

16S rRNA Gene Sequence-based Microbial Diversity Analyses of the Geothermal Areas of Cisolok, Kamojang, and Likupang in Indonesia

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Microbial diversity analyses were performed in several geothermal areas in Indonesia using a culture-independent approach with 16S rRNA gene sequencing. All areas and the majority of samples were noted as being affiliated with *Proteobacteria*. In addition, unclassified bacteria with no phylum affiliation were detected at an incidence rate of 20.0-26.5% in every location. The majority groupings in the geothermal hot stream in Cisolok belonged to β -*Proteobacteria* (27.1%) and *Cyanobacteria* (11.0%), whereas the majority from the volcanic area in Kamojang was γ -*Proteobacteria* (51.5%) followed by *Aquificales* (12.9%). The predominant groups around an underwater thermal vent in the sea at Likupang were γ -*Proteobacteria* (33.3%) and then *Bacteroidetes* (27.6%). This detailed microbial community analyses of each area strongly support a possible association with plausible community groups and environmental habitats, such as extremely geothermal or marine habitats. This study has significantly contributed to the expansion of scientific knowledge of the microbial community in Indonesia.

Keywords: Indonesia, microbial diversity, 16S rRNA gene

Over the past few decades, the numerous studies on microbial diversity have been reported to investigate a microbial community as well as to isolate and identify novel microorganisms in nature. However, the extent of microbial diversity has been limited by culture-dependent approach which has permitted to isolate barely a few microorganisms, suggesting that over 99% of microorganisms present in nature could be hard to be cultivated with standard culture methods [2]. Recently, the modified culture-dependent approach has been tried using synthetic media and nutrient enrichments to reduce the limitation [7, 13]. On the other hand, recombinant DNA and molecular phylogenetic techniques has provided a culture-independent approach with 16S rRNA gene sequencing to identify the microbial diversity directly [15]. In addition to 16S rRNA gene, other molecular markers such as β -subunit of RNA polymerase (*rpoB*) and β -subunit of DNA gyrase (*gyrB*) gene have been also employed to analysis the

microbial community to address the inherent problem of intra-species heterogeneity between 16S rRNA genes [8, 28]. However, the approach based on 16S rRNA gene has been still used as a powerful and convenient technique to identify the uncultured microorganisms and investigate the microbial ecosystem because of the extensive database of 16S RNA gene sequences that has been accumulated so far [25].

Microbial communities in extreme nature have attracted much attention because of the considerable biotechnological potential in extreme environments. In this connection, there have been many studies on thermophile diversity in geothermal areas among which one of the most successful researches was the study on diversity in Yellowstone hot spring [14]. Since then, a variety of hot springs have been investigated to elucidate the microbial diversity, especially with culture-independent approach, consequently discovering numerous evolutionary lineages of microorganisms [10]. Recent studies were also reported in geothermal environments, for example in Indonesia and Malaysia [3, 11].

Indonesia is one of the most tectonically active countries in the world with a number of volcanoes and geothermal

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regions. Despite these specific geographical characteristics of Indonesia, there have been few studies on microbial diversity from Indonesian geothermal areas [1, 3]. In this study, we therefore analyzed the microbial diversity of geothermal areas in Indonesia. The analysis was based on culture-independent approach using 16S rRNA gene sequencing after direct extraction of environmental DNA.

Environmental samples were collected from geothermal areas (Cisolok, Kamojang, and Likupang) in Indonesia (Fig. 1). The environmental temperature and pH of each sample was 70°C and pH 7.5 for Cisolok, 80°C and pH 4.0 for Kamojang, and 80°C and pH 7.0 for Likupang. After collecting the samples, genomic DNA was directly extracted from each environmental sample using SDS-based DNA extraction method as previously described [29]. Partial 16S rRNA gene was amplified using a set of bacterial specific universal primers; 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The PCR condition included initial denaturing at 94°C for 1 min followed by 30 cycles of 94°C for 1 min, 55°C for 30 sec, 72°C for 1 min, and a final extension step of 72°C for 5 min. The amplified PCR products of expected size were extracted and purified from agarose gel with QIAquick Gel Extraction kit (Qiagen, CA, USA). The purified DNA fragments were cloned into the pGEM-T vector (Promega, WI, USA) and transformed into *E. coli* TOP10 cells to construct the 16S rRNA gene libraries. After sequencing each 16S rRNA gene from the libraries, too short sequences of less than 350 bp in length were excluded for the further analysis. The resulting sequences were compared to 16S rRNA gene sequence from the nonredundant sequence database by using BLASTN program at National Centre of Biotechnological Information (NCBI) to determine their

