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Molecular characterization and evaluation of potential isolated bacteria in optimizing former coal mining land as a planting media in East Kalimantan, Indonesia

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Abstract. *Rahayu YS, Yuliani, Asri MT, Rahayu DA. 2024. Molecular characterization and evaluation of potential isolated bacteria in optimizing former coal mining land as a planting media in East Kalimantan, Indonesia. Biodiversitas 25: 4059-4066.* East Kalimantan is an extensive province in Indonesia dedicated to coal mining activities. Despite the potential, several coal mining enterprises do not implement land reclamation procedures before abandoning the mines. This situation often leads to low soil fertility and challenges in effectively converting the resulting marginal land into productive terrain. Several reports have shown the importance of concerted efforts to address those challenges. Therefore, this study aims to isolate, characterize, and evaluate endophytic microorganisms in optimizing former coal mining areas using the partial sequence of 16S rRNA. The construction of the phylogenetic tree topology between samples and the close relatives from GenBank was analyzed using the Neighbor-Joining (NJ) and Maximum-Likelihood (ML) methods with the Kimura 2-Parameter (K2P) calculation model (with 1,000 bootstraps). The resulting cladogram could be divided into two major clusters, with cluster 1 comprising two distinct clades, namely the *Bacillus* genus clade and clade 2 consisting of the Genus *Achromobacter* and *Pseudomonas*. Meanwhile, cluster 2 was divided into two clades: *Bacillus alvei* and *Bacillus pantothenticus*. Each group in these clades was then further categorized based on the species, with a bootstrap value of 100%. The molecular data identification revealed five variations of nucleotide bases, and the genetic distance average value within the ingroup was 1.0%. The results revealed that species similarities existed between *Pseudomonas guariconensis, Bacillus mycoides, Bacillus pantothenticus, Bacillus sphaericus, Bacillus mycoides, Achromobacter xylosoxidans, Bacillus sphaericus, Bacillus firmus, Bacillus stearothermophilus, Bacillus alvei*. Based on these results, the 16S ribosomal RNA (16S rRNA) sequences had successfully differentiated bacteria in optimizing former coal mining areas in East Kalimantan.

Keywords: 16S rRNA, identification, molecular genetics, phylogenetic

INTRODUCTION

Coal is the rock sedimented from the deposition of organic matter, including plant remains, widely known for its applicability as fossil fuels. Indonesia, particularly East Kalimantan, is a key area for coal mining due to its promising productivity potential. However, mining activities significantly affect the environment, causing physical, chemical, and biological harm to the soil. These activities often lead to decreased soil quality, depleted nutrient levels, and heightened acidity (Kesumaningwati and Urnemi 2017; Rahman et al. 2021).

Therefore, to overcome these challenges, several studies proposed applying bioremediation methods to rehabilitate previously used mining areas effectively. The crucial microorganisms participating in the process are fungi and bacteria (Gustiano et al. 2021; Rahayu et al. 2023). Diverse soil microorganisms have been reported to be essential in preserving soil fertility and can be harnessed in bioremediation methods; these comprise phosphate-solubilizing bacteria, bacteria proficient in degrading hydrocarbon compounds, and nitrogen-fixing bacteria. Several studies showed that phosphate-solubilizing bacteria could serve as bioagents due to their ability to enhance the availability of phosphorus (P) nutrients in the soil for plants through mineralization and solubilization (Billah et al. 2019; Chen and Liu 2019; Timofeeva et al. 2022; Long and Wasaki 2023). Lin et al. (2018) and Yang et al. (2018) also reported that these microorganisms could improve the phytoremediation efficiency of *Wedelia trilobata* plants in metal-contaminated soil, exert a positive effect on soil microflora, enhance soil quality, promote plant growth, and act as phytoremediation agents. Meanwhile, hydrocarbon-degrading bacteria play an important role in enzymatically breaking down abundant hydrocarbon compounds into shorter, non-toxic forms, which enhances soil nutrient content resulting from those breakdown process (Das and Chandran 2011; Rahayu et al. 2018; Rahayu 2020). Nitrogen-fixing bacteria can be harnessed for their ability to provide nitrogen (Widawati 2019) essential for protein synthesis and growth hormone formations (Zaidi et al. 2017; Anas et al. 2020).

