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IDENTIFICATION AND DIVERSITY OF ENDOPHYTIC FUNGI ASSOCIATED WITH THE SEAGRASSES OF CEBU, CENTRAL PHILIPPINES

VENUS B. KINAMOT[1, 2] AND ALVIN P. MONOTILLA[1]

[1] *Department of Biology, University of San Carlos, 6000 Cebu, Philippines*
[2] *Biology Department, Negros Oriental State University, 6217 Dumaguete, Philippines*

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ABSTRACT

Endophytic fungi in seagrasses were poorly documented, especially in the Philippines, despite having large areas of seagrass meadows. Thus, this study was conducted to isolate and identify endophytic fungi associated with common seagrasses: *Enhalus acoroides, Cymodocea serrulata,* and *Thalassia hemprichii* from the Philippines by morphologic and molecular methods. Morphologic identification was carried out based on the fungal colony, somatic and reproductive structures. Seven species were identified in this study: *Aspergillus tamarii, A. ochraceopetaliformis, A. sydowii, Penicillium citrinum, Xylaria* sp., *Beauveria bassiana,* and *Eutypella* sp. *Aspergillus* spp. had white to brown colonies, septate hyphae, biseriate conidiophore, and smooth to rough globose conidia. *Penicillium citrinum* had a green colony, biverticillate conidiophore, and smooth, globose conidia. *Beauveria bassiana* had white to cream colonies with irregular edges and a powdery appearance. The conidiogenous cells had a zigzag rachis in which a chain of conidia emerged. *Eutypella* sp. had a white, filariform, and plumose colony. *Xylaria* sp. had a white colony with conspicuous radial lines and a plumose margin. Phylogenetic analysis using 80 ITS rDNA sequences by neighbor-joining revealed the clustering of our isolates with the closest match taxa in the same clade with 100% bootstrap value. The estimate of evolutionary divergence between our isolates and their closest match taxa by pairwise distance showed no nucleotide base substitution suggesting high sequence identity between sequences. The most diverse endophyte is *Aspergillus*, which is ubiquitously adapted to the marine environment. The potential of these species as valuable sources of bioactive metabolites are deemed for further investigations.

Keywords: diversity, endophytic fungi, Philippines, seagrass

INTRODUCTION

Endophytic fungi are considered important biological components of plants (Supaphon *et al.* 2017). They provide fitness to their host including response to environmental stresses (Bacon & White 2015; Khare *et al.* 2018), recycling of nutrients (Saikkonen *et al.* 2015; Wolfe & Ballhorn 2020), and decreased susceptibility to pests and pathogens (Pal *et al.* 2020).

Endophytic fungi also produce secondary metabolites which have an allelopathic inhibitory effect on other organisms or serve as a communication medium to their host (Alam *et al.* 2021). These metabolites have unique molecular structures in the marine environment due to enormous selective pressure (El-Bondkly *et al.* 2021). Many of these secondary metabolites are highly bioactive which could be potential sources of medicine like *Xylariphilone*, a new azaphilone derivative from seagrass-derived *Xylariales* (Arunpanichlert *et al.* 2016). Azaphilone is a polyketide compound with various proven bioactivities including enzyme inhibitions, anti-microbial, cytotoxic, antioxidant, and anti-inflammatory activities (Chen *et al.* 2020).

In the Philippines, endophytic fungi are reported from terrestrial plants like sweet potato (*Ipomoea batatas*) (Hipol 2012), pandan (*Pandanus amoryllifolius*) (Bungihan *et al.* 2013), pili nut

*Corresponding author, email: vdbbio@yahoo.com

(*Canarium ovatum)* (Torres & dela Cruz 2015; General & Guerrero 2017), fig (*Ficus* sp.) (Solis *et al.* 2016), pines (*Casuarina equisetifolia* and *Pinus kesiya*) (de Mesa *et al.* 2020) and palm *Cycas* sp. (Pecundo *et al.* 2021).

Little is known about marine plants. The estimated total fungal species on earth range from 2.2 to 3.8 million based on host association (Hyde 2022) and even higher with high-throughput sequencing techniques (Wu *et al.* 2019; Hyde 2022). However, fungi from the marine environment represent only < 1% out of 1.5 million known species, even if marine habitats are accounted for 70% of the earth's surface (Gonclaves *et al.* 2022). This indicates that fungal diversity needs to be explored from a lot of marine habitats.

Seagrasses are aquatic angiosperms contributing to primary production in shallow water environments, which also serve as important breeding, nursery, and foraging ground for marine species, recycling nutrients and mitigating the impact of climate change (Fourqurean *et al.* 2012). Due to the immense importance of seagrasses in the aquatic ecosystem and the potential role of endophytic fungi in seagrass ecology and physiology, it is essential to study fungal diversity in seagrasses.

The documentation of endophytic fungi in seagrasses is very limited compared with documentation in terrestrial plants and other marine flora (Fahimipour *et al.* 2017; Hurtado-McCormick *et al.* 2019; Ettinger & Eisen 2020). The lack of documentation is possibly because of the notion that seagrasses have low fungal diversity.

Several fungal species in seagrasses are prolific sources of metabolites, such as *Aspergillus, Penicillium,* and *Xylaria* (Supaphon *et al.* 2013; Notarte *et al.* 2018). Endophytic fungi could also have an important ecological role in seagrasses, like nutrient uptake of minerals, as observed in dark septate endophytes in the roots of *Posidonia oceanica* (Vohnik *et al.* 2017). Thus, endophytic fungi in seagrasses are equally important to be studied as in other marine organisms. Moreover, seagrasses are fragile and acceleratingly losing, especially in Southeast Asia (Fortes *et al.* 2018) which may equate to the loss of its associated mycobiota without being discovered and assayed for potential importance.

