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The phylogenetic placement of *Ernakulamia cochinensis* within Pleosporales (Dothideomycetes, Ascomycota)

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Abstract – The phylogenetic affinities of the anamorphic fungus *Ernakulamia cochinensis* are investigated based on a representative specimen recently collected on *Astrocaryum standleyanum* (Arecaceae) in Panama. Molecular phylogenetic analyses using nuclear ribosomal DNA sequence data of the large subunit and the internal transcribed spacer region together with a fragment of the β-tubulin gene suggest that the fungus belongs to the Dothideomycetes (Ascomycota) where it groups with members of the family Tetraplosphaeriaceae in Pleosporales. Morphologically, this placement is further supported by the presence of an internal hyphal structure found within the conidia of the Panamanian collection and an isotype specimen of the fungus similar to species of closely related genera within Tetraplosphaeriaceae, e.g., *Quadricrura* and *Polyplosphaeria*. The putative phylogenetic position of the morphologically similar *Piricaudilium lobatum* in Tetraplosphaeriaceae is proposed based on examination of its type specimen.

palmicolous / *Petrakia* / *Piricauda* / saprobic / taxonomy

INTRODUCTION

Palm trees (Arecaceae) harbor a wide range of microfungi exhibiting a variety of life strategies such as saprobic, parasitic and endophytic ones (Fröhlich *et al.*, 2000; Fröhlich & Hyde, 2000; Hyde *et al.*, 2000; Taylor & Hyde, 2003). The monotypic genus *Ernakulamia* Subram. (Subramanian, 1994) is one of the saprobic taxa commonly found associated with palm hosts. *Ernakulamia cochinensis*

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(Subram.) Subram., the type species, is characterized by semimacronematous, simple conidiophores, monotretic, integrated, terminal or intercalary, cicatrized conidiogenous cells with a well-defined pore in the middle of each scar and muriform, dark brown, euseptate conidia of variable shape that are verrucose at their base and possess numerous straight, unbranched appendages (Ellis, 1976). Subramanian (1957) first described this peculiar anamorph without illustration within the genus *Petrakia* (*Pe*.) Syd. & P. Syd. as *Pe. cochinensis* Subram. based on a specimen collected on a dead spathe of *Cocos nucifera* L. in India. He considered it congeneric with *Pe. echinata* (Peglion) Syd. & P. Syd., the generic type, based on similar conidial shape, muriform septation and appendiculate conidia. Later, Ellis (1976) illustrated the fungus and transferred it to *Piricauda* (*P*.) Bubák as *P. cochinensis* (Subram.) M.B. Ellis following the generic concept of Hughes (1960) who previously had redescribed its type species *P. uleana* (Sacc. & P. Syd.) Bubák (≡ *P. paraguayensis* (Speg.) R.T. Moore). According to this concept, the micronematous, arched conidiophores developing on superficial hyphae and the tretic conidia arising singly from a pore on the conidiogenous cell are the most distinctive features of the genus (Mercado *et al.*, 2005; da Silva *et al.*, 2016). Holubová-Jechová (1988) commented on the similarity of *P. cochinensis* with the morphologically close fungus *Piricaudilium* (*Pi*.) *lobatum* Hol.-Jech. She also noted the impossibility to prove the presence of an internal hyphal structure within the strongly melanized conidia of *P. cochinensis* similar to the one found in the conidia of *Pi. lobatum*. She further suggested that a detailed study of *P. cochinensis* was needed to confirm if the fungus was congeneric with *P. paraguayensis* despite sharing the same conidiogenesis. Subramanian (1994) introduced *Ernakulamia* after deciding that the fungus cannot be retained in either *Petrakia* or *Piricauda* because of morphological and ecological differences such as conidial septation, conidiogenesis and habitat. In a revision of *Piricauda* following Hughes' and Ellis' criteria, Mercado *et al.* (2005) accepted *P. cochinensis* together with seven other species probably unaware of Subramanian's publication as the name *Ernakulamia* has been overlooked by most authors reporting this fungus (Capdeet & Romero, 2010).

Literature and online records show that *E. cochinensis* is common in tropical and subtropical areas where it has been mostly collected on petioles and dead leaves of palm species belonging to twenty different genera of Arecaceae and several other undetermined palm trees (Bhat & Sutton, 1985; Holubová-Jechová & Mercado, 1986, 1989; Mercado *et al.*, 1997b, 2005; Cybertruffle's Robigalia, 2017; HerbIMI Database, 2017; Mycoportal, 2017). Taylor & Hyde (2003) considered its host range restricted to this family of monocots. However, the fungus has also been recorded on a broader host spectrum including *Benthamidia japonica* (Siebold & Zucc.) H. Hara (Cornaceae), *Stewartia monadelpha* Siebold & Zucc. (Theaceae), *Ilex* sp. (Aquifoliaceae), *Ocotea leucoxylon* (Sw.) De Laness. (Lauraceae), *Freycinetia multiflora* Merr., *Pandanus tectorius* Parkinson ex Du Roi, *P. monticola* F. Muell., *Pandanus* sp. (Pandanaceae) and *Vitex* sp. (Lamiaceae) (Delgado & Mena, 2004; Whitton *et al.*, 2012; Farr & Rossman, 2017). Nakagiri & Ito (1995) first isolated and described *E. cochinensis* on corn meal agar (CMA) from a specimen collected on a dead petiole of the palm tree *Satakentia liukiuensis* (Hatus.) H.E. Moore in Japan. They also conducted scanning electron microscopy studies on conidiogenesis and conidia showing ultrastructural details of the pores at the apex of tretic conidiogenous cells and the conidia basal cells. Phylogenetic relationships using molecular data, on the other hand, have not been previously assessed for *Ernakulamia* and DNA sequence data are still lacking in GenBank database. Teleomorph connections are currently unknown and the genus is tentatively considered

