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A new *Ernakulamia* (Pleosporales, Tetraplosphaeriaceae) species found on palm in Brazil

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Abstract

During a survey of microfungi on decaying leaves of *Syagrus coronata* (licuri palm), a species related to the Tetraplosphaeriaceae was found on this native palm from northeast Brazil. Tetraplosphaeriaceae comprises ten genera and the hyphomycetous asexual morph is characterized by the production of appendages on the conidial body. The new isolate also has the same morphological features on the conidial body, related to genus *Ernakulamia*. A comprehensive morphological and phylogenetic analysis revealed that this specimen represents an undescribed taxon within the genus *Ernakulamia*. Combined phylogenetic analyses (ITS-LSU-SSU-*RPB2*) positioned this *Ernakulamia* strain in a well-supported lineage basal to the *Ernakulamia* clade.

Keywords – Ascomycota – Brazilian fungi – Molecular phylogeny – Novel taxon – Taxonomy

Introduction

Ernakulamia is characterized by its semi-macronematous, simple, brown conidiophores; simple, brown, monotretic conidiogenous cells, and solitary, brown, dry, muriform, euseptate, appendiculate conidia with simple appendages (Delgado et al. 2017, Dong et al. 2020, Hyde et al. 2020). The genus is typified by *E. cochinensis*, and subsequent species added to this genus exhibit similar characteristics, with conidiophores essentially reduced to conidiogenous cells (Subramanian 1994, Delgado et al. 2017). Notably, only *E. cochinensis* and *E. krabiensis*, described by Jayasiri et al. (2019), are recognized to have well-developed conidiophores.

Currently, five species are recognized within the genus *Ernakulamia*, all of which have molecular data available. The type species, *E. cochinensis*, was recently placed in the order Pleosporales, within the family Tetraplosphaeriaceae, according to Delgado et al. (2017). This classification is based on recent material rather than Subramanian's original type collections. The family Tetraplosphaeriaceae is characterized by Tetraploa-like fungi, which feature conidia with several appendages, and includes *Aquatisphaeria* (Li et al. 2021, Zhao et al. 2024), *Byssolophis* (Yu et al. 2024), *Ernakulamia* (Delgado et al. 2017, Dong et al. 2020, Hyde et al. 2020), *Polyplosphaeria* (Phookamsak et al. 2022), *Pseudopolyplosphaeria* (Zhang et al. 2023), *Pseudotetraploa* (Zhao et al. 2024), *Quadricrura* (Tanaka et al. 2009), *Shrungabeeja* (Dong et al. 2020), *Tetraploa* (Li et al. 2023), and *Triplosphaeria* (Tanaka et al. 2009). Members of this family are known to exhibit saprobic lifestyles, typically occurring on Poaceae (bamboo or grasses), palm trees, and diverse hosts (Tanaka et al. 2009, Tang et al. 2023, Zhao et al. 2024).

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While surveying substrates of *Syagrus coronata* (Arecaceae), in Bahia state, Brazil, we found a distinctive species related to *Ernakulamia* genus. Phylogenetic and morphological analysis indicated that it represents a new species, which is described and illustrated herein.

Materials & Methods

Sampling site, isolation and morphological identification

Expeditions at the Universidade Estadual de Feira de Santana in Bahia, Brazil, collected dead leaf samples from *Syagrus coronata* specimens. Following the method of Castañeda-Ruiz et al. (2016), these samples were monitored daily for 21 days using a Leica S8APO stereomicroscope to observe fungal development. Conidia were isolated by transferring them to Petri dishes containing Cornmeal Carrot Agar media (30 g of cornmeal, 30 g of carrot, and 13 g of agar per liter) and incubated at approximately 25°C. Microscope slides with fungal reproductive structures were prepared using lactic acid or PVL (polyvinyl alcohol and lactophenol) resin. The reproductive structures were documented, and their sizes were measured using 40–100× objective lenses on an Olympus BX-51 light microscope equipped with differential interference contrast (DIC), an Olympus DP25 camera, and cellSens Imaging Software (Olympus Corporation, Tokyo, Japan).

