Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of nitrification bacteria in tropical rain forest and oil palm plantation

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Abstract. Denaturing gradient gel electrophoresis (DGGE) is a method used for structure community analysis of microbes without requires a process of cultivation. The aim of this research was to determine soil microbes diversity with its role in nitrification in tropical rain forest and oil palm plantation using DGGE. This research was conducted on June to February 2017. DNA samples from tropical rain forest soil and oil palm plantation was extracted. Isolation of DNA samples used FastDNATM SPIN Kit for soil (Bio 101, Inc., Vista, CA, USA). The DNA sample was amplified using a primer set of genes that encode amoA and profiled using Denaturing Gradient Gel Electrophoresis (DGGE) DCode Mutation Detection System (Biorad, Hercules, CA, USA). Statistical analysis of DGGE results was carried out by using index Shannon-Wiener diversity (He'), Simpson index and Domination index to determine the similarity pattern of band between samples. Quantification data was analysis used PAST3 software. DGGE DNA bands was analyzed by using software CLIQS 1D. The results showed that DGGE technique could be used to determine diversity of soil microbes by using gene amoA from tropical rain forest and oil palm plantation. The PCR-DGGE method provided insights regarding the structures of nitrifying bacterial community from tropical rain forest and oil palm plantation.

1. Introduction
Denaturing gradient gel electrophoresis (DGGE) is a molecular technique used to separate DNA fragments from PCR products that have the same size of base pair but different sequence arrangement. The polymerase chain reaction of environmental DNA can generate templates of differing DNA sequence that represent many of the dominant microbial organisms. It is separated by acrylamide gel with a gradient denaturant from low to high [1]. DGGE usually encompasses a four-step process: (1) DNA or RNA extraction, (2) amplification, (3) separation and visualization, and (4) sequence identification. The amplification step uses polymerase chain reaction (PCR) to generate a multitude of copies of a variable region within a target gene. The DNA sequence of this variable region is different for each type of bacteria. Thus, the PCR step generates a mixture of the gene segments each
representing a species present in the original sample. The third step of DGGE uses an electric current (electrophoresis) and a denaturing process to separate this mixture based on the DNA sequence, producing a profile, or fingerprint, of the microbial community [1].

Alteration in the use of land into oil palm plantations can change the domination and activity of soil bacteria. Alteration in soil microbial communities can directly affect soil ecosystem function, particularly on carbon and nitrogen cycles. Nitrogen can be a limiting nutrient and the availability of nitrogen in the soil environment becomes a major factor in controlling the production of biomass. This research aimed to study the abundance and diversity of nitrifying bacteria community based functional genes amoA in tropical rainforest at Taman Nasional Bukit Duabelas (TNBD) and oil palm plantations in Sarolangun Jambi.

Nitrification is the process of oxidation ammonia to be hidroksilamine (NH₂OH), oxidation hydroxylamine to be nitrite, and oxidation nitrite to be nitrate with side product NO gas and N₂O [2, 3, 4]. Stages reaction in this process involve different enzymes, namely ammonia monoxygenases (AMO) that converts ammonia to be hydroxylamine, hydroxylamine oxidoreductase (HAO), which change hydroxylamine to be nitrite, and nitrite oxidoreductase (NO) which converts nitrite to be nitrate [5, 6].

The use of gene amoA as marker (marker) can revealed changes of composition community oxidizing NH₃⁺ bacteria as response to environment changes such as concentration of NH₃⁺ [3, 7] and pH [8]. Primary AmoA-1F and AmoA-2R has many used for finding the diversity NH₃ oxidizing bacteria. Research from Francis et al. [9] showed that with the primary could revealed diversity NH₃ oxidizing bacteria in the Chesapeake Bay, North America and it was known that NH₃ oxidizing bacteria dominated by group bacteria Nitrosospira and Nitrosomonas. Results of Chu et al. [10] using the same primer report that diversity NH₃ oxidizing bacteria in the land agriculture with application fertilization and addition organic dominated by group bacteria Nitrosospira.

