Phytotaxa 362 (1): 077–086
http://www.mapress.com/j/pt/
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Article

ISSN 1179-3155 (print edition)
PHYTOTAXA
ISSN 1179-3163 (online edition)

https://doi.org/10.11646/phytotaxa.362.1.6

Neolinocarpon phayaoense sp. nov. (Linocarpaceae) from Thailand

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Abstract

A new species of *Neolinocarpon*, *N. phayaoense* was found on a branch of *Hevea brasiliensis* in northern Thailand. The new species is introduced in this paper, with evidence from morphology and phylogenetic analyses. Descriptions, illustrations and notes are provided for the new taxon. Maximum-likelihood and Bayesian inference analyses of a combined LSU and ITS sequence data clarified the phylogenetic affinity in *Neolinocarpon*, with the species separated from *Linocarpon* in Linocarpaceae. *Neolinocarpon* is morphologically distinct from *Linocarpon* in having immersed ascomata.

Keywords: 1 new species, Para rubber, Sordariomycetes, taxonomy

Introduction

The genus *Neolinocarpon* was introduced to accommodate linocarpon-like species by Hyde (1992a, b), with *N. globosicarpum* K.D. Hyde as the type species. The genus is characterized by a clypeus with a dense blackened, shiny central papilla, deeply immersed, oval to globose ascomata, 8-spored, unitunicate asci, with a reflective apical ring and filiform, fasciculate ascospores, containing refringent bands, with or without appendages (Hyde 1992b, Hyde *et al*. 1998, Vitoria *et al*. 2013, Konta *et al*. 2017). *Neolinocarpon* is morphologically similar to *Linocarpon*, in the apical structure of the ascus and in ascospore morphology, but differs in having deeply immersed ascomata forming below a slightly raised or flattened clypeus, and with a refractive globose body below the ascus apical ring (Hyde 1992b), while in *Linocarpon* ascomata are superficial or slightly immersed, and lack a refractive globose body (Hyde 1992a, 1997, Hyde *et al*. 1998, Konta *et al*. 2017). Previous studies reported that most species of *Neolinocarpon* have been reported on Arecaceae, except *N. penniseti*, a species from a different host family (Poaceae) (Hyde 1992b, Hyde *et al*. 1998, Hyde & Alias 1999, Bhilabutra *et al*. 2006, Vitoria *et al*. 2013, Wijayawardene *et al*. 2017).

Neolinocarpon was placed in Xylariaceae (Hyde 1992b). Hyde (1997) assigned *Neolinocarpon* to Hyponectriaceae. Wang & Hyde (1999) excluded the genus from Hyponectriaceae based on the apical ring morphology. Kirk *et al*. (2001) and Eriksson (2006) transferred *Neolinocarpon* to Sordariomycetes genera *incertae sedis*. Bahl (2006) indicated that *Neolinocarpon* has relationships with the Xylariales according to phylogenetic analysis of LSU and RPB2 sequence data. Based on morphology, the genus *Neolinocarpon* cannot be placed in any family within Xylariales with certainty and thus is placed as Xylariales genera *incertae sedis* (Jones *et al.* 2009). The family placement of *Neolinocarpon* was not determined in Maharachchikumbura *et al*. (2015, 2016). Vitoria *et al*. (2013) introduced a new species of *Neolinocarpon* on Arecaceae based on morphology. Based on phylogenetic analyses of selected reliable strains from GenBank and new species, Konta *et al.* (2017) introduced Linocarpaceae (Chaetosphaeriales) comprising *Linocarpon* and *Neolinocarpon* and this was accepted in the outline of ascomycetes by Wijayawardene *et al*. (2018).

The present research is part of a broader study where we are establishing the fungi involved with disease and decay of rubber in southern China and Thailand (Liyanage *et al.* 2016, Senwanna *et al*. 2017). Para rubber was introduced to Asia and is now grown in large areas of China, Indonesia, Laos, Malaysia, Thailand, Vietnam, Cambodia,

Accepted by Sajeewa Maharachchikumbura: 20 May 2018; published: 23 Jul. 2018

and Myanmar (Fox & Castella 2013, Ahrends *et al.* 2015). The aim of the present study is to introduce a new species, *Neolinocarpon phayaoense* from *Hevea brasiliensis*, based on phylogenetic analyses and morphological characters together with descriptions and illustrations and comparison with similar taxa.

