CHARACTERISATION, PATHOGENICITY, AND MYCOTOXIN ANALYSIS OF FUNGAL ENDOPHYTES OF CORN (*Zea mays* L.) IN PENINSULAR MALAYSIA

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by

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LIST OF SYMBOLS

α	Alpha
β	Beta
g	Gram
mg	Milligram
kcal	Kilocalories
γ	Gamma
µg	Microgram
mL	Milliliter
kg	Kilogram
°C	Degree celcius
µL	Microliter
µM	Micromole
V	Volt
mA	Milliampere
µg/g	Microgram per gram
nm	Nanometer
cm	Centimeter
d	Day
s	Second

min	Minute
h	Hour
rpm	Revolutions per minute
bp	Base pairs
mm	Millimeter

LIST OF ABBREVIATIONS

TEF	Translation Elongation Factor
ITS	Internal Transcribed Spacer
LSU	Large Subunit
SSU	Small Subunit
ACT	Actin
CaM	Calmodulin
AFs	Aflatoxins
OTs	Ochratoxins
DON	Deoxynivalenol
ZEA	Zearalenone
PDA	Potato Dextrose Agar
CLA	Carnation Leaf Agar
SA	Soil Agar
MEA	Malt Extract Agar
CYEA	Czapek Yeast Extract Agar
DG18	Dichloran 18% Glycerol Agar
OA	Oat Meal Agar
DNA	Deoxyribonucleic Acid
rDNA	Ribosomal Deoxyribonucleic Acid

TEF-1α	Translation Elongatiotion Factor - 1 Alpha
β-tubulin	Beta-Tubulin
AA-tRNA	Aminoacyl-Tranfer Ribonucleic Acid
EF1	Elongation Factor 1
EF2	Elongation Factor 2
GAPDH	Glycerol-3-Phosphate Dehydrogenase
SAR	Systemic Acquired Resistance
FB1	Fumonisin B1
FUMs	Fumonisins
OTA	Ochratoxin A
GPS	Global Positioning System
GIS	Geographic Information System
PDB	Potato Dextrose Broth
TBE	Tris Borate-EDTA (Ethylenediamine Tetraacetic Acid)
MEGA	Molecular Evolutionary Genetics Analysis
BLAST	Basic Local Alignment Search Tool
BIC	Bayesian Information Criterion
AIC	Akaike Information Criterion
ML	Maximum Liklihood
PCR	Polymerase Chain Reaction
DSI	Disease Severity Index

ANOVA	Analysis of Variance
HSD	Honestly Significant Difference
FAA	Formalin Acetic Acid
TEM	Transmission Electron Microscopy
LM	Light Microscopy
HPLC	High Performance Liquid Chromatography
UPLC	Ultra Performance Liquid Chromatography
OPA	*Ortho*-Phthalaldehyde
PTFE	Polytetrafluoroethylene
CWDEs	Cell Wall Degrading Enzymes
NO_3^-	Nitrate
$MgCl_2$	Magnesium chloride
dNTP	Deoxynucleotide triphosphate
MeOH	Mercapto Ethanol
KCl	Potassium chloride
$Na_2B_4O_7$	Sodium tetra borate

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PENCIRIAN, KEPATOGENAN, DAN ANALISIS MIKOTOSIN KULAT ENDOFIT JAGUNG (*Zea mays* L.) DI SEMENANJUNG MALAYSIA

ABSTRAK

Endofit adalah mikrob yang menjangkiti tisu dalaman tanaman perumah tumbuhan pada keseluruhan atau sebahagian daripada kitaran hidupnya, tanpa menyebabkan gejala penyakit yang nyata. Bergantung pada beberapa faktor seperti tahap perkembangan tanaman tumbuhan perumah dan kulat, faktor persekitaran, dan tindak balas pertahanan perumah, kulat endofit yang terdapat di dalam perumah tumbuhan boleh menjadi patogen laten. Di Semenanjung Malaysia, kerja-kerja berkaitan kulat endofit yang dijalankan setakat ini tertumpu kepada peranan ekologi dan fungsi mereka dalam meningkatkan pertumbuhan tumbuhan dan sifat kemandirian yang lain, sementara masih banyak yang tidak diketahui mengenai permulaan penyakit, dan potensi penghasil mikotoksin bagi endofit kulat terutamanya jagung, dan tanaman bijirin yang lain. Kajian ini dijalankan untuk mengenal pasti dan mencirikan, menilai sifat patogenik, dan menyiasat potensi penghasil mikotoksin kulat endofit yang terdapat dalam tanaman jagung yang ditanam di ladang-ladang di Semenanjung Malaysia. Dengan menggunakan kombinasi teknik morfologi dan molekul, kulat endofit yang dipencilkan dari tisu tanaman jagung dikenal pasti kepada tujuh genera, iaitu *Fusarium*, *Penicillium*, *Aspergillus*, *Aureobasidium*, *Cladosporium*, *Epicoccum*, dan *Curvularia*. Berdasarkan carian BLAST dan analisis filogenetik gabungan jujukan β-tubulin dan TEF-1α, spesies endofitik *Fusarium* yang dipencilkan dari tanaman jagung dikenal pasti secara molekul sebagai *F. pseudocircinatum*, *F. verticillioides*, *F. andiyazi*, *F. sacchari*, *F. mangiferae*, *F. fujikuroi*, *F. proliferatum*, dan *F. incarnatum*. Spesies endofitik *Penicillium* dikenal pasti sebagai *P. oxalicum*, *P. polonicum*, dan *P.*

