



## Phylogenetic reappraisal and epitypification of *Laccaria macrocystidiata* (Hydnangiaceae, Basidiomycota)

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### Abstract

*Laccaria macrocystidiata*, a marasmioid species, originally described as *Laccaria affinis* f. *macrocystidiata* from Central Italy, was synonymised with the North American taxon *Laccaria laccata* var. *pallidifolia*, but only on a morphological basis. In this paper, the independent position of *L. macrocystidiata* from *L. laccata* var. *pallidifolia* and other species of *Laccaria* is pointed out on the basis of ITS-LSU phylogenetic analyses. According to our results, *Laccaria macrocystidiata* var. *longispinosa* is considered a synonym of *Laccaria macrocystidiata*. *Laccaria macrocystidiata* holotype consists of only one basidioma in poor condition; thus, a new epitype from an Italian collection was designated.

**Keywords:** Agaricomycetes, ectomycorrhizal fungi, Italy, taxonomy

### Introduction

*Laccaria* Berkeley & Broome (1883: 370) is a large and widely distributed genus (Kirk *et al.* 2008) capable of forming mycorrhizal associations with numerous plant hosts belonging to Pinaceae, Myrtaceae, Salicaceae, Fagaceae, Dipterocarpaceae, Nothofagaceae and Fabaceae (Mueller, 1992). A recent study by Wilson *et al.* (2016), using a phylogenetic analysis of four independent DNA regions (ITS, 28S, *RPB2* and *EF1- $\alpha$* ), has shown that there are at least 116 phylo-species of *Laccaria*. Some of these species are still not well-known, while others have still not been described. Although in the past, many taxa have been considered synonymous on morphological basis, many of the phylogenetic relationships among different taxa have not yet been resolved. Migliozi & Lavorato (1988) described a pink-red *Laccaria* from Lazio, Italy, which they named *Laccaria affinis* (Singer 1967: 111) Bon (1983: 49) f. *macrocystidiata* Migl. & Lavorato (1988: 6). It is a relatively robust species that is characterised mainly by its globose basidiospores with short spines up to 1.3  $\mu$ m, 4-spored basidia, and clavate to cylindrical cheilocystidia. Six years after the description of this variety, Pazmany (1994) proposed a new combination of *L. affinis* f. *macrocystidiata* in *Laccaria macrocystidiata* (Migl. & Lavorato) Pázmány (1994: 8), despite the fact that two years earlier Mueller (1992) had considered this taxon to be a synonym of *Laccaria laccata* var. *pallidifolia* (Peck 1890: 84) Peck (1912: 92). It is also remarkable to note that Bon & Contu (1985) described *Laccaria affinis* var. *sardoa* Bon & Contu (1985: 53), mainly on the basis of the pink colour of its basidiocarps without considering the presence of cheilocystidia and caulocystidia. Three years after, performing an accurate microscopic analysis of the species by Vellinga & Mueller (1987), the presence of conspicuous cheilocystidia and cystidia-like elements at the stipe apex was pointed out. However, Vellinga & Mueller (1987) didn't feel that these two characteristic elements were sufficient to describe a new taxon, so they placed *L. affinis* var. *sardoa* in the synonymy of *L. laccata* var. *pallidifolia*. Conversely, Contu (2003) placed *L.*

*affinis* var. *sardoa* in the synonymy of *Laccaria macrocystidiata* after re-evaluating the presence of cheilocystidia and caulocystidia, and excluding that it could be *L. laccata* var. *pallidifolia* due to the presence of large cheilocystidia and caulocystidia. The present paper aims to assess the taxonomic status of *L. macrocystidiata* to evaluate its relationship with *Laccaria laccata* var. *pallidifolia*. The genetic analysis includes several European samples of *L. macrocystidiata*, ranging from Italy to Greece, and new data for typical habitats and iconographic material are added. Additionally, the holotype of *L. macrocystidiata* consists of a single sample in a poorly conserved state, the amplification of said sample's ITS region has failed, and the microscopic analysis has shown partial deterioration of the sample. For this reason, based on the original description, one Italian collection was chosen as the epitype of *L. macrocystidiata* (see Table 1).

