The potential of bacterial key species as a tool for monitoring peatland quality

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Abstract. Bacterial key species (BKS) is unique and found only in peat secondary forest, but not in converted peat areas. Its presence helps in biomonitoring of peatland quality. BKS candidates were detected based on the 16S rRNA gene sequence using the Next Generation Sequencing method. The 16S rRNA gene sequencing data were obtained from DNA isolated from peat soil of the secondary forest (SF), acacia plantations (AP), and rubber plantations (RP) in the Giam Siak Kecil – Bukit Batu (GSK-BB) Biosphere Reserve, Riau. The natural vegetation of peat swamp forest dominates the SF, which was relatively heterogeneous with anaerobic conditions and water level at 60-120 cm. The RP locations were planted with 6-7 year old rubber, water level was 20 cm, and the garden was not maintained. The AP locations were planted with A. crassicarpa and peat thickness was 9 m. The peat soil was sampled in August 2019. BKS candidates were selected based on a phylogenetic tree using MEGA 6.06 by observing the grouping of DNA sequences obtained only from secondary forests. Furthermore, the selection was also conducted using BLASTn: Align Two or More Sequence analysis to determine the similarity between selected BKS candidates. Based on the detected BKS candidate, a specific primer was designed to amplify the BKS sequence, and the specificity was tested in silico with FastPCR to detect that the primer was only for the amplification of the BKS target. Two BKS candidates with the same sequence length of 455 bp were discovered in the secondary forests and there were successfully amplified by 2 pairs of specific primers. The 16S rRNA gene sequences of the two BKS candidates could be used to monitor the peat quality that has been converted into plantation areas molecularly.

1. Introduction
Indonesia has tropical peatlands scattered on the islands of Sumatra, Borneo, and Papua, partially at ±3.86 million ha are located in Riau province [1]. The peatlands have degraded significantly due to deforestation, fires, and land conversion [2,3]. Peatland of Riau province has mostly been converted into wood pulp plantation, rubber plantation, and agricultural fields [4]. This conversion resulted in significant changes in the ecosystem and the quality of peat soil. Generally, peatland quality is monitored through the physical, chemical, and biological characteristics of the soil. Biological characteristics reflect the response of soil microbes on the environmental changes, hence it is potential for soil quality indicator. The molecular approach, based on the 16S rRNA gene marker sequence, has been widely used in all aspects of the biological sciences, one of which is the identification of bacteria.
The application of DNA sequences indicator has also proven as a reliable bacterial genetic marker because it does not change over time [5].

DNA sequences were proposed as markers for each species to enable new species identification [6] and also to differentiate them based on the sequence of nucleotide bases. The unique DNA sequence region is used as an optimal taxonomic characteristic [7] which is relied upon the tracking and identification of soil bacteria. Soil bacteria play an important role in the ecosystem, such as support biogeochemical cycles and stimulating plant growth [8]. These bacteria are known as bacterial key species (BKS). BKS is a key species to determine the relationship between environmental changes and microbial communities. According to Mouquet et al. [9], BKS is very sensitive to disturbances that occur in the ecosystem, thus the presence of BKS can be used as an indicator to detect disturbances exposing the ecosystem.

Identification of BKS requires specific primers in the amplification process to duplicate the desired DNA sequence. Specific primers are quite important in the determination of biodiversity or unknown diversity of environmental samples. The concept of key species, which is a determinant in the characterization of bacterial communities in ecosystems, is not only discovered abundant bacterial communities, but is determined based on its role, structure, and function [9]. For example, Desulfosporosinus spp. plays an important role in supporting the sustainability of sulfur and carbon cycles, but its abundance is relatively low at around 0.06% of the total bacterial community in the ecosystem [10]. According to Xu et al. [11], a bioinformatics approach in revealing the composition of soil bacterial communities using the 16S rRNA gene sequence can be used to determine BKS. In silico DNA sequence analysis is the solution for determining BKS. The determined BKS can then be used as a peat quality monitoring tool. Based on this, it is necessary to search for BKS candidates that are only present in secondary forests but do not exist in acacia and rubber plantations by using the “in silico” method.