DNA extraction, amplification (PCR) and sequencing

Colonies were cultured on CMCA medium for up to 30 days at approximately 25°C under a natural light-dark cycle, after which they were used for DNA extraction. The mycelial content was removed and transferred to an extraction tube. Genomic DNA was extracted using the DNeasy® Plant Mini Kit (Qiagen), following the manufacturer's instructions. The internal transcribed spacer (ITS) region, the nuclear ribosomal large subunit (LSU) and nuclear ribosomal small subunit (SSU) of rDNA, and part of the RNA polymerase largest subunit (*RPB2*) were amplified using primers ITS4/ITS5 (White et al. 1990), LR0R/LR5 (Vilgalys & Hester 1990, Vilgalys & Sun 1994), NS1/NS4 (White et al. 1990) and fRPB2-5f/fRPB2-7cR (Liu et al. 1999). Amplification reactions for ITS, LSU, SSU gene regions followed the protocol described by Barreto et al. (2023) and for *RPB2*, Yilmaz et al. (2021) was used. PCR products were purified with 20% polyethylene glycol (PEG) (Paithankar & Prasad 1991) and sequenced using the same primers at the Fundação Oswaldo Cruz (Fiocruz) in Bahia, Brazil.

Phylogenetic analyses

DNA consensus sequences were assembled using BioEdit (version 5.0.9) (Hall 1999) and compared with data from NCBI GenBank using the BLASTn tool. Following the methodology of phylogenetics analyses from Delgado et al. (2017) and Li et al. (2021), a combined dataset was constructed comprising sequences of species within the Tetraplosphaeriaceae, Pleosporales. This dataset was aligned using the MAFFT online program (version 7) (Katoh & Standley 2013) to confirm the phylogenetic relationship of our strain within the *Ernakulamia* genus. Subsequently, a combined ITS-LSU-SSU-*RPB2* alignment was created to examine the phylogenetic relationship of our strain with related taxa in the Tetraplosphaeriaceae. Each gene matrix was individually aligned using default parameters in the MAFFT software.

The Maximum Likelihood analysis (ML) was performed via IQ-TREE (ver. 1.6.12, https://www.iqtree.org/; Nguyen et al. 2015). The analysis involved 100 ML searches, starting from one randomized stepwise addition parsimony tree. Branch supports were calculated using the Ultrafast bootstrap method (Hoang et al. 2018) with 1,000 replications. To determine the best-fit evolution model and best partition strategy for the dataset, we employed ModelFinder (Kalyaanamoorthy et al. 2017) within the IQ-TREE, based on the Akaike information criterion (AICc). Bayesian inference (BI) analysis was conducted using MrBayes (ver. 3.2.7a) in the CIPRES Science Gateway (see http://www.phylo.org/; Miller et al. 2010), employing a Markov Chain Monte Carlo (MCMC) algorithm. The models and partitions were the same as those used in ML analysis. Four independent Metropolis-coupled MCMC chains were run twice, with 10 million

generations, and trees were saved every 1000th generation. A burn-in of 25 % was set for the initially sampled trees, and the remaining trees were used to reconstruct a 50 % majority-rule consensus tree and calculate Bayesian posterior probabilities (BPP) of the clades.

The bootstrap support (BS ≥ 70%) and Bayesian posterior probability (BPP ≥ 0.95) values were plotted at the nodes. The phylogenetic tree was visualized via FigTree (ver. 1.4.4, see http//tree.bio.ed.ac.uk/, Rambaut 2012) and edited using Inkscape (ver. 1.1.1, see www.inkscape.org). The newly generated sequences and others retrieved from GenBank are presented in Table 1.