In line with previous studies, the isolation and identification of endophytic bacteria isolates from former coal mining soil have been carried out as an initial step to obtain indigenous bacteria from these areas (Rahayu et al. 2021). Therefore, molecular studies are typically required to confirm the identification results obtained from the

process. One commonly used molecular marker in this context is the 16S rRNA gene, often used in bacterial identification and classification (Titilawo et al. 2020; Saidu et al. 2021). In addition, it is a component of the small subunit of prokaryotic ribosomes with many variable regions to distinguish between different bacterial species. The 16S rRNA gene is also a component of the bacterial ribosome and essential for protein synthesis (Bibiana et al. 2018; Clarridge 2004; Meliah et al. 2020; Mazalan et al. 2020). Although 16S rRNA gene sequencing provides information about the identity and phylogeny of bacteria, it does not provide direct insight into their functional capabilities. The bioinformatics tools can be used to predict the potential functions of the identified bacteria based on the taxonomic affiliations to overcome this limitation. The information obtained from 16S rRNA gene sequencing and subsequent analyses is then used to evaluate the potential of the isolated microorganisms in optimizing the abandoned coal mining soil. Comparing 16S rRNA gene sequences enables the classification of strains at several levels, including species and subspecies, and facilitates discrimination between organisms at the genus level across all major phyla of bacteria (Johnson et al. 2019; Hou et al. 2018). Therefore, this study aims to evaluate the molecular identity and phylogeny of bacteria successfully isolated from former coal mining areas.

The results are essential for developing an optimization model for former coal mining areas as planting media using microorganisms to shape the soil nutrient dynamics. The success of bioremediation processes is, in part, determined by their interaction with plants, which have the potential to act as bioremediation in their native environment. This ensures the successful implementation of the model, emphasizing the concept of maintaining a balanced and sustainable environmental equilibrium.

MATERIALS AND METHODS

Isolation of bacteria from former coal mining

An investigative study was conducted in former coal mining areas in East Penajam Paser Regency, East Borneo, Indonesia. This study included three specific sites from the previous coal mining activities, and in each site, 5 locations were selected as samples, which resulted in a total of 15 soil sample locations. Bacteria were isolated from those soil samples, and then to initiate this process, 10 mL sterile distilled water was used to mix 1 gram of soil sample, followed by a homogenization process. Subsequently, 1 mL mixture was extracted and placed in a test tube containing Czapex Broth without sucrose, which underwent an incubation process for 7 days at 30°C (Rahayu et al. 2021). During incubation, 1 mL from each sample was inoculated using a pour plate method on Petri dishes containing nutrient agarmedia and then incubated at 30°C. Simultaneously, a test tube containing nutrient agar media was prepared to facilitate the identification of the growing bacteria inoculated. Once pure isolates were obtained, morphological gram staining and physiological bacterial colony tests were conducted to analyze the results comprehensively.

Cultivation of test bacteria and extraction of DNA

A volume of 1.5 mL bacteria in media was extracted and then transferred to a 1.5 mL tube. Centrifugation was conducted at 10,000 rpm for 2 minutes then the supernatant was carefully discarded, leaving the pellet. Next, 180 µL of Buffer GT1 was added to the tube and vortexed. Subsequently, 200 µL buffer GT2 and 20 µL Proteinase K were added, followed by mixing using a vortex. The mixture was incubated at a temperature of 56°C for 10 minutes, with the tube being inverted every 5 minutes during the incubation. Next, 200 µL absolute ethanol was added to the incubated mixture and briefly vortexed. The sample was then loaded onto the spin column and centrifuged at a speed of 13,000 rpm for 1 minute, discarding the flow-through, and 500 µL buffer W1 was added to the spin column, then centrifuged for 1 minute at a speed of 13.000 rpm. The flow-through was discarded, and 700 µL buffer W2 (previously mixed with ethanol) was added, followed by centrifugation for 1 minute at 15.000 rpm. The flow-through result was discarded, and the spin column was centrifuged for another 2 minutes at 13,000 rpm. DNA was transferred to the 1.5 mL tube using a spin column. Subsequently, 50-100 µL of elution buffer was added, and the mixture was incubated for 1 minute at room temperature. After centrifugation for 1 minute at a speed of 13.000 rpm, the DNA spin column was discarded, and the DNA was purified for the next step. DNA was stored at -20°C for a few days and at -70°C for longer-term storage.

