BIOTROPIA Vol. 28 No. 3, 2021: 221 - 230 DOI: 10.11598/btb.2021.28.3.1339

DIVERSITY OF ENDOPHYTIC FUNGI ASSOCIATED WITH FRUITS AND LEAVES OF TAMARIND (*Tamarindus indica* L.) BASED ON ITS RIBOSOMAL DNA SEQUENCES

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Received 18 February 2020 / Accepted 2 May 2020

ABSTRACT

Plant-associated microbes are among essential natural resources that abundantly exist in a natural environment, such as endophytic fungi. Studies on endophytic fungi in medicinal plants have allowed the discovery of numerous fungi species and their hidden potentials. Therefore, this study focused on the isolation and identification of endophytic fungi from several plant parts of tamarind (*T. indica*), such as leaves and fruits. A total of 69 fungal cultures were successfully isolated and identified into 31 distinct species from 15 genera based on morphological characteristics and internal transcribed spacer (ITS) sequence analysis using a Maximum Likelihood method. A high diversity of endophytic fungi associated with *T. indica* were observed by Shannon–Wiener index H' (3.083). There were six different species obtained from the genus *Colletotrichum* (*C. aenigma, C. brevisporum, C. cobbittiense, C. fructicola, C. gloeosporioides* and *C. siamense*), and *Diaporthe* (*D. arecae, D. ceratozamiae, D. phaseolorum, D. pseudomangiferae, D. pseudooculi* and *D. pseudophoenicicola*), four species of *Aspergillus* (*A. aculeatus, A. carbonarius, A. flavus* and *A. tubingensis*), two species of *Curvularia/Cochliobolus* (*C. geniculatus* and *C. lunata*) and *Nigrospora* (*N. lacticolonia* and *N. oryzae*), two species of *Lasiodiplodia* (*L. pseudotheobromae* and *L. theobromae*) and *Penicillium* (*P. rolfsii* and *P. verruculosum*). Other fungal species that were also identified are *Botryosphaeria mamane, Fusarium solani, Truncospora tephropora, Phyllosticta fallopiae, Sarcostroma bisetulatum, Trichoderma asperellum* and *Xylaria feejeensis.*

Keywords: Endophytic fungi, internal transcribed spacer (ITS), phylogenetic tree, tamarind

INTRODUCTION

Endophytic fungi are microorganisms inhabiting plant tissues in a part of their life without showing any harm toward the host plants. The species of endophytic fungi are expected in over a million species, which arisen from the natural surroundings (Mishra *et al.* 2018). They are widely distributed, which have been found in many plant species that can grow in natural environments such as terrestrial plant communities (Nisa *et al.* 2015). Endophytic fungi such as *Aspergillus, Colletotrichum, Fusarium, Penicillium* and *Trichoderma* may colonize several parts of plants, including fruits and leaves (Hanada *et al.* 2010). There are many research studies reported the abundance of fungi associated with plants, however, there is a lack of study in the endophytic fungi associated with *T. indica.*

Bourou *et al.* (2010) reported, three genera of arbuscular mycorrhizal fungi (*Acaulospora, Glomus* and *Scutellospora*) were associated with *T. indica.* Tamarind tree has been reported to be infected by some wood decay fungi such as *Daldinia concentrica, Schizophyllum commune, Flavodon flavus, Irpex hydnoides,* and *Phellinus fastuosus* (Nnagadesi & Arya 2015). In previous reports, *Aspergillus niger, Rhizopus stolonifer, Ulocladium chartarum, Penicillium chrysogenum, P. citrinum* and *Phomopsis liquidambaris* were associated with infected-tamarind fruit

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(Danggomen *et al.* 2013; Peter & Patrick 2017). *Penicillium chrysogenum* and *P. citrinum* are confirmed pathogens and caused spoilage in fruits (Peter & Patrick 2017). Recently, *Aspergillus niger* has been proven as a pathogen that causes black pod of tamarind (Meena *et al.* 2018).