2. Methods

2.1. Materials

The material used in this study was 16S rRNA gene sequence data from the results of sequencing using the Illumina HiSeq 2500 PE250 platform. The universal primers employed were 338F (5'–ACTCCTACGGGAGGCAGCA-3') and 806R (5'GGAC-TACHVGGGTWTCTAAT-3') which amplify the V3-V4 region. The data obtained from the DNA sequencing analysis are in the form of FastQ. FastQ files are used to store the results of DNA sequencing analysis. The 16S rRNA gene sequencing data were obtained from DNA isolated from peat soil of the secondary forest (SF), acacia plantations (AP), and rubber plantations (RP) in the Giam Siak Kecil Biosphere Reserve (GSK–BB), Riau. The GSK–BB Biosphere Reserve is a massive peatland crossing over Bengkalis and Siak Regencies, covering 705,271 hectares and divided into core zones, transition zones, and buffer zones. The natural vegetation of peat swamp forest dominates the SF location, which is relatively heterogeneous and has anaerobic conditions. Palaquium sp., Shorea sp., Camnosperma sp., and Cinnamomum sp. are among typical plants. The SF is located in the core zone at N 01°26'13.68", E 101°51'11.1 and has a water level of 60-120 cm. The RP locations are planted with 6-7 year old rubber that is located at coordinates N 01°38'07", E 101°44'02.7" and occupies a transition zone. The water level is 20 cm, and the garden was not maintained. The AP locations are located at N 01°29'43.3", E 101°47'47.0", and are also in a transition zone. Peat thickness was 9 m, and the area was planted with A. crassicarpa.

2.2. Bacterial key species detection

Raw data was processed into Fasta format using the Galaxy program from the website of http://usegalaxy.eu/. DNA sequences in Fasta format were analyzed and aligned for the construction of phylogenetic trees using MEGA version 6.0 with the Neighbor-Joining Tree method [12]. A total of 2 phylogenetic trees were constructed with sequences from the secondary forest - acacia plantation and
the secondary forest - rubber plantation. BKS candidates were detected using BLASTn: Align Two or More Sequence analysis which can be accessed on the website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). BKS was selected based on DNA sequences that were only present in secondary forests but were not found in acacia or rubber plantations. The analysis aims to eliminate and group BKS candidates based on 97-100% similarities in DNA sequences. Therefore, the confirmed BKS candidates were aligned and edited using the MEGA version 6.0 to obtain a consensus sequence.

2.3. Primer design
In silico primer design aims to obtain specific primers using the FastPCR 6.7 application to obtain specific primers for the amplification of the targeted DNA. DNA sequences of the BKS candidate consensus were used as templates for primer design. The primary design work step begins with entering the consensus sequence on the FastPCR page and running the data to produce the best primer candidate, and this is saved by clicking the "File" menu then "Save as" and "OK" [13].

2.4. Primer specificity test
The specificity test aims to find primers that only amplify BKS candidate sequences using in silico PCR, and this was conducted through the help of the FastPCR. The FastPCR main screen was selected “in silico PCR”, then sequences from the acacia and rubber plantations were moved in the “General Sequence(s)” column. The BKS primer candidates which have been designed were tested one after the other in the “Additional sequence(s) or pre-designed primers (probes) list” column, preceding to “Start” the main process. In addition, the results were displayed in the "in silico PCR result" column, and it showed the specific primers were only attached to BKS candidate sequences (targets) and not to sequences from the secondary forest and plantation areas (not targets). This is indicated by a negative and positive signs, which shows the absence and presence of an amplicon formed in the column, respectively [13].

3. Results and discussion
3.1. Total sequencing 16s rRNA information
The number of 16S rRNA gene sequences obtained from sequencing by the NGS method is presented in Table 1. The sequences were derived from DNA isolated from soils in the secondary forests, acacia, and rubber plantations. The highest number of sequences was from the secondary forest, while acacia plantations and rubber plantations had relatively the same number of sequences.