**TABLE 1.** Collections of *L. macrocystidiata* examined and sequenced in this study and their GenBank codes.

Species	Origin, and collection data	Habitat	Herbarium n.	ITS (LSU) GenBank acc. n.
<i>Laccaria macrocystidiata</i>	GREECE, Parnitha (Attica), 21.10.2009, leg. Vasileios Kaounas	<i>Abies cephalonica</i> and <i>Pinus nigra</i>	GDOR 5075	MW584893
<i>L. macrocystidiata</i>	ITALY, Piemonte, Giaveno (Torino), 13.10.2010, leg. Francesco Dovana	<i>Alnus</i> sp., <i>Betula pendula</i> and <i>Larix decidua</i>	GDOR 5076	MW584898
<i>L. macrocystidiata</i>	ITALY, Piemonte, Giaveno (Torino), 06.10.2015, leg. Francesco Dovana	<i>Alnus</i> sp., <i>Betula pendula</i> and <i>Larix decidua</i>	GDOR 5077	MW584897
<i>L. macrocystidiata</i>	ITALY, Piemonte, Voltaggio (Alessandria), 06.10.2010, leg. Francesco Dovana	<i>Pinus sylvestris</i> , <i>Pinus nigra</i> and <i>broad-leaf</i>	GDOR 5078	MW584892
<i>L. macrocystidiata</i>	ITALY, Calabria, Acri (Cosenza), 9.10.2015, leg. Carmine Lavorato	<i>Cedrus atlantica</i> and <i>Cedrus libani</i>	GDOR 5079	MW584891
<i>L. macrocystidiata</i> epitype	ITALY, Liguria, Bolzaneto (Genova), 17.11.2010, leg. Francesco Dovana	<i>Quercus ilex</i>	GDOR 5080	MW584890
<i>L. macrocystidiata</i> var. <i>longispinosa</i>	ITALY, Calabria, Vuturino (Cosenza), 14.10.2010, leg. Orlando Marsico	<i>Pinus</i> spp.	GDOR 5081	MW584889 (LSU MZ267722)
<i>L. macrocystidiata</i>	ITALY, Toscana, Rimigliano di San Vincenzo (Livorno), 04.12.2014, leg. Roberto Para	<i>Pinus</i> spp. and <i>Quercus ilex</i>	GDOR 5082	MW584895
<i>L. macrocystidiata</i>	ITALY, Toscana, Torrente Milia di Monterotondo Marittimo (Grosseto), 06.12.2014, leg. Roberto Para	<i>Quercus</i> spp.	GDOR 5083	MW584894
<i>L. macrocystidiata</i> var. <i>longispinosa</i>	ITALY, Sardegna, Madonna della neve (Olbia-Tempio), 16.11.2008, leg. Marco Contu	<i>Abies cephalonica</i>	GDOR 5084	MW584896
<i>L. macrocystidiata</i> var. <i>longispinosa</i>	ITALY, Sardegna, Madonna della neve (Olbia-Tempio), 16.11.2008, leg. Marco Contu	<i>Abies cephalonica</i>	AH 38996	MW751682
<i>L. macrocystidiata</i>	ITALY, Sardegna, Madonna della neve (Olbia-Tempio), 01.11.2008, leg. Marco Contu	<i>Cupressus</i> sp.	AH 38993	MW751684
<i>L. macrocystidiata</i>	ITALY, Sardegna, Monte Limbara (Valliciola), (Olbia-Tempio), 25.11.2006, leg. Marco Contu	<i>Pseudotsuga menziesii</i> and <i>Abies cephalonica</i>	AH 38995	MW751683

