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Trichocybe, a new genus for *Clitocybe puberula* (Agaricomycetes, Agaricales)

Alfredo Vizzini, Enzo Musumeci, Claude Murat

Abstract

Trichocybe, a new genus of *Agaricales*, is described to accommodate the rare species *Clitocybe puberula* characterized by an isolated phylogenetic position. *Trichocybe* is distinguished from its allied clitocyboid genera by a unique combination of macro- and micromorphological characters, including a *Gymnopus*-like habitus, a pubescent pileus with squamulose flocci, an eccentric stipe growing on wood debris, a putrescent non-reviviscent context, a strongly farinaceous odour, rare but clearly-differentiated cheilocystidia, an unusual, strongly differentiated pileipellis, a subpellis with vesiculose-physaloid elements, a stipitipellis with diverticulate hyphae, and smooth, acyanophilous, inamyloid basidiospores. *Trichocybe* is so far known only from northwestern Europe. Based on a LSU- and 5.8S-rDNA Bayesian phylogenetic analysis, *Trichocybe* clusters within the Tricholomatoid clade, where it occupies an isolate position. Furthermore, according to the present analysis, also *Clitocybe phaeophthalma* seems to represent an independent evolutive line in the clitocyboid fungi, which suggests adopting the genus *Singerocybe* Harmaja for this taxon as well as similar ones.

Keywords

Basidiomycota *Clitocybe* *Singerocybe* *Trichocybe puberula* Tricholomatoid clade Taxonomy

Electronic supplementary material

The online version of this article (doi:[10.1007/s13225-010-0030-8](https://doi.org/10.1007/s13225-010-0030-8)) contains supplementary material, which is available to authorized users.

Introduction

Clitocybe (Fr.) Staude is one of the less appealing fungal genera, whose members are mostly insubstantial in size, drab in appearance and dull in pileus colours. *Clitocybe* is a character-poor genus; in fact, many species differ only in very subtle features, such as basidioma macromorphology, pileipellis structure and basidiospores size (e.g. Harmaja [1969](#); Bigelow [1982](#), [1985](#); Kuyper [1982](#), [1995](#); Cléménçon [1984](#); Singer [1986](#); Raithelhuber [1990](#), [2004](#); Bon [1997](#); Takahashi [2000](#), [2003](#); Gregory [2007](#)). From a morphological perspective, this often makes species identification difficult or even daunting. The paucity of characters of *Clitocybe* species has been detrimental to the establishing of a non-artificial taxonomic framework rationalizing the relationships among infrageneric groupings. Moreover, a worldwide critical monograph of the genus is so far lacking and is badly needed.

With the advent of molecular techniques, phylogenetic analyses of fungi mainly based on sequences of nuclear ribosomal DNA are beginning to shed new, revelatory light on generic and infrageneric circumscriptions. Recent molecular analyses (Moncalvo et al. [2002](#); Matheny et al. [2006](#)), including a significant number of *Clitocybe* species, have shown that *Clitocybe* is a non-

monophyletic assemblage. In fact, the genus represents a heterogeneous, artificial set of disparate and in many cases phylogenetically-unrelated taxa, sharing the so-called clitocyboid habit. It encompasses saprotrophic members of the *Agaricales* (Euagarics) that produce fleshy basidiomata with lamellae that are broadly attached to, or run down, the stipe (adnate-decurrent lamellae), convex to funnel-shaped pilei and a usually whitish, yellowish or pinkish spore print (Singer [1986](#); Bas [1990](#)).

As a consequence, over the past few years, former *Clitocybe* species have been transferred to other genera or segregated in new ones based on various combinations of morphological and molecular markers. New genera that have recently been erected to accommodate former *Clitocybe* species are: *Ampulloclitocybe* (Redhead et al. [2002](#)), *Infundibulicybe* (Harmaja [2003](#)) and *Cleistocybe* (Ammirati et al. [2007](#)). It is likely that more *Clitocybe* species will be transferred from the genus as further molecular analyses including other taxa of *Clitocybe* will be performed.

In the present paper, recent Swiss collections of *Clitocybe puberula* Kuyper, a very rare and distinctive species, are described and compared to the original description (diagnosis) and the data from the type revision. Based on morphological and molecular analyses, *Clitocybe puberula* appears to be aberrant within *Clitocybe* and un-subsumable into any of the extant genera. Accordingly, we propose to erect a new genus, *Trichocybe*, to circumscribe the peculiar, unique combination of features characterizing this fungus, and a necessary new combination.

Materials and methods

Morphology

Macroscopic characters were examined from fresh material. Microscopical analyses are based on dried material using a Leica DM 4500 B and an Olympus BX50 light microscope with a magnification up to 1000 \times . Observations were made on mounts in these reagents: Congo red in 10% ammonia, 3% KOH, Cresyl-blue in water and Melzer's reagent. Measurements of the basidiospores do not include the apiculus. The following abbreviations were used: [X, Y, Z] indicates that measurements were made on X spores, in Y samples from Z collections; Q = the quotient of length and width of the spores in side view; Qm = the mean value of Q values in all collections studied; L = number of entire lamellae; l = number of lamellulae between each pair of entire lamellae. Herbarium abbreviations refer to Holmgren and Holmgren ([1998](#)). The Latin description of the new genus and the new combination were deposited in MycoBank (<http://www.mycobank.org/DefaultPage.aspx>).

