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Characteristics of limestone soil collected from Gunung Lang, Perak and metagenomic analysis of the soil microbial community

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ABSTRACT

Aims: This project was aimed to study the microbial diversity of the limestone soil and its correlation with the environment.
Methodology and results: The study was carried out using samples obtained from Gunung Lang, Ipoh, Perak in August 2013. X-ray diffraction analysis of the rock structure confirmed that the samples were of limestone origin. Besides that, soil analysis revealed that this area was fertile and rich in nutrients. It therefore served as a suitable habitat for microorganismal diversity to flourish. This was proven by the 16S rDNA metagenomic analysis which targeted on 16S rDNA variable region V3-V5 using Illumina MiSeq sequencer. Using this approach, a variety of microorganisms was identified and many yet to be characterized microorganisms were detected from this area.
Conclusion, significance and impact of study: This is the first study in Malaysia that aimed to study the microbial diversity of limestone soils through metagenomic approach. The study showed that limestone is rich in microbial diversity and it is a place worth looking for novel microbes and genes of interest in biotechnology.

Keywords: Limestone, soil, rock, 16S rDNA metagenomic analysis

INTRODUCTION

Limestone is a type of sedimentary rock that consists of more than 50% of carbonate minerals such as calcium carbonate ($CaCO_3$). Most limestones are composed of skeletal fragments of marine organisms such as corals. These marine organisms are the main grain producers that help in the sedimentation of carbonate grains (Scoffin, 1987). Limestone is one of the most important geological formations in Malaysia. They can be found abundantly in Peninsular Malaysia especially in the state of Pahang, Langkawi islands, Kinta Valley in Perak and Klang Valley in Selangor (Tan, 2001; Bakhshipouri *et al*., 2009; Zabidi and Freitas, 2011). In Malaysia, limestone has metamorphosed into marbles that possess higher density and strength and this metamorphism occurred due to the exposure of limestone to high pressure and temperature (Tan, 2001).

In the construction industry, limestone plays an important role as a raw material due to its versatility and low cost. It is widely used in the construction of dams and bridges, port developments, buildings and roads. Besides that, it is also being utilized as ornamental stones. Apart from the industry, limestone hills are generally picturesque and rich in geo-heritage values. Indeed, these limestone hills have become popular tourist attractions throughout the country. Among the limestone hills famous for eco-tourism are Gua Tempurung, Batu Caves, Perak Cave and Gua Niah (Tan, 2001).

Over the years, the significance of limestone biodiversity has not been given enough attention by the government and researchers as compared to other habitats such as rainforest. Nevertheless, quarrying activity has been rapidly undergoing in the limestone area, causing destruction to the natural habitats (Vermeulen and Whitten, 1999). Numerous international environmental organizations such as World Wide Fund for Nature (WWF), BirdLife International, *International Union for Conservation of Nature* (IUCN) as well as Fauna and Flora International (FFI) have come together to instill awareness on the importance of limestone biodiversity amongst the community. Reports published by these organizations show that limestone-restricted biodiversity is specifically vulnerable towards cement extraction activity. Limestone has a complex structure that requires millions of years of formation. Once this habitat is damaged, it is almost impossible for restoration to be performed. Moreover, the organisms living in this area are confined to this unique environment. They may not be able to adapt to other environment if their original habitat is destroyed (BirdLife *et al*., 2014). Hence, the conservation of limestone-restricted biodiversity is essential.

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Previously, a few studies have been carried out on the microbial diversity in limestone region (McNamara *et al.*, 2006; Villa *et al.*, 2014; Srivastava *et al.*, 2015). However, information related to these studies is still limited and not extensively available especially in Malaysia. Therefore, the exploration into the microbial diversity of limestone area in Malaysia is needed to gain further understanding. Therefore, the aim of this project was to study the microbial diversity of the limestone soil and its correlation with the environment.

MATERIALS AND METHODS

Soil sample collection and pre-treatment

Soil sample collection was done at Gunung Lang, Ipoh, Perak in August 2013. Gunung Lang is a recreational park surrounded by greeneries nestled between limestone hills. It is located at Jalan Kuala Kangsar, about 5 km away from the city centre. The coordinates of the location were detected using eTrex Vista® HCxGlobal Positioning System (GPS) receiver (Garmin, USA). An autoclaved hammer was used to break and collect the rocks from the limestone hill and the samples were kept in sterile zip-lock bags. At the same time, an autoclaved spatula was used to collect the soil under the limestone hill. A thermometer was used to measure the temperature of the soil. The weather condition at the sampling site on that particular day was also recorded. The soil sample collection was done at a depth of approximately 5 cm from the top layer of the ground surface. Soil samples were collected from several different sites around the area. The samples were stored in sterile 50 mL falcon tubes and then transferred back to the laboratory. During the transferring process, all the samples were kept in an ice-box**.** In the laboratory, the plant roots and humus were removed from the soil samples using an autoclaved clipper in the laminar flow to ensure the sterility of the entire process. The samples were then stored in a −20 °C freezer.

