DNA barcoding reveals three *Rhodocybe* species new to Britain

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Introduction

As part of routine surveys during 2019 and 2020, several *Rhodocybe* species were collected from both Buckinghamshire and Sussex. Within *Rhodocybe*, the brown to pinkish colouration of three of our collections suggested a connection to section *Rufobrunnea*, a section for which only 2 species are currently recorded from the UK, neither of which appeared to be good matches to these collections. However, several new species in this section have recently been described from Turkey, Italy and Estonia (Vizzini *et al*., 2016, Sesli & Vizzini, 2017; Vizzini *et al*., 2018), which had some resemblance to our collections but had not yet been confirmed from the UK.

To investigate whether the three *Rhodocybe* collections did correspond to these newly described species, the collections were flagged up as candidates for DNA extraction, amplification, and sequencing by both the Sussex Fungus Group and the Hampshire Fungus Group. This DNA work was done as part of a field mycology DNA barcoding initiative set up in collaboration with the Lost and Found Fungi (LAFF) project (Royal Botanic Gardens, Kew).

Methods

Specimens were photographed in situ, microscopically examined and imaged in their fresh state, mounted in water, Congo red, Melzer's reagent and Cotton blue in lactophenol.

DNA extraction, amplification, visualisation, sequencing, and sequence analysis was conducted as described in Box 1 below.

Results

For one specimen, the newly generated ITS sequences produced a 99.5% full length match with the sequence derived from the holotype of

BOX 1
Molecular Methods

DNA extraction was performed using a slightly modified version of the dipstick protocol described by Zou *et al*. (2017). Briefly, an approximately 2 mm3 section of clean dried gill tissue was macerated in 200 ul of lysis buffer (20 mM Tris [pH 8.0], 25 mM NaCl, 2.5 mM EDTA, 0.05% SDS) using a plastic pestle, before diluting the crude extract with a further 300 µl of lysis buffer. A homemade filter-paper dipstick with wax handle was used to extract DNA from the crude extract; to wash the DNA in wash buffer (10 mM Tris [pH 8.0], 0.1% polysorbate 20); and to release the DNA into the PCR reaction mix – three dips of the dipstick were used at each step as recommended by Zou *et al*. (2017).

The internal transcribed spacer (ITS) region was amplified using the primer pair ITS1F and ITS4. Thermocycling was done with a Bento Lab (Bento Bioworks Ltd., London, United Kingdom), using the following protocol: 4 minutes at 95 ˚C, then 35 cycles of: 95 ˚C for 30 seconds, 52 ˚C for 30 seconds, 72 ˚C for two minutes followed by a final extension step for 10 min at 72°C and hold at 15 ˚C. 10 ul of PCR products were visualised on the Bento Lab electrophoresis unit on a 40 ml agarose gel stained with StainIN™ green (Client Life Science, Stourbridge, United Kingdom). Sequencing was done at the Institute of Biological, Environmental & Rural Sciences (IBERS) of the University of Aberystwyth.

Nucleotide traces were checked manually for quality and errors in FinchTV 1.4.0 (Yang *et al*., 2017). Approximate taxonomic affiliation was determined using a BLAST search in Genbank (httpscbi phylogeny was created based on the ITS phylogeny in Vizzini *et al*. (2018). All phylogenetic work was done in AliView (Larsson, 2014). Sequences were aligned using the MAFFT E-INS-I algorithm; the alignment was trimmed; and phylogenies were calculated using the FastTree algorithm and visualised in FigTree. A final phylogeny was produced using RAxML. *Clitopilus prunulus* AFTOL-ID_522 (DQ202272) was selected as an outgroup.

Rhodocybe asyae (KX834266), differing in only 1 single nucleotide polymorphism (SNP) and two gaps; for another, a 99.6% full length match with the sequence from the holotype of *R. asanii* (NR_154442) (2 SNPs); and for the third, a 99% match with the sequence from the holotype of *R. fumanellii* (NR_166243) (2 SNPs and 1 gap). Within our phylogeny these sequences clearly clustered with the holotype and paratype sequences of these species. In contrast, no other available sequences were more than 86% similar to the collections. All three collections were therefore considered conspecific with the type collections.