Ascomycota incertae sedis (Wijayawardene *et al.*, 2012). Tanaka *et al.* (2009) suggested that species of *Piricauda* sensu Mercado *et al.* (2005) including *E. cochinensis* have conidia morphologically similar to those present in some members of Tetraplosphaeriaceae, a pleosporalean family they introduced for *Massarina*-like ascomycetes with appendiculate anamorphs resembling *Tetraploa* Berk. & Broome. They also pointed out that molecular studies are necessary to clarify their phylogenetic affinities and their morphological resemblance may be the result of convergent evolution.

During field sampling in south-western Panama one of us (O.K.) collected *E. cochinensis* on rotten leaves of a palm tree. The fungus grew on agar media and the isolate was characterized by morphological, cultural and molecular data. In order to test the morphology-based hypotheses outlined above and to elucidate a phylogenetic placement for *E. cochinensis* within the current classification of Ascomycota (Schoch *et al.*, 2009) DNA sequence data of two different gene regions were analyzed. Results are presented here along with morphological and cultural studies of the Panamanian collection and a revision of an isotype specimen. Comments on the putative phylogenetic placement of *Pi. lobatum* are also provided based on morphological examination of its type material.

MATERIALS AND METHODS

Morphological and cultural study

The specimen of *E. cochinensis* studied here was collected on rotten leaves of the palm tree *Astrocaryum standleyanum* L.H. Bailey, the black palm, during field work carried out in Chiriquí Province, Panama, in July 2016. A first isolation was made on 2% malt extract agar (MEA) by removing single conidia from the substrate surface with a sterile needle. Pieces of mycelium were later transferred aseptically to different culture media e.g. MEA, potato carrot agar (PCA), modified cellulose agar (MCA), water agar with sterile wooden toothpicks, and incubated at room temperature (22-25°C) for cultural characterization and to induce sporulation. Conidia from natural substrate were first bleached in 1% or 3% sodium hypochlorite solutions (NaClO) following Tanaka *et al.* (2009) to detect the presence of internal structures. Partially or fully bleached conidia were then transferred to a drop of Lactocotton Blue to obtain semi-permanent slides. Voucher specimens are deposited in the Herbarium of the Faculty of Science of the Charles University, Prague (PRC) and the University of Panama Herbarium, Panama (PMA). A living culture was also deposited in the Charles University Culture Collection of Fungi (CCF). An isotype specimen of *E. cochinensis* and the holotype specimen of *Pi. lobatum* were borrowed from the Fungarium of the Royal Botanic Gardens, Kew (IMI) and the Herbarium of the National Museum, Prague (PRM), respectively, for comparison and observation of the internal conidial structure. Line drawings were made with the aid of a drawing tube (Carl Zeiss, Oberkochen, Germany). Fungal names across the text followed Index Fungorum and host plant names followed International Plant Names Index (www.ipni.org). Herbaria or culture collection acronyms are cited according to Index Herbariorum (http://sweetgum.nybg.org/science/ih/).

DNA extraction, PCR amplification & sequencing

Genomic DNA was extracted from 2 weeks old cultures growing on MEA using a Zymo Research Fungal/Bacterial Kit (Zymo Research, Orange, USA) following the manufacturer´s protocols. Nuclear rDNA containing the ITS1-5.8S-ITS2 region and the highly variable D1/D2 domains of the 28S (further referred to as ITS-LSU) was amplified with primer sets ITS1F/NL4 (O'Donnell, 1993) and a fragment of the β-tubulin gene was amplified with primer set T1/T22 (O'Donnell & Cigelnik, 1997). The PCR products were viewed by means of electrophoresis on 1% (w/v) TAE agarose gel stained with ethidium bromide. The PCR products were purified with the Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech, Bade City, Taiwan). Both strands of the PCR fragments were sequenced with the primers used for amplification at the Sequencing Laboratory of the OMICS Core Facility, BIOCEV (Vestec, Czech Republic).

