Molecular Identification of Endophytic Fungi Associated with *Cynometra ramiflora* L. and *Wrightia pubescens* (R. Br.) Using the Internal Transcribed Spacer (ITS) Region of rDNA and Its Morphotypes

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Abstract

In this study, endophytic fungi from Philippine medicinal plants namely, *Cynometra ramiflora* and *Wrightia pubescens* were isolated and identified. The endophytic fungi were isolated using potato dextrose agar, and were identified by amplifying a fragment of their internal transcribed spacer (ITS) regions of rDNA using polymerase chain reaction. The identified endophytic fungi were *Colletotrichum karsti, Diaporthe longicolla, Phomopsis columnaris, D. pseudomangiferae, C. acutatum,* and *D. inconspicua.* All of the isolates belonged to phylum Ascomycota. Two isolates (*C. karsti* and *C. acutatum*) belonged to Glomerellaceae family and four isolates (*D. longicolla, P. columnaris, D. pseudomangiferae,* and *D. inconspicua*) were under the Diaporthaceae family. In addition, based on cultural morphology, the isolated endophytic fungi were described as irregular and circular morphotypes. This is the first report on the isolation of endophytic fungi associated with healthy leaves of *C. ramiflora* and *W. pubescens.*

Keywords: Cynometra ramiflora; *Wrightia pubescens*; endophytic fungi; Diaporthaceae; Glomerellaceae
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1. Introduction

Endophytic fungi are types of microorganisms inhabiting the inner tissues of plants. It has been found that a variable relationship exists between endophytes and their host plants, ranging from either mutualism or symbiosis to antagonism or slightly pathogenic [1]. Although some endophytes

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can be termed pathogens, most are in an inactive state within the host tissue. Some saprobes can also be facultative parasites. Moreover, endophytic microorganisms tend to become pathogenic when the host plant is in a stressed condition [2].

Generally, most endophytic fungi are found to be beneficial to their host plants and produce or initiate the development of important bioactive compounds that have been used in many applications [3-4]. Interestingly, the bioactive compounds of endophytes not only play an important role in ecology or environment, but also have a positive impact in the field of medicine [5].

Recently, there has been extensive research on endophytic fungi from different plants including trees, vegetables, fruits, and crops. However, less research has been conducted on the leaves of tropical plants and specifically on medicinal plants. *Cynometra ramiflora*, also known as Katong laut in Malaysia and known as Balitbitan in the Philippines, is a small erect tree known to have anticancer, antioxidant, and antiviral activities [6]. On the other hand, *Wrightia pubescens*, known as Lanete in the Philippines, is a medicinal plant that was found to have anti-inflammatory and anticancer activities [7, 8].

Among the DNA barcoding markers available, the internal transcribed spacer (ITS) regions of rDNA has been found to have the highest probability of successful identification of the broadest range of fungi [9]. rDNA-ITS was also found to have high resolution in some taxonomic groups and has been used as the standard DNA barcode for fungi and other biological groups such as algae, protist, and animals [10]. Nowadays, the ITS regions are the most accepted DNA barcode for fungi [11] and provide important information on fungal diversity [12] and uniqueness because they have the highest resolving power to discriminate closely related species [9]. Aside from morphotype determination of the endophytic fungi using the cultural characteristics, the identification of the specific endophytic fungi associated with healthy leaves of *C. ramiflora* and *W. pubescens* was also performed using the ITS region in this study.

2. Materials and Methods

2.1 Collection of samples

Healthy leaves of *C. ramiflora* and *W. pubescens* were collected in the Science City of Muñoz, Nueva Ecija, Philippines. Ten to fifteen pieces of leaves were randomly picked around the tree and placed in a clean polyethylene bag, which was immediately transported to the laboratory.

