Calocybella, a new genus for Rugosomyces pudicus (Agaricales, Lyophyllaceae) and emendation of the genus Gerhardtia

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Abstract: Calocybella is a new genus established to accommodate Rugosomyces pudicus. Phylogenetic analyses based on a LSU-ITS sequence dataset place Calocybella sister to Gerhardtia from which it differs morphologically in the presence of clamp-connections and reddening context. The genus Gerhardtia is emended to also include taxa with smooth spores. According to our morphological analysis of voucher material, Calocybe juniccola s. auct. is shown to be Calocybella pudica.

Key words: Agaricomycetes
Calocybe
Lyophyllaceae
Lyophyllum
tricholomatoid clade
LSU and ITS sequences
taxonomy

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INTRODUCTION

The generic name Rugosomyces, typified by Agaricus orychinus, was established by Raithelhuber (1979) for the lyophyllloid species (taxa with siderophilous basidia) previously placed in Calocybe with a collybioid habit, bright colourations (vacular pigment) and a pileipellis consisting of inflated, pseudoparenchymatic elements. Later, the circumscription of the genus was emended and broadened by Bon (1991a) to include, besides the taxa with a more or less subcellular pileipellis (sect. Rugosomyces), also species with a trichodermic pileipellis formed by short, catenulate elements (sect. Carnioviolacei), mixed parietal and intracellular pigments and distinct from Calocybe which was restricted to the species with paler colorations, a cutis-like pileipellis and a tricholomatoid habit. A few years later, the genus Rugosomyces was monographed by Bon (1999) and accepted by Kalameres (1995, 2004, 2012a) and Horak (2005). Phylogenetic analyses of Lyophyllaceae (Hofstetter et al., 2002, 2014) based on nuclear and mitochondrial rDNA sequences showed that the generic concept based only on morphological characters (Singer 1986, Bon 1999, Consiglio & Contu 2002, Kalameres 2004, 2012a–c) was artificial and should be re-framed; in particular, Rugosomyces species form a single clade together with species of Calocybe. Rugosomyces pudicus, a striking species of Rugosomyces sect. Rugosomyces, was not included in the molecular work by Hofstetter et al. (2002, 2014).

It was described by Contu & Bon (2000) on the basis of a collection from Sardinia (Italy) and characterized by a Collybia- or Callistosporium-like habit, the context turning blood-red on cutting or bruising, and red-violaceous after applying a drop of NH₃ or KOH, and verruculose spores. Since these features appeared aberrant within Rugosomyces, they established the new subsect. Rubescentes of sect. Rugosomyces for it. As this puzzling taxon combines features of several genera within Lyophyllaceae, the taxonomic position of this species has been greatly debated and was far from clear. Contu & Ortega (2001) provided SEM micrographs of the spores showing evident Rhodocybe-like verruculose ornamentation, and elevated subsect. Rubescentes to the rank of section. They recognized an affinity of the species with taxa placed in the genus Gerhardtia, which, however, are devoid of clamp-connections. Contu & Consiglio (2004) recombined Rugosomyces pudicus into Lyophyllum which they employed in a broad sense to include Calocybe. Arnoldo (2006), following the statements of Hofstetter et al. (2002) and Moncalvo et al. (2002) based on molecular markers, did not recognize Rugosomyces as independent from Calocybe and recombined R. pudicus into Calocybe.

Picillo & Contu (2009) reported R. pudicus also from a littoral site in Latium (Sabaudia), and adopted the concept of Arnoldo (2006), highlighting affinities with Calocybe and not with Tephrocybe, due to the vascular pigment. Finally, Vizzini et al. (in Vizzini 2014) combined R. pudicus into Gerhardtia because of the spore ornamentation.

As this rare enigmatic species had not been studied molecularly so far, the aim of this paper was to determine its phylogenetic position within Lyophyllaceae based on LSU and ITS rDNA analyses as well as to fully describe and illustrate it on the basis of recent collections.
MATERIALS AND METHODS

Morphology

All the collections studied were photographed in situ, using a Nikon D90 digital camera, with a tripod and in natural light. Macromorphological features were described from fresh specimens. The microscopic structures were examined in both fresh and dried material, in different mountants: water, L4 (7.2 g KOH, 160 mL glycerine, 840 mL dH2O, 7.6 g NaCl and 5 mL Invadin (Ciba-Geigy), Clémencéon 1972), Melzer’s reagent, Congo red, and Cotton blue. Cotton blue was utilized to highlight the siderosphoral granulation in the basidium, following Baroni (1981). Dried fragments were rehydrated in water and mounted in L4. All microscopic measurements were carried out under oil immersion at ×1000 with a Zeiss Axioscope 40.

