BOTANY
Research Journal
MEDWELL PUBLICATIONS

Molecular Characterization, Phylogeny and Flavonoid Secretions of Fungal Endophytes Isolated from Species of Tree Fern, *Cyathea contaminans* (Hook) Copel from the Philippines

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Key words: Endophytes, eurotiales, flavonoids, hypocreales, cyathea

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Page No.: 37-41
Volume: 12, Issue 3, 2019
ISSN: 1995-4751
Botany Research Journal

Abstract: This study was conducted to identify and describe the phylogenetic positions of the species of fungal endophytes isolated from *Cyathea contaminans* (Hook) Copel using the molecular character, the ITS1-5.8sRNA-ITS2 gene sequence. A total of nine species were identified by comparing the isolates gene sequence with the sequences from the genbank.The phylogeny of the species was also described using the Maximum Parsimony Tree (MP). Moreover, the fungal extracts were also subjected to Shinoda's Test and UV-Vis spectrophotometry to determine their flavonoid secretions. The species identified include the: *Aspergillus sydowii*, *Colletotrichum gloeosporioides*, *Fusarium kyushuense*, *Fusarium pseudensiforme*, *Fusarium solani*, *Nigrospora sphaerica*, *Nectria hematococca*, *Penicillium citrinum* and *Penicillium griseofulvum*. The MP tree revealed the clustering of three species belonging to *Fusarium solani* species complex (*N. hematococca*, *F. pseudensiforme* and *F. solani*) and with *Fusarium kyushuense* they are held under order Hypocreales Another group the *A.sydowii*, *P. citrinum* and *P. griseofulvum* represented the order Eurotiales. *N. sphaerica* under order Trichosphaeriales was positioned basal to the Hypocreales and *C. gloeosporiodes* under Phyllachoralles was basal to Eurotiales. Phytochemical analysis revealed that all the endophytic fungal isolates except *A. sydowii* produced flavonoids. There were five flavonoid types secreted by the isolates namely: Band B flavones and flavonols, Band A flavones, Band A flavonols, Band A flavanones and Band B flavanones. All isolates produced the flavonoids with *N. sphaerica* positive to all the five types and *A. sydowii* negative to all. Here, the endophytic fungal isolates from *C. contaminans* can be identified to species level by the ITS1-srRNA-ITS2 gene sequence and are good prospects for bioprospecting.

INTRODUCTION

All plant species surveyed for the presence of fungi so far harbor one or more endophytic fungi symbionts in their photosynthetic tissues (Stone *et al*., 2000; Arnold and Lutzoni, 2007). These are organisms whose infections are internal and inconspicuous and the infected host tissue are transiently symptomless (Banerjee, 2011). Some of them exhibit a complex interactions with host plants and could act as chemical synthesizers (Owen and Handly, 2004; Joseph and Priya, 2011) of substances with strong antioxidant properties (Joseph and Priya, 2011). According to Bhagobathy and Joshi (2011) these endophytic fungi could be the actual producers of chemical compounds produced by these plants.

The endophytic fungal diversity, requires readily available identification tools for species delimitations. Traditionally morphological attributes were used. However, morphological species recognition criteria have been difficult to develop for many endophytes are phenotypically simple and often do not develop taxonomically informative vegetative, sexual (Gazis and Chaverri, 2010) or asexual (Reynolds, 1993; Taylor *et al*., 1999; Gazis, *et al*., 2011) reproductive structures. This necessitates the the use of molecular data such as the ITS srRNA genes. Within the rDNA locus, the ITS region has been particularly useful for analysis of closely related fungal species (Bernardi-Wenzel *et al*., 2010). Shafiquzzman etc. suggested that the best conceivable connection between morphological character and molecular character is to use a sequence based analysis of ITS 1and 2 regions of the rDNA.

C. contaminans is an endemic and threatened species of tree fern (Monilophyta) with disjunct distribution in some islands of the Philippine archipelago and used as alternative medicine to cure wounds and scratches (Taguiling, 2013). The plants may harbor endophytes which provide them adaptibility to survive against adverse environmental factors (Selvanathan *et al*., 2011). They are also considered as prolific sources of new bioactive natural products (Sunitha *et al*., 2013) thus it is important to identify its endophytic symbionts and their secretions.

The study was aimed to identify the fungal endophytes of *C. contaminans* and describe their phylogenetic positions using the ITS1-5.8 srRNA-ITS2 gene sequence and determine the types of flavonoids secreted by each endophytic species.

