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ORIGINAL ARTICLE

The Caloscyphaceae (Pezizomycetes, Ascomycota), with a new genus

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Abstract The family Caloscyphaceae with a single genus, *Caloscypha*, has been considered to include a single species, *C. fulgens*. Study of an overlooked second species, *Caloscypha incarnata* from North Africa and Italy, using SSU, LSU rDNA, and morphology allows placement of this species in a new genus, *Kallistoskypha*, in the Caloscyphaceae. This fungus is found in association with *Eucalyptus* species. The species was recently redescribed from Spain under the name *Marcelleina parvispora. Caloscypha fulgens*, the type species of the genus *Caloscypha*, shows sequence variation from across its range.

Keywords *Eucalyptus* · *Marcelleina parvispora* · Conifer pathogen

Introduction

The family Caloscyphaceae, a family in the Pezizomycetes, was introduced by Harmaja (2002) to accommodate a single genus, *Caloscypha* Boudier. The type species of *Caloscypha, C. fulgens* (Pers.:Fr.) Boudier, is a distinctive vernal species of north temperate regions; principally, it grows under conifers and on calcareous soil, with an anamorphic state represented by *Geniculodendron pyriforme* (Paden et al. 1978). *Caloscypha fulgens* has been the only species generally recognized in the genus. Harmaja's characterization of the family centered on ascospores that lack secondary wall layers; an ascus wall that may thicken distally; paraphyses that taper apically; an anamorph, *Geniculodendron pyriforme*; and a distinctive phylogenetic placement first pointed out by Landvik et al. (1997), that has been confirmed by subsequent workers (Hansen and Pfister 2006, Perry et al. 2007). Earlier placements of *C. fulgens* suggested an alliance with the Pyronemataceae (Arpin 1969; Korf 1972, 1973). Recent work suggests that *Caloscypha* is allied to the families Karstenellaceae (Hansen et al. 2008), Helvellaceae, Tuberaceae, and Discinaceae (Perry et al. 2007).

A specimen, photographs, and drawings of a distinctive discomycete associated with *Eucalyptus* spp. from Italy was initially sent by one of the authors (Carlo Agnello) to the senior author (Donald H. Pfister) for identification. This initiated an in-depth study of the Caloscyphaceae when it was discovered, through comparing SSU and LSU rDNA sequences, that this fungus was close to *Caloscypha fulgens*. Additional specimens and commentary by Angela Lantieri and a search of the literature led to the other named species of *Caloscypha*, *C. incarnata* Duvernoy & Maine. Although well characterized in the original description, this species has been largely overlooked, and in fact it has recently been described as *Marcelleina parvispora* Rubio, Tabarés & Martinez (Rubio et al. 2010). Collections under both names have been made around *Eucalyptus* spp. in the Mediterranean region. The present phylogenetic and morphological study suggests that this species is best referred to a new genus. Several collections of *C. fulgens* from around the world were included in this study.

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Materials and methods

Morphological analyses

Examination of macro- and microscopic features is based on fresh material. Microscopic studies used a IOS trinocular optical microscope, cold light, and an Olympus BX40 compound microscope. The following mounting reagents were used: Melzer's reagent, Cotton blue in lactic acid, and Congo red in water and ammonia. Water mounts were used for observation of the colors and also for measurement of spores. At least 30 spores were measured from a spore deposit on a glass slide. The reaction of the asci were also tested in the dry state after rehydration in water and in 5 % KOH.

DNA samples

DNA was isolated and PCR conducted using the following specimens: DHP 10-686, collected by Carlo Agnello in Italy and sent to D.H. Pfister; *C. incarnata,* AL 18-01-2010 and AL 24-01-2011, both collected by Salvatore Spata and Santo Scandurra in Balestrate (Palermo), Le Macchie, Italy and determined by Angela Lantieri; *Marcelleina parvispora,* 23-01-2010, collected by Manuel Tabarés, Spain; *C. fulgens,* JK-11052605, collected by Jason Karakehian in the Olympic National Park, WA, USA; *C. fulgens,* KH.06.02 (FH), collected by Kitty Griffin in Norwich, VT, USA, and determined by Karen Hansen; and *C. fulgens*, DHP 08-05-1994 (Farlow Herbarium) collected by D.H. Pfister, Pratts Island, ME, USA.

