



## Article

# *Calocybe pilosella* sp. nov., a distinctive new lyophylloid agaric collected near Trento (Italy)

Marco Floriani<sup>1\*</sup>, Alfredo Vizzini<sup>2</sup>

<sup>1</sup> Via dei Caldonazzi 44, Serso, 38057 Pergine Valsugana (TN), Italy

<sup>2</sup> Dipartimento di Scienze della Vita e Biologia dei Sistemi, Università di Torino, Viale P.A. Mattioli 25, 10125 Torino, Italy

### Key words

- Tricholomataceae
- Lyophyllaceae
- Calocybe
- ITS phylogeny
- taxonomy
- Trentino

### Parole chiave

- Tricholomataceae
- Lyophyllaceae
- Calocybe
- filogenesi basata su sequenze ITS
- tassonomia
- Trentino (Italy)

\* Corresponding author:

e-mail: [marco.floriani@gmail.com](mailto:marco.floriani@gmail.com)

### Summary

*Calocybe pilosella*, a remarkable agaric collected in the forest of Dosso San Rocco, near Trento, is described as a new species based on repeated collections made in 2010, 2012 and 2013. The species is described and illustrated in detail, and its taxonomic placement is discussed, based both on morphological and molecular data. The newly described species is a typical lyophylloid agaric, because of the combination of white, inamyloid and cyanophilous spores and siderophilous basidia with granulation of the macro-type. The medium to large basidiomata, very small spores, and relatively short length of the basidia, as well as the intracellular pigment, suggest its placement in the genus *Calocybe*; this position is also supported by a phylogenetic analysis based on ITS sequences. Within this genus the clearly velutinate cap surface and the grey-brown colors represent a very peculiar character combination.

### Riassunto

*Calocybe pilosella*, una notevole nuova specie agaricoide raccolta nel bosco del Dosso San Rocco, vicino a Trento, viene presentata sulla base di ripetute raccolte fatte negli anni 2010, 2012 e 2013. La specie viene descritta e illustrata in dettaglio e ne viene discussa la collocazione tassonomica, sulla base di dati morfologici e molecolari. La nuova entità è un tipico rappresentante degli agarici liofilloidi, per la combinazione di spore bianche, non amiloidi e cianofile, nonché per i basidi siderofili con granulazioni del tipo "macro". I basidiomi di medio-grandi dimensioni, le spore molto piccole, i basidi relativamente corti e il pigmento di natura intracellulare ne suggeriscono la collocazione nel genere *Calocybe*, come confermato dall'analisi delle sequenze ITS; in seno a questo genere la superficie della cuticola evidentemente tomentosa e le colorazioni grigio-brune rappresentano una combinazione di caratteri peculiare e unica.

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## Introduction

In the course of different visits to the forest of the Dosso San Rocco, a remarkable agaric was collected in October 2010, September 2012 and October 2013, growing with *Ostrya carpinifolia* and *Quercus pubescens*. The macro- and micromorphological features of this fungus (in particular the inamyloid and cyanophilous spores and the presence of siderophilous granulation in the basidia) allowed us to place it in the *Lyophyllaceae* sensu Matheny et al. 2006, but without the possibility to assign it to any known species. Within *Lyophyllaceae*, its placement in the genus *Calocybe* was inferred from a phylogenetic analysis of ITS sequences representative of this family. It is therefore described as new under the name *Calocybe pilosella*, because of the characteristically hairy surface of the pileus.

## Study area

Within an ongoing project devoted to the recording and mapping of macromycete species in the Province of Trento (North-Eastern Italy), we recently focused our attention on a few areas in the near surroundings of Trento, characterised by a kind of vegetation which is not very common in this province; these areas include the city park of Gocciadoro (explored in detail also by Giacomo Bresadola in the early 20th century), the Doss Trento, a small mount located just West of the Trento city centre, and finally the Dosso San Rocco, another small hill which hosts a large city park, recently restored by the Province of Trento.

