MycoKeys 7: 1–22 (2013)
doi: 10.3897/mycokeys.7.4508
www.pensoft.net/journals/mycokeys

RESEARCH ARTICLE

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DNA barcode identification of lichen-forming fungal species in the *Rhizoplaca melanophthalma* species-complex (Lecanorales, Lecanoraceae), including five new species

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Academic editor: *I. Schmitt* | Received 13 December 2012 | Accepted 17 April 2013 | Published 9 May 2013

Citation: Leavitt SD, Fernández-Mendoza F, Pérez-Ortega S, Sohrabi M, Divakar PK, Lumbsch TH, St. Clair LLS (2013) DNA barcode identification of lichen-forming fungal species in the *Rhizoplaca melanophthalma* species-complex (Lecanorales, Lecanoraceae), including five new species. MycoKeys 7: 1–22. doi: 10.3897/mycokeys.7.4508

Abstract

Recent studies using sequence data from multiple loci and coalescent-based species delimitation have revealed several species-level lineages within the phenotypically circumscribed taxon *Rhizoplaca melanophthalma* sensu lato. Here, we formally describe five new species within this group, *R. occulta*, *R. parilis*, *R. polymorpha*, *R. porterii*, and *R. shushanii*, using support from the coalescent-based species delimitation method implemented in the program Bayesian Phylogenetics and Phylogeography (BPP) as the diagnostic feature distinguishing new species. We provide a reference DNA sequence database using the ITS marker as a DNA barcode for identifying species within this complex. We also assessed intraspecific genetic distances within the six *R. melanophthalma* sensu lato species. While intraspecific genetic distances within the five new species were less than or equal to the lowest interspecific pairwise comparison values, an overlap in genetic distances within the *R. melanophthalma* sensu stricto clade suggests the potential for additional

phenotypically cryptic lineages within this broadly distributed lineage. Overall, our results demonstrate the potential for accurately identifying species within the *R. melanophthalma* group by using molecular-based identification methods.

Key words
Bio-monitoring, BPP, coalescence, cosmopolitan distribution, cryptic species, molecular identification, symbiosis

Introduction

The lichen symbiosis has been highly successful with more than 18,000 currently accepted species of lichen-forming fungi (Feuerer and Hawksworth 2007) and an estimated diversity of more than 28,000 species (Lücking et al. 2009). However, robust species delimitations and accurate identification of lichenized fungal species remains challenging (reviewed in Crespo and Perez-Ortega 2009; Crespo and Lumbsch 2010; Printzen 2010; Lumbsch and Leavitt 2011). In many cases, characters used for identification may be subtle or difficult to discern, diagnostic morphological and chemical characters may be lacking in juvenile or fragmentary samples, and the traditional phenotype-based approach to species recognition in lichenized fungi has been shown in some cases to substantially misrepresent diversity (e.g. Taylor et al. 2000; Divakar et al. 2010a; Kelly et al. 2011; Leavitt et al. 2011a; Leavitt et al. 2011b; Molina et al. 2011a; Schmull et al. 2011; Pino-Bodas et al. 2012). As in other groups of fungi, molecular data have also revolutionized our understanding of evolution and species delimitation in lichenized fungi (Crespo and Perez-Ortega 2009; Crespo and Lumbsch 2010; Printzen 2010; Lumbsch and Leavitt 2011). Furthermore, molecular-based identification methods now provide an accessory approach for specimen identification (Seifert 2009; Begerow et al. 2010; Del-Prado et al. 2010; Kelly et al. 2011; Schoch et al. 2012).

Using DNA barcoding, a reference DNA sequence database generated from expertly identified specimens can provide an effective alternative to phenotype-based identification of lichen-forming fungal species (Kelly et al. 2011; Schoch et al. 2012). However, the practicality of DNA barcoding as a major tool for identification of lichenized fungi is largely dependent on the development of high-quality sequence databases that are thoroughly curated by taxonomists and systematists (Begerow et al. 2010; Orock et al. 2012). Previous studies have demonstrated that a high proportion of lichen-forming fungal species can be correctly identified by comparing sequence similarity using BLAST-based identification from fragments of the internal transcribed spacer region of the nuclear ribosomal RNA cistron (ITS: ITS1-5.8S-ITS2) against custom databases (Kelly et al. 2011; Schoch et al. 2012). These results highlight the potential utility of DNA-based identification as a valuable tool in lichen systematics research. Furthermore, the ITS region was recently proposed for adoption by the Consortium for the Barcode of Life as the first fungal barcode marker (Schoch et al. 2012). However, its accuracy is largely dependent on the taxonomic knowledge and

Figure 1. Variation in morphology and habit within *Rhizoplaca melanophthalma* sensu lato. Scale bar = 5 mm.

the sample coverage of the group (Roe et al. 2010; Kelly et al. 2011; Kiss 2012; Orock et al. 2012; Schoch et al. 2012).

Rhizoplaca Zopf, as currently circumscribed, is a morphologically diverse, polyphyletic genus (Arup and Grube 2000) represented by ca. 19 lichen-forming fungal species (Index Fungorum: http://www.indexfungorum.org/). Within the past decade a number of studies have indicated that traditional phenotype-based species circumscriptions fail to recognize multiple species-level lineages within this genus (Zhou et al. 2006; Leavitt et al. 2011a). The *Rhizoplaca melanophthalma* species-complex (sensu Leavitt et al. 2011a) includes a morphologically diverse assemblage of species, including individuals ranging from placodioid crustose and umbilicate forms, to completely vagrant, or obligatory unattached forms. Substantial chemical variation has been identified within this group, including at least four chemotypes within *R. melanophthalma* sensu lato (s.l.) (McCune 1987; Ryan 2001); and eight previously unrecognized extrolites recently identified from specimens within this complex (Leavitt et al. 2011a).

Within *R. melanophthalma* s. l. (Fig. 1), Leavitt et al. (2011a) circumscribed six 'candidate' species that were supported using multiple lines of evidence from molecular sequence data, including: fixed nucleotide characters, genealogical exclusivity, Bayesian population clustering, and the coalescent-based species delimitation program Bayesian Phylogenetics and Phylogeography (BPP; Yang and Rannala 2010). The lat-

ter, has recently been shown to outperform other species delimitation methods under a variety of scenarios (Camargo et al. 2012). In spite of a high degree of morphological and chemical variation within this species complex, most of the candidate species identified in this study were morphologically and/or chemically polymorphic, and diagnostic morphological/chemical characters were not identified for the majority of the independent species-level lineages (Leavitt et al. 2011a). Recently, additional data, including broader geographic sampling and three additional genetic markers, provide additional support that the candidate species identified in Leavitt et al. (2011a) represent species-level lineages (Leavitt et al. 2013).

While morphological and chemical character differences have traditionally served as proxies for identifying reproductively isolated groups, multilocus coalescent-based species delimitation methods can provide a more direct assessment of gene flow and independent lineage status through genetic analysis. Coalescent-based methods can provide a more direct and replicable approach for assessing hypotheses of evolutionary independence, regardless of whether putative lineages differ in potentially subjective phenotypic character systems (Fujita et al. 2012). Character evolution in lichens is still poorly understood, leading to potentially confounding morphological/chemical taxonomic features (e.g. Leavitt et al. 2011b; Leavitt et al. 2011c; Pino-Bodas et al. 2011; Pérez-Ortega et al. 2012; Pino-Bodas et al. 2012). While we are strong advocates for the application of independent data types (i.e. ecology, geography, morphology, genetics, and chemistry) in developing an integrative taxonomy, there is an increasing need to formally recognize the existence of phenotypically cryptic species-level lineages in lichen-forming fungi (Hibbett et al. 2011).

In this paper we use support from the coalescent-based species delimitation method implemented in the program BPP (Yang and Rannala 2010) as the diagnostic feature distinguishing new species from other taxa. While in practice, most modern species descriptions include a character-based diagnosis, it has been argued that coalescent-based diagnosis serve the same purpose as a standard diagnosis when the species in question is not diagnosable on the basis of morphology alone (Leache and Fujita 2010; Fujita and Leache 2011; Fujita et al. 2012).

Rhizoplaca melanophthalma s.l. is frequently used in air quality bio-monitoring studies (Dillman 1996; Ugur et al. 2004), and differences in pollution accumulation patterns among closely related species have not been tested. In addition, *R. melanophthalma* s.l. has been shown to have pharmaceutical potential for treating drug genotoxicity in human blood (Geyikoglu et al. 2007). Therefore, accurate specimen identification may have important implications for bio-monitoring and pharmaceutical research using *R. melanophthalma* s.l. Furthermore, several lineages within the *R. melanophthalma* species-complex are broadly distributed (Leavitt et al. 2013), and these may potentially serve as valuable groups for assessing dispersal capacity and landscape-level genetics in response to changing climatic conditions, assuming accurate specimen identification. Given the overall importance of accurate specimen identification, including phenotypically cryptic lineages, the objectives of this study are to (1) formally describe five new species within this group and (2) provide a reference DNA sequence database using the ITS marker as a DNA barcode for identifying species within this complex.

Methods

Candidate species and taxon sampling

Using multilocus sequence data generated from *Rhizoplaca* specimens collected throughout the Intermountain region of western North America, Leavitt et al. (2011a) circumscribed six candidate species, 'C2', 'C3', 'C4a', 'C4b', 'C4c', and 'C4d', within *R. melanophthalma* s.l. Based on initial sampling, these six species-level lineages were strongly supported by a variety of operational criteria for species delimitation (Leavitt et al. 2011a). Furthermore, many of the candidate species within *R. melanophthalma* s.l. were shown to occur sympatrically with strong evidence of reproductive isolation among lineages (Leavitt et al. 2011a), and thus *de facto* species status. However, these candidate species were not formally described due to the limited geographical sampling. Increased geographic sampling, including collections from Antarctica, Central Asia, Europe, and North and South America, along with additional genetic markers corroborate the previously recognized candidate species (Leavitt et al. 2013).

Data analysis

In this study, we used the ITS alignment and phylogeny reported in Leavitt et al. (2013) (Fig. 2A; supplementary file 1; TreeBase ID 13903). This data consisted of 228 sequences and 524 aligned nucleotide position characters. A full description of multiple sequence alignment and phylogenetic reconstruction methods is given in Leavitt et al. (2013). In short, the multiple sequence alignment was performed using the program MAFFT v6 (Katoh et al. 2005; Katoh and Toh 2008), and phylogenetic relationships were estimated using maximum likelihood using the program RAxML v7.2.8 (Stamatakis 2006; Stamatakis et al. 2008).

In the present study, we calculated pairwise distances to characterize both intra- and interspecific variation within and among candidate species-level lineages. Pairwise distances can be viewed as a rough measure for the overall sequence divergence (DelPrado et al. 2010). Average genetic distances were computed using PAUP* (Swofford 2002) based on pairwise comparisons of all sequences within each candidate species individually, overall intraspecific distances from all species, and pairwise interspecific distances. Pairwise distances between different haplotypes were reported as the number of nucleotide substitutions per site (s/s).

The Barcode of Life Data Systems (BOLD; Ratnasingham and Hebert 2007) provides an informatics workbench aiding the acquisition, storage, analysis, and application of DNA barcode data, including a BLAST-based identification tool for fungi using the ITS region. Data from the candidate species circumscribed in Leavitt et al. (2011a), including ITS sequences, electropherograms, and collection information, were submitted to the BOLD database, project name '*Rhizoplaca melanophthalma-*

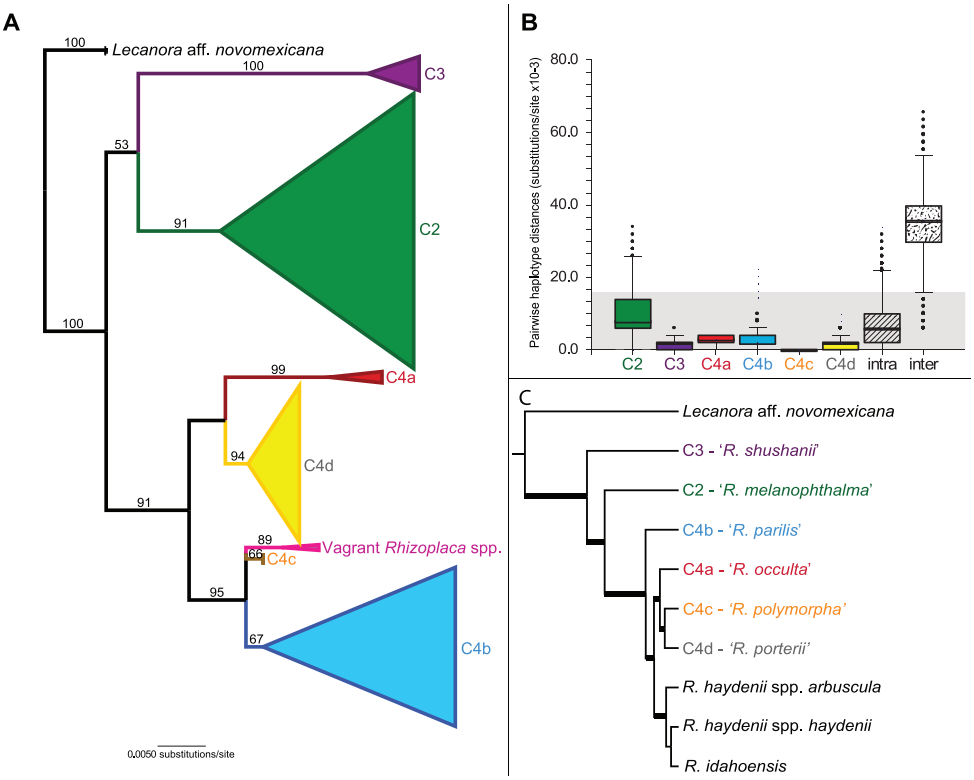

Figure 2. A Cartoon representation of the maximum likelihood ITS topology obtained from 240 *Rhizoplaca melanophthalma* sensu lato specimens in Leavitt et al. (in review). Values at each node indicate non-parametric-bootstrap support; only support values > 50% are indicated **B** Box plots of ITS genetic distances within each new species, all intraspecific distances, and all interspecific distances. In each box plot, the box shows the interquartile range (IQR) of the data. The IQR is defined as the difference between the 75th percentile and the 25th percentile. The solid and dotted line through the box represent the median and the average length, respectively; and **C** The coalescent-based species-tree for the *Rhizoplaca melanophthalma* species-complex estimated from five genetic markers (ITS, IGS, group I intron, β-tubulin, and MCM7 loci) in Leavitt et al. 2011a.

species complex'. This custom database includes collections form the western USA representing the six previously recognized candidate species.

We assessed the utility of the ITS region for BLAST-based specimen identification in the *R. melanophthalma* group by conducting searches of the newly reported ITS sequences in Leavitt et al. (2013) against the custom BOLD database. The queried sequences were generated from *R. melanophthalma* s.l. specimens from Antarctica, Austria, Chile, China, the Czech Republic, Iran, Kazakhstan, Kyrgyzstan, Russia, Spain, Switzerland, and additional specimens from the United States of America (supplementary file 1). Top species matches obtained from the BLAST searches against the BOLD database for each specimen were recorded and compared with results from a standard, tree-based method

to address accuracy of identification in a thoroughly sampled phylogeny. Species were scored as successfully discriminated if the samples were recovered within monophyletic clades in the ML analysis corresponding to candidate species and if top species matches for each new sequence obtained from the BLAST searches against the BOLD database corresponded to the monophyletic clade where it was recovered.

The marginal posterior probability of speciation (speciation probability) was estimated in Leavitt et al. (2013) from multi-locus sequence data using the program BPP v2.1 (Yang and Rannala, 2010). This method accommodates the species phylogeny as well as lineage sorting due to ancestral polymorphism. BPP has recently been shown to outperform other coalescent-based species delimitation methods, with robust performance using a modest number of genetic markers (Camargo et al., 2012). Full details are reported in Leavitt et al. (2013) and summarized only briefly here. The multi-locus species tree, representing the six candidate species, was used as the fully resolved guide tree. Because the prior distribution of θ and τ_0 can result in strong support for models containing more species (Leaché & Fujita, 2010), Leavitt et al. (2013) explored three combinations of priors, including a moderate and conservative combination. The most conservative combinations of priors favors fewer species by assuming large ancestral population sizes, and relatively shallow divergences among species. The moderately conservative set of priors assumed intermediate ancestral population sizes, and relatively shallow divergences among species. High speciation probabilities (SP ≥ 0.95) were estimated at all nodes using both the default prior gamma distributions for θ and τ_0 and a more moderate combination of these priors, with the exception of the split between the two vagrant species (*R. haydenii* and *R. idahoensis*). Under the most conservative combination of priors for θ and τ_0, speciation probabilities match those supported using default priors, with the exception of lower probabilities for a split between 'C4d' and 'C4c' (SP < 0.50).

Results

For each candidate species, the pairwise distances among the different ITS haplotypes were estimated and the distribution of distances plotted (Fig. 2B). Intraspecific genetic distances in candidate species-level lineages 'C3', 'C4a', 'C4b', 'C4c' and 'C4d' were less than or equal to the lowest interspecific pairwise comparison values (0.006 substitutions/site), with the exception of outlier values in 'C4b' and 'C4d' (Fig. 2B). The outlier values within clade 'C4b' were based on comparisons from two sequences retrieved from GenBank (EF095278 and EF095287). We were unable to verify the quality of these sequences. Within candidate lineage 'C2', intraspecific values largely fell below the lower quartile of interspecific pairwise comparison values (Fig. 2B), although there was substantial overlap between intra- and interspecific genetic distance values. Mean distance values, standard deviations and the range of intraspecific distances within the six candidate species are reported in Table 1.

Table 1. Mean genetic distance values (given as number of nucleotide substitution per site) and range of intraspecific distances for the *Rhizoplaca melanophthalma* species-complex. Numbers within parentheses indicate the number of sampled individuals/lineage; and values following mean genetic distance represent standard deviations.

Species/Clade	Mean	range
R. melanophthalma 'C2' (98)	0.009 ± 0.006	0.0 – 0.034
R. occulta 'C4a' (5)	0.003 ± 0.001	0.0 – 0.004
R. parilis 'C4b' (56)	0.003 ± 0.004	0.0 – 0.022
R. polymorpha 'C4c' (6)	0.0 ± 0	0.0 – 0.0
R. porterii 'C4d' (57)	0.002 ± 0.002	0.0 – 0.010
R. shushanii 'C3'(13)	0.001 ± 0.002	0.0 – 0.006
Intraspecific	0.007 ± 0.006	0.0 – 0.034
Interspecific	0.033 ± 0.009	0.006 – 0.066

In all BLAST-based searches against the BOLD database, the sequences with the highest overall similarity to the query sequence were also recovered in the corresponding monophyletic clade.

