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Analysis of tropical soil bacterial communities in Sabah using high-throughput sequencing

Grace Xin Hui Lau[1], Hui Yin Fan[2] and Clemente Michael Vui Ling Wong[1]*

[1]Biotechnology Research Institute, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia.
[2]Food Safety and Security Research Group, Faculty of Food Science and Nutrition, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia.
Email: michaelw@ums.edu.my

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ABSTRACT

Aims: Soils contain a diverse bacterial population that is essential in various ecological processes such as biogeochemical cycling, soil quality improvement, plant growth and the maintenance of a balanced ecosystem. Hence, there is a need to understand the bacterial diversity and sustainability of various geographical regions. Nonetheless, despite growing knowledge of soil microbial diversity in many parts of the world, there is little data on the bacterial diversity of tropical soils such as Malaysia. Hence, this research was conducted to determine the bacterial communities of soils from selected areas in East Malaysia using the 16S rRNA gene-based metagenomic high-throughput sequencing and analysis approach.
Methodology and results: Soil samples (n=3) were collected from three sites within the same vicinity in Kota Kinabalu, Sabah. DNA was extracted from bacteria in the soil samples and the mix of bacterial 16S rRNA genes was amplified, sequenced and aligned to those in the Genebank. A total of 39 different bacteria phyla were found in the soil samples. The most abundant phyla in the analysed soils were Proteobacteria (19.90%), followed by Acidobacteriota (15.73%), Actinobacteriota (12.79%), Firmicutes (9.40%), Chloroflexi (9.23%), Planctomycetota (7.19%), Verrucomicrobiota (5.53%), Myxococcota (5.43%), Latescibacterota (2.72%) and Desulfobacterota (2.38%).
Conclusion, significance and impact of study: Research findings provided an overview of the most prevalent bacterial phyla in the selected Sabah soils, allowing the effects of environmental change on bacterial population shift to be tracked in the future.

Keywords: Bacterial diversity, Kota Kinabalu, Sabah, tropical soil

INTRODUCTION

Soil bacterial diversity and composition in soils are greatly influenced by environmental factors (Zheng *et al.*, 2019), such as soil type, moisture content, climate condition, geographical location, vegetation, land use practice and organic matter availability. Hence, it is crucial to keep a reliable record of soil bacterial diversity to track and forecast potential changes brought on by environmental changes in the future. Since Woese and Fox (1977) first developed the 16S rRNA gene-based molecular analysis, many investigations have been conducted to identify the bacterial diversity in soils; many studies have been conducted to discover the soil bacterial fingerprints of different regions in the world (Yergeau *et al.*, 2007; Chu *et al.*, 2010; Kerfahi *et al.*, 2016; Chin and Wong, 2022). Soil bacterial diversity data from many countries are now available, including the remote regions of the Arctic and Antarctica (Yergeau *et al.*, 2007; Chu *et al.*, 2010; Kerfahi *et al.*, 2016; Chin and Wong, 2022). Malaysia has one of the most megadiverse biodiversity in the world (Tong, 2020) and is believed to have soils that harbour a massive population of diverse bacterial species.

Malaysia is located in Southeast Asia and covers a total area of 329,847 km^2 with coordinates of 2°30' N 112°30' E (CountryReports, 2023). It is divided into two main parts: West Malaysia (also known as Peninsular Malaysia) and East Malaysia by the South China Sea. Malaysia, located near the equatorial region, generally experiences a tropical climate with hot and humid conditions throughout the year. The average annual temperature is 26.37 °C with an annual precipitation of 3001.98 mm (Climate Change Knowledge Portal, 2021). In Malaysia, there are three main soil groups: residual soils of granite, residual soils of sedimentary rock and coastal alluvial soils. Over 70% of the land is covered by residual soil, whereas the remaining 30% is coastal alluvial soil (Ashraf *et al.*, 2017). The moisture content of

***Corresponding author**

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Malaysia's soils is generally 60% to 70% for the whole year, which enables microbial growth and activity, especially during dry seasons. Soils in Malaysia have pH values ranging from 4.5 to 5.5, varying depending on land use type. Tripathi *et al.* (2012) sampled several types of soils in Malaysia and they found out the soil pH ranged from 3.0 to 8.0. Overall, tropical soils provide a favourable environment for soil microbes to thrive. However, to date, the information on Malaysia's tropical soil microbes, especially in Sabah, East Malaysia, is relatively sparse when compared to other parts of the world.