Soil analysis

The collected soil sample was sent to ALS Technichem (M) Sdn. Bhd, Selangor for analysis. The pH value, total alkalinity, cation exchange capacity, total organic carbon, hydrolysis acidity, total nitrogen, nitrate, ammoniacal nitrogen, total phosphorus, soluble calcium, soluble magnesium, soluble potassium, soluble sodium, chloride and sulphate content of the soil were determined. Hydrometer sedimentation was also done to identify the soil texture. All these tests were carried out according to the guidelines established by the American Public Health Association (APHA) and the United States Environmental Protection Agency (USEPA).

Rock analysis

The collected rock samples were sent to the Centre for Global Archaeological Research, Universiti Sains Malaysia (USM), for X-Ray Diffraction (XRD) analysis.

Recovery of DNA from limestone soil

DNA extractions were done using a sodium dodecyl sulphate (SDS)-based method for DNA recovery from soil with some modifications (Zhou *et al.*, 1996). In this method, hexadecyltrimethylammonium bromide (CTAB) was used to reduce the humic acids contamination. Further DNA isolation was done using agarose gel purification method with Wizard® SV Gel and PCR Clean-Up System (Promega, USA).

16S rDNA phylogenetic analysis

After extraction, the genomic DNA from soil was sent to Genomics BioSci and Tech. Co., Ltd for 16S rDNA amplicon sequencing and bioinformatics analysis. The set of primers for the reaction were targeted on the bacterial 16S rDNA variable region V3~V5 (Nossa *et al.*, 2010). The sequences of the primers used are as listed below:

16S-517F - 5' GCCAGCAGCCGCGGTAA 3'
16S-926R - 5' CCGTCAATTYYTTTRAGTTT 3'

PCR reactions were carried out with the following conditions: one cycle of 94 °C for 3 min and 35 cycles of 92 °C for 15 sec, 55 °C for 15 sec, 68 °C for 1 min, incubation at 68 °C for 6 min and a final incubation at 4 °C. Agarose gel slabs containing the desired amplified DNA fragments of sizes ranging from 350-450 bp were excised and the DNA was purified using QIAquick Gel Extraction Kit (Qiagen, Netherlands). TruSeq DNA sample preparation kit (Illumina, USA) was used for the library construction. The library was then quantified using GeneRead Library Quant Kit (Qiagen, Netherlands) before loading into the MiSeq (Illumina, USA) sequencer.

MiSeq sequencing reads were analyzed for their quantities and qualities. Due to the huge amount of data, the paired-reads were merged into amplicon sequences and then checked for the existence of the primers. The duplicated sequences in the amplicon sequences were removed. Short sequences were also filtered. Subsequently, the raw unassembled sequences were uploaded to the MG-RAST database for further analysis (Meyer *et al.*, 2008). The data was generated by referring to the Greengenes database with a maximum Expect Value (E) of 30, minimum identity of 70% and minimum alignment length of 50 (DeSantis *et al.*, 2006; Sturgeon *et al.*, 2013).