DNA extraction, PCR amplification, and DNA sequencing

Genomic DNA was isolated from 1 mg of herbarium specimens (Table [1](#)) by using the DNeasy Plant Mini Kit (Qiagen, Milan Italy) according to the manufacturer's instructions. Universal primers ITS1f/ITS4 were used for the ITS region amplification (White et al. [1990](#); Gardes and Bruns [1993](#)) and primers LR0R/ LR6 (Vilgalys and Hester [1990](#); Vilgalys lab, unpublished, <http://www.botany.duke.edu/fungi/mycolab>) for the LSU rDNA amplification. Amplification reactions were performed in PE9700 thermal cycler (Perkin-Elmer, Applied Biosystems) in a 25 μ l reaction mixture using the following final concentrations or total amounts: 5 ng DNA, 1 \times PCR buffer (20 mM Tris/HCl pH 8.4, 50 mM KCl), 1 μ M of each primer, 2.5 mM MgCl₂, 0.25 mM of each dNTP, 0.5 unit of *Taq* polymerase (Promega). The PCR program was as follows: 3 min at 95°C for 1 cycle; 30 s at 94°C, 45 s at 50°C for both ITS and LSU primers, 2 min at 72°C for 35 cycles, 10 min at 72°C for 1 cycle. PCR products were resolved on a 1.0% agarose gel and visualized by staining with ethidium bromide. The PCR products were purified and sequenced by DiNAMYCODE srl (Turin, Italy).

Table 1 Sources and accession numbers of own isolates and sequences used in this study

| Species | GenBank accession numbers | | Source, substrate and locality |
|---|---------------------------|----------|---|
| | LSU | ITS | |
| <i>Trichocybe puberula</i> | FM877680 | FM877683 | TO-HG 1148, on decaying straw bales mixed to wood debris, Ettingen, Switzerland |
| <i>Trichocybe puberula</i> (typus) | FM877681 | FM877682 | L 0053903, on sawdust, Vaclusotte, France |
| <i>Clitocybe phaeophthalma</i> (Pers.) Kuyper | FM877679 | FM877684 | TO-HG 1147, on bare soil, Trieste, Italy |

Sequence alignment and phylogenetic analysis

The sequences obtained in this study were compared to the GenBank Database (<http://www.ncbi.nlm.nih.gov/Genbank/>) using the blastn algorithm. Sequences included in the phylogenetic analyses were either generated by us or obtained from GenBank. Based on the blastn results, sequences were selected according to the outcomes of other phylogenetic studies of *Agaricales* (Moncalvo et al. 2002; Matheny et al. 2006). Multiple alignments were performed with CLUSTAL W (Thompson et al. 1994) using default settings and manually optimized with BioEdit version 5.0.9 (Hall 1999). Two independent analyses, one with only LSU sequences and a second with a combined analysis of LSU and 5.8S sequences, have been carried out. Bayesian inference (BI) based upon the posterior probability distribution of trees was performed with MrBayes (Ronquist and Huelsenbeck 2003) with the following settings for the LSU sequences: outgroup *Infundibulicybe gibba*; Lset nst = 6 rates = invgamma; Prset statefreqpr = fixed (equal); mcmc ngen= 10,000,000 samplefreq= 1,000; other settings = default. For the combined LSU and 5.8S analysis the following settings have been changed: mcmc ngen= 1,000,000. The sump burnin= 2,500 for LSU and 250 for combined analysis was used to verify the stationarity of the analysis. The sumt command with respective burnin values was used to produce summary statistics for trees sampled during the Bayesian analysis. The consensus tree was visualized and edited with FigTree v1.1.2 (Rambaut 2008).

Results

Phylogenetic analyses

Amplification and sequencing of the ITS and LSU rDNA regions was successful for all the specimens selected for molecular study (Table 1). Comparing these sequences with those from GenBank (supplementary Tab. 1) revealed that *Trichocybe puberula* belongs to the Tricholomatoid clade as defined by Matheny et al. (2006), encompassing *Lyophyllaceae* Jülich, *Entolomataceae* Kotl. & Pouzar, *Tricholomataceae* R. Heim ex Pouzar s.str., the *Catathelasma* clade and *Mycenaceae* Overeem; accession numbers are given in Table 1 and in the supplementary Table 1. A similarity of 98.94% was found between the two *T. puberula* ITS sequences.

The main clades found by Matheny et al. (2006) have been identified in the LSU phylogenetic tree realized with 73 species belonging to the Tricholomatoid clade (Fig. 1). In the LSU phylogenetic tree *T. puberula* belongs to the *Clitocybeae* clade, but with a low posterior probability value (Fig. 1), whereas in the combined LSU/5.8S phylogenetic tree it does not belong to any of the known clades (Fig. 2).

