ISOLATION AND CHARACTERIZATION OF ENDOPHYTIC FUNGI ASSOCIATED WITH FRESH BLOOMING FLOWER OF *Rafflesia kerri* IN LOJING HIGHLANDS, KELANTAN

by

ALIA NABILA BINTI MAHADI

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DECLARATION

I declare that the thesis entitled "Isolation and Characterization of Endophytic Fungi Associated with Fresh Blooming Flower of *Rafflesia kerri* in Lojing Highlands, Kelantan" is the result of my research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature for any other degree.

Signature :

Name : ALIA NABILA BINTI MAHADI

Date : 23/ 7 / 25

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Isolation and Characterization of Endophytic Fungi Associated with Fresh Blooming Flower of *Rafflesia kerri* in Lojing Highland, Kelantan

ABSTRACT

This study's main aim was to study and identify endophytic fungi obtained from flower elements of *Rafflesia kerri* in the Lojing Highlands, Kelantan, Malaysia, a rare parasitic plant that may possibly have an association with the internal microorganisms including fungi. The primary concern in this area of research is the very little knowledge about the types of endophytic fungi associated with *R. kerri*, their roles, and the effects of these fungi on plant health and survival in tropical climates. Through the use of culture techniques on Potato Dextrose Agar (PDA) medium combined with microscopic examination of the morphology of the isolated fungi, the purpose of this study was to determine the isolates of endophytic fungi and their characteristics from flower elements of *R. kerri*. Samples consisting of petal A, petal B, and ramenta were sterilized and then incubated in PDA medium for approximately two weeks before being studied. Fungi from the samples exhibited a great degree of morphological variation including colour coloration, texture, speed of growth, size of spores and hyphae, and several species of endophytic fungi appeared to contribute to processes related to the breakdown of organic matter, the cycling of nutrients, and the defence against diseases. These results indicate that additional studies need to be conducted utilizing methods that will allow for the accurate identification of fungi species such as molecular techniques (DNA markers), and further investigate the potential applications of biotechnology in the fields of agriculture, medicine, and ecosystem management.

Pengasingan dan Pencirian Kulat Endofit yang Dikaitkan dengan Bunga Segar *Rafflesia kerri yang Mekar* di Tanah Tinggi Lojing Highland, Kelantan

ABSTRAK

Matlamat utama kajian ini adalah untuk mengkaji dan mengenal pasti kulat endofit yang diperoleh daripada unsur bunga *Rafflesia kerri* di Tanah Tinggi Lojing, Kelantan, Malaysia, tumbuhan parasit yang jarang ditemui yang mungkin mempunyai kaitan dengan mikroorganisma dalaman termasuk kulat. Kebimbangan utama dalam bidang penyelidikan ini ialah pengetahuan yang sangat sedikit tentang jenis kulat endofit yang dikaitkan dengan *R. kerri*, peranan mereka, dan kesan kulat ini terhadap kesihatan dan kelangsungan hidup tumbuhan di iklim tropika. Melalui penggunaan teknik kultur pada medium Agar Dekstrosa Kentang (PDA) digabungkan dengan pemeriksaan mikroskopik morfologi kulat terpencil, tujuan kajian ini adalah untuk menentukan pengasingan kulat endofit dan ciri-cirinya daripada unsur bunga *R. kerri*. Sampel yang terdiri daripada kelopak A, kelopak B, dan ramentum telah disterilkan dan kemudian diinkubasi dalam medium PDA selama kira-kira dua minggu sebelum dikaji. Kulat daripada sampel mempamerkan tahap variasi morfologi yang besar termasuk warna, tekstur, kelajuan pertumbuhan, saiz spora dan hifa, dan beberapa spesies kulat endofit nampaknya menyumbang kepada proses yang berkaitan dengan pecahan bahan organik, kitaran nutrien, dan pertahanan terhadap penyakit. Keputusan ini menunjukkan bahawa kajian tambahan perlu dijalankan menggunakan kaedah yang akan membolehkan pengenalpastian tepat spesies kulat seperti teknik molekul (penanda DNA), dan menyiasat lebih lanjut potensi aplikasi bioteknologi dalam bidang pertanian, perubatan dan pengurusan ekosistem.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

R.kerri	*Rafflesia kerri*
R. arnoldii	*Rafflesia arnoldii*
R. cantleyi	*Rafflesia cantleyi*
FSB	Faculty of Earth Science
DNA	Deoxyribonucleic Acid
PDA	Potato Dextrose Agar
pH	Potential of Hydrogen
DH2O	Distilled Water
FIAT	Faculty of Agro-Based Industries
rRNA	Ribosomal Ribonucleic Acid
ITS	Internal Transcribed Spacer
gDNA	Genomic Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction
SSU rRNA	Small Subunit Ribosoamal Ribonucleic Acid
T. harzianum	*Trichoderma harianum*
T. lixii	*Trichoderms lixii*

LIST OF SYMBOLS

Cm	Centimeter
˚C	Degree(s) Celcius
%	Percent
KM^2	Square Kilometer
ml	Mililiter
µm	Micrometers
Sec	Second
Min	Minute

CHAPTER 1

INTRODUCTION

1.1 Background of Study

The *Rafflesia kerri* is the largest flower in the world, and it has been promoted internationally as a symbol of unique beauty and is becoming increasingly popular as a tourist icon. This flower is located only in Southeast Asia, including Peninsular Malaysia and southern Thailand, Borneo, Sumatra, Java, and the Philippines. In Peninsular Malaysia and southern Thailand, the genus is endemic; therefore, *R. kerri* is the only species from this genus. The plant genus Rafflesiaceae contains some very interesting genera, with Rafflesia being the most well-known obligate holoparasitic plants (Wicaksono et al., 2020). It has a very strange way of growing, without true leaves, stems or roots; however, the only portion of the plant that is visible outside of its host is the flowers that form the large flower clusters. R. arnoldii produces flowers that can reach 1 m across; however, the blooms of *R. kerri* are usually 50-90 cm in diameter and have an extremely foul odor to attract flies for pollination during the reproductive process.

In Malaysia, Rafflesia is also referred to as "*Bunga Pakma*" (Molina et al., 2021), and there are fourteen types of Rafflesias in Malaysia. A new type of Rafflesia called *R. kerri* was identified in 2002 by Forest Gan and (Haji Adam et al., 2013) Matthew Wong in the Main Range area at the border of Kelantan and Perak in Peninsular Malaysia (Hamzah et al.) and it is considered the second largest type of *R. kerri*, after *R. arnoldii* (Latshaw & Jensen, 1970).

As a member of the Endoparasites Class, Rafflesia grows within its host plant until approximately 160 days after germination, when the host vine is destroyed (Wicaksono et al., 2020). A host-specific parasite, Rafflesia will only infect vines of the genus Tetrastigma (Molina et al., 2025)

The Rafflesia was first identified in 1818 during an expedition in Bengkulu, Indonesia. The species was named after Sir Thomas Stamford Raffles, leader of the expedition, along with his fellow scientist Dr. Joseph Arnold and their local guide, who were all credited with the identification of the genus. The genus Rafflesia is found in Northern Regions of the world and includes the Kra Isthmus, Malaysia, the Philippines, Borneo, Java, and Sumatra (Grismer & Davis, 2018). Rafflesia have been described to have a diameter of a meter or more; there are forty-two known species of Rafflesia. *R. arnoldii* is distinguished from other Rafflesia species by the recorded bloom diameters of up to 107 cm as such, it is considered the largest known species of Rafflesia. It is a unique example of floral gigantism and is an Indonesian endemic. The smaller species, *R. cantleyi* is endemic to Peninsular Malaysia alone. The limited distribution of this species highlights the uniqueness of the Malaysian rainforests and emphasizes the need for conservation efforts of this parasitic plant (Demetrio et al., 2025). The native inhabitants of Peninsular Malaysia used Rafflesia to treat several health problems even before it had been named. As a parasitic plant, Rafflesia grows on the Tetrastigma vine (Vitaceae) growth begins as a small lump located just above the root of the host vine. After approximately 6-9 months, the lower portion of the bud containing the ovary of the male flower develops into a fruit filled with thousands of seeds. These seeds are dispersed by animals including but not limited to squirrels and tree cigars.

In less than twelve months, the "maxi cabbage" paired with the flower will develop either pink or brown colour with no leaves, or leaves that are fibrous, or roots that appear to be attacking the grapevine tissue (Bosma et al., 2000). It is interesting to note, however, that the flowers are unisexual rather than bisexual and they emit scents that appear to be attracting flies like Lucilia and Chrysomya, Calliphoridae (Zhang et al., 2018). The fact that the vine contains chlorophyll and is therefore presumably a finer filament due to the course of evolutionary development, was noted as well.

This is but one of many examples of how life is complex and often quite surprising, as is the case with Rafflesia, the largest flower in the world that also has an intimate relationship with fungi that is full of the joys of evolutionary peculiarity. Since Rafflesia is a holoparasite, 100% of its carbon and nutrient supply comes from the host plant (usually some type of Tetrastigma) on to which it attaches itself at various stages of its life cycle. Like many plants, some species form mutualistic relationships with mycorrhizal fungi that assist the plant in acquiring necessary nutrients. The enhanced nutrient status of the host root system may therefore indirectly benefit *R. kerri* via this symbiotic system. Second, endophytic fungi (such as endophytic Unga), endophytic fungi that live within plant tissues, can enhance the host's tolerance to environmental stresses and resistance to pathogens. Although it appears unlikely that Rafflesia contains endophytic fungi, its host plants are very likely to contain them. The endophytes appear to facilitate Rafflesia parasitism indirectly by increasing the host plant's resilience. After flowering, the Rafflesia flowers die and the fungi play a crucial role in breaking down the organic matter. Therefore, the areas in which Rafflesia occurs are likely to serve as reactors for the potential recycling of nutrients generated by the activity of decomposer fungi that would improve soil health and potentially offer benefits to both host plants and other nearby flora. In addition to that, the relationship of *R. kerri* with fungi is a mutualistic relationship. It is also possible that fungi could be the cause of infection of Rafflesia, or that a fungus infecting one of Rafflesia's host plants. The interaction of fungal pathogens and plants in tropical ecosystems, as well as the interactions of those same pathogens with parasites of plants is an area of study that is just beginning however, based on what we do know, if a fungus were to discover either Rafflesia or one of its hosts it would likely decrease the likelihood of finding Rafflesia, as stated by Marcell, would allow us to package Rafflesia more easily. Conversely, there are also fungi that are beneficial to plants and can potentially reduce the growth of pathogens. These relationships can be quite dynamic.

Other than this, there are micro fungi called endophytic fungi that live within plant tissues and cause no visual harm to the plant. There are many reports of the existence of endophytes in the tissues of woody plants and grasses (Jha et al., 2023). All plants will have some type of endophyte since endophytes produce compounds that aid in the survival of the plant (Afzal et al., 2019). The fact that endophytic compounds are being isolated and characterized is particularly important since they may eventually become part of modern medicine such as antibiotics and antimycotics as well as treatments for various types of cancers (Zhang et al., 2018). Tropical and temperate rainforests are considered two of the most ecologically rich ecosystems in terms of biological diversity found on land. Many plants found in these regions are likely to have endophytic fungi and biosynthetic organisms present in them that are unexplainable due to the unique environment of the plants, as well as their ethnobotanical or endemic history (Chowdhary et al., 2012). Rafflesia is a species native to the Southeast Asian region, and like many other species in the region, there has been little research on Rafflesia and its endophytic flora.

The possible association of Rafflesia with endophytic fungi highlights the dependence of tropical ecosystems on each other and illustrates the importance of fungi in the cycling of nutrients, the maintenance of plant health, and the establishment of ecological balance, all of which can have a significant indirect impact on the life cycle of Rafflesia. To further understand the relationship between Rafflesia and its potential endophytic flora, additional studies need to be conducted to provide a basis of understanding of these associations, especially in areas where Rafflesia grows in dense tropical environments with many different species.

1.2 Problem Statement

Rafflesia kerri is an unusual and interesting flowering parasitic plant that grows only in Southeast Asia (Lojing Highlands Kelantan). The *R. kerri* has a few distinct features such as large flowers and a stinking odor that is a feature of all parasitic plants. It is these unique qualities that make *R. kerri* especially of interest to scientists. A symbiotic relationship has developed among many parasitic plants (including *R. kerri*) with endophytic fungi that grow within plant tissues without harming the plant. Endophytic fungi create compounds that enhance plant health and improve a plant's ability to withstand environmental stressors, and also increases a plant's ability to resist local disease and to survive in extreme environments. Although there have been numerous investigations of parasitic plants, there is still much that is unknown about the types of endophytic fungi that live in *R. kerri*. This lack of information limits scientists' ability to understand *R. kerri's* ecological roles and *R. kerri's* possible use in agriculture, medicine, and biotechnology.

It is imperative to identify whether *R. kerri* has a symbiotic relationship with endophytic fungi due to the relatively low amount of data available. The main goal of this proposal is to collect fresh blooming flowers from *R. kerri*, and then to identify/isolate the endophytic fungi living within the flowers. The focus of the research will be on identifying the morphological and physical characteristics of the fungi associated with the plant as well as the role of the fungi in the biological interaction of the fungi and the plant. By studying these fungi, researchers can gain a greater understanding of the fungi themselves as well as how they have been able to affect the life cycle and ecological adaptability of *R. kerri*. Furthermore, research will broaden the scientific community's understanding of the multiple forms of synergy that exist in nature, specifically mutualistic relationships between parasitic plants and fungi.

