Mycosphere 8(10): 1869–1892 (2017) www.mycosphere.org ISSN 2077 7019
Article
Doi 10.5943/mycosphere/8/10/11

Can ITS sequence data identify fungal endophytes from cultures? A case study from *Rhizophora apiculata*

Doilom M[1,2,3,7], Manawasinghe IS[1,3], Jeewon R[4], Jayawardena RS[1,3], Tibpromma S[2,3], Hongsanan S[3], Meepol W[5], Lumyong S[6], Jones EBG[7], Hyde KD[2,3,8]

[1]*Beijing Key Laboratory of Environmental Friendly Management on Fruits Diseases and Pests in North China, Institute of Plant and Environment Protection, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, People's Republic of China*
[2]*Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Science, Kunming 650201, Yunnan, People's Republic of China*
[3]*Center of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai 57100, Thailand*
[4]*Department of Health Sciences, Faculty of Science, University of Mauritius, Reduit, Mauritius*
[5]*Mangrove Forest Research Center, Mangrove Conservation Office Department of Marine and Coastal Resources, Ranong 85000, Thailand*
[6]*Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand*
[7]*Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand*
[8]*World Agroforestry Centre, East and Central Asia, Kunming 650201, Yunnan, People's Republic of China*

Doilom M, Manawasinghe IS, Jeewon R, Jayawardena RS, Tibpromma S, Hongsanan S, Meepol W, Lumyong S, Jones EBG, Hyde KD 2017 – Can ITS sequence data identify fungal endophytes from cultures? A case study from *Rhizophora apiculata*. Mycosphere 8(10), 1869–1892, Doi 10.5943/mycosphere/8/10/11

Abstract

Culture-based studies have recovered fungal endophytes from numerous plant hosts, while direct examination of sporulating cultures has enabled identification. However, many endophytes cannot be identified due to the fact that they only form mycelia sterilia in culture. Although next generation sequencing (NGS), as well as ITS sequence analyses have been used to identify endophytes, identification is still rudimentary. In this study, we isolated fungal endophytes from *Rhizophora apiculata* in Thailand and established how many can be identified to species level based on ITS sequence data. Endophytic fungi were isolated from leaves, petioles and aerial roots of *R. apiculata* in four provinces of Thailand. One hundred and fifty four isolates were obtained and initially grouped into 20 morphotypes based on cultural characteristics. Nine were sporulating morphotypes, which were assigned to seven genera (*Colletotrichum*, *Diaporthe*, *Hypoxylon*, *Neopestalotiopsis*, *Neodevriesia*, *Pestalotiopsis* and *Phyllosticta*), and eleven morphotypes were non-sporulating mycelia sterilia. Sequence similarity comparison and phylogenetic analysis of the ITS regions were further used to identify taxa. While ITS sequence data is reliable to assign isolates at the generic rank, and can be useful to identify taxa to species level in a small number of fungal genera, it cannot generally be used to determine specific species in most genera. ITS analysis classified 30 representative isolates into 20 taxonomic units residing in 15 known genera: *Allophoma* sp., *Colletotrichum* spp., *Diaporthe* spp., *Hortaea werneckii*, *Hypoxylon griseobrunneum*, *Hypoxylon* sp., *Pestalotiopsis* sp., *Phanerochaete* sp., *Phyllosticta* spp., *Pseudopithomyces maydicus*, *Preussia* sp., *Nemania* sp., *Neodevriesia* sp., *Neopestalotiopsis* sp.,

Submitted 11 2017, Accepted 29 November 2017, Published 4 December 2017
Corresponding Author: Kevin D Hyde e-mail – kdhyde3@gmail.com and
Saisamorn Lumyong – e-mail – scboi009@gmail.com

Rigidoporus vinctus, *Schizophyllum* sp. and one unidentified genus. Of the morphotypes, four were identified to species. The results from Blast searches and ITS phylogeny of the 15 genera and the one unidentified genus are discussed. Twenty-five of the 30 isolates could not be identified and thus an estimated 20 isolates are likely to be new species and one a new genus. This is remarkable, as endophytes of a single host in Thailand, may yield 75% or more of new species. This is not akin to the OTUs generated in NGS platforms, which are generally short sequences with high possibility to not match with sequences in GenBank, not because they are new, but because of the quality of the sequence data.

Key words – cultural characteristics – culture-dependent technique – ITS – mangrove – morphotype – mycelia sterilia – phylogenetic analysis

Introduction

Rhizophora apiculata (*Rhizophoraceae*) is a mangrove tree species which occurs in the intermediate estuarine zone in the mid-intertidal region. The species is also widespread throughout most tropical coastal areas of the western Pacific region to east Africa (Duke 2006). Mangrove ecosystems play important roles in estuarine systems (Thrush et al. 2013). For example, the root system of *R. apiculata* serves as a habitat and nursery ground for numerous organisms, such as juvenile fish and crustaceans (MFF 2011). They also provide erosion mitigation and stabilization for adjacent coastal landforms (Harty 1997). Mangrove species also have potential to absorb and fix heavy metals (Lian et al. 1999, Ravikumar et al. 2007).

Endophytes are microorganisms, which colonize and live in internal plant tissues without causing apparent symptoms of disease to the host plant (Petrini 1991, Photita et al. 2001, Kumar & Hyde 2004, Promputtha et al. 2005, Hyde & Soytong 2008, Yoo & Eom et al. 2012). Fungal endophytes are important in studies on plant ecology and evolutionary relationships as they can be latent pathogens, saprobes or beneficial symbionts (Photita et al. 2004, Rodriguez et al. 2004, Hyde et al. 2007, Promputtha et al. 2007, Sieber 2007, Slippers & Wingfield 2007, Hyde & Soytong 2008, Aly et al. 2010, Saikkonen et al. 2010, Thirunavukkarasu et al. 2011). Endophytes have possible uses in biotechnology as they can produce bioactive secondary metabolites, which have potential for anti-microbial and anti-cancer use (Chaeprasert et al. 2010, Devi et al. 2012, Joel & Bhimba 2012, Job et al. 2015).

The traditional study and identification of fungal endophytes has been based on promoting sporulation of isolates growing out from sterilized asymptomatic plant tissues (Guo et al. 1998, Taylor et al. 1999). Many studies on endophytes have isolated strains which sporulate on different artificial media, under different cultural conditions (Carroll 1986, Paul et al. 2012). However, many do not sporulate in culture and are known as mycelia sterilia. This makes identification difficult. Thus several methods have been developed to stimulate sporulation of fungal endophytes, e.g. exposure at 20–25 °C under a 12 hours alternating near UV-light and darkness (Guo et al. 1998, Gomes et al. 2013) and transferring non-sporulating isolates to chemically defined media such as Czapek's agar, or on natural media such as corn meal agar (CMA), malt extract agar (MEA), oat meal agar (OMA), potato dextrose agar (PDA) and vegetable juice agar (V8A) (Paul et al. 2012). Sometimes growing mycelium of fungal endophytes with sterilized host substrates will induce sporulation (Guo et al. 1998, Taylor et al. 1999, Fröhlich et al. 2000, Gomes et al. 2013). Many taxonomic concepts have also been discussed for non-sporulating fungal isolates, e.g. grouping mycelia sterilia isolates into morphological species on the basis of similarity of cultural characteristics (Bills 1996). Isolates having similar cultural characteristics are grouped into the same morphotype, but such a taxonomic concept may be erroneous as isolates of the same morphotype may not be closely related (Guo et al. 2003).

Molecular based sequence data have also been used for phylogenetic placement of endophytic mycelia sterilia. Many studies have traditionally used sequence data from the ITS region to identify non-sporulating colonies and evaluate morphotaxonomic delimitation of endophytic fungi (Guo et al. 2000, 2001, 2003, Arnold 2002, Lacap et al. 2003, Promputtha et al. 2005, Tejesvi et al. 2011,

Jeewon et al. 2013, Haghighi & Shahdoust 2015). The internal transcribed spacer (ITS) region is the most widely sequenced genetic marker for fungi (Nilsson et al. 2008, 2014), and ITS has been formally proposed as the standard fungal barcode marker (Schoch et al. 2012, 2014). Comparisons of ITS sequence data are comprehensively used for molecular phylogenetic studies because the region is easy to amplify, even when small quantities of DNA are used, due to the high copy number of rRNA genes available in the genome (Haghighi & Shahdoust 2015). ITS region is highly variable although with a few exceptions (Gazis et al. 2011, Lindahl et al. 2013).

The aim of the present study was to establish how many species of endophytes from a study of *Rhizophora apiculata* in Thailand can be resolved to species based on ITS sequence data. *Rhizophora apiculata* is commonly found in mangrove forests in Thailand. However, there are few reports on the species composition and biodiversity of endophytic fungi from Thai mangrove plants (Chaowalit 2009, Buatong 2010, Chaeprasert et al. 2010, Sakayaroj et al. 2010). Cultural characteristics were initially used to group the endophytes, while ITS sequence data was used to identify and establish if the endophytes can be assigned to species level.

