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Isolation and Identification of Fungal Endophytes Associated with Leaves of *Rhizophora mucronata* Lamk.

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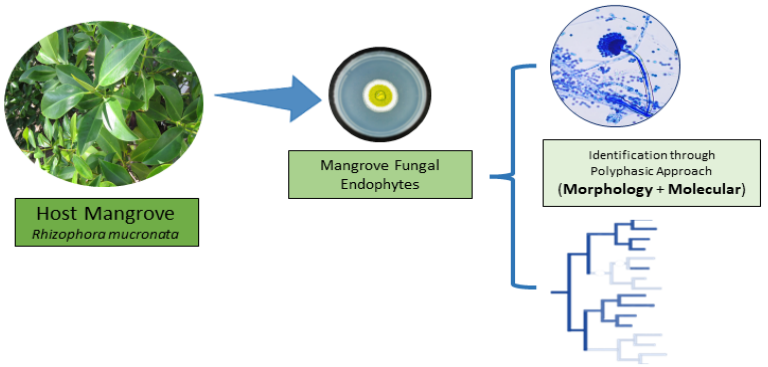
Graphical Abstract



Abstract

Mangrove-associated fungal endophytes (MFE) produce structurally diverse secondary metabolites with promising pharmaceutical applications. In our effort to search for these valuable fungi, reported herein is our isolation and molecular identification of fungal endophytes associated with mature leaves of the host mangrove *Rhizophora mucronata*. The isolated MFE were characterized based on their colonial growth on three culture media - potato dextrose agar (PDA), Czapek Dox agar (CDA), and malt extract agar (MEA), and these were grouped into 16 morphospecies. Analysis of the ITS genes confirmed the identities of the isolated mangrove fungal endophytes as belonging to the genera *Aspergillus*, *Cladosporium*, *Curvularia*, *Diaporthe*, *Fusarium*, *Nigrospora*, *Penicillium*, *Pestalotiopsis*, and *Schizophyllum*. Our study showed the species richness of fungal endophytes associated with Philippine mangroves.

Keywords: mangrove forests, molecular phylogeny, Philippines, species identification, tropical fungi

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Introduction

Rhizophora mucronata, commonly known as red mangrove, loop root mangrove, or Asiatic mangrove, is described as a true mangrove species that belongs to the mangrove family Rhizophoraceae [1]. The species is a small to medium-sized evergreen tree that grows on the banks of rivers and the fringes of the seas. The trees of *R. mucronata* tree have numerous aerial stilt roots that support the trunk, while its leaves are elliptical and with elongated tips [2]. There are also corky warts on the pale undersides of the leaves. The flowers develop in axillary clusters on the twigs. Each has a hard cream-colored calyx with four sepals and four white, hairy petals. The seeds are viviparous and start to develop while still attached to the tree. *Rhizophora mucronata* is widely distributed in Indo-West Pacific region, and from Eastern African shorelines to the Western Pacific [3].

Just like other mangrove species, trees of *R. mucronata* offer protection of inland areas by stabilizing the shoreline as it traps suspended solids of the upland runoff [4,5], thereby safeguarding the coral reefs and seagrass beds from the adverse effect of suspended particles. It also acts as a barrier to strong winds and waves from storm surges, hurricanes, and tsunamis [6] and can sequester vast amounts of carbon [7], thus counteracting global heating and reducing global warming. The root system serves as a breeding area for prawns and fishes, and a shelter for their juveniles [5,8]. Aside from its ecological significance, *R. mucronata* is an important source of timber and charcoal [9], food and natural products [10,11] including secondary metabolites that target multi-drug resistant bacteria [12]. In ethnomedicine, *R. mucronata* treats diarrhea, inflammation, angina, and hematuria [13]. Furthermore, extracts derived from the leaves of *R. mucronata* have been scientifically proven to possess an antibacterial [14], antiviral [15], larvicidal [16], anti-inflammatory [17], antioxidant and anti-cholinesterase [18], and even anticancer [19] properties.

In many mangrove species, the production of various natural products is not just through plant biosynthesis but also through microbial interaction [20]. One of these interacting microorganisms are the fungal endophytes. These fungi produce inconspicuous infection upon colonization of healthy plant tissues [21]. As mutualistic partners of plants, fungal endophytes produce secondary metabolites that help the host plants adapt to unfavorable conditions [22]. The secondary metabolites produced by mangrove-associated fungi were also known to have antibacterial, antifungal, and cytotoxic activities [23–27]. Thus, it is vital to isolate and identify various species of fungal endophytes that are associated with Philippine mangroves, i.e. *R. mucronata*, for they may become potential sources of compounds with promising pharmaceutical and biotechnological applications. This study reports the successful isolation and identification through DNA-based methods of fungal endophytes associated with leaves of the host mangrove *Rhizophora mucronata*.