The species

***Rhizoplaca melanophthalma* (DC.) Leuckert & Poelt Nova Hedwigia 28: 72 (1977)**
MycoBank no. MB 343580
http://species-id.net/wiki/Rhizoplaca_melanophthalma

Basionym. *Lichen melanophthalma* Ramond in Lamarck & de Candolle, Fl. Franc. Ed. 3.2:377 (1805).

Epitype. Spain, Teruel, Noguera de Albarracín, carretera A-1521 hacia Orihuela del Tremedal, antes de la Peña del Castillo, pista a "Ruta Laguna", "Peña Aguada", 30TXK16815, 1590 m alt., on quartzite in a *Quercus pyrenaica* and *Pinus sylvestris* forest, 04 October 2010, M. Vivas & J. Rico, Vivas 94, MAF –Lich 16805 (Epitype: MAF-Lich). We were unable to obtain fresh material from the Pic du Midi de Bigorre in the French Pyrenees (location of original type collection of *R. melanophthalma*). In order to fix the application of the name, we selected a specimen from Teruel, Spain (MAF-Lich 16805) as the epitype. The epitype shares an identical ITS haplotype with specimens collected in Chile, China, Spain, Switzerland, and the USA, and thus appropriately represents the cosmopolitan distribution of *R. melanophthalma* s.s. The ITS sequence of the epitype is deposited in GenBank under accession no. JX948232.

Description. A morphological description can be found in Leuckert et al. (1977) and Ryan (2001). *Rhizoplaca melanophthalma* consists of specimens recovered within 'clade II' in Leavitt et al. (2011a), which is supported as a lineage distinct from all other populations according to coalescent-based genetic analysis of multiple genetic loci. The mean genetic distances among ITS haplotypes was estimated to be 0.009 ± 0.006.

Chemistry – Usnic acid (major); usually with psoromic (major), constipatic (minor), dehydroconstipatic (minor), dehydroprotocetraric (minor), and 2'-*O*-demethylsubpsoromic (minor or trace) acids; occasionally with subpsoromic (minor) and 2'-*O*-demethylpsoromic (minor) acids.

Reference phylogeny. Supplementary file 2 (terminal label: '6604') & Leavitt et al. 2011a (Fig. 5, 'clade II').

Reference sequence. GenBank no. JX948232.

Phylogenetic notes: Strongly supported as monophyletic lineage in both concatenated multilocus gene tree (ML bootstrap = 95%: posterior probability = 1.0) and the ITS gene topology (ML bootstrap = 91%, this study); and strong speciation probability inferred from multiple loci (BPP speciation probability = 1.0).

Ecology and distribution. In its narrower circumscription, this taxon is known from Antarctica, Asia (including Central Asia and China), Europe, North and South America. The species has also been recorded from alpine areas in the tropics. However, additional studies are required to verify the identity of these populations. It typically occurs on exposed calcium-poor rock (e.g. basalt, granite, schist), but sometimes on calcium rich sandstone and limestone. It ranges in distribution from arid lowland woodlands into upper montane coniferous forests and the lower portions of the alpine tundra.
Specimens examined. See supplementary file 1.

Rhizoplaca occulta **S. Leavitt, F. Fernández-Mendoza, Lumbsch, Sohrabi & L. St. Clair, sp. nov.**
MycoBank no. MB 803475
http://species-id.net/wiki/Rhizoplaca_occulta

Type. USA, Nevada, White Pine County, on Cave Mountain, north of antenna site, Humboldt-Toiyabe National Forest, 39.1734°N, -114.6130°W, on basalt, 3150 m alt., July 2010, S. D. Leavitt & Larry L. St. Clair BRY-C55076 (holotype BRY).

Description. Consists of specimens recovered within 'clade IVa' in Leavitt et al. (2011a), which is supported as a lineage distinct from all other populations according to coalescent-based genetic analysis of multiple genetic loci. This species is morphologically variable. While some individuals are morphologically similar to *R. melanophthalma* sensu stricto, vagrant forms, including *R. cerebriformis* ined. and *R. subidahoensis* ined. which have been suggested to be distinct species based on morphology (Arup and Grube 2000; Figs. 7 & 9), also belong within this species. The mean genetic distance among ITS haplotypes was estimated to be 0.003 ± 0.001.

Chemistry – Usnic (major), constipatic (minor), dehydroconstipatic (minor), 2'-*O*-demethylpsoromic (minor), and 2'-*O*-demethylsubpsoromic (minor or trace) acids; usually with psoromic acid (major); and occasionally with dehydroprotocetraric acid (minor).

Reference phylogeny. Leavitt et al. 2011a (Fig. 5, 'clade IVa').

Reference sequences. GenBank Nos. HM577307 (ITS), HM577081 (IGS), HM577210 (group I intron), HM577441 (*MCM7*), and HM576952 (β-tubulin).

Phylogenetic notes: Strongly supported as monophyletic lineage in both concatenated multilocus gene tree (ML bootstrap = 100%: posterior probability = 1.0) and the ITS gene topology (ML bootstrap = 99%, this study); and strong speciation probability inferred from multiple loci (BPP speciation probability ≥ 0.98). *R. occulta* belongs to a closely related, and well-supported, monophyletic lineage including *R. paralis*, *R. polymorpha*, *R. porterii*, and the obligatory vagrant species *R. haydenii* and *R. idahoensis.*

Ecology and distribution. Growing usually on exposed calcium-poor rock (e.g. basalt, granite, schist) in pinyon-juniper woodlands but also occurs free on soil. So far known only from collections in western North America. *R. occulta* included a total of five individuals from Idaho (3 individuals), Nevada (1), and Utah (1), USA, and included GenBank accessions identified as *R. cerebriformis* ined. (AF159942) and *R. subidahoensis* ined. (AF159944).

Etymology. The name is derived from the Latin "occultus," meaning hidden, and refers to the fact that this species was hidden within the phenotypically circumscribed taxon *Rhizoplaca melanophthalma* sensu lato.

Specimens examined. See supplementary file 1.

***Rhizoplaca parilis* S. Leavitt, F. Fernández-Mendoza, Lumbsch, Sohrabi & L. St. Clair, sp. nov.**
MycoBank no. MB 803476
http://species-id.net/wiki/Rhizoplaca_parilis

Type. USA, Utah, Sevier County, Thousand Lakes Mountain, north of 'Flat Top', 38.5111°N, -111.4732°W, on basalt, 2875 m alt., October 1997, Lyndon D. Porter BRY-C55077 (holotype BRY).

Description. Morphologically similar to *R. melanophthalma* sensu stricto, but consists of specimens recovered within 'clade IVb' in Leavitt et al. (2011a), which is supported as a lineage distinct from all other populations according to coalescent-based genetic analysis of multiple genetic loci. Within the *R. melanophthalma* species-complex, the occurrence of orsellinic, lecanoric, and gyrophoric acids appear to be restricted to *R. parilis*. However, the occurrence of these compounds varies widely within this species, with the proportional occurrence of each compound ranging between 0.43 – 0.64 (Leavitt et al. 2011a). The mean genetic distance among ITS haplotypes was estimated to be 0.003 ± 0.004.

Chemistry – Usnic (major) and psoromic (major) acids; usually with constipatic (minor), dehydroconstipatic (minor), dehydroprotocetraric (minor), lecanoric (major), orsellinic (minor), and subpsoromic (minor) acids; occasionally with gyrophoric (trace), 2'-*O*-demethylsubpsoromic (minor or trace) and 2'-*O*-demethylpsoromic (minor) acids.

Reference phylogeny. Leavitt et al. 2011a (Fig. 5, 'clade IVb').

Reference sequences. GenBank Nos. HM577308 (ITS), HM577082 (IGS), HM577211 (group I intron), HM577442 (*MCM7*), and HM576953 (β-tubulin)

Phylogenetic notes: Strong to moderate support as monophyletic lineage in both concatenated multilocus gene tree (ML bootstrap = 83%: posterior probability = 0.93), and with weak statistical support in the ITS gene topology (ML bootstrap = 67%, this study); and strong speciation probability inferred from multiple loci (BPP speciation probability = 1.0). *R. parilis* belongs to a closely related, and well-supported, monophyletic lineage including *R. occultum*, *R. polymorpha*, *R. porterii*, and the obligatory vagrant species *R. haydenii* and *R. idahoensis.*

Ecology and distribution. This species usually occurs on exposed calcium-poor rock (e.g. basalt, granite, schist), but sometimes on calcium rich sandstone and limestone. Its habitat ranges from pinyon-juniper woodlands to montane coniferous forests and the lower portions of alpine tundra. This taxon is currently known from Asia (including Central Asia and China), Europe, and North and South America

Etymology. The specific epithet is chosen from the Latin *parilis*, meaning equivalent, like, or similar, in reference to the morphological similarity between the new species and the other species within the *R. melanophthalma* species-complex.

Specimens examined. See supplementary file 1.

***Rhizoplaca polymorpha* S. Leavitt, F. Fernández-Mendoza, Lumbsch, Sohrabi & L. St. Clair, sp. nov.**
MycoBank no. MB 803477
http://species-id.net/wiki/Rhizoplaca_polymorpha

Type. USA, Idaho, Owyhee County, 43.3202°N, -116.9795°W, 1291 m alt., 04 July 2008, S. D. Leavitt, H. C. Leavitt & J.H. Leavitt BRY-C55093 (holotype BRY).

Description. *Rhizoplaca polymorpha* consists of specimens recovered within 'clade IVc' in Leavitt et al. (2011a), which is supported as a lineage distinct from all other populations according to coalescent-based genetic analysis of multiple genetic loci. This species is morphologically quite variable. While some individuals are morphologically similar to *R. melanophthalma* sensu stricto, vagrant forms partly embedded in badland soils in western Idaho also belong within this species. The mean genetic distance among ITS haplotypes was estimated to be 0.0 ± 0.

Chemistry – Usnic (major), constipatic (minor), dehydroconstipatic (minor), 2'-*O*-demethylsubpsoromic (minor or trace) and 2'-*O*-demethylpsoromic (minor) acids; occasionally with dehydroprotocetraric (minor) and psoromic acid (major).

Reference phylogeny. Leavitt et al. 2011a (Fig. 5, 'clade IVc').

Reference sequences. GenBank Nos. HM577324 (ITS), HM577097 (IGS), HM577227 (group I intron), HM577458 (*MCM7*), and HM576968 (β-tubulin).

Phylogenetic notes: Strongly supported as monophyletic lineage in both concatenated multilocus gene tree (ML bootstrap = 82%: posterior probability = 1.0), and

weak statistical support in the ITS gene topology (ML bootstrap = 66%, this study); and strong speciation probability inferred from multiple loci (BPP speciation probability ≥ 0.97). *R. polymorpha* belongs to a closely related, and well-supported, monophyletic lineage including *R. occulta*, *R. parilis*, *R. porterii*, and the obligatory vagrant species *R. haydenii* and *R. idahoensis.*

Ecology and distribution. Currently only known from collections in western North America. Its habitat includes pinyon-juniper woodlands and montane coniferous forests, but unattached forms are also known from the McBride Creek Badlands in Western Idaho.

Etymology. The specific epithet was selected based on the morphologically polymorphic forms within this species, including both umbilicate and vagrant forms.

Specimens examined. See supplementary file 1.

Rhizoplaca porterii **S. Leavitt, F. Fernández-Mendoza, Lumbsch, Sohrabi & L. St. Clair, sp. nov.**
MycoBank no. MB 803478
http://species-id.net/wiki/Rhizoplaca_porterii

Type. USA, Utah, Wayne County, Thousand Lakes Mountain, vicinity of 'Flat Top', near summit, 38.4432°N, -111.4703°W, on basalt, 3400 m alt., October 1997, Lyndon D. Porter BRY-C55096 (holotype BRY)**.**

Description. Morphologically similar to *R. melanophthalma* sensu stricto, but consists of specimens recovered within 'clade IVd' in Leavitt et al. (2011a), which is supported as a lineage distinct from all other populations according to coalescent-based genetic analysis of multiple genetic loci. This species is also characterized by the absence of a group I intron in the nuclear SSU rDNA at the 1516 position (Gutiérrez et al. 2007), which is present in all other species within the *R. melanophthalma* species-complex. The mean genetic distances among ITS haplotypes was estimated to be 0.002 ± 0.002.

Chemistry – Usnic acid (major); usually with psoromic (major), constipatic (minor), dehydroconstipatic (minor), dehydroprotocetraric (minor), subpsoromic (minor), demethylpsoromic (minor), and 2'-*O*-demethylsubpsoromic (minor or trace) acids.

Reference phylogeny. Leavitt et al. 2011a (Fig. 5, 'clade IVd').

Reference sequences. GenBank Nos. HM577327 (ITS), HM57710 (IGS), HM577461 (*MCM7*), and HM576971 (β-tubulin).

Phylogenetic notes: A monophyletic lineage in both concatenated multilocus gene tree with weak statistical support (ML bootstrap < 50%; posterior probability < 0.5), and with strong statistical support in the ITS gene topology (ML bootstrap = 94%, this study); and strong speciation probability inferred from multiple loci (BPP speciation probability ≥ 0.97). *R. porterii* belongs to a closely related, and well-supported, monophyletic lineage including *R. occulta*, *R. parilis*, *R. porterii*, and the obligatory vagrant species *R. haydenii* and *R. idahoensis.*

Ecology and distribution. This species usually occurs on exposed calcium-poor rock (e.g. basalt, granite, schist), but sometimes on calcium rich sandstone and limestone. Its habitat ranges from pinyon-juniper woodland into montane coniferous forests and lower alpine tundra. This taxon is currently known only from the western USA (Idaho and Utah).

Etymology. The new taxon is named in honor of Dr. Lyndon D. Porter, whose research on *Rhizoplaca melanophthalma* proved invaluable to the present work.

Specimens examined. See supplementary file 1.

***Rhizoplaca shushanii* S. Leavitt, F. Fernández-Mendoza, Lumbsch, Sohrabi & L. St. Clair, sp. nov.**
MycoBank no. MB 803479
http://species-id.net/wiki/Rhizoplaca_shushanii

Type. USA, Utah, Wayne County, Thousand Lakes Mountain, vicinity of 'Flat Top', near summit, 38.4366°N, -111.4677°W, on basalt, 3270 m alt., October 1997, Lyndon D. Porter BRY-C55061 (holotype BRY).

Description. Morphologically similar to *R. melanophthalma* sensu stricto, but consists of specimens recovered within 'clade III' in Leavitt et al. (2011a), which is supported as a lineage distinct from all other populations according to coalescent-based genetic analysis of multiple genetic loci. The mean genetic distances among ITS haplotypes was estimated to be 0.001 ± 0.002.

Chemistry – Usnic (major), psoromic (major), subpsoromic (minor), and 2'-*O*-demethylsubpsoromic (minor or trace) acids; usually with constipatic (minor) and 2'-*O*-demethylpsoromic (minor) acids; and occasionally with dehydroconstipatic (minor) and dehydroprotocetraric (minor) acids.

Reference phylogeny. Leavitt et al. 2011a (Fig. 5, 'clade III').

Reference sequences. GenBank Nos. HM577282 (ITS), HM577058 (IGS), HM577187 (group I intron), HM577416 (*MCM7*), and HM576927 (β-tubulin).

Phylogenetic notes: A monophyletic lineage in both concatenated multilocus gene tree with strong statistical support (ML bootstrap = 100%; posterior probability 1.0), and with strong statistical support in the ITS gene topology (ML bootstrap = 100%, this study); and high speciation probability inferred from multiple loci (BPP speciation probability = 1.0).

Ecology and distribution. Found growing only on sun-exposed basalt boulders in subalpine meadows in southwestern USA. Currently known only from subalpine habitats on the Aquarius Plateau in southern Utah, USA.

Etymology. The new taxon is named in honor of the late Dr. Sam Shushan, a pioneer in western North American lichenology.

Specimens examined. See supplementary file 1.

Discussion

In this study we described five new species within *Rhizoplaca melanophthalma* s.l. Our results indicate that a molecular-based approach for specimen identification in the common lichen-forming *R. melanophthalma* species-complex can effectively assign individuals from cosmopolitan populations to previously circumscribed 'candidate' species (Leavitt et al. 2011a; Leavitt et al. 2013). Molecular data indicate that the genus *Lecanora*, as currently circumscribed, is not monophyletic (Arup and Grube 1998; 2000; Lumbsch 2002; Grube et al. 2004). The *Rhizoplaca melanophthalma* species-complex clearly falls outside of the core group of *Lecanora* sensu stricto (including the type species *L. allophana*; see Brodo and Vitikainen 1984), and we choose to describe the new species within the heterogeneous, and also non-monophyletic, genus *Rhizoplaca*, pending future taxonomic revisions.

In spite of the limitations in delimiting taxa using molecular data, the effective use of genetic data appears to be essential to appropriately and practically identify natural groups in some phenotypically cryptic lichen-forming fungal lineages (Divakar et al. 2010b; Leavitt et al. 2011b; Leavitt et al. 2011c; Molina et al. 2011b; Pino-Bodas et al. 2011; Pino-Bodas et al. 2012), including *R. melanophthalma* s.l. This does not preclude the fact that additional studies investigating morphological and chemical characters may potentially identify independent characters, or combinations of characters, supporting species circumscribed using molecular data. In fact, under the general lineage species concept (GLC; de Queiroz 1998, 1999, 2007), more independent properties associated with putative species boundaries are associated with a higher degree of corroboration, resulting in a truly integrative approach to species discovery. However, robust species delimitations using molecular data in phenotypically cryptic species can provide working hypotheses about what constitutes separately evolving metapopulation lineages (de Queiroz 1998; 1999; Mayden 1999; de Queiroz 2007; Fujita et al. 2012). DNA barcoding provides an objective approach for specimen identification within these taxonomically difficult groups.

Within the *Rhizoplaca melanophthalma* species-complex, DNA barcoding can be performed in a variety of ecological, bio-monitoring and population genetic studies in order to quickly sort specimens into genetically divergent groups. In *R. melanophthalma* s.l., this barcode application for specimen identification may provide a valuable framework for assessing biogeographic patterns, bio-monitoring research, and prove to be an important tool in making critical conservation-related decisions. The application of molecular-based identification could also be used as a way for both specialists and non-specialists alike to discriminate species that are otherwise difficult to identify, making specimen identification more accessible and more accurate at the same time. In spite of the progress in recognizing independent species-level lineages within *R. melanophthalma* s.l., high intraspecific distances within *R. melanophthalma* sensu stricto, suggest additional species-level lineages may potentially be hidden within this lineage. In order to address this question, we are currently developing novel genetic markers (i.e. microsatellites) specific for this group in order to assess population structure and gene flow within this broadly distributed species.