Due to the information regarding endophytic fungal diversity associated with *T. indica* is lacking, this study will provide important information regarding the diversity of fungal endophytes associated with *T. indica*. This study was aimed to determine the culturable endophytic fungal diversity associated with *T. indica* using molecular phylogenetic analysis of ITS rDNA sequences.

MATERIALS AND METHODS

Plant Samples

Collection of leaves and fruits samples of *T. indica* was completed in 2018 and 2019 at Jalan Asam Jawa, Universiti Putra Malaysia, Serdang Selangor located at 3°00'09.0"N 101°42'34.8"E (Fig. 1). The fruits and leaves samples were collected using fruit picker from 20 *T. indica* trees with 2 m apart. All samples were further placed in paper bags, properly labeled, and brought to the Mycology Laboratory, Department of Biology for fungal isolation.

Isolation, Purification and Preservation of Microfungi

All plant samples were washed in running tap water for 30 min to remove any debris or soil before being processed. The leaves were cut into segments of 5 × 5 mm. Then, the surface of the leaves and fruits was surface sterilized by following the method described by Ravindran *et al.* (2012) by immersing in 70% ethanol (5 sec), 4% sodium hypochlorite (NaOCl) (90 sec), rinsed with sterile distilled water (30 sec) and blotted dry with sterile filter paper. All of the segments were placed (3 segments each plate) on potato dextrose agar (PDA) supplemented with streptomycin (0.05 g/ml) and neomycin (0.01 g/L) using sterilized forceps. The culture plate was incubated at room temperature (27 ± 2°C) for 5 to 7 days or until there was an appearance of mycelium or colony from the sample fragments.

The fungal mycelia grown from the parts of the sample were streaked on 4% water agar (WA) for purification. The WA plate was incubated for another 24 hours. Then, the single tip of hyphae was cut and transferred onto a new PDA plate and incubated at 27±2°C for seven days. The pure isolated fungi were preliminarily identified by examining their morphological characteristics. All isolates were maintained and preserved at -20 °C using a modified filter paper method for working and stock cultures with slight modifications (Fong *et al.* 2000).

Figure 1 Samples of fruits (A) and leaves (B) of *T. indica* were collected in Persiaran Asam Jawa, Universiti Putra Malaysia

DNA Extraction, PCR Amplification and Sequencing

All isolates were cultured on PDA and incubated for 5 days. DNA of the isolates was extracted using UltraClean® Microbial DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) according to manufacturer's instruction. Amplification of the ITS regions was conducted using Polymerase Chain Reaction (PCR) machine (Hercuvan Lab Systems, California, USA) involved primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.* 1990). The PCR master mix was prepared from 4 µL of 5×PCR buffer, 2 µL of 2 mM dNTP, 2 µL of 25 mM $MgCl_2$, 1 µL of 10 mM for each primer, 0.1 µL of Taq DNA polymerase with concentration 5 U µL, 6.9 µL of nuclease-free water and 3 µL of DNA in a total volume of 20 µL. The PCR protocol with initial denaturation step was done for 30 sec at 95 °C, followed by 35 cycles of denaturation (95 °C for 10 sec), annealing (59 °C for 15 sec) and extension (72 °C for 30 sec), and was completed by final extension step at 72 °C for 5 min. Then, the PCR product was prepared for gel electrophoresis or stored at -20 °C.

The PCR products were gel-electrophoresed using 1.5% agarose gel. The mixture of 2.5 µL of 6× loading dye (blue/orange) and 2.5 µL of 100 bp DNA marker were used as a ladder. The DNA and ladder were pipetted with 5 µl in volume into the holes using a micropipette and electrophoresed. The amplicon size was visualized under a UV trans-illuminator. The PCR products were purified using a QIAquick gel extraction kit (QIAGEN, USA), following the manufacturer's instructions. The purified PCR products were sequenced by using an Applied Biosystem 3730xl DNA Analyzer (MyTACG Bioscience Company, MY).

