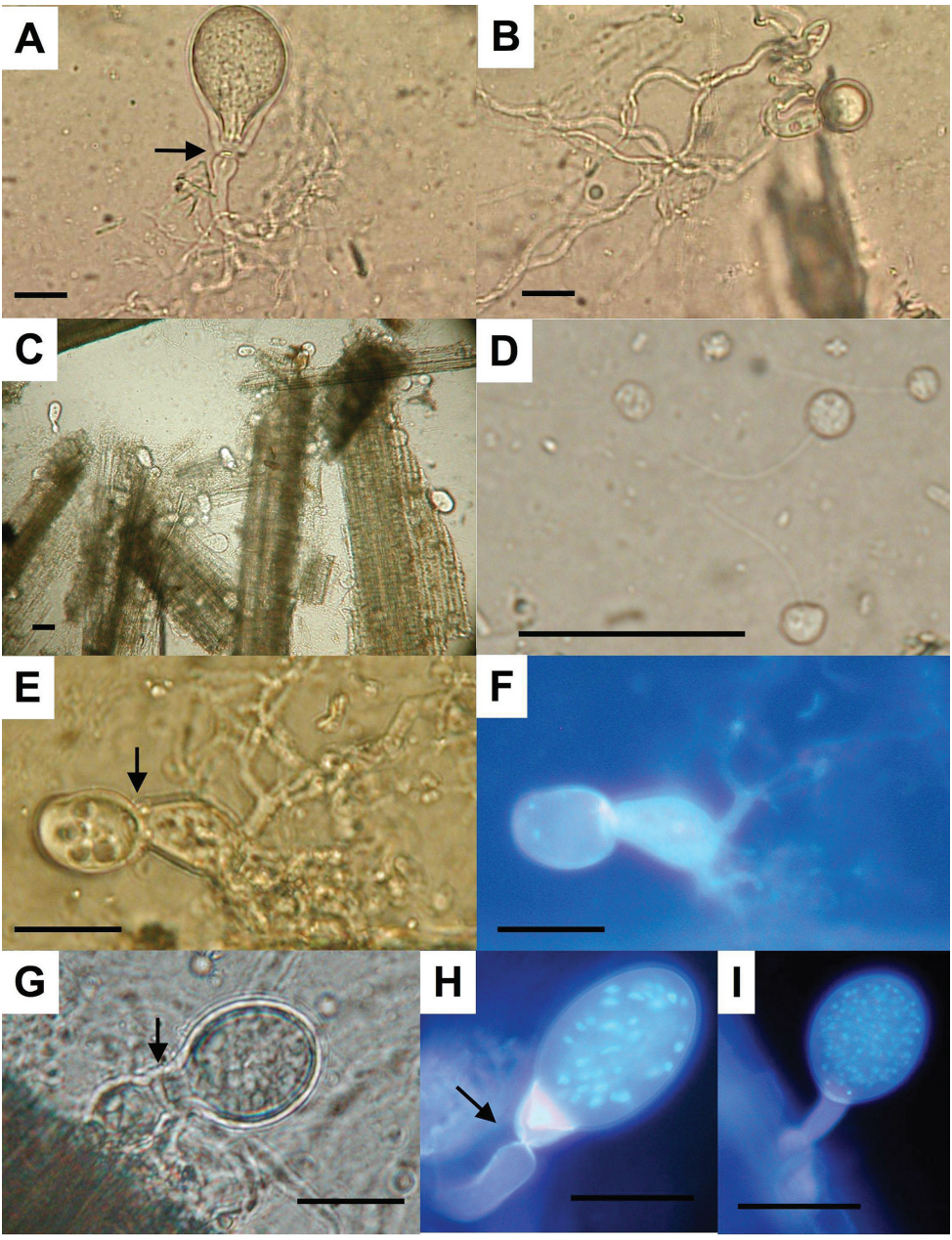

Figure 1. Morphology of *Buwchfawromyces eastonii*. Sporangia are ovoid (**A**) to spherical (**B**), tending to be more elongate when growing on straw particles (**C**). Zoospores are uniformly monoflagellate (**D**). A distinct septum is visible where the sporangium is attached to the sporangiophore (**A, E, G, H** arrowed) and sporangiophores are often swollen (**E–H**). Nuclei were not observed in sporangiophores or rhizoids (**F, H, I**). Scalebar indicates 50 μm.

where the sporangium joined the sporangiophore (Fig. 1A, E–I). The sporangiophore was contiguous with the rhizoids which tapered (from 20 μm to 5 μm) and branched (Fig. 1B). The proximal rhizoids (within 100 μm of the sporangium) were often contorted (Fig. 1B). DAPI staining was used to observe the location of nuclei within the thalli (Fig. 1F, H, I); these were abundant in sporangia but none were observed in the sporangiophores or rhizoids.

The swollen sporangiophores and twisted rhizoids observed here are very similar to those noted by Ho et al. (1993b) in *Piromyces spiralis* (Suppl. material 1C, D). Also similar are the swollen sporangiophores reported in *P. mae* (Li et al. 1990) (isolate PN11 from horse; Fig. 25). It is also noteworthy that *Anaeromyces* (formerly *Piromyces*) *polycephalus* (Suppl. material 1E) (Chen et al. 2002; Kirk 2012), whilst forming multiple rather than single sporangia, also forms a distinctly swollen sporangiophore. It is possible that such structures play some role in physical disruption of the substrate, as is the case for the bulbous holdfasts formed by *Caecomyces* and *Cyllamyces* spp.

Cultures of GE09 maintained viability, and could be subcultured, after incubation at 39 °C for periods of several weeks. This raised the possibility that these cultures may form long-term survival structures (McGranaghan et al. 1999; Struchtemeyer et al. 2014). Thick-walled and septate structures (3–4 septa; 30–40 μm long × 10–15 μm wide), very similar to those previously observed in *Anaeromyces* sp. EO2 (Brookman et al. 2000) were seen in wheat straw cultures incubated for 28 d (Suppl. material 1F, G) and never observed in younger (3–5 d old) cultures. However, detailed examination of the development of these putative resting structures was not undertaken.

Phylogenetic analysis

Detailed examination of isolate GE09 was not undertaken when it was first isolated. However, it was used as a reference sample in a study of the colonisation of forage by anaerobic fungi (Edwards et al. 2008), in the course of which the ITS1/2 spacer regions were amplified and cloned. The sequences of the five clones were submitted to GenBank (EU414755–EU414759) under the generic name *Anaeromyces*, since these sequences clustered close to the *Anaeromyces* clade at that time. However, more detailed analysis has since suggested that this isolate is quite distinct from *Anaeromyces* (Kittelmann et al. 2012).

The sequence of the D1/D2 domains (ca. 750 bp) of the LSU of GE09 and the three other isolates were identical (submitted to GenBank as KP205570). These sequences were aligned with 36 other LSU sequences (from GenBank) covering all the known genera of Neocallimastigomycota (700 bp alignment; 188 phylogenetically informative sites), and including the outgroup taxon *Gromochytrium mamkaevae* (Chytridiomycota). Phylogenetic reconstruction consistently recovered *Buwchfawromyces* isolates as a distinct clade (85% bootstrap support). LSU sequences from *Anaeromyces*, *Neocallimastix* and *Orpinomyces* were also recovered as distinct clades with strong (≥80%) bootstrap support (Fig. 2). *Neocallimastix* and *Orpinomyces* were more

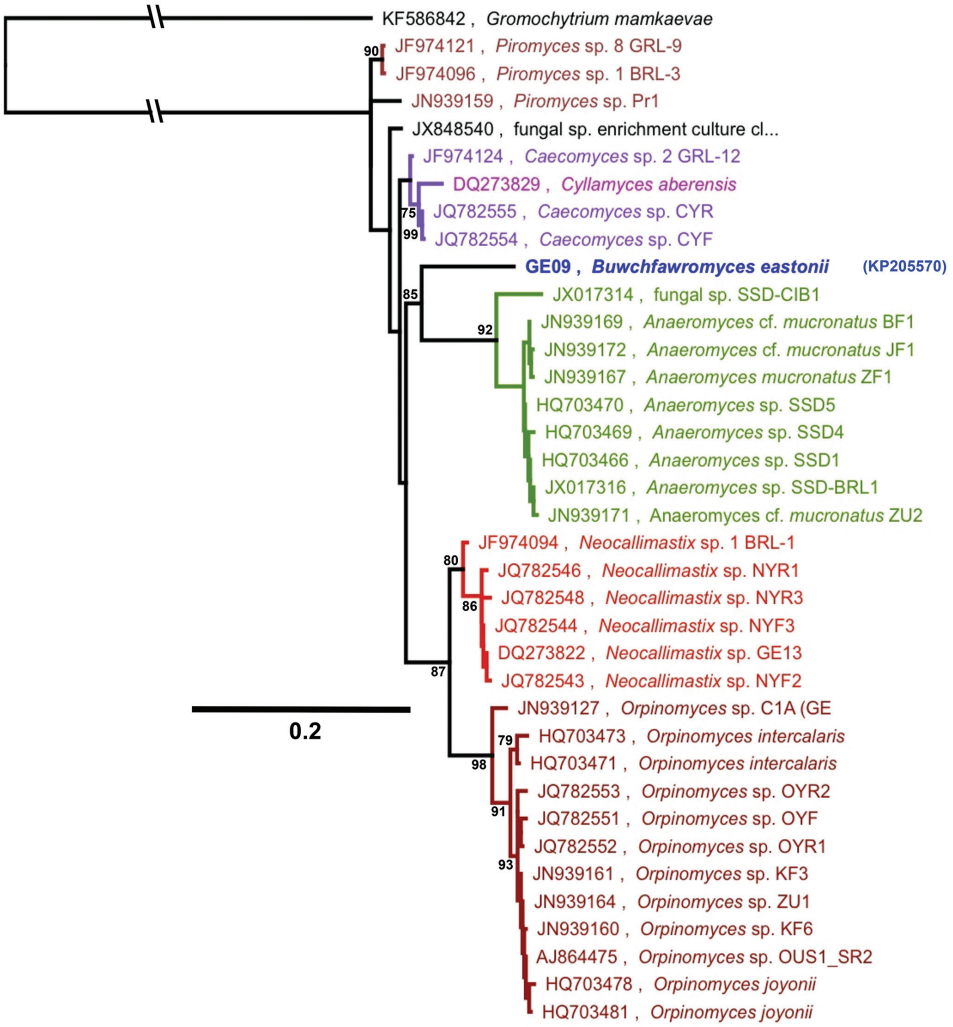

Figure 2. Maximum likelihood tree based on alignment of the D1/D2 region of the Large Ribosomal Subunit (700 bp alignment; 37 sequences; 188 phylogenetically informative sites; TrN+gamma model). Bootstrap values over 70% are shown (1000 replicates). Scale bar indicates number of substitutions per site.

closely related to each other than to other genera, consistent with the occurrence of polyflagellate flagella in these genera, a feature not found in other flagellate fungi (James et al. 2006). The genera forming bulbous holdfasts (*Caecomyces*, *Cyllamyces*) also formed a distinct clade, and *Piromyces* isolates occupied a basal position but without strong bootstrap support.

Whilst analysis of the LSU proved informative to confirm the distinctiveness of the GE09 clade, there are relatively few LSU sequences available in GenBank for comparison. Therefore, phylogenetic analysis of the ITS1 internal transcribed spacer region, for which there are hundreds of published sequences, was conducted (357

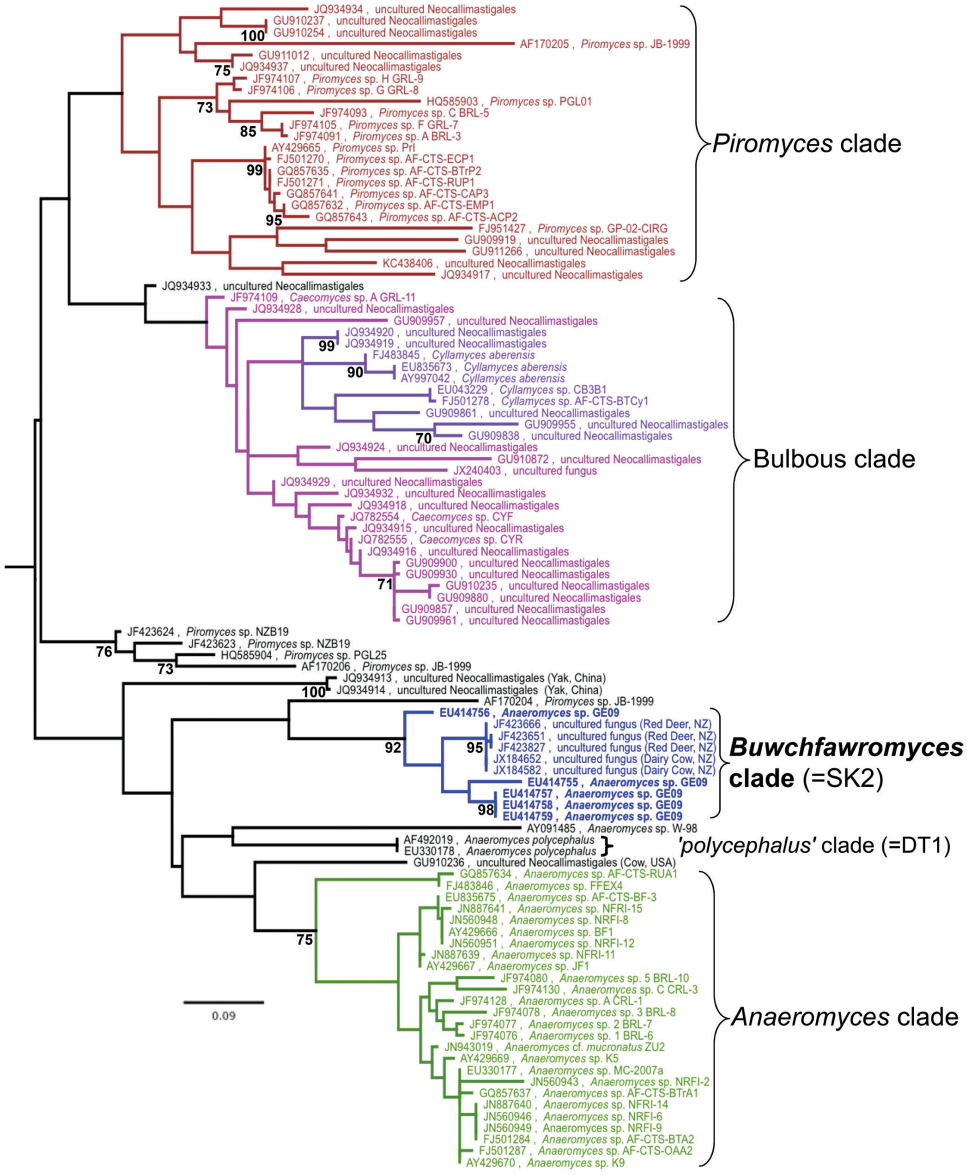

Figure 3. Maximum likelihood tree based on alignment (357 bp) of the ITS1 region. Midpoint rooting was used to root the tree and bootstrap values over 70% are shown (1000 replicates). Scalebar shows the number of substitutions per site. Clades corresponding to the known genera, the new *Buwchfawromyces* clade and also the '*polycephalus*' clade are labelled. Codes in brackets indicate the novel clades identified by Koetschan et al. (2014).

bp alignment; 271 phylogenetically informative sites, 101 sequences) (Fig. 3). Since analysis of the LSU sequences had shown members of the genera *Neocallimastix* and *Orpinomyces* to be quite distinct, these were omitted from analyses of the ITS1 region

in order to improve the quality of the alignments (fewer gaps). Additionally, 'environmental' sequences obtained from clone library studies, (mostly labelled as "uncultured Neocallimastigales") were also included, as recommended by Nilsson et al. (2011).