This research will take the scientific and technological aspects of discovery to previously unseen levels. This proposed investigation on *R. kerri* and endophytic fungi may offer new insights into novel compounds, mechanisms, and/or properties that may be useful in biotechnology, biosciences, agriculture, etc. or aid in solving environmental problems. More importantly, the study seeks to fill the void in knowledge by describing the unexplored world of endophytic fungi therefore, providing the needed information to further the understanding of plant-fungi interactions. Overall, the research described above seeks to establish a new area of exploration of the ecological significance and potential uses of endophytic fungi to describe their presence in nature and their contributions to science.

1.3 Objectives

The present investigation is expected to accomplish the following purposes.

1. To identify endophytic fungi that are associated with fresh blooming flowers of *Rafflesia kerri*.
2. To identify and characterize the endophytic fungi that were identified from the flower of *Rafflesia kerri* through morphological identification and 18S rRNA sequencing.

1.4 Scope of Study

This study aims to collect, identify and characterize the endophytes of flowering *Rafflesia kerri.* The greatest difficulty is to sample the flowers in the field and to carry out the fungi isolation process - the first step of isolating endophytic fungi being the culturing of tissues from the flowers in the lab using appropriate microbiological conditions.

Therefore, the separation of endophytic fungi will be carried out on *R. kerri* flower samples collected during the spring from different types of flower organs at Lojing Highland, Kelantan. Biodiversity by species of these fungi will be estimated using the morphological structure and sequence data.

This part will contain data about the length, the morphological features of the endophytic (Chowdhary et al., 2012) fungi isolated that will allow to make an examination of the fungi. Observations of fungi parts like hyphae, spores, etc. are made possible through microscopy and other characteristics of fungi are visible with the help of staining and imaging methods. A problem of detail is apparent in the identification and characterization of fungi because the authors only use morphology and not genetic or physiological methods (Jha et al., 2023).

1.5 Significance of Study

The importance of this research lies in the exploration of specific endophytic fungi in *Rafflesia kerri* an extremely rare, endangered Corpse Flower found in the Lojing Highlands, Kelantan. The role of endophytic fungi in assisting a plant's health includes providing enhanced nutrient and water absorption, disease protection, and improved growth of the plant (Afzal et al., 2019). In some cases, endophytic fungi develop a symbiotic relationship with their host by existing between the cell spaces of the plant, which allows them to grow and function together (Afzal et al., 2019). Identifying and characterizing the fungi present on Rafflesiaceae plants provides additional understanding of the diversity of fungi and potentially identifies new taxa.

In addition, the findings of this study provide substantial knowledge regarding Rafflesiaceae flowers and endophytic fungi that are associated with them, specifically the *R. kerri* flower. Understanding how the fungi interact with the host plant, and the functions of the fungi within the host plant, under extreme environmental conditions enhances our knowledge of the relationship between the fungi and the host plant.

This research adds to the body of scientific knowledge by providing more detailed information about the morphology of endophytic fungi, ultimately creating potential opportunities for applications of endophytic fungi in ecological and biotechnological studies. This research could also provide opportunities for the use of endophytic fungi in numerous areas of human endeavour, such as medicine, agriculture and ecosystem management thereby contributing to the betterment of society and the environment.

CHAPTER 2

LITERATURE REVIEW

2.1 History of *Rafflesia kerri*

This plant has been recognized to only grow in very specific regions of the world. *Rafflesia kerri* is only found in a small region of the mountainous areas of peninsular Malaysia and the southern parts of Thailand, specifically in the Titiwangsa mountain range. In addition to this limited geographical location, *R. kerri* is very susceptible to habitat loss due to the effects of climate change and habitat fragmentation. One of the main populations of *R. kerri* can be located in the Lojing Highlands, which supports a large population of *R. kerri*. This region provides ideal growing conditions for *R. kerri*, including a good environment and enough host plants. (Asfarina et al., 2022)

R. kerri is dependent on other plants of the genus Tetrastigma, similar to all other species of the Rafflesiaceae family. As a result, *R. kerri's* ability to grow is only in those locations where Tetrastigma species grow. Although *R. kerri* produces flowers of impressive size, they do not last long, as the gestational period of *R. kerri* is nine months. Flowers in the attic/petals on the wind, and the flower itself blooms for only about a week.

The primary reasons that *R. kerri* is considered to be threatened include deforestation and human activities, such as the removal of its buds for alleged medicinal purposes. Habitat loss and destruction of habitats resulting from logging and land use changes to agricultural uses represent another serious threat to the continued existence of this species. Due to these factors, *R. kerri* is currently listed as vulnerable. Therefore, protecting what remains of the habitat of this species is becoming increasingly important for conservation efforts to preserve remaining habitat. Protecting the Lojing Highlands is critical for ensuring the continued existence of *R. kerri*, as it is one of the few areas where the species is still present. (Asfarina et al., 2022). Additionally, this species plays a vital role in the diversity of local flora (Cross et al., 2020). It adds to the unique characteristics of the area and serves as a food source for various fauna and pollinators.

To protect *R. kerri*, we need to work towards mitigating the current threats to the species' existence while increasing our knowledge of the ecological functions of *R. kerri*, specifically related to interactions between *R. kerri* and its Tetrastigma hosts. *R. kerri* is (Zhang et al., 2018) a rare inshore species, with limited occurrences in unique environments. We must implement conservation measures for tropical ecosystems to protect not only *R. kerri*, but also to conserve many other unusual species found in a biodiversity-rich area.

2.2 Taxonomy and Morphology of *Rafflesia kerri*

2.2.1 Taxonomy of *Rafflesia kerri*

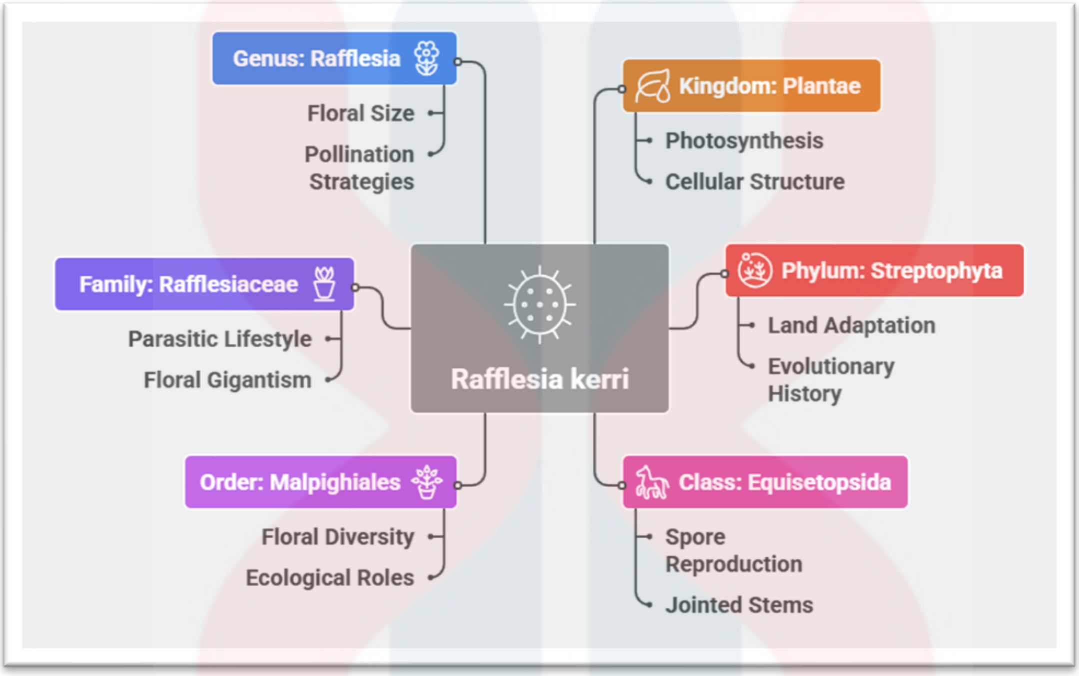


Figure 2. 1: Taxonomy of *Rafflesia kerri*

2.2.2 Morphology and Anatomy of *Rafflesia kerri*

Research on the Rafflesia has shown a number of different characteristics such as morphology, anatomy, and other interesting features related to the flower and endophyte (Hidayati & L. Walck, 2016). There have been a number of recent studies which show many interesting aspects of the flower's anatomy and the endophyte of Rafflesia. Developmental comparison and gene expression studies of (Kagan & Hejnol, 2024) demonstrated that despite their similarities in morphology, the flowers of Rafflesia and Sapria are not homologous due to significant differences in their structure (Nikolov et al., 2013). The endophyte grows through an embryo and the flowering shoot originates from the same type of undifferentiated cells as the endophyte (Nikolov et al., 2014). The host plant responds to the parasite only once the parasite develops into a flower. The endophyte may be either commensal (Nikolov et al., (2014) or mutualistic with respect to the host plant (Patra et al., 2023).

There was a positive correlation observed between flower diameter and ovule size within species (Burd et al., 2009). On the contrary, they did not observe an overall trend between these two variables for *R. azlanii, R. cantleyi, R. hasseltii* and *R. kerri* (Hidayati & L. Walck, (2016). The ovules of *R. azlanii and R. cantleyi* were significantly larger than the ovules of *R. kerri*, however *R. kerri* had the largest flower among the four species studied (Sofiyanti & Yen, 2012). Typically, the seeds of the Rafflesia are chestnut shaped, brown when fresh, approximately 500-1,500 µm in length (Hidayati & L. Walck, 2016).

2.3 Symbiotic Relationships between Endophytic Fungi and *Rafflesia kerri*

Endophytic fungi are microscopic fungi that live in plant tissue. These fungi do not typically cause disease in their host; they have an essentially neutral symbiosis with plants. The literature describes many potential benefits to the host plant of endophytic fungi including growth enhancement, increased resistance to pathogens, and increased tolerance to both biotic factors (insects) and abiotic factors (drought, salinity). Many of the fungi have mutualistic relationships with the plant where each partner receives a benefit from the relationship. However, among some fungi, are opportunistic pathogens that can use a stressed or compromised immune system in a plant to establish a mutually beneficial relationship and harmful relationship (Marcos et al., 2016).

In previous research, endophytic fungi were shown to influence the growth, health and ability of a plant to withstand its environment, in addition to various aspects of the plant's soil (Toppo et al., 2023). In order to isolate and characterize any fungi that provide a benefit to *R. kerri*, this study will investigate in detail the association between the fungi communities in the soil of *R. kerri* and the plant itself. Once the fungi are identified, they may be able to identify compounds that would be useful for developing medicine or as a biotechnology product. Investigating the endophyte community in the habitat of *R. kerri* has the added value of providing an ecological function by providing a better understanding of the interaction between the plant and its microbiome. As such, the discovery of endophytic fungi in the habitat of *R. kerri* will aid in developing effective conservation plans for *R. kerri*, as well as other plants with similar ecological relationships.

2.4 Protocol Used to Isolate Endophytic Fungi from *Rafflesia kerri*

While the procedures for extracting endophytes from Rafflesiaceae (*Rafflesia kerri*) have already been developed, most of them mainly focus on avoiding contamination by external organisms; this is why only the inner parts can be extracted. The first step is to collect tissue samples from fresh flowers, followed by using surface sterilization methods to remove any external microorganisms. Sterilized tissues are then cultured on selective media (for example, potato dextrose agar – PDA), where fungi growth is promoted. Afterward, fungi pure culture was obtained, and the isolates were used to successfully identify tropical and rare plants (Demeni et al., 2025). These studies collectively offer a starting point for the isolation of the various fungi communities found in *R. kerri* to identify the members that are best adapted to its parasitic lifestyle and to its plant host as well as its surroundings in the Lojing Highland region. Molecular techniques (such as DNA sequencing of the ITS regions of rRNA genes) can generate additional data at an extremely high level of resolution to facilitate identification of fungi (Tedersoo & Nilsson, 2016) when the association between a fungus and its host is ambiguous. A molecular technique could potentially reveal *R. kerri* specific fungi species or previously reported fungi with possibly novel bioactive properties.

2.5 Morphological, Physiological, and Molecular Characteristics of Endophytic

2.5.1 Fungi Isolated from *Rafflesia kerri*

Morphological and physiological traits of fungi indicate the possible extent of functional diversity when isolated. For example, using microscope to study colony morphology, spores, and hyphae is a useful way to determine taxonomy for each fungus (Norhazlini et al., 2020). Ideally, traditional methods of morphological classification will be used along with new data generated by modern molecular techniques to realize the overall objective of identifying each fungi isolate.

Physiological characterization involves assessing the ability of each isolate to grow in different environmental settings, as well as measuring the growth of different isolates in response to varying levels of nutrition or stressful conditions (Zulhazman et al., 2021). These assessments give some indication of how well the fungi may have adapted to an environment that would likely be representative of the highland region in which *R. kerri* blooms.

2.6 Bioactive Potential of Endophytic Fungi Isolated from *Rafflesia kerri*

Identification of bioactive potential is critical for candidate discovery with possible pharmaceutical and agricultural applications. Various kinds of bioactive metabolites, such as those active against microorganisms, fungi, as well as antioxidant agents from these endophytic species, appear to function as protective agents against the host from pathogenic and various environmental stressors. A previous study reported that the fungi isolated from Malaysian medicinal plants were good sources of bioactive compounds, suggesting that isolates from these endophytes of *R. kerri* may also be potential novel sources for bioactive compounds (Yap et al., 2017). To explore potential medicinal use, extracts from each fungi isolate are subject to bioactivity screening against typical pathogens and free radicals (Asomadu et al., 2024).

2.6.1 Endophytic Fungi

Microorganisms referred to as endophytic fungi grow within plant types (Wen et al., 2022), which may stimulate a plant's growth by inhibiting phytopathogens and insect pests or by producing compounds that promote plant growth (Jha et al., 2023). Some of these endophytic fungi function as saprophytes, many genera of which function in the process of leaf and plant maturity and leaf decomposition (Wen et al., 2022).