The vegetation covering the Dosso San Rocco can be classified as a whole as a thermophilic deciduous oak forest, broadly corresponding to the association *Fraxino ornii-Ostryetum carpinifoliae* Br.-Bl. 1961. The dominant tree species are in fact Hop Hornbeam (*Ostrya carpinifolia*), Pubescent Oak (*Quercus pubescens*), South European flowering ash (*Fraxinus ornus*), Scots pine (*Pinus sylvestris*), and Whitebeam (*Sorbus aria*). The calcareous matrix, characterised by a high permeability caused by karstification and fracture, and the mainly South to South-western exposition of the slopes have favored poor arid soils, on which many xerotolerant plants have established with time. Some good examples are the abundance, among others, of the Wayfaring tree (*Viburnum lantana*), smoke tree (*Cotinus coggygria*), Barberry (*Berberis vulgaris*), rock Buckthorn (*Rhamnus saxatilis*) in the shrub layer, and of *Sesleria varia*, *Carex humilis* and *Erica carnea* in the herb layer of clearings. Historical use of the soil, related to intensive firewood collection and grazing, has contributed to the general conditions of edaphic poverty and scarce forest evolution. Some mesophilic forest associations are nevertheless noted in some dells and North exposed areas, with the presence of mixed oak forests with Turkey oak (*Quercus cerris*), Sweet Chestnut (*Castanea sativa*) and service tree (*Sorbus domestica*). Over the last decades, past intensive uses of the soil have ceased, and the whole forest system is generally evolving, with a growing amount of standing and fallen dead wood.

The soil is usually of the rendzinform type, humus-rich and shallow, with an A-AC-C profile and very rich in structure. Partial decarbonations of the superficial A horizon and a more prominent edaphic evolution are noted only in the less steep areas, where an



**Fig. 1** - Basidiomata in various stages of development from the holotype collection of *Calocybe pilosella*. Holotype collection TR gmb 00931, Oct. 2nd, 2012, Dosso San Rocco. Scale bar = 1 cm. / Basidiomi in vari stadi di sviluppo dalla raccolta olotipica di *Calocybe pilosella*. Raccolta olotipica TR gmb 00931, 2 ottobre 2012, Dosso San Rocco. Barra = 1 cm.

intermediate B horizon of finer, ochraceous material (forest Brown Earth) can be observed and a more exacting vegetation grows. The humus layer is usually characterised by the presence of non-decomposed litter on the surface and of underlying consistent OH horizons (moder).

## Methods

### Morphology

The morphological description of the species was done on the basis of the observation of fresh specimens from the holotype collection and of other material. Colours are coded according to the Flora of British Fungi Colour Identification Chart (1969), referred in the text as 'Bc'. Microscopic description and drawings were obtained examining both fresh and dried material. Spores were observed in water, Melzer's reagent and Cotton blue; hymenial elements were observed in L4 and Congo red; elements of the pileipellis were observed both in water and in Congo red; spore measurements were made in side view at 1000 × magnification; basidia length excludes sterigmata length.

### DNA extraction, PCR amplification and DNA sequencing

Genomic DNA was isolated from 10 mg of dried herbarium specimens (TR gmb 00931, holotype collection, and TR gmb 00697), using the DNeasy Plant Mini Kit (Qiagen, Milan) according to the manufacturer's instructions. Universal primers ITS1F/ITS4 were used 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) following Vizzini et al. (2011). The PCR products were purified with the AMPure XP kit (Beckman) and sequenced by MACROGEN (Seoul, Republic of Korea). The sequences are deposited in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) under the accession numbers given in paragraph 4.3.

### 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 the GenBank database by using the Blastn algorithm. Based on the Blastn results, sequences were selected according to the outcomes of recent phylogenetic studies on *Lyophyllaceae* (Hofstetter et al. 2002) and retrieved from GenBank and UNITE (<http://unite.ut.ee/>) databases. The alignment was generated using MAFFT (Katoh et al. 2002) with default conditions for gap openings and gap extension penalties and 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 v. 5.0 (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 the default settings. The phylogenetic analysis was performed using the Maximum Likelihood (ML) approach. An *Entoloma prunuloides* sequence (DQ206983) was used as outgroup. 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 MLB over 70 % are reported in the resulting tree (Fig. 6).

## Results

### Phylogenetic analysis

The ITS data matrix comprises a total of 84 sequences (including 73 from GenBank and 9 from UNITE). This dataset is 755 base pairs long and contains 430 (56.9%) variable sites. In the obtained ML phylogram (Figure 6), our sequences cluster together and fall in the genus *Calocybe*, where they occupy an isolated position.

