doi:10.5598/imafungus.2015.06.01.01

IMA FUNGUS · 6(1): 1–11 (2015)

ARTICLE

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

Alfredo Vizzini[1], Giovanni Consiglio[2], Ledo Setti[3], and Enrico Ercole[1]

[1]Department of Life Sciences and Systems Biology, University of Torino, Viale P.A. Mattioli 25, I-10125 Torino, Italy; corresponding author e-mail: alfredo.vizzini@unito.it

[2]Via Ronzani 61, I-40033 Casalecchio di Reno (Bologna), Italy

[3]Via C. Pavese 1, I-46029 Suzzara (Mantova), Italy

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 juncicola* s. auct. is shown to be *Calocybella pudica*.

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

Article info: Submitted: 12 January 2015; Accepted: 10 March 2015; Published: 23 March 2015.

INTRODUCTION

The generic name *Rugosomyces*, typified by *Agaricus onychinus*, was established by Raithelhuber (1979) for the lyophylloid species (taxa with siderophilous basidia) previously placed in *Calocybe* with a collybioid habit, bright colourations (vacuolar 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. *Carneoviolacei*), 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 Kalamees (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, Kalamees 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 NH_3 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*. Arnolds (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 Arnolds (2006), highlighting affinities with *Calocybe* and not with *Tephrocybe*, due to the vacuolar 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.