Quantification of genomic DNA

The gel electrophoresis was performed using a 1% agarose gel prepared with 0.5 g of agarose and 50 mL of TAE buffer to visualize the genomic DNA. Additionally, 4 µL of Ethidium Bromide (EtBr) was incorporated as a dye into the gel. The next step was to mix 3 µL of genomic DNA with 1 µL of loading dye before putting the mixture in an agarose well. The electrophoresis process was performed using a machine with a voltage of 220 V and a current of 400 mA for 25 minutes.

Amplification and purification of 16S rRNA gene

The isolated results were subsequently amplified using a Biorad PCR machine in a 30 µL solution, which consisted of 15 µL PCR Master Mix Nexpro, 2.5 µL DNA Template sample (100 ng/µL), 7.5 µL water, and 2.5 µL of each primer (10 pmol for both forward and reverse primers). The primers were 16S 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3'). The amplification process involved the following temperature settings: pre-denaturation at 95℃ for 5 minutes, followed by 40 cycles comprising denaturation at 95℃ for 45 seconds, annealing at 58℃ for 45 seconds, and extension at 72℃ for 45 seconds. Subsequently, the post-elongation process was conducted at 72℃ for 7 minutes. PCR results were then subjected to electrophoresis on a 1% agarose gel. Finally, the sequencings of PCR products were performed by 1st BASE Laboratories Sdn Bhd sequencing services.

Sequencing, blasting, and phylogenetic analysis of the amplified DNA

A dataset comprising over 800 base pairs was obtained from 11 bacteria isolated from former coal mining land in East Kalimantan, representing the final dataset. Following nucleotide sequencing, chromatogram analysis was conducted using Finch TV software, and the sequences were translated into amino acid sequences through the ExPASy website (Duvaud et al. 2021). Subsequently, all sequences underwent verification through the BLAST (Basic Local Alignment Search Tool) method (Boratyn et al. 2012) to compare the results with closely related species. Multiple sequence alignment was performed using Clustal X (Larkin et al. 2007) and manually checked through BioEdit software (Hall 1999). The partial 16S rRNA gene sequences were submitted to GenBank with assigned accession numbers, as indicated in Table 2. Taxonomic characteristics and GenBank accession numbers were associated with a specific code.

Calculation of similarity values was executed using the formula, similarity percentage = (1-Genetic Distance)×100%. The substitution of transitions and transversions of nucleotide bases was determined using the Kimura 2-Parameter (K2P) model. Genetic diversity analysis, pertinent to phylogenetic reconstruction, included nucleotide diversity (Pi), the number of polymorphic sites (S), haplotype analysis (haplotype diversity (Hd), and the number of haplotypes (nHap) (Saitou and Nei 1987). MEGA X version 10.2.6 (Kumar et al. 2018) was used to compute nucleotide frequencies, transition/transversion ratio (k), transition/conversion rate ratio bias (R), and probabilities.