DNA isolation, PCR, and sequencing techniques

DNA was extracted from the specimens using the Qiagen DNeasy Plant Mini Kit (cat. no. 69104). A 1/10 and 1/100 dilution of the DNA was used for PCR amplification of the SSU and LSU rDNA (ribosomal DNA) regions. The SSU was amplified using the NS1, NS2, NS4 (White et al. 1990) and SL1,SL122, and SL344 (Landvik et al. 1996) primers. Amplification of the LSU rDNA region utilized the primers LROR and LR5 (Moncalvo et al. 2000). All PCR reactions were done in a Peltier Thermal cycler PTC-200 (MJ Research, Watertown, MA, USA) and used Econo Taq DNA Polymerase (Lucigen, Middleton, WI, USA). PCR amplification, purification, and sequencing were as previously described in Hansen et al. (2005).

Sequence analysis

The data matrix used for the phylogenetic analyses included the combined SSU and LSU sequences obtained for DHP 10-686 (GenBank JX560173, JX560169), *C. incarnata,* AL 18-01-2010 (GenBank JX560171, JX560167) and AL 24-01-2011 (GenBank JX560172, JX560168), *Marcelleina parvispora,* 23-01-2010 (GenBank JX560174, JX560170), and a subset of the alignment from Hansen and Pfister (2006) available from TreeBASE (www.treebase.org) as accession S1598. Additional sequences of *Caloscypha fulgens* from GenBank (SSU and LSU, respectively) were included in the phylogenetic analysis: *Caloscypha fulgens* 1 DQ247807+DQ247799/Oregon, USA and *Caloscypha fulgens* 2 (U53374/Norway+FJ499390/Denmark). The outgroup taxa were *Orbilia auricolor* (DQ471001+DQ470953) and *Orbilia vinosa* (DQ471000+DQ470952).

A second dataset of LSU rDNA sequences was constructed for the phylogenetic analyses of *C. fulgens* isolates from different geographical locations. In addition to the sequences of *C. fulgens* (*C. fulgens*/JK-11052605, JX560164; *C. fulgens*/KH.06.02, JX560165; and *C. fulgens*/DHP 08-05-1994, JX560166), *C. incarnata*, and *M. parvispora* determined in this study, the following GenBank sequences were included in this matrix: *C. fulgens*/DED 6107, DQ220318; *C. fulgens*/AFTOL 152, DQ491483; *C. fulgens*/KH-97-06, DQ220319; *C. fulgens*/JV94-112, FJ499390. *Peziza badiofusca* (AF335132) and *Peziza vesiculosa* (AY500552) were used as outgroup taxa.

Sequencher 4.6 (GeneCodes, Ann Arbor, MI, USA) was used to edit the DNA sequences obtained. Alignment of the DNA sequences was done using Se-Al v.2.0a8 (Rambaut 1996). As previously described (LoBuglio and Pfister 2010), DNA sequence alignments were analyzed using: MrBayes v.3.0b4 (Ronquist and Huelsenbeck 2003) for obtaining Bayesian posterior probabilities (PP); Maximum Parsimony using PAUP 4.0b10 (MP; Swofford 2002); and Maximum Likelihood with RAxML-HPC2 on Abe through the Cipres Science Gateway (ML; Miller et al. 2009). Branch support for MP and ML analyses was determined by 1,000 bootstrap replicates.

Results

The combined SSU and LSU alignment of 77 taxa, belonging to the Pezizomycetes, included 2,697 bp. One region, 2,576–2,590 bp, was excluded in the phylogenetic analyses due to ambiguous alignment. Results from MP, Bayesian, and ML analyses identified a well-supported monophyletic Caloscyphaceae lineage (99, 95, and 75 %, respectively) that was positioned in all 3 analyses as basal within a clade that contains all of the Pezizales, excluding Pezizaceae and Ascobolaceae (Fig. 1). Two highly supported lineages were identified (Fig. 1) in the Caloscyphaceae clade. One included the two specimens of *C. fulgens*, and a second included the three *C. incarnata* specimens from Italy as well as the specimen of *Marcelleina parvispora* from Spain. DNA sequence comparison of the three *C. incarnata* specimens and