The *Buwchfawromyces* (GE09) clade was again recovered with high (92%) bootstrap support, as was the *Anaeromyces* clade. Five environmental sequences, all from New Zealand (from cow or red deer; Fig. 2 and listed in Suppl. material 4) also fall into the *Buwchfawromyces* clade. This clade was also identified (and denoted as clade SK2) in the recent study by Koetschan et al. (2014), who were able to create a reliable phylogeny by using predictors of ITS1 folding to decrease the effects of gaps in anaerobic fungal ITS1 alignments.

The largest survey of anaerobic fungi conducted to date is that of Liggenstoffer et al. (2010). They obtained ca. 250,000 ITS1 sequences, from the faeces of 30 herbivore species kept in Oklahoma zoos (Liggenstoffer et al. 2010), including three of the four herbivore species from which we cultured *Buwchfawromyces*. It was surprising that such a large dataset should yield no sequences falling into the *Buwchfawromyces* clade. However, examination of the primers used by Liggenstoffer et al. (2010) revealed the presence of mismatches between the forward primer (MN100modified; 5'-TCCTACCCTTTGTGAATTTG-3') and the cognate sequences found in members of the *Buwchfawromyces* clade (TCCTACCCTTTGTGAATT***GT*** or TCCTTACCCTTTGTGAA***C***T***GA***) (Suppl. material 2). These mismatches would very likely have caused significant primer bias and thus poor amplification of any *Buwchfawromyces* spp. present.

Five cloned ITS1/2 PCR amplicons of GE09 were originally submitted to GenBank (EU414755–EU414759). These reveal an extremely high level of sequence divergence (<27 polymorphisms within the ca. 200bp ITS1 region; 87.1%–99.5% identity, (Suppl. material 3). High levels of intragenomic variation has also been found in other anaerobic fungi (Ozkose 2001), and also in some other fungal taxa, for instance phylum Glomeromycota (Pawlowska and Taylor 2004; Pringle et al. 2000). For GE09, the most divergent of these clones (EU414756) was more distantly related to the other GE09 clones than were sequences from New Zealand (Fig. 3). A cut-off level of 97% identity is often used to define species or OTUs (operational taxonomic units) in mycology (Nilsson et al. 2008; Yamamoto and Bibby 2014). However, such high levels of intragenomic variation, make it very difficult to generate reliable species hypotheses (Koljalg et al. 2013) for the Neocallimastigomycota based on ITS sequences alone, although the delineation of different genera is still possible.

We consider that isolate GE09 and the three other similar fungi also isolated in the Aberystwyth area represent a new genus *Buwchfawromyces* within the phylum Neocallimastigomycota. It is not possible for *Buwchfawromyces* to be placed within the related genus *Anaeromyces*, since it forms a monocentric thallus not consistent with the circumscription of this genus (Breton et al. 1990) which comprises species with polycentric thalli. Close to *Buwchfawromyces* and *Anaeromyces*, is the species currently known as *Anaeromyces* (formerly *Piromyces*) *polycephalus*. This species has a distinctive morphology (Suppl. material 1E), with multiple sporangia arising from a swollen spo-

rangiophore, and with an anucleate rhizoidal system. It was originally isolated and described from buffalo in Taiwan (isolate W-33; (Chen et al. 2002)) and has since been reported from India (isolate CTS-47 from zebra faeces; GenBank EU330178); this clade was denoted DT1 (Fig. 3) by Koetschan et al. (2014). Given that '*A. polycephalus*' is both morphologically and genetically distinct, this species should be renamed.

The unusually high level of intragenomic variation in ITS1 makes it difficult to nominate a single reference sequence, therefore two are presented (EU414755 and EU414756).

Diagnosis

***Buwchfawromyces* Callaghan, Tony & G.W. Griff., gen. nov.**
Registration identifier: IF550797

Note. Strictly anaerobic fungus with determinate, monocentric thallus with single, spherical to ovoid terminal sporangium (often with swollen sporangiophore) and forming uniflagellate zoospores. The clade is defined by the sequences EU414755 and EU414756 (ITS1, 5.8S, ITS2 complete), and also KP205570 (LSU, partial sequence). The most genetically similar genus is *Anaeromyces*, which is defined as forming a polycentric thallus (Breton et al. 1990. FEMS Microbiol. Lett. 58, p.177), in contrast to the monocentric *Buwchfawromyces*.

***Buwchfawromyces eastonii* Callaghan, Tony & G.W. Griff., gen. nov., sp. nov.**
Registration identifier: IF550798

Note. An obligately anaerobic fungus with determinate monocentric thallus and spherical to ovoid sporangia. Thalli often with a distinctly swollen sporangiophore and twisted rhizoids. Extensive rhizoidal system but sporangiphore and rhizoids lacking nuclei. Sporangia ovoid to spherical (30–80 µm × 20–60 µm), non-papillate. Zoospores formed abundantly, spherical (9–11 µm diameter) with single flagellum (30–40 µm long). The reference sequences for this species are EU414755 and EU414756 (ITS1, 5.8S, ITS2), and KP205570 (LSU, D1/D2 regions). Since intragenomic variation in ITS1 sequence is present, the ITS1 sequence *B. eastonii* is defined as the least inclusive clade containing both EU414755 and EU414756. The type culture (isolate GE09) is stored cryogenically in liquid nitrogen at Aberystwyth University. Type material from 3 d old cultures and preserved in 5% glutaraldehyde is lodged in the biorepositories at: Aberystwyth University (code ABS) with isotype material at Royal Botanic Gardens, Kew, London (K); and Friedrich-Schiller-Universität Jena, Germany (JE).

Etymology. From the Welsh words for large cow ('buwch fawr'), since the original isolate GE09 was isolated from a buffalo for which there is no Welsh word. The specific epithet in honour of our former colleague Gary Easton who isolated this fungus.

Nomenclature. *Buwchfawromyces* Callaghan, Tony & G.W. Griff., gen. nov. Strictly anaerobic fungus forming a monocentric thallus with a single sporangium, usually borne on a swollen sporangiophore connected to a branching and twisted rhizoidal system. Zoospores are spherical and uniflagellate. "*Buwch fawr*" means large cow in Welsh.

Conclusions

It has been apparent since the widespread use of DNA sequence data to identify anaerobic fungi that many of the sequences currently lodged with GenBank do not fall into any of the currently recognised genera of the Neocallimastigomycota. The situation is further complicated by the presence of many sequences from isolates which are very likely misidentified, a phenomenon which is also problematic for other groups of Fungi (Schoch et al. 2014).

A new genus is described based on on a pure culture which was isolated a decade ago and for which ITS1 sequence has been lodged with GenBank since 2008. The RefSeq project aims to resolve this, and other related issues, by linking ITS and LSU sequences from vouchered reference specimens to accepted names (Schoch et al. 2014). As shown above, ITS sequences for anaerobic fungi can be problematic due to intragenomic sequence variation. Similar problems have not been found for the more conserved LSU region of the rRNA locus (Dagar et al. 2011; Eckart et al. 2010), highlighting the synergy of using both loci. We also note that analysis of LSU data does have the distinct advantage of yielding robust alignments across a wide range of basal fungal taxa (including Chytridiomycetes) and being amenable to direct sequencing of PCR products.

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Supplementary material 1

SuppFig. 1

Authors: Tony Martin Callaghan, Sabine Marie Podmirseg, Daniel Hohlweck, Joan Elizabeth Edwards, Anil Kumar Puniya, Sumit Singh Dagar, Gareth Wyn Griffith

Data type: Adobe PDF file

Explanation note: Putative fused zoospores in isolate GE09 (A) and the similar structure reported by Orpin (1975) (B). Swollen sporangiophores and twisted rhizoids, as found in isolate GE09, were also reported for *Piromyces spiralis* (C, D) by Ho et al. (1993), whilst *Anaeromyces* (formerly *Piromyces*) *polycephalus* also forms a swollen sporangiophore (E). In older cultures of GE09, thick walled putative spore structures were frequently observed (F, G). Scalebar indicates 20 μm. A video of the putative fused zoospore is found at: https://www.youtube.com/watch?v=im14hz1jiX0.

Supplementary material 2

SuppFig. 2

Authors: Tony Martin Callaghan, Sabine Marie Podmirseg, Daniel Hohlweck, Joan Elizabeth Edwards, Anil Kumar Puniya, Sumit Singh Dagar, Gareth Wyn Griffith

Data type: Adobe PDF file

Explanation note: Alignment of part of the ITS1 region across a range of anaerobic fungi from all the known genera. The sequences of the modified MN100 primer used by Liggenstoffer et al. (2010) (TCCTACCCTTTGTGAATTTG) is indicated (green). For all clades except *Buwchfawromyces*, there is a good match for this primer. However, for members of the *Buwchfawromyces*, there are several mismatches at the 3' end of the primer binding site which are likely to impede PCR amplification.

Supplementary material 3

SuppFig. 3

Authors: Tony Martin Callaghan, Sabine Marie Podmirseg, Daniel Hohlweck, Joan Elizabeth Edwards, Anil Kumar Puniya, Sumit Singh Dagar, Gareth Wyn Griffith

Data type: Adobe PDF file

Explanation note: Intragenomic variation in ITS sequences among the five clones sequenced from isolate GE09. 18S boundary ends with GATCATTA and 5.8S begins with CAACTTT, according to the convention of Hibbett et al. (1995).

Supplementary material 4

SuppTable 1

Authors: Tony Martin Callaghan, Sabine Marie Podmirseg, Daniel Hohlweck, Joan Elizabeth Edwards, Anil Kumar Puniya, Sumit Singh Dagar, Gareth Wyn Griffith

Data type: Microsoft Excel file

Explanation note: Details of ITS sequences falling into the *Buwchfawromyces* clade. The first five are all derived from the isolate GE09.

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RESEARCH ARTICLE

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Freshwater Ascomycetes: *Jahnula purpurea* (Jahnulales, Dothideomycetes), a new species on submerged wood from Martinique Island, Lesser Antilles

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Abstract

Jahnula purpurea J.Fourn., Raja & Shearer, a new species in the Jahnulales (Dothideomycetes) collected from submerged wood in a freshwater river in Martinique Island, Lesser Antilles, is described and illustrated. The characteristic features of the new species are: globose to subglobose, brownish black ascomata with broad, golden brown, subtending hyphae which stain the underlying wood purple; a peridial wall composed of large pseudoparenchymatic cells, which are *textura angularis* to *prismatica* in surface view; sparsely septate pseudoparaphyses embedded in a gel matrix; clavate to obclavate asci with a short pedicel; brown, one-septate, ellipsoidal, rough-walled ascospores without a gelatinous sheath or appendages. Unfortunately, because limited material was available from the type collection, we were unable to obtain molecular data. *Jahnula purpurea* is distinct from all previously described species of *Jahnula* in its ability to stain the wood purple and in a combination of ascomal, ascus, and ascospore size and morphology.

Key words

Aquatic fungi, Lotic, Submerged wood, Systematics, Morphology

Introduction

The genus *Jahnula*, typified by *Jahnula aquatica* (Plöttner and Kirschst.) Kirschst., is the largest genus in the Jahnulales, Dothideomycetes and currently contains 15 species (Hyde 1993; Hawksworth 1984; Hyde and Wong 1999; Pinruan et al. 2002; Raja and Shearer 2006; Raja et al. 2008; Sivichai and Boonyuen 2010; Suetrong et al. 2011). All species of *Jahnula* have been reported from submerged decorticated wood in freshwater habitats, mostly from tropical/subtropical habitats. Exceptions are *J. aquatica*, *J. apiospora* A. Carter, Raja & Shearer, and *J. sangamonensis* Shearer & Raja, which have been described and reported from fresh water in temperate geographical locations (Hawksworth 1984; Raja and Shearer 2006; Raja et al. 2008). Members of the Jahnulales are primarily reported from freshwater habitats, except *Manglicola guatemalensis* Kohlm. & E. Kohlm, that is a mangrove species (Suetrong et al. 2010).

In this paper, we describe and illustrate a new species of *Jahnula* that was found on submerged wood collected from a freshwater river on Martinique Island, Lesser Antilles.

Methods

The methods for collection, morphological characterization and illustration are outlined in Fournier and Lechat (2010) and Réblová et al. (2015). Asci and hamathecia were mounted in black ink (Pelikan brand) for illustrations. Culturing was attempted, but without success. Since the fungus has been collected only from the type locality thus far and limited material was available to serve as a holotype specimen, attempts made to extract DNA and obtain molecular data from a very small number of ascomata were also unsuccessful. The holotype specimen is deposited in the Illinois Natural History Survey Fungarium (ILLS).

Results

Taxonomy

***Jahnula purpurea* J. Fourn., Raja & Shearer, sp. nov.**
Mycobank: MB811212
Figs 1, 2

Holotype. FWI, Martinique: Prêcheur, Anse Couleuvre, Couleuvre River, coastal rainforest, 14°50'13.05"N, 61°13'22.40"W, on submerged decorticated branch, 03 Jun. 2014, J. Fournier, MJF 14016; **(ILLS 72402)**.