### Taxonomy

#### *Calocybe pilosella* Floriani & Vizzini, sp. nov.

MycoBank: MB 808361 Figura 1-5, 7

Etymology: from the Latin adjective *pilosellus*, finely hairy, referring to the surface of the cap.

*Pileus* 50-80 (-100) mm, convexus, deinde planus, griseo-brunneus, cum decolorationis albis, cuticula pilosella. Lamellae albae, confertae, in vetustis interdum brunneo-maculatae. Stipes solidus, basi aequalis vel leviter bulbosus, albus, dein leviter et pallidissime luteus. Caro alba, odore grato, sapore leviter farinaceo. Sporae hyalinae, subellipticae, 3,9-4,1 x 2,5-3,1 μm, inamyloideae, cyanophilae. Basidia 18-24 x 4,8-5,5 μm, cum granulis siderophilis. Cystidia absentia. Pileipellis ex hyphis repentibus, elementis cylindraceutis usque ad 10-18 μm latis. Fibulae praesentes. Habitatione in nemoribus frondosis, sub *Ostryis carpinifoliis* et *Quercubus pubescentibus*, autumnno.

HOLOTYPE: ITALY, Trento, Dosso San Rocco, 2 October 2012, TR gmb 00931 (TO, isotype).

### Examined collections

Dosso San Rocco (Trento), 29.9.2010, a single young specimen collected in a mixed woodland with *Quercus pubescens*, *Ostrya carpinifolia* and scattered *Pinus nigra*, 46°02'08" N, 11°08'24" E, 400 m a.s.l., leg. M. Floriani (TR gmb 00693). Dosso San Rocco (Trento), 29.9.2010, five specimens collected in a mixed woodland with *Quercus pubescens*, *Ostrya carpinifolia* and scattered *Pinus nigra*, 46°02'05" N, 11°08'18" E, 400 m a.s.l., leg. M. Floriani (TR gmb 00697, GenBank accession no. KJ883237). Dosso San Rocco (Trento), 2.10.2012, five specimens collected in a mixed woodland with *Quercus pubescens* and *Ostrya carpinifolia*, with *Cotinus coggygria*, 46°01'52" N, 11°08'25" E, 400 m a.s.l., leg. A. Valdagni & M. Floriani (TR gmb 00931, holotype, GenBank accession no. KJ576811). Dosso San Rocco (Trento), 2.10.2012, two specimens collected in a mixed woodland with *Quercus pubescens* and *Ostrya carpinifolia*, 46°02'02" N, 11°08'19" E, 400 m a.s.l., with *Cotinus coggygria*, leg. M. Floriani (TR gmb 00932). Dosso San Rocco (Trento), 31.10.2013, four specimens collected in a mixed woodland with *Quercus pubescens* and *Ostrya carpinifolia*, 46°01'54" N, 11°08'22" E, 400 m a.s.l., leg. M. Floriani & A. Valdagni (TR gmb 01081).

### Description

*Cap*: 50-80 (-100) mm, convex at first, then plane or slightly depressed at centre, at maturity almost without an umbo, regularly shaped, more or less discoid, only in one carpophore with a lobate outline. Margin not striate. Surface definitely matt, with a hairy-wooly covering, somehow reminiscent of *Tricholoma* subsect. *Terrea*, grey (Bc 34 *Smoke grey*), beige-grey (Bc 32 *Clay buff*) with some darker grey areas and some decolorated, whitish areas (Bc 2 B), especially towards the margin. Felty covering light grey.

*Lamellae*: crowded, with numerous lamellulae (1 L = 3-5 l), notched, easily detachable from the cap flesh, with slightly undulate but entire edge, uniformly whitish to pale cream (Bc 2 B), unchanging when touched or rubbed, but with some dark brown spots (Bc 18 *Umber*) in old specimens.



**Fig. 2-5** - Morphological features of *Calocybe pilosella*. 2. Basidiomata of collection TR gmb 00697, showing the typical white discoloration at the cap margin. 3. Basidiomata of collection TR gmb 01081; note the white discoloration even in the very old and water-soaked specimen on the left. 4. Close-up of the cap cuticle in the holotype collection TR gmb 00931, showing the velutinate surface. 5. Close-up of the gills in collection TR gmb 01081, showing the gill attachment and the dark brown spots in the old specimen. 3. Basidiomi della raccolta TR gmb 01081; si noti la decolorazione bianca anche nell'esemplare vecchio e imbevuto d'acqua sulla sinistra. 4. Ingrandimento della cuticola della raccolta olotipica TR gmb 00931, che evidenzia la superficie vellutata. 5. Ingrandimento delle lamelle nella raccolta TR gmb 01081, che mostra l'attaccatura delle lamelle al gambo e le macchie bruno scure nell'esemplare adulto. Barre = 1 cm.

