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A new cryptic species in the genus *Tubariomyces* (*Inocybaceae*, *Agaricales*)

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Abstract

A new *Tubariomyces* species from Italy, *T. similis*, is described and illustrated. It is phenotypically very close to *T. hygrophoroides* but, based on a combined ITS-LSU rDNA analysis, phylogenetically distinct.

Keywords

Basidiomycota Agaricomycetes Inocybe Phylogeny Taxonomy

Introduction

Inocybaceae Jülich, a family recently resurrected by Matheny (2005) on molecular basis, is a lineage of ectomycorrhizal fungi encompassing taxa formerly included in *Inocybe* (Fr.) Fr. s.l. (Matheny et al. 2009; Ryberg et al. 2010). These taxa are characterized by small- to medium-sized basidiomata with a central stipe, veil present or not, adnexed (almost free) to subdecurrent lamellae, a usually brown spore-print, mainly with a cutis to trichoderm pileipellis, usually without well-differentiated pileocystidia, spores binucleate, smooth, nodulose or spinulose, almost always without a germ pore, heteromorphic cheilocystidia (thin-walled paracystidia mixed with thick-walled and often encrusted cystidia), pleurocystidia absent in several lineages and when present, then of subhymenial origin, and presence of clamp-connections (Kuyper 1986; Kobayashi 2002; Matheny et al. 2002; Matheny 2009; Ryberg et al. 2010); they are, according to Matheny (2005), Matheny et al. (2006, 2009) and Ryberg et al. (2010), sister to the saprobic *Crepidotaceae* s.s., even though Petersen et al. (2010) included *Inocybaceae* in *Crepidotaceae* s.l.

Seven major lineages were recently recognized within *Inocybaceae* (Matheny 2009; Matheny et al. 2009). Four of these clades, namely Inocybe s.s. (= genus *Inocybe* s.s.), Pseudosperma (= section *Rimosae* s.s., Larsson et al. 2009; Ryberg 2009), Mallocybe (= *Inocybe* subgenus *Mallocybe* Kuyper) and Inosperma, are widespread and relatively well studied in Europe. The lineage informally labeled Nothocybe consists so far of only a single collection from southern India (Matheny 2009; Matheny et al. 2007, 2009). The geographically more restricted Auritella clade

(recently recognized as a new genus, *Auritella* Matheny & Bougher) includes eight species known only from Africa, India, and Australia (Matheny and Bougher 2006a,b, 2010; Matheny et al. 2012). Finally, the relict Mallocybella clade is based only on two species, *Inocybe inexpectata* M. Villarreal et al. from European Mediterranean areas (Spain and Corse, Moreau et al. 2007; Villarreal et al. 1998), and an undescribed species from Zambia (Matheny et al. 2009).

Alvarado et al. (2010) recently established the new genus *Tubariomyces* Esteve-Rav. & Matheny to accommodate *Inocybe inexpectata*, the new taxon *T. hygrophoroides* Esteve-Rav., P.A. Moreau & C.E. Hermos., and two undescribed species, *Tubariomyces* sp_1 (voucher RFS 0805, Spain) and *Tubariomyces* sp_2 (voucher BB6018 (PC), Zambia). *Tubariomyces* species are distinguished by small basidiomata usually resembling *Tubaria*/*Flammulaster-Phaeomarasmius* specimens, a squamose pileus reminding of *Mallocybe*, a subtrichodermal to trichodermal pileipellis, necropigmented basidia, abundant short caulocystidia and, above all, by the presence of typically suballantoid to boletoid spores. All the species from the Mediterranean area are characteristically associated with *Cistaceae*, whereas *Tubariomyces* sp_2 from Africa (Zambia) was collected under *Phyllanthaceae* and/or *Fabaceae*.

In the present paper, we describe, on the basis on morphological and ITS-LSU rDNA analysis, a new *Tubariomyces* species from Tuscany, Italy. The collection, which at first, after macro- and microscopic analysis, has been considered by part of the authors a new taxon within the genus *Flammulaster* Earle, is molecularly identical to the *Tubariomyces* sp_1 in Alvarado et al. (2010) and is a cryptic species, since it shows morphological features reminding those of *T. hygrophoroides*.