RESULTS AND DISCUSSION

Soil sample collection

Based on the GPS reading, the coordinate of the sampling site is 4.622°N, 101.0888°E. It was located below a limestone hill as shown in Figure 1. This area was chosen because it is less explored by people compare to the others. Therefore, it is less disturbed by human activity.
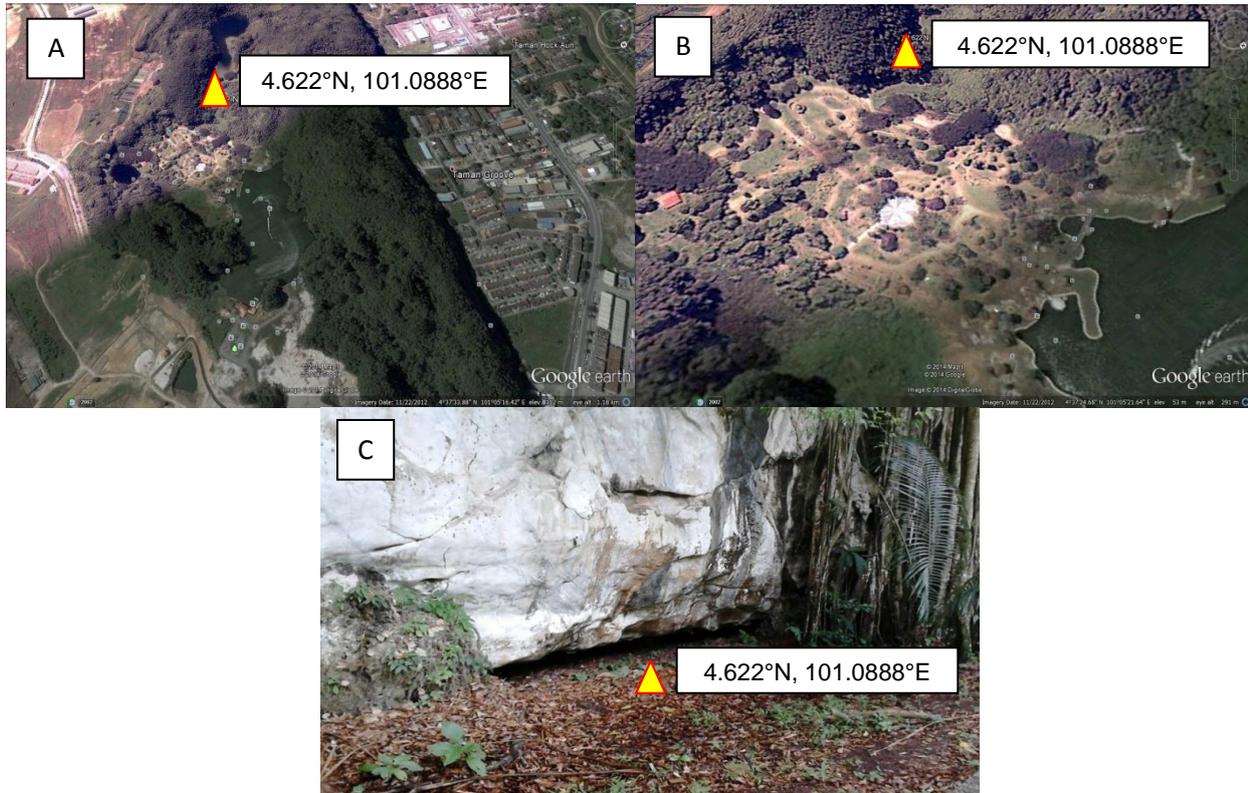
Figure 1: (A) Zoomed-out view and (B) Zoomed-in view of Gunung Lang using Google Earth (C) Soil samples collection site under a limestone hill in Gunung Lang. The coordinate of the site is 4.622°N, 101.0888°E.

Soil analysis

Soils are associated to everything around us and they play many crucial roles in supporting life on Earth. A handful of soil may contain billions of organisms and thus, soils have been considered as one of the many complex living ecosystems of their own. Soil can be divided into a few horizons where the texture, structure, composition, colour and number of living organisms differ among one another. The 'O' horizon, only a few centimetres thick is the top layer of the soil where decomposed materials are abundant. The next layer of the soil, known as the 'A' horizon or topsoil is the nearest to the surface. It contains more organic matter compared to horizons 'B' and 'C', making it darker in colour due to the accumulation of organic matters. Moreover, the fact that there are intense occurrences of biological activity in this horizon due to the high abundance of living organisms had also contributed to the darker tone of the soil (Brady and Weil, 2008; Monroe and Wicander, 2009). Since both 'O' and 'A' horizons are similarly rich in organic matters, the soil samples were collected from the top of the 'O' horizon to the 'A' horizon.

The soil analysis results are shown in Table 1. According to literatures, solution of limestone soils usually has high concentrations of $CaCO_3$ and HCO_3^- but no exchangeable H+ ions. Other than that, the pH of the soil is also high (Isra and Tyler, 1999). Based on the results obtained, the pH value of the soil was 7.4, which was slightly alkaline.

The total alkalinity, reported as milligrams per litre of calcium carbonate (mg/L $CaCO_3$), refers to the ability of a solution to neutralize acid to the equivalence point of carbonate or bicarbonate. Alkalinity is contributed by the weathering process of geologic materials and soil respiration. One of the most common example is the dissolution of carbonate rocks: $CaCO_3(s) + H_2CO_3 \rightarrow Ca^{2+} + 2HCO_3^-$ (Kaushal *et al.*, 2013). For the soil sample in this study, the total alkalinity value was 22 mg/kg.