Taxon sampling and phylogenetic analyses

The newly obtained sequence from the freshly isolated strain of *E. cochinensis* (CCF 5738) was first aligned with an ITS-LSU sequence from the morphologically well-characterized Japanese isolate studied by Nakagiri & Ito (1995) and accessed through the website of the Biological Resource Center (NBRC) of the National Institute of Technology and Evaluation of Japan (NITE) (http://www.nbrc.nite.go.jp/). Both sequences were identical and BLAST searches of the consensus including also the newly obtained β-tubulin sequence showed close affinities with members of the family Tetraplosphaeriaceae (Pleosporales, Dothideomycetes). Closest hits and sequences from each genus within the family were selected from previous phylogenetic studies (Tanaka *et al.*, 2009; Ariyawansa *et al.*, 2015; Li *et al.*, 2016) and used to build datasets. Additional taxa from related families in Pleosporales (Hyde *et al.*, 2013; Tibpromma *et al.*, 2016) were also included. Details of strains and sequences used in this study are listed in Table 1. Three separate datasets (ITS, LSU, β-tubulin) were assembled and aligned using the MUSCLE algorithm implemented in Geneious v.6.1.5 software and manually edited in the same software. The best-fit substitution model for each gene was determined using jModeltest v.2.1.5 (Darriba *et al.*, 2012) and the selected models for the ITS, LSU and β-tubulin regions employing the Akaike Information Criterion were TIM2 + G, TIM2 + I + G and HKY + I + G, respectively. The three datasets were tested for combinability by using the partition homogeneity test (Farris *et al.*, 1994) implemented in PAUP*4.0b10 (Swofford, 2002), which showed that there was no significant incongruence only between the ITS and LSU datasets (1,000 artificial data sets, P = 0.41). Phylogenetic analyses of the ITS-LSU dataset with both regions set as separate partitions and β-tubulin were performed by Bayesian inference using MrBayes v.3.2 (Ronquist *et al.*, 2012) and Maximum likelihood (ML) running on the RAxML Web Server v.7.7.1 (Stamatakis *et al.*, 2008). For Bayesian analyses two independent runs of 3,000,000 generations were ran with sampling every 100th generation. The first 25% of samples were discarded as burn-in and the remaining trees were used to compute a 50% majority rule consensus tree with posterior probabilities (PP) as Bayesian branch support. The average standard deviation of split frequencies estimating convergence reached the level of 0.004 and 0.001 at the end of analysis of ITS-LSU and β-tubulin, respectively. The GTRCAT approximation implemented in the ML analysis and nonparametric bootstrapping (BS) with 1000 replicates were used for branch support.

Table 1. Strains included in this study and their GenBank accession numbers. Newly generated sequences are written in bold

Taxon	*Strain*	*Country of origin*	*GenBank accession numbers*			*Reference*
			ITS	*LSU*	*β-tubulin*	
Aquasubmersa japonica	KT 2863	Japan	LC061593	LC061588	–	Ariyawansa *et al.* (2015)
Aquasubmersa japonica	KT 2813	Japan	LC061591	LC061586	–	Ariyawansa *et al.* (2015)
Ernakulamia cochinensis	CCF 5738	Panama	LT964671	LT964670	LT964672	This study
Ernakulamia cochinensis	NBRC 32666	Japan	03266601*	03266601*	–	Unpublished
Hermatomyces krabiensis	MFLUCC 16-0249	Thailand	KX525750	KX525742	–	Tibpromma *et al.* (2016)
Hermatomyces sphaericus	HMAS 42922	P.R. China	KU999956	KX033549	KX036229	Unpublished
Hermatomyces subiculosa	MFLUCC 15-0843	Thailand	KX259521	KX259523	–	Hyde *et al.*(2016)
Hermatomyces tectonae	MFLUCC 14-1140	Thailand	KU144917	KU764695	–	Doilom *et al.* (2017)
Hermatomyces tectonae	MFLUCC 14-1141	Thailand	KU144918	KU764696	–	Doilom *et al.* (2017)
Hermatomyces thailandica	MFLUCC 14-1143	Thailand	KU144920	KU764692	–	Doilom *et al.* (2017)
Hermatomyces thailandica	MFLUCC 14-1144	Thailand	KU144921	KU764693	–	Doilom *et al.* (2017)
Lepidosphaeria nicotiae	CBS 559.71	Algeria	GQ203760	DQ384106	–	Kruys *et al.* (2006)
Lophiotrema neoarundinaria	KT 856	Japan	AB524786	AB524596	AB524848	Tanaka *et al.* (2009)
Lophiotrema neoarundinaria	KT 2200	Japan	AB524787	AB524597	AB524849	Tanaka *et al.* (2009)
Lophiotrema nucula	JCM 14132	Sweden	–	AB619021	–	Hirayama & Tanaka (2011)
Lophiotrema vagabundum	JCM 14138	Sweden	–	AB619025	–	Hirayama & Tanaka (2011)
Paraphaeosphaeria parmeliae	CBS 131728	Belgium	–	–	KP170703	Trakunyingcharoen *et al.* (2014)
Polyplosphaeria fusca	JCM 13175	Japan	AB524789	AB524604	AB524850	Tanaka *et al.* (2009)
Polyplosphaeria fusca	JCM 13173	Japan	AB524788	AB524603	AB524851	Tanaka *et al.* (2009)
Polyplosphaeria thailandica	MFLUCC 15-0840	Thailand	KU248766	KU248767	–	Li *et al.* (2016)
Pseudotetraploa curviappendiculata	JCM 12852	Japan	AB524792	AB524608	AB524854	Tanaka *et al.* (2009)
Pseudotetraploa curviappendiculata	MAFF 239496	Japan	AB524793	AB524609	AB524855	Tanaka *et al.* (2009)

Table 1. Strains included in this study and their GenBank accession numbers. Newly generated sequences are written in bold *(continued)*