Results

Phylogenetic analyses

After excluding ambiguously aligned regions, the combined alignment included 34 sequences from Tetraplosphaeriaceae, and *Amniculicola immersa* (CBS 123083) and *A. parva* (CBS 123092) from Amniculicaceae were used as the outgroup (Table 1). The alignment comprised 3220 characters, including gaps (LSU: 787; ITS: 473; SSU: 966 and *RPB2*: 994), of which 2446 were conserved, 755 were variable, and 481 were parsimony informative. The evolutionary models applied were GTR+I+G for both ITS and LSU, HKY + I for SSU and GTR + I for *RPB2*. The best-scoring maximum likelihood tree had a final optimization likelihood value of -*ln* = -6480.933. For Bayesian inference, the average standard deviation of split frequencies was 0.007177 after 10 million generations. Both the ML and BI analyses produced trees with identical topologies (Fig. 1). In the combined (ITS-LSU-SSU-*RPB2*) dataset, our specimen was grouped into a strongly supported independent clade (BS: 100%; BPP: 1), with *Ernakulamia syagri* forming an independent lineage at the base of *Ernakulamia* clade.

Ernakulamia syagri G.G. Barreto, L.M.S. Lima & Gusmão sp. nov. Fig 2

Index Fungorum number: IF904155; Facesoffungi number: FoF18018

Etymology – '*syagri'* refers to the host *Syagrus coronata*.

Saprobic on dead leaves of *Syagrus coronata*. **Sexual morph:** Undetermined. **Asexual morph**: Hyphomycetous. *Colonies* on natural substrate effuse, black, punctiform. *Mycelium* partly superficial, partly immersed, brown, branched, septate verrucose. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* monotretric, determinate, intercalary or terminal, globose, smooth, brown to dark brown, 6–11 µm diam. ($\bar{x}$ = 8.9 µm, n = 50). Schyzolitic secession. *Conidia* 42–63 × 30–50 µm ($\bar{x}$ = 55.2 × 43.4 µm, n = 50), dictyoseptate, solitary, dry, variable in form, oval to ellipsoid, subglobose or fusiform, mainly obconic or piriform, breaks easily, pale brown to dark brown, verruculose, schizolytic. Conidial appendages in the middle or at the apex of conidia, 4–8 appendages, brown, septate, 57–125 (140–) × 5–7.5 µm ($\bar{x}$ = 85.7 × 6.3 µm, n = 50).

Material examined – Brazil, Bahia, Feira de Santana, State University of Feira de Santana, on dead leaves of *Syagrus coronata*, 3 Jul 2023, L.M.S. Lima, (HUEFS 268787, holotype permanent slides), ex-type living culture CCMB 742.

Notes – The genus *Ernakulamia* has five described species (Table 2). In the blast search, the ITS region has the closest hits with *Ernakulamia cochinensis* (MFLUCC 18-1237), with identities 497/509 (98%) and gaps 2/509 (0%), and with *Ernakulamia* sp. (SZ-2024a) with identities 496/509 (97%), and gaps 1/509 (0%). The LSU region shows similarities with *Ernakulamia tanakae* (LSP02), having identities of 823/825 (99%) and gaps 0/825 (0%), and with *Ernakulamia tanakae* strain NFCCI 4617 having identities 823/825 (99%) and gaps 0/825 (0%). The SSU region shows similarities with *Ernakulamia* sp. (SZ-2024a), with identities of 983/984 (99%) and gaps 1/984 (0%), and with *Ernakulamia cochinensis* strain MFLUCC 18-1237 with identities of 977/978 (99%), and gaps 1/978 (0%). The *RPB2* region shares with *Ernakulamia* sp. (SZ-2024a), with identities of 1047/1061 (99%) and gaps 1/1061 (0%), and with *Ernakulamia krabiensis* strain MFLUCC 18-0237, with identities of 1048/1069 (98%), and gaps 1/1069 (0%). *Ernakulamia syagri* is morphologically similar to *E. cochinensis* (37–68 × 28–42 µm) in terms of conidia.