Cation exchange capacity (CEC) is defined as the sum total of the exchangeable cations that a soil can absorb and it is usually expressed as the number of moles of positive charge such as calcium (Ca^{2+}), magnesium (Mg^{2+}), and potassium (K^+) adsorbed per unit mass. It is affected by both pH and the ionic strength of the soil solution. The CEC value for the soil sample in this study was 22 meq/100g. This CEC value can be considered as an indicator of soil quality and productivity because these exchangeable cations can be used by the higher plants and microorganisms for survival (Rhoades, 1982; Brady and Weil, 2008).

Table 1: Soil analysis results of limestone soil from Gunung Lang, Ipoh, Perak.

Test parameters	Results	Method reference
Soil temperature	25 °C	-
pH value	7.4	Leaching, APHA 4500 H+ B
Total alkalinity as $CaCO_3$	22 mg/kg	Leaching, APHA 2320B
Hydrolytic acidity	ND (<10 mg/kg)	Leaching, APHA 2310B
Cation Exchange Capacity	22.2 meq/100g	In-house method
Total organic carbon	1.2%	USEPA 9060
Total nitrogen	8480 mg/kg	Acid digestion/titration
Nitrate	60 mg/kg	Leaching, APHA 4500 NO_3-H
Ammoniacal nitrogen	ND (<0.5 mg/kg)	Leaching, APHA 4500 NH_3-G
Total phosphorus	831 mg/kg	USEPA 6010B
Soluble potassium	3 mg/kg	Leaching, USEPA 6010 B
Soluble calcium	116 mg/kg	Leaching, USEPA 6010 B
Soluble magnesium	15 mg/kg	Leaching, USEPA 6010 B
Chloride	12 mg/kg	Leaching, APHA 4500 Cl-B
Sulphate	ND (<3 mg/kg)	Leaching, APHA, 4500 SO_4^{-2} E
Soluble sodium	9 mg/kg	Leaching, USEPA 6010 B

Total soil acidity is sum of two types of acidity: exchange acidity (contributed by free hydrogen ion and acidic cations) and hydrolytic acidity (contributed by acidic hydroxyl group or carboxyl group). It is the total base consumed when the natural pH of the soil changes to its final pH after reaction with the base. The soil acidity is caused by the presence of hydrogen ions in the soil solution and of exchangeable hydrogen and aluminium ions in the soil adsorbing complex. Normally, very little of hydrogen ions are present in the acidic soils. If the soil acidity is too high, it will be detrimental to plant development and microbial growth (Chesworth, 2008). Hydrolytic acidity in the soil sample was not detected. The low alkalinity of this soil could cause the acidic hydroxyl groups that normally contribute to soil acidity too low to be detected.

The total organic carbon of the soil sample was 1.2%. Although the percentage of soil organic matter was small, these organic matters still play important roles in the functioning of soil. For example, the CEC of the soil and its quality are affected by the percentage of organic matter. Carbon is known as the foundation of life since most of the compounds in the living organisms are made up of carbon. Most of the carbon present in soil is due to the decomposition of dead organisms such as microorganisms, plant and animal residues which result in the production of humus (Nelson and Sommers, 1996; Robertson et al., 1999).

Different nutrients are required for the survival of organisms and all of these nutrients can be divided into two groups: macronutrients and micronutrients. The macronutrients available in the soil sample are nitrogen, potassium, phosphorus, calcium and magnesium. All of these nutrients are essential in sustaining life of microorganisms. Among these nutrients, nitrogen is one of the most essential for the living organisms since it is a major component of amino acids and they will be used as building blocks of protein. Total nitrogen consisted of three forms of nitrogen, which are ammonia, nitrates and nitrites (Brady and Weil, 2008). The total nitrogen of the collected soil sample in this study is 8480 mg/kg. However, ammoniacal nitrogen cannot be detected. Therefore, the nitrogen sources in this soil were most likely composed of nitrates and nitrites.

Other than nitrogen, phosphorus is one of the most important nutrients for the survival because it is also an essential component of adenosine triphosphate that drives most of the biochemical processes. It is also a component of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). The phosphorus content in the soil sample was high (831 mg/kg). Rocks are big reservoirs of phosphorus and could influence the phosphorus content of the surrounding soil (Rodríguez and Fraga, 1999). Potassium exists in soil as K+ ions and they are useful for plants and animals. It acts as an activator for cellular enzymes and plays an important role in different processes such as energy metabolism, photosynthesis, starch synthesis and sugar degradation. Besides, it helps plants in the adaption towards environmental stresses (Brady and Weil, 2008). However, the amount of potassium in soil is normally fairly low, which is only 3 mg/kg (Alexander, 1961).