Using ITS sequence data, specimens within the *Rhizoplaca melanophthalma* species group can be identified by means of DNA barcoding using the publicly available database in BOLD (http://www.boldsystems.org/). Based on our broad intercontinental sampling, it appears that *Rhizoplaca melanophthalma* s.l. specimens can be accurately identified to species using the BLAST-based identification tool for fungi in BOLD. This provides an objective approach for a broad array of researchers to accurately identify species within this group using ITS sequence data from their collections, regardless of their level of taxonomic expertise.

Although in some cases the ITS region has been shown to be effective for molecular identification using DNA barcoding (Kelly et al. 2011; Schoch et al. 2012), including species within the *Rhizoplaca melanophthalma* species-complex, the reliance on a single locus for inferring relationships and circumscribing species is problematic because the history of a single gene might not be representative of the organismal history. Within the *R. melanophthalma* species-complex relationships among species-level lineages are largely unsupported in the ITS topology (Fig. 2A) and differ greatly from the coalescence-based species tree estimated from multilocus sequence data (Fig. 2C; Leavitt et al. 2011a; Leavitt et al. 2013). Additionally, genealogical concordance among independent genetic markers can provide strong evidence that distinct clades represent reproductively isolated lineages among well-separated groups (Dettman et al. 2003; Pringle et al. 2005). Although different datasets and operational criteria may give conflicting or ambiguous results due to the multiple evolutionary processes associated with speciation, the use of multilocus sequence data and multiple empirical methods are known to establish robust species boundaries in many lichen-forming fungal lineages (reviewed in Lumbsch and Leavitt 2011). Within the *R. melanophthalma* species-complex, the majority of candidate species showed high levels of genealogical concordance among three ribosomal and two protein-coding markers (Leavitt et al. 2011a). The coalescent-based species delimitation method BPP (Yang and Rannala 2010) also supported the distinctness of all new *Rhizoplaca* species described here (Leavitt et al. 2013) and, under a variety of scenarios, has been shown to be among the most accurate coalescent-based species delimitation methods (Camargo et al. 2012).

While in some cases data have supported the taxonomic use of secondary metabolic characters for delimiting lichen taxa (Tehler and Kallersjo 2001; Blanco et al. 2004; Schmitt and Lumbsch 2004; Molina et al. 2011a), other studies found no correlation between chemotypes and lineages identified using molecular phylogenetic reconstructions (Articus et al. 2002; Buschbom and Mueller 2006; Divakar et al. 2006; Nelsen and Gargas 2009; Velmala et al. 2009; Myllys et al. 2011). In spite of some general patterns in the distribution of secondary metabolites among species within the *Rhizoplaca melanophthalma* species-complex, it appears that chemical characters cannot consistently be used to diagnose independent species-level lineages. For example, within this complex the occurrence of orsellinic, lecanoric, and gyrophoric acids appear to be restricted to *R. parilis*. However, the occurrence of these compounds varies widely within this species, with the proportional occurrence of each compound ranging between 0.43 – 0.64 (Leavitt et al. 2011a). Additionally, all sampled specimens

of vagrant *Rhizoplaca* species only produced usnic acid, although additional morphological differences are required to accurately identify distinct vagrant species. Previous studies have used TLC to characterize lichen secondary metabolic products within *Rhizoplaca* (McCune 1987; Zhou et al. 2006). However, it appears that within *R. melanophthalma* s.l. some extrolites would be masked by other compounds, or likely found at levels undetectable by TLC; and HPLC has been shown to provide a more sensitive approach for determining secondary metabolite diversity within the *R. melanophthalma* complex (Leavitt et al. 2011a). In spite of the increased sensitivity of HPLC, unambiguous secondary metabolic characters corroborating most of the species within *R. melanophthalma* s.l., including the most genetically divergent clades, were not identified (Leavitt et al. 2011a).

As molecular sequence data become more readily available, they will allow us to better understand the diversity of lichenized fungi. Their use in identifying species will become increasingly important and routinely applied. Other disciplines such as ecology, conservation and physiology will benefit from a more objectively based species circumscription, enabling us to interpret distribution and ecological patterns better and more accurately monitor environmental disturbance and climate change.

Acknowledgements

We thank Roger Rosentreter, Johnathon Fankhauser, Leigh Johnson, Christopher Jones, Dean Leavitt, Hailey Leavitt, Jackson Leavitt, Lyndon Porter, Monica Proulx, and Peter Ririe for invaluable assistance with this research. This study was supported, in part, by funds from Brigham Young University graduate mentoring and graduate research fellowship awards, the USDA National Forest Service, the Negaunee Foundation and the National Science Foundation (DEB-0949147). PKD thanks to the Ministerio de Ciencia e Innovación, Spain for financial support (CGL2010-21646/BOS, RYC02007-01576). SPO thanks the JAE-Doc program (CSIC) for financial support.

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Appendix 1

Supplementary Table S1. (doi: 10.3897/mycokeys.7.4508.app1) Microsoft Excel Document (xls).

Explanation note: Collection information for all *Rhizoplaca melanophthalma* sensu lato specimens included in the present study. Modified from Leavitt et al. (2013).

Citation: Leavitt SD, Fernández-Mendoza F, Pérez-Ortega S, Sohrabi M, Divakar PK, Lumbsch TH, St. Clair LLS (2013) DNA barcode identification of lichen-forming fungal species in the *Rhizoplaca melanophthalma* species-complex (Lecanorales, Lecanoraceae), including five new species. MycoKeys 7: 1–22. doi: 10.3897/mycokeys.7.4508.app1

Appendix 2

Supplementary Fig S1. (doi: 10.3897/mycokeys.7.4508.app2) File format: Adobe Portable Document Format (pdf).

Explanation note: Maximum likelihood ITS gene tree of the 240 sampled *Rhizoplaca melanophthalma* sensu lato specimens. Bootstrap support indicated at nodes. Modified from Leavitt et al. (2013).

Citation: Leavitt SD, Fernández-Mendoza F, Pérez-Ortega S, Sohrabi M, Divakar PK, Lumbsch TH, St. Clair LLS (2013) DNA barcode identification of lichen-forming fungal species in the *Rhizoplaca melanophthalma* species-complex (Lecanorales, Lecanoraceae), including five new species. MycoKeys 7: 1–22. doi: 10.3897/mycokeys.7.4508.app2

MycoKeys 7: 23–30 (2013)
doi: 10.3897/mycokeys.7.5182
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RESEARCH ARTICLE

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Cortinarius bovarius (Agaricales), a new species from western North America

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Academic editor: *K. Hosaka* | Received 21 March 2013 | Accepted 30 April 2013 | Published 14 May 2013

Citation: Liimatainen K, Niskanen T (2013) *Cortinarius bovarius* (Agaricales), a new species from western North America. MycoKeys 7: 23–30. doi: 10.3897/mycokeys.7.5182

Abstract

Cortinarius bovarius **sp. nov.**, a conifer associated taxon growing on calcareous ground, is described from western North America. Phylogenetic relationships and species limits were investigated using rDNA ITS and nuclear *rpb*2 sequences, morphological and ecological data. The species belongs to section *Bovini* and its closest relative is European *C. bovinus*.

Key words

ITS, MrBayes, *rpb*2, taxonomy, *Telamonia*

Introduction

Cortinarius is the most species rich genus of the Agaricales with a worldwide distribution. *Cortinarius* species are important ectomycorrhizal fungi associated with different trees and shrubs belonging e.g. to the order Fagales and families Pinaceae and Salicaceae. Lately it has also been suggested that they have a key role in the carbon cycling of boreal forests (Bödeker et al. 2011).

In recent years there have been a number of publications on the taxonomy, evolution and biogeography of species found in North America (Seidl 2000, Moser and Peintner 2002, Matheny and Ammirati 2006, Garnica et al. 2009, 2011, Bojantchev 2011a, b, Bojantchev and Davis 2011, Harrower et al. 2011, Ammirati et al. 2012, 2013, Niskanen et al. 2012, 2013a, in press a). These studies show several patterns of species distributions. There are species common to North America and Europe, especially those species from more northern and montane conifer forests, i.e. *Corti-*

narius aureofulvus M. M. Moser and *C. napus* Fr. There are also presumably endemic species in both Western North America, eastern North America and Europe, i.e. *C. elegantio-occidentalis* Garnica & Ammirati and *C. californicus* A.H. Sm. in western North America, *C. hesleri* Ammirati, Niskanen, Liimat. & Matheny in eastern North America, and *C. puniceus* P.D. Orton in Europe.

Niskanen et al. (in press b) studied *Cortinarius bovinus* Fr. and morphologically similar species occurring in boreal coniferous forests in rich forest soils in northern Europe. Seven species were recognized, all belonging to section *Bovini* (subgenus *Telamonia*). Four of them, *C. bovinus* Fr., *C. bovinaster* Niskanen, Kytöv. & Liimat., *C. bovinatus* Kytöv., Liimat., Niskanen & H. Lindstr., and *C. oulankaënsis* Kytöv., Niskanen, Liimat. & H. Lindstr., formed a well-supported (PP 1.00) clade inside sect. *Bovini* (*Bovini* s. str.). The species are characterized by brown to dark brown basidiomes without bluish colors and exsiccatae with a dark brown to blackish brown pileus. The universal veil is white, brownish white or grayish white, in some species becoming grayish brown with age, and the odor is indistinct or slightly raphanoid. To date, species are only known from Europe, except *C. oulankaënsis* which also occurs in Canada in British Columbia. By studying more material from western North America, we wanted to determine if *C. bovinus* found from Alaska, U.S.A. and Alberta, Canada is conspecific with European samples or does it represent an autonomous species.

Methods

Material gathered by the authors from North America was studied morphologically, ecologically and sequenced to infer phylogentic relationships with other species in *Bovini*. DNA was extracted from dried material (a piece of lamella) with the NucleoSpin Plant kit (Macherey-Nagel, Düren, Germany). Primers ITS 1F and ITS 4 (White et al. 1990, Gardes and Bruns 1993) were used to amplify ITS regions, and specific primers cort6F and b7.1R (Frøslev et al. 2005) for the *rpb*2 region. The same primer pairs were used in direct sequencing. PCR amplification and sequencing followed Niskanen et al. (2009). Sequences were assembled and edited with Sequencher 4.1 (Gene Codes, Ann Arbor, Mich., USA). Using a BLAST query of the public databases (GenBank: http://www.ncbi.nlm.nih.gov/ and UNITE: http://unite.ut.ee/), we checked if identical or similar sequences for our species exist in public databases. For the phylogenetic analysis ITS and *rpb2* sequences of the species belonging to the well-supported ingroup of section *Bovini*, *C. bovinus, C. bovinaster, C. bovinatus*, and *C. oulankaënsis*, were included. *Cortinarius anisatus*, *C. neofurvolaesus*, and *C. sordidemaculatus* were chosen as outgroup species.

The combined ITS and *rpb2* alignment of 11 specimens was produced with the program MUSCLE (Edgar 2004) under default settings. The alignment comprised 1286 nucleotides (including gaps). The alignment is available at TreeBASE under S14159 (http://www.treebase.org/treebase-web/home.html).

Bayesian inference (BI) was performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). The best substitution model for the alignment was estimated by both the

Table 1. Specimens included in DNA analysis. Sequences produced in this study marked in bold. For acronyms of biological provinces see e.g. Knudsen and Vesterholt 2008: Funga Nordica: 32–35. * = GenBank Accession Numbers

Species	Voucher	Herb	Locality	ITS*	rpb2*
C. bovarius (type)	11-188	H	U.S.A. Alaska, Fairbanks	**KC905156**	**KC905160**
C. bovarius	11-255	H	U.S.A. Alaska, Fairbanks	**KC905158**	**KC905162**
C. bovarius	11-298	H	Canada, Alberta, Hinton	**KC905157**	**KC905161**
C. bovarius	11-373	H	Canada, Alberta	**KC905159**	**KC905163**
C. bovinaster (type)	04-669	H	Finland, PeP, Ylitornio	JX407264	JX407340
C. bovinatus	09-1520b	H	Finland, ES, Kerimäki	JX407267	JX407341
C. bovinus	10-006	H	Norway, Oppl, Lunner	JX407282	JX407343
C. oulankaënsis	09-535	H	Norway, NTr, Steinkjer	JX407295	JX407345
C. anisatus	04-550	H	Finland, PeP, Runteli	DQ120754	JX407346
C. neofurvolaesus	04-001	H	Finland, U, Helsinki	DQ139997	JX407367
C. sordidemaculatus	04-003	H	Finland, U, Kirkkonummi	DQ139991	JX407368

Akaike information criterion and the Bayesian information criterion with jModelTest version 0.1.1 (Posada 2008). A GTR model, including a gamma shape parameter, was chosen for both DNA regions. Two independent runs with four chains in each were performed for 1 000 000 generations sampling every 100th generation. All trees sampled before stationarity were discarded with a 25% safety margin (burn-in of 2 500 trees [250 000 generations]). Sampled trees from both runs were combined in a 50% majority rule consensus phylogram and posterior probabilities (PP) were calculated. The analysis was run with computer clusters of the CSC, IT Center for Science, Espoo, Finland.

Morphological descriptions are based on material collected by the authors including specimens in all stages of development. Color notations in the description follow Munsell (2009) soil color charts. Microscopic characteristics were observed from dried material mounted in Melzer's reagent (MLZ). Measurements were made in MLZ with an ocular micrometer using 100× oil-immersion lens. Basidiospores were measured from the veil or top of the stipe, 20 spores from one basidiocarp. The length and width were measured for each spore, and their length/width ratios (Q value) were calculated. The lamellar trama and basidia also were examined, and the pileipellis structure was studied from scalp sections taken from the pileus center.

Results

The 50% majority rule phylogram resulting from the BI analysis is shown in Fig. 1. *Cortinarius bovarius* is supported as a new taxon (PP 1.00). It clusters together with *C. bovinus* (PP 0.90) but differs from it by at least 18 substitutions and indel positions in the ITS regions and 3 substitutions in the *rpb2* region. The four ITS sequences of *C. bovarius* have altogether 1 base and 2 length intragenomic polymorphisms. No sequences of this species exist in public databases.

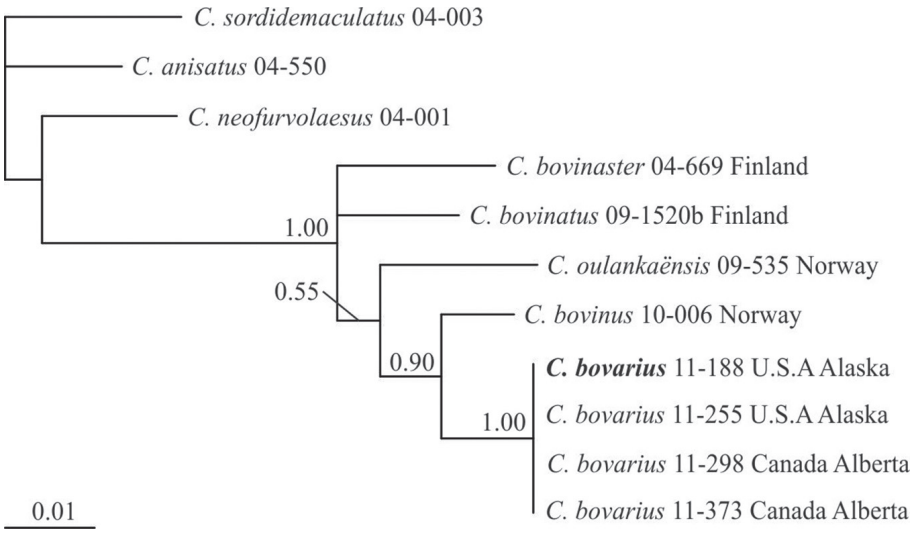

Figure 1. The Bayesian 50% majority-rule consensus tree inferred from combined ITS and *rpb2* regions. PP > 0.50 are indicated above branches.

Taxonomy

Cortinarius bovarius **Liimat. & Niskanen, sp. nov.**
MycoBank MB 804030
http://species-id.net/wiki/Cortinarius_bovarius
Figures 2 and 3

Diagnosis. Basidiomata medium-sized to large, pileus reddish brown, smell in lamellae indistinct or slightly raphanoid. Universal veil at first white, becoming pale brown. Basidiospores 8.5–10 × 5.5–6(–6.5) µm, amygdaloid to weakly ellipsoid. In coniferous forests with *Picea*, on rich, calcareous ground. Belongs to sect. *Bovini*.

Description. Pileus 3.5–7 cm diam., hemispherical at first, then low convex to almost plane, sometimes with a low, broad umbo, weakly fibrillose when young, later more apparent fibrillose only on the margin, somewhat waxy-glossy when moist; when young light reddish brown (5YR 6/4) to yellowish red (5YR 5/6-4/6) to reddish brown (2.5YR 5/4–4/4, 5YR 5/4–4/4), later dark red (2.5YR 3/6) to dark reddish brown (5YR 3/4–4/4, 2.5YR 3/3–3/4) and often with black spots; hygrophanous, soon drying from the center like *Kuehneromyces mutabilis* to lighter and more reddish brown, in dry condition reddish yellow (5YR 6/6, 7.5YR 7/6–6/6). Lamellae medium spaced to almost distant, adnexed to emarginate, fairly broad to broad, light reddish brown (5YR 6/4), light brown (7.5YR 6/3–6/4) to yellowish red (5YR 4/6), later dark reddish brown (2.5YR 3/4, 5YR 3/4–4/4), edge paler or concolorous. Stipe 5–11 cm long, 0.8–1.7 cm wide at apex, 1–3.5 cm wide at base, clavate to almost bulbose, rarely cylindrical, grayish white (silky) fibrillose, soon light reddish brown (5YR 6/3–6/4) to reddish brown (5YR 5/4) when older. Universal veil at first white, becoming pale

Figure 2. Photo of *Cortinarius bovarius* 11-188 (H). Photograph by K. Liimatainen.

brown, forming a girdle and thin sock-like sheath or rarely incomplete girdles on stipe surface, almost completely lost with age. Basal mycelium white. Context marbled hygrophanous, in pileus and upper part of the stipe light reddish brown (5YR 6/3–6/4) to reddish brown (5YR 4/4, 5/3), darkening towards the base of the stipe, in base reddish brown (5YR 5/3) when young, dark reddish brown (2.5YR 3/4 to 5YR 3/3–3/4) when old. Odor indistinct or slightly raphanoid. Exsiccatae: pileus brown (7.5YR 4/2–4/3) to dark brown (7.5YR 3/2–3/3), sometimes with a black center; stipe very pale brown (10YR 8/2) to light gray (10YR 7/2), in older basidiomes often darker, from grayish brown (10YR 5/2) to dark brown (10YR 4/2).