Materials and Methods

Collections, isolation and identification

Fresh materials of Para rubber (*Hevea brasiliensis* (Willd.) Muell. Arg) were collected from Chiang Rai, Thailand in 2016. Fungal structures were examined with a Motic SMZ 168 series stereomicroscope and photographed with an Axio camera on a Zeiss Discover V8 stereomicroscope. Micro-morphological structures were photographed by a Nikon ECLIPSE 80i microscope with a Canon 600D digital camera. The measurement of fungal structures (e.g. ascostromata, perithecia, peridium, paraphyses, asci and ascospores) were obtained by using Tarosoft (R) Image Framework program v.0.9.0.7. Photographic plates were made by using Adobe Photoshop CS6 version 13.0 (Adobe Systems. U.S.A.). Cultures were obtained by single spore isolation following the method of Chomnunti *et al*. (2014). Germinating ascospores were aseptically transferred to malt extract agar (MEA) and incubated at room temperature. The holotype specimens are deposited in the herbarium of Mae Fah Luang University (MFLU), Phayao, Thailand. Ex-type living cultures are deposited in Mae Fah Luang University Culture Collection (MFLUCC) in Chiang Rai, Thailand. Facesoffungi and Index Fungorum numbers are registered as in Jayasiri *et al*. (2015) and Index Fungorum (2018) respectively.

DNA extraction, PCR amplification and DNA sequencing

Pure cultures were grown on MEA at 25–30 °C for two weeks. The fungal genomic DNA obtained from mycelium was extracted by the Biospin Fungus Genomic DNA Extraction Kit (BioFlux®, China) following the manufacturer's instructions (Hangzhou, P.R. China). DNA amplifications were performed by polymerase chain reaction (PCR). The partial large subunit nuclear rDNA (LSU) was amplified with primer pairs LROR and LR5 (Vilgalys & Hester 1990). The internal transcribed spacers (ITS) was amplified with primers pairs ITS5 and ITS4 (White *et al*. 1990). The partial small subunit nuclear rDNA (SSU) was amplified with primer pairs NS1 and NS4 (White *et al*. 1990). The fragment of translation elongation factor 1-α (TEF1-α) was amplified with primer pairs EF1-983F and EF1-2218R (Rehner 2001).

PCR reactions were performed in 25 µl final volumes containing 8.5 µl of sterilized water, 12.5 µl of 2 × Easy Taq PCR Super Mix (mixture of Easy Taq TM DNA Polymerase, dNTPs, and optimized buffer (Beijing Trans Gen Biotech Co., Chaoyang District, Beijing, PR China), 1 µl of each forward and reverse primers (10 pM), and 2 µl of DNA template. The PCR thermal cycle program for LSU, SSU and ITS gene were amplified as: initial denaturation 95 ºC for 3 minutes, followed by 35 cycles of denaturation at 95 ºC for 30 seconds, annealing at 55 ºC for 50 seconds, elongation at 72 ºC for 50 seconds, and final extension at 72 ºC for 10 minutes. The PCR thermal cycle program for TEF1-α was amplified as: initially denaturation at 95 °C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 52°C for 50 seconds and extension at 72°C for 5 minutes, with a final extension step at 72 °C for 10 minutes. PCR products were sent to sequence at Sangon Biotech Co., Shanghai, China. Generated new sequences of LSU, ITS, SSU and TEF1-α regions are deposited in GenBank.

Phylogenetic analyses

The most closely related taxa were determined using nucleotide BLAST searches online in GenBank and also from recent publications (Konta *et al*. 2017). *Gelasinospora tetrasperma* (CBS 178.33) and *Sordaria fimicola* (CBS 508.50) were selected as the outgroup taxa (Table 1). LSU and ITS sequence datasets were selected to construct the phylogenetic tree. The combined LSU and ITS sequence data were initially aligned by using MAFFT version 7 (Katoh *et al*. 2017; http://mafft.cbrc.jp/alignment/server/). The alignment was checked and improved in BioEdit v. 7.0.5.3 (Hall 1999). The individual gene alignments were analyzed separately for comparing any conflicts of tree. A combined LSU and ITS sequence data was analyzed and inferred the phylogenetic tree based on maximum likelihood (ML), and Bayesian inference (BI) analyses.

