ISOLATION OF BACTERIAL DNA FROM RHIZOPHERE OF SELECTED GESNERIACEAE PLANTS

by

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DECLARATION

I declare that this thesis entitled "Isolation of Bacterial DNA from Rhizophere of selected Gesneriaceae Plant" is the result of my own research except as cited in references. The thesis has not been accepted to any degree and is not concurrently submitted in candidature of any other degree.

Signature : 

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Date : 06 Jun 2025

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بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

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ISOLATION OF BACTERIAL DNA FROM RHIZOPHERE OF SELECTED GESNERIACEA PLANTS

ABSTRACT

This study aimed to isolate and analyze bacterial DNA found in the rhizosphere of three plant species from the Gesneriaceae family which is *Cytandra cupulata Ridl*., *Codonoboea rugosa*, and Cytandra sp. in the Sungai Tiang area, Royal Belum, Perak. The rhizosphere refers to the soil layer inhabited by plant roots. This study aimed to determine the best method for extracting bacterial DNA from rhizosphere soil. To do this, the study used two distinct techniques to assess the quantity and quality of DNA extracted: the spin-column approach (MACHEREY-NAGEL NucleoSpin® Soil kit, Germany) and the traditional method (bead beating). Samples of rhizosphere soil were gathered from Sg. Papan, Sg. Kejar, and Sg. Kenarong, three distinct localities. The Faculty of Earth Science's Microbiology Laboratory served as the site for the DNA extraction procedure. The integrity of the collected DNA was also assessed using agarose gel electrophoresis, and a Nanodrop spectrophotometer was utilised at Biology Laboratory 1 of the Faculty of Agro-based Industry to determine the purity of the DNA. The results of the study showed that the spin-column (NucleoSpin® Soil kit MACHEREY-NAGEL, Germany), protocol kit method was more effective in producing high quality DNA, with A260/A280 values in the range of 1.8–2.0 and clearer DNA bands on agarose gel. Therefore, the conventional method showed inconsistent purity values and possible contamination or degradation of DNA, with negative DNA concentration and unrealistic A260/A280 purity ratio (up to 28.09), indicating contamination or interference of the reading. These results show that the spectrophotometer readings are being interfered with, most likely because of substantial contamination from substances like Tris-EDTA or partially removed organic soil components. This study shows that the bacterial community in the rhizosphere of Gesneriaceae plants can be studied metagenomically. Therefore, it is advisable to use the spin-column (NucleoSpin® Soil kit MACHEREY-NAGEL, Germany), protocol kit method to extract bacterial DNA for soil biodiversity studies. This is because a deeper understanding of the role of microbes in ensuring fertile soil and sustainable agriculture will increase.

PENGASINGAN DNA BAKTERIA DARIPADA TANAH RIZOSFERA TUMBUHAN TERPILIH GESNERIACEAE

ABSTRAK

Kajian ini bertujuan untuk mengasingkan dan menganalisis DNA bakteria yang terdapat dalam rizosfera dari tiga spesies tumbuhan daripada keluarga Gesneriaceae iaitu *Cytandra cupulata Ridl*., *Codonoboea rugosa*, and Cytandra sp. di kawasan Sungai Tiang, Royal Belum, Perak. Rizosfera merujuk kepada lapisan tanah yang dihuni oleh akar tumbuhan. Kajian ini juga bertujuan menentukan kaedah terbaik untuk mengekstrak DNA bakteria dari tanah rizosfera. Bagi mencapai tujuan ini, dua teknik berbeza telah digunakan untuk menilai kuantiti dan kualiti DNA yang diekstrak: kaedah spin-column (NucleoSpin® Soil dari MACHEREY-NAGEL, Jerman) dan kaedah tradisional (bead beating). Sampel tanah rizosfera telah dikumpulkan dari tiga lokasi berbeza iaitu Sg. Papan, Sg. Kejar, dan Sg. Kenarong. Proses pengekstrakan DNA telah dijalankan di Makmal Mikrobiologi, Fakulti Sains Bumi. Ketulenan DNA yang diperoleh dinilai menggunakan spektrofotometer Nanodrop di Makmal Biologi 1, Fakulti Industri Asas Tani, manakala integriti DNA dianalisis menggunakan elektroforesis gel agarosa. Keputusan kajian menunjukkan bahawa kaedah kit spin-column (NucleoSpin® Soil dari MACHEREY-NAGEL, Jerman) adalah lebih berkesan dalam menghasilkan DNA yang berkualiti tinggi, dengan nilai A260/A280 dalam julat 1.8–2.0 serta jalur DNA yang lebih jelas pada gel agarosa. Sebaliknya, kaedah konvensional menunjukkan nilai ketulenan yang tidak konsisten dan kemungkinan pencemaran atau degradasi DNA, dengan kepekatan DNA negatif dan nisbah A260/A280 yang tidak realistik (sehingga 28.09), menunjukkan gangguan terhadap bacaan spektrofotometer, berkemungkinan besar disebabkan pencemaran daripada bahan seperti Tris-EDTA atau komponen organik tanah yang tidak dibuang sepenuhnya. Kajian ini membuktikan bahawa komuniti bakteria dalam rizosfera tumbuhan Gesneriaceae boleh dikaji secara metagenomik. Oleh itu, adalah disarankan untuk menggunakan kaedah kit spin-column (NucleoSpin® Soil dari MACHEREY-NAGEL, Jerman) bagi pengekstrakan DNA bakteria dalam kajian biodiversiti tanah. Ini kerana pemahaman yang lebih mendalam tentang peranan mikrob dalam memastikan kesuburan tanah dan pertanian lestari akan dapat ditingkatkan.

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LIST OF ABBREVIATION

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
PCR	Polymerase Chain Reaction
EDTA	Ethylenediaminetetraacetic acid
SDS	Sodium Dodecyl Sulphate
CTAB	Cetyltrimethylammonium bromide
TE buffer	Tris-EDTA buffer
TAE buffer	Tris-acetate-EDTA
Tris-HCI	Tris hydrochloride
NaCI	Sodium chloride
EtBr	Ethidium Bromide
rpm	Revolutions per minute
UV	Ultraviolet
PVP	Polyvinyl Pyrrollidone
HGP	Human Genome Project
SW1 and SW2	Wash Buffer
SX	Enhancer
SL1, SL2, and SL3	Lysis Buffer
SB	Binding Buffer
SE	Elution Buffer

P.N	Sungai Papan
K.R	Sungai Kejar
K.G	Sungai Kenarong

LIST OF SYMBOLS

Symbol	Meaning
cm	Centimetre
g	Gram
%	Percent
µg	Microgram
mL	Millilitre
µL	Microliter
pH	Potential of hydrogen
nm	Nanometer
mm	Millimetre
bp	Base pair
A	Amperes

CHAPTER 1

INTRODUCTION

1.1 Background of Study

On 31 July 2003, Royal Belum was declared by His Majesty Sultan Azlan Shah, Sultan of Perak as Royal Belum State Park in Perak. (Coordinates: 5°32′58.91″N 101°20′52.4″E). The park is managed by the Perak State Park Corporation. The Royal Belum area consists of pristine tropical rainforest, there are many River systems and small grassland areas as well as some abandoned agricultural areas. In Royal Belum there is also a lake known as Tasik Temengor. Lake Temengor is a large man-made lake. Royal Belum also has its own northern element because this area borders Thailand. Royal Belum is part of a large forest block in Peninsular Malaysia (Belum - Temengor). Royal Belum is also an important habitat for flora and fauna. The Royal Belum area is also recognized in the National Ecotourism Plan as a priority site for ecotourism (Azman *et al.,* 2013).

Gesneriaceae is a family of plants, just like Orchidaceae (orchids), Poaceae (grasses), Rosaceae (roses, apples, strawberries) and so on. Gesneriaceae is a large family with more than 3500 species and 150 genera. This species is widely distributed and occurs on all continents. Gesneriaceae is also like several other families, including orchids and roses that have very attractive flowers and foliage and have attracted human attention because of their beauty. The majority of gesneriads are tropical or subtropical plants

(Stefanello *et al.,* 2024). The Gesneriad family also includes many popular houseplants with succulent levels from the genera Aeschynanthus, Columnea, Gloxinia, Nematanthus, Saintpaulia and Streptocarpus. These plants are mostly non-xerophytic and require regular watering and frost-free conditions, but most do not. Some genera are adapted to alpine or temperate climates and can live under snow to bloom in spring. Gesneriad is also established in many places such as mountainous areas of Europe, Southeast Asia, Malaysian islands and India. Australia, New Zealand and the Pacific Islands and most of South America. The growth form of this species also varies. All gesneriads have fibrous roots, some also develop scaly rhizomes and other tubers. As a result, when conditions are suitable again, the plant can periodically die back and grow from the roots. Some plants, especially cacti and succulents, are even grown for their tubers and (for some) attractiveness. However, Gesneriad tubers may grow quite large and often sprout partially above ground (Jorge Caus *et al.,* 2019).

The rhizosphere is an area of soil surrounded by plant roots. The plant's roots are affected by the biology and chemistry of the soil. This area of the rhizosphere is also a place where biological and chemical activities are strongly influenced by roots and microorganisms. Root exudates include amino acids, organic acids, carbohydrates, sugars and vitamins and proteins. Exudates act to stimulate biological and physical interactions between roots and soil organisms. They modify the biochemical and physical properties of the rhizosphere and contribute to root growth for survival (Kelly *et al.,* 2005). These microorganisms play an important role in plant fertility and the acquisition of plant nutrients.

To study further, this study has used a sophisticated technique known as metagenomics. Soil metagenomic analysis is useful for revealing environmental interactions in various genetic microorganisms that live around plant roots, as well as their potential in supporting plant growth. Metagenomics is the study of the structure and function of entire nucleotide sequences isolated and analyzed from all the organisms (typically microbes) in a bulk sample. Metagenomics is often used to study a specific community of microorganisms, such as those residing on human skin, in the soil or in a water sample. Metagenomics allows the analysis of genetic material that can be extracted directly from environmental samples. The microbial network of the rhizosphere plays an important role in analysis (Julie *et al.* 2012*)*.

1.2 Problem Statement

The rhizosphere is the area of the soil that is directly affected by plant roots. The rhizosphere of Gesneriaceae plants also has a diverse microbial community as it plays an important role in plant health and growth. However, Methods for the isolation of soil microbes especially for Gesneriaceae are not optimized because this study aims to analyze and optimize bacteria from the rhizosphere of Gesneriaceae plants to obtain information about the plant's potential in promoting plant growth and disease suppression.

1.3 Objectives

1. To optimize the isolation of soil DNA from the rhizosphere of selected Gesneriaceae plant.
2. To analyze quality of isolated bacterial DNA from the rhizophere of selected Gesneriaceae plant.

1.4 Scope of Study

- To identify and analyze the species of bacterial DNA found in the rhizophere.

1.5 Significance of Study

The main goal of this study is to understand the abundance of soil microbes found in the area and provide additional information on bacterial species that are beneficial for soil conservation. In addition, it can increase knowledge about the interaction between soil and plants with microbes.

CHAPTER 2

LITERATURE REVIEW

2.1 General introduction of Royal Belum, Perak

In 1971, Royal Belum State Park, Perak Malaysia (coordinates: 5°32′58.91″N 101°20′52.4″E) was gazetted as Belum Forest Reserve and in 2007, it was gazetted again according to the Perak State Park Corporation Enactment 2001 as a State Park, which is called the Peninsular Glory (Razak *et al*., 2015). Topographically and geologically, the Royal Belum State Park represents the northern tip of the Main Range (Banjaran Titiwangsa), the backbone of Peninsular Malaysia where nature (exogenic or surface processes) had crafted the landforms dynamically for over 200 million years. It is believed that the forest reserve coverage is about 290,000 hectares and with more than 146,000 hectares of virgin forest (Azman *et al.,* 2013). In 2012, it was also gazetted under the Perak State Forestry Enactment (Perak State Forestry Enactment) as well as a National Heritage Site which is the highest recognition given by the Malaysian Government (Stefanello *et al.,* 2017).