Materials & Methods

Collection and isolation

Plant samples of *Rhizophora apiculata* were collected from five sites in four provinces in Thailand (Chanthaburi, Phetchaburi, Prachuap Khiri Khan and Rayong Provinces). At each site, asymptomatic leaves, petioles and aerial tip roots were randomly collected and placed in separate brown paper bags. The samples were brought to the laboratory and when feasible isolated on the same day. Where necessary, samples were refrigerated at 4 °C and isolation was carried out the following day.

The isolation of endophytic fungi was adapted from Bharathidasan & Panneerselvam (2011). Asymptomatic leaf, petiole and aerial stilt root samples were rinsed in tap water to remove debris and dirt. Root samples were cut into small pieces (0.5–0.7 $cm^{2)}$ using a sterilized blade. Leaf samples were cut using a sterilized hole puncher size 6 mm diam. Petiole samples (0.5–0.7 cm long) were cut using a sterilized blade. A total of 200 segments recovered from the different plant tissues (70, 70 and 60 segments of leaves, petioles and aerial stilt roots, respectively) were used. Each set of plant material was surface sterilized by slowly shaking for 1 minute in 3% sodium hypochlorite (NaOCl) followed by a minute in sterile water. The samples were then disinfected in 70% ethanol for 1 minute followed by three rinses with sterile distilled water. The plant pieces were dried by blotting on sterile filter paper. Four to five segments were placed in each Petri-dish on water agar (WA) supplemented with 100 mg/ ml streptomycin. The dishes were sealed with parafilm and incubated at 27 °C ± 2 °C for one or two weeks at room temperature until the onset of fungal growth. Fungal colonies were selected and purified by transferring single hyphal tips daily onto potato dextrose agar (PDA) plates throughout a 2-week period. Pure cultures were maintained for further study on PDA. Living cultures of different isolates were deposited in the Mae Fah Luang Culture Collection, Thailand (MFLUCC).

Comparison of cultural characteristics

The cultural characteristics of each isolate were compared based on the morphotype concept (Bills 1996, Lacap et al. 2003). Five mm diameter plugs were cut from the edge of actively growing colonies and placed onto fresh PDA. Three replicates of each isolate were incubated in the dark at 25 °C. The average colony diameter (mm) was determined after seven days. Colony characteristics, including colour (above and below), elevation, margin, shape and surface were observed after 14 days of incubation (Lacap et al. 2003). Colony colour was defined with the Methuen Handbook of Colour (Kornerup & Wanscher 1967) and colonies photographed. Those cultures which sporulated on PDA after 1–2 months were identified based on morphological characteristics from general reference sources as mentioned in Doilom et al. (2017), and recent relevant literature.

DNA extraction, PCR amplification and sequencing

Twenty morphotypes of the 30 representative isolates (where available, 2–5 isolates from each morphotype) were selected for the molecular analyses. The selection was based on different cultural characteristics after purification. Total genomic DNA was extracted from fresh mycelium (500 mg) scraped from the margin of a colony on a PDA plate incubated at 25 °C ± 2 °C for 7–10 days. Biospin Fungus Genomic DNA Extraction Kit–BSC14S1 (BioFlux, P.R. China) was used to extract genomic DNA from the mycelium following the manufacturer's protocol. The ITS regions were amplified and sequenced with the primers ITS5/ITS4 (White et al. 1990).

The amplification PCR reactions were performed in a total volume of 25 µl. PCR mixtures contained TaKaRa Ex-Taq DNA polymerase 0.3 µl, 12.5 µl of 2 × PCR buffer with 2.5 µl of dNTPs, 1 µl of each primer, 9.2 µl of double-distilled water and 100–500 ng of DNA template. PCR reactions were run on a BIORAD 1000 Thermal Cycler (Applied Biosystems, Foster City, CA, U.S.A.) under the following conditions. Initial denaturation of 5 minutes at 95 °C, followed by 35 cycles of 30 seconds at 94 °C, 45 seconds annealing at 58 °C, 1 minute at 72 °C, and a final extension of 10 minutes at 72 °C. Positive amplicons were visualised on 1% agarose gel under UV light using a Gel Doc^{TM} XR+ Molecular Imager (BIO-RAD, USA). Sequencing of the positive amplicons was carried on a Sun-biotech Company Sequencer (Beijing, China).

Phylogenetic analysis

Consensus sequences were generated using Geneious® R7 (Biomatters Ltd., New Zealand) and BioEdit (Hall 1999) to ensure sequence integrity. Representative sequences were individually blasted using the MegaBLAST search of NCBIs GenBank nucleotide database (www http://blast.ncbi.nlm.nih.gov/) to retrieve similar taxa for further analyses. The maximum identity with maximum score taxa from the blast searches were selected, and available type strains were included for building the phylogenetic trees. Two different datasets were analysed in this study. First, 104 taxa with *Rhizopus oryzae* CBS 112.07 as the outgroup taxon were used in the phylogenetic analysis for most of the endophytic isolates recovered. Second, 26 *Pseudopithomyces* taxa were analysed with *Deniquelata barringtoniae* as an outgroup taxon to determine species relationships of our putative *Pseudopithomyces* endophytic isolate. Datasets of ITS regions were aligned online using the MAFFT version 7.221 server. Phylogenetic trees were inferred with maximum likelihood (ML) and Bayesian inference (BI). Maximum likelihood (ML) analysis was calculated online using The CIPRES Science Gateway v. 3.3 (http://www.phylo.org/portal2/, Miller et al. 2010). Bayesian inference was conducted using the Markov Chain Monte Carlo (MCMC) method with MrBayes v. 3.2.2 (Ronquist et al. 2011). The methodologies of these two analyses were performed as described in Doilom et al. (2017). In Bayesian inference, the GTR+I+G was selected as the best-fit nucleotide substitution models under the Akaike information criterion (AIC) for overview phylogeny of the ITS sequence data. Five million generations were selected with a sampling frequency every 100 generations. Phylogenetic trees were visualized using Treeview (Page 1996) and formatted using PowerPoint 2010 (Microsoft Corporation, WA, USA). Sequence data in this study are deposited in GenBank. Accession numbers for each strain are provided in Fig. 3.

Results and Discussion

Cultural characteristics

Based on cultural characteristics such as colony shape, colour of hyphae and surface, 154 isolates were grouped into 20 morphotypes. Of these, nine morphotypes sporulated on PDA and they were identified to seven genera viz. *Colletotrichum*, *Diaporthe*, *Hypoxylon*, *Neopestalotiopsis*, *Neodevriesia*, *Pestalotiopsis* and *Phyllosticta*. The remaining eleven groups were mycelia sterilia on PDA. Different endophytic taxa were selected to be potentially identified to assess our current taxonomic concept based on morphotypes. Growth rate and colony were observed and noted (Table 1, Figs 1, 2). These characters are provided as they may be useful for primary identification of endophytic fungi from mangroves and/or other host plants, at least for certain taxonomic groups. However, the colour of mycelium, and growth rates, can vary under different conditions such as

media, light and temperature. Moreover, grouping of taxa into morphotypes does not reflect species phylogeny as morphotypes are not real taxonomic units (Guo et al. 2003). Surprisingly, some isolates having different cultural characters were found to belong to the same genus (Figs 2a, 2b). Two replicates of the same morphotype showed slightly different cultural characteristics (Figs 2g, 2h).

Phylogenetic analysis

The blast search results of ITS sequence data are shown in Table 2. The ITS alignments length including gaps comprised 901 and 708 characters for the two datasets investigated (Figs. 3, 4, respectively). The RAxML analysis resulted in a best scoring likelihood tree selected with a final value for the ITS dataset = −11994.67091 (Fig. 3), and −1963.727247 (Fig. 4). The likelihood of the final tree was evaluated and optimized under GAMMA model parameters, with 694 distinct alignment patterns and 38.29% of completely undetermined characters and gaps (Fig. 3), and 167 distinct alignment patterns and 21.66% undetermined characters or gaps (Fig. 4). The ITS phylogeny resulted in the detection of 20 taxonomic units residing in 15 known genera (*Allophoma*, *Colletotrichum*, *Diaporthe*, *Hortaea*, *Hypoxylon*, *Pestalotiopsis*, *Phanerochaete*, *Phyllosticta*, *Pseudopithomyces*, *Preussia*, *Neodevriesia*, *Neopestalotiopsis*, *Nemania*, *Rigidoporus*, *Schizophyllum*) and one unidentified ascomycete genus from 30 representative strains out of the 154 isolates (Fig. 3). Thirteen of the genera belong to Ascomycota, while three genera, *Phanerochaete*, *Rigidoporus* and *Schizophyllum* are basidiomycetes. Basidiomycetous fungi such as *Polyporales* and *Rigidoporus* have also been found as endophytes in leaves of coniferous trees and *Paphiopedilum villosum* (Yoo & Eom 2012, Khamchatra et al. 2016). Details of representative strains of the determined endophytic fungal genera on *R. apiculata* are listed (Table 3). The results of Blast search and ITS phylogeny of these 16 genera as well as additional data and genes are discussed below.