Sabah is one of the states in East Malaysia on the island of Borneo. It represents the second-largest state in Malaysia, with an area of 73,711 km^2. Its daily temperatures ranged between 23 °C and 34 °C year-round. Primarily covered by tropical rainforest, Sabah has a remarkable range of soil types and a high level of biodiversity. Among the major types of soils found in Sabah are Fluvisols and Gleysols, Lithosols, Regosols and Cambisols, Luvisols and Nitosols, Acrisols and Ferralsols, as well as Histosols (Ashraf *et al.*, 2017). Thus far, only a few studies have contributed to the bacterial diversity in West Malaysia (Tripathi *et al.*, 2012; Miyashita *et al.*, 2013; Chan *et al.*, 2015; Jayanthi *et al.*, 2016; Kerfahi *et al.*, 2016) and East Malaysia (Chin and Wong, 2022), in which the areas covered in the tropics are limited. Expanding our coverage of tropical regions is crucial to better understanding terrestrial tropical bacteria diversity and their roles in the soil ecosystem. This data serves as the foundation for tracking species fluctuations in response to environmental disturbances and climate changes, without which the monitoring and development of mitigation steps would be challenging. Thus, to increase the bacterial diversity database for a better understanding of these intricate microbial communities, their interactions, and their impact on the environment, this study was carried out to determine the diversity of bacteria from selected soils in Sabah, East Malaysia.

MATERIALS AND METHODS

Soil sampling and storage

Three soil samples were collected in Universiti Malaysia Sabah, Kota Kinabalu Campus at the following location: S1(6°2'17.326"N, 116°6'48.334"E), S2 (6°2'17.394"N, 116°6'48.384"E) and S3(6°2'17.423"N, 116°6'48.478"E) (Sabah Diversity Center (SaBC) access and export license reference nos. JKM/MBS.1000-2/2 JLD.14(92), (93) and (94) and JKM/MBS.1000-2/3 JLD.5(10), respectively). Approximately 7 g of soil with three replicates at each location was collected from the top two to three cm of the soil layer using a tubular soil sampler and put inside sterile 50 mL centrifuge tubes. The soil samples were sieved using a 2-mm mesh and stored at -20 °C before DNA extraction.

Total DNA extraction

DNeasy PowerSoil Kit was used to extract total genomic DNA from soil core samples (QIAGEN, Hilden, Germany). Each of the soil core replicates had its DNA extracted independently, which was then pooled according to their sampling location to create the mixed total DNA for the downstream analysis. The integrity of the genomic DNA extracted was verified using agarose gel electrophoresis. Their concentration and purity were measured using a spectrophotometer (Implen NanoPhotometer® N60/N50).

Amplification and sequencing of 16S rRNA gene

The purified genomic DNA was amplified using locus-specific sequence primers GC S-D-Bact-0341-b-S-17 (5'-GC*CCT ACG GGN GGC WGC AG-3') and S-D-Bact-0785-a-A-21 (5'-GAC TAC HVG GGT ATC TAA TCC-3') targeting the V3-V4 regions of 16S bacterial rRNA gene. All the PCR reactions were carried out with REDiant 2× PCR Master Mix, which contains reaction buffer, 0.06 U/µL of Taq DNA polymerase, 3 mM $MgCl_2$ and 400 µM of each deoxynucleotide triphosphates. Then, bacterial 16S rRNA gene of the selected regions (16S V3-V4) were amplified using locus-specific sequence primers with forward overhang adapters 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locus-specific sequence]-3' and reverse overhang adapters 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[locus-specific sequence]-3'. All the PCR reactions were carried out with KOD-Multi and Epi-® (Toyobo). Dual indices were attached to the amplicon PCR using Illumina Nextera XT Index Kit v2 following the manufacturer's protocols. The quality of the libraries was measured using the Agilent Bioanalyzer 2100 System by Agilent DNA 1000 Kit and fluorometric quantification by Helixyte GreenTM Quantifying Reagent. The libraries were normalized and pooled according to the protocol recommended by Illumina and a high throughput sequencing was performed using the Illumina MiSeq platform with 300 paired-ends.