Materials and methods

We isolated fungal endophytes from mature and healthy leaves of *R. mucronata* collected from two mangrove forest sites in Sta. Ana, Cagayan Province in Northern Philippines. Isolation of the fungal endophytes followed the surface sterilization protocol for mangrove leaves as described by Apurillo et al. [24]. Briefly, leaf explants, approximately 6 mm in diameter, were cut with a one-hole puncher from the leaves of *R. mucronata*. Only leaves with no visible signs of diseases were used in this study. The leaf explants were then surface sterilized by the sequential washing of 75% EtOH (for 1 min), followed by commercial bleach (5% NaOCl diluted into 1:10, for 1 min), and finally with 75% EtOH (for 30 seconds). These were washed with double-distilled water (ddH_2O) three times to remove any adhering chemicals. Following surface sterilization, five surface-sterilized leaf explants were plated on one petri plate containing potato dextrose agar (PDA, Carl Roth GmbH, Karlsruhe, Germany) supplemented with 500 mg/L streptomycin sulfate (Sigma-Aldrich, Burlington, MA, USA). The addition of antibiotics aims to suppress any leaf-associated bacteria. A total of 200 leaf explants were placed on PDA plates for this study. Tissue prints were also prepared by touching leaf fragments on PDA for 10 seconds to test the efficacy of the surface sterilization method. If fungal or bacterial growth is absent on the tissue prints, this indicates that the leaf explants were effectively surface sterilized and that the fungi growing from the explants were indeed fungal endophytes. The culture plates were incubated at room temperature (25–28 °C) and checked for fungal growth daily for up to one week. All fungi growing out of the leaf explants were subcultured on freshly prepared PDA plates for isolation and purification through subsequent subculturing. The pure cultures of the fungal isolates were maintained on PDA slants at Visayas State University in Visca, Baybay City, Leyte Province.

We initially characterized the colonial growth and spore morphologies of the isolated mangrove fungal endophytes. Cultural characterization was done by cultivating the fungi in potato dextrose agar (PDA), Czapek-ox agar (CDA), and malt extract agar (MEA). On the other hand, slide cultures of the fungal isolates were stained with lactophenol cotton blue and viewed under a light microscope to visualize spore morphologies. With these, we grouped the isolated mangrove fungal endophytes into morphospecies. To confirm species identities, pure cultures of representative isolates of each morphospecies were sent to Macrogen, Inc. in South Korea for the outsourced sequencing of the nuclear ribosomal ITS sequences using the ITS5 and ITS4 primer pairs. The sequences were then checked for quality using MEGA 11 [28] and assembled using CAP3 Sequence Assembly Program [29]. The assembled ITS sequence for each isolate was run in BLASTn [30] to search for sequence homology and identify their possible genera. Backbone trees, whenever available in the literature, were then used to construct the respective phylogenetic tree for each genus through multiple sequence alignment using ClustalW followed by phylogenetic analysis through maximum likelihood using the Tamura-Nei model with 1000 bootstrap replicates. The alignment and tree construction were done using MEGA 11 [28]. All assembled sequences for all isolates were deposited in GenBank.

Results and Discussion

We isolated a total of 42 mangrove fungal endophytes from mature and healthy leaves of *Rhizophora mucronata* and analysis of their colonial appearance and spore morphologies grouped these into 16 morphospecies (Table 1). Several strains of MFE were also isolated, but failed to grow further, and hence were excluded in this report.

Based on our combined morpho-cultural characterization and molecular identification, MFE1, MFE2, and MFE8 belong to the genus *Penicillium* (Fig. 1). Though we were not able to fully resolve the species identity of MFE1, we identified our other isolates as *Penicillium commune* (MFE2) and *P. citrinum* (MFE8). *Penicillium* spp. have been constantly reported as endophytes of several plant species including mangroves [31]. For instance, *P. commune* has been isolated from various plant species like the semi-mangrove species *Hibiscus tiliaceus* [32], the mangrove *Kandelia candel* [33], the terrestrial plants *Vitis vinifera* [34] and *Tylophora ovata* [35]. *P. citrinum*, on the other hand, was isolated as endophytes of *Ceratonia siliqua* [36], *Ocimum tenuiflorum* [37], *Azadirachta indica* [38], and *Swertia chirayita* [39].

MFE3 and MFE9 belong to the genus *Diaporthe* (Fig. 2), but with only MFE9 as fully resolved and identified as *Diaporthe tectonendophytica*. The species under the genus *Diaporthe* are also commonly reported either as saprobic or pathogenic fungi. However, studies have also shown that some species occur as non-pathogenic endophytes of various plant species [40]. For example, *D. tectonendophytica* was reported as a pathogen of red-fleshed dragon fruit (*Hylocereus polyrhizus*) causing stem gray blight disease [41] but was also isolated as an endophyte of a medicinal plant *Oroxylum indicum* [42].