Description. Ascomata 125–185 (–220) µm diam, globose to subglobose, scattered or clustered in small groups, immersed-erumpent, the base remaining immersed in the substrate, brownish black appearing black when dry, attached to subtending

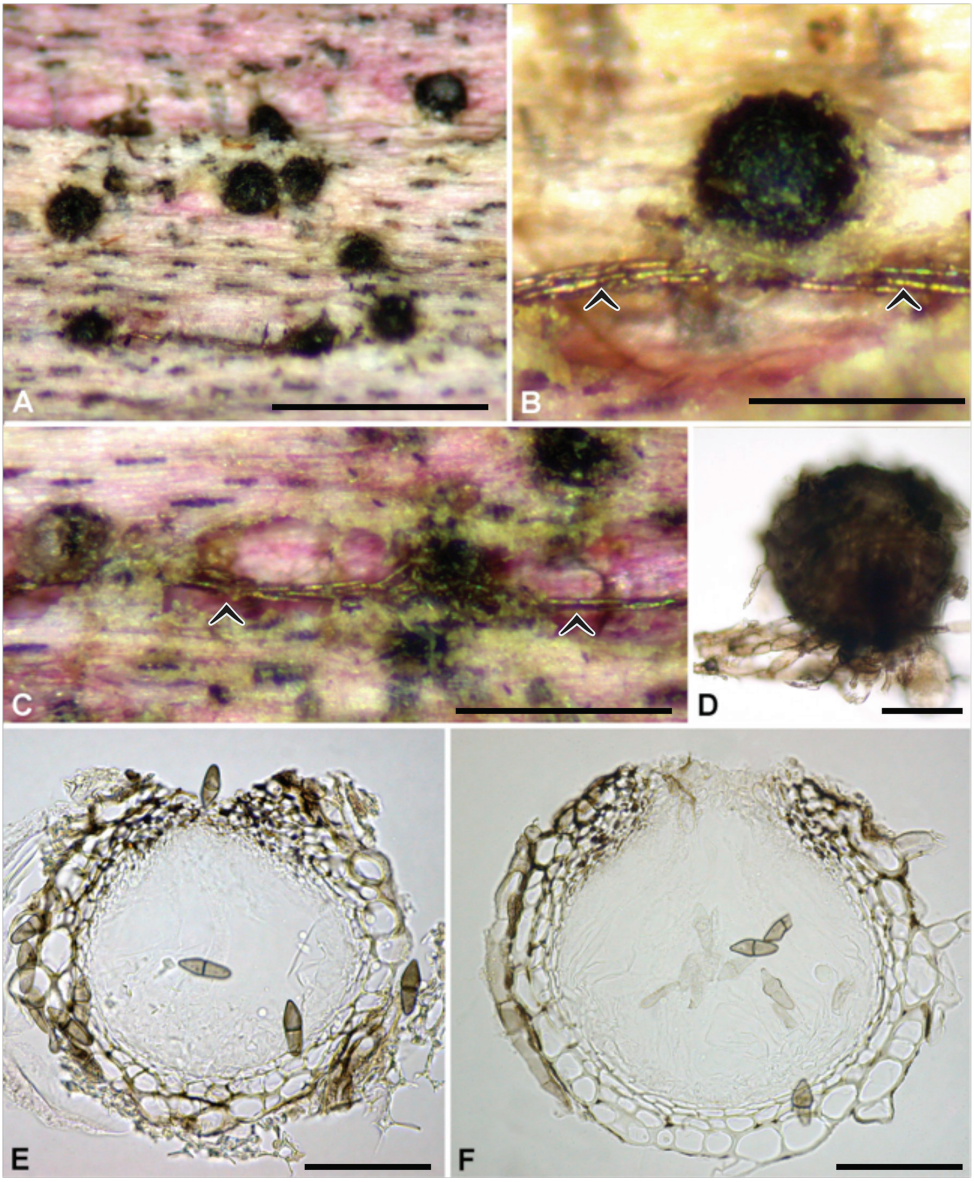

Figure 1.A–F *Jahnula purpurea* (from the HOLOTYPE; MJF 14016, ILLS 72402). **A–C** Ascomata on submerged wood. Note the purple stain. Arrowheads indicate the subtending superficial hyphae on wood, which connect multiple ascomata on wood **D** Ascoma in water showing broad hyphae emerging from the base of the fruiting body **E, F** Longitudinal section through ascoma. Note broad pseudoparenchymatic cells comprising the peridial wall. Scale bars: **A, C** = 500 µm; **B** = 1 mm; **D** = 100 µm; **E–F** = 20 µm;

golden brown hyphae 8–22 µm diam, smooth, slightly constricted at septa; hyphae form cords developing under the wood surface and linking adjacent ascomata (Fig. 1A–D); wood beneath ascomata or at the periphery of the colony stained purple; ostiole

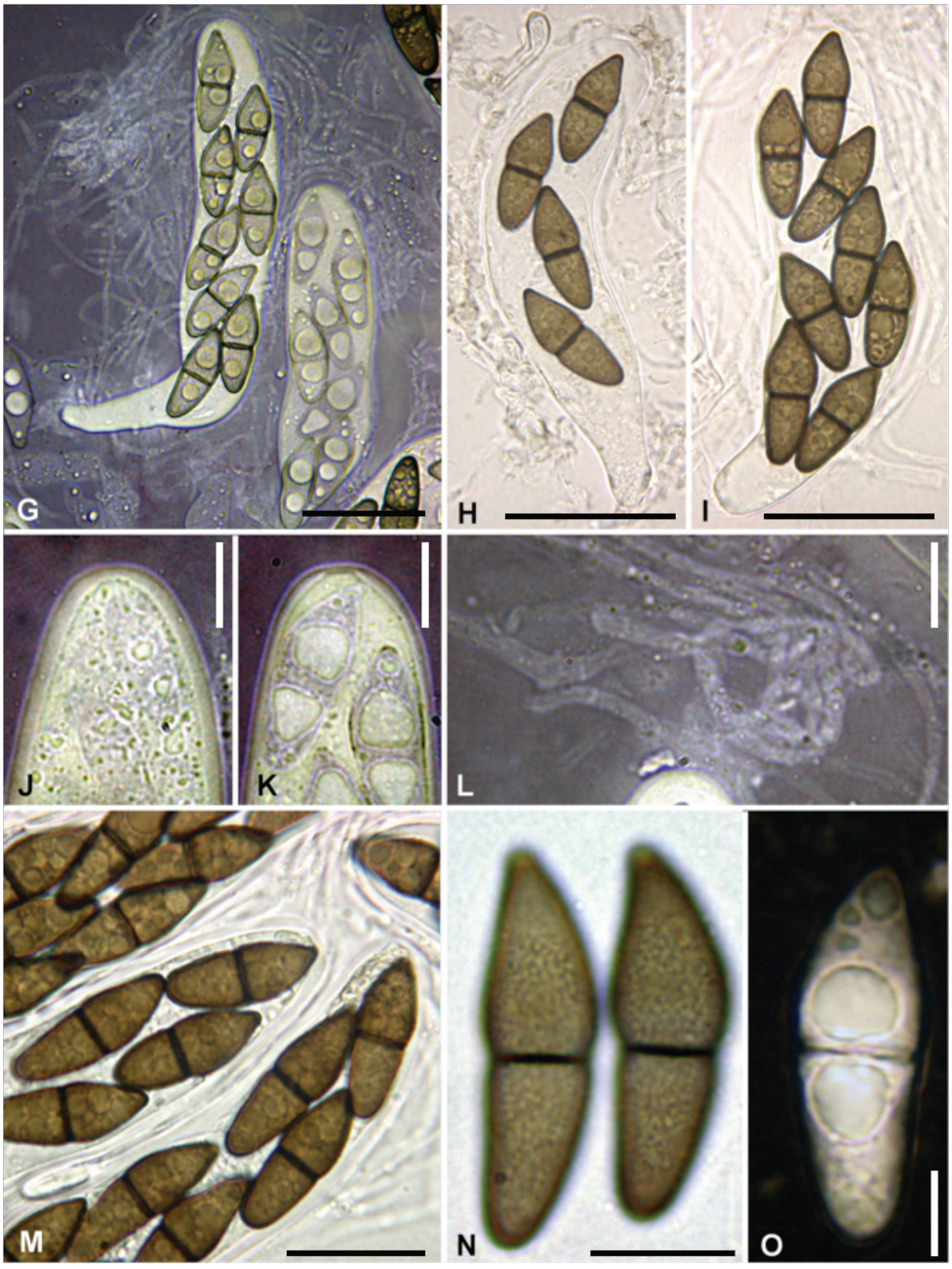

Figure 2. G–I Clavate to obclavate asci. **J, K** Ascus apex showing faint truncate ocular chamber **L** Pseudoparaphyses **M–N** Multiguttulate brown ascospores. Note ascospores showing minutely verrucose warts forming a loose reticulate pattern **O** Immature ascospore in India ink. Scale bars: **G–I, M** = 20 μm; **J–L** = 5 μm; **N, O** = 10 μm.

non-papillate, slightly depressed, pallid, rounded, minute, filled with hyaline periphyses (Fig. 1F). Peridium appearing roughened by protruding cells (Fig. 1B, D), 22–35 µm wide, two-layered: outermost layer *textura angularis,* composed of 1–2 rows of large thick-walled cells 8–18 µm in their greatest dimension, extending into hyphal appendages in places (Fig. 1F), more pigmented in upper half, inwardly lined by smaller hyaline flattened cells forming a *textura prismatica* (Fig. 1E, F). Asci 90–98 × 22.5–25 µm, bitunicate, with fissitunicate dehiscence occurring rarely, clavate to slightly obclavate, shortly pedicellate, 4–8-spored, ascospores 1–3 seriate (Fig. 2G–I); apex without (Fig. 2J) or with a faint truncate ocular chamber (Fig. 2K). Pseudoparaphyses 1–2.5 µm wide, cellular, often contorted, sparsely septate, rarely anastomosing, embedded in a gel matrix (Fig. 2G, L). Ascospores (23) 24–28 (31) × (7) 8–9 µm, (mean = 26 × 8 µm; n = 60), ellipsoid-fusiform, 1-septate, septum median to slightly submedian (0.53, N = 20), slightly constricted at the septum, upper cell wider and often apically pinched, lower cell obtusely rounded (Fig. 2H, I, M), contents densely guttulate; wall medium brown, minutely verrucose with warts partially in contact and forming a loose reticulate pattern (Fig. 2N), visible in hyaline immature ascospores; no sheath or appendages observed in aqueous nigrosin or India ink (Fig. 2O).

Etymology. From Latin "purpureus" referring to the characteristic staining of the substrate purple by this species.

Anamorph. Not known.

Known distribution. Martinique, Lesser Antilles (Known only from type locality thus far).

Discussion

Jahnula purpurea differs from all other species of *Jahnula* in that it stains the wood on which it grows purple. In addition, it is one of the species of *Jahnula*, which has minute ascomata (125–185 µm diameter). The only other species reported to have minute ascomata is *Jahnula marakotii* Sivichai & Boonyeun, a species reported from a submerged wood test block of *Azadirachta indica* from a peat swamp in Thailand (Sivichai and Boonyeun 2010). *Jahnula purpurea* differs from *J. marakotii* in a number of morphological characters such as shape and size of asci and ascospores. The asci of *J. purpurea* are clavate to obclavate (90–98 × 22.5–25 µm), while those of *J. marakotii* are cylindrical (107.5–120 × 9–11.5 µm). The ascospores of *J. purpurea* do not possess apical appendages and are larger in size (24–28 × 8–9 µm), while those of *J. markotii* are equipped with bipolar, hyaline apical appendages and are shorter (17.5–20 × 5–6.5) in size.

Jahnula purpurea should also be compared to the type species of the genus, *J. aquatica*, in that the ascospores of the two species look morphologically similar at first glance. The two species are however, quite distinct. *Jahnula purpurea* has smaller ascomata, clavate asci, and smaller ascospores, while *J. aquatica* has larger ascomata, cylindrical

asci, and larger ascospores (Hawksworth 1984; Hyde and Wong 1999; Raja and Shearer 2006). In addition, *J. purpurea* stains its wood substrate purple (Fig. 1A–C), which has never been reported for substrates on which *J. aquatica* occurs (Hawksworth 1984; Raja and Shearer 2006). Another species of *Jahnula* that is morphologically similar to *J. purpurea* in overall ascomata and ascospore morphology includes *J. australiensis*. On closer examination, however, the species are quite distinct. *Jahnula purpurea* stains subtending wood purple, a character not observed in *J. australiensis;* the asci in *J. purpurea* are clavate to obclavate, while those of *J. australiensis* are cylindrical; the ascospores of both the species are some what similar in size (24–28 × 8–9 µm in *J. purpurea* vs. 19–30 × 6–8 µm in *J. australiensis*) but those of *J. purpurea* are slightly smaller and wider.

Several species of freshwater ascomycetes in the family Amniculicolaceae are capable of staining underlying wood substrates purple (Zhang et al. 2009a; Zhang et al. 2009b; Zhang et al. 2012), a characteristic similar to *J. purpurea*. However, Amniculicolaceae is phylogenetically related to the Pleosporales, while based on morphological data presented herein *J. purpurea* belongs to the Jahnulales (Pang et al. 2002; Campbell et al. 2007; Suetrong et al. 2011).

In addition, species of *Massariosphaeria* such as *M. phaeospora* (E. Müll.) Crivelli has the ability to stain wood purple (Zhang et al. 2012). *Lophiostoma purpurascens* (K.D. Hyde & Aptroot) Aptroot & K.D. Hyde reported from submerged wood in freshwater habitats in Australia and Papua New Guinea also stains wood purple (Hyde and Aptroot 1998; Hyde et al. 2002). However *M. phaeospora*, and *L. purpurascens* are phylogenetically unrelated to the Amniculicolaceae (Zhang et al. 2012). Another recently described species, *Massariosphaeria fridae* M. Spooren stains its substrate (dead stalks of *Alisma plantago-aquatica*) red (Spooren 2007). This suggests that unrelated freshwater Dothideomycetes have the ability to stain the vicinity of their substrate red or purple. It would be interesting to study the secondary metabolites from these taxa in the future to understand if there is an underlying ecological significance to the production of bright pigments by species of both terrestrial and freshwater ascomycetes within the Dothideomycetes. Additional collections and molecular sequence data from *J. purpurea* in the future would certainly shed light on the hypothesis of purple pigment being a true phylogenetic informative character at the family-level within the Dothideomycetes as suggested by Zhang et al. (2012). The production of purple pigment on wood by *J. purpurea* suggests that pigment production might be a case of convergent evolution that could be functionally significant within the Dothideomycetes. It is also likely that different colors might be characteristic of different phylogenetic clades and might have similar or dissimilar ecological functions.

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RESEARCH ARTICLE

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Transatlantic disjunction in fleshy fungi III: *Gymnopus confluens*

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Abstract

Phylogeographic data indicate that DNA differences consistently exist between the North American and European allopatric populations of *Gymnopus confluens*. Conversely, pairing experiments show that collections from both populations were sexually compatible *in vitro* and detailed morphological examinations of numerous fresh and dried basidiomata do not produce qualitative differences. Percent ITS sequence divergence between Europe and North American collections of *G. confluens* was 3.25%. Species delineation metrics including Rosenberg's P_{AB} statistic, P_{ID} metrics, R_{RD} (randomly distributed) and PTP (Poisson Tree Processes) gave mixed indications that North American and European populations were distinct at species rank. The North American populations are described as *Gymnopus confluens* subsp. *campanulatus* (Peck) R.H. Petersen.

Key words

Allopatric speciation, biogeography, disjunct distributions, phylogenetic species, species delineations

Introduction

North American species numbers for fleshy fungi are certainly underestimated by the historical practice of assigning European names to North American taxa but the extent to which North American taxon numbers are underestimated is unknown and is dependent on species concepts used by investigators. A number of studies documenting cryptic geographical species argue that species may be more localized and more numerous than historical treatises might suggest. A recent study by Talbot et al (Talbot et al. 2014) demonstrated that most soil fungi are regionally endemic and limited by

factors such as climate and dispersal ability. Geographical partitioning may be the rule for basidiomycete taxa, rather than the exception (Taylor 2008; Taylor et al. 2000).

In agaric systematics, discrepancy among parameters used to make taxonomic judgments at species rank is becoming more widely recognized. Three such standards, DNA sequence data, sexual compatibility and morphological characters of basidiocarps have evolved as important in taxonomic judgments, including proposal of new taxa [*Lentinus* (Grand et al. 2011); *Megacollybia* (Hughes et al. 2007); *Lentinellus* (Hughes and Petersen 2004; Petersen and Hughes 2004); *Sparassis crispa* complex (Hughes et al. 2014); *Artomyces* (Lickey et al. 2003)], and revision of generic or species complexes [(*Panellus stypticus* (Jin 2001); *Marasmius scorodonius* (Gordon and Petersen 1998); *M. androsaceus* (Gordon and Petersen 1997); *Strobilomyces* (Sato et al. 2007); *Omphalotus* (Kirchmair et al. 2004; Petersen and Hughes 1998); *Pleurotus* (Vilgalys et al. 1993; Vilgalys and Sun 1994); *Gymnopus* s. l. (Mata et al. 2007)]. As phylogenetic analyses based on molecular data have increased, it has become increasingly clear that genetic differentiation of fungi may proceed in the absence of observable morphological change in basidiocarps. Taylor et al. (2006) note that eukaryotic microbes (predominantly fungi) have few discriminating morphological characters on which to base species assessments. Further, the basidiomycete fungal mating system acts to slow or prevent establishment of reproductive barriers (James et al. 1999). In the absence of morphological change and reproductive isolation, delineation of taxa may rest on evaluation of observed genetic change among populations.