Materials and methods

Morphology

The macroscopic characters were observed on fresh specimens and described by evaluating more than 50 basidiomata. The microscopic structures were observed on both fresh and dried material, through several examinations, mounted in water, 5 % KOH, Congo red and Melzer's reagent, separately. Dried fragments were rehydrated in 5 % KOH. All microscopic measurements were carried out through × 1,000 oil immersion objective. In the description below, average sizes were reported as result of measurements on at least 50 different elements, randomly selected from five basidiomata. Statistical treatments and notations follow Fannechère (2005). Dimensions of the microscopic characters are given as follows: (minimum value) first decile – average value – nineth decile (maximum value). The width of basidia was measured at the widest part, and the length was measured from the apex (sterigmata excluded) to the basal septum. The following abbreviations are used in text: L = number of entire lamellae; l = number of lamellulae between each pair of entire lamellae; Q = the quotient of length and width of the spores in side view; Qm = average quotient. Colour terms in CAPITAL letters (e.g. Plate XIV Cinnamon-Rufous) are those of Ridgway (1912). Herbarium acronyms follow Thiers (2011). Author citations follow Index Fungorum (http://www.indexfungorum.org/authorsoffungalnames.htm). The type collection is housed at MCVE. The name and description of the new species are 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 specimen (MCVE 27371, type collection), 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/LR7 (Vilgalys and Hester 1990; Vilgalys Lab, unpublished, http://www.botany.duke.edu/fungi/mycolab) for the LSU rDNA amplification. Amplification reactions were performed in a PE9700 thermal cycler (Perkin-Elmer, Applied Biosystems) following Vizzini et al. (2010). The PCR products were purified with the AMPure XP kit (Beckman) and sequenced by MACROGEN Inc. (Seoul, Republic of Korea). The sequences were assembled and edited with the phred/phrap/consed software suite. The sequences were submitted to GenBank and their accession numbers are reported in Fig. 1.