Phylogenetic Analysis

Evolutionary analyses of ITS sequences were conducted in Molecular Evolutionary Genetics Analysis (MEGA) 6.0 software to obtain alignment sequences (Tamura *et al.* 2013). Homologous sequences were obtained from The GenBank database NCBI (http://blast.ncbi.nlm.nih.gov/) using BLASTN search (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) of the ITS sequences. The phylogenetic analysis was conducted using the Maximum Likelihood method based on the Tamura-Nei model with 1000 bootstrap test (Tamura & Nei 1993) in MEGA version 6.0. *Saccharomyces cerevisae* CBS 1171 (AB018043) was used as an outgroup (Fig. 3). The GenBank accession number of new sequences were listed in Table 1.

Species Diversity

The species diversity was calculated by using the Shannon-Weiner Index (Spellerberg 2008) as formula below:

$$H' = -\sum_{i=1}^{s} pi \ln pi$$

where:
H' = Value of Shannon Wiener's diversity index
pi = Proportion of species
s = Number of species in community
I = Number of individuals in species

RESULTS AND DISCUSSION

A total of 69 isolates of fungi were obtained from 20 fruit and leaf samples of *T. indica,* and were identified based on their morphological characteristics (Fig. 2) and ITS sequence analysis (Table 1 and Fig. 2). Thirty-two species belong to 15 genera were found in the present study including *Aspergillus* (4 species), *Botryosphaeria* (a single species), *Colletotrichum* (6 species), *Cochliobolus/Curvularia* (2 species), *Diaporthe* (6 species), *Fusarium* (a single species), *Lasiodiplodia* (2 species), *Nigrospora* (2 species), *Penicillium* (2 species), *Truncospora* (a single species), *Phyllosticta* (a single species), *Sarcostroma* (a single species), *Trichoderma* (a single species), and *Xylaria* (a single species) (Table 1).