Royal Belum is in the Hulu Perak District, bordering Thailand on the north side (Figure 1.1) and the East-West Highway on the south side and bordering the state of Kelantan on the east side. Bordering Bang Lang National Park, Thailand & Hala Bala Wildlife Sanctuary, Thailand (Hamdan *et al.*, 2002).

The Royal Belum is composed mainly of pristine tropical rainforest, with many river systems, and small grassland areas, some abandoned agricultural plots, and Tasik Temengor, a large man-made lake. The Royal Belum is part of the large Belum-Temengor forest landscape, one of the largest blocks of forest in Paninsular Malaysia. However, the entire Belum and Temenggor forests were considered 'black areas' and were placed under Emergency from 1948 to 1989 (Azman *et al.*, 2013). In Royal Belum there is also a place known as Sungai Tiang. In the Sungai Tiang area, there are various plant species including *Gesneriaceae* spesies (Hamdan *et al.,* 2020).

The park is also home to a wide variety of flora and fauna, including over 3,000 species of flowering plants, 300 species of birds, and various mammals such as elephants, tigers, and tapirs. (Stefanello *et al.*, 2017).

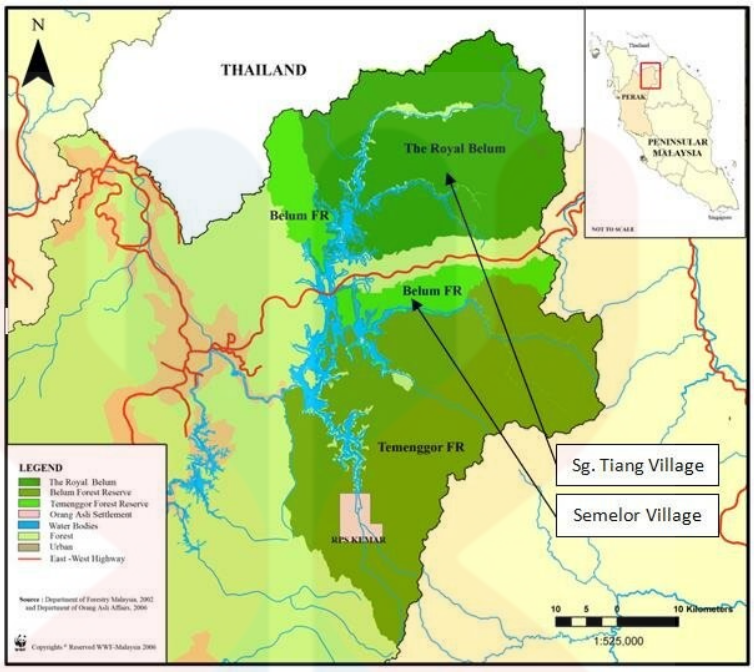

Figure 2.1: Map of the Royal Belum State Park

(Rozaimi, 2015)

The Royal Belum State Park, which is situated in the northern region of Perak, Malaysia, is physically situated on the map. Part of the wider Belum-Temengor Forest Complex, one of the world's oldest and most biodiverse tropical rainforests, this area is located close to the southern Thai border.

2.2 Gesneriaceae Plants

Gesneriaceae, one of 23 families in the flowering plant order Lamiales, consisting of 147 genera and about 3200 species of mostly tropical plants, such as African violet gloxinia and subtropical herbaceous or slightly woody plants. The family is characterized by bilaterally symmetrical, bisexual flowers, each of which has a two-lipped corolla of

five fused petals, five-lobed calyx (sepals), two or four, rarely five, anthers that are joined lightly together or in pairs and a superior are partly inferior ovary with a single chamber in which numerous small seeds are produced on the walls of the ovary (parietal placentation).

Alternativey of Gesneriaceae is a nomen conservandum meaning that although alternative, less well used names for the family were published earlier, the international code of Nomenclature for algae, fungi, and plants specifies this as the name to be used. From about 1997 onwards, molecular phylogenetic studies led to extensive changes in the classification of the family Gesneriaceae and its genera, many of which have been re-circumscribed or synonymized. The family of flowering plants known as Gesneriaceae is a member of the order Lamiales, which also contains the mint and olive families. Gesneriaceae belongs to the subfamily Gesnerioideae of this order (Figure 2.2). The family is separated into around 150 years and has a complicated taxonomy. In the Gesneriaceae family (Figure 2.3), Streptocarpus, Saintpaulia (African Violets), Episcia, Gloxinia, and Sinningia are some of the most famous genera. This family also includes some other less famous but no less interesting genera, such as Boea, Titanotrichum and Petrocodon. Taxonomists are still researching and revising the relationships between the many genera and species in the Gesneriaceae. Lamiaceae, Plantaginaceae, and Bignoniaceae are other related plant families (A. *et al.,* 2020).

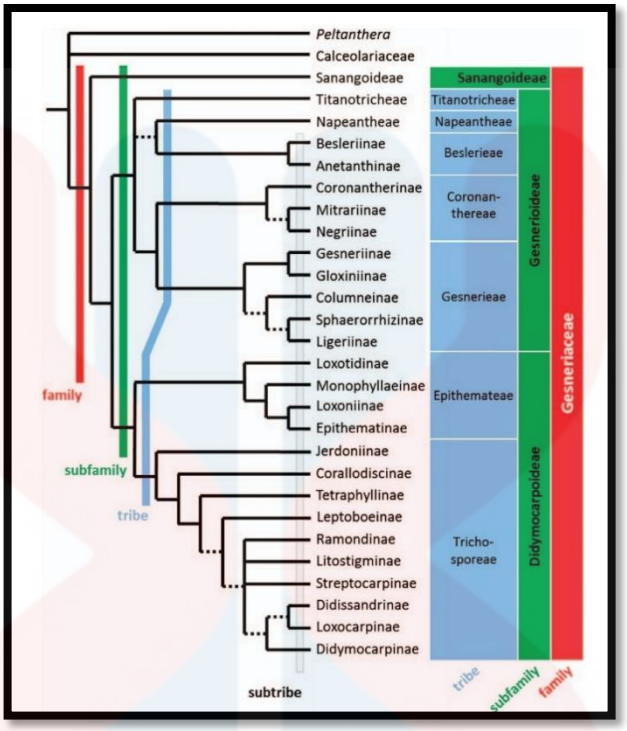

Figure 2.2: The family Gesneriaceae

(Selbyana, 2013)

The family Gesneriaceae, which is divided into three major subfamilies like Sanangoideae, Gesnerioideae, and Didymocarpoideae is taxonomised in this figure. There are several tribes within each subfamily, and certain tribes, like Trichosporeae, have even been further subdivided into smaller groups. This graphic shows how different plant groupings in the Gesneriaceae family have evolved together.

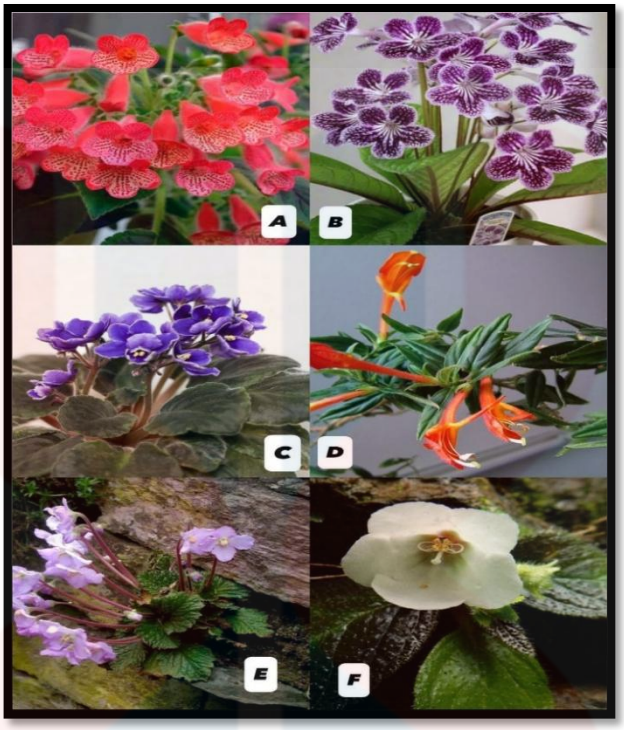

Figure 2.3: Among the species found in the Gesneriaceae family are, **A:** *Kohleria*, **B:** *Cape primrose*, **C:** *African violets*, **D:** *Columnea*, **E:** *Romonda nathaliae*, **F:** *Phinaea*.

(Michael, 2019)

Additionally, the Gesneriaceae family is well known for its rich therapeutic history in addition to its aesthetic appeal. Many species in this family have been used for millennia in traditional medical systems across the world. The Gesneriaceae family is primarily valued for its horticultural properties; many species and hybrids are grown locally or commercially for their exquisite foliage and blooms. Several species are used in traditional neotropical medicine to treat various conditions such as *Drymonia coriacea*, and *Codonanthopsis* dissimulate, Amazonian tribes use *Columnea rubria*cuta to treat toothache. Several species of *Columnea* are used to cure eczema and used to burn injuries. *Columnea sanguinea* leaves also known as *Columnea* Indian tribes in lowland Ecuador

smoke picta as a stimulant. Numerous *Drymonia* and *Columnea* species have been used to cure snake bites (Verdan *et al.,* 2012).

2.2.1 Distribution and Habitat of Gesneriaceae Plants

The flowering plant family Gesneriaceae is widely distributed (Figure 2.5), with representatives found in tropical and subtropical climates as well as other parts of the world. At low altitudes, gesneriads are predominantly bound to forest habitats (Figure 2.4), growing in damp, shady places on slopes or moist rocks, often near streams or waterfalls (Piter Shalit *et al.,* 2023). Southeast Asia has the largest concentration of species variety, especially in Malaysia and Indonesia, home to some of the most well-known genera, including Petrocodon, Chirita, and Oreocharis. Additionally, Madagascar, Africa, and Central and South America are home to many Gesneriaceae species. Certain species have evolved to thrive in certain environments, including rocky outcrops, woods, or even sandy beaches. Because of this, they may be found growing in a variety of environments, including dry deserts, alpine meadows, and rainforests. Climate, soil type, elevation, and competition from other plant species all affect the spread of Gesneriaceae plants. Numerous species have evolved to flourish in certain ecological niches, allowing them to occupy distinct niches and evade competition (Sheridan *et al.,* 2024).

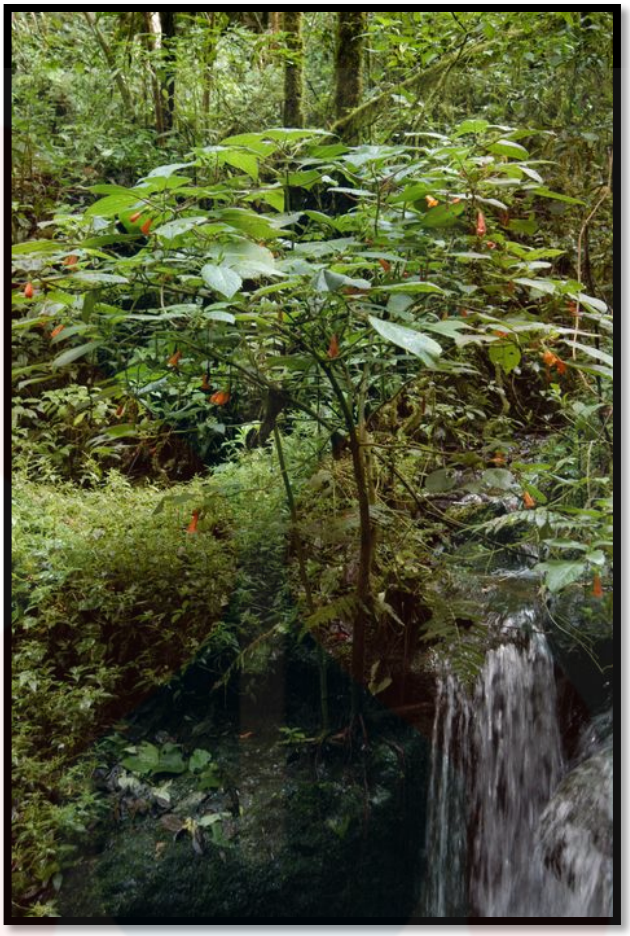

Figure 2.4: Gesneriaceae plant habitats.

