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Original article

Mahabalella pernambucana, a new hyphomycete from Brazil with notes on its phylogenetic positioning

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ABSTRACT

A new species of *Mahabalella* B. Sutton & S. D. Patil was isolated from decaying leaves of an unidentified plant, during a survey in the Charles Darwin Ecological Refuge in Igarassu, Pernambuco, Brazil, in June 2018. The new species is morphologically distinguishable from the other *Mahabalella* species by the size of their conidia, conidiogenous cells and setae. In this study, the morphological descriptions, illustrations and cultural characteristics for *Mahabalella pernambucana* are provided, as well as a key to species of this genus. Total DNA from the isolate was extracted, and four genes were partially sequenced (nLSU, ITS cluster, TEF1 alpha, and beta tubulin). Based on the nLSU analysis, the phylogenetic position of the new species was determined within the Sordariomycetes, Ascomycota, as a sister genus to *Neotracylla*, in the Tracyllaceae family.

Keywords: asexual Ascomycota, Atlantic Rainforest, leaf litter, phylogeny, taxonomy.

Introduction

The genus *Mahabalella* B. Sutton & S.D. Patil was described from decomposing bamboo leaves by Sutton & Patil (1966) and typified by *Mahabalella acutisetosa* B. Sutton & S. D. Patil. It is characterized by flat, pustuliform, setose sporodochial conidiomata. Setae straight, erect, aseptate, unbranched, smooth-walled, medium to dark brown, base darker, bulbous, tapering toward the acute apex. Conidiogenous cells phialidic, ampulliform, doliform or globose, smooth, discrete walls. The conidia are straight, hyaline, unicellular, obtuse at the ends, with a filiform, rigid, unbranched appendage at both ends.

Four species have been described in this genus: *Mahabalella acutisetosa* B. Sutton & S.D. Patil. is the type species, *M. cubensis* R.F. Castañeda (Castañeda-Ruiz 1985) isolated from decomposing leaves of unidentified Lauraceae, *M. dimorpha* Matsush. (Matsushima 1995), described from leaf litter, and *M. stiriaca* Melnik, Scheuer & Heftb. (Mel'nik *et al.* 2003), observed from a species of sedge. The latter, however, has not been validly published according to the Art. 40.6 of the International Code of Nomenclature for algae, fungi, and plants (Shenzhen Code, Turland *et al.* 2018).

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This genus is kept as *incertae sedis* in the classification of Fungi (Index Fungorum 2022). This is due to the lack of information regarding its possible sexual state, and also because there is no DNA sequence deposited for any gene of its accepted species (https://www.ncbi.nlm.nih.gov/ on March 4th, 2022) that would allow a phylogenetic position to be inferred.

During a mycological survey of microfungi associated with leaf litter in a Brazilian Atlantic Forest, a conspicuous fungus was collected. It is described here as a new *Mahabalella* species, with inference to its phylogenetic position.

Materials and methods

Study area, sampling and morphological analyses

Sampling of leaf litter was carried out at the Ecological Refuge Charles Darwin, a rain forest fragment located in the municipality of Igarassu, north coast of the state of Pernambuco, Brazil. The site is within an area of about 60 ha, crossed by the Jacoca River (also called Tabatinga River), which is perennial and belongs to the main drainage basin of the municipality. Individual collections were placed in plastic bags, taken to the laboratory, and treated according to Castañeda-Ruiz *et al.* (2016). Mounts were prepared in polyvinyl alcohol-glycerol (8 g PVA in 100 ml water, plus 5 ml glycerol) and lactofuchsin (0.1 g acid fuchsin, 100 ml 85% lactic acid) following Carmichael (1955) or in lactic acid (90%). Measurements were taken at a magnification of ×1000 under an Olympus CX41 microscope with phase contrast, and photomicrographs were taken using a Nikon Eclipse Ni-U microscope with DIC optics and a Nikon DS-Fi2 camera. Fungal reproductive structures were isolated in water-agar, pH 6.3 with chloramphenicol, and after 2-5 days the colonies were transferred to V8 agar, pH 6.3 for DNA extraction. The holotype was deposited in the Herbarium URM and a culture voucher in the Micoteca URM of the Universidade Federal de Pernambuco, Recife, Brazil.