The estimated evolutionary model of Bayesian inference and maximum likelihood were performed independently for each locus using MrModeltest v. 2.3 (Nylander 2008). The best-fit model is resulted as GTR+I+G model for each locus under the Akaike Information Criterion (AIC). Maximum likelihood (ML) analysis was performed by Randomized

Axelerated Maximum Likelihood (RAxML) (Stamatakis 2006) version 7.4.2 (released by Alexandros Stamatakis on November 2012) implemented in raxmlGUI v.1.0 (Stamatakis *et al.* 2008, Silvestro & Michalak 2011). The search strategy was set to rapid bootstrapping at 1,000 replicates and the analysis carried out using the GTR+GAMMAI model of nucleotide substitution. Bootstrap values greater than 60% were accepted. Bayesian Inference analysis was performed by MrBayes v. 3.2.2 (Ronquist *et al.* 2011) with the best-fit model of sequences evolution under the Akaike Information Criterion (AIC). Bayesian posterior probabilities (BYPP) (Rannala & Yang 1996, Zhaxybayeva & Gogarten 2002) were determined by Markov Chain Monte Carlo Sampling (BMCMC). Six simultaneous Markov chains were run from random trees for 1,000,000 generations and trees were sampled every 100th generations. The first 2,000 trees, representing the burn-in phase of the analyses, were discarded and the remaining 8,000 trees (post burn-in) were used for calculating posterior probabilities (PP) in the majority rule consensus tree (critical value for the topological convergence diagnostic set to 0.01). Bayesian posterior probabilities (BYPP) values greater than 0.95 were accepted. The phylogenetic trees were figured in FigTree v.1.4.3 (Rambaut 2016) and edited using Microsoft PowerPoint 2013 and converted to TIFF file in Adobe Photoshop CS6 version 13.0 (Adobe Systems. U.S.A.). The newly sequences generated in this study were submitted in GenBank (Table 1). The finalized alignment and tree are deposited in TreeBASE, submission ID: 22147 (http://www.treebase.org/). We follow the recommendations of Jeewon and Hyde (2016) for introducing new taxa based on morphology and molecular data.

Results

Phylogenetic analyses

Phylogenetic analyses of the combined LSU and ITS sequence data comprises 26 taxa including the newly generated sequences and the outgroup taxa (Table 1). The dataset was analyzed by maximum likelihood (ML) and Bayesian Inference analyses. The dataset consists of 1417 total characters, with 654 distinct alignment patterns and ML 36.62% of undetermined characters or gaps. The best scoring of RAxML analysis shown in Fig. 1, with the final ML optimization likelihood value of -7866.801054. Bayesian posterior probabilities were evaluated by MCMC with a final average standard deviation of split frequencies = 0.008231.

Phylogenetic trees generated from maximum likelihood (ML) and Bayesian Inference analyses were similar in overall topologies with were not significant difference. In the phylogenetic tree (Fig. 1), our new taxon forms a distinct lineage, sister to *Neolinocarpon rachidis* and *N. arengae* within the family Linocarpaceae.

Taxonomy

Neolinocarpon phayaoense Senwanna & K.D. Hyde, *sp. nov.* (Fig. 2)

Index Fungorum number: IF554038, *Facesoffungi number*: FoF 03882

Etymology:—The epithet "*phayaoense*" refers to Phayao Province in Thailand where the fungus was found.