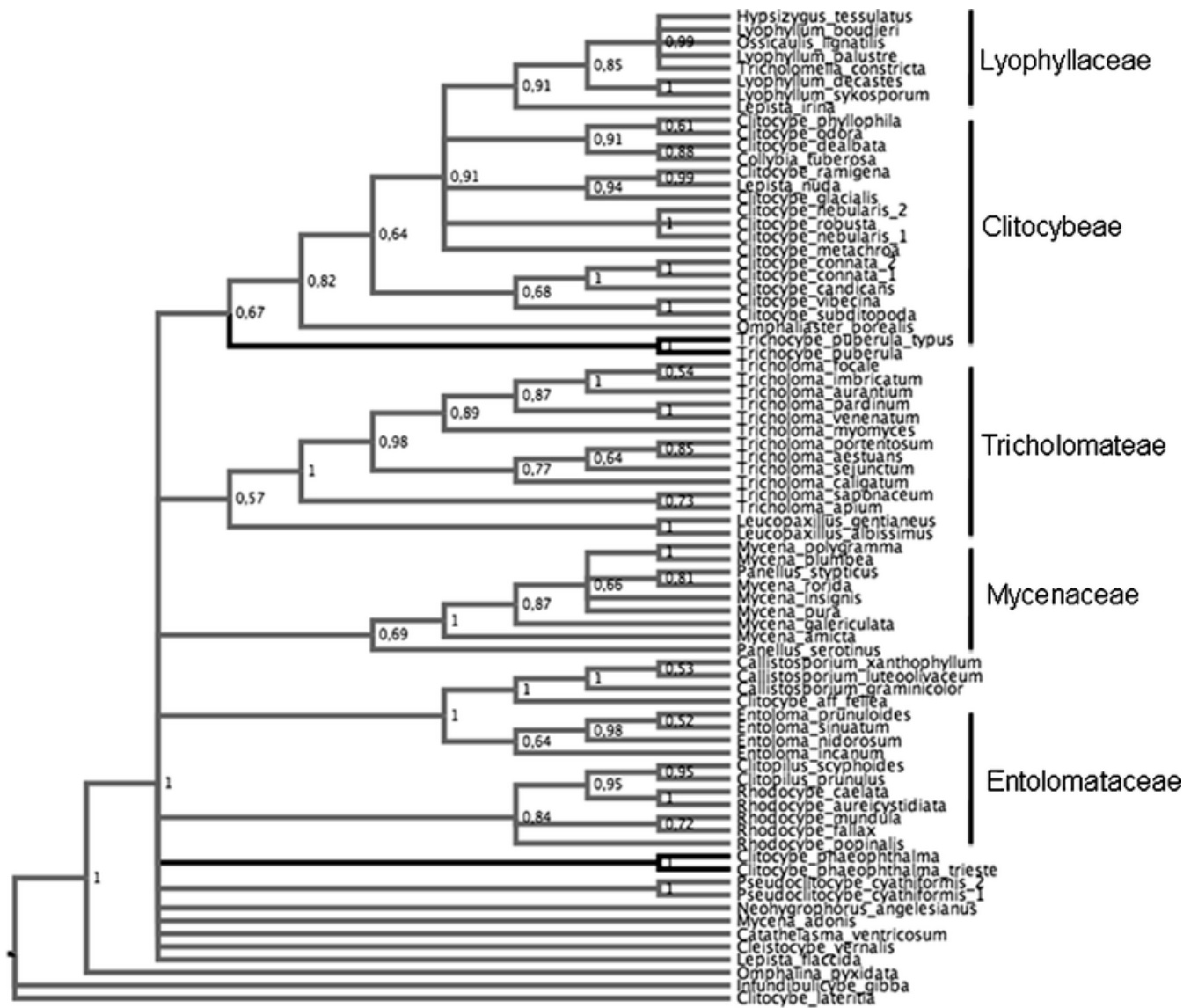


Fig. 1 LSU rDNA phylogeny inferred by Bayesian analysis corresponding to the consensus tree. Values of posterior probabilities are shown