*Spore print*: purely white.

*Stipe*: 50-65 × 10-12 mm, thickened at the base up to 20 mm, cylindrical with a small basal bulb, solid, with a fibrose texture. Surface finely fibrillose, whitish, slightly darker, light brownish, towards the base.

*Flesh*: soft, fibrose in the stipe, white in the cap, whitish in the stipe, light beige close to the stipe cortex or where it is more wet. Smell fungoid, pleasant (with a slight *Lepiota cristata*-like component) to distinctly mealy; taste mild, with a slight mealy aftertaste.

*Spores*: [ $n = 30$ ] (3.6-) 3.9-4.1 (-4.2) × (2.2-) 2.5-3.1 (-3.2)  $\mu\text{m}$ , on average 4.0 × 2.8  $\mu\text{m}$ ,  $Q_{av} = 1.45$ , smooth, ovoid to ellipsoid, cyanophilous, non amyloid.

*Basidia*: clavate, with abundant siderophilous granules of the 'macro' type (Cléménçon 1978), (size 0.2-0.5  $\mu\text{m}$ , rarely up to 1  $\mu\text{m}$ ), clearly visible also when observed in Congo red, mostly 4-spored, (18-) 18.7-23.1 (-24) × (4.8-) 4.9-5.3 (-5.5)  $\mu\text{m}$ , with sterigmata 2-3  $\mu\text{m}$  long. *Cystidia*: not observed.

*Subhymenium*: cellular, composed of more or less isodiametric cells.

*Gill trama*: regular, composed of long, cylindric to barrel-shaped elements of 40-80 (-100) × 10-15 (-20)  $\mu\text{m}$ , sometimes with clamps at septa.

*Cap cuticle*: a cutis of more or less cylindrical, hyaline to brownish, thin-walled, 6-10 (-18)  $\mu\text{m}$  wide, radially oriented to interwoven

