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Neohelicosporium fusisporum sp. nov. (Tubeufiaceae) and a first record of a sexual morph within *Neohelicosporium*

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Abstract

Both sexual and asexual morphs (holomorph) are known in several genera of Tubeufiaceae whereas in others, mainly the asexual or rarely the sexual morph is known. The genus *Neohelicosporium* is known only by its asexual morph, we have recently collected the sexual morph of the genus, which is characterized by fleshy, superficial ascomata, bitunicate asci, and hyaline to pale brown, fusiform ascospores. Analyses of combined ITS, LSU and *tef1* sequence data placed the taxon in *Neohelicosporium.* In this paper, we introduce the new taxon *N. fusisporum* based on both morphological characteristics and phylogenetic data.

Keywords – Bitunicate – fusiform – new species – phylogenetic data

Introduction

The family Tubeufiaceae is an interesting and important group of fungi with 23 genera, that includes saprobic, hyperparasitic or hypersaprobic species on ascomycetes and scale insects (Boonmee et al. 2011, 2014, Hyde et al. 2016, Brahamanage et al. 2017, Chaiwan et al. 2017, Lu et al. 2017a, b, c, d, Luo et al. 2017, Tanney & Miller 2017). They are characterized by brightly pigmented, fleshy, superficial ascomata, bitunicate asci, and mostly hyaline to pale brownish, narrowly-elongate, obovoid or oblong septate ascospores (Boonmee et al. 2011, 2014). The asexual morphs of Tubeufiaceae have been well-studied and are mostly related to helicosporous taxa, such as *Helicoma*, *Helicomyces* and *Helicosporium* (Tsui et al. 2006, 2007, Hyde et al. 2011). However, helicosporous asexual morphs of Tubeufiaceae are now known to be present in several genera including *Acanthohelicospora*, *Chlamydotubeufia*, *Helicangiospora*, *Helicoma*, *Helicomyces*, *Helicosporium*, *Neoacanthostigma*, *Neohelicomyces*, *Neohelicosporium* and *Tubeufia* (Boonmee et al. 2014, Hyde et al. 2016, Brahamanage et al. 2017, Lu et al. 2017a, b, c, d, Luo et al. 2017).

In this study we introduce a sexual morph of a new species which was placed in *Neohelicosporium* according to the combined ITS, LSU and *tef1* phylogenetic data. The asexual morph of this new *Neohelicosporium* species was also found in culture. Lu et al. (2017d) introduced *Neohelicosporium* as an asexual genus in Tubeufiaceae and in our study, we record both the sexual and asexual morph of the new *Neohelicosporium* species. Most Tubeufiaceae species are commonly found on woody litter, however some species can also be found on leaf litters or even

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decaying cloths, and some are associated with other fungi or scale insects (Barr 1980, Rossman 1987, Kodsueb et al. 2006, Promputtha & Miller 2010, Sánchez & Bianchinotti 2010). In this paper, the new species *Neohelicosporium fusisporum* is introduced from Thailand which was found on a decaying fruit of a Malvaceae forest tree.

Material and Methods

Sample collection and specimen examination

The specimen was collected from Krabi, Thailand in 2015. Fruits collected were brought to the laboratory and observed using a Motic SMZ 168 Series microscope. Hand sections of fruiting structures were mounted in water for microscopic studies and photomicrography. The fungus was examined with a Nikon ECLIPSE 80i compound microscope and photographed with a Canon 450D digital camera connected to the microscope. Measurements were made with the Tarosoft (R) Image Frame Work program and images used for the figures were processed with Adobe Photoshop CS6 Extended version 10.0 software (Adobe Systems, USA). Isolations were made from single ascospores, following a modified method of Chomnunti et al. (2014).