However, *E. cochinensis* has the production of 3–15 appendages (Subramanian 1994; Delgado et al. 2017), while *E. syagri* has 4–8 appendages. *Ernakulamia krabiensis* is described as producing conidia in conidiophore tips and has fewer appendages in number (3–4) and smaller in size (up to 32 µm) (Jayasiri et al. 2019). *Ernakulamia tanakae* (Hyde et al. 2020) has similar conidial and appendage sizes but differs phylogenetically. *Ernakulamia xishuangbannaensis* (Dong et al. 2020) has more globose conidia, differing easily from *E. syagri* conidia, which are more obconic, and larger than the other species.

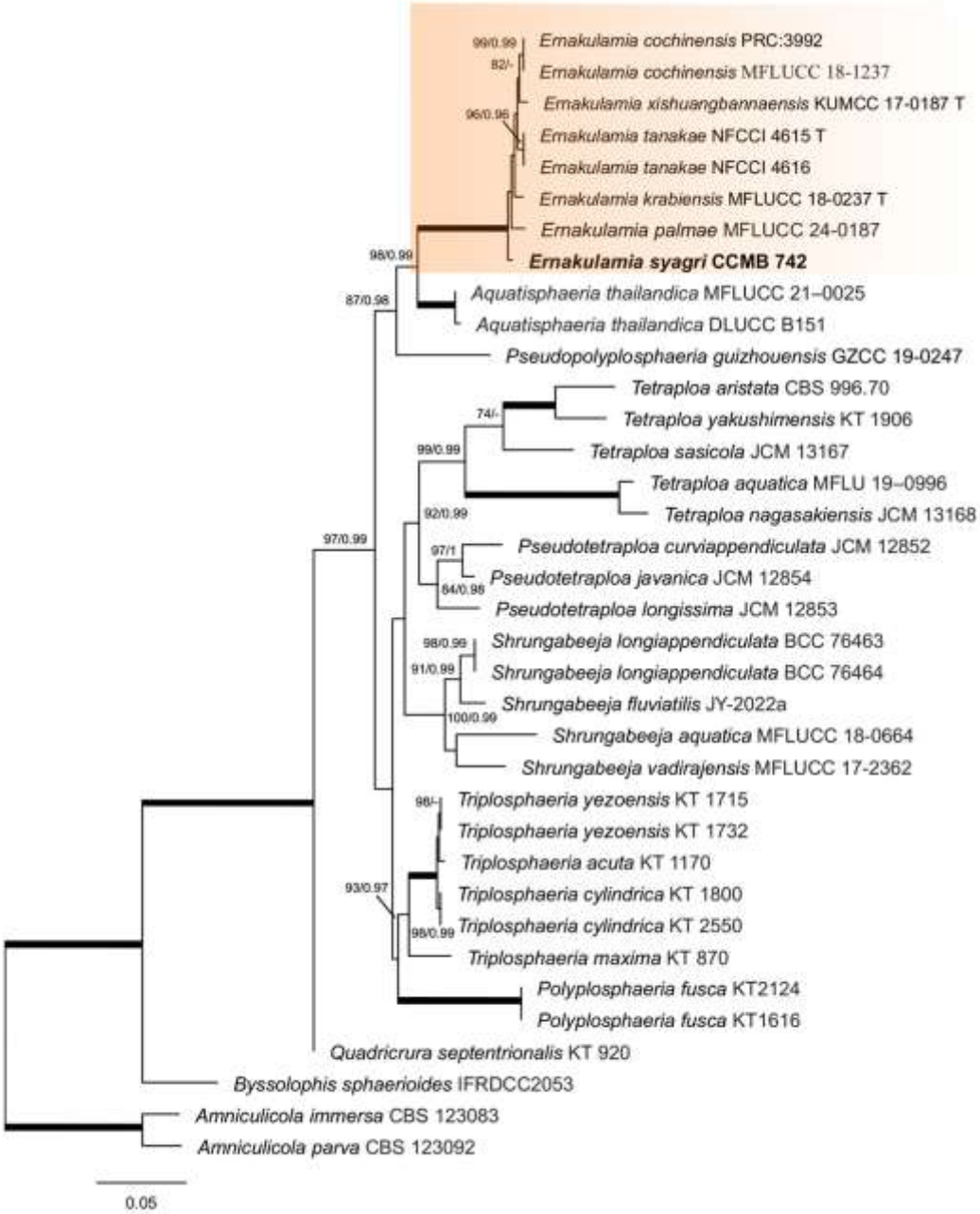

Fig. 1 – Maximum Likelihood phylogenetic tree based on the combined ITS, LSU, SSU and *RPB2* datasets. Confidence values for MLBS ≥ 70% and BPP ≥0.95 are included near the nodes, the "-" indicating a lack of statistical support. The scale bar represents the expected number of changes per site. Full statistical support (MLBS = 100% and BPP= 1.0) is highlighted by thickened branches. The newly generated sequence is in bold and the ex-type strains are marked with a "T" after the culture number. The tree was rooted to *Amniculicola immersa* (CBS 123083) and *A. parva* (CBS 123092) from Amniculicaceae.

Table 1. Species, strains, vouchers, and accession numbers of the specimens used in phylogenetic analyses. Newly generated sequences are in bold.

Species	Strain/Voucher	Country	GenBank Accession no.				References
			ITS	LSU	SSU	*RPB2*	