In order to remove impurities and eliminate epiphytic microorganisms, the leaves were subjected to a surface-sterilization procedure. Each plant tissue was cut with a sterile blade into 1-cm segments. Each part of the samples was thoroughly washed under running tap water, after which, the surface was sterilized by submerging the part in 75% ethanol for 2 min, 5.3% sodium hypochlorite for 5 min, and 75% ethanol for 30 sec, and finally, rinsed with sterile distilled water for 1 min. Thereafter, the samples were dried on sterile filter paper.

2.2 Media preparation and sterilization

Potato Dextrose Agar (PDA) media were prepared by dissolving 9.75 grams of PDA powder (HIMEDIA) in a 250 ml of distilled water. After sterilization, the bottled media were allowed to cool. Twenty-five ml of prepared medium was aseptically dispensed into newly sterilized dried petri dishes. They were allowed to stand up open for a few min to allow the moisture to evaporate in order to avoid contamination. These petri dishes were observed for 24 h to ensure that these were free from contaminants before inoculation.

2.3 Isolation of endophytic fungi

The freshly picked plant samples were placed aseptically into the PDA medium, with 100µl of streptomycin sulfate (30 mg/l) to inhibit the growth of bacteria, using a clean and sterile blade. The plates were incubated and monitored at room temperature approximately 20 to 25°C for 5-7 days. The isolated fungal endophyte was revived and was aseptically transferred onto new PDA plates. The cultures were incubated 27°C for 3-5 days to allow mycelia proliferation.

Once the mycelial growth from the revived culture had proliferated, a small portion of the colony was aseptically transferred using the inoculation needle onto the sterilized PDA slants in test tubes for the stock culture. The mycelia from the stock culture were inoculated using the small block of agar containing mycelial structures on sterilized PDA plates and incubated at 27°C until profuse growth was observed for morphological characterization. These cultures were incubated and stored as pure cultures readily available for molecular identification.

2.4 Genomic DNA extraction, gel electrophoresis and PCR amplification

The mycelia of endophytic fungi were scraped off using a sterile inoculating needle and were transferred immediately to 1.5 ml tube that contained 500µl cetyltrimethylammonium bromide (CTAB). The samples were ground using pipette tip. The samples were then mixed using a vortex every 5 min. Samples were then incubated at room temperature and 500µl of chloroform isoamyl-alcohol (24:1 ratio) was added and thoroughly mixed using vortex. Then, it was spun using a centrifuge at 10,000 rpm for 30 min.

The supernatant (upper phase) of each sample was transferred to a new tube (2 ml) and immediately added with the 500µl of cold isopropanol and incubated at -20ºC overnight. After incubation, the samples were spun on a centrifuge at 10,000 rpm for 10 min. The isopropanol (supernatant or the upper phase) was decanted and the pellet was washed with 500µl of 70% ethanol and it was spun on a centrifuge at 12,000 rpm for 3 min and the ethanol was removed. The addition of ethanol and spinning were repeated twice. The pellet was drained dry by inverting the tube on a paper towel to get rid of excess liquid. The pellet was dissolved in 100µl of 1× TE buffer and was incubated at room temperature for 3-4 h until pellet was dissolved completely.

The DNA quality was checked by mixing 1µl of the DNA samples with loading dye and dispensed on a well with 1% agarose gel containing 1µl of Biotium GelRed™ Nucleic Acid Gel Stain. The DNA was run for 30 min in the electrophoresis tank (ENDURO™ Horizontal Gel Electrophoresis System) with 100V along with the standard ladder. After electrophoresis, it was viewed in the gel documentation system (ENDURO™ Gel Documentation System) for imaging. The genomic DNA was diluted 1:100 µl using sterilized distilled water before rDNA-ITS amplification.