Basidiospores 8.5–10 × 5.5–6(–6.5) µm, Q = 1.45–1.65, av. = 8.9–9.5 × 5.7–6.1 µm, Qav. = 1.49–1.62 (80 spores, 4 specimens, Fig. 3), amygdaloid to weakly ellipsoid, moderately verrucose, somewhat more strongly so at the apex, moderately dextrinoid. Lamellar trama hyphae smooth to very finely scabrous, sometimes with sepia colored spots. Basidia 4-spored, 30–40 × 7.5–9.5 µm, almost concolorous with the background to olivaceous brownish. Pileipellis duplex, epicutis thin, hyphae 3–9 µm wide, unevenly pale brown, pigment in granules or in walls of hyphae, hypoderm distinct, elements 30–55 × 15–25(–30) µm, hyaline and smooth. Clamp connections present.

Ecology and distribution. In mesic coniferous forests with *Picea*, on rich, calcareous soil. Known from U.S.A, Alaska and Canada, Alberta. Fruiting from late August to September.

Etymology. *bovarius* for its affinity to *C. bovinus*.

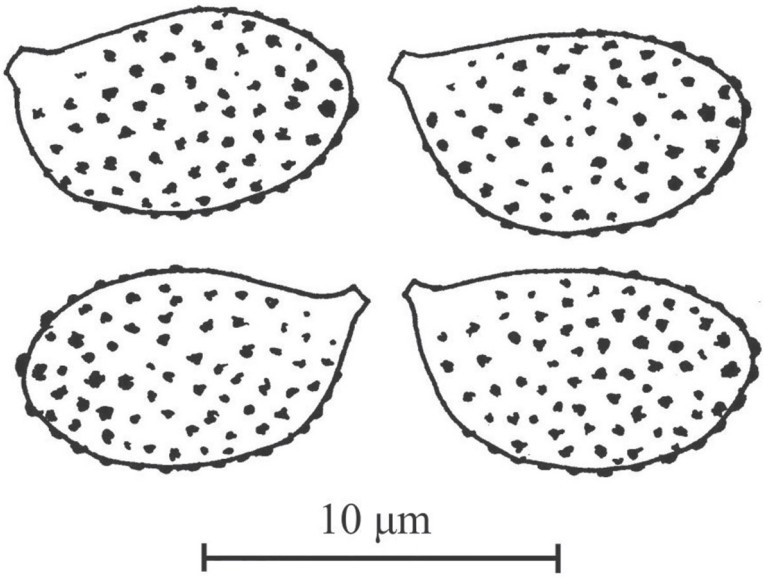

Figure 3. Spores of *Cortinarius bovarius* 11-188 (H) in Melzer's reagent. Drawing by T. Niskanen.

Type. U.S.A. Alaska: Fairbanks, University campus NW, trails starting from the end of Yukon road, mesic, mossy, partly needle/leaf covered *Picea* dominated forest with some *Populus, Betula, Alnus* and *Salix*, on rich ground, 64°51'33"N, 147°49'29"W, 22 Aug 2011, Niskanen & Liimatainen 11-188 (H, holotype; NY, isotype). GenBank no. KC905156 (ITS), KC905160 (*rpb2*).

Other specimens examined. Canada, Alberta, Hinton, S of center, Road to Percotte Creek, old mossy *Picea* dominated forest with some *Populus*, on calcareous ground, 53°21'53"N, 117°33'29"W, 30 Aug 2011, Liimatainen & Niskanen 11-298 (H). Alberta, Hinton, N of Athabasca river, *Populus* dominated forest with some *Picea*, 53°22'48"N, 117°51'35"W, 1040 m a.s.l., 5 Sept 2011, leg. L. Gagnon, Niskanen 11-373 (H). U.S.A. Alaska, Fairbanks, Wedgewood Resort trails, mesic *Picea* dominated forest with some *Betula* and *Populus*, on calcareous ground, 64°51'41"N, 147°42'46"W, 25 Aug 2011, Liimatainen & Niskanen 11-255 (H).

Discussion. *Cortinarius bovarius* is a typical member of section *Bovini*, a brown species with at first a white universal veil that later becomes brownish, indistinct or slightly raphanoid smell, and occurrence on calcareous ground. It differs from its European sister species, *C. bovinus*, by on average narrower, less dextrinoid and less verrucose spores (those of *C. bovinus* on average 6.1–6.4 µm wide, fairly strongly to strongly verrucose at the apex, and fairly strongly dextrinoid). The other known species of section *Bovini* s. str. from western North America, *C. oulankaënsis*, has a more grayish brown pileus, more distant lamellae, and relatively narrower spores (Qav. = 1.61–1.65). *Cortinarius bovarius* is a well-defined species based on morphology and molecular data, and therefore, is here describe as new to science.

Acknowledgements

We are grateful to Martin Osis for the hospitality and all the help he provided to us during our field work in Alberta. We thank Gary Laursen for providing information on collections sites in Fairbanks. Lorraine Gagnon is thanked for collecting *C. bovarius* during the Alberta Mycological Society's foray. Finally we thank both referees for their helpful comments and suggestions. This work was supported by the Academy of Finland (project # 129052).

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MycoKeys 7: 31–44 (2013)
doi: 10.3897/mycokeys.7.4710
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RESEARCH ARTICLE

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Three new species of foetid *Gymnopus* in New Zealand

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Academic editor: *S. Redhead* | Received 18 January 2013 | Accepted 24 June 2013 | Published 26 June 2013

Citation: Cooper JA, Leonard PL (2013) Three new species of foetid *Gymnopus* in New Zealand. MycoKeys 7: 31–44. doi: 10.3897/mycokeys.7.4710

Abstract

We describe three new species, *Gymnopus imbricatus*, *G. ceraceicola* and *G. hakaroa,* from New Zealand that are similar to *G. foetidus (= Micromphale foetidum*), growing on wood, with an insititious stipe and foetid odour. The position of these species within the /gymnopoid clade is confirmed by ITS sequence analysis.

Key words

Gymnopus, *Micromphale*, New Zealand

Introduction

The new species we describe are members of the family Omphalotaceae Matheney et al. (2006) and have the morphological characteristics of *Gymnopus* section *Vestipedes* subsection *Impudicae* (Antonín & Noordeloos 2010) which contains *Micromphale foetidum* (Sowerby) Singer, the type species of the formerly recognised genus *Micromphale* (e.g. in the sense of Singer 1986). Fruitbodies of the group often have a foetid odour when crushed, described as like rotting cabbage or garlic. The only existing records of this group in New Zealand were found to be misapplications of names applied to northern hemisphere species.

Moncalvo et al. (2002) investigated nLSU rDNA sequence data for a large number of agarics and recognised a /micromphale clade containing *M. foetidum* (AF261328). The clade also contained *Gymnopus* pro parte, *Caripia*, *Setulipes* and *Micromphale*.

Their /micromphale clade was nested within a broader /lentinuloid clade including *Rhodocollybia*, *Marasmiellus ramealis* (Bull.) Singer, *Marasmius scorodonius* (Fr.) Fr. and *Lentinula*. Mata et al. (2004), based on an LSU analysis, also identified a clade containing sequences of *M. foetidum* and material named as *Setulipes androsaceus* (L.) Antonín and *Gymnopus fusipes* (Bull.) Gray, the type species of those respective genera. They adopted a broad concept of *Gymnopus* incorporating these genera together with *Marasmiellus*. Wilson and Desjardin (2005) used LSU to examine the group and identified a /gymnopus clade containing *G. fusipes*, *M. foetidum*, *S. androsaceus* at its core with *Micromphale perforans* (Hoffm.) Gray lying on its boundary. These results were broadly supported by Mata et al. (2006) in their analysis using ITS1–5.8–ITS2 but they demonstrate clustering of *G. fusipes*, *S. androsaceus* and *Micromphale* on the periphery of a concentration of *Gymnopus*-labelled samples. On the basis of these results the currently generally accepted concept of *Gymnopus* is broad (e.g. Noordeloos 2012), and incorporates a number of previously recognised genera. Hughes et al. (2010) erected the genus *Connopus* to accommodate the *Gymnopus acervatus* group within the gymnopoid clade and presented LSU and ITS data indicating its placement close to *Rhodocollybia*. Their LSU analysis supports a core gymnopoid clade containing *G. fusipes*, *S. androsaceus*, which once again places *Micromphale foetidum* and *M. perforans* on a boundary with a sister group containing *Rhodocollybia*, *Marasmiellus juniperinus* Murrill and various *Gymnopus* species. The /gymnopus, clade as interpreted by Hughes et al., contains significant substructure. A multi-gene analysis including more representatives may indicate the recognition of further segregates at genus-level. In this paper we accept our newly described species within the current broad concept of *Gymnopus* whilst recognising their close alliance to the historical concept of the genus *Micromphale*.

For this study we analysed ITS1–5.8–ITS2 data for related New Zealand collections together with representative sequences from Genbank, many from the studies cited above. The structure of our ITS tree is consistent with these previous analyses, and once again identifies a /micromphale clade closely linked to core *Gymnopus* species. ITS data generated for a number of representative collections of our newly described taxa support species concepts based on morphology.

Materials and methods

Morphological protocols

Spore dimensions are stated as the mean ± 1.5 SD of 20 measurements, thus covering 86% of measurements under an assumed normal distribution model. Fresh or dried material was examined mounted in 10% KOH or Melzer's reagent. Material was hand-sectioned. Some micrographs were obtained under DIC conditions. Measurements were always taken without DIC optics and an extended objective iris in order to maximise boundary contrast.

Phylogenetic protocols

DNA extraction and sequencing followed the protocols outlined in Cooper and Leonard (2012). We downloaded from Genbank selected sequences used in cited publications, together with close BLAST matches, Table 1. General sequence management was carried out using Geneious (Drummond et al. 2011). Data exchange between applications was facilitated using Alter (Glez-Peña et al. 2010). Sequence alignment was carried out using MAFFT within Geneious (Katoh et al. 2002). A maximum likelihood analysis was executed using RAxML (Stamatakis 2006), with 100 bootstrap runs, launched from Topali 2.5 (Milne et al. 2004). The substitution model of GTR+G was recommended by Topali 2.5. We selected a sequence of *Anthracophyllum archeri* (Berk.) Pegler as the outgroup.

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

Genbank #	Collection #	Organism	PDD Voucher#	Country
DQ444308	TENN50049	*Anthracophyllum archeri*		New Zealand
DQ480112	TENN58672	*Gymnopus alkalivirens*		Greenland
DQ480114	TENN55834	*Gymnopus alpinus*		Scotland
AY256691	TENN57012	*Gymnopus aquosus*		Germany
DQ449971	TENN59738	*Gymnopus aquosus*		USA
KC248409	**PL6304**	***Gymnopus ceraceicola***	**PDD 101750**	New Zealand
KC248389	**PL126406**	***Gymnopus ceraceicola***	**PDD 101754**	New Zealand
KC248400	**PL189402**	***Gymnopus ceraceicola***	**PDD 76358**	New Zealand
KC248403	**JAC9334**	***Gymnopus ceraceicola***	**PDD 80771**	New Zealand
KC248405	**JAC10084**	***Gymnopus ceraceicola***	**PDD 87181**	New Zealand
KC248404	**JAC10336**	***Gymnopus ceraceicola***	**PDD 87424**	New Zealand
KC248394	**JAC10395**	***Gymnopus ceraceicola***	**PDD 87483**	New Zealand
KC248408	**JAC10817**	***Gymnopus ceraceicola***	**PDD 87661**	New Zealand
KC248392	**RHP13063**	***Gymnopus ceraceicola***	**PDD 90101**	New Zealand
KC248391	**KWH12891**	***Gymnopus ceraceicola***	**PDD 90119**	New Zealand
KC248393	**RHP12871**	***Gymnopus ceraceicola***	**PDD 90132**	New Zealand
KC248397	**JAC11005**	***Gymnopus ceraceicola***	**PDD 95459**	New Zealand
KC248395	**JAC11093**	***Gymnopus ceraceicola***	**PDD 95544**	New Zealand
AY256690	TENN57012	*Gymnopus dryophilus*		USA
DQ449974	TENN58087	*Gymnopus dryophilus*		Costa Rica
AF505778	TENN 59141	*Gymnopus dysodes*		Costa Rica
AY256694	TENN59457	*Gymnopus earleae*		USA
DQ449973	TFB10718	*Gymnopus exculptus*		Greenland
AF505780	FB11434	*Gymnopus foetidum*		USA
AY256710	TENN59217	*Gymnopus fusipes*		France
KC248407	**JAC9585**	***Gymnopus hakaroa***	**PDD 81086**	New Zealand
KC248410	**JAC10225**	***Gymnopus hakaroa***	**PDD 87315**	New Zealand
KC248411	**PL25404**	***Gymnopus imbricatus***	**PDD 101753**	New Zealand
KC248406	**JAC10089**	***Gymnopus imbricatus***	**PDD 87186**	New Zealand

Genbank #	Collection #	Organism	PDD Voucher#	Country
KC248398	**JAC10310**	***Gymnopus imbricatus***	**PDD 87398**	New Zealand
KC248401	**JAC10322**	***Gymnopus imbricatus***	**PDD 87410**	New Zealand
KC248399	**JAC10815**	***Gymnopus imbricatus***	**PDD 87659**	New Zealand
KC248402	**JAC10816**	***Gymnopus imbricatus***	**PDD 87660**	New Zealand
KC248396	**JAC10495**	***Gymnopus imbricatus***	**PDD 87675**	New Zealand
KC248390	**JAC11038**	***Gymnopus imbricatus***	**PDD 95489**	New Zealand
AF505779	TENN56658	*Gymnopus impudicus*		Costa Rica
DQ449986	Duke RV94154	*Gymnopus iocephalus*		USA
AY256693	TENN59532	*Gymnopus junquilleus*		USA
DQ449960	TENN50620	*Gymnopus ocior*		Switzerland
DQ449972	TENN56321	*Gymnopus subsulphureus*		USA
AY263453	AWW115	*Gymnopus vitellinipes*		Java/Bali
AY256708	TENN59540	*Marasmiellus juniperinus*		USA
GU234007	JB14	*Marasmius androsaceus*		Sweden
DQ444312	TENN50482	*Marasmius androsaceus*		UK
DQ444311	TENN50704	*Marasmius androsaceus*		USA
DQ449990	TENN59293	*Micromphale brassicolens*		Austria

Results

Our analysis places the New Zealand taxa in a monophyletic clade close to *G. foetidum* and *G. brassicolens* historically recognised in the genus *Micromphale* (Fig. 1). The combination of sequence data and morphological analysis of many collections indicate two major groups which we equate with the newly described species *G. imbricatus* and *G. ceraceicola*. In addition we recognise a further species, *G. hakaroa*, which is poorly distinguished from *G. imbricatus* on the basis of ITS sequences but which is morphologically consistently different. Minor sequence variation in the *G. ceraceicola* group does not correlate with morphology and we choose to recognise these specimens as a single species. More information and images of collections may be found on the Landcare Research website (Systematics Collections Data).

Gymnopus ceraceicola **J.A. Cooper & P. Leonard, sp. nov.**
http://species-id.net/wiki/Gymnopus_ceraceicola
Holotype: PDD 87181. Registration identifier: IF550091

Diagnosis. *G. ceraceicola* is distinguished from related New Zealand species by the combination of pruinose, central stipe and dark pileus.

Macromorphology. Pileus 5–20 mm, generally broadly convex to applanate, but sometimes campanulate when young, brick to purplish chestnut, minutely felty, radially furrowed and striate towards the margin, margin slightly fimbriate. Lamellae cream, creamy yellow to vinaceous buff, waxy, adnate. Lamellae present, in series of

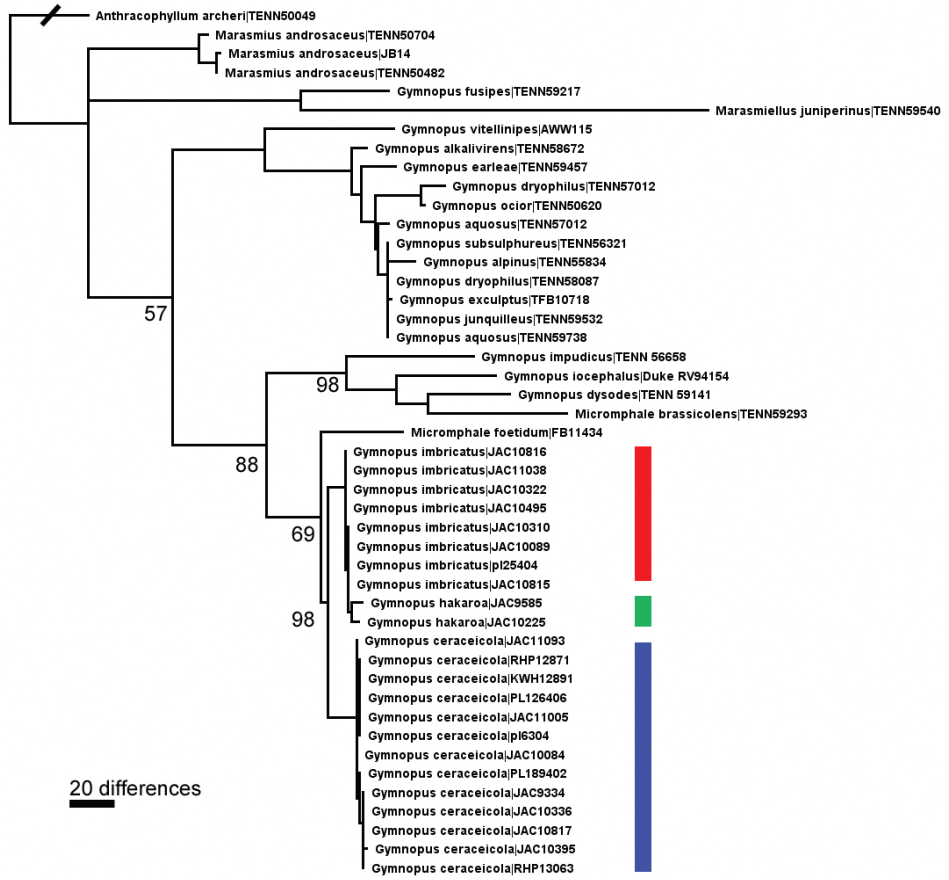

Figure 1. Maximum likelihood cladogram of selected ITS sequences, with bootstrap proportion. Red bar = *Gymnopus imbricatus*, green bar = *G. hakaroa*, blue bar = *G. ceraceicola*

three: intercalated short/long/short. Stipe central, cartilaginous, 10–20 × 1–2 mm, equal, brown vinaceous, sometimes paler towards apex or base, always entirely finely pruinose. Stipe base insititious and always associated with a thin waxy to chalky cream layer of partially gelatinised hyphae covering the substrate. This layer is often extensive, with a distinct margin, and often green with algal cells. Fruitbodies with garlic/rotten cabbage smell, especially when crushed.