Our collection data are provided here in order to contribute to the knowledge of this poorly studied group, and to confirm the presence of these three species within the UK

Rhodocybe asyae Sesli & Vizzini

Collected 13/10/19, Tilgate Park, Crawley, East Sussex (VC 14). UK grid ref: TQ 2739 3425. Genbank accession number: MN840644. Herb: NA131019.

Pileus: To 35 mm, smooth, dry, minutely felty, reddish brown in centre, paler pinkish towards margin.

Lamellae: Narrowly adnate to subdecurrent, white or very pale buff, even at maturity; discolouring pinkish when handled, the number of lamellae reaching from cap margin to stipe 40-45.

Spore print: pinkish brown (surprising considering the pale gills!).

Stipe: to 30 x 5 mm, cylindrical, hollow, beige to reddish brown, covered with a white pruina (especially at apex) which disappears with age/handling.

Odour: Indistinct on collection, farinaceous on drying.

Basidiospores: 5.2–6.8 x 3.5–4.6 µm, subangular, hyaline, contents minutely guttulate/granular, inamyloid, acyanophilus.

Cystidia: Not observed, despite a deliberate search ("rare" and "versiform" in original description)

Basidia: (2-) 4-spored, simple-septate at base.

Cap cuticle: A cutis with light golden yellow pigment, hyphae with light incrustation.

Clamps: Not observed.

Habitat: In short grass under *Pinus* sp.

Figure 1: NA131019 *Rhodocybe asyae*. Photograph © N. Aplin.

Figure 2: HFRG_PC200928_1 *Rhodocybe fumanellii.* Photograph © P. Cullington

Rhodocybe fumanellii Vizzini & Fellin

Collected 28/9/2020, Rushbeds Wood, Buckinghamshire. UK Grid ref: SP 667 156. Genbank accession number: MW401761, Herb: HFRG_PC200928_1

Pileus: To 50 mm, smooth, dry, appearing +/- pruinose, covered with a fine tomentum, radially striate, reddish cocoa brown but in larger specimens beginning to dry out paler from centre outwards. Not umbonate, slightly rounded at first then +/- flat with downturned margin.
Lamellae: Crowded, subdecurrent, pale cream when young, gradually becoming more pinkish. Spore print: Distinctly pink.
Stipe: To 60 mm x 10 mm, cylindrical, concolorous with gills, with fine white striate tomentum which where rubbed reveals a pinker surface beneath; base slightly broader where firmly attached to substrate with mycelial strands.
Odour: Pleasant on drying, fruity and sweet, aromatic, when cut strong with acidic component.
Basidiospores: 6-6.5 x 4-5 µm subangular, hyaline, contents minutely guttulate/granular.
Cheilocystidia: Sparse, cylindrical, slightly flexuose and septate. Basidia: 4-spored.
Cap cuticle: A cutis.
Clamps: Not observed.
Habitat: On fallen wood in a decomposing log pile (mainly composed of *Fraxinus excelsior*).

Rhodocybe asanii Sesli & Vizzini

Collected 13/10/20, Tilgate Park, Crawley, East Sussex (VC 14). UK grid ref: TQ 2734 3437. Genbank accession number: MW375030, Herb: NA13102020

Pileus: To 50 mm, cocoa brown in centre, beige elsewhere, margin almost white, smooth, dry, rounded at first and sometimes with an indistinct umbo, later becoming shallowly cup or funnel-shaped.
Lamellae: Moderately crowded, pale pink, adnate to subdecurrent. Spore print: Light pink.
Stipe: To 40 × 12 mm, cylindrical to bulbous at base, pruinose, discolouring grey- brown with handling.
Odour: fruity and sweet.
Basidiospores: 5-7.8 × 4.1-5.2 µm, subangular, hyaline, contents minutely guttulate/granular, inamyloid.
Cheilocystidia: Cylindrical to clavate, slightly flexuose and septate (absent in type material).
Basidia: 2 to 4-spored, simple septate at base.
Cap cuticle: A cutis, hyphae lightly incrusted.
Clamps: Not observed.
Habitat: On bare soil amongst needle litter under *Picea*.

Figure 3: NA13102020 *Rhodocybe asanii.* Photograph © N. Aplin.