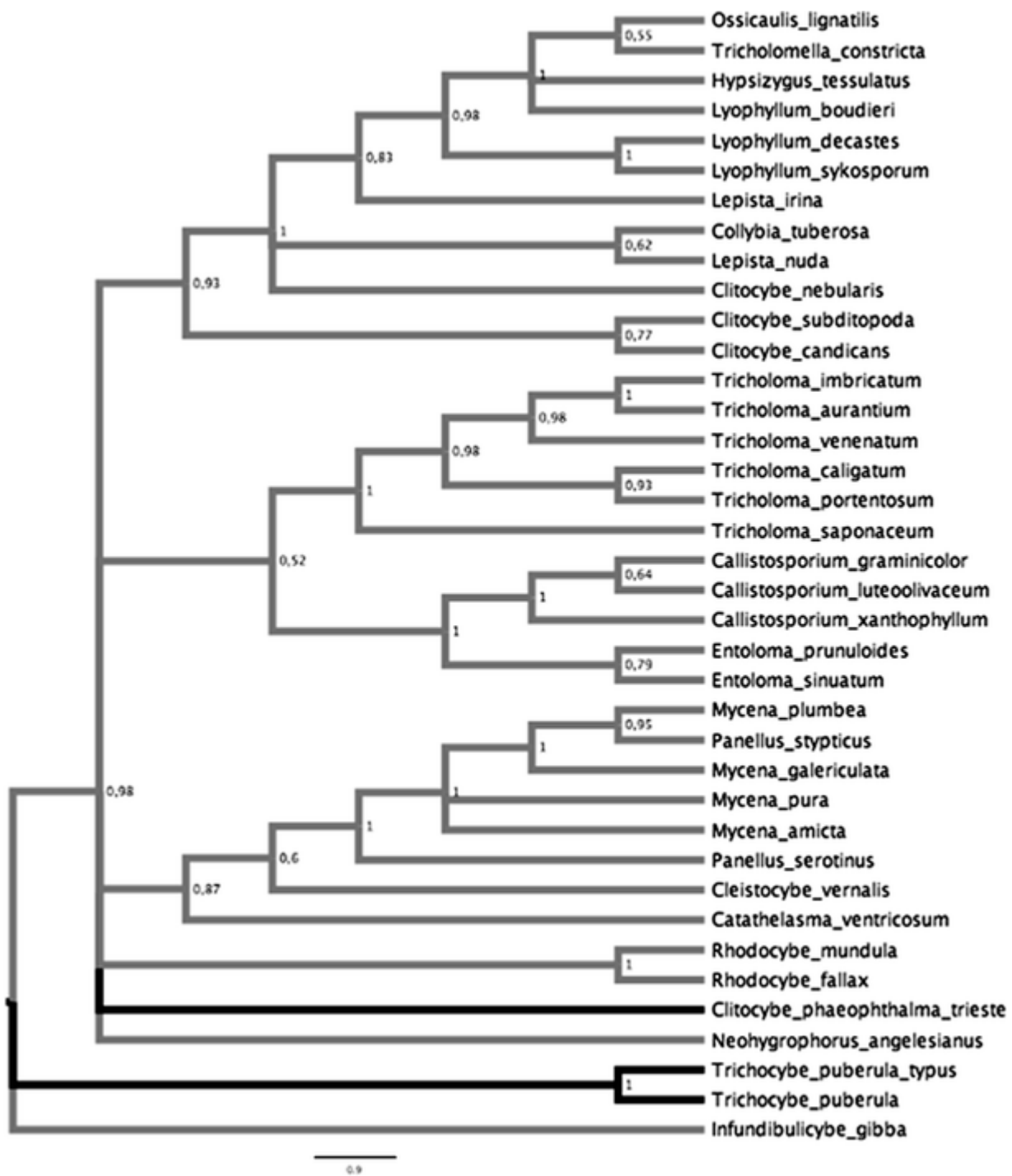


Fig. 2 LSU/5.8S rDNA phylogeny inferred by Bayesian analysis corresponding to the consensus tree. Values of posterior probabilities are shown

Taxonomy

Trichocybe Vizzini, **gen. nov.**

[= *Clitocybe* sect. *Puberulae* Kuyper, Sydowia 36: 173 ([1983](#))].

MycoBank: MB 512688.

Etymology: the name derives from the Greek words *trichós*/τριχός ‘hair’ and ‘kúbe/κύβη head’, in reference to the complex trichodermic structure of the pileipellis.

Habitus collybio-clitocyboideus, lamellae adnexae; velum membranosum vel arachnoideum deest. Sporae in cumulo albae, sub lente hyalinae, laeves, haud amyloideae, haud destrinoideae, tenuitunicatae. Basidia haud hygrophoroidea, granulis siderophilis destituta; cheilocystidia saepe praesentes, oleiferae raras; pilei pellis e squamulis pyramidalibus et ex hyphis fasciculatis erectisque constitutis efformata; subpellis ex hyphis ventricosis, frequenter inflatis, vel ampullaceis ad instar *Singerocybei phaeophthalmae* constituta; stipitipellis ex hyphis diverticulatis constituta, haud sarcomdimitica. Fibulae praesentes. Ad matricem ligneam.

Typus: *Clitocybe puberula* Kuyper.

Trichocybe puberula (Kuyper) Vizzini, **comb. nov.**

MycoBank: MB 512689.

(Figs. [3](#), [4](#) and [5](#))