DNA extraction, PCR amplification and sequencing

The mycelia collected from culture on V8 media (pH 6.3) were used for total DNA extraction performed using the method of Góes-Neto *et al.* (2005). DNA was precipitated with isopropanol, washed with 70% ethanol, dried at 37 °C and re-suspended in ultrapure water. DNA concentration was estimated by electrophoresis in 1% agarose gel, at 3 V cm-1 in 1X Tris-Acetic acid-EDTA (TAE), by comparison with the 1 Kb plus DNA size marker (Thermo Fisher Scientific).

Amplification reactions of the fungal ITS, nLSU rDNA, beta-Tubulin and Elongation Factor 1-alpha were carried out in a final volume of 25 µl using the Top Taq Master Mix kit (Qiagen) and the primers ITS4-ITS5 (White *et al.* 1990), LR0R-LR5 (Vilgalys & Hester 1990), BT2A-BT2B (Glass & Donaldson 1995) and TEF-1 alpha EF-1 - TEF-1 alpha EF-2 (O'Donnell *et al.* 1998), respectively, at 0.40 µM each, and 25 ng of DNA template. All amplification had an initial denaturation at 94 °C for 5 min and final extension at 72 °C for 7 min, and the cycling program consisted of 94 °C for 30 s, 53 °C for 30 s, 72 °C for 1 min (37 cycles) for ITS, 94 °C for 30 s, 48 °C for 30 s, 72 °C for 1 min (35 cycles) for nLSU, 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min 30 s (37 cycles) for beta-tubulin and 95 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min (35 cycles) for TEF1-alpha. The product of the amplification was visualized in agarose gel (1%). Purification was carried out with the GeneJet PCR purification kit (Thermo Scientific) according to the manufacturer instructions and the products were sent for sequencing in the DNA Sequencing Platform of the Universidade Federal de Pernambuco, in an ABI-310 Capillary Sequencer (PerkinElmer, Wellesley Massachusetts, USA). Primers ITS4/ITS5, LR0R/LR5, BT2A/BT2B and TEF-1αEF-1/TEF-1αEF-2 were used for sequencing the respective genes. The sequences were deposited in the GenBank (Benson *et al.* 2013).

Phylogenetic analysis for taxonomical positioning

The sequences obtained in this study were used to BLAST for sequences of the closest related taxa in the GenBank. The best matches for the new sequences were retrieved from GenBank, along with relevant sequences, including *Saccharata proteae* (GenBank EU552145) that was used as the outgroup based on Crous *et al.* (2018) and added to the final matrix. The final nLSU dataset was aligned with Clustal X (Thompson *et al.* 1997) and manually adjusted as necessary with BioEdit (Hall 1999). The best nucleotide substitutions model for each gene was estimated using the AIC (Akaike Information Criterion). Two phylogenetic routines were used to reconstruct the relationships' hypothesis, using the dataset: Maximum Likelihood (ML) and Bayesian Inference (BI). ML analysis was carried out using MEGA 7.0 (Kumar *et al.* 2016), applying the GTR+GAMMA+I model, with all parameters estimated by the software. The best-scored likelihood tree from all searches was kept. To access the reliability of the nodes, multiparametric bootstrapping replicates (1000) under the same model were computed. The bootstrap values were then annotated to the best likelihood tree found.

BI was performed using Mr. Bayes as implemented in the Topali v2.5 (Milne *et al.* 2004). The analysis was implemented by two MCMC independent runs, each one starting from random trees and with four simultaneous independent chains, performing 10 million generations, keeping one tree every 1000th generation. Five rate categories were used to approximate the gamma distribution, and the nucleotide substitutions rates were fixed to the estimated values. The first 25% of the sampled trees was discarded as burn-in, while the remaining ones were used to reconstruct a 50% majority-rule consensus tree and to estimate Bayesian Posterior Probabilities (BPP) of the branches. A node was

considered to be strongly supported if it showed a BPP ≥ 0.95 and/or BS ≥ 90%, while moderate support was considered when BPP ≥ 0.9 and/or BS ≥ 70%.