Few primary literature sources are available for identification of *Gymnopus s.str*. Peck's proposals of new species, often under *Marasmius*, were included by Halling (1983) in a publication intended to summarize the taxa (under *Collybia*) of northeastern USA and adjacent Canada. Murrill (Kimbrough 1972) accepted *Gymnopus* Roussel to represent the "collybioid" taxa of Florida and described several taxa of *Gymnopus* (i.e. *Collybia*) and *Marasmius* s.l. from central Florida [see Kimbrough (1972); Halling (1983)]. This left a geographical hiatus between the New England region and Florida. Without publishing a summary of his study, Hesler included observations on Murrill specimens in his (Hesler) notebooks (http://trace.tennessee.edu/utk_hesler/), and Smith [see (Petersen et al. 2014)] also examined Murrill's type specimens. Lennox (1979) published her dissertation on Pacific Northwest collybioid genera, but included only a single species under *Collybia* s. str. Desjardin (1987) published a summary of collybioid fungi from California which further expanded geographical and taxonomic coverage of Gymnopus.

In Europe, several compendia were available (Kühner and Romagnesi 1953; Moser 1978); but Antonín and Noordeloos (Antonín and Noordeloos 1993; Antonín and Noordeloos 2010) and Antonín et al. (2013) summarized *Gymnopus* for Europe and arranged the taxa according to Mata et al.'s recent phylogenetic treatment (Mata et al. 2007).

A molecular discontinuity between European and eastern North American populations of *Gymnopus confluens* has been known for some years (Mata et al. 2007), most recently implied in a phylogeny placing *G. eneficola* from Newfoundland, Canada (Petersen et al. 2014). Additional data now permit a more detailed report as part of a wider project on transatlantic disjunctions in fleshy fungi.

In this study, we evaluate morphology, ability to dikaryotize *in vitro* and ITS-LSU sequence divergence to determine whether intercontinental allopatric populations of *Gymnopus confluens* represent separate taxa at some rank. In this paper, we consider the term population to comprise a group of currently interbreeding individuals. This paper is third in a series of papers exploring the nature of transatlantic disjunctions. The first in the series was Hughes et al. (2014), the second is Petersen et al. (in press).

Methods

Collections

Field collections made by the authors were dried overnight on the day they were collected. Prior to drying, a fragment was stored in silica gel for later DNA extraction and spores were deposited on malt extract agar (MEA) plates to obtain cultures. Collections were accessioned into TENN, cultures into CULTENN.

Pairing experiments

Establishment of single basidiospore isolates (SBIs) and pairing experiments were performed as described in Gordon and Petersen (1992). All SBIs were examined microscopically to determine monokaryon status before mating experiments.

Macromorphology

Macromorphological characters observed were stipe length, stipe vesture, color of living and dried material, and lamella structure (distance between lamella, number, narrow vs. broad, attachment). "Complete" lamellae (those which extended from pileus margin to attachment juxtaposed to stipe) were interspersed with numerous lamellulae, usually of at least two and occasionally three ranks. The most accurate assay counted all lamellae (and lamellulae) which reached the pileus margin. This tally was performed by counting lamellae for approximately ¼ pileus circumference and multiplying by four.

Micromorphology

Basidiospore statistics were gathered for "European" vs. "American" collections. Two spore metrics were especially examined: Q^m (median ratio of spore length to width) and L^m (median spore length). Pileipellis hyphae were examined for presence of short side branches reported by Antonín and Noordeloos (2010). Terminal cell shape of

these side branches was noted. Cheilocystidia vary within subg. *Vestipedes*, and were evaluated for *G. confluens* in both size and shape (i.e.non-strangulate to strangulate).

Molecular studies

DNA was extracted from dried specimens and/or cultures, and ITS and LSU sequences were amplified and sequenced as described in Hughes et al. (2013). Sequences were deposited in GenBank (Table 1). Sequences were manually aligned using GCG (2000). PhyML with 100 bootstrap replicates was performed in Geneious V9. *Gymnopus eneficola* (Petersen et al. 2014) was selected as the outgroup because it is the most closely related species and is sister to *G. confluens*. *G. eneficola* is often mistaken for *G. confluens* in the field. Trees were visualized in TreeView (Page 1996) and deposited in Dryad (Petersen and Hughes 2015).

Species-delineation metrics

Several species-delineation metrics including Rosenberg's P_{AB} statistic (Rosenberg 2007), P_{ID} (strict) and P_{ID}(liberal) (Ross et al. 2008), P_{RD} (Rodrigo et al. 2008) and PTP (Zhang et al. 2013). P_{AB}, P_{ID} (strict), P_{ID} (liberal) and P_{RD} were implemented in Geneious V9 (Geneious 2005; Masters et al. 2011). PTP was implemented at http://species.h-its.org/ using web default settings for generations (100,000) burn-in (0.1, MCMC convergence was reached) and thinning (100). Rosenberg's P_{AB} statistic is the probability that a putative species will be monophyletic with respect to a sister clade under the model of random coalescence. The null hypothesis is that monophyly is a chance outcome of random branching. The P_{ID} statistics provide the frequency with which a member of a putative species can be correctly identified given a specific alignment of sequences. P_{ID}(strict) requires that an unknown specimen falls within but not sister to the species clade. P_{ID}(liberal) requires that an unknown specimen falls either sister to or within the species clade. P_{RD} (Probability Randomly Distributed) is the probability that a clade has the observed degree of distinctiveness due to random coalescent processes. A probability value less than 0.05 rejects the null hypothesis of random coalescence and suggests that the clade is a cryptic species. PTP (Poisson Tree Processes; Zhang et al. 2013) estimates the number of species using both maximum likelihood and Bayesian approaches.

Specimens examined for morphological analysis

NORTH AMERICA, **CANADA**, **New Brunswick**, Fundy National Park, vic. Alma, Caribou Plains Trail, 45°38.59'N, 65°06.94'W, 25.IX.2013, coll Stephen Clayden, TFB14409 (TENN-F-69073); Fundy National Park, vic. Alma, Maple Grove Backroad, 45°35.34'N, 64°59.014'W (stop 1), 24.IX.2013, coll. Unknown, TFB 14389

Table 1. Sequences used in phylogenetic reconstructions.

Herbarium No.	Collection No.	GenBank ITS	GenBank LSU	Location1
Gymnopus eneficola				
MICH PK6975 (as *G. confluens*)	PK6975	KP710270	KP710304	USA, AK
MICH PK6976 (as *G. confluens*)	PK6976	KP710271	KP710305	USA, AK
No specimen	MS4-007	KJ416257	No sequence	Canada, NL
TENN-F-69120	MR3-016	KJ128262	No sequence	Canada, NL
TENN-F-69122	10-09-21 AV04	KJ128265	KJ189590	Canada, NL
TENN-F-69123	09-09-26 AV13	KJ128264	KJ189586	Canada, NL
TENN-F-69127	06-09-02 AV01	KJ128267	KJ189588	Canada, NL
TENN-F-69128	09-09-26 AV12	KJ128268	KJ189589	Canada, NL
Gymnopus confluens				
No specimen	House dust[c]	AM901885	No sequence	Finland
No Specimen	MS4-009	KP710277	No sequence	Canada, NL
BRNM734005[d]	BRNM734005	JX536124	No sequence	Czech Rep.
Culture LE(BIN)	10977ss7[a] and LE(Bin)183	DQ450047	No sequence	Russia, Southern Urals
Culture LE(BIN)	LE(BIN)1178	KP710282	KJ189580	USA, NC
Culture LE(BIN)	LE(BIN)1212	KP710290	KJ189575	Russia, Leningrad area
Culture LE(BIN)	LE(BIN)2294	KP710291	KJ189576	Russia, Altai Region
Culture LE(BIN)	LE(BIN)2357	KP710287	KJ189577	Russia, Altai Region
EIU ASM10643	ASM10643	KP710303	No sequence	Russia, Samara Region
MICH PK6820	PK6820	KP710286	KP710311	USA, AK
MICH PK6943	PK6943	KP710285	KP710312	USA, AK
Not given	H21[b]	JX029935	No sequence	Czech Rep.
Private collection, Michael Burzynski	BUR1	KP710275	KJ189583	Canada, NL
TENN-F-50524	3787	DQ450044	No sequence	Sweden
TENN-F-52248	5824	DQ450053	No sequence	USA, WA
TENN-F-53522	7219	KP710283	KP710309	USA, NC
TENN-F-55695	9048ss2[a]	DQ450050	No Sequence	USA, CA
TENN-F-55879	6960ss3[a]	KP710302	No sequence	Scotland
TENN-F-55879	6960ss4[a]	KP710301	No sequence	Scotland
TENN-F-55880	6962	DQ450051	No Sequence	Scotland
TENN-F-58239	10650ss4[a]	DQ450046	No sequence	Russia, Leningrad
TENN-F-58242	10653	AY256697	No sequence	Russia, Leningrad
TENN-F-59219	11335ss2[a]	DQ450045	No sequence	France, Rhône-Alpes
TENN-F-59285	11400ss1[a]	KP710299	No sequence	Switzerland
TENN-F-59500	9875	AY505773	No sequence	USA, WA
TENN-F-59578	11615	DQ450048	No sequence	Russia, Novgorod
TENN-F-59582	11619	DQ450049	No sequence	Russia, Novgorod
TENN-F-59603	11641	KP710300	No sequence	Russia, Novgorod
TENN-F-60062	12134	DQ450052	No Sequence	USA, NC, GSMNP
TENN-F-60736	11852	KP710289	No sequence	Russia, Kedrovaya Res.

Herbarium No.	Collection No.	GenBank ITS	GenBank LSU	Location1
TENN-F-61147	12587	FJ596784	No Sequence	USA, NC, GSMNP
TENN-F-63806	PBM2991	KP710276	KP710310	USA, VA
TENN-F-65121	13744h1[a]	KP710297	KJ189572	Belgium
TENN-F-65121	13744h2[a]	KP710298	KJ189572	Belgium
TENN-F-65131	13754	KP710288	KJ189571	Belgium
TENN-F-65835	13939	KP710284	KJ189579	USA, NY
TENN-F-67819	14072	KP710280	KJ189258	USA, NC
TENN-F-67822	14075	KP710281	KJ189581	USA, NC
TENN-F-67864	14114h1[a]	KP710295	KJ189573	Germany, Thuringia
TENN-F-67864	14114h2[a]	KP710296	KJ189573	Germany, Thuringia
TENN-F-67865	14115	KP710292	KJ189578	Germany, Thuringia
TENN-F-67882	14132h1[a]	KP710293	KJ189574	Germany, Thuringia
TENN-F-67882	14132h2[a]	KP710294	KJ189574	German, Thuringia
TENN-F-69053	14389	KP710279	KJ189584	Canada, NB
TENN-F-69073	14409	KP710278	KJ189585	Canada, NB
WTU005	WTU005	KP710273	KP710307	USA, AK
WTU394	WTU394	KP710274	KP710308	USA, AK
WTU514	WTU514	KP710272	KP710306	USA, AK

[a]ss=single spore culture, available from the culture collection of the University of Tennessee (CulTENN); h1, h2=haplotypes deduced from forward and reverse sequences; [b] Baldrian et al. 2013; [c] Pitkaranta et al. 2007; [d] Antonín et al. 2013

(TENN-F-69053); **Newfoundland**, Moccasin Lake, Abitibi Trail, 10.ix.2008, coll. Maria Voitk, mixed woods, MS4-009 (TENN-F-69133). **UNITED STATES**, **New York**, Tompkins Co., Ringwood Preserve, 42°27.03'N, 76°21.80'W, 4.IX.2013, coll TJ Baroni & RHP, TFB 14357 (TENN-F-69006); Tompkins Co., vic. Dryden, Ringwood Preserve, 42°28.11"N, 76°19.06"W, 13.IX.1984, leg. & det. R.E. Halling, REH no. 3851 (dupl. NY), TENN-F-47030; **North Carolina**, Macon Co., vic. Highlands, Shortoff Mt. area, 35°05.47'N, 083°11.25'W, 18.VII.1994, coll. J. Johnson, TFB7219 (TENN-F- 53522)[annot. R.E.Halling as C. confluens]; **Tennessee**, Sevier Co., GSMNP, Rainbow Falls Parking Area, 28.VII.1989, coll RHP (as Collybia ?acervata), TFB 2033 (TENN-F-48376); Sevier Co., vic. Gatlinburg, GSMNP, "Mt. LeConte," 8.VIII.1941, coll L.R. Hesler & S.L. Meyer, LRH 13883 (TENN-F-13833); **Virginia**, Smyth Co., vic, Sugar Grove, Mt. Rogers National Recreation Area, Appalachian Trail, 8.VIII.2008, coll. P.B. Matheny, PBM 2991 (TENN-F-63806).

EUROPE, **BELGIUM**, Domain Massembre vic. Hevre, 50°09.62' N, 4°51.48'E, 7.IX.2010, coll RHP, TFB13754 (TENN-F-65131). **FINLAND**, Hämeenlinna, Torronguo National Park, 60°44.32'N, 23°38.63'E, 8.VIII.2002, coll. J.L. Mata, TFB 11055 (TENN-F-59469). **FRANCE**, Rhône-Alpes, Dpt. Savoie (73), commune St. Germain laChambotte, 45°46.62'N, 5°53.07'E, 10.IX.2001, RHP & Pierre-Arthur Moreau, TFB11335 (TENN-F-59219). **GERMANY**, Thuringia, vic. Menteroda, 51°18.04'N, 10°31.44'E, 28.VIII.12, coll RHP 2012, TFB14132 (TENN-F-67882); Thuringia, vic. Schlotheim by Pöthen, 51°16.90'N, 10°33.434' E, 27.VIII.2012, coll. RHP, TFB14115

(TENN-F-67865). **RUSSIA**, Leningrad Reg., vic. Lodeynoe Pole, 60°41.70'N, 33°17.98'E, 30.VIII.1999, coll RHP, TFB10650 (TENN-F-58239); Novgorod Region, Valdai District, National Park Valdaiski, vic. Road to National Park, 57°57.88'N, 33°19.32'E, 19.VIII.2003, coll. RHP, TFB 11615 (TENN 59578); Valdai National Park at resort, 58°00.511' N, 33°21.543' E, 22.VIII.2003, coll. RHP, TFB11641 (TENN-F-59603); Samara Region, Bakhilovo district, vic. Bakhilovo, Shiryayeva Valley, 53°24.30'N, 49°55.03'E, 17.VIII.2004, coll. RHP, TFB12171 (TENN-F-60109). **SWEDEN**, Uppland, vic. Uppsala, Gottsundaborgen, 59°48.8'N, 17°37.40'E, 7.IX.1994, coll Svenngunnar Ryman, TFB7262 (TENN-F-53546). **SWITZERLAND**, Graubunden, Chur, Lenzerheide, 46°40.17'N, 9°38.97'E, 19.IX.2001, coll. E. Horak, TFB11400 (TENN-F-59285).

Abbreviations used in table and figures

North America — AK=USA, Alaska, GSMNP=USA, Great Smoky Mountains National Park (TN or NC); NB=Canada, New Brunswick; NC=USA, North Carolina; NL=Canada, Newfoundland; TN=USA, Tennessee; VA=USA, Virginia; WA=Washington. **Europe** — BE=Belgium; FI=Finland; FR=France; GE=Germany; RU=Russia; SZ=Switzerland; SW=Sweden. SBI=single-basidiospore isolates; h=haplotype; c=clone.