Spore measurements were made by photographing, from time to time, all the spores (taken from the hymenophore of mature specimens) occurring in the visual field of the microscope using Mycomètre software (Fannechère 2011). Spore dimensions excluded the hilar appendix and the ornamentation, and are given as: (minimum–) average minus standard deviation – average plus standard deviation (–maximum) of length × (minimum–) average minus standard deviation – average plus standard deviation (–maximum) of width; Q = (minimum–) average minus standard deviation – average plus standard deviation (–maximum) of ratio length/width; Q_m = average ± standard deviation of ratio length/width; V = (minimum–) average minus standard deviation – average plus standard deviation (–maximum) of the volume [μm³]; V_m = average ± standard deviation of the volume (in μm³). The approximate spore volume was calculated as that of an ellipsoid (Gross 1972, Meerts 1999). The notation ‘n/m/p’ indicates that measurements were made on ‘n’ randomly selected spores from ‘m’ basidiomes of ‘p’ collections. The width of the basidium was measured at the widest part, and the length was measured from the apex (stereigmata excluded) to the basal septum. ‘i’ = number of lamellae between each pair of lamellae.

Microscopic pictures were taken on a Nikon Coolpix 4500 digital camera connected to a Zeiss Axioscope 40 microscope with both interferential contrast and phase-contrast optics. Scanning electron micrographs were made under a Zeiss DSM 950 SEM following Moreno et al. (1995).

Colour terms in capital letters (e.g., Peach Red, Plate I) are those of Ridgway (1912).

DNA extraction, PCR amplification, and DNA sequencing

Genomic DNA was isolated from 10 mg of six dried voucher specimens (AMB 15993–AMB 15997 and LIP-MB 991027), by using the DNeasy Plant Mini Kit (Qiagen, Milan) according to the manufacturer’s instructions. Primers LR0R/LR8 (Vilgalys & Hester 1990, Vilgalys lab. http://www.botany.duke.edu/fungi/mycolab) were used for the LSU rDNA amplification and universal primers ITS1/ITS4 for the ITS region amplification (White et al. 1990, Gardes & Bruns 1993). Amplification reactions were performed in a PE9700 thermal cycler (Perkin-Elmer, Applied Biosystems, Norwalk) in 25 μL reaction mixtures using the following final concentrations or total amounts: 5 ng DNA, 1 × PCR buffer (20 mM Tris/HCl pH 8.4, 50 mM KCl), 1 mM of each primer, 2.5 mM MgCl2, 0.25 mM of each dNTP, 0.5 unit of Taq polymerase (Promega, Madison). The PCR program was as follows: 3 min at 95 °C for 1 cycle; 30 s at 94 °C, 45 s at 50 °C, 2 min at 72 °C for 35 cycles, 10 min at 72 °C for 1 cycle. PCR products were resolved on a 1 % agarose gel and visualized by staining with ethidium bromide. The PCR products were purified with the AMPure XP kit (Beckman Coulter, Pasadena) and sequenced by MACROGEN (Seoul). The sequences were submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and their accession numbers are reported in Table 1 and Figs 1–2.

Sequence alignment and phylogenetic analysis

The sequences obtained in this study were checked and assembled using Geneious v. 5.3 (Drummond et al. 2010) and compared to those available in GenBank using the Blastn algorithm. Based on the BLASTn results (sequences were selected based on the greatest similarity) and outcomes of recent phylogenetic studies focused on Lyophyllaceae (Hofstetter et al. 2002, 2014), sequences were retrieved from GenBank and UNITe (http://unite.ut.ee/) databases for the comparative phylogenetic analysis. Alignments were generated for each single LSU and ITS dataset using MAFFT (Katoh et al. 2002) with default conditions for gap openings and gap extension penalties. The two alignments were then imported into MEGA v. 5.0 (Tamura et al. 2011) for manual adjustment. The influence of ambiguously aligned sites in the ITS alignment was tested by conducting a neighbor-joining (NJ) analysis in MEGA 5 (2000 bootstrap iterations) and comparing it with a similar analysis using a conservative alignment obtained with GBLOCKS 0.91b (Castsensana 2000) through its online server using default settings. ITS alignment was partitioned into ITS1, 5.8S and ITS2 regions.