Amniculicola immersa	CBS 123083	Denmark	-	NG056964	NG062796	GU456358	Zhang et al. (2009)
A. parva	CBS 123092	France	-	NG056970	NG016504	-	Schoch et al. (2009)
Aquatisphaeria thailandica	MFLUCC 21–0025	Thailand	MW890969	MW890763	MW890967	-	Li et al. (2021)
A. thailandica	DLUCC B151	Thailand	-	MW890764	MW890968	-	Li et al. (2021)
Byssolophis sphaerioides	IFRDCC2053	France	-	GU301805	GU296140	GU456348	Schoch et al. (2009)
Ernakulamia cochinensis	PRC:3992	Panama	LT964671	LT964670	-	-	Delgado et al. (2017)
E. cochinensis	MFLUCC 18-1237	Thailand	MT627670	MN913716	MT864326	-	Dong et al. (2020)
E. krabiensis	MFLUCC 18-0237	Thailand	NR_163341	NG_066314	-	MK434872	Jayasiri et al. (2019)
E. palmae	MFLUCC 24-0187	Thailand	PP592382	PP593412	PP627533	PP761054	Li et al. (2023)
E. syagri	**CCMB 742**	**Brazil**	**PP889895**	**PP889896**	**PQ726987**	**PQ732966**	**This paper**
E. tanakae	NFCCI 4615	India	NR_168845	MN937211	-	-	Hyde et al. (2020)
E. tanakae	NFCCI 4616	India	MN937227	MN937209	-	-	Hyde et al. (2020)
E. xishuangbannaensis	KUMCC 17-0187	China	NR_168184	MH260314	MH260354	-	Tibpromma et al. (2018)
Polyplosphaeria fusca	KT2124	Japan	AB524791	AB524607	AB524466	-	Tanaka et al. (2009)
P. fusca	KT1616	Japan	AB524789	AB524604	AB524463	-	Tanaka et al. (2009)
Pseudotetraploa curviappendiculata	JCM 12852	Japan	AB524792	AB524608	AB524467	-	Tanaka et al. (2009)
P. javanica	JCM 12854	Japan	AB524795	AB524611	AB524470	-	Tanaka et al. (2009)
P. longissima	JCM 12853	Japan	AB524796	AB524612	AB524471	-	Tanaka et al. (2009)