The Philippines has the highest seagrass species diversity in Southeast Asia with 19 out of 21 seagrass species. Pacific turtle grass (*Thalassia hemprichii)* and serrated ribbon grass (*Cymodocea serrulata*) are among the most widespread species, whereas eelgrass (*Enhalus acoroides)* is a climax species dominating in the muddy substrate. Philippines also has the largest territorial seas with seagrass meadows (Fortes *et al.* 2018). Cebu, Philippines is among the areas where major seagrass beds are located, yet, there has been no report published on the endophytic fungi associated with seagrasses from Cebu.

Records on fungal endophytes associated with seagrasses were done to only a few species, such as *E. acoroid*es and *Syringodium pyriformis* from Negros Oriental (Notarte *et al.* 2018). Relatively, fewer endophytic fungi were recorded from seagrasses in the Philippines than in other countries, like India (Venkatachalam *et al.* 2015) and Thailand (Sakayaroj *et al.* 2010; Supaphon *et al.* 2017). Hence, there is a need to account for the diversity of mycoflora associated with seagrasses in the Philippines.

This study aimed to isolate and identify the fungal endophytes associated with *E. acoroides, C. serrulat*a, and *T. hemprichii* from Cebu, Philippines by morphological and molecular methods. The findings of this study are expected to enhance the record of fungal diversity associated with the marine flora in the Philippines. The identification of endophytic fungi is preliminary in understanding their roles in seagrass functional ecology and their potential as sources of novel bioactive compounds.

MATERIALS AND METHODS

Site Selection and Sample Collection

Seagrass samples were collected from the coast of Hilutungan Channel, Central Philippines during low tide from October to December 2021. Mactan Island bounds this channel on the eastern side and Olango Island on the western side. On the southern coast of Mactan Island lies Cordova Island (Fig. 1.). These sites are known for having rich marine resources in Central Cebu, Philippines including seagrass beds. It has a vast reef flat dominated by seagrasses *Cymodocea, Thalassia,* and *Enhalus.*

In Olango Island, 8 species of seagrass are listed, including extensive mangrove forests, macrobenthic algae, coral reefs, fishes, and invertebrates.

Fifteen (15) sampling stations were distributed along the coast of the three islands. In each station, one 100-m-transect line with 5 quadrats (30 x 30 cm) was laid perpendicular to the shore. The whole existing seagrass plants classified as *E. acoroides*, *C. serrulata,* and *T. hemprichii* (Fig. 2.) inside the quadrat were collected using a sterilized razor and brought to the USC Marine Station in sealed plastic bags for processing.

Figure 1 Sampling sites of the three seagrass species, *Enhalus acoroides*, *Cymodocea serrulata,* and *Thalassia hemprichii* along Hilutungan Channel, Philippines (indicated in red dots)

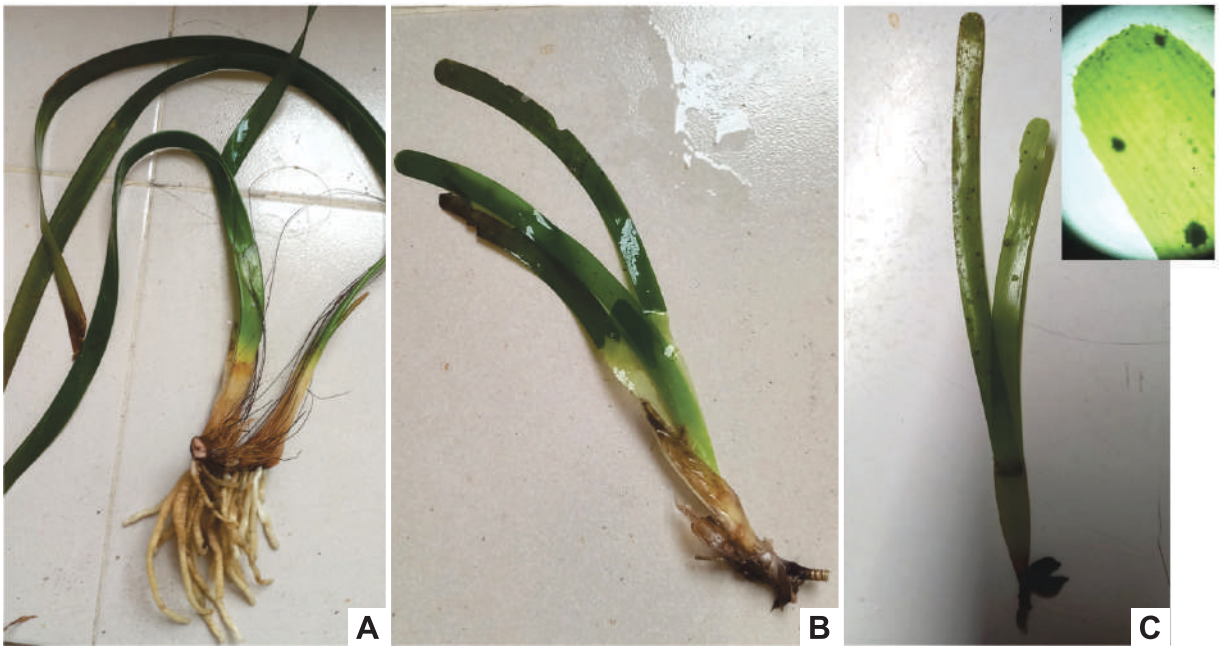

Figure 2 Photograph of (A) *Enhalus acoroides*, (B) *Thalassia hemprichii*, (C) *Cymodocea serrulata*
Note: Inset showing the serrated tip of the leaf of *Cymodocea serrulata*

Isolation of Endophytic Fungi Associated with Seagrass

The samples were rinsed with filtered seawater until the epiphytes were removed. Leaves, rhizomes, and roots were cut to 3 - 5 mm explant using a sterilized scalpel. All explants were surface-sterilized using 10% ethanol (EtOH) for 3 minutes, 3% sodium hypochlorite (NaClO) for 10 seconds; 10% EtOH for 3 minutes, and finally rinsed twice with sterile distilled water and blotted dry with sterile tissue paper (Supaphon *et al.* 2014).