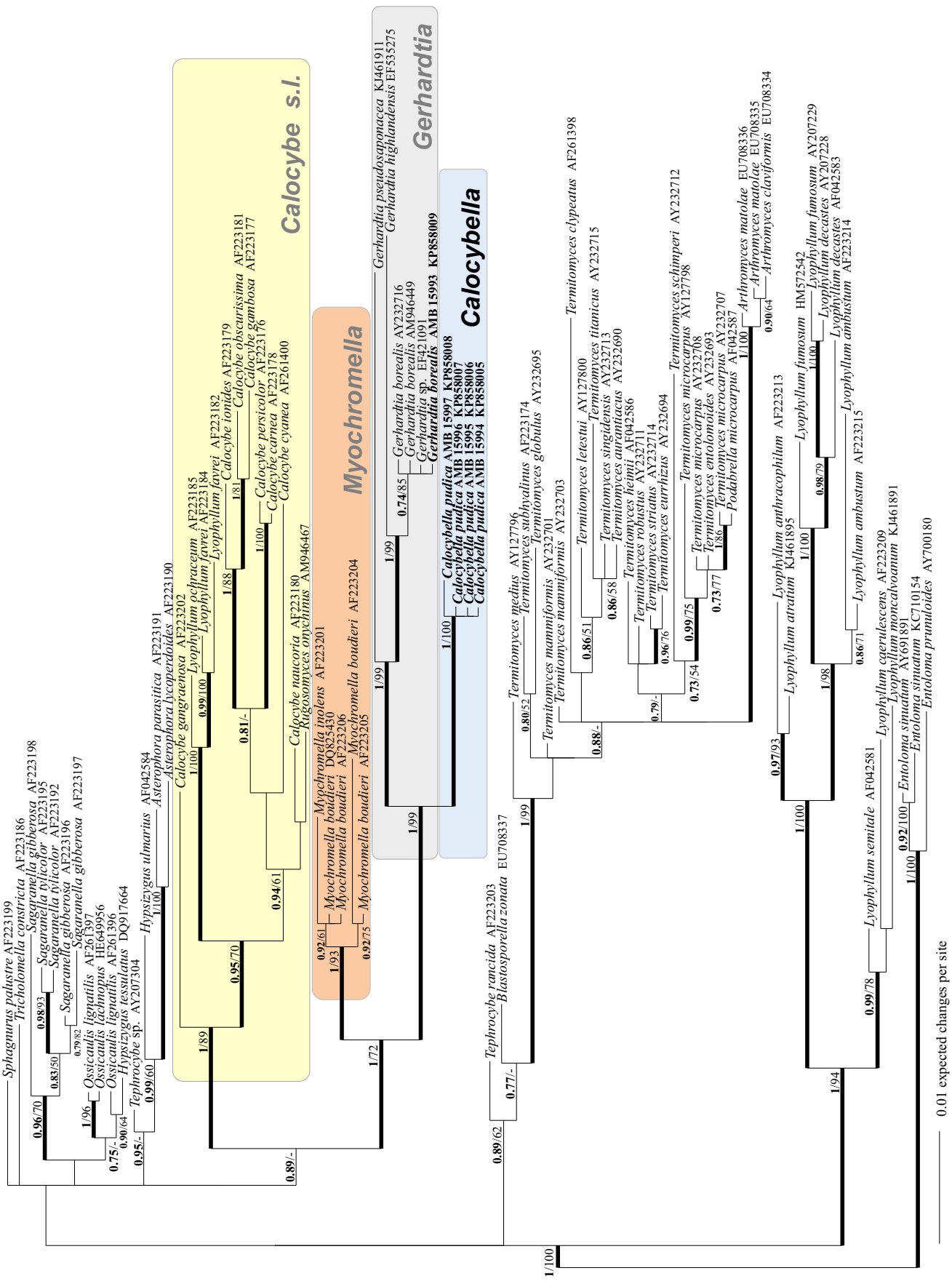

Fig. 1. Bayesian phylogenetic analysis based on the LSU sequences of *Lyophyllaceae*, with *Entoloma sinuatum* and *E. prunuloides* as outgroup taxa. BPP values (in bold) ≥ 0.7 and MLB values ≥ 50 % are shown on the branches. Thickened branches indicate Bayesian posterior probability > 0.95 and ML bootstrap support > 70 %. For each sequenced taxon the Genbank/UNITE number is given. Newly sequenced collections are in bold.

Table 1. Samples sequenced for the present study.

Species	GenBank accession no.		Source and country
	LSU	ITS	
Calocybella pudica	KP858005	KP858000	AMB 15994, Lido di Ostia (RM), Latium, Italy
Calocybella pudica	KP858006	KP858001	AMB 15995, Sabaudia (LT), Latium, Italy
Calocybella pudica	KP858007	KP858002	AMB 15996, Bassa del Bardello (RA), Emilia-Romagna, Italy
Calocybella pudica	KP858008	KP858003	AMB 15997, Bassa del Bardello (RA), Emilia-Romagna, Italy
Gerhardtia borealis	KP858009	KP858004	AMB 15993, Castelìr, Bellamonte (TN), Trentino-Alto Adige, Italy

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 dH_2O, 7.6 g NaCl and 5 mL Invadin (Ciba-Geigy), Clémençon 1972], Melzer's reagent, Congo red, and Cotton blue. Cotton blue was utilized to highlight the siderophilous granulation in the basidia, 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 [μm^3]; V_m = average ± standard deviation of the volume (in μm^3). 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 basidia was measured at the widest part, and the length was measured from the apex (sterigmata excluded) to the basal septum. 'l' = number of lamellulae 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/LR6 (Vilgalys & Hester 1990, Vilgalys lab. http://www.botany.duke.edu/fungi/mycolab) were used for the LSU rDNA amplification and universal primers ITS1F/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 mL 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), 1mM 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 (Castresana 2000) through its online server using default settings. ITS alignment was partitioned into ITS1, 5.8S and ITS2 regions.