Soil sampling was performed randomly at three points representing each area of tropical rainforests and 7 to 8 year old oil palm plantations. Soil samples were collected using a soil sample core from 0 to 15 cm below the surface with the depth strata of 0 to 5 cm, 5 to 10 cm and 10 to 15 cm. Composite was conducted on samples from each point corresponding to the depth strata. Soil samples were stored at -20°C prior testing. Diversity of bacteria was analyzed by PCR-DGGE method.

2. Methods

2.1. Site description and sampling.
Samples were taken from the tropical rain forest Bukit Dua Belas National Park (TC, TD) and oil palm plantation (SA, SB) in Sarolangun District, Jambi Province, Sumatera, Indonesia. All spatial geographical coordinates and altitudes were recorded using GPS (eTrex Venture, Garmin, Lenexa, KS, USA). Soil samples were collected using corers (10cm diameter) in October 2015. At each site, a total of 24 soil samples at a 0–5cm, 5-10cm and 10-15cm depth was collected. The samples were transported to the laboratory immediately after collection and stored at 4 °C for soil physicochemical analysis and at −20 °C for total genomic DNA extraction [11].

2.2. Determination of physicochemical parameters of the soil sample
Physicochemical parameters of the soil sample were determined according to the instructions, International Soil Reference and Information Centre (ISRIC) standards [12].

2.3. DNA extraction, purification, and quantification.
Total genomic DNA was extracted from the soil samples using a Fast DNA SPIN Kit for soil (Bio 101, Inc. Vista, CA, USA) by following the manufacturer's instructions. In this procedure, cell lyses was performed by vigorous shaking in a mini-beadbeater (Biospec product, Wakenyaku, Co., Tokyo, Japan) with intense speed of 4.8 for 30 sec. DNA extracts were stored at -20 °C before used as a template for subsequent PCR reaction. DNA quality was assessed using the values of 260 nm/280 nm
and 260 nm/230 nm ratios, and final DNA concentrations were quantified by Nano drop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

2.4. Amplification of amoA gene
The first PCR was carried out with the forward primer AmoA-1F (5′-GGG GTT TCT ACT GGT GGT-3′) AmoA-1F GC clamp (5′-CCG CCG CGC GGC GGG CCG GGC AGC GGG GGG GTT TCT ACT GGT GGT-3′) and AmoA-2R (5′-CCC CTC KGS AAA GCC TTC TTC-3′) [10, 13], generating a product of 490 bp. The 30 µL reaction mix contained: 1 µL of template DNA (50–100 ng), 5 µL of 10xPCR buffer (100mM Tris–HCl, pH 9; 500 mM KCl), 1.5mM MgCl2, 0.5mM of each primer, 200 mM of each dNTPs and 2.5 U of Taq DNA polymerase. The amplification conditions used were: 30 cycles consisting of denaturation at 94 °C for 5 min, annealing for 1min at 58 °C, primer extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min. Negative controls were run in all amplifications. PCR products were run in 1.5% agarose gel electrophoresis followed by staining with ethidium bromide for visualization to confirm their sizes.

2.5. Denaturing Gradient Gel Electrophoresis (DGGE)
DGGE was performed using Bio-Rad DCode system (Bio-Rad, Hercules, CA, USA) in 8% (w/v) polyacrylamide gel (acrylamide-bisacrylamide, 37.5:1) with 30% to 70% denaturing concentrations for nifH gene DGGE analysis (100% denaturant corresponding to 7M urea and 40% deionized formamide). Electrophoresis was performed at 150 V and 60°C for 6 hour in 1X Tris-acetate EDTA (TAE). The gel was stained for 15 min with EtBr gel stain (Molecular Probes, Invitrogen, Cergy Pontoise, France). Gel was rinsed with 500 mL of TAE buffer and scanned by G:BOX gel documentation (Syngene, Frederick, MD, USA). Band profile image was analyzed using CLIQS 1D software (Total Lab) to estimate the total bands that appeared on polyacrylamide gel. Single appeared band was excised using sterile scalpel and put into micro tube containing 100 µL ddH2O. The micro tube was incubated at 4 °C overnight and 60 °C for 2 h [14, 15]. Ten µL (~50 ng µL⁻¹) of template was used for re-PCR using primer without GC-clamp. The condition of re-PCR was the same as the previous PCR condition.