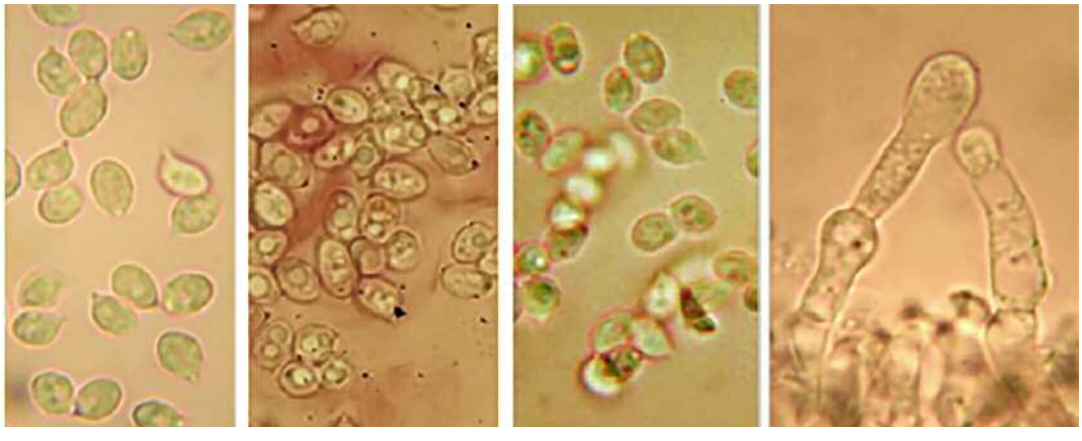

Figure 4: from left to right: Spores of *R. asyae**, *R. fumanellii***, *R. asanii** and cheilocystidia of *R. asanii**

*Mounted in water. Photographs © N.Aplin.

**Mounted in Congo red dye. Photograph © P. Cullington.

Discussion

Given that these three species were only described in 2017 and 2018 and are not yet present in any field guides or keys for this region, it is hardly surprising that there have been no previous UK reports. Until there is evidence of further collections it will be unclear whether they are in fact widely distributed in Great Britain and Ireland. Given the frequency of new species descriptions from other parts of Europe in the *Rhodocybe* group (Vizzini *et al*., 2016, Sesli & Vizzini, 2017, Vizzini *et al*., 2018) we suspect there is great potential to further expand and refine the British and Irish records. We follow Henrici (2020) in suggesting that unusual specimens not fitting to known species concepts be documented and retained for DNA sequencing in the future, as described in Harries (2017). We also apologetically refrain from offering a dichotomous key (for the same reasons offered in Henrici, 2020), and note that whilst some morphological characteristics seem to remain stable across collections, others do not.

Whilst these three species are superficially

Figure 5: *Rhodocybe* sp., HFRG_EJ171117_1 fruitbodies. Photograph © E. Janke.

similar, especially in microscopic anatomy, their habits and habitats may be enough to separate them. *R. asyae* and *R. asanii* seem to prefer soil under conifers (Sesli & Vizzini 2016) whereas *R. fumanellii* is most frequently described under broadleaf trees (Vizzini *et al.* 2018). Furthermore, whilst *R. asyae* has a smaller and slender collybioid/clitocyboid habit, *R. asanii* and *R. fumanellii* have a stockier lactarioid/trichalomatoid appearance. We note that in at least one of our collections the presence of cystidia is somewhat conflicting with the type descriptions, suggesting this characteristic may be an unreliable tool for identification.

A further unidentified collection

In addition to these 3 collections in the section *Rufobrunnea*, one of us (EJ) recalled an earlier collection made at Exbury Gardens in 2017, which was identified as *R. caelata* using the literature available at the time. In the light of the above discoveries he decided to sequence that collection and found that the ITS sequence (listed as HFRG_EJ171119_1 in Fig. 6) formed a basal clade outside Section *Rufobrunnea*, along with *R. pallidogrisea* and *R. tugrulii*, from which the collection is morphologically and genetically distinct.

Rhodocybe sp.

Collected 17/11/2017 Exbury Gardens, S. Hampshire (VC11) UK grid ref SU424002. Genbank accession numbers: ITS region - MW397197, LSU region - MW397521, Herb: HFRG_EJ171117_1

Pileus: Purple-brown 10–20 mm.