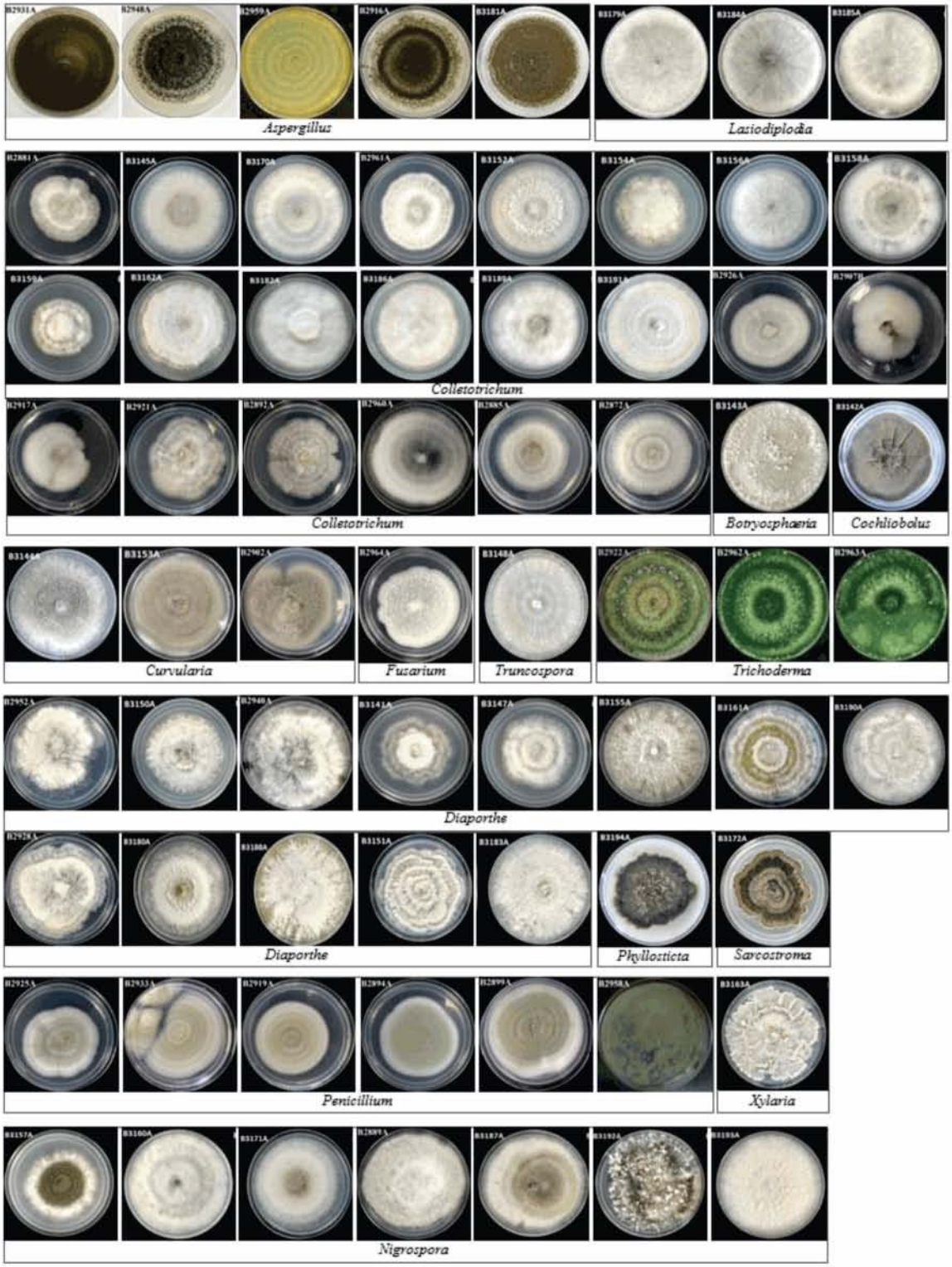

Figure 2 Fungal morphological retrieved in the culture media isolation procedure. Endophytic fungi were isolated from fruits and leaves of *T. indica*. Fungi were cultivated in PDA medium at 27 °C for 7 days

Based on phylogenetic analysis of ITS sequences of the 69 endophytic fungi isolated from tamarind fruits and leaves, two major clades (A and B) were generated (Fig. 3). The first clade (Clade A) comprises isolates of fungi under Phylum Ascomycota and Clade B contains *Truncospora tephropora* B3148 (Phylum Basidiomycota). Clade A was divided into 2 sub-clades; Clade A1 represents isolates of *Aspergillus, Botryoshaeria, Colletotrichum, Diaporthe, Fusarium, Nigrospora, Sarcostroma, Trichoderma, Penicillium, Phyllosticta*, and *Xylaria*, whereas Clade A2 represents isolates of *Lasiodiplodia* and *Curvularia/Cochliobolus.*

Table 1 ITS sequences GenBank accession number of deposited fungal isolates from fruits and leaves of *T. indica*