For the effectivity of surface sterilization and sterility check, tissue printing and exposure of uninoculated plates to air were performed, respectively. A total of 2,090 sterile explants from the three tissues of each seagrass species were placed in each culture plate using cornmeal agar/CMA (cornmeal 50 g, agar 15 g, pH 6.0 ± 0.2), malt extract agar/MEA (malt extract 30 g, agar 15 g, mycological peptone 5 g, pH 5.4 ± 0.2 at 25 °C) and potato dextrose agar/PDA (potato peptone 200 g, glucose 20 g, agar 15 g, pH 5.6 ± 0.2) at salinities of 0, 8, 16, 24, and 32 ppt (Ettinger & Eisen 2020).

Filtered and sterilized seawater was used to prepare the different salt concentrations of the culture media. Each culture plate was supplemented with chloramphenicol (150 mg/L) to suppress the growth of bacteria, then incubated at 25 ± 2 °C for at least 14 days. Fungal colonies were then subcultured in PDA and purified by the hyphal tip method.

Morphological Identification of the Fungal Endophytes

Morphological identification was carried out based on macroscopic and microscopic characteristics. The macroscopic characteristics include colony color, form, texture, elevation, and margin. Microscopic characteristics of each purified strain were examined using a light microscope and characterized based on the types of hyphae, the diameter of hyphae, conidia, and conidiophore. Scientific articles were used as references in the morphological identification (Houbraken *et al.* 2010; Chen *et al.* 2013; Samson *et al.* 2014; Visagie *et al.* 2014; Campos *et al.* 2019; Dhar *et al.* 2019; Bich *et al.* 2021; Yee *et al.* 2022). Measurement of the somatic and reproductive structures was done using ImageJ software.

DNA Extraction, Sequencing, and Sequence Alignment

Using a sterile loop, the mycelia of a 1-week pure culture was transferred to a 1.5 µL microcentrifuge with nuclease-free water and

sent to Macrogen (Korea) for DNA extraction, amplification, and sequencing. The genomic DNA of each endophytic fungal isolate was extracted using InstaGene Matrix (Bio-Rad). The DNA fragments were amplified in DNA Engine Tetrad 2 Peltier Thermal Cycler using Internal Transcribed Region (ITS). ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') was used as a forward primer and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as a reverse primer (White *et al.* 1990). The PCR cycle started with an initial denaturation at 95 °C for 5 minutes, 35 cycles of denaturation at 95 °C for 30 seconds, primer annealing at 55 °C for 30 seconds, extension at 72 °C for 1 minute, and final extension at 72 °C. PCR products were purified using a multiscreen filter plate and sequenced using BigDye(R) Terminator v3.1 Cycle Sequencing Kit in an ABI PRISM 3730XL Analyzer following the manufacturer's protocol.

Sequences were edited using BioEdit Sequence Alignment Editor 7.2 and the contig assembly of sequences was undertaken using the cap contig assembly program. The sequences were then analyzed for the most probable closely related taxa using BLAST search. The most similar sequences (99 - 100%) and sequence data from publications were downloaded from GenBank and used for subsequent phylogenetic analysis. To confirm the identity of the fungal isolate, our sequences and reference sequences were aligned using MUSCLE in MegaX software.

Phylogenetic Tree Construction and Analysis

For phylogenetic analysis, a total of 80 ITS rDNA sequences were aligned using MUSCLE in MegaX software. The reference sequences were composed of sequences from the closest match taxa and those isolated from the marine organisms (Supplementary Table 1). The phylogenetic tree was constructed using neighbor-joining based on the Kimura-2 parameter model with 1000 bootstrap replications. The estimate of evolutionary divergence between sequences was determined by the number of base substitutions per site based on pairwise distance.

Fungal Diversity

The isolation rate (IR) was calculated according to Supaphon *et al.* (2014) using the formula:

$$\% \text{ IR} = \frac{\text{Total number of isolates yielded}}{\text{Total number of sample segments}} \text{ x } 100$$

Fungal diversity was calculated using Shannon-Weiner's index (*H*) and Simpson's index (D) according to Supaphon *et al.* (2014). Shannon Weiner's index was calculated using the formula:

$$H = -\sum (P_i) (\ln P_i)$$

where:

P_i = relative abundance of fungal species in seagrasses.

The Simpson's index is calculated using the formula:

$$D = \frac{1-\sum n (n-1)}{N (N-1)}$$

where:

n = number of individuals of a specific species.
N = total number of individuals of all species.

RESULTS AND DISCUSSION

Seventy-seven (77) isolates of endophytic fungi were obtained from 2,090 seagrass explants. Seven species belonging to 5 genera were recorded in this study, i.e., *Aspergillus tamarii, A. ochraceopetaliformis, A. sydowii, Penicillium citrinum, Beauveria bassiana, Eutypella* sp., and *Xylaria* sp. (Fig. 3).