citrinum, berdasarkan kawasan ITS dan gen β-tubulin. Gabungan jujukan ITS, gen β-tubulin, dan Calmodulin digunakan untuk mengenal pasti spesies endofitik *Aspergillus* sebagai *A. flavus*, dan *A. tubingensis*, ITS dan gen β-tubulin untuk pengenalpastian *A. pullulans*, dan gen ACT untuk mengenal pasti *C. tenuissimum*, sementara gabungan kawasan ITS dan gen LSU digunakan untuk mengenal pasti *E. sorghinum* dan *C. lunata*. Ujian kepatogenan menunjukkan bahawa semua kulat endofit menghasilkan pelbagai tahap gejala penyakit pada tanaman jagung yang sihat, di mana *F. verticillioides* merupakan kulat endofit yang paling patogenik. Penilaian histopatologi tisu jagung yang dijangkiti melalui Mikroskopi Elektron Transmisi menunjukkan kolonisasi tisu antara sel dan intrasel tisu jagung yang dijangkiti oleh kulat endofit. Keratan rentas akar, batang dan daun tanaman jagung yang dijangkiti kulat endofit menunjukkan pengurangan dan pengherotan ketara saiz dan bentuk sel epidermis, sel korteks dan sel vaskular. Pengesanan dan analisis gen mikotoksin menunjukkan kulat endofitik *F. verticillioides*, *F. fujikuroi*, dan *F. proliferatum* mengandungi gen biosintetik FUM, *FUM1*, dan menghasilkan FB1 dalam kepekatan antara 11.20 - 18.47 μg/g. Kajian itu mendedahkan bahawa tumbuhan jagung di Semenanjung Malaysia menjadi ~~tuan~~ perumah kepada kulat endofit yang mampu mencetuskan penyakit, dengan mengubah struktur selular normal dan komposisi tisu tumbuhan jagung yang dijangkiti. Keupayaan beberapa kulat endofit dalam genus *Fusarium* untuk menghasilkan mikotoksin fitotoksik, FB1 menunjukkan kemungkinan peranan yang dimainkan oleh mikotosin dalam mempertingkatkan kolonisasi tisu dan perkembangan gejala dalam tisu jagung yang dijangkiti. Langkah kawalan bersepadu yang melibatkan penggunaan varieti jagung manis yang tahan penyakit, kawalan biologi, dan penggunaan racun kulat sistemik diperlukan untuk meningkatkan kesihatan tanaman dan memelihara hasil jagung manis di Semenanjung Malaysia.

CHARACTERISATION, PATHOGENICITY, AND MYCOTOXIN ANALYSIS OF FUNGAL ENDOPHYTES OF CORN (*Zea mays* L.) IN PENINSULAR MALAYSIA

ABSTRACT

Endophytes are microbes that infect internal tissues of host plants for all or part of their life cycles, without causing any visible symptoms of disease. Depending on a number of factors such as the developmental stage of both host plant and fungus, environmental factors, and host defence responses, fungal endophytes indwelling tissues of host plants may become latent plant pathogens. In Peninsular Malaysia, works on endophytic fungi carried out so far have focused mainly on their ecological and functional roles in enhancing plant growth and other survival attributes, while much remains unknown regarding the disease initiation, and mycotoxin-producing potentials of fungal endophytes especially of corn and other cereal crops. Thus, the present study was carried out to identify, evaluate the pathogenicity, and investigate the mycotoxin-producing potentials of endophytic fungi resident in corn plants grown on different fields in Peninsular Malaysia. Using a combination of morphological and molecular techniques, endophytic fungi recovered from tissues of corn plants were identified in seven genera, namely *Fusarium, Penicillium, Aspergillus, Aureobasidium, Cladosporium, Epicoccum,* and *Curvularia.* Based on BLAST search and phylogenetic analysis of combined β-tubulin and TEF-1α sequences, endophytic *Fusarium* species isolated from corn plants were molecularly identified as *F. pseudocircinatum, F. verticillioides, F. andiyazi, F. sacchari, F. mangiferae, F. fujikuroi, F. proliferatum,* and *F. incarnatum.* Endophytic *Penicillium* species were identified as *P. oxalicum, P. polonicum,* and *P. citrinum,* based on ITS region and β-

tubulin gene. Combined sequences of ITS region, β-tubulin, and Calmodulin genes were used to identify endophytic *Aspergillus* species as *A. flavus,* and *A. tubingensis,* ITS region and β-tubulin gene for the identification of *A. pullulans,* ITS region and ACT gene for identification of *C. tenuissimum,* while combined ITS region and LSU gene were used for identification of *E. sorghinum* and *C. lunata*. Pathogenicity test showed that all endophytic fungi produced varying levels of disease symptoms on healthy corn plants, with *F. verticillioides* being the most pathogenic. Histopathological assessment of infected corn tissues via light and Transmission Electron Microscopy showed both intercellular and intracellular colonisation of infected corn tissues by endophytic fungi. Cross-sections of endophyte-infected roots, stems and leaves of corn plants showed significant reductions and distortions in sizes and shapes of epidermal, cortical and vascular cells. Mycotoxin gene detection and analysis showed that endophytic *F. verticillioides, F. fujikuroi,* and *F. proliferatum* contained the FUMs biosynthetic gene, *FUM1,* and produced FB1 in concentrations ranging from 11.20 - 18.47 µg/g. The study revealed that corn plants in Peninsular Malaysia play host to endophytic fungi which are capable of disease initiation, by altering the normal cellular structure and tissue composition of infected corn plants. The ability of some endophytic fungi in the genus *Fusarium* to produce the phytotoxic mycotoxin, FB1 is indicative of the possible role played by the mycotoxin in advancing tissue colonisation and symptom development in infected corn tissues. Integrated control measures involving use of resistant sweet corn varieties, biological control, and use of systemic fungicides are required to enhance crop health and preserve yield of sweet corn in Peninsular Malaysia.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Corn (*Zea mays* L.) is an annual crop belonging to the grass family *Poaceae*, the fourth largest flowering plant family, containing approximately 11,000 species in nearly 800 genera worldwide (Kumar *et al.,* 2013). The corn crop is regarded as the third most important cereal crop in the world after wheat and rice with respect to cultivated area and total production (Ansari *et al.,* 2017). Presently, the United States is the highest corn producer, with a production volume of about 370.96 million metric tons, followed by China (215.89 million metric tons) and Brazil (82 million metric tons) (Shahbandeh, 2021). In addition to being the world's highest producers of corn, the United States, China, and Brazil are also reported as the world's highest consumers of corn and corn products (Statistica, 2021).

In Malaysia, domestic maize production is barely enough to meet the overwhelming demand for human and livestock consumption, and over 70% of corn consumed in Malaysia is imported (Nazmi *et al.,* 2021). Fungal infections are major threats to maize production in Malaysia, often resulting in poor crop yield and product quality (Afsah-Hejri *et al.,* 2013; Shaik, 2016). Although much is yet to be understood regarding the factors responsible for infection of corn plants by fungi in Malaysia, the conditions of high relative humidity and warm temperatures often prevalent in most parts of Malaysia are ideal for field and storage infection of corn plants by a vast array of fungi, including pathogenic, mycotoxigenic, and endophytic fungi (Arnold & Herre, 2003; Yazid *et al.,* 2021). Thus, studies on fungal infections and their effects on corn health and yield could provide useful information that may foster the establishment of

appropriate disease control measures aimed at the preservation of corn yield and enhancement of post-harvest grain quality of corn in Malaysia.

