Folia Cryptog. Estonica, Fasc. 61: 1–11 (2024)
https://doi.org/10.12697/fce.2024.61.01

Tylopilus dunensis (Boletaceae, Basidiomycota): notes on morphological, phylogenetical and distributional aspects

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Abstract: *Tylopilus* is a worldwide distributed genus of boletes with about 100 known taxa, of which at least 16 are from Brazil and Guyana. *Tylopilus dunensis*, a species originally described from sand dune habitats in the state of Rio Grande do Norte in northeastern Brazil, has now been recovered in a 'tabuleiro' (i.e., tableland forest) from Paraíba. The main phenetic features of this still poorly known species are the orange to orange-ochraceous pileus with yellowish brown margins, unchanging pileus context, the pale cream hymenophore with wide pores, the yellowish stipe, the small and narrow basidiospores, and the long and frequent dextrinoid pseudocystidioid pleurocystidia. After the discovery of the phylloporoid tube trama in our specimens, we emended tube trama type of *T. dunensis*.

Keywords: Agaricomycetes, Boletales, Neotropic, taxonomy

INTRODUCTION

Tylopilus P. Karst. is a genus of Boletaceae, that is distributed worldwide and includes about 100 known taxa (He et al., 2019; Wijayawardene et al., 2020). The modern concept of genus considers as characteristics the subtomentose to glabrous pileus, unchanging to sometimes rufescent context when bruised or exposed, white to pinkish hymenophore, trichodermial pileipellis, and smooth basidiospores (Wu et al., 2016). Based on nrITS, nrLSU (28S), and RPB2 phylogenies, the genus can be treated as a monophyletic lineage within the subfamily Boletoideae (Gelardi et al., 2019). *Tylopilus* is an ectomycorrhizal (ECM) fungus (Smith & Read 2008), and it is well known in the Neotropics as symbiotic with members of *Aldina* Endl., *Coccoloba* P. Browne, and *Dicymbe* Spruce ex Benth. in tropical South America (Singer et al., 1983; Henkel, 1999; Barbosa-Silva, et al., 2017), and with members of *Fagus* L., *Pinus* L., and *Quercus* L. in Central America and Mexico (Singer et al., 1983; Halling & Mueller, 2001; Rodríguez-Ramírez et al., 2020; Montoya et al., 2023).

In the Neotropic, *Tylopilus sensu lato* has considerable species richness. Barbosa-Silva et