Micromorphology. Pileipellis a partially gelatinised radially arranged clamped cutis of smooth hyphae to 5 µm diameter, with brown extra-cellular encrustation. Epidermal layer to 140 µm. Subepidermis of thick glassy–walled non-gelatinised smooth hyaline hyphae, weakly dextrinoid. Basidia clavate to 40 × 8 µm. Sterigmata to 7 µm, 4–spored. Basidioles cylindrical, tapering towards apex, 40 × 4 µm. Spores hyaline, lacrymoid, 7.9 ± 1 × 4.5 ± 0.6 µm, Q = 1.8 ± 0.1 including apiculus. Cheilocystidia and pleurocystidia not observed. Stipitipellis a cutis of brown parallel hyphae, to 5 µm wide. Caulocystidia smooth, hyaline, agglutinated into fascicles.

Figure 2. *Gymnopus ceraceicola* Holotype, PDD 87181. Fruitbodies.

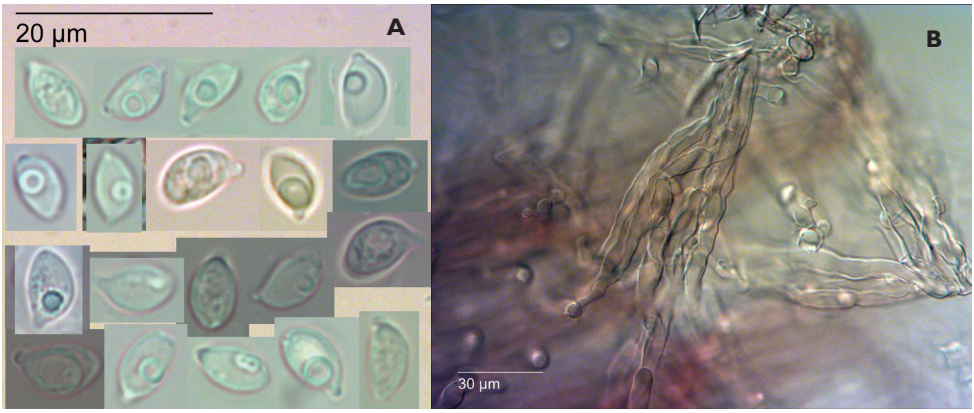

Figure 3. *Gymnopus ceraceicola* Holotype, PDD 87181. **A** Spores (KOH) **B** Agglutinated fascicles of caulocystidia on stipe (KOH).

Habitat. Colonies of a few to hundreds of fruitbodies on bark of fallen, dead branches and twigs, especially *Nothofagus*.

Distribution. Broadly distributed and common in both North and South Islands of New Zealand.

Etymology. Ceraceicola, indicating association with a basal waxy layer, although this feature is common to the three species described here.

Notes. Sequence data indicate variability in the taxon but the morphological details are constant and we choose to recognise a single species. New Zealand records of *Gymnopus* (*Micromphale*) *foetidum* and *Gymnopus* (*Micromphale*) *brassicolens* are attributable to *G. ceraceicola*. Authentic New Zealand material of these two species has not been identified. *Gymnopus brassicolens* has paler pileus colours, non-gelatinized pileipellis, cheilocystidia and pileipellis elements with lateral projections, and larger basidiospores. *Gymnopus foetidus* is macroscopically similar but does not possess the agglutinate fascicles of caulocystidia of *G. ceraceicola*.

Specimens examined. New Zealand, North Island: PDD 40852, on dead wood, Anawhata Rd., Waitakare Ranges, Collector P.R. Johnston & G. Samuels, 9 June 1981. PDD 80771, on dead wood of *Beilschmiedia tawa*, Erua Forest, Taupo, Collector J.A. Cooper (JAC9334), 4 April 2005. PDD 87382, on dead wood of *Nothofagus fusca*, Mt Holdsworth, Gentle Annie Track, Wairarapa, Collector J.A. Cooper (JAC10294), 11 May 2007. PDD 87483, on wood, Mt Holdsworth, Donnelly Flat Loop Track, Wairarapa, Collector G. Gates & D. Ratkowsky (JAC10395), 7 May 2007. PDD 87424, on dead bark of *Nothofagus*, Mt Holdsworth, Gentle Annie Track, Wairarapa, Collector J.A. Cooper (JAC10336), 11 May 2007. PDD 95544, on bark of *Nothofagus fusca*, Rimutaka Forest Park, Wellington, Collector J.A. Cooper (JAC11093), 14 May 2009. PDD 95545, on bark of dead branch of *Nothofagus fusca*, Rimutaka Forest Park, Wellington, Collector J.A. Cooper (JAC11094), 14 May 2009.

New Zealand, South Island: PDD 76357, on dead twig of *Nothofagus*, Canaan Road Track, Nelson, Collector P.L. Leonard, 30 April 2002. PDD 96730, on dead wood, Wangapeka, Nelson, collector P.L. Leonard (PL126406), 14 April, 2006. PDD 90101=TENN 061068, on bark, vicinity of Seddonville, Charming Creek Track, Nelson, Collector R.H. Petersen (RHP 13063), 11 May 2006. PDD 76358, on bark on dead branch of *Nothofagus menziesii*, Lake Daniels Track, Nelson, Collector P.L. Leonard (PL189402), 2 April 2002. PDD 95459, on bark of dead branch of *Nothofagus solandri*, Kowai Bush, Springfield, Mid Canterbury, Collector J.A. Cooper (JAC11005), 2 May 2009. PDD 95462, on bark of dead branch of *Nothofagus solandri,* Kowai Bush, Springfield, Mid Canterbury, Collector J.A. Cooper (JAC11008), 2 May 2009. **Holotype** PDD 87181, on dead branch of *Nothofagus fusca*, Hinewai Reserve, Akaroa, Mid Canterbury, Collector J.A. Cooper (JAC10084), 3 June 2006. PDD 87661, on dead twigs of *Leptospermum scoparium*, Government Track, Waipori Falls Road, Dunedin, Collector K. Soop (JAC10817), 12 May 2008. PDD 96636, on dead wood of *Nothofagus solandri*, Lake Hauroko, Fiordland, Collector P. White (JAC12522). 7 May 2012. PDD 90119 =TENN061007, on twigs, Vicinity of Te Anau, Kepler Track from Rainbow Reach, Fiordland, Collector K.W. Hughes (KWH12891), 30 April 2006. PDD 90132=TENN060986, vicinity Manapouri, Borland Lodge, Nature Track, Fiordland, Collector R.H. Petersen (RHP12871), 29 April 2006.

***Gymnopus imbricatus* J.A. Cooper & P. Leonard, sp. nov.**
http://species-id.net/wiki/Gymnopus_imbricatus
Holotype: PDD 95489. Registration identifier: IF550092

Diagnosis. *G. imbricatus* is distinguished from related New Zealand species by the smooth stipe, larger basidiospores, and imbricate habit.

Macromorphology. Pileus 3–20 mm in diameter convex, cream to fawn, minutely felty, radially furrowed and striate towards the margin, margin fimbriate. Lamellae cream to creamy yellow, adnate. Lamellae present, in series of two: short/long. Stipe mostly eccentric, cartilaginous, to 3 × 0.5 mm, equal, umber to black, sometimes paler

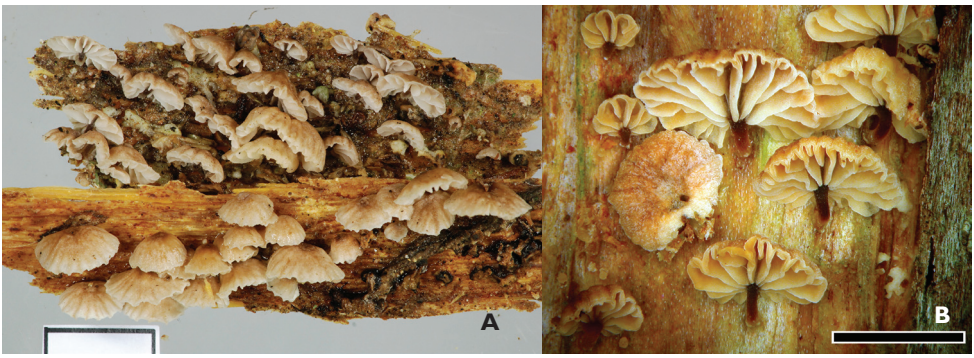

Figure 4. *Gymnopus imbricatus*. **A** Holotype PDD 95489. Fruitbodies, scale 1 cm **B** PDD 87186. Scale 1 mm.

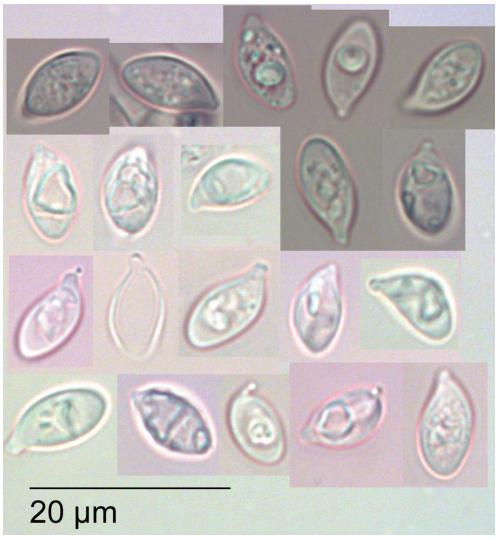

Figure 5. *Gymnopus imbricatus* Holotype PDD 95489. Spores (in KOH).

towards base, always entirely smooth. Stipe base insititious and usually associated with a thin waxy to chalky cream layer of partially gelatinised hyphae covering the substrate, usually green with algal cells. Fruitbodies with garlic/rotten cabbage smell, especially when crushed.

Micromorphology. Pileipellis a partially gelatinised irregular clamped cutis of hyphae 4 µm diameter, without intra or extracellular pigmentation, terminal layer with gelatinised coralloid elements, to 2 µm wide, and occasional small finger-like trichodermal elements to 20 µm. Epidermal layer to 25 µm. Subepidermis of thick glassy-walled non-gelatinised smooth hyaline hyphae, weakly dextrinoid. Basidia clavate to 50 × 10 µm. Sterigmata to 5 µm, 4–spored. Basidioles to 50 × 6 µm cylindrical and tapered towards apex. Spores hyaline, lacrymoid 9.8 ± 1.2 × 5.1 ± 0.4 µm, Q = 1.9 ± 0.3 including apiculus. Cheilocystidia and pleurocystidia not observed. Stipitipellis a cutis of parallel brown hyphae, to 6 µm wide. Caulocystidia absent.

Habitat. Forming imbricate colonies of dozens to hundreds of fruitbodies on bark and decorticate wood of dead branches and twigs, especially *Kunzea* and *Leptospermum* but occurs with other trees. Also occurs at the stem base of live trees.

Distribution. Broadly distributed and common in both North and South Islands of New Zealand.

Etymology. Imbricatus, pertaining to the often tiered and overlapping eccentrically stemmed caps.

Specimens examined. New Zealand, North Island: PDD 80766, on bark of *Beilschmedia tawa*, Erua Forest, Taupo, collector J.A. Cooper (JAC9329), 4 April, 2005. PDD 87398, bark on dead branch of *Nothofagus*, Waiohine Gorge, Wairarapa, Collector J.A. Cooper (JAC10310), 10 May 2007. PDD 87410, dead stems of *Ripogonum scandens*, Waiohine Gorge, Wairarapa, Collector J.A. Cooper (JAC10322), 10 May 2007.

New Zealand, South Island: PDD 101753, dead branches of *Nothofagus menziesii*, Riwaka Resurgence, Nelson, Collector P.L. Leonard (PL25404), 10 April, 2006. PDD 96141, dead twigs of *Kunzea ericoides*, Mt Fyffe Track, Kaikoura, collector J.A. Cooper (JAC11734), 26 Feb. 2011. PDD 80154, dead log of *Nothofagus menziesii*, Lewis Pass, Buller, collector J.A. Cooper (JAC8287), 24 November, 2001. PDD 80157, on dead de-corticate log, Lyell Walkway, Nelson, collector J.A. Cooper (JAC80157), 25 November, 2001. PDD 87675, living stem of *Fuchsia excorticata*, Saddle Hill, Mid Canterbury, Collector J.A. Cooper (JAC10495), 22 May 2005. **Holotype** PDD 95489 (Figs 4 and 5), base of live trees of *Kunzea ericoides*, Kennedy's Bush, Mid Canterbury, Collector J.A. Cooper (JAC11038), 24 May 2009. PDD 79799, bark of dead tree, Kennedy's Bush, Mid Canterbury, Collector J.A. Cooper (JAC8921), 20 March 2004. PDD 87186, on bark of living tree of *Kunzea ericoides*, Hinewai Reserve, Akaroa, Mid Canterbury, Collector J.A. Cooper (JAC10089), 3 June 2006. PDD 87660, fallen log, Racemans Track, Silverstream Valley, Dunedin, Collector S. Dodd (JAC10816), 13 May 2008. PDD 87659, on dead twigs of *Kunzea ericoides*, Evansdale Glen, Dunedin, Collector P.R. Johnston (JAC10815), 12 May 2008.

***Gymnopus hakaroa* J.A. Cooper & P. Leonard, sp. nov.**
http://species-id.net/wiki/Gymnopus_hakaroa
Holotype: PDD 87315. Registration identifier: IF550093

Diagnosis. *G. hakaroa* is distinguished from *G. ceraceicola* by smaller stature and a pruinose stipe lacking fascicles of agglutinate caulocystidia. It is distinguished from *G. imbricatus* by non-imbricate growth, a consistently central stipe, and smaller basidiospores.

Macromorphology. Pileus 3–10 mm diam. convex, rusty tawny to umber, minutely felty, weakly radially furrowed and striate towards the margin. Lamella cream to yellow, waxy. Lamellae present, in series of three: intercalated short/long/short. Stipe central, cartilaginous, to 5 × 0.6 mm, equal, umber to black, paler towards base, smooth to minutely pruinose. Stipe base insititious and always associated with an obvious waxy to chalky cream layer of partially gelatinised hyphae covering the substrate,

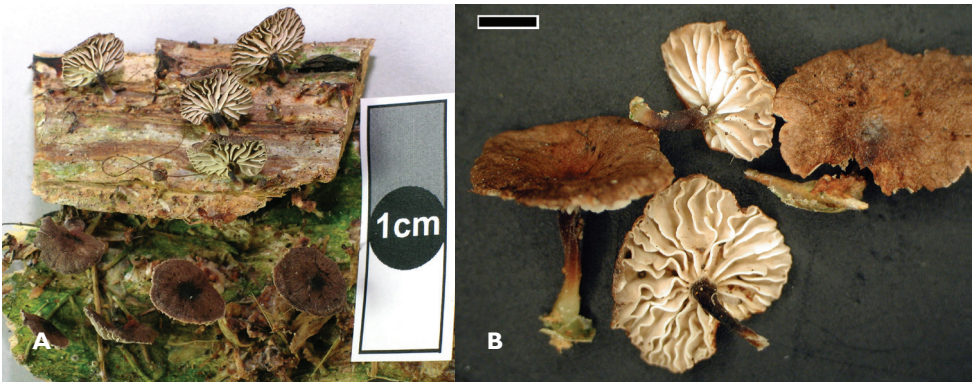

Figure 6. *Gymnopus hakaroa* **A** PDD 81086. Fruitbodies **B** scale= 2 mm

Figure 7. *Gymnopus hakaroa* Holotype PDD 87315. Fruitbodies, showing waxy substratum.

usually green with algal cells. Fruitbodies with garlic/rotten cabbage smell, especially when crushed.

Micromorphology. Pileipellis a partially gelatinised radially arranged clamped cutis of smooth hyphae to 3 µm in diameter, with brown extra-cellular encrustation. Epidermal layer to 80 µm. Subepidermis of thick glassy-walled non-gelatinised smooth hyaline hyphae, to 3 µm in diameter, weakly dextrinoid. Basidia clavate to 40 × 8 µm.

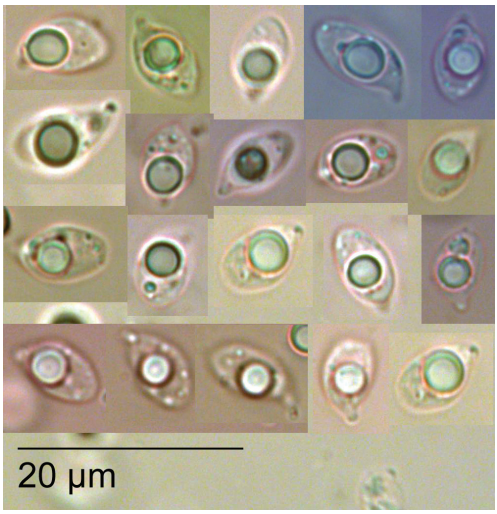

Figure 8. *Gymnopus hakaroa* Holotype PDD 87315. Spores (KOH).

Sterigmata to 7 µm, 2–4-spored. Basidioles cylindrical and tapered towards apex 40 × 6 µm. Spores hyaline, lacrymoid 8.3 ± 1 × 4.8 ± 0.3 µm, including apiculus, Q = 1.7 ± 0.2. Cheilocystidia and pleurocystidia not observed. Stipitipellis a cutis of hyaline to pale brown hyphae, to 5 µm wide. Stipe without caulocystidia.

Habitat. Forming imbricate colonies of dozens to hundreds of fruitbodies on decorticate dead wood.

Distribution. Currently *G. hakaroa* is only known from a single location on the Canterbury Port Hills in the South Island of New Zealand.

Etymology. Hakaroa, a Maori name for the Bank's Peninsula region of New Zealand.

Notes. Sequence data (Fig 1) indicates a close phylogenetic relationship to *G. imbricatus* but there are consistent and substantial morphological differences.

Specimens examined. New Zealand, South Island: Holotype PDD 87315 (Figs 6 and 7) on dead log, Kennedys Bush Reserve, Port Hills, Mid Canterbury, Collector J.A. Cooper (JAC10225), 11 Feb. 2007. PDD 81086 (Fig. 8), on dead wood of *Kunzea ericoides*, Kennedys Bush Reserve, Port Hills, Mid Canterbury, Collector J.A. Cooper (JAC9585), 23 July, 2007. PDD 96390, on dead decorticate log of *Melicytus ramiflorus*, Kennedys Bush Reserve, Port Hills, Mid Canterbury, Collector J.A. Cooper (JAC11301), 17 April, 2010.

