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# Nitrogen-fixing bacterial community in sago palm roots in different soil environments of East Malaysia and South Thailand

Koki Asano [1,2,3*], Kanokkorn Sinma [4], Jutamas Romkaew [5],
Siraj Munir Bin Mohammad [6], Margaret Chan Kit Yok [6], Toshiyuki Isoi [7],
Mana Kano-Nakata [3] and Hiroshi Ehara [1,3]

[1] Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan

[2] Program of Agricultural Sciences, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Nakhon Pathom, 73140, Thailand

[3] International Center for Research and Education in Agriculture, Nagoya University, Nagoya 464-8601, Japan

[4] Department of Soil Science, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University Nakhon Pathom, 73140, Thailand

[5] Department of Agronomy, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University Nakhon Pathom, 73140, Thailand

[6] Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA, Cawangan Sarawak, 94300 Samarahan, Sarawak, Malaysia

[7] Faculty of Agriculture, Meijo University, 1-501 Shiogamaguchi, Tempaku-ku, Nagoya 468-8502, Japan

*e-mail: asano.koki.96@gmail.com

**Abstract:** The diversity and taxonomic composition of endophytic bacteria and nitrogen-fixing bacteria (NFB) in sago palm roots were examined from two sites in East Malaysia (clay loam of mineral soil, and shallow peat soil) and five sites in South Thailand (clay loam, sandy clay loam, sandy clay, and clay soils) by the amplicon sequencing of 16S rRNA and *nifH* gene. As a result, Shannon diversity and Simpson's evenness of bacteria and NFB were not different among sampling sites (although the root sample in shallow peat soil had a low value). The soil bulk density, clay content, volumetric water content, pH, EC, exchangeable cation contents, and total N affected both communities of endophytic bacteria and NFB. As a result of phylogenetic analysis of NifH (translated from *nifH* gene amplicon), NifH adjacent to *Bradyrhizobium*, *Burkholderia*, *Cupriavidus*, *Frankia*, *Geobacter*, *Anaeromyxobacter*, *Desulfovibrio*, *Clostridium*, and *Spirochaeta* were highly detected. Surprisingly, NifH close to *Burkholderia xenovorans* was dominant (> 30% relative abundance) in the strong acidity (pH 4.1) of shallow peat soil in Malaysia. The relative abundance of aerobic or facultative anaerobic NFB (*Bradyrhizobium, Burkholderia, Frankia,* and *Cupriavidus* genera) was negatively correlated with the relative abundance of anaerobic NFB (*Clostridium, Geobacter, Anaeromyxobacter, Desulfovibrio,* and *Spirochaeta*). It is suggested that the key players of root endophytic NFB in sago palm roots shifted by the oxygen level in the root interior affected by waterlogging in the soil.

**Keywords:** Biological nitrogen-fixation, *Bradyrhizobium, Burkholderia, nifH* gene, Soil physicochemical properties, Waterlogging

## Introduction

Sago palm (*Metroxylon sagu* Rottb.) is an underutilized starch-producing crop. It accumulates over 200 kg of dry starch per plant in its trunk and is used variously for fresh and processed food or floured for noodle making (Ehara., 2018). It is distributed across Southeast Asia, including Malaysia, Indonesia, the Southern part of Thailand, Brunei, the Southern

part of the Philippines, and northwestern Melanesia, including Papua New Guinea and the Solomon Islands (Bintoro et al., 2018). It is reported that starch resources obtained from this plant were stapled food since around 3000 BC in South China (Yang et al., 2013), and it is still a staple food in East Indonesia and Papua New Guinea. Sarawak, Malaysia, has huge sago palm plantations (Bintoro et al., 2018; Kueh et al., 1991). Although there are no vast sago palm plantations in Thailand, sago palm leaves as roofing materials are commercially utilized (Charungsutjaritkul et al., 2018). Sago palms can grow in various types of soil, acidic, waterlogged, saline, and peaty soil (The society of sago palm, 2015).