Endophytes are microbes that live all or part of their life cycles asymptomatically in the intercellular and intracellular spaces of living and apparently healthy host plant tissues, while the inhabited host tissues remain intact and functional most of the time (Petrini, 1991). Fungal endophytes are found living within all plant parts, and appear to have a symbiotic relationship with the host plant. Endophytic fungi are highly diverse, and can have profound impacts on plant communities through increasing fitness, by conferring abiotic and biotic stress tolerance, and increasing biomass. They also decrease fitness by altering resource allocation (Clay, 1988; Lind *et al*., 2013).

Fungal endophytes show extensive functional diversity ranging from pathogenesis to mutualism, depending on the fungal strain, host genotype, and growth conditions (Eaton *et al.*, 2011). Although a number of studies have revealed the presence and identity of fungal endophytes belonging to different fungal genera in tissues of different parts of the corn plant (Fisher *et al.*, 1992; Yates *et al.*, 1997; Pinto *et al.,* 2000; Amin, 2013; Russo *et al.,* 2016; Renuka & Ramanujam, 2016), endophytic colonisation of corn plants in Malaysia is yet to be reported. Therefore, the present study shall provide information on the identification and occurrence of various groups of endophytic fungi in corn plants in selected states of Peninsular Malaysia.

Isolation and identification are generally considered the first steps in the studies of endophytic fungi. Plant materials considered for isolation of endophytic fungi are usually collected from apparently healthy and disease-free tissues (without obvious signs of disease), in order to minimize the presence of pathogens and other saprobic fungi which may be present in diseased tissues (Strobel, 2003). For accurate

identification of endophytic fungi, a polyphasic approach involving both morphological and molecular characterisation is recommended (van Nieuwenhuijzen *et al.,* 2016).

Morphological identification is often based on the observation of both macroscopic and microscopic characteristics of endophytic fungi. The major macroscopic characteristics considered are colony colour, texture, and pigmentation, while microscopic characteristics such as shapes, sizes, pigmentation, and septation of conidia, type and branching pattern of conidiophores, as well as the orientation of hyphae, are important microscopic characteristics considered in the morphological delineation of endophytic fungi (Petrini *et al.,* 1982). Compared to macroscopic characteristics, microscopic characteristics exhibit minimal variation, and are thus considered more reliable for morphological identification of endophytic fungi (Karki, 2018). However, morphological characteristics are unsuitable for accurate species identification of many endophytic fungi, since there are limited numbers of morphological characters that can be considered for exhaustive fungal identification of several species (Hyde & Soytong, 2008; Ko *et al.*, 2011). To enable accurate identification of endophytic fungi, molecular identification is recommended (Guo *et al.*, 2000).

Molecular identification of endophytic fungi often relies on sequence and phylogenetic analyses of DNA, amplified using specific genetic markers (Huang *et al.,* 2009). Due to advantages such as ease of amplification, vast usage, and significantly wide barcode gap between inter- and intraspecific variation, the internal transcribed spacer (ITS) region was proposed as the barcode for fungal identification by the International Fungal Barcoding Consortium. However, since ITS region shows non-uniformity in variability in a number of fungal groups, especially Ascomycetes

such as *Alternaria, Aspergillus, Cladosporium, Penicillium,* and *Fusarium,* the ITS region is regarded as unsuitable for the molecular identification of endophytic fungi in these genera. To address the limitations of the ITS region in the molecular identification of endophytic fungi, the use of protein-coding genes such as actin (ACT), translation elongation factor (TEF-1α), β-tubulin, and calmodulin (CaM), has been recommended. These protein-coding genes are able to distinguish between closely related species, cryptic species, and also reveal the phylogenetic relationships among different fungal species within the same genus (Tekpinar & Kalmer, 2019).

Phylogenetic identification involves the use of sequence data to infer the relationships that exist between organisms, and to confirm their species identity by grouping same species together in same clade (Ziemert & Jensen, 2012). Although phylogenetic trees created using sequences from single genes are quite informative, combination of two or more genes is often required to achieve greater species discrimination, especially in species such as *Colletotrichum* and *Diaporthe* (Castlebury *et al.,* 2003; Weir *et al.,* 2012). Multi-loci phylogenetic analyses involving genes such as the TEF-1α, CaM, β-tubulin, and ACT, have also been useful in the species delineation of endophytic fungi from several genera such as *Fusarium, Aspergillus, Penicillium, Cladosporium, Epicoccum, Aureobasidium, Alternaria, Bionectria, Xylaria, Pestalotiopsis,* and *Trichoderma* (dos Santos *et al.,* 2016; Zakaria *et al.* 2016; Tibpromma *et al*., 2018; Azuddin *et al.* 2021).

The symptomless relationship between endophytic fungi and host plants is maintained by the balance between biotic factors such as host genotype, and abiotic factors such as temperature, and relative humidity. Hence, a disturbance in this balanced relationship often results in plant stress, leading to the occurrence of disease (Schulz & Boyle, 2005; Bacon *et al.*, 2008). *Fusarium verticillioides* is a known

example of an endophytic fungus that is able to switch from endophyte to pathogen of corn plants, resulting in devastating disease symptoms such as stem and ear rots of infected corn plants (Kuldau & Yates, 2000). A number of studies have investigated the pathogenicity of endophytic fungi in different host plants such as wild banana (*Musa acuminata*) (Photita *et al.,* 2004), tropical almond (*Terminalia mantaly* and *Terminalia catappa*) (Begoude *et al.*, 2011), Japanese knotweed (*Fallopia japonica*) (Kurose *et al.,* 2012), and black cottonwood of the Pacific Northwest (*Populus trichocarpa*) (Raghavendra & Newcombe, 2013), nevertheless, such studies are yet to be reported on corn plants in Malaysia. Therefore, the present study shall provide information on the pathogenicity of endophytic fungi from corn plants in different states of Peninsular Malaysia.