To identify the isolated fungal endophytes from the leaves of *C. ramiflora and W. pubescens*, total DNAs were run in a Polymerase Chain Reaction (PCR) machine (Applied Biosystems®, 2720 Thermal Cycler) with the primers, ITS 1F (5'-CTTGGTCATTTAGAGGAA GTAA-3') [13], and ITS 4R (5'-TCCTCCGCTTATTGATATGC-3') [14]. A volume of 1 µl of diluted DNA was mixed with PCR components (volume depends on brand's instruction). The PCR was performed fitted with a heated lid using the following PCR profile: for initial denaturation, 95°C for 1 min followed by 35 cycles of 95°C for 15 s, 50.1°C for 15 s, and 72°C for 15 s, with final extension step of 72°C for 5 min. The amplified products were checked again using gel electrophoresis system using 3µl run for 30 min in the electrophoresis tank with 100V along with the standard ladder using gel doc system and were sent to 1st BASE Laboratory at Malaysia for purification of the PCR product

and sequencing procedure. The sequences were used for BLAST (Basic Local Alignment Search Tool) [15] analysis and the related gene sequences available from NCBI (National Center for Biotechnology Information) were used for identification and phylogenetic analysis.

2.5 Phylogenetic analysis

The rDNA-ITS sequences were aligned using Clustal W provided in the default parameter of MEGA X [16]. Phylogenetic tree was constructed by applying the Neighbor-Joining (NJ) and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model [17, 18].

3. Results and Discussion

3.1 Morphotypes of endophytic isolates from *W. pubescens* and *C. ramiflora*

There were six endophytic fungi that were isolated in this study (Figure 1). One endophytic fungus (Wp1) was isolated from *W. pubescens*, while there were five endophytic fungal isolates (Cr1, Cr2, Cr3, Cr4 and Cr6) from *C. ramiflora* . The isolated endophytic fungi were separated in three morphotypes based on their cultural characteristics as shown in Table 1.

The colony of Wp1 mycelia appeared as a white, circular form and has an entire margin. The conidia appeared hyaline, cylindrical, and aseptate as also described by Tahere *et al.* [19]. On the other hand, the isolate Cr1 appeared as a white, irregular form and undulate margin. The conidiogenous cells were hyaline and unbranched as also described by Yang *et al.* [20]. The colony of isolate Cr2 was also observed to be white, irregular form and undulate margin. The conidiogenous cells appeared hyaline with multicellular columns as reported by Farr *et al.* [21]. The colony of isolate Cr3, on the other hand, had white mycelia in a circular form and entire margin. The hyphae appeared septate hyaline and branched; currently, there has been no available concrete detailed description of

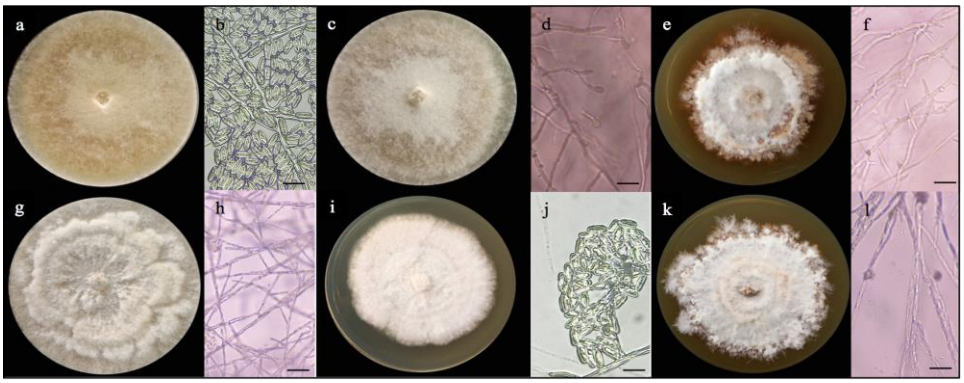

Figure 1. Morphological characteristics of the six isolates on PDA, **(**a**)** colony of *Colletotrichum karsti* (b) conidia of *C. karsti* (c) colony of *Diaporthe longicolla* (d) conidiogenous cells of *D. longicolla* (e) colony of *Phomopsis columnaris* (f) conidiogenous cells of *P. columnaris* (g) colony of *D. pseudomangiferae* (h) hyphae of *D. pseudomangiferae* (i) colony of *C. acutatum* (j) conidia of *C. acutatum* (k) colony of *D. inconspicua* (l) conidiogenous cells of *D. inconspicua* Scale bar = 1µm