Biological nitrogen fixation (BNF) is a process of fixing atmospheric nitrogen ($N_2$) into ammonium ($NH_3$) under average temperature and pressure by bacteria. Utilizing the potential BNF activity is important for getting N sources for plants. A wide range of bacterial genera performs BNF, called nitrogen-fixing bacteria (NFB). BNF is actively conducted in the root nodule for leguminous plants, while for non-leguminous plants, free-living endophytic NFB within the root interior plays a role in BNF. Various species of bacteria can be detected as endophytic NFB in rice, and major species in phylum Proteobacteria division belonging to the alphaproteobacterial (*Azospirillum, Agrobacterium, Gluconacetobacter, Azorhizobium, Bradyrhizobium,* and *Rhizobium*), betaproteobacterial (*Burkholderia* and *Herbaspirillum*), gmaproteobacterial (*Klebsiella, Pantoea* and *Azotobacter*), and deltaproteobacterial (*Anaeromyxobacter*, and *Geobacter*) genera (Muthukumarasamy et al., 2005; Boddey et al., 2003; Govindarajan et al., 2008; Loiret et al., 2004; Kumar et al., 2021; Xing et al., 2006; Subramanian et al., 2015; Wei et al., 2014; Pedraza et al., 2009; Mårtensson et al., 2009; Elbeltagy et al., 2008; Manapure et al., 2021; Yanni et al., 1997). Furthermore, NFB belonging to the phylum Actinobacteria, Firmicutes, and Bacteroidetes have been detected in rice roots (Alishahi et al., 2020; Wang et al., 2017). Within the palm species (Arecaceae genera), Tang, et al., (2010) isolated *Burkholderia vietnamiensis* from the root tissues of nipa palm (*Nypa fruticans*) in Sarawak, Malaysia, and Dobereiner and Baldani (1998) reported that *Herbaspirillum seropedicae* works as an endophytic nitrogen fixer in oil palms. In the case of sago palm, *Klebsiella pnuemoniae, Pantoea agglomerans, Enterobacter cloacae, Burkholderia* sp., and *Bacillus megaterium* were isolated from the roots (Shrestha et al., 2006).

Nitrogen availability in the soil is suggested as an important nutrient to get better growth of sago palms (Kakuda et al., 2000). The recent advanced technology of next-generation sequencers allows us to understand the taxonomic composition of microbial communities. In this study, we tried to reveal the endophytic bacterial and NFB community structure, which are assumed to support the nitrogen acquisition of sago palms.

## Materials and Methods

### *2.1. Sampling sites 1 (East Malaysia)*

#### 2.1.1 Sample collection

The same soil and root samples from sago palms grown in mineral soil (MS) and shallow peat soil (SPS) collected by Asano et al., (2021) were used for this study. In September 2019, 5 individual palms were randomly selected from sago palm fields grown in mineral soil in Kota Samarahan (1º 26.26'N, 110º 27.11'E) and shallow peat soil in Dalat (2º 51.14'N, 111º 49.38'E) (peat layer: 0 to 30 cm). Fine roots at a 1 m distance from the mother palms were collected using a shovel at a soil depth of 0-15 cm. The root samples were then washed in sterile water to remove soil and stored at -20°C for subsequent staining and DNA extraction. For each of the five trees, MS samples were taken at a depth of 15 cm from the soil surface using a shovel, while SPS samples were collected at a depth of 50 cm from the surface using a peat sampler (Eijkelkamp Co., Netherlands).

2.1.2. Soil physicochemical analysis

Soil physical properties (Bulk density and volumetric water content) and chemical properties (pH, C, and N content, and available P content) were analyzed by the method written in Asano et al., (2021).

2.1.3 DNA extraction and amplicon sequencing

Cetyltrimethylammonium bromide (CTAB) and polyvinylpyrrolidone (PVP) method (Porebski et al., 1997) was used for extracting DNA. The five DNA solutions were pooled in equal proportions by concentration before the analysis following the method of Osborn et al., (2018). The pooled DNA was used as a template for amplifying 16S rRNA and *nifH* gene sequences. Primer pairs of 515F/806R (Caporaso et al., 2011) and PolF/PolR (Poly et al., 2001) were attached via the Illumina MiSeq adapter sequences were used, respectively. PCR was performed using GoTaq Green Master Mix (Promega Co., USA) with the thermal cycle as follows: 16S rRNA gene [94°C 3 min; 28 cycles of (94°C 30s, 52°C 40s, 72°C 1 min); and 72°C 5 min], and *nifH* gene [94°C 3 min; 30 cycles of (95°C 1 min, 55°C 1 min, 72°C 1 min); and 72°C 10 min]. The PCR products were electrophoresed on 2% agarose gel and visualized on a FASIV ultraviolet transilluminator (Nippon Genetics Co., Japan); they were then sent for next-generation sequencing to Bioengineering Lab Co. in Japan. PCR products were purified using AMPure XP magnetic beads (Beckman Coulter, CA, USA). Then, $2_{nd}$ PCR was conducted by following the instructions of the manufacturer. PCR mixtures were 2 $\mu$L of PCR products, 1.0 $\mu$L of 10× Ex buffer, 0.8 $\mu$L of dNTPs (each 2.5 mM), 0.5 $\mu$L of 10 $\mu$M forward and reverse primer, Ex Taq HS (TaKaRa Bio Inc., Shiga, Japan), and 5.1 $\mu$L of $ddH_2O$ water. Thermal cycle condition was 94°C 2 min; 12 cycles of (94°C 30 min, 60°C 30s, 72°C 30s); and 72°C 5 min. Illumina libraries were constructed, and pared-end sequenced (2×300 bp) on the Illumina MiSeq platform following the manufacturer's instructions (Illumina Co., USA).