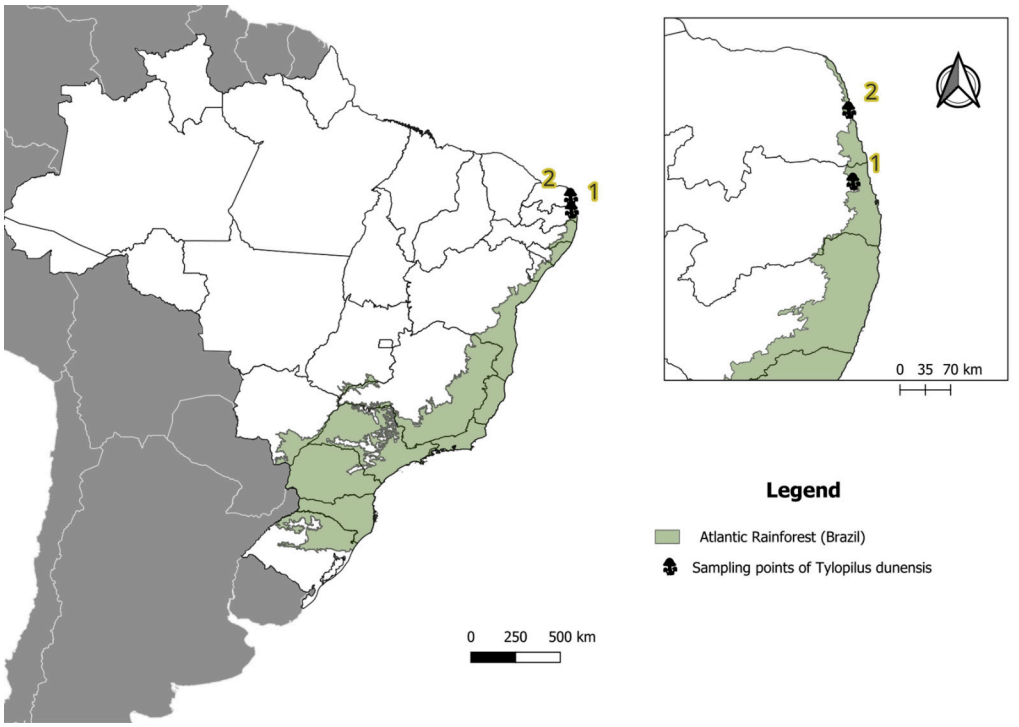

Fig. 1. Map showing the distribution of *Tylopilus dunensis* (including the holotype and paratypes described by Magnago et al., 2017) from Parque das Dunas (1) and Biological Reserve Guaribas (2).

Fig. 2. General view of the 'tabuleiro' forest in Biological Reserve of Guaribas.

al. (2020) listed 16 species from Brazil and Guyana and other 21 taxa from other countries in Central and South America. Among them, *T. dunensis* (Magnago et al., 2017) was discovered from the Brazilian Atlantic Forest in a white sand dune habitat (sensu Roy et al., 2016).

During our field trips, we found a fungus with orange pileus and wide angular pores that turn more radially elongate near the stipe then adnate. Microscopically, we observed a 'phylloporoid' tube trama, i.e., hyphae of the lateral tube trama not abruptly recurved but only slightly divergent, with little or no differentiated mediostratum (Singer, 1986). In this study we analyze these additional specimens and present new distributional data on this species, as well as additional information on its morphology and habitat.

MATERIAL AND METHODS

Study area

Tylopilus basidiomata were collected in two conservation units in northeastern Brazil: Parque Estadual das Dunas' (Natal municipality, Rio Grande do Norte state), and Biological Reserve Guaribas (Mamanguape municipality, Paraíba state). We used GeoCAT (Bachman et al., 2011), and QGIS (QGIS Development Team, 2021) tools to develop the map of collecting localities (Fig. 1).

In Parque das Dunas, *Guapira pernambucensis* (Casar) Lundell (Nyctagynaceae), *Coccoloba alnifolia* Casar., *C. brasiliensis* Nees & Mart., and *C. laevis* Casar (Polygonaceae) were found in a floristic study (Freire, 1990). Other collections were found in a savannoid lowland forest (also known as 'tabuleiro') in the Guaribas Biological Reserve at western part of the dune regions, with altitude ranging to 70–120 m – characteristically plane landscape, covered by mostly open savanna on poor, sandy soil (Thomas & Barbosa, 2008) (Fig. 2). Barbosa et al. (2011) published a list of tree species from this reserve and reported *Guapira opposita* (Vell.) Reitz, *G. pernambucensis* (*Nyctagynaceae*), *Coccoloba alnifolia*, *C. arborescens* (Vell.) R.A. Howard, *C. laevis*, *C. mollis* Casar., *C. ramosissima* Wedd., and *C. scandens* Casar. (*Polygonaceae*). The genera *Coccoloba* and *Guapira* are treated as ectomycorrhizal (ECM) trees genera in the Neotropic (e.g., Smith & Read, 2008).

Morphology

Microscopic characteristics were observed using the Leica DM500™ light microscope. The material was mounted in 3% KOH, Melzer's reagent, Congo red and sulphovaniline solutions (Singer, 1986). Photomicrographs were taken using a camera and software (ZEN Microscopy Software™) connected to a Primo Star Zeiss™ microscope. Color codes followed Kelly (1965) and Kramer (2004), abbreviated as 'K' and 'OAC' respectively. For basidiospores data we followed the slightly modified methodology of Tulloss et al. (1992), that include the abbreviations L(W) = average basidiospores length (width), Q = the length : width ratio range as determined from all measured basidiospores, and Qm = the Q value averaged from all basidiospores measured. Measurements and statistics are based on 30 basidiospores. The studied specimens are deposited in the Herbarium JPB (Department of Systematic and Ecology, Federal University of Paraíba) (Thiers, 2023).