(Peter, 2023)

Characteristics of Gesneriaceae plant habitats. These plants grow in damp and shady places on slopes or damp rocks, often near rivers or waterfalls.

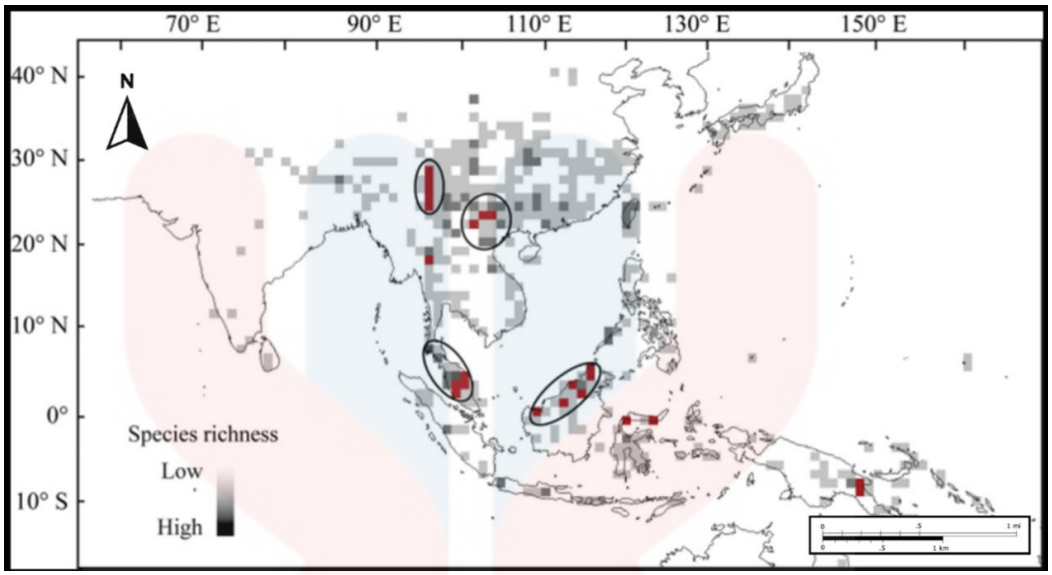

Figure 2.5: Species distributions pattern of the Asian Gesneriaceae.

(Tan, 2020)

Black circles indicate diversification centers with highest species richness and the red grids are the evolutionary hotspots (at least 25% species are neoendemics).

2.2.2 Morphology of Gesneriaceae Plants

Plants of the Gesneriaceae family exhibit a variety of morphologies, but they are known for their unique and prominent flower shapes. Although the blooms vary in size, colour, and form, they typically have two or emply stamens and five fused petals, making them bilaterally symmetrical. The primary components (Figure 2.6) of Gesneriaceae flowers are four whorls of floral organs, two carpels (always combined to form a syncarpus pistil), one whorl of stamens, petals (always fused to make a sympetalous corolla), and sepals (calyx), (Flower Structure 1 *et al.,* 2024). Whilst some species have

complex or whorled leaves, Gesneriaceae plants typically have simple, oppositely orientated leaves. Gesneriaceae plants usually have simple, oppositely oriented leaves (Figure 2.6). Gesneriaceae plants have leaf characteristics such as smooth, hairy leaf surfaces. Gesneriaceae plants also have leaves that are arranged oppositely on the stem. This Gesneriaceae plant has flowers that feature various colors including purple, red and white (Figure 2.7). Due to the beautiful shape and color of this gesneriaceae plant, this plant also attracts the attention of insects such as bees and butterflies. Furthermore, many plants in the Gesneriaceae family have evolved to flourish in certain ecological niches. For example, they may grow on other plants without becoming parasitic thanks to their epiphytic growth habits. *Saintpaulia* (African violets) and the well-known houseplant genus *Episcia* are two noteworthy examples of epiphytic genera in this family. Specialised hairs on the stems or leaves of plants in the Gesneriaceae family are another noteworthy adaptation that helps the plant retain moisture and ward off herbivores (Sheridan *et al.,* 2024).

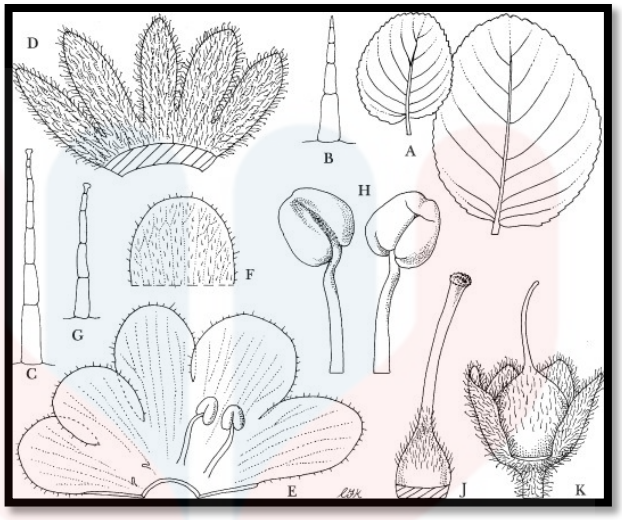

Figure 2.6: Morphology of Gesneriaceae family. Drawing of parts of *Streptocarpus ulugurensis*, from Haston et al (2009); **A:** leaf outlines, **B:** hair from upper surface of leaf, **C:** hair from petiole, **D:** calyx, exterior, **E:** corolla, interior, **F:** corolla lobe, exterior, **G:** hair from corolla, **H:** stamen, **J:** gynoecium, **K:** developing capsule.

(Haston, 2019)

Figure 2.7: Characteristics for flowers and fruits in the Gesneriaceae. **A, B**. Resupinate flower of *Glossoloma tetragonoides* (ampliate on upper surface and medial lobe upward). **C, D.** Non-resupinate flower of *Drymonia croatii* (ampliate on lower surface and medial lobe downward). **E, F.** Dehisced (left) and immature (right) fruits featuring a display capsule in *Drymonia brochidodroma*. **G.** Mature berry in *Columnea tecta*. **H.** Mature berry featuring black seeds and translucent outer fruit wall in *Corytoplectus speciosus*.

(Clark, 2025)

2.3 Rhizophere bacterial DNA

Deoxyribonucleic acid molecules in cells contain genetic information responsible for the development and function of an organism. DNA molecules allow this information to be passed from one generation to the next. DNA consists of a double-stranded helix held together by weak hydrogen bonds between purine-pyrimidine nucleotide base pairs. The rhizophere is a complex environment with dynamic signaling mechanisms between plant roots and root-inhabiting microbes in a continuous flux of biochemical and physiological processes which collectively contribute to the cumulative effects on plant nutrition and bioprotection (Roossinck *et al,* 2019).

Root exudates include amino acids, organic acids, carbohydrates, sugars and vitamins and proteins. Exudates act to stimulate biological and physical interactions between roots and soil organisms. They modify the biochemical and physical properties of the rhizosphere and contribute to root growth for survival (Kelly *et al.,* 2005). Two techniques are frequently employed in the lab to carry out this DNA isolation. Traditional and contemporary segregation are two examples of them. The streak plate approach, which is used for the conventional isolation of DNA from soil, is probably going to take a long time to extract the bacterial DNA. To solve this strategy, a lot of labour is also needed. Because it makes use of sophisticated instruments called the metagenome approach, the contemporary isolation technique is quick. When compared to conventional procedures, the process requires less personnel (García *et al.,* 2018).

Certain soil bacteria that have been separated from root zone plants are known to promote plant development. The term "plant growth promoting rhizobacteria" (PGPR)

refers to these advantageous, free-living soil bacteria. These include microorganisms that fix nitrogen and those that dissolve phosphate, among others. Either a direct or indirect mechanism mediates the positive PGPR effects. Direct effects are typically linked to the generation of plant hormones such cytokinin, auxin, and gibberellin, or to the supply of biologically constant nitrogen. By suppressing bacteria, fungus, and nematode pathogens and producing siderophores, hydrogen cyanide (HCN), ammonia, antibiotics, volatile metabolites, and other substances, PGPR also promotes growth indirectly (Glick *et al.,* 1995).

2.4 Soil sample collection protocol

In this work, rhizosphere soil sampling was carried out in accordance with the guidelines provided by Novello et al. (2023) and Hannes Schmidt et al. (2018). To guarantee the existence of active microbial communities, Schmidt et al. state that rhizosphere soil must be taken from plants that are robust and actively developing. Three sites in the Royal Belum region of Perak—Sungai Kejar, Sungai Kenarong, and Sungai Papan were used to sample a few species of the Gesneriaceae family together with the rhizosphere soil that goes with them. In accordance with Novello et al., dirt was collected at a depth of 5 to 20 cm after the top 5 cm of soil was initially removed perpendicularly along the root axis to remove surface pollutants. The loose surrounding dirt, often known as bulk earth, was gently brushed off after the plants were delicately removed. Only the soil that was firmly attached to the roots, known as the rhizosphere, was collected by shaking or lightly brushing it into sterile sample bags while wearing sterile gloves. After

that, these samples were brought to the lab in a cold environment for microbial investigation and DNA extraction. This technique guarantees that the microbial communities examined are those that are intimately related to the root zone and accurately represent interactions between plants and microbes.

2.5 Application of DNA Isolation from Microorganisms

The ability to extract DNA is of primary importance to studying the genetic causes of disease and for the development of diagnostics and drugs. It is also essential for carrying out forensic science, sequencing genomes, detecting bacteria and viruses in the environment and for determining paternity. Arccording of Smith (2024), the polymerase chain reaction, or PCR for short, is a lab method that quickly creates millions to billions of copies of a certain DNA sequence so that it may be further examined. In PCR, certain genome segments are chosen to be amplified using brief synthetic DNA fragments known as primers, and those segments are subsequently amplified by many rounds of DNA synthesis.

Typically, the goal of PCR is to make enough of the target DNA region that it can be analyzed or used in some other way. For instance, DNA amplified by PCR may be sent for sequencing, visualized by gel electrophoresis, or cloned into a plasmid for further experiments (Khan *et al.,* 2021).

PCR-produced DNA may be utilised in a wide range of laboratory processes after it has been amplified. For instance, PCR is used in most of the Human Genome Project's (HGP) mapping methods. A few laboratory and clinical procedures, including DNA

fingerprinting, the identification of bacteria or viruses (particularly AIDS), and the diagnosis of genetic abnormalities, benefit from the use of PCR (Eric *et al.,* 2020).