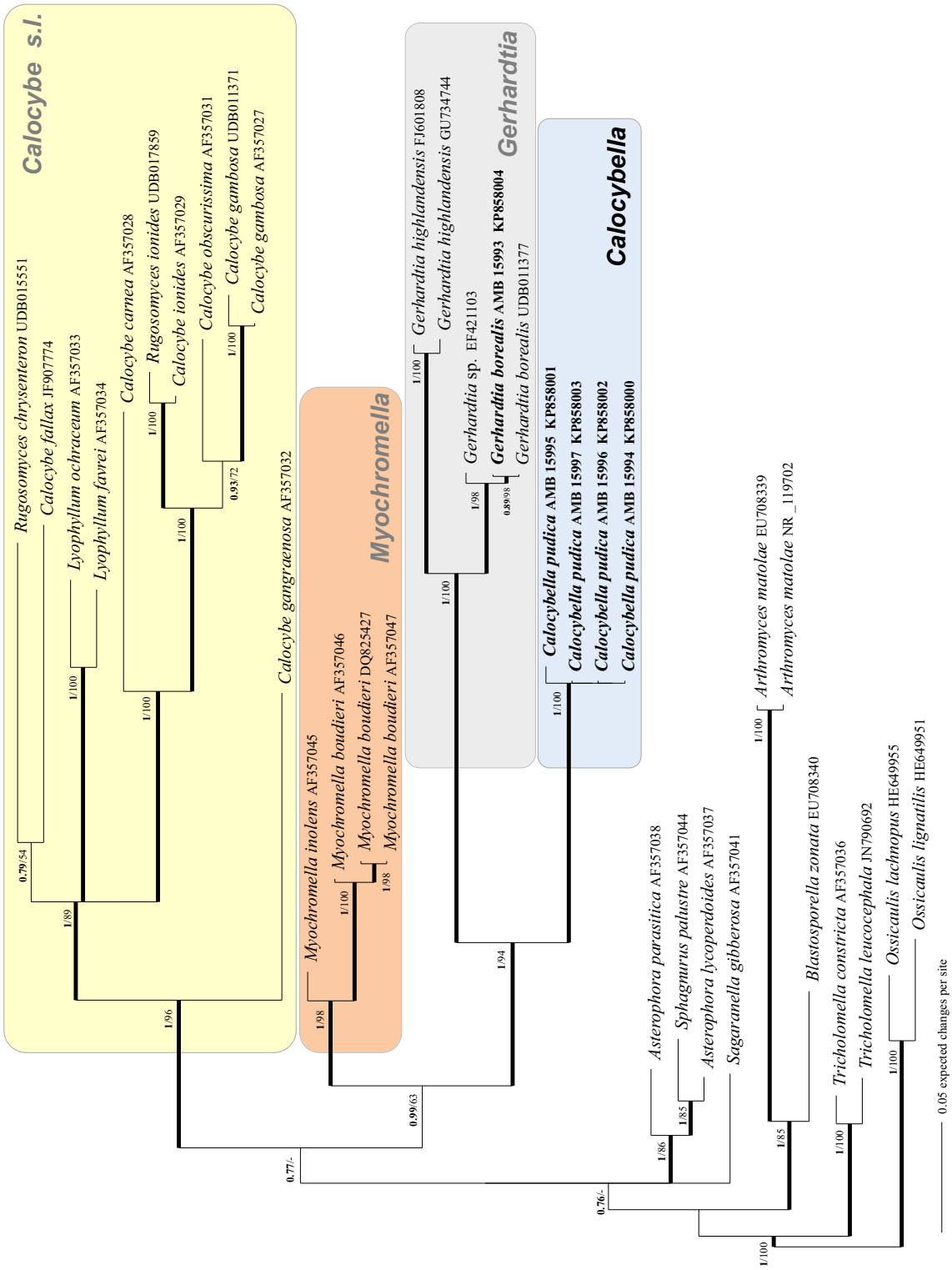

Fig. 2. Bayesian phylogenetic analysis based on selected ITS sequences of *Lyophyllaceae*, with *Ossicaulis lachnopus* and *O. lignatilis* as outgroup taxa. BPP values (in bold) ≥ 0.7 and MLB values ≥ 50 % are shown on the branches. Thickened branches indicate Bayesian posterior probability > 0.95 and ML bootstrap support > 70 %. For each sequenced taxon the Genbank/UNITE number is given. Newly sequenced collections are in bold.

The best-fit models were estimated by the Bayesian Information Criterion (BIC) using jModelTest v. 2.1.7 (Darriba *et al.* 2012) to provide a substitution model for each single alignment. GTR+G models were chosen for both the LSU and partitioned ITS alignments.

Phylogenetic analyses were performed using the Bayesian Inference (BI) and Maximum likelihood (ML) approaches. *Entoloma sinuatum* (AY691891 and KC710154) and *E. prunuloides* (AY700180) were chosen as outgroup taxa in the LSU analysis, and *Ossicaulis lachnopus*

(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 Bayesian trees with both BPP and MLB values are shown (Figs 1–2). In both the LSU and ITS sequence analysis, the sequences of *Calocybella 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). *Calocybella pudica* plus the *Gerhardtia* clade formed a small sister clade to species of *Myochromella* (*viz*. *M. boudieri* and *M. inolens*), a genus recently segregated from *Tephrocybe* (Hofstetter *et al*. 2014).

TAXONOMY

Calocybella Vizzini, Consiglio & Setti, **gen. nov.**
MycoBank MB811739

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

Synonym: *Rugosomyces* sect. *Rubescentes* Bon & Contu, *Doc. Mycol*. **29** (116): 36 (2000).

Diagnosis: The genus is distinguished from *Gerhardtia* Bon 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.

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

Basionym: *Rugosomyces pudicus* Bon & Contu, *Doc. Mycol*. **29** (116): 35 (2000).

Synonyms: *Lyophyllum pudicum* (Bon & Contu) Consiglio & Contu, *Micol. Veg. Medit*. **19**: 159 (2005) ["2004"].

Calocybe pudica (Bon & Contu) Arnolds, *Acta Mycol*. **41**: 38 (2006).

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

Misapplied names: *Tricholoma chrysenteron* var. *juncicola* R. Heim, *Treb. Mus. Ciènc. Nat. Barcelona, ser. Bot*. **15**: 101 (1934).