Table 1. Samples sequenced for the present study.

<table>
<thead>
<tr>
<th>Species</th>
<th>GenBank accession no.</th>
<th>Source and country</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calocybella pudica</td>
<td>KP858005</td>
<td>AMB 15994, Lido di Ostia (RM), Latium, Italy</td>
</tr>
<tr>
<td>Calocybella pudica</td>
<td>KP858006</td>
<td>AMB 15995, Sabaudia (LT), Latium, Italy</td>
</tr>
<tr>
<td>Calocybella pudica</td>
<td>KP858007</td>
<td>AMB 15996, Bassa del Bardello (RA), Emilia-Romagna, Italy</td>
</tr>
<tr>
<td>Calocybella pudica</td>
<td>KP858008</td>
<td>AMB 15997, Bassa del Bardello (RA), Emilia-Romagna, Italy</td>
</tr>
<tr>
<td>Gerhardtia borealis</td>
<td>KP858009</td>
<td>AMB 15993, Castelir, Bellamonte (TN), Trentino-Alto Adige, Italy</td>
</tr>
</tbody>
</table>
The best-fit models were estimated by the Bayesian phylogenetic analysis based on selected ITS sequences of Lyophyllaceae, with Ossicaulis lachnopus and O. lignatilis as outgroup taxa. EPP values (in bold) ≥ 0.7 and ML values ≥ 60% are shown on the branches. Thickened branches indicate Bayesian posterior probability > 0.95 and ML bootstrap support > 70%. For each sequenced taxon the GenBank/GenBank number is given. Newly sequenced collections are in bold.

Fig. 2. Bayesian phylogenetic analysis of selected ITS sequences of Lyophyllaceae, with Ossicaulis lachnopus and O. lignatilis as outgroup taxa. EPP values (in bold) ≥ 0.7 and ML values ≥ 60% are shown on the branches. Thickened branches indicate Bayesian posterior probability > 0.95 and ML bootstrap support > 70%. For each sequenced taxon the GenBank/GenBank number is given. Newly sequenced collections are in bold.
(HE649955) and O. lignatilis (HE649951) in the ITS analysis (Figs 1–2).

Bayesian Inference and ML inferences were performed online using the CIPRES Science Gateway website (Miller et al. 2010) and both methods were implemented as single software usage. BI phylogeny using Monte Carlo Markov Chains (MCMC) was carried out with MrBayes v. 3.2.2 (Ronquist et al. 2012). Four incrementally heated simultaneous MCMC were run over 10 M generations. Trees were sampled every 1000 generations resulting in an overall sampling of 10,001 trees. The first 2500 trees were discarded as burn-in (25 %).

For the remaining trees, a majority rule consensus tree showing all compatible partitions was computed to obtain estimates for Bayesian Posterior Probabilities (BPP). Branch lengths were estimated as mean values over the sampled trees. ML estimation was performed through RAxML v. 7.0.4 (Stamatakis 2006) with 1000 bootstrap replicates (Felsenstein 1985) using the GTRGAMMA algorithm to perform a tree inference and search for a good topology. Support values from bootstrapping runs (MLB) were mapped on the globally best tree using the ‘f a’ option of RAxML and -x 12345 as a random seed to invoke the novel rapid bootstrapping algorithm. Only BPP values over 0.70 and MLB values over 50 % are reported in the resulting trees (Figs 1–2).

RESULTS

Amplification and sequencing of the LSU and ITS rDNA regions were successful for all specimens selected for molecular study, with the exception of LIP-MB 991027, the isotype of Rugosomyces pudicus, which was in a poor state of conservation and unsuitable for DNA extraction. The PCR product was 864–907 bp (LSU) and 651–653 bp (ITS). The LSU data matrix comprised 81 sequences (including 76 from GenBank). This dataset was 904 bp long and contained 289 (31.9 %) variable sites. Of these, 185 (64.0 %) sites were parsimony informative. The ITS data matrix comprised 35 sequences (including 25 from GenBank and 5 from UNITE). This dataset was 686 bp long and contained 369 (53.8 %) variable sites. Of these, 307 (83.2 %) were parsimony informative.