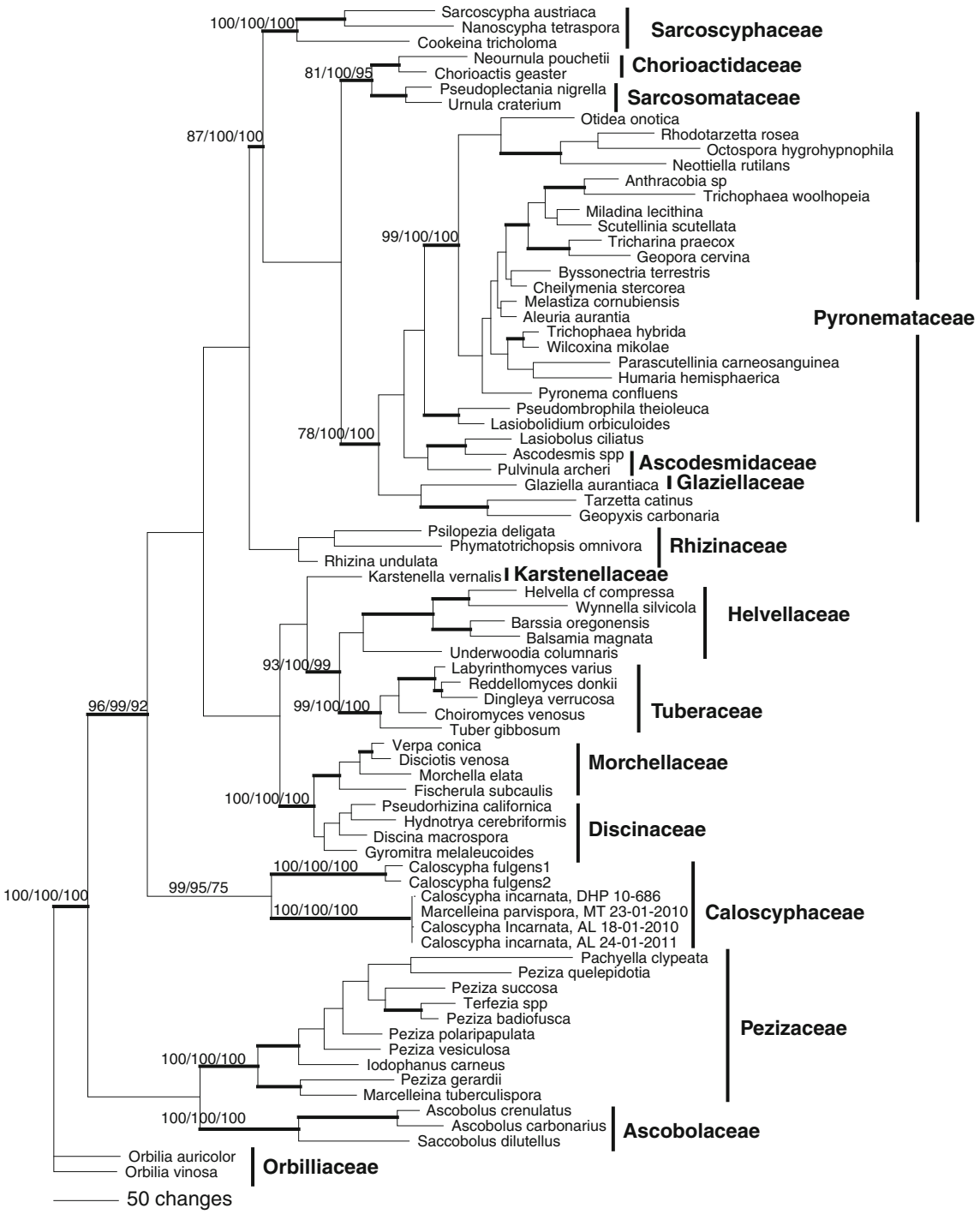

Fig. 1 Phylogenetic placement of the Caloscyphaceae within the Pezizomycetes. One of 18 most parsimonious trees from phylogenetic analyses of the 18S and 28S rDNA regions. Parsimony Bootstrap (MP) ≥75 %, Bayesian posterior probability (PP) ≥95 %, and RAxML (ML) bootstrap support ≥75 % are shown respectively above the branches. *Dashes* indicate support below these MP, PP and ML values. Internal branches that received MP, PP, and ML support ≥75, 95, and 75 %, respectively, are in *bold*. *Orbilia auricolor* (DQ471001 + DQ470953), and *Orbilia vinosa* (DQ471000 + DQ470952) were used as outgroup species

M. parvispora showed that these four specimens were 100 % identical in SSU and LSU rDNA sequences.

Phylogenetic analyses of the 28S rDNA region showed significant diversity among the isolates of *C. fulgens* examined (Fig. 2). The seven isolates formed two well-supported clades that correspond to geographic origins of the collections. The two isolates from New England formed one clade, while the second clade included samples originating from the western USA and Denmark.