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

Calocybe juncicola (R. Heim) Singer, *Sydowia* **15**: 47 (1962) ["1961"].

Calocybe chrysenteron var. *juncicola* (R. Heim) G. Moreno, *Cryptog. Mycol*. **15**: 240 (1994).

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

Rugosomyces chrysenteron var. *juncicola* (R. Heim) Bon, *Doc. Mycol*. **21** (82): 65 (1991).

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 l = (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 XVII) or brownish yellow (Cream-Buff, Chamois, Plate XXX), finely white-pruinose, fibrillose-striate; base subtended by yellow rhizoids. *Context*

Fig. 3. *Calocybella pudica*. Basidiomes. **A. (**AMB 15995). **B.** (AMB 15997). **C.** (AMB 15998). **D.** (AMB 15999). Bars = 10 mm.

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 % NH_3 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), Q_m = 1.63 ± 0.17, V = (16.7–) 23.7–40.6 (–61.4) µm³, V_m = 32.2 ± 8.4 µm³, ellipsoid to elongate-ellipsoid to oblong, abaxially 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 misshapen basidioles or basidia, exhibiting apical extroflexions (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), Q_m = 1.82 ± 0.21, V = (21.0–) 22.6–41.5 (–60.3) µm³, V_m = 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 anticlinally 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.