Taxon	*Strain*	*Country of origin*	*GenBank accession numbers*			*Reference*
			ITS	*LSU*	*β-tubulin*	
Pseudotetraploa curviappendiculata	CBS 125426	Japan	–	–	AB524856	Tanaka *et al.* (2009)
Pseudotetraploa javanica	JCM 12854	Japan	AB524795	AB524611	AB524857	Tanaka *et al.* (2009)
Pseudotetraploa longissima	JCM 12853	Japan	AB524796	AB524612	AB524858	Tanaka *et al.* (2009)
Quadricrura bicornis	CBS 125427	Japan	AB524797	AB524613	AB524859	Tanaka *et al.* (2009)
Quadricrura meridionalis	CBS 125684	Japan	AB524798	AB524614	AB524860	Tanaka *et al.* (2009)
Quadricrura septentrionalis	CBS 125428	Japan	AB524801	AB524617	AB524862	Tanaka *et al.* (2009)
Quadricrura septentrionalis	CBS 125430	Japan	AB524800	AB524616	AB524863	Tanaka *et al.* (2009)
Shrungabeeja longiappendiculata	BCC 76463	Thailand	KT376474	KT376472	–	Ariyawansa *et al.* (2015)
Shrungabeeja longiappendiculata	BCC 76464	Thailand	KT376475	KT376473	–	Ariyawansa *et al.* (2015)
Tetraploa aristata	CBS 996.70	Japan	AB524805	AB524627	AB524867	Tanaka *et al.* (2009)
Tetraploa sasicola	JCM 13167	Japan	AB524807	AB524631	AB524869	Tanaka *et al.* (2009)
Tetraploa yakushimensis	CBS 125435	Japan	AB524808	AB524632	AB524870	Tanaka *et al.* (2009)
Triplosphaeria acuta	JCM 13171	Japan	AB524809	AB524633	AB524871	Tanaka *et al.* (2009)
Triplosphaeria cylindrica	JCM 14425	Japan	AB524810	AB524635	AB524872	Tanaka *et al.* (2009)
Triplosphaeria cylindrica	NBRC 106247	Japan	AB524811	AB524636	AB524873	Tanaka *et al.* (2009)
Triplosphaeria maxima	JCM 13172	Japan	AB524812	AB524637	AB524874	Tanaka *et al.* (2009)
Triplosphaeria sp.	NBRC 106248	Japan	AB524815	AB524640	AB524877	Tanaka *et al.* (2009)
Triplosphaeria sp.	NBRC 106249	Japan	AB524816	AB524641	AB524878	Tanaka *et al.* (2009)
Triplosphaeria yezoensis	CBS 125436	Japan	AB524813	AB524638	AB524875	Tanaka *et al.* (2009)
Triplosphaeria yezoensis	CBS 125437	Japan	AB524814	AB524639	AB524876	Tanaka *et al.* (2009)
Verruculina enalia	CBS 304.66	Liberia	GQ203796	DQ678079	–	Kruys & Wedin (2009)

*Sequence ID retrieved online from http://www.nbrc.nite.go.jp.
Abbreviations: **BCC**: BIOTEC Culture Collection, Bangkok, Thailand; **CBS**: Centraalbureau voor Schimmelcultures-Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; **CCF**: Culture Collection of Fungi, Charles University, Prague, Czech Republic; **HMAS**: Institute of Microbiology, Chinese Academy of Sciences, Beijing, People's Republic of China; **JCM**: Japan Collection of Microorganisms, RIKEN BioResource Center, Tsukuba, Japan; **KT**: Kazuaki Tanaka; **MAFF**: Ministry of Agriculture, Forestry, and Fisheries Culture Collection, Tokyo, Japan; **MFLUCC**: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; **NBRC**: NITE Biological Resource Center, Kisarazu, Japan.

RESULTS

Molecular analyses

The final concatenated ITS-LSU dataset consisted of 1471 characters, out of which 269 were parsimony informative and 359 variable, and 41 taxa including the outgroup. The 50% majority rule consensus tree resulting from the Bayesian analysis was similar in topology to the most likely ML tree and show that both our isolate of *E. cochinensis* CCF 5738 and the Japanese strain NBRC 32666 clustered together with strong support (PP > 0.95, BS 99%). They grouped with members of Tetraplosphaeriaceae in Pleosporales (Fig. 1a) and occurred within a moderately supported monophyletic lineage (PP 0.94) containing species of *Polyplosphaeria* (*Po*.) Kaz. Tanaka & K. Hiray. and *Quadricrura* Kaz. Tanaka, K. Hiray. & Sat. Hatak. Both genera are characterized by producing globose, appendiculate conidia with internal hyphal structure that are born on monoblastic conidiogenous cells. The only exception also producing this type of conidia, *Shrungabeeja longiappendiculata* Sommai, Pinruan, S. Nuankaew & Suetrong, was placed basal to a *Tetraploa-Pseudotetraploa* clade. The remaining genera, *Triplosphaeria* Kaz. Tanaka & K. Hiray., *Pseudotetraploa* Kaz. Tanaka & K. Hiray. and *Tetraploa* were resolved as monophyletic clades with moderate or strong PP and BS supports. Tetraplosphaeriaceae was also recovered as monophyletic with strong PP support (PP > 0.95).