Data resources

The data underpinning the analyses reported in this paper are deposited in the Dryad Data Repository at doi: 10.5061/dryad.8239h.

Results

Gymnopus confluens basidiomata from North America and Europe are shown in Figs 1, 2.

Morphological parameters – macromorphology

Macromorphological characters readily distinguish *G. confluens* (at least in Europe and North America) from other *Gymnopus* taxa. In nature and in herbarium specimens, basidiomata generally exhibit long stipes compared to pileus diameter.

Lamellae: Lamellae in *G. confluens* appear to be quite consistent; crowded, narrow and significantly seceding upon drying. In an attempt to statistically measure the first two items, lamellae in numerous collections were carefully examined for breadth (rarely exceeding two mm) and number. The number of lamellae reaching pileus margin ranged from 116–147, consistently more than 120, and with no discernible intercontinental difference. In similar morphological taxa (i.e. *G. subnudus*, *G. eneficola*, etc.) this number ranged from 65–87, significantly fewer than in *G. confluens*.

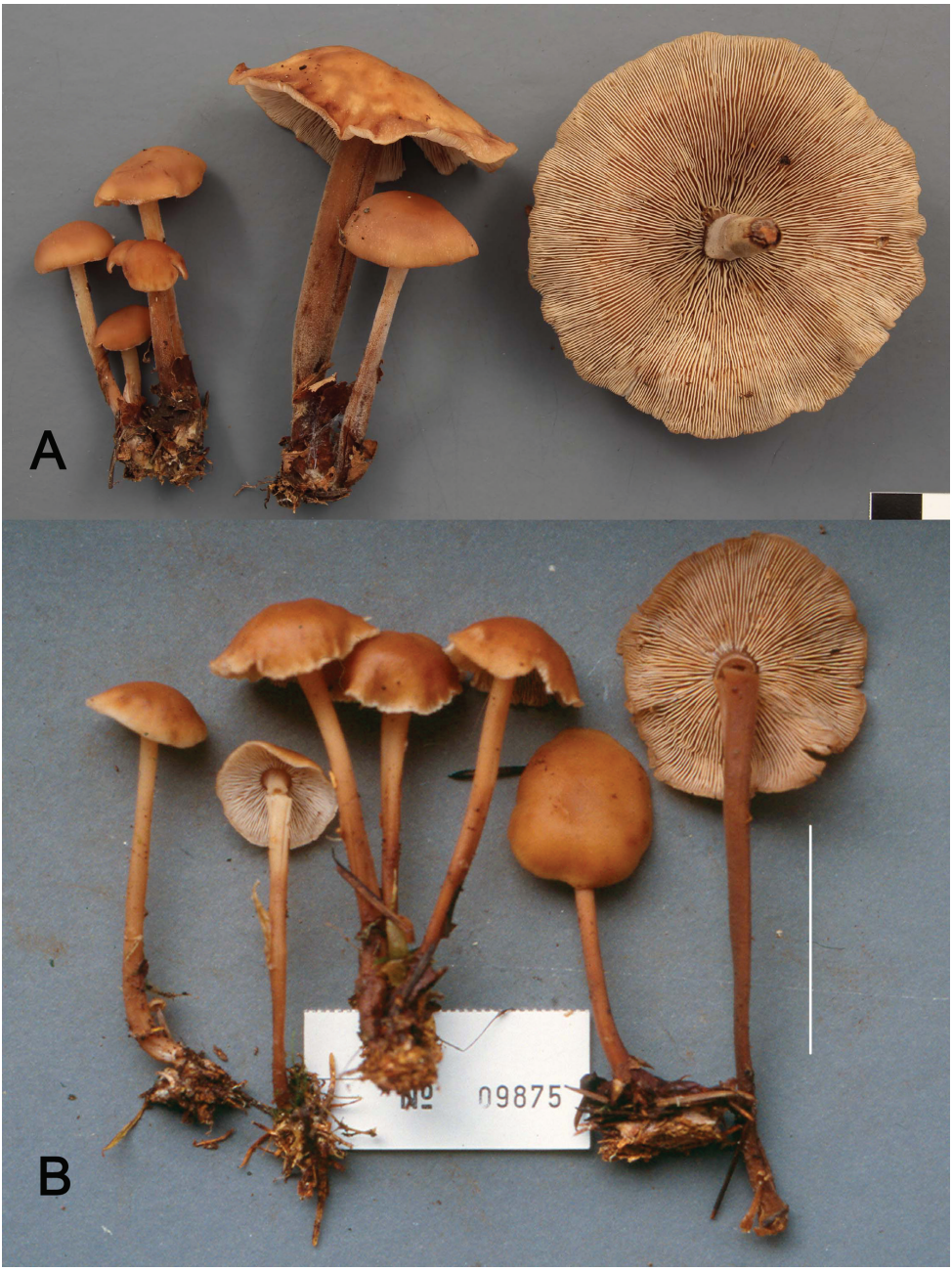

Figure 1. *Gymnopus confluens* subsp. *campanulate* basidiomata, North America. **A** TENN-F-69073 (TFB14409) NB. Photo courtesy Roger Smith. Black box = 10 mm **B** TENN-F-59500 WA. Standard bar = 50 mm. Basidiomata in both images exhibit a tendency toward campanulate pileus margin.

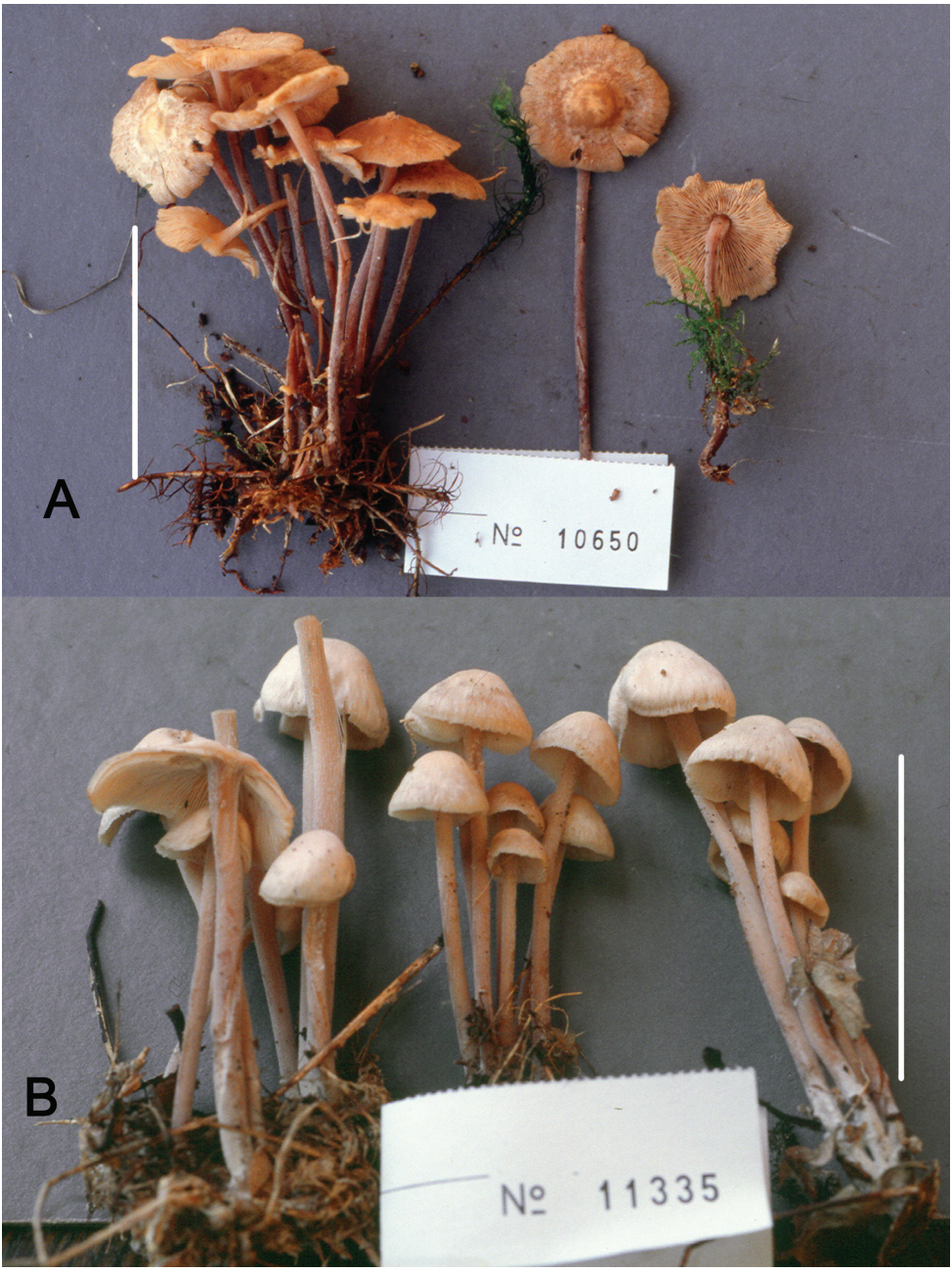

Figure 2. *Gymnopus confluens* basidiomata, Europe. **A** TENN-F-58239 (TFB10650) RU **B** TENN-F-59219 (TFB11335) FR. Standard bars = 50 mm.

Stipe: Pileus Ratio: Overall, stipe length:pileus diameter usually exceeded 3:1 (with range from 1.5:1 to 6:1). This ratio varied little between North American and European populations, but both clades exhibited some ratios downward, usually explainable due to dry weather or poor nutrition.

Stipe vesture: In most cases, stipe vesture is most sparse upward on the stipe and there (at 10X) exhibiting densely distributed spikes. Downward, vesture becomes denser, and toward the stipe base, a felty subiculum subsumes individual spikes and often is strigose. In dried material, stipe vesture takes on a gray coloration, sometimes with a very slight olive tint. Stipe vesture varied considerably in both populations/clades. In fact, vesture variation within the major populations exceeded that between clades. No suitable metric was devised to summarize this situation, but macroscopic vesture characters did not prove distinctive.

Morphological parameters – micromorphology

Variation in spore shape is shown in Figs 3 and 4. Spores of at least three European collections were shorter (and somewhat narrower) than those of eastern North American material, and also tapering more obviously proximally (TENN-F-67865 GE, TENN-F-67882 GE, TENN-F-59212 FR). Spores of most European collections approach the metrics of eastern North American material (Table 2). A few collections of otherwise mature basidiomata were devoid of spores. Whether this is a function of *in vivo* drying followed by mechanical but not biological resuscitation is not known. Counter to this hypothesis is the presence on such basidiomata of mature (sterigmate), turgid basidia which appear to be fecund.

When spore statistics from numerous collections were compared, little difference was apparent, and spore statistics were concluded to be inconclusive for morphological separation of the phylogenetic clades of *G. confluens*. Median spore length for North American collections was 7.72 µm (n=10 collections); for European collections it was 7.52 µm (n=10 collections) (Table 2). This was not significantly different based on a 2-tailed T-test (P=0.40, df=19). Q^m for North American collections was 2.34 (n=10 collections); for European collections it was 2.30µ (n=14 collections). Q^m values were not significantly different based on a two-tailed T-test (P=0.71). The two collections from Alaska (MICH139598 and MICH139602) which clearly fell within the European clade by ITS sequence, were included in calculations of spore statistics for Europe.

Cheilocystidia: Cheilocystidum size and shape vary within *Gymnopus* subg. *Vestipedes*, and this variation was closely examined for numerous collections of *G. confluens* from both continents. Variation in both size and shape (i.e.non-strangulate to strangulate) was expected based on previous reports and illustrations. As expected, cheilocystidia varied in abundance, size and shape, but without correlation to geographic origin. Although cheilocystidia of European collections generally appear to be longer and longer-stalked than those from eastern North American specimens, the range of sizes and complexities seems parallel across the two populations. Overall variation of cheilo-

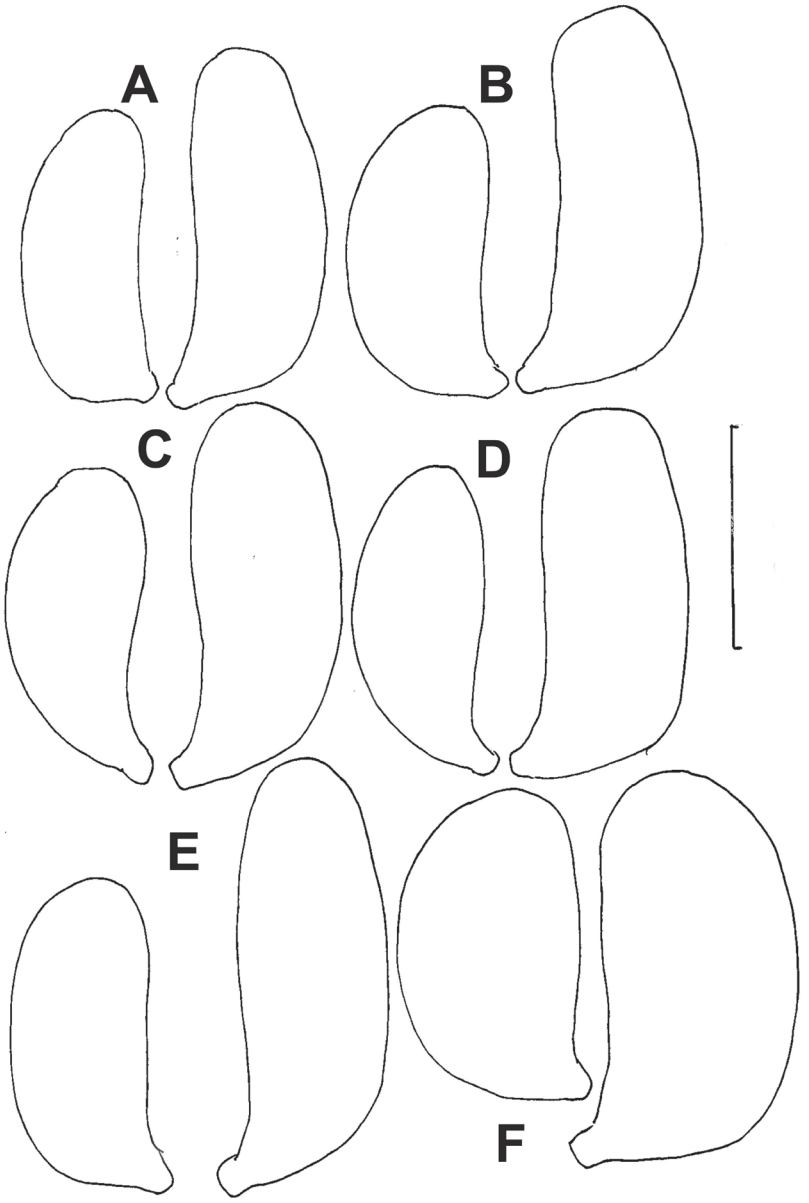

Figure 3. *Gymnopus confluens* Europe. Outlines of basidiospores showing variation in sizes and shapes. **A** TENN-F-59219 FR **B** TENN-F-67865 GE **C** TENN-F-65131 BE **D** TENN-F-59578 RU **E** TENN-F-53546 SW **F** TENN-F-59285 SZ. Standard bar = 5 μm.