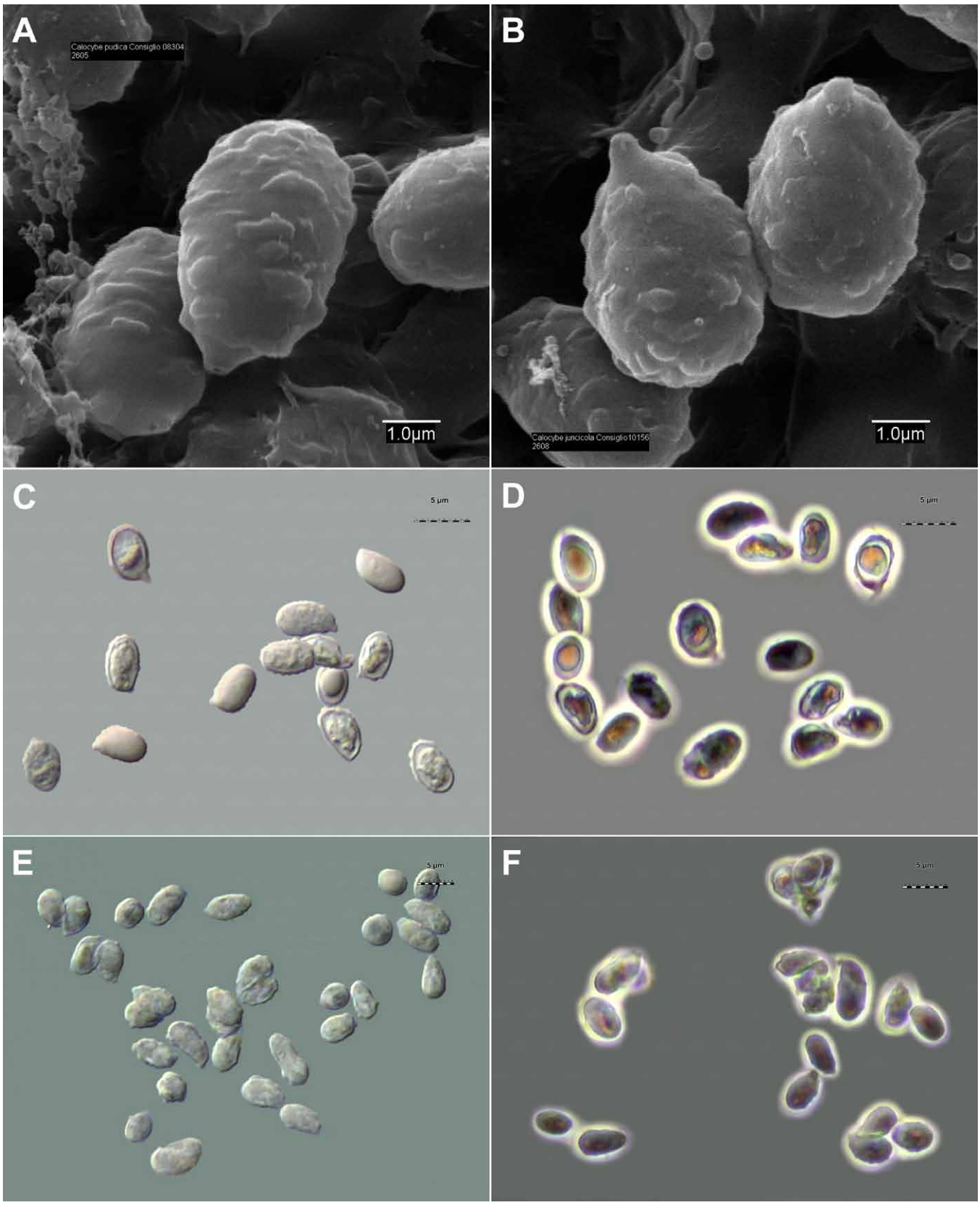

Fig. 4. *Calocybella pudica*. Spores. **A**, **C–D** (AMB 15995). **B** (AMB 15997). **E–F** (LIP-MB 991027 – isotype). Bars: A–B = 1 µm, C–F = 5 µm.

Material examined: *Calocybella pudica*. **– Italy**: *Sardinia*, Olbia (SS), "F. Noce Sport Club", in small groups, in moist places under poplars (*P. nigra*), on acid soil, 28 Oct. 1999, *M. Contu* (LIP-MB 991027 – isotype of *Rugosomyces pudicus*); *Emilia-Romagna*, Bassa del Bardello (RA), 9 Nov. 1992, *A. Zuccherelli* (WU 11229, sub *Calocybe juncicola*); *ibidem*, 6 Nov. 1994, *A. Hausknecht* (WU 13458, sub *C. juncicola*); *ibidem*, 6 Nov. 2000, *A. Hausknecht et al*. (WU 20752, sub *C. juncicola*, identified E. Arnolds); *ibidem*, numerous small groups, in a grassy clearing with *Juncus* sp., 18 Nov. 2010, *G. Consiglio et al.* (AMB 15996 and 15997); *ibidem*, some small groups, 30 Oct. 2013,

G. Consiglio & A. Zuccherelli (AMB 15998); *ibidem*, some small groups, 19 Nov. 2014, *G. Consiglio & A. Zuccherelli* (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*, Castelìr (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).

Gerhardtia Bon, *Doc. Mycol.* **24** (93): 66 (1994).

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

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

DISCUSSION

Phylogeny and delimitation of *Calocybella 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 *Lyophyllopsis*. Bon (1994) raised subgenus *Lyophyllopsis* to the generic level under the new generic name *Gerhardtia* (a name not preoccupied by *Lyophyllopsis* 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 hyphae 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 *Lyophyllaceae* 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 *Lyophyllaceae*. 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 *Lyophyllaceae* 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 *Clitocybe highlandensis*) to a cutis/trichoderm (*G. borealis*, Contu & Consiglio 2004) or a hymeniderm (*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, *Calocybella* 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 *Myochromella* differs from *Calocybella* in having mycenoid basidiomes, striate and hygrophanous pilei, free to nearly free lamellae, brown-grey pigments, an unchanging context, and smooth spores (Hofstetter *et al*. 2014).

Calocybella 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. chrysenteron* and *C. naucoria*, two species which otherwise are somewhat similar. First and foremost, the basidiomes of *C. pudica* redden 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 of all other tissues in mounts of dried material rehydrated with these solutions.