Table 1. Total 16S rRNA gene sequences from each location.

| Location           | Total sequences | Average base length (bp) |
|--------------------|----------------|--------------------------|
| Secondary forest   | 2013           | 406                      |
| Acacia plantation  | 2009           | 428                      |
| Rubber plantation  | 2008           | 425                      |

Total sequences obtained between locations were relatively similar. The number of sequences found at each location was related to the analysis results obtained from the NGS sequencing. These number sequences had not yet been identified and matched based on the Operational Taxonomic Unit (OTU) in the GenBank database. After being matched and identified with the database, the number of OTUs in the secondary forest, acacia, and rubber plantations were 1104, 1092, 1230 OTUs, respectively. The number of OTUs indicates the level of bacterial diversity. The sequences obtained from the secondary forest, acacia, and rubber plantation locations had an average base length of 406 bp, 428 bp, and 425 bp. The large number of sequences obtained from the secondary forests is associated with relatively heterogeneous vegetation with anaerobic conditions, acidic pH, and low air
humidity. The results of this study are in line with what was found by Lin et al. [14] that the heterogeneous peat secondary forest environment supports soil bacterial diversity. The relatively diverse and aged secondary forest vegetation produces a variety of root exudates and nutrients released in the soil. The nutrients support the growth of soil bacteria [15].

Compared to the secondary forest, the area of acacia and rubber plantations with homogeneous vegetation leads to a low number of sequences. The relative monoculture vegetation in both plantations produces similar composition and amount of root exudates, which results in low bacterial diversity and an increase in the population of certain bacteria. Vegetation is homogeneous with aerobic conditions, pH tends to be neutral, and the decomposition process of organic matter increases, causing some biodiversity to decrease [16,17]. That soil bacteria significantly decreased during land conversion due to decreasing nutrient content in the form of C, N, and P in the soil. This condition promotes the loss or elimination of soil microbes unable to adapt to a changing environment [18].

3.2. BKS candidate

The selection of BKS candidates was by constructing two phylogenetic trees using the Neighbor-Joining Tree method. The first phylogenetic tree consisted of the sequences from the secondary forest-acacia plantations (4022 sequences) and the others were a combination of sequences in the secondary forest-rubber plantations (4021 sequences). The construction of a phylogenetic tree was carried out for helping the detection of BKS candidates that only grouped in sequence clusters originating from the secondary forests. Based on the selection of the two phylogenetic trees, 72 and 97 BKS candidates were found. The BKS existed only in the secondary forests but did not exist in acacia and rubber plantations (Table 2).

| Phylogenetic tree | Total BKS candidates | BKS candidate group | Total sequences of BLAST analysis results | Base size (bp) |
|-------------------|----------------------|---------------------|------------------------------------------|---------------|
| A                 | 72                   | BKS-SA₁             | 5                                        | 458           |
|                   |                      | BKS-SA₂             | 4                                        | 458           |
|                   |                      | BKS-SA₃             | 3                                        | 455           |
|                   |                      | BKS-SA₄             | 3                                        | 453           |
|                   |                      | BKS-SA₅             | 8                                        | 455           |
| B                 | 97                   | BKS-SR₁             | 2                                        | 453           |
|                   |                      | BKS-SR₂             | 3                                        | 455           |
|                   |                      | BKS-SR₃             | 2                                        | 453           |
|                   |                      | BKS-SR₄             | 2                                        | 453           |
|                   |                      | BKS-SR₅             | 13                                       | 453           |
|                   |                      | BKS-SR₆             | 3                                        | 455           |
|                   |                      | BKS-SR₇             | 2                                        | 458           |
|                   |                      | BKS-SR₈             | 2                                        | 458           |
|                   |                      | BKS-SR₉             | 2                                        | 435           |

Description: BKS = bacterial key species; SA = secondary forest and acacia plantations; SR = secondary forest and rubber plantations; 1…n indicates the order of the BKS groups.