*2.2. Sampling sites 2 (South Thailand)*

2.2.1. Sample collection

Collections of roots and soils in sago palm fields in Thailand were conducted in two upland sites beside the water canal in Trang Province from Nayong and Koksaba district (T1 and T2: 7°30.528'N, 99°43.032'E, and 7°31.805'N, 99°41.875'E) in May (the end of the dry season) 2021, two waterlogging sites near the seashore in Phrom Khiri and Tha Ngio district (N1 and N2: 8°32.337'N, 99°55.888'E, and 8°29.362'N, 99°55.362'E), and one upland site in Tha Sara district (N3: 8°32.465'N, 99°54.933'E) on January 25th, 2022 (the end of wet season). Additionally, we collected soil samples in the oil palm plantation in Na Khian district (N4: 8°26.753'N, 99°56.517'E) in January (the beginning of the dry season) as a reference. Waterlogging was partially seen in the location of N1 and N2. Sago palms (T1, T2, N1, N2, and N3) were cultivated without fertilizer application, while oil palms (N4) were cultivated with a general amount of chemical fertilizers.

2.2.2. Soil physicochemical analysis

Soil texture was determined by Bouyoucos (Hydrometer) method after digesting with $H_2O_2$ for soil samples with high organic matter content (Bouyoucos et al., 1962). Bulk density was determined by measuring weight after oven drying at 105°C for one day, and volumetric water content was calculated from weight loss after oven drying divided by the volume of the cylindrical soil core (100 $cm^3$). The density of soil particles was measured by the liquid-displacement method, and three phases of soil were calculated. Organic matter content and total C content were determined by the Walkley-Black acid digestion method (Walkley and Black, 1934). The pH ($H_2O$) and EC were measured in a suspension (1:2.5 and 1:5) with a pH meter and EC meter (Mettler Toledo, Ohio, US), respectively. Total N (digested with $H_2SO_4$) was measured by the Kjeldahl method. Total P (digested with $H_2SO_4$) and available P (Bray II) were measured by molybdenum blue colorimetry (Bray and Kurtz,

1945). The p absorption coefficient was also determined. 25.0 g of soil was put in a 100 mL flask and soaked with 50 mL of 13.44 g $L^{-1}$ $P_2O_5$ solution for 24 hours in the shaker at 5 to 10 rpm. Then, the P content of the filtrate solution was determined by molybdenum yellow calorimetry (Kitson and Mellon, 1944). Exchangeable cations (K, Na, Mg, and Ca) were measured using atomic absorption spectrophotometry (200 series AA systems, Agilent Technologies, California, United States) after digestion with $HNO_3$. All soil physicochemical properties were calculated based on the unit cubic meter ($m^3$) since soil bulk densities were widely different.

2.2.3. DNA extraction and amplicon sequencing

The CTAB method was used for root DNA extraction. After surface sterilization by soaking with 80% ethanol and sonication for 5 min, 3 to 4 replicates of roots were mixed equally and grounded by mortar and pestle. Ground samples were incubated in a 2 × CTAB buffer [2% CTAB; 1.4 M NaCl; 100 mM Tris HCl pH 8.0; 20 mM ethylenediamine tetraacetic acid (EDTA)] with 20 μL of 2% β-mercaptoethanol at 65°C for 60 min. Separation of DNA and protein by adding one volume of chloroform-isoamyl (24:1), treatment with 15 μL of RNase A (20 mg $mL^{-1}$), precipitation with one volume of isopropanol, washing with ethanol (70%), and dissolution in Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 7.6) was conducted to extract the DNA.