Colonisation of the inter- and intra-cellular spaces of plant tissues by endophytic fungi is often accompanied by varying degrees of histological responses which indicate growth improvement or disease of host plants. These cellular responses are evaluated using histological methods involving light and electron microscopy, which also play important roles in the structural description of the infection process in several endophyte-host plant associations (Hinton & Bacon, 1985; Stone, 1987). In light microscopy, endophytic fungi and infected tissues are visualized in thin sections of plant tissues (about 10 µm thick) (Livingston *et al.*, 2009). Light microscopy reveals the cellular distribution of endophytic fungi within infected tissues, and enables the observation and investigation of the cellular effects of endophytic colonisation of host tissues. Whereas, in electron microscopy, greater detail of infection structures and the precise location of endophytic fungi could be observed within ultrathin sections (<0.1µm) of endophyte-infected plant tissues (Guzmán *et al*, 2014).

Histological studies involving the visualisation of endophytic fungi in corn and other tropical grasses using light and electron microscopy techniques have been reported (White *et al.,* 1995; White *et al.*, 1997; Yates *et al.,* 1997; Bacon *et al.,* 2008). However, such studies are yet to be carried out in Malaysia.

Mycotoxigenic endophytes, especially within the genera *Aspergillus, Penicillium*, and *Fusarium,* have been reported in several plants (Bennett & Bentley, 1989). Mycotoxins produced by endophytic fungi include aflatoxins (AFs), ochratoxins (OTs), deoxynivalenol (DON), zearalenone (ZEA), and fumonisins (FUMs) (Anjorin & Inje, 2014; Ismail, 2014; Lofgren *et al.*, 2018). These mycotoxins are known to play key roles of fungal defence against other microbes, but they are best known for their toxicity to humans and other animals upon consumption of mycotoxin-contaminated foods and feeds, respectively (Etzel, 1999; Peraica *et al.*, 1999). Although the corn crop is considered the most susceptible to mycotoxin contamination compared to other cereals (Chulze, 2010), studies on the mycotoxin-producing potentials of endophytic fungi recovered from the corn plants are lacking.

1.2 Objectives of the study

To address the paucity of information regarding the occurrence of endophytic fungi from corn plants, the general objective of this study was to determine the fungal isolates residing in different parts of the corn plants, with the following specific objectives:

1) To isolate and identify endophytic fungi from husks, silks, and kernels of field grown corn from corn fields in selected states of Peninsular Malaysia, using morphological and molecular characteristics,

2) To determine the pathogenicity of isolated fungal endophytes on sweet corn,

3) To investigate the colonisation pattern of infected corn tissues by fungal endophytes using light (LM) and transmission electron microscopy (TEM),

4) To determine the mycotoxin-producing potentials of selected endophytic fungi using mycotoxin gene detection assays and ultra performance liquid chromatography (UPLC) analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Origin and Distribution of Corn (*Zea mays* L.)

The word corn is linked to an Indo-European word, *grn,* used to refer to the plant that produces small worn out particles known as *gnorm* (later known as grain), and also the word *kurnam* used for the plant in pre-historic German, which was later developed into the English word *corn*, used today mainly in America, Canada, Australia, and other parts of the world (Culinarylore, 2013). Although the name "corn" for the plant, is used and quite common in many parts of the world, its synonym "maize" derived from the word "mahiz", which is the Spanish form of the indigenous Taíno word for the plant, is also quite popular in many countries (Verheye, 2004; Dean, 2013).

The origin of the corn plant (*Zea mays* L.) has been a subject of much controversy over the years, but only two theories of origin have received serious consideration. One states that teosinte (*Zea mexicana* Schrad.) is the wild progenitor of corn, while the other asserts that a wild pod corn, now extinct, was the ancestor of domesticated corn. It is however generally accepted that the centre of origin of the corn plant is located in Mesoamerica, primarily Mexico and the Caribbean (Singh & Kumar, 2016). The crop was introduced to Europe in the 16th century, from where it spread to Africa and Asia. The crop has now spread to more than 100 countries, and is regarded as one of the most widely-grown crops around the world in both temperate and tropical regions (Verheye, 2004; Hersant, 2013).

The ability of the corn plant to grow well in various agroecologies is credited to its wide climatic adaptability, and it is propagated in more diverse regions than any other crop (Verheye, 2004; Singh & Kumar, 2016). This ability to grow in a wide range

of environments is reflected in the high diversity of morphological and physiological traits obtainable in the crop. Brown *et al*. (1985) reported that the corn plant has become so domesticated that seeds cannot be separated from the cob and disseminated without human intervention.

In Malaysia, corn (*Jagung* in Malay) was first introduced to Melaka state during the Portuguese and Dutch occupations in the 16th century (Wong, 1992). Grain corn varieties such as flint and dent corn were the first to be introduced, and were cultivated by smallholder enterprises in river valleys in Kelantan, Terengganu, and Pahang, until they were eventually phased out and replaced by sweet corn. Sweet corn is preferred over other corn types in Malaysia because of its reduced production costs and shorter cultivation period (Wong, 1992; Rosali *et al.,* 2019). At present, sweet corn is grown in all parts of Malaysia (60,000 metric tons in total), with Selangor producing the most (15,600 metric tons), followed by Johor (12,000 metric tons), Sarawak (10,200 metric tons), Perak (6,600 metric tons), and both Perak and Sarawak (3,300 metric tons) (USDA, 2022).

Despite the increase in total cultivated area for corn in Malaysia, domestic corn production accounts for only about 20% of available corn, which is inadequate in meeting the huge demand especially in the livestock and food industry. Hence, over 70% of corn consumed in Malaysia is imported, mainly from countries such as Argentina, Brazil, and the United States (Rosali *et al.,* 2019; Nazmi *et al.,* 2021). The challenges of corn production in Malaysia include the prevailing climatic conditions of high temperature and relative humidity in most parts of Malaysia, which are unfavourable for uptimum productivity and yield of corn crops. High temperatures and relative humidity also enhance the infection of corn plants by pathogenic and

mycotoxigenic fungi, resulting in disease and consequent crop loss (Arnold & Herre, 2003; Yazid *et al.,* 2021).

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2.1.1 Economic importance of corn

2.1.1(a) Food uses

Only about 15% of corn production worldwide is used for food consumption, with most production going to animal feed. In developing countries, the proportion of corn production for food is higher at 25% and even higher in regions such as South-East Asia, where it is estimated between 30-40%. In parts of Sub-Saharan Africa, food usage of corn can be as high as 70-80% (De Groote & Kimenju, 2012; Hersant, 2013).