Holotype:—MFLU17-1980

Saprobic on branches of *Hevea brasiliensis*. **Sexual morph**: *Ascostromata* 250–550 µm diam., 120–300 µm high, solitary to gregarious, with 1–3 locules, immersed to semi-immersed, becoming raised to erumpent through the host tissue, globose to subglobose, dark brown to black, with a central, papillate, blackened, shiny ostiole. *Ostiolar canal* 60–120 µm wide, 105–265 µm high, central, not prominent, cylindrical, straight, black, periphysate. *Peridium* 5–50 µm wide, outer cells merging with the host epidermal cells, composed of dark brown to black cells of *textura angularis*. *Hamathecium* 3–7 µm wide, comprising dense, hyaline, septate, unbranched, filamentous paraphyses, tapering towards the apex. *Asci* (108–)120–165(–180) × (8–)10–14(–17) µm ($\bar{x}$ = 143 × 12 µm, n = 15), 8-spored, unitunicate, with a refractive J-, wedge-shaped, subapical ring (2–3 µm × 1–2 µm), cylindrical, long pedicellate, thin-walled. *Ascospores* (77–)83–92 × (2–)4–5 µm ($\bar{x}$ = 85 × 4 µm, n = 20), fasciculate, elongate subcylindric-clavate, hyaline to pale brown, aseptate, mostly curved, containing refringent septum-like band, tapering towards the base and with a narrow rounded apex, smooth-walled. **Asexual morph**: Undetermined.