Moreover, *Calocybella 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

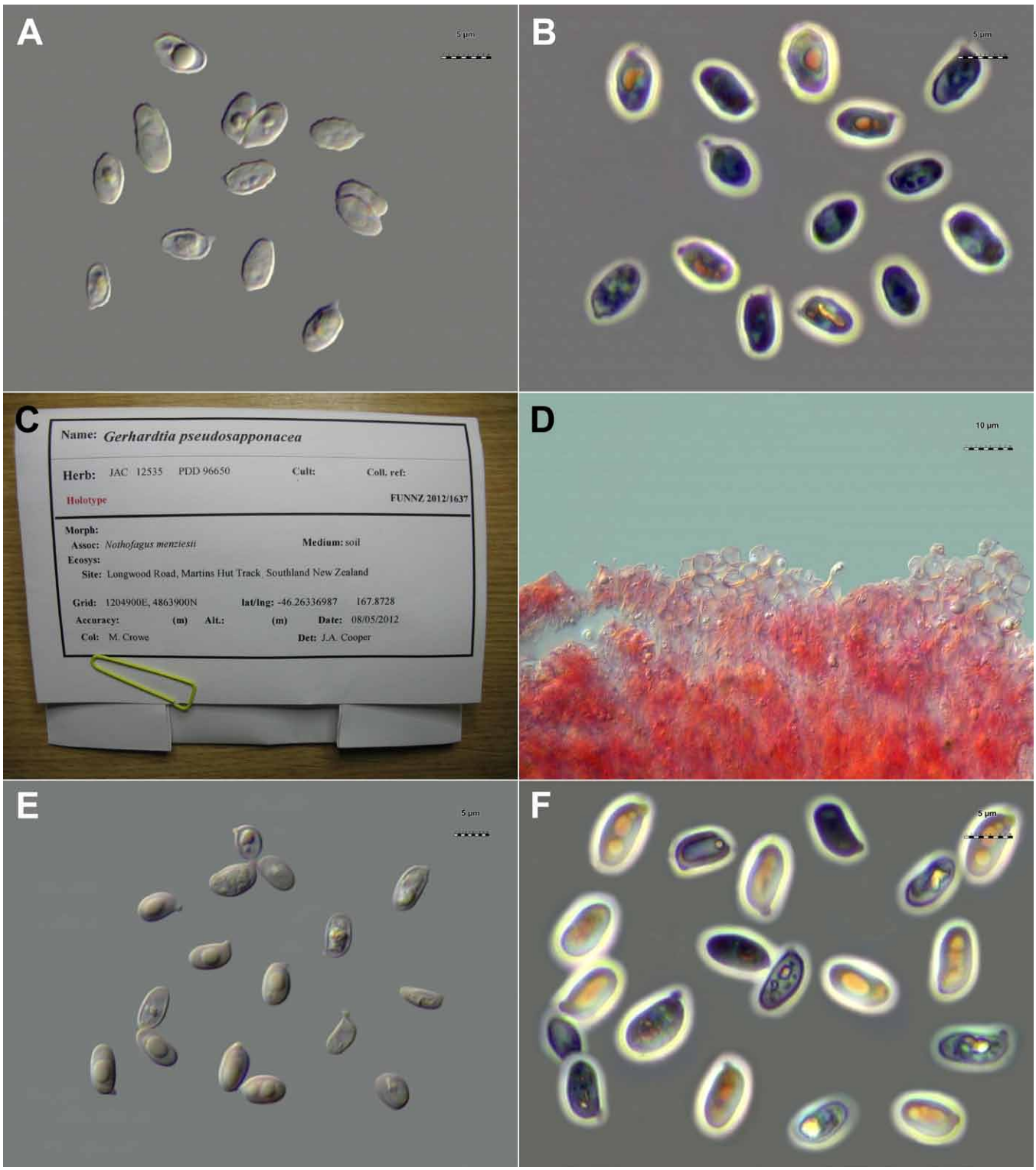

Fig. 5. *Gerhardtia* species. **A–B.** Spores of *G. borealis* (AMB 15993). **C–F.** Holotype of *G. pseudosaponacea* (PDD 96650): **C.** Label. **D.** Hymenidermic pileipellis (in Congo Red). **E–F.** Spores. Bars: A–B, E–F = 5 µm, D = 10 µm.

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, Antonín *et al*. 2009, Aron 2014,

Halama & Rutkowski 2014). *Callistodermatium* differs from *Calocybella* 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, lamellae 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), Hausknecht & 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 *at 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 immutable. 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 Hausknecht & 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 Hausknecht & 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 *Calocybella 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 *Calocybella pudica*.

ACKNOWLEDGMENTS

We express our gratitude to Gabriel Moreno Horcajada (University of Alcalà, Madrid), who provided us with the SEM photos of *Calocybella pudica*, Anton Hausknecht (Maissau,) and Mauro Marchetti (Pisa) for help and advice, Adler Zuccherelli (Ravenna) and Merrisiano Caldironi (Ravenna) for their assistance in fieldwork, and Edmondo Grilli (Pescara) for improving the English text.

REFERENCES

Antonín V, Beran M, Dvorák D, Holec J (2009) First records of *Callistosporium pinicola* in the Czech Republic and new findings on its ecology. *Czech Mycology* **61**: 1–12.

Arnolds E (2006) A confusing duo: *Calocybe cerina* and *Callistosporium pinicola. Acta Mycologica* **41**: 29–40.

Aron C (2014) *Callistosporium pinicola* on National Fungus Day at Treborth Botanic Gardens, Bangor. *Field Mycology* **15**: 49–50.

Baroni TJ (1981) The genus *Rhodocybe* Maire (*Agaricales*). *Beihefte zur Nova Hedwigia* **67**: 1–194.

Bigelow HE (1985) North American species of *Clitocybe*, part II. *Beihefte zur Nova Hedwigia* **81**: 281–471.