Corn grain is a rich source of starch (72%), proteins (10%), vitamins A and B (3 – 5%), oil (4.8%), fibre (5.8%), sugar (3.0%) and ash (1.7%). One hundred g of fresh grain contains about 361 cal of energy, 9.4 g protein; 4.3 g fat, 74.4 g carbohydrate, 1.8 g fibre, 1.3 g ash, 10.6% water, 140 mg vitamins, 9 mg calcium, 290 mg, phosphorus and 2.5 mg iron (Arain, 2013). In addition to its nutritional qualities, corn grain has been reported to supply energy density as high as 365 Kcal/100 g (Hersant, 2013).

Corn grains can either be eaten raw, cooked, roasted, fried, ground, pounded or crushed to prepare various types of food like corn flakes, popcorn, taco, corn grits, and corn chips (Kochhar, 2016). Corn flour can be used in similar ways as wheat flour in making bread and other breakfast meals. It is highly rich in protein, dietary fibre and very low in fat, and is by far the most widely eaten flour after wheat and rice flour. Fortified corn flour has been employed in tackling food security challenges such as malnutrition in some parts of the world (Orhun, 2013). Starch extracted from corn grain is used in making confectionary and noodles. Corn syrup from corn is rich in fructose

and acts as sweetener when added to certain foods. Oil extracted from corn grain is an all-purpose culinary oil (Oladejo & Adetunji, 2012; Kumar & Jhariya, 2013).

2.1.1(b) Livestock feeds

Bulk of the concentrates fed to farm animals in the tropics consist grains of which corn is the most important component, making up 40-75% of the rations (IITA, 1982). Corn grain yields the highest conversion ratio to meat, milk and eggs when compared with other grains used as livestock feed. The high conversion ratio is attributed to its high starch and low fibre content, which makes it a highly concentrated source of energy for livestock production. Yellow corn supplemented with vitamins and proteins is preferred for livestock feed, and is used either as whole grains, cracked or coarse ground, dry, wet, or steamed (Orhun, 2013).

2.1.1(c) Industrial uses

The industrial uses of corn can be categorized under four major processes; dry milling, wet milling, distillation and fermentation. Dry milling is focused on the production of corn meal, corn flour, grits and breakfast cereals, while the wet millers manufacture starch, feed, syrup, sugar, oil and dextrines. The focus of the distillation and fermentation industries is the manufacture of ethyl alcohol, butyl alcohol, propyl alcohol, acetaldehyde, acetic acid, acetone, lactic acid, citric acid, glycerol, and whisky (IITA, 1982; Arain, 2013). Approximately 5% of total corn production in the United States is utilized in the manufacture of high fructose corn syrup, a popular substitute for

sucrose (found in sugar), also used in soft drinks and other processed foods (Abbassian, 2007).

The rapid expansion in the ethanol industry has brought about a sudden spike in the demand for distillers grains. Furthermore, corn starch is used as an adhesive in pigment coating in the manufacture of paper and paper boards (Orhun, 2013). Corn is also used in the manufacture of photographic film, plastics, alcohol, ink, paint, glue, shoe polish, fireworks, and rust blockading (Milind & Isha, 2013).

2.1.1(d) Medicinal uses

Corn silk extracts have been reported to possess potent diuretic, hypoglycemic, anti-fatigue, nephrotoxicity-reduction, anti-inflammatory, and anti-oxidant activities. Other traditional uses of corn silk extracts as anti-diarrheal, anti-dysentery, anti-tumour, anti-prostatitis, and anti-gonorrhoeal, have also been documented (Wang *et al.,* 2012; Zao *et al.,* 2012; Milind & Isha, 2013).

Corn is also believed to have potential anti-HIV activity due to the presence of galanthus nivalis agglutinin (GNA), lectin, or GNA-corn. Decoctions of corn silks, roots, leaves, and cobs are used for treatment of bladder problems, nausea, vomiting, and stomach complaints (Shah *et al.*, 2016). Corn is also used in the treatment of erectile dysfunction in men (Kumar & Jhariya, 2013).

2.2 Fungal Endophytes of Crop Plants

The term endophyte was first described by de Barry (1866) from two Greek words, "endon" meaning within, and "phyton" meaning plant, and since then, several

definitions have been put forward by different authors. Carroll (1986) defined endophytes as mutualistic organisms that colonise aerial parts of living plant tissues without causing any symptoms of disease. Petrini (1991) proposed an expansion of the definition of Carroll (1986) to include all organisms which inhabit plant tissues at some point in their life cycles, without causing apparent injury to the host plant. Wilson (1995) described endophytes as fungi or bacteria which invade the tissues of living plants for all or part of their life cycles, causing asymptomatic and unapparent infections, entirely within plant tissues. Bills (1996) added that since endophytes and certain types of mycorrhizae such as ericoid mycorrhizae, ectendomycorrhizae, and pseudomycorrhizae, are indistinct, certain mutualistic root-inhabiting or mycorrhizal fungi can be referred to as endophytes. Despite the variations in definitions of endophytes by different authors, the definition of Petrini (1991) has been most commonly used in endophyte studies (Sun & Guo, 2012).

Endophytes are ubiquitous and have been isolated from all parts of nearly 300,000 land species, ranging from mosses, ferns, grasses, shrubs, deciduous and coniferous trees, to complex associations such as lichens (Petrini *et al.*, 1982; Swatzell *et al.,* 1996; Müller & Krauss, 2005). Although endophytic fungi consist mainly of members of the Ascomycota, large numbers of fungi belonging to the Basidiomycota, Zygomycota and Oomycota have also been identified (Zheng & Jiang, 1995). There are not less than 1 million species of endophytic fungi alone, and their assemblages are influenced by different factors such as age, specificity, and geographical location of the colonised tissues (Dreyfuss & Chapela, 1994; Arnold, 2007).

Endophyte-host plant associations are dynamic and complex. Endophytic fungi often contribute to the improved health and development of host plants in exchange for a relatively privileged niche. Although fungal endophytes have been isolated from

nearly all plants, knowledge of the extent of fungal endophytism is still relatively new, and much is yet to be understood concerning endophyte-plant interactions (Torres *et al.,* 2011).

On the bases of evolutionary relationships, differences in plant hosts, and ecological functions, endophytic fungi are generally grouped under two major groups, the clavicipitaceous (C-endophytes), and non-clavicipitaceous (NC-endophytes) endophytes. Clavicipitaceous/C-endophytes are infective on some grasses, whereas the non-clavicipitaceous/NC-endophytes target mostly tissues of non-vascular plants, ferns, conifers, and angiosperms (Rodriguez *et al.,* 2009).