No.	Isolates	Species	Plant part	GenBank accession number
1.	B2931	*Aspergillus aculeatus*	Fruit	MK204304
2.	B2948	*A. carbonarius*	Fruit	MK204302
3.	B2959	*A. flavus*	Fruit	MK204299
4.	B2916	*A. tubingensis*	Fruit	MK204311
5.	B3181	*A. tubingensis*	Leaf	MT043791
6.	B3143	*Botryosphaeria mamane*	Leaf	MT043767
7.	B2881	*Colletotrichum aenigma*	Leaf	MK204314
8.	B3145	*C. brevisporum*	Leaf	MT043769
9.	B3170	*C. cobbittiense*	Leaf	MT043786
10.	B2961	*C. fructicola*	Leaf	MK204289
11.	B3152	*C. gloeosporioides*	Leaf	MT043774
12.	B3154	*C. gloeosporioides*	Leaf	MT043776
13.	B3156	*C. gloeosporioides*	Leaf	MT043778
14.	B3158	*C. gloeosporioides*	Leaf	MT043780
15.	B3159	*C. gloeosporioides*	Leaf	MT043781
16.	B3162	*C. gloeosporioides*	Leaf	MT043784
17.	B3182	*C. gloeosporioides*	Leaf	MT043792
18.	B3186	*C. gloeosporioides*	Leaf	MT043796
19.	B3189	*C. gloeosporioides*	Leaf	MT043799
20.	B3191	*C. gloeosporioides*	Leaf	MT043801
21.	B2926	*C. siamense*	Fruit	MK204291
22.	B2907	*C. siamense*	Fruit	MK204292
23.	B2917	*C. siamense*	Fruit	MK204293
24.	B2921	*C. siamense*	Fruit	MK204294
25.	B2892	*C. siamense*	Leaf	MK204295
26.	B2960	*C. siamense*	Leaf	MK204296
27.	B2885	*C. siamense*	Leaf	MK204297
28.	B2872	*C. siamense*	Leaf	MK204298
29.	B3144	*Curvularia lunata*	Leaf	MT043768
30.	B3153	*C. lunata*	Leaf	MT043775
31.	B3142	*Cochliobolus geniculatus*	Leaf	MT043766
32.	B2902	*C. lunata*	Leaf	MK204312
33.	B2952	*Diaporthe arecae*	Fruit	MK204301
34.	B3150	*D. ceratozamiae*	Leaf	MT043772
35.	B2940	*D. phaseolorum*	Fruit	MK204303
36.	B3141	*D. phaseolorum*	Leaf	MT043765
37.	B3147	*D. phaseolorum*	Leaf	MT043770
38.	B3155	*D. phaseolorum*	Leaf	MT043777
39.	B3161	*D. phaseolorum*	Leaf	MT043783
40.	B3190	*D. phaseolorum*	Leaf	MT043800
41.	B2928	*D. pseudomangiferae*	Fruit	MK204305
42.	B3180	*D. pseudooculi*	Leaf	MT043790
43.	B3188	*D. pseudooculi*	Leaf	MT043798
44.	B3151	*D. pseudophoenicicola*	Leaf	MT043773
45.	B3183	*D. pseudophoenicicola*	Leaf	MT043793
46.	B2964	*Fusarium solani*	Fruit	MK204285
47.	B3184	*Lasiodiplodia pseudotheobromae*	Leaf	MT043794
48.	B3179	*L. theobromae*	Leaf	MT043789
49.	B3185	*L. theobromae*	Leaf	MT043795
50.	B3157	*Nigrospora lacticolonia*	Leaf	MT043779
51.	B3160	*N. lacticolonia*	Leaf	MT043782
52.	B3171	*N. lacticolonia*	Leaf	MT043787
53.	B2889	*N. oryzae*	Leaf	MK204313
54.	B3187	*N. oryzae*	Leaf	MT043797
55.	B3192	*N. oryzae*	Leaf	MT043802
56.	B3193	*N. oryzae*	Leaf	MT043803
57.	B2925	*Penicillium rolfsii*	Fruit	MK204306
58.	B2933	*P. rolfsii*	Fruit	MK204307
59.	B2919	*P. rolfsii*	Fruit	MK204308
60.	B2894	*P. rolfsii*	Leaf	MK204309
61.	B2899	*P. rolfsii*	Leaf	MK204310
62.	B2958	*P. verruculosum*	Fruit	MK204300
63.	B3194	*Phyllosticta fallopiae*	Leaf	MT043804
64.	B3172	*Sarcostroma bisetulatum*	Leaf	MT043788
65.	B2922	*Trichoderma asperellum*	Fruit	MK204286
66.	B2962	*T. asperellum*	Fruit	MK204287
67.	B2963	*T. asperellum*	Fruit	MK204288
68.	B3148	*Truncospora tephropora*	Leaf	MT043771
69.	B3163	*Xylaria feejeensis*	Leaf	MT043785