The voucher specimen was deposited in the herbarium of Mae Fah Luang University (Herb. MFLU) and New Zealand Fungal & Plant Disease Collection (PDD). The living cultures were deposited in the culture collection of Mae Fah Luang University (MFLUCC), Thailand with duplicates in BIOTEC Culture Collection (BCC), Bangkok, Thailand. Faces of fungi and IF numbers were obtained as in Jayasiri et al. (2015) and Index Fungorum (2017).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from the growing mycelium after 30 days on MEA at 18°C using the Biospin Fungus Genomic DNA Extraction Kit (BioFlux®) following the manufacturer's protocol (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 spacer region of rDNA was amplified with primer pairs ITS1 and ITS4 (White et al. 1990). The translation elongation factor 1-alpha gene (*tef1*) was amplified by using primers EF1-983F and EF1-2218R (Rehner & Buckley 2005). The amplification procedure was carried in a 50 µl reaction volume containing 2 µl DNA, 25 µl PCR mix, 19 µl distilled water 2 µl of each primer. The PCR reactions for amplification of ITS, LSU and *tef1* were performed under standard conditions (White et al. 1990). Purification and sequencing of PCR products were carried at Shanghai Sangon Biological Engineering Technology and Services Co. (China).

Sequence alignment and phylogenetic analysis

All sequences acquired from GenBank were used in Lu et al. (2017b, d). Multiple sequence alignments were generated with MAFFT v. 6.864b (http://mafft.cbrc.jp/alignment/server/index.html) and further improved manually where necessary and datasets analyzed under different optimality criteria as outlined by Jeewon et al. (2013). All introns and exons were aligned individually. Ambiguously aligned regions with many leading or trailing gaps were excluded in alignments prior to tree building. Sequences generated from the ITS, LSU and *tef1* gene regions were carefully verified before further analyses. The final phylogenetic tree used to infer the taxonomic placement of our new taxon was generated based on DNA sequence analyses of a concatenated dataset of ITS, LSU and *tef1*. A maximum likelihood analysis was performed at CIPRES using RAxML v.7.2.8 as part of the "RAxMLHPC2 on TG" tool (Stamatakis et al. 2008, Miller et al. 2010). The general time reversible model (GTR) using proportion of invariable sites were applied with a discrete gamma distribution and four rate classes. The best scoring tree was selected with a final likelihood value of -28217.368593. Maximum likelihood bootstrap support (MLBS) equal or greater than 60% are given near to each node (Fig. 1).

The model of evolution was performed using jModeltest 2.1.7 (Guindon & Gascuel 2003, Darriba et al. 2012). Posterior probabilities (PP) (Rannala & Yang 1996, Zhaxybayeva & Gogarten 2002) were determined by Markov Chain Monte Carlo sampling (MCMC) in MrBayes v. 3.0b4 (Huelsenbeck & Ronquist 2001). Six simultaneous Markov chains were run for 1,000,000 generations and trees were sampled every 100th generation. MCMC heated chain was set with a "temperature" value of 0.15. All sampled topologies beneath the asymptote (25%) were discarded as part of a burn-in procedure, the remaining trees were used for calculating posterior probabilities in the majority rule consensus tree. Bayesian Posterior Probabilities (BP) equal or greater than 0.90 is given near to each node (Fig. 1). Phylogenetic trees were drawn using FigTree v. 1.4 (Rambaut & Drummond 2008). The sequences of novel species are deposited in GenBank.

Results

Phylogenetic analyses

Multiple genes (ITS, LSU and *tef1*) were used for the phylogenetic analyses. The topologies of the obtained trees for each gene were compared manually, to verify that the overall tree topology of the individual datasets was congruent with the tree obtained from the combined alignment. The Bayesian analyses showed similar tree topologies and were congruent to those obtained in the ML analysis. The combined gene analysis of ITS, LSU and *tef1*sequence data representing the genera of family Tubeufiaceae is shown in Fig. 1, which included 62 strains, representing 42 species and consisted of 3677 characters. *Botryosphaeria dothidea* (CBS 115476) is the outgroup taxon. The Bayesian analysis resulted in 7500 trees after 1,000,000 generations. The first 2500 trees, representing the burn-in phase of the analyses were discarded, while the remaining tree was used for calculating posterior probabilities in the majority rule consensus tree and is shown in Fig. 1. A best scoring RAxML tree resulted with the value of likelihood: -28217.368593 Phylogenetic trees obtained from ML and Bayesian analysis yielded trees with similar overall topology at the species level and in agreement with previous studies based on maximum likelihood and Bayesian analysis (Lu et al. 2017c, d). The new strain of *Neohelicosporium* species forms a sister clade to other *Neohelicosporium* spp. with moderate statistical support in maximum likelihood and high statistical support in Bayesian analyses (64% MLBS, 0.94 BPP). Therefore, a new species is introduced to accommodate this taxon in the genus *Neohelicosporium* (*N. fusisporum*).