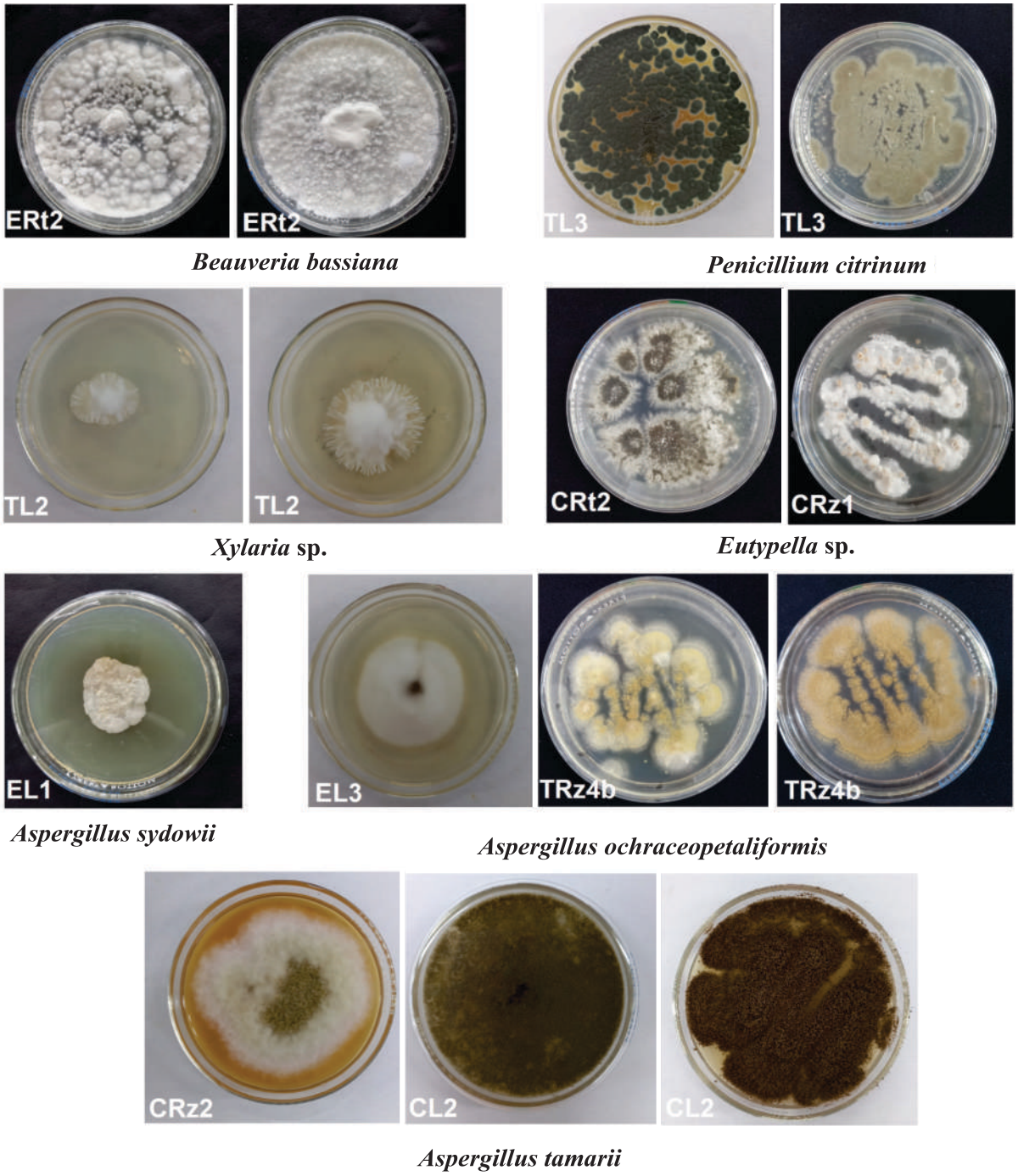

Figure 3 Colony morphology of endophytic fungi from seagrasses within 14 days of incubation at 25 ± 2 °C in PDA, CMA and MEA culture media
Note: Isolate codes are written below the picture.

Aspergillus tamarii had two morphologically distinct isolates coded as CL2 and CRz2. CL2 had a green colony which turned brown when mature (Table 1). The hyphae were septate with a diameter of 2.093 µm. The fungi were highly sporulating in culture. The conidiophore was biseriate measuring 20.714 µm in diameter, the metulae were 3.713 µm and the phialide was 1.759 µm in diameter. The vesicle was globose and measured 11.246 µm in diameter. The stalk was septate and hyaline.

Table 1 Colony characteristics of each species of endophytic fungi from seagrasses

Species	Isolate Code	Macroscopic (Colony) Features					
		Color		Form	Margin	Texture	Elevation
		Surface	Reverse				
Aspergillus tamarii	CL2	Green to Brown	Brown	Circular	Entire	Powdery	Slightly Raised
	CRz2	Green with white edge	White to Light Yellow	Circular	Filiform	Plumose and Cottony	Slightly raised
Aspergillus ochraceopetaliformis	EL3	White	White	Circular	Entire	Cottony	Raised
	TRz4b	Yellow to Brown	Yellow to Brown	Circular	Undulate	Powdery to Cottony	Flat to slightly raised
Aspergillus sydowii	EL1	White	Light Brown	Circular	Entire	Powdery	Flat
Penicillium citrinum	TL3	Ash green	Ash green	Circular with dispersed growth	Entire	Powdery	Flat
Beauveria bassiana	ERt2	White	Cream	Circular with dispersed growth	Irregular	Powdery	Slightly Raised
Eutypella sp.	CRz1	White	Cream	Circular	Entire	Velvety	Flat
	CRt2	White to green	White	Circular	Filiform	Plumose and Cottony	Flat
Xylaria sp.	TL2	White with radial lines	White	Circular with conspicuous radial lines	Filiform	Plumose	Raised, slightly raised and flat

The mean diameter was 2.163 µm. The conidia were green to brownish green in color, sub-globose to globose with a spinous surface, and measured 1.809 µm in diameter (Table 2). CRz2 was sterile mycelia (did not sporulate). The colony of *A. tamarii* was green with a white edge, circular in form, filiriform, and plumose to cottony in texture.