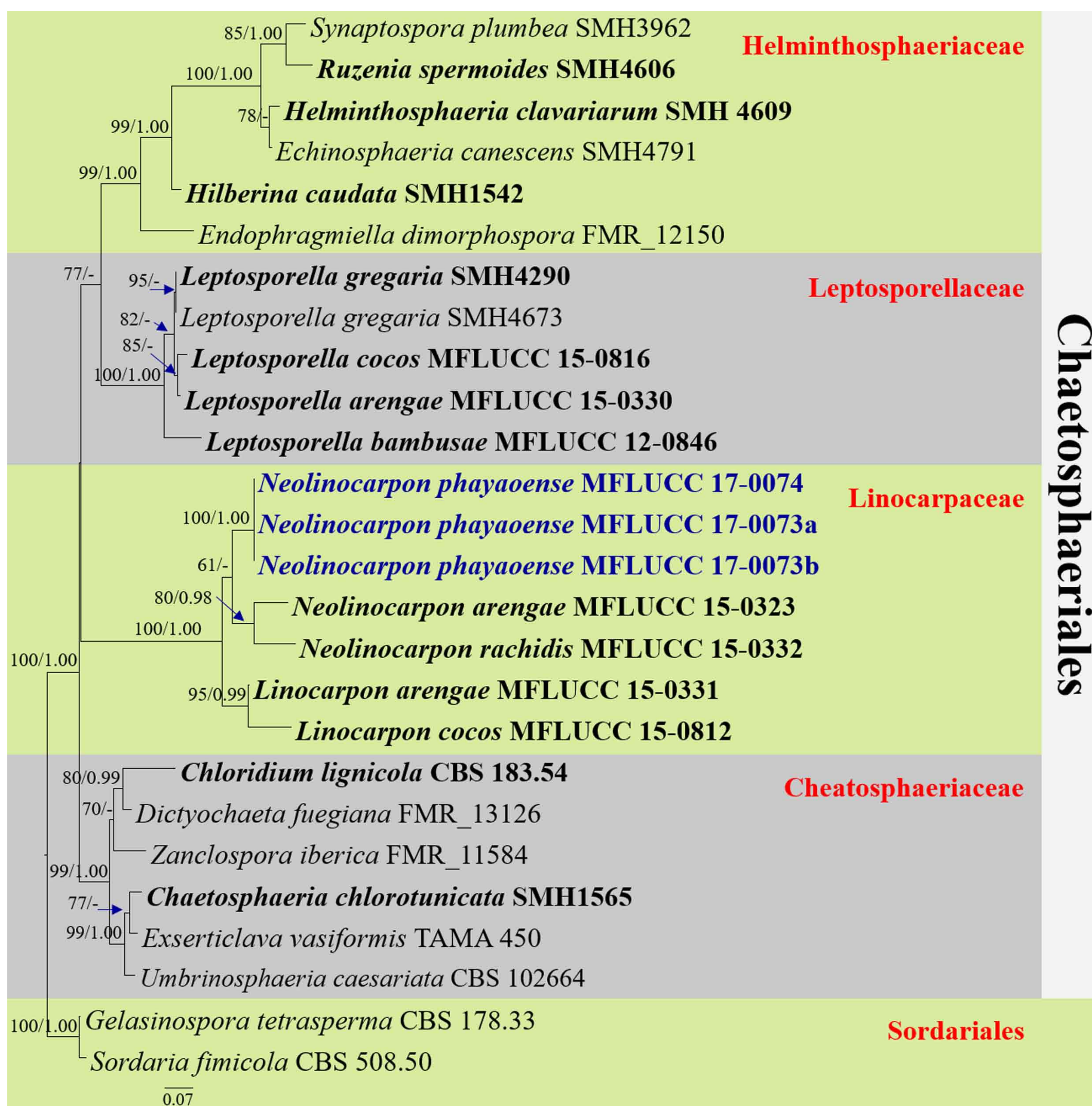

FIGURE 1. Phylogram of RAxML analysis based on a combined LSU and ITS sequence data. Bootstrap support values for maximum likelihood (ML, left) greater than 60% and Bayesian posterior probabilities (PP, right) equal to or greater than 0.95 are indicated at the nodes. The tree is rooted to *Gelasinospora tetrasperma* (CBS 178.33) and *Sordaria fumicola* (CBS 508.50). All sequences from ex-type strains are in bold. The newly generated sequences are in blue.

Culture characteristics:—Ascospores germinating on MEA within 24 hours and germtube produced from one or both sides or middle of the ascospores. Colonies on MEA reaching 2–3 cm diam. after 2 weeks at 25–30°C, colonies circular, medium dense, surface smooth with edge entire, velvety to woolly, colony from above white; from below: white at the margin, yellowish to light brown in the center; not producing pigmentation in agar.

Material examined:—THAILAND, Phayao Province, Mae Ka Subdistrict, Mueang District, on branch of *Hevea brasiliensis* (Euphorbiaceae), 1 November 2016, C. Senwanna, RBCR014 (MFLU 17-1980, **holotype**!), ex-type living culture, MFLUCC 17-0074! living culture MFLUCC 17-0073!

Addition GenBank numbers:—SSU = MG581936, MFLUCC 17-0073a; MG581937, MFLUCC 17-0073b; MG581938, MFLUCC 17-0074; TEF 1-α = MG739512, MFLUCC 17-0073a; MG739513, MFLUCC 17-0073b; MG739514, MFLUCC 17-0074