For detailed observation of the basidiospores shape and ornamentation of the spore wall, Scanning Electron Microscopy (SEM) images were made from three small sections made in the hymenium of dry basidiomata, and mounted directly on aluminum stubs using carbon adhesive tabs. Fragments were coated with gold using a sputter coater and examined in a Shimadzu SSX-550™.

Molecular analysis

For identification of our specimens, whole genomic DNA was extracted from one specimens of each sampling using the DNeasy Plant Mini Kit (Qiagen, Germany). PCR amplifications were performed for the complete internal transcribed spacers 1 and 2 and the 5.8S rDNA (nuc-ITS-rDNA) limited by primers ITS1 and ITS4 (White et al., 1990). PCR conditions followed Halling et al. (2008). PCR products were unidirectionally sequenced in ABI 3130 Genetic Analyzer (Applied Biosystems). We used GENEIOUS v 9.1.3 (Kearse et al., 2012) to check the sequence quality of the strands by comparison with their respective chromatograms and to assemble and edit as necessary.

To identify our samples, we assigned them to a species using a nucleotide BLAST (Ashtul et al., 1990) approach performed in GenBank (http://blast.ncbi.nlm.nih.gov) and by the

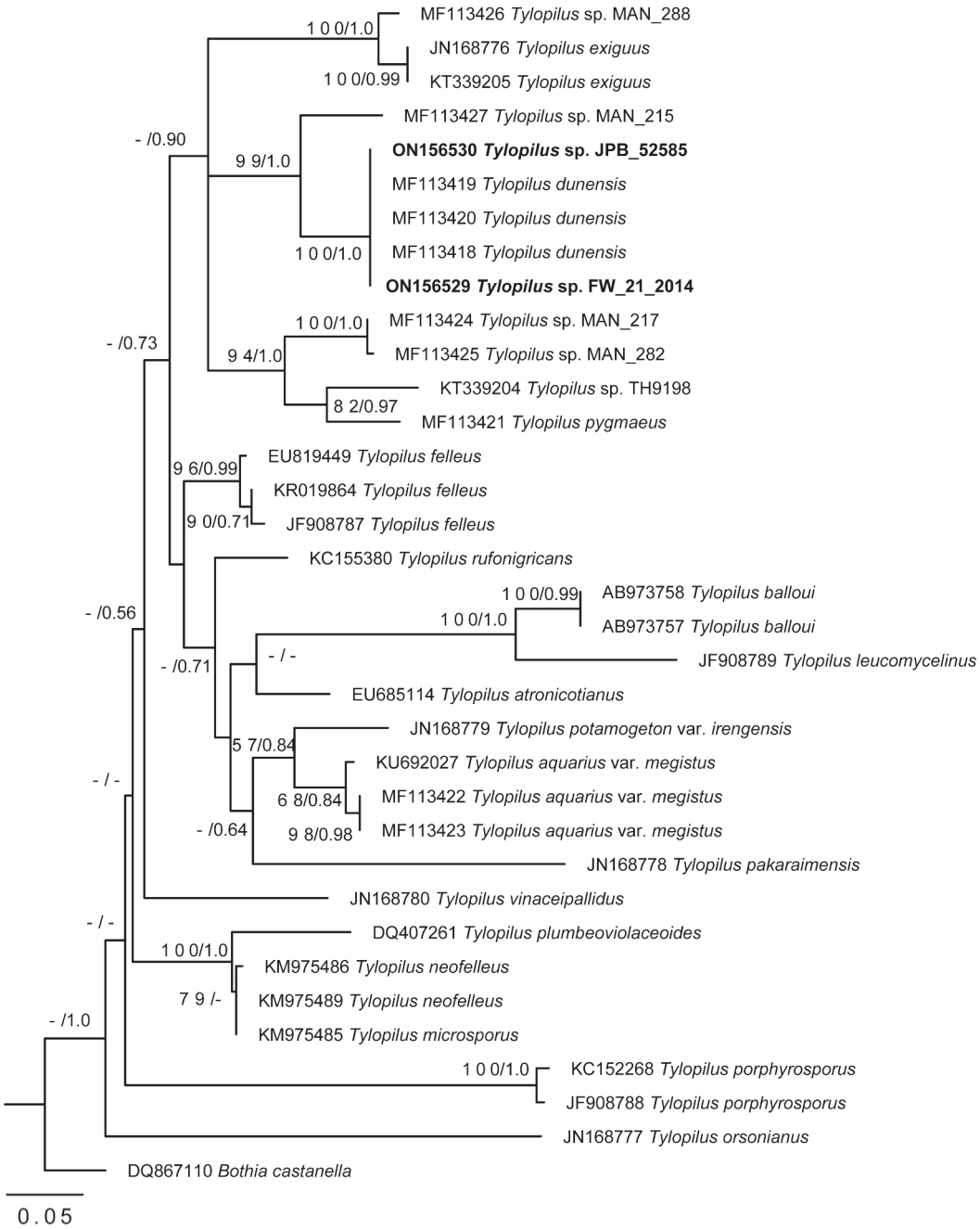

Fig. 3. Phylogenetic tree generated by the maximum likelihood method from the nuc-ITS rDNA region. The numbers near the branches indicate the values supported by the maximum likelihood method (bootstrap percentages) and Bayesian inference (posterior probabilities), respectively (> 50% and > 0.5). A dash (-) indicates that a node has a low value or is not supported in the analysis. *Bothia castanella* was included as an outgroup.