approximate taxonomic identifications and investigate the microbial diversity.

Analysis of Microbial Diversity in Cisolok

Cisolok located in West Java has the extreme environment with high temperature and high sulfate concentration [12]. Based on 155 representative 16S rRNA gene sequences, two most major abundant phyla were shown in Cisolok; *Proteobacteria* (38.7%) with major class, β -*Proteobacteria* (27.1%) and *Cyanobacteria* (11.0%) with major genus, *Thermosynechococcus* (10.3%) (Table 1 and Fig. 2). Unclassified bacteria with no phylum affiliation also accounted for 26.4% of all 16S rRNA gene sequences. Concerning the evolution of tolerance, *Cyanobacteria* have been established in diverse environmental habitats showing wide ranges in temperature, salinity, pH, and irradiance [23]. As a good example, *Cyanobacteria* community containing *Synechococcus* in hot spring flows with marked temperature gradients was developed. Similarly, the diversity analysis of Cisolok showed that *Thermosynechococcus* was distributed with 10.3%. The 16S rRNA gene sequencing in Cisolok also exhibited the presence of chemolithotrophic thermophiles including the sulfate reducer *Thermodesulfobacteria* and hydrogen-oxidizing *Aquificae*. The occurrence of such microorganisms is consistent with the environment of Cisolok where sulfate-rich hot spring is, suggesting the lithotrophic base for primary productivity in Cisolok through sulfate reduction and hydrogen oxidation [14].

Sample collection from geothermal hot stream in Cisolok expected the resulting sequences to be majorly derived from thermophiles. Accordingly, some divisions were detected including *Deinococcus-Thermus*, *Thermodesulfobacteria*,

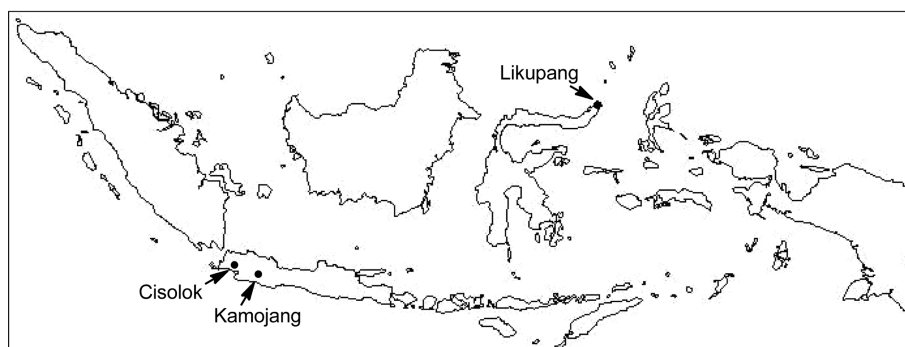


Fig. 1. Map of Indonesia showing the sampling sites (colored circles) for analysis of microbial diversity in this study. Environmental samples were collected from geothermal hot stream at Cisolok (6°57'23"S, 106°28'46"E), volcano area at Kamojang (7°07'30"S, 107°47'60"E), West Java, and thermal vent under the sea water at Likupang (1°41'35"N, 125°00'47"E), north Sulawesi, Indonesia.

Table 1. Frequency and phylogenetic affiliation of 16S rRNA gene libraries from the geothermal areas of Cisolok, Kamojang, and Likupang in Indonesia.