Dicussion

Gymnopus imbricatus, as its name suggests forms dense populations of small imbricate fruitbodies. It is most commonly associated with tea-tree (*Kunzea ericoides* and *Leptospermum scoparium*) and often found on the bark at the base of living trees. *Gymnopus hakaroa* is larger, with a dark minutely pruinose cap and again forms dense populations

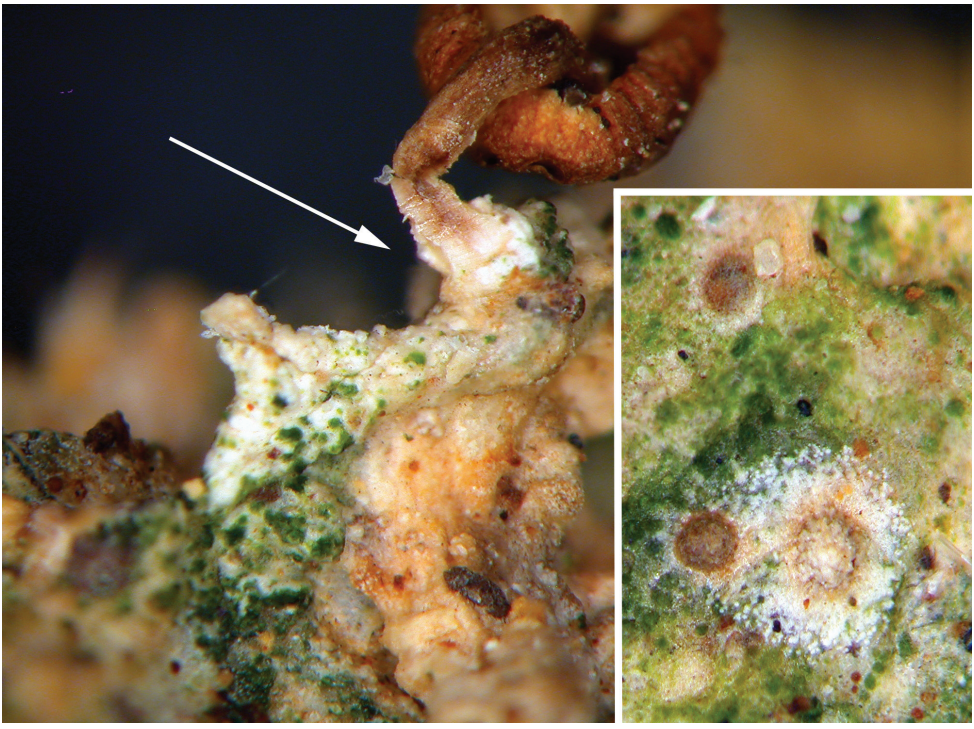

Figure 9. *Gymnopus hakaroa* PDD 96390. Stipe base (arrow) with surrounding algal mat. Inset, primordial arising from algal mat.

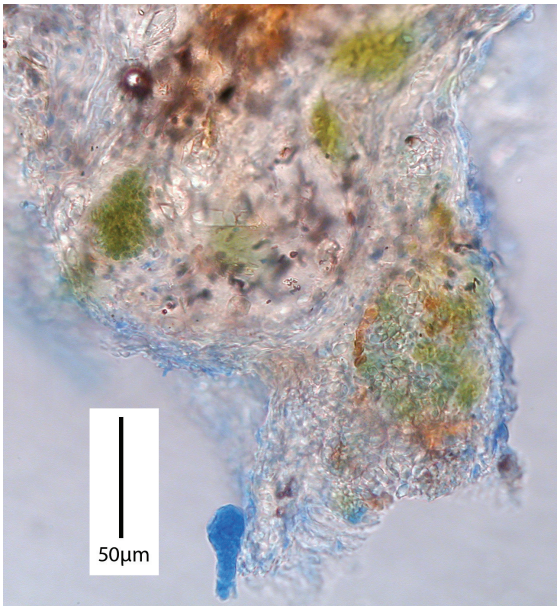

Figure 10. *Gymnopus hakaroa* PDD 96390. Pockets of algal cells embedded in hyphal tissue of stem base (cotton blue stain).

on the bark of dead logs. These two species have smooth stems. *Gymnopus ceraceicola* is distinguished by larger fruitbodies, a pruinose stipe, and is more commonly associated with southern-beech forests on dead fallen logs. The species of *Gymnopus* described here belong in the /micromphale clade of Moncalvo et al. (2002) and share the diagnostic feature of this clade of a foetid odour likely due to the presence of mercaptan-like compounds. In New Zealand this feature is shared with *Mycetinis curraniae* (G. Stev.) J.A. Cooper & P. Leonard, a marasmioid fungus distinguished by its ornamented hymeniderm pileipellis. Another very distinctive character common to all three *Gymnopus* species, and visible in the accompanying photographs (Figs 2 and 6), is the presence of a waxy layer of partially gelatinised hyphae on the substrate from which the fruitbodies emerge. This layer is usually green from the presence of embedded algal cells. Interestingly, some published images of *G. foetidus* in the northern hemisphere also show a similar layer, e.g. Antonín and Noordeloos (2010). Detailed examination of our material does show algal cells deeply embedded within the context of the waxy layer and the basal portion of the stipe (Figs 9 and 10), but it would seem unlikely that algal cells are present in sufficient numbers to confer any significant nutritional benefit to the fungus. The morphologically similar *Marasmiellus affixus* (Berk.) Singer, described from Australia and commonly known as the 'little stinker', is also associated with a waxy algae-infected layer. The association of *M. affixus* with alga was noted by Singer (1973) and has been speculated to be a basidio-lichen, although this has not proven (Lepp 2011). A partial, poor quality ITS1 sequence *for M. affixu*s obtained during this work (not deposited) suggests it has affinity with *Marasmiellus ramealis* (Bull.) Singer rather than the taxa treated here.

Acknowledgements

Thanks to Dukchul Park, Landcare Research, for DNA extraction and sequencing, and to Sapphire McMullen-Fisher for material of *Marasmiellus affixus*. The New Zealand Department of Conservation is thanked for permission to collect specimens from reserves and national parks that they manage. The first author was supported through the Landcare Research Systematics Portfolio, with Core funding support from the Science and Innovation Group of the New Zealand Ministry of Business, Innovation and Employment.

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MycoKeys 7: 45–62 (2013)
doi: 10.3897/mycokeys.7.5860
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DNA barcoding and morphological studies reveal two new species of waxcap mushrooms (Hygrophoraceae) in Britain

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Academic editor: *Scott Redhead* | Received 25 June 2013 | Accepted 15 August 2013 | Published 9 September 2013

Citation: Ainsworth AM, Cannon PF, Dentinger BTM (2013) DNA barcoding and morphological studies reveal two new species of waxcap mushrooms (Hygrophoraceae) in Britain. MycoKeys 7: 45–62. doi: 10.3897/mycokeys.7.5860

Abstract

Rigorous diagnostics and documentation of fungal species are fundamental to their conservation. During the course of a species-level study of UK waxcap (Hygrophoraceae) diversity, two previously unrecognized species were discovered. We describe *Gliophorus europerplexus* **sp. nov.** and *G. reginae* **sp. nov.**, respectively orange–brown and purple–pink waxcap mushrooms, from nutrient-poor grasslands in Britain. Both share some morphological features with specimens assigned to *Gliophorus* (=*Hygrocybe*) *psittacinus*. However, analysis of sequences of the nuclear ITS DNA barcode region from these and related taxa confirms the phylogenetic distinctness of these lineages. Furthermore, we demonstrated that the holotype of *Hygrophorus perplexus*, a North American species morphologically resembling *G. europerplexus*, is phylogenetically divergent from all our collections. It is likely that further collections of *G. europerplexus* will be revealed by sequencing European material currently filed under *G. perplexus* and its synonyms. However, two such collections in the Kew fungarium yielded sequences that clustered together but were divergent from those of *G. europerplexus*, *G. perplexus* and *G. psittacinus* and may represent a further novel taxon. By contrast, *G. reginae* is morphologically distinct and can usually be recognized in the field by its purplish viscid pileus and relatively stout, flexuose, pale stipe. It is named to commemorate the diamond jubilee of Her Majesty Queen Elizabeth II in 2012 and the 60th anniversary of her coronation in 2013.

Keywords

Conservation, cryptic species, diamond jubilee, DNA barcoding, fungi, *Gliophorus*, *Glutinosae*, Hygrophoraceae, parrot waxcap, taxonomy, waxcap grasslands

Introduction

In Europe, waxcap mushrooms (Hygrophoraceae, *Hygrocybe* s.l.) are conspicuous and often attractive features of nutrient-poor short turf. Hotspots of waxcap taxonomic diversity are usually biocide-free, unfertilised or semi-improved, grazed grasslands or mown lawns with low levels of soil disturbance and long periods of ecological continuity. Such sites are often, but not always, of low botanical interest and this has undoubtedly delayed their recognition as sites of international conservation importance. Historically, therefore, even the best "waxcap grasslands" (using Arnolds' (1980) terminology) have rarely received adequate long-term protection. More recently, waxcap assemblages are increasingly being recognized as useful bioindicators for identifying sites of conservation priority (e.g., Boertmann 1995, 2010, Nitare 2000, McHugh et al. 2001, Griffith et al. 2002, Newton et al. 2003, Evans 2004, Genney et al. 2009). Some species, such as the pink waxcap *H. calyptriformis*, have emerged as flagships for the still nascent practice of targeted conservation of fungi, and waxcaps as a group are becoming mascots for fungal conservation in general.

Waxcaps are regarded as nitrogen-sensitive organisms because fruiting is inhibited by applications of nitrogenous fertilizers (Arnolds 1989). However, their below-ground ecology, in particular their nutritional mode(s), remains unclear despite recent attention by several researchers. Indirect evidence from carbon and nitrogen stable isotope ratios suggests that at least some taxa are biotrophic (Seitzman et al. 2011), and further evidence of a mycorrhiza-like association with plants has been demonstrated recently (Halbwachs et al. unpublished data).

Taxonomic treatments of European waxcaps have recognized from one to seven genera (e.g. Kummer 1871, Orton 1960, Orton and Watling 1969, Kühner 1980, Moser 1983, Kovalenko 1989, Arnolds 1990, Bon 1990, Boertmann 1995, 2010, 2012, Candusso 1997, Krieglsteiner 2001, Bresinsky 2008, Vizzini and Ercole 2012). Molecular phylogenetic analysis indicates that these fungi are not monophyletic and that at least two major phylogenetic clades can be recognized (Babos et al. 2011, Lodge et al. in press). Basidiomata of one group are characterized by vivid yellow, orange and red colours whereas those of the second group lack muscaflavin pigments and are pallid to brown, sometimes showing olive, pink or purple tints (Babos et al. 2011). At least three major groups can be recognized based on hyphal arrangement and compartment lengths within the hymenophoral trama (Boertmann 1995, 2010). These categories are partly supported by phylogenetic evidence (Babos et al. 2011, Lodge et al. in press).

Waxcap identification in Britain and Ireland currently adheres to Boertmann's (1995, 2010) taxonomic concepts. In turn, these concepts are based on basidiomatal macroscopic and microscopic morphology, although it is accepted that some taxa can show overlapping variation. As a result, 51 species (plus eight infraspecific taxa) of *Hygrocybe* s.l. are currently accepted in the online *Checklist of the British and Irish Basidiomycota* (CBIB; http://www.basidiochecklist.info/). However, only a handful of these are individually recognised as species of conservation concern. Five waxcaps were assessed as Vulnerable or Near Threatened in the Great Britain & Isle of Man

unofficial Red Data List (Evans et al. 2006) and only the date waxcap, *H. spadicea*, is currently recognised as a priority species in the UK Biodiversity Action Plan. Not only does morphological identification of waxcaps underpin their current RDL assessment (Evans et al. 2006), it also contributes to the designation of UK sites as Important Fungus Areas (Evans et al. 2001) and Sites of Special Scientific Interest (SSSI). Indeed, waxcaps are currently one of the few groups of fungi for which SSSIs can be designated; any site with at least 18 recorded *Hygrocybe* s.l. species "should be considered for SSSI status" (Genney et al. 2009). Waxcap taxonomy and identification are, therefore, fundamental to their effective conservation.

Recent developments in DNA-based methods of identification ("DNA barcoding") are revolutionizing rapid diagnosis of diversity in mushrooms and other *Fungi* (Dentinger et al. 2011, Schoch et al. 2012). This study is part of a UK-wide initiative that is applying a DNA barcoding approach to waxcaps and revealing surprising levels of unknown diversity. We currently believe that at least 96 species are present in the UK as defined by DNA sequence-based methods (Defra science and research project WC0787). This has involved morphological and molecular analysis, or reanalysis, of 83 fungarium specimens in K whose sequences were published by Brock et al. (2009), 124 newly-sequenced specimens from K, E, and MICH, and more than 600 new field collections mostly from 2011 and 2012.

This paper focuses on our treatment of two unusual waxcaps that, because of their viscid pilei and subregular hymenophoral tramal hyphae, are assigned to the segregate genus *Gliophorus*. They share some morphological characters with the parrot waxcap *G. psittacinus*, which encompasses a wide range of basidiomatal pigmentation based on current concepts. Numerous colour forms can be recognised (Boertmann 2001) but, partly because this character is known to be influenced by ageing and weather conditions, formal taxonomic resolution into recognisable segregate species has proved more challenging. Four varieties are listed in Index Fungorum (http://www.indexfungorum.org). Our unusual collections lacked green pigments and one group matched the type description of *Hygrophorus perplexus* A.H. Sm. & Hesler, a North American species. This is recorded in Europe where, as one of the few accepted parrot waxcap segregates, it is currently recognised as *Hygrocybe psittacina* var. *perplexa* (Boertmann 2012). Molecular analysis, including sequences derived from type specimens of *Hygrophorus perplexus*, collections filed as *H. psittacina* var. *perplexa* in K and downloaded from GenBank labelled as *H. psittacina*, confirmed the presence of two new species lacking green pigments, which we describe here.

Methods

Taxon and specimen sampling

A total of 20 collections corresponding to the *G. psittacinus* complex were sequenced and morphologically examined in the current study. These comprised 12 recent UK field collections now in K, four existing K collections from UK and Jersey and four US type collec-

tions in MICH. Table 1 shows the relevant collection details. Further details for specimens of *G. europerplexus* and *G. reginae* are provided in the taxonomic treatment below. Geographical coordinates of collections were converted from Ordnance Survey National Grid References, based on the OSGB36 datum, to latitude and longitude (WGS84 datum).

Morphological analysis

Spore measurements are rounded to the nearest half micron and preceded by associated data in square brackets. For example, [60, K(M)181128*, K(M)181129] would indicate that 60 spores in total were measured either in water from prints (*G. reginae*) or in Melzer's reagent from lamellar squashes (*G. europerplexus*) from the collections K(M)181128 and K(M)181129. Collections sequenced during this study, such as K(M)181128 in this example, are denoted throughout by *. Measurements of basidia and other hyphal elements are rounded to the nearest micron. Colours given in parentheses refer to those shown in a standard mycological identification chart (Anon 1969).

DNA extraction and sequencing

DNA was extracted using either an enzymatic digestion-glass fiber filtration protocol in 96-well plate format with a vacuum-manifold or the Whatman FTA® card method described in Dentinger et al. (2010). Full and partial nuclear ribosomal internal transcribed spacer regions (ITS) were amplified and sequenced with primers ITS1F/ITS3 and ITS2/ITS4 (White et al. 1990, Gardes and Bruns 1993) or with primers ITS8F and ITS6R (Dentinger et al. 2010) following the cycling conditions in Dentinger et al. (2010). PCR products were visualized by UV fluorescence after running out 2 µL PCR products in a 1% agarose gel containing 0.005% ethidium bromide. Prior to sequencing, positive PCRs were cleaned by adding 0.5 µL ExoSAP-IT to every 2.5 µL PCR reaction mix and incubating this mix for 15 min at 37 C followed by 15 min at 80 C. Unidirectional dye-terminator sequencing used the ABI BigDye kit (Foster City, CA) following the manufacturer's instructions except reducing the total reaction volume to 5 µL. Sequencing reactions were cleaned using ethanol precipitation and resuspended in distilled water before loading into an ABI PRISM 3730 DNA Analyzer in the Jodrell Laboratory, Royal Botanic Gardens, Kew. Complementary unidirectional reads were aligned and edited using Sequencher4.2 (GeneCodes, Ann Arbor, MI). All new sequences have been deposited in the International Nucleotide Sequence Database (Accession numbers: KF218257–KF218275).

Phylogenetic analysis

Six additional sequences labelled as *H. psittacina* (Brock et al. 2009, Babos et al. 2011) were downloaded from GenBank and combined with our dataset (Table 1). The se-

Table 1. Collection and voucher information for the specimens used in this study.