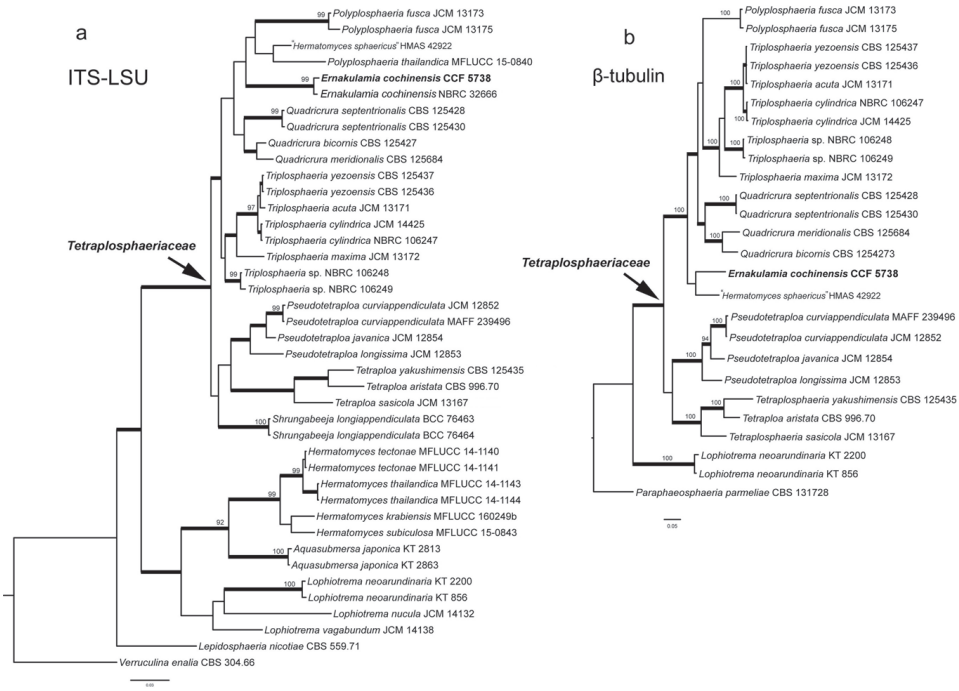

Fig. 1. **a.** Phylogenetic trees inferred from Bayesian and ML analyses of the a) ITS-LSU nrDNA and b) β-tubulin showing the placement of *Ernakulamia cochinensis* among Tetraplosphaeriaceae (Pleosporales). Thickened branches indicate posterior probabilities > 0.95% and numbers above branches represent ML bootstrap support values BS > 90%. The new strain obtained during this study is in bold.

The β-tubulin dataset consisted of 673 characters, out of which 296 were parsimony informative and 323 variable, and 27 taxa including the outgroup. Tetraplosphaeriaceae and each genus within the family received strong support (PP 1, BS 100%) in the resulting Bayesian and ML trees although the placement of *E. cochinensis* differed (Fig. 1b). Our isolate CCF 5738 grouped with moderate support (PP 0.92) with a strain named *Hermatomyces sphaericus* (Sacc.) S. Hughes HMAS 42922 (unpublished) basal to the *Polyplosphaeria*, *Triplosphaeria* and *Quadricrura* clade. This strain was placed sister to *Po. thailandica* C.G. Lin, Yong Wang bis & K.D. Hyde in the ITS-LSU tree and apparently represents an incorrectly identified entry because the genus *Hermatomyces* Speg. is placed outside Tetraplosphaeriaceae (Fig. 1a).

Taxonomy

Ernakulamia cochinensis (Subram.) Subram., Kavaka 22/23: 67 (1996) [1994]
Figs 2-3

≡ *Petrakia cochinensis* Subram., Beih. Sydowia 1: 15 (1957)
≡ *Piricauda cochinensis* (Subram.) M.B. Ellis, More Dematiaceous Hyphomycetes: 367 (1976)

Colonies on natural substrate effuse, black. *Conidiophores* and *conidiogenous cells* not seen. *Conidia* variable in shape, subglobose, obconical, broadly ellipsoidal to broadly pyriform, muriform, dark brown to blackish brown, verrucose at the base where a pore is often seen, 24-60 × 18-53 μm, internally filled with a mass of