Taxonomy

Neohelicosporium fusisporum Jayasiri & K.D. Hyde, sp. nov. Figs 2–3

Index fungorum number: IF553908; Facesoffungi number: FoF03785

Holotype – MFLU 16–0950

Etymology – "*fusisporum*" refers to the fusiform ascospores

Saprobic on the decaying fruit of Malvaceae sp. Sexual morph: *Ascomata* 340–400 µm high × 235–290 µm diam. ($\bar{x}$ = 380 × 255 µm), superficial, solitary, scattered, subglobose, ellipsoidal-ovate, with few hyphae developing from ascomatal base on substrate, pale brown to dark brown, velvety, ostiolate. *Peridium* 25–30 µm wide, comprising 3–4 layers, composed of cells of *textura angularis*, with inner cells brown and outer cells dark brown. *Hamathecium* comprising 1–2 µm wide, numerous, filiform, pseudoparaphyses. *Asci* 130–165 × 9–12 µm ($\bar{x}$ = 148 × 11 µm, n = 20), 8-spored, bitunicate, cylindrical, apically thickened and rounded, with a pedicel. *Ascospores* 43–65 × 1.6–4.6 µm ($\bar{x}$ = 53 × 3.2 µm, n = 20), overlapping fasciculate, fusiform, with tapering and rounded ends, straight to slightly curved, 11–13-septate, not constricted at septa, hyaline, smooth-walled. Asexual morph: hyphomycetous, helicosporous. *Colonies* on the MEA media superficial, effuse, gregarious, white to light pink. *Mycelium* composed of partly immersed, partly superficial, hyaline to pale brown, septate, abundantly branched hyphae, with masses of crowded, glistening conidia. *Conidiophores* micronematous, mononematous, flexuous, cylindrical, long, branched, septate, pale brown, smooth-walled. *Conidiogenous cells* 12–20 µm long, 1.5–2.5 µm wide,