In this study, a relatively high amount of calcium (116 mg/kg) was recorded in the soil sample. This may be due to the location of the sampling site which is rich in calcium carbonate. When weathering of the limestone happens, calcium disperses into the soil. Calcium is essential because it strengthens the supportive system of plants and animals. On the other hand, in comparison with the amount of calcium taken up by plants, the amount of magnesium absorbed is lower. Despite that, magnesium is of equal importance as calcium for plants and animals. It is found that magnesium is the central component of chlorophyll and thus, making it crucial for plant photosynthesis. In the soil sample of this study, magnesium was found to be the second most abundant mineral at 15 mg/kg. According to some agriculturists, they believe that the best ratio of calcium to magnesium is 6:1 for optimum plant growth and the results obtained in this study are in accordance with this ratio (Brady and Weil, 2008).

Other than the macronutrients, plants and animals also require micronutrients such as chloride, iron and manganese. These micronutrients are equally important as the macronutrients despite having needed in rather smaller amounts. The absence of these micronutrients will cause dramatic effects especially for plants. The amount of chloride in the soil sample collected was 12 mg/kg. Chloride is crucial for the plant growth since it plays an important role in photosynthesis and enzyme activation (Alexander, 1961; Terry, 1977).

Sodium may affect the salinity and the pH of soil. The presence of salts in soils is mostly due to the weathering of rock. The amount of sodium in the collected soil sample was 9 mg/kg. A high concentration of sodium may be detrimental to plants. On the other hand, the soil analysis results showed that the sulphur content of the soil sample was undetectable. Although this element is abundant in the earth's crust, it is only present in negligible amount in soil (Alexander, 1961).

After hydrometer sedimentation was carried out to determine the soil particle size, it was found that this soil sample contained 56% of clay, 40% of silt and 4% of sand (Table 2). The particle size of the soil ranged from 0.002 mm to 0.06 mm. The texture of the soil collected from the sampling area was smooth and sticky. With the soil texture triangle as reference, it can be concluded that the texture of limestone soil from Gunung Lang belongs to the silt clay category.

Table 2: Types and percentage of soil particles present in limestone soil from Gunung Lang, Ipoh, Perak. It can be concluded that the texture of limestone soil from Gunung Lang belongs to the silt clay categoryby referring to the soil texture triangle.

Types	Percentages (%)
Gravel	0
Sand	4
Silt	40
Clay	56
Total	100

Rock analysis

Limestone rock X-Ray Diffraction (XRD) analysis

The powder diffraction file (PDF) is commonly used as a reference and serves as a "fingerprint" for the identification of unknown materials. It is determined through the angle of diffraction and the intensity that are dependent on the arrangement of elements in the crystal structure. Based on Figure 2, the patterns in diffractogram confirmed that the sample was made up of calcite (PDF 01-071-3699).
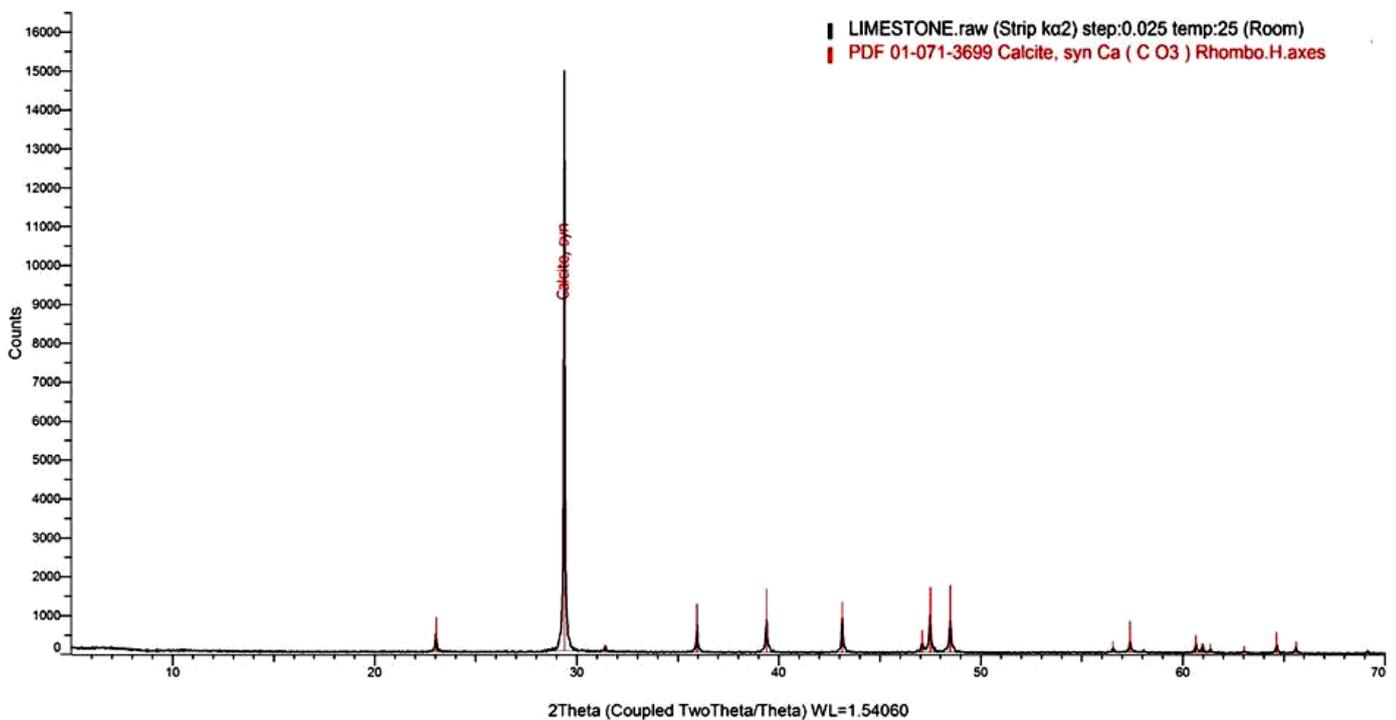