Allophoma – MFLUCC 17-0003 had 100% matches with many isolates in the MegaBLAST search of NCBIs GenBank nucleotide database. The maximum score was 981, which is similar to e.g. *Stagonosporopsis cucurbitacearum* E-271 (Table 2). Our ITS based phylogeny does not clearly separate *Allophoma* from *Stagonosporopsis* (Fig 3: K). Strain MFLUCC 17-0003 is assigned to *Allophoma* as the ITS sequence was most similar to this genus. However, *S. cucurbitacearum* E-271 has not been formally published. A combined multi-locus phylogenetic analysis based on ITS, large subunit rDNA (LSU), second largest subunit of RNA polymerase II (RPB2) and β-tubulin (Tub2), and morphological studies are needed for a better generic delimitation for these closely related genera as has been the case for the *Didymellaceae* (Chen et al. 2015, 2017, Hyde et al. 2016).

Colletotrichum – Three representative isolates (MFLUCC 17-1943, MFLUCC 17-1944, MFLUCC 17-0004) were identified as *Colletotrichum* based on morphological and cultural characteristics. ITS sequence data of MFLUCC 17-1943 and MFLUCC 17-1944 were identical, but differed from MFLUCC 17-0004. Based on a MegaBLAST search, the ITS of MFLUCC 17-1943 matched 100% with many isolates of *Colletotrichum* and fungal endophytes, but had max score (1074) with *C. gloeosporioides* AAP-018 and Fungal sp. SF3 (Table 2). Isolate MFLUCC 17-0004 also shared 99% with many *Colletotrichum* species, but had a 1088 max score with *C. brevisporum* PC-1 (Table 2). However, it is known that many species of *Colletotrichum* cannot be distinguished reliably using ITS (Hyde et al. 2009, Weir et al. 2012), and hence the three representative isolates are named as *Colletotrichum* spp. Multi-locus gene regions would help determine the current species. Cai et al. (2009) provided detailed protocols for studying *Colletotrichum* species. Multilocus phylogenetic analyses have typically been used to resolve *Colletotrichum* species (e.g. Talhinhas et al. 2002, Weir et al. 2012, Damm et al. 2013, Tao et al. 2013). Recommended genetic markers were suggested (Hyde et al. 2014). Genes and combination of genes that can be used for identification of the *Colletotrichum* species complexes were recommended (Cannon et al. 2012, Hyde et al. 2014) and have been summarized by Jayawardena et al. (2016). For example, a combined of ITS, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), chitin synthase 1

Table 1 Cultural characteristics of the 20 morphotypes from *Rhizophora apiculata* on potato dextrose agar (PDA) at 25 °C in the dark.

Morphotype	Representative strain	Size (mm) of colony after 7 days	Colour[a,b]		Form or Shape	Elevation	Margin	Density	Figure
			Above	Below					
1	MFLUCC17-0025	27–35	Orange white (6A2) mix with white (6A1)	Light brown (6D6)[a], Pale orange (5A3)[b]	Irregular	Raised to lower convex	Erose or dentate	Spare	1a
2	MFLUCC 17-1941	60–65	White (5A1)[a], light orange (5A4)[b]	Orange white (5A2)[a], light orange (5A5)[b]	Irregular	Convex with papillate surface	Undulate	Spare	1b
3	MFLUCC 17-0005	14–20	Brownish grey (7D2) mix with white (7D1)	Black mix with orange white (6A2)	Irregular	Flat	Erose or dentate	Spare	1c
4	MFLUCC 17-0020	37–45	Brown (7E5) mix with white (7A1)	Light brown (7D6) mix with white (7A1)	Irregular, punctiform	Raised	Undulate	Spare	1d
5	MFLUCC 17-1945	40–50	Brownish grey (6F2), brownish orange (6C4), alternate white (6A1)	Brownish grey (6F2), white (6A1)	Irregular to circular	Raised to convex with papillate surface	Undulate	Medium	1e
6	MFLUCC 17-0004	52–60	Greyish brown (6F3) to (6E3) alternate white (6A1)	Greyish brown (7F3), white (7D1) and greyish brown (7D3)	Circular	Flat or effuse to raise	Entire edge	Dense	1f
7	MFLUCC 17-1943	50–60	Brownish grey (7C2)[a], white (7A1)[b]	Brownish grey (7D2)[a], white (7A1)[b]	Circular	Raised to convex with papillate surface	Undulate	Medium	1g
8	MFLUCC 17-0035	70–80	Yellowish white (4A2)	Light yellow (4A4)	Irregular	Raised convex with papillate surface	Lobate, with concentric rings	Dense	1h
9	MFLUCC 17-1942	37–45	Pale yellow (4A3)[a], white (4A1)[b]	Yellowish brown (5F8)[a], pale yellow (4A3)[b]	Irregular	Raised to lower convex	Lobate to undulate	Dense	1i
10	MFLUCC 17-0003	22–31	Greyish orange (5B4)[a], white (5A1)[b]	Light brown (6D5)[a], white (6A1)[b]	Circular	Raised to lower convex	Entire edge	Medium	1j

Table 1 Continued.

Morphotype	Representative strain	Size (mm) of colony after 7 days	Colour[a,b]		Form or Shape	Elevation	Margin	Density	Figure
			Above	Below					
11	MFLUCC 17-0028	40–45	Light brown (6D4) alternate brown (6E4)	Brown (6F4) alternate brown (6E5)	Circular	Raised to lower convex	Undulate	Medium	1k
12	MFLUCC 17-0022	7–18	Pale orange (5A3)[a], orange white (5A2)[b]	Orange white (5A2)	Irregular	Flat	Entire edge	Spare	1l
13	MFLUCC 17-0030	10–15	Olive brown (4E2)[a], yellowish white (4A2)[b]	Greyish beige (4D2)[a], yellowish white (4A2)[b]	Irregular	Flat to lower convex	Crenated to lobate	Medium	2a
14	MFLUCC 17-1937	15–22	Olive grey (3F2)	Olive (3F3)	Irregular	Flat or effuse	Crenated to lobate	Medium	2b
15	MFLUCC 17-1938	8–13	Olive grey (2F2)	Olive grey (2F2)	Irregular	Raise	Undulate to Entire edge	Medium	2c
16	MFLUCC 17-1940	11–13	Olive grey (3F2)	Olive brown (4F3)	Irregular	Flat	Undulate	Spare	2d
17	MFLUCC 17-1939	7–10	Grey (3F1)	Olive grey (2F2)	Irregular	Lower convex	Undulate	Spare	2e
18	MFLUCC 17-0007	Completely covering the Petri-dish	White (4A1)	Yellowish white (4A2)	Circular	Raised	Entire edge	Dense	2f
19	MFLUCC 17-0002	60–70	Yellowish white (4A2)[a], violet brown (11F4)[b]	Yellowish white (4A2)[a], violet brown (11F4)[b]	Irregular	Flat or effuse	Crenated	Spare	2g, h
20	MFLUCC 17-1946	Completely covering the Petri-dish	White (4A1)	Yellowish white (4A2)	Circular	Raised, convex in center	Crenated	Medium dense	2i

[a]Center of culture, [b]edge of culture.