PCR is a three-step process that is carried out in repeated cycles. The initial step is the denaturation, or separation, of the two strands of the DNA molecule. This is accomplished by heating the starting material to temperatures of about 95 °C (203 °F). Each strand is a template on which a new strand is built. In the second step the temperature is reduced to about 55 °C (131 °F) so that the primers can anneal to the template. In the third step the temperature is raised to about 72 °C (162 °F), and the DNA polymerase begins by adding nucleotides onto the ends of the annealed primers. At the end of the cycle, which lasts about five minutes, the temperature is raised, and the process begins again. The number of copies doubles after each cycle. Usually, 25 to 30 cycles produce enough DNA. In the original PCR procedure, one problem was that the DNA polymerase had to be replenished after every cycle because it is not stable at the high temperatures needed for denaturation. This problem was solved in 1987 with the discovery of a heat-stable DNA polymerase called *Taq,* an enzyme isolated from the thermophilic bacterium *Thermus aquaticus*, which inhabits hot springs. *Taq* polymerase also led to the invention of the PCR machine.

Because DNA from a wide range of sources can be amplified, the technique has been applied to many fields. PCR is used to diagnose genetic disease and to detect low levels of viral infection. In forensic medicine it is used to analyze minute traces of blood and other tissues in order to identify the donor by his genetic "fingerprint." The technique has also been used to amplify DNA fragments found in preserved tissues, such

as those of a 40,000-year-old frozen woolly mammoth or of a 7,500-year-old human found in a peat bog (Britannica *et al.,* 2019).

2.6 Application of DNA Extraction using CTAB-based Buffer.

Cetyltrimethylammonium bromide (CTAB)–based methods are widely used to isolate DNA from plant tissues, but the unique chemical composition of secondary metabolites among plant species has necessitated optimization. The study reviewed literature for a modified CTAB protocol used for plant DNA isolation. The study found that each step of the CTAB protocol was modified, and we summarize those modifications to provide recommendations for extraction optimization. Future genomic studies will rely on optimized CTAB protocols (Schenk *et al.,* 2023).

Besides that, DNA isolation using CTAB is one of the preferred DNA isolations methods especially for plant samples. This is because CTAB solves the limitation problem of collecting good quality DNA from mature plants that are composed of high concentration of secondary metabolites. CTAB buffers are effective at removing polysaccharides and polyphenols from plant DNA preparations. CTAB based extraction buffers are widely used when purifying DNA from plant tissues. The hazard with traditional CTAB protocols is the protein component of plant lysates is usually removed using phenol and chloroform. These two solvents are generally considered hazardous. Apart from that, DNA isolation using this method also helps to deal with problems involving DNA contamination, degradation as well as low yield of DNA as a result from irreversible attachment of phenolic compound with DNA molecules from plant samples

(Naveed *et al.,* 2021). Additionally, this technique yields a consistent and repeatable amplification output, which amply demonstrates the DNA's appropriateness for PCR use. Additionally, the use of CTAB for DNA extraction has produced a very high complete genomic DNA since the isolation procedure does not hydrolyse the DNA. In contrast to phenol-chloroform, this technique is also safe to employ (Hosseinpour *et al*., 2013). Extraction technique that seriously jeopardises human health.

It's interesting to note that only the activity of other chemicals in CTAB buffer may produce DNA of high quality. This comprises the removal of phenolic compounds using polyvinyl pyrrollidone (PVP) and β-mercaptoethanol. Apart from that, chloroform-isoamyl alcohols are crucial for controlling cellular proteins and eliminating various colouring agents including pigments, dyes, and chlorophyll (Naveed *et al.,* 2021). In addition to filtering the result, the use of ethanol facilitates the extraction of the DNA pellet from the liquid solution.

2.7 Isolation of Pure Genomic DNA from Gesneriaceae Plants

There is currently a dearth of scientific study on Gesneriaceae, primarily in the field of biotechnology. After DNA is collected, it may be documented for use in upcoming investigations and utilised to further genome sequencing and PCR research. PCR procedures are appropriate for methods that provide high DNA purity and don't employ hazardous chemicals. (Mahuku *et al.,* 2004). This process involves precipitating polysaccharides in a high salinity environment and inactivating proteins. This would be

the best way to extract DNA for the Gesneriaceae study before using the PCR procedure to continue the results.

To get genetic material in a somewhat pure state for use in further research, including PCR and sequencing, DNA extraction from plants is typically utilised. This procedure uses a mix of physical and chemical techniques to purify DNA from cells (Hasan *et al*., 2020). Because there aren't enough studies on Gesneriaceae DNA, DNA extraction from plants will result in new research.

They both have pros and cons when it comes to the approach. The most effective technique for isolating pure genomic DNA is genomic, which is helpful for coding certain gene clones in prokaryotes with tiny genomes. Because it may be used to change an organism's genome for a variety of objectives, this gene coding approach is crucial to modern biology. The next benefit of this genomic approach is that it facilitates further investigation into the composition and operation of the organism's genome. Genetic mutations are also studied with it. The technique is also highly helpful for sequencing DNA. Knowing the genetic information stored in DNA is aided by DNA sequencing, which is the process of organising DNA molecules. Furthermore, genomics aids in the identification of significant genes from pharmacological drugs by employing this technique.

One notable drawback of the Pure Genomic DNA Isolation approach is that it necessitates a significant amount of processing power and complex software. Moreover, this procedure is prone to mistakes. This approach is also less than ideal since eukaryotic

genomes with very big genomes contain a lot of non-protein-coding DNA in addition to non-coding DNA such repetitive DNA and regulatory areas (Aakash *et al.,* 2021).

CHAPTER 3

MATERIALS AND METHOD

3.1 Materials and Apparatus

DNA was extracted and analysed from rhizosphere soil in this investigation using a variety of lab apparatus and chemical reagents. Beakers, test tubes, a spatula, micropipette (Eppendorf, Germany), gloves, a thermometer, ziplock paper bags, kimtech (Science), tag labels, and ice to keep the temperature low were among the supplies. Rhizosphere soil samples were gathered and kept in ice storage. The NucleoSpin® Soil Column, NucleoSpin® Inhibitor Removal, two millilitre collection tubes, and a variety of buffers, including Wash Buffer (SW1 and SW2), Enhancer (SX), Lysis Buffers (SL1, SL2, SL3), Binding Buffer (SB), and Elution Buffer (SE), were used in the spin-column (MACHEREY-NAGEL NucleoSpin® Soil, Germany),protocol kit method (MN Bead Tube Type A) to extract DNA. Chloroform, 70% ethanol, 1kb DNA ladder (Promega, United States) and runSAFE stain (Cleaver Scientific, United Kingdom), agarose powder (Promega, United States) cold isopropanol, sodium dodecyl sulphate (SDS) solution (Promega, United States), Tris-HCl-EDTA, sodium chloride (NaCl) (Promega, United States, sterile distilled water, CTAB Buffer, TAE Buffer, and TE Buffer were among the other chemicals utilised. An electrophoresis apparatus thidium bromide as a staining agent were utilised to see the outcomes of DNA amplification.

We used essential scientific equipment to measure the amount and purity of bacterial DNA. DNA fragments were separated on a 1% agarose gel made using TAE buffer and heated in a lab microwave (Samsung, South Korea) as part of agarose gel electrophoresis, dry water bath (Hangzhou Allsheng Instruments Co. Ltd, China), microcentrifuge (Eppendorf Corperate, Germany), vortexed mixer (VELP SCIENTIFICA, Italy) which includes a chamber, power supply, and gel tray (Major Science Co. Ltd, Taiwan). Following electrophoresis, the purity and integrity of the DNA bands were examined by shining a transilluminator under UV light (Major Science Co Ltd, Taiwan). A microvolume UV-Vis spectrophotometer (DeNovix®, USA) evaluated absorbance at 260 and 280 nm to quantify and purify DNA. The A260/A280 ratio for purity (~1.8, suggesting low protein contamination) and DNA content in ng/µL or µg/mL were calculated from a tiny amount of DNA sample, providing crucial information for additional molecular research.

3.2 Soil sample collection for selected Gesneriaceae Plants for whole Microorganisma DNA Isolation

The sample plan for the rhizosphere soil of Gesneriaceae plants in the Royal Belum region of Perak, Malaysia, is described in this paper. In figure 3.1, three different locations within the Belum-Temengor Forest Complex, Royal Belum Rainforest, namely Sungai Kejar (K.R), Sungai Kenarong (K.G), and Sungai Papan (P.N) also table 3.1 shows the abbreviation of sample, were used to collect rhizosphere soil samples.A few healthy Gesneriaceae species that usually thrive in damp, shady forest habitats, especially

along riverbanks and rocky outcrops microhabitats that are frequent in Royal Belum were linked to these soils. The coordinates of the general sampling area are approximately 5°N latitude and 101°E longitude, placing it in northern Perak near the Thailand border. Royal Belum is a protected state park known for its rich biodiversity and pristine tropical rainforest, making it a highly suitable location for ecological and microbiological research (Schwabe *et al*., 2014).

Abbreviation	Sample	Locality
P.N	Soil sample from Cytandra sp.	Sungai Papan
K.R	Soil sample from *Cytandra cupulata Ridl.*	Sungai Kejar
K.G	Soil sample from *Codonoboea rugosa*	Sungai Kenarong

Table 3.1: List of abbreviations with sample and locality. Three soil samples were collected from Sungai Cytandra sp. (P.N), *Cytandra cupulata Ridl.* (K.R), and *Codonoboea rugosa* (K.G), with each sample labeled using specific abbreviations for consistent reference throughout the study.

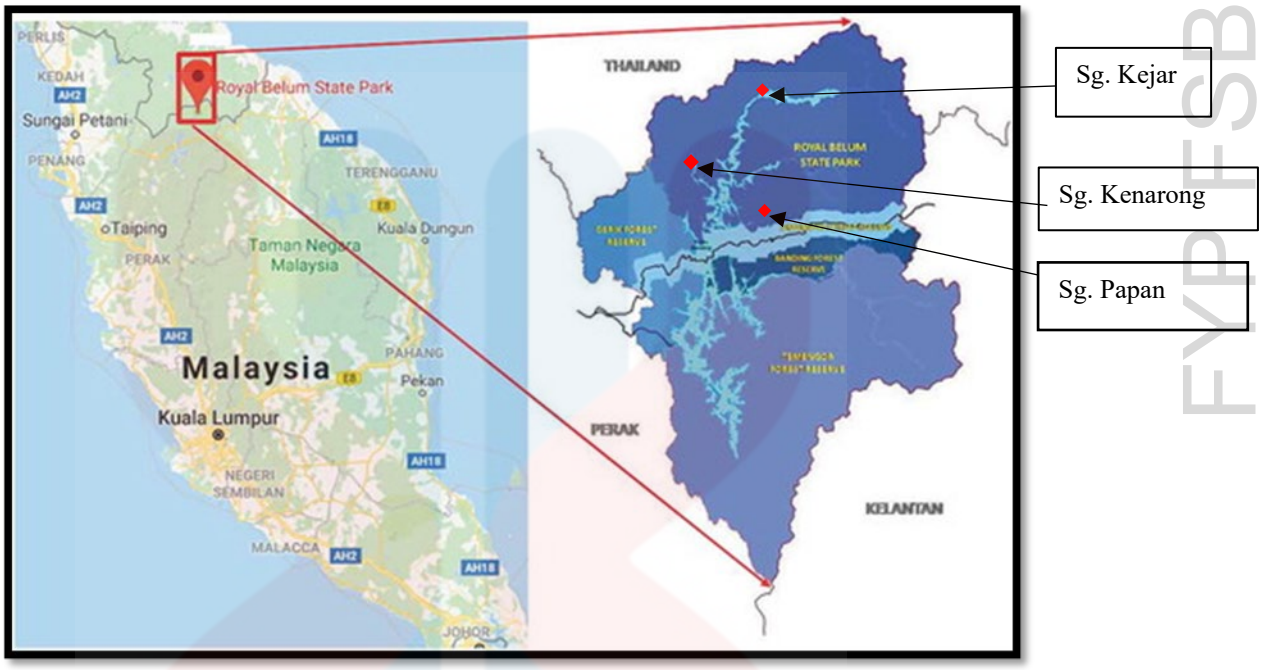

Figure 3.1 The research area's geographic environment is shown in the figure.