MATERIALS AND METHODS

Isolation of fungal endophytes: Fresh fronds of the fern plant *C. contaminans* were collected from 6 different areas of the Philippine archipelago namely: Mt. Province, Sorsogon, Cebu, Siquijor, Zambianga del Sur and Davao Provinces. The leaf samples were washed with running tap water for 15 min and cut into small segments.

Surface sterilization utilized the triple sterilization method (Bussaban *et al*., 2001) done by immersing the cut segments in 95% ethanol for 15 sec, followed by 1% sodium hypochlorite (NaOCl) for 5 min and immersed again in 95% ethanol for 15 min. The segments were rinsed three times with sterile distilled water and air dried inside a clean bench.

The sterilized segments were inoculated on plates containing Potato Dextrose Agar (PDA) supplemented with 100 ug mL^{-1} of 500 mg streptomycin to prevent bacterial contamination and incubated at 25°C for 7 days. Monocultures, were obtained by growing hyphal discs from PDA to Malt Extract Agar (MEA) medium and subjected to alternating 12 h light and dark phases inside an ecological chamber for 15 days.

Molecular identification of the endophytic fungal sequences: Molecular identification was done by comparison of the ITS1-5.8 small ribosomal RNA-ITS2 gene sequence with the Genbank sequences using the BLAST software. The genus and species match were accepted whenever identity between sequences and that of the database was 95-100%.

DNA Extraction, PCR (Polymerase Chain Reaction) amplification and ITS-rDNA Sequencing of the Endophytic Fungi: For each species a pinch of mycelia from fungal endophytic monoculture was used for DNA extraction. The DNA extraction procedure used the QiaAMP mini kit. PCR amplification followed the Qiagen one-step RTPCR using ITS4 and ITS 5 forward and reverse primers. The sequencing used the ABI PRISM® BigDye® Terminator v1.1 Cycle Sequencing.

Reconstruction of phylogenetic trees: Maximum Parsimony tree was constructed to assess the phylogenetic relationships of the fungal endophytes. The DNA sequences of the fungal endophytes with some of the downloaded fungal sequences were subjected to sequence alignment using the MEGA 5. The analysis utilized 1000 bootstrap replications.

Phyto-chemical analysis

Preparation of the crude extracts: Mycelia from the solid culture were harvested and air dried for 2 days. The dried mycelia were cut and pounded into granules. For each preparation, about 100 g of the granules was placed in 500 mL Erlenmeyer flask containing 250 mL of 90% methanol. This was allowed to stand for 7 days. The

preparation was then filtered in whatman filter paper. The residue was kept for future extractions while the filtrate was concentrated under reduced pressure through a rotary evaporator at 150°C. The concentrate was then evaporated to dryness on a water bath.

Extraction of the flavonoids: Shinoda's test was performed for the presence of flavonoids. The 2 mg of the methanolic extract was dissolved in 5 mL of ethanol. To this mixture dilute HCL and small granules of magnesium were added. The appearance of reddish brown color revealed the presence of flavonoids.

Chromatographic separation: Separation of the different types of flavonoids was done through paper chromatography. The 10 microliter of the methanolic extract was spotted on a paper chromatogram. The 5 mL of the mobile phase made up of ethyl acetate-methanol-water (1:1:1 mixture) was poured into a 200 mLbeaker. The spotted chromatogram was placed over the mobile phase which was allowed to migrate 10 cm from the starting line. The spots were sprayed with iodine and dried. Under the visible light flavonoids appeared yellow, black green or violet. The spots were cut and dissolved in pure laboratory grade ethanol.

Spectrophotometric (UV-Vis) scanning: Identification of the types of flavonoids was carried out with a spectrophotometer on the basis of the UV spectra through absorption maxima (λ_m) at at specific wavelength. The ethanolic solution containing the separated flavonoids were scanned in the wavelength ranging from 200-700 nm with pure ethanol as the blank reference. To identify the substances the values were compared with the existing absorbance data from literatures.

RESULTS

Molecular identification: A total of nine fungal endophytic species were isolated and identified from the fern plant, *C. contaminans.* Table 1 shows the identities of *F.sydowii, P.citrinum, P.griseofulvum, N. spaherica* and *C. gloeosporioides* that matched completely (100%) with the genbank sequences. The species *F. kyushuense* and *F. solani* closely matched with 99% sequence similarity with their genbank counterparts. The compared sequences of *F. kyushuense* consisted of 505 base-pairs with genetic distance of 6 base-pairs located at 5.8 *srRNA* gene sequence while *F. solani* sequences consisted of 521 base-pairs with genetic distance of 4 base pairs located at ITS1, 5.8 srRNA and ITS 2. The compared sequences of *N. hematoccocca* consisted of 542 base-pairs with 98% sequence similarity with genetic distance of eight base-pairs located at ITS1 and ITS 2. *F. pseudensiforme* compared sequences consisted of 321 base-pairs with genetic distance of 15 base-pairs located at ITS1, 5.8 srRNA and ITS2 genes.