2.6.2 Classification of Endophytes Fungi

Two primary classifications exist for endophytes; based on their differing taxonomic classifications, host ranges, patterns of colonization and transmission, ecological functions and tissue specificity (Rodriguez et al., 2009). The first classification includes the two categories of non-cultivated (NC) endophytic fungi of the grass (Clavicipitaceae) while the second classification includes the non-clavicipitaceae NC endophytic fungi in vascular and non-vascular plants (Li et al., 2025), create an alternate classification system using phylogenetic data and life history characteristics where endophytic fungi are classified into four classes (Chowdhary et al., 2012). They typically belong to either the grass family Poaceae, or less commonly Cyperaceae, and are typically transmitted via seed vertically (Afzal et al., 2019) and most of the time the seed belongs to the Hypocreales order of the Ascomycota class of the phylum. Most of the time, clavicipitaceous fungi of this class colonize intracellular spaces of leaf sheaths, rhizomes and leaf blades (Afzal et al., 2019). Clavicipitaceous fungi are very important as insecticides and mammalian herbivores repellents due to chemical compounds such as loline, peramine (Chowdhary et al., 2012) and ergot and lolitrem alkaloids (Chen et al., 2011). Some research indicates that there are many types of fungi that can be used to manage tiny worm parasites of plants known as nematodes. For example, when a species of grass known as Festuca arundinacea is infected with the fungus Acremonium coenophialum, the population of nematodes in the soil is reduced (Sunitha et al., 2013). Additionally, some fungi of the Epichloe festucae group produce compounds like indole derivatives, sesquiterpenes, and acetamide, which provide protection against pathogens (Niones & Takemoto, 2014).

2.6.3 Biodiversity of Endophytic Fungi

Despite that we know endophytic fungi are very varied with over 1 million yet to be discovered (Wen et al., 2022) it has been shown in studies of endophytic fungi of various hosts for the past 20-30 years that endophytic fungi occur in many land plants (Li et al., 2025). For the past 25+ years, researchers have investigated the areas where endophytes exist and the biological/ecological function of endophytes in Europe and North America (Petrini, 1996). Additionally, endophytes have been detected in plants in several types of environments, e.g. tropical, temperate, and boreal forest ecosystems (Selim, K., 2012). Many of the endophytic fungi are in gymnosperms in temperate regions (Asfarina et al., 2022) however, they have also been detected in grasses, liverworts (Azuddin et al., 2023), nonvascular plants, algae, ferns, and plants closely related to ferns as well as mosses (Rodriguez et al., 2009). Tropical forests contain the largest number of different endophytic fungus types and include woody flowering plants (Rodriguez et al., 2009).

Endophytic fungi represent one of the most significant components of natural world fungi diversity. Studies have indicated that virtually all plants that were studied have endophytic fungi; and many tree species have numerous endophytic species, with numbers ranging from hundreds to thousands (Li et al., 2025). Much of the initial research was focused on estimating the number of fungi species and the relationships between plants and fungi (Priyashantha et al., 2023). Initially scientists estimated the number of fungi to be about 1.5 million species based on the idea that for every type of plant there would be six species of fungi. This estimate was later increased to 2.27 million (Blackwell, 2011). It was proposed that the 270,000 known plant species could support up to 1.38 million unique species of fungi that reside in those plants.

Conversely, the latest discoveries of fungi especially through new technology, provide evidence that the total number of fungi species is much smaller than prior estimates.

The primary groups of fungi that are considered endophytic fungi are those that belong to Ascomycota, Basidiomycota, and Zygomycota (Mucormycota). The dispersal of the fungi depends on environmental conditions such as temperature, humidity, and the type and age of the plant tissue (Priyashantha et al., 2023). Further investigation of older plant tissue will show that old plant tissue contains more endophytic fungi than young plant tissue (Volk et al., 2022). More recent studies have also demonstrated that Dothideomycetes and Sordariomycetes are two of the most important orders of fungi that contribute to the endophyte community from medicinal plants; however, the number of endophytic fungi can vary greatly depending on the region.

It is likely that there are significantly more undiagnosed endophytic fungi than there are diagnosed ones since most research on endophytic fungi diversity was done using culture dependent techniques. High-throughput sequencing provides a method for recovering additional endophytic fungi resulting in a reliable record of endophytic fungi diversity (Chen et al., 2020). There have been several studies regarding the distribution and biodiversity of endophytic fungi in host plants in the last 20 years. Some of the most reported species of endophytic fungi are Alternaria, Aspergillus, Fusarium, Cladosporium, Penicillium, Trichoderma, Acremonium, Chaetomium, Neurospora, Epicoccum, and 12 species of Curvularia, Arthrinium (Rashmi, 2019).

Sterilizing the outer surfaces of plants and dividing the plant parts into smaller sections to allow for sterilization of those areas in addition to isolating the fungi are two key steps in identifying the types of fungi that grow within a host plant's tissues. The effectiveness of surface sterilization will depend upon the method(s) used as well as the area of the tissue or plant from which the sample was taken; this is due to variations in the effectiveness of different methods for killing the epiphytic fungi growing on the external layers of the plant as well as promoting the growth of the mucosa growing internally in the tissues (Sahu et al., 2022).

2.6.3.1 Multifaceted Interaction between Endophytic Fungi and the Plants

Interactions between endophytes and host plants. Endophytes can be symbionts, commensals, decomposers or latent bacterial pathogens (Akram et al., 2023). In addition to being plant associated for some portion of their life cycle, most endophytes provide a benefit to the plant and help to defend the plant against disease (Li et al., 2025). Most endophytic fungi spend the greater portion of their host's lifetime in a latent phase (Beachy) and will remain non-pathogenic unless the environmental conditions of the fungi are such that the transition to a pathogen is triggered; or if the host has reached an appropriate developmental stage to support the growth of the fungi (Peng et al., 2021). However, very little information exists regarding what the external or internal stimuli would be to induce the endophyte to become a pathogen. For this reason, comparative studies of gene expression are required to understand the dynamic nature of endophytism, how the same microbial species can act as a mutualist or pathogen (Khare et al., 2018).

Important endospheric colonizers - 13 LGT (Lateral Gene Transfer) (Abby et al., 2012). The number of bioactive compounds found in endophytes from tropical regions is significantly higher than those from temperate regions and include unique metabolisms (Abby et al., 2012), thus emphasizing that the host plant plays a major role in influencing the metabolism of endophytic microbes.

2.6.3.2 Secondary Metabolites Production of Endophytic Fungi

Plant-based natural products include a variety of compound types, including fatty acids; acetylenes; monoterpenoids, iridoids, sesquiterpenoids, diterpenoids and triterpenoids (as part of terpenoids), steroids, phenolic-rich essential oils, which contain mainly terpenoids and phenylpropanoids, phenols, phenylpropanoids, flavonoids, tannins, anthocyanins, quinones, coumarins, lignans, alkaloids, and also saponins and cardiac glycosides, among the numerous other examples of glycosidic derivatives (such as germacrone or sesterterpenoids) (Coyago-Cruz et al., 2023). In view of the fact that endophytes produce secondary metabolites through chemo-synthesis in plants, it is possible to use them as an alternative source of these chemical groups for therapeutic applications and agriculture (Ogbe et al., 2020). The lack of toxicity towards cultured eukaryotic cells may have been due to a symbiotic relationship between the EF and the plant host if there were to be secondary metabolite drugs that have toxic side effects in humans, then the relationship between the EF and the plant could be one reason why there would be no toxic side effects (Rajamanikyam et al., 2017). Secondary metabolites produced by fungi provide them a benefit as well, such as being used against competitors or in plant-pathogen interactions when stressed (Ogbe et al., 2020).

2.7 History of DNA Sequence-Based Fungal Identification

Although the molecular marker was available from early 1970s, the use of DNA sequence for fungi evolution did not begin until the early 1990's when PCR and DNA sequencing were developed along with development of the fungal primer system by (Bergeron & Drouin, 2008) utilizing ITS1 and ITS4 primers which are currently being used.

By the mid 1990's DNA sequencing had become a common method for identifying fungi. The large datasets generated on the D1/D2 rDNA domains of hundreds of yeast species and demonstrated that they could be used to identify between species that were very closely related (Yap et al., 2017). Another study performed by (Bergeron & Drouin, 2008) on basidiomycete yeasts demonstrated that ITS separated between very closely related species more effectively than D1/D2; whereas, IGS separated between species and strains.

These advancements in identifying species led to an explosion of the number of new species identified as well as increased ecological data on fungi. At present, both the D1/D2 domain and ITS are standards in yeast identification. For filamentous fungi and macro-fungi, mushrooms, ITS is commonly used because it is more variable (Zain et al., 2020).

Although ITS has been used for decades prior to it being formalized as a "fungal barcode" in 2012, ITS remains one of the most widely used tools in the mapping and identification of fungi species in public databases.

CHAPTER 3

MATERIALS AND METHOD

3.1 Materials

3.1.1 The Study Area

This study is located within the Rafflesia Conservation Area in Lojing Highlands, Kelantan (Figure 3.1). The Lojing Highlands is famous for *Rafflesia kerri*, which is the second largest Rafflesia species in the world, second only to *Rafflesia arnoldii* found in West Sumatra, Indonesia. There is also an Orang Asli (indigenous people) community of Temiar tribe living in the vicinity of the conservation area in Kampung Jedip, Lojing Highlands. The Rafflesia Conservation Area is about 15 kilometres away from Cameron Highlands, another well-known tourist area in the neighbouring State of Pahang. Located at the foot of the Main Range (also known as Titiwangsa Range), this area is rich with tropical rainforests that have abundant species of plants and animals and inhabited by indigenous peoples that coexist with nature.

Data analysis for this project was completed at the Research Laboratory 1, Faculty of Agro-Based Industries (FIAT), University Malaysia Kelantan (Jeli Campus) between December 2024 through July 2025. The reason for selecting this laboratory was the availability of specialized equipment such as a laminar flow hood, growth chamber, autoclave and refrigerator to create optimal laboratory conditions.

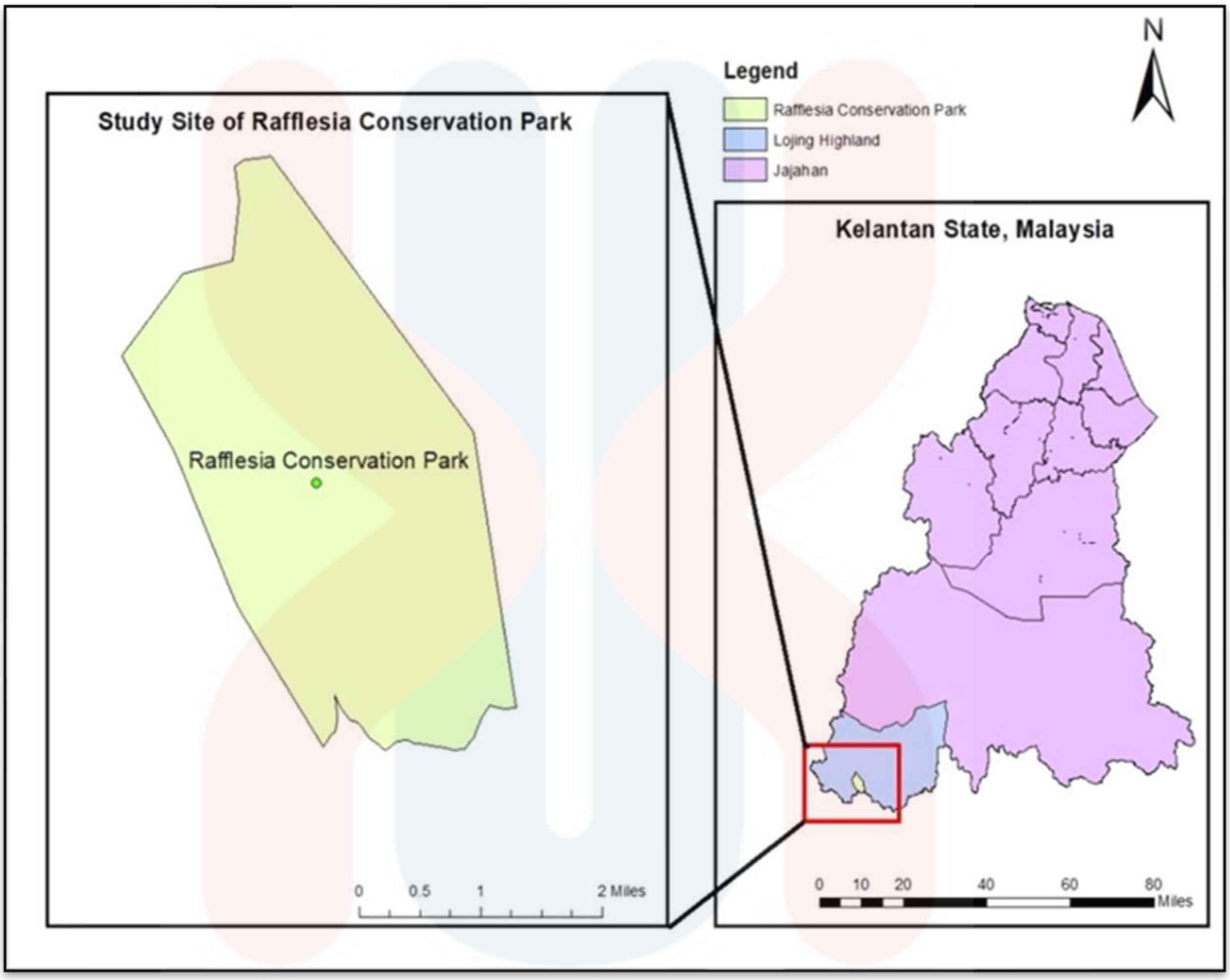


Figure 3. 1: Map of Lojing Highland, Kelantan

3.2 Materials and Apparatus

3.2.1 List of Chemicals

The chemicals used in this study are listed in Tables 3.1

Table 3. 1: List of Chemicals

No.	Items	Usage/Function
1.	70% ethanol	Sterilization of the sample to remove contaminants from outside the sample.
2.	Sodium hypochlorite solution (5.25%)	Disinfection of microorganisms present on the surface of the sample.
3.	Culture Media: Potato Dextrose Agar (PDA)	Growth medium for fungi growth.
4.	Solvent	Dissolving the sample and test material.
5.	Oil Immersion	Improves quality and clarity of images taken.