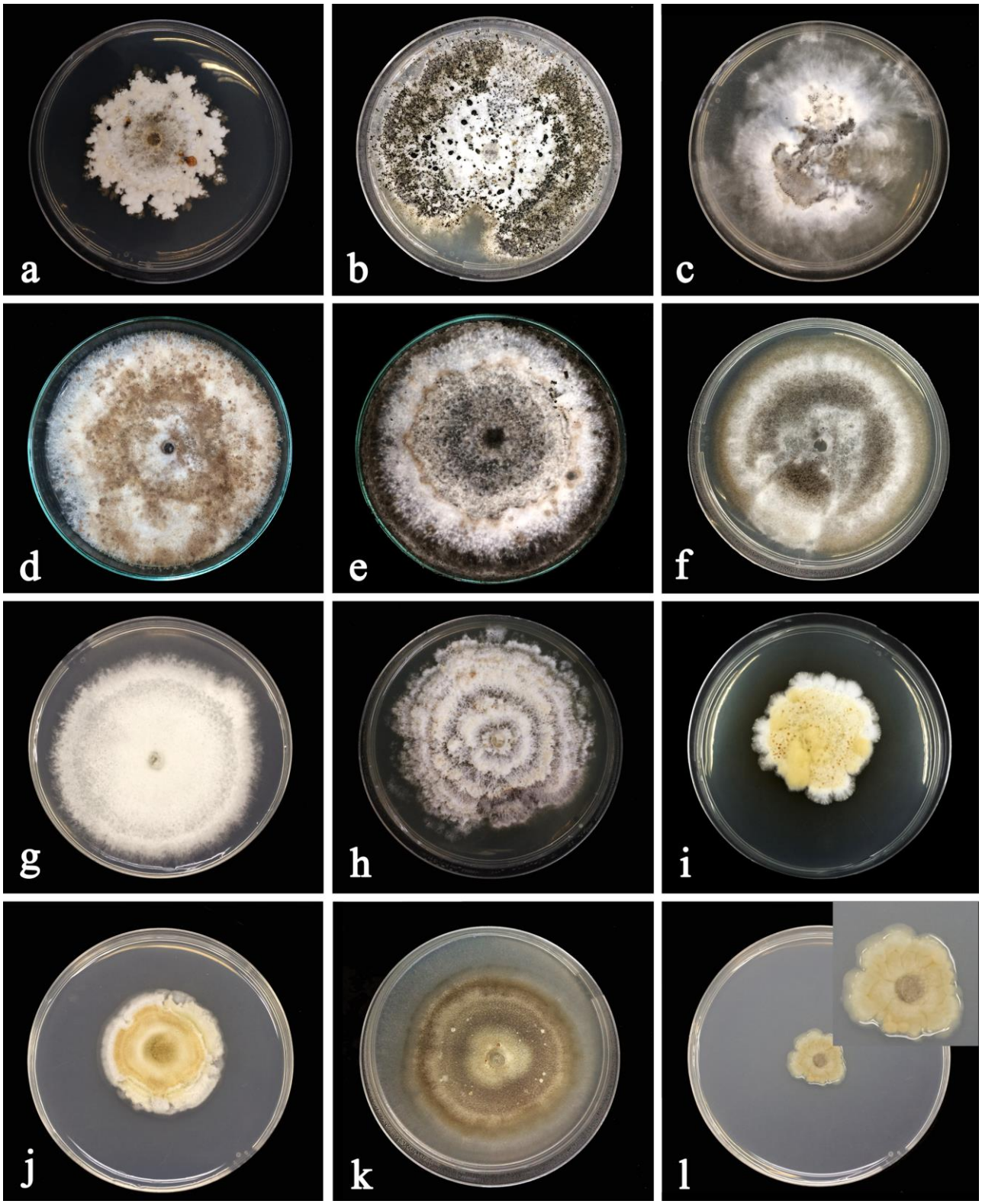

Figure 1 – Colony morphology of fungal endophytes on PDA, at 25 °C in the dark, isolated from *Rhizophora apiculata*. a *Pestalotiopsis* sp. MFLUCC 17-0025 (22 d). b *Neopestalotiopsis* sp. MFLUCC 17-1941 (30 d). c *Nemania* sp. MFLUCC 17-0005 (22 d). d *Hypoxylon griseobrunneum* MFLUCC 17-0020 (22 d). e *Hypoxylon* sp. MFLUCC 17-1945 (22 d). f *Colletotrichum* sp. MFLUCC 17-0004 (25 d). g *Colletotrichum* sp. MFLUCC 17-1943 (14 d). h *Diaporthe* sp. MFLUCC 17-0035 (5 d). i *Diaporthe* sp. MFLUCC 17-1942 (14 d). j *Allophoma* sp. MFLUCC 17-0003 (14 d). k *Pseudopithomyces maydicus* MFLUCC 17-0028 (25 d). l *Preussia* sp. MFLUCC17-0022 (14 d).

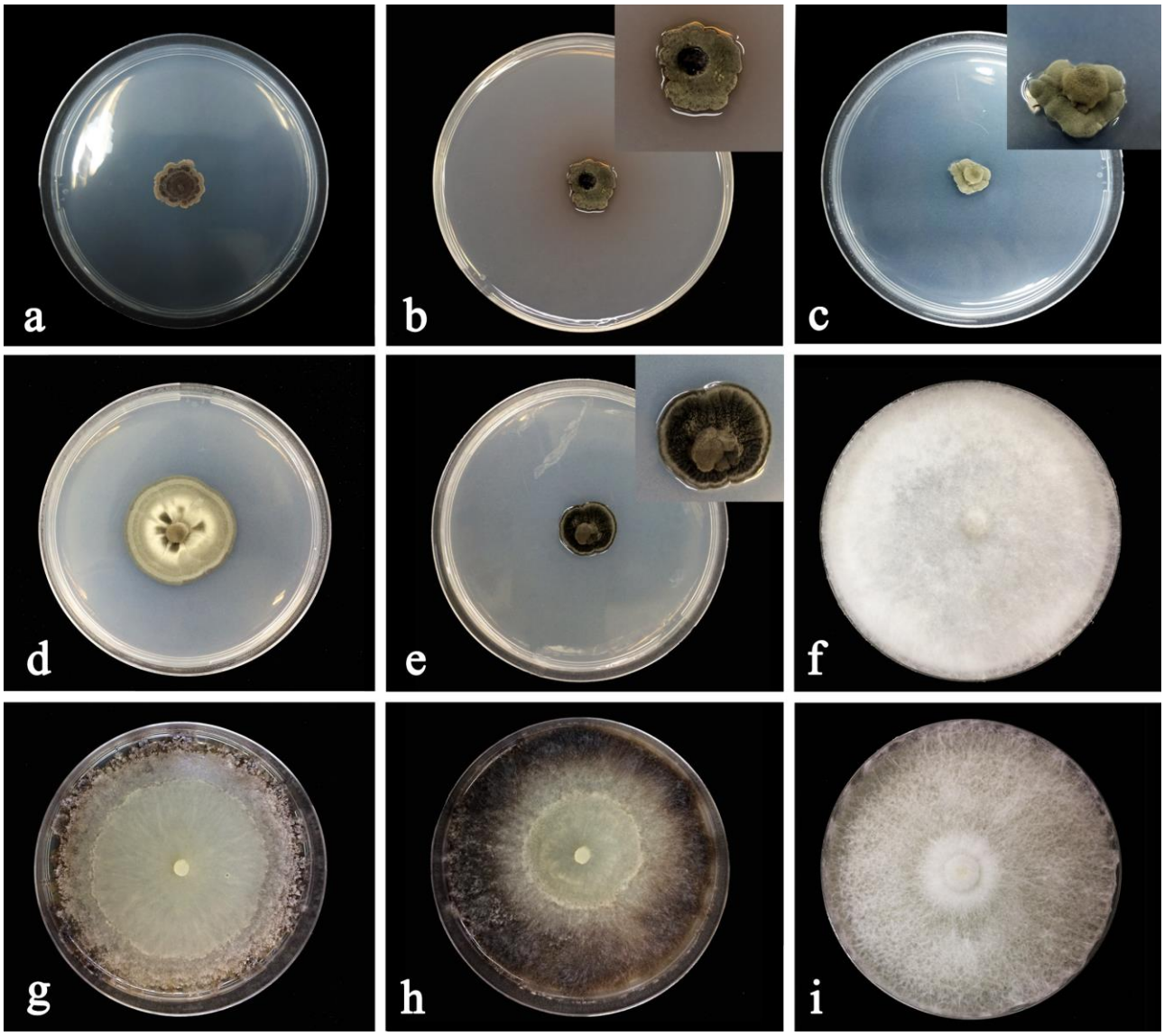

Figure 2 – Colony morphology of fungal endophytes on PDA, at 25 °C in the dark, isolated from *Rhizophora apiculata*. a *Phyllosticta* sp. MFLUCC 17-0030 (14 d). b *Phyllosticta* sp. MFLUCC 17-1937 (14 d). c Fungal endophyte (unidentified) MFLUCC 17-1938 (14 d). d *Hortaea werneckii* MFLUCC 17-1940 (30 d). e *Neodevriesia* sp. MFLUCC 17-1939 (30 d). f *Rigidoporus vinctus* MFLUCC 17-0007 (30 d). g, h *Phanerochaete* sp. MFLUCC 17-0002 (25 d). i *Schizophyllum* sp. MFLUCC 17-1946 (30 d).

(CHS-1), histone H3 (HIS3), actin (ACT) and Tub2 sequences data was used to identify species in the *Colletotrichum acutatum* species complex (Jayawardena et al. 2016).

Diaporthe – Comparison of nucleotide polymorphism of the ITS sequences data between MFLUCC 17-1942 and MFLUCC 17-0035 showed the major differences over a span of c. 29 polymorphisms. These consist of two deletions, five insertions, 12 transitions and 10 tranversions. Based on the recommendations of Jeewon & Hyde (2016), we consider these two isolates to be distinct. Blast searches of the ITS, MFLUCC 17-0035 and MFLUCC 17-1942 are provided in Table 2. ITS phylogenetic analysis showed that MFLUCC 17-1942 grouped with *Phomopsis* sp. MA194 with weak bootstrap support, and next to *Diaporthe arengae* CBS 114979 (ex-type) with 95% ML and 0.95 PP (Fig 3: F). These three isolates grouped with *Diaporthe arecae* CBS 161.64 (ex-isotype) and MFLUCC 17-0035 with 100% ML and 1.00 PP (Fig. 3, F). However, multiple gene analyses are required to improve the accuracy of species delimitation of these two isolates. Identification of *Diaporthe* species is not always appropriate using morphological characters,

because of their plasticity and overlap between different species (Santos & Phillips 2009). ACT, calmodulin (CAL), HIS3, ITS, translation elongation factor 1-alpha (TEF) and Tub2 should be used in combined analyses with at least 4–5 genes with recommended primers (Udayanga et al. 2012, 2014a, b, Gomes et al. 2013, Hyde et al. 2014, Dissanayake et al. 2017). Santos et al. (2017) suggested that if only four loci can be sequenced these should be TEF, Tub2, CAL and HIS3; if three loci these should be TEF, Tub2 and CAL; if two loci TEF and Tub2; if only one locus TEF as TEF sequence data is the most informative for species separation. ITS sequence data is the least informative to resolve *Diaporthe* species (Santos et al. 2017). However multi-loci sequence analysis (MLSA) provides more accurate estimation of phylogeny and has less separation errors than single locus analysis, if reasonable loci are used (Gadagkar et al. 2005, Mirarab et al. 2016, Santos et al. 2017).