Table 1. Morphotypes and cultural characteristics of the isolates on PDA

Morphotypes	Isolates	Host	Size (cm) of colony; 14 days	Shape	Color	Edge
1	Wp1	*W. pubescens*	7.6	Circular	White	Entire
	Cr3	*C. ramiflora*	>A	Circular	White	Entire
2	Cr1	*C. ramiflora*	7.2	Irregular	White	Undulate
	Cr2	*C. ramiflora*	6.0	Irregular	White	Undulate
	Cr4	*C. ramiflora*	7.0	Irregular	White	Undulate
3	Cr6	*C. ramiflora*	6.4	Irregular	Light brown to white	Filiform

Note: >A Completely covering plate

sexual morphology of this isolate so far. The colony of isolate Cr4 had white mycelia, irregular form, and undulate margin. The conidia appeared hyaline, ellipsoid, and fusiform [22]. The mycelia of the isolate Cr6, had light brown to white mycelia, irregular form, and filiform margin. The conidiogenous cells were phialidic and hyaline [23] (Figure 1 and Table 1).

3.2 Molecular identity of the endophytic fungi based on ITS gene sequence

The identity of the endophytic fungi was resolved using the rDNA-ITS region (Table 2). The isolate Wp1 was identified as *C. karsti* (KX578794.1) with 99.67% identity. The isolate Cr1 was identified as *D. longicolla* (LN552212.1) with 97.95% identity. The isolate Cr2 had 100% identity to *Phomopsis columnaris* (KU204512.1), while the isolate Cr3 was identified as *D. pseudomangiferae* (MG576128.1) with 97.72% identity. The isolate Cr5 was identified as *C. acutatum* (LC194224.1) with 99.66% identity, and lastly, the isolate Cr6 was identified as *D. inconspicua* (MF495476.1) with 98.07% identity. Similarly, the phylogenetic study based on the rDNA-ITS region revealed that the isolates belonged to three genera as delineated in the constructed phylogeny tree. Wp1, and Cr4 were from *Colletotrichum*, while Cr1, Cr3, and Cr6 belonged to *Diaporthe*, and Cr2 was in the genus *Phomopsis* (Figure 2). The molecular identification of the isolates was confirmed using their morphological description, also taking into consideration the mycelia, conidia, or their conidiogenous cells or hyphae.

Table 2. Identities of the endophytic fungi based on the ITS region

Isolates	Scientific name	%Identity	Accession number from NCBI GenBank
Wp1	*Colletotrichum karsti*	99.67%	KX578794.1
Cr1	*Diaporthe longicolla*	97.95%	LN552212.1
Cr2	*Phomopsis columnaris*	100.00%	KU204512.1
Cr3	*Diaporthe pseudomangiferae*	97.72%	MG576128.1
Cr4	*Colletotrichum acutatum*	99.66%	LC194224.1
Cr6	*Diaporthe inconspicua*	98.07%	MF495476.1