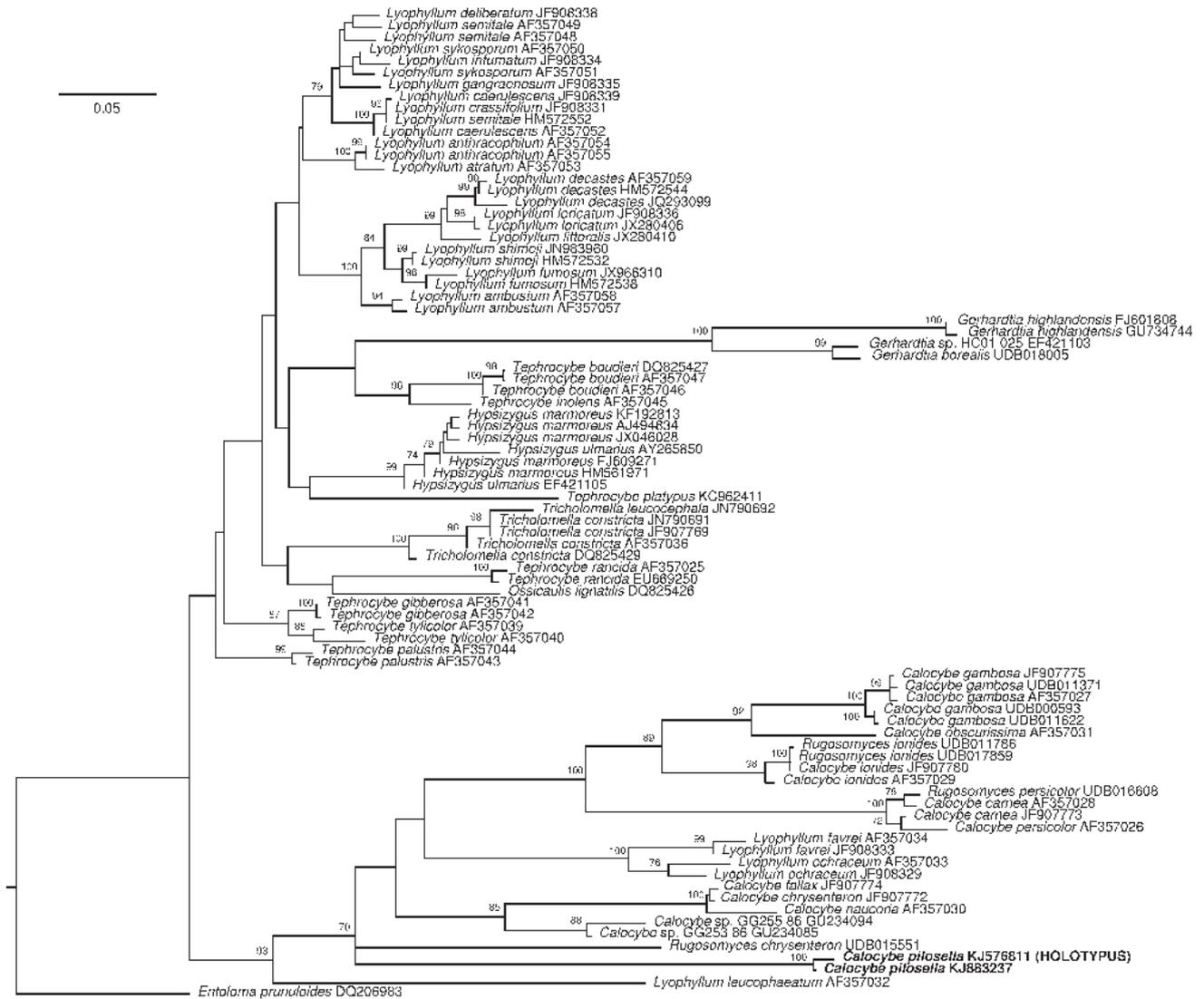
hyphae, sometimes with ascending hyphae forming a transition to a trichoderm; pigment intracellular, light brownish when observed in water.

*Ecology*: on the ground, in warm and shady sites, growing with *Ostrya carpinifolia*, *Quercus pubescens*, often observed near *Cotinus coggygria*. Known from five different spots in the same locality, at approximately 400 m a.s.l.

## Discussion

*Calocybe pilosella* appears as an easily recognizable fungus in the field: its key characteristics are the medium to large basidiomata, with a flattened cap and a nearly cylindrical stipe (*Melanoleuca*-like habit), the greyish brown, felted cap, with whitish discolorations observed especially towards the cap margin. In case of doubt, the very small (circa 4  $\mu\text{m}$  in length), ellipsoid and smooth spores are distinctive characteristics.

Our first gathering of *Calocybe pilosella* was tentatively assigned, on the basis of some macromorphological features (in particular the easily detachable gills and the habit of the fungus), to the genus *Lepista* (Fr.) W.G. Sm., reminiscent in particular of *Lepista tomentosa* M.M. Moser, or of other taxa in the *L. luscina*-complex; microscopically, however, the very small and smooth spores made



**Fig. 6** - Maximum Likelihood phylogram obtained from the ITS (ITS1-5.8S-ITS2) sequence alignment of Lyophyllaceae. *Entoloma prunuloides* was used as outgroup. MLB values over 70 % are given above branches. The newly sequenced collections are in bold. / Albero filogenetico di massima verosimiglianza ottenuto dall'allineamento delle sequenze ITS (ITS1-5.8S-ITS2) di diverse Lyophyllaceae. *Entoloma prunuloides* è stato utilizzato come outgroup. Valori di MLB superiori al 70 % sono indicati sopra i rami. Le nuove sequenze sono indicate in grassetto.