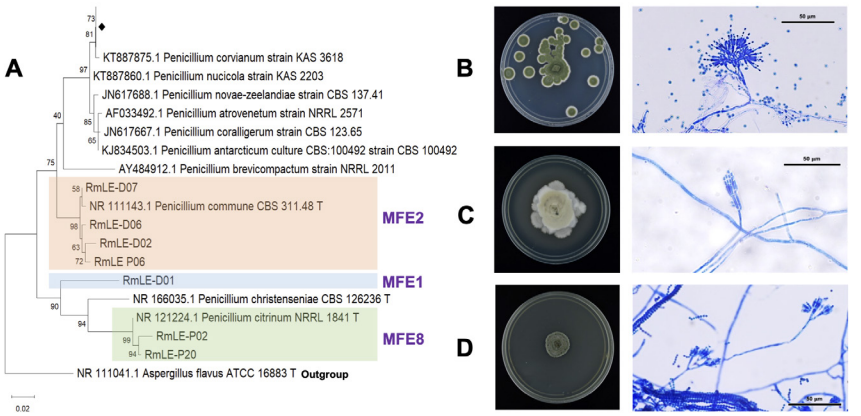

Figure 1. Maximum likelihood tree for Penicillium (A) showing MFE2 which was identified as *Penicillium commune* and MFE8 as *Penicillium citrinum*. The unresolved species, MFE1 and reported only as *Penicillium* sp., is also shown and clearly grouped within species of *Penicillium*. Photo inserts are the colony and microscopic features of *P. commune* RmLE-D07 (B), *Penicillium* sp. RmLE-D01 (C), and *P. citrinum* RmLE-P02 (D).

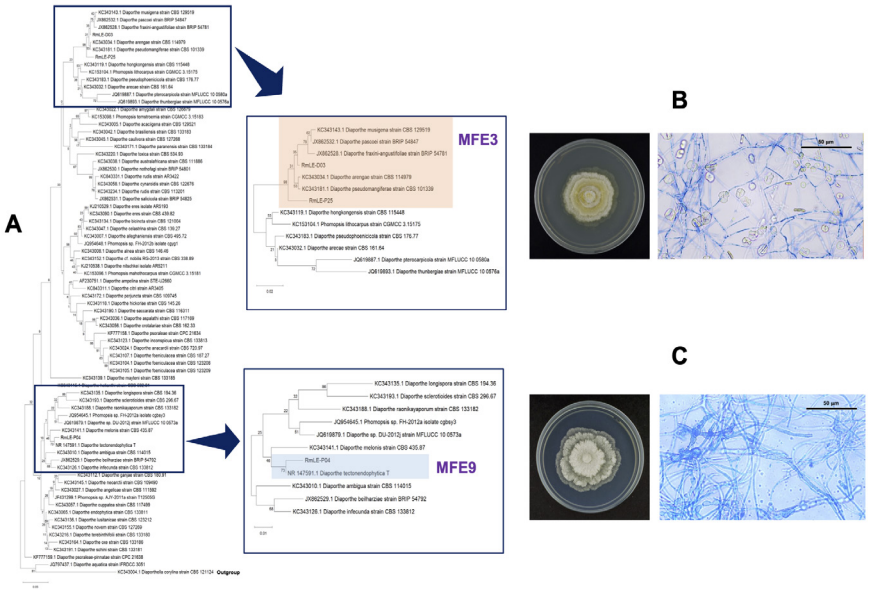

Figure 2. Maximum likelihood tree for *Diaporthe* (A) showing the unresolved species MFE3 and identified as *Diaporthe* sp., and MFE9 as *Diaporthe tectonendophytica*. Photo inserts are the colony and microscopic features of *Diaporthe* sp. RmLE-D03 (B) and *D. tectonendophytica* RmLE-P04 (C).

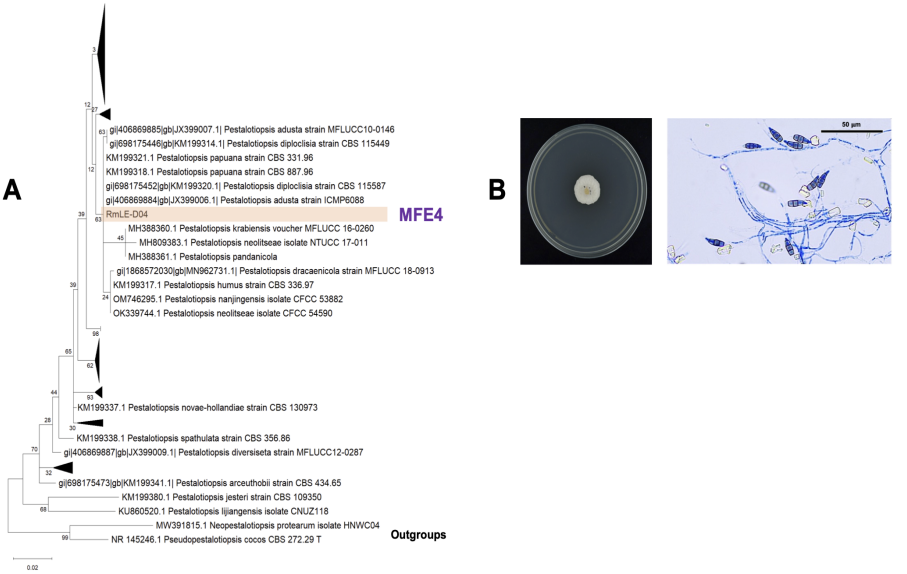

Figure 3. Maximum likelihood tree for *Pestalotiopsis* (A) showing MFE4, here identified only as *Pestalotiopsis* sp. Photo inserts are the colony and microscopic features of *Pestalotiopsis* sp. RmLE-D04 (B).