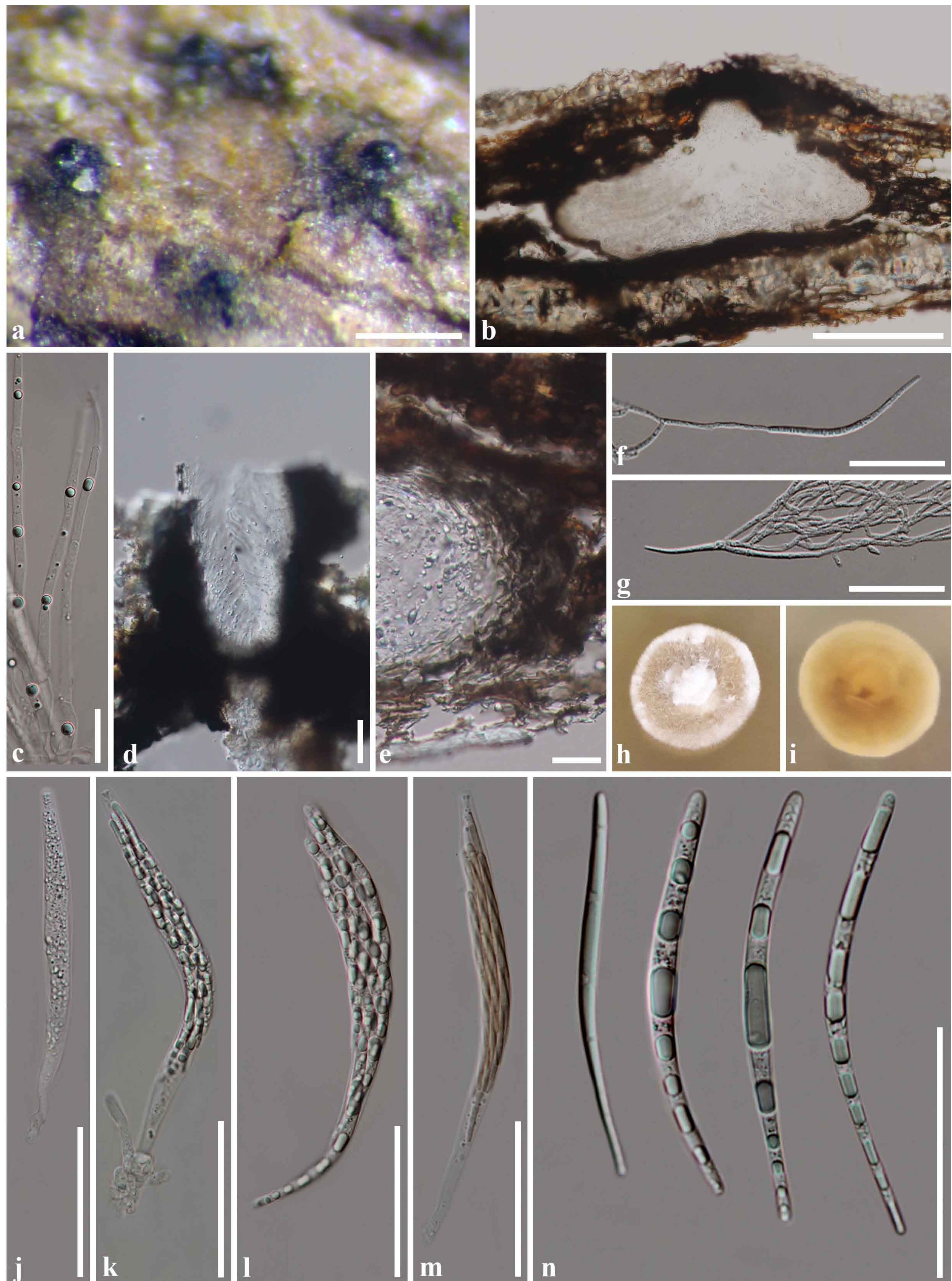

FIGURE 2. *Neolinocarpon phayaoense* (MFLU 17-1980, holotype!). **a** Habit of ascostromata on host substrate. **b** Section through ascoma. **c** Paraphyses. **d** Ostiole with periphyses. **e** Peridium. **f, g** Germinated ascospores. **h, i** Culture characteristic on MEA after 21 days (h = colony from above, i = colony from below). **j, m** Asci (j, l = mouthed in water; m = mouthed in Melzer's reagent). **n** Ascospores (mouthed in water). Scale bar: a**,** b = 200 µm, d, j, n = 50 µm, c, e**,** g = 20 µm.

TABLE 1. GenBank accession numbers of the sequences used in phylogenetic analysis.

Species name	Strain	GenBank accession number	
		LSU	ITS
Chaetosphaeria chlorotunicata	**SMH1565**	**AF466064**	-
Chloridium lignicola	**CBS 143.54**	**AF178544**	**AF178544**
Dictyochaeta fuegiana	FMR_13126	KY853500	KY853440
Echinosphaeria canescens	**SMH4666**	**KF765605**	-
Endophragmiella dimorphospora	FMR 12150	KY853502	KY853442
Exserticlava vasiformis	TAMA 450	AB753846	-
Gelasinospora tetrasperma	CBS 178.33	DQ470980	-
Helminthosphaeria clavariarum	**SMH4609**	**AY346283**	-
Hilberina caudata	**SMH1542**	**KF765615**	-
Leptosporella arengae	**MFLUCC 15-0330**	**MG272246**	**MG272255**
Leptosporella bambusae	**MFLUCC 12-0846**	**KU863122**	**KU940134**
Leptosporella cocois	**MFLUCC 15-0816**	-	**MG272256**
Leptosporella gregaria	**SMH 4290**	**AY346290**	-
Leptosporella gregaria	SMH 4673	HM171287	-
Linocarpon arengae	**MFLUCC 15-0331**	**MG272247**	-
Linocarpon cocois	**MFLUCC 15-0812**	**MG272248**	**MG272257**
Neolinocarpon arengae	**MFLUCC 15-0323**	**MG272249**	**MG272258**
Neolinocarpon rachides	**MFLUCC 15-0332**	**MG272250**	-
Neolinocarpon phayaoense*	MFLUCC 17-0073a	MG581933	-
Neolinocarpon phayaoense*	MFLUCC 17-0073b	MG581934	-
Neolinocarpon phayaoense*	**MFLUCC 17-0074**	**MG581935**	-
Ruzenia spermoides	SMH4606	AY436422	-
Sordaria fimicola	CBS 508.50	AY681160	AY681188
Synaptospora plumbea	SMH3962	KF765621	-
Umbrinosphaeria caesariata	CBS 102664	AF261069	-
Zanclospora iberica	FMR_11584	KY853544	KY853480