3.2.2 List of Apparatus and Equipment

Table 3. 2: The List of Apparatus and Equipment

No.	Items	Quantity	Usage/Function
1.	Laminar and hood	1 unit	Provides a sterile workspace to prevent contamination during sample handling.
2.	Autoclave	1 unit	Sterilizes equipment, culture media, and materials using high pressure and temperature (121°C).
3.	Incubator	1 unit	Maintains controlled temperatures for optimal fungi culture growth.
4.	Scott bottle 500 ml	2 units	Container for distilled water.
5.	pH meter	1 unit	Measures and adjusts the pH of solutions and culture media to ensure optimal growth conditions.

Table 3.2: Continued

No.	Items	Quantity	Usage/Function
6.	Sterile petri dishes	100 pieces	Used to culture microorganisms on agar media.
7.	Sterile test tubes	50 pieces	Holds or incubates liquid samples under sterile conditions.
8.	Measuring cylinders (500 ml)	2 pieces (each)	Accurately measures the volume of liquids.
9.	Conical tubes (5 ml and 50 ml)	50 pieces (each)	Stores samples or reagents for centrifugation or temporary storage.
10.	Sterile forceps	3 pairs	Handles or transfers materials and samples in a sterile manner.
11.	Sterile scalpel blades and handles	20 pieces	Cut or slices tissue samples in a sterile environment.
12.	Bunsen burner	1 unit	Provides a flame for sterilizing metal tools and maintaining a sterile workspace.
13.	Light microscope	1 unit	Observes fungi morphology, including hyphae and spores.
14.	Camera for the microscope	1 unit	Captures images of specimens observed under the microscope for documentation.
15.	Glass slides and cover slips	100 pieces	Prepares thin samples for microscopic examination.
16.	Micro centrifuge tube	30 unit	Storing small-sized laboratory samples.
17.	Beaker	3 units	Used for measuring and mixing solutions for media preparation and sterilization processes.

Table 3.2: Continued

No.	Items	Quantity	Usage/Function
18.	Distilled Water	As needed	Used to prepare solutions and media, ensuring no impurities affect plant growth
19.	Autoclave bags	20 pieces	Holds materials and equipment for sterilization in the autoclave.
20.	Laboratory notebook	2 units	Records all data, observations, and experimental steps conducted during the study.
21.	Marker pens (permanent)	5 pieces	Label equipment, samples, or materials clearly and permanently.
22.	Adhesive labeling tape	3 rolls	Used to label samples and equipment in the laboratory easily and clearly.
23.	Safety gear (gloves, masks, lab coats)	As needed	Protects researchers from hazardous chemicals and reduces the risk of contamination.
24.	Tissue paper	As needed	Used for cleaning and drying tools.

3.3 Methodology

3.3.1 Isolation of Endophytic Fungi from *Rafflesia kerri*

A number of small endophytic fungi were isolated from freshly bloomed *Rafflesia kerri* plants which were collected from Lojing Highland and brought back to the lab under sterile conditions. The samples were cleaned by washing the sample in running water for 2-3 minutes to remove the soil. The sample was then treated with 70% alcohol for 4 minutes, then 5.25% Sodium Hypochloride (Bipruch) solution for 3 minutes. After treatment, the sample was washed 3 times using deionised water to sterilise the sample.

After this process, the *R. kerri* flower samples were allowed to dry completely before being plated on PDA agar medium to prevent contamination. The sterilised *R. kerri* samples were then carefully plated onto the PDA + Chloramphenicol agar plates to avoid contaminating the agar plates to ensure fungi growth would be unimpeded. At last, after incubated for 5 – 8 days, the fungi grown from *R. kerri* samples were identified. The temperature of incubator was controlled between 25°C - 28°C for optimal fungi growth.

3.3.2 Morphological Test for Isolated Endophytic Fungi from *Rafflesia kerri*

The first step in the Culture Section process is to prepare a small quantity of fungi culture from an agar plate using a sterilization method. After the fungi culture is prepared the next step is the preparation of the slide. To prepare the slide, add a small quantity of sterile distilled DH20 onto the glass slide. Add a small portion of the endophytic fungi into the DH20 droplet. Add one drop of coloured cotton blue indophenol solution to the fungi sample located on the slide. Allowing the indophenol solution to diffuse into the sample for 1-2 minutes. Remove the excess indophenol solution using tissue paper. Next, place a cover slip over the sample to prevent air bubbles from forming. The sample should now be viewed under the microscope.

Once the fungi colony has grown the next step is to make a morphological description. Fungi morphology is described both macroscopically and microscopically. Recordings were taken of the colony shape, surface colour and bottom colour, colony texture, and growth rate macroscopically. A compound microscope was used to observe microscopic features such as the types of hyphae, spores, and specific characteristics of the fungi. A staining agent, such as lactophenol blue cotton solution, was applied during the microscopic examination. Characteristics of the obtained fungi were described and compared to standard fungi characteristic charts to classify. Short-term preservation of pure cultures can be achieved by preserving them on PDA slopes. Long-term preservation can be achieved through cryopreservation or drying methods.

3.3.3 Fungi DNA Barcoding (Endophytic Fungi)

Each sample is prepared with three small agar cubes placed into a tube that has been sterilized through an autoclave prior to use. After preparation, each sample tube is wrapped in parafilm and placed inside a clearly labeled 5 ml conical tube to avoid any confusion during analysis.

Samples were stored at 4˚c in a laboratory refrigerator to preserve the quality of the fungi. However, if the sample is sent to a different laboratory for analysis, and the transport time exceeds 2 days, the sample may be stored in a cooler with temperature control either at room temperature or frozen depending on the needs and protocol of the

Study. The goal of this is to ensure the sample does not degrade prior to analysis due to microbial contamination or DNA fragmentation.

Cross-contamination can be avoided through sterilization and aseptic handling throughout the process. To amplify the ITS region of the fungi genome, the universal primers ITS1 and ITS4 were used. The components of the 25 µL PCR mixture included in-house optimized DNA (gDNA) that had been purified from the samples, 0.5 pmol of each primer (ITS1 and ITS4), 0.5 U of a thermostable DNA polymerase, 0.2 mM dNTPs (each dATP, dGTP, dCTP, and dTTP), supplied PCR buffer, and DH2O. The PCR procedure consisted of one cycle of 98˚C for 2 minutes for denaturation, followed by 25 cycles of 98˚C for 15 seconds for denaturation, 60˚C for 30 seconds for annealing, 72˚C for 30 seconds for extension, and one cycle of 72˚C for 10 minutes for final extension. The PCR products were cleaned up according to the standard PCR cleanup procedure. The purified PCR products were subjected to bidirectional Sanger sequencing with universal primers M13F (-20) and M13R-pUC (-26) using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

3.3.4 Data Analysis

In order to obtain the first goal (obtaining fungi endophytes from *Rafflesia kerri* flower) we must relate the number of fungi isolates to the percentage of successful cultures grown on PDA plates. The results are recorded in the form of a table that presents the number of isolates along with the percentage of successful cultures of each sample. In the same manner, for instance, if a cumulative extraction of five isolations were conducted from one sample and it was reported that all the extracted plates showed growth; then the record of the extraction will be included with the percentage of growth, 100%.

In regard to the second goal, which describe the morphology of the separated endophytic fungi, we focused our attention on both gross and minute characteristics of these fungi. Gross characteristics include the forms, colours, textures, etc. of the fungi colonies and their rate of growth, whereas minute characteristics, such as the types of hyphae, the morphological characteristics of the spores and other specific structures of the fungi are observed with the compound microscope. These characteristics are then compared to standardized fungi morphology tables or charts for easier and more accurate identification of species.

The above methodology allows us to organize our sampling of data in a coherent and unified way with our research objectives, and at the same time make it easy to understand the results.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation of Endophytic Fungi Associated with Fresh Blooming Flowers of *Rafflesia kerri*

Three sample types, Sample A (1st petal), Sample B (2nd petal), Sample C (ramenta), were obtained from Lojing Highlands to extract fungi from the *Rafflesia kerri* flower. Samples were thoroughly washed with plain water when received in the lab and after being treated with 70% ethanol for one minute and sodium hypochlorite solution for two minutes, to remove surface contaminants. These samples were individually cut into smaller pieces using aseptic techniques and equipment. After each piece was triple rinse with sterile distilled water and dried in the air for several hours, the pieces were plated as individuals on Potato Dextrose Agar (PDA) media contained within Petri dishes, which were immediately sealed with parafilm to ensure continued sterility. All of the cultures were incubated at temperatures ranging from 25-28°C for one to four weeks, allowing observation of the fungi growth. The procedures used for all sample types, included the ramenta samples, were identical throughout the study. This uniformity eliminated the opportunity for cross contamination and provided consistent isolation conditions during the entire study period.

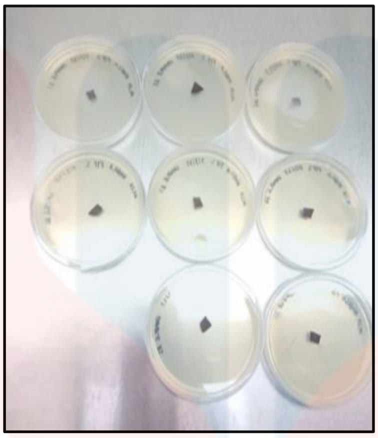
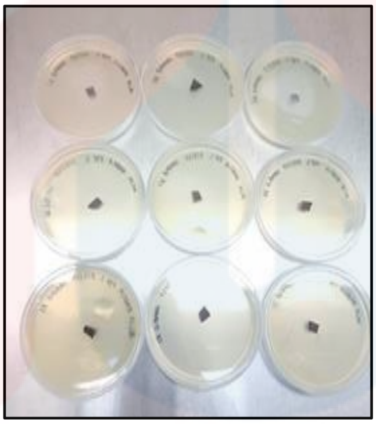
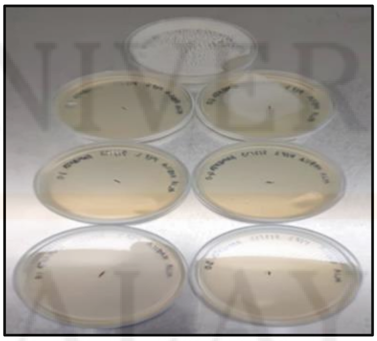
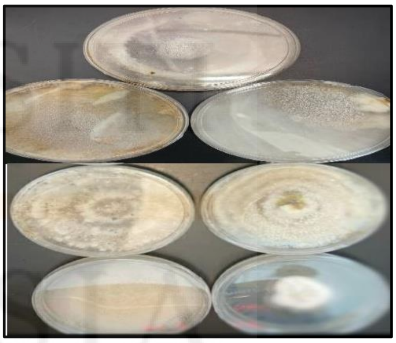
Bil.	Sample	Week 1	Week 4
1.	Sample A (Petal 1)		
2.	Sample B (Petal 2)		
3.	Ramenta		

Figure 4. 1: Isolation of Fungi Isolates from Rafflesia kerri a) isolates 1 (Petal A), b) isolates 2 (Petal B) and c) isoletes 3 (Ramenta).

A Sample A (Petal 1) from *R. kerri* was divided into eight sections to extract fungi using a Potato Dextrose Agar (PDA) medium. It is a universal culture medium that will grow both endophytes and saprophytes. After a week, no fungi were evident on the petri dish and the PDA medium remained colourless. This suggests that if there were fungi on the Petal, they would have been in the lag phase and undergoing physiological adjustments before initiating growth. At Week Four, a few fungi colonies were evident on the PDA petri dish. Each of the colonies exhibited a variety of characteristics including a range of morphologies, texture (dust to cotton) and colours (white, grey, light brown and dark brown). The colony growth rates were also significantly variable; some colonies grew extremely quickly and took up a major portion of the petri dish while others were slower growing and smaller. When mature, the colonies produced areas of colour (yellow and brown) associated with sporulation, and evidence of competition between the colonies was evident; the overlapping colonies formed either physical barriers or inhibition zones indicative of potential antagonism between species. Overall, the data demonstrates that *R. kerri* provides a habitat for a diverse array of endophytic and saprophytic fungi many of which may produce bioactive compounds, and demonstrates the ecological and biotechnological significance of the flower.

The total area of fungi growth on Plate 1 was almost entirely across the petri dish surface. The colonies on Plate 1 were primarily spherical in appearance and while somewhat irregular in shape in areas, the colonies' borders were distinct. The primary colour of the fungi colonies was yellow white/cream, indicating the fungi produced little dark pigment. The texture of the fungi colonies on the agar surface was characterized as being woolly/cottony, which is characteristic of dense mycelial growth on the agar surface. The growth rate of the fungi was relatively quick and the majority of the substrate was covered by the fungi in four weeks, which is consistent with the expectation of high degree of suitability for growth of the substrate.