Pseudopolyplosphaeria guizhouensis	GZCC 19-0247	China	-	-	NG_242985	OR146944	Zhang et al. (2023)
Quadricrura septentrionalis	KT 920	Japan	AB524801	AB524617	AB524476	-	Tanaka et al. (2009)
Tetraploa aquatica	MFLU 19–0996	China	MT530449	MT530453	MT530454	-	Li et al. (2020)
T. aristata	CBS 996.70	Japan	AB524805	AB524627	AB524486	-	Tanaka et al. (2009)
T. nagasakiensis	JCM 13168	Japan	AB524806	AB524630	AB524489	-	Tanaka et al. (2009)
T. sasicola	JCM 13167	Japan	AB524807	AB524631	AB524490	-	Tanaka et al. (2009)
T. yakushimensis	KT 1906	Japan	AB524808	AB524632	AB524491	-	Tanaka et al. (2009)
Triplosphaeria acuta	KT 1170	Japan	AB524809	AB524633	AB524492	-	Tanaka et al. (2009)
T. cylindrica	KT 1800	Japan	AB524810	AB524635	AB524494	-	Tanaka et al. (2009)
T. cylindrica	KT 2550	Japan	AB524811	AB524636	AB524495	-	Tanaka et al. (2009)
T. maxima	KT 870	Japan	AB524812	AB524637	AB524496	-	Tanaka et al. (2009)
T. yezoensis	KT 1715	Japan	AB524813	AB524638	AB524497	-	Tanaka et al. (2009)
T. yezoensis	KT 1732	Japan	AB524814	AB524639	AB524498	-	Tanaka et al. (2009)
Shrungabeeja aquatica	MFLUCC 18-0664	Thailand	NR_171977	NG_074463	-	-	Dong et al. (2020)
S. fluviatilis	JY-2022a	China	OP377804	OP377903	-	OP473080	Yang et al. (2023)
S. longiappendiculata	BCC 76463	Thailand	KT376474	KT376472	KT376471	-	Ariyawansa et al. (2015)
S. longiappendiculata	BCC 76464	Thailand	KT376475	KT376473	-	-	Ariyawansa et al. (2015)
S. vadirajensis	MFLUCC 17-2362	Thailand	MT627681	MN913685	-	-	Dong et al. (2020)