(Gwee, 2018)

The three sampling sites Sg. Kejar, Sg. Kenarong, and Sg. Papan are depicted on a comprehensive map of the Royal Belum rainforest, along with a generic map of Malaysia that highlights Perak. The precise areas where rhizosphere soil samples were taken are shown by red dots and lines, giving a clear visual representation of the sampling distribution.

3.3 DNA Extraction of genomic from Gesneriaceae Plants

3.3.1 DNA extraction of genomic from Gesneriaceae Plants using Conventional Method (Bead Beating)

DNA was extracted at Universiti Malaysia Kelantan Campus Jeli's Microbiology and Biochemistry Laboratory (Mohanty et al., 2013). Using a pestle and mortar, genomic DNA was extracted from frozen rhizophere soil samples to acquire DNA from the rhizophere soil of Gesneriacea plants. DNA extraction kits provide all essential components, including chemicals, buffers, columns, magnetic particles, and instructions for effective DNA extraction (Inzhenernaya st et al., 2025). The extraction result is shown in figure 3.2 below.

A soil extraction method, according to Sela (2024), is a laboratory technique used to determine the concentration of certain nutrients that are accessible to plants. These techniques imitate the circumstances under which plants take nutrients from the soil sample from Sungai Papan (P.N) by extracting them using a variety of chemical solutions. Using 100 ml of extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], and 1.5 M NaCl) were mixed with 100 g (wet weight) of soil. The samples were agitated for two minutes in a Bio-Spec Products Bead-Beater following the addition of 100 g of glass beads (Bio-Spec Products, Bartesville, USA). Ten millilitres of 20% sodium dodecyl sulphate (SDS) were added, and mixing was continued for five more seconds. The samples were incubated at 65 °C for an hour before being moved to a 250 ml centrifuge container and centrifuged at 6000 g for 10 minutes. The soil pellet was

extracted once more using a 100 ml extraction buffer after the supernatant was collected. It was then incubated for 10 minutes at 65 °C and centrifuged as previously mentioned.

Following its transfer to a 50 ml centrifuge tube containing half a volume of sodium chloride (1.6 M) and polyethylene glycol (30%), the supernatant was left to stand at room temperature for two hours. Following a 20-minute centrifugation at 10,000 g, the partially purified nucleic acid pellet was resuspended in 20 millilitres of TE (10 mM Tris-HCl, 1 mM sodium EDTA, pH 8.0). Samples were centrifuged at 4 degrees Celsius for 30 minutes at 16,000 g after being on ice for five minutes to precipitate proteins and polysaccharides. After the aqueous phase was eliminated using chloroform/isoamyl alcohol and phenol/chloroform, 0.6 litres of isopropanol were added to precipitate the DNA. The DNA was resuspended in one millilitre of TE after being centrifuged at 16,000 g for 30 minutes to pellet it after two hours at room temperature. (Yeates *et* al., 1998). The two rhizosphere soil samples from Sungai Kejar (K.R) and Sungai Kenarong (K.G) were also subjected to this procedure.

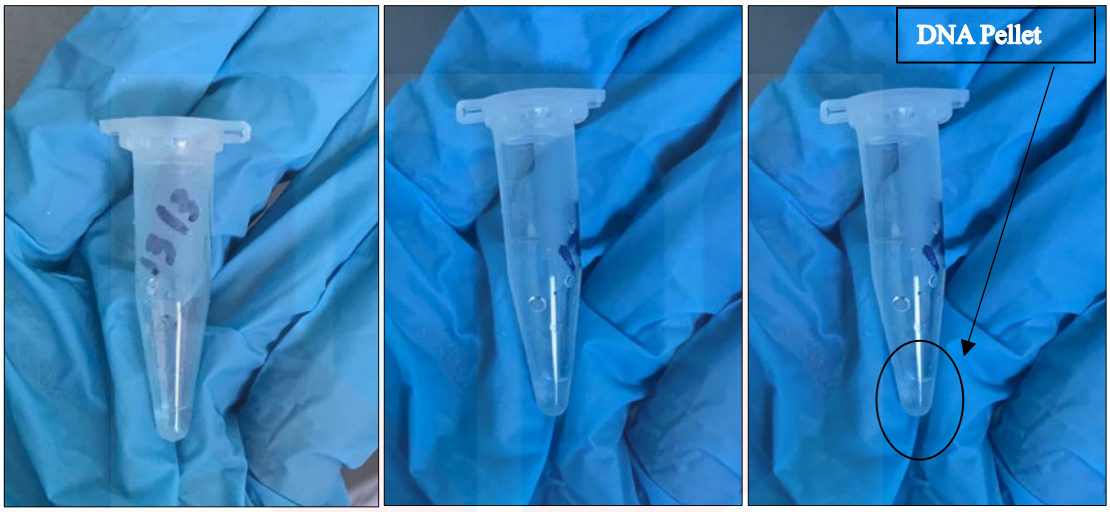

Figure 3.2: Results of DNA extraction from rhizosphere soil from all three sites Sungai Papan (P.N), Sungai Kejar (K.R), and Sungai Kenarong (K.G) using Conventional techniques (bead beating).

3.3.2 DNA extraction of genomic from Gesneriaceae Plants using Spin-column (MACHEREY-NAGEL NucleoSpin® Soil, Germany), Protocol Kit Method.

DNA was extracted at University Malaysia Kelantan Campus Jeli's Microbiology and Biochemistry Laboratory (Mohanty et al., 2013). Using a pestle and mortar, genomic DNA was extracted from frozen rhizosphere soil from Sungai Papan (P.N) samples to obtain DNA from rhizosphere soil of Gesneriacea plants. DNA extraction spin-column (MACHEREY-NAGEL NucleoSpin® Soil, Germany), protocol kit method provides all essential components, including chemicals, buffers, columns, magnetic particles, and instructions for effective DNA extraction (Inzhenernaya st et al., 2025).

After pipetting around 500 µL, 250 µL of SB was added. After that, the mixture was vortexed gently for five seconds. The supernatant was then moved to another tube, which was the DNA binding column, a green filter tube. To enable the DNA to attach to

the column, the tube was centrifuged for 30 seconds at 13,000 rpm. The identical green-ring tube was kept, but the flow-through was thrown away. After adding 500 µL of SB, the tube was centrifuged once more for 30 minutes at 13,000 rpm. They threw away the supernatant. Following the addition of 400 µL of SW1, the tube was centrifuged for 30 seconds at 13,000 rpm, and the flow-through was disposed of. After the sample was cleaned with 300 µL of SW2 (ethanol), the tube was centrifuged for 30 seconds at 13,000 rpm. The supernatant was then discarded. To make sure that impurities were eliminated from the DNA sample, this washing procedure with SW2 was carried out twice. The tube was then allowed to dry for two to three minutes by centrifuging it at 13,000 rpm. After adding 65 µL of SE, the tube was centrifuged for one minute at 13,000 rpm to extract the DNA from the column. To preserve its purity over time, the isolated DNA was kept at -20°C after the green filter was disposed of (Sigma Aldrich et al., 2025). All three of the rhizosphere soil samples from Sg. Papan, Sg. Kejar, and Sg. Kenarong were subjected to this procedure. This process was also applied to the two rhizosphere soil samples from Sungai Kejar (K.R.) and Sungai Kenarong (K.G.). The extraction result is shown in figure 3.3 below.

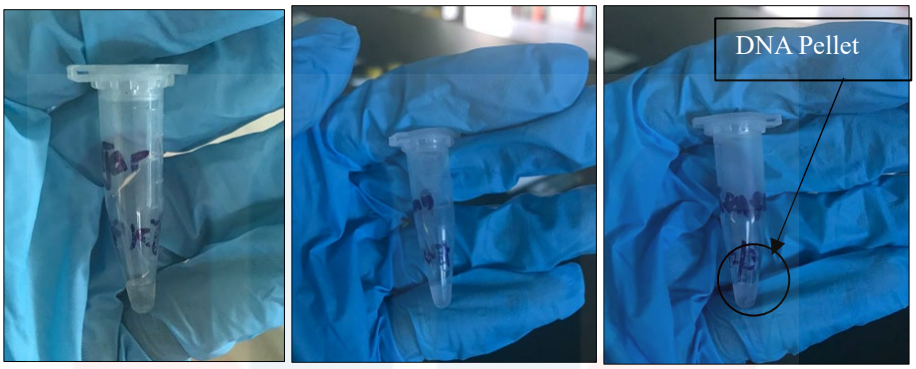

Figure 3.3: Results of DNA extraction from rhizosphere soil from all three sites Sungai Papan (P.N), Sungai Kejar 9K.R), and Sungai Kenarong (K.G) using Spin-column (MACHEREY-NAGEL NucleoSpin® Soil, Germany), protocol kit method.

3.4 DNA of genomic from selected Gesneriaceae Plants Quality and Quantity checking using Agarose Gel Electrophoresis and Nanodrop Spectrophotometer

This method was carried out in the Faculty of Earth Sciences' Microbiology Laboratory. Weighing up the right quantity of agarose and dissolving it in TAE buffer produced a 1% agarose solution. Then, using the agarose gel electrophoresis method, water samples from Sungai Papan (P.N.) were added to the agarose solution for examination.

After that, the mixture was steadily cooked in a microwave until the agarose was completely dissolved and the mixture turned transparent. The solution was heated and then allowed to cool to around 60°C before being transferred onto a gel tray that had a comb attached to it. The gel was either chilled to hasten the gelling process or allowed to fully harden at ambient temperature. After solidifying, the gel was put in a gel box and

coated with a 1X TAE buffer, which was the same buffer that was used to prepare the gel. After properly loading the DNA samples onto the gel wells. As a size marker, 2 µL of DNA ladder and 1 µL of RedSafe were combined and added to one of the wells. Two microlitres of RedSafe were combined with ten microlitres of each DNA sample, which was then put into the remaining wells. For 40 minutes, the gel electrophoresis was operated at a steady 100 V voltage. The power was switched off when the run was finished, and the gel was gently taken out of the gel box. Lastly, a UV transilluminator was used to see the DNA bands, which showed up on the gel as discrete bands (Addgene *et al*., 2018). This method has been carried out for both methods, namely the conventional method with the spin-column (kit method).

Two distinct extraction techniques were used to produce three DNA samples: the kit-based spin-column approach and the traditional bead-beating method. Each sample was analysed using a microvolume spectrophotometer (DeNovix, USA) in accordance with a meticulous protocol to guarantee the precision of DNA concentration results. It was carried out in the Biology Laboratory 1 of the Faculty of Agro-based Industry. The Nanodrop instrument's pedestal surface was cleaned with distilled water and lint-free lab tissue prior to any measurements being taken. The purpose of this cleaning procedure was to remove any cross-contamination from earlier samples that could have impacted the outcomes. Depending on the kind of buffer used for extraction, the blanking procedure was then carried out to provide baseline reading. One microlitre of TE buffer was used to blank DNA samples from the traditional approach, and one microlitre of SE buffer (elution buffer) was used to blank samples from the kit method. To confirm system stability and instrument cleanliness, an additional control of 1 µL of pure water was

measured. Following blanking, 1 µL of each sample was used to quantify the DNA concentration. The instrument's arm was lowered, the sample was carefully set on the pedestal, and the "Measure" function was turned on. The output contained the purity ratios A260/A280 and A260/A230, which were used to evaluate any protein or chemical contamination in the samples, as well as the DNA content in ng/µL. Accuracy and cleanliness were maintained throughout the procedure by cleaning the pedestal with distilled water after each measurement and then applying the subsequent sample. This process allowed for the effective acquisition and comparison of accurate and legitimate data on DNA concentration and purity from both approaches (Erk *et al*., 2022). The NanoDrop 1000 spectrophotometer was used to quantify the quantity of extracted DNA. All things considered; UV spectrophotometry is a widely used method to evaluate the quality of DNA extracts (Davis et al., 2014).