Both Bayesian and Maximum likelihood analyses produced the same topology; therefore, only the sampled trees with both BPP and MLB values are shown (Figs 1–2). In both the LSU and ITS sequence analysis, the sequences of Calocybe pudica clustered in a well-supported clade (BPP = 1, MLB = 100), sister (BP = 1, MLB = 99, in the LSU analysis; BP = 1, MLB = 94 in the ITS analysis) to a clade consisting of Gerhardtia sequences (BP = 1, MLB = 99, in the LSU analysis; BP = 1, MLB = 100 in the ITS analysis). Calocybe pudica plus the Gerhardtia clade formed a small sister clade to species of Mychromellla (viz. M. boudieri and M. inolens), a genus recently segregated from Tephrocybe (Hofstetter et al. 2014).

TAXONOMY

Calocybe Vizzini, Consiglio & Setti, gen. nov. MycoBank MB811739

Etymology: Calocybe = a small Calocybe, with reference to the habit shared with some species of that genus and the size of the basidiomes.


Diagnosis: The genus is distinguished from Gerhardtia by the context turning red on bruising and violaceous-red in alkaline solutions, the presence of clamp-connections and different ITS and LSU sequences.

Type species: Rugosomyces pudicus Bon & Contu 2000.

Calocybe pudica (Bon & Contu) Vizzini, Consiglio & Setti, comb. nov. MycoBank MB811740 (Figs 3–4)


Gerhardtia pudica (Bon & Contu) Vizzini et al., Index Fungorum 155: 1 (2014).


Calocybe juncicola (R. Heim) Singer, Ann. Mycol. 41: 109 (1943); nom. inval. (Art. 35.1).


Lyophyllum juncicola (R. Heim) Kühner & Romagn., Fl. Champ. Sup.: 162 (1953); nom. inval. (Art. 41.5).


Description: Pileus 5–25 mm diam, hemispherical-campanulate, then plano-convex, occasionally umbonate, non-hygrophanous, dry, smooth, orange to brownish-orange (Peach Red, Plate I; Bittersweet Orange, Grenadine Red, Mars Orange, Orange Rufous, Plate II; Capucine Orange, Mikado Orange, Cadmium Orange, Plate III; Apricot Buff, Rufous, Apricot Orange, Plate XIV; Zinc Orange, Ochraceous Orange, Tawny, Plate XV; Orange-Cinnamon, Plate XXIX), sometimes paler (Antimony Yellow, Yellow Ocher, Plate XV) over the margin. Lamellae medium crowded, adnate-smarginate, lamellulae 1 = (0) 1–2 (3), bright yellow (Pale Orange-Yellow, Light Orange-Yellow, Plate III) or ochraceous yellow (Buff-Yellow, Apricot Yellow, Plate IV); edge paler. Stipe 25–60 × 2–8 mm, cylindrical, sometimes tapered at the base, firm, pale yellow (Massicot Yellow, Naphthalene Yellow, Plate XVI; Pale Chalcedony Yellow, Plate XVIII) or brownish yellow (Cream-Buff, Chamois, Plate XXX), finely white-pruinose, fibrillose-striate; base subtended by yellow rhizoids. Context
firm, elastic, yellowish, more or less rapidly changing to red on bruising or when exposed and to violaceous-red after applying a drop of 10% NH₃ or 5% KOH; odour mealy, taste mealy then slightly astringent.