Statistical analysis of DGGE profile was conducted by using alpha diversity (Shannon-Wiener/He) to estimate the diversity within each sample and beta diversity (Dice similarity coefficient/SD) to estimate the similarity of band pattern between the samples. The quantification of statistical analysis was conducted using PAST3 Software based on the estimation analysis of band intensity using CLIQS 1D software. The index was calculated by following equation:

\[ SD = 2Nc/(Na + Nb) \]

\[ He = \sum_{i=1}^{s} Pi \ln Pi \]

Where, Na represented the number of bands detected in sample a; Nb represented the number of bands detected in sample b; Nc represented the number of bands detected in both samples; s represented the number of species in the sample; Pi represented the proportion of species i in the sample [16].

3. Results and Discussion
3.1. Results
Total genomic DNA obtained was used as a template for amplification of amoA genes. Results of amoA gene amplification-490 bp (Figure 1).
Diversity bacteria nitrification was determined based on DGGE results of product amoA gene amplification. DGGE analysis of gene amoA consisted as many as 14 different bands on different positions (Figure 2). This showed that in the tropical forest and oil palm plantation consisted of community nitrification bacteria with number of 14 diversity. From the DNA bands which succeed cut, there is nothing works amplified repeated allegedly this is because DNA results little elution, impurity, or influence from UV exposure during gel eluting.

Figure 2. DGGE band profiles amplified PCR of amoA genes from tropical forest (TC; TD) and oil palm plantations (SA, SB) (left). DGGE ribbon illustrations using CLIQS 1D software (right)

Clustering analysis using binary data (Figure 3) showed that gene amoA community in soil of SA had close similarity of bacterial community structure with soil of SB. Meanwhile, soil of SA and SB had similar community pattern but their cluster separated with soil sample of TC and TD which indicates that they have different community pattern with soil samples of SA and TC.
Figure 3. Dendogram of amoA gene resemblance levels between oil palm plantations (SA, SB) and tropical rainforests (TC, TD)

Shannon-Wiener, Simpson Index and dominance index for gene amoA showed the pattern of soil bacteria diversity with diversity from low - medium (1-2) (Figure 4). Shannon-Wiener index showed that the highest diversity was in tropical rain forest TC (2.126) and the lowest was in oil palm plantation SB (1.755). Simpson index showed that highest diversity was in Tropical rain forest TC (0.860) and the lowest was in oil palm plantation SB (0.792). The highest dominance index was in oil palm plantation SB (0.207) and the lowest was in tropical rain forest TC (0.139). Thereby, the highest bacterial diversity was in tropical rain forest followed by that of oil palm plantation.

Figure 4. Indexs Shannon-Wiener, Simpson and Dominant diversity of gene amoA DGGE from oil palm plantation (SA, SB) and tropical rain forest (TC, TD)

Abundance ratio of amoA gene indicated the rank of abundance equally for the level of dominance from all samples were below 30%. Dominance of sample SA are 11% - 28%, sample SB are 9% - 20%, sample TC are 4% - 26% and sample TD are 9% - 26% (Figure 5)

If the overall number of dominant percentages is always present in each location, the tropical rainforest soil have higher levels of dominance and abundance than that of the oil palm plantation soil (Table 1).
Figure 5. Rank abundance curve of *amoA* gene from oil palm plantation (SA, SB) and tropical rain forest (TC, TD)

Based on *amoA* genes abundance (Table 1), tropical rain forest (TC) with OTU = 11 showed the highest abundance followed by oil palm plantation with OTU = 10. OTU dominance at each location are number 3, 4, and 8. Percentage dominant OTU on soil of palm plantation are 43-47 % and soil of tropical rain forest are 56-74%