The colonies on Plate 2 appeared to have grown similarly to the colonies on Plate 1. They were circular and nearly symmetrical, and they had a uniform colour of a yellowish-white or creamy white. There was no indication of dark pigment within the colonies. The colonies also exhibited a fluffiness and thickness to their texture, indicating a developed and healthy mycelium. The size of the colonies and their growth rate were like the colonies found on Plate 1, with the colonies covering much of the plate, like Plate 1.

As was the case for the first two plates, the colonies on Plate 3 were large and circular however, the colonies on Plate 3 had slight variations. The colour of the colonies on Plate 3 was uniform across the entire colony and ranged from a yellowish white to creamy white. While the texture of the colonies on Plate 3 appeared to be fluffy, it appeared less dense or possibly slightly different in density than the colonies found on Plates 1 and 2. Also, in several areas of the colonies on Plate 3, the colonies appear to be flat. The colonies on Plate 3 grew as quickly as the previous colonies, and the colonies grew and filled nearly the whole surface area of the agar on the plate, demonstrating consistent growth among the three colonies.

The colonies on Plate 4 were obviously different from the colonies on the first plate. The colonies completely covered the petri dish. The colonies on Plate 4 were rounded in shape but were slightly irregular at the edges due to the extreme growth of the colonies. One of the most distinctive features of the colonies on Plate 4 was their colour, which was dark, dark brown or blackish, especially around the center of the colonies, which could be attributed to spore development or some other type of fungi growth. The colonies on Plate 4 had flat or wavy surfaces, rather than hairy, and the colonies were spotted where they appeared to be wet or slimy. The colonies on Plate 4 grew extremely quickly and filled nearly the entire surface area of the plate, showing a high level of activity of the fungus in the same media. The colonies on Plate 5 were not as circular and possibly did not have clear borders, looking instead as if they were made up of diffuse growth. The colonies were white or yellowish, although there were some areas or spots that may have been darker or unevenly dense. The texture of the colonies on Plate 5 was not uniform, with the possibility of having a hairy appearance in some places and being flat in others. This suggests that the colonies' growth may have been somewhat variable. The colonies' growth rate was acceptable, but the colonies were not as round or as dense as some of the other colonies. This may indicate differences in the colonies' growth rates or patterns.

The colonies on Plate 6 generally do not spread throughout the petri dish. The colonies were rounded, and the thickness of the fuzz was apparent. The colonies were yellowish white, with a grayish tint, and were even. The colonies had a very hairy and dense texture, like cotton, and had a well-developed mycelium. The colonies on Plate 6 grew as well as the other colonies, indicating that the conditions for the colonies to grow optimally were met.

The colonies on Plate 7 were large and rounded with distinct margins. The colonies were yellow and white, with a slightly denser center. The colonies were woolly or cottony, and the characteristic filamentous mycelium was evident. The colonies' growth rate was good, with the colonies covering most of the surface area of the agar, as in the colonies on Plates 1, 2, 3, and 4.

The colonies on Plate 8 were spreading over the petri dish, with many of the colonies covering the entire surface of the petri dish, and with an irregular elongated or creeping shape that was different from the other colonies. The colonies had a white cotton alternate with orange, and a darker grey in the center. While the colonies' colour was brown or brownish, it indicated that the colonies may contain pigments or may be a mixed culture. The colonies' texture was heterogeneous, with both fluffy and flat textures and possibly even wavy. The colonies' growth rate was very wide and active, and therefore this fungus would be able to grow in abundance in the medium, and possibly there is more than one type of fungus growing.

Sample B was obtained from the second petal (Petal 2) of *R. kerri* and was used for fungi isolation on Potato Dextrose Agar (PDA) medium, divided into nine segments. Like Sample A, no fungi growth was observed during the first week, with all PDA plates remaining clear, indicating that any present fungi were in the lag phase, a time of metabolic adaptation prior to active growth. After four weeks of incubation, Sample B demonstrated various fungi colonies with varied morphology.

Many fungi types were successfully isolated, differentiated based on variations in colour (white, light grey and blackish brown), surface texture (cottony, dense, dusty, or granular), colony margins (smooth, irregular, or radial), and growth rates. The colour variation suggested variations in colony maturity and species identity: white colonies likely represented viable mycelium without spore development; whereas the presence of darker pigmentation (blackish-brown) indicated the presence of spores or secondary metabolites produced by the colonies, typical of genera such as Aspergillus, Penicillium, or Fusarium.

Plate 1 colonies had grown well, however, they didn't cover the full petri dish by the fourth week. They are circular, yet have an irregularly shaped perimeter for reasons of their relatively weak competitive ability. Colonies are predominantly white, with some possible darker colour s towards the center. Colonies are very fluffy and densely packed; they feel like cotton and demonstrate very robust mycelial development. Their overall growth is very strong but relatively slow.

The growth in Plate 2 colonies is equally abundant as in Plate 1. The colonies are also round and have a clearly defined margin. The colour of the colony is a yellowish white and has no signs of dark pigmentation. The texture is very hairy and compact, much like cotton, and demonstrates a highly developed and robust mycelium. The overall growth rate of the fungus in Plate 2 is extremely high, and aggressive during the course of a specified time frame.

Like Plate 1, Plate 3 colonies were rounded and simple, they didn't cover the full petri dish. Colonies are yellowish white or creamy in colour. Like the other two plates, the texture of the colonies is fluffy and compact, much like cotton, and display traits associated with strong mycelial growth. The overall growth rate of the fungi in Plate 3 was excellent but only moderately aggressive, the same as in Plate 1, this indicates the fungi will require additional time to fill the PDA media.

Plate 4 stands out from the others and has a number of morphologically distinct features. The colonies are large and round with a very smooth border. The colonies are a uniform white throughout and the texture of the fur is thin in the middle and thicker at the sides. The growth rate of the fungi in Plate 4 is very good and even across the petri dish.

Colonies in Plate 5 were typically round in shape, and had nearly filled the full petri dish. Colonies were primarily yellowish white or cream colour ed and contained tiny dark spots or slightly denser areas in the center. The texture of the colonies was both thick and hairy and also had a wavy appearance on the surface. The growth rate of the fungi in Plate 5 was very good and large and even.

Colonies in Plate 6 were very large and round and completely covered the petri dish. The colonies were uniformly yellowish-white in colour and contained no black pigmentation. The texture of the colonies was very fluffy and dense, similar to cotton and demonstrated a very healthy and vigorous mycelial growth. The growth rate of the fungi in Plate 6 was very high, one of the highest observed, and the colonies completely covered almost all of the agar surface, demonstrating ideal growth conditions.

Colonies in Plate 7 were very large in diameter, rounded, but irregular at the edges, with a great deal of growth. In the center of each colony there is a colour zone that is yellowish white, and the outer area is very dark, blackish grey with many long, thick hairs, likely produced as a result of the formation of dark spores or changes in the peripheral metabolism of pigments. The texture of the colonies is cotton-like and very dense, throughout. The growth rate of the fungi in Plate 7 is very high and expansive, demonstrating that the fungi are very healthy and growing rapidly.

Plate 8 is particularly interesting because the colonies grow in concentric or layered, spherical patterns and show several layers of growth that can be seen visually. The colour pattern of the colonies is yellowish white in the central area and dark, blackish-grey in the new outer area that is developing, typical of some species of fungi. The texture of the colonies is different between bands, possibly hairy in light areas and flat or slimy in dark areas. The growth rate of the fungi in Plate 8 is very high and extensive and covers virtually the whole plate, with growth forms that are unique and characteristic.

The next images show that colonies in Plates 7-9 are all circular and have clearly defined edges. Colonies appear white, because they are completely clear, and therefore can be easily observed when placed on a petri dish. In addition, each of these colonies has an extremely high density of fine hairs that resemble cotton; this suggests that there has been a tremendous amount of healthy mycelia produced by the fungus. As such, the growth rate of the fungi in Plates 7-9 was comparable to the fungi in Plates 1-6, indicating that the fungi grew as expected in the PDA medium.

Colonies with fuzzy/cotton-like surfaces were indicative of vigorous mycelial growth, while colonies with a powdery/dusty appearance indicated spore production, as seen in Cladosporium spp. and Penicillium spp. Colony edges showed variation in terms of their smoothness or irregularity/ radial nature; the presence of smooth edges suggested that the fungi had grown uniformly, while the presence of irregular/radial edges implied competition among the fungi for nutrients and/or metabolite-mediated antagonism between different fungi species. In addition, many of the fungi developed what is referred to as a "zone" of growth; the zone consisted of a central area that was darker and denser than the surrounding area, which represented the initial site of inoculation and subsequent sporulation. A lighter coloured, radiating area around the central zone represented areas of active mycelial growth.

Colonies that overlapped on the Petri dishes demonstrated evidence of ecological interactions, including inhibition zones or growth suppression; this suggested that some of the fungi were producing metabolites that inhibited the growth of other fungi. Furthermore, the growth rates of the fungi varied significantly among the colonies, ranging from those that rapidly covered the petri dishes to those that remained confined to a small area; the latter likely resulted from differences in nutrient uptake/utilization and/or environmental adaptation.

Together, the data presented here demonstrate a remarkable level of diversity among the endophytic and saprophytic fungi associated with *R. kerri*, which included a range of morphologies that corresponded to multiple genera of fungi. The evidence of metabolite-mediated antagonism and the variety of pigmented metabolites suggest that these fungi may represent a source of bioactive compounds for use in biotechnology, medicine and agriculture.

Plates 1 through 7 were inoculated with the Ramenta sample of *R. kerri* and contained fungi cultures that displayed significant differences in colony growth, colour ,texture and morphology over the course of 28 days.

Plate 1 had a high degree of growth, almost filling the entire petri dish, and the circular outline of the colony had a slight irregularity at its border. Overall, the colour of the colony was white, although the center and ends of the colony were thicker than the rest of the colony. The texture of the colony was distinctly fluffy and densely hairy in the middle of the colony, similar to cotton, and was indicative of a very healthy mycelial growth. This colony was able to cover nearly the entire surface of the agar in the petri dish during the 28 day incubation period, and as such indicated that the fungi would grow very well under PDA media.

Plate 2 had a large growth of fungi that was round in shape and had a clear edge about one-third of the diameter of the colony. The colony was generally light brownish-white in colour and did not display any signs of dark pigmentation. The texture of the colony was firm and hairy and thick, similar to cotton wool, and indicated a good mycelium and a high degree of development. This colony had a high degree of growth and was able to cover a very large portion of the petri dish during the 28 day incubation period, indicating that it was growing at a highly optimal and aggressive rate for a certain period of time.

Plate 3 had a large number of round colonies, that filled the petri dish. The colour of the colony was white and even. The texture was fluffy and very dense, consistent with the characteristics of luxuriant mycelial growth. In general, the growth of fungi on Plate 3 was very good and rapid, almost identical to the growth of fungi on Plates 1 and 2, indicating that this fungi will grow very well in PDA media.

The colonies shown in Plates 4 through 7 show differences in size and colour. Plates 4 through 5 show colonies that have grown extensively and are almost completely developed. Both plates show the same type of colony, with a symmetrical rounded appearance and the center area is more highly developed than the periphery. The overall colour of both plates is pale grayish white but show alternating patterns of dark gray or black patches with light-colour ed patches. The colonies are fluffy or fuzzy on the outer areas of the plate and become denser towards the center of the plate. The colony development in both plates is extremely dense and fills nearly all the available space.

Plate 6 consists of two different types of growth zones. Most of the agar plate is covered by fairly uniform light brown to beige colonies. These colonies grow out from their initial points of origin and have dense, hair-like mycelia and appear to be almost flat against the agar surface. They have smooth and somewhat powdery (sandy) surfaces.

Plate 7 represents colonies which are moderately developed and were present in the center of the agar plate while growing outwards toward the edges of the plate. The colonies are round and have sharp edges. The upper surface of the colonies is bright and pure white in colour and appears to be snowy and fluffy. The texture of the colonies is fluffy and airy (cotton-like). Even though they did not grow completely across the entire plate as some of the other samples did, they had a great deal of aerial mycelium and developed into large masses of colony material.

This variability indicates that there are likely multiple fungi species within this ramenta sample, each having their own unique growth rates and physical characteristics.

4.2 Characteristics of the Endophytic Fungi Isolated from the Flower of *Rafflesia kerri* via Morphological Identification

4.2.1 Staining

Three sample types are shown in Figure 4.1 to illustrate the method used for preparing samples for microscopy of fungi. These sample types include Sample A (Petal 1: Plates A1, A3, A4, and A8), Sample B (Petal 2: Plates B1, B6, B7, and B8), and Sample C (Ramenta: Plates 1, 4, 6, and 7). The method for preparing these samples involved cutting small pieces from fungi grown on PDA media using a sterile surgical blade. Each piece was then transferred to a slide and viewed through a microscope. Following the transfer of the sample, a few drops of lactophenol cotton blue solution were added to the sample. Lactophenol Cotton Blue is an excellent fixative, it has several uses; the phenol acts as a disinfectant to kill bacteria and other microorganisms, the lactic acid will help maintain the morphology of the fungus and the cotton blue dye will cause the fungi to become visible when viewed through a microscope. Once the staining solution had been applied, a coverslip was placed over the sample to create optimal viewing conditions. Any extra stain was removed with lint free absorbent paper prior to placing the coverslip over the sample. This method provided consistent sample quality for the purpose of precise microscopic identification and morphological examination of the isolated fungal specimens. All procedures were performed in aseptic conditions to prevent contamination and maintain sample integrity throughout the testing process.

Figure 4.2: Slide Preparation for Microscopic Identification

4.2.2 Microscopic Identification

The tables (Tables 4.2, 4.3 & 4.4) listed below present a comparison of the morphology based endophytic fungi isolates obtained from *Rafflesia kerri* flower via morphological characterization of three samples: Petal A, Petal B and Ramenta with respect to their spore and hyphae characteristics.