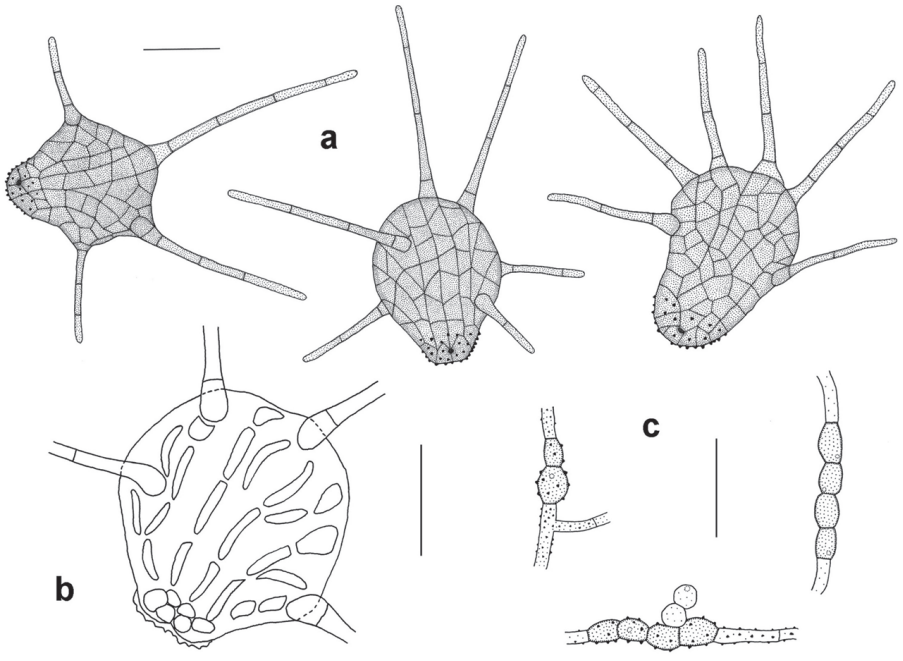

Fig. 2. *Ernakulamia cochinensis* (PRC 3992). **a.** Conidia. **b.** Internal structure of a conidium. **c.** Tretic conidiogenous cells and chlamydospore-like cells on MEA. Scale bars: a-c = 20 μm.

hyaline, septate, 1.5-2 µm wide hyphae sometimes with swollen cells up to 5 µm wide, appendiculate, with 3-13 cylindrical, straight or flexuous, septate, brown, smooth appendages, up to 132 µm long, 3-5 µm wide, 4.5-7 µm wide at base, 2-3 µm wide at the apex.

Colonies on MEA moderately slow growing, reaching 12-16 mm diam. after 14 days at room temperature (22-25°C), circular, velvety, gray, slightly darker

Fig. 3. *Ernakulamia cochinensis* (PRC 3992 = CCF 5738). On natural substrate. **a.** Colonies (arrows) **b.** Conidium. **c.** Detail of a pore at the verrucose base. **d.** Bleached conidium showing internal hyphae. In culture (PCA). **e.** Colonies after 14 days. **f.** Conidium initials born on chlamydospore-like cells. **g.** Conidium attached to conidiogenous cell (arrow). **h-i.** Conidia. *Ibid.* (IMI 114626, isotype). **j.** Packet. **k.** Herbarium material. **l.** Colony. **m-n.** Conidia. **o-p.** Bleached conidia showing internal hyphae. Scale bars: a = 500 µm b-d, f-i, m-n = 20 µm, k = 10 mm, l = 100 µm, o-p = 10 µm.

in the center and raised 1-2 mm, margin entire, reverse dark gray, sporulation starting late after 6-8 weeks. *Colonies* on PCA moderately slow growing, reaching 18-21 mm diam. after 14 days at room temperature (22-25°C), circular, velvety and gray in the center, flat and creamy-white toward the edge, margin entire, sporulation late. *Colonies* on MCA very slow growing, reaching 12-15 mm diam. after 2 months at room temperature (22-25°C), circular, velvety, gray, margin diffuse, reverse black, sporulation not observed even after 3 months. *Mycelium* composed of branched, septate, smooth, finely rough or verruculose hyphae, subhyaline to pale brown or brown in mass, 1-3 µm wide, frequently forming terminal or intercalary, sub-cylindrical or inflated, pale brown to brown, thick-walled, smooth or verrucose, 0-1 septate chlamydospore-like cells, 4-14 × 3.5-7 µm, single or adjacent to each other in rows of up to 9 and often constricted at the septa between them, with 0-1 pore-like conidiogenous locus. *Conidiophores* absent or inconspicuous, short, cylindrical or obconical, 5-8 × 4 µm. *Conidiogenous cells* monotretic, non-cicatrized, globose or subglobose, smooth or verruculose, determinate, subhyaline to brown, 4-8 × 5-7 µm, acropleurogenous, born directly on the hyphae or intercalary between the chlamydospore-like cells, rarely in short chains of 2-3 cells. *Conidia* variable in shape, sometimes similar to those on natural substrate but more often irregularly or aberrantly shaped, with several lobes and spherical protrusions, also cheiroid, with 1-5 diverging columns of cells 10-32 µm wide, muriform, brown, verrucose at base or along the columns of cells when cheiroid, 36-81 × 19-56 µm, with 0-5 appendages, 17-83 × 3-5 µm, often swollen at the base and 6-12 µm wide.

Materials examined: Panama, Chiriquí Province, Los Algarrobos village, along a path to Río Majagua, on rotten leaves of *Astrocaryum standleyanum*, 103 m a.s.l. (8°29'20.1"N 82°26'01.0"W), 12 July 2016, coll. P. Zehnálek & O. Koukol (PRC 3992, PMA); ex-living culture KZP240 = CCF 5738; *ibid.* 11 July 2015, coll O. Koukol (PRC 3730); India, Kerala, Ernakulam, on dead spathe of *C. nucifera*, 16 May 1953, coll. C.V. Subramanian (IMI 114626, isotype of *Pe. echinata*). ***Piricaudilium lobatum*** Hol.-Jech., Cuba, Santiago de Cuba, Sierra de la Gran Piedra, Isabelica Norte Nature Reserve, on dead branches of an undetermined liana, 23 May 1985, coll. V. Holubová-Jechová (PRM 842755, holotype).