GenBank Accession No.	Taxon	Fungarium Accession No.	Collection code/ seq. literature ref.	Source	Notes
KF218268	*Gliophorus europerplexus*	K(M)181241	E.J.M.Arnolds WX359	UK, Wales, Merionethshire	
KF218266	*Gliophorus europerplexus*	K(M)181245	D.J.Harries DJH064A WX663	UK, Wales, Pembrokeshire	
KF218267	*Gliophorus europerplexus*	K(M)181246	D.J.Harries DJH064B WX664	UK, Wales, Pembrokeshire	Holotype
KF218272	*Gliophorus perplexus*	MICH10924	A.H.Smith 21491	USA, Michigan, Cheboygan Co.	*Hygrophorus perplexus* holotype, part
KF218270	*Gliophorus perplexus*	MICH45363	T.E.Brooks 1098	USA, Michigan, Cheboygan Co.	*Hygrophorus perplexus* paratype
KF218271	*Gliophorus perplexus*	MICH45364	T.E.Brooks 1099	USA, Michigan, Cheboygan Co.	*Hygrophorus perplexus* paratype
KF218274	*Gliophorus perplexus* aff.	K(M)121495	E.W.Brown	Channel Islands, Jersey	as *Hygrocybe psittacina* var. *perplexa*
KF218273	*Gliophorus perplexus* aff.	K(M)166625	N.W.Legon	UK, England, South Somerset	as *Hygrocybe psittacina* var. *perplexa*
KF218269	*Gliophorus perplexus*	MICH45365	A.H.Smith 34029	USA, Michigan, Chippewa Co.	Hygrophorus perplexus paratype
EU784339	*Gliophorus psittacinus*	K(M)127070	Brock et al. 2009	UK, N. Ireland, Derry	
EU784340	*Gliophorus psittacinus*	K(M)127194	Brock et al. 2009	UK, N. Ireland, Tyrone	
EU784341	*Gliophorus psittacinus*	K(M)90029	Brock et al. 2009	UK, England, Buckinghamshire	
EU784342	*Gliophorus psittacinus*	K(M)90674	Brock et al. 2009	UK, England, East Sussex	
FM208875	*Gliophorus psittacinus*		Babos et al. 2011	Hungary, Kétvölgy	
FM208895	*Gliophorus psittacinus*		Babos et al. 2011	Hungary, Apátistvánfalva	
KF218259	*Gliophorus reginae*	K(M)156265	R.Winnall	UK, England, Worcestershire	Holotype
KF218258	*Gliophorus reginae*	K(M)181115	R.D.Foster WCS15 WX115	UK, England, Derbyshire	
KF218260	*Gliophorus reginae*	K(M)181116	R.D.Foster WCS26 WX126	UK, England, Derbyshire	
KF218263	*Gliophorus reginae*	K(M)181117	R.Winnall WX459	UK, England, Worcestershire	
KF218265	*Gliophorus reginae*	K(M)181124	J.E.Hodges DJH055 WX535	UK, Wales, Pembrokeshire	
KF218264	*Gliophorus reginae*	K(M)181126	R.Winnall WX673	UK, England, Worcestershire	
KF218275	*Gliophorus reginae*	K(M)181127	R.Winnall & A.M.Ainsworth WX694	UK, England, Worcestershire	
KF218257	*Gliophorus reginae*	K(M)181128	R.Winnall & A.M.Ainsworth WX695	UK, England, Worcestershire	
KF245883	*Gliophorus reginae*	K(M)181129	R.Winnall & A.M.Ainsworth WX696	UK, England, Worcestershire	
KF218262	*Gliophorus reginae*	K(M)181227	R.Winnall WX461	UK, England, Worcestershire	
KF218261	*Gliophorus reginae*	K(M)41524	C.Lovatt	UK, England, Staffordshire	

quences were trimmed to minimize uneven ends across the dataset and aligned using the RNA structure-based algorithm Q-INS-i implemented in MAFFT v7.023b (Katoh et al. 2002, Katoh and Toh 2008, Katoh and Standley 2013). Phylogenetic analysis under the maximum likelihood criterion was performed using the Pthreads-parallelized version of RAxML v7.0.3 (Stamatakis 2006, Ott et al. 2007) with a GTR-GAMMA model. Branch support was assessed using nonparametric bootstrapping with the "thorough" option and 1000 replicates. The final alignment and phylogenetic tree are available from TreeBase (#14384; http://purl.org/phylo/treebase/phylows/study/TB2:S14384).

Results

The full ITS region was amplified and sequenced for 14 specimens. Only the ITS1 region was sequenced for all specimens from MICH, while only the ITS2 region was sequenced for K(M)181124. The ITS1 and ITS2 regions were amplified and sequenced separately for K(M)181245, and the two non-overlapping regions were concatenated and separated by 66 gaps corresponding to the 5.8S ribosomal subunit in the final alignment. Phylogenetic analysis resulted in a highly resolved tree with most nodes receiving strong bootstrap support (Fig. 1). Both *G. psittacinus* and *G. perplexus* were found to be polyphyletic. Two distinct clades were strongly supported (100%): 1) *G. reginae* (99%) and a subclade (73%) consisting of *G. europerplexus* (99%) and a single sequence from a paratype specimen of *G. perplexus*, and 2) all other sequences, including three *G. psittacinus* clades (87%, 100%, 100%) comprising the GenBank sequences, one *G.* aff. *perplexus* clade (100%), and one clade composed of sequences from the holotype and two paratype specimens of *G. perplexus* (100%).

Taxonomic treatment

Gliophorus reginae **Dentinger, A.M.Ainsw., & P.F.Cannon, sp. nov.**
Registration Identifier: IndexFungorum IF 550184
http://species-id.net/wiki/Gliophorus_reginae
Figures 2, 3, 6

Holotype. UNITED KINGDOM. England. Worcestershire (vice county 37): Bewdley, Willow Bank, 52°21.46'N, 2°22.42'W (Nat. Grid Ref. SO746733), 24 Jan 2008, R.Winnall (K(M)156265)

Description. Pileus 15–55 mm diam., hemispherical to broadly conical or campanulate, initially with incurved margin, becoming applanate, often retaining broad umbo and irregular, lobed outline with indentations, folds and pleats, sometimes becoming radially furrowed, or split and flared, margin faintly to strongly translucently striate to half-way and becoming reflexed to highly revolute, viscid with gelatinous

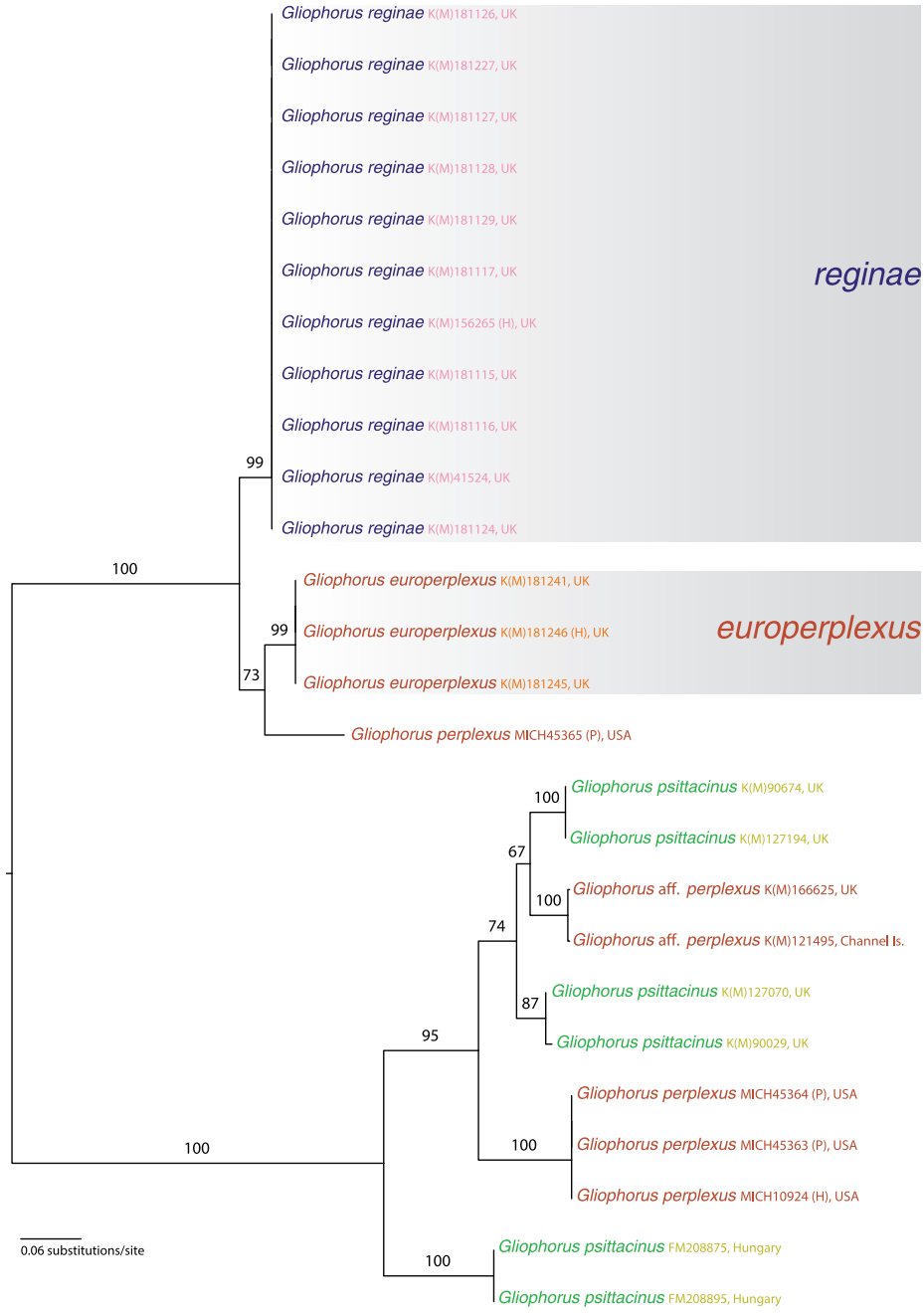

Figure 1. Maximum likelihood phylogram using full and partial nuclear ribosomal internal transcribed spacers (ITS) sequences. Numbers above branches are nonparametric bootstrap values. Tree is arbitrarily rooted at the midpoint. Two well-supported terminal clades representing the new species *G. reginae* and *G. europerplexus* are superimposed over light grey boxes. Species names for specimens from which sequences were derived are followed by fungarium or INSD accession number, and geographic location. Notations (H) and (P) indicate specimens used were holotypes or paratypes, respectively.

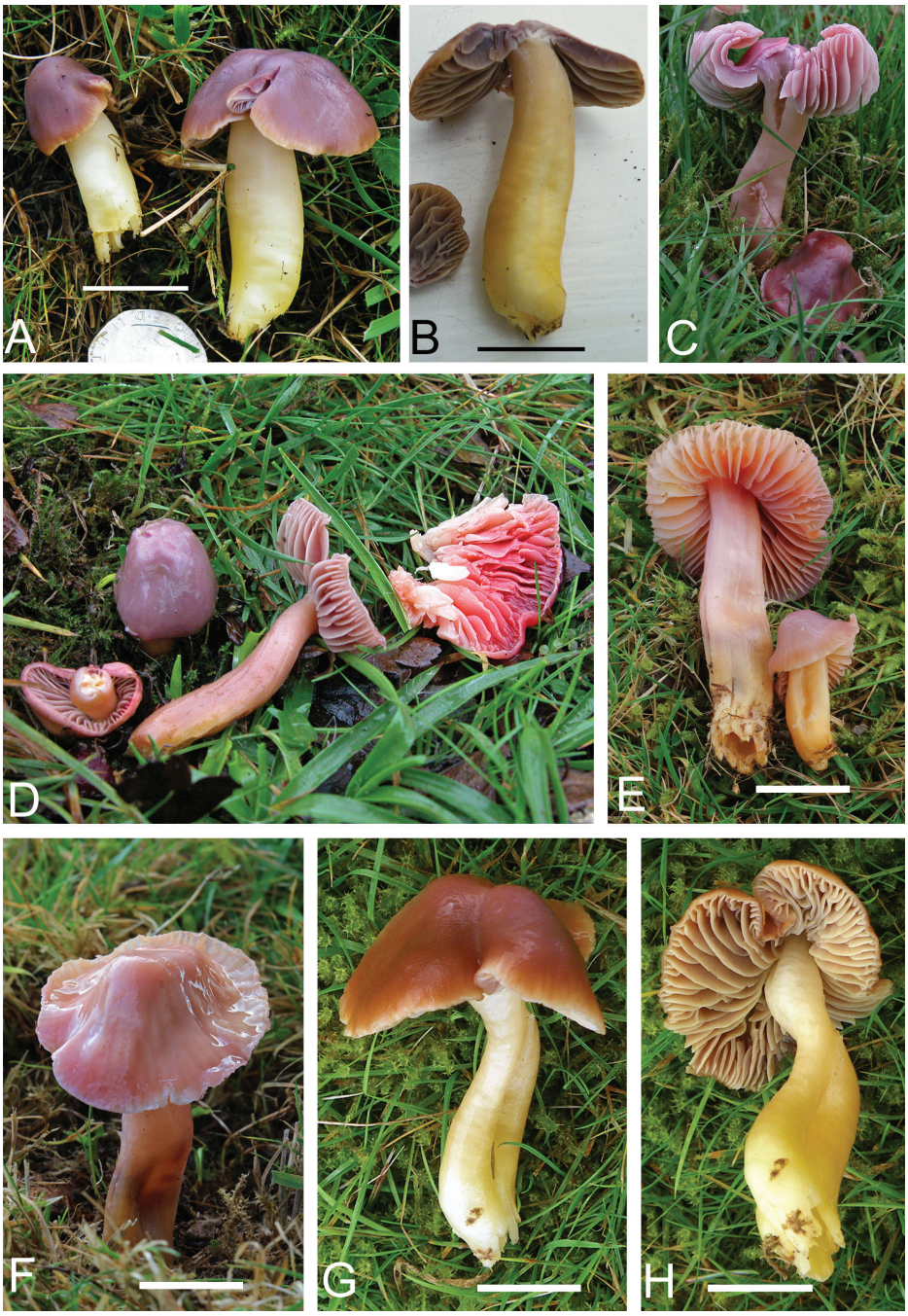

Figure 2. Basidiomata of *G. reginae* showing pileal colour range: **A–D** purple, **E–F** pink and **G–H** reddish brown, scale bars represent 20 mm. Photographs **C–F** taken *in situ* at the type locality. **A, B** K(M)181115* photographed by R.D. Foster. **C** K(M)181117* and **D** K(M)181123 photographed by R. Winnall. **E** (left), **F** K(M)181128* and **E** (right) K(M)181127* photographed by A.M.A. **G, H** K(M)181124* photographed by D.J. Harries.

pellicle, sometimes with minutely rugose texture, at first usually dull violet purple (vinaceous grey to purple) with areas of pink, darker red or red-brown tones (rose, blood red to rusty tawny), sometimes more brownish (purplish date to dark brick), becoming paler and pinkish especially around margin which can also develop yellow (luteous) or yellow-brown (fulvous) tints, hygrophanous, dried pilei characteristically pale orange (saffron) flushed pink (rose). Lamellae ventricose, mostly narrowly adnate with some free, sinuate or broadly adnate elements, intervenose, concolorous with pileus near pileal attachment, becoming paler towards free edge, sometimes with yellow (luteous) or orange (saffron) tints. Stipe 15–70 × 5–15 mm, relatively stout, sometimes tapering upwards from the clavate base, hollow, often flexuose or tortuous, compressed or grooved, viscid but usually slightly less so than pileus, white often apically tinged with pileal colour and basally yellow (luteous) to pale orange (saffron) or becoming so, sometimes with purplish (vinaceous grey) blotches if frosted. Outer tissues of context concolorous with adjacent external surfaces, inner tissues paler. Dried lamellar trama (lens) often conspicuously dark pink (coral), contrasting with paler subhymenium and lamellar surfaces. Green pigments entirely absent. Without distinctive taste or smell. Spores [120, K(M)181126*, K(M)181127*, K(M)181128*, K(M)181129*] 6.0–8.5(–9.0) × 4.0–5.5(–6.0) µm, per-basidioma mean values 7.0–7.5 × 5.0 µm, Q = 1.2–2.0, mean 1.5, short-ellipsoidal to ellipsoidal, not constricted. Basidia predominantly 4-spored, clavate, relatively long and slender with long attenuated base, (37–)40–63(–67) × 6–10 µm excluding sterigmatal length (4.0–8.0 µm). Clamp connections on basidia, within lamellar trama and pileipellis often with conspicuously looped hook cells (medallion clamps). Lamellar trama subregular with some interwoven elements, compartments 24–183 × 4–24 µm. Stipitipellis and pileipellis are ixotrichoderms.

Distribution. Known from a cemetery in West Wales (Pembrokeshire) and fields in central England (Worcestershire, Staffordshire and Derbyshire). The earliest known collection was made by C. Lovatt in Staffordshire in 1996 who noted that she had recorded similar specimens in 1994. It has fruited on private land at Willow Bank (Worcestershire) almost every year from 2000 onwards and recorded there in five discrete fruiting patches in a single field of ca. 0.8 ha.

Ecology. In unimproved short (grazed or mown) acid-neutral rough pasture or other grassland. This species is often a relatively late fruiter and can continue producing basidiomata in January, long after other waxcaps have finished.

Etymology. Latin *reginae* meaning "of a queen", named for the royal purple colour of the basidiomata and to celebrate the diamond jubilee of Her Majesty Queen Elizabeth II in 2012 and the 60th anniversary of her coronation in 2013.

Conservation status. Collectors noted that although basidiomata of this species resembled *G. psittacinus*, some characters such as pileal colour and radial splitting, were more characteristic of *H. calyptriformis.* Furthermore, dried collections of the latter and *G. reginae* often attained a similar reddish-coral tint in the fungarium that was distinct from the pale saffron of *G. psittacinus*. This similarity facilitated a rapid search of the British *G. psittacinus* collections at Kew, but no additional *G. reginae* specimens were

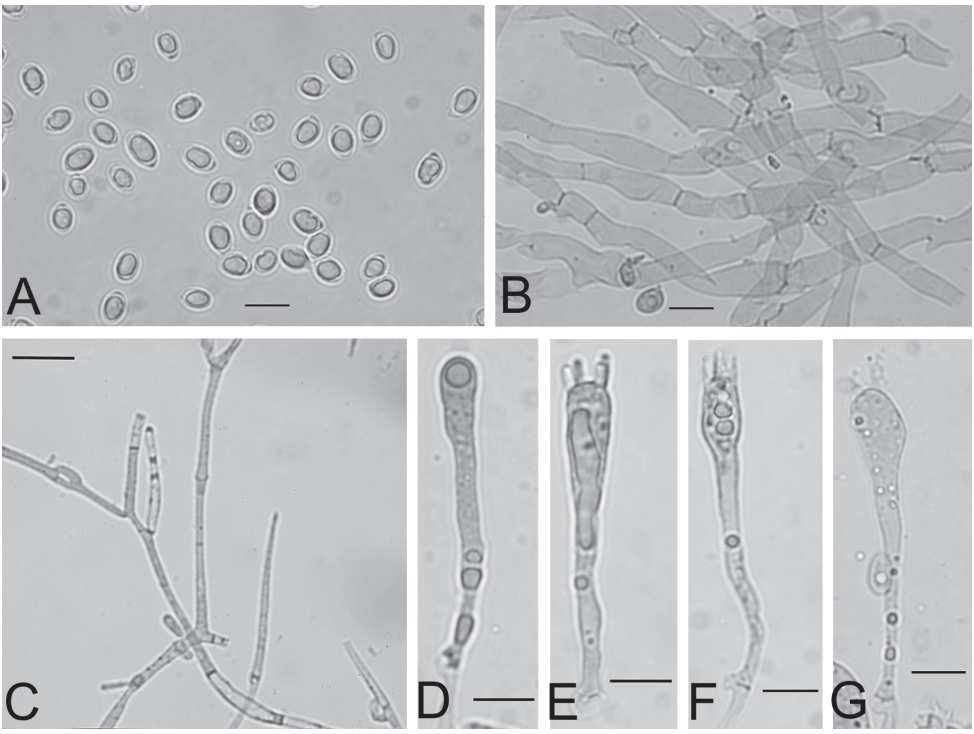

Figure 3. Microscopic characters of *G. reginae* collected from the type locality, **B**–**G** mounted in Congo Red, scale bars represent 10 µm. **A** Spores from water mount of spore print K(M)181127* **B** Subregular lamellar trama from squash mount K(M)181129* **C** Pileipellis hyphae showing medallion clamp connections K(M)181127* **D–F** Basidial developmental series K(M)181129* **G** Basidium K(M)181127*.

discovered. This suggests that it is genuinely rare in Britain and Endangered (EN D, <250 mature individuals) might be the current regional conservation assessment following IUCN guidelines, categories and criteria (IUCN 2012a, b, 2013). However, we think that this species is so poorly known at present that it should be assessed as Data Deficient pending further survey work.