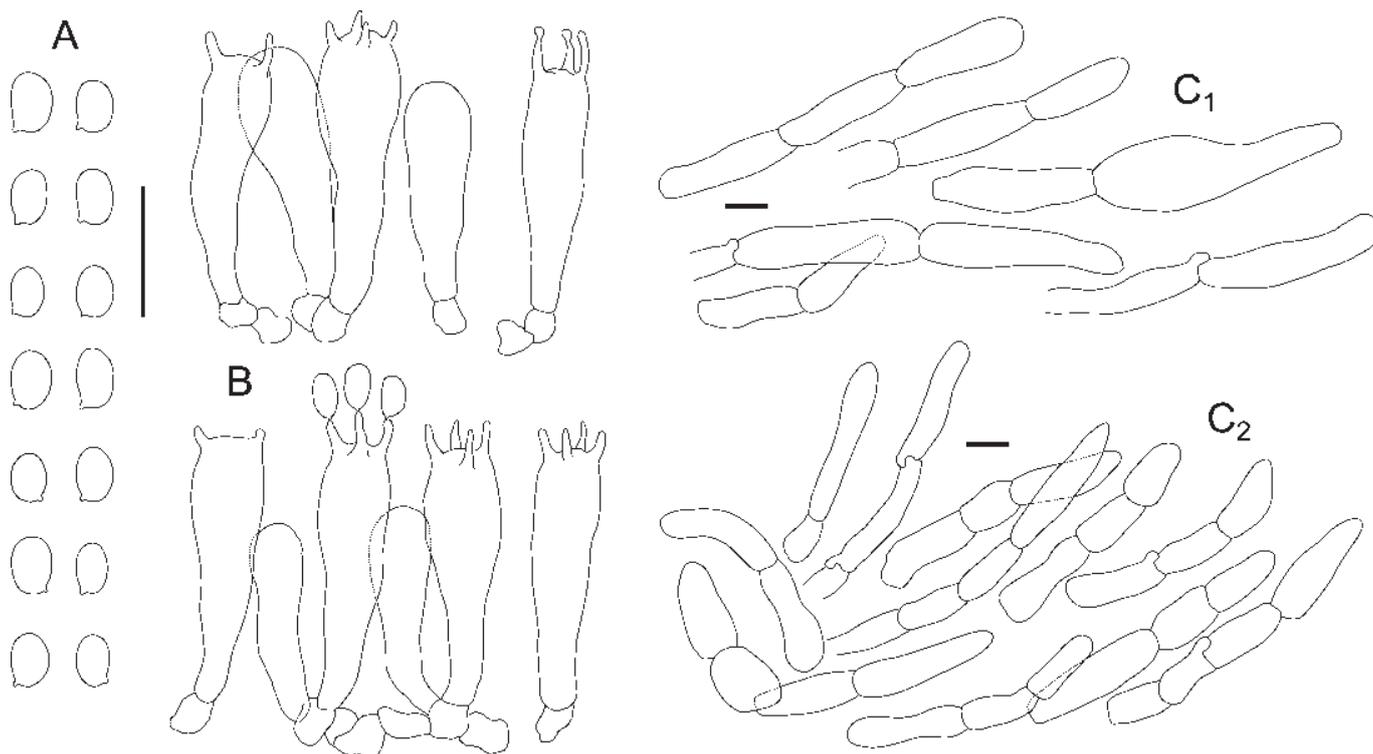
us exclude this hypothesis. Further microscopical observations revealed that the spores are inamyloid, cyanophilous and that the basidia show distinct siderophilous contents, the latter character, in particular, pointing to the lyophylloid genera in the family *Tricholomataceae*. This complex agaric group, by some authors (e.g. Knudsen & Vesterholt 2012) regarded as a family of its own (*Lyophyllaceae* Jülich), comprehends several genera whose delimitation is still insufficiently clear (see e.g. Hofstetter et al. 2002; Vesterholt & Ludwig 2012).

The phylogenetic analysis confirmed this affiliation and supported the inclusion of the new taxon in the genus *Calocybe* Kühner ex Donk, following a delimitation of this genus corresponding to clade 13A in Hofstetter et al. 2002, therefore including (i) *C. gambosa*, (ii) the brightly colored species formerly transferred by Bon to the genus *Rugosomyces* and (iii) the rare *Calocybe favrei* and *Calocybe ochracea*. Morphologically, the very small spores, the distinct siderophilous granulation of the basidia (of the 'macro' type following Cléménçon 1978) and the relatively short length of the basidia, as well as the intracellular pigmentation (Bon 1999: 16) all agree with an assignment to the genus *Calocybe*.