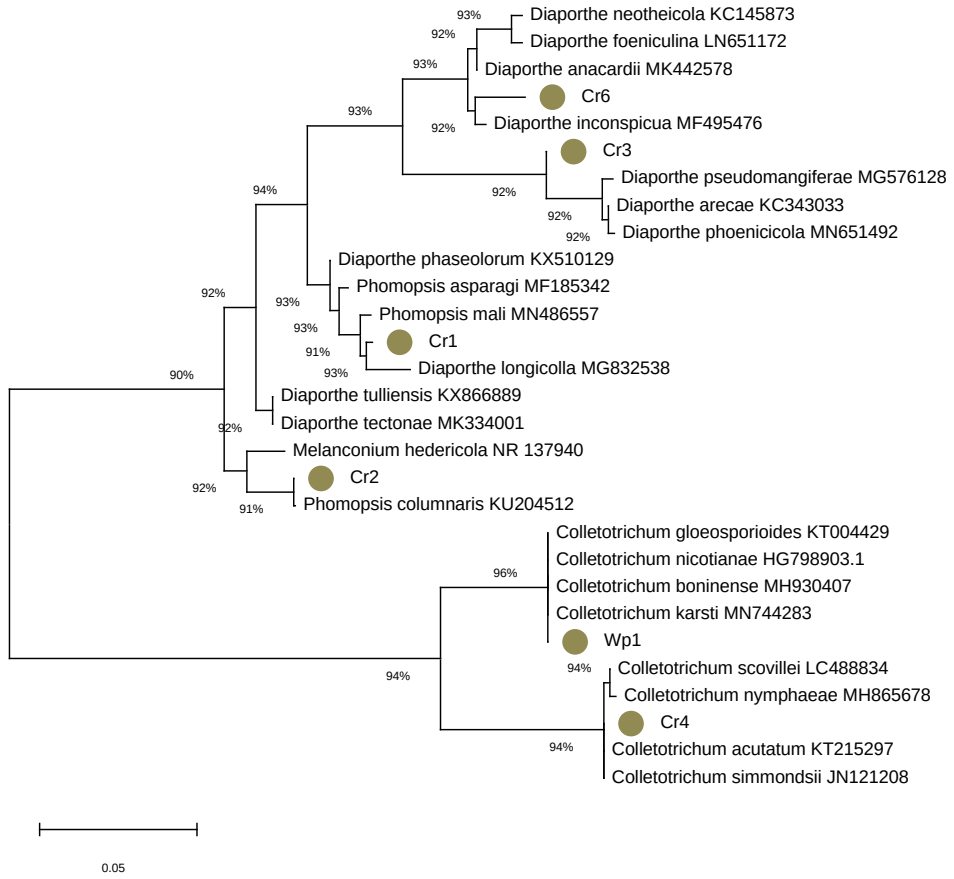

Figure 2. Phylogenetic tree based on the nucleotide sequences of rDNA-ITS region. The isolates and their phylogenetic position based on the ITS region according to neighbor joining method. The phylogeny involved 29 nucleotide sequences; the NCBI GenBank accession numbers from the reference strains are shown after the name of the strain.

Colletotrichum karsti causes anthracnose of *Capsicum anuum* in mango in northeastern Brazil [24]. In this study, *C. karsti* was found on the healthy leaves of *W. pubescens*, suggesting that it benefits the growth and development of *W. pubescens*. In the study of Gangadevi and Muthumary [25], the relative family of *C. karsti*, which is the *C. gloeosporioides*, a novel endophytic taxol-producing fungus, was isolated from the leaves of a medicinal plant, *Justicia gendarussa*. In addition, *Colletotrichium* species, in particular *C. gloesporioides*, produces taxol (163.4 μg/l), which is a secondary metabolite capable of inhibiting cell division or proliferation. Based on its taxonomy, *C. karsti* belongs to subkingdom Dikarya, phylum Ascomycota, subphylum Pezizomycotina, class Sordiaromycetes, subclass Hypocreomycetidae, order Glomerellales, family Glomerellaceae, and genus *Colletotrichum*.

Diaporthe longicolla causes the black zone lines on the lower stems of mature soybean plants [26]. According to Preeti *et al.* [27], through mutualism, both plants and endophytic fungi

release some similar secondary metabolites. This could be an indication that the isolated endophytic fungus identified molecularly as *D. longicolla* can play a vital role regarding the chemical and pharmacological properties of *C. ramiflora* and offers protection from pathogens and grazing animals. It belongs to subkingdom Dikarya, phylum Ascomycota, subphylum Pezizomycotina, class Sordiaromycetes, subclass Sordiaromycetidae, order Diaporthales, family Diaporthaceae, and genus *Diaporthe*.