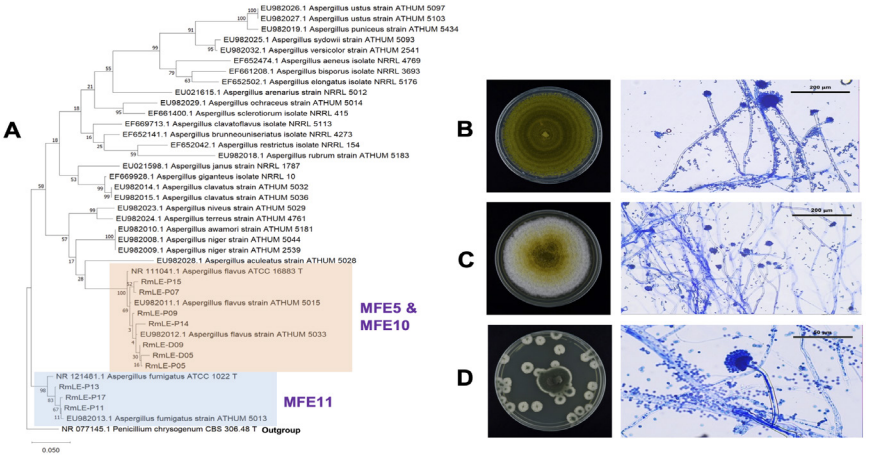

Figure 4. Maximum likelihood tree for *Aspergillus* (A) showing the monophyletic clade of MFE5 and MFE10 both identified as *Aspergillus flavus*, and MFE11 identified as *Aspergillus fumigatus*. Photo inserts are the colony and microscopic features of A. flavus RmLE-D09 (B), A. flavus RmLE-P14 (C), and A. fumigatus RmLE-P11 (D).

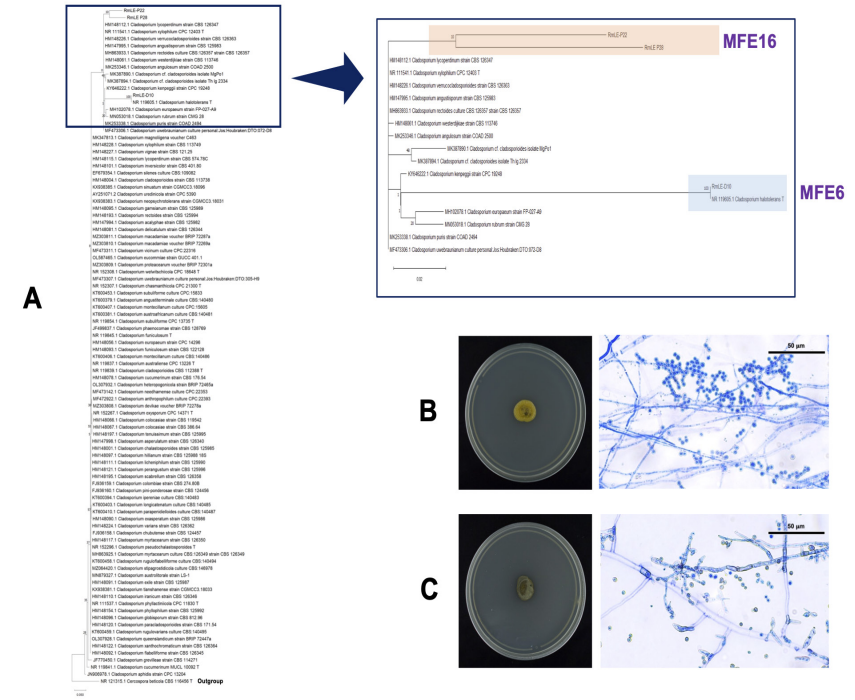

Figure 5. Maximum likelihood tree for *Cladosporium* (A) showing MFE16 identified as *Cladosporium* sp. and MFE6 identified as *Cladosporium halotolerans*. Photo inserts are the colony and microscopic features of *Cladosporium* sp. RmLE-P28 (B) and *C. halotolerans* RmLE-D10 (C).