Spores [163, 5, 5] (3.8–) 4.9–6.0 (–6.7) × (2.8–) 3.0–3.7 (4.3) μm, Q = (1.3–) 1.46–1.80 (–2.1), Qₘ = 1.63 ± 0.17, V = (16.7–) 23.7–40.6 (–61.4) μm³, Vᵅ = 32.2 ± 8.4 μm³, ellipsoid to elongate-ellipsoid to oblong, axially or adaxially often flattened, even sub-lacrymoid in front view, hilar appendix rather long and prominent, content granular or mono- to multi-guttulate, inamyloid, cyanophilous, smooth to verrucose. Basidia 25–33 × 6.9–7.9 μm, four-spored, rarely two-spored, clavate, occasionally with pre- apical or central constriction, siderophilous (with internal siderophilous/cyanophilous granules); sterigmata up to 6 μm long. Hymenophoral trama regular to subregular, made up of hyphae 3–10 μm wide, becoming red in L4 and yellow in Melzer’s. Subhymenium constituted by short, septate elements, 2–4 μm wide. Cheilocystidia none. It was observed the occurrence of numerous missshapen basidiobolous or basidia, exhibiting apical extraflaxes (to 20 × 1.5–2 μm), constrictions, or the upper part may be swollen or subcapitate. Pleurocystidia none. Pileipellis: suprapellis a cutis of subparallel, variously interwoven hyphae, 2–8 μm wide, with slightly gelatinized outermost layer, yellow in Melzer’s, smooth, cylindrical, with smooth, undifferentiated to slightly enlarged terminal elements, up to 6 μm wide; pigment intracellular, of more or less dark yellow colour, some with a very fine epiparietal pigment; subpellis consisting of elongate hyphae, 4–12 μm wide; trama hyphae cylindrical, to 12 μm wide. Stipe hyphae cylindrical, 8–14 μm wide within the stipe; cortical hyphae 2.5–5 μm wide, smooth, with smooth, undifferentiated to slightly enlarged, round-tipped terminal elements. Clamp-connections present throughout.

Isotype of Rugosomyces pudicus (LIP-MB 991027; Fig. 4 E–F): Spores [64, 2, 1] (4.6–) 5.1–6.5 (–8.7) × (2.8–) 3.0–3.5 (–4.6) μm, Q = (1.50–) 1.6–2.0 (–2.3), Qᵅ = 1.82 ± 0.21, V = (21.0–) 22.6–41.5 (–60.3) μm³, Vᵅ = 32.0 ± 9.4 μm³, ellipsoid to elongate-ellipsoid, to oblong, with opaque, granular content, non-amyloid, cyanophilous, rugose-bumpy. Basidia all collapsed, basidioles clavate. Hymenophoral trama regular to subregular, composed of hyphae up to 23 μm wide, hyaline in L4 and yellow in Melzer’s. Cheilocystidia none. Pleurocystidia none. Pileipellis: suprapellis a cutis of smooth, subparallel to variously interwoven, slightly gelatinized, cylindrical hyphae, 4–7 μm wide, yellow in Melzer’s, with undifferentiated, occasionally antically oriented terminal elements; pigment intracellular, of a more or less dark yellow colour; trama hyphae shortly cylindrical, to 17 μm wide. Clamp-connections present everywhere.

Habitat and distribution: Gregarious, in small groups, usually fasciculate, in grassy clearings with Juncus sp.; so far known from Italy, France, and Spain.

G. Consiglio & A. Zucherelli (AMB 15998); ibidem, some small groups, 19 Nov. 2014, G. Consiglio & A. Zucherelli (AMB 15999); Latium, Lido di Ostia (RM), two specimens in a grassy clearing of the backdune, 2 Dec. 2001, G. Consiglio et al. (AMB 15994); Sabaudia (LT), numerous specimens in a grassy clearing of the backdune, 22 Nov. 2008, G. Consiglio et al. (AMB 15995).– France: Prabert, Coldes Ayes, in groups or subcaespitose, in a grassy clearing near Picea sp. and Abies sp., 24 Sept. 1980, J. Vast (LIP No. 80092406, sub C. cerina cf. var. juncicola, identified M. Bon).

Additional material examined: Gerhardtia borealis:– Italy: Trentino-Alto Adige, Castelir (Bellamonte, TN), two specimens under Picea abies, 19 Aug. 1998, G. Consiglio (AMB 15993).– Gerhardtia pseudosaponacea:– New Zealand: Southland; Longwood Road, Martins Hut Track, in groups under Nothofagus menziesii, 8 May 2012, M. Crowe (PDD96650 – holotype).


Type species: Gerhardtia borealis (Fr.) Contu & Ortega 2002 (syn. G. incarnatobrunnea (Ew. Gerhardt) Bon 1994).

Emended diagnosis: Genus of Lyophylloaceae with smooth or verruculose spores and without clamp-connections. Pileipellis organized as a cutis, trichoderm or hymenidem (see Clémençon 2004 for diagrams and definitions of tissue types).