Sequence alignment

The raw amplicon paired-end reads were demultiplexed by grouping them according to their barcode sequences. Fastqc was used to assess the quality of raw reads after primers and adaptors were removed using Cutadapt 3.5. Using DADA2 V1.18, paired-end reads were processed and merged. The SILVA non-redundant database V138.1 was used for chimera screening and taxonomic assignment. The 16S rRNA gene sequences were aligned using the Basic Local Alignment Search Tool (BLAST) against the NCBI's nucleotide database.

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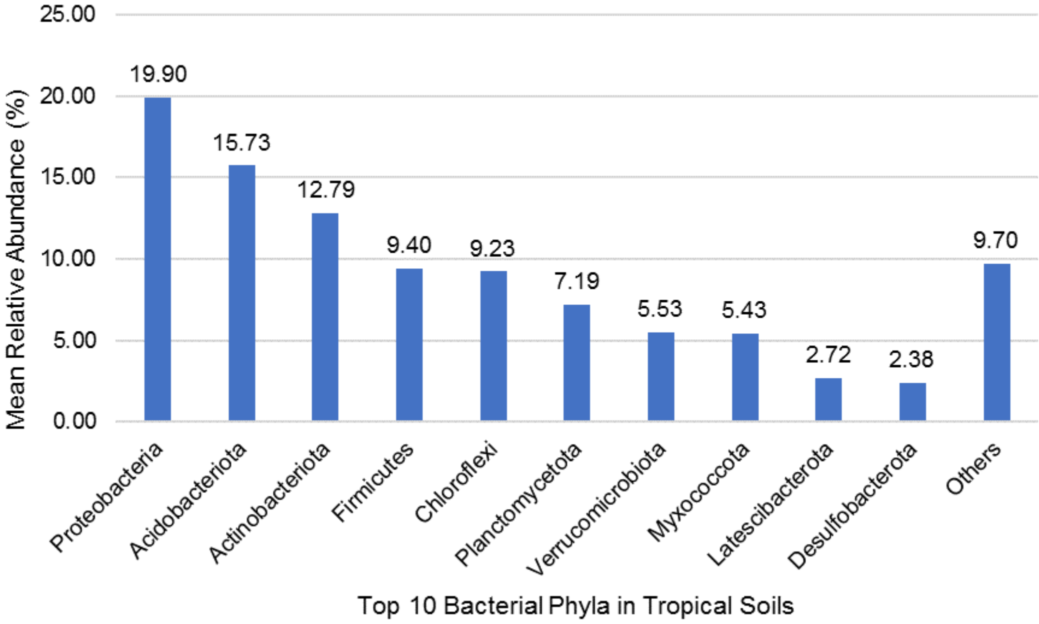

Figure 1: Mean relative abundance of top 10 bacterial phyla in tropical soils at sample sites.

Table 1: Concentration and purity of soil bacterial DNA.

No.	Sample name	Nanodrop (water as blank)		
		Concentration (ng/µL)	$OD_{260/280}$	$OD_{260/230}$
1	S1	33.45	1.934	1.582
2	S2	45.35	1.963	1.721
3	S3	45.45	1.922	1.530

RESULTS

The concentration of genomic DNA of the bacteria extracted directly from the three soil samples without enrichment and culturing steps yielded between 33.45 and 45.45 ng/µL (Table 1). The genomic DNA had $OD_{260/280}$ and $OD_{260/230}$ ratios ranging between 1.922-1.963 and 1.530-1.721, respectively, indicating that the extraction method used in this research was suitable for soil samples.

DNA sequence analysis

A total of 39 different bacteria phyla were found in the soil samples. In general, the dominant phyla from the Sabah soil samples were Proteobacteria (19.90%), followed by Acidobacteriota (15.73%), Actinobacteriota (12.79%), Firmicutes (9.40%), Chloroflexi (9.23%), Planctomycetota (7.19%), Verrucomicrobiota (5.53%), Myxococcota (5.43%), Latescibacterota (2.72%) and Desulfobacterota (2.38%). The remaining 9.7% of the OTUs were not grouped into any of the known phyla (Figure 1). The Shannon Diversity Index (also known as the Shannon-Wiener Index) of the three samples were recorded at 6.5625, 6.5952 and 6.9087, respectively.