Division	Sampling area Number of clones (%)		
	Cisolok	Kamojang	Likupang
Actinobacteria			
Acidimicrobiae		1 (0.6%)	
Aquificae			
Aquificales	1 (0.6%)	22 (12.9%)	
Bacteroidetes/Chlorobi group			
Bacteroidetes			
Sphingobacteria	3 (1.9%)		7 (6.7%)
Flavobacteria			5 (4.8%)
Unclassified Bacteroidetes		1 (0.6%)	17 (16.2%)
Chlorobi	3 (1.9%)		
Environmental samples	2 (1.3%)		
Chloroflexi			
Chloroflexales	3 (1.9%)		
Unclassified Chloroflexi	1 (0.6%)		
Cyanobacteria			
Thermosynechococcus	16 (10.3%)		
Unclassified Cyanobacteria	1 (0.6%)		
Deferribacteres			
Defferribacterales	2 (1.3%)		
Deinococcus-Thermus			
Thermales	4 (2.6%)		
Dictyoglomi			
Dictyoglomales	1 (0.6%)		
Firmicutes			
Bacilli			
Bacillales	4 (2.6%)	1 (0.6%)	1 (1.0%)
Unclassified Bacilli			1 (1.0%)
Clostridia			
Clostridiales	5 (3.2%)		2 (1.9%)
Unclassified Firmicutes			4 (3.8%)
Fusobacteria			
Fusobacteriales	2 (1.3%)		
Nitrospirae			
Nitrospirales	1 (0.6%)		
Planctomycetes			
Planctomycetales	2 (1.3%)		
Proteobacteria			
α -Proteobacteria	1 (0.6%)		3 (2.9%)
Caulobacteriales		1 (0.6%)	
Rhodobacterales	1 (0.6%)		1 (1.0%)
Rhizobiales	2 (1.3%)		1 (1.0%)
Unclassified α -Proteobacteria	3 (1.9%)		
β -Proteobacteria			
Burkholderiales	15 (9.7%)	6 (3.5%)	
Hydrogenophilales	6 (3.9%)	4 (2.3%)	
Nitrosomonadales		1 (0.6%)	
Rhodocyclales	7 (4.5%)	1 (0.6%)	1 (1.0%)
Unclassified β -Proteobacteria	14 (9.0%)	7 (4.1%)	1 (1.0%)

Table 1. Continued.

Division	Sampling area Number of clones (%)		
	Cisolok	Kamojang	Likupang
γ -Proteobacteria			1 (1.0%)
Acidithiobacillales		79 (46.2%)	
Alteromonadales			16 (15.2%)
Oceanospirillales			6 (5.7%)
Pseudomonadales	2 (1.3%)	8 (4.7%)	
Thiotrichales			7 (6.7%)
Unclassified γ -Proteobacteria	1 (0.6%)	1 (0.6%)	5 (4.8%)
Environmental samples	1 (0.6%)		
δ -Proteobacteria	7 (4.5%)	1 (0.6%)	2 (1.9%)
Unclassified δ -Proteobacteria			3 (2.9%)
Thermodesulfobacteria			
Thermodesulfobacteriales	3 (1.9%)		
Unclassified Bacteria	41 (26.5%)	37 (21.6%)	21 (20.0%)
Total clones	155 (100%)	171 (100%)	105 (100%)

Aquificae, and *Dictyoglomi* which could be cultured from the geothermal habitats. However, several other microbial divisions characterized by mesophiles were also detected, including *Proteobacteria*, *Firmicutes*, and *Planctomycetes*, suggesting the possible contamination from groundwater flow into the hot stream in Cisolok [14].