## Materials and methods

### *Morphology*

Macroscopic descriptions were made based on observations of fresh material. The specimens were dried with an electric dryer at 30°C. Voucher specimens were deposited in the herbarium Museo Civico di Storia Naturale Giacomo Doria (GDOR), and in the Herbarium AH (Plant Biology herbarium of the University of Alcalá, Madrid, Spain). The terminology used in the descriptions follows Vellinga (1988). Descriptions of the micro-morphological characteristics are based on the study of fresh and dried material rehydrated in KOH or NH<sub>4</sub>OH, while other reagents used were Congo Red, Phloxin B and Melzer's reagent. The description is based on the collections shown in Table 1. All observations and measurements were carried out with an optical trinocular Nikon Eclipse E200. Photographs of spores by SEM (critical point) were obtained with the use of a Zeiss-DSM 950 microscope (Moreno *et al.* 2018). Basidiospores were measured from both fresh spore deposits and dried specimens. Basidiospore dimensions are expressed as (a) b–c (d), where (a) = minimum value, b = (average – standard deviation), c = (average + standard deviation), and (d) = maximum value.

### *DNA extraction, PCR amplification and DNA sequencing*

The DNA was extracted with NaOH following the procedures of Dovana *et al.* (2017), the CTAB procedure by Doyle & Doyle (1987) or by using the Qiagen Mini Kit following the manufacturer's instructions. The nrITS region was amplified with primers ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990) and nrLSU region with primers LR0R and LR7 (Vilgalys & Hester 1990). PCR products were purified and sequenced by IGA Technology Services (Udine, Italy), Bio-Fab Research s.r.l. and Macrogen Inc. (Seoul, Republic of Korea). The collections that were analysed are reported in Tab. 1.

### *Sequence alignment, dataset assembly and phylogenetic analysis*

The sequences were checked and assembled with Geneious v. R 11.1.5 (<http://www.geneious.com>, Kearse *et al.* 2012) and compared to those available in the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>) using the BLASTN search method. Our dataset includes sequences of *Laccaria* from the Northern Hemisphere and tropical regions retrieved from previous phylogenetic studies (Wilson *et al.* 2013, Popa *et al.* 2014, 2016, Luo *et al.* 2016, Vincenot *et al.* 2017, Ramos *et al.* 2017, Cho *et al.* 2018, Wang *et al.* 2019). Other sequences with the greatest similarity to *L. macrocystidiata* available in GenBank were also selected based on BLASTN search, and one sequence of *Laccaria ambigua* K. Hosaka, A.W. Wilson & G.M. Mueller from Wilson *et al.* (2016: 1869) was added. *Mythicomyces corneipes* (Fr.) Redhead & A.H. Sm. (1986: 643) was used as the outgroup taxon following Luo *et al.* (2016). ITS and LSU sequences were independently aligned using MAFFT v 7.017 (Katoh *et al.* 2002) with default conditions for gap openings and gap extension penalties and subsequently concatenated. Partition Finder 2 (Lanfear *et al.* 2017) in CIPRES (Miller *et al.* 2010) was used to estimate the best partitioning schemes and evolution models for each subset with the MrBayes option. Datasets were analysed using Maximum Likelihood (ML) and Bayesian Inference (BI). The ML analysis was inferred with RAxML v. 8.2.11. (Stamatakis 2014) in Geneious v. R 11.1.4, GTR+G model was chosen for both ITS1-5.8S-ITS2 partitions, and a total of 1000 bootstrap replicates (with Rapid Bootstrapping and searches for the best-scoring ML tree algorithm) were performed to assess the relative robustness of the branches of maximum likelihood (Felsenstein 1985). BI was performed with MrBayes v.3.2 (Ronquist *et al.* 2012) in the CIPRES server; the SYM model was selected for 5.8S, GTR+G was selected for the ITS1 and ITS2, and HKY+I+G was selected for LSU regions, and two independent analyses of four MCMC chains were run for 20 million generations, sampling trees every 1000 generations. The first 25% of trees were discarded as “burn-in”, and for the remaining trees, a majority rule consensus tree was computed to obtain estimates for Bayesian Posterior Probabilities (BPP). Chain mixing and convergence were evaluated in Tracer v1.5 (Rambaut *et al.* 2018), considering ESS values >200 as a good indicator. Pairwise % identity values of the nrITS sequences were calculated using MEGA version X (Kumar *et al.* 2018). Significant support is considered to be ≥ 70% for bootstrap values in the ML analysis and ≥ 0.95 for BPP values in the Bayesian analysis.