The two rhizosphere soil samples taken from the Royal Belum Forest Reserve, namely Sungai Kejar (K.R), and Sungai Kenarong (K.G), were also subjected to the same procedure to determine the amount and quality of bacterial DNA.

CHAPTER 4

RESULT AND DISCUSSION

4.1 DNA Extraction results of Genomic

4.1.1 DNA Extraction using Conventional Method (Bead Beating)

DNA isolation from three samples that had been treated according to the classical bead punching procedure. To break the cell walls and release the DNA into the solution, mechanical force was applied in this method through shaking with small glass beads. Material was added to a previously used tube containing glass beads and lysis solution to start the process of extraction. To thoroughly agitate the cells, the tube was then shaken for a few minutes with a bead beater machine. To separate the solid from the supernatant rich in DNA, the mixture was then centrifuged. In order to enable the DNA to precipitate, the resultant supernatant was taken and combined with a precipitating solution, such as isopropanol. A DNA pellet was created and collected at the tube's bottom resulting from a subsequent centrifugation step that followed the process of precipitation. The DNA pellet which is created at the bottom of the tube in each sample is very prominent.

The presence of pellets shows that the extraction of DNA in each of the samples was carried out properly. The initial composition of the DNA or the degree of efficiency of the process of lysis carried out in each sample may have resulted in a slight difference in the size and the density of the pellets between the samples. All things considered have

shown that the bead punching technique always produces positive DNA extraction outcomes. The reason why this DNA pellet was successfully formed is because the DNA is now ready for analysis by means of techniques like NanoDrop spectrophotometry, which determines concentration and purity. This graphic also confirms that the extraction process that was carried out was successful, and it is also simpler to use to visually compare the three samples.

4.1.2 DNA Extraction using Spin-column (MACHEREY-NAGEL NucleoSpin® Soil, Germany), Protocol Kit Method

Using the spin column kit protocol to clean DNA from three samples. This was a process involving a few steps based on DNA adsorption onto a silica membrane contained in a spin-column used in the purification and isolation of DNA. To confirm that the cells had been disrupted and DNA fully released, a lysis solution provided with the kit was added to all samples before incubation. To make the DNA adsorb onto the silica membrane once the impurities were washed away, the sample was thereafter dropped into a spin column and centrifuged. To facilitate the washing away of contaminants such as proteins, RNA, and salts, several steps of washing were used. To obtain a solution that is free from DNA impurities, the tube was centrifuged after the DNA was eluted in SE (elution buffer) solution.

This means that the purification and extraction procedures were done successfully. The existence of an elution solution suggests that the DNA was successfully separated and ready for spectrophotometer analysis or other modern techniques, even in the absence

of pellet formation as with the bead beating technique. The spin-column technique might produce DNA free and clear of contamination, as observed.

4.1.3 Comparison results extraction between the Conventional Method (Bead Beating) and the Spin-column (MACHEREY-NAGEL NucleoSpin® Soil, Germany), Protocol Kit Method.

DNA was isolated from three samples using two different techniques the traditional method (bead beating) and the spin-column kit method. In the conventional approach, glass beads were vigorously shaken to mechanically lyse the cells. Alcohol was then added to enrich the extracted DNA, followed by centrifugation. The efficiency of the lysis and sedimentation processes resulted in a visible DNA pellet at the bottom of the tube, varying in size and volume among the three samples. However, the final product from this method appeared turbid (cloudy), indicating possible contamination by proteins or bacteria.

In contrast, the spin-column kit method involved chemical lysis of cells, followed by binding of the resulting DNA to a silica membrane within a spin-column tube. A series of washing steps was performed before the DNA was eluted using SE buffer. This method yielded DNA from all three samples with consistently high clarity and purity. Unlike the conventional method, the DNA obtained using the spin-column technique was noticeably clear, suggesting minimal interference from bacterial or protein contaminants. Therefore, the spin-column method proved to be more consistent, convenient, and capable of producing high-quality DNA suitable for downstream analysis (Ressmann et al., 2015).

4.2 Agarose Gel Electrophoresis of Extracted Genomic DNA of Microbes

An electrophoresis machine, according to Smith (2021), is a laboratory equipment used to distinguish biomolecules such as DNA, RNA, or proteins based on size and charge. The ideal method for DNA fragment separation in the range of 100 bp to 25 kb is agarose gel electrophoresis (Lee et al., 2012). The electrophoresis device is placed at 100 amperes (A) and for 40 minutes to achieve the outcome of DNA movement. Negatively charged DNA migrates through pores in agarose gel to the positively charged end of the gel when a current of electricity is applied, with the smaller fragments moving earlier (Steward et al., 2022).

RedSafe™ Nucleic Acid Staining Solution is a new and safer nucleic acid stain reagent, an alternative to EtBr (ethidium bromide). Redsafe is used for DNA fluorescence using UV-Transilluminator for viewing isolated DNA bands. RedSafeTM Nucleic Acid Staining Solution is superior in sensitivity compared to EtBr and easy and simple to use. Because of its significantly lower genotoxicity than EtBr, it is safe for use by users as well as in laboratories. It glows at 520–540 nm when bound to nucleic acids (a 530 or 535 nm filter is appropriate), (Sheep et al., 2024).

DNA extraction through the kit process and bead beating process was verified using agarose gel electrophoresis. Two agarose gel electrophoresis were performed, one of the kit processes (Figure 4.1) and the other of the bead beating process (Figure 4.2). There were 3 samples of varying locations and selected Gesneriaceae plants in each gel. The successful extraction of DNA could be verified by the presence of bands on the agarose gel from a UV-transilluminator.

To estimate DNA fragments of size between 1,000-base pairs (bp), a one kb DNA ladder is employed in molecular biology laboratories. It involves separating the under-study DNA sample on an agarose gel with some DNA fragments of known sizes, which are usually from 500 bp to 10,000 bp. According to their size, the sample's DNA fragments run faster for smaller fragments than for larger fragments (Lan et al., 2012). Negatively charged DNA/RNA can move through pores in an agarose gel to the positively charged end of the gel when an electric current is employed, with faster movement for smaller fragments (Steward et al., 2022). There is a marker marked as M in figure 4.1 and figure 4.2 which is an indicator of the DNA fragment size in the other lane.

The extractions of DNA that were carried out were identified and authenticated presence of DNA by agarose gel electrophoresis. 1 µL volume of DNA extractions from three samples to be tested were taken using agarose gel electrophoresis. The obtained bands on the gel when UV analyzer visualized confirmed presence of DNA. The 1kb DNA marker was used to determine the size of the bands. The range of this ladder marker is 10k bp to 250 bp.

Figure 4.1 and 4.2 showed the agarose gel electrophoresis result for three extraction samples of varying methods. Regarding quality and integrity of the DNA, the two DNA extraction methods in the gel electrophoresis photographs show their respective strengths and weaknesses. The three samples (Lanes 1–3) showed DNA bands approximately around 10 kb using the bead-beating method (Figure 4.2). However, in comparison to the kit method, sharpness and strength of bands were relatively inferior, even though they were defined. This suggests that even if DNA isolation was carried out effectively, the purity or concentration of the material isolated could not be as good as

predicted. Conversely, the kit approach (Figure 4.1) revealed DNA bands also some ~10 kb for every sample but brighter and sharper in intensity, though some smearing was evident. This, therefore, means that DNA can still be produced via the kit approach with satisfactory results and good-quality outputs. All samples yielded comparable results, showing this procedure is still valid. While there was sporadic decline, the kit method generally held very good potential for generating high-quality DNA with strong band intensity.

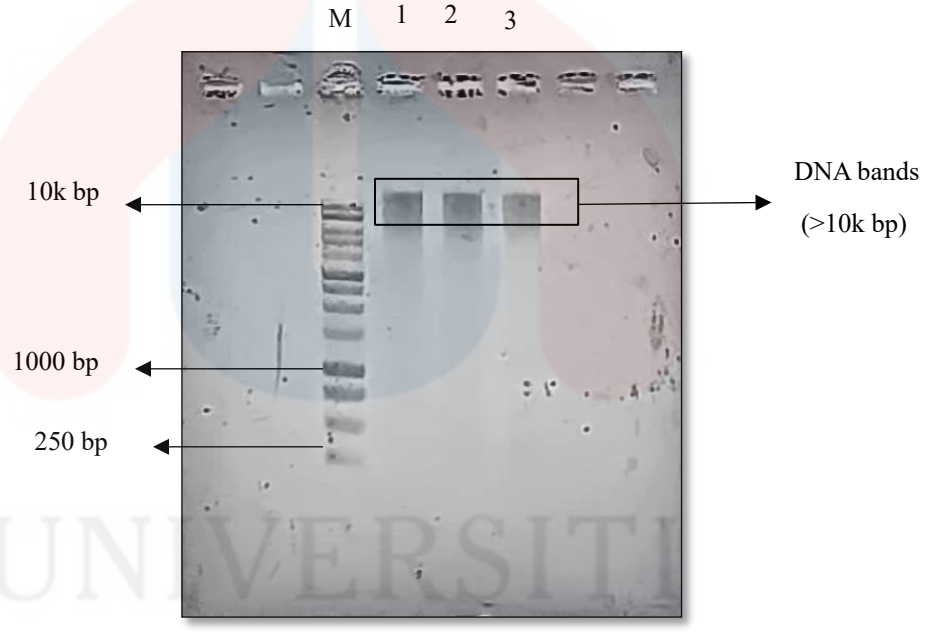

Figure 4.1: Gel Electrophoresis of DNA from kit method. The figure shows that there were DNA bands present in Lane 1 to Lane 3 after being visualized under UV transilluminator of 1% agarose gel 90V for 10 minutes. **Lane 1** (Sample A): Sungai Papan (P.N), **Lane 2 (**Sample B): Sungai Kejar (K.R), and **Lane 3 (**Sample C): Sungai Kenarong (K.G).

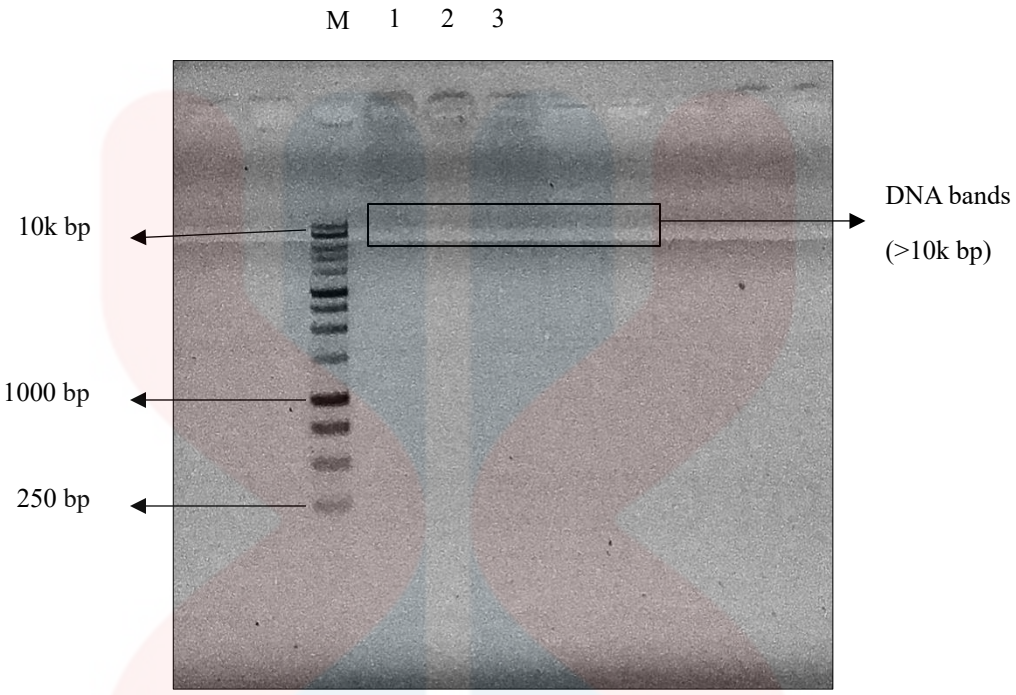

Figure 4.2: Gel Electrophoresis of DNA from bead beating method. The figure shows that there were DNA bands present in Lane 1 to Lane 3 after being visualized under UV transilluminator of 1% agarose gel 90V for 10 minutes. **Lane 1 (**Sample A): for Sungai Papan (P.N), **Lane 2 (**Sample B): Sungai Kejar (K.R), and **Lane 3 (**Sample C): Sungai Kenarong (K.G).