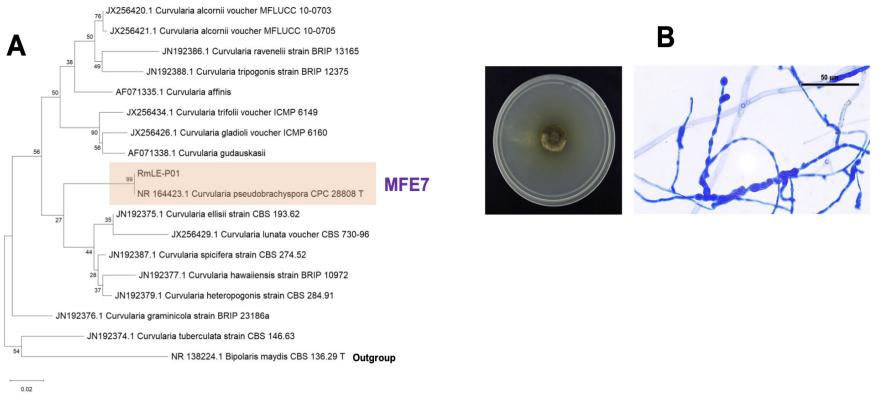

Figure 6. Maximum likelihood tree for *Curvularia* (A) showing MFE7 identified as *Curvularia pseudobrachyspora*. Photo inserts are the colony and microscopic features of *C. pseudobrachyspora* RmLE-P01 (B).

Figure 7. Maximum likelihood tree for *Schizophyllum* (A) showing MFE12 identified as *Schizophyllum commune*. Photo inserts are the colony and microscopic features of *S. commune* RmLE-P12 (B).

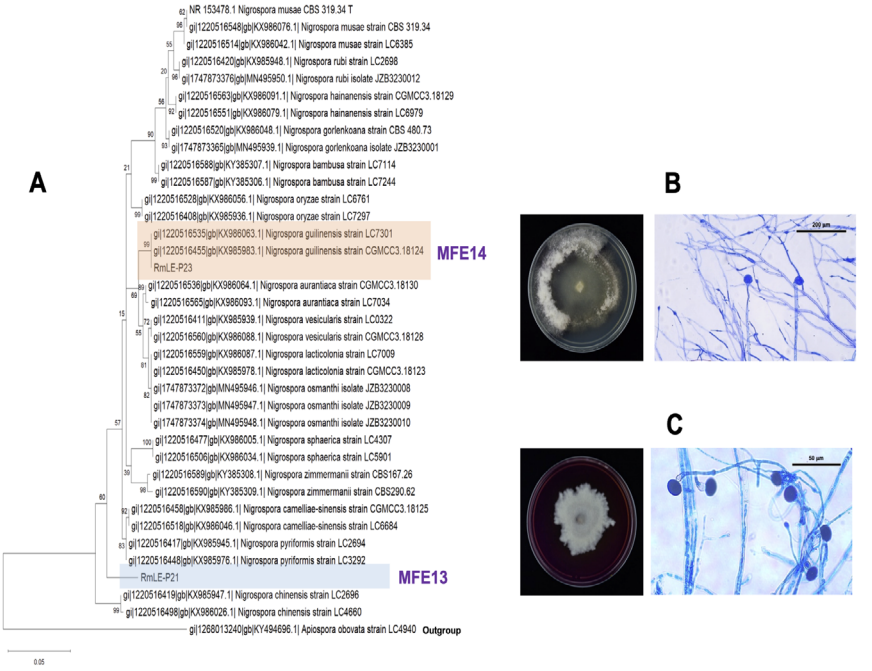

Figure 8. Maximum likelihood tree for *Nigrospora* (A) showing MFE14 identified as *Nigrospora guilinensis* and MFE13 identified only as *Nigrospora* sp. Photo inserts are the colony and microscopic features of *N. guilinensis* RmLE-P23 (B) and *Nigrospora* sp. RmLE-P21 (C).

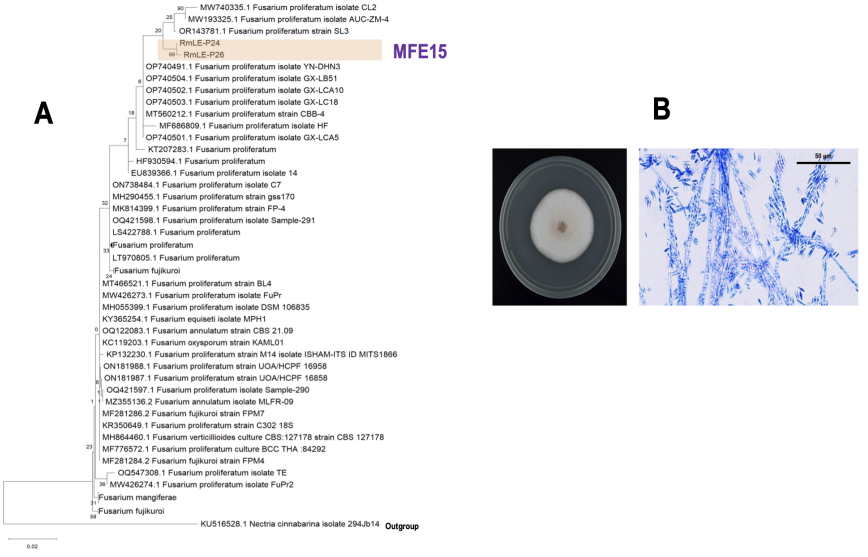

Figure 9. Maximum likelihood tree for *Fusarium* (A) showing MFE15 which grouped with the polyphyletic clade of *Fusarium proliferatum*. Photo inserts are the colony and microscopic features of *Fusarium* sp. RmLE-P26 (B).