Table 1. Rank-abundance of *amoA* gene based on OTU from oil palm plantation (SA, SB) and tropical rain forest (TC, TD)

| No | Location | Total OUT | Percent of OTU dominance *amoA* gene | OUT in location | Percentages total |
|----|----------|-----------|--------------------------------------|----------------|------------------|
| 1  | SA       | 7         | 4 (28%), 3 (15%), 2 (14%), 5 (12%), 6 (11%) | 4, 3, 8        | 43 %            |
| 2  | SB       | 11        | 8 (20%), 9 (19%), 4 (17%), 3 (10%), 11 (9%) | 4, 3, 8        | 47 %            |
| 3  | TC       | 8         | 8 (26%), 3 (25%), 4 (23%), 7 (6%), 11 (4%) | 4, 3, 8        | 74 %            |
| 4  | TD       | 10        | 4 (26%), 3 (16%), 8 (14%), 11 (10%), 14 (9%) | 4, 3, 8        | 56 %            |

3.2. Discussion
The results of DGGE of *amoA* gene profiles could be used to see a community diversity of soil bacteria in an environment (Ferris et al. 1996). Similarity of DNA bands from DGGE profiles were compared between the four locations (SA, SB, TC, TD). The samples were distributed to two main clusters (Figure 3). SA and TD samples derived from oil palm plantations and tropical rain forests form a single cluster. Similarities indicated an evolutionary relationship, spread, and interaction among the soil bacterial communities.
Diversity index can be determined using DGGE [17]. DGGE DNA bands are translated into the total number of bands (s), bandwidth peak area (ni), and the sum of the entire peak band (N). This information is used to calculate the Shannon, Shimpson, and dominance index [18]. Results of analysis of variance showed that the highest diversity Shannon-Wiener index was located on tropical rain forest TC (2.126) and the lowest from soil oil palm plantation SB (1.755). According to Odum [19] the value of H'<1 is including low diversity and value of 1 <H' <3 is including moderate and H' > 3 high. It was shown that, the Shannon index of four location both in oil palm plantations and tropical rainforests indicated a moderate diversity [19], which means the distribution of the individual species of each species is moderately stable. Differences in soil bacterial diversity of the tropical rain forest and oil palm plantations could be influenced by several factors including environmental factors e.g soil erosion, the addition of fertilizers that can affect the parameters of land, other than that according to Le-Cruz et al. [20]. The addition of fertilizers that can be decrease the competition of nutrients so, it will produce a variety of soil bacteria.

The Shimpson index is used to denote the proportion of individuals within the species [17]. The value of the Shimpson index at this study site ranged from 0.79-0.86, this suggested that the species in the location were not dominated by one species, it might eventually distributed. The dominance index is a measure to determine the degree of mastery of the community species. For dominance index, the highest was seen in plantation palm SB (0.207) and the lowest was seen in Tropical rain forest TC (0.139). The lower dominance index means the location is not dominated by one species.

The abundance ranking provided an overview of the relationship between abundance and equity in each sample community (Fig 3.5). Each DNA bands from DGGE results represent an operational taxonomic unit (OTU) [13]. In general, the abundance rank from 4 locations showed the normal pattern for the bacterial community, characterized by at least OTU high abundance and more OTU low abundance [22].

Competition and dominance in the soil bacterial community is high because of the limited variety of available nutrients limited to certain organic compounds. The growth of nitrifying bacteria was strongly affected by the content of organic compound. Organic compound was one of nitrogen source in the forest resulted from organic material decomposition to form NH4+ which could be used as the initial substrate of nitrification process. On soil plantation, the application of nitrogen fertilizer might increase the presence of NH4+ which could increase the rate of nitrification.

4. Conclusion
PCR-DGGE technique could be used to determine diversity of soil microbes by using gene amoA from tropical rain forest and oil palm plantation. The PCR-DGGE method provided insights regarding the structures of nitrifying bacterial community from tropical rain forest and oil palm plantation. Shannon-Wiener index and DGGE profiles showed the diversity of bacteria in tropical rain forest were higher than that of palm oil plantation, while the diversity of bacteria were not varied among the samples. The dominant bacteria for nitrification based on amoA gene showed a variation, in which the dominat bacteria in palm oil plantations and tropical rain forests were highly varied.

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