Ex-type strains are in bold. The new taxon is indicated with an asterisk.

Abbreviation: CBS: Centraalbureau voor Schimmelcultures, The Netherlands; FMR: Facultat de Medicina i Ciencies de la Salut, Reus, Spain; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; SMH: Sabine M. Huhndorf.

Notes:—*Neolinocarpon phayaoense* is similar to other species in the morphological characters of the immerse ascomata with an ostiole, filiform ascospores. However, it differs from *Neolinocarpon* species in its size of ascomata, asci and ascospores, ascomata shape and lacking mucilaginous appendages in the ascospores (Table 2). *Neolinocarpon phayaoense* differs from *N. australiense*, *N. calami*, *N. enshiensis*, *N. eutypoides* and *N. globosicarpum* as it lacks a mucilaginous sheath at the apices of the ascospores. Ascospores of *Neolinocarpon phayaoense* have similar dimension to *N. attaleae* [(52.5–)57.5–93(–105) × 3–4(–5) μm] and *N. inconspicuum* [76–98 × 2–3 μm], but differs in size of ascomata [(350–880 × 220–650 μm) and (200–255 × 365–410 μm)] and asci [(137.5–227.5 × 7.5–14(–15) μm) and (105–156 × 7.5–12 μm)] (Table 2). In addition, *N. phayaoense* was isolated from different host family (Euphorbiaceae) with all *Neolinocarpon* species. The phylogenetic analysis indicates that *N. phayaoense* grouped together with *N. rachidis* and *N. arengae* (Fig. 1).

TABLE 2. *Neolinocarpon* species with host, location, synopsis of the characteristics and relevant references

Species	Habitat/ Host family	Country	Ascomata (µm)	Asci (µm)	Ascospores (µm)	Appendage	Reference
Neolinocarpon arengae	Saprobe/ Arecaceae	Thailand	230–490 × 336–566	168–214 × 15–24	114–134 × 3–4	Polar mucilaginous appendage at apex	Konta *et al*. 2017
Neolinocarpon attaleae	Saprobe/ Arecaceae	Brazil	350–880 × 220–650	137.5–227.5 × 7.5–14(–15)	(52.5–)57.5–93(–105) × 3–4(–5)	None	Vitoria *et al*. 2013
Neolinocarpon australiense	Saprobe/ Arecaceae	Australia	560–616(–760) × 204–296(–380)	125–165 × 11–15	81–107(126) × 2.5–3.5	Keel-like appendage at narrower end	Hyde *et al*. 1998
Neolinocarpon calami	Saprobe/ Arecaceae	Brunei	448–500 × 292–336	115–138 × 10.5–13	68–85 × 2.5–3.5	Crescent-shaped mucilaginous pad at apex	Hyde *et al*. 1998
Neolinocarpon enshiensis	Saprobe/ Arecaceae	China	225–335 ×200–260	74–108 × 8–13	42–64 × 2–3.5	Mucilaginous pad at narrow truncated base	Hyde *et al*. 1998
Neolinocarpon eutypoides	Saprobe/ Arecaceae	Australia, Brunei Darussalam, Hong Kong, Indonesia, Malaysia	204–321 × 544–576	108–138 × 6–8.5	(73–95(–106) × 1.5–2.2	Mucilaginous appendage at base	Hyde *et al*. 1998
Neolinocarpon globosicarpum	Saprobe/ Arecaceae	Brunei	155–400 × 310–520	136–170 × 6–8.5	70–119.5 × 2–3	Mucilaginous appendage at one end	Hyde 1992b
Neolinocarpon phayaoense	Saprobe/ Euphorbiaceae	Thailand	250–500 × 120–300	(108–)120–165(−180) × (8–)10–14(−17)	(77–)83–92 × (2–)4–5	None	This study
Neolinocarpon inconspicuum	Saprobe/ Arecaceae	Australia	200–255 × 365–410	105–156 × 7.5–12	76–98 × 2–3	None	Hyde *et al*. 1998
Neolinocarpon nonappendiculatus	Saprobe/ Arecaceae	Australia	637–710 × 375–520	134–190 × 8.5–12	114–138 × 2–2.5	None	Hyde *et al*. 1998
Neolinocarpon nypicola	Saprobe/ Arecaceae	Malaysia	600–1000 (diam.)	100–164 × 8–10	92–117 × 2–3.8	None	Hyde & Alias1999
Neolinocarpon penniseti	Saprobe/ Poaaceae	Hong Kong	–	–	(52.5–)57–64(–84) × 2.5–3	None	Bhilabutra *et al*. 2006, Vitoria *et al*. 2013
Neolinocarpon rachidis	Saprobe/ Arecaceae	Thailand	320–390 × 508–590	157–205 × 9–19	123–140 × 2–4	Mucilaginous appendage at apex	Konta *et al*. 2017