## Results

### Phylogenetic analysis

The ITS-LSU dataset consisted of 130 sequences and 1621 base-pair positions. Ten *L. macrocystidiata* and three *Laccaria macrocystidiata* var. *longispinosa* Contu (2003: 21) sequences produced in this study and nineteen sequences retrieved from GenBank were grouped in a strongly independently supported clade named “/macrocystidiata clade” (MLB = 100%, BPP=1; ITS average pairwise % identity value = 99.8%) (Fig. 1). Within the /macrocystidiata clade, the newly generated sequences grouped with six sequences retrieved from GenBank (EU816633, MT730600, KC686872, MK028444, KX886047 and KM576421) in “subclade A” (MLB=58\*, BPP=0.99) (Fig. 1), and they were characterised by the presence of a -T- insertion in the ITS2 region.

## Taxonomy

*Laccaria macrocystidiata* (Migl. & Lavorato) Pázmány, *Z. Mykol.* 60(1): 8 (1994)

Basionym: *Laccaria affinis* f. *macrocystidiata* Migl. & Lavorato, *Micol. Ital.* XVII(2): 6, (1988)

Holotype—Italy, Lazio, Roma, Castelfusano, 30 November 1987, V. Migliozi, M.V.148/87 (now stored in TR, MUSE - Museo delle Scienze) Fig. 2.

=*Laccaria affinis* var. *sardoa* M. Bon & Contu in *Doc. Mycol.* fasc. 59: 53, (1985)

=*Laccaria macrocystidiata* var. *longispinosa* Contu, *Bollettino del Gruppo Micologico “G. Bresadola”* 46 (1): 21 (2003)

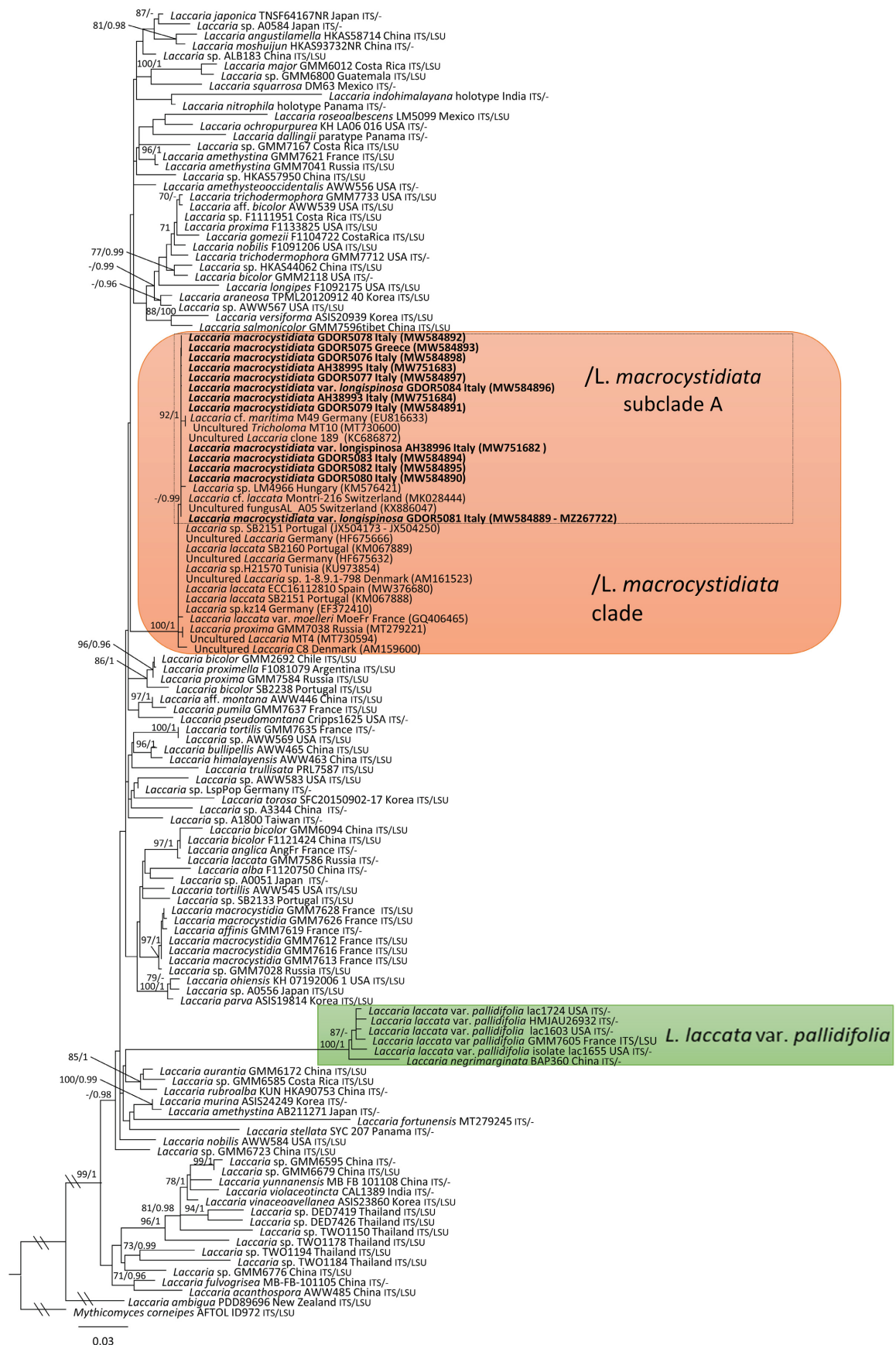
Epitype (*hic designatus*) (MycoBank typification number: MBT 10001563):—Italy, Liguria, Genova, Bolzaneto, (GDOR 5075) Fig. 3,4,5.