C-endophytes, also known as Class 1 endophytes are a group of phylogenetically related clavicipitaceous species, which form systemic intercellular infections in mainly shoots of cool-and warm-season grasses (Bischoff & White, 2005). Three types of Class 1 endophytes (C-endophytes) have been recognised on the basis of their virulence behaviour, namely Type I which are the symptomatic and pathogenic species, Type II which show a mixed-interaction between symptomatic and asymptomatic, and Type III representing the asymptomatic endophytes (Clay & Schardl, 2002). Class 1 endophytes are transmitted vertically from parent plants to their offsprings especially through seed infections, and remain dominant in the colonised plant (Wille *et al.,* 1999; Saikkonen *et al.,* 2002). Depending on host species, host genotype, and prevailing environmental conditions, the benefits of plant infection by Class 1 endophytes may include increased biomass, abiotic stress resistance, and production of toxic chemicals to reduce herbivory by animals (Clay, 1988). An example of a Class 1 endophyte is the grass endophyte, *Colletotrichum endophytica,* which was isolated from the dwarf napier (*Pennisetum purpureum*) and lemon grass (*Cymbopogon citratus*) in Thailand (Manamgoda & Udayanga, 2013).

Based on host colonisation pattern, transmission mechanism, ecological function, and *in planta* biodiversity levels, NC-endophytes have been grouped under three functional classes, namely Class 2, Class 3, and Class 4. Class 2 endophytes are usually distributed in both above- and below-ground tissues, while Class 3 are confined to above-ground tissues, and Class 4 restricted within the roots of infected plants. Also, while tissue infection by class 3 endophytes are highly localized, Class 2 and Class 4 endophytes are able to colonise tissues more extensively. Furthermore, although the diversity of Class 4 endophytes within individual host plants is yet to be conclusively evaluated, Class 2 endophytes are generally known to be limited in individual host plants, while Class 3 endophytes often show extreme diversity in host plants (Arnold *et al.*, 2003; Rodriguez *et al*., 2008).

In addition to horizontal transmission by both Class 2 and Class 3 endophytes, vertical transmission of Class 2 endophytes via seed coats, seeds or rhizomes, is also common, and plants infected by Class 2 endophytes have been found to exhibit enhanced habitat-specific stress tolerance (Rodriguez *et al*., 2008). Redman *et al.* (2011) reported that *F. culmorum,* a Class 2 endophyte isolated from the coastal plant *Leymus mollis* (American dune grass), was able to enhance salt, cold, and drought stress tolerance in rice (*Oryza sativa*).

Class 3 endophytes are defined by their ability to exclusively infect above-ground tissues, exhibit horizontal transmission, produce highly localized infections, confer non-habitat-specific advantages on hosts, and exceptionally high *in planta* biodiversity. Examples of Class 3 endophytes include the highly diverse community of endophytic fungi associated with leaves, fruits, and flowers of tropical trees, tissues of nonvascular plants, and seedless vascular plants, conifers, as well as woody and herbaceous angiosperms in biomes spanning from tropical forests to the boreal and

Arctic/Antarctic plant communities (Petrini, 1986; Gamboa & Bayman, 2001). Despite being horizontally transmitted, Class 3 endophytes are usually distinguished from pathogens associated with the same host species, and epiphyllous fungi on the same plant part (Ganley *et al.,* 2004; Santamaría & Bayman, 2005). Majority of Class 3 endophytes are Ascomycetes, while a few are Basidiomycetes (Hyde & Soytong, 2008) which reproduce either by hyphal fragmentation or through the production of sexual or asexual spores on dead or senescent plant tissues (Herre *et al.,* 2005). Class 3 endophytes such as *Phyllosticta* spp. rely on high humidity occasioned by rainfall, dew or fog, for effective colonisation and dispersal (Arnold & Herre, 2003).

Class 4 endophytes also known as dark septate endophytes, are generally described as ascomycetous fungi that are confined to the roots of infected plants, and characterized by the presence of melanised structures such as inter- and intra-cellular hyphae and microsclerotia. Class 4 endophytes are generally regarded as non-pathogenic, and known to have low host or habitat specificity, with a vast habitat range, spanning the Antarctic, Arctic, to the African coastal plants and lowlands. Owing to their presence in soils and plant roots, transmission of Class 4 endophytes is most presumed to be horizontal (Jumpponen & Trappe, 1998).

A classic example of a Class 4 endophytic infection is the colonisation of plant roots by endophytic *Phialocephala fortinii,* which begins with the formation of a loose network of superficial and/or runner hyphae on the root surface. Hyphal formation on root surfaces is followed by the growth of individual hyphae along the root's main axis, between cortical cells, and within the intercellular depressions of the epidermis (O'Dell *et al.,* 1993). During intracellular colonisation of root tissues by *P. fortinii*, the endophyte forms clusters of closely packed thick-walled sclerotial bodies in the cortical cells, without causing any distortion to the host roots (Wang *et al.,* 1985).

Although Class 4 endophytes are often found in healthy roots of plants growing in high-stress environments, little is known about the roles played by Class 4 endophytes in the ecophysiology of host plants (Rodriguez *et al.,* 2009). However, Mandyam & Jumpponen (2005) proposed that colonisation of plants by Class 4 endophytes could deter pathogens, by minimizing available rhizospheric carbon. The authors further suggested that toxicity to herbivores could also be achieved by the production of antagonistic secondary metabolites from the abundant melanin pigments in the endophyte's cells.

2.2.1 Morphological identification of endophytic fungi

Traditionally, the two basic techniques recognised for observation and identification of endophytic fungi in plant tissues are direct observation, and cultivation-dependent methods. Direct observation involves the direct microscopic examination of fungal structures within living plant tissues. This method is particularly useful for the observation of biotrophic fungi that cannot be cultured on standard growth media (Deckert *et al.,* 2001; Lucero *et al.*, 2011).

Cultivation-dependent identification of fungal endophytes is based on cultural and microscopic observation of growth and reproductive features of fungal isolates on artificial growth media. Cultivation-dependent techniques are however more routinely used in studies relating to endophyte diversity, owing to the fact that an overwhelming majority of fungal endophytes are non-biotrophic, and can be obtained as microbial resources for further use (Petrini *et al.,* 1982; Rodrigues & Samuels, 1990; Guo *et al.,* 2000; Vieira *et al.,* 2011). Limitations of cultivation-dependent methods in the study of fungal endophytes include (i) a number of endophytic fungi are not adapted to growth

and sporulation on artificial growth media, and (ii) slower growing species are usually outcompeted by fast-growing fungal species on artificial growth media (Taylor *et al.,* 2000; O'Brien *et al.,* 2005).