The new phylogenetic tree consisted of the sequences of BKS candidates and a random sequence taken from plantation areas as outgroups was built (unpublished data). Based on the results, the BKS
candidate sequences form a separate cluster and were not closely related to the sequences originating from the plantation area. The detected BKS candidates were then grouped and eliminated based on similarity in the range of 97-100% using BLASTn analysis by observing the closest similarity relationship between the selected BKS candidate sequences. The BLASTn analysis between BKS candidate sequences from the secondary forest - acacia plantations (BKS-SA) resulted in five groups and nine groups from the secondary forest - rubber plantations (BKS-SR) (Table 2).

Based on Hagstrom et al. [19], bacteria were grouped as the same species if the similarity of 16S rRNA gene sequence was in the range of 97-100%, while sequences with the similarity of 93-97% indicated that the bacteria belonged to the same genus. The sequences of each BKS group were then aligned and edited to determine the consensus sequence of the BKS candidates. A total of five consensus sequences from BKS-SA and nine consensus sequences from BKS-SR were obtained. Each BKS group produces one BKS candidate consensus sequence. The consensus sequence is used as a template for the primer design. BKS candidate consensus sequences can be used as DNA barcodes. Bacterial identification generally employs the 16S rRNA gene which includes a hypervariable conservation region [20]. The hypervariable area is a conservative area used for metagenomic studies of microbial communities in assessing biodiversity and species identification. Metagenomic studies improve quantitative data on the acquisition of the total composition of cultured and non-culturable soil microbes [21].

3.3. Primer and primary specificity

The detected BKS candidate consensus sequence was used as a template to design a specific primer. The primer is expected to amplify the DNA of the BKS candidate inimitably. There were 50 designed primers from the BKS-SA candidate sequences template and 90 primers from the BKS-SR candidate sequences template. For each BKS candidate template, ten pairs of primers were obtained with a length of about 20-25 bp. The primer length has met the ideal primer standard at about 18-30 bp [22]. The melting point temperature obtained from each primer is around 54.2-57.0°C and the GC content is about 55%. The primer should have an optimal melting temperature of around 52-58°C and a GC content of 40-60% [22]. The specificity primers designed were tested by employing sequences from the secondary forest and plantation areas. Specific primers are defined as primers that only amplify sequences originating from the secondary forest as targets. A total of 2 BKS candidates namely BKS-SA3 and BKS-SR2 candidates were successfully amplified by each of 2 specific primers (Table 3).

Based on the primer specificity test, BKS-SA3 and BKS-SR2 candidates are being amplified by specific primers. The primer specificity test aims to detect primers that can only amplify BKS candidate sequences. A study in determining specific primers was also conducted by Humbert et al. [23] to identify anaerobic bacteria originated from wetlands. The specific primers that were successfully designed to detect these bacteria were Amx694f/Amx960r and A438f/A684r. A similar study by Thijs et al. [24] successfully detected contaminated and uncontaminated soil bacteria by using the specific primer designed (341f/785r). Sequences of confirmed BKS candidates are presented in Table 4.
Table 3. Specificity of primer designed.

| BKS candidate | Primary name | Target sequence$^1$ | Sequence, not target$^2$ | Description |
|---------------|--------------|---------------------|---------------------------|-------------|
| BKS-SA3       | BKS-SA3-P1   | +                   | +                         | Not Specific|
|               | BKS-SA3-P2   | +                   | +                         | Not Specific|
|               | BKS-SA3-P3   | +                   | +                         | Not Specific|
|               | BKS-SA3-P4   | +                   | +                         | Not Specific|
|               | BKS-SA3-P5   | +                   | +                         | Not Specific|
|               | BKS-SA3-P6   | +                   | +                         | Not Specific|
|               | BKS-SA3-P7   | +                   | +                         | Not Specific|
|               | BKS-SA3-P8   | +                   | -                         | Specific    |
|               | BKS-SA3-P9   | +                   | -                         | Specific    |
|               | BKS-SA3P10   | +                   | +                         | Not Specific|
| BKS-SR2       | BKS-SR2-P1   | +                   | +                         | Not Specific|
|               | BKS-SR2-P2   | +                   | +                         | Not Specific|
|               | BKS-SR2-P3   | +                   | +                         | Not Specific|
|               | BKS-SR2-P4   | +                   | +                         | Not Specific|
|               | BKS-SR2-P5   | +                   | +                         | Not Specific|
|               | BKS-SR2-P6   | +                   | +                         | Not Specific|
|               | BKS-SR2-P7   | +                   | +                         | Not Specific|
|               | BKS-SR2-P8   | +                   | -                         | Specific    |
|               | BKS-SR2-P9   | +                   | -                         | Specific    |
|               | BKS-SKKS2-P10| +                   | +                         | Not Specific|