Phylogenetic reconstruction

The partial sequence of the 16S rRNA gene was used to reconstruct the phylogenetic tree, including 11 sequences, using MEGA X version 10.2.6. Minimum Evolution (ME) and Maximum-Likelihood (ML) were the 2 methods applied to ascertain the grouping of different species. For ME phylogenetic tree reconstruction, a bootstrap consensus tree was inferred from 1,000 replicates, with branches represented by partitions, reproduced in less than 50% of bootstrap replicates being eliminated (Felsenstein 1985). Evolutionary distances were calculated using the K2P method, with a gamma distribution (shape parameter = 63) modeling rate variation among sites. ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm at search level 2, and the first tree was generated by the Neighbor-Joining (NJ) algorithm (Saitou and Nei 1987). The K2P substitution model (Saitou and Nei 1987) with the rate variation among sites modeled using a Gamma distribution was used for maximum likelihood phylogenetic tree reconstruction. A bootstrap consensus tree was inferred from 1,000 replicates (Felsenstein 1985). The percentage of replicate trees was displayed next to the branches in which associated taxa clustered in the bootstrap test (1,000 replicates).

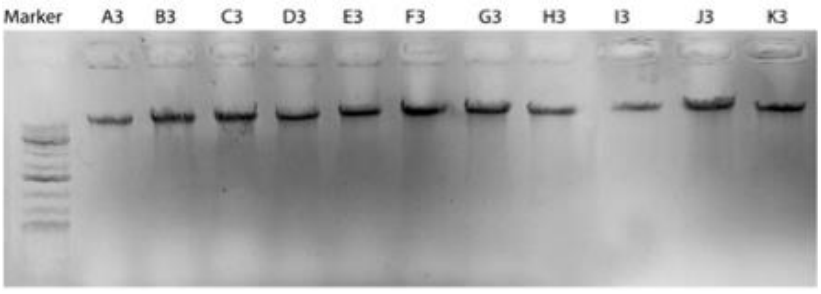
RESULTS AND DISCUSSION

Quantification and purity of the extracted DNA

The amplification of the target 16S rRNA gene was conducted through conventional PCR (Figure 1) with universal primers, namely 16S 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3'). The amplified DNA of the target 16S rRNA gene was subsequently subjected to 1% agarose gel electrophoresis and visualized using a UV transilluminator. The successfully amplified 16S rRNA gene target was indicated by a distinct and thick DNA band without any smearing. After sequencing, nucleotide bases were obtained from the eleven research samples, each exceeding 800 bp. The sequencing results were analyzed using Finch TV to interpret the DNA chromatogram. The analysis indicated similarity among each research sample and the ingroup species from GenBank, as detailed in Table 1.

Identification similarity using the BLAST

The nucleotide base of bacteria in optimizing former coal mining land in East Kalimantan as planting media samples were identified as eleven species with 98.8-100% similarity. These sequencing results were processed using Expasy by translation into protein until no stop codon was found in the middle of the nucleotide base. Expasy results were subsequently analyzed using the BLAST (Table 1) to determine the highest degree of relationship of the study samples.



***used 1 kb marker**

Figure 1. Electropherogram of the DNA band from bacterial samples in a 1% agarose gel (Note: M: DNA Ladder 2 kb)

Table 1. Similarity values of the samples with the Gene Bank (Blast)

Code sample	Code acc number sample	Species in group	Code acc num	Similarity
8 III B4 (gram-positive)	OR506111	*Pseudomonas guariconensis*	LC575133.1	99.20%
7 III (B3) gram-positive	OR506110	*Bacillus mycoides*	AB368963.1	98.80%
4 III E3 (gram-positive)	OR506093	*Bacillus pantothenticus*	D78477.1	98.80%
I D3 (gram-positive)	OR506139	*Bacillus sphaericus*	L38654.1	100.00%
IE 5 (Gram-positive)	OR507845	*Bacillus mycoides*	AB368963.1	98.68%
2 IA3 (gram-positive)	OR506091	*Achromobacter xylosoxidans*	LK391696.1	99.05%
II A4 (gram-positive)	OR507846	*Bacillus sphaericus*	L38654.1	100.00%
3 IB4 (gram positive)	OR506092	*Bacillus firmus*	AB650511.1	99.72%
5 IB3 (gram-positive)	OR506094	*Bacillus stearothermophilus*	AJ005760.1	100.00%
1 IA4 (gram-positive)	OR506090	*Bacillus alvei*	X57304.1	99.14%
6 II B3 (gram-positive)	OR506109	*Bacillus pantothenticus*	D78477.1	98.30%