Aspergillus ochraceopetaliformis had also two distinct isolates coded as TRz4b and EL3. TRz4b had a yellowish colony when young and became brown and cottony when mature (Table 1). The hyphae were septate and measured 1.128 µm in diameter. The conidiophore was biseriate with a mean diameter of 12. 466 µm, metulae measuring 1.187 µm, and a whorl of phialide at 0.695 µm. The conidia were globose and smooth with a mean diameter of 0.577 µm (Table 2). EL3 had a white, circular, cottony colony, and septate hyphae. This isolate was also sterile mycelia.

Beauveria bassiana had white to cream colonies with irregular edges and a powdery appearance (Table 1). The conidiogenous cells had a mean diameter of 5.197 µm with a zigzag rachis in which a chain of conidia emerged. The conidia were globose measuring 0.533 µm in diameter. The vegetative hyphae were septate and measured 1.152 µm in diameter (Table 2).

Eutypella sp. had two isolates coded as Crz1 and CRt2, both were sterile mycelia. CRz1 had a white colony on the surface, cream on the reverse side, with a flat, velvety, and circular colony. CRt2 also had a white colony which turned ash green when mature, with filiriform and plumose texture.

Xylaria sp. had a white colony that turns black when mature. The colony was slightly raised and cottony. It had conspicuous radial lines and a plumose margin as well as septate hyphae with a mean diameter of 0.711 µm. The conidia were ovate measuring 2.93 µm (SD = 0.46 µm). Sporulation was very low which was observed after 28 days of culture in malt extract agar.

Culture and conidial characteristics are among taxonomic importance of identification. The conidiophore pattern of *Aspergillus* was described as uniseriate and biseriate (Samson *et al.* 2014). In this study, the conidiophore of *A. tamarii* was biseriate and conidia were sub-globose to globose with rough texture having similar morphology to *A. tamarii* isolated from oregano (*Plectranthus amboinicus*) (Campos *et al.* 2019). In *Penicillium* sp. the conidiophore pattern ranged from having solitary phialide to complex like monoverticillate, biverticillate, terviticillate, and quadriverticillate (Vesagie *et al.* 2014).

Table 2 Features of the somatic and reproductive structures of the 8 species of endophytic fungi after 14-28 days of incubation at 25±2 °C

Species	Isolate Code	Sporulation	Conidiophore		Diameter (µm) of Metulae	Diameter (µm) of Phialide	Vesicle		Vegetative Hyphae		Conidia	
			Type	Diameter (µm)			Shape	Diameter (µm)	Type	Diameter (µm)	Shape	Diameter (µm)
Aspergillus tamarii	CL2	Highly	Biseriate	20.714 (7.14)	3.713 (1.12)	1.759 (0.29)	globose	11.246 (3.13)	Septate	2.093 (0.45)	Sub-globose to globose with spinous surface	1.809 (0.36)
	CRz2	Not-sporulating	*	*	*	*	*	*	Septate	0.72 (0.25)	*	*
A. ochaceopetaliformis	EL3	Not-sporulating	*	*	*	*	*	*	Septate	1.04 (0.36)	*	*
	TRz4b	Highly	Biseriate	12.466 (3.03)	1.187 (0.28)	0.695 (0.27)	globose	4.20 (1.54)	Septate	1.128 (0.37)	globose with smooth surface	0.577 (0.21)
A. sydowii	EL1	Not-sporulating	*	*	*	*	*	*	Septate	1.2 (0.43)	*	*
P. citrinum	TL3	Highly	Biverticillate	3.53 (1.16)	0.820 (0.41)	0.808 (0.06)	Absent	-	Septate	0.767 (0.20)	Globose	0.424 (0.11)
Beauveria bassiana	ERt2	Highly	Dense cluster of conidia with zigzag rachis	5.197 (1.24)	*	*	*	*	Septate	1.152 (0.29)	Globose	0.533 (0.219)
Eutypella sp.	CRz1	Not-sporulating	*	*	*	*	*	*	Septate	3.54 (0.58)	*	*
	CRT2	Not-sporulating	*	*	*	*	*	*	Septate	1.13 (0.43)	*	*
Xylaria sp.	TL2	Very Low	*	*	*	*	*	*	Septate	0.711 (0.28)	Ovate	2.93 (0.46)

Notes: * = Not observed; measurements presented are mean values (standard deviation).

In this study, the conidiophore pattern of *P. citrinum* was biverticillate similar to the fungal isolates reported by Houbraken *et al.* (2010).

White to cream powdery colonies with a zigzag arrangement of conidigenous cells observed in *B. bassiana* are distinct characteristics of *Beauveria* as observed in other studies (Dhar *et al.* 2019; Bich *et al.* 2021). The mean diameter of the conidiogenous cells and conidia of *B. bassiana* was comparable with the *B. bassiana* isolated in the study of Bich *et al.* (2021). With these resemblances in morphological features, our isolates were identified. However, in most instances, isolates from seagrasses did not sporulate in culture despite the use of different culture media. This provides difficulty in identification, hence, the use of molecular taxonomic techniques.

The BLASTn analysis using ITS gene sequences showed that the endophytic fungi associated with seagrasses had 99 - 100% nucleotide similarity to the closest match taxa. Both CL2 and CRz2 had 99.83% nucleotide similarity with *Aspergillus tamarii*. EL3 and TRz4b were 99.83% and 99.67% similar to the nucleotide sequence of *Aspergillus ochraceo-petaliformis,* respectively. CRt2 and CRz1 were > 99% similar to *Eutypella* sp. Four isolates had > 99% nucleotide similarity to *A. sydowii, P. citrinum, Xylaria* sp., and *Beauveria bassiana*, respectively (Table 3).