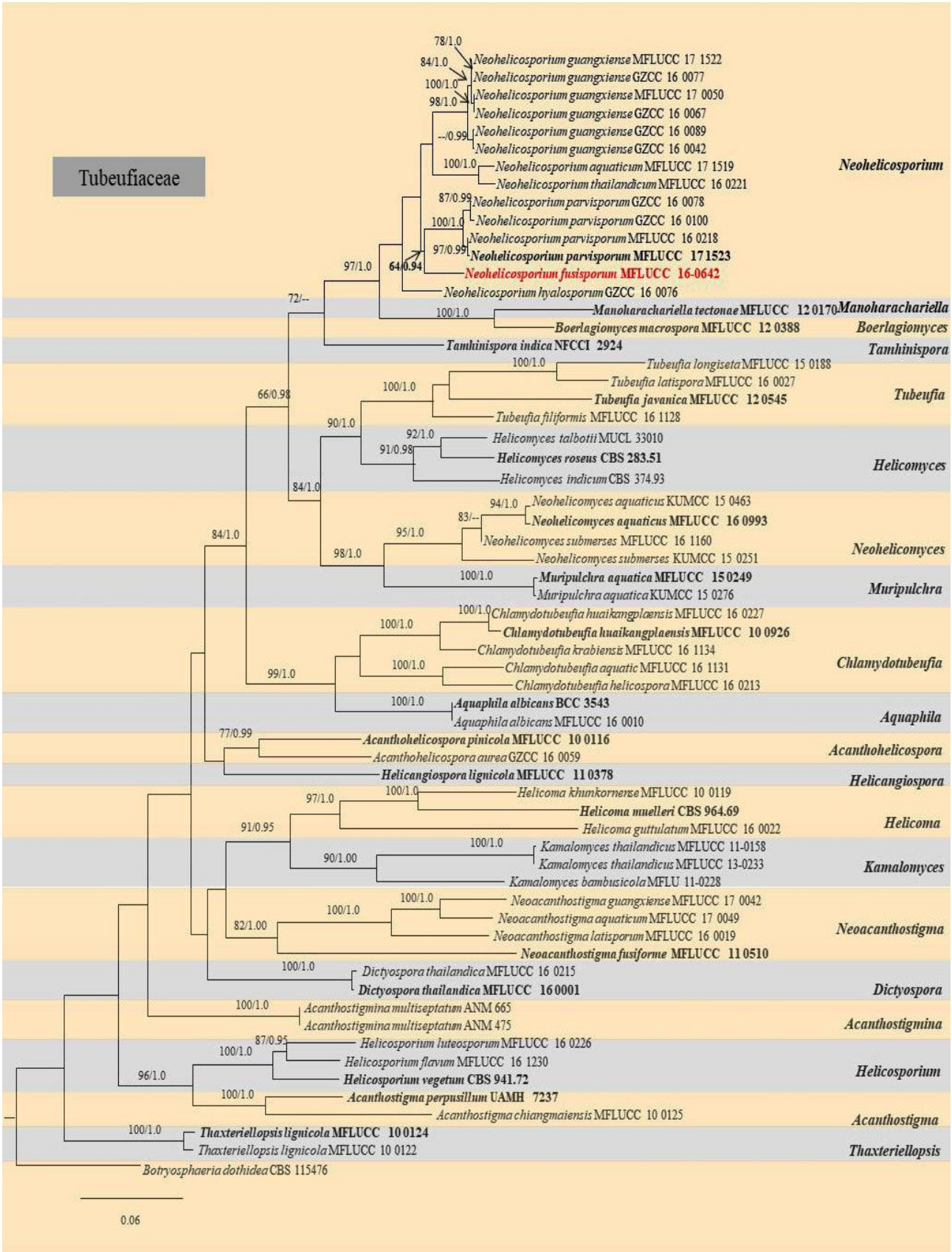

Fig. 1 – Simplified phylogram showing the best RAxML maximum likelihood tree obtained from the combined multigene (ITS, LSU and *tef1*) matrix of 62 taxa including genera in Tubeufiaceae. MLBS above 60 % and Bayesian posterior probabilities above 0.90 are given at each branch. The tree is rooted with *Botryosphaeria dothidea* (CBS 115476) (Botryosphaeriaceae). The type species of each genus are in bold and the new fungal isolate is in bold red.