Phomopsis columnaris causes the twig dieback of *Vaccinium vitis-idaea* (lingon berry) [21]. The isolated endophytic fungus *P. columnaris* could promote growth and nutrient uptake capability of the plant *C. ramiflora*. *Phomopsis columnaris* belongs to subkingdom Dikarya, phylum Ascomycota, subphylum Pezizomycotina, class Sordiaromycetes, subclass Diaporthomycetidae, order Diaporthales, family Diaporthaceae, and genus *Phomopsis*.

Diaporthe pseudomangiferae was first reported causing inflorescence rot, rachis canker, and flower abortion in *Mangifera indica* in Puerto Rico [28]. Furthermore, the ethanol extract of *C. ramiflora* leaves has cytotoxic effects on HeLa, T47D, and WiDr cell-lines [29]. The cytotoxic properties of *C. ramiflora* could be due to the association with the endophytic fungus *D. pseudomangiferae*. Through mutualism, the plants and endophytic fungi were found to secrete some similar secondary metabolites [28]. *D. pseudomangiferae* belongs to subkingdom Dikarya, phylum Ascomycota, subphylum Pezizomycotina, class Sordiaromycetes, subclass Sordiaromycetidae, order Diaporthales, family Diaporthaceae, and genus *Diaporthe*.

On the other hand, like *C. karsti*, *C. acutatum* also causes anthracnose [30]. In another study, *C. acutatum* was isolated from anthracnose lesions in key lime, star fruit, mango, and leatherleaf fern [31]. The presence of endophytic fungi on the development of plants might be an indicator of potential medicinal properties of the plant. In addition, the relative family of *C. acutatum*, which is *C. gloeosporioides*, showed cytotoxic activity against numerous cell lines including MCF-7, NCI-H460, HepG-2, and SF-268 tumor cell lines [32]. *Colletotrichum acutatum* belongs to subkingdom Dikarya, phylum Ascomycota, subphylum Pezizomycotina, class Sordiaromycetes, subclass Hypocreomycetidae, order Gomerellales, family Glomerellaceae, and genus *Colletotrichum*.

Zang *et al.* [33] reported that the secondary metabolite isochromophilone X isolated from *Diaporthe* sp. was found to have moderate cytotoxic activities. Moreover, kampanol A, R-mevalonolactone, ergosterol, and ergosterol peroxide were isolated from the endophytic fungus *Phomopsis archeri* [34]. In addition, another isolated compound from *P. archeri*, phomoarcherin B showed antimalarial activity against *Plasmodium falciparum* with an IC_{50} value of 0.79 μg/ml. The isolated endophytic fungus *D. inconspicua*, associated with *C. ramiflora* leaves, is a close relative of *Diaporthe* sp. and *Phomopsis archeri*, which are all under the family Diaporthaceae. Therefore, it could be possible for *D. inconspicua* to have a cytotoxic and anti-malarial activity. *Diaporthe inconspicua* belongs to subkingdom Dikarya, phylum Ascomycota, subphylum Pezizomycotina, class Sordiaromycetes, subclass Diaporthomycetidae, order Diaporthales, family Diaporthaceae, and genus Diaporthe.

4. Conclusions

This study performed the isolation of endophytic fungi from the known medicinal plants in the Philippines namely, *C. ramiflora* and *W. pubescens*. With the application of modern biotechnological tools such as DNA barcoding in plants, the endophytes were identified with robust results and accuracy. The internal transcribed spacer (ITS) region was found to have a high accuracy for the identification of the broadest range of fungi. The isolated endophytic fungi in this

study are important materials to study on their secondary metabolites, close association with plants, and important enzymes, all of which may have biotechnological application and medicinal uses.

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