The analysis revealed the similarities among the eleven bacteria samples with related species with 98.8-100%. This similarity value indicated that these samples have been identified at the species level. Subsequently, the BLAST determines the highest degree of relationship of the research samples (Johnson et al. 2019; Janda and Abbot 2007). This indicated similarities between the eleven bacteria samples and related species, with similarities ranging from 98.8 to 100%. This high level of similarity suggested that the samples have been accurately identified at the species level, which included the *Pseudomonas guariconensis, Bacillus mycoides, Bacillus pantothenticus, Bacillus sphaericus, Bacillus mycoides, Achromobacter xylosoxidans, Bacillus sphaericus, Bacillus firmus, Bacillus stearothermophilus, Bacillus alvei.* In this investigation, two samples were identified as belonging to the same species based on their 16S rRNA gene sequences. Both samples (OR506093 and OR506109) were determined to be *Bacillus pantothenticus*. In another study, Titilawo et al. (2020) demonstrated that BLAST analysis revealed ≥99% homology of six isolates with reference PGP strains of *Bacillus, Escherichia, Citrobacter, Serratia, Exiguobacterium,* and *Microbacterium,* while two strains showed 94 and 91% homology with Proteus. The almost complete 16S rRNA gene sequence comparison has been widely used to establish taxonomic relationships between prokaryotic strains, and the 98.65% similarity is currently recognized as the cut-off for delineating species (Janda and Abbott 2007; Johnson et al. 2019). Therefore, using low-throughput methods, 16S sequences have been used to differentiate strains, often referred to as subspecies, by analyzing variations in the gene. Single-nucleotide polymorphisms (SNPs) were used to identify clinically relevant strains or, when consistently associated with other segments of the bacterial haplotype, to anticipate phenotypic traits (Johnson et al. 2019). In another study by Sadiqi et al. (2022), the identified species had the potential to produce antimicrobial compounds that could be used for the 16S rRNA gene. The consistency in species identification and the high similarity values obtained through the nucleotide base composition analysis had the accuracy and reliability of the identification process. The BLAST tools facilitated the precise identification of the bacterial species present in the samples, which provided valuable insights into the microbial composition of the former coal mining land in East Kalimantan. Understanding the specific bacterial species in these environments was crucial for optimizing reclamation strategies and promoting ecosystem restoration efforts.

Composition of nucleotide bases

Based on the results of the eleven samples, the average G+C nucleotide composition was 43.12%, while the average A+T nucleotide base composition was 56.87%. These results indicated that the nucleotide base composition of G+C was low compared to the nucleotide base composition of A+T (Table 2).

The nucleotide base composition of DNA sequences of the bacteria in optimizing former coal mining land in East Kalimantan as a planting media obtained nucleotide base composition values that did not differ between the three samples. This was because the composition of the nucleotide bases of the study samples was still in the same species. The average nucleotide base composition of the sample obtained the percentage value of G+C nucleotide bases to be lower than the A+T nucleotide base composition (Table 2).

Table 2. Composition of nucleotide bases of bacteria in optimizing former coal mining land in East Kalimantan as a planting media

Sample name	A (%)	C (%)	T (%)	G (%)
8 III B4 (gram-positive)	27	24.02	27	21.98
7 III (B3) gram-positive	26.2	21.28	31.98	20.54
4 III E3 (gram-positive)	25.72	22.53	31.24	20.51
I D3 (gram-positive)	26.05	22.15	31.41	20.39
IE 5 (gram-positive)	26.2	21.28	31.98	20.54
2 IA3 (gram-positive)	25.7	22.53	31.24	20.53
II A4 (gram-positive)	26.05	22.15	31.41	20.39
3 IB4 (gram-positive)	24.61	25.32	29.73	20.34
5 IB3 (gram-positive)	23.1	24.9	34.06	17.94
1 IA4 (gram-positive)	27.85	21.29	30	20.86
6 II B3 (gram-positive)	25.14	22.51	31.91	20.44
Average	25.78	22.72	31.08	20.40
A+T	56.87			
G+C	43.12			

Note: A: Adenine; C: Cytosine; G: Guanine; T: Thymine

This showed that it followed the consistency of the features of the mitochondrial base composition (Chen 2023). The nucleotide base composition of DNA sequences of bacteria in optimizing former coal mining land in East Kalimantan exhibited consistent values across three samples as the planting media, indicating a similarity in species composition. This composition revealed a lower percentage of G+C nucleotide bases than A+T nucleotide bases, aligning with the features of the bacterial base composition (Clarridge 2004).