Results

Taxonomic treatment

Mahabalella pernambucana W.L. Tavares, Malosso & R.F. Castañeda ***sp. nov.***

Type: Brazil, Pernambuco, Igarassú, RPPN Refúgio Ecológico Charles Darwin, 7°48'50.95" S 34°57'17.32" W, on decaying leaves of an unidentified plant, 08 Jun 2018, W.L. Tavares (Holotype Herbarium URM 94154), ex-type culture in Micoteca URM 8056. MycoBank number: MB 834444 (Fig. 1).

Conidiomata on natural substrate, sporodochial, flatted, blister-shape, setose, scattered or confluent, mostly epiphyllous, dark brown. Mycelium superficial composed of branched, septate, pale yellowish brown, smooth-walled hyphae, 1–2 µm diam. Setae cylindrical, bulbous or inflated at the base, acute at the apex, straight, continuous, smooth-walled, dark brown to black, 25–30 × 5–6 µm. Conidiophores reduced to conidiogenous cells. Conidiogenous cells monophialidic, globose to broadly ampulliform, 2.5–5×2.5–3 µm (n=20). Conidia seriate, cylindrical, rounded at the ends, sausage-shaped, unicellular, hyaline, smooth, 6–8 × 1.5–2 µm (n=30), with an appendage filiform, 7–10 µm length, at the ends, arranged forming white masses around the setae and covering the conidiomata.

Cultural characters on V8 agar – After 15 days at room temperature, in the dark, the colonies developed dark aerial mycelium, yellowish brown, velvety, flat and sparse, entire margin; reverse dark brown. In the culture collection, the specimens are preserved in PDA under mineral oil and using Castellani's method.

Distribution and habitat: *Mahabalella* spp. are known from decomposing leaf litter in Austria (temperate forest), Cuba (tropical forest), and Japan (subtropical forest), and it was now found in the Northeast of Brazil (tropical forest). The Ecological Refuge Charles Darwin is a private property of about 60 ha, crossed by the river Jacoca. This perennial watercourse is part of the main basin in the municipality, the river Botafogo catchment. This wildlife refuge is used as shelter for animals rescued by IBAMA and presents a main unpaved road for visiting and service cars, and accessory trails destined to visitors hiking to denser forest (Santiago & Barros 2003). The vegetation is characteristic of Pernambuco's Atlantic Forest zone, with coastal hygrophilous broadleaf evergreen forest formation composed of an arboreal stratum varying from 8 to 15 meters in height and a shrubby herbaceous stratum. The climate is Am-type with transition to As-type (Andrade-Lima 1960). The average temperature in the region between 2018 and 2019 ranged from 20.5 °C (Jul/2018) to 24.2 °C (May/2019). And the average monthly rainfall ranged from 86.8 mm (Jul/2018) to 62.5 mm (May/2019).

Conservation status: The distribution and size population of *M. pernambucana* are still unknown to adequately assess its conservation status under the IUCN (2012) categories and criteria. Thus, *M. pernambucana* is here categorized as Data Deficient (DD), until more information from the species becomes available. However, the wildlife refuge where the species was recorded is immersed in the metropolitan area matrix and suffers for plausible threats (e.g. air pollution, high population density, sugarcane plantation) that may compromise the maintenance of species.

Etymology: Latin, pernambucana, based on the name of the Pernambuco State of Brazil, where the fungus was found.

Key to ***Mahabalella*** species

1. Conidia unicellular 2

1. Conidia 1-septate, broadly subfusiform or cylindrical, 11–14 × 2 µm ..... *M. cubensis*

2. Conidia cylindric, 16–18.5 × 2 µm ..... *M. acutisetosa*

2. Conidia cylindric, 6–8 × 1.5- 2 µm ..... *M. pernambucana*

Phylogenetic analyses

New DNA sequences for four genes were obtained and are in the GenBank under accession numbers MT027233 (nLSU), MT027234 (ITS), MT027235 (beta-tubulin), MT036366 (TEF 1-α). The nLSU alignment was deposited in TreeBASE (http://www.treebase.org/treebase/index.html), under accession ID: S29817.