phylogenetic methods described below. The sequences obtained were analyzed by Standard Nucleotide analysis BLAST to find the most closely related species. The sequences of closely related species indicated by Magnago et al. (2017) were downloaded from the GenBank database (Benson et al., 2012). For phylogenetic analysis, we first aligned these reference sequences with our obtained sequences using Muscle v. 3.8.425 (Edgar, 2004), a module implemented in GENEIOUS v. 9.1.3 with default settings. In our analysis, we considered nuc-ITSrDNA sequences longer than 400 bp. Conservative regions were selected using Gblocks v. 0.91b (Castresana, 2000) with the less stringent option (383 bp).

The Maximum Likelihood (ML) trees were constructed using RAxML v. 8.2.12 (Stamatakis, 2014) with GTR GAMMA model and 1,000 bootstrap (BP) replicates. Bayesian tree (BS) was created using MrBayes 3.2.6 (Huelsenbeck & Ronquist, 2001). For the best available evolutionary model jModel Test 3.0.4 was used (Posada, 2008) (nuc-ITSrDNA, HKY+G). Two independent parallel runs were performed, sampling every 1000th generation for a total of 50 million generations. Convergence of parameters was assessed using Tracer v.1.6.082. Effective sample sizes (ESS) were within acceptable ranges (ESS>200). After discarding the first 10% of the sample trees as burn-in, the remaining trees were used to calculate a majority rule consensus tree and posterior probabilities of bipartitions. Our sequences were deposited in GenBank (NCBI) under accession numbers ON156529 and ON156530.

RESULTS

Molecular analysis

Our nucleotide BLAST analysis of the rDNA ITS showed that both specimens presented here match the sequence from the type of the species *Tylopilus dunensis* (100% identity, E-value 0.00; NR_156624.1) described by Magnago et al. (2017). After alignment and Gblocks adjustment, our dataset contained 390 sites. In our phylogenetic tree (Fig. 3), the generated sequences were monophyletic, and the posterior probability values of the nodes were high for *Tylopilus dunensis* and formed a sister-group with an unidentified sequence named '*Tylopilus* sp. MAN_215'.