For the 1st PCR procedure, the same primer pairs and thermal cycles were applied, although PCR was performed using MyTaq Red Mix (Meridian Bioscience Inc., Ohio, United States) with the thermal cycles. After the confirmation of the PCR products by electrophoresis on 2% agarose gel and visualization on UV, they were sent to Bioengineering Lab Co., Japan, for amplicon sequencing. PCR products were purified using AMPure XP magnetic beads (Beckman Coulter, CA, USA). Then, 2nd PCR products was conducted following the instructions of the manufacturer. PCR mixtures were 2 μL of PCR products, 1.0 μL of 10× Ex buffer, 0.8 μL of dNTPs (each 2.5 mM), 0.5 μL of 10 μM forward and reverse primer, Ex Taq HS (TaKaRa Bio Inc., Shiga, Japan), and 5.1 μL of dd$H_2O$ water. Thermal cycle condition was 94°C 2 min; 10-12 cycles of (94°C 30 min, 60°C 30s, 72°C 30s); and 72°C 5 min. Illumina libraries were constructed, and pared-end sequenced (2×300 bp) on the Illumina MiSeq platform following the manufacturer's instructions (Illumina Co., USA). However, one replicate of 16S rRNA and *nifH* gene sequence in T2 and three replicates in the *nifH* gene sequences of oil palm samples (N4) had no amplification; thereby, we analyzed a total of seventeen 16S rRNA gene amplicons and fourteen *nifH* gene sequences.

*2.3. Bioinformatic processing of obtained sequence reads*

All obtained paired-end raw reads (including both sampling sites) were processed by using QIIME2 (version 2022.2; http://qiime2.org/). After the removal of the reads before 5' end by *cutadapt* function in QIIME2, the forward and reverse primers' sequences and chimeric sequences were removed using the DADA2 plugin. Then, obtained amplicon sequence variant (ASV) was used for the following analysis.

For 16S rRNA sequences, the feature-classifier plugin was used to estimate phylogeny by comparing the obtained representative sequences with amplicon sequence variants (ASVs) using SILVA database (ver.138.1) (Quast et al., 2012). Sequences classified as chloroplast, mitochondria, Archaea, and less than 5 reads' sequences were removed for the following analysis. For NFB (*nifH* gene), we followed the procedure of Okamoto, et al., (2021). Representative sequences were translated into amino acid sequences, and sequences without Cys 97 and Cys 132 amino acids were removed. To assess the phylogenetic distribution of *nifH* gene sequences, obtained sequences with more than 1% of relative abundance (RA) were aligned together with reference sequences of *nifH*_database_2012 (Gaby and Buckley, 2014) using ARB program version 6.1 (http://www.arb-home.de/) (Ludwig et al, 2004). After the translation

of DNA into amino acid sequences, a neighbor-joining phylogenetic tree with 1000 bootstrap replicates was created using MEGA11 software (Tamura et al., 2021).

Diversity metrics for both bacteria and NFB were calculated after the random sampling of rarefaction analysis at the sequencing depth of the lowest reads (Figure 1). The number of observed ASVs, Shannon diversity index (natural log scale), and Simpson's evenness were calculated by using *phyloseq* package. Statistical differences within the same sampling sites with three replicates were tested using a one-way ANOVA with Tukey's honestly significant difference test. For *nifH* gene sequence analysis, a Venn diagram was created to show the ASV overlap in major NFB among the sampling sites using *gplots* package in RStudio (Version 2022.07.02, https://posit.co/). For beta diversity analysis, unweighted UniFrac (Lozupone et al., 2005; Lozupone et al., 2006) distance was calculated by QIIME2. Non-metric multi-dimensional scaling (NMDS) was performed to display the ordination of the distances using *phyloseq* package in RStudio. Correlation between beta diversity distances and environmental variables were calculated and plotted using *vegan* package in RStudio. Permutational Multivariate Analysis of Variance (PERMANOVA) (999 permutations) was used to test the unweighted UniFrac distance between all the pairs of sampling sites with three replicates, vegetations (sago palm and oil palm), and location (Trang and Nakhon Si Thammarat). The raw data sequences have been deposited at the Sequence Read Archive of the DNA Data Bank of Japan (DDBJ) under Bio Project Accession number PRJDB15222.

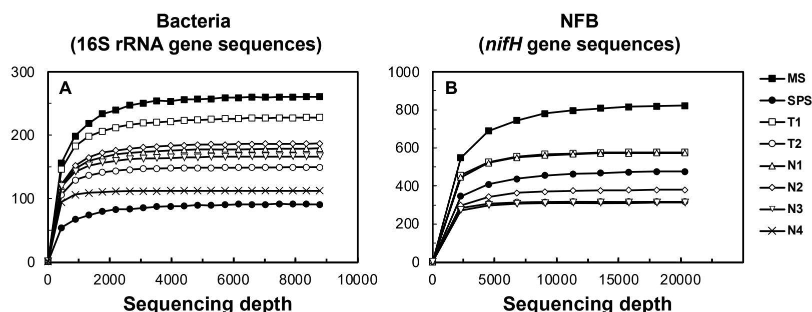



**Fig. 1.** The relationships between depths of Illumina amplicon sequencing of (A) bacteria (16S rRNA gene sequences), (B) NFB (nifH gene sequences) and observed ASV numbers (average value). Sequences were rarefied at 8,779 and 20,326 reads, respectively.