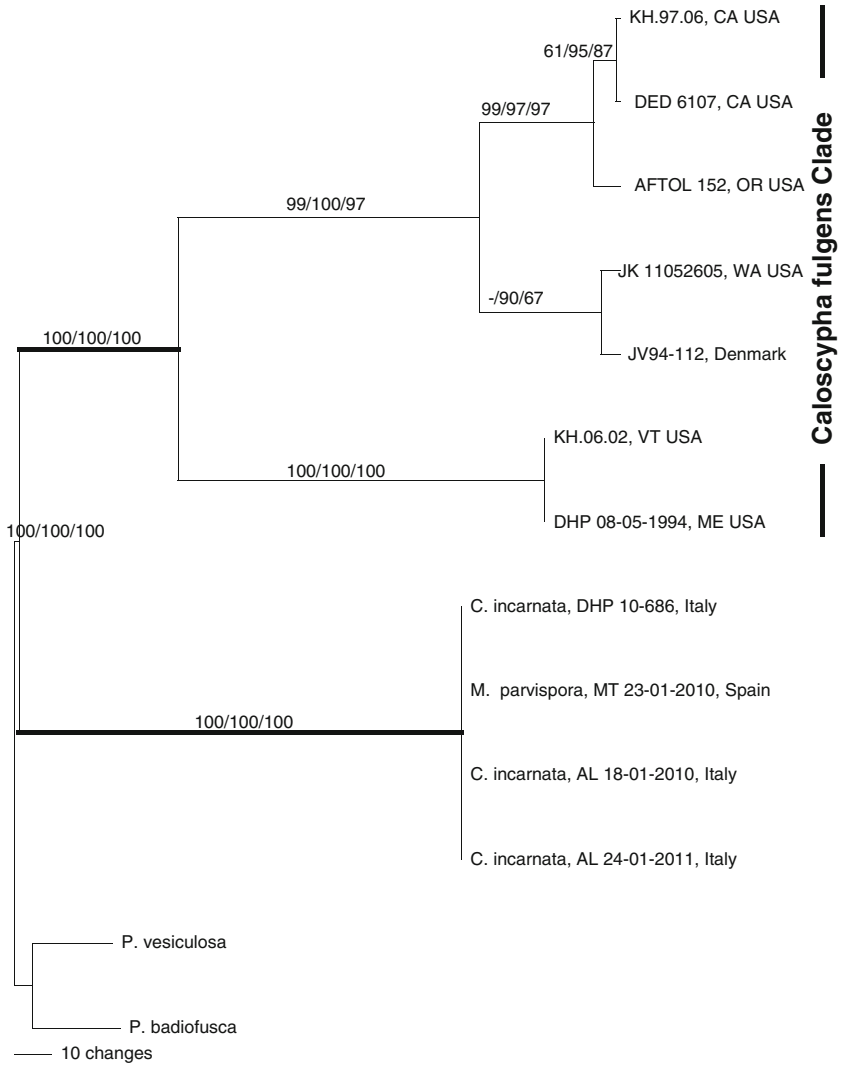

Fig. 2 Phylogenetic diversity within *Caloscypha fulgens*. One of 2 most parsimonious trees from phylogenetic analyses of the 28S rDNA regions. Parsimony bootstrap (MP) ≥60 %, Bayesian posterior probability (PP) ≥95 %, and RAxML (ML) bootstrap support ≥75 % are shown respectively above the branches. *Dashes* indicate support below these MP, PP, and ML values. *Peziza badiofusca* (AF335132) and *Peziza vesiculosa* (AY500552) were used as outgroup taxa

Morphological results are reported below in the description of the species

***Kallistoskypha* Pfister, Agnello, Lantieri, & LoBuglio gen. nov.**

Mycobank: MB 802268

Diagnosis: Apothecial ascomata up to 16 mm diam, hymenium pink, salmon to yellow-cream, often with a short stipe. Outer surface granulose or furfuraceous. Ascospores globose, guttulate. Asci 8-spored, inamyloid, operculate, often with gelatinous granules on the outside. Paraphyses septate, branched only slightly enlarged at the apex, vacuoles and cytoplasm with pigments. Medullary excipulum of several layers of globose to angular cells. Ectal excipulum of globose angular cells arranged in parallel rows, some of the outer cells giving rise to hyaline, often encrusted hairs.