DISCUSSION

Soil is a complex ecosystem that harbours a vast array of microorganisms, including bacteria. A single gram of soil can contain billions of bacterial cells from thousands of different species. These bacteria are significant in nutrient cycling, organic matter decomposition, soil fertility and overall ecosystem functioning. Their diversity is influenced by factors such as soil type, soil pH, moisture content, temperature, climate condition, geographical location, vegetation, land use practice and organic matter availability.

In this study, Universiti Malaysia Sabah, Kota Kinabalu Campus was chosen as the sampling site for studying soil bacterial diversity. This site has a rich and diverse ecosystem, making it an ideal location to study bacterial diversity. In addition, it is easily accessible to facilitate regular sampling and data collection. While this study has focused on sampling only three sites, it serves as a valuable starting point for exploring bacterial diversity in this specific region. By combining the research data with findings from other researchers (Tripathi *et al.*, 2012; Miyashita *et al.,* 2013; Chan *et al.*, 2015; Jayanthi *et al.*, 2016; Kerfahi *et al.*, 2016; Chin and Wong, 2022), this collaborative effort has the potential to yield a more

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representative assessment when compared to the limited data available at present.

Initial diversity of bacterial phyla of the soil samples collected in this study showed that the soil samples were dominated by Proteobacteria (19.90%), followed by Acidobacteriota (15.85%), Actinobacteriota (12.13%), Firmicutes (9.33%), Chloroflexi (7.72%), Planctomycetota (7.10%), Verrucomicrobiota (5.19%), Myxococcota (5.35%), Latescibacterota (2.83%) and Desulfobacterota (2.76%). The result was consistent with previous study of tropical soil bacterial diversity done by Chin and Wong (2022), in which the dominant phyla from the Sabah soil samples were Proteobacteria (32%), followed by Acidobacteria (24%), Firmicutes (12%), Actinobacteria (11%), Planctomycetes (6%), Verrucomicrobia (5%), Chloroflexi (2%) and Bacteroidetes (1%), except the ranking of the bacterial phyla were different. Myxococcota (5.35%), Latescibacterota (2.83%) and Desulfobacterota (2.76%) are three new bacterial phyla found in the soil samples in this study. The difference may be due to factors such as soil type, pH, moisture content, temperature, climate condition, geographical location, vegetation, land use practice and organic matter availability (Chin and Wong, 2022).

The Shannon Diversity Index (also known as the Shannon-Wiener Index) of the three samples ranged between 6.5625 and 6.9087, showing the diversity of bacterial species in the soil samples. The hot and humid weather in Malaysia provided a favourable environment for the survival of soil microorganisms. Chin and Wong (2022) reported that the Shannon Diversity Index ranged between 3.34 and 5.48 for 10 soil samples in Sabah. The varied diversity indices reported may be due to different environmental conditions, which resulted in distinctive prevalent bacterial communities. Compared with tropical soils, polar soils are predicted to have a lower diversity as they come from a harsh environment with minimal nutrient availability that is unsuitable for microorganisms to survive (Chin and Wong, 2022). Choon *et al.* (2010) reported the Shannon-Wiener diversity index between 2.63 and 3.15 for the bacterial diversity of soil samples in Fildes Peninsula, King George Island. Thus, soil bacterial diversity is highly dependent on biotic and abiotic factors interacting with each other.

The most abundant bacterial phyla in Sabah soil samples collected are Proteobacteria (19.90%) (Figure 1). This finding is in line with the results reported by Chin and Wong (2022), in which Proteobacteria covered up to 32% of the total bacterial phyla found in the soil samples. Proteobacteria are Gram-negative bacteria commonly found in rhizospheres, saline soils and semiarid soils (Mhete *et al.*, 2020). They occupied the highest richness of all soil samples, in line with previous studies done by Cheah *et al.* (2018) and Mhete *et al*. (2020). In general, Proteobacteria break down numerous hazardous compounds in soil, act as nitrogen fixers (for example, *Rhizobium* and *Mesorhizobium*) and play an important part in the carbon cycle (Aislabie *et al.*, 2013). They induce plant growth by contributing to nutrient acquisition and protecting against disease through the production of inhibitory substances or increasing the natural resistance of the plant (Lugtenberg and Kamilova, 2009; Verma *et al.*, 2010). Their corresponding abundance increases with high organic carbon levels in soil, as reported by Fierer *et al.* (2007), Eilers *et al.* (2010) and Mhete *et al*. (2020). There are six classes of Proteobacteria: Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria and Zetaproteobacteria; each plays a vital role in carbon, nitrogen and sulphur cycling. In this study, only Alphaproteobacteria (8.19%) and Gammaproteobacteria (11.71%) are found in the soil samples. Chin and Wong (2022) observed 44.94% of Alphaproteobacteria, 22.58% of Deltaproteobacteria, 19.35% of Betaproteobacteria and 16.13% of Gammaproteobacteria among the Proteobacteria found in Sabah soils.