## Results and Discussion

### *3.1. Soil physicochemical properties*

For the sampling site 1 in Malaysia, the soil physicochemical properties were shown in (Table 1). The soil pH ($H_2O$) in Malaysia was low (4.0 to 4.6), showing the feature of strong acidic soil. The soil volumetric water content was lower in MS (49.0%) than in SPS (ranging from 64.8 to 78.5%). The soil bulk density in SPS in the 0–15 cm and 15–30 cm layers was lower (0.20, 0.23 g/cm$^3$), as compared with that in the 30–50 cm layer (0.96 g cm$^{-3}$) and MS. The soil C content per soil volume was similar in all soil samples (ranging from 52.7 to 68.7 kg m$^{-3}$). However, the total N content was higher in the 30–50 cm soil layer in SPS than in MS.

For sampling site 2 in Thailand, we collected the soil from Ultisols (T1, T2, and N4), Entisols (N1 and N2), and Inceptisols (N3). Five different textural soil classes were obtained from the six different fields (sandy loam, sandy clay loam, sandy clay, clay loam, clay) (Table 1). Soil bulk density was higher than 1 g cm$^{-3}$ in T1, T2, and N4, while N1, N2, and N3 showed lower bulk density (lower than 1 g cm$^{-3}$) with relatively higher organic matter content. Volumetric water content (%) was the highest in waterlogging fields in N2, followed by N1 and N3, T1, T2, and N4. Soil pH ($H_2O$) in sampling sites ranged from 4.6 to 5.6, showing all sampling sites were acidic soils, and a higher pH of mean 0.6 U was measured in N2 than in T2 and N1. Total C and N contents were significantly highest in N2. N4 showed a higher available P content

**Table 1.** Soil physicochemical properties in each sampling sites. The soils were collected from 0 to 15 cm depth.

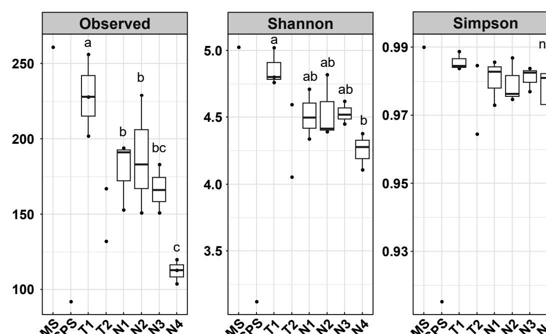
| Location | | East Malaysia | | South Thailand | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | MS | SPS | T1 | T2 | N1 | N2 | N3 | N4 (Oil palm) |
| USDP soil taxonomy | | - | Histosols | Ultisols | Ultisols | Entisols | Entisols | Inceptisols | Ultisols |
| Soil textural class | | Clay loam (1) | - | Clay loam | Sandy clay loam | Sandy clay | Sandy clay loam | Clay | Sandy loam |
| Bulk density | (g $cm^{-3}$) | 1.03 (2) | 0.20 (2) | 1.22 | 1.17 | 0.72 | 0.38 | 0.92 | 1.39 |
| Gaseous phase ratio | (%) | - | - | 9.4 | 25.9 | 7.1 | 4.9 | 2.0 | 16.6 |
| Volumetric water content | (%) | 49.0 (2) | 78.5 (2) | 42.4 | 29.1 | 64.6 | 80.4 | 62.0 | 29.0 |
| Organic Matter content | (%) | 11.1(2) | 60.9 (2) | 2.5 | 3.2 | 5.6 | 26.8 | 4.2 | 2.0 |
| Sand | (%) | 45 (1) | - | 35.4 | 55.0 | 44.1 | 48.0 | 11.4 | 64.3 |
| Silt | (%) | 30 (1) | - | 32.5 | 14.9 | 12.6 | 27.7 | 35.2 | 24.9 |
| Clay | (%) | 25 (1) | - | 32.1 | 30.0 | 43.3 | 24.3 | 53.4 | 10.8 |
| pH ($H_2O$) (2) | | 4.6 | 4.1 (2) | 5.3 | 4.9 | 4.9 | 5.5 | 5.2 | 5.1 |
| Total C | (g $kg^{-1}$) | 22.3 (1) | 352.2 (2) | 14.4 | 18.7 | 32.8 | 155.8 | 24.6 | 11.6 |
| Total N | (g $kg^{-1}$) | 1.4 (1) | - | 0.8 | 0.7 | 1.9 | 5.0 | 1.1 | 0.6 |
| C/N | | 16 (1) | - | 17.4 | 25.2 | 17.5 | 31 | 23.3 | 19.1 |
| Available P (Bray Ⅱ) | ($P_2O_5$ mg $kg^{-1}$) | 1.6 (2) | 9.7 (2) | 9.1 | 5.6 | 3.4 | 11.2 | 2.8 | 27.4 |
| Total P | ($P_2O_5$ mg $kg^{-1}$) | 156 (1) | - | 476 | 430 | 884 | 1945 | 900 | 1409 |
| EC (1:5) | (dS $m^{-1}$) | - | - | 0.40 | 0.40 | 0.90 | 2.00 | 0.50 | 0.30 |
| Exchangeable K | (cmol $kg^{-1}$) | 0.16 (1) | - | 0.16 | 0.20 | 0.28 | 0.83 | 0.23 | 0.22 |
| Exchangeable Ca | (cmol $kg^{-1}$) | 0.44 (1) | - | 0.89 | 0.81 | 2.08 | 7.05 | 2.55 | 0.17 |
| Exchangeable Mg | (cmol $kg^{-1}$) | 0.42 (1) | - | 0.54 | 0.32 | 0.50 | 2.14 | 0.77 | 0.10 |
| Exchangeable Na | (cmol $kg^{-1}$) | 0.54 (1) | - | 0.16 | 0.12 | 0.22 | 0.63 | 0.24 | 0.12 |