This consistency in nucleotide base composition among the samples suggested that the bacteria in the study samples share similarities in their genetic relationship. The similarity in base composition reflected the genetic relatedness of these bacteria at the molecular level. The lower percentage of G+C nucleotide bases compared to A+T nucleotide bases was consistent with the typical base composition observed in bacterial DNA (Table 3). This further supports the notion that the bacterial species in the samples share common genetic characteristics (Clarridge 2004).

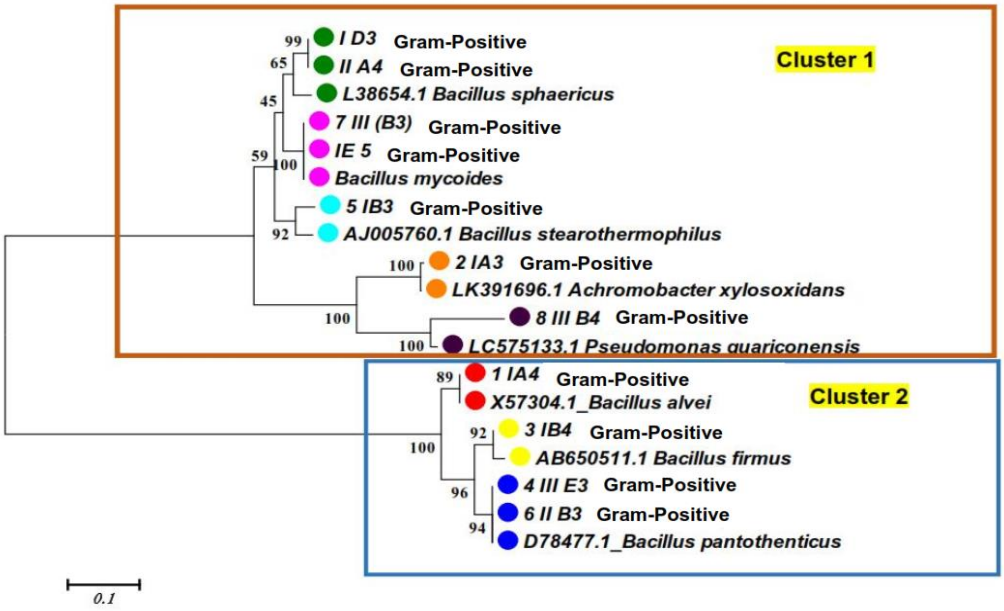


Figure 2. Phylogenetic tree of *bacteria* based on 16S rRNA gene sequences using Neighboor Joining Tree