$^1$The target sequence is a BKS candidate sequence and the sign (+) indicates that the specific primer only attaches to the BKS candidate sequence.

$^2$The non-target sequence is from the plantation areas and the sign (-) indicates the primer is not attached to the sequence from the plantation areas.
Table 4. Sequence data of confirmed BKS candidates.

| BKS candidate | BKS candidate sequence                                                                 | Base size (bp) |
|---------------|----------------------------------------------------------------------------------------|----------------|
| BKS-SA3       | GCCAATCTATGGGATGCCAGCTGGGGAATCTTGGAGCAATGCGGTAATATCGCRTGTGAGTGAAGAAGGGCAATGCTTTCG  | 455            |
|               | GGGCGAAGGCCCGGTAAAGACGGAGGGGGGGGAAGGCTTTTGGTCGAGTGCGAGTGGGCGAAAGCCCGATCCAGCAATATCGCGTGAGTGAAGAAGGGCAATGCCGCTTGTAAAGCTCTTTCGTCGAGTGCGCGATCATGACATGACTCGAGGAAGAAGCCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAAAGACGGGGGGGGCAAGTGTTCTTCGGAATGACTGGGCGTAAAGGGCACGTAGGCGGTGAATCGGGTTGAAAGTGAAAATCGCCAAAACTGGCGGAATGCTCTCGAAACCAATTCACCTTGAAGATGACAGAGGAGAGTGGAATTTCGTGAGGAGGGGTGAAATCCAAAGATCTACGAAGGAACGC| 455 |
| BKS-SR2       | GCCAATCTATGGGCGCAGCTGGGGAATCTTGGAGCAATGCGGTAATATCGCRTGTGAGTGAAGAAGGGCAATGCTTTCG  | 455            |
|               | GGGCGAAGGCCCGGTAAAGACGGAGGGGGGGGAAGGCTTTTGGTCGAGTGCGAGTGGGCGAAAGCCCGATCCAGCAATATCGCGTGAGTGAAGAAGGGCAATGCCGCTTGTAAAGCTCTTTCGTCGAGTGCGCGATCATGACATGACTCGAGGAAGAAGCCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAAAGACGGGGGGGGCAAGTGTTCTTCGGAATGACTGGGCGTAAAGGGCACGTAGGCGGTGAATCGGGTTGAAAGTGAAAATCGCCAAAACTGGCGGAATGCTCTCGAAACCAATTCACCTTGAAGATGACAGAGGAGAGTGGAATTTCGTGAGGAGGGGTGAAATCCAAAGATCTACGAAGGAACGC| 455 |

4. Conclusion
Two BKS candidate sequences were obtained from the secondary forest and from converted land into acacia and rubber plantation areas. The two BKS candidates have base lengths of 455 bp: BKS-SA3 and BKS-SR2. The BKS candidates were successfully amplified using specific primers designed with the FastPCR program. BKS-SA3 candidate can be amplified using primers BKS-SA3-P8 and BKS-SA3-P9 and BKS-SR2 can be amplified using primers BKS-SR2-P8 and BKS-SR2-P9. The 16S rRNA gene sequences of the two BKS candidates are DNA barcodes to be used for monitoring the quality of peat soils being converted into plantation molecularly.

Acknowledgment
Acknowledgment is addressed to the Directorate of Research and Community Service (DRPM) of the Ministry of Education, Culture, Research, and Technology for funding this research through the PDUPT scheme for the 2021 Fiscal Year. The authors also thank the Microbiology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Riau University for the permission to use its facilities.

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