The first process in the cultivation-dependent identification of endophytic fungi, is the macromorphological characterisation of the endophytes through the observation of their cultural features. This process involves the measurement of growth rate, colony colour, texture, shape, and the presence of pigments and secretion of metabolites in growth media. In some fungi, sexual fruiting bodies such as perithecia, apothecia and cleistothecia, or asexual fruiting bodies such as sporodochia and aservuli, are produced on the surfaces of growth media (Watanabe, 2010). Although cultural characteristics are important in fungal identification, they are usually limited by the inconsistency in growth behaviour of fungi on different growth media (Karki, 2018).

Microscopic characteristics of fungi are generally considered more stable, as they exhibit minimal variation compared to cultural characteristics (Karki, 2018). Shape, colour, septation, and size of conidia and conidiogenous cells, hyphal septation and branching pattern, and the presence of other survival structures such as chlamydospores and sclerotia, are considered as useful features in the microscopic delineation of endophytic fungal species (Seifert & Gams, 2011).

Media used for the morphological characterisation of endophytic fungi are usually selected based on the nutrient requirement for growth, sporulation, and the production of other identification features by the examined fungi (Samson *et al.*, 2010). Generally, Potato Dextrose Agar (PDA), a carbohydrate-rich medium which supports the growth of a vast majority of fungi is considered a standard medium for isolation and cultivation of endophytic fungi. Other growth media used in the morphological studies of fungal endophytes include; Carnation Leaf Agar (CLA), and Soil Agar (SA), for

Fusarium spp. (Leslie & Summerell, 2006), Malt Extract Agar (MEA), Czapek Yeast Extract Agar (CYEA), and Dichloran 18% Glycerol Agar (DG18), for *Aspergillus, Penicillium,* and *Cladosporium* spp. (Samson *et al.,* 2010), and Oat Meal Agar (OA) for *Epicoccum, Curvularia,* and *Aureobasidium* spp. (Williamson & Duncan, 1975; Alvindia & Hirooka, 2011; de Lima *et al.,* 2011).

Although endophytic fungi are largely ascomycetous, some have been observed not to produce reproductive structures even after several months in culture, and are usually encouraged to sporulate by the incorporation of host plant tissues or extracts in growth media (Matsushima, 1971; Huang *et al.,* 2001). Endophytes that fail to sporulate in growth media are referred to as mycelia sterilia (Lacap *et al.*, 2003; Andersen *et al.,* 2021).

2.2.2 Molecular identification and phylogenetic analysis of endophytic fungi

Molecular techniques have been adopted for detection and identification of fungi within their natural habitats (Liew *et al.,* 1998; Ranghoo *et al.,* 1999; Azuddin *et al.,* 2021). These techniques have enabled a more accurate species identification of fungi, and particularly improved our recognition of non-sporulating fungi (mycelia sterilia) that are not easily classified into known taxonomic groups based on morphological techniques alone (Guo *et al.*, 2000; Sun & Guo, 2012). Other limitations of morphological identification methods include (i) morphological characters in some fungal lineages can present problems even for trained mycologists, as they may be inadequate in providing accurate groupings within an evolutionary framework, especially at the species level (Geiser, 2004; Raja *et al.,* 2017), (ii) morphological characterisation is often unreliable in critical situations of hybridisation, the occurrence

of cryptic species, and convergent evolution (Olson & Stenlid, 2002; Hughes *et al.*, 2013; Liu *et al.,* 2017), (iii) limited numbers of morphological characters for some species that can be considered for exhaustive fungal identification (Hyde & Soytong, 2008; Ko *et al.*, 2011), (iv) morphological variables of the asexual structures of sporulating fungi, such as shape and size of conidia, are often non-uniform, making identification challenging (Raja *et al.,* 2017).

Molecular identification of endophytic fungi often relies on sequence and phylogenetic analyses of DNA amplified using specific genetic markers (Huang *et al.,* 2009; Azuddin *et al.,* 2021). A genetic marker is defined as a gene or DNA sequence with a known location on a chromosome, which can be used in species identification (Khan, 2014; NHGRI, 2022). An ideal marker for the study of fungal communities should have the following properties; (i) possess primer sites that are common in all fungi, (ii) length of the marker should be appropriate for efficient amplification and sequencing, (iii) the marker should have high interspecific and low intraspecific variations, and should be able to align across all fungi (Lindahl *et al.,* 2013; Egydio Brandao *et al.,* 2020).

Although no known markers meet the stated requirements, components of the nuclear ribosomal repeat unit (rDNA) which comprises the small subunit (SSU:16S/18S) and large subunit (LSU:23S/25S/28S) separated by the ITS region, are by far the most commonly used genetic markers for taxonomic and phylogenetic identification of fungi and other microbes (Herrera *et al.,* 2009; Raja *et al.,* 2017).

In phylogenetic identification of fungi, sequence data are used to infer the relationships that exist between organisms and the genes they possess, and the evolutionary relationships established through phylogenetic analysis are often depicted in branching and tree-like diagrams known as phylogenetic trees (Ziemert & Jensen,

2012; Choudhuri, 2014). The methods used in the phylogenetic resolution of species are broadly classified as distance based and character based methods (Burr, 2010; Haber & Velasco, 2021).

Distance-based methods organise sequences based on their overall similarity by computing the number of nucleotide substitutions between pairs of sequences (Pardi & Gascuel, 2016). The neighbour-joining method is a widely used distance-based method, and does not draw any inferences about the evolutionary processes (Hong *et al.,* 2020). Whereas, character-based methods consider specific nucleotides and take into account the number of insertions and deletions at each site. The advantage of character-based over distance-based methods is in their ability to ignore uninformative changes by separately weighing the significance of different nucleotide changes in the sequences. Examples of character-based phylogenetic analysis methods are maximum parsimony, maximum likelihood, and Bayesian inference (Saitou & Nei, 1987; Money, 2016).