DISCUSSION

Phylogeny and delimitation of Calocybeella versus Gerhardtia

Gerhardt (1982) described the new species Lyophyllum incarnatobrunneum as characterized by the absence of clamp-connections, and placed it in the new subgenus Lyophyllopis. Bon (1994) raised subgenus Lyophyllopis to the generic level under the new generic name Gerhardtia (a name not preoccupied by Lyophyllopis Sathe & J.T. Daniel 1981), highlighting also the presence of minutely verruculose spores as the characterizing feature of the genus. He recognized two species within Gerhardtia, G. borealis and G. piperata.

Contu & Consiglio (2004) included four additional species in the genus, G. highlandensis, G. leucopaxilloides, G. marasmioides, and G. suburens. Subsequently, a further new species, G. pseudosaponacea has been described from New Zealand (Cooper 2014), with clampless hypheal but smooth spores. The pileipellis was originally described as a simple cutis, but a re-examination of the holotype (Fig. 5) revealed the presence of inflated cells (Fig. 5D); the spores were confirmed as smooth by both phase-contrast and bright field microscopy (Fig. 5 E–F).

From a molecular point of view, Gerhardtia is still poorly studied. No species of Gerhardtia were included in the molecular study of Lyophylloaceae by Hofstetter et al. (2002). The analyses by Frøslev et al. (2003), Saar et al. (2009), and Cooper (2014), focused on Termitomyces, Cystoderma s. lat. and lyophylloid taxa from New Zealand, respectively, and showed that Gerhardtia belonged to Lyophylloaceae. In Hofstetter et al. (2014), the one Gerhardtia included in the analysis, Gerhardtia sp., clustered basally (but with low support) to Calocybe.

According to our LSU and ITS analyses (Figs 1–2), Gerhardtia is a strongly supported genus of Lyophylloaceae and includes species with clearly verruculose (G. borealis, G. highlandensis) or smooth (G. pseudosaponacea) spores. Consequently, the circumscription of the genus is emended here to also include species with smooth spores. The pileipellis also seems variously structured in the species of Gerhardtia, ranging from a cutis (G. highlandensis, Bigelow 1985, as Citocybe highlandensis) to a cutis/trichoder (G. borealis, Contu & Consiglio 2004) or a hymenidem (G. pseudosaponacea, Fig. 5D). This heterogeneity in the pileus covering is similar to that found in Calocybe s. lat. (Bon 1999, Consiglio & Contu 2002).

The phylogenetic position of the remaining morphologically circumscribed species of Gerhardtia (G. leucopaxilloides, G. marasmioides, and G. suburens, all with verruculose spores and a pileipellis as a cutis, Bigelow 1985) will have to be assessed on the basis of future molecular studies.

In our molecular analyses, Calocybeella appeared as a sister group to Gerhardtia (Figs 1–2) from which it differs in the context reddening on bruising or turning violaceous red in alkaline solutions, and the presence of clamp-connections. The phylogenetically related Mychorromella differs from Calocybeella in having mycenanoid basidiomes, striate and hygrophanous pilei, free to nearly free lamellae, brown-grey pigments, an unchanging context, and smooth spores (Hofstetter et al. 2014).

Calocybeella pudica and morphologically allied taxa

This species shares with members of the genus Calocybe the general habit and presence of colourful mainly vacuolar pigments. But it exhibits a suite of peculiar characters that distinguish it from both C. chrysonerion and C. naucoria, two species which otherwise are somewhat similar. First and foremost, the basidiomes of C. pudica redder more or less strongly and clearly when bruised or cut. The speed and intensity of the red colour change are not the same across collections. The collections from Latium (AMB 15994 and 15995) exhibited an immediate red colour change when touched, while in those from Emilia-Romagna (AMB 15996 to 15999), the reddening appeared only after sustained bruising. Such a peculiar chemical property is matched by the quick violet-red colour change of the outer surfaces when 5 % KOH or 10 % ammonia are applied, as well as all other tissues in mounts of dried material rehydrated with these solutions.