## Description

*Pileus* 20–120 mm diam., at first hemispheric then convex to plano-convex, generally with central depression, rarely striate, hygrophanous, covered with fine squamules or tufts, pale brown, orange to orange-brown, pallescent to white or cream on the centre upon drying. *Lamellae* emarginate, sometimes with decurrent tooth, up to 12 mm broad, pale flesh-pink when young, later sordid pink-brown or concolourous with pileus. *Stipe* 30–130 × 3–10 mm, cylindrical, sometimes broadened towards the base, solid to fistulose in early-stage, orange-brown or brown, generally darker than pileus, sometimes fibrillose-striate, white furfuraceous in upper part, with white basal tomentum. *Context* hygrophanous and concolourous with or paler than the surface. Smell fungoid to herbaceous-fungoid. Taste mild. Spore print white. *Basidiospores* (7.0–)7.5–10.0(–12.0) × (6.5–)7.4–9.7(–11.0) μm, Q = 0.99–1.05(–1.09), globose to subglobose, with spines variable in shape and dimension, from pyramidal to finely conical, the second type’s longest spine up to 2.5 μm in length after rehydration. *Basidia* 25–45 × 10–16 μm, clavate, rarely flexuose, hyaline, 4-spored, sterigmata up to 9 μm long. *Cheilocystidia* 35–150 × 6–16 μm, cylindrical to flexuose, rarely coralloid, generally obtuse, sometimes tapered towards the apex, colourless. *Stipitipellis* a cutis of 3–6 μm wide cylindrical hyphae with caulocystidia frequent, scattered or in clusters, 10–140 × 6–16 μm, variable in shape and dimension, clavate, cylindrical to flexuose, sometimes they are lateral extroflexions of hyphae. *Pileipellis* a cutis of radially arranged cylindrical hyphae, often with uplifted bundles of hyphae; terminal elements are similar to cheilocystidia, sometimes bifurcate.