Analysis of Microbial Diversity in Kamojang

Kamojang is one of the geothermal areas in West Java, Indonesia and is around the environment with an extremely low pH, high sulfate concentration, and high temperature [1]. After sequencing 171 representatives, the two most abundant microbial orders were *Acidithiobacillales* (46.2%) belonged to γ -*Proteobacteria*, followed by *Aquificales* (12.9%), whereas unclassified bacteria also accounted for a high portion (21.6%) (Table 1 and Fig. 2). The major distribution of *Acidithiobacillales* is responsible for the extreme conditions including acidic environment and high concentrations of sulfates and ferric irons, being supported by the previous report showing that the iron- and sulfur-oxidizing *Acidithiobacillus ferrooxidans* and sulfur-oxidizing *A. thiooxidans* are present in these extreme conditions [20]. Concerning the iron cycle, *A. ferrooxidans* utilizes the reduced ferrous iron as an energy source, producing the oxidized ferric iron which is relatively stable at acidic pH in the presence of elemental sulfur [6]. Therefore, the abundance of *Acidithiobacillales* seems to play an important role in the generation and maintenance of the extremely acidic conditions of the

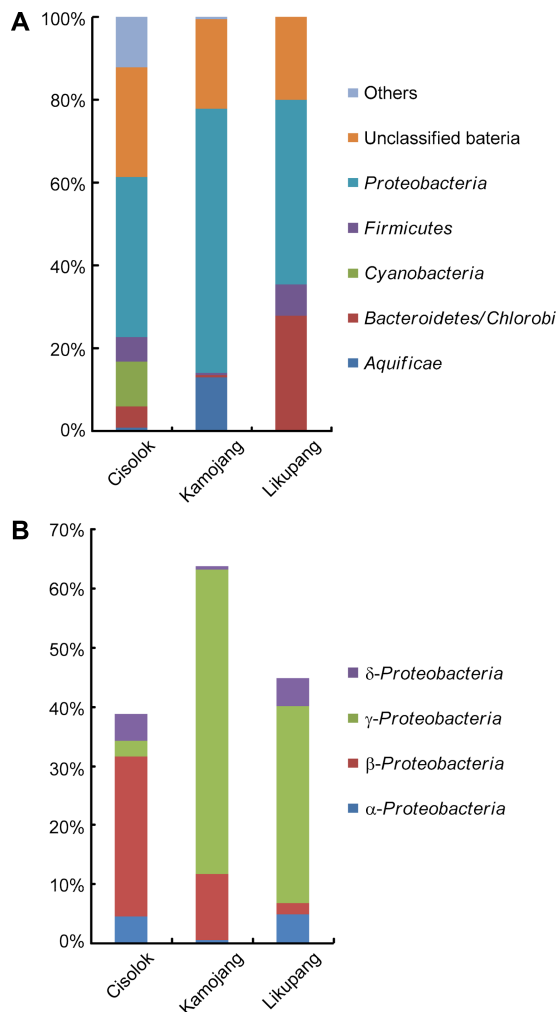


Fig. 2. Distribution of the predominant phyla (A) and detailed *Proteobacteria* (B) identified from 16S rRNA gene libraries. Four classes (α , β , γ , and δ -*Proteobacteria*) belonged to *Proteobacteria* phylum were used in the case of *Proteobacteria*. The combined distribution of minor phyla affiliated below 7% of total sequenced clones (except *Proteobacteria*) was represented to be "Others".

ecosystem in Kamojang area.

The explanation of extremely acidic condition based on the distribution of *Acidithiobacillales* could be strongly supported by the previous study on microbial diversity of acidic hot spring (Kawah Hujan B) in Kamojang area [1]. The study described that the majority of the sequences was closely related to *Crenarchaeota* frequently found in extremely acidic environments [19] and capable to utilize elemental sulfur as energy source [5].