On the other hand, Acidobacteria, the second largest soil bacteria phyla in Sabah soils, were the most dominant bacterial phyla found in soils in Peninsular Malaysia (Kerfahi *et al.*, 2016). The differences in the relative abundance of bacteria phyla between the soil in West and East Malaysia were probably caused by the differences in biotic and abiotic characteristics of the soil environment or the geographical locations (Chin and Wong, 2022). Acidobacteria members are Gram-negative and non-spore formers. They are ubiquitous in diverse terrestrial environments ranging from tundra soils to desert soils, from peatland soils and sediments to grasslands, forests and agricultural lands (Janssen, 2006; Eichorst *et al.*, 2018). They are oligotrophs that can sustain low nutrient environments and changes in soil moisture as well as perform nitrate and nitrite reduction (Ward *et al.*, 2009). The phylum Acidobacteria is phylogenetically classified into 26 subdivisions according to the analysis of major 16S rRNA gene sequence clades, in which only members of subdivisions 1, 3, 4, 6, 8, 10 and 23 are represented by taxonomically described members (Dedysh and Yilmaz, 2018; Eichorst *et al.*, 2018). They facilitate the modulation of vital biogeochemical nutrient cycles, which involve carbon, nitrogen and sulfur cycles. Exopolysaccharides (produced by almost all cultured members) help to form soil matrix and promote plant growth by facilitating water and nutrient uptake by plants (Kalam *et al.*, 2020).

Actinobacteria have also been found to be dominant in tropical soils, especially in saline and garden soils. Their diversity is highly correlated with soil salinity. They are Gram-positive bacteria with three common subphyla: Actinobacteridae, Acidomicrobidae and Rubrobacteridae (Aislabie *et al.*, 2013). They show antimicrobial characteristics (Aislabie *et al.*, 2013), can withstand ionising radiation (Holmes *et al.*, 2000) and act as acid-tolerant ferrous iron oxidizers (Clark and Norris, 1996). They play vital roles in the carbon cycle and produce secondary metabolites, including the production of melanin, which is associated with soil humic acid (Shivlata and Satyanarayana, 2015).

Similar to Actinobacteria, Firmicutes are also Gram-positive bacteria that are widely found in the forest soil of

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Kashmir, India (Ahmad *et al.*, 2009) and in soils of the pasture and grassland of the Netherlands (Kuramae *et al.*, 2012). In Malaysia, they are more readily found in Borneo's soils compared to soils in Peninsular Malaysia. Firmicutes are mostly related to human diseases, for example, the genus *Bacillus* and *Clostridium*. Their diversity is correlated with their capability to create endospores and refrain from desiccation, which enables them to survive under extreme conditions (Aislabie *et al.*, 2013). On the other hand, the Bacteroidetes phylum, which was also found in tropical soils as reported by Cheah *et al.* (2018) as well as Chin and Wong (2022), are related to human health, as Bacteriodetes and Firmicutes are two important bacteria involved in gut health. In contrast with the weight gain and obesity brought by Firmicutes, Bacteroidetes aid in weight loss by reducing calories derived from food and increasing the calories eliminated in stool. In soils, members of Bacteroidetes phylum function as decomposers of polymeric organic matter. They have been detected to be the major phyla living under anaerobic conditions existing in the rhizosphere and nearby surface soil ecosystems (Martínez-Alonso *et al.*, 2010).