Observations of the *L. macrocystidiata* holotype

The holotype consists of a single basidiome in a bad state of conservation (Fig. 2). There are few gills generally without their edge portions or in a bad state of preservation, so cheilocystidia were not found in the examined parts of the lamellae. Spores don’t show spines (they are most likely collapsed), but cystidia-like elements up to 40 μm long were found on the stipe surface, and their shape is in accordance with what has been described above.



**FIGURE 1:** Maximum likelihood phylogenetic tree inferred from the dataset of ITS1-5.8S-ITS2-LSU sequences. Support values (MLB  $\geq 70\%$  / BPP  $\geq 0.95$ ) are given above branches. Newly generated sequences are in bold characters. In /*macrocystidiata* clade GenBank accession numbers given in brackets. When more than one GenBank accession numbers are present, the first is ITS and the second is LSU.



FIGURE 2. *Laccaria macrocystidiata* specimen from the holotype (M.V. 148/87) and label. Photo by F. Dovana.



FIGURE 3. *Laccaria macrocystidiata* specimens from the epitype (GDOR 5075). Photo by F. Dovana.  
The epitype of *Laccaria macrocystidiata*

The macro-micromorphological features of collection GDOR5080 designated here as the epitype fit well with the original description reported in Migliozi & Lavorato (1988) and the above description reported here. The particular measures of the epitype (GDOR5080) are the following: pileus 20–100 mm diam., spores (8.0–)8.6–10.2(–11.5) × (8.0–)8.6–10.1(–11.0) μm, Q = 1.00–1.02(–1.06), cheilocystidia 50–140 × 8–14 μm, caulocystidia 10–130 × 7–15 μm.



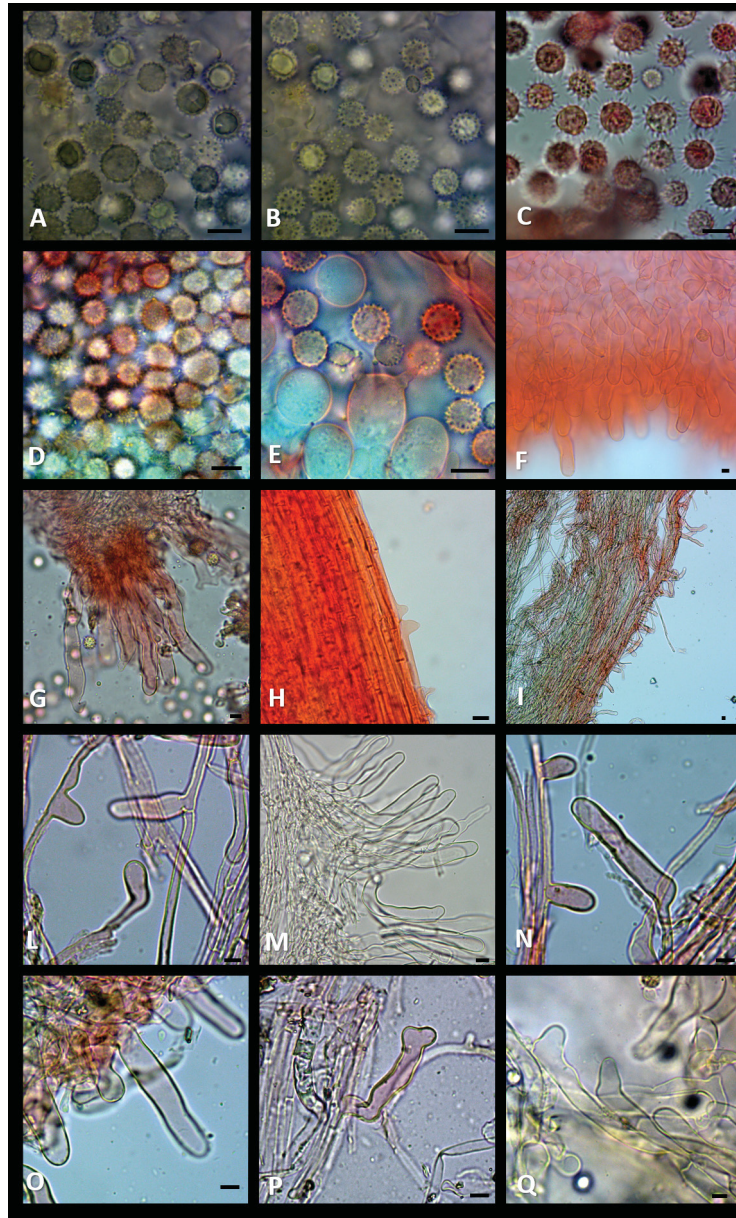
**FIGURE 4.** *Laccaria macrocystidiata*. A–F Fresh basidiomata. A–B Coll. GDOR 5080. C Coll. GDOR 5078. D Coll. GDOR 5082. E Coll. GDOR 5079. F. Coll. GDOR 5077. Photos: A–C,F by F. Dovana; D by R. Para; E by C. Lavorato.