Based on the phylogenetic analysis of 80 ITS rDNA sequences by neighbor-joining, two major clades were generated, i.e., Eurotiomycete and Sordariomycete (Fig. 4). Ascomycota is reportedly the most common fungal endophyte in which Eurotiomycetes and Sordariomycetes are among the main classes of fungi associated with seagrasses (Raja *et al.* 2016b; Wainwright *et al.* 2018). The same finding was reflected in our study, in which Eurotiomycete had 4 subclades composed of *Aspergillus tamarii, A. ochraceo-petaliformis*, *A. sydowii,* and *Penicillium citrinum.*

CRz2 and CL2 were clustered with the clade of *A. tamarii* having 100% bootstrap value. The estimate of evolutionary divergence between these sequences was 0.0 based on p-distance, suggesting that these two isolates were both *A. tamarii*. These isolates shared the same subclade with *A. tamarii* from the endophytes of terrestrial plants (Accession Numbers KP784375 & JX110981).

Table 3 Maximum nucleotide match identity for the endophytic fungal isolates by BLASTn search in GenBank

Isolate code	GenBank accession ID	Match identity (%)	Identification	GenBank accession of the closest match species
CL2	ON507744	99.83	*Aspergillus tamarii*	MK638758
CRz2	ON627706	99.83	*Aspergillus tamarii*	MK638758
EL3	ON627707	99.83	*Aspergillus ochraceopetaliformis*	MH857406
TRz4b	ON507753	99.67	*Aspergillus ochraceopetaliformis*	MH857406
EL1	ON507753	100.00	*Aspergillus sydowii*	MH233983
TL3	ON507748	99.64	*Penicillium citrinum*	MH858073
ERt2	ON507746	100.00	*Beauveria bassiana*	MT530083
CRz1	ON627708	99.32	*Eutypella* sp.	MK775825
CRt2	ON627708	99.84	*Eutypella* sp.	MK775825
TL2	ON644448	99.48	*Xylaria* sp.	DQ480355

Note: The sequences of these fungal isolates were deposited in the GenBank database.

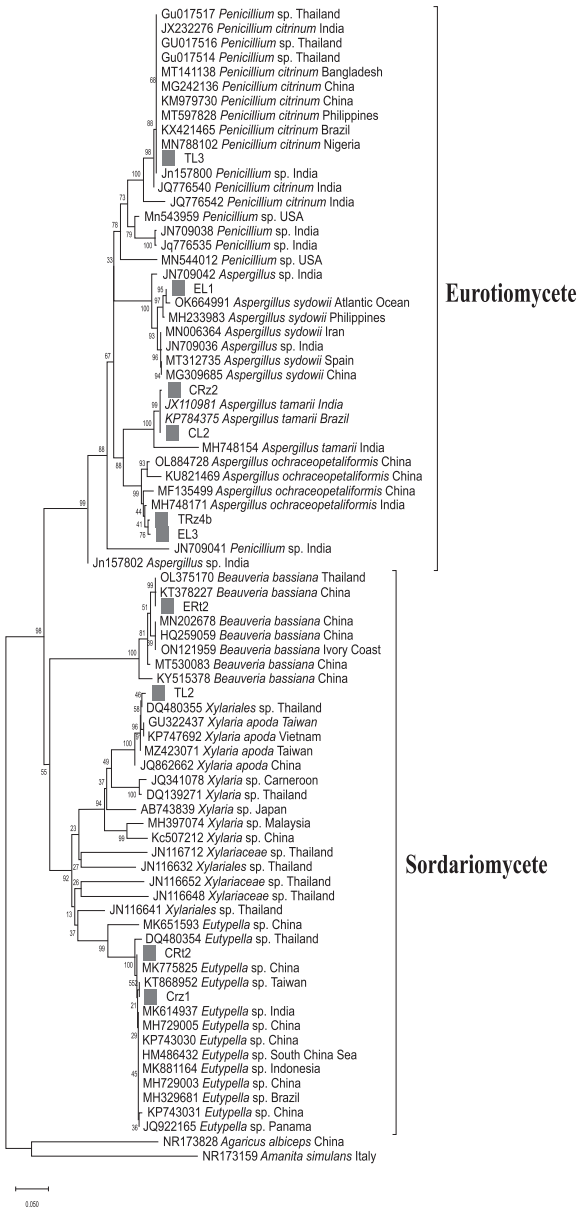

Figure 4 Phylogenetic tree of ITS sequences inferred by neighbor-joining
Notes: The phylogenetic tree was rooted with the *Agaricus albiceps* and *Amanita simulans* as outgroups. The isolates from the current study were designated in red boxes.

Table 4 Isolation rate and diversity index of the endophytic fungi in seagrasses

Species	Number of isolates	%	Isolation rate	Shannon-Wiener Index (H')	Simpson Diversity Index (D)
Aspergillus tamarii	16	20.78	0.77	-0.33	0.04
Aspergillus ochraceopetaliformis	19	24.68	0.91	-0.35	0.06
Aspergillus sydowii	5	6.49	0.24	-0.18	0.00
Beauveria bassiana	6	7.79	0.29	-0.20	0.01
Eutypella sp.	8	10.39	0.38	-0.24	0.01
Penicillium citrinum	17	22.08	0.81	-0.33	0.05
Xylaria sp.	6	7.79	0.29	-0.20	0.01
Total	77			**1.82**	**0.83**

TRz4b and EL3 had high affinity to *Aspergillus ochraceopetaliformis* having a bootstrap value of 99%. The estimate of evolutionary divergence between TRz4b and EL3 supported that these two isolates were both *A. ochraceopetaliformis*. These isolates formed a sister subclade with *A. ochraceopetaliformis* from a red alga, *Acanthophora spicifera* (Acc. No. MH748171). The subclade of *A. sydowii* had 100% bootstrap value. *A. sydowii* in this study, which was coded as EL1, formed a subclade with *A. sydowii* isolated from an amphipod of the Atlantic Ocean (Accession Number OK664991). This subclade branched with *A. sydowii* isolated from *Perna viridis* of the Philippines (Acc. No. MH233983) at 97% bootstrap support. Isolation and high nucleotide affinity of these fungal species isolated from marine organisms indicate their wide distribution and adaptation in the marine environment (Notarte *et al.* 2018).