Table 1. Key morphological traits of fungal endophytes associated with leaves of the *R. mucronata*. The number of isolates and accession numbers of sequenced MFEs are provided.

M	NI	RI	GBAN	Key Morphological Traits	
				Colonial Morphology	**Spore Morphology**
MFE1	1	RmLE-D01	OR978449	Colonies are fast-growing, in shades of white when grown in potato dextrose agar (PDA) and Czapek Dox agar (CDA). Colonies grow in the shade of deep concentric olivaceous green with white margins as they mature. Colonies mostly consist of a dense felt of conidiophores.	Chains of single-celled conidia are produced in basipetal succession from the branched, single phialide. Conidia are formed in a basocatenate manner with size ranging from 3-5 µm. Branches are biverticilliate. Conidiophores are hyaline, fusiform, olivaceous green to hyaline in color. Sclerotia not observed.
MFE2	8	RmLE-D02 RmLE-D06 RmLE-D07 RmLE-P06	OR978450 OR978454 OR978455 OR978462	Colonies are scattered and rapidly growing, in shades of green to olivaceous, when grown in potato dextrose agar (PDA) and Czapek Dox agar (CDA). The underside colonies show white, and yellow to creamy yellow color. When grown in malt extract agar (MEA), it displays white granular colonies. No exudates were formed.	Conidia are smooth, globular with size ranging from 3 – 5 µm attached in an irregular form of conidiophores with rough-walled stalks. Stems that contain conidia are formed in fascicles with stem size ranging from 150 – 300 µm.
MFE3	2	RmLE-D03 RmLE-P25	OR978451 OR978476	Colonies are fluffy with smooth margin that appears to be white to off-white in color (obverse) while underside colonies are yellowish to white in color. Spores are black to soft brown in color and appears in clusters. Conidial masses are hyaline to creamy or yellowish.	Conidiomata was not observed on our isolates. Alpha-conidia ranges from 7 – 10 µm; aseptate, hyaline, fusoid, smooth-walled and truncate.
MFE4	1	RmLE-D04	OR978452	It exhibits rapid growth on a PDA medium, with a filamentous colony having a smooth to slightly undulating edge and a somewhat raised and fluffy white appearance. Usually, conidiomata arises from mycelia and produces black slimy and shiny cell mass.	The conidia are fusiform to clavate and mostly straight to slightly curved, thin and smooth-walled, made up of four -five segments, with the upper three cells having a darker color and the lower median cell being pale. Conidial size ranges from 5 – 9 µm. At the top of the conidia, there are 2-3 tubular, unbranched appendages, arranged at the center, with or without a knob-like structure.
MFE5	6	RmLE-D05 RmLE-D09 RmLE-P05 RmLE-P15	OR978453 OR978456 OR978461 OR978469	On CDA, the colony is flat, with radial grooves, yellow at first but quickly becoming bright to dark yellow green with age. PDA and MEA colonies share the same features, with fluffy to cottony growth, margin is entire. Underside colonies form leathery crevices and usually yellow to deep yellow in color.	Conidial heads are radiate with sizes ranging from 200 – 300 µm. Heads are biseriate with phialides borne directly on the vesicle. Conidiophore is hyaline and coarse. Conidia are globose to sub-globose with sizes ranging from 3 – 6 µm in diameter, usually pale, olivaceous green, echinulate. No sclerotia observed.
MFE6	1	RmLE-D10	OR978457	Colonies are slow growing, mostly olivaceous-brown to blackish-brown, buff or brown, suede-like to floccose, becoming powdery due to the production of abundant conidia. The reverse is olivaceous-black. Vegetative hyphae are equally pigmented.	Conidiophores exhibit a discernible separation from the vegetative hyphae, presenting as either upright or flexuous structures. They remain unbranched or display branching exclusively in the apical region, featuring a geniculate sympodial elongation. Conidia are generated in acropetal chains with branching, appearing smooth, verrucose, or echinulate, typically consisting of one to four cells and featuring a distinctive dark hilum. The conidia nearest to the conidiophore, especially at the branching points, commonly take on a characteristic "shield-shaped" morphology.
MFE7	1	RmLE-P01	OR978458	Colonies are fast growing, suede-like to downy, brown to blackish brown with a black reverse. Colonies are grey to blackish-brown and suede-like in surface texture.	Conidiophores erect, unbranched, septate, flexuose in the apical part, with flat, dark brown scars. Conidia smooth-walled, olivaceous brown, end cells somewhat paler; conidia obovoidal to broadly clavate, curved at the subterminal cell, sizes ranging from 8 -10 µm.
MFE8	3	RmLE-P02 RmLE-P20	OR978459 OR978471	Colonies exhibit moderately slow growth. Surface texture is soft to floccose. The colonial growth appears radially sulcate. The mature colony has a central greyish to greyish-orange color with a white periphery. The reverse is a pale yellow to a light-yellow brown.	Hyphae is septate, hyaline hyphae. Smooth-walled conidiophores stipes are rather long (100 – 300 µm) and is biverticillate. Metulae are 12 – 15 µm in length which are found in whorls of 3 – 5 divergent structures. Phialides are ampuliform and about 7 – 12 µm in length. Conidia are globose to sub-globose and are smooth or have a finely roughened surface.