Analysis of Microbial Diversity in Likupang

Total of 105 representative clones from thermal vent under the sea water at Likupang, north Sulawesi, Indonesia were sequenced and analyzed, resulting that the highest proportion was γ -*Proteobacteria* (33.3%), followed by *Bacteroidetes* (27.6%) and unclassified bacteria group (20%). The minor groups were also affiliated to *Firmicutes* (7.6%) and α -*Proteobacteria* (4.8%) (Table 1 and Fig. 2). In fact, these populations could be common characteristics exhibited in the microbial communities of marine environments with hydrothermal vents. For example, the microbial diversity analysis of hydrothermal vent (Black point) in the Southern Tyrrhenian Sea, Italy, showed γ -*Proteobacteria* and *Firmicutes* to be the dominant populations [22]. In γ -*Proteobacteria* group, *Alteromonadales* were accounted with a high proportion (15.2%), followed by *Thiotrichales* (6.7%) and *Oceanospirillales* (5.7%). *Alteromonadales*, which is prevalent in different oceanic regions, but more frequently in deep sea [4], is primarily composed of heterotrophic marine bacteria playing a significant role in the biogeochemical cycle of carbon, nitrogen, and sulfur [16]. *Thiotrichales* group including *Thiomicrospira*, *Thiothrix*, and *Beggiatoa* has been identified to be chemolithotrophic sulfur-oxidizing bacteria group [26]. Considering the previous studies that these sulfur-oxidizing bacteria were frequently observed in the hydrothermal vents and wide range of marine habitats, these microbial communities could be typical components in the thermal vent under the sea water at Likupang where they can utilize the reduced sulfur compounds as electron donors [21].

Bacteroidetes phylum has been known to occur ubiquitously in marine habitats and their colonies known to exhibit specific color pigmentations due to the presence of flexirubin-type pigments [18]. Our diversity analysis of Likupang also showed that *Bacteroidetes* group had the second proportion (27.6%) where *Sphingobacteria* and *Flavobacteria* classes accounted with 6.7% and 4.8% proportion, respectively.

In summary, we analyzed and compared the microbial communities of several geothermal areas in Indonesia via culture-independent approach with 16S rRNA gene sequencing (Fig. 2). Regardless of sampling areas, there was a large portion of unclassified bacteria (20.0-26.5%), representing the possible existence of a novel sub-class or even higher class in these geothermal areas [27]. However, the fact that 16S rRNA gene sequence-based analysis cannot completely represent the environmental habitats

with just classifying the microbial distribution roughly, will encourage to further studies on microbial diversity with fluorescence in-situ hybridization analysis (FISH) as well as other specific genes such as *rpoB* and *gyrB* as well as fluorescence *in-situ* hybridization [9]. Nevertheless, the detail microbial diversity analysis from each sampling area indicates that the majority of libraries appear to be plausible community groups corresponding to each environment of sampling area. From the point that there have been just few studies reporting the microbial community of geothermal areas in Indonesia [1, 3], the present study could contribute to expand the knowledge of the microbial diversity and provide a basic ecosystem of geothermal areas in Indonesia. Additionally, this study could support the big challenge looking for novel biomaterials and functional enzymes for industrial applications [17, 24].

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국문초록

16S rRNA 분석을 통한 인도네시아의 Cisolok, Kamojang, Likupang 지열지대 내 미생물 다양성 분석

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인도네시아 지열지대의 미생물 다양성을 16S rRNA 염기서열 분석을 통해 조사하였다. 전체적으로 어떠한 phylum 계통군에도 포함되지 않는 unclassified bacteria가 20.0-26.5% 존재하였으며 sampling 지역에 상관없이 *Proteobacteria*가 우점 phylum 계통군으로 나타났다. Cisolok 주변의 지열 지역을 조사한 결과 β -*Proteobacteria* (27.1%)와 *Cyanobacteria* (11.0%)가 높은 비율을 차지한 반면 Kamojang의 화산 주변 지역의 경우에는 γ -*Proteobacteria* (51.5%) 그리고 *Aquificales* (12.9%)가 우점 계통군으로 나타났다. 또한 Likupang 열수구의 경우에는 γ -*Proteobacteria* (33.3%)와 *Bacteroidetes* (27.6%)가 높은 비율로 나타났다. 본 연구를 통해 인도네시아 각 지열지대에 분포하는 미생물 군집은 각 지역의 환경적인 특징 (극한 지열 및 해양 서식지)과 밀접한 연관성이 있음을 알 수 있었다.