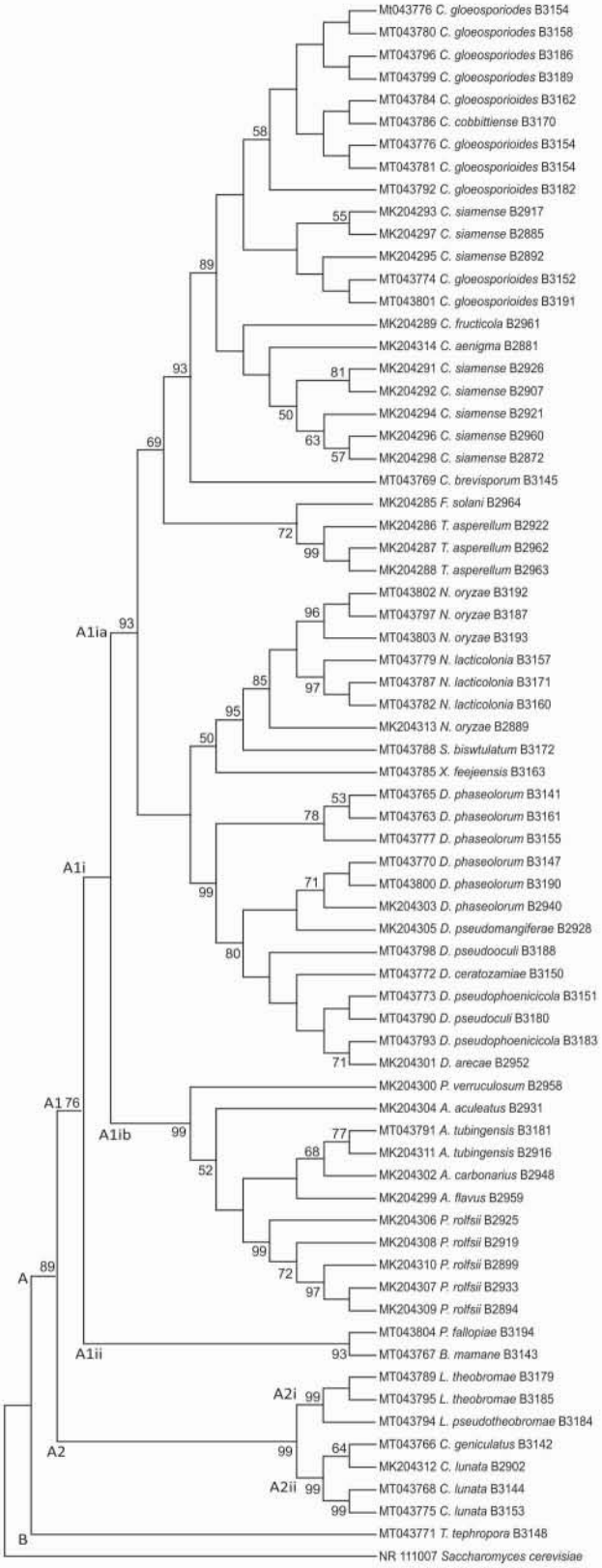

Figure 3 Phylogenetic tree generated from the Maximum Likelihood method based on the ITS sequences of 69 fungal endophytes sequences associated with *T. indica*. The tree generated using Tamura-Nei model with 1000 Bootstrap replications. All Bootstrap scores with less than 50% are not shown in the tree

The Shannon index (H' = 3.083) indicated that the tamarind fungal community possesses a vast diversity of endophytic fungi (Table 2). The most diverse fungal genera isolated from tamarind leaves was *Colletotrichum* and *Diaporthe* (Fig. 3, Table 1).