Bon M (1991a) Les genres *Echinoderma* (Locq. ex Bon) st. nov. et *Rugosomyces* Raithelhuber ss. lato. *Documents Mycologiques* **21**(82): 61–66.

Bon M (1991b) *Flore Mycologique d' Europe.* Vol. 2. *Les Tricholomes er resemblants*. [Documents Mycologiques, Mémoire hors-série no. 2.] Amiens: Centre régional de documentation pédagogique de l'académie d'Amiens.

Bon M (1994) Deux *Lyophylloideae* interessantes et le genre *Gerhardtia* st. et nom. nov. *Documents Mycologiques* **24**(93): 65–68.

Bon M (1999) *Flore Mycologique d' Europe* Vol. 5. *Les Collybio-marasmioïdes et ressemblants*. [Documents Mycologiques, Mémoire hors-série no. 5.] Amiens: Centre régional de documentation pédagogique de l'académie d'Amiens.

Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution* **17**: 540–552.

Clémençon H (1972) Zwei verbesserte Präparierlösungen für die microskopische Untersuchung von Pilze. *Zeitschrift für Pilzkunde* **38**: 49–53.

Clémençon H (2004) *Cytology and Plectology of the Hymenomycetes*. [Bibliotheca Mycologica vol. 199.] Berlin: J. Cramer.

Consiglio G, Contu M (2002) Il genere *Lyophyllum* P. Karst. emend. Kühner, in Italia. *Rivista di Micologia* **45**: 99–181.

Contu M, Bon M (2000) Une nouvelle espèce de *Rugosomyces* rougissant. *Documents mycologiques* **29**(116): 35–36.

Contu M, Consiglio G (2004) Il genere *Gerhardtia* (*Lyophyllaceae*) in Europa, con osservazioni sulle rimanenti specie conosciute. *Micologia e Vegetazione Mediterranea* **19**: 151–162.

Contu M, Ortega A (2001) Studi sulle *Lyophyllaceae* della Sardegna. V. Morfologia sporale di *Rugosomyces pudicus* ed implicazioni sulla sua posizione sistematica. *Boletín de la Sociedad Micológica de Madrid* **26**: 171–176.

Cooper JA (2014) New species and combinations of some New Zealand agarics belonging to *Clitopilus*, *Lyophyllum*, *Gerhardtia*, *Clitocybe*, *Hydnangium*, *Mycena*, *Rhodocollybia* and *Gerronema*. *Mycosphere* **5**: 263–288.

Darriba D, Taboada GL, Doallo R, Posada D (2012) "jModelTest 2: more models, new heuristics and parallel computing". *Nature Methods* **9**(8): 772.

Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, *et al*. (2010) Geneious v5.3. Available from http://www.geneious.com/

Fannechére G (2011) *Mycomètre, logiciel d'aide à la mesure et de traitement statistique*. http://mycolim.free.fr/DOC_SML/mycm202/Charg_Mycm202.htm

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.

Frøslev TG, Aanen DK, Læssøe T, Rosendahl S (2003) Phylogenetic relationships of *Termitomyces* and related taxa. *Mycological Research* **107**: 1277–1286.

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**: 113–118.

Gerhardt E (1982) Über zwei neue Tricholomataceen: *Collybia hebelomoides* und *Lyophyllum incarnatobrunneum*, gefunden in Berlin. *Zeitschrift für Mykologie* **48**: 239–243.

Gross G (1972) Kernzahl und sporenvolumen bei einigen Hymenogasterarten. *Zeitschrift für Pilzkunde* **38**: 109–158.

Halama M, Rutkowski R (2014) *Callistosporium pinicola* (*Basidiomycota*), a fungus species new to Poland. *Acta Mycologica* **49**: 189–197.

Hausknecht A, Zuccherelli A (1994) Ritrovamenti interessanti dal Ravennate. 3a parte. *Agaricales* a polvere sporale bianca. *Bollettino del Gruppo micologico G. Bresadola* **37**: 67–95.

Heim R, Font Quer P, Codina J (1934) Fungi Iberici. Observations sur la flore mycologique Catalane. *Treballs del Museu de Ciències Naturals de Barcelona*, *sèrie Botanica* **15**(3): 1–146.

Hofstetter V, Clémençon H, Vilgalys R, Moncalvo J-M (2002) Phylogenetic analyses of the *Lyophylleae* (*Agaricales*, *Basidiomycota*) based on nuclear and mitochondrial rDNA sequences. *Mycological Research* **106**: 104–1059.

Hofstetter V, Redhead SA, Kauff F, Moncalvo J-M, Matheny PB, *et al.* (2014) Taxonomic revision and examination of ecological transitions of the *Lyophyllaceae* (*Basidiomycota*, *Agaricales*) based on a multigene phylogeny. *Cryptogamie*, *Mycologie* **35**: 399–425.