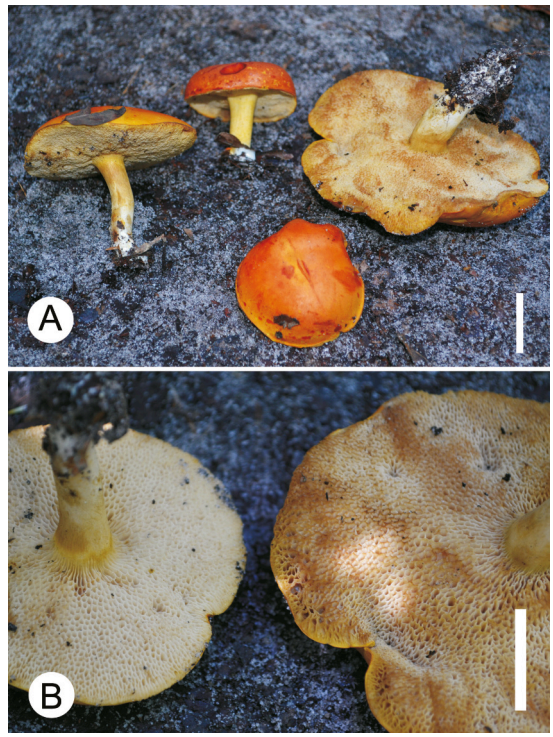

Fig. 4. *Tylopilus dunensis*. A – basidiomata *in situ*. B – details of the hymenophore. From Wartchow 142/2012. Photos by F. Wartchow. Bars = 30 mm.

Morphological description

Tylopilus dunensis A.C. Magnago & M.A. Neves, Phytotaxa 316: 255. 2017, emend. Valões-Araújo, Barbosa-Silva & Wartchow (Figs. 4-5.)

Note: The morphological description of this species is emended here, adding phylloporoid tube trama. (Figure 5D, E).

Basidiomata subgregarious to gregarious, medium to sometimes large sized. *Pileus* 65–125 mm in diam., hemispheric to flattened-convex, orange ochraceous (OAC 761–762; K 48.v.O) in mature basidiomes ochraceous (OAC 692; K 50.s.O) with yellowish beige/buff tints (OAC 791; K 67.brill.O Y); surface dry, smooth, shiny; margin entire, smooth; context cream, 9–20 mm, soft, thick, unchanging when exposed to air. *Hymenophore* tubulose; tubes adnate with short

decurrent tooth, cream (K 92. y White) then pale buff (OAC 761–762; K 48.v.O) after bruising, with concolorous edge, not distinctly staining; pores mostly pentagonal or hexagonal, up to 1.5 mm in diam., turning more elongate (sublamellate) near stipe. *Stipe* 40–55 × 9–16 mm, central, slightly more attenuate toward base; yellowish to yellowish buff (OAC 812-813; K 83.brill.y), smooth but longitudinally fibrillose (under hand lens); context solid, yellowish (paler than OAC 858; K 89.p.Y), unchanging when bruised or exposed to air. Odor pleasant, similar to lemon detergent. No chemical reactions observed.

Basidiospores (6–)6.5–7.6(–8.5) × 3.5–4 µm, L = 7 µm, W = 3.7 µm, Q = (1.63–)1.71–2.14(–2.50); Qm = 1.91, inamyloid, pale, smooth (Fig. 5F), thin-walled, phaseoliform in side view, short cylindrical in face view; hilar appendix small, sublateral; guttules as large oil drop. *Basidia* 22–33 × 5–8.5 µm, clavate, mostly with 4 sterigmata, each up to 2.5–4.5 µm long, clampless. *Pleurocystidia* abundant, (29–)56–100 × 7.5–12 µm, mostly ventricose-rostrate, frequently with elongated neck, mostly pallid to melleous or rarely almost hyaline, brownish in water, dextrinoid but sometimes inamyloid, dark blackish blue in sulphovaniline, thin-walled. *Cheilocystidia* difficult to see due the somewhat collapsed edge but appearing similar to pleurocystidia. *Tube trama* phylloporoid, with undifferentiated mediostratum; hyphae mostly parallel, 5–10 µm wide, thin walled, pallid with apparent refractive helical contents, shallowly divergent near edge, thin-to sometimes slightly thick-walled (up to 0.9 µm). *Pileipellis* a loosely arranged trichodermium of parallel and anticlinal to sometimes suberect, interwoven, colorless hyphae in KOH, 4–8.5 µm wide, yellowish brown with some greenish tints in water, colorless in KOH, thin-walled; terminal elements 24–44 × 3–6.5 µm, cylindric, subcylindric to sometimes slender ventricose-rostrate, pale beige, thin-walled. *Pileus context* hyphae 3.7–7.5 µm, densely interwoven; devoid of any color in KOH, thin-walled, not gelatinized. Stipe context hyphae 3.7–7.5 µm wide, heavily interwoven; colorless in KOH, thin walled, not gelatinized. *Stipitipellis* with longitudinally oriented, colorless hyphae, not projecting; caulobasidia 19.5–30 × 6–8 µm, abundant at apex, with four sterigmata; organized in tufts, up to 18–29 × 5–8 (–10.5) µm, clavate to occasionally clavate-capitulate, colorless, thin walled. *Clamp connections* absent from all tissues examined.