cystidia is shown in Figs 5 and 6, where cheilocystidia are arranged according to increasing complexity. Tentacularly branched cheilocystidia (TENN-F-53546 SW) and the formation of a thatch out of the withered cheilocystidial apices (TENN-F-60109 RU) seem unique in *Gymnopus* sect. *Vestipedes*. Cheilocystidia may be involved in mi-

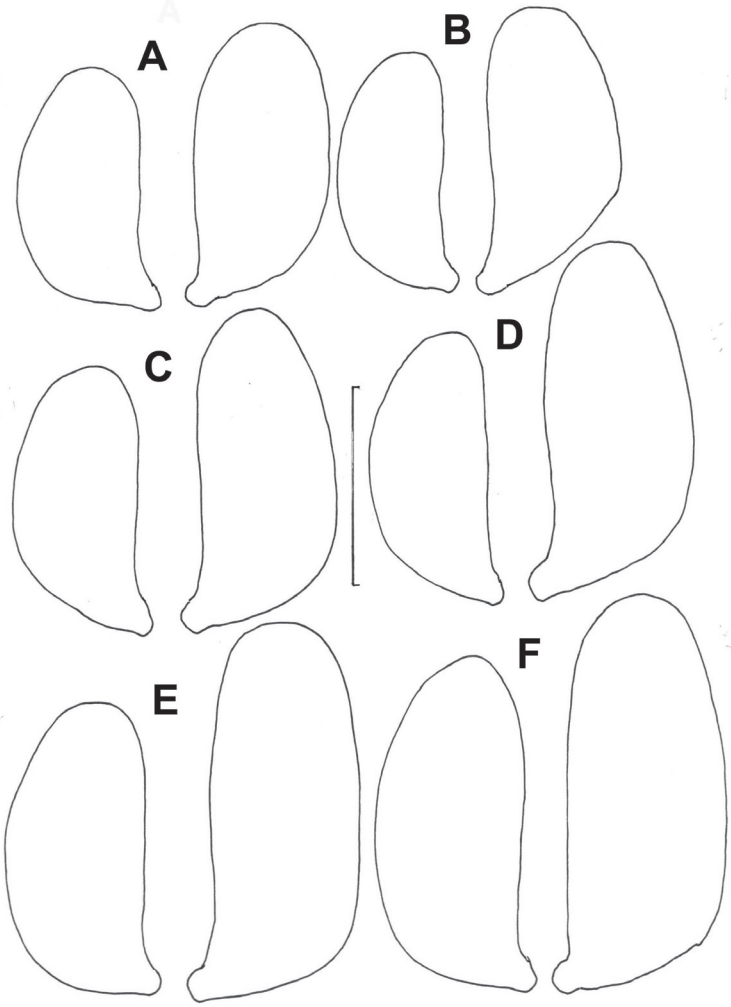

Figure 4. *Gymnopus confluens* subsp. *campanulate*. North America. Outlines of basidiospores showing variation in sizes and shapes: **A** TENN-F-47030 NY **B** TENN-F-48376 TN **C** TENN-F-63806 VA **D** TENN-F-48376 TN **E** WTU-F-021394 AK **F** WTU-F-024005 AK. Standard bar = 5 μm.

croscopic exudate of slime which conserves water as a microscopic moist chamber in which the apical portion of cheilocystidia can proliferate.

Caulocystidia: In observing caulocystidial hyphae composing stipe vesture, especially those gathered to form the characteristic vesture "spikes," some variation was perceived in the shape of the terminal cells, whether equal (parallel-sided and bluntly rounded at apex), tapering distally (and therefore narrowly rounded at apex), or some variation in shape (i.e. subsagitate, lobed, etc.). Once noted, special care was taken to observe this character. Caulocystidial hyphae are invariably clamped within their emergent length. Caulocystidia, correctly depicted by Antonín and Noordeloos (2010)

Table 2. Basidiospore metrics for European and North American collections of *Gymnopus confluens*.

Collection Accession Number[4]	**Spore Length × width (µm)**	**L^m (µm)** (median length)	**Q** (ratio length to width)	**Q^m** (median Q values)
TENN-F-50359 SZ[1]	6.5–8.5(–9) × (2.5–)3–3.5	7.30	1.86–3.00	2.35
TENN-F-53546 SW[1]	(6–)7–8(–8.5) × (3.5–)3.5–4.5	7.40	1.71–2.67	2.02
TENN-F-53546 SW[2]	7.5–9.5(–10) × 3.5–4	8.55	2.13–2.71	2.31
TENN-F-59219 FR	(5.5–)6–7(–8) × (2.5–)3–3.5	6.50	2.00–2.33	2.13
TENN-F-59282 SZ[1]	7.5–8.5 × 3.5–4	7.90	1.88–2.14	2.06
TENN-F-59282 SZ[2]	(7.5–)8.5–10 × 3.5–4	8.70	2.00–2.71	2.32
TENN-F-59578 RU	7–9 × 3–4	8.00	2.13–2.83	2.37
TENN-F-65131 BL	(6–) 6.5–8 × 3–3.5(–4)	7.15	1.71–2.50	2.11
TENN-F-67865 GE	(5.5–)6–7 × 2.5–3.5	6.40	1.86–2.80	2.32
TENN-F-67882 GE	6–7 × 3–3.5	6.40	1.71–2.00	1.94
TENN-F-62904 SW	(5.5–)7.5–9.5 × 2.5–3.5(–4)	7.85	2.00–3.20	2.61
TENN-F- 50565 SW	7.5–9 × 2.5–3.5	8.20	2.33–3.60	2.85
MICH 139598 AK[3]	6.5–8.5 × 3–4	7.45	1.88–2.50	2.17
MICH 139602 AK[3]	7.5–9(9.5) × 3–3.5(4)	8.25	2.13–3.17	2.60
		$L^{m\ ave}$ = 7.52		**$Q^{m\ ave}$ = 2.30**
TENN-F-69053 NB	6–7 × (2.5–)3–3.5	6.75	2.00–2.33(–2.80)	2.16
TENN-F-48376 TN	(6–)6.5–9 × 2.5–3.5	7.40	2.00–3.20	2.59
TENN-F-47030 NY[2]	6.5–8.5 × 3–3.5	7.60	2.14–2.67	2.42
TENN-F-63806 VA[1]	6.5–9 × (3–)3.5–4.5	7.75	1.86–2.43(–3.00)	2.20
TENN-F-69073 NB	7–8.5 × 3.5–4	7.80	2.00–2.67	2.21
TENN-F-53522 NC	7–9.5 × 3–3.5	8.05	2.14–3.00	2.53
TENN-F-63806 VA[2]	7–9 × 4–4.5	8.05	1.67–2.25	1.95
WTU 021394 AK	7–8.5 × (2.5–)3–3.5	7.70	2.00–3.20	2.36
WTU 024005 AK	(6.5–)7–8.5 × (2.5–)3–3.5	7.85	2.17–2.85	2.58
WTU 021152 AK	(7–)8–9 × 3–4	8.30	2.13–2.67	2.42
		$L^{m\ ave}$ = 7.72		**$Q^{m\ ave}$ = 2.34**

[1] From pileipellis; [2] From hymenium; [3] Alaskan collections with European ITS sequences; [4] Herbarium designations according to Index Herbariorum (Thiers continuously updated)

often include small side lobes with narrow attachments to parent hyphae. It is easy to observe the erect hyphae which compose the spikes, but more difficult is observation of the subicular hyphae which produce the hyphal complexes which elongate into the spikes. The complexity and depth of the subiculum vary from arachnoid (and then revealing the color and glassy surface of the dried stipe cortex) (TENN-F-59282 SZ; TENN-F-59219 FR) to opaque-felty with subsumed spikes (TENN-F-67865 GE) to shaggy (TENN-F-67882 GE). The felty subiculum increases downward and appears as a sheath toward the stipe base, somewhat reminiscent of *Connopus acervatus*.

Pleurocystidia: Whether pleurocystidia are commonly present has not been thoroughly investigated. Structures resembling cheilocystidia were observed in three collections (TENN-F-59578 RU, TENN-F-67865 GE, TENN-F-59212 FR) among basidia rather than being clustered at the temini of lamellar tramal hyphae.

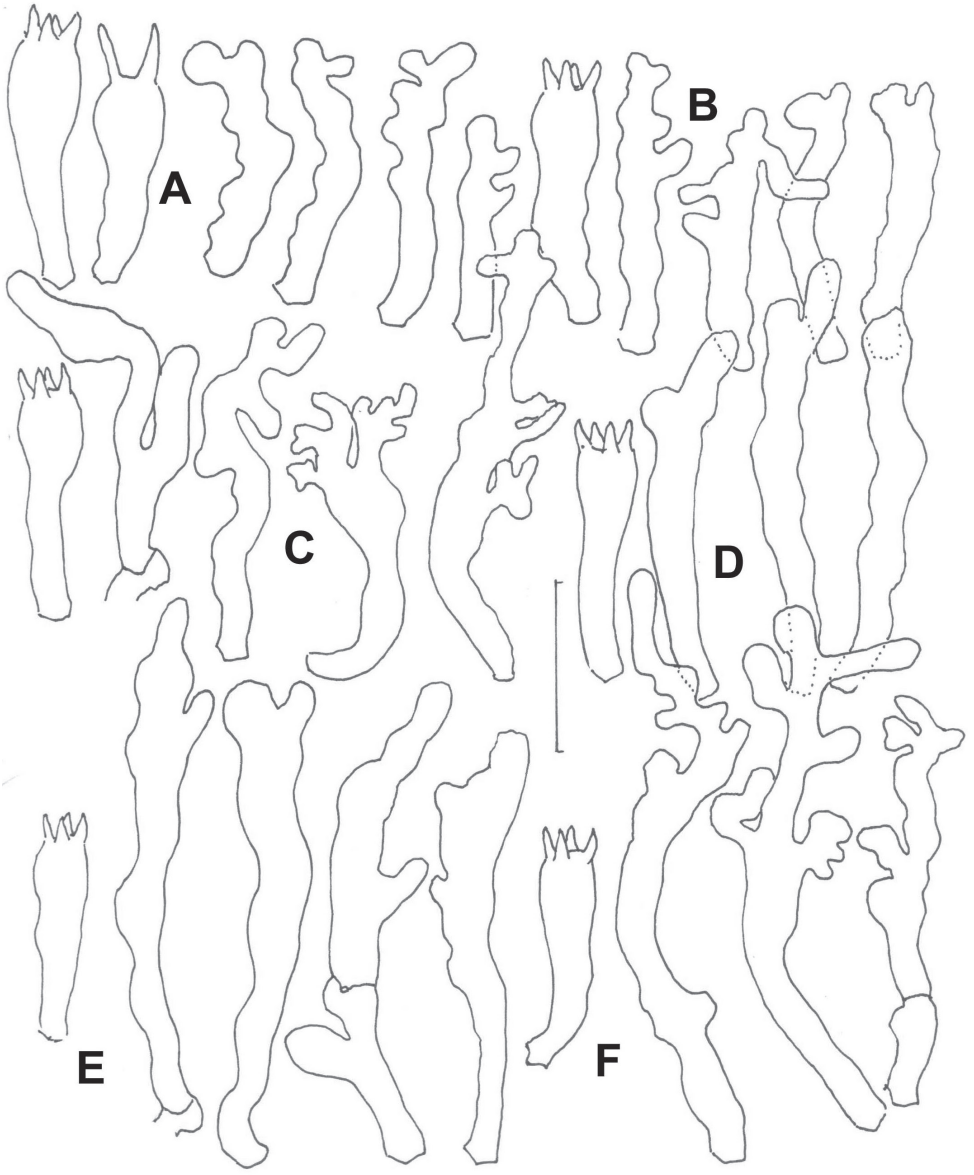

Figure 5. *Gymnopus confluens*. Europe. Outlines of cheilocystidia, showing variation in sizes and complexity. Basidia furnished for size comparison. **A** TENN-F-59285 SZ **B** TENN-F-59469 FI **C** TENN-F-53546 SW **D** TENN-F-59219 FR **E** TENN-F-67882 GE **F** TENN-F-60109 RU. Standard bar = 20 μm.

Basidia: Basidia varied little across both continents. Rarely, an individual two-spored basidium was detected, and four-spored basidia accounted for almost all mature basidia observed (Figs 5, 6).

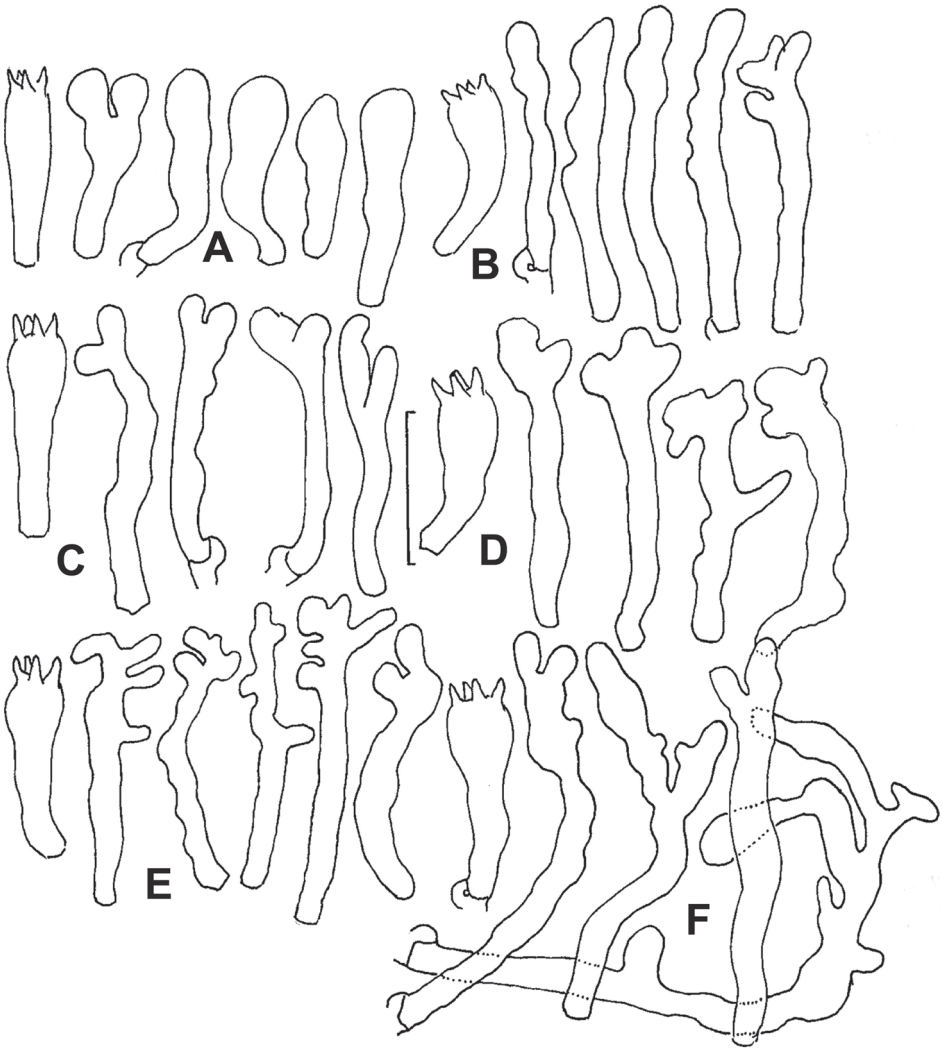

Figure 6. *Gymnopus confluens* subsp. *campanulatus* North America. Outlines of cheilocystidia, showing variation in sizes and complexity. Basidia furnished for size comparison. **A** MS4-009 NF **B** TENN-F-47030 NY **C** TENN-F-69053 NB **D** TENN-F-63806 VA **E** TENN-F-48376 TN **F** TENN-F-69074 NB.Standard bar = 20 μm.