## Discussion

Macroscopically, *Laccaria macrocystidiata* is characterised by a medium-sized basidiomata with pink or orange to orange-brown pileus covered with minute scales and a generally darker stipe, especially towards the base, generally furfuraceous near apex, lamellae with a sterile edge, and white basal mycelium. Microscopically, it can be recognised by 4-spored basidia, globose to rarely subglobose spores with aculei variable in shape and dimension, cheilocystidia generally numerous and caulocystidia variable in shape. According to Contu's classification (2003) of *Laccaria*, the macro and micro-morphological characteristics of the basidiomata would place *L. macrocystidiata* in *Laccaria* sect. *Laccaria* subsect. *Amethystinae* (M. Bon 1983 : 46) Contu (1992 : 81). However, our molecular phylogenetic results did not support this attribution, and suggested that the European species with cheilocystidia are not monophyletic. In 2003, Contu described a new taxon, *Laccaria macrocystidiata* var. *longispinosa*, which differs mainly from *Laccaria macrocystidiata* var. *macrocystidiata* by its fawn-reddish colours and basidiospores with longer spines of up to 2,5 µm. In this phylogenetic analysis, one collection of *Laccaria macrocystidiata* var. *longispinosa* from Southern Italy and two collections from locus classicus collected and identified by Marco Contu grouped in the */macrocystidiata* clade with *L. macrocystidiata*, and didn't show phylogenetic differences with respect to other Italian collections of *L. macrocystidiata*. Our observations of *L. macrocystidiata* reveal a high variability in the shape and dimension of basidiospore spines among different specimens and even among different spores of the same basidiome.

In this study, *Laccaria macrocystidiata* var. *longispinosa* is treated as a synonym of *Laccaria macrocystidiata*. In some spores, the long conical spines are visible under the optical microscope only after long rehydration (Fig. 5: c-d), while under SEM microscope in the non-rehydrated samples, they appear mostly as aberrant spines appressed to the sporal wall (Fig. 6: C). This hypervariability in spine dimension has also been previously observed in *L. tortilis* (Bolton 1788: 41) Cooke (1884: 70) (Vizzini *et al.* 2011). In our phylogenetic tree, the */macrocystidiata* clade (Fig. 1) includes sequences from twelve countries: Denmark, France, Germany, Greece, Hungary, Italy, Poland, Portugal, Russia, Spain, Switzerland and Tunisia; this result supports the extensive diffusion of this species. Records of this species outside of Europe had already been reported from Turkey by Sesli *et al.* (2012) but without molecular confirmation. *Laccaria macrocystidiata* appears to have the capacity to form mycorrhizal associations with a wide range of host species, including Betulaceae, Ericaceae, Fagaceae, Pinaceae, and Salicaceae. Six sequences retrieved from GenBank