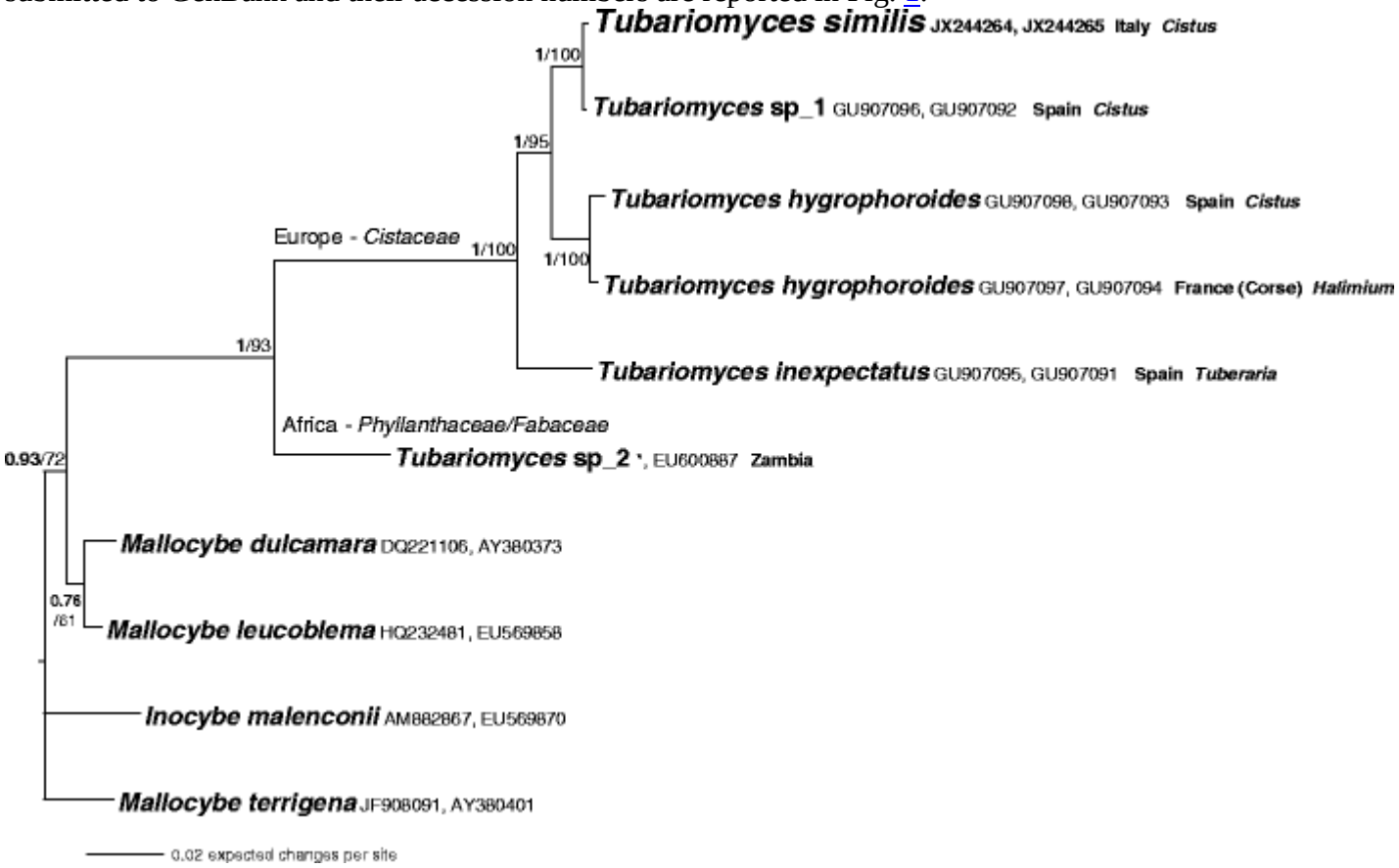

Fig. 1

Bayesian phylogram obtained from the combined ITS-LSU sequence alignment. Support values for clades that are supported in either the Bayesian (Posterior Probabilities values – BPP) and Maximum likelihood (ML Bootstrap percentage – MLB) analyses are indicated. Only **BPP** values over 0.70 and MLB values over 50 % are given above branches

Sequence alignment and phylogenetic analysis

The sequences obtained in this study were checked and assembled using Geneious v5.3 (Drummond et al. 2010), and compared to those available in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/) using the Blastn algorithm. Based on the Blastn results, sequences were selected according to Alvarado et al. (2010). A combined analysis of ITS and LSU sequences was carried out using, when possible, sequences from the same strain or specimen. *Inocybe malenconii* (AM882867, EU569870), *Mallocybe terrigena* (JF908091, AY380401), *Mallocybe leucoblema* (HQ232481, EU569858) and *Mallocybe dulcamara* (DQ221106, AY380373) were used as outgroup taxa according to Alvarado et al. (2010). Alignments were generated using MAFFT (Katoh et al. 2002) with default conditions for gap openings and gap extension penalties. The sequence alignment, its manual adjustment, and the best-fit models

estimation follow Vizzini et al. (2010). The GTR + Γ substitution model was used for the combined ITS and LSU dataset. Molecular analyses were performed using the Bayesian Inference (BI) and Maximum Likelihood (ML) approaches. BI using Monte Carlo Markov Chains (MCMC) was carried out with MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). Four incrementally heated simultaneous MCMC were run over 10,000,000 generations, under model assumptions. Trees were sampled every 100 generations resulting in an overall sampling of 100,001 trees. The "burn-in" value was evaluated using Tracer 1.5 (Rambaut and Drummond 2007). The first 20 % of trees were discarded as "burn-in". For the remaining trees, a majority rule consensus tree showing all compatible partitions was computed to obtain estimates for Bayesian Posterior Probabilities (BPP). ML estimation was performed through RAxML v.7.0.4 (Stamatakis 2006) with 1,000 bootstrap replicates (Felsenstein 1985) using the GTRGAMMA algorithm (for all partitions, respectively) to perform a tree inference and search for a good topology. Support values from bootstrapping runs (MLB) were mapped on the globally best tree using the "-f a" option of RAxML and "-x 12345" as a random seed to invoke the novel rapid bootstrapping algorithm. Only BPP values over 0.70 and MLB over 50 % are reported in the resulting tree (Fig. 1). Pairwise % identity values of ITS sequences were calculated using MEGA 5.0 (Tamura et al. 2011).