Penicillium citrinum in this study coded with TL3 formed a well-supported subclade with *P. citrinum* isolated from *Enhalus acoroides* Acc. No. (JQ776540), *Cymodocea rotundata* (Acc. No. JQ776542), red alga, *Laurencia okamurai* (Acc. No. MG242136) and mangrove, *Avicenia alba* (Acc. No. MT141138). Interestingly, *Penicillium* sp. from the *E. acoroides* of Thailand (Acc. No. GU17514, GU17516, GU17517) and India (Acc. No. JN157800) was within the subclade of *P. citrinum*. This observation supported the report of Sakayaroj *et al.* (2010) that 9 fungal assemblages from *E. acoroides* identified as *Penicillium* spp. had 100% sequence similarity with *P. citrinum*.

However, *Penicillium* sp. from *Zostera marina* formed a separate clade from *P. citrinum* suggesting a subtle variation in the fungal species composition between seagrass species. This could be due to host identity and ecological processes' influence on the endophytic fungal assemblage as observed in *Conocycea*, *Urostigma Ficus* (Liu *et al.* 2019).

Different host plant species release different compounds which can alter the colonization of endophytes (Chen *et al.* 2020). For instance, the phytochemical analysis of the crude extracts of *C. serrulata, Thalassia hemprichii,* and *E. acoroides* showed that the type of phenol and phenylpropanoid derivatives in these three seagrass species vary. *E. acoroides* had coumaric acid but *Cymodocea* spp. and *Thalassia hemprichii* had none instead they had caffeic acid. Both *E. acoroides* and *T. hemprichii* had protocatechic acid, while *Cymodocea* spp. had vanillic acid.

Furthermore, the flavonoids of *E. acoroides* and *T. hemprichii* were sulfated (Subhashini *et al.* 2013). The dominant group of phenolic compounds in *Zostera marina* were mono and disulfated flavonoids and phenylpropanoic acids, particularly rosmarinic acid (Li *et al.* 2022).

Sordariomycete was divided into subclades of *Beauveria bassiana, Xylaria* sp., and *Eutypella* sp. Isolate ERt2 clustered in a well-supported subclade with *B. bassiana* at 100% bootstrap value. TL2 had a high affinity to *Xylaria apoda* including *Xylariales* at 100% bootstrap value. Within the subclade were *X. apoda* (JQ862662) from *Dendrobium* spp. (Acc. No. JQ862662) and red alga, *Pterocladiella capillacea* (Acc. No. MZ423071).

Our isolate and *Xylariales* species had p-distance = 0.0, suggesting no nucleotide base substitution per site. However, the p-distance between our isolates and *X. apoda* was 0.01, suggesting the presence of evolutionary divergence between these sequences. Thus, our isolate was identified as *Xylaria* sp. The

identification of CRt2 and CRz1 as *Eutypella* sp. had high bootstrap support and the p-distance was 0.0, suggesting that these two isolates were both *Eutypella* sp. CRt2 and CRz1 formed a well-supported subclade with *Eutypella* sp. from the endophytes of *Garcinia* sp. (Acc. No. DQ480354, DQ480355), *Nelumbo nucifera* (Acc. No. KT868952) and *Camellia sinensis* (Acc. No. KP743031, KP743030).

Diatrypaceous fungi like *Xylaria* sp. and *Eutypella* sp. are common wood saprophytes. *Xylaria* sp. was a significant decomposer of organic compounds in tropical woods by prolifically producing lignocellulosic enzymes (Osono 2020). However, species of *Xylaria* sp. were also isolated as endophytes of *Dendrobium* spp. (Chen *et al.* 2013), lichens (Santiago *et al.* 2021), mangroves (Hamzah *et al.* 2018), and seagrasses (Supaphon *et al.* 2014). This supported the finding of our study that *Xylaria* sp. occurred as endophyte.

On the other hand, *Eutypella* sp. was isolated from the dead branches of *Vitis vinifera, Ficus carica,* and *Citrus paradisi* (Trouillas *et al.* 2011). But, in this study, *Eutypella* sp. was isolated from seagrass as an endophyte of the marine environment. This indicated an occurrence of several life strategies in endophytic fungi, in which fungi can shift their lifestyle and ecological role from being an endophyte to saprophyte and vice-versa.

For instance, *Peroneutypa scoparia*, a closely related species of *Eutypella* sp. was found to colonize the tissues of *Broussonetia papyrifera* as endophytes. But when the organ fell off due to senescence, they became saprotrophs utilizing the available resources (de Errasti *et al.* 2014). *Eutypella* sp. could have a similar lifestyle to *Peroneutypa scoparia* but it deserves further investigation. Endophyte-saprophyte lifestyle shift in these species suggests their possible role in decomposition and nutrient cycling in seagrass beds, which can be elucidated in future studies.