Table 2 Endophytic fungal percentage and Shannon-Wiener Index obtained from culture media isolation using fruits and leaves of *T. indica*

No.	*Species*	Number of isolate	Percentage (%)	Shannon-Wiener Index (H')
1.	*Aspergillus aculeatus*	1	1.45	0.061
2.	*A. carbonarius*	1	1.45	0.061
3.	*A. flavus*	1	1.45	0.061
4.	*A. tubingensis*	2	2.90	0.103
5.	*Botryosphaeria mamane*	1	1.45	0.061
6.	*Colletotrichum aenigma*	1	1.45	0.061
7.	*C. brevisporum*	1	1.45	0.061
8.	*C. cobbittiense*	1	1.45	0.061
9.	*C. fructicola*	1	1.45	0.061
10.	*C. gloeosporioides*	10	14.49	0.280
11.	*C. siamense*	8	11.59	0.250
12.	*C. lunata*	3	4.35	0.061
13.	*Cochliobolus geniculatus*	1	1.45	0.136
14.	*Diaporthe arecae*	1	1.45	0.061
15.	*D. ceratozamiae*	1	1.45	0.061
16.	*D. phaseolorum*	6	8.70	0.212
17.	*D. pseudomangiferae*	1	1.45	0.061
18.	*D. pseudooculi*	2	2.90	0.103
19.	*D. pseudophoenicicola*	2	2.90	0.103
20.	*Fusarium solani*	1	1.45	0.061
21.	*Lasiodiplodia theobromae*	2	2.90	0.103
22.	*L. pseudotheobromae*	1	1.45	0.061
23.	*Nigrospora lacticolonia*	3	4.34	0.136
24.	*N. oryzae*	4	5.79	0.165
25.	*Penicillium rolfsii*	5	7.25	0.190
26.	*P. verruculosum*	1	1.45	0.061
27.	*Truncospora tephropora*	1	1.45	0.061
28.	*Phyllosticta fallopiae*	1	1.45	0.061
29.	*Sarcostroma bisetulatum*	1	1.45	0.061
30.	*Trichoderma asperellum*	3	4.34	0.136
31.	*Xylaria feejeensis*	1	1.45	0.061
	Total	69	100	3.083

In this study, the most abundant fungal (26 isolates) species obtained from *T. indica* leaves was from genus *Colletotrichum* where 10 isolates were identified as *C. gloeosporioides* with 14.49% (H' = 0.280). Endophytic *C. fructicola* and *C. siamense* have been recovered from healthy *Cymbopogon citratus* (Manamgoda *et al.* 2013). Weir *et al.* (2012) stated that *C. siamense* is geographically diverse with a varied host range and is a common saprobe or endophyte. *Colletotrichum* species can be found abundantly forming its association with temperate plants and they are widely distributed in the tropical and subtropical areas (Cannon *et al.* 2012), but no report on associations with *T. indica*. A study by Boddy (2016) also reported that *Colletotrichum* species could be existed within plant tissues without causing any harm while it is in an inactive state. These studies showed that members of *Colletotrichum* exhibit a multiple life styles.

Six isolates of endophytic *Diaporthe phaseolorum* have been isolates from healthy fruits and leaves of *T. indica*. *Diaporthe* spp. are known to be existed symbiotically alongside plants as saprobic, endophytic or phytopathogenic (Udayanga *et al.* 2011; Tan *et al.* 2013; Gomzhina & Gannibal 2018). According to González and Tello (2011), endophytic *Diaporthe* species are commonly isolated from several hosts in the temperate and tropical region. Research on *Diaporthe* species by Gomes *et al.* (2013) collected several species of *Diaporthe* from *Vaccinium* growing regions in Europe including *D. phaseolorum* and *D. arecae*. *Diaporthe pseudomangiferae* has been reported cause inflorescence rot, rachis, canker, and flower abortion of mango (Serrato-Diaz *et al.* 2014).

Endophytic *C. lunata* and *Cochliobolus geniculatus* (telemorph of *C. geniculata*) have been isolated from leaves of *T. indica*. Two distinct species from genus *Lasidioplodia* that were isolated from the leaves of tamarind were *Lasidioplodia theobromae* and *Lasidioplodia pseudotheobromae* with a similarity percentage of 99% and 97% respectively. Similar to *Colletotrichum* species, *Curvularia/Cochliobolus* and *Lasiodiplodia* are well-known plant pathogens and can also be endophytes.