Specimens examined: Brazil. Paraíba, Mamanguape, Biological Reserve Guaribas, SEMA II, (-6.741°S, -35.140°W), alt. 180 m, 30.06.2012, F. Wartchow FW 142/2012 (JPB 52585, GenBank ON156530); Rio Grande do Norte, Natal, Parque das Dunas, Trilha da Geologia (-5.843°S, -35.194°W), alt. 60 m, 24.07.2014, F. Wartchow FW 21/2014 (JPB 60536, GenBank ON156529).

Distribution: Brazil, states of Rio Grande do Norte (Magnago et al., 2017) and Paraíba (this work) (Fig. 1).

Habitat: Gregarious and scattered on soil of 'tabuleiro' forest in tropical Atlantic Forest trees, such as *Coccoloba* spp. (pers. obs.).

DISCUSSION

Our material of *T. dunensis* agrees in many aspects with the protologue of Magnago et al. (2017). It is characterized by the medium to large sized basidiomata, orange to orange ochraceous pileus, unchanging context when bruised or exposed, mostly cream hymenophore, yellow and unchanging stipe, somewhat small and pale basidiospores sized (6–)6.5–7.6(–8.5) × 3.5–4 µm, that are adaxially concave, and a loosely arranged trichodermal pileipellis. Our phylogeny supports previous results (e.g., Magnago et al., 2017; Chakraborty et al., 2018), which point to a monophyletic branch that includes sequences of *T. dunensis* and *T. pygmaeus* from Brazil, a sequence from Guyana named *T. balloui* sensu Henkel (1999), and some additional unidentified sequences (Magnago et al., 2017). An interesting feature of the known taxa of this group is the width of basidiospores measuring 3–4 (–5) µm. Unfortunately, we do not have information on the morphological features of the specimen corresponding to the sequence with voucher '*Tylopilus* sp. MAN_215' available for phylogenetic analyses. The reported material matches *T. dunensis* phylogenetically and morphologically. However, we found a different tube trama, namely phylloporoid (Fig. 5D–E) instead of boletoid as described in Magnago et al. (2017).