The diversity of endophytic fungi in Philippine seagrasses based on Shannon-Weiner and Simpson's diversity indices were H = 1.82 and D = 0.83, respectively (Table 4). Compared to other marine substrates, e.g., mangroves, the diversity of endophytic fungi in seagrasses is low because of a combination of physical, biological, and chemical factors.

High salinity and low oxygen in the submerged condition of seagrasses reduce the diversity of endophytic fungi (Sakayaroj *et al.* 2010). Biological traits of the host species influence the colonization and composition of endophytic fungi. For instance, a wide leaf area in *Cymodocea serrulata* is assumed to influence a higher colonization rate of endophytic assemblages compared with *Halodule pinifolia* and *Halophila ovalis* (Raja *et al.* 2016a).

A similar observation was reported in *Thalassia testudinum* compared with *Halodule wrightii* (Mata & Cebrian 2013). The presence of thick-walled hypodermis and root hairs in *Posidonia oceanica* was believed to explain the colonization of dark septate endophytes and not in the neighboring seagrass, *Cymodocea nodosa* (Vohnik *et al.* 2017).

The antifungal compounds produced by seagrasses for defense also reduce the diversity of endophytic fungi. For instance, crude extracts of *Thalassia testudinum, Halodule wrightii*, and *Syringodium filiforme* prevent the growth of a saprophyte, *Dendryphiella salina,* and fungal pathogens, *Lindra thalassiae* and *Fusarium* spp. (Ross *et al.* 2008). Further, the amount of anti-fungal compounds produced varies with the species of seagrass, age, examined tissues, and environmental conditions. For instance, phytochemical analysis of crude extract of *C. serrulata, Enhalus acoroides,* and *Thalassia hemprichii* showed that *C. serrulata* contained the highest amount of phenol, tannin, and flavonoids. *E. acoroides* had the lowest (Kannan *et al.* 2013).

Defensive needs from epiphytes in the leaves resulted in high phenolic concentration than in the rhizomes and roots. Metal contamination, pollution in the environment, and interactions with herbivores also increase the amount of phenolics production (Baby *et al.* 2017). The variation in the concentration of the phenolic compounds due to both biotic and abiotic factors is another factor that determines the endophyte species composition.

Aspergillus spp. was the dominant group in the endophytic community in which the most diverse was *A. ochaceopetaliformis* (IR = 0.91). *Penicillium citrinum* was the second most diverse endophyte (IR = 0.81) (Table 4). *Aspergillus* and *Penicillium* are ubiquitous in the marine environment in which these genera are

commonly isolated from several marine organisms (Cha *et al.* 2021).

A. ochraceopetaliformis was isolated from *Gracilaria crassa, Caulerpa scafeliformis,* and *Acanthophora specifera*. *Aspergillus tamarii* was isolated *from G. crassa, Chaetomorpha antenina, Padina tetrastomatica* and *Acanthophora specifera* (Sahoo *et al.* 2021). The halotolerant and halophilic characteristic of *Aspergillus* may explain their close association with marine organisms (Cha *et al.* 2021).

Likewise, several authors reported the isolation of *Penicillium* spp. in seagrasses (Devarajan *et al.* 2002; Sakayaroj *et al.* 2010; Supaphon *et al.* 2014; 2017; Venkatachalam *et al.* 2015; Kirichuck & Pivkin 2015; Raja *et al.* 2016b; Ettinger & Eisen 2020). *P. citrinum* was isolated from *Zostera marina*, *Enhalus acoroides*, and *Cymodocea rotundata* as well as mangroves like *Bruguiera sexangular* var. *rhynchopetala* (He *et al.* 2017) and *Avicennia alba* (Rahaman *et al.* 2020).

Wide host range and repeated isolation of *Aspergillus* and *Penicillium* species from marine hosts classified them as marine fungi (Jones *et al.* 2015). The role of these endophytes in seagrasses is not yet well-elucidated, but both *Aspergillus* and *Penicillium* produce secondary metabolites which may be used to protect their hosts. It was also suggested that *Penicillium* may have a role in carbon cycling as observed in macroalgae (Cha *et al.* 2021).

The 7 endophytic fungal species identified in this study were the first report from seagrasses of the Philippines. *B. bassiana* was previously reported from *Tedania* sp., a mangrove-associated sponge in Panay Island, Philippines (Calabon *et al.* 2019) but not in seagrass. Although this species was reported in *Posidonia oceanica* and *Z. marina,* these were collected from the Mediterranean (Panno *et al.* 2013) and Japan, respectively (Kirichuck & Pivkin 2015).

Eutypella sp. was never reported in seagrasses. The previous reports on *Eutypella* sp. were on the terrestrial plants, *Garcinia* species (Phongpaichit *et al.* 2006), lotus, *Nelumbo nucifera* (Chen & Kirschner 2018), *Camellia sinensis* (Wang *et al.* 2016) and *Centella asiatica* (Radiastuli *et al.* 2019). Likewise, species of *Xylaria* sp. was not reported from the Philippine seagrasses, though reported in mangroves (Apurillo *et al.* 2019), lichen (Santiago *et al.* 2021) and terrestrial plants (de Mesa *et al.* 2020).

Aspergillus and *Penicillium* were previously reported in *E. acoroides* and *Syringodium isoetifolium* from Negros Oriental, Philippines by Notarte *et al.* (2018) but the species identified in this study were not mentioned. Therefore, this study presents new updates on the list of endophytic fungi present in Philippine seagrass.

CONCLUSION

Morphological and molecular methods identified 5 genera with 7 species of endophytic fungi in Philippine seagrasses: *Aspergillus* (3 species), *Penicillium* (1 species), *Xylaria* (1 species), *Beauveria* (1 species), *Eutypella* (1 species). *Aspergillus spp.* were the most diverse. These species deserve further investigation as promising source of bioactive metabolites and novel natural products.

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