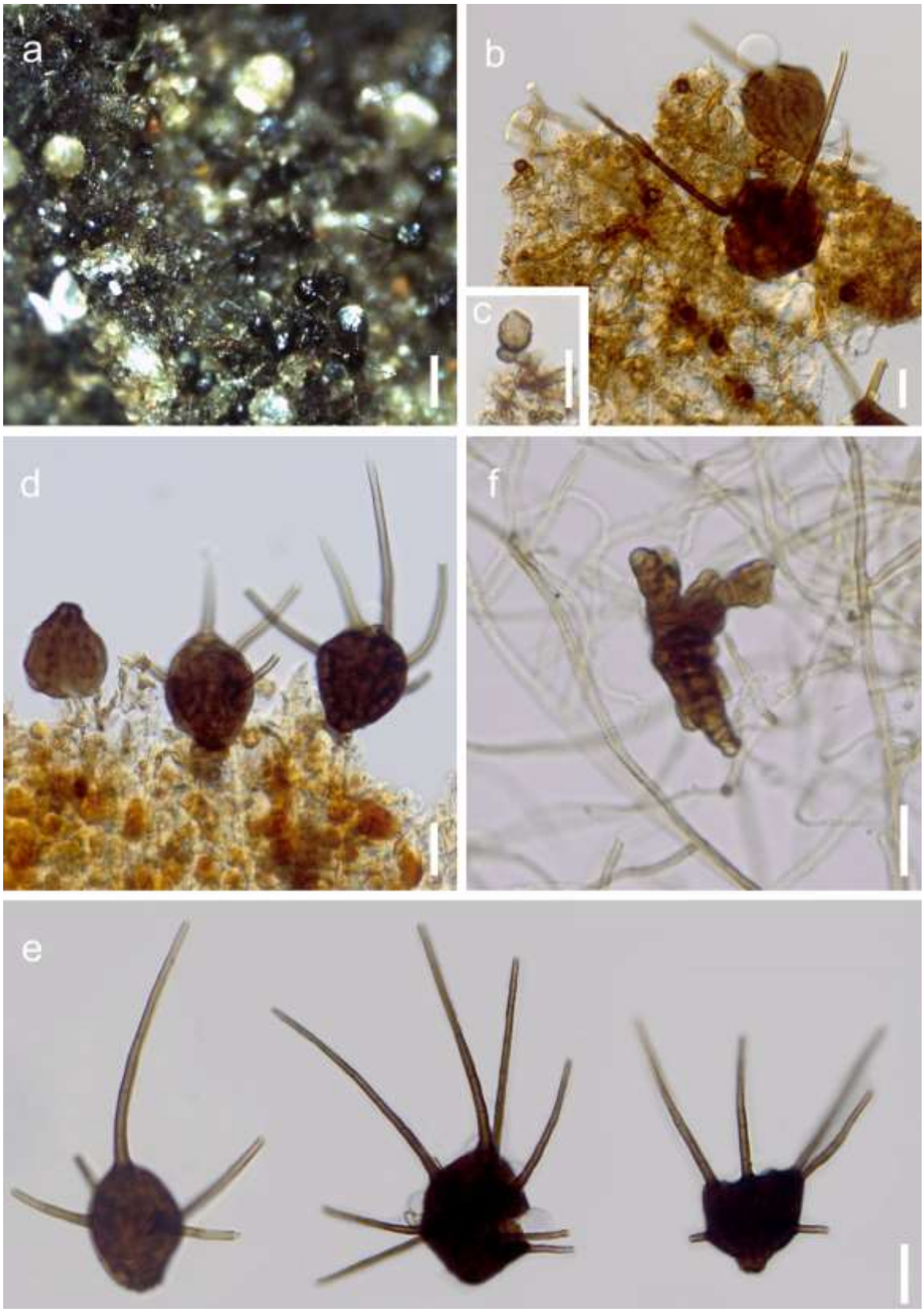

Fig. 2 – *Ernakulamia syagri* (HUEFS 268787, holotype permanent slides). a Colony on dead leaves of *Syagrus coronata*. b–e Mature conidia, conidiogenous cells and young conidium from natural substrate. f Conidia and verrucose hyphae from CMCA culture media (CCMB 742). Scale bars: a = 100 μm, b–f = 20 μm.

Table 2. Summary table for morphological characteristics of asexual *Ernakulamia* species.

Species	Appendage number/size (µm)	Conidia morphology/size (µm)	References
E. cochinensis	3–15/up to 140 long	Obconical, ovoid, broad fusiform, subglobose, verrucose, dictyoseptate, dark brown, 37–68 × 28–42	Subramanian (1957, 1994), Delgado et al. (2017)
E. krabiensis	3–4/21–32 × 3.3–3.7	Obconical, ovoid, broad-fusiform or subglobose, verrucose, dictyoseptate, brown 46–55 × 36–49	Jayasiri et al. (2019)
E. syagri	4–8/57–125 (140–) × 5–7.5	Oval to ellipsoid, subglobose or fusiform, mainly obconic or piriform, verrucose, dictyoseptate, pale brown to dark brown 42–63 × 30–50	This study
E. tanakae	(4–)6–13/30–87 × 3–4.5	Obconical to irregular, dictyoseptate, verrucose, dark brown, 30–53 × 22–50	Hyde et al. (2020)
E. xishuangbannaensis	5–9/75–132 × 4–7	Subglobose, solitary, verrucose, black, 53–115 × 45–105	Dong et al. (2020)