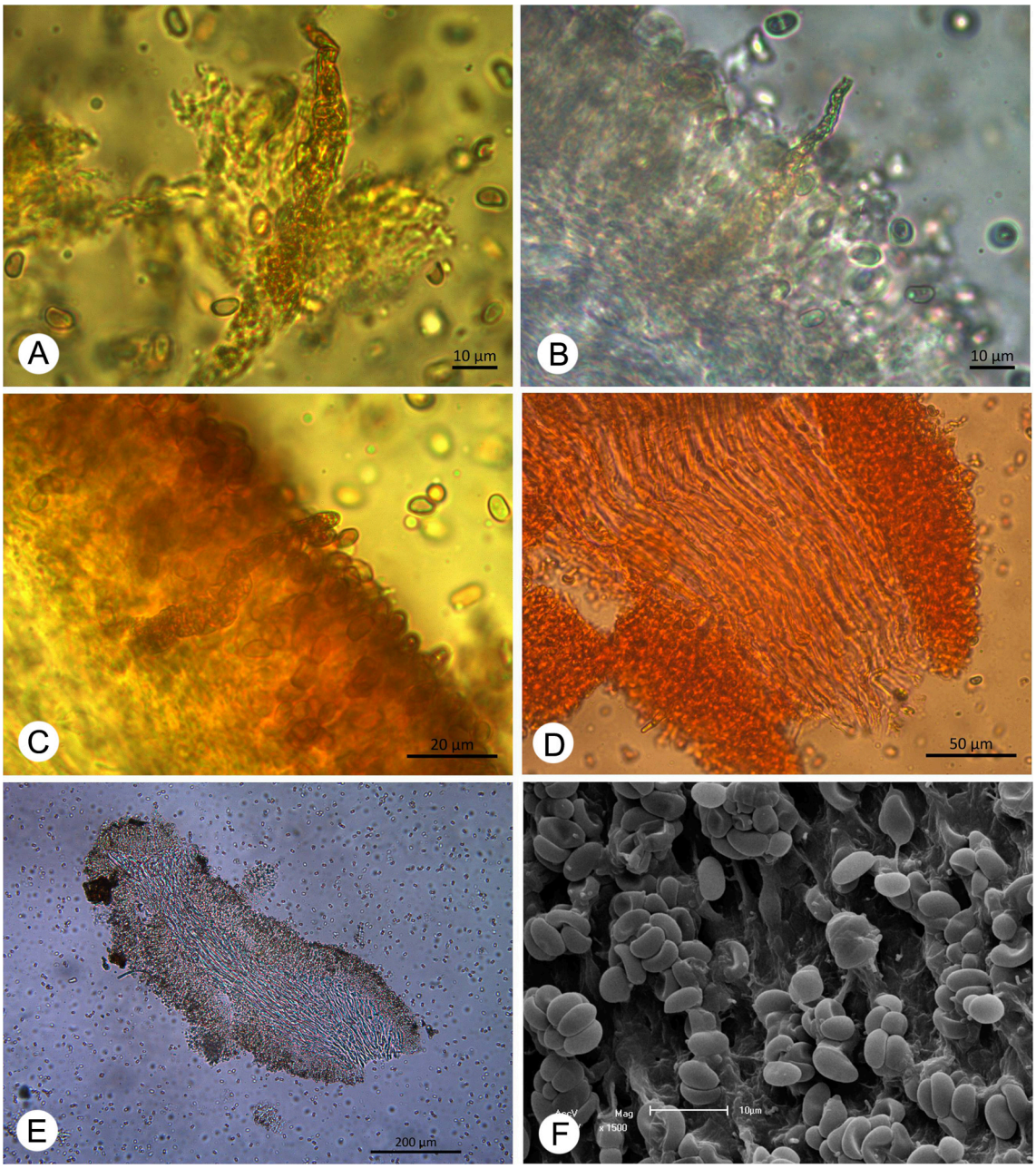

Fig. 5. *Tylopilus dunensis*. A – Cystidium and basidiospores in Melzer's reagent. B – Cystidium and basidiospores in 3% KOH. C – Hymenium in Melzer's reagent. D – Hymenophoral trama with 3% KOH and Congo red. E – Slice of the tube after crushed showing the phylloporoid trama. F – SEM micrograph from hymenial surface with basidiospores. From Wartchow 142/2012.

The uncommon phylloporoid tube trama morphology (as it was observed in our specimens) has been noticed since the earliest classification of the order Agaricales, where *Tylopilus* was described as having a 'truly bilateral-divergent trama of the *Boletus*-subtype' (Singer, 1951: 681). This characteristic was even used in the last Agaricales' system, in the key to distinguish the subfamily Xerocomoideae ('*Phylloporus*-type' containing species) from the rest of the *Boletaceae* (Singer, 1986: 739). The discussion about the importance of the tube trama characteristics among boletes was reintroduced by Šutara (2008), who considered the truly phylloporoid tube trama as characteristic of *Xerocomus* Quél. differentiating from the 'intermediate between the boletoid and phylloporoid types' for the newly erected genus *Xerocomellus* Šutara. The presence of phylloporoid tube trama was also used as part of the diagnosis of some new recently described genera, e.g., *Singerocomus* Henkel & A.H. Sm., and *Neotropicomus* A.C. Magnago, Alves-Silva & T.W Henkel (Henkel et al., 2016; Magnago et al., 2022). In *Tylopilus*, although not explicitly mentioned in the modern genus description, the boleoid/divergent tube trama has been referred to recently described species (Wu et al., 2016; Magnago et al., 2017; Chakraborty et al., 2018; Gelardi et al., 2019; Montoya et al., 2023). Thus, our discovery of parallel, i.e., phylloporoid, tube trama brings insights into the morphology of *Tylopilus*.