Hortaea – Based on a MegaBLAST search of NCBIs GenBank nucleotide database, the closest hit using the ITS sequence of MFLUCC 17-1940 matched 100% and had max score (966) with many isolates such as *Hortaea werneckii* MCw215 (Table 2). This latter isolate is from a sample of water, collected during the pre-monsoon summer season from mangroves at Santa Cruz, India (Nayak et al. 2012). The ITS phylogeny grouped MFLUCC 17-1940 with *H. werneckii* MCw215 and CBS 107.67 (ex-neotype) with 100% ML and 1.00 PP (Fig. 3, H), and is therefore considered to be *Hortaea werneckii*.

Hypoxylon – ITS sequence data of MFLUCC 17-0020 was identical to MFLUCC 17-0027. The closest hits using a MegaBLAST search of the ITS DNA sequence data of MFLUCC 17-0020 had 100% similar to several strains, but had a 935 max score to *H. anthochroum* EGJMP20 and *Xylariaceae* sp. AT4 (unpublished in GenBank 2017) (Table 2). The types and published strains of *H. griseobrunneum* were also included in the ITS phylogeny based on the information from Kuhnert et al. (2014a). Two isolates in this study grouped with *H. griseobrunneum* agrEK07, STMA06148, CBS 331.73 (ex-type), BCRC 34050 (as *H. anthochroum*), and *H. anthochroum* EGJMP20 with 93% ML and 0.99 PP support (Fig. 3, D). MFLUCC 17-0020 and MFLUCC 17-0027 are identified as *H. griseobrunneum* based on similarity of ITS sequence data and morphology of the asexual morph being similar to *H. griseobrunneum* (Kuhnert et al. 2014a). Blast searches of another isolate MFLUCC 17-1945 are provided in Table 2. This isolate grouped sister to *H. lechatii* MUCL 54609 and CBS 123577 (ex-type) (Kuhnert et al. 2014b), and *Xylariaceae* sp. D11a4 with 87% ML and 0.97 PP. Comparison of nucleotide polymorphism of the ITS sequence data differs between our collection MFLUCC 17-1945 and *H. lechatii* CBS 123577 (ex-type) strain with eight polymorphisms consisting of five transitions, two deletions and one insertion. Thus, MFLUCC 17-1945 is considered to be *Hypoxylon* sp., however, it is necessary to compare the Tub2 gene to clarify species level reliably (Kuhnert et al. 2014a, b, Daranagama et al. 2017).

Nemania – MFLUCC 17-0005 was closely related to *Nemania diffusa* BCC 18754 (Okane et al. 2012) with 100% sequence similarity and 1003 max score based on a MegaBLAST search (Table 2). *Nemania diffusa* BCC 18754 has been reported as a xylariaceous endophytic fungus from *Pteris decrescens* in Thailand (Okane et al. 2012). There are many isolates of *N. diffusa* with ITS sequences data available in GenBank, but the type strain is not sequenced. MFLUCC 17-0005 had high ITS sequence similarity to *N. diffusa* BCC 18754. However, *N. diffusa* BCC 18754 (Okane et al. 2012) showed multiple nucleotide differences from other *N. diffusa* isolates GZ AT-F006 and FR AT-113 (Tang et al. 2007, 2009). MFLUCC 17-0005 is named as *Nemania* sp. (Fig. 3, C). The isolate should be compared with ex-type cultures for resolving the species name. An epitype for this species should be designated.

Neodevriesia – The ITS of MFLUCC 17-1939 was 99% similar to *Neodevriesia pakbia* CBS 139914 (ex-type) (Table 2). This isolate grouped close to, but was distinct from *N. pakbia* CBS 139914 (ex-type) with 100% ML and 1.00 PP. MFLUCC 17-1939 is determined to be *Neodevriesia* sp. (Fig. 3, I). LSU, TEF and Tub2 should be used for further species identification coupled with morphological characterization (Crous et al. 2014).

Neopestalotiopsis – Blast searches of the ITS for isolate MFLUCC 17-1941 is provided in Table 2. The ITS phylogeny suggests it is closely related to *Neopestalotiopsis aotearoa* CBS

367.54 (ex-type), *N. piceana* CBS 394.48 (ex-type), *N. eucalypticola* CBS 264.37 (ex-type), and *Pestalotiopsis* sp. LH162 (from blast search) with 90% ML and 1.00 PP (Fig. 3, A), Morphological examination of characters also pointed to the genus *Neopestalotiopsis* and hence we refer isolate MFLUCC 17-1941 to *Neopestalotiopsis* sp. However, we refrain from assigning a species name because of morphological plasticity among those species (Jeewon et al. 2002, 2003, Maharachchikumbura et al. 2014). Analysis of combined sequence data of ITS, Tub2 and TEF gene regions is necessary to delimit the species according to Maharachchikumbura et al. (2014, 2016).

Pestalotiopsis – ITS sequence data of MFLUCC 17-0016, MFLUCC 17-0018, MFLUCC 17-0019, MFLUCC 17-0024, MFLUCC 17-0025, MFLUCC 17-0026 and MFLUCC 17-1941 were aligned together in MAFFT. All of these isolates shared identical sequences, except MFLUCC 17-1941. Isolate MFLUCC 17-0019 was selected as the representative strain of this group and blasted using the GenBank BLAST option. The ITS of MFLUCC 17-0019 had 100% similarity to several strains, and one of these was *Pestalotiopsis* sp. SC5A8 with 1075 max score (Table 2). MFLUCC 17-0016, MFLUCC 17-0018, MFLUCC 17-0019, MFLUCC 17-0024, MFLUCC 17-0025 and MFLUCC 17-0026 are named as *Pestalotiopsis* sp. as they were similar to *Pestalotiopsis* sp. based on morphological and cultural characteristics, and ITS phylogeny (Fig. 3, B). Most of the key conidial characters are unstable for species level separation as they vary with host range, generation, culture and other environmental conditions (Jeewon et al. 2003, Hu et al. 2007). Naming of species based on morphological characters should be taken into account rather than host association (Jeewon et al. 2004, Maharachchikumbura et al. 2011). Maharachchikumbura et al. (2012) utilized ten gene regions (ACT, Tub2, CAL, GAPDH, glutamine synthetase (GS), ITS, LSU, largest subunit of RNA polymerase II (RPB1), SSU and TEF) to resolve cryptic *Pestalotiopsis* species, and showed that ITS, Tub2 and TEF were better markers. The other gene regions were less useful owing to poor success in PCR amplification and/or in their ability to determine species delimitation. TEF appeared to be an ideal candidate and functions well to determine species delimitation due to its better species resolution and PCR success. Tub2 showed fairly good differences among species. Combined ITS, Tub2 and TEF gene regions has given a high number of strongly supported nodes at the terminal clades as compared to single gene analysis (Maharachchikumbura et al. 2012, 2014).

Phanerochaete – ITS of MFLUCC 17-0002 had similarity (97% identity) with 946 max score to *Phanerochaete stereoides* He2309 (Table 2). ITS phylogeny of MFLUCC 17-0002 grouped sister to *P. stereoides* He2309 (Liu & He 2016) with bootstrap support of ML (96%) and PP (1.00) (Fig. 3O). Isolate MFLUCC 17-0002 is therefore assigned to *Phanerochaete* sp. RPB1, RPB2, ITS and LSU were used to revised the taxonomy of *Phanerochaete* (*Polyporales*, Basidiomycota) (Floudas & Hibbett 2015).

Phyllosticta – Isolate MFLUCC 17-0030 had 92% similarity with many species of *Phyllosticta*, but showed 845 max score with *P. aristolochiicola* BRIP 53316a (Table 2). MFLUCC 17-0030 and MFLUCC 17-0031 grouped together with 100% ML and 1.00 PP, but separated from *Guignardia* sp. 1-3-5-1-3-1 with weak bootstrap support, and next to *P. styracicola* CGMCC 3.14985 (ex type) with 70% ML and weak support of PP (Fig. 3J). Another isolate, MFLUCC 17-1937 was 99% similar to many isolates, but had max score (1064) with Fungal sp. isolate 59815 (Table 2). MFLUCC 17-1937 grouped 100% ML and 1.00 PP with Fungal sp. isolate 59815 and *P. fallopiae* MUCC 0113. There are two species of *Phyllosticta* from *Rhizophora apiculata* in this study based on ITS sequence data. However multiple genes are required to clarify species names reliably. Wikee et al. (2013a) compared phylogenies of five genes analysis (ACT, GAPDH, ITS, LSU and TEF) with two genes analysis (ACT and ITS). They indicated that the combined ACT and ITS gene loci is sufficiently robust to distinguish most taxa in *Phyllosticta*, except those closely related to *P. capitalensis*. Wikee et al. (2013b) used analysis of combined ITS, ACT and TEF gene data to confirm the identity of all isolates of *P. capitalensis.* ITS gene and combined analyses of ITS, TEF, GAPDH and ACT sequence data are recommended for generic level and inter-specific delineation respectively (Hyde et al. 2014). A polyphasic approach including morphological, molecular and proteomic techniques were used to improved species identification and delimitation

(Wulandari et al. 2009, Glienke et al. 2011, Wicht et al. 2012, Wong et al. 2012, Guarnaccia et al. 2017).