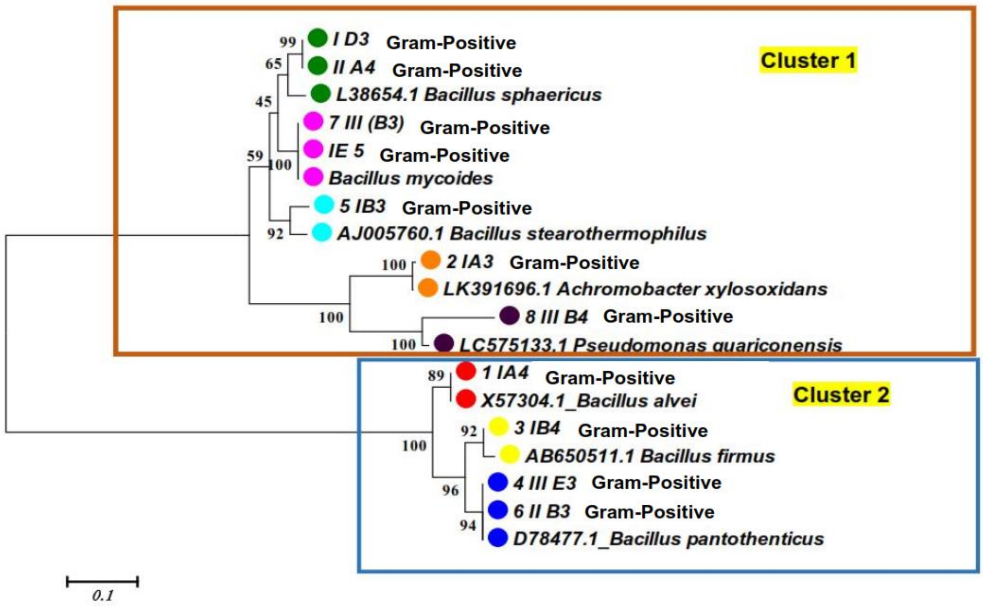


Figure 3. Phylogenetic tree of *bacteria* based on 16S rRNA gene sequences using Neighboor Joining Tree

Table 3. Genetic distance of bacteria in optimizing former coal mining land in East Kalimantan, Indonesia as a planting media with ingroup

1_IA4_(gram_positive)																		
X57304.1_Bacillus_alvei	0.000																	
3_IB4_(gram_positive)	0.095	0.095																
4_III_E3_(gram_positive)	0.100	0.100	0.051															
6_II_B3_(gram_positive)	0.100	0.100	0.051	0.000														
D78477.1_Bacillus_pantothenticus	0.100	0.100	0.051	0.000	0.000													
AB650511.1_Bacillus_firmus	0.110	0.110	0.013	0.065	0.065	0.065												
5_IB3_(gram_positive)	1.052	1.052	1.123	1.110	1.110	1.110	1.142											
AJ005760.1_Bacillus_stearothermophilus	1.069	1.069	1.081	1.078	1.078	1.078	1.100	0.050										
I_D3_(gram_positive)	1.034	1.034	1.097	1.106	1.106	1.106	1.116	0.117	0.123									
II_A4_(gram_positive)	1.034	1.034	1.097	1.106	1.106	1.106	1.116	0.117	0.123	0.000								
L38654.1_Bacillus_sphaericus	1.027	1.027	1.078	1.087	1.087	1.087	1.097	0.112	0.115	0.046	0.046							
7_III_(B3)_gram_positive	1.059	1.059	1.109	1.098	1.098	1.098	1.128	0.094	0.087	0.060	0.060	0.072						
IE_5_(gram_positive)	1.059	1.059	1.109	1.098	1.098	1.098	1.128	0.094	0.087	0.060	0.060	0.072	0.000					
Bacillus_mycoides	1.059	1.059	1.109	1.098	1.098	1.098	1.128	0.094	0.087	0.060	0.060	0.072	0.000	0.000				
2_IA3_(gram_positive)	1.210	1.210	1.232	1.255	1.255	1.255	1.243	0.314	0.317	0.317	0.317	0.307	0.307	0.307	0.307			
LK391696.1_Achromobacter_xylosoxidans	1.209	1.209	1.210	1.232	1.232	1.232	1.221	0.311	0.314	0.313	0.313	0.303	0.304	0.304	0.304	0.004		
8_III_B4_(gram_positive)	1.348	1.348	1.385	1.387	1.387	1.387	1.414	0.461	0.444	0.407	0.407	0.411	0.412	0.412	0.412	0.295	0.295	
LC575133.1_Pseudomonas_guariconensis	1.243	1.243	1.305	1.291	1.291	1.291	1.330	0.359	0.339	0.318	0.318	0.325	0.319	0.319	0.319	0.206	0.204	0.112