Horak E (2005) *Röhrlinge und Blätterpilze in Europa.* 6th edn. München: Elsevier Spektrum Akademischer Verlag.

Kalamees K (1995) On *Rugosomyces fallax* and allied species (*Tricholomatales*). *Documents Mycologiques* **25**(98-100): 229–236.

Kalamees K (2004) Palearctic *Lyophyllaceae* (*Tricholomataceae*) in northern and eastern Europe and Asia. *Scripta Mycologica* **18**: 3–134.

Kalamees K (2012a) *Rugosomyces* Raithelh. In: *Funga Nordica: agaricoid, boletoid, clavarioid, cyphelloid and gastroid genera* (Knudsen H, Vesterholt J, eds) **2**: 592–594. 2nd edn. Copenhagen: Nordsvamp.

Kalamees K (2012b) *Gerhardtia* Bon. In: *Funga Nordica: agaricoid, boletoid, clavarioid, cyphelloid and gastroid genera* (Knudsen H, Vesterholt J, eds) **2**: 582. 2nd edn. Copenhagen: Nordsvamp.

Kalamees K (2012c) *Calocybe* Donk. In: *Funga Nordica: agaricoid, boletoid, clavarioid, cyphelloid and gastroid genera* (Knudsen H, Vesterholt J, eds) **2**: 581–582. 2nd edn. Copenhagen: Nordsvamp.

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 Research* **30**: 3059–3066.

Meerts P (1999) The evolution of spores in agarics: do big mushrooms have big spores? *Journal of Evolutionary Biology* **12**: 161–165.

Miller MA, Pfeiffer W, Schwartz T (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. *Proceedings of the Gateway Computing Environments Workshop (GCE). 14 Nov. 2010, New Orleans, LA*: 1–8. New Orleans: Institute of Electrical and Electronics Engineers.

Moncalvo J-M, Vilgalys R, Redhead SA, Johnson JE, James TY, *et al.* (2002) One hundred and seventeen clades of euagarics. *Molecular Phylogenetics and Evolution* **23**: 357–400.

Moreno G, Altés A, Ochoa C, Wright JE (1995) Contribution to the study of the *Tulostomataceae* in Baja California, Mexico. I. *Mycologia* **87**: 96–120.

Moreno G, Arenal F, González V (1994) Algunos *Agaricales* de las playas de Espana peninsular. *Cryptogamie, Mycologie* **15**: 239–254.

Picillo B, Contu M (2009) *Calocybe pudica* (*Basidiomycota*) osservata nel Lazio. *Micologia e Vegetazione Mediterranea* **24**: 23–29.

Raithelhuber J (1979) *Calocybe* Kühner - Eine Sammelgattung? *Metrodiana* **8**: 9–10.

Redhead SA (1982) The systematics of *Callistosporium luteo-olivaceum*. *Sydowia* **35**: 223–235.

Ridgway R (1912) *Color Standards and Color Nomenclature*. Washington D.C.: R. Ridgway.

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, *et al.* (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61**: 539–542.

Saar I, Põldmaa K, Kõljalg U (2009) The phylogeny and taxonomy of genera *Cystoderma* and *Cystodermella* (*Agaricales*) based on nuclear ITS and LSU sequences. *Mycological Progress* **8**: 59–73.

Singer R (1961) Diagnoses fungorum novorum agaricalium II. *Sydowia* **15**(1-6): 45–83.

Singer R (1981) New genera of *Agaricales*. *Mycologia* **73**: 500–510.

Singer R (1986) *The Agaricales in Modern Taxonomy.* 4th edn. Koenigstein: Koeltz Scientific Books.

Stamatakis A (2006) RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**: 2688–2690.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, *et al.* (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**: 2731–2739.

Vesterholt J, Holec J (2012) *Callistosporium* Singer. In: *Funga Nordica: agaricoid, boletoid, clavarioid, cyphelloid and gastroid genera* (Knudsen H, Vesterholt J, eds) **1**: 449–450. 2nd edn. Copenhagen: Nordsvamp.

Vilgalys R, Hester M (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* **172**: 4238–4246.

Vizzini A (2014) Nomenclatural novelties. *Gerhardtia pudica* Vizzini, Consiglio & Setti, new combination. *Index Fungorum* **155**: 1.

White TJ, Bruns TD, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: a guide to methods and applications* (Innis MA, Gelfand D, Sninsky J, White T, eds): 315–322. San Diego: Academic Press.