Discussion

The family Tetraplosphaeriaceae predominantly comprises asexual morphs (hyphomycetous), including genera such as *Aquatisphaeria*, *Ernakulania*, *Quadricrura*, and *Shrungabeeja* (Hyde et al. 2024). Sexual representatives are limited to *Byssolophis* and *Pseudopolyplosphaeria*, while *Pseudotetraploa*, *Tetraploa*, *Triplosphaeria*, and *Polyplosphaeria* exhibit both sexual and asexual morphs. In hyphomycetous representatives, conidia are cylindrical to obpyriform, composed of 3–8 columns or an internal hyphal structure (Tang et al. 2023). These conidia are typically verrucose at the base and bear 2–8 setose appendages.

Our phylogenetic analyses placed *Ernakulamia syagri* in a strong-supported clade, basal to *Ernakulamia* species, and related to *Pseudopolyplosphaeria*, and *Aquatisphaeria*. *Pseudopolyplosphaeria* is characterized by the production of only sexual morph with cylindric-clavate asci and hyaline, fusiform, one-septate ascospores (Zhang et al. 2023), while *Aquatisphaeria* was only described based on its asexual morph. *Aquatisphaeria* is characterized by globose conidia with pale appendages radiating to their apex and also producing conidiophores (Li et al. 2021). Regarding *Ernakulamia* distribution and ecology, all species were first described occurring in the Asian continent, being *E. cochinensis* and *E. tanakae* described on dead spathes of *Cocos nucifera* (India) (Subramanian 1957, 1994, Hyde et al. 2020); *E. krabiensis* occurring on decaying pods septum of *Acacia* sp. in Thailand (Jayasiri et al. 2019), and *E. xishuangbannaensis* described occurring on dead leaves of *Pandanus* sp. in Yunnan, China (Dong et al. 2020). Recently, a new species *Ernakulamia palmae* was also described in palm substrate, occurring on *Calamus* sp., this species was also the first on *Ernakulamia* genus having its sexual morph described (Zhang et al. 2024). It appears that *Ernakulamia* prefers palm tree substrates. This is evidenced by the descriptions of *E. cochinensis*, *E. palmae* and *E. tanakae* on palms, with *E. cochinensis* consistently being found on the same substrates, being also recorded on palms of the genera *Archontophoenix*, *Arenga*, *Bactris*, *Calyptronoma*, *Livistona*, *Phoenix*, and *Roystonea*, supporting this theory (Lu et al. 2000, Delgado-Rodriguez & Mena-Portales 2004, Delgado 2008, Tianyu 2009, Capdeet & Romero 2010).

Over the past decades, fungi on palm trees have been extensively studied. This substrate is known to host numerous fungi, and new collections of native species from tropical regions such as South America have described numerous novelties (Fröhlich et al. 1999, Taylor et al. 2000, Pereira & Phillips 2023). In Brazil, studies of fungi on this plant are rare despite the rich diversity of palm species that are endemic (Souza et al. 2008, Vitoria et al. 2020). Monteiro (2019) analyzed the fungal community on palm leaf litter and identified 107 fungal species corresponding to 79 genera and 25 families. The palms studied were *Attalea* sp., *Astrocaryum gynacanthum*, *A. murumuru*, *Bactris* sp., *Euterpe oleracea*, *Geonoma* sp., *Oenocarpus* sp., *Socratea* sp. and *Syagrus* sp.

Syagrus is considered underestimated in terms of biodiversity, specifically *Syagrus coronata*, an endemic plant from Brazil. Some studies have investigated this plant for fungal diversity, but the methods recovered mainly endophytic fungi (Souza et al. 2008, Vitória et al. 2020, da Silva & Vitória 2024). Therefore, new collections are necessary to investigate the real potential of fungal diversity on these substrates.

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Accessibility of data

The data generated here are deposited on public repositories. The sequences are available at GenBank NCBI (https://www.ncbi.nlm.nih.gov/).

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