Results

Phylogenetic analysis

The combined dataset comprises a total of 10 taxa (including nine from GenBank) and is 1811 base pairs long. Both Bayesian and Maximum likelihood analyses produced the same topology; therefore, only the Bayesian tree with both BPP and MLB values is shown (Fig. 1). In both analyses, *Tubariomyces similis* clusters with the sequence of *Tubariomyces* sp_1 (BPP = 1.0, MLB = 100 %, pairwise % identity value = 100) indicating a conspecific status. This taxon is sister to *T. hygrophoroides* (BPP = 1.0, MLB = 95 %). The ITS pairwise % identity value between the *T. similis* and *T. hygrophoroides* sequences is 95.2; accepting an intraspecific variability lower than 3 % (Nilsson et al. 2008), *T. similis* and *T. hygrophoroides* should be considered distinct species. *Tubariomyces similis* and *T. hygrophoroides* are sister to *T. inexpectatus*. In our analyses *Tubariomyces* sp_2 from Zambia is sister to the other *Tubariomyces* species with a high sequences divergence due to the branch length. The /Tubariomyces clade is monophyletic with high BPP and MLB support.

Taxonomy

Tubariomyces similis Della Maggiora, Tolaini & Vizzini, sp. nov. Figs. 2 – 3

Fig. 2
Tubariomyces similis. Basidiomata (from MCVE 27371, holotype). *Scale bar* 10 mm