Notes: Bleached conidia of the material of *E. cochinensis* from Panama (PRC 3992) and the isotype specimen of *Pe. cochinensis* from India (IMI 114626) revealed the presence of an internal hyphal structure similar to the one found in species of *Polyplosphaeria* and *Quadricrura* (Figs. 2b, 3d, o-p). The morphological study of the holotype specimen of *Pi. lobatum* from Cuba (PRM 842755, Fig. 4) also indicated a strong affinity of this taxon with Tetraplosphaeriaceae particularly in the presence of numerous conidial appendages, internal hyphae and the newly detected peel-like outer wall of conidia.

DISCUSSION

The present collections represent the first record of *Ernakulamia cochinensis* from Panama based on specimens including complete collection data. They are morphologically consistent with a well preserved isotype specimen of *Pe. cochinensis* from India (Figs. 3j-l) of which an ex-type living culture of this or the holotype specimen is currently unavailable. On artificial media the isolate CCF 5738 exhibits phenotypic plasticity and frequently produced irregularly shaped conidia often

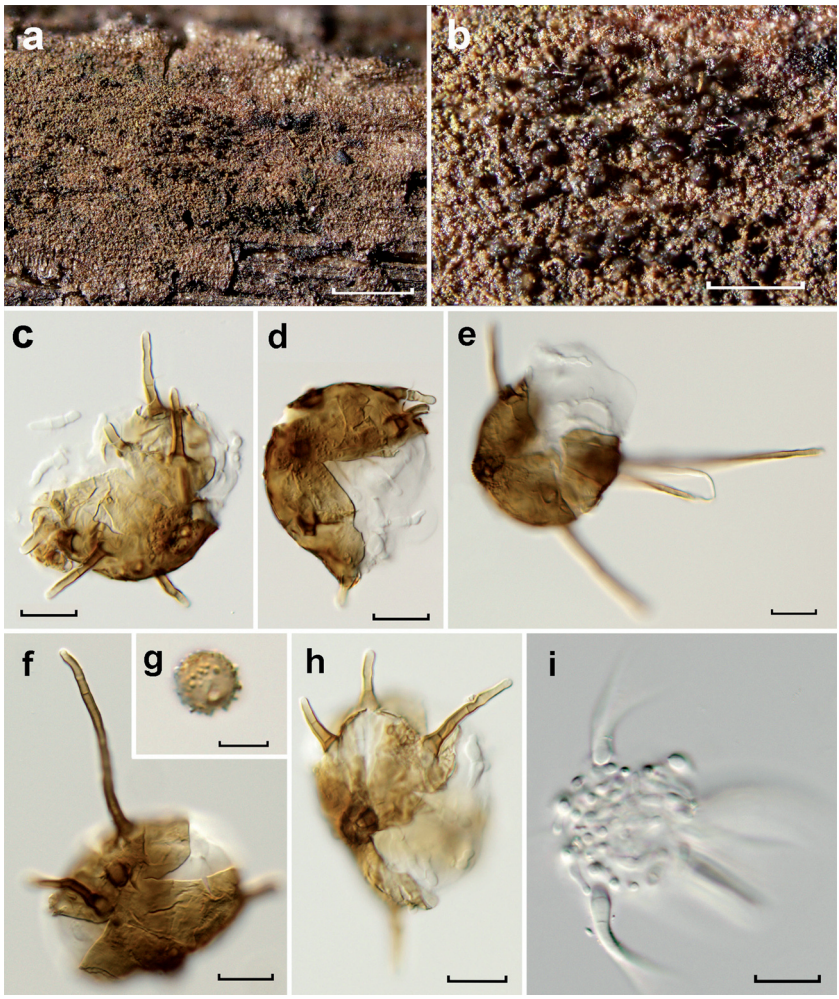

Fig. 4. *Piricaudilium lobatum* (PRM 842755, holotype). **a-b.** Colonies on natural substrate. **c-f.** Mature conidia with peel-like outer wall. **g.** Young conidium with verrucose surface. **h.** Detail of verrucose base. **i.** Bleached conidia showing internal hyphae. Scale bars: a-b = 500 µm, c-j = 20 µm.

observed as aberrant, multi-cellular masses with several lobes and spherical protrusions. They were also cheiroid or cheiroid-like in shape with widely divergent columns of cells or rarely closely appressed around the base and distally diverging or curving at the apical part of the columns. Appendages were often lacking and when present they were shorter and in less number compared with conidia on natural substrate or the type specimen with up to 15 of them and up to 140 µm long (Subramanian, 1957). Thick-walled, often verrucose chlamydospore-like cells, terminal or intercalary on the hyphae, were formed on MEA. They were found to be conidiogenous and showed a single, inconspicuous pore-like locus per cell with conidium initials arising from them (Fig. 3f). Distinct but non-cicatrized conidiogenous cells were also found arising directly on the hyphae or intercalary

between the chlamydospore-like cells of the mycelium. They were more or less similar in shape and disposition to the ones illustrated by Ellis (1976) on natural substrate and curiously, although very rarely, they were present in short chains of 2 or 3 with the apical cell showing an inconspicuous pore (Fig. 2c). Conidiophores were not observed on natural substrate or the isotype specimen and were rarely seen in our isolate. Nakagiri & Ito (1995) also described several differences between their specimen on natural and artificial conditions similar to those observed in our strain. They included the presence of verrucose or tuberculate hyphae, irregularly shaped conidia surrounded by a thin membrane having short, 1-4 appendages only and absence of conidiophores when growing on CMA.