Occurring on soil and plant debris around *Eucalyptus* spp. Type species: *Caloscypha incarnata* Duvernoy & Maine, *Bull. Soc. Hist. Nat. Afrique N.* **8**: 179 (1917)

Etymology: from the Greek "kallistos" – most beautiful and "skyphos," Greek, referring to cup.

***Kallistoskypha incarnata* Pfister, Agnello, Lantieri, & LoBuglio, comb. nov., Fig.** 3, 4, and 5

Mycobank: MB 802269

≡ *Caloscypha incarnata* Duvernoy & Maine, *Bull. Soc. Hist. Nat. Afrique. N.* **8**: 179. Maire 1917.

≡ *Barlaeina incarnata* (Duvernoy & Maire) Sacc., *Syll. Fung.* (Abellini) 24 (2): 1168. 1928.

= *Marcelleina parvispora* Rubio, Tabarés & Martínez, Revista Catalana Micol. 32: 31. 2011.

Apothecia deeply cupulate to cupulate, often compressed on one side, never completely flat even in age, up to 15–16 mm diam. **Hymenium** smooth, coral pink to cherry red in young specimens, changing with age to pale pink, fleshy pink, yellowish-cream, yellowish, with pinkish spots. **External surface** from white to whitish-yellowish to concolorous with hymenium at maturity, finely granulose, often with mycelial strands "entrapping" the substrate, hyaline hairs are visible with hand lens, especially at the margin. **Stipe** sometimes clearly visible, 3–4 mm long, with mycelial tufts present and firmly attached to soil remnants. **Flesh** waxy, very

Fig. 3 *Kallistoskypha incarnata*. Apothecia in living condition in situ. From collection of C. Agnello, 13 Feb 2010 (FH)

fragile, thin and without layers visible to naked eyes; no milk or exudate noted, smell and taste not significant. **Ascopores** globose, 7.31–8.70 µm (Vm 7.89), smooth, thick-walled, multi-guttulate with several small oil guttules, rarely with one large guttule, in deposit white. **Asci** 145–215×9–11.5 µm, cylindrical, 8-spored, inamyloid, operculate, aporinchous; close to the base, some asci have a more or less prominent "spur," or rudimentary bifurcation; along the walls granular/gelatinous lumps could be seen. **Paraphyses** as long as the asci, cylindrical, septate and branched, often several times, simple and slightly enlarged at the apex or more or less pointed, 2–3(–3.5) µm diam., sinuate, in a few cases, the cells of the paraphyses swollen and nearly moniliform, intracellular,