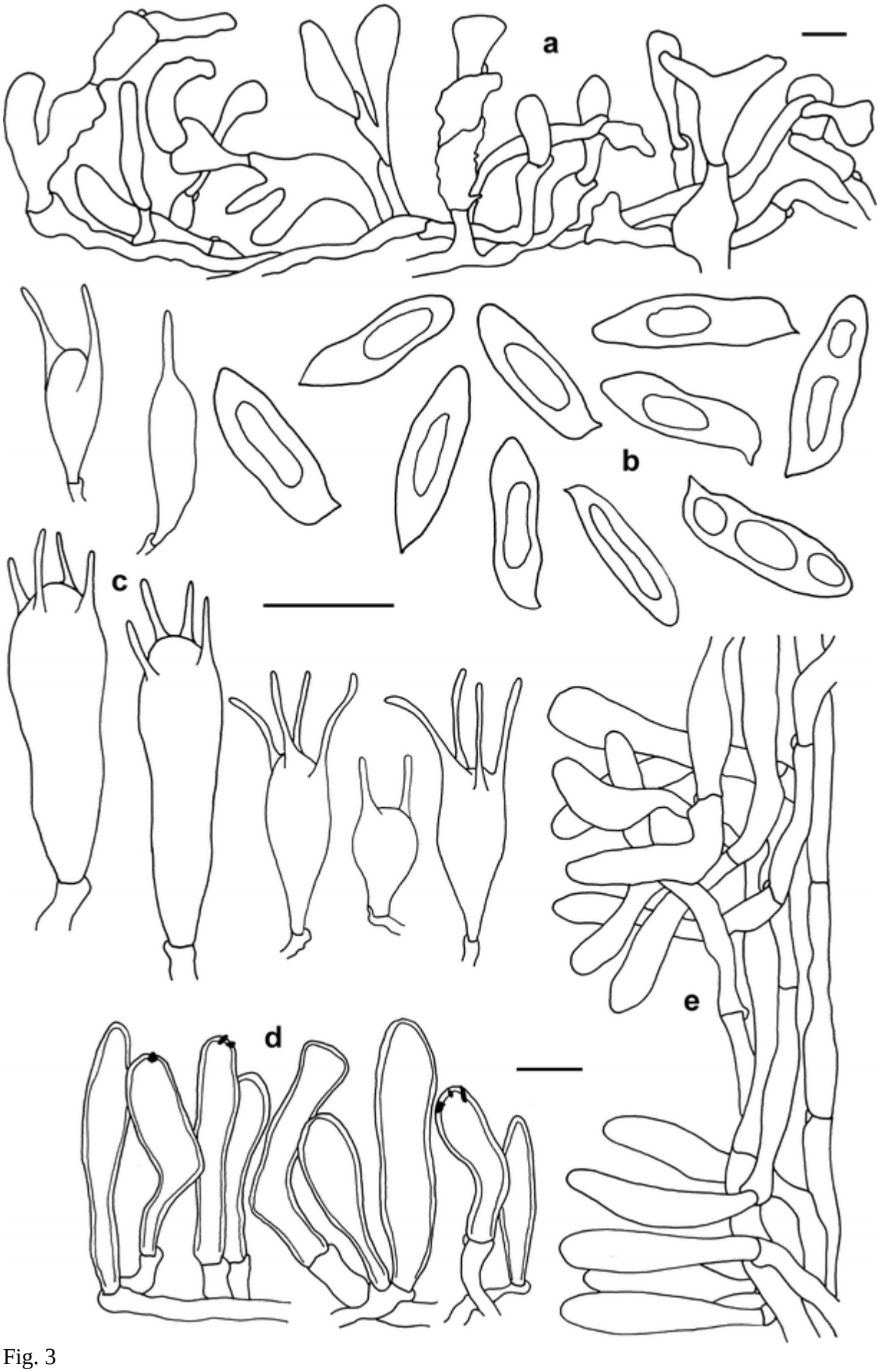

Fig. 3

Tubariomyces similis. Micromorphological features (from MCVE 27371, holotype). **a** Elements of the pileipellis; **b** spores; **c** basidia; **d** cheilocystidia; **e** caulocystidia (from stipe apex). *Scale bars* 10 μm

MycoBank MB 800837

It differs from *T. hygrophoroides* in having a clearly trichodermal pileipellis, narrower spores (Qm > 3.0), different ITS and LSU sequences, and by fruiting in spring.

Type: Italy, Tuscany, Parco Migliarino San Rossore, Massaciuccoli (PI), MCVE 27371.

Etymology

The epithet, derived from the Latin word *similis* (similar, like, undistinguished), refers to the morphological features of the basidiomata, that are very close to those of *T. hygrophoroides*.

Macrocharacters

Pileus 5–15 mm, convex, hemispherical to plano-convex, sometimes obtusely umbonate, dry, surface furfuraceous squamulose, owing to the presence of small concolorous scales, slightly cracked in old specimens; brown-orange, rusty-brown (Plate XIV, Ferruginous, Cinnamon-Rufous; Plate XXVIII, Terra Cotta, Testaceous, Vinaceous-Tawny; Plate XXIX, Orange-Cinnamon), not hygrophanous; margin slightly projecting, not striate, incurved when young. Lamellae distant [L = 18–24, l = 0–1(2)] and thick, slightly arcuate and shortly decurrent with a tooth, cream-beige, ochraceous in young specimens (Plate XXIX, Pinkish Buff, Cinnamon-Buff) soon brownish (Plate XIV, Cinnamon-Rufous; Plate XXVIII, Cacao-Brown; Plate XXIX Mikado Brown), with an eroded whitish edge. Stipe 15–30 × 1–2 (3) mm, quite cylindrical, larger at apex, slightly flexuose at the base, solid, concolorous with pileus, slightly paler at apex (Plate XXIX, Cinnamon, Cinnamon-Buff); surface fibrillose and furfuraceous owing to the presence of minute scattered scales. Context pale ochraceous in the whole basidiome, with no significant smell and taste.

Microcharacters

Spores (12.0) 12.4–**13.6**–15.3 (16.5) × (3.5) 3.6–**4.1**–4.5 (4.7) μm, Q = (3.0) 3.07–**3.32**–3.56 (3.72), irregularly cylindrical-fusiform to suballantoid, typically boletoid, smooth, with a clearly visible hilar appendage, sometimes with vestigial remnants of sterigmata, without germ pore, with one or more guttules, neither amyloid nor dextrinoid. Basidia mostly (11.0) 11.8–**13.5**–17.7 (20.0) × (5.5) 5.6–**6.0**–6.8 (7.0) μm, clavate, but very variable in size and shape, most of them rather short and squat, others more soaring, up to 25–30 × 9 μm, always not very protruding and concealed in the hymenium, one- to four-spored, with yellowish necropigment, with basal clamp connections, with flexuous sterigmata up to 8 μm long. Lamellar edge sterile with numerous cheilocystidia arranged like a fence and shortly overflowed on the faces of the lamellae, (25.0) 26.8–**33.0**–39.9 (45.0) × (3.5) 4.1–**7.8**–9.5 (10.5) μm, cylindrical to clavate or with irregular shape, hyaline, walls up to 1 μm thick, rare elements poorly crystalliferous at apex; rare squat shaped, nearly spheropedunculate cheilocystidia also observed. Pleurocystidia not observed. Caulocystidia present throughout the stipe, (15.0) 16.5–**24.5**–28.1 (33.0) × (3.5) 3.9–**5.2**–7.3 (8.0) μm, clavate to cylindrical, prevalently arranged in tufts at stipe apex, elsewhere scattered. Pileipellis a trichoderm consisting of absolutely irregular elements, (10.1) 10.3–**20.2**–28.6 (35.4) × (5.5) 6.4–**9.2**–13.6 (15.3) μm, often with swollen terminal elements, usually thin-walled or thickened up to 1.5 μm (wall thickness not drawn in Fig. 3 for better clearness), in limited zones presenting a jigsaw-puzzle-like appearance; scattered protruding elements, resembling irregular-cylindrical pileocystidia, have been