A basidium is produced as a terminal cell of a subhymenial hypha. The hyphae then proliferates through the subtending clamp connection and another basidium is produced in the same fashion. After several such proliferations and basidial discharge (usually leaving little or no residue), the subhymenial hypha appears asymmetrically notched, and superficially resembles some cheilocystidia (TENN-F-67865 GE), for which they are easily mistaken, especially as seen in considerable numbers in older hymenia.

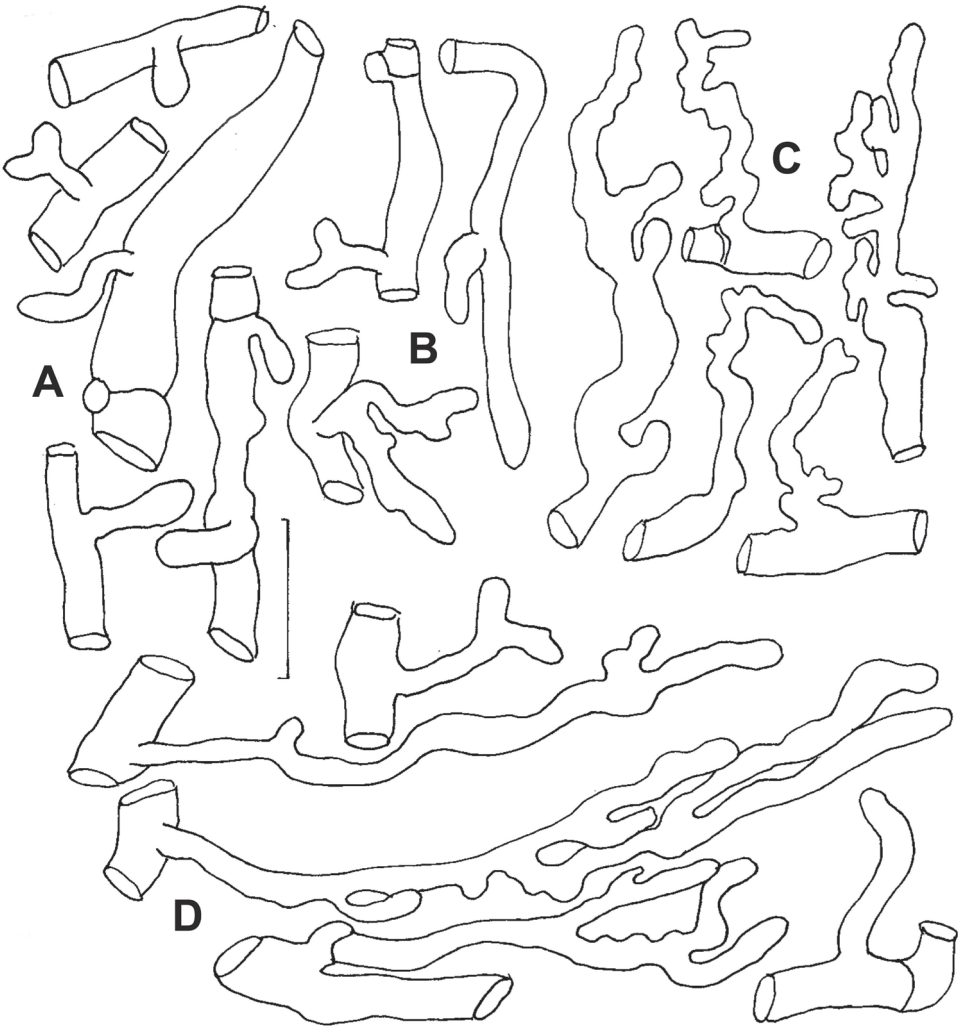

Figure 7. *Gymnopus confluens*. Europe. Outlines of side branches of pileipellis hyphae showing variation in size and complexity. **A** TENN-F-65131 BE **B** TENN-F-60109. RU **C** TENN-F-59469 FI **D** TENN-F-67865 GE. Standard bar = 20 μm.

Side branches from pileipellis: The side branches from pileipellis hyphae reported and illustrated by Halling (Halling 1983) and Antonín and Noordeloos (2010) were consistently observed. Shapes ranged from short, simple and digitate (i.e. TENN-F-13883, TENN-F-48376, TENN-F-69133, TENN-F-53522 from North America; TENN-F-65131, TENN-F-60109, TENN-F-67882, TENN-F-59469 from Europe) to over 100 μm long and branched in a tentacular fashion (i.e. TENN-F-63806 from North America; TENN-F-67865 from Europe). Quantitatively, however, these side branches seem longer (TENN-F-67882 GE, TENN-F-60109 RU) and usually more complex (i.e. branched in a coralloid fashion (TENN-F-67865 GE, TENN-F-59469

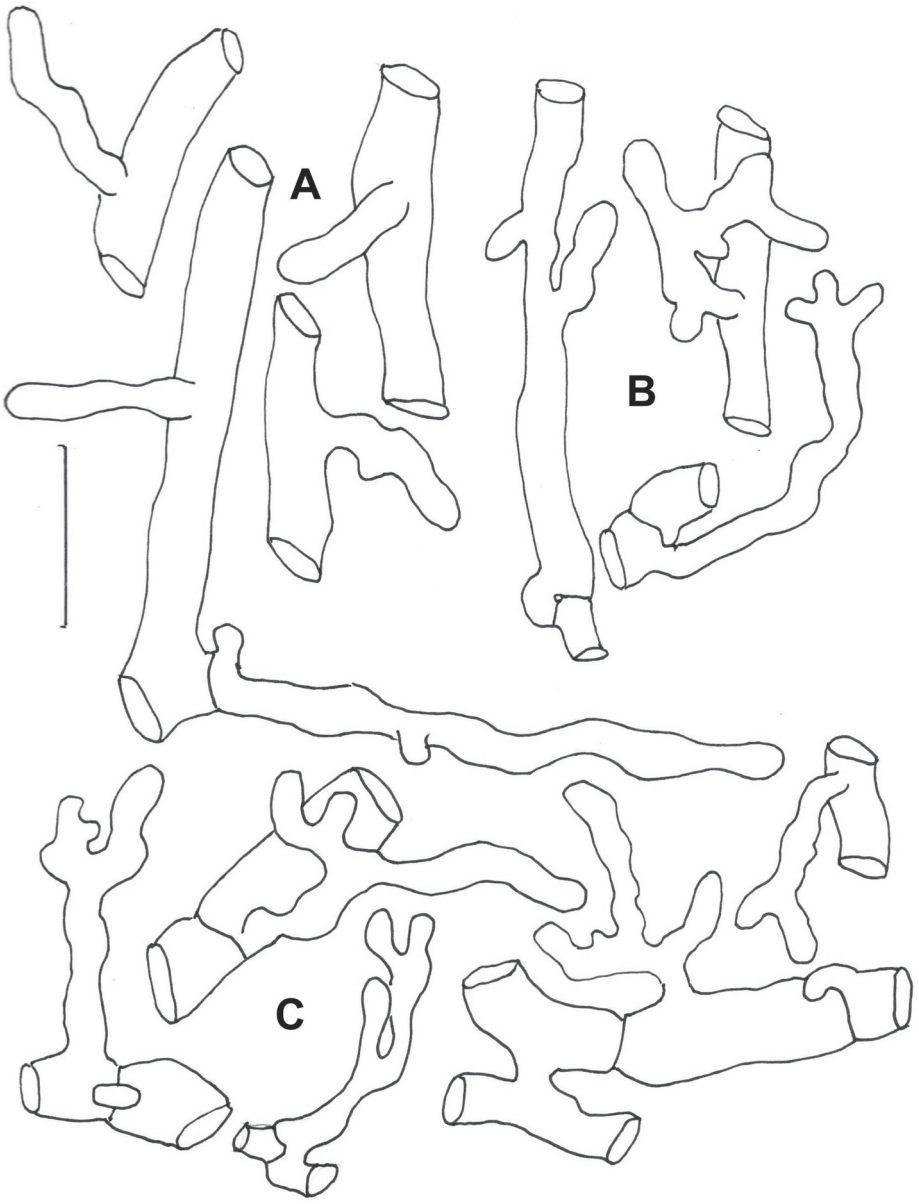

Figure 8. *Gymnopus confluens* subsp. *campanulate*. North American. Outlines of side branches of pileipellis hyphae, showing variation in size and complexity. **A** TENN-F-53522 NC **B** TENN-F-69133 NL **C** TENN-F-63806 VA. Standard bar = 20 μm.

FI) in European specimens than in American. It may be that this proliferation is aided by microscopic local moisture idiosyncratic to individual conditions (including some mucoid matrix if present). The variation was wide both within and between clades, and no clade separation was possible based on this character (Figs 7, 8).

Morphological summary: Morphological characteristics based on lamellae, side branches from pileipellis hyphae, cheilocystidia, caulocystidia, and pleurocystidia, varied considerably but observed differences were not continent-specific. Basidia and basidiospores showed little variability and did not differ between continents.

Sexual recognition experiments

Results of three self-crosses of *G. confluens* (collections TFB 7219, NC; 9048, CA; 11400, SZ) were reported (Mata et al. 2007) as showing tetrapolarity, not unusual in Omphalotaceae. This study added TENN-F-69053 (TFB14389), New Brunswick, Canada, which we also determined to be tetrapolar. In all cases, distribution of mating types was unbalanced and in most of these self-crosses, some SBIs were found which exhibited unexplained mating results, mostly ability to dikaryotize two opposing mating types, most easily explained by either harvesting of two hemicompatible basidiospore germlings together, or occurrence of two hemicompatible nuclei within a single basidiospore (Petersen 1995). Such results have also been found in other members of *Gymnopus* subg. *Vestipedes*, such as *G. subnudus* (Murphy 1992; Murphy and Miller 1993; Petersen 1995) which has been reported as bipolar and tetrapolar (Petersen, ined.)

In the sexual compatibility study using *G. confluens* [(Mata et al. 2007);Fig. 14], a total of 11 collections were used, seven from Europe and four from North America. Five intercontinental intercollection pairings were performed, all universally sexually compatible. If clamp connection production is accepted as a proxy for sexual reproduction, no apparent prezygotic reproductive barrier exists between the continents. It was concluded, based on limited evidence, that only a single sexual recognition species was involved. No attempt to segregate morphological entities was offered in that study.

Phylogenetic analyses

A PhyML tree based on ribosomal ITS sequences is given in Fig. 9. North American and European collections segregated largely into two distinct clades which share an average sequence identity of 96.75%. Collections from Alaska were placed in both clades. A collection from California was also affiliated with the European clade. For a smaller data set, both ribosomal ITS and LSU sequences were available and were concatenated. Results of the PhyML analysis are given in Fig. 10.

For the ITS data set, Rosenberg's P_{AB} statistic for both European and North American clades was $P=1.6 \times 10^{-8}$. Thus, a null hypothesis of reciprocal monophyly under a random coalescence model can be rejected. The probability of correctly identifying an unknown member of a putative species is given by P_{ID} statistics. P_{ID} (strict) European clade = 0.95 (σ=0.89,1.00) and P_{ID} (strict) North American clade = 0.98 (σ=0.93, 1.00). P_{ID} (strict) is the more stringent of the P_{ID} statistics. The probability P_{RD} that a clade has the observed distinctiveness under a null hypothesis of random coalescence for North

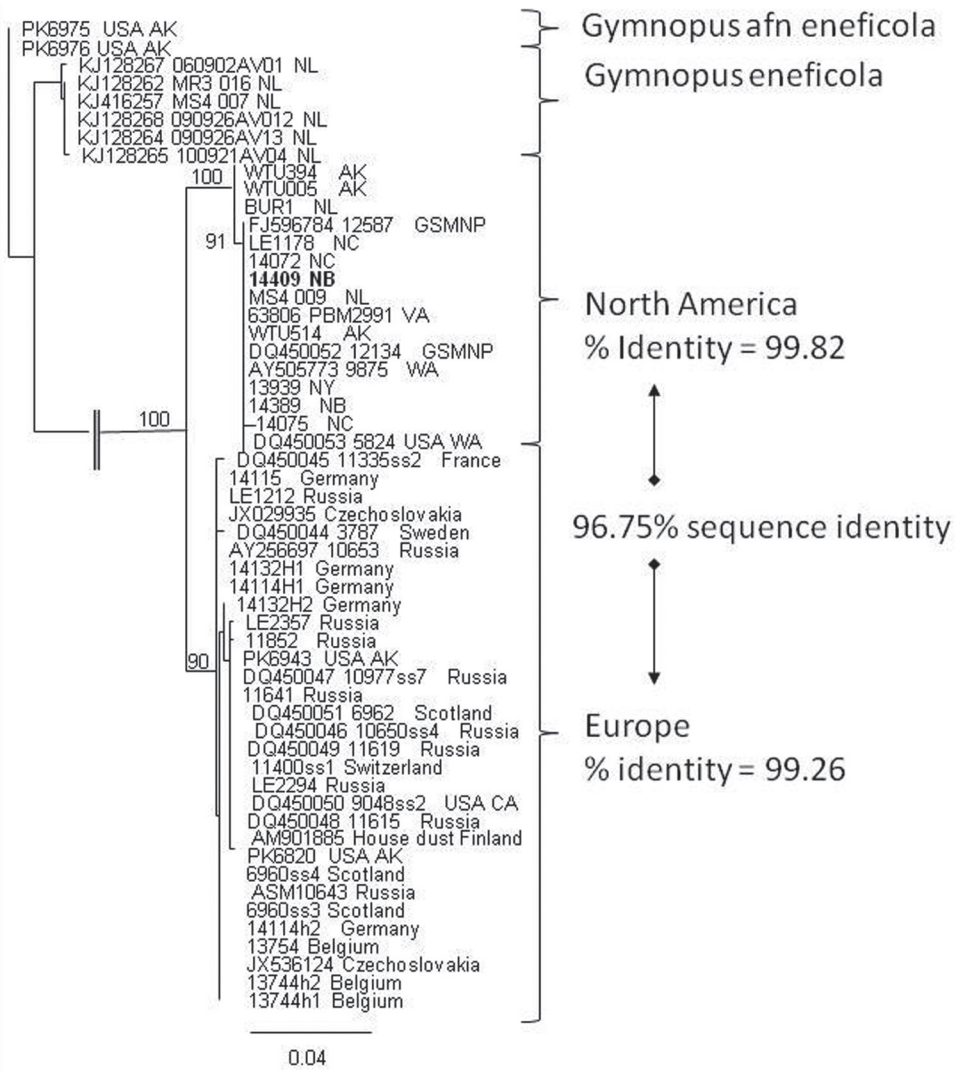

Figure 9. PhyML tree based on ribosomal ITS sequences. Bootstrap values based on 100 bootstrap replicates are at the left of the supported node. Analysis assumed the GTR model of evolution with the transition/transversion ratio, number of invariable sites and shape of the gamma distribution estimated. The log likelihood of the tree was -1776.7. Bold type = holotype of *Gymnopus confluens* subsp. *campanulatus*. Percent identity was based on the entire ITS1-5.8S-ITS2 sequence.

America is 0.05 and for Europe is 0.21. Neither of these probabilities reject the null hypothesis. PTP species-delimitation results produce both maximum likelihood and Bayesian estimates of the number of species. PTP for both analyses partitions *G. confluens* European and North American populations into two species groups but without significant support (bootstrap support for both analyses was 0.23 for North America and 0.55 for Europe. Outgroup taxa are also partitioned into two species groups.