The search for the most similar sequences in the NCBI/GenBank nucleotide database for the four sequenced genes resulted in four lists of the closest hits (Tab. 1). Using the nLSU sequence of the new fungus, the highest similarity is to *Neotracylla pini* (GenBank MN567636.1; Identities = 773/798 (97%), 2 gaps (0%)). Closest hits using the ITS sequence is also *N. pini* (GenBank MN562129.1; Identities = 338/375 (90%), 5 gaps (1%)). The beta-tubulin sequence matches *Talaromyces siamensis* (GenBank JX091379.1; Identities = 328/332 (99%), no gaps) and the TEF 1-α closest hit is *Myxospora crassiseta* (GenBank KU846521.1; Identities = 140/143 (98%), no gaps).

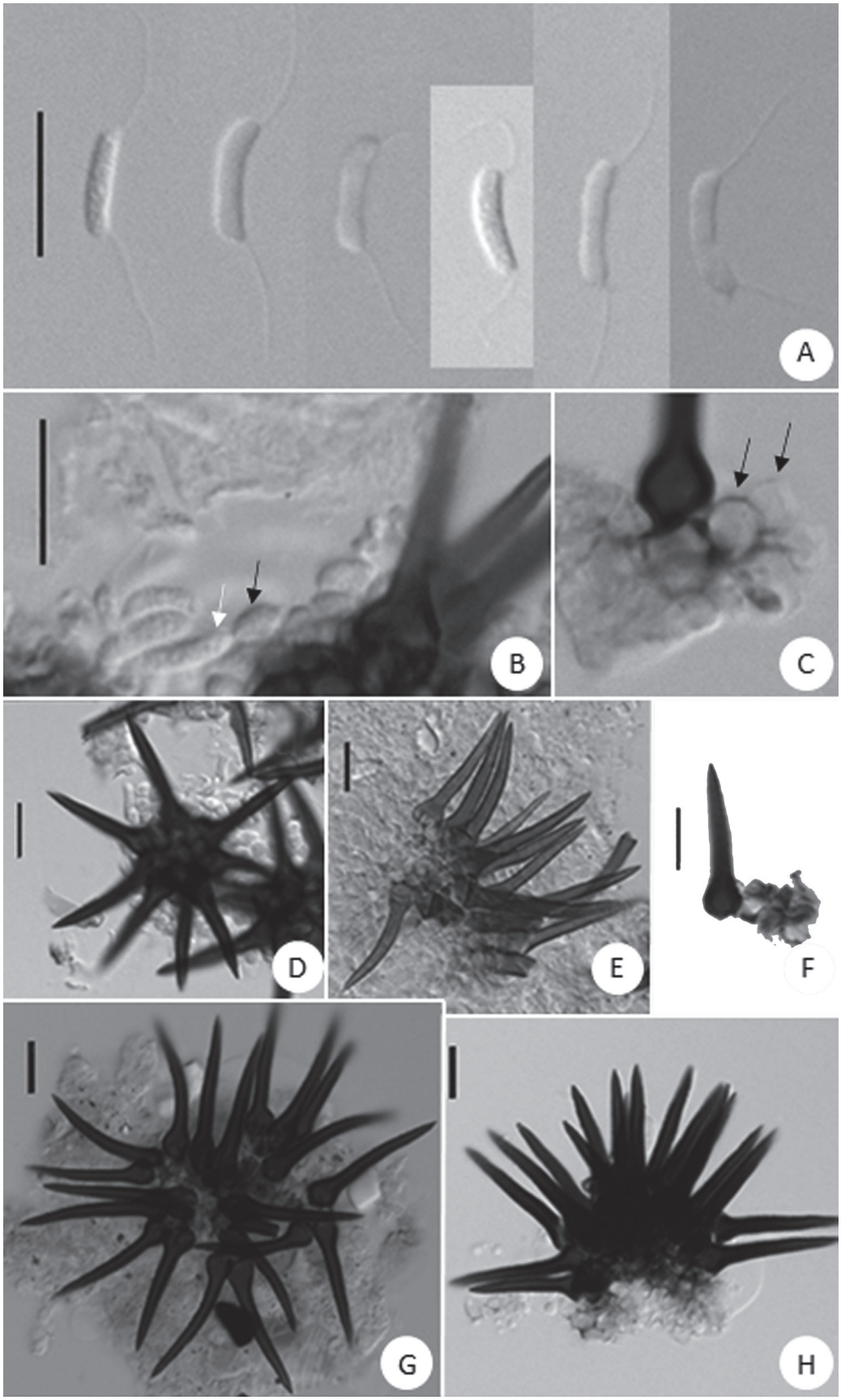

Figure 1. *Mahabalella pernambucana* sp. nov. Conidia (**A**), conidium (white arrow) inserted in conidiogenous cells (black arrow) (**B**), conidiogenous cells (black arrows) (**C**), setae (**D – F**), conidiomata on substrate (**G – H**). Scale bars = 10 µm.

Table 1. Some of the most similar sequences recovered from BLAST search for each of the four genes sequenced for *Mahabalella pernambucana* sp. nov.

Description	E-value	Per. Ident	Accession
nLSU rDNA			
Neotracylla pini **culture CPC:36731**	0.0	96.87%	MN567636
Tracylla eucalypti **culture CPC:31806 EX-TYPE**	0.0	95.61%	MH327846
Tracylla aristata **CPC 25500 TYPE material**	0.0	95.24%	NG_059699
Rhexodenticula cylindrospora **strain CBS 318.95**	0.0	93.36%	KM485039
Camaropella pugillus **strain CBS 128346**	0.0	93.29%	MH876340
Camarops ustulinoides **isolate AFTOL-ID 72**	0.0	93.17%	DQ470941
Camaropella pugillus	0.0	93.04%	EU481406
Camarops amorpha **strain SMH1450**	0.0	92.90%	AY780054
Chaetosphaeria curvispora **strain CBS 125782**	0.0	92.89%	MH875199
Chaetomidium subfimeti **culture-collection CBS:169.71**	0.0	92.62%	FJ666357
Cryptophialoidea fasciculata **voucher MFLU:18-1499**	0.0	92.41%	MH758208