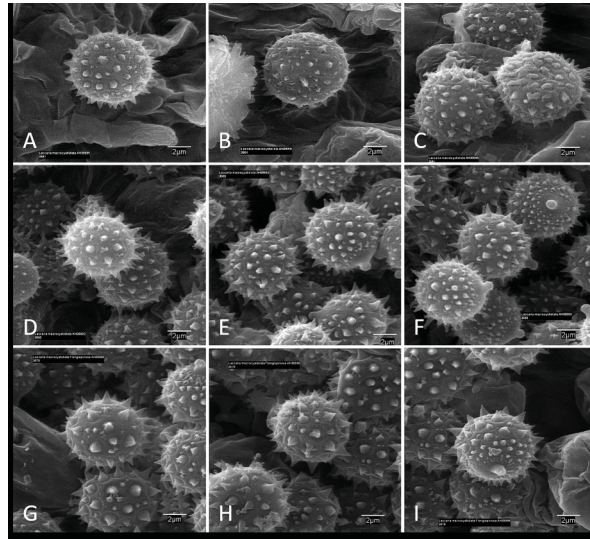
(KC686872, MT730594, EF372410, KM576421, AM161523 and KX886047) came from samples obtained from mycorrhizal roots of *Abies alba*, *Populus deltoides*, *Pyrola minor*, *Quercus petraea* and *Fagus sylvatica* (Kjøller 2006, Zimmer *et al.* 2007, Ważny & Kowalski 2017, GenBank source). In our field observations, *L. macrocystidiata* specimens were found under *Abies cephalonica*, *Pinus nigra*, *Pinus sylvestris*, *Cedrus atlantica*, *Cedrus libani*, *Pseudotsuga menziesii*, and *Larix decidua*, as well as under the broad-leaf species *Alnus* spp., *Betula pendula* and *Quercus* spp.



**FIGURE 5.** *Laccaria macrocystidiata*. **A–B** Spores (GDOR5080). **C–D** Spores (GDOR5081). **E** Basidia (GDOR5080). **F** Cheilocystidia (GDOR5080). **G** Cheilocystidia (GDOR5078). **H** Stipitipellis (M.V.148/87). **I–P** Caulocystidia (GDOR5080). **Q** Terminal elements of pileipellis (GDOR5080). Scale bars: **A–H, L–Q** 10  $\mu$ m. **I** 20  $\mu$ m. Photos by F. Dovana

In our phylogenetic analysis, five sequences that were labelled “*Laccaria macrocystidia*” (GMM7612, GMM7613, GMM7616, GMM7626, GMM7628), one *Laccaria* sp. (GMM7028) and one *Laccaria affinis* (GMM7619) grouped in a strongly supported clade (MLB=92, BPP=1) isolated from the *L. macrocystidiata* clade. “*Laccaria macrocystidia*” doesn’t seem to be a valid name for these taxa, due to the fact that as of 20<sup>th</sup> January 2021, no accession number with this name was available in Mycobank or Indexfungorum. Thus, *L. affinis* could represent a more appropriate name for this clade, but a future study that includes the holotype and/or topotypical material of *L. affinis* will be necessary to confirm this hypothesis. Previously, *Laccaria laccata* var. *pallidifolia* was considered by Mueller (1992) to be a synonym of *Laccaria macrocystidiata* based only on morphology without providing molecular evidence, but in our phylogenetic analysis, the former is shown to be phylogenetically distant from *Laccaria macrocystidiata*. Furthermore,

*L. laccata* var. *pallidifolia* presented as sister to *Laccaria negrimarginata*, a species originally described in mixed temperate alpine conifer forests of China with black scales on the pileus and a blackish lamellae edge (Wilson *et al.* 2013). From a morphological point of view, *Laccaria macrocystidiata* is distinguished from *Laccaria laccata* var. *pallidifolia* by larger cheilocystidia and abundant caulocystidia, while the other characters seem not to be useful in the separation of the two taxa. In conclusion, *Laccaria macrocystidiata* represents a well-characterised species from a genetic and morphological point of view; however only future studies that will include the holotypes of European species will be able to clarify the real relationships among different species belonging to this genus.



**FIGURE 6.** *Laccaria macrocystidiata*. Spores (SEM photographs). **A–C** Collection AH38995. **D–F** Collection AH38993. **G–I** Collection AH38996. Scale Bars = 2  $\mu$ m. Photos by G. Moreno.

## Acknowledgments

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