Discussion

The family Linocarpaceae was introduced by Konta *et al*. (2017) based on analysis of combined LSU and ITS DNA sequence data and comprised the genera *Linocarpon* and *Neolinocarpon*. Species of *Linocarpon* and *Neolinocarpon* are identified based on the ascostromata position, the size of the asci and ascospores, the shape of ascospores, and the presence of ascospore appendages (Yanna & Hyde. 2003, Konta *et al*. 2017). Many sequences of *Linocarpon* and *Neolinocarpon* are available in GenBank. In our pre-analyses, these strains were shown to be polyphyletic due to their phylogenetic affinities distant from *Neolinocarpon* and *Linocarpon* in Linocarpaceae. We therefore do not include this sequence data in our analyses as we cannot verify these data using morphology. Hence, we use our sequence data together with reliable sequences from GenBank to determine the taxonomic placement of our new species. In this study, we introduced a new species, *Neolinocarpon phayaoense*, found on branches of *Hevea brasiliensis*, based on morphological characters and phylogenetic analyses as recommended by Jeewon and Hyde (2016). (Fig. 1, Table 2). Phylogenetic analyses indicated that *Neolinocarpon phayaoense* is clustered with *N. rachidis* and *N. arengae*, albeit with low bootstrap support. However, they can be distinguished based on the size of asci and ascospores and with or without mucilaginous appendages (Table 2, Konta *et al.* 2017). This is the first record of *Neolinocarpon* on Euphorbiaceae and may suggest it has jumped from another host. Details of host and distribution of all *Neolinocarpon* species are listed in Table 2.

Acknowledgements

The authors extend their appreciation to the International Scientific Partnership Program ISPP at King Saud University for funding this research work through ISPP#0089. We would like to thank the Center of Excellence in Fungal Research, Mae Fah Luang University and Kunming Institute of Botany for facilities for carrying out taxonomic work and molecular work. K.D. Hyde would like to thank National Research Council of Thailand (Mae Fah Luang University) grant no 60201000201 entitled ‘‘Diseases of mangrove trees and maintenance of good forestry practice’’ and Thailand Research Fund (TRF) grant no RSA5980068 entitled ‘‘Biodiversity, phylogeny and role of fungal endophytes on above parts of *Rhizophora apiculata* and *Nypa fruticans*’’. R. Phookamsak expresses appreciation to the Research Fund from China Postdoctoral Science Foundation (grant no. Y71B283261) and Chiang Mai University for financial support. R. Cheewangkoon would like to thank Thailand Research Fund (MRG5580163) for financial support. We thank to Dr. Sinang Hongnan, Dr. Mingkwan Doilom, Sirinapa Konta, Qui-Ju Shang, Saowaluck Tibpromma and Nimali Indeewari de Silva for their valuable suggestions and helping in phylogenetic analyses.

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