(1) Source: Mohidin et al., (2007)
(2) Source: partially modified from Asano et al., (2021)
(3) Soil pH was determined with soil to water ratios of 1:7.5 for soils in Malaysia, 1:2.5 for soils in Thailand

(Bray II), total P content. The highest EC value was observed in N2, followed by N1. These two sites exhibited highly to severely saline conditions. This probably occurred due to the geographical feature that N1 and N2 have located lowlands near the seashore.

### 3.2. Alpha and beta diversity metrics

A total of 424,144 and 538,454 sequence reads (average 22,323 and 33,653 per sample) for 16S rRNA gene sequences (bacteria) and *nifH* gene sequences (NFB) were obtained. Observed ASV numbers, Shannon diversity, and Simpson's evenness were shown in Figure 2. Shannon diversity of bacteria ranged from 4.47 to 7.29 (average 6.51) except for SPS, with similar diversity and evenness scores of 4.5 to 6.0 with endophytic bacterial diversity in rice roots (Samuel et al., 2022). Among all bacterial and NFB diversity indices except for observed ASV numbers, SPS showed the lowest scores. For NFB in SPS, there was a highly abundant ASV (ASV1: 30.1% of RA) belonging to *Burkholderia* sp., and this ASV seems to reduce the alpha diversity scores. Anyango, et al. (1995) surveyed *nifH* copies in the root nodule of the common bean grown in different pH (6.8 and 4.5)



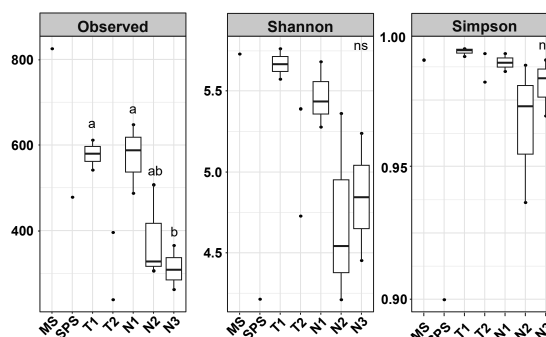




**Fig. 2.** Alpha diversity indices of (A) Bacteria (16S rRNA gene sequences) and (B) *nifH* gene sequences. Dots represent each value; boxes represent the interquartile range; lines within boxes represent median values; whiskers represent the range between minimum and maximum values. Different letters in each figure show significant differences ($P$<0.05) according to Tukey's multiple comparison test among the sites (A: T1, N1, N2, N3, and N4; B: T1, N1, N2, and N3). Observed: observed ASV numbers, Shannon: Shannon diversity index, Simpson: Simpson's evenness

soils, and they found that a single copy of the *nifH* gene was dominant for the sample from the soil pH 4.5. It is suspected that the strong acidity in SPS dramatically reduced both bacterial and NFB diversity in this study. Observed ASV numbers and Shannon diversity index in bacteria were the higher trends in sago palms (except for SPS) than in oil palms (N4), although the Simpson's evenness is not significantly different. We revealed that the Shannon diversity (not in Simpson's evenness) of endophytic root bacteria in sago palms that are not grown with strong acidic SPS was higher compared to oil palms grown in the sandy loam.