Chaetomium madrasense **strain CBS 126663**	0.0	92.38%	MH875653
Farlowiella carmichaeliana	0.0	92.38%	KF836062
Rhexodenticula acaciae **CPC 29590 TYPE material**	0.0	92.36%	NG_066376
Cryptophiale udagawae **voucher MFLU 19-0209**	0.0	92.30%	MN104619
Internal Transcribed Spacer ribosomal gene			
Fungal sp. W3 isolate W3-d	5e-132	90.13%	KP122265
Neotracylla pini **culture CPC 36731**	2e-135	90.03%	MN562129
Sordariomycetes sp. EF878	4e-133	89.84%	LC131027
Uncultured fungus clone RA2_E06	4e-138	89.80%	JN561598
Sordariomycetes sp. voucher ARIZ:YLH0014	2e-136	89.38%	KP991996
Uncultured fungus clone L049676F03	2e-136	89.36%	JX135771
Uncultured fungus clone L049495C01	2e-135	89.30%	JX135678
Coniochaeta **sp. strain NW-FVA2332**	8e-135	89.28%	MG098277
Fungal endophyte voucher ARIZ:DM0187	8e-135	89.28%	KF673725
Fungal endophyte voucher ARIZ:DM0183	8e-135	89.28%	KF673721
Lecythophora **sp. strain VD030**	4e-133	89.05%	KT588470
Fungal sp. isolate 20A1	4e-133	89.05%	MH497172
Coniochaeta **sp. strain LH1**	4e-133	89.03%	MK734072
Vermiculariopsiella immersa **var.** *spiralis* **strain CBS 523.93**	5e-132	88.78%	MH862440
Lecythophora **sp. strain P5_B2_479**	5e-132	88.70%	KU325210
Beta tubulin			
Talaromyces siamensis **strain CBS 475.88**	3e-167	98.80%	JX091379
Talaromyces siamensis **strain CGMCC 3.18214**	4e-171	97.98%	KX961219
Talaromyces cnidii **strain CNU 100149**	2e-164	96.83%	KF183641
Talaromyces cnidii **strain DI16-138**	8e-163	96.54%	LT559077
Talaromyces xishaensis	4e-151	94.78%	KU644581
Talaromyces aculeatus **strain CBS 282.92**	2e-138	94.69%	KF741914
Talaromyces argentinensis **isolate NRRL 28750**	4e-151	94.56%	MH792917
Talaromyces flavus **voucher BYD07-13**	4e-151	94.56%	KF917584
Trichocomaceae sp. KJ-2012e strain GZU-BCECGYN42-3	4e-141	93.29%	JQ965108
Talaromyces verruculosus **isolate A1S1-6**	2e-139	93.00%	KJ767046
Talaromyces fuscoviridis **isolate NRRL 66370**	4e-141	92.86%	MH792965