The placement of *E. cochinensis* within Tetraplosphaeriaceae as previously suggested by Tanaka *et al.* (2009) was confirmed by molecular data. It also supports the recognition of *Ernakulamia* as a distinct, well delimited taxon and its previous placements within other genera based on morphological characters (Subramanian, 1957; Ellis, 1976) were rejected. The genus *Petrakia* was recently emended and *Pe. echinata*, its type species, was found to be a member of Melanommataceae, an unrelated family in Pleosporales (Jaklitsch & Voglmayr, 2017). In the case of *Piricauda*, on the other hand, a representative specimen of *P. paraguayensis* from Brazil was found to belong to Capnodiales (da Silva *et al.*, 2016) and therefore this genus is distantly related to Tetraplosphaeriaceae in Pleosporales. The remaining *Piricauda* species still lack DNA sequence data and their phylogenetic affinities are currently unknown although some taxa resembling *Ernakulamia* and sharing a palmicolous habitat e.g. *P. longispora* Mercado, Gené & Guarro and *P. mexicana* Mercado, Heredia & J. Mena (Mercado *et al.*, 1997a, c) might be congeneric upon recollection and sequencing. In the absence of an ex-type culture of *E. cochinensis* to be included in the molecular analyses further evidence of relatedness was found in the presence of an internal conidial structure similarly to species of *Polyplosphaeria, Quadricrura* and *Triplosphaeria*. These and other genera were previously delimited among Tetraplosphaeriaceae based on molecular data and morphological differences of the anamorph and partly of the teleomorph (Tanaka *et al.*, 2009, Ariyawansa *et al.*, 2015). Our ITS-LSU phylogeny containing all currently sequenced genera of Tetraplosphaeriaceae including the newly added *Ernakulamia* strains (Fig. 1a) showed as well a high support for their delimitation based on molecular data and conidial morphology. A first lineage includes *Polyplosphaeria*, *Ernakulamia* and *Quadricrura* characterized by globose, appendiculate conidia. A second one includes *Triplosphaeria* with distoseptate conidia composed of three columns and a third lineage includes *Tetraploa* and *Pseudotetraploa* having both eu- and distoseptate, obpyriform conidia composed of four columns. Interestingly, *S. longiappendiculata* having subglobose, appendaged conidia forms a separate fourth lineage. Our placement of *Shrungabeeja* based on ITS-LSU sequence data is consistent with the analysis of SSU-LSU regions made by Ariyawansa *et al.* (2015) but differs from an ITS phylogeny presented in the same study where *Shrungabeeja* was placed as a basal clade to *Triplosphaeria*. Therefore the position of this genus should be revised using also protein-coding genes. Additionally, *Shrungabeeja* species are distinct in having macronematous, erect and cylindrical conidiophores bearing determinate or percurrent, lageniform conidiogenous cells (Rao & Reddy, 1981; Zhang *et al.*, 2009) in contrast with the remaining genera in Tetraplosphaeriaceae including *Ernakulamia* having reduced or absent conidiophores.

Interestingly, the *Polyplosphaeria*, *Ernakulamia* and *Quadricrura* lineage retrieved from the ITS-LSU phylogeny contains species with both septate (*Po. thailandica*, *E. cochinensis*, Figs. 2-3) and nonseptate conidia (*Po. fusca*,

Quadricrura spp.) suggesting limited importance of this character for generic delimitation. Unfortunately, only *Po. fusca* has a known teleomorph which precludes comparison of further phenotypic characters that may show diagnostic differences among them. Tanaka *et al.* (2009) noted a morphological similarity between *Quadricrura* and *Piricaudilium* but did not provide clear delimiting characteristics among them. Based on examination of the holotype of *Pi. lobatum, Quadricrura* and *Piricaudilium* seem related and might be considered congeneric. *Quadricrura* species and *Pi. lobatum* both produce subglobose conidia with internal hyphal structure (Fig. 4i), external appendages and peel-like outer wall (Fig. 4c-h). This latter character was not mentioned by Holubová-Jechová (1988) in her original description but it was detected during our study of the type material and supports their affinity. The only clear demarcating difference between them is conidiogenesis that is holoblastic in *Quadricrura* (Tanaka, pers. com.) but monotretic in *Piricaudilium* (Holubová-Jechová, 1988). However, this characteristic needs revision because distinct pore-like structures are seen at the base of *Quadricrura* conidia (Tanaka *et al.*, 2009 Figs. 14H, 15G) indicating tretic conidiogenesis rather than holoblastic. Molecular data are therefore necessary to confirm a putative phylogenetic placement of *Piricaudilium* among Tetraplosphaeriaceae and its affinity to *Quadricrura*. With the addition of *Ernakulamia* and possibly *Piricaudilium* to the family tretic conidium ontogeny becomes another diagnostic feature for its anamorphs besides the predominantly monoblastic conidiogenesis. This type of conidial formation is nevertheless quite common among the anamorph-rich Pleosporales (Zhang *et al.*, 2009; 2012).

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