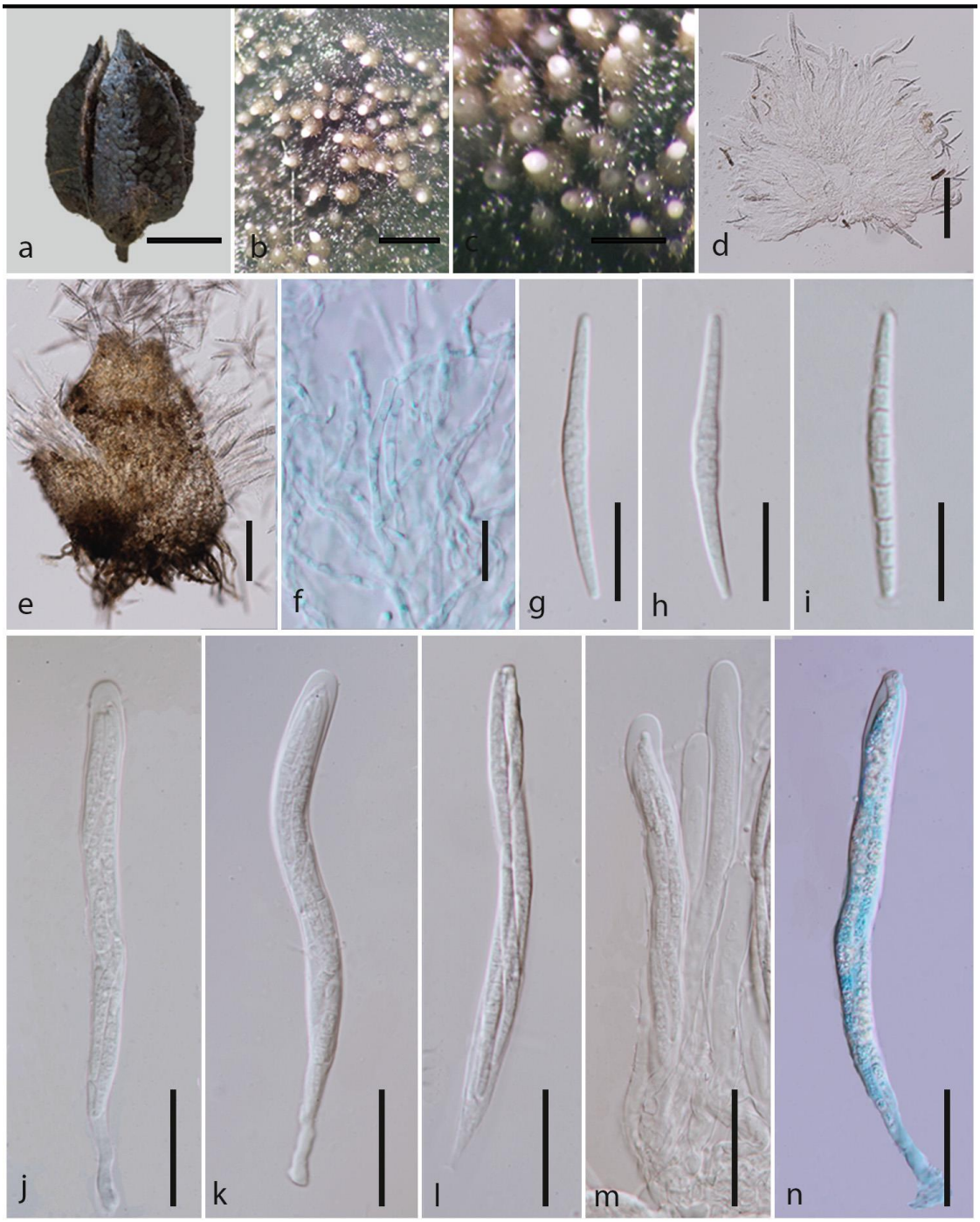

Fig. 2 – *Neohelicosporium fusisporum* (holotype). a The host fruit. b, c Superficial ascomata on substrate, white oozing mass of ascospores at apex of ascomata. d White mass of ascomata. e View of ascoma with peridium cells. f Pseudoparaphyses. g-i Ascospores j-n Asci. Scale bars: f = 10 μm, g-i = 20 μm, j-n = 30 μm.

holoblastic, mono- to polyblastic, integrated, intercalary, cylindrical, with denticles, pale brown, smooth-walled. *Conidia* 18–22 μm diam. and conidial filament 1.5–2.5 μm wide ($\bar{x}$ = 18 μm × 2 μm, n = 50), 100–150 μm long, tightly coiled 2½–3¼ times, loosely coiled in water, rounded at the ends, multi-septate, verrucose, guttulate, hyaline.

Colony morphology – Ascospores germinated on malt extract agar medium (MEA). On MEA colonies are appressed, circular, flat surface, edge entire, first cream then become dark brown and rise in the centre with mycelium, reverse brown reaching 10 mm in 2 weeks at 18 °C.

Material examined – Thailand, Krabi, Thanon Phet Kasem decaying fruit of Malvaceae sp. tree. Dec. 2015, Kevin D. Hyde, C 123 (MFLU 16–0950, holotype; PDD, isotype), ex-type living culture (MFUCC 16–0642, BCC). GenBank numbers – LSU: MG017613, ITS: MG017612, *tef1*: MG017614