Note: **bold** are samples in this research

Phylogenetic tree

Figures 2 and 3 shows the Phylogenetic tree of the bacteria calculation model (1,000 bootstrap iterations). The resulting cladogram was divided into 2 major clusters, further splitting into 2 sub-clusters. Cluster 1 comprised the *Bacillus* and Clade 2, consisting of the *Achromobacter* and *Pseudomonas* genera. Each type in these clades was further categorized based on the species, with a bootstrap value of 100%. Meanwhile, the second cluster was divided into the *Bacillus alvei* and *B. pantothenticus* clades. The Neighbor-Joining and Maximum-Likelihood methods demonstrated consistent relatedness between bacteria and the relatives, differing only in bootstrap values. According to Madduppa et al. (2017) and Hesterberg (2015), a bootstrap percentage of over 80% in branching indicated robust results, strongly supporting that samples in the same branch were indeed of the same species. The few instances in which 16S rRNA gene sequencing was not beneficial were typically associated with multiple well-known species with identical or strikingly similar sequences. One of the key benefits of molecular identification was the capacity to precisely and specifically identify bacterial species. Using the 16S rRNA sequencing method differentiated microorganisms based on distinct genetic characteristics (Iskandar et al. 2021). The examination of 16S rRNA at the molecular level could be used for identification purposes, particularly to pinpoint the bacterial rRNA gene investigated at the species level (Jenkins et al. 2012).

Phylogeny investigated the evolutionary relationships among organism groups, providing a hypothesis about the evolutionary history and their connections (Kanojia et al. 2019; Mittal et al. 2013). A phylogenetic tree can estimate the relationships between taxa (or sequences) and their hypothetical common ancestors (Hall 2013). Bootstrap analysis was employed to analyze cluster homogeneity, offering a framework for validating the arrangement of branches in a phylogenetic tree (Studer 2021). The bootstrap method was crucial for determining the number of repetitions in sequence alignment, enhancing the accuracy of identifying species relationships in a phylogenetic tree (Hikam et al. 2021). The widely used Neighbor-Joining method constructed phylogenetic trees based on the genetic distance between the species (Hong et al. 2021). The Neighbor-Joining algorithm formed the basis for phylogenetic reconstruction in various bioinformatic pipelines (Clausen 2022). Additionally, the Neighbor-Joining method has been used in studies such as the reconstruction of phylogenetic trees based on CO1 partial gene sequences (Jeon et al. 2022) and in the construction of phylogenetic trees in various research contexts (Brihuega et al. 2021). This method was preferred due to the minimal assumptions, rapid operations, and high accuracy, relying on the distance between taxa (Hong et al. 2021). Therefore, by the Neighbor-Joining method (Figure 3) the phylogenetic tree results revealed that the study sample, distinct 2 clusters based on the genus, exhibited a bootstrap value of 100. Bootstrap values ≥70 were considered well-supported and reliable, usually corresponding to a highly probable "real" clade (Esfahani et al. 2016; Yang et al. 2017).

Maximum-Likelihood method observed sequence changes or mutations resulting from sequence variations. This method maximized the probability of data fitting onto a tree under a given evolutionary model, necessitating explicit assumptions about sequence evolution, such as mutation rates (Bawono and Heringa 2014). The phylogenetic tree results using Maximum-Likelihood (Figures 2 and 3) indicated that the study sample bacteria from East Kalimantan was in the same clade with related species, with a bootstrap value of 59 and 100. Therefore, the 16S rRNA gene sequencing was valuable in identifying isolated bacteria in optimizing former coal mining land. The results of this study were crucial as a foundation for developing an optimization model for former coal mining areas as planting media, using soil microorganisms that play a role in shaping the dynamics of soil nutrients. This study demonstrated that conducting 16S rRNA PCR assays could significantly aid in determining coal mining bacteria by identifying the most suitable bacteria. The results suggested that the assay's sensitivity depended on the amplified 16S rRNA gene fragment size. This study aimed to advance the field by exploring novel microbial strains or bioremediation techniques, improving our understanding of microbial community dynamics in contaminated environments, or integrating bioremediation with other remediation strategies for enhanced effectiveness.

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