In this study, *Aspergillus tubengensis* was found associated with the *T. indica* leaves. This species was found to form an association with many plant species such as the mangrove plant, Sonora desert plant (Nadumane *et al.* 2016), and strawberry (Palmer *et al.* 2019). Previously, other species of *Aspergillus* which is *Aspergillus niger* was isolated from diseased-fruits of *T. indica* and caused black pod (Meena *et al.* 2018). Two species of *Penicillium*, *P. rolfsii* and *P. verruculosum* have been isolated from healthy fruits and leaves of *T. indica*. *Penicillium* spp. are common pathogens and caused spoilage in fruits (Peter & Patrick 2017). The assemblage of endophytic fungi in healthy tissue of *T. indica* may indicate that some of the fungi are possible latent pathogens and some may saprophytic.

The other genus dominated the *T. indica* leaves was *Nigrospora* sp. Wang *et al.* (2017) claimed that *Nigrospora* sp. is a common in forming symbiosis with plants as pathogens, endophytes or saprophytes. *Nigrospora sphaerica* (synonym of *N. oryzae*) was found inhabiting numerous hosts such as the *Zea*, *Andropogon* and *Cymbopogon* as reported by Wang *et al.* (2017). Supaphon and Preedanon (2019) also claimed, the species was isolated from *Helianthus annus* as an endophyte. *Botryosphaeria mamane* was only one isolate obtained from this genus. According to Phillips *et al.* (2013), this species that belonged to the Botryosphaeriaceae is existed diversely in nature as pathogenic, endophytic or saprobic with more preferable to woody plants. A study by Li *et al.* (2018), also recorded the discovery of species of Botryosphaeriaceae from plantation trees including *Cunninghamina lanceolata, Dimocarpus longan, Melastoma sanguineum* and *Phoenix hanceana,* which were growing adjacent to *Eucalyptus.*

Phyllostica species have been known to form their association with plants widely and can be either pathogens or endophytes. In this study, one isolate of *Phyllosticta fallopiae* with a 100% percentage of similarity with the established sequence in the GenBank database. The morphology of the isolate characterized as *P. fallopiae* also fit the description of this species by Zhang *et al.* (2013). One isolate was identified as *Xylaria feejeensis* which was isolated from healthy leaves samples with 98.90% similarity to the GenBank sequences. According to Chen *et al.* (2013) xylariaceous fungi are dominantly associated with the *Dendrobium* species of class Orchidaceae. This finding had supported the existence of *Xylaria* sp. as an endophyte. *Truncospora tephropora* (synonym of *Perenniporia tephropora*) was the only basidiomycete found associated with healthy *T. indica* leaves with similarity percentage of 99.84% from the sequence from GenBank database.

CONCLUSION

This study revealed that various endophytic fungi were isolated from the fruits and leaves of tamarind. The 31 species that have been successfully identified were *A. aculeatus, A. carbonarius, A. flavus, A. tubingensis*, *B. mamane, C. aenigma, C. brevisporum, C. cobbittiense, C. fructicola, C. gloeosporioides*, *C. siamense*, *C. geniculatus*, *C. lunata*, *D. arecae, D. ceratozamiae, D. phaseolorum, D. pseudomangiferae, D. pseudooculi, D. pseudophoenicicola*, *F. solani*, *L. pseudotheobromae*, *L. theobromae*, *N. lacticolonia, N. oryzae*, *P. rolfsii*, *P. verruculosum*, *T. tephropora* , *P. fallopiae, S. bisetulatum, T. asperellum* and *X. feejeensis.*

ACKNOWLEDGEMENTS

The authors thank all staff of the Department of Biology, Faculty of Science, Universiti Putra Malaysia for their facilities during the study. This work was partially supported by the Fundamental Research Grant Scheme (FRGS/1/2018/STG03/UPM/02/12/5540129).

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