Moreover, Calocybeella pudica is characterized by ornamented spores, whereas all the Calocybe species (with the exception of C. gangraenosa which has spores with verrucae dissolving in alkaline solutions) have smooth spores (Singer 1986, Kalamees 1995, 2004, 2012a, c, Bon 1999, Consiglio & Contu 2002). The spore ornamentation is not always easily seen, however, in light and bright field microscopy. Further, in some collections the ornamented spores are mixed with apparently smooth spores, so much
so that at first sight they can be mistaken as alien. In other mounts, the spores are practically all ornamented. It is most likely that, as in many other cases and in diverse genera, the prominence of the ornamentation depends on the stage of spore maturation. In mounts observed in interference and in phase contrast (Fig. 4C–F), the ornamentation is seen much more easily, and all the more so in SEM micrographs (Fig. 4A–B).

Finally, *C. pudica* has a pileipellis with a cutis structure similar to that of *G. borealis* and different from those, hymenidermic and trichodermic respectively, of the apparently closely allied *Calocybe naucoria* and *C. chrysenteron*.

Other tricholomatoid taxa with a violaceous red discoloration of tissues in alkaline solutions are *Callistodermatium* (Singer 1981, 1986) and *Callistosporium pinicola* (Arnolds 2006, Antonin et al. 2009, Aron 2014,

Halama & Rutkowski 2014). *Callistodermatium* differs from *Calocybe* in having a context not staining red on bruising, smooth spores (some with an ochraceous resinous incrustation covering the wall), non-siderophilous basidia, a bilateral hymenophoral trama of the *Phylloporus*-type, and cystidia on pileus, lamellassae and stipe (Singer 1981). *Callistosporium pinicola*, as all *Callistosporium* species, is distinguished by the smooth spores containing yellow pigmented bodies, non-siderophilous basidia, and absence of clamp-connections (Redhead 1982, Singer 1986, Bon 1991b, Arnolds 2006, Vesterholt & Holec 2012).

In the same biotope (Emilia-Romagna, Bassa del Bardello, RA, among debris of *Juncus* sp.) where the collections of *C. pudica* were made (AMB 15996 to 15999), Hauskncht & Zuccherelli (1994) made a few collections they determined as *Calocybe juncicola*. That species was originally published on the basis of a collection from Girona (Spain) growing on debris of *Juncus acutus*, as *Tricholoma chrysenteron var. juncicola* (Heim et al. 1934) with a French description that was relatively detailed but with no indication of a type. Based on the protologue and the colour plate provided by Heim et al. (1934), the taxon resembles *C. pudica* quite well, even though the spores are described as smooth and the context as immutab. Subsequently, *Tricholoma chrysenteron var. juncicola* was transferred to *Calocybe* by Singer (1961), and then recombined by Moreno (Moreno et al. 1994) as *C. chrysenteron var. juncicola*. The species has not been recollected in the area of Girona, the type locality (Perez-de-Gregorio, in litt.) and the few subsequent records relate to the collection by Moreno et al. (1994), a French collection by Bon (see above, as *Calocybe cerina* cf. var. *juncicola*), and those from Emilia-Romagna by Hauskncht & Zuccherelli (1994). The species has recently been exhaustively described by Arnolds (2006) based on the same collection (WU 20752) as that studied by us (see Material examined). All the cited authors describe the spores as smooth. Moreno (Moreno et al. 1994) and Arnolds (2006) pointed out that the tissues change to violet-red in alkaline solution. According to our observations, the collections cited in Hauskncht & Zuccherelli (1994), Arnolds (2006), and the French collection by Bon (LIP 80092406) all have verruculose spores. We could not carry out a micromorphological analysis of the material named as *C. chrysenteron var. juncicola* (Moreno et al. 1994) since the relevant collection is no longer available (Moreno, pers. comm.) but the staining violet-red of the tissues in alkaline solution, the pileipellis structured as a cutis and growth near *Juncus maritimus* do not leave any doubt as to the identity of that collection with *C. pudica*. Consequently, we consider all these collections referred to as *Calocybe juncicola* to represent *Calocybe pudica*. As there appear to be no extant original specimens, we can only suspect, but not confirm, that *Tricholoma chrysenteron var. juncicola* in the original sense might be the same as *C. pudica*. In the absence of new collections of *Calocybe juncicola*, however, we prefer to treat that name as misapplied and of uncertain application, and to refer the above mentioned collections to *Calocybe pudica*.

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