observed. Subpellis formed by shorter and broader articles (× 5–25 µm). Pigment yellowish-brown, intracellular, intraparietal, but also encrusting and forming little plates, zebra stripes or granules on some external elements of pilei- and stipitipellis. Clamp connections abundant, observed in all parts of the basidiome.

Habitat

Gregarious, in inland dunes, under *Cistus*; known fructifying only in spring (May).

Collections examined

ITALY, Tuscany, Parco Migliarino San Rossore, Massaciuccoli (PI), 0 m a.s.l., 09. 05. 2004, over 50 basidiomes on sandy soil, near *Cistus salvifolius*, inland dunes with *Quercus ilex*, *Pinus pinea*, *Erica scoparia*, *Phillyrea angustifolia*, *Rubus ulmifolius*, *leg.* S. Matteucci (MCVE 27371); *ibidem*, 13. 05. 2004, *leg.* M. Della Maggiora & F. Tolaini (TO AV000S10).

Discussion

According to Alvarado et al. (2010) the two so far described species of *Tubariomyces* are morphologically closely related but differing for some features: *T. inexpectatus* is characterized by an omphalinoid habit (subdistant to normally spaced and strongly decurrent lamellae, L = [(20) 24–28], a typical trichodermal pileipellis (with erect terminal elements), cystidia covered by evident and abundant yellow-brown mucoid deposits, a sporal Qm = 3.1, and by fruiting in spring under *Tuberaria guttata* (annual herbaceous species of *Cistaceae*); *T. hygrophoroides* is distinguished by a tubarioid habit (arcuate-decurrent lamellae or nearly straight with decurrent tooth), distant lamellae (L = 18–22), a subtrichodermal pileipellis with more prostrate elements, cystidia not covered apically with mucoid deposits, a sporal Qm = 2.55, and by growing in autumn under *Cistus* and *Halimium* (perennial shrub species of *Cistaceae*). *T. similis* clusters with *Tubariomyces* sp_1 (pairwise % identity value = 100) both in ITS and LSU analyses, and it is sister to *T. hygrophoroides* (Fig. 1). *Tubariomyces* sp_1, based on a Spanish collection labelled by Monedero García and Fernández Sasia (2009) as *T. inexpectatus*, shows features fitting well with our new species (Monedero García and Fernández Sasia 2009; Alvarado et al. 2010), and it should be regarded as conspecific to *T. similis*.

Apart from molecular differences (our analyses show only a 95.2 % pairwise ITS sequence identity between *T. similis* and *T. hygrophoroides*), *T. hygrophoroides* is very similar morphologically to *T. similis* and is distinguished only by a subtrichodermal pileipellis with numerous prostrate hyphae, wider spores [× 4.4–5.1–5.8 (−6) µm, Qm < 3.0], and by fruiting in autumn (Hermosilla and Sánchez 1998; Moreau et al. 2007; Alvarado et al. 2010). These two taxa are clearly cryptic species (phylogenetic species); in fact, even if they are phylogenetically well defined, they are not supported by morphological characters so as distinguishing features can not be identified. The existence of cryptic species among fungi has been repeatedly demonstrated using sexual intercompatibility tests and/or molecular data (Aanen and Kuyper 1999; Sato et al. 2007; Vellinga 2007; Hedh et al. 2008; Crespo and Pérez-Ortega 2009; Grebenc et al. 2009).

All the European species of *Tubariomyces* are strictly associated with *Cistaceae* in Mediterranean areas; future work will assess if this association is a derived or ancestral condition compared with the *Phyllanthaceae*/*Fabaceae* connection of *Tubariomyces* sp_2 from dry tropical Africa (Zambia) (Fig. 1).

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