Table 1. Cont.

Description	E-value	Per. Ident	Accession
Talaromyces angelicus **strain FMR 15490**	2e-139	92.57%	LT898317
Talaromyces californicus **isolate NRRL 58661**	2e-139	92.55%	MH792932
Talaromyces pratensis **isolate NRRL 62126**	1e-136	92.44%	MH792937
Penicillium verruculosum **strain ATHUM 5186**	8e-138	92.29%	FJ004439
Translation elongation factor 1-alpha			
Myxospora crassiseta **strain CBS 121141**	6e-62	97.90%	KU846521
Trichoderma **sp. strain TC614**	1e-63	97.32%	MF371225
Trichoderma sichuanense **strain HMAS 248739**	1e-63	97.32%	KX344430
Trichoderma rossicum **strain S586**	1e-63	97.32%	KJ665703
Trichoderma medusae **strain GJS 01-166**	1e-63	97.32%	HQ342215
Dimorphiseta terrestris **strain CBS 127345**	5e-63	97.30%	KU846401
Trichoderma albolutescens **strain KUC21168**	2e-62	97.28%	KX912194
Trichoderma barbatum **strain TC700**	6e-62	96.64%	MF095877
Trichoderma verticillatum **strain HMAS 248742**	6e-62	96.64%	KX344433
Trichoderma virens **strain TS86**	6e-62	96.64%	KU301729
Allantonectria miltina **strain CBS 125499**	6e-62	96.64%	KM231974
Trichoderma patella **strain G.J.S. 91-141**	6e-62	96.64%	KJ665630
Trichoderma ceramicum **strain S353**	6e-62	96.64%	KJ665445
Hypocrea parestonica **strain C.P.K. 2427**	6e-62	96.64%	FJ860666
Smaragdiniseta bisetosa **strain CBS 459.82**	2e-61	96.62%	KU847303

The phylogenetic analysis of the nLSU sequence of *Mahabalella pernambucana* (Fig. 2) places this fungus in the tree as a sister genus to *Neotracylla*, in the Tracyllaceae family (bootstrap 98%, posterior probability 1.00). The analysis of the ITS fragment did not give as good resolution as LSU to the new species, probably due to its intrinsic variability; however, it also places *M. pernambucana* in the same clade of the Tracyllaceae.

The closest hit for the beta-tubulin gene sequence is a species in the Eurotiomycetes (Tab. 1). *Talaromyces siamensis* is considered *nomen invalidum*, according to the Art. 40.1 of the Nomenclature Code, Melbourne (Index Fungorum 2022), however, other sequences from species of the same genus, like *T. cnidii* and *T. aculeatus* are also good matches, being these in the Aspergillaceae, Eurotiales.

Discussion

The main morphological characteristics of *Mahabalella pernambucana* are aligned with those of the genus, like the absence of conidiophores, straight non-septate setae, phialidic conidiogenous cells and conidia straight with appendages at each end.