Phylogenetic positions of the fungal endophytic species: Figure 1 shows the phylogenetic positions of 12 fungal endophytes. Nine sequences were from the endophytic isolates of *C. contaminans* while three were from the genbank sequences. The phylogenetic position of three species namely: *N. hematococca*, F. pseudensiforme and *F. solani* had a very high bootstrap support of 100%. *N. sphaerica* was also clustered basal to the Fusaria and had a high bootstrap support of 88%. The phylogenetic position of three species *Aspergillus sydowii*, *P. citrinum* and *P. griseofulvum* was resolved by 98% bootstrap support. *P. citrinum* and *P. griseofulvum* were considered sister taxa with bootstrap support of 100%. It turned out that *C. gloeosporioides* was basal to all species.

Flavonoid secretions of the fungal endophytes: Table 2 shows the different types of flavonoids screened using the fungal endophytic extracts. These include: band B flavones and flavonols, band A flavones, band A flavonols, band A flavanones and band B flavanones. Table 2 further shows that eight of the nine fungal endophytes observed were positive of the flavonoids.*N. sphaerica* was positive to all flavonoid types with the highest maximum absorbance (λ_m) of 3.0 at wavelength absorbed by band B flavanones while *A. sydowii* was negative of all the types. Maximum absorbances of 2.8 and 2.3 by *P. citrinum* was suggestive of band B flavones and flavonols and band B flavanone respectively. *P. griseofulvum* was positive of band B flavones and flavonols (λ_m 3.0), band A flavones (λ_m 3.0) and band A flavanones (λ_m 1.1) and band B flavanones (λ_m 3.0). *F. kyushuense* was positive of band B flavones and flavonols (λ_m 2.9), band A flavones (λ_m 0.519) and band A flavanones (λ_m 1.0). *F. pseudensiforme* was positive of band A flavones and

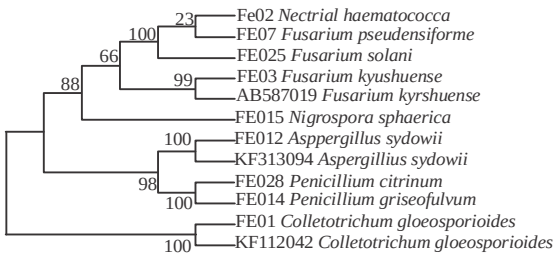

Fig. 1: The Maximum Parsimony tree (MP) showing the Phylogenetic positions of the fungal endophytes. (Bootsrap, % are indicated above and below the lines

Table 1: Comparison of the Fungal Endophytic ITS1-5.8srRNA-ITS2 with Genbank Sequences: Number of Base-pairs, % similarity, Genetic Distance and Location of Variable Base-pairs

Species code	Fungal Endophytes	Genbank Sequence Accession Number	# of base- pairs	Similarity Coefficient(%)	GeneticDistanc	Location in the egene sequence
FEO 12	*Aspergillus sydowii*	KF 313094.0	535	100	0	-
FEO 28	*Penicillium citrinum*	JGU 56673.1	512	100	0	-
FEO 14	*Penicillium griseofulvum*	FJ 441620.1	505	100	0	-
FEO 3	*Fusarium kyushuense*	AB 587019.1	505	99	6	5.8 sr RNA and ITS2
FEO 7	*Fusarium pseudensiforme*	KC 691584.1	321	95	15	ITS1,5.8 srRNA,ITS2
FEO 25	*Fusarium solani*	KC 577186.1	521	99	4	ITS1,5.8 srRNA, ITS2
FEO 15	*Nigrospora sphaerica*	GU 199423.1	304	100	4	5.8 srRNA
FEO 1	*Colletotrichum gloeosporioides*	JX 258802.1	532	100	0	-
FEO 2	*Nectria hematococca*	AB 513852.1	542	98	8	ITS, ITS2

Table 2: Wavelength (nm) and Maximum Absorbances (λ_m) of the Flavonoid Extracts from the Fungal