Abbreviations: M: Morphospecies; NI: Number of Isolates; RI: Representative Isolates; GBAN: GenBank Accession Numbers

Table 1. Key morphological traits of fungal endophytes associated with leaves of the *R. mucronata*. The number of isolates and accession numbers of sequenced MFEs are provided. ***(cont'd)***

M	NI	RI	GBAN	Key Morphological Traits	
				Colonial Morphology	**Spore Morphology**
MFE9	1	RmLE-P04	OR978460	Colonies on PDA exhibits margins of entire, velvety, initially white, azonate, turning pale brown from the center, reverse similar.	Conidiophores hyaline, branched, cylindrical, arise from swollen pigmented cells. Conidia is hyaline, filiform, curved, cream-yellow in mass, size ranging from 19-30 µm.
MFE10	6	RmLE-P07 RmLE-P09 RmLE-P14	OR978463 OR978464 OR978468	On PDA, colony is fluffy, with radial grooves, yellow at first but quickly becoming bright to deep yellow green with age. PDA and MEA colonies share the same features, with cottony colony growth, margin is entire. Underside colonies form leathery crevices and usually yellow to deep yellow in color.	Conidial heads are radiate with sizes ranging from 150 – 200 µm. Heads are biseriate with phialides borne directly on the vesicle. Conidiophore is hyaline and coarse. Conidia are globose to sub-globose with sizes ranging from 5 – 8 µm in diameter, usually pale, olivaceous green, echinulate. No sclerotia observed.
MFE11	5	RmLE-P11 RmLE-P13 RmLE-P17	OR978465 OR978467 OR978470	On CDA, colonies are blue-green consisting of suede-like surface and a dense felt of conidiophores.	Conidial heads are typically columnar, size varies from 30 – 50 µm and uniseriate. Conidiophore stipes are short, smooth-walled and have conical-shaped terminal vesicles which support a single row of phialides on the upper two thirds of the vesicle. Conidia forms long chains and are globose to subglobose with varying sizes form 2.5 – 3.0 µm in diameter, color is green and finely roughened.
MFE12	1	RmLE-P12	OR978466	Colonies are spreading, woolly, whitish to pale greyish-brown when grown in PDA and MEA. This soon will form macroscopically visible fruiting bodies.	Hyphae are hyaline, wide and have clamp connections. Basidia contains four basidiospores on erect sterigmata. Basidiospores are hyaline, smooth-walled, elongate with lateral scar at lower end, 6-7 µm.
MFE13	1	RmLE-P21	OR978472	Culture on PDA plates grew rapidly and produced white colonies, initially, and then became brown to dark brown due to the abundance of sporulation.	Colonies produced a single-cell conidium with varying sizes from 14 – 16 µm in diameter; each conidium was borne on a hyaline vesicle at the tip of the conidiophore. The conidium shape was ranging from spherical to black subspherical.
MFE14	1	RmLE-P23	OR978474	Culture on PDA plates grew rapidly and produced white colonies, initially, and then became brown to dark brown due to the abundance of sporulation. Growth on MEA and CDA are slow with white, thick mycelia.	Colonies produced single-cell conidium at the attenuate apex of conidiophores which varies in size of 7 – 9 µm in diameter, spherical to oblate.
MFE15	2	RmLE-P24 RmLE-P26	OR978475 OR978477	The colony has a fluffy and floccose texture with visible aerial mycelia in all three-culture media.	The macroconidia are elongated and initially straight but can also have a curved, sickle-like shape. The microconidia are shaped like a club (clavate) or obovoid. There are no chlamydospores present.
MFE16	2	RmLE-P22 RmLE-P28	OR978473 OR978478	The colony transforms in color from a dark, dull green to a deep green shade. It possesses a velvety or cushiony texture and features a pale green margin with a white to clear boundary.	The conidiophore is distinct from the hyphae, showing branching and several enlargements with brown or olivaceous brown color. Conidia form in chains, branching out and have a cylindrical to ellipsoidal shape.
Total	42				

Abbreviations: M: Morphospecies; NI: Number of Isolates; RI: Representative Isolates; GBAN: GenBank Accession Numbers

MFE4 was identified only as *Pestalotiopsis* sp. (Fig. 3) while three morphospecies, i.e., MFE5, MFE10, and MFE11, were identified as within the genus *Aspergillus* (Fig. 4). Both MFE5 and MFE10 were *Aspergillus flavus*. MFE11 was identified as *Aspergillus fumigatus*. *Aspergillus flavus* was also reported as an endophyte of *Ficus elastica* [43], *Aegle marmelos* [44], and *Solanum lycopersicum* [45], while *A. fumigatus* was an endophyte of *Cynodon dactylon* [46], *Juniperus communis* [47], and *Melia azedarach* [48].