Other specimens examined. United Kingdom. England. Derbyshire (vice county 57): Edale, Lower Hollins Farm, 53°21.84'N, 1°47.96'W (Nat. Grid Ref. SK134852), 5 Oct 2010, R.D.Foster WCS8 WX108 (K(M)181114). Ibid. 17 Oct 2010, R.D.Foster WCS15 WX115 (K(M)181115*). Woodlands Valley, Rowlee Bridge Fields, 53°23.99'N, 1°46.60'W (Nat. Grid Ref. SK149892), 9 Nov 2010, R.D.Foster WCS26 WX126 (K(M)181116*). Staffordshire (vice county 39): Danebridge (near), 53°10.25'N, 2°3.99'W (Nat. Grid Ref. SJ956637), 22 Oct 1996, C.Lovatt (K(M)41524*, sub *H. psittacina*). Worcestershire (vice county 37): Bewdley, Bowcastle Farm cherry orchard, 52°22.38'N, 2°20.40'W (Nat. Grid Ref. SO769750), 3 Nov 2004, R.Winnall WX461 (K(M)181227*). Bewdley, Willow Bank, 52°21.46'N, 2°22.42'W (Nat. Grid Ref. SO746733), 4 Nov 2001, R.Winnall (K(M)92058, sub *H.* cf. *psittacina*). Ibid. 11 Nov 2004, R.Winnall WX459 (K(M)181117*). Ibid.

15 Dec 2004, R.Winnall WX460 (K(M)181123). Ibid. 18 Oct 2012, R.Winnall WX673 (K(M)181126*). Ibid. 15 Jan 2013, R.Winnall & A.M.Ainsworth WX694 (K(M)181127*), WX695 (K(M)181128*), WX696 (K(M)181129*). Wales. Pembrokeshire (vice county 45): Fishguard Cemetery, 51°59.30'N, 4°57.64'W (Nat. Grid Ref. SM96803634), 11 Nov 2011, J.E.Hodges DJH055 WX535 (K(M)181124*).

Gliophorus europerplexus **Dentinger, A.M.Ainsw., & P.F.Cannon, sp. nov.**
Registration Identifier: IndexFungorum IF 550185
http://species-id.net/wiki/Gliophorus_europerplexus
Figures 4–6

Holotype. UNITED KINGDOM. Wales. Pembrokeshire (vice county 45): Hundleton, Somerton Farm, 51°39.88'N, 4°59.54'W (Nat. Grid Ref. SM931004),11 Oct 2012, D.J.Harries DJH064B WX664 (K(M)181246)

Description. Pileus 10–25 mm diam., hemispherical to conical or campanulate, sometimes with incurved margin, becoming plano-convex or remaining broadly conical, often umbonate, sometimes with irregular, lobed outline, margin faintly to strongly translucently striate to half-way, viscid or at least very lubricous, sometimes partially flared, at first usually pink-brown to orange-brown (brick, rusty tawny to fulvous), margin paler sometimes with orange (sienna to apricot) tints, hygrophanous, dried pilei dull orange (saffron to rust). Lamellae ventricose, mostly narrowly to broadly adnate with some slightly decurrent elements, intervenose, concolorous with pileus near pileal attachment, becoming paler towards free edge. Stipe 12–60 × 2–8 mm, cylindrical or compressed, sometimes with clavate base, hollow, sometimes flexuose, viscid but usually slightly less so than pileus, apically concolorous with pileus, paler below, sometimes basally tinted orange (apricot). Dried lamellar trama (lens) often darker than subhymenium and lamellar surfaces. Green pigments entirely absent. Without distinctive taste or smell, although one specimen [K(M)181241*] was noted to have a faint rubbery smell reminiscent of *G. laetus*. Spores [70, K(M)181241*, K(M)181245*, K(M)181246*] (6.5–)7.0–9.0 × (4.0–)4.5–5.5(–6.0) µm, per-basidioma mean values 7.5–8.0 × 5.0 µm, Q = 1.3–1.8, mean 1.6, short-ellipsoidal to ellipsoidal, not constricted. Basidia predominantly 4-spored, clavate with attenuated base, (30–)34–57 × 5–9 µm excluding sterigmatal length (3.0–7.0 µm). Clamp connections on basidia, within lamellar trama and pileipellis usually normal, occasionally with conspicuously looped hook cells (medallion clamps). Lamellar trama subregular, compartments 20–120 × 4–21 µm. Stipitipellis and pileipellis are ixotrichoderms.

Distribution. Identified from two sites in west Wales (Merionethshire and Pembrokeshire) supported by DNA sequence data. Fruiting was probably observed at the type locality by D.J. Harries on 6 August 2009 but no material was kept.

Ecology. In unimproved short acid-neutral rough pasture in Merionethshire and found fruiting on bare soil near mosses on an almost vertical south-facing earth bank on farmland in Pembrokeshire.

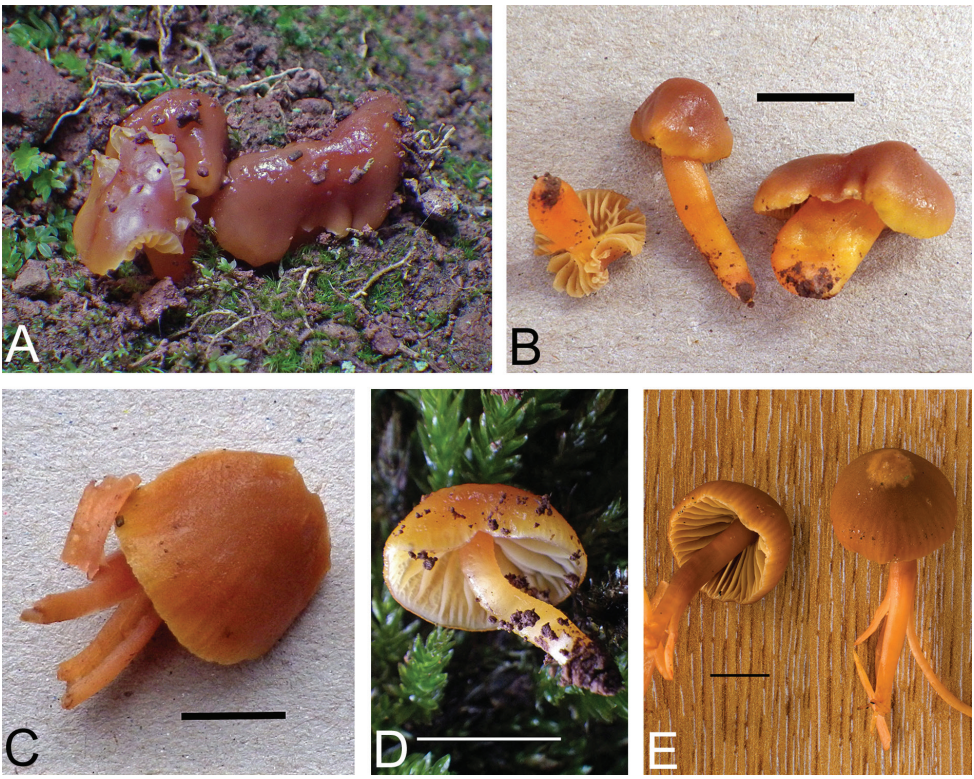

Figure 4. Basidiomata of *Gliophorus europerplexus*, scale bars represent 10 mm. Photographs **A**–**D** by D.J. Harries taken *in situ* at, or of collections from, the type locality, and **E** by B.T.M.D. **A, B** K(M)181245* **C, D** K(M)181246* holotype **E** K(M)181241*.

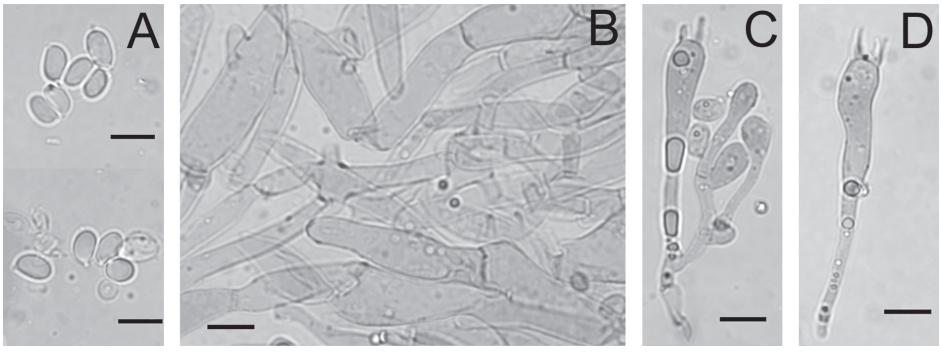

Figure 5. Microscopic characters of *G. europerplexus*, **A** mounted in Melzer's Reagent and **B–D** (holotype) in Congo Red, scale bars represent 10 µm. **A** Spores from lamellar squash K(M)181241* **B** Subregular lamellar trama from squash mount K(M)181246* **C–D** Basidia K(M)181246*.

Etymology. Named to distinguish this European taxon from the morphologically similar *Hygrophorus perplexus* A.H.Smith & Hesler, a species with North American type material.

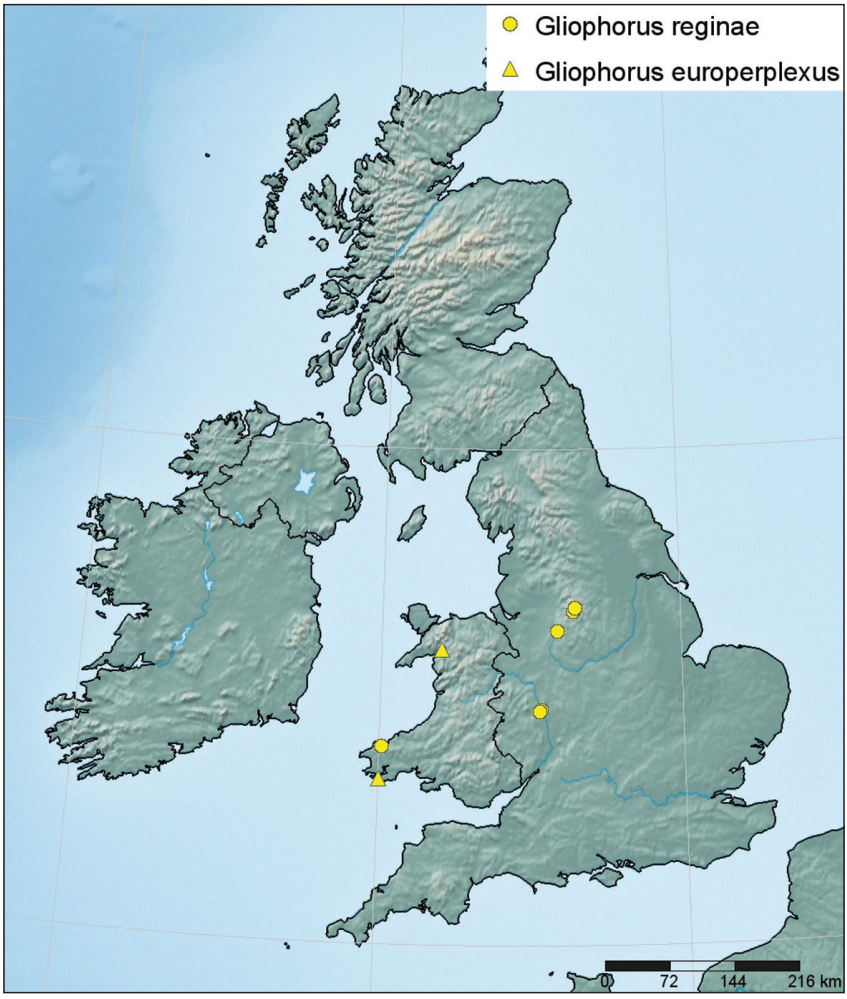

Figure 6. Distribution of *G. reginae* (●) and *G. europerplexus* (▲) in Britain based on sequenced collections and plotted using SimpleMappr (Shorthouse 2010).

Conservation status. Initially, it seemed likely that historic British collections assigned to *H. psittacina* var. *perplexa* would be redetermined as *G. europerplexus* following DNA sequencing. However, two specimens filed in K under the former name yielded distinct ITS sequences (Fig.1). Therefore, the distribution of *G. europerplexus* is currently unknown and it should be assessed as Data Deficient.

Other specimens examined. United Kingdom. Wales. Merionethshire (vice county 48): Croesor, Cnicht, 52°58.34'N, 4°0.22'W (Nat. Grid Ref. SH64 but estimated to be SH655435 for conversion to latitude and longitude), 11 Oct 2011, E.J.M.Arnolds WX359 (K(M)181241*). Pembrokeshire (vice county 45): Hundleton, Somerton Farm, 51°39.88'N, 4°59.54'W (Nat. Grid Ref. SM931004), 19 Aug 2012, D.J.Harries DJH064A WX663 (K(M)181245*; immature).

Discussion

The traditional *Gliophorus* (=*Hygrocybe*) *psittacinus* species concept is relatively broad (Boertmann 2001), mainly due to difficulties in defining unambiguous morphological discontinuities in basidiomatal characters. Our ITS sequence analysis revealed a clade comprising two terminal clusters, *G. reginae* and *G. europerplexus*, together with a singleton, a paratype of *H. perplexus*, that is clearly divergent from that comprising the currently-accepted European taxon *G. psittacinus* and the N. American *G. perplexus* (Fig. 1). It is clear that the name *G. psittacinus* currently represents a species complex and further work is required to characterise and describe the component taxa. We have assigned our two new species to the segregate waxcap genus *Gliophorus* Herink based on recent supporting molecular phylogenetic evidence (Babos et al. 2011, Lodge et al. in press).

One of the novel taxa, *G. reginae*, is recognisable in the field having a relatively stout stipe, sometimes yellowing at the base, and distinctive deep purple or reddish-brown pileus. Our field observations suggest that a colour form of this might be shown in Boertmann's photograph of Danish specimen DB 2000/33 taken at Lysnet, E. Jylland, on 17 Oct. 2000 (Boertmann 2001 fig. 4, 2010 p.91). The remaining currently-accepted European taxon in the parrot waxcap group is *H. psittacina* var. *sciophanoides* (Boertmann 2010, 2012). This species was originally described as *Hygrophorus sciophanoides* by Rea (1922) based on a painting of an English specimen found in 1909 in Derbyshire designated as Rea 937, but no type specimen is preserved at Kew. Rea's "uncommon" fungus had a rosy pink striate pileus 1-3 cm diam. with pale pink lamellae, a concolorous stipe 2–5 cm × 2–3 mm and flesh described as pale yellow becoming white. Although Rea did not use the word "viscid" in his description, nevertheless he synonymised Cooke's (1889) concept of *Hygrophorus sciophanus*, a slightly viscid-pileate species with decurrent gills, with *H. sciophanoides*. Cooke (1889) quoted a description of two Scottish specimens, one pale and sterile and the other darker and yielding "very pale clay-coloured" spores from *Notices of British Fungi No. 1560* (Berkeley and Broome 1876). The latter authors also recorded some small Welsh specimens in *Notices of British Fungi No. 1885*, again noting the existence of light and dark forms (Berkeley and Broome 1881). Cooke's (1886–1888) six illustrations (No. 905 Plate 937A) of English material from Kendal resemble the specimen depicted by Rea. *G. reginae* can be similarly pink and striate (Fig. 2F), but Rea and Cooke both described and illustrated basidiomata that were strikingly more slender than those of *G. reginae.* This together with the lack of type material of *H. sciophanoides* and the existence of other colour forms of the *H. psittacina* complex that can develop pink tints with age, leads us to conclude that *H. sciophanoides* should be regarded as a *nomen dubium*. Our attempts to sequence Welsh material collected in 1950 (K(M) 69657) and determined by Pearson as *H. sciophanoides* were unsuccessful.

In Europe, *Hygrophorus sciophanus* (Fr.) Fr. is currently regarded as a synonym of *H. psittacina* var. *perplexa* with *Hygrophorus perplexus* A.H. Sm. & Hesler as basionym. By contrast, Hesler and Smith (1963) argued that their taxon had a very similar lamellar attachment, "never decurrent", to that of *Hygrophorus psittacinus*, a character

that distinguished it from *H. sciophanus*. Indeed Fries distinguished the lamellar attachment of *Agaricus psittacinus*, described as "adnatis", from that of *A. sciophanus*, "decurrentibus" (Fries 1821) and, later, of *H. sciophanus*, "subdecurrentibus" (Fries 1836–1838). Rea (1922), on the other hand, described the attachment in *H. sciophanus* as "attenuato-adnate", as shown in Rea 936, a painting of a French collection, and he cited an illustration approved by Fries. The latter painting (Fries 1877–1884, Plate 167.1) appears to bear out Rea's description, but in the same volume (p. 66), Fries used the word "decurrentibus" in the diagnosis and commented that the illustration showed "lamellarum insertio minus typica". The original concept of *H. sciophanus* thus is unclear and various interpretations exist in the literature. Three collections originally filed as *Hygrophorus sciophanus* preserved in K were sequenced and determined to be highly divergent from the *Gliophorus* sequences in our dataset, belonging instead to *Hygrocybe* sensu stricto (data not shown). In our view, *H. sciophanus* should be regarded as a *nomen dubium*.

Our analysis showed that the ITS sequence derived from the holotype specimen of *H. perplexus* is certainly distinct from the second of our new species, *G. europerplexus*. Two specimens identified as *H. psittacina* var. *perplexa* (Table 1) collected in 2003 and 2008 were also sequenced, but they are phylogenetically distinct, forming a clade near to *G. psittacinus* (Fig. 1) and may represent a further novel taxon. The single anomalous sequence from a paratype of *H. perplexus*, which comes near *G. europerplexus* in our analysis, reveals additional cryptic diversity within this species complex in North America and highlights the difficulty in correctly naming waxcap species using morphology alone. Attempts should be made, therefore, to sequence additional European and North American specimens currently filed as *G. perplexus*, *Hygrophorus perplexus*, *Hygrocybe perplexa* and *H. psittacina* var. *perplexa* to gain a better understanding of the distribution of *G. europerplexus* and other emerging segregate taxa.

Acknowledgements

We would like to thank Defra, Natural England and Scottish Natural Heritage for financial support and all those who collected and sent specimens of the two species described herein: E.J.M. Arnolds, R.D. Foster, D.J. Harries, J.E. Hodges and R. Winnall. Thanks also to R.D.F., D.J.H. and R.W. for allowing us to use their photographs.

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