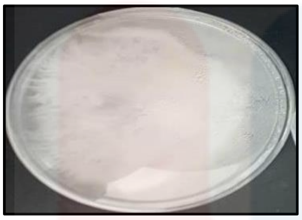
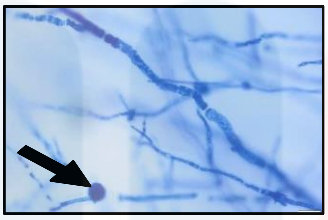
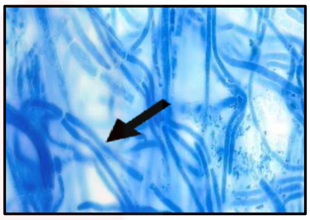
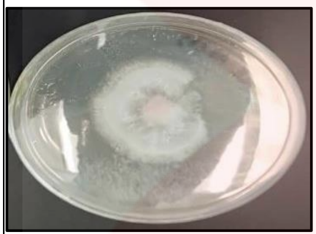
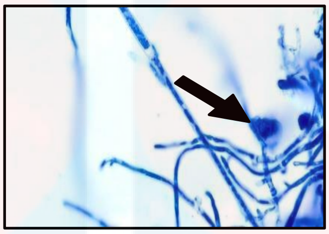
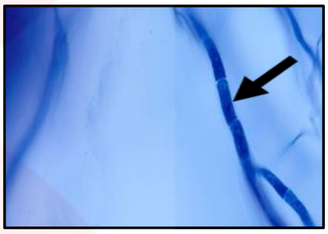
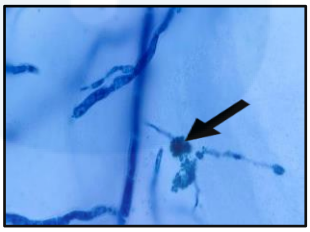
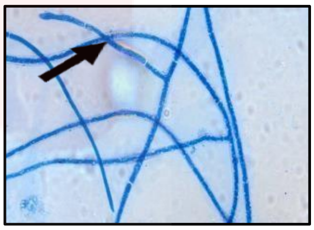
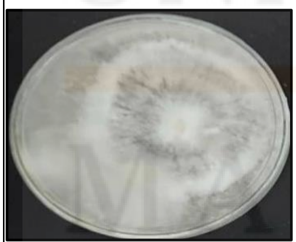
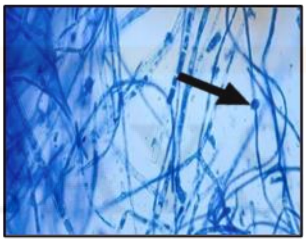
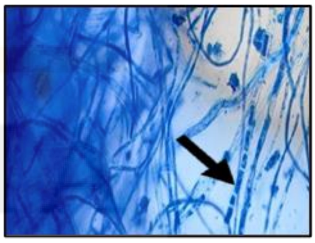
Bil.	Media	Spores	Hyphae
1.	 Plate A1		
2.	 Plate A3		
3.	 Plate A4		
4.	 Plate A8 (1.1)		

Figure 4. 3: Microscopic Identification for Petal A under Magnification of 100× (Oil Immersion).

The variation of fungi in sample Petal A, as shown by plates A1, A3, A4, and A8 (1.1) is demonstrated by differences in both macroscopic and microscopic features. One reason why these four media were selected is that the macroscopic features of spore and hyphae development are easily observable than in most other media. For example, from plate A1, all the fungi colonies that were developed presented an obvious macroscopic morphological feature; they were circular and symmetrical and were completely covered in mycelium. They were white colour with new and developing growth. The fungi colonies were very hairy and fluffy in appearance, and the colonies were a great deal of cotton-like material and thus are typical of filamentous fungi that develop rapidly. From the microscopic examination of the fungi in plate A1, the spores developed were spherical and smooth with a diameter of 6.25 µm.

The spores were located at the end of the spore bearing structure, and they were grouped together at the tip of the spore bearing structure in a manner that resembled a conidiophore. Staining the hyphae revealed them to be brightly colour ed blue in colour and had a diameter of 39.38 µm. The hyphae were septate, branched, and had the ability to stain. Based upon the macroscopic and microscopic morphological features of the fungi in plate A1, it appears that the fungi in this plate represent species of the group Ascomycota that reproduce via a sexual mode using the formation of ascospores and/or by asexual reproduction using the formation of conidia. On the basis of the colony morphology and micro-morphology of plate A1, it would appear that the fungi in this plate represents invasive and vigorous fungi that are in the process of developing. In comparison, the colony of fungi that was grown in plate A3 was round and had clearly defined concentric zones on the surface of the medium.

The center of the colony was gray in colour and had a white colour ed outer layer, showing the two distinct developmental phases of the fungi, namely, the early, immature center and the growing edge of the colony. At the center of the colony there was compact growth, while at the periphery of the colony there was moderate amounts of hairiness, which is consistent with the fact that many fungi that produce secondary metabolites have the same characteristics. Microscopically, the spores were ovate to elongated in form, with a tapering apex of 11.88 µm. The spores were attached to each other in a network arrangement on an uncharacteristic phialide-like spore-bearing structure, which is a type of asexual spore called phialoconidia. The hyphae were septate and straight in nature and measured 13.13 µm in length. The walls of the hyphae stained dark blue and therefore could be visualized. It is likely that the fungi that were cultured in plate A3 are part of a genus of fungi, such as Penicillium or Aspergillus, that produce conidia by means of asexual structures. Plate A3 generally showed the characteristics of a mature and stable fungi with a well-developed and complex microscopic structure. In terms of the growth of the fungi, plate A4 produced circular-shaped colonies that were symmetrical and had visible concentric zones of growth.

The colour of the colonies was white with a dense and slightly seeded center, which suggests that a mature growth zone or the formation of spores has occurred. The colonies had a relatively coarse and dry surface and contained a fine dust-like texture in the center of the colonies, which indicates the formation of a reproductive structure. Microscopically, the spores were small in length of 9.38 µm, spherical in shape, and randomly located. The spores were possibly produced by short and simple conidiophores. The hyphae were long, thin, and septate in nature and measured 27.5 µm in length and were intensely blue in colour due to effective staining, which indicated that the hyphae structure was healthy and active. The hyphae were minimally branched and had a fine nature.

Therefore, it is possible that the fungus that was cultured in plate A4 belongs to a genus such as Fusarium or Cladosporium with zonal growth and the conidia structure being simple. In terms of the colony morphology of plate A8 (1.1), it was characterized by a large, round, and symmetrical colony that grew uniformly across the entire colony. The colony colour was whitish-gray at the center and faded towards the periphery. The colony center had denser growth, which indicated a more mature growth zone. The colony texture was velvety, which indicated a high density of hyphae growth on the surface of the media. Microscopically, the spores were rounded in shape, smooth surface, and were either alone or in pairs. The spores were produced by conidiophores that were 3.75 µm in length and branched. The septate hyphae measured 6.88 µm in length and were long, actively branched and brightly blue in colour after staining. These characteristics are typical of the genera Penicillium or Trichoderma, which are well-known for their vigorous growth and unique conidia morphology. Generally speaking, the overall microscopic morphology of the fungi present in plate A8 indicated that the fungi were fertile, mature, and filamentous in the media.

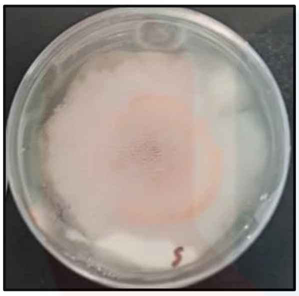
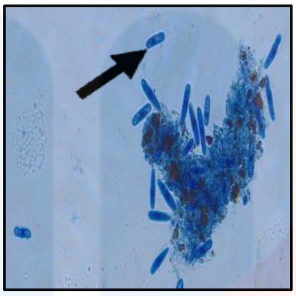
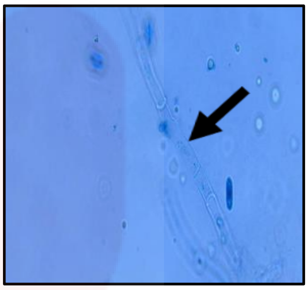
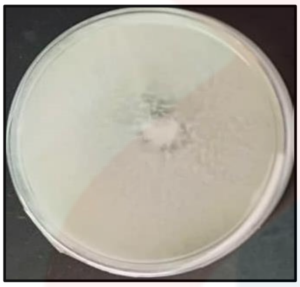
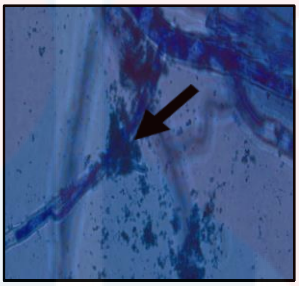
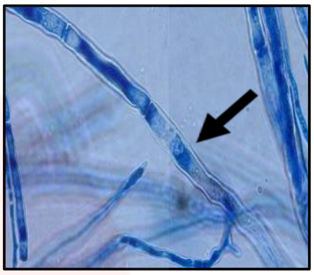
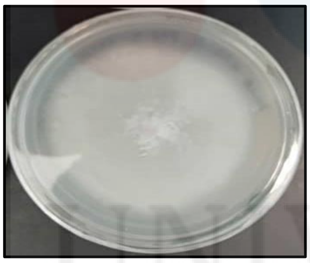
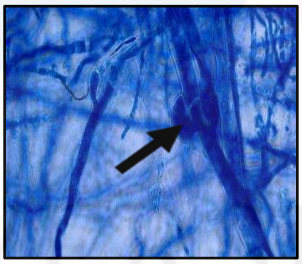
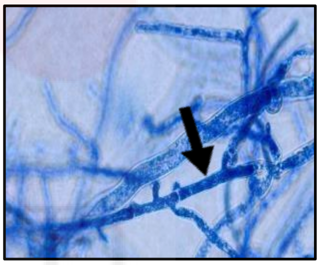
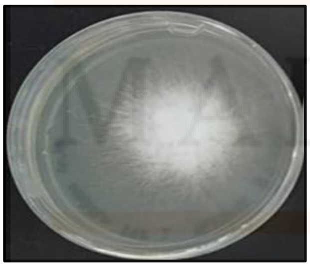
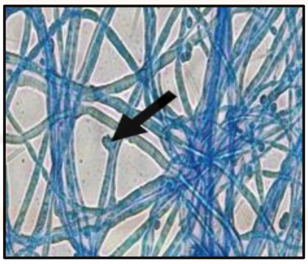
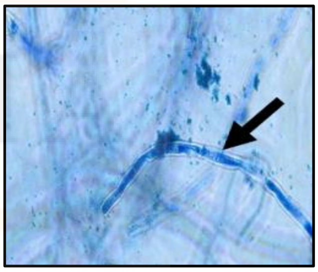
Bil.	Media	Spores	Hyphae
1.	 Plate B1		
2.	 Plate B6		
3.	 Plate B7		
4.	 Plate B8		

Figure 4.4: Microscopic Identification for Petal B under Magnification of 100× (Oil Immersion).

Sample petal B as demonstrated by plate B1, B6, B7 and B8 show in the microscopic view and as an example for the outer morphology of various types of fungi colonies developed. The reason for selecting these four was due to the ability of forming spores and hyphae that is more observable than others. Plate B1 displays a fungi colony on top of the culture medium showing a symmetrical and rounded growth. It is orange in the center and brownish white around the perimeter. The colour s demonstrate an area of growth moving outward toward the center and the area in the center is mature/spored. The colony's texture is dense and has fine hairs on the top with moderate and not rapid growth demonstrating stability/slow fungi. Microscopically, the spores that are oval cylindrical and 9.38μm in size, occur in clusters in the form of conidia by way of specialized structures (phialides or conidiophores) that bear branches. The spores stain blue with a thick and clear cell wall. The hyphae are septate; long and thin (21.25μm) and have not too crowded branching. The hyphae are blue in colour and have a clear septum. Based upon the above characteristics, it would appear the fungi of plate B1 would be most likely to represent a genus such as Fusarium or Trichoderma, colour ed and highly aggregated asexual conidia structures.

Colonies are flat and rounded with flat edges. Colonies are pale brownish white in colour and have a texture that is cottony or fluffy, indicative of high density mycelium growth. The surface is flat and homogeneous. The growth of the colonies is moderate, and they cover the media space evenly with good symmetry. Also, microscopically irregular spherical spores are evident that are stained with lactophenol cotton blue staining and have a diameter of 40μm. Furthermore, the fine presence of conidia or phialides can be viewed. Branching and septate hyphae structure is also evident with a diameter of 39.38μm. Also, the long fine hyphae structure is evident based on the blue nature of lactophenol cotton blue staining. The fungi morphology is a close network of hyphae, and therefore, is a genus such as Trichoderma.

The colonies on Plate B7 are round and have a relatively flat edge. The colonies are white and less than those on plate B6. The texture is more dense than that on plate B6. The colonies are shiny in the center indicating active growth. The colonies grow very quickly and nearly fill the media. Microscopically, spores are oval or spherical, and are dark blue with heavy walls. The spores are 13.13 µm in diameter. Based on the hyphae structure, the hyphae are clear, septate, and branched extensively and measured 31.25 µm. Hyphae are packed tightly and conidia are present in chains. A phialide-like structure is also evident along with conidia formation. The morphology is consistent with that of the genera such as Penicillium or Aspergillus.

The colonies on Plate B8 are circular and have a very fluffy and dense center. The colonies are deep white and have a grayish tint around the edges. The colony growth is very active and of a high growth rate. Microscopic observation indicates that the spores are spherical in shape and small in diameter 2.5 µm. The hyphae structure is elongated, fine, and branched with a length of 13.75 µm. Septate hyphae and conidia are present as short chains. Staining clearly shows a cell wall, and at times, structures such as vesicles or sterigmata may be distinguishable. These characteristics are compatible with the genus Trichoderma or possibly Fusarium.