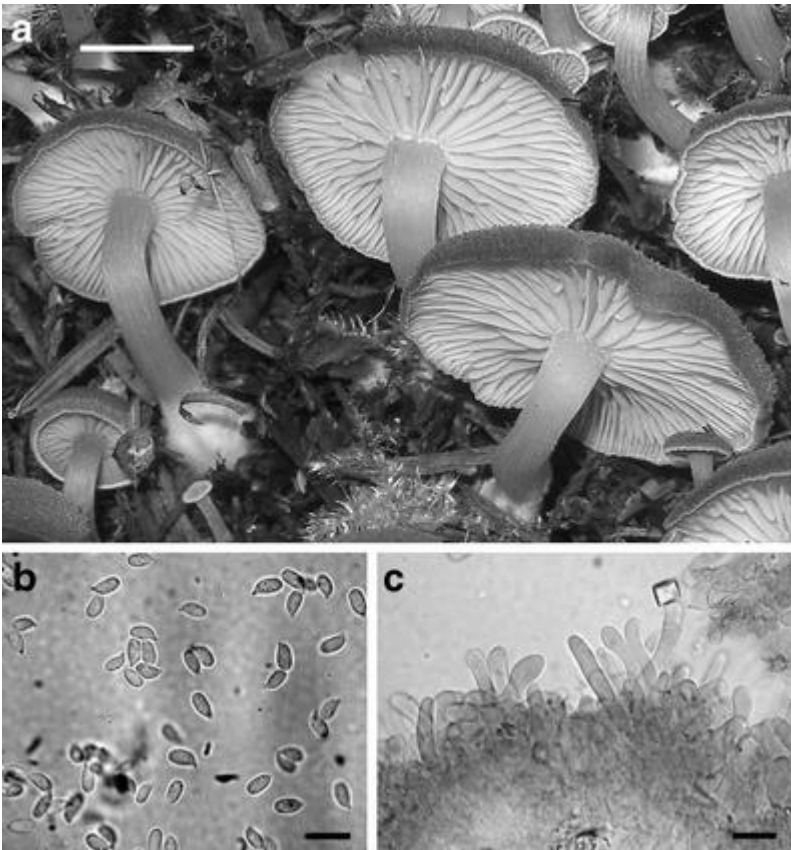


Fig. 3 *Trichocybe puberula* (TO-HG 1148, Musumeci 7092-08). **a** Basidiomes. **b** Basidiospores. **c** Cheilocystidia. Bars: a = 10 mm, b–c = 10 μ m

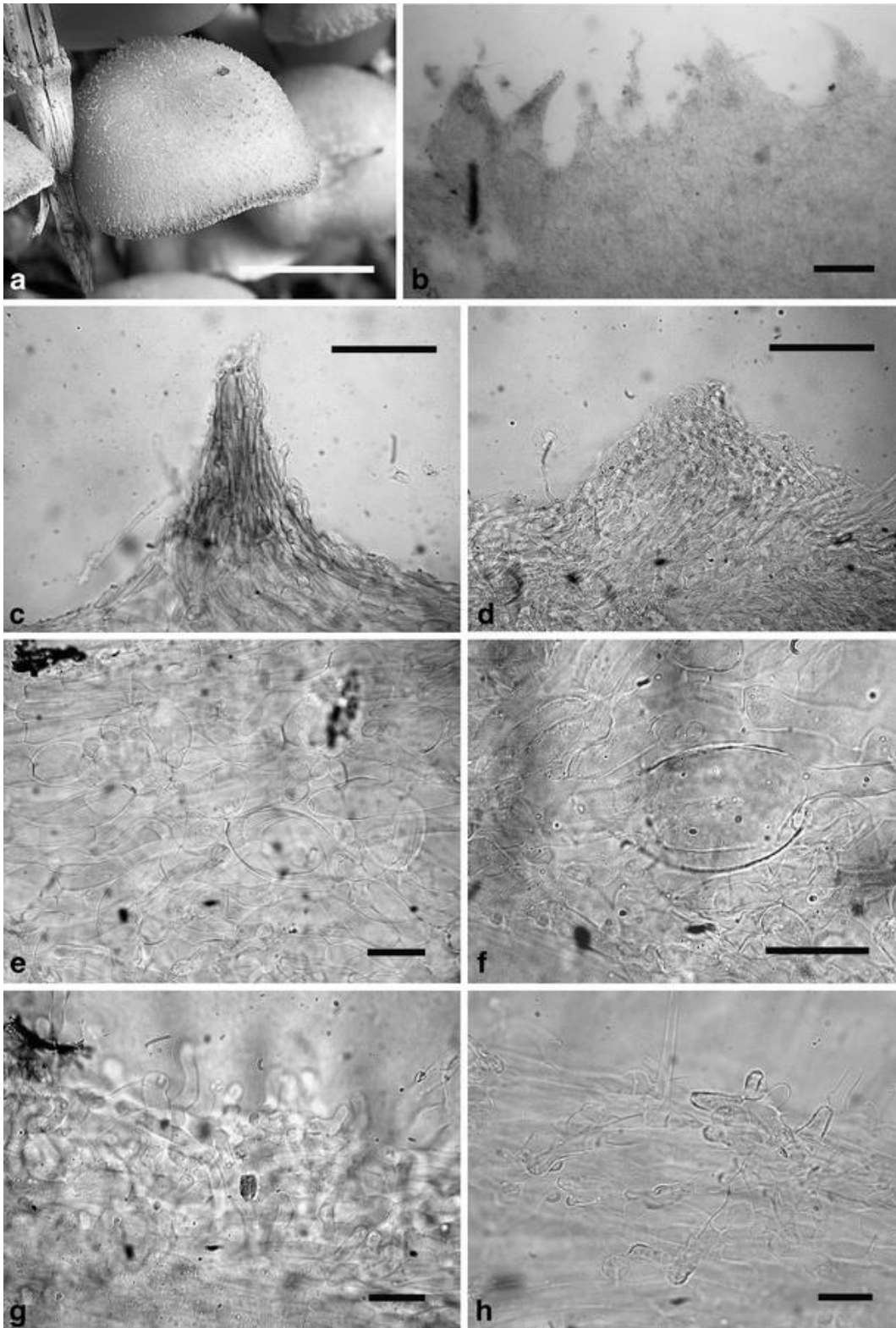


Fig. 4 *Trichocybe puberula*. **a** Young basidiomes with well-developed squamules (TO-HG 1148, Musumeci 7092-08). **b, c, d** Pileipellis: ascending bunches of hyphae (**b, c** TO-HG 1148, Musumeci 7092-08; **d typus**). **e, f** Subpellis: physaloid-vesiculose elements (**e** TO-HG 1148, Musumeci 7092-08; **f typus**). **g** Diverticulate hyphae of the pileipellis (**typus**). **h** Diverticulate hyphae of the stipitipellis (**typus**). Bars: **a** = 10 mm, **b–d** = 100 μ m, **e–h** = 10 μ m