Of the four species that have been previously reported in this genus, *Mahabalella acutisetosa* B. Sutton & S.D. Patil., *M. cubensis* R.F. Castañeda, and *M. dimorpha* Matsush. are currently accepted while *M. stiriaca* Melnik, Scheuer & Heftb., although the description matches the genus, has not been validly published (Nom. inval., Art. 40.6, Shenzhen, Turland *et al.* 2018; Index Fungorum 2022). *M. dimorpha*, however, presents some morphological features that support its exclusion from the genus, differing from the other species by having narrow cylindrical, sometimes branched, conidiogenous cells and septate setae (Mel'nik *et al.* 2003). Mel'nik *et al.* (2003) also comment on the conidiogenous cells that can also be produced in short conidiophores, which suggest this species to be non-congeneric. Our species differs from *Mahabalella acutisetosa* by its smaller conidia, conidiogenous cells and setae.

Mahabalella pernambucana also differs from closed related genus *Minimidochium* B. Sutton by the shape of the sporodochia, the shape of the conidiogenous cells and shape of conidia.

The best inferred phylogenetic position for *M. pernambucana* to date is with the Tracyllaceae, however, more DNA sequencing of related species are necessary to better support these findings.

The recently erected Order Tracyllales presents to date one family and two genera (Crous *et al.* 2018; 2019) that remained resolved in the phylogenetic tree when the new *Mahabalella* sequence was included in the analysis (Fig. 2) within Sordariomycetes.