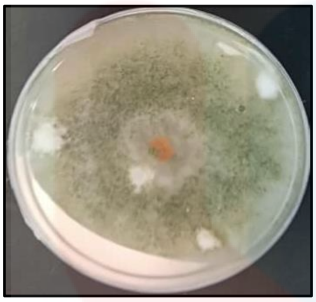
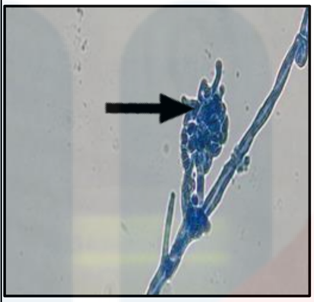
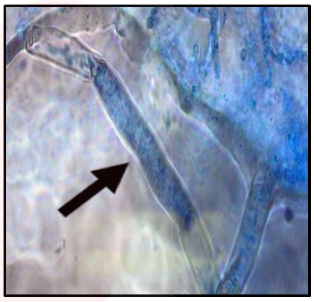
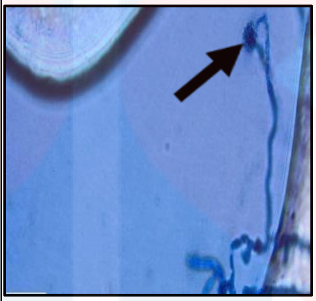
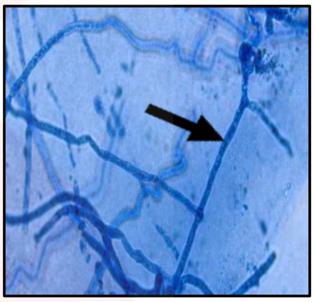
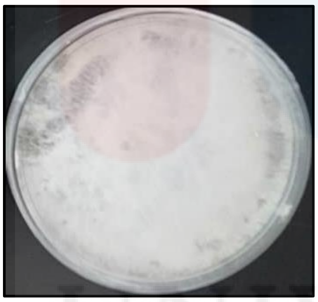
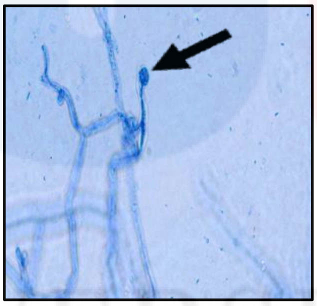
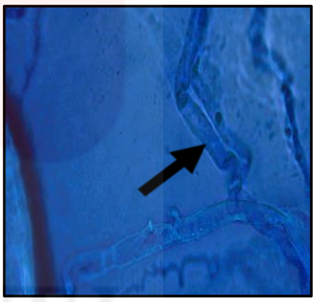
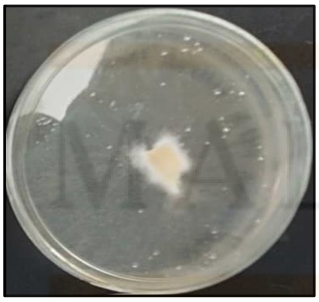
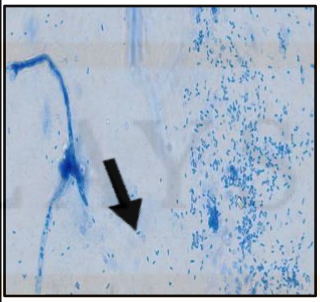
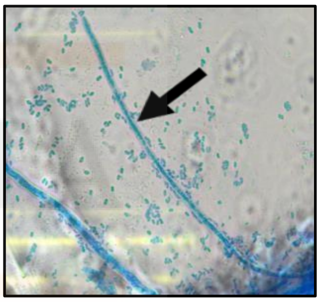
Bil.	Media	Spores	Hyphae
1.	 Plate 1		
2.	 Plate 4		
3.	 Plate 6		
4.	 Plate 7		

Figure 4. 5: Microscopic Identification for Ramenta under Magnification of 100× (Oil Immersion).

Microscopic examination of the Ramenta portion of the *Rafflesia kerri* flower exhibited various features of endophytes that were structural and intriguing. All four of the media used for inoculating the colonies provided better visualization of the spore and hypha formations when compared to the other media used.

The colonies on plate 1 were rounded with uneven and irregular edges. The center of the colonies was orange-red and surrounded by a heavy, dark green ring around the periphery and a lighter colour ed zone at the perimeter of the colony. These characteristics indicated that the colony's growth and spore production occurred at the growing center of the colony. The colony's texture was powdery and grainy; it demonstrated large quantities of conidia on the surface of the colony. The colony developed rapidly to moderately and expanded uniformly in all directions. Examination with a microscope revealed densely-packed, round to oval-shaped spores measuring approximately 6.88 µm. The hyphae were septate, 50 µm in length and branching. Conidia structures were located at the tip of the conidiophore and were attached in chains or clusters. This morphological structure could potentially indicate a genus similar to Penicillium or Aspergillus. The fungi cell wall was clearly visible and the fine branched mycelium structure was visible in the fungi examined. The colony on plate 4 was rounded and had a more solidly formed and slightly raised center of the colony. The colony generally was white.

The colony was fluffy, fibrous in texture with aerial mycelium radiating outwards from the center towards the perimeter of the colony. The center of the colony was more coarse and denser. Colonies grew slowly to moderately. On microscopic examination, longer, round and chain-like spore formations (conidia) were observed and were single, measuring 6.25 µm in length. Upon staining with lactophenol cotton blue, blue spores were seen in the microscope. Septate and dichotomously branching hyphae were observed, measuring 27.5 μm and possessing relatively thicker walls. Larger oval conidia were observed, not occurring in long chains. The morphology of the conidia and their location could be related to genera such as Fusarium or Cladosporium. The mycelium structure was compact with variability in the diameter of hyphae.

The colonies on plate 6 were flat, even and had sharp, circular edges. The colonies were entirely pure white and were quite homogeneous, suggesting they were either new colonies or had produced few spores. The colonies were soft and cottony in appearance and stuck uniformly to the agar surface. Rapid growth of the colonies caused them to cover the majority of the media surface. Upon microscopic examination, the colonies yielded spherical spores of 6.88 μm in diameter with internal structure and could be associated with a genus such as Trichoderma. Characteristic structures of vesicles were absent but conidia formation was evident. The hyphae were septate, thin, and showed branching in a parallel mode, measuring 25.63 μm. Small, spherical conidia arranged singly or in small clusters were observed on conidiophores. The fungi colonies on plate 7 were round with a yellow-white center and a clear white border area that looked moist. The center of the colony was moderately dense and raised with an active growth zone. The growth was moderate with colonies limited to the central portion of the media and slow. The colony texture was cottony/semi-floccose and soft with a lightly and slightly fibrous mycelium in the center.

Upon microscopy, there were numerous oval to spherical spores closely packed into 1.25 μm clusters. Conidia stained blue-green upon staining and were typical of a genus such as Trichoderma sp. since this genus produces green or blue-green conidia. Structures including conidiophores were observed, and at the distal ends of the conidiophores, conidia were observed. Conidia were produced in clusters or short chains. Septate and dichotomously branching hyphae were observed under microscopical staining with smooth and well-defined walls measuring 21.88 µm. In certain areas, hyphae were closely packed and overlaid one another, suggesting the presence of common mycelial growth.

4.3 DNA Sequencing

All five isolates were DNA-barcoded, but only three (S1-Ramenta, S4-B8 and S5-B7), whilst S2-A4 and S3-A8 (1.1) were unable to be characterized by virtue of category 5 problem, a failure to amplify in the target region.

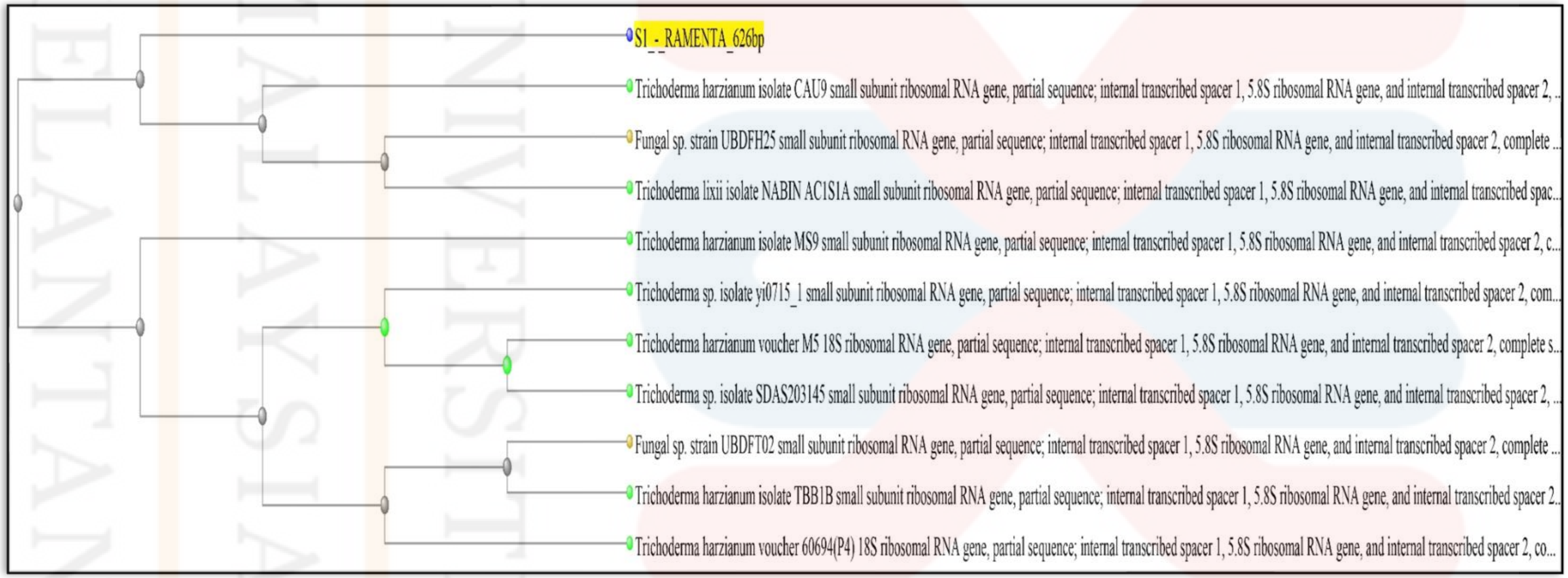


Figure 4.6: Phylogenetic Tree Generated from Fungi Isolates Obtained from the Ramenta of *Rafflesia kerri*

Sample S1 - RAMENTA_626bp shows an extremely close relationship of evolution with certain species of the genus Trichoderma, including *T. harzianum* and other related species, based upon the provided phylogenetic tree. That S1 is placed in the same clade with *T. harzianum* isolate CAU9, *T. lixii* and other species, clearly establishes that S1 comes from the genus Trichoderma. The short branch length between S1 and these species illustrates a low amount of genetic divergence and supports the possibility that S1 may be genetically almost identical to the group.

The fact that S1 branches out from the same common node (shared common node) as several Trichoderma species which have been previously molecularly identified, provides evidence for high levels of taxonomic identification. Stable grouping of the investigated ramenta isolates into a single clade with Trichoderma species also demonstrates that the ramenta sample from flowers of *Rafflesia kerri* contains a population of fungi of the Trichoderma genus.

Therefore, the molecular analysis using the small subunit ribosomal gene (SSU rRNA) and the ITS spacer region provide substantial molecular evidence that the fungi present in the ramenta sample were most likely members of the genus Trichoderma, and more specifically, near species of *T. harzianum* and *T. lixii*. These results are consistent with the general morphological characteristics of Trichoderma fungi which can be found as endophytes or saprophytes in various plant tissues, including flower tissues such as those of *Rafflesia kerri.*

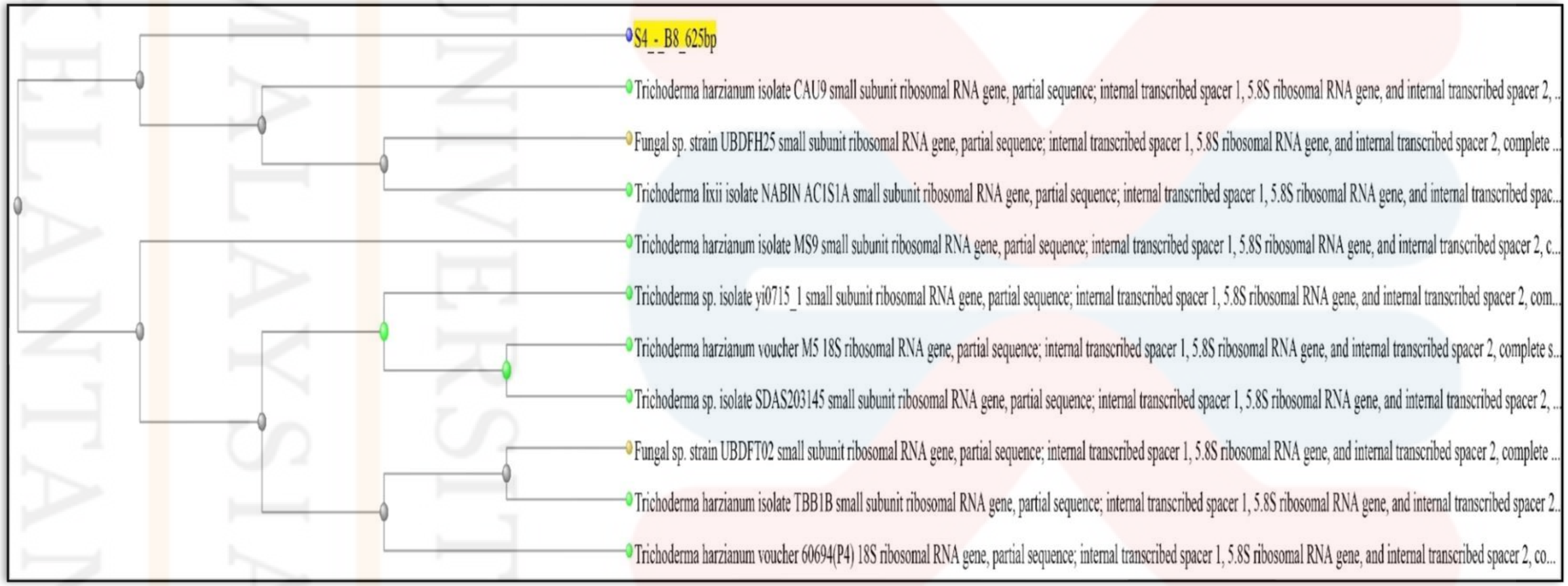


Figure 4.7: Phylogenetic Tree Generated from Fungi Isolates Obtained from the Petal B8 of *Rafflesia kerri*

DNA Barcoding revealed that the S4-BB_626bp sample has an extremely close evolutionary relationship with members of the Trichoderma genus (specifically the species *Trichoderma harzianum* and *Trichoderma lixii)*. S4 appears in the same clade and at the same node with different Trichoderma isolates such as *T. harzianum* isolate CAU9, T. lixii NABRN AC1S1A, and *T. harzianum* MS9; indicating the S4 branch is at an extremely low genetic divergence from these species, which suggests the S4 sample and the above mentioned species have identical taxonomic identities.