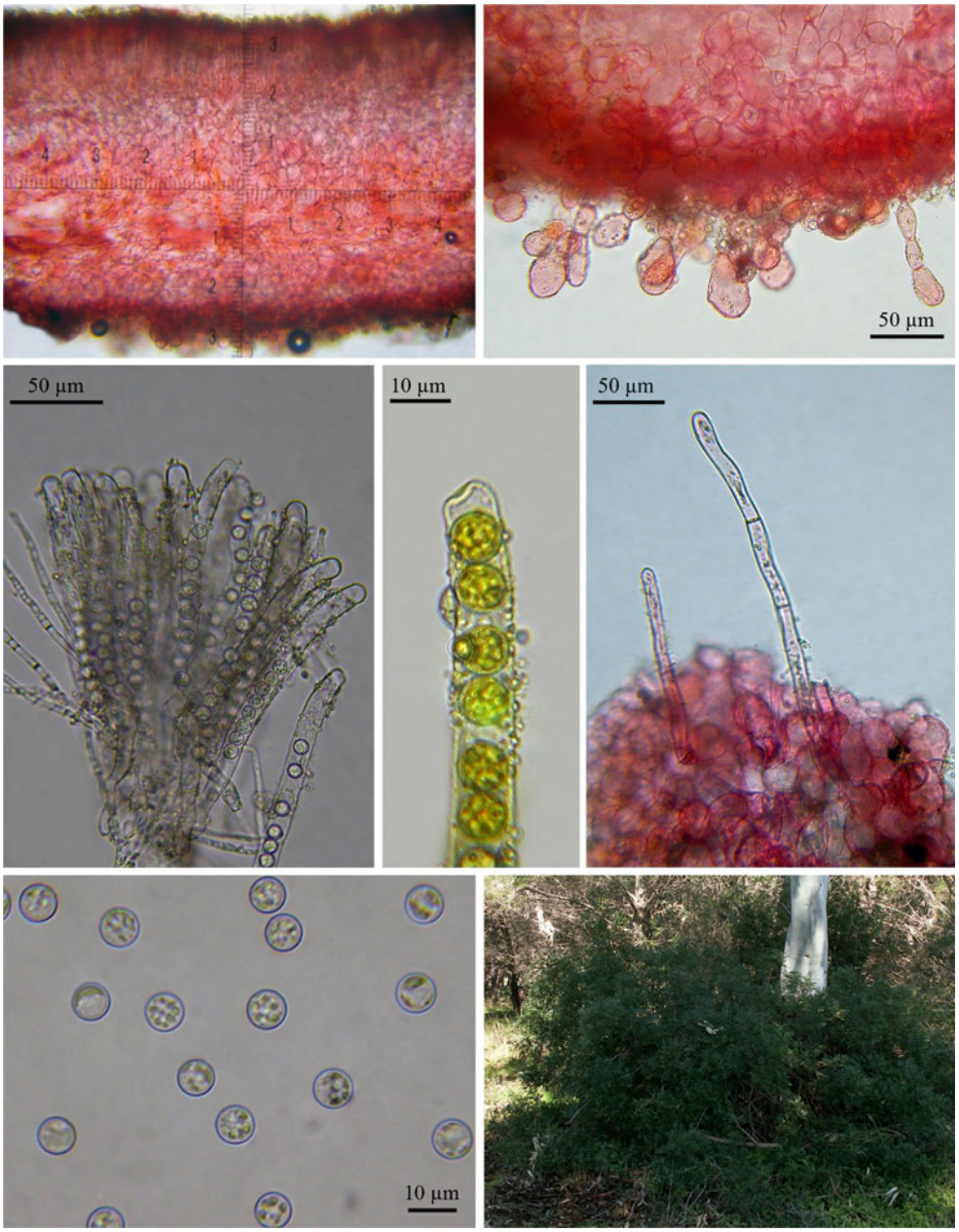

Fig. 4 *Kallistoskypha incarnata*. **a** Section mounted in Congo Red. **b** Outer cells of the ectal excipulum mounted in red congo. **c**. Asci, water mount. **d** Ascus in Melzer's, with gelatinous lumps. **e** Hairs from the ectal excipulum near the margin, mounted in red congo. **f** Spores in water mounts. **g** *Eucalyptus camaldulensis* and *Pistacia lentiscus* (with leaves removed in the *bottom left*). *Scale bars* as indicated. From collection of C. Agnello, 6 Feb 2010 (FH)