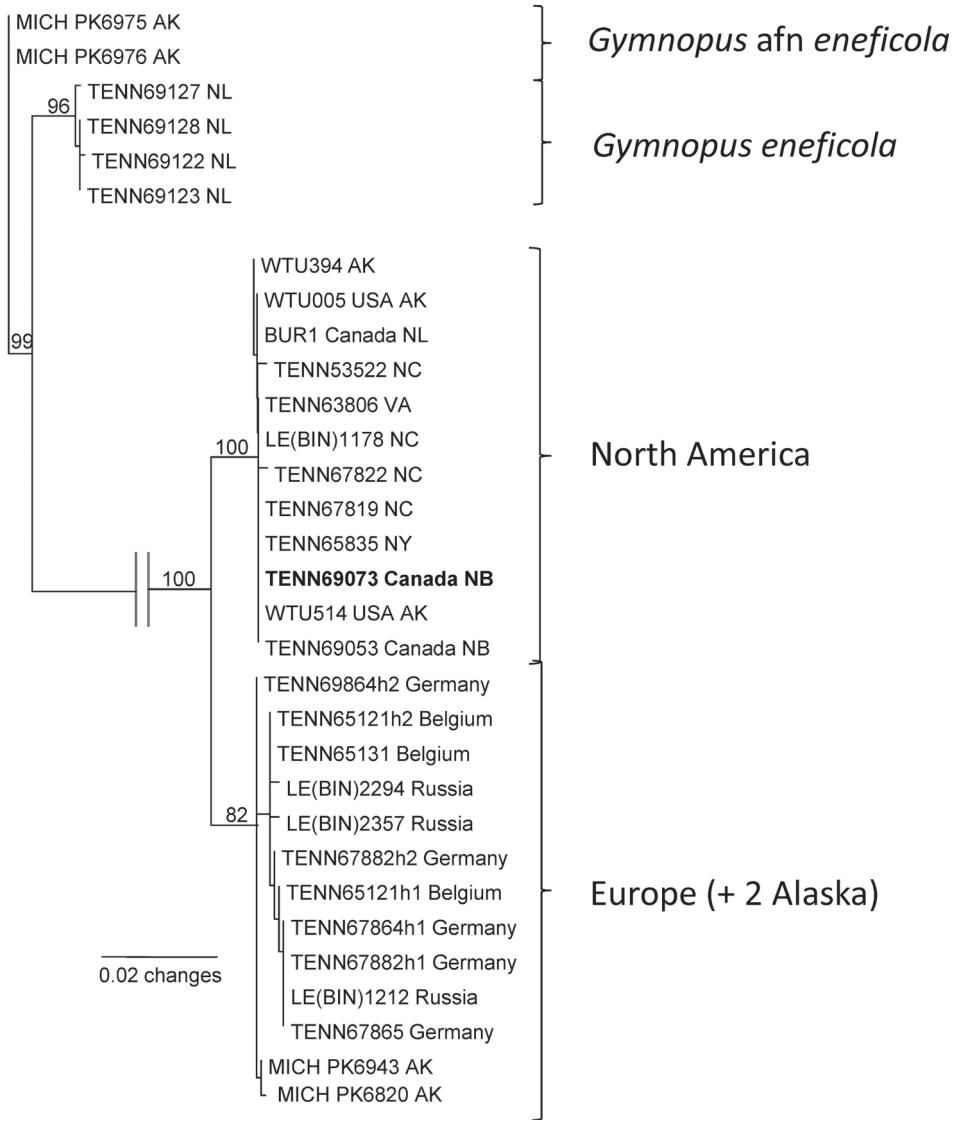

Figure 10. PhyML tree based on concatenated ribosomal ITS + LSU sequences. Bootstrap values based on 100 bootstrap replicates are at the left of the supported node. Analysis assumed the GTR model of evolution with the transition/transversion ratio, number of invariable sites and shape of the gamma distribution estimated. The log likelihood of the tree was -3135.8. Bold type = holotype of *Gymnopus confluens* subsp. *campanulatus*.

Discussion

Allopatric speciation may be the most common mode of speciation in fungi and other organisms, and separation of populations on different continents would, in the absence of significant gene flow, lead gradually to accumulation of genetic differences

and ultimately speciation. The mode and tempo of speciation, however, must vary with reproductive strategies and selection pressures. The point at which two allopatric populations become new species is often a matter of judgment but important in terms of evaluating conservation status and estimating species diversity for a given region. Numerous studies suggest that in basidiomycete fungi, ability of allopatric populations to intercross *in vitro* is conserved as a function of the unique multiple allelic mating systems even while genetic divergence (usually indicated by differences in nuclear ribosomal ITS sequences) proceeds (Gordon and Petersen 1997; 1998; Grubisha et al. 2012; James et al. 1999; Lickey et al. 2002; Lickey et al. 1999; Taylor et al. 2006; Vilgalys and Sun 1994). It should be noted, however, that *in vitro* compatibility examines only one aspect of reproductive intercompatibility and that failure to produce F_1 fruitbodies, reduced fertility of F_1 hybrids, lack of or inviability of F_2 progeny and competition failure at any level may also be involved in reproductive isolation. These factors have not been evaluated as isolated mechanisms between basidiomycete populations to date.

In *Gymnopus confluens*, two of three criteria used to evaluate delineation of species (morphology and ability to intercross *in vitro*) show no significant intercontinental separation. ITS sequences, however, are divergent (3.25% base pair difference), a level often used to suggest different species (Hughes et al. 2009), and European and North American clades are well-supported. The combination of a barcode gap, Rosenberg's P_{AB} statistic results and P_{ID} (strict) suggest that North American and European clades are monophyletic, that the observed differences are not due to coalescence in gene trees and that they are distinct phylogenetic species. In contrast, P_{RD} and PVP do not reject the possibility that the observed result (Fig. 9) is due to random coalescence. The question then becomes whether to assign these populations nomenclatural rank on the basis of ribosomal ITS and LSU sequences alone. To identify these populations as comprising a single species ignores significant ITS + LSU sequence divergence indicative of speciation processes, and underestimates diversity. To identify these populations as separate species may overstate the degree of genetic divergence. A middle-ground solution seems to be to assign these populations subspecies rank, thus recognizing genetic differentiation. We do so below adhering to, insofar as possible, procedures suggested by Tripp and Lendemer (2014) for naming taxa when molecular evidence is the only evidence available.

The finding that ITS sequences for collections from Alaska represented two distinct ITS entities, one of which falls within the European clade (Fig. 9) suggests a dual origin for collections from this region. An ITS sequence of a collection from California also falls within the European clade. Possibly, these collections represent human-mediated transfer of material from Europe to North America. Alternately, movement from Eurasia to Alaska thence to California may have been feasible via the Bering land bridge during periods of glaciation, but without an understanding of *G. confluens* from Asia, neither hypothesis be substantiated. Intra-continental geographical partitioning is not clearly evident for either European or North American populations of *Gymnopus confluens*. This contrasts with findings in some other basidiomycete taxa (Geml et al. 2008; Hughes et al. 2014; Zhao et al. 2013) but intracontinental biogeographical distributions have not been extensively examined and are likely to be species-specific.

Taxonomy

***Gymnopus confluens* subsp. *campanulatus* (Peck) R.H. Petersen, comb. et stat. nov.**
MycoBank no. 811950

***Basionym*:** *Collybia confluens* var. *campanulatus* Peck. "1901" (1902). Bull. N.Y. State Mus. 54: 963.

Type material. Holotype. United States, New York, Bolton, IX.1900, coll. C.H. Peck (NYS). **Epitype.** CANADA, New Brunswick, Fundy Nat. Park, vic. Alma, Caribou Plains Trail, 45°38.587' N, 65°06.937' W, 25.IX.2013, coll Stephen Clayden, TFB14409 (TENN-F-69073)

Taxon diagnosis. 1) ITS nrDNA sequence significantly different from sequence of *Gymnopus confluens* subsp. *confluens*; 2) basidiomata densely gregarious to subcespitose; 3) basidiomata apparently persistent beyond spore production and discharge; 4) stipe:pileus diameter ration from 2-5:1 (stipe significantly longer than pileus diameter); 5) pileus hygrophanous, brown where moist, pallid tan to pinkish buff where dry, drying to more uniform pallid color; 6) lamellae very crowded (total lamellae at pileus margin 110-140), shallow, seceding upon drying; 7) lamellar edge entire (smooth) to delicately fimbriate; 8) stipe grooved or compressed, stiff, with brown cortex (rind); 9) stipe vesture concolorous with pileus when moist and fresh, easily bleaching on drying to pallid gray shades; 10) basidiospores generally elongate-ellipsoid to sublacrymiform; 11) cheilocystidia stalked, usually lobed or strangulate, sometimes branched; 12) pileipellis hyphae smooth, firm-walled, with occasional to common side branches appearing digitate to long and branched. 13) Distribution in North America.

Description. Gymnopus confluens subsp. campanulatus; taxon description:

Pileus: Pileus 7–33 mm broad, thin (parchment-like and brittle when dry), often generally truncate-conical to shallowly convex with downturned margin when young becoming applanate to somewhat flaccid campanulate by maturity, occasionally with very shallow umbo or flattened over disc, minutely suede-like (not glabrous); disc "cinnamon buff" (6B4; dry), "sayal brown" (6C5) to "tawny olive" (5C5; moist); limb and margin "pinkish buff" (6A3) to "tilleul buff" (7B2) occasionally in hygrophanous zones; margin entire to somewhat lobate, sometimes subtly closely striate when dry. **Lamellae:** Lamellae very crowded, free to adnexed but significantly seceding upon drying and leaving a pale, off-white ring around the stipe apex, with relatively numerous lamellulae, very shallow (1 mm or less deep), slightly thickish, "tilleul buff" (7B2), "light buff" (3A2) to "deep olive buff" (3C3); lamellar edge never totally smooth, minutely fimbriate to minutely serrulate and usually paler than lamellar face. **Stipe:** Stipe of mature basidiomata 35–80(-95) mm long, 2.5–4 mm broad, stiff, equal except for slightly expanded base and slightly flaring apex, consistently grooved or fluted (but not compressed), stuffed to profoundly hollow; cortex (rind) tough, russet to mahogany ("Mars brown" 8F7, "tawny olive" 5C5), glassy; medulla (interior), lightly stuffed, nearly hollow, grayish cream colored, loose; vesture more or less uniform over

entire stipe length, consistently "tilleul buff" (7B2) to "pale olive buff" (3B2) when dry, detersile when fresh, easily disarticulated by handling when dry into minute chaff. Vesture of luxuriant form (New Brunswick, TFB 14409) delicately pruinose, apically concolorous with gills, soon "sayal brown"(6C5) to "Verona brown" (6E5). **Odor** none to faintly fresh; **taste** negligible to mild, perhaps weakly acidic, NOT acrid.

Habitat and phenology. on duff under *Quercus* and other hardwoods including *Acer* (TENN 63806); gregarious on leaf litter under *Fagus* (TENN 47030) and occasionally *Pinus*; hardwood duff (TENN 48376).

Pileipellis a thin layer of generally radially oriented hyphae; hyphae 4–11 µm diam, firm-walled, smooth (unornamented) to hardly ornamented (minute grit with suggestion of stripes or rings), conspicuously clamped, with infrequent, erect, side branches, ->75 µm long, 1.5–2.5 µm diam, simple to branched similar to cheilocystidial apices, arising from clamp connection or between clamps, often terminating in gradually tapering (2–4 µm diam at terminus) hyphal tips; contents heterogeneous, from amorphous sludge to coarsely spotted (PhC). **Pileus and lamellar trama hyphae** 3-9 µm diam, thin- to firm-walled, with occasional cheilocystidioid branches which seem to arise from clamp connections, conspicuously clamped, essentially free-form (TENN 53522), often anastomosing in "H" connections, when squashed often liberating minute debris in a subsoluble mucoid substance. Basidioles 22–25 × 5-7 µm, narrowly fusoid to torpedo-shaped, arising from a clamp. **Basidia** 21–30 ×7–9(-10) µm, clavate to broadly clavate, seldom bulbo-clavate, obscurely clamped, 4-sterigmate, arising from an obscure clamp; contents more or less homogeneous. **Basidiospores** (6-)6.5–9 × (2.5-)3–3.5(-4) µm (Q = 2.00-3.20; Q^m = 2.59; L^m = 7.40 µm), elongate ellipsoid, somewhat flattened adaxially to slightly sway-back, thin-walled, smooth; contents homogeneous. In TFB 14409 (NB), spores plump ellipsoid to plump pip-shaped; contents 1-several guttulate. TFB 14389 (NB) produced somewhat smaller basidiospores [6–7 × (2.5-)3–3.5 µm (Q = 2.00-2.33(-2.80); Q^m 2.16]. Lamellar edge entire to minutely fimbriate or minutely serrulate with cheilocystidia (64X), under magnification, lamellar edge fertile, with cheilocystidia locally abundant to sparsely scattered amongst fertile basidia; **cheilocystidia** typically (23-)34–77 × 2.5–4(-15) µm, hyphal, often 2-celled (with internal clamp), simple and substrangulate to usually branched with apical or subapical lobes or coralloid, contorted branches. Usually an accumulation of subsoluble mucoid material (with granular inclusions and embedded spores) surrounding cheilocystidial apices, perhaps exuded by the cheilocystidia themselves; cheilocystidia occasionally ramifying into slender (-1.5 µm diam), branched, arbuscular hyphal tips seemingly embedded in the mucoid matrix. Stipe surface hyphae 3.5–9 µm diam, strictly longitudinal and tightly parallel, occasionally but conspicuously clamped, often irregularly beset with small side lobes and short branchlets, sometimes arising from a clamp with a very thin mucoid sheath (with abundant embedded granular or globular material). **Stipe vesture** juxtaposed to stipe surface a thick, tightly interwoven thatch of thick-walled (wall -0.7 µm thick), very frequently branched, abundantly clamped hyphae 3.5–4.5 µm diam from which vesture columns and/or spikes arise; columns or spikes -100 µm tall, do not appear coherent, nor do

they seem gathered from neighboring hyphae, but seem to arise in groups to form columns; **caulocystidial hyphae** -150 × 3.5–5 µm, thick-walled (wall -0.7 µm thick) at origin, soon branched (at a clamp) to produce two individuals, often with an additional internal clamp and further unbranched, firm-walled, conspicuously clamped, replete with numerous small lobes or branches, terminating in a bluntly rounded apex.

Conclusions

Gymnopus confluens in Europe and North America shows intercontinental but not intracontinental divergence in ITS and LSU sequences but European and North American populations do not differ morphologically and retain the ability to dikaryotize *in vitro*. Intercontinental ITS/LSU sequence divergence is sufficient to recognize differences taxonomically. The North American population is described as *G. confluens* subsp. *campanulatus*.

Acknowledgements

We thank Dr. Nadezhda Psurtseva (Komarov Botanical Institute of the Russian Academy of Sciences) for arranging field work in Russia and for cultures from LE-BIN. We thank Stephen Claydon for donating specimens found during the Fundy Foray (2013) and Dr. Felix Hampe for collections found at the Russulales Congress in Germany 2012. MICH and WTU loaned critical specimens for analysis. Mr. Matt Aldrovandi helped with sequencing. We thank Roy Halling, Karl-Henrik Larsson, Christian Wurzbacher and an unknown reviewer for their reviews and suggestions.

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