Stipe: Brown, fibrillose, 10 x 2 mm.

Spores: Ellipsoid 6–9 x 4–5.5 µm verrucose, cyanophylic.

Pseudocystidia with yellowish content in KOH.

Habitat: Near pathway in parkland, in disturbed, sandy soil near base of fallen *Pinus*.

The role of citizen science in mycology

This work adds to the growing body of evidence that field mycologists can undertake DNA-based

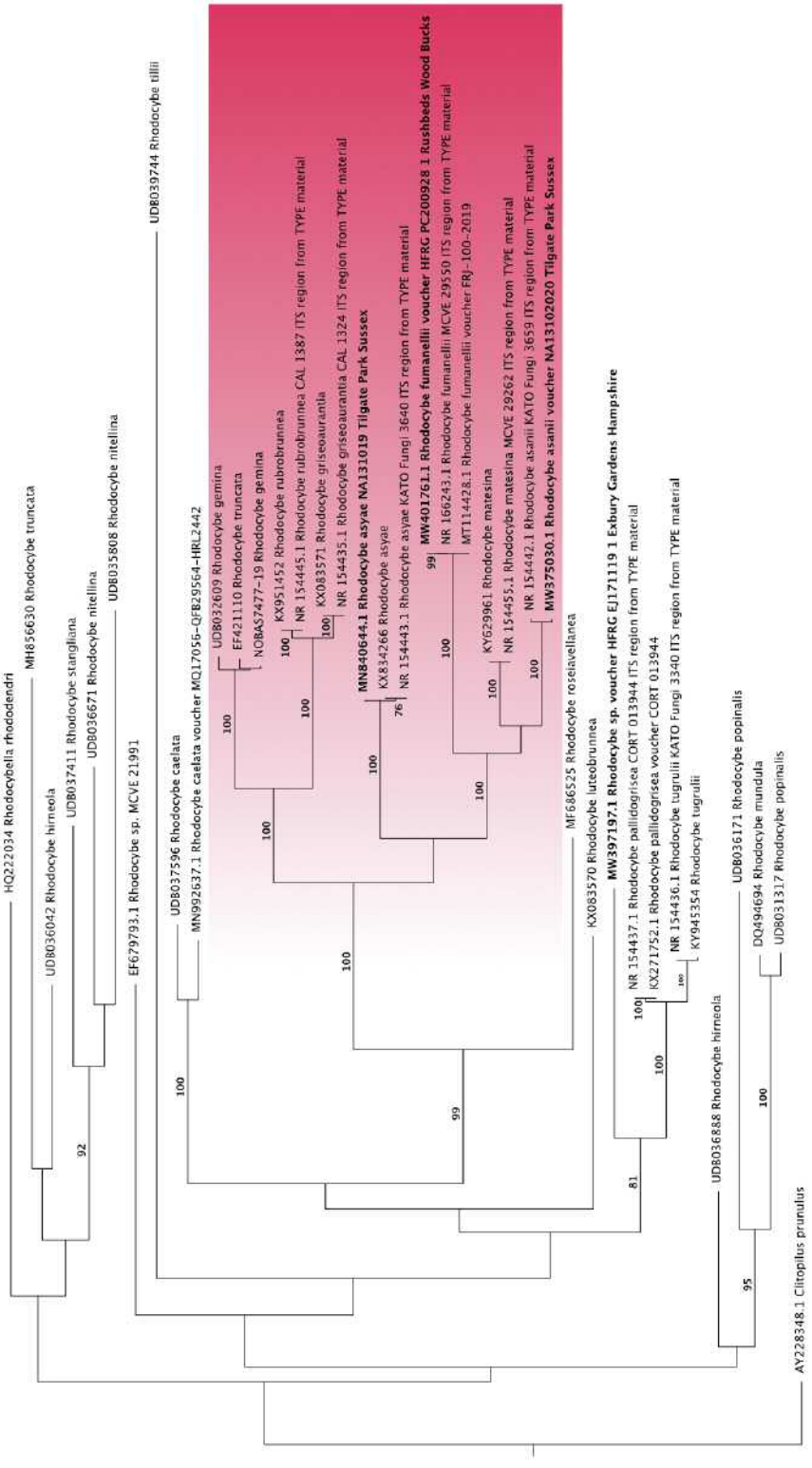

Figure 6: Maximum likelihood tree of *Rhodocybe*, with emphasis on sect. *Rufobrunnea* (shaded in red), rooted with *Clitopilus prunulus*. Our collections described in this article are displayed in bold.

identification of fungi themselves, and that this methodology can help make important contributions to the discovery and identification of fungal biodiversity, including that of novel species to Britain.