MFE6 and MFE16 belong to the genus *Cladosporium* (Fig. 5). However, only MFE6 was resolved as *Cladosporium halotolerans*. Although *C. halotolerans* is commonly isolated from hypersaline environments, it was also isolated as an endophyte of terrestrial plants like *Citrullus colocynthis* [49] and *Vitis labrusca* [50]. On the other hand, MFE7 was identified as *Curvularia pseudobrachyspora* (Fig. 6) while MFE12 was identified as *Schizophyllum commune* (Fig. 7). *C. pseudobrachyspora* commonly occurs as a plant pathogen causing leaf spots in *Cocos nucifera* [51], *Areca catechu* [52], *Cannabis sativa* [53], *Musa acuminata* [54], and bulb rot in *Lilium brownii* var. *viridulum* [55]. *S. commune* was the only basidiomycete isolated in this study. It is a common endophyte of terrestrial plants like *Aloe vera* [56], *Panax ginseng* [57], and *Gastrodia elata* [58].

MFE13 and MFE14 belong to the genus *Nigrospora* (Fig. 8). However, species identification was only possible for MFE14 which was resolved as *Nigrospora guilinensis*. *N. guilinensis* is not a commonly reported endophyte as compared to other *Nigrospora* species. Some studies isolated this species as an endophyte of macroalgae [59] and *Carissa carandas* [60]. Finally, MFE15 was identified under the genus *Fusarium* (Fig. 9). Its species identity was not fully resolved. Although it grouped with *Fusarium proliferatum*, the clade was found to be polyphyletic.

Species identification of fungal endophytes may require other gene markers. Hence, additional markers are recommended to resolve their species identities. For instance, in *Fusarium*, the combination of several markers like rDNA cluster, and the *β-tub*, *EF-1α*, and *lys2* genes can successfully classify *Fusarium* into 7 clades [61]. Apurillo et al. [24] also utilized multiple gene markers for the confirmation of the identities of the mangrove fungal endophytes they isolated from *Sonneratia alba* J. Smith., *Rhizophora mucronata*, *Aegiceras floridum* Roemer & Schultes, and *Avecinnia marina* (Forssk.) Vierh., all collected in Leyte and Samar, Eastern Philippines. In addition to ITS (internal transcribed spacer region) which was also used in this study, the other gene markers included CAL (partial calmodulin gene), HIS (partial histone H3 gene), GAPDH (partial glyceraldehyde-3-phosphate dehydrogenase gene), TEF (partial translation elongation factor 1-alpha gene), TUB (partial beta-tubulin gene), ApMAT (Apn2/MAT locus), and ACT (partial actin gene). As similarly observed in this study, most of the MFE isolates reported in the paper of Apurillo et al. [24] was successfully identified with the single ITS gene marker. However, for other MFE isolates, other gene markers were needed. For example, *Pestalotiopsis* required ITS, TUB, and TEF genes while *Diaporthe* and *Phomopsis* also needed these three genes in addition to the HIS gene marker. Owing to the gene similarities between closely related species of *Colletotrichum*, Apurillo et al. [24] utilized six gene markers: ITS, TUB, CAL, GAPDH, ApMAT, and ACT. We therefore suggest the use of multigene markers to support the identification of mangrove fungal endophytes.

Conclusion

Our study reported 42 MFE grouped into 16 morphospecies that are associated with mature leaves of the mangrove *R. mucronata*. This showed that this single mangrove species already hosts a variety of fungal endophytes. The isolated MFE were identified through a combined polyphasic approach of colonial growth description, spore morphologies, and gene sequence analysis. MFE from *R. mucronata* were reported to occur as fungal endophytes of various terrestrial and other aquatic plants, thereby providing insight into the ubiquity of these microorganisms. We suggest exploring these fungal endophytes for various applications.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization, J.G.B. and T.E.E.D.C.; methodology, J.G.B., J.K.S.J., and T.E.E.D.C.; data collection, J.G.B. and J.K.S.J.; analysis and interpretation of data, J.G.B., J.K.S.J., and T.E.E.D.C.; original draft preparation, J.G.B.; review and editing of the draft, J.G.B., J.K.S.J., and T.E.E.D.C. All authors have read and agreed to the final version of the manuscript.

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