Preussia – The ITS sequences of MFLUCC 17-0022 and MFLUCC 17-0023 were identical. Isolate MFLUCC 17-0023 was selected to blast and it matched 99% with many isolates, but had max score (983) with *Preussia* sp. CY218 (Table 2). The ITS phylogeny, grouped MFLUCC 17-0022 and MFLUCC 17-0023 close to *Preussia* sp. CY218 with 100% ML and 0.97 PP, and sister to *P. persica* CBS 117680 (ex-type) with 100% ML and 1.00 PP (Fig. 3L). Nucleotide differences comparison of the ITS sequence data between our isolates and *P. persica* CBS 117680 (ex-type) strain reveal nine polymorphisms. Thus, the two isolates are described herein as *Preussia* sp. Additional genes and morphological characteristics are needed for more precise species delimitation. Arenal et al. (2007) used morphological characters and combined ITS, LSU and TEF loci to erect and identify new species of *Preussia*.

Pseudopithomyces – Isolate MFLUCC 17-0028 had the highest similarity (100%) to many isolates, but had max score (1040) with *Pithomyces maydicus* UTHSC 06-1549 (da Cunha et al. 2014) However, *Pithomyces maydicus* (Sacc.) M.B. Ellis was combined as *Pseudopithomyces maydicus* (Sacc.) J.F. Li, Ariyawansa & K.D. Hyde by Ariyawansa et al. (2015). The genus *Pseudopithomyces* comprises *P. chartarum* (Berk. & M.A. Curtis) M.B. Ellis, *P. maydicus*, *P. sacchari* (Speg.) M.B. Ellis and some unidentified *Pithomyces* strains, which group together in *Didymosphaeriaceae* (Ariyawansa et al. 2015). The isolate in this study (MFLUCC 17-0028) grouped with *P. maydicus* UTHSC 06-1549 and UTHSC 06-3954 with high statistical support (Fig. 3, M). *Pseudopithomyces* species can be separated based on ITS phylogeny in this study (Fig. 4). Isolate MFLUCC 17-0028 is therefore considered as *P. maydicus*. A combined dataset of ITS, LSU, GAPDH and RPB2 has also been reported to resolve taxonomy of *Pseudopithomyces* species (Crous et al. 2016).

Rigidoporus – MFLUCC 17-0007 had a high sequence similarity (100%) identity and 1158 max score with *Rigidoporus vinctus* FRIM142 (Table 2). ITS phylogeny placed this isolate with *R. vinctus* FRIM142, PAPH04 and N_L7_E6 with 100% ML and 1.00 PP (Fig. 3N), and it is therefore considered to be *Rigidoporus vinctus*.

Schizophyllum – ITS sequence data of MFLUCC 17-1946 was selected to blast as it was identical to the ITS sequence of MFLUCC 17-1947. Isolate MFLUCC 17-1946 showed 99% sequence similarity and 1146 max score with *Schizophyllum commune* isolate UZ1552_14 (Table 2). Other published isolates and reference strains of *Schizophyllum* were also included in the ITS phylogeny (Siqueira et al. 2016). The isolates in this study could not be well separated from *S. commune* and *S. radiatum* (Fig. 3P). Therefore, the two isolates are determined to be *Schizophyllum* sp. Siqueira et al. (2016) noted that the phylogenetic analyses of the individual ITS and LSU genes were much conserved and did not discriminate well between the closely related species *S. commune* and *S. radiatum*. The LSU, TEF, and RPB2 markers showed consistency and were used to perform a concatenated study.

Unidentified – MFLUCC 17-1938 showed a sequence similarity (99%) and max score (983) with *Mycosphaerellaceae* sp. MA12 (Table 2). Isolate MA12 is an endophyte on a mangrove plant in the South of Thailand, and Buatong (2010) placed it in *Mycosphaerellaceae* and reported that the isolate displayed strong antifungal activity against *Cryptococcus neoformans* ATCC90112. ITS phylogeny groups our isolate (MFLUCC 17-1938) with *Mycosphaerellaceae* sp. MA12 with 100% ML and 1.00 PP (Fig. 3G). MFLUCC 17-1938 and *Mycosphaerellaceae* sp. MA12 are nested in between the genera *Devriesia* and *Hortaea* which are classified in the *Teratosphaeriaceae* (Fig. 3G). We could not observe morphological details on culture of MFLUCC 17-1938 as it did not produce spores. Thus, isolate MFLUCC 17-1938 remains an unidentified taxon.

The use of highest nucleotide similarity in MegaBLAST searches of NCBIs GenBank nucleotide database is a preliminary step towards determining fungal endophyte species. The top score match may not necessarily indicate the same species (Kang et al. 2010). It is difficult to make conclusions for isolates within or related to species complexes based on MegaBLAST searches and phylogenetic analysis of ITS sequences data. However, the ITS region is still useful in some cases

for reconstruction of interspecific relationships (Cai et al. 2009). Although ITS has been formally proposed as the primary barcoding marker for fungi by the Consortium for the Barcode of Life, there is the possibility that supplementary barcodes may be developed for particularly narrowly circumscribed taxonomic groups (Schoch et al. 2012). It is better to use multigene analysis to accurately identify species of fungal endophytes (Guo et al. 2001, Huang et al. 2009, Ko et al. 2011, Sun et al. 2011). Moreover, most of the sequences named in GenBank are erroneously named, such as *Colletotrichum* and *Curvularia lunata* (Cai et al. 2009, 2011, da Cunha et al. 2013). A comparison of sequence data from fungal endophytes with ex-type cultures of named species must be considered (Dayarathne et al. 2016). This will prevent misidentification of endophytes isolated from various hosts and localities (Ko et al. 2011). Correct identification of fungi is important for understanding the biology, ecology, evolutionary relationships, for controlling plant diseases, and useful for future application in biotechnology (Santos & Phillips 2009, Ko et al. 2011, Udayanga et al. 2011, Hyde et al. 2014).

Comparison between culture-dependent and culture-independent techniques

ITS is the targeted region in culture-dependent or NGS analysis. In this study, we identified the fungal endophytes based on ITS sequence data using the culture-dependent approach, which is different from culture-independent methods (e.g. study using NGS that reads into operational taxonomic units (OTUs)). The identification of bacteria and fungi by culture-dependent methods has resulted in lower numbers of microorganisms than culture-independent methods (Carraro et al. 2011, Stefani et al. 2015). This is because culture conditions used (aeration, nutritional, temperature, etc.) can affect growth of organisms. Artificial medium usually allows growth of only a small fraction of the organisms (Carraro et al. 2011). Fast-growing species suppress growth of others, thus slow-growing organisms are out-competed (Nocker et al. 2007). Culture-independent methods can also reveal the community of unculturable organisms. Nevertheless, culture-dependent methods have higher taxonomical accuracy (discriminative power), and ease of performance and interpretation when compared to culture-independent methods ((denaturing gradient gel electrophoresis (DGGE) and 454 Pyrosequencing) (Vaz-Moreira et al. 2011). Moreover, culturing is essential for future applications in agricultural, industrial, food and medicine.

New high-throughput methods, in the culture-independent approach, are a useful strategy to estimate diversity of the mycobiome from any substrate. However, methodological biases, limitations of the markers and bioinformatic analysis, may lead to misleading conclusions (Lindahl et al. 2013, Vaz et al. 2017). Operational taxonomic units are based on sequences with ≥ 97% similarity. However, several problems can be seen in this method. If the ITS sequences of two species are ≥ 97% similar they can be assigned into one OTU instead of two. Another problem is that the same species that has a similarity less than 97% can be assigned as two different OTUs. This can be observed clearly in species complexes such as *Colletotrichum* and *Diaporthe*. Also, in the OUT dataset, species that occur only once are considered as rare OTUs or singletons considering that they may have originated from sequencing errors. In most cases, the fungal identifications from both methods can be consistent. However, inconsistent identifications can occur due to the lower power of taxonomic assignment in the culture-independent (amplicon sequencing) as compared with culture dependent approaches. This will result in identifying the organisms to family, order or class level instead of species level.

Conclusions

We resolved four endophyte species out of an estimated 20 morphotypes in this study based on ITS sequence data and cultural characteristic as well as support with available morphological study of sporulating species. These were *Hortaea werneckii*, *Hypoxylon griseobrunneum*, *Pseudopithomyces maydicus* and *Rigidoporus vinctus* and accuracy depends on the fungal group. These four species are first reports on *Rhizophora apiculata*. Both Ascomycota and Basidiomycota genera were found from *Rhizophora apiculate*, with Ascomycota being the most abundant. ITS

sequence data can be used to identify taxa that have identical nucleotide sequence data to those of ex-types of species. ITS can separate species or indicate new species when having nucleotide

Table 2 Closest match for endophytes following MegaBLAST search of NCBI GenBank nucleotide database. Max identity and max score are shown.