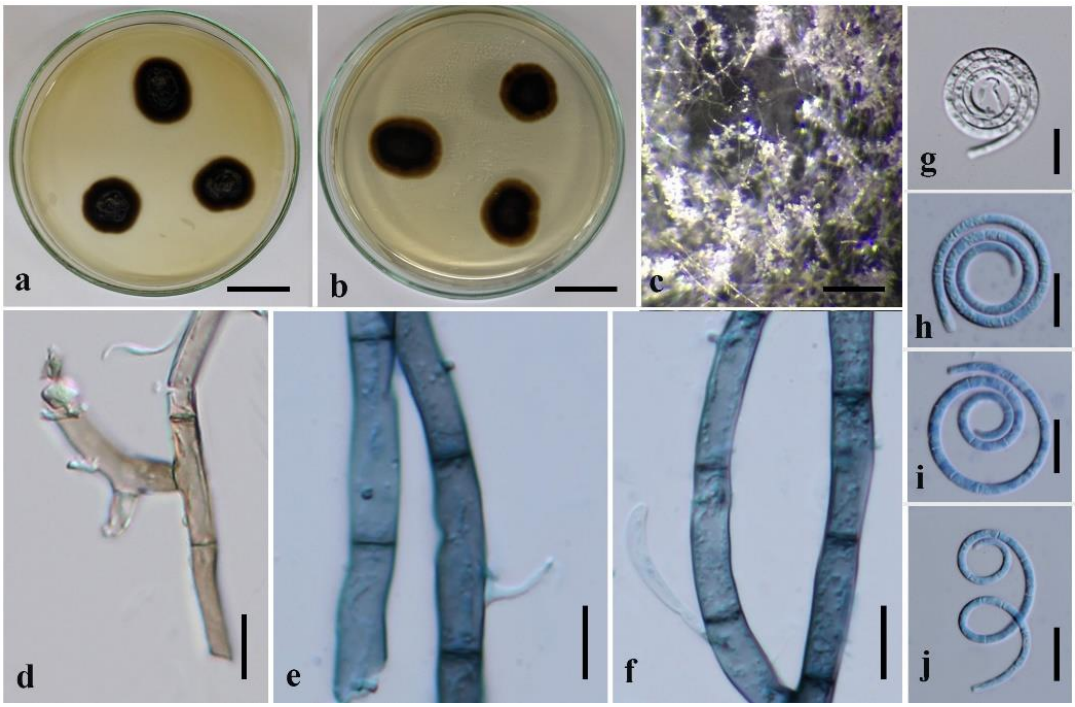

Fig. 3 – *Neohelicosporium fusisporum* asexual morph (from ex-type culture). a, b Upper and reverse view of the culture. c Appearance of fruiting bodies in culture. d-f Conidiophores with conidiogenous cells. g-j Conidia. Scale bars: a, b = 1 cm, c = 200 μm, d-j = 10 μm

Discussion

In this study, we introduce a new species in the genus *Neohelicosporium* based on morphological and multigene phylogenetic analyses. *Neohelicosporium* comprises five other species: *N. aquaticum*, *N. guangxiense*, *N. hyalosporum*, *N. parvisporum* (type species) and *N. thailandicum.* This genus was recently introduced as an asexual genus with helicoid conidia. *Neohelicosporium* is morphologically similar to *Helicosporium* species in conidial features but has distinct conidiophores. The conidiophores of *N. parvisporum* are flexuous, branched, hyphae-like, very long that makes measuring them difficult, while the conidiophores of *Helicosporium* species are erect, unbranched, fertile in the middle, sterile and tapering towards narrow subacute apex (Moore 1957, Goos 1989, Zhao et al. 2007, Boonmee et al. 2014).

Neohelicosporium fusisporum fits with the generic description of *Neohelicosporium* (*N. parvisporum*) (Lu et al. 2017d), however, it differs in having narrow conidiogenous cells (1.5–2.5 μm vs 3.5–4.5 μm) and conidial filaments (1.5–2.5 μm vs 2–4 μm). Following the recommendations of Jeewon & Hyde (2016) for delimitation of a new species, we noted eleven base pair differences between *N. fusisporum* and *N. parvisporum* in the ribosomal ITS sequences. In addition to six and 33 base pair differences in LSU and *tef1* sequences respectively. Therefore, we confirm *N. fusisporum* as a distinct new species within the genus *Neohelicosporium.*

The family Tubeufiaceae is known by both sexual and asexual genera, and some genera with both morphs. The sexual morph of *N. fusisporum* is reported from a decaying fruit and the asexual morph was found in a culture of the isolate. The sexual morph is similar to *Tubeufia* but differs in the morphology of the ascospores. *Tubeufia* species comprise elongate cylindric-subfusiform or narrowly oblong ascospores, however *N. fusisporum* is comprise fusiform ascospores.

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