NMDS ordination visualized the closeness of diversity patterns (Figure 3). The diversity pattern of SPS was far from other sampling sites. Since SPS has a characteristic of strong acidity, it is considered that the dramatically different beta-diversity patterns in SPS are due to this unique soil condition compared to other sites. Pairwise PERMANOVA test (999 times of permutation) of beta diversity among sampling sites in Thailand (For bacteria: T1, N1, N2, N3, and N4; NFB: T1, N1, N2, and N3) did not show significant differences. However, there were significant differences ($p < 0.05$) between sago palms and oil palms (bacteria: df=16, F=2.70, P=0.01), Trang and Nakhon Si Thammarat (df=13, F=2.53, P<0.01; NFB: df=13, F=2.74, P<0.01). The significant difference between the two locations (Trang and Nakhon Si Thammarat province) of beta diversity may not be resulted from the genetic variation because all root samples were collected from spinless sago palms, and sago palms in Thailand were not so geographically far. Hence, we suspect that the soil, i.e., the root surrounding environment, is the major factor that changes the diversity of endophytic bacteria and NFB.

### *3.3. The relationships between diversity metrics and soil physicochemical properties*

The soil is the primary source of root endophytic bacteria (Papik et al., 2020). Therefore, soil physicochemical properties can indirectly change the diversity of root endophytic bacterial communities. In this study, correlations between soil physicochemical properties and beta diversity for both bacteria and NFB were examined (Figure 4). Both soil physical parameters (bulk density [BD], volumetric water content [VWC], volumetric clay content [Clay], and organic matter content [OM]) and chemical parameters (pH, total C, total N, EC, and exchangeable cations) were significantly correlated for bacteria. Beta diversity of NFB was also correlated with the same parameters except for pH (although there is a correlation at $p < 0.10$ level). Soil bacterial community structure is highly affected by soil texture and water potential (Carson et al., 2010; Chau et al., 2011; Fang et al., 2005). Especially fine-textured soil (clayey and silty soil) increases the population and diversity of bacterial communities (Seaton et al., 2020; Rutherford

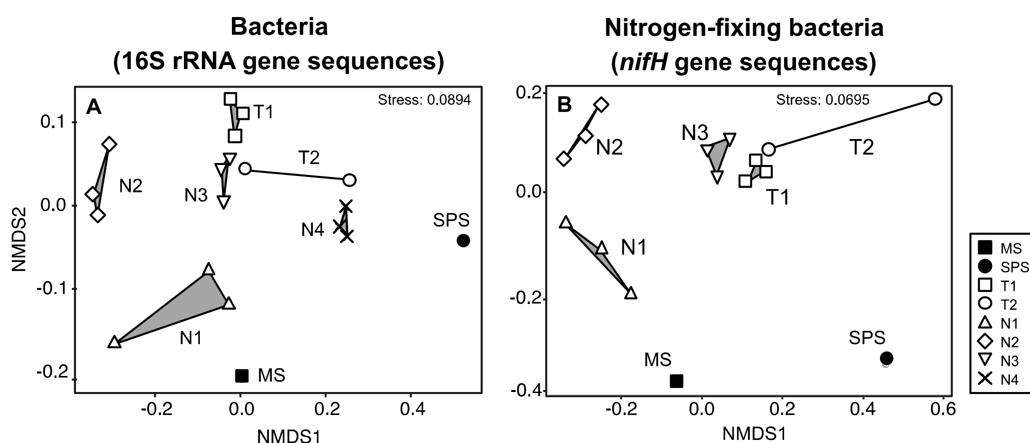



**Fig. 3.** Non-metric multidimensional scaling (NMDS) of unweighted UniFrac distances of (A) bacteria (16S rRNA gene sequences) and (B) nitrogen-fixing bacteria (*nifH* gene sequences) in sago palm roots.