Isolate number in this study	Nearest match BLAST search result	Voucher/ Culture	ITS GenBank accession number	Max score	Max identity (%)
Ascomycota					
MFLUCC 17-0003	*Stagonosporopsis cucurbitacearum*	E-271	KU059901	981	531/531(100%)
	Stagonosporopsis cucurbitacearum	SE5	AB714984	981	531/531(100%)
	Didymella bryoniae	MA71	GU592001	981	531/531(100%)
MFLUCC 17-0004	*Colletotrichum brevisporum*	PC-1	KX756146	1088	602/608(99%)
MFLUCC 17-0005	*Nemania diffusa*	BCC 18754	AB625422	1003	543/543(100%)
MFLUCC 17-0019	*Pestalotiopsis* sp.	SC5A8	KU252287	1075	582/582(100%)
	Pestalotiopsis sp.	SC3A14	KU252282	1075	582/582(100%)
	Pestalotiopsis sp.	SC3A4	KU252277	1075	582/582(100%)
MFLUCC 17-0020	*Hypoxylon anthochroum*	EGJMP 20	KF192825	935	506/506(100%)
	Xylariaceae sp.	AT4	KX953392	935	506/506(100%)
MFLUCC 17-0023	*Preussia* sp.	CY218	HQ608038	983	539/542(99%)
MFLUCC 17-0028	*Pithomyces maydicus*	UTHSC 06-1549	HG933801	1040	563/563(100%)
MFLUCC 17-0030	*Phyllosticta aristolochiicola*	BRIP 53316a	NR_111791	845	574/627(92%)
MFLUCC 17-0035	*Diaporthe arecae*	CBS 161.64	KC343032	1026	560/562(99%)
MFLUCC 17-1937	Fungal sp.	59815	KP890364	1064	592/600(99%)
MFLUCC 17-1938	*Mycosphaerellaceae* sp.	MA 12	GU591997	983	542/547(99%)
MFLUCC 17-1939	*Neodevriesia pakbiae*	CPC 25044/ CBS 139914	KR476742	998	557/565(99%)
MFLUCC 17-1940	*Hortaea werneckii*	MCw215	HQ711621	966	523/523(100%)
	Fungal endophyte	2789	KR015340	966	523/523(100%)
	Hortaea werneckii	RY 51	KM014604	966	523/523(100%)
	Hortaea werneckii	JY 54	KM014589	966	523/523(100%)
	Hortaea werneckii	Hw5	JN997374	966	523/523(100%)
	Hortaea sp.	F47	FJ755827	966	523/523(100%)
	Hortaea werneckii	IFM 4988	AB087199	966	523/523(100%)
MFLUCC 17-1941	*Pestalotiopsis* sp.	LH162	HQ832816	1003	547/549(99%)
	Pestalotiopsis sp.	MA129	GQ254681	1003	547/549(99%)
	Pestalotiopsis sp.	MA165	GU592005	1003	547/549(99%)
	Pestalotiopsis sp.	14JAES	EF451799	1003	547/549(99%)
MFLUCC 17-1942	*Phomopsis* sp.	MA194	GU592007	1029	572/579(99%)
MFLUCC 17-1943	*Colletotrichum gloeosporioides*	AAP-018	KU534983	1074	581/581(100%)
	Fungal sp.	SF3	MF962538	1074	581/581(100%)
MFLUCC 17-1945	*Xylariaceae* sp.	D11a4	JQ341090	1068	604/616(98%)
	Hypoxylon lechatii	MUCL 54609	KF923407	1068	606/619(98%)
Basidiomycota					
MFLUCC 17-0002	*Phanerochaete stereoides*	He2309	KX212219	946	547/564(97%)
MFLUCC 17-0007	*Rigidoporus vinctus*	FRIM 142	HQ400710	1158	627/627(100%)
MFLUCC 17-1946	*Schizophyllum commune*	UZ1552_14	KP326577	1146	625/627(99%)

Note: When more than one isolate shared similarity of ITS sequence alignment then a representative isolate was blasted.

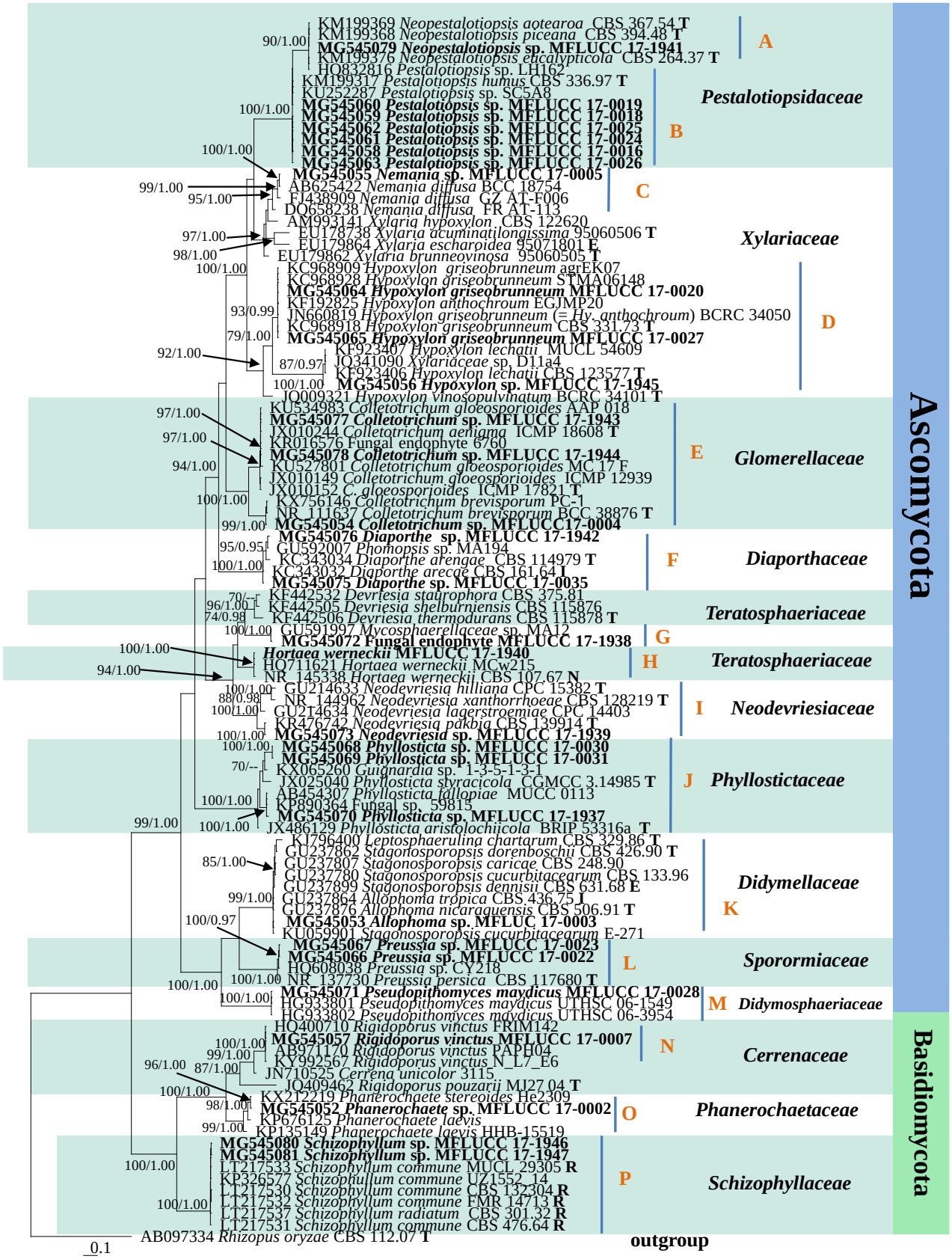

Figure 3 – Phylogram generated from maximum likelihood analysis (RAxML) based on ITS sequence data. The tree is rooted to *Rhizopus oryzae* CBS 112.07. Maximum likelihood bootstrap values (MLBS) ≥70% and Bayesian posterior probabilities ≥0.95, (MLBS/PP) are given at the nodes. Ex-epitype, ex-isotype, ex-neotype, ex-type and reference strains are marked with E, I, N, T and R, respectively. The new isolates are in bold.