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References

Baroni, T. J., & Mattock, G. (2001). *Rhodocybe roseiavellanea* (Murr.) Singer, a new record for Britain and Europe. *Mycologist*, 4 (15): 151–152.

Bjorbækmo, M. F. M., Carlsen, T., Brysting, A *et al*. (2010). High diversity of root associated fungi in both alpine and arctic *Dryas octopetala*. *BMC Plant Biology*, 10 (1): 244.

Breitenbach J., Kränzlin, F. (1995). *Fungi of Switzerland*. Vol. 4. 4th ed. Lucerne, Switzerland: Verlag Mykologia.

Crous, P. W., Wingfield, M. J., Burgess, T. I., Hardy *et al*. (2017). Fungal Planet description sheets: 558–624. *Persoonia*: *Molecular Phylogeny and Evolution of Fungi*, 38: 240.

Harries, D. (2017). DNA and the field mycologist: part 1, *Field Mycology*, 18 (1): 20–23.

Harries, D. (2017). DNA and the field mycologist: part 2, *Field Mycology*, 18 (3): 92–96.

Henrici, A. (2020). What's been happening to *Rhodocybe*? *Field Mycology*: 21 (3), 108–111.

Humphrey, C. (1938). Notes on Some Basidiomycetes from the Orient. *Mycologia*, 30 (3): 327–335.

Katoh K, Misawa K, Kuma K, Miyata T (2002). MAFFT: A novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res*. 30: 3059–3066.

Kluting, K.L., Baroni, T.J. & Bergemann, S.E. (2013). Toward a stable classification of genera within the *Entolomataceae*: a phylogenetic re-evaluation of the *Rhodocybe-Clitopilus* clade. *Mycologia*. 106 (6): 1127–1142.

Knudsen H, Vesterholt J (2008). *Funga Nordica. Agaricoid, Boletoid and Cyphelloid Genera*. Copenhagen, Denmark: Nordsvamp.

Larsson, A. (2014). AliView: a fast and lightweight alignment viewer and editor for large data sets. *Bioinformatics* 30(22): 3276–3278.

Murrill, W. A. (1938). New Florida Agarics. *Mycologia* 30(4): 367.

Pearson, A. A. (1952). New records and observations. V. *Trans Brit. Mycol.Soc*, 35(2), 97–122.

Price, M. N., Dehal, P. S., & Arkin, A. P. (2010). FastTree 2–approximately maximum-likelihood trees for large alignments. *PloS one*, 5 (3).

Sesli, E & Vizzini, A. (2016). Two new *Rhodocybe* species (sect. *Rufobrunnea*, *Entolomataceae*) from the East Black Sea coast of Turkey. *Turkish Journal of Botany*, 41 (2): 200–210.

Vizzini A, Baroni TJ, Sesli E, Antonín V, Saar, I. (2016). *Rhodocybe tugrulii* (*Agaricales*, *Entolomataceae*), a new species from Turkey and Estonia based on morphological and molecular data, and a new combination in *Clitocella* (*Entolomataceae*). *Phytotaxa* 267: 1–15.

Vizzini, A., Ferrari, R. J., Ercole, E., & Fellin, A. (2018). A new species of *Rhodocybe* sect. *Rufobrunnea* (*Entolomataceae*, *Agaricales*) from Italy. *MycoKeys*, (36): 21.

Yang, X., Tyler, B. M., & Hong, C. (2017). An expanded phylogeny for the genus *Phytophthora*. *IMA fungus*, 8(2): 355.

Zou, Y., Mason, M. G., Wang, Y *et al*. (2017). Nucleic acid purification from plants, animals and microbes in under 30 seconds. *PLoS biology*, 15 (11).