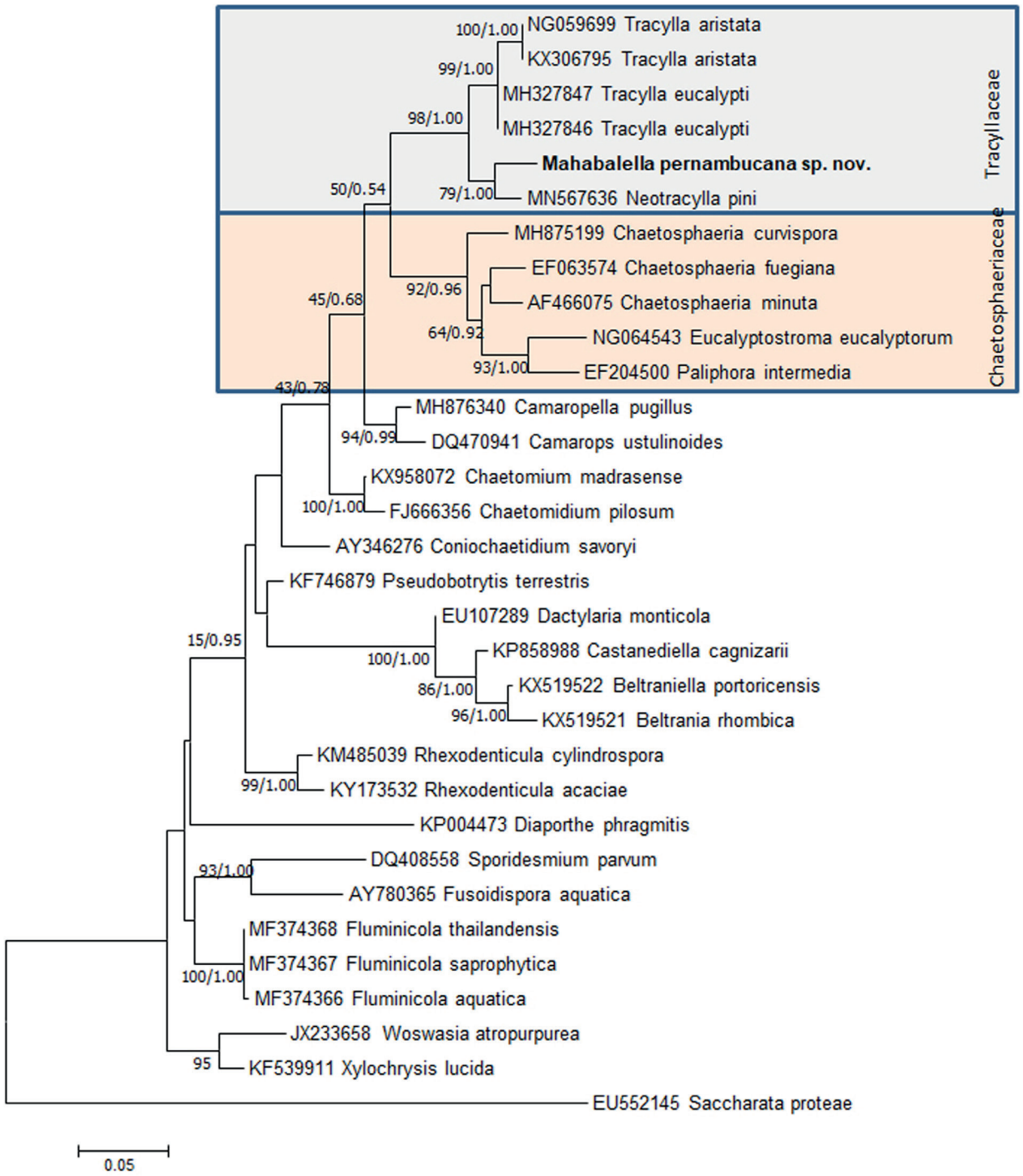

Figure 2. Phylogenetic analysis of *Mahabalella pernambucana* sp. nov. and related fungi. The tree is a maximum likelihood cladogram constructed with the GTR model plus gamma distribution and invariant sites factors and is based on an alignment of 811 bases of the LSU ribosomal region. Numbers closest to the branches indicate % bootstrap recovery/Bayesian posterior probabilities.

The closest sequence to the TEF 1-α gene is also a species of the Sordariomycetes (*Myxospora crassiseta*, Stachybotryaceae, Hypocreales). With our choice of primers it was possible to sequence completely the second and third exons of the TEF 1-α gene, considering the sequence of nucleotides and peptide presented by Nakazato (2005) for *Metarhizium anisopliae* (GenBank AY445082). The regions identified as introns in the sequenced fragment show some variability. However, the conserved domains of the putative peptide (Fig. 3) that was possible to derive from the sequence with confidence using ExPASy (Artimo *et al.* 2012), aligned perfectly with *Metarhizium anisopliae*, *Trichoderma virens* and *Dactylonectria novozelandica* (GenBank AY445082, KU301729 and MK602815, respectively), indicating high conservation over these two different orders.

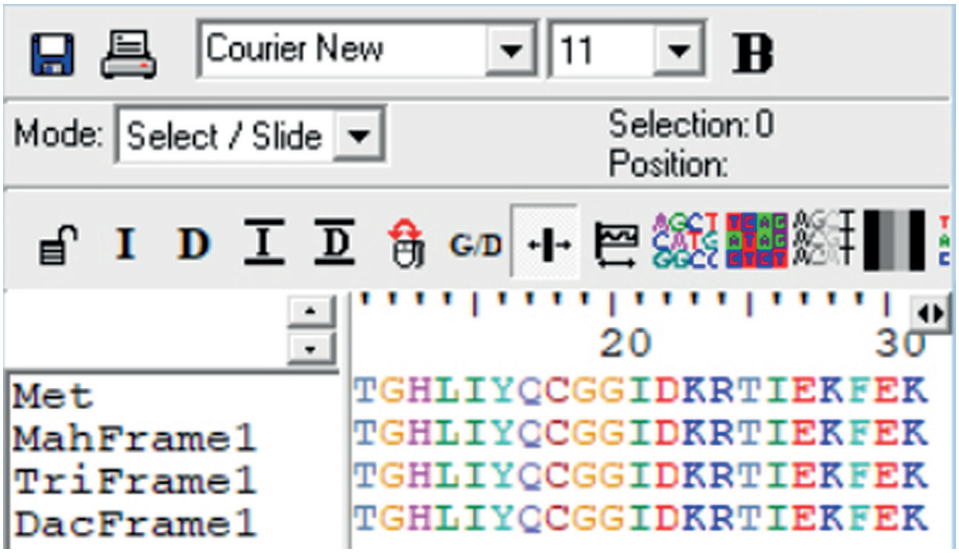

Figure 3. Alignment of TEF 1-α conserved aminoacid residues of the second and third exons for *Metarhizium anisopliae* (Met) *Mahabalella pernambucana* (MahFrame1), *Trichoderma virens* (TriFrame1) and *Dactylonectria novozelandica* (DacFrame1).

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