Further evidence of the evolutionary stability of this group of organisms within the clade under examination comes from the placement of S4 near many other Trichoderma isolates. Additionally, the presence of high bootstrap values on each of the respective nodes (indicated by green circles) provides additional statistical support for this group of organisms. Also, the grouping of S4 along with all the other voucher strains of *T. harzianum* isolates indicates that the BB sample (possibly a portion of a flower or another type of tissue) is a fungus of the genus Trichoderma, and likely a member of the *T. harzianum* complex.

In summary, using the 18S rRNA gene and ITS regions to perform phylogenetic analysis we were able to determine that the S4-BB_626bp sample is a member of the Trichoderma genus and is closely related to both *T. harzianum* and *T. lixii.* Notably, the Trichoderma genus has been associated with a variety of ecologically important functions including acting as a plant endophyte, a biological control agent, and an organic matter degrader. Therefore, the presence of Trichoderma in these BB tissues could have important ecological implications for the interactions between microorganisms and their host plants, *Rafflesia kerri.*

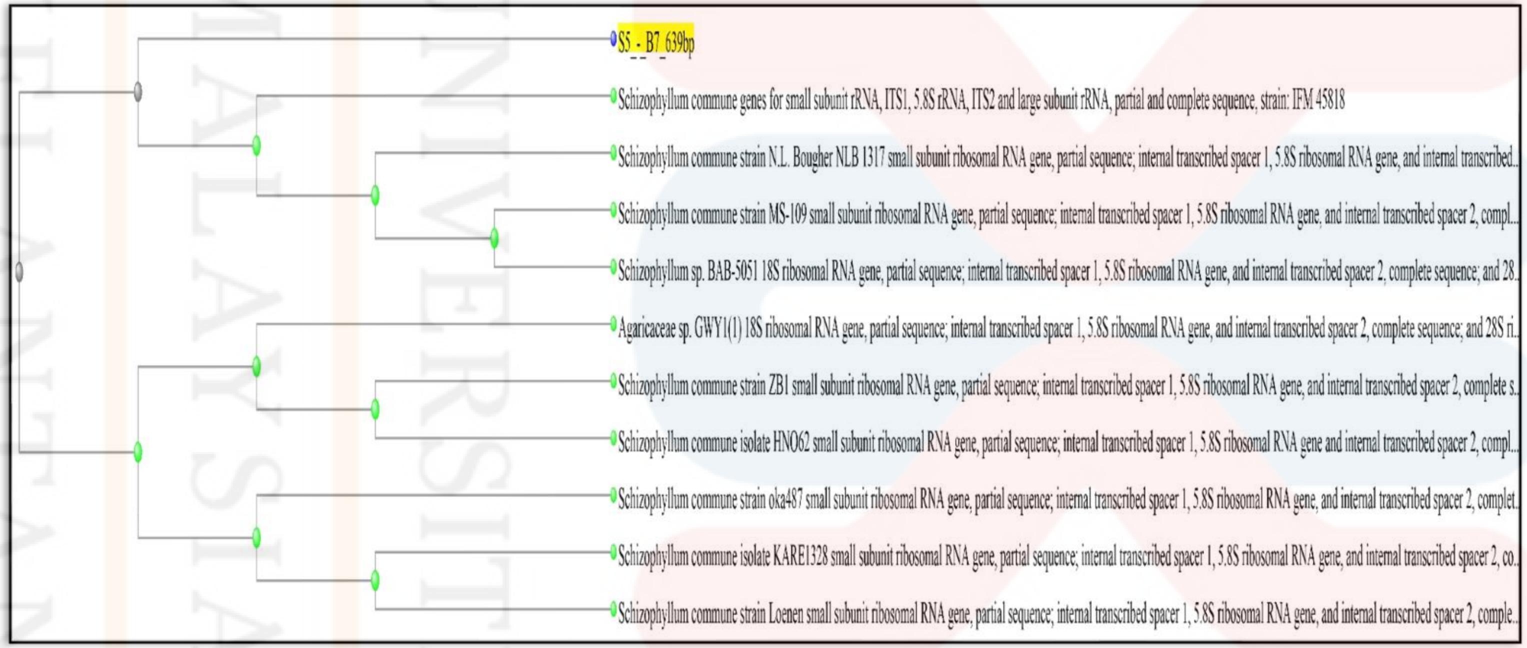


Figure 4.8: Phylogenetic Tree Generated from Fungi Isolates Obtained from the Petal B7 of *Rafflesia kerri*

The phylogenetic tree presented in Figure S5-B7_639bp has demonstrated via DNA barcoding that the sample (S5) belongs to the genus Schizophyllum and demonstrates a recent evolutionary past with *Schizophyllum commune*. The sample S5 was found to form a consistent clade with multiple strains of *Schizophyllum commune* isolated from different geographical locations including MS-109, ZSB1, HN002, and Locem. It appears that the sample S5 possesses a short evolutionary history and expresses minimal genetic diversity relative to the rest of the samples.

This grouping of the sample S5 along with *Schizophyllum commune* and the presence of green circle nodes at each branching point indicate a high level of statistical support (bootstrap value) that suggest sample S5's molecular identity is extremely similar to that of the basidiomycota fungus with split-gilled (fan-shaped) spores, S. commune. The finding of this sample in B7 (assumedly from the flower tissue or vegetative part of *Rafflesia kerri*) may suggest that this fungi functions as a potential decomposer to break down dead organic matter or possibly as an endophyte living inside plant tissues without any harm.

In addition to the above, further evidence of correct species classification were obtained by using specific genetic markers such as 18S rRNA, ITS1, 5.8S rRNA and ITS2. Therefore, based on these findings, we conclude that sample S5 is an isolate of *Schizophyllum commune* and therefore contributes additional knowledge to the body of information regarding the microbial community associated with *Rafflesia kerri*, in addition to identifying the functional roles of the sample as a decomposer and as an intermediary between microorganisms and plants.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The primary objective of this study has been fulfilled by isolating and identifying endophytes from flowering *Rafflesia kerri* found blooming at the Lojing Highlands in Kelantan. Isolation of endophytic fungi was achieved using the combination of surface sterilization methods with culturing on PDA medium and then examining morphologically with both macroscopic (colour, texture, growth rate) and microscopic (shape of spores, shape of hyphae) examinations of the endophyte cultures. The outcomes of this investigation suggest that a high density of endophytic fungi live within the *R. kerri* plant and potentially play an important role in maintaining the persistence of the *R. kerri* plant through processes such as nutrient cycling, breakdown, and protection against pathogens and stresses. This research provides a new area of study into the relationship between the parasite plant (in this case *R. kerri*) and its symbiotic internal microorganism relationships. It will provide scientists with the opportunity to develop applications in the areas of medicine, agriculture and biotechnology. Finally, it provides evidence for the need to protect and conserve the natural environment and endemic species such as *R. kerri*.

5.2 Recommendations

Future research must include biological studies of isolated endophytes to examine how well they survive and grow in a variety of conditions, including studies at different temperature ranges, levels of pH, and amounts of various nutrients. In addition, studies of resistance of endophytes to abiotic stressors such as heavy metals and/or drought could provide useful data for evaluating the potential of endophytes to remediate soils and contaminated sites. A particularly exciting area of research would be the examination of how endophytes may function in organic decomposition processes post-Rafflesiaceae senescence. Such research would likely require an examination of the production of decomposition enzymes (cellulase, pectinase, ligninase) by fungi which are involved in nutrient cycling within forest ecosystems. Furthermore, the biochemical pathways of these enzyme biosyntheses have commercial significance and could be used in a variety of agricultural applications, food processing, and bioremediation of organic waste.

Additionally, it would be of interest to conduct research into the interactions between the isolated endophytic fungi and other microbes in the environment or soil of the host roots of Tetrastigma. Laboratory based co-culture experiments and/or the co-breeding of the endophytic fungi with other microbes to determine if the interaction was synergistic or antagonistic would allow researchers to demonstrate the contributions of the fungi to the natural microbiome network. These types of studies could also lead to finding biocontrol agents that could protect other plants from pathogens. Therefore, it is highly recommended that future research is done through the use of ecological/physiological methods and the study of microbe-microbe interactions to clearly define the roles of endophytic fungi in the Rafflesia ecosystem and possibly to develop a method of conservation and a biotechnology application for the endophytes.

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APPENDIX A

A1: Fresh and mature *Rafflesia kerri*.

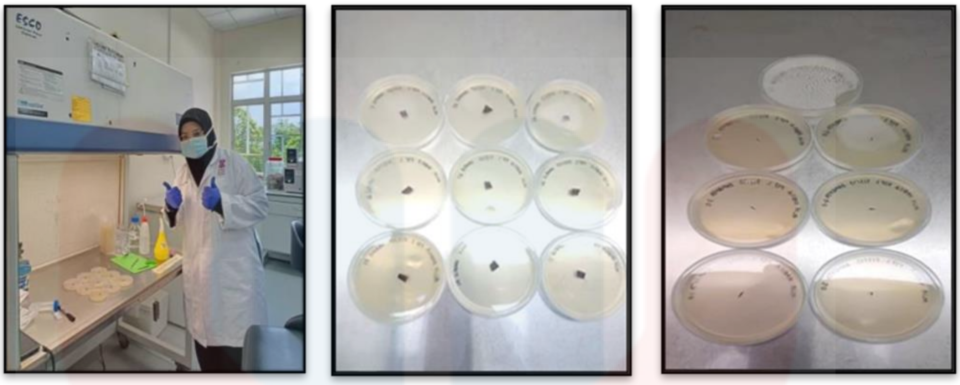
A2: Coordinate of *Rafflesia kerri*.

A3: Soil pH.

A4: Soil Temperature.

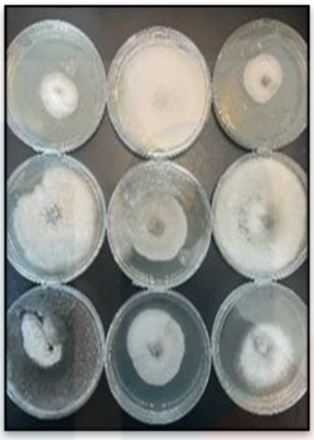
A5: *Rafflesia kerri* was collected.

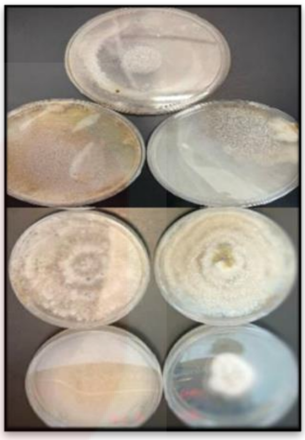
A6: Preparation for PDA media.



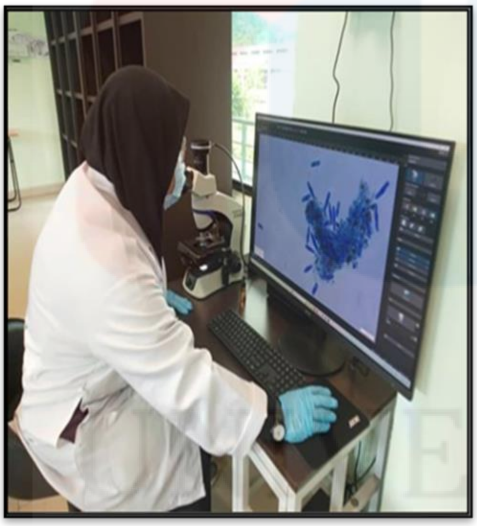
A7: Isolation of *Rafflesia kerri* samples for Petal A, Petal B, and ramenta in PDA media.

A8: Samples of *Rafflesia kerri* for Petal A, Petal B, and ramenta in PDA media are placed in an incubator with a temperature of 25˚C to 28˚C for good growth of endophytic fungi.





A9: Development of fungi growth on *Rafflesia kerri* flower samples for flower Petals A, flower Petals B and ramenta in the third and fourth weeks.



A10: Observing spores and hyphae using a microscope camera.

A11: Simple preparations for DNA Barcoding.