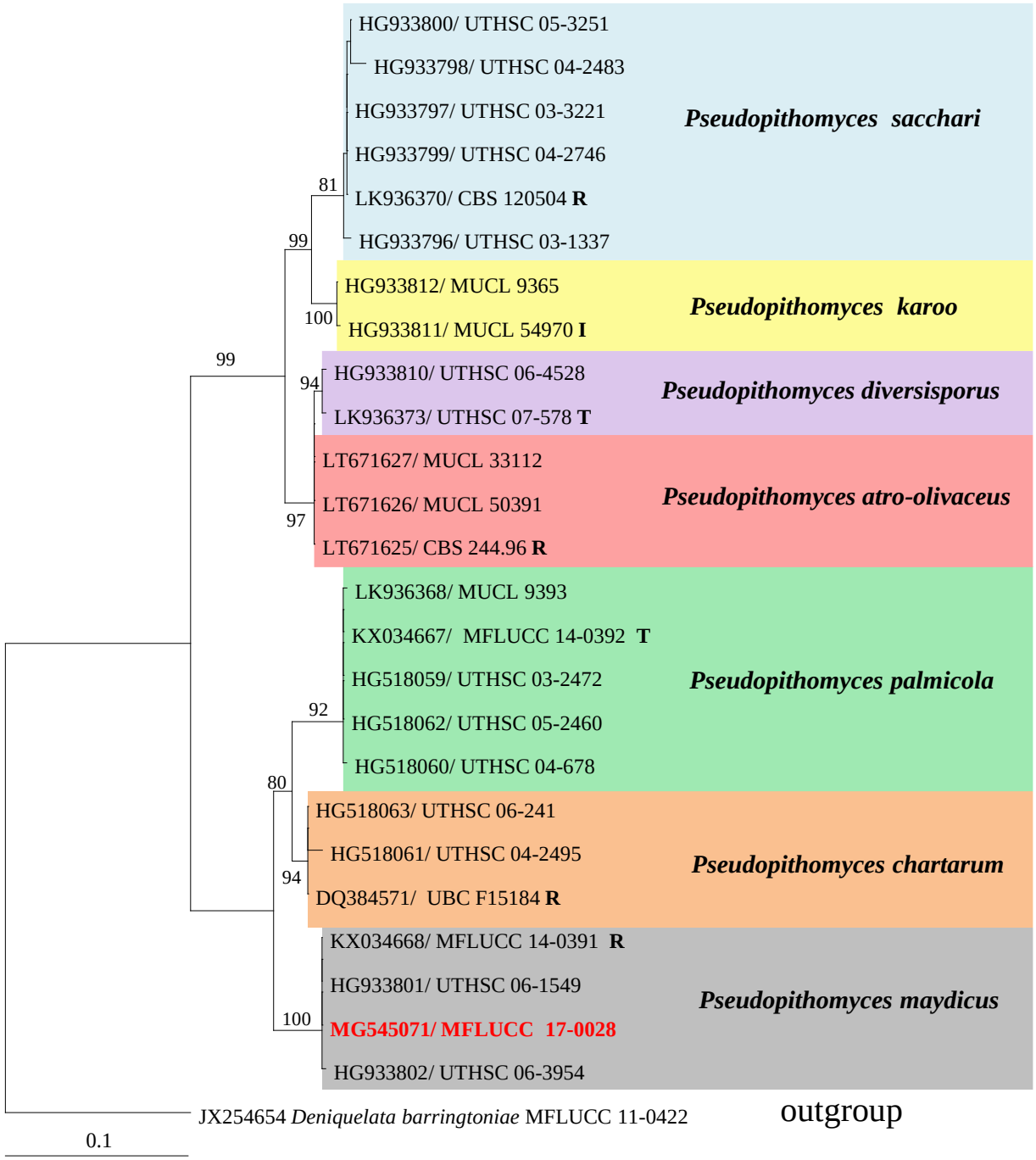

Figure 4 – Phylogram generated from maximum likelihood analysis (RAxML) based on ITS sequence data in *Pseudopithomyces* species. The tree is rooted to *Deniquelata barringtoniae* MFLUCC 11-0422. Maximum likelihood bootstrap values (MLBS) ≥70% are given at the nodes. Ex-isotype, ex-type and reference strains are marked with I, T and R, respectively. The new isolate is in red bold.

sequence clearly diverges from previous described species. Individual ITS can clearly discriminate *Pseudopithomyces* species. ITS sequence data are hardly enough to identify the species boundary for species complex such as *Colletotrichum* and *Diaporthe*, in fact for most genera isolated in this study, but can provide an idea for taxonomic groups at least to genus level in most cases. Additional data and analysis, especially from multiple gene loci, are required to identify fungal endophytes.

Table 3 Details of representative strains of isolated endophytic fungi from *Rhizophora apiculata*.

	Classification	Strain no	Locality	Habitat
Ascomycota, Dothideomycetes	***Botryosphaeriales, Phyllostictaceae***			
	Phyllosticta spp.	MFLUCC 17-0030	Kram Subdistrict, Kleang District, Rayong Province	Leaf
		MFLUCC 17-0031	Phra Samut Chedi Klang Nam, Mueang District, Rayong Province	Leaf
		MFLUCC 17-1937	Phra Samut Chedi Klang Nam, Mueang District, Rayong Province	Petiole
	Capnodiales, Neodevriesiaceae			
	Neodevriesia sp.	MFLUCC 17-1939	Sirinart rajini, Pak Nam Pran Sub district, Pranburi District, Prachuap Khiri Khan Province	Leaf
	Capnodiales, Teratosphaeriaceae			
	Hortaea werneckii	MFLUCC 17-1940	Ao Khung Kraben, Khlong Khut Sub district, Tha Mai District, Chanthaburi Province	Leaf
	Pleosporales, Didymellaceae			
	Allophoma sp.	MFLUCC 17-0003	Sirindhorn, Cha-am Subdistrict, Cha-am District, Phetchaburi Province	Leaf
	Pleosporales, Didymosphaeriaceae			
	Pseudopithomyces maydicus	MFLUCC 17-0028	Sirinart rajini, Pak Nam Pran Subdistrict, Pranburi District, Prachuap Khiri Khan Province	Aerial stilt root
	Pleosporales, Sporormiaceae			
	Preussia sp.	MFLUCC 17-0022 and MFLUCC 17-0023	Sirindhorn, Cha-am Subdistrict, Cha-am District, Phetchaburi Province	Aerial stilt root
	Ascomycota genera, incertae sedis			
	Fungal endophyte (unidentified)	MFLUCC 17-1938	Ao Khung Kraben, Khlong Khut Sub district, Tha Mai District, Chanthaburi Province	Leaf
Ascomycota, Sordariomycetes	***Amphisphaeriales, Pestalotiopsidaceae***			
	Pestalotiopsis spp.	MFLUCC 17-0016, MFLUCC 17-0018, MFLUCC 17-0019, MFLUCC 17-0024, MFLUCC 17-0025 and MFLUCC 17-0026	Sirindhorn, Cha-am Subdistrict, Cha-am District, Phetchaburi Province	Aerial stilt root
	Neopestalotiopsis sp.	MFLUCC 17-1941	Sirindhorn, Cha-am Subdistrict, Cha-am District, Phetchaburi Province	Aerial stilt root

Table 3 Continued.

	Classification	Strain no	Locality	Habitat
Ascomycota, Sordariomycetes	***Diaporthales, Diaporthaceae***			
	Diaporthe sp.	MFLUCC 17-0035	Kram Subdistrict, Kleang District, Rayong Province	Aerial stilt root
	Diaporthe sp.	MFLUCC 17-1942	Kram Subdistrict, Kleang District, Rayong Province	Aerial stilt root
	Glomerellales, Glomerellaceae			
	Colletotrichum sp.	MFLUCC 17-0004	Sirindhorn, Cha-am Subdistrict, Cha-am District, Phetchaburi Province	Leaf
	Colletotrichum sp.	MFLUCC 17-1943	Kram Subdistrict, Kleang District, Rayong Province	Leaf
	Colletotrichum sp.	MFLUCC 17-1944	Sirinart rajini, Pak Nam Pran Subdistrict, Pranburi District, Prachuap Khiri Khan Province	Leaf
	Xylariales, Xylariaceae			
	Hypoxylon griseobrunneum	MFLUCC 17-0020 and MFLUCC 17-0027	Sirinart rajini, Pak Nam Pran Subdistrict, Pranburi District, Prachuap Khiri Khan Province	Leaf
	Hypoxylon sp.	MFLUCC 17-1945	Sirinart rajini, Pak Nam Pran Subdistrict, Pranburi District, Prachuap Khiri Khan Province	Leaf
	Nemania sp.	MFLUCC 17-0005	Sirindhorn, Cha-am Subdistrict, Cha-am District, Phetchaburi Province	Leaf
Basidiomycota, Agaricomycetes	***Agaricales, Schizophyllaceae***			
	Schizophyllum sp.	MFLUCC 17-1946 and MFLUCC 17-1947	Sirinart rajini, Pak Nam Pran Subdistrict, Pranburi District, Prachuap Khiri Khan Province	Aerial stilt root
	Polyporales, Cerrenaceae			
	Rigidoporus vinctus	MFLUCC 17-0007	Sirindhorn, Cha-am District, Phetchaburi Province	Petiole
	Polyporales, Phanerochaetaceae			
	Phanerochaete sp.	MFLUCC 17-0002	Sirindhorn, Cha-am District, Phetchaburi Province	Leaf

Acknowledgements

This work was financially supported by Thailand Research Fund (TRF) grant no RSA5980068 entitled ‘‘Biodiversity, phylogeny and role of fungal endophytes on above parts of *Rhizophora apiculata* and *Nypa fruticans*’’. 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’’. K.D. Hyde is adjunct professor at Chiang Mai University. We are appreciative and thankful to staff at Ao Kung Krabaen Bay Nature Center, The Sirindhorn International Environment Park and Sirinart Rajini Ecosystem Learning Center for giving us the knowledge, and granting us permission to study and collect mangrove

specimens. Mingkwan Doilom is grateful to Dr. Joanne E. Taylor (Royal Botanic Garden Edinburgh, Scotland, UK) and Assoc. Prof. Dr. JianKui Liu (Guizhou Institute of Biotechnology, Guizhou Academy of Agricultural Sciences, Guiyang, Guizhou, China) for kind suggestions on the manuscript.

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