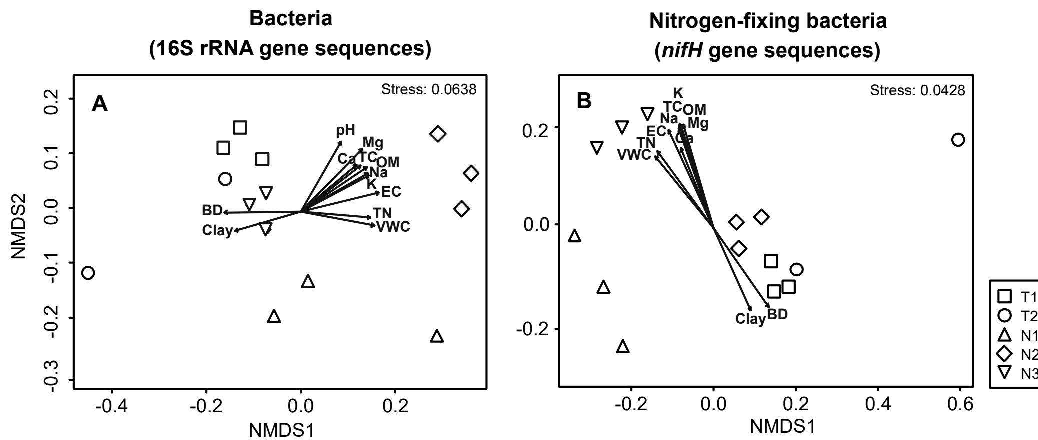



**Fig. 4.** Non-metric multidimensional scaling (NMDS) of unweighted-UniFrac distances of (A) bacteria (16S rRNA sequences) and (B) nitrogen-fixing bacteria (*nifH* gene sequences) for sago palm roots in sampling site 2. Arrows in the figures represent loadings of soil environmental variables ($P$<0.05). BD, bulk density; VWC, volumetric water content; Clay, volumetric clay content; OM, organic matter content; TC, total carbon; TN, total nitrogen; EC, electric conductivity; Na, exchangeable Na content; Mg, exchangeable Mg content; Ca, exchangeable Ca content; K, exchangeable K content.

and Juma, 1992; Hamarashid et al., 2010). BD was negatively correlated with OM (r=-0.827, $P$<0.001) and TC (r=-0.782, $P$=0.001). In general, OM alters soil bacterial diversity (Landa et al., 2013; Zhang et al., 2022). Soil pH and EC have marked effects on soil bacteria and NFB diversity (Canfora et al., 2014; Li et al., 2021); however, whether the effect of soil OM, pH, and EC reach the root interior is not clear. At least in this study, we could confirm the significant correlation between them. Since endophytic bacteria is mainly originated from the soil (Cocking, 2003), it can be easily understood that the difference in soil texture (Clay) and VWC, pH, and EC in this study indirectly affect the endophytic bacteria and NFB communities through the change of soil bacterial community. Since the source of nitrogen is an important element for bacterial community assembling, it is understandable that soil nitrogen availability correlates to endophytic bacterial and NFB diversity.

### *3.4. Identification of nitrogen-fixing bacteria and their relationships with soil factors*

*nifH* genes are grouped into four clusters, clusters I-IV (Zehr, 2003; Raymond 2004). Cyanobacteria, Frankia, Proteobacteria, and some are affiliated with Clostridia, Bacilli, and Nitrospirae are, fallen into Cluster I, and anaerobic bacteria such as methanogens, spirochetes, sulfate reducers, purple non-sulfur bacteria, green sulfur bacteria, and Clostridia are confirmed in Cluster III. A neighbor-joining tree (Figure 5) revealed that NifH neighboring to *Bradyrhizobium, Burkholderia, Cupriavidus, Frankia, Anaeromyxobacter, Geobacter* (Cluster I), *Clostridium, Desulfovibrio,* and *Spirochaeta* (Cluster III) showed high occurrence. These NFB were previously isolated from other plants as well. *Bradyrhizobium japonicum* is a root nodule formulating bacteria of leguminous plants (Subramanian et al., 2015), and it is also isolated from rice and sweet potato (Tan et al., 2001; Terakado-Tonooka et al., 2008). *Burkholderia* is not only NFB but also known as important Plant Growth Promoting Rhizobacteria (Poupin et al., 2016). Although *Burkholderia* spp. are obligate aerobic species, many of them are isolated from paddy rice roots under waterlogging conditions (Ikeda et al., 2014). Especially, *Burkholderia xenovorans* (which is phylogenetically close to ASV1 found in SPS) had been isolated from nipa palms (*Nypa fruticans*) that grow in a neighboring niche with sago palm in Sarawak, Malaysia (Tang et al., 2010). *Burkholderia vietnamiensis* (which is phylogenetically close to ASV2 found in N2) had also been isolated from the roots of sweet potato and sugarcane (Govindarajan et al., 2018; Shinjo et al., 2020). *Cupriavidus* and *Franka* are known as