4.3 Quantification of Extracted genomic DNA of Microbes from Gesneriaceae Plans using Conventional Method (Bead Beating) and Spin-column (MACHEREY-NAGEL NucleoSpin® Soil, Germany), Protocol Kit Method

Quantification of genomic DNA is highly dependent on the yield and quality of DNA obtained through extraction from two different methods (Milavec *et al*., 2023), namely the Spin-column protocol kit method and the conventional method (bead beating). The table 4.1, show the value quantification from extracted DNA using Conventional method and Spin-column method (MACHEREY-NAGEL NucleoSpin® Soil, Germany).

SAMPLE	CONVENTIONAL METHOD		SPIN-COLUMN METHOD	
	A260/280	DNA MASS (NG/50UL)	A260/280	DNA MASS (NG/50UL)
Sungai Papan (P.N)	6.60	NA	1.72	521.3
Sungai Kejar (K.R)	10.39	NA	1.50	503.5
Sungai Kenarong (K.G)	13.50	NA	1.89	300.1

Table 4.1: Quantification values from DNA extracted using the Conventional method and the Spin-column method (MACHEREY-NAGEL NucleoSpin® Soil, Germany) from three samples, namely Sungai Papan (P.N), Sungai Kejar (K.R) and Sungai Kenarong (K.G).

Table 4.2 provides the outcome of quantification of DNA extraction samples (A, B, and C) using a Nanodrop spectrophotometer. The method determines the absorbance of extracted solutions in different wavelengths and, accordingly, estimates the DNA content and purity (García-Alegría et al., 2020). The 260/280 nm ratio of absorbance and DNA concentration (µg/ml) are two vital parameters for the estimation of extraction results. The 260/280 ratio is an indication of DNA purity, and a reading around 1.8–2.0 represents pure DNA without protein contamination. Concentration is the indication of the amount of DNA obtained from the samples. (Frostegård et al., 1999).

The purpose of this study was to compare the efficiencies of two methods of DNA extraction with the assistance of rhizosphere soil samples collected from Papan, Kejar, and Kenarong, three separate sites. Protocol 2 employed the traditional method (bead beating) with Tris-HCl-EDTA buffer as the blank buffer, while Protocol 1 employed the spin-column kit method with SE buffer blanking.

Protocol 1 utilizes a spin column-based DNA extraction kit, a new approach. Due to its speed, sanitation, and propensity for producing high-quality DNA, the technique can be applied to isolate nucleic acids from environmental samples. SE buffer, a normal buffer with little impact on optical reading under spectrophotometer, is used as the blank buffer. Protocol 2, on the other hand, employs the traditional bead beating technique, where glass or ceramic beads are employed to physically lyse the microbial cells in an aqueous sample. Tris-HCl-EDTA, a routine blank buffer for the majority of the molecular techniques, is used in this process. However, Tris and EDTA, if not properly cleaned after extraction, may absorb light of wavelengths shared by DNA and contribute to skewed spectrophotometer readings of DNA concentration. DNA extraction results of

the three samples Kejar, Kenarong, and Papan showed obvious differences between the two methods employed.

Protocol 1 using the spin-column method gave a ratio of 260/280 = 1.72 and a DNA concentration of 10.426 µg/ml for sample A (Papan), which indicates good results in terms of both DNA quality and amount.

But the ratio value dropped severely to 6.60 when the same sample was run with Protocol 2 (the bead beating method), and the DNA concentration yielded a negative value of -5.802 µg/ml. This meant that the spectrophotometer reading had been negatively impacted, most likely because of interference from the Tris-EDTA buffer or other contaminants that were not extracted out during extraction. The same trend was observed in sample B's (Kejar) readings. The DNA content varied from 10.070 to 13.200 µg/ml, while measurements for 260/280 ratio, under Protocol 1, were between 1.50 to 1.57.

This ratio of purity is nonetheless deemed fit for most molecular biology uses, although lower than the ideal value by a fraction of about 1.8. With the use of Protocol 2, though, the obtained concentration recorded values in the range of -2.942 to -3.168 µg/ml, and the purity ratio rose unnecessarily to 28.09. These values were evidently in error, and the measurement would have been most likely grossly inaccurate because of interference by foreign substances that absorb wavelengths close to DNA. For sample concentrations between 6.001 and 8.302 µg/ml and purity ratios between 1.64 and 1.89, Sample C (Kenarong) also demonstrated the effectiveness of Protocol 1. These readings showed that the spin-column method would yield rewarding extraction results. However, with

Protocol 2 again, irrational values were obtained, such as negative DNA amounts of approximately -5 µg/ml and ratios up to 13.50.

These results confirmed that Protocol 2 produced excessive interference with the spectrophotometer readings, and they were consistent with those from the first two samples. In summary, Protocol 1 was more effective and precise in this test for isolating DNA from river water samples, whereas Protocol 2 demands much effort before it can be utilized effectively. The results of this study's comparison of the two DNA extraction protocols were that Protocol 1, employing a spin-column procedure and blanked with SE buffer, was far more efficient than Protocol 2, employing Tris-HCl-EDTA buffer and regular bead beating. Positive and correctly quantified DNA concentrations, consistent results, and DNA purity within the correct range (260/280 ratio about 1.8) all validated Protocol 1's efficiency.

A more effective purification method, like the use of a silica membrane and various steps of washing that assisted in removing impurities like proteins, metabolites, and other impurities that can sully the purity of DNA, was also partly to blame for the breakthrough. Protocol 2, however, exhibited lethal flaws that sufficiently demonstrated interference at the level of spectrophotometry, including irrational ratios of 260/280 and negative concentration results. The UV absorption spectrum of the prepared Tris-HCl-EDTA buffer is most likely the cause of this interference since it shows the ability to skew wavelength measurements at 260 and 280 nm. The sample is further contaminated by the more violent bead beating technique used to disrupt the cells, which degrades other organic material such as proteins and water-soluble chemicals that co-extract with DNA. This is not controlled since Protocol 2 lacks extra purifying protocols. Accordingly, it is

apparent from the study findings that Protocol 1 is better suited to research involving the extraction of DNA from water samples obtained from environmental sources like rivers or waterfalls, whereas Protocol 2 needs further adjustment, e.g., inclusion of purification steps or the utilization of more appropriate buffers, prior to being used successfully (Barnard et al., 2022).

Samples		Spin-column (Protocol 1)		Conventional (Protocol 2)	
		260/280 ratio	**Conc. (µg/ml)**	**260/280 ratio**	**Conc. (µg/ml)**
A	A	1.72	10.426	6.60	-5.802
(Sg. Papan)	*	1.69	12.407	8.12	-6.228
B	A	1.50	10.070	10.39	-2.942
(Sg. Kejar)	*	1.57	13.200	28.09	-3.168
C	A	1.89	6.001	13.50	-5.507
(Sg. Kenarong)	*	1.64	8.302	8.88	-4.963

(*) – same samples repeated for all the 2 protocols.

Table 4.2: Results Sample Quantities DNA Extraction using Nanodrop Spectrophotometer from Spin-column method (MACHEREY-NAGEL NucleoSpin® Soil, Germany), protocol kit method (**Protocol 1**), and conventional (bead beating) method (**Protocol 2**).

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The best technique for the extraction of bacterial DNA from the rhizosphere soil of Gesneriaceae plants sampled from the natural environment zones of Sungai Papan, Sungai Kejar, and Sungai Kenarong in Royal Belum, Perak, has been established by this study. The existence of a varied soil environment and rich biodiversity in this area makes it convenient for research on microbial communities in the rhizosphere. The traditional beating method, relying on the mechanical disruption of cells through glass beads, and the recent spin-column kit method, wherein the principle of DNA adsorption onto silica membranes with stepwise purification is involved, were compared in the current research. The analysis results revealed that the kit method was much better, more consistent, and could produce high-quality DNA. The 260/280 purity ratio measurements done by the kit method varied from 1.50 to 1.89, which is near the ideal value of ~1.8, and this showed that the DNA extracted was clean and almost free of protein contamination. For instance, sample B (Sg. Kejar) gave the ratio of 1.57 and DNA concentration of 13.200 µg/ml, while sample C (Sg. Kenarong) yielded a ratio of 1.64 and concentration of 8.302 µg/ml. On the other hand, the conventional method gave fluctuating 260/280 ratios (ranging from 6.50 to 28.09) and very low or even negative DNA concentrations, i.e., -6.228 µg/ml for

sample A (Sg. Papan) and -4.963 µg/ml in sample C (Sg. Kenarong). Moreover, spectrophotometric measurements of DNA concentration using the kit method always resulted in positive values.Conversely, the bead beating procedure was less accurate and tended to yield inconsistent results.

Extreme interference during reading using the spectrophotometer is indicated by negative DNA concentrations and purity ratio readings that are either too high (greater than 10) or beyond the optimum range. The likely explanation for this is optical interference from contaminating protein, polyphenols, or remaining Tris-HCl-EDTA buffer from the extraction procedure. Since tris and EDTA are UV-absorbing wavelengths (260 and 280 nm), their inability to be removed during purification may cause inaccurate or misleading results. Further, vigorous bead beating procedures can not only lyse microbial cells but also other organic matter from the soil, including proteins and metabolites, which may contaminate the DNA sample. This observation was also confirmed visually through agarose gel electrophoresis results. Better DNA integrity was reflected in the brighter, clearer, and more uniform DNA bands yielded by the kit method. With the traditional methods, the DNA bands were blurrier or irregular in shape and sometimes also reflected degradation that may have been caused by contamination or DNA breakage during extraction. This visual examination is significant since it gives a good measure of the DNA sample's quality and aptness for subsequent molecular analysis. Not just technological, but also biological variables influence DNA extraction results.

Intimate interactions with plant roots are experienced by many microorganisms in rhizosphere soil.

Cell lysis and DNA extraction are interfered with by the presence of degradation enzymes, root exudate compounds, and the range of microbial cell morphologies, i.e., thick walls (gram-positive). Thus, the biological properties of the soil and the properties of the target microorganisms must be considered in choosing an extraction method. Overall, the results of the study clearly show that the choice of the best DNA extraction method is very important for both the attainment of high DNA quality and purity and also the success of any other downstream molecular application, including PCR, sequencing, and metagenomic analysis. For plant rhizosphere soil studies, the use of the spin-column kit method is suggested as the method of choice, particularly in complex tropical rainforest ecosystems like Royal Belum.

This technique minimizes contamination and technical fault risks in analysis and also increases the efficacy and validity of the study results.

5.2 Recommendations

Based on this study's findings, a number of recommendations might be considered to enhance the precision and efficiency of future studies associated with the isolation of rhizosphere soil microbial DNA. First, since the spin-column approach has been established to provide cleaner and purer DNA results, it is advised that the kit be employed on a routine basis in metagenomic research or microbial biodiversity research. Higher DNA purity can be obtained by adding more steps of purification like protein precipitation, phenol/chloroform contamination removal, and using buffers that will not

interfere with spectrophotometer readings if the traditional bead beating method still needs to be used because of financial or technical limitations.