Tylopilus pygmaeus A.C. Magnago is the only other species with a complete morphological description of this monophyletic branch. It differs in many aspects: the much smaller pileus 11–26 mm in diameter with velutinous surface, pale then turning pinkish tubes, boletoid hymenophoral trama, wider basidiospores 7–9 × 4–5 µm, and trichodermial pileipellis with cylindrical to fusoid, golden brown and dextrinoid elements, measuring 28–73 × 8–10 µm (Magnago et al., 2017). Phylogenetically, this species is well separated from *T. dunensis*, with a good differential support value and closer to the specimens '*Tylopilus* sp. MAN_217' and '*Tylopilus* sp. MAN_282'.

Chakaborty et al. (2018) included three additional sequences named '*Tylopilus balloui* (Peck) Singer' from Guyana. One of these, the material with voucher number TH6385 was studied by Henkel (1999, as *T. balloui*). This specimen had orange-red to brick-red glabrous pileus, broader basidiospores (5.5–75 × 4–5 µm), hyaline cystidia, and boletoid tube trama.

The epithet "*dunensis*" chosen by Magnago et al. (2017) corresponds to the habitat in the Atlantic Forest where the type specimen was collected, and now we found it in a 'tabuleiro' forest (Fig. 5) in the same biome. This landscape is located in the western part of the dune regions, at an elevation of 70–120 m, and is characteristically plane and covered by mostly open savanna on poor, sandy soils (Thomas & Barbosa, 2008). Sandy soils possibly select for a similar flora. Our data on the distribution of *T. dunensis* are consistent with observations from herbaria in Brazil, showing similarities among the different sand associated forests (Roy et al., 2016). Considering that few distribution maps are available for ectomycorrhizal fungi in South America, collecting evidence on their occurrence and ecology is a priority to better determine their conservation status.

ACKNOWLEDGEMENTS

The authors thank Dr. Iuri G. Baseia for allowing SEM studies, Dr. Maria Regina V. Barbosa and TAXON laboratory for providing facilities, Felipe G.B. Pinheiro and Dr. Clark L. Ovrebo for accompanying them in field trips, Dr. Rivete S. Lima ('Laboratório de Anatomia Vegetal'-UFPB) for helping in the preparation of microphotographs. We thank the 'Conselho Nacional de Desenvolvimento Científico e Tecnológico' (CNPq) for funding the project through support for the 'Programa de Pesquisas em Biodiversidade' (PPBio Proc. 60/2009) and the projects 'Fungos agaricoides em áreas de Mata Atlântica e Caatinga no Estado da Paraíba' (Edital Universal Proc. 420.448/2016-0) and 'Produtividade em Pesquisa' grant for FW (Proc. 307922/2014-6, Proc. 307947/2017-3 and Proc. 309652/2020-0). We also thank the 'Universidade Federal da Paraíba', wich was recognized for funding this project by 'Chamada Interna Produtividade em Pesquisa' (PROPESQ/UFPB Nº 06/2021 Cód. PVA13212-2020). RK received financial support (BLD-PDRP) nº 2022/2003 from the 'Fundação de Apoio à Pesquisa do Estado da Paraíba' (FAPESQ); JCVA (Proc. DS 88887.702560/2022-00) and ABS (Proc. DS 88882.380049/2019-01) received a grant from Coordenação de

Aperfeiçoamento de Pessoal de Nível Superior' (CAPES); MR an "investissement d'avenir" grant from the Agence Nationale de la Recherche (CEBA, ref ANR-10-LABX-25-01). The authors declare no conflits of interest and emphasize that all were involved in design of this study.

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