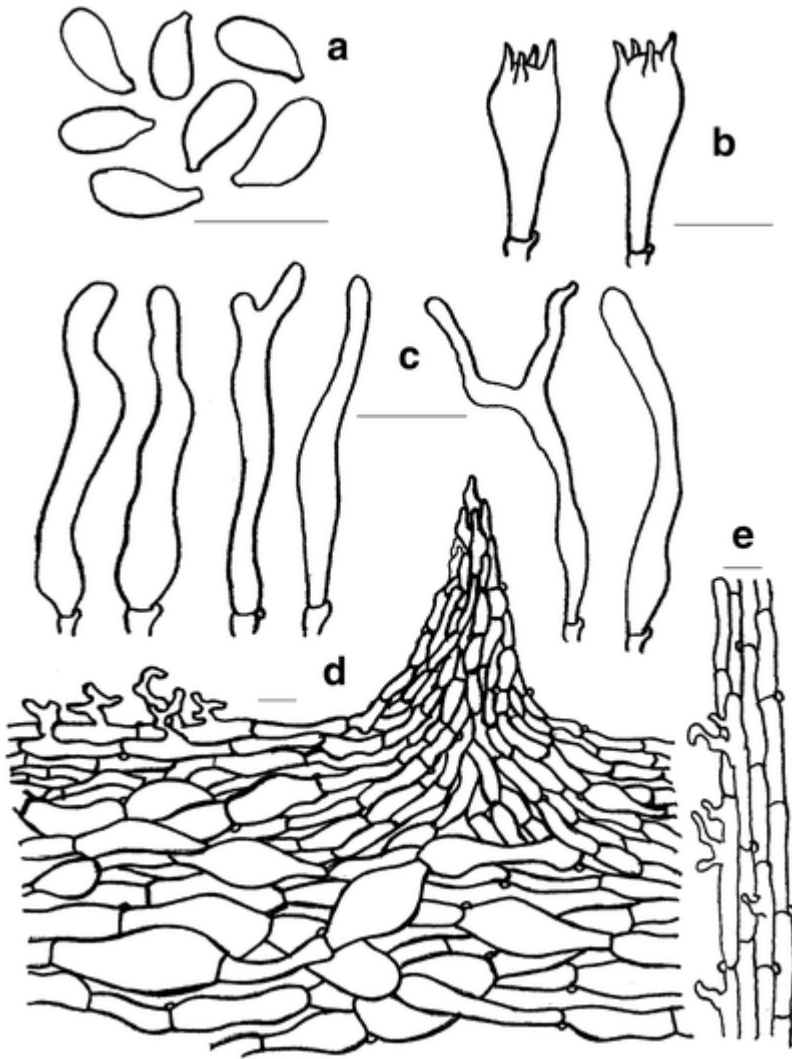


Fig. 5 *Trichocybe puberula* (TO-HG 1148, Musumeci 7092-08). **a** Basidiospores. **b** Basidia. **c** Cheilocystidia (marginal cells of lamellae). **d** Pileipellis and subpellis. **e** Stipitipellis. Bars = 10 μ m

Basionym: *Clitocybe puberula* Kuyper, Sydowia 36: 173 (1983).

Pileus 10–45 mm broad, at first hemispherical, broadly conical-trapezoidal to convex, expanding with age to plano-convex, then nearly applanate, slightly depressed at centre to funnel-shaped when old; with involute, then straight margin, finally with irregularly undulate marginal zone, sometimes fimbriate, pubescent, membranaceous to thin-fleshed, translucently striate when moist; dry, hygrophanous, at first roughly pruinose-pubescent overall due to minute white squamulose flocci persisting towards margin; when young grey-ochraceous, ochre-brown, then brown, vinaceous-brown, with margin darker, dark brown to blackish brown, with vinaceous-violaceous tinges, pallescent when wet to cream-yellowish, whitish-cream. **Lamellae** L= 26–30, l = (1) 2–3(–4), up to 4–5 mm broad, moderately distant, sinuate to broadly adnate to slightly decurrent, often slightly intervenose, sometimes partially fused with the lamellulae and then forked, firm, flexible-greasy to the touch, fairly thick, with entire concolorous edge; whitish-cream with lilac hues, then yellowish-ochre. **Stipe** 25–60 \times 3–7 mm, central to clearly eccentric, rarely lateral, cylindrical or slightly broadened towards the base, often curved or flexuous, fistulose, with abundant white mycelium at base, dry, finely white fibrillose-granulose at apex, minutely whitish-striate downwards; cream-whitish, ochre-brown, concolorous with lamellae at apex, distinctly darkening towards the base. Rhizomorphs absent. **Context** rather firm, non-reviviscent, tough in the pileus, fibrous in the stipe,

cream-whitish then ochre-brown, in the pileus 1.5–2.5 mm thick. **Smell**, strongly farinaceous, subrancid; taste mild, subfarinaceous, subrancid. **Spore-print** whitish.