Fungal endophytes	Band B Flavones and Flavonols λ_m at 250-270	Band A Flavones λ_m at 310-350	Band A Flavanones λ_m at350-385	Band A Flavanones λ_m at 300-330	Band BFlavonols λ_m at 277-295
A. sydowii	-	-	-	-	-
P. citrinum	2.8	-	-	-	2.3
P. griseofulvum	3.0	0.520	-	1.1	3.0
N. sphaerica	2.8	2.3	1.3	2.5	3.0
F. kyushuense	2.9	0.519	-	1.0	-
F. pseudensiformi	3.0	0.518	-	1.0	3.0
F. solani	2.8				
C. gloeosporioides	2.0	-	-	-	-
N. haematocca	2.8	-	-	-	-

flavonols (λ_m 3.0), band A flavones (λ_m 0.518) and band A (λ_m 1.0) and Bflavanones (λ_m 3.0). *C. gloeosporioides*, *F. solani* and *N. hematococca* were positive of the band B flavones and flavonols with maximum absorbances of 2.8, 2.0 and 2.8, respectively.

DISCUSSION

The nuclear ITS 1-5.8 small ribosomal RNA-ITS2 sequence is the most popular region in molecular phylogenetic studies because it is multicopied and contains highly conserved genes 18S, 5.8S and 28S as well as variable domains ITS1 and ITS2 (Krimitras *et al*., 2013). The variable domains, ITS1 and ITS2 regions have high interspecific variability which have been useful for identification of *Colletotrichum* species (Martinez-Culebras *et al*., 2000). This is considered as the formal DNA barcoding region for molecular identification of fungi (Blaalid *et al*., 2013). In this study, this molecular character was used to resolve the identities of fungal endophytic species, *A. sydowii*, *F. kyushuense*, *F. solani*, *pseudensiforme*, *P. griseofulvum*, *P. citrinum*, *N. haematococca*, *N. sphaerica* and *C. gloeosporioides* The Maximum Parsimony tree further confirmed the identities of the fungal species and resolved their phylogenetic positions. In fact, the phylogenetic position of *F. pseudensiforme*, *N. hematococca* and *F. solani* resolved their close affinity, all being under the *Fusarium solani* species complex and *N. hematococca* as teleomorph of *F. solani* (Mehl and Epstein, 2007). The high bootstrap support of the cluster consisting *Fusarium* species support their morphological classification under Hypocreales.

Another cluster with bootstrap support of 98% consists of *A. sydowii*, *P. citrinum* and *P. griseofulvum* resolved their affinities because all are members of order Eurotiales (Houbraken and Samson, 2011). The Maximum Parsimony tree (Fig. 1) also placed *N. sphaerica*, under order Trichosphaeriales, closer to the Hypocreales and *C. gloeosporioides*, under order Phyllachorales which is basal to Eurotiales. Endophytic fungi associated with traditionally used medicinal plants especially of the tropics could be rich sources of functional metabolites (Suryanarayanan *et al*., 2009).

Several studies reported a large number of antimicrobial compounds isolated from endophytes belonging to structural classes like alkaloids, peptides, steroids, terpenoids, phenols, quinines and flavonoids (Pimentel *et al*., 2011). In this study seven types of flavonoids were identified from species of fungal endophytes from *C. contaminans*. For example, *N. sphaerica* produced all types of flavonoids tested. This species was also described in the work of Suryanarayanan *et al.* (2009) as excellent producers of bioactive compounds. Frisvad *et al*. (2008), mentioned *Fusarium* strains to have produced the highest amount of phenolics. The 2 species supported their findings, *F. kyushuense* produced band B flavones and flavonols and band A flavones and flavanones while *F. pseudensiforme* produced band B flavones and flavononones, band A flavones and band A and B flavanones. Penicillium species also produced a much diversified array of secondary metabolites (Petit *et al.*, 2009). This is true to *P. griseofulvum* which in this

study, produced four types of flavonoids namely: band B flavones and flavononones, band A flavones and band A and B flavanones and *P. citrinum* which produced band B flavones and flavonols and band B flavanones. *N. hematococca*, *F. solani* and *C. gloeosporioides* produced band B flavones and flavones and flavonols. These findings indicated that fungal endophytes are rich sources of flavonoids.

CONCLUSION

Molecular characterization confirmed the morphological groupings of fungal endophytes. Specifically, the *ITS1-srRNA-ITS2* gene sequence confirmed the morphological groupings of *Fusarium* species under Order Hypocreales and *Aspergillus* and *Penicillium* species under Order Eurotiales. The phytochemical analysis revealed that the fungal endophytes produced flavonoids similar to their host plants, hence, good subjects for bioprospecting.

ACKNOWLEDGEMENT

The Molecular Biology laboratory of the Biology Department of the University of San Carlos provided the venue and equipment used for the entire research.

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