Figure 2: Chromatogram of XRD analysis on limestone with 2-Theta value of 29.5°. The patterns in chromatogram confirmed that the sample was made up of calcite (PDF 01-071-3699). A sharp peak with high intensity was detected, which showed that the concentration of calcite was high.

By definition, carbonate rocks must contain more than 50% of carbonate minerals (Scoffin, 1987). This is because limestone is composed of seashells in its origin and thus, it is rich in calcium carbonate. Calcium carbonate exists in two forms: aragonite and calcite. Since aragonite is unstable, it usually changes into calcite, the main component of limestone (Monroe and Wicander, 2009). In the diffractogram, a sharp peak with high intensity was detected at the 2-Theta value of 29.5°, which showed that the concentration of calcite was high. With such high amount of calcite, the limestone in this study can be categorized as a high-calcium limestone (Missouri Department of Natural Resources, 2011).

Study on biodiversity of limestone soil in Gunung Lang, Ipoh, Perak through 16S rDNA metagenomic analysis

Little is known of the biodiversity in the limestone area compared to the other habitats such as mangroves, forests and seawater. Not many studies have been carried out but it is believed that this area will be rich in biodiversity and unique microorganisms can be identified in this area with the help of metagenomic tools. Therefore, 16S rDNA metagenomic analysis was done on samples collected from this area with the aim of studying the microbial diversity of the soil at Gunung Lang.

After the sequencing, 1,334,194 pairs of reads (250 bp for each read) were generated and 1,143,158 were successfully converted into merged amplicon sequences (Table 3). Among these merged amplicon sequences, about 97% of them were with length between 400-413 bp, and 99.5% were with length above 365 bp. There were 96.20% of bases with Q score above 20. Merged sequences containing both or either of the 5' or 3' primer consisted of 99.7% of the total sequences. Therefore, sequences without any primer sequence were discarded resulting in 1,139,740 valid sequences.

Table 3: Amount of data generated for 16S rDNA metagenomic analysis using NGS sequencer, Miseq (Illumina).

Input	1,334,194	Pairs
Output including	1,143,158	Converted (85.7%)
	489,310	Exact overlaps (36.67%)
Discarded due to	58,940	Not aligned (4.42%)
	123,675	Too many diffs (max=10) (9.27%)
	8,421	Staggered (0.63%)

For further analysis, files with raw reads (709 MB each) were uploaded onto MG-RAST and 98.2% of the sequences have passed the quality control. The MG-RAST ID for this data is 4542505.3.