Spores [90, 6, 3] (5.0)6.5–8.5(–9) × (3.0) 3.5–4.8(–5.5) μm, on average 7.5 × 4.2 μm, Q = (1.3)1.6–1.9(–2.0), Q_m = 1.75, smooth, hyaline, thin-walled, inamyloid, non-dextrinoid, acyanophilous, only a small minority in tetrads, subellipsoid-subcylindric in side-view, oblong-ellipsoid in front-view.

Basidia 22–30 × 6–7 μm, 4-spored, claviform to pear-shaped, clamped, with basal clamp connection, lacking siderophilous granules. **Pileipellis** a slightly gelatinized plagiotrichoderm (sensu Clémenton 2004), i.e. a cutis of 3–11 μm wide, cylindrical to physaloid periclinal hyphae, which patchily has a tendency to form a trichoderm; the erect, repent elements rising from the cutis may be isolated, tufted into squamules or even coalesce into dense, 120–450 μm high, pyramidal or oblique bunches of yellow-pigmented hyphae. The hyphae may have 10–80 μm long lateral outgrowths. **Subpellis** made up of largely cylindrical to physaloid-vesiculose intercalary elements, 5–30 μm wide, sometimes sphaerocyte-like. **Stipitipellis** consisting of slightly gelatinized hyphae, (2.5)4–8(–10) μm wide, sometimes with a fine yellow pigment and with yellow, often minutely incrustated, walls, often diverticulate and with long outgrowths. **Stipititrama** composed of longitudinally running, thin-walled, up to 30 μm wide, cylindrical, unbranched, smooth, colourless, inamyloid hyphae. **Cheilocystidia** 35–60 × 3–5 μm, scattered, mostly cylindrical, sublageniform, often curved and flexuous, sometimes forked at apex, with branched apex, hyaline, thin-walled.

Pleurocystidia very rare, in the form of occasional cylindrical-filiform (thread-like) elements.

Hymenophoral trama subparallel, subregular, consisting of 2.5–18 μm wide hyphae. **Pigment** intracellular and minutely incrusting. **Clamp connections** present in all tissues.

Habitat: gregarious, saprobic on saw-dust heaps, marcescent straw bales mixed with wood debris, stumps.

Known distribution: so far known only from northwestern Europe, viz. France, Switzerland, Germany and Scandinavia (Norway).

Material examined: FRANCE, dept. Doubs: Vaclusotte, Dessoubre valley, on sawdust, 01 May 1969, leg. M. Poulain, det. Th.W. Kuyper (L 0053903; **typus**); SWITZERLAND, Ettingen: on marcescent, decaying straw bales mixed with wood debris, in a *Picea abies/Abies alba* forest on calcareous soil, 480 m a.s.l., 05 April 2008, leg. E. Musumeci & K. Minder, det. A. Vizzini (TO-HG 1148, Musumeci 7092-08); ibidem, 26 April 2008 (TO-HG 1149, Musumeci 7565-08); ibidem 26 April 2008, on an *Abies alba* stump (Musumeci 7650-08).

Discussion

Clitocybe puberula was originally described by Kuyper (1983) on the basis of a French collection made by Poulain, in 1969, and later forwarded to Huijsman who was the first to point out that the species had non-revivicent basidiomata, in contrast to most *Clitocybe* species (Huijsman 1971). Furthermore, Kuyper (1983), in order to emphasize the unique features of the species, established the new section *Puberulae* to accommodate this deviating taxon.

Afterwards, new collections of the species were reported from France by Poulain (1990, in the same original locality), Cheype (2005) and from Germany by Lohmeyer et al. (1993); Bon (1997) mentioned some collections from Norway. It seems to be usually associated with conifer wood debris. The specimens from Italy, identified by Alessio (1991) as *Clitocybe puberula*, are of doubtful taxonomic attribution: the Herbarium specimens at TO are lacking, and, in our opinion, judging from the original description they represent a taxon within the tricky *Gymnopus dryophilus* complex.

The original description of *C. puberula* (Kuyper 1983) fits well the Swiss collections but for the fact that the Dutch mycologist gives an incomplete sketch-like description of the pileipellis (indicated as simply trichodermic), without reporting squamules, diverticulate hyphae and physaloids. On revising the type collection (L), however, it was possible to observe the distinctive hyphal bunches of the pileipellis squamules, its diverticulate hyphae, and the vesiculose elements of the subpellis (Fig. 4d, f, g, h). The hyphal outgrowths in pilei- and stiptipellis (up to 80 µm long) were found to be slightly longer in comparison with the Swiss material, but the differences are here considered to fall within the phenetic plasticity of the species: our DNA analyses, in fact, clearly demonstrate that the Swiss specimens are conspecific with the type material (Figs. 1, 2).