We tried to compare the present species to any agaric described in modern treatments of the lyophylloid genera for Europe (Bon 1999; Ludwig 2000, 2001; Consiglio & Contu 2002; Kalamees 2004, 2012; Vesterholt & Ludwig 2012): none fits, even vaguely, the characteristics of our collection. Unfortunately, recent monographic treatments for other continents are very scarce – a recent treatment of *Lyophyllum* for North America (Cléménçon & Smith 1983) concerning only the staining species of the genus –, so we tried to consult standard floras and general works (in particular Singer 1977, 1986; May & Wood 1997), once again without finding similar species.

In particular, the very small size of the spores, measured in four collections and on spores obtained from good spore prints, dramatically reduces the number of candidate species among lyophylloid agarics. The following small-spored species are compared to *C. pilosella*, even if their resemblance is in all cases far from striking:

- within the genus *Calocybe*, several species previously assigned to the genus '*Rugosomyces*', namely *Calocybe onychina* (Fr.) Donk, *Calocybe fallax* (Sacc.) Redhead & Singer (nom. illeg.), *Calocybe chrysenteron* (Bull.: Fr.) Singer have spores with similar size, but they are all macroscopically very different from *C. pilosella*: they



**Fig. 7** - Microscopic features of *Calocybe pilosella*. A. Spores. B. Basidia and basidioles. C<sub>1</sub>, C<sub>2</sub>. Elements of the pileipellis. (A, B, C<sub>1</sub> from holotype collection TR gmb 00931, C<sub>2</sub> from paratype collection TR gmb 01081). Scale bars = 10 µm. / Caratteri microscopici di *Calocybe pilosella*. A. Spore. B. Basidi e basidioli. C<sub>1</sub>, C<sub>2</sub>. Elementi della pileipellis. (A, B, C<sub>1</sub> dall'holotipus TR gmb 00931, C<sub>2</sub> dal paratipus TR gmb 01081). Barre = 10 µm.

are characterised by a smaller size and the occurrence of bright yellow colors at least in the gills; microscopically, the pileus covering is hymeniform (Bon 1999; Consiglio & Contu 2002; Kalamees 2004; Vesterholt & Ludwig 2012);

- *C. obscurissima* (A. Pearson) Mos., is easily distinguished by the smaller size, smooth and darker cap surface, distinctly larger and differently shaped spores (Bon 1999; Consiglio & Contu 2002; Kalamees 2004; Vesterholt & Ludwig 2012);
- also within *Calocybe*, the rare *Calocybe favrei* (R. Haller Aar. & R. Haller Suhr) Bon and *C. ochracea* (R. Haller Aar.) Bon have very similar spores and a medium to large size, comparable to that of *C. pilosella*; the colours of the basidiomata are very different, and they have a strongly reddening, then blackening context (Bon 1999; Consiglio & Contu 2002; Kalamees 2004; Vesterholt & Ludwig 2012);
- among the extra-European *Calocybe* species, *C. bipigmentata* Singer from Brazil and *C. alneti* from Argentina are both distinguished by smaller subglobose spores, 2.5-3 × 2-2.5 µm, a subglabrous cap surface, ochre to yellowish lamellae and growth on wood debris (Singer 1977).

Finally, none of the species considered above has a cap cuticle similar to that of *C. pilosella*, which therefore seems to be a morphologically very well delimited taxon among lyophylloid agarics.

Extending our analysis to other genera, macroscopically, as already stated, the genus *Lepista* presents several morphological affinities, but it can be excluded because of the occurrence of distinctly siderophilous basidia. The habit of *C. pilosella* is similar to that of the genus *Melanoleuca* Pat., but in this genus the spores are amyloid and distinctly verrucose, and clamp connections are absent. Siderophilous granules, if present, are very small ('micro' type, Cléménçon 1978) and hardly noticeable in light microscopy. Also the genus *Tricholoma* (Fr.) Staude may be excluded at least by the absence of siderophilous

granules and by the non cyanophilous spores.

*Porpoloma spinulosum* (Kühner & Romagn.) Singer, recorded in analogous habitat conditions and having similar stature and colours, as well as a rough cap surface, was suggested by a correspondent as a possible lookalike (its depiction in Lange 1936, under the name *Tricholoma guttatum*, for example, is reminiscent of our fungus); the cap surface is however more scaly than felty or velutinate, especially near the cap margin. Moreover, the spores are distinctly larger and amyloid. The in-amyloid spores and, once again, the occurrence of siderophilous basidia, definitely rule out the genus *Porpoloma* Singer.

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