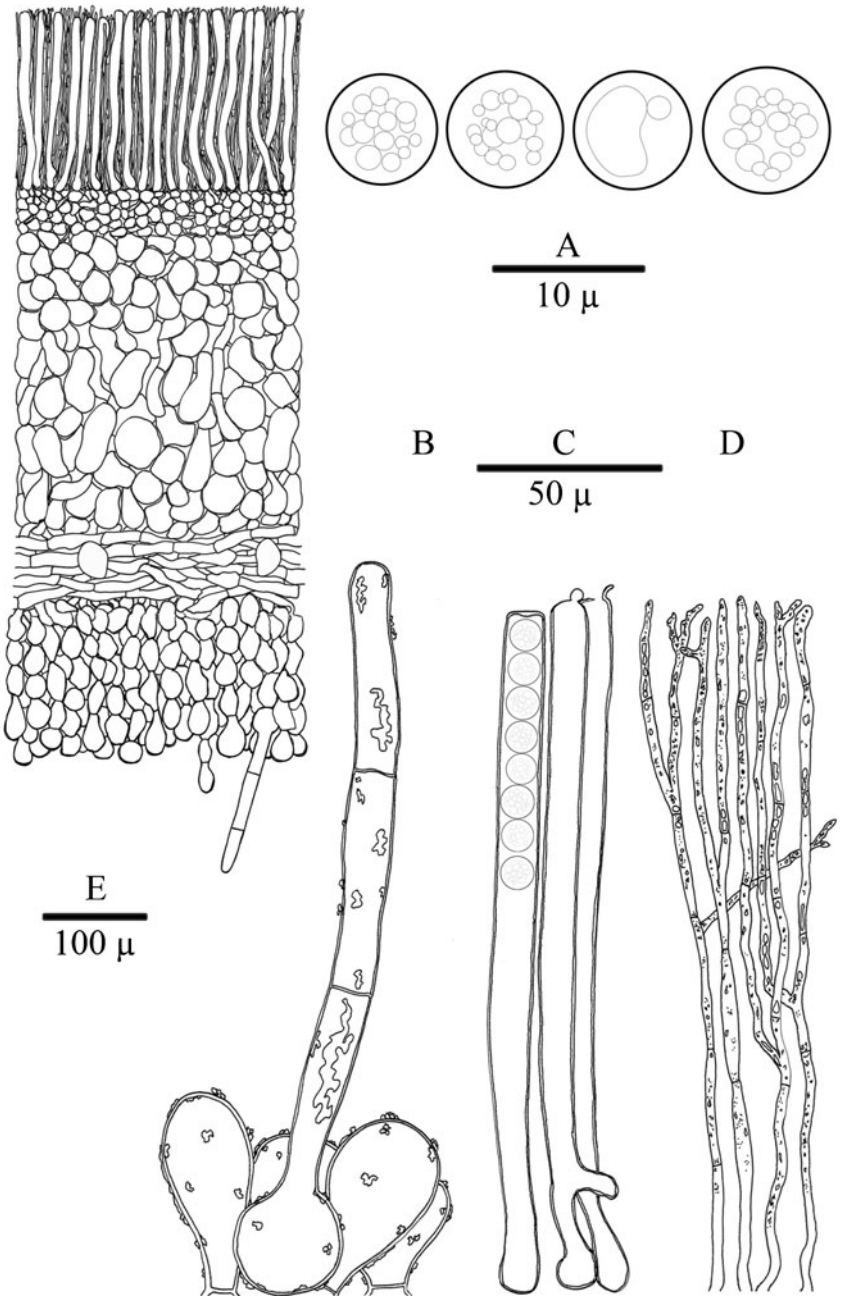

Fig. 5 *Kallistoskypha incarnata*. **a** Ascospores, **b** Excipular hairs, **c** Asci, **d** Paraphyses, **e** Cross section of ascomata. *Scale bars* as indicated. From collection of C. Agnello, 6 Feb 2010 (FH)

vacuolar and cytoplasmic pigments present, are brown-yellowish (yellow-green in Melzer's), pigments at low magnification give the hymenium a brown-reddish coloration in the young apothecia, hymenium becoming brown-yellowish in age. **Subhymenium** *textura globulosa-angularis* composed of cells 8–15 µm diam. **Medullary excipulum** upper part a *textura globulosa-angularis* composed of elements 20–35(–40) µm, below this region more elongate club-shaped, utriform cells, up to 80×40 µm mixed with others smaller and narrower hyphal cells that are parallel in orientation, below this there is *textura intricata*, 35–70×10–15 µm. **Ectal excipulum** thick, about 90–120 µm, of *textura globulosa-angularis*, made-up of chain of 3–5(–6) cells, with the terminal cell expanded, subglobose to club-shaped, thick-walled, incrusted and with granules of refractive granular/gelatinous matter, up to 30 µm wide. The terminal cells infrequently giving rise to hyaline, septate, incrusted, cylindrical hairs, with blunt apices. The hairs are up to 300 µm long and 11–15 µm wide, with walls up to 1.2 µm thick.

Habitat and ecology

In rich soil mixed with rotten wood debris; a few apothecia found on small pieces of wood or on dried leaves. Apothecia generally on soil under leaves of *Eucalyptus* species. Species of *Eucalyptus* that have been reported in the vicinity

of collections are: *E. camaldulensis* (reported here), *E. rudis, E. rostrata*, *E. globulus* (Maire 1917*), and E. gomphocephala* (Bertault MPU-Bert_01- 119-07). In Italy *Pistacia lentiscus* L. and *Pinus halepensis* Mill. were also in the area where the fungus was collected. *Plectania rhytidia* (Berk.) Nannf. & Korf was also found (on 13.02.2010) in this area.