Organisms are grouped into three domains, namely bacteria, archaea and eukaryotes, where each of these domains contain two or more kingdoms. Based on the result generated by MG-RAST (Figure 3), the organisms were classified into three domains, which were bacteria, eukaryotes and archaea. Among all these three, bacteria domain was the most dominant. Besides that, unassigned or unclassified sequences were detected as well. These sequences can be considered as either potentially novel organisms which have not been studied or organisms that cannot be categorized into any known domain.
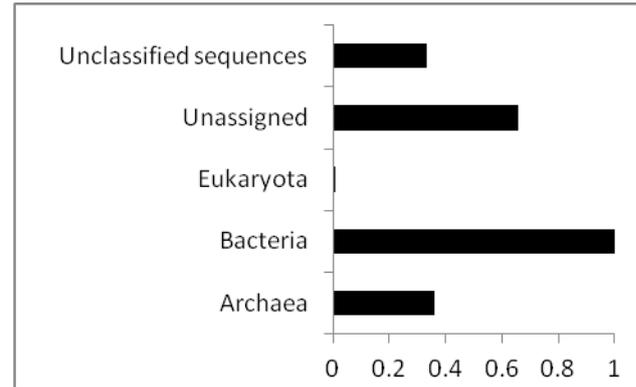

Figure 3: Domain of organisms present in limestone soil from Gunung Lang, Perak. Bacteria domain is the most dominant, followed by archaea and eukaryote. The unassigned and unclassified sequences indicate the presence of organism which have not been studied or those that cannot be categorized into any known domain.

The main objective of this study was to observe the archaea and bacteria present in this area. Archaeans are different from bacteria in their cell wall components. Other than that, archaeans spool their DNA around histones which are similar to eukaryotes (Starr *et al.*, 2013). Figure 4 shows the phyla of archaea present in this area. Crenarchaeota, Euryarchaeota and Thaumarchaeota represented the archaea domain. Previous study showed that Thaumarchaeota adapted well in environment with low ammonia concentrations (Pester *et al.*, 2011). Therefore, Thaumarchaeota was the most abundant phylum among all the archaea phyla although ammonia concentration in this soil was very low.
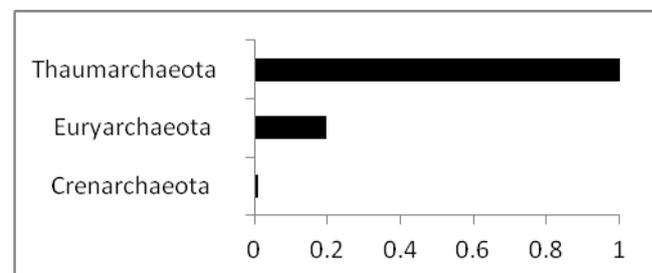

Figure 4: Phyla of archaea present in limestone soil from Gunung Lang, Perak, which were Thaumarchaeota, Euryarchaeota and Crenarchaeota.

Based on Figure 5, the domain bacteria contained a number of unclassified species. This proves that there are many unknown bacteria in this area which are worth exploring. More studies have to be carried out to identify all these unique bacteria. Other than the unclassified bacteria, the phyla found in this area were Acidobacteria, Actinobacteria, Aquificae, Bacteroidetes, Chlamydiae, Chlorobi, Cyanobacteria, Deinococcus-Thermus, Firmicutes, Fusobacteria, Nitrospirae, Planctomycetes, Proteobacteria, Spirochaetes, Tenericutes, Thermodesulfobacteria and Verrucomicrobia. Figure 6 shows the order of bacteria present in each phylum. Proteobacteria was the most dominant and diverse phylum within the bacteria domain. This phylum consists of 5 major groups known as Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria and Epsilonproteobacteria. Bacteria from all these groups are important as they are actively involved in carbon, sulfur and nitrogen cycles on our planet (Kersters *et al.*, 2006). On the other hand, Spirochaetes, Thermodesulfobacteria, Aquificae, Chlamydiae, Chlorobi, Deinococcus-Thermus, Fusobacteria, Nitrospirae and Planctomycetes are phyla that are less diverse.