For buffers, however, Tris-HCl-EDTA should not be used as a blank buffer since it can absorb UV light at wavelengths 260 and 280 nm and therefore distort DNA purity and concentration values.

However, for spectrophotometric reading, it is preferable and more appropriate to use a more neutral SE (elution buffer). Besides, it is strongly suggested to utilize a tool like a Qubit fluorometer for more sensitive and accurate DNA quantitation, particularly if the likelihood of optical interference or low DNA concentration is high. Also, this research has great potential to be developed into a complete metagenomic analysis to explore the microbial community diversity in the rhizosphere of Gesneriaceae plants to its fullest.

This will pave the way for a better comprehension of the involvement of microorganisms in the soil ecosystem and how they contribute to agricultural sustainability and soil fertility.

Lastly, it is suggested that more samples from diverse Gesneriaceae species and geographical areas be added in the future. In order to provide greater statistical precision and data reliability, this should be supported with adequate technical and biological replication. Subsequent research in plant biotechnology and soil microbiome can be carried out more effectively and meaningfully if these recommendations are implemented.

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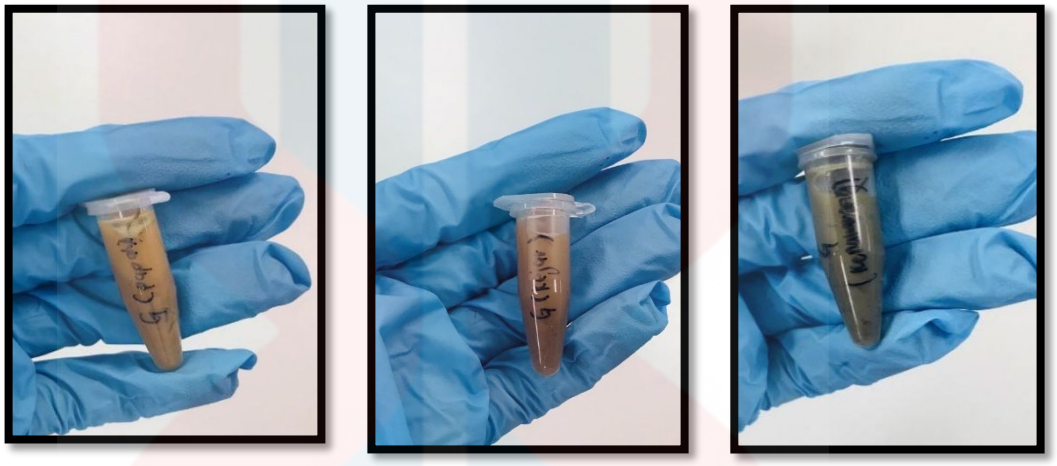
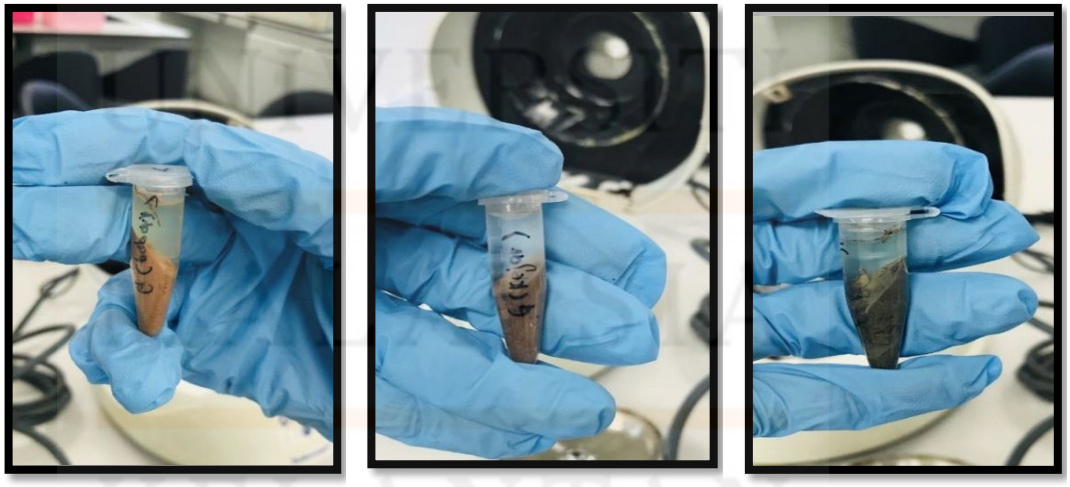
APPENDICES

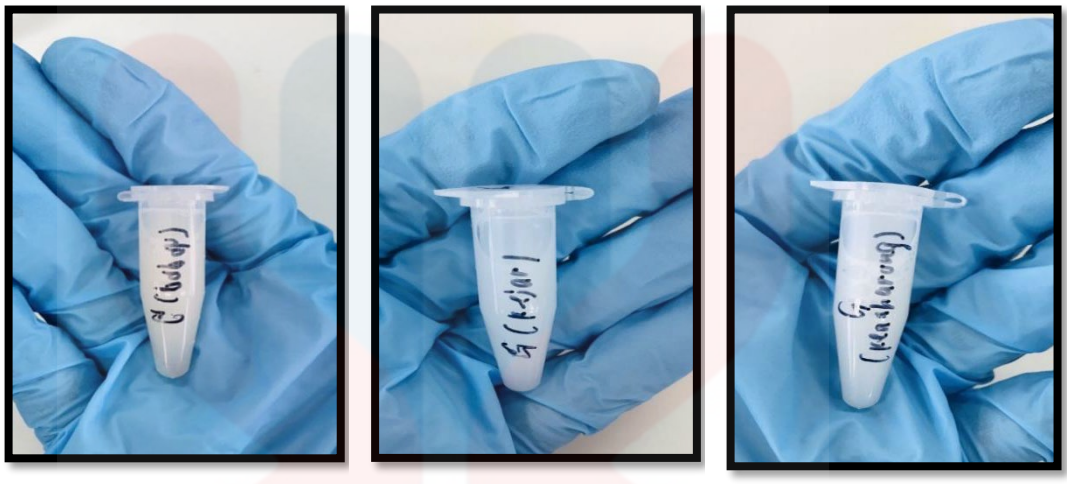
APPENDIX A



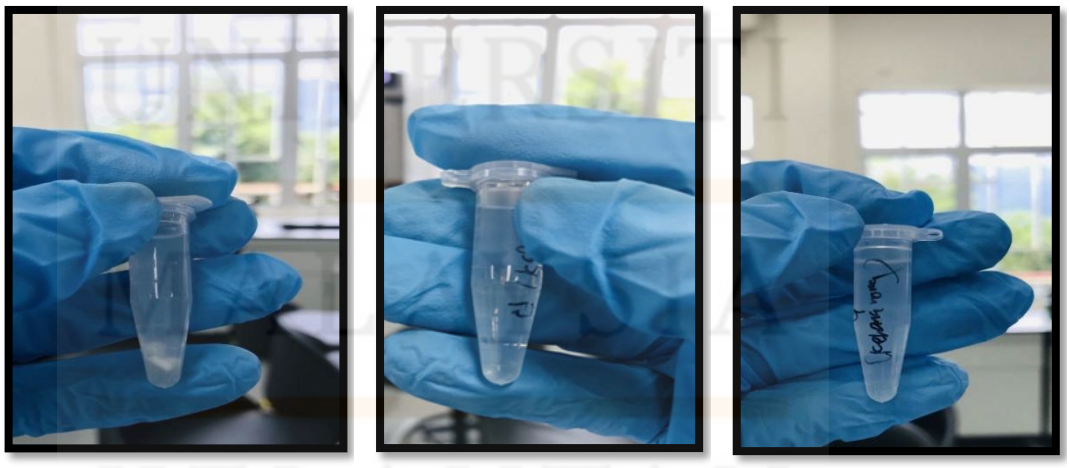
APPENDIX B



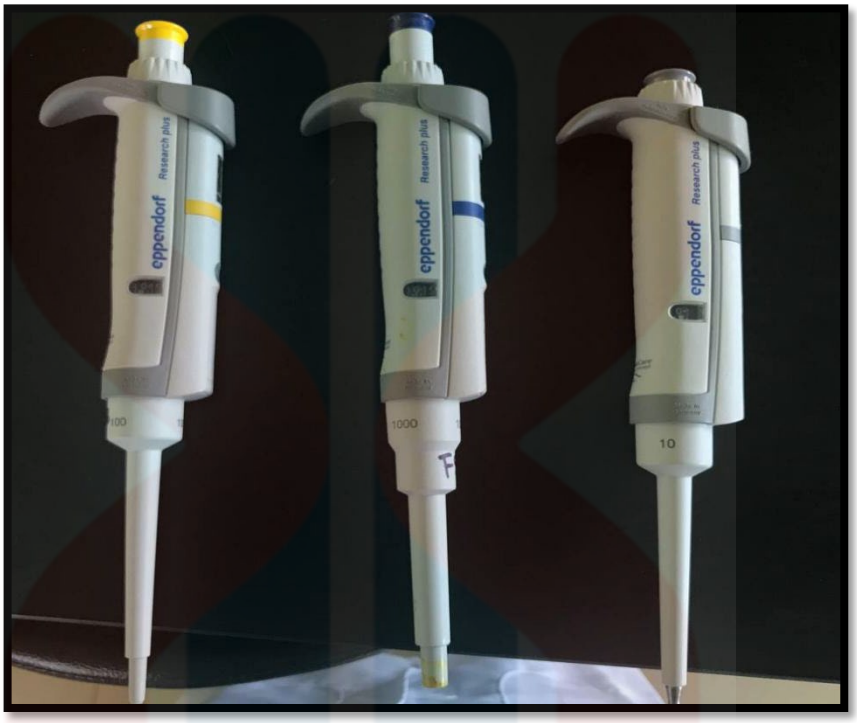
APPENDIX C



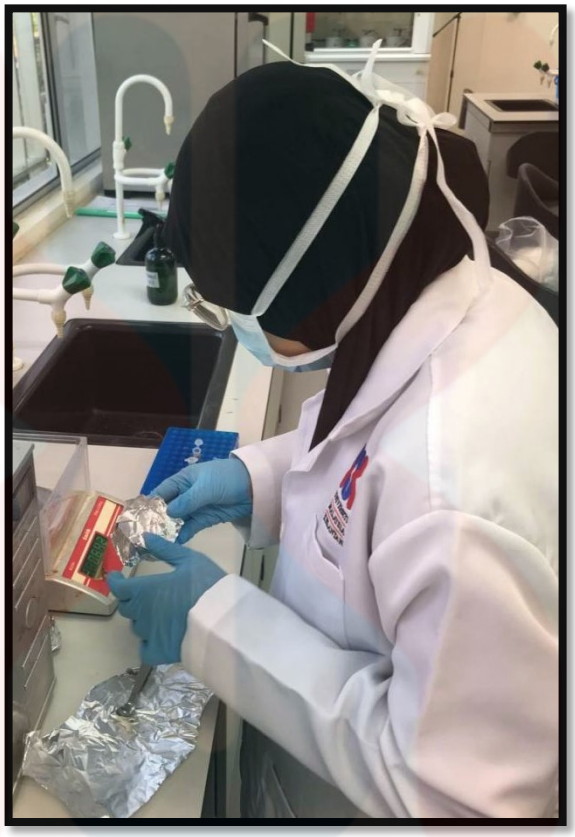
APPENDIX D



APPENDIX E



APPENDIX F



APPENDIX G

APPENDIX H

APPENDIX I