Several independent clitocyboid lineages have recently been identified in multigene phylogenetic studies of the *Agaricales* (Matheny et al. 2006; Ammirati et al. 2007). Four clitocyboid lineages are distributed in the Tricholomatoid clade, one out of the six major clades of *Agaricales* identified by Matheny et al. (2006): (1) *Neohygrophorus* Singer (see Redhead et al. 2000) with amyloid spores; (2) *Clitocybe* (Fr.) Staude *sensu stricto* [(typified by *C. nebularis* (Batsch) P. Kumm.)], close to *Lepista* (Fr.) W.G. Sm. and *Collybia* (Fr.) Staude s.str. and together forming the tribe *Clitocybeae* Fayod; (3) a cluster including *Clitocybe candicans* (Pers.) P. Kumm. and *C. subditopoda* Peck, possibly the sister-group to the *Entolomataceae* and *Lyophyllaceae*; and (4) *Cleistocybe* Ammirati, A.D. Parker & Matheny (Ammirati et al. 2007), a genus including the new species *C. vernalis* (type species) and the comb. nov. *C. gomphidioides* (A.H. Sm.) Ammirati, A.D. Parker & Matheny, which clusters within the *Catathelasma* clade [(together with *Catathelasma* Lovejoy, *Macrocybe* Pegler & Lodge, *Callistosporium* Singer and *Pleurocollybia* Singer). It is distinguished by an interwoven hymenophoral trama with divergent elements when young, and a distinct or ephemeral fibrillose to submembranous partial veil. Three additional lineages include: (1) *Ampulloclitocybe* Redhead, Lutzoni, Moncalvo & Vilgalys (= *Clavicybe* Harmaja 2002), which is closely related with taxa in the Hygrophoroid clade. Among clitocyboid taxa, *A. clavipes* (Pers.) Redhead, Lutzoni, Moncalvo & Vilgalys is uniquely characterized by the formation of coprine-like compounds (fatty acid relatives acting as in vitro aldehyde dehydrogenase inhibitors and inducing an antabuse-like syndrome, Kawagishi et al. 2002) and, ultrastructurally, by the minutely roughened basidiospores (in contrast to the smooth-spored *Clitocybe* or echinate-spored *Lepista*). *A. clavipes* also produces a new class of tyrosine kinase inhibitors, named *clavilactones* (Cassinelli et al. 2000); (2) *Cantharocybe* H.E. Bigelow & A.H. Sm., which appears related to the *Pleurotaceae*, and is characterized by large, oblong to subcylindric spores, and the presence of cheilocystidia; and (3) *Infundibulicybe* Harmaja, which currently occupies an unresolved position in the *Agaricales*

(Bigelow and Smith [1973](#); Redhead et al. [2002](#); Harmaja [2003](#)), a genus erected by Harmaja ([2003](#)) where to place *Clitocybe sensu lato* species that differed in having mycelia that cannot reduce nitrate, lacrymoid spores with confluent bases, a cyanophobic spore wall, and spores that do not adhere together in tetrads.

In both our phylogenetic trees (Figs. [1](#), [2](#)) *Clitocybe puberula* (= *T. puberula*) is neither closely related to *Clitocybe nebularis*, the typus of the genus *Clitocybe* (Redhead et al. [2002](#)) nor to other *Clitocybe* species with high posterior probability value. Based on macro- and micromorphological data and DNA analyses (Figs. [1](#), [2](#)), *Clitocybe puberula* represents a new phyletic line of clitocyboid fungi and is sufficiently different from other taxa within *Agaricales* to warrant the erection of a new genus: *Trichocybe*.

Trichocybe is clearly circumscribed from both the other clitocyboid lineages and the *Agaricales* (Euagarics) in toto, by having a unique combination of macro- and micromorphological characters that includes: a) a *Gymnopus*-like habitus with adnate to slightly decurrent gills; b) a pubescent pileus with squamulose, pyramidal flocci; c) a subcentral to eccentric stipe; d) a putrescent non-revirescent context with a strongly farinaceous odour; e) rare but well-differentiated cheilocystidia; f) a peculiar, highly differentiated trichodermal pileipellis; g) a subpellis with vesiculose-physaloid elements; h) the presence both in the pileipellis and stipitipellis of diverticulate hyphae; i) a white spore deposit, and smooth, acyanophilous, inamyloid basidiospores; l) a preference for wood debris (sawdust, straw bales, stumps).

With regard to the peculiar *Trichocybe* pileipellis structure, the distinctive hyphal bunches giving rise to the squamules are strongly reminiscent of the cylindrical to conico-pyramidal and gravitropic hyphal projections observed in the hymenium of some polyporoid fungi such as *Epithele* (Pat.) Pat., *Polyporus* P. Micheli ex Adans., *Lentinus* Fr., called hyphal pegs or hyphal papillae (Eriksson and Ryvarden [1975](#), [1976](#); Pegler [1983](#)). However these structures are evidently not homologous.

The globosely inflated hyphae in the subpellis of *Trichocybe* call to mind, to some extent, the abruptly swollen elements described in the *Clitocybe* species of the section *Bulluliferae* Singer and the sect. *Cystoclitus* Singer of the subgenus *Cystoclitus* Singer [(type species = *C. phaeophthalma* (Pers.) Kuyper; Singer [1986](#))], as apical, terminal and/or intercalary elements, respectively (Singer [1986](#); Takahashi [2003](#)), but they differ in having no inner granular inclusions. Based on our phylogenetic analyses (Figs. [1](#), [2](#)), clearly *C. phaeophthalma* is not closely related either to *Trichocybe* or other taxa of the Tricholomatoid clade, consequently we accept this lineage to represent a distinct genus and suggest resurrecting the genus *Singerocybe* Harmaja ([1988](#)) for *C. phaeophthalma* and allied species.

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