The abundance of bacteria could be important in maintaining the fertility of the soil. The high nutrient content in this limestone area had indirectly contributed to the richness of its biodiversity. All these elements were crucial for plant growth. Although nitrogen was abundant in the atmosphere (about 80%), it was not readily available for the use of living organisms. Ammonia, nitrate and nitrite were nitrogenous compounds that were required and this turnover of nitrogen occurred through the nitrogen cycle (Stanier *et al.*, 1986). Based on the soil analysis, the soil was rich in total nitrogen content and this was contributed by the nitrogen fixing bacteria that were present in this area such as Nitrosomonadales, Cyanobacteria, Nostocales, Desulfovibrionales, Nitrospirales, Rhizobiales and Clostridiales. Both aerobes and anaerobes were present in this area (Coyne, 1999; Tate, 2000). Other than that, phosphorus solubilizing bacteria such as Flavobacteriales and Pseudomonadales were also present in this area. They helped in the conversion of insoluble organic phosphorus compounds into forms that were available for the plants. Iron bacteria such as Caulobacterales and Chlamydiales were present as well to help in the transformation of iron in soil (Alexander, 1961).
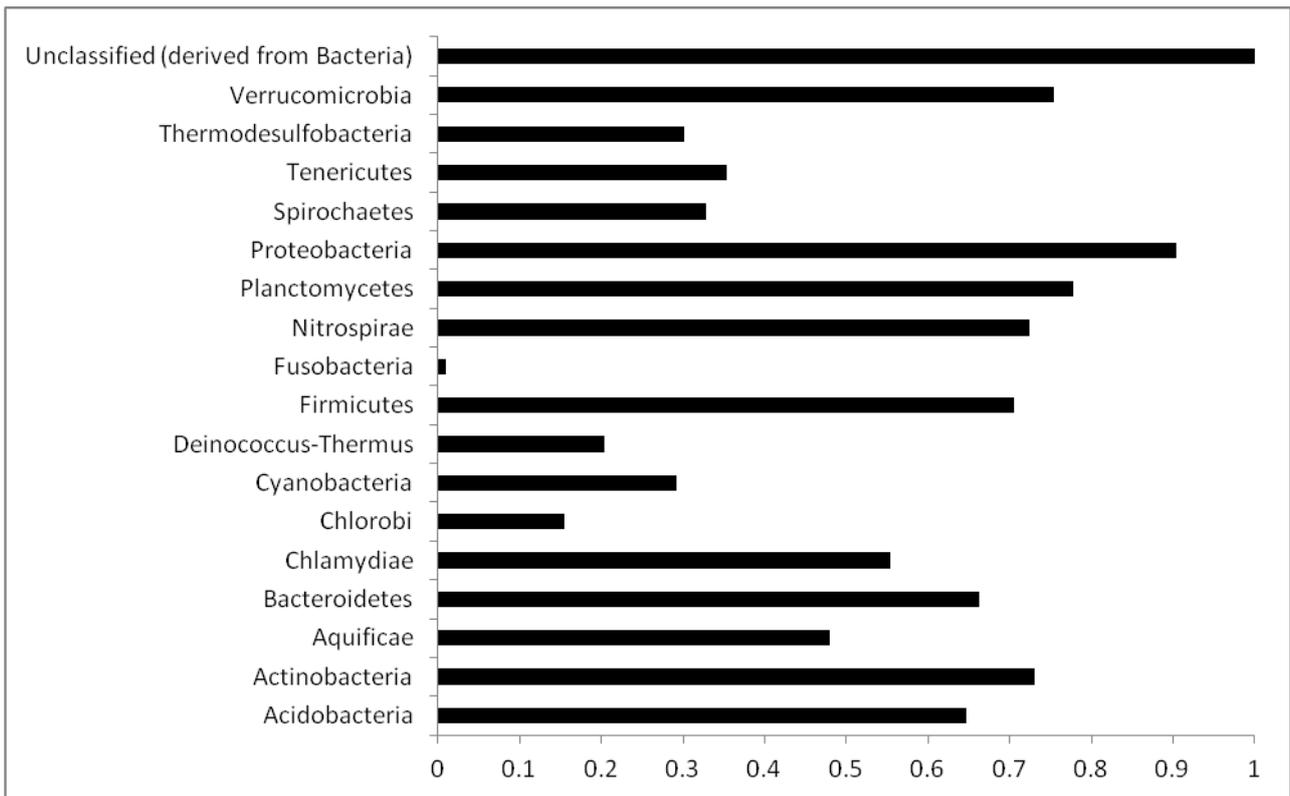

Figure 5: Phyla of bacteria present in limestone soil from Gunung Lang, Perak, which were Verrucomicrobia, Thermodesulfobacteria, Tenericutes, Spirochaetes, Proteobacteria, Planctomycetes, Nitrospirae, Fusobacteria, Firmicutes, Deinococcus-Thermus, Cyanobacteria, Chlorobi, Chlamydiae, Bacteroidetes, Aquificae, Actinobacteria, Acidobacteria and unclassified bacteria.

Figure 6: Order of bacteria present in each phylum limestone soil from Gunung Lang, Perak (MG-RAST ID: 4542505.3). Proteobacteria (purple) was the most dominant and diverse phylum within the bacteria domain.

CONCLUSION

In conclusion, this study has shown that this limestone area is diverse in microbial community and many of them are unique microorganisms that are worth exploring further. Therefore, the screening of biotechnologically useful genes from this limestone area is currently being carried out in our laboratory.

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