Phylum Chloroflexi, which occupied 9.23% of total bacteria phyla in Sabah soils, is one of the significant phyla containing bacteria with various metabolic features. This phylum is further divided into six classes: Chloroflexi, Anaerolineae, Caldilineae, Ktedonobacteria, Dehalococcoidetdia and Thermomicrobia (Hanada, 2014). The class Chloroflexi is the only phototroph (filamentous anoxygenic phototrophic bacteria) in this phylum. Three families (Chloroflexaceae, Oscillochloridaceae and Roseiflexaceae) in the class have the same phenotypic characteristics: multicellular filamentous morphology, gliding motility and anoxygenic photosynthetic activity. *Chloroflexus* species are abundant in neutral to alkaline hot springs (Hanada, 2014). They are mostly found in the top 2 cm of the soil, suggesting the potential phototrophic properties displayed by the bacteria (Freeman *et al.*, 2009). Chloroflexi provides the filamentous matrix around which form strong flocs with rapid settling properties to feed on the debris from lysed bacterial cells, to utilize carbohydrates to obtain energy and to breakdown other complex polymeric organic substances into low molecular weight substrates to sustain their growth (Speirs *et al.*, 2019). They utilize higher wavelengths to penetrate much further into the soil for photosynthesis. Cyanobacteria, eukaryotic algae and Chloroflexi communities in soils are believed to interact with each other for microbial photosynthesis, which requires additional research in the future.

Planctomycetes are bacterial phyla not only found in soils but also in aquatic environments such as marine and freshwater habitats. They are usually dominated by peatlands (Rakitin *et al.*, 2022). Their intracellular compartmentalization and absence of peptidoglycan in their cell walls make them unusual among bacterial species. Since they have no peptidoglycan in their cell walls, Planctomycetes inherently resist antibiotics that prevent cell wall synthesis, such as the β-lactams and vancomycin (Fuerst and Sagulenko, 2011). On the other hand, Verrucomicrobiota is a phylum of Gram-negative bacteria which can be found in freshwater, marine and soil environments as well as human faeces. Both bacterial phyla, together with Chlamydiae and Lentisphaerae are members of the PVC superphylum. From the evolutionary perspective, Verrucomicrobiota and Planctomycetota displayed high affinities (Butler *et al.*, 2007). The relative abundance of Planctomycetota in soils was affected by the available phosphorus (AP) content, like Proteobacteria, while the relative abundance of Verrucomicrobiota was more affected by the total nitrogen (TN) content. However, there is little knowledge regarding the relationship between nutrient availability and microorganisms, so further study is required to investigate the biological implication behind microbial community succession and its impact on the intercropping system (Shi *et al.*, 2021).

Three bacterial phyla, namely Myxococcota (5.35%), Latescibacterota (2.83%) and Desulfobacterota (2.76 %), were identified in this study but have not been reported in any of the studies regarding soil bacterial diversity. The Myxococcota are a phylum of Gram-negative bacteria known as the fruiting gliding bacteria. They are predominantly aerobic soil-dwelling microorganisms that are capable of releasing myxospores in unfavourable environments. They have separated from class Deltaproteobacteria due to their highly coordinated predation and cellular differentiation capacities (Murphy *et al.*, 2021). Myxococcota act as predators, which release secondary metabolites and extracellular enzymes to lyse prey cells. Similar to the other bacteria phyla, such as Latescibacterota and Desulfobacterota, which are also found in tropical soils, less information could be found due to a poor understanding of these phyla. Given the importance of soil bacteria to the community and niche as well as the potential risk of pathogenic bacteria in soils, it is important to keep a good record of soil bacterial diversity for conservation purposes or to track changes due to various environmental factors such as global warming.

CONCLUSION

We can conclude from this study that the major dominant bacterial phyla found in tropical soil samples are similar to those found in Peninsular Malaysia. Nonetheless, the relative abundances differ, with Acidobacteria being the most dominant phylum in Peninsular Malaysia and the Proteobacteria phylum dominating in selected sites in Sabah. Given the importance of soil microbes to the community and niche as the primary player in biogeochemical cycling, this record of bacterial diversity, in combination with those reported by other researchers, can be used as a baseline population for future reference. For example, we can simulate warming to determine how it affects the bacterial population, particularly beneficial bacteria that contribute to biogeochemical cycling and promote agricultural plant growth. This will allow us to take mitigation measures that maintain important bacteria

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in place, preventing ecosystem dysfunction and disruption. With this additional baseline data for comparison, other researchers will be encouraged to conduct similar studies covering bacterial diversity in other Malaysian niche areas, with the ultimate goal of contributing to creating a comprehensive Malaysia microbial diversity map. Additionally, the precise extent of bacterial contribution to soil ecosystem processes can be revealed in the future when the activities of all soil community members are studied together.

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