Although phylogenetic trees created using sequences from single genes are quite informative, trees based on single genes are often insufficient in resolving important evolutionary relationships, therefore, it is recommended to combine data from more than one gene to provide sufficient data for the complete resolution of fungal taxa (Peterson, 2008; Johnston *et al.,* 2019). Multi-loci phylogenetic analyses involving genes such as the TEF-1α, CaM, β-tubulin, and ACT, have been useful in the species delineation of endophytic fungi from several genera such as *Fusarium, Aspergillus, Penicillium, Cladosporium, Epicoccum, Aureobasidium, Colletotrichum, Alternaria, Bionectria, Xylaria, Pestalotiopsis, Trichoderma* and *Diaporthe* (dos Santos *et al.,* 2016; Zakaria *et al.,* 2016; Tibpromma *et al*., 2018; Azuddin *et al.,* 2021).

2.2.2(a) Internal transcribed spacer region

The International Fungal Barcoding Consortium proposed the use of the ITS region of the nuclear ribosomal DNA (rDNA) gene cluster, as the primary barcode for fungal identification. The choice of ITS as fungal identification barcode was based largely on its ease of amplification, vast usage, and significantly wide barcode gap between inter- and intraspecific variation. Other advantages of the ITS region include its relatively low intraspecific and high interspecific variation, high polymorphism, non-protein coding attributes, and the presence of adequate taxonomic units in the ITS region that are able to separate sequences to species level (Schoch *et al*., 2012). More so, the enormity of reference sequences of the ITS region in public databases makes the region a preferred marker for easy molecular identification of fungi (Samson *et al.*, 2010).

The ITS region comprises two spacer regions ITS1 and ITS2, with 5.8S gene occurring at an intercalary position between ITS1 and ITS2 (Figure 2.1). Several universal primers have been designed for the amplification of the ITS region as shown in Figure 2.1 (White *et al.*, 1990; Gardes & Bruns, 1993; Schoch *et al.*, 2012).

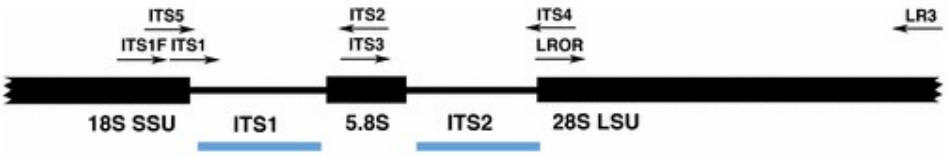

Figure 2.1. Location of the ITS, SSU, and LSU regions and universal primers within the rDNA cassette (Raja *et al.,* 2017).

Sequencing of ITS regions has been useful in the identification of numerous isolates of endophytic fungi in several studies. Deepthi *et al.* (2018) identified endophytic *Nigrospora* sp., *Pestalotiopsis* sp., *Colletotrichum* sp., and *Fusarium* sp.

from leaves of the medicinal plants *Elaeocarpus sphaericus,* and *Myristica fragrans*, using the ITS region. The ITS region was used in the identification of endophytic *Arthrobotrys foliicola* from leaf blades of paddy plants (*Oryza sativa*) (Zakaria *et al.,* 2010). Similarly, endophytic fungi in the *genera Pestalotiopsis*, *Alternaria*, *Cladosporium*, *Periconia, Pithomyces*, *Xylaria*, *Curvularia, Diaporthe, Epicoccum, Fusarium, Leptosphaeria, Lophiostoma, Nigrospora, Phaeosphaeriopsis, Phoma, Phomopsis, Schizophyllum*, and *Stagonosporopsis,* from the leaves of the mangrove plant *Rhizophora mucronata* were also identified using the ITS region (Hamzah *et al.,* 2018). Furthermore, Maadon *et al.* (2018) isolated and identified endophytic *Daldinia* sp., *Lentinus* sp., *Rigidoporus* sp., and *Polyporales* sp. from forest trees using the ITS region.

Despite the advantages of ITS sequences in the molecular identification of fungi, studies of ITS sequences in the International Nucleotide Sequence Database have revealed the non-uniformity of variability in the ITS regions of some fungal groups, especially members of the Ascomycota such as *Alternaria, Aspergillus, Cladosporium, Penicillium,* and *Fusarium,* which have narrow or no gaps in the barcodes of their ITS regions (Lindner & Banik, 2011; Schoch *et al.*, 2012). To address the limitations in the use of the ITS as universal barcode for the identification of some fungal groups, the use of several protein-coding regions such as ACT, TEF-1α, β-tubulin, and CaM genes, has been suggested as secondary barcodes. The concatenated alignment of the ITS region with one or more of other protein-coding genes has been found to be more efficient in the precise species identification of various fungal groups (Tekpinar & Kalmer, 2019).

2.2.2(b) Large subunit of ribosomal DNA

In a vast majority of fungi, the rDNA comprises the small subunit (SSU, 18S), ITS (ITS1+5.8S+ITS2), and large subunit (LSU, 25-28S) regions. Although the ITS region is recognised as the official barcode for fungal identification, the LSU region may be specifically targeted in amplicon-based sequencing studies of fungal populations. A prominent advantage of the LSU over the ITS region is the occurrence of sequence variation in the LSU divergent domains (D1 and D2). More so, the presence of large collections of LSU reference sequences in repositories such as GenBank, makes the LSU region a suitable and convenient choice for genus or higher level taxonomic classification of a vast diversity of fungi, including yeasts and non-sporulating fungal groups (Mycelia sterilia) (Fell *et al.*, 2000).

The nuclear ribosomal LSU region is situated immediately downstream of the ITS region in the rDNA cassette (Figure 2.1), and comprises highly variable domains (D1 and D2) which are flanked by relatively conserved regions. Several primers have been designed for the amplification of the variable regions of the LSU in different fungal genera (Vilgalys & Hester, 1990; Hibbett & Vilgalys, 1993; Hopple & Vilgalys, 1999).

The LSU region has been used independently, and in combination with other loci for the phylogenetic identification of endophytic fungi from various plant parts. Crozier *et al*. (2006) identified various types of endophytic fungi from different genera such as *Hypocreales*, *Clavicipitaceae*, *Bionectria*, *Nectriaceae*, and *Xylaria*, from stems and pods of cocoa (*Theobroma cacao*) in natural forest ecosystems and agroecosystems of Latin America and West Africa, using the LSU region. Endophytic *H. endiandrae*, *H. livistonae*, and *P. terricola*, isolated from the spines of rattan (*Calamus castaneus*) were identified using combined LSU and ITS regions (Azuddin *et al.*, 2021). Combined