Specimens examined ITALY, Puglia, Taranto, Manduria, in vicinity of Torre Colimena, at sea level, 1,800 m. inland, under *Eucalyptus camaldulensis*, 40°17′0″N, 17°44′0″E, 6 Feb 2010, leg. Carlo Agnello (FH); from the same locality, 13 Feb 2010 and 23 Feb 2010; Sicily, Balestrate, Palermo, in vicinity of Le Machie, on the ground in a reforested area with *E. camaldulensis*, 18 Jan 2010, leg. S. Spata et S. Scandurra, det. A. Lantieri (K(M) 169881); from same locality, 28 Jan 2010 (FH); from the same locality, 24 Jan 2011, K(M) 169880. SPAIN, Girona, Llagostera, bajo humus de *Eucalyptus* sp., 23 Jan 2010, leg. Manuel Tabarés et A. H. Tarin [?] (FH). Other specimens not consulted: a terre sous *Eucalyptus*, Oued Cherrat, Maroco, 16 Jan 1961 (MPU as *Barlaeina incarnata*); a terre sous *Eucalyptus gomphocephala*, El-Jadida, Maroco, R. Bertault, 5 Mar 1963 (MPU as *Barlaeina incarnata*)

Discussion

In this study. we show that the Caloscyphaceae is positioned basal to all the Pezizomycetes except the families Pezizaceae and Ascobolaceae—those with asci that react positively in iodine solutions. Landvik et al. (1997) first showed a placement of *C. fulgens*, the only species commonly recognized in *Caloscypha*, outside the Pyronemataceae where it had traditionally been placed. Subsequent phylogenetic studies have also pointed to a placement outside the Pyronemataceae among the families Discinaceae, Helvellaceae, Karstenellaceae, Morchellaceae, and Tuberaceae—the so-called "B lineage" of Landvik et al. (1997). The presence of carotenoid pigments and the small globose ascospores of *C. fulgens* suggested a placement in the Pyronemataceae. The pigments present in *K. incarnata* have not been characterized; those of *C. fulgens* were studied by Arpin (1969). In that species, he found beta-carotene and a carotenoid named P. 444. This was said to be a new pigment. The presence of carotenoid pigments in *C. fulgens* and its discoloration when bruised or in age led Korf (1972) to place the genus in a pyronemataceous tribe, Sowerbyelleae, with *Sowerbyella* and *Acervus* (including *Phaedropezia*) and *Byssonectria* (as *Pseudocollema*). The tribe as he delimited it, even with the removal of *Caloscypha*, is not monophyletic (Perry et al. 2007).

Kallistoskypha incarnata differs from *C. fulgens* in several critical ways that we use to segregate the new genus. The ascomata are smaller in *K. incarnata* than in *C. fulgens* and pigments seem quite different. There are never hints of yellow or orange in *C. fulgens* whereas in *K. incarnata* the salmon to pink pigments fade to beige or whitish. When bruised, *K. incarnata* does not change color as is so characteristic of *C. fulgens*. Differences in hosts have also been used in considering the generic boundaries. The anatomy of the excipular tissues in these two species is quite similar, both having rows of cells that are perpendicular to the outer surface. In this character, the two genera are similar in excipular construction to species in the Hevellaceae, Discinaceae, and Morchellacaea.

Collections of *K. incarnata* were all made in association with *Eucalypus* species*,* a genus native to Australasia. In our search for the identity of this fungus, we studied the pertinent literature, in particular the treatment of Rifai (1968), in a bid to connect this fungus with its host in its native range. We were unable to find the species in those sources and herbaria searches without a name were fruitless. We know nothing of the biology of this species, but the regular association with a single host genus might be suggestive of a symbiotic relationship between the two.

No comprehensive study of *C. fulgens* has been made from across its range. Descriptions are available from around the world; in these, there is some variation in ascospore size and in associated coniferous plants. In our study, highly supported geographical groups emerge. Collections from western North America and those from eastern North America are significantly different. We will pursue the question of whether these geographical strains deserve recognition. Since *C. fulgens* is known as a conifer pathogen, it might be that there are distinctive strains on different species of trees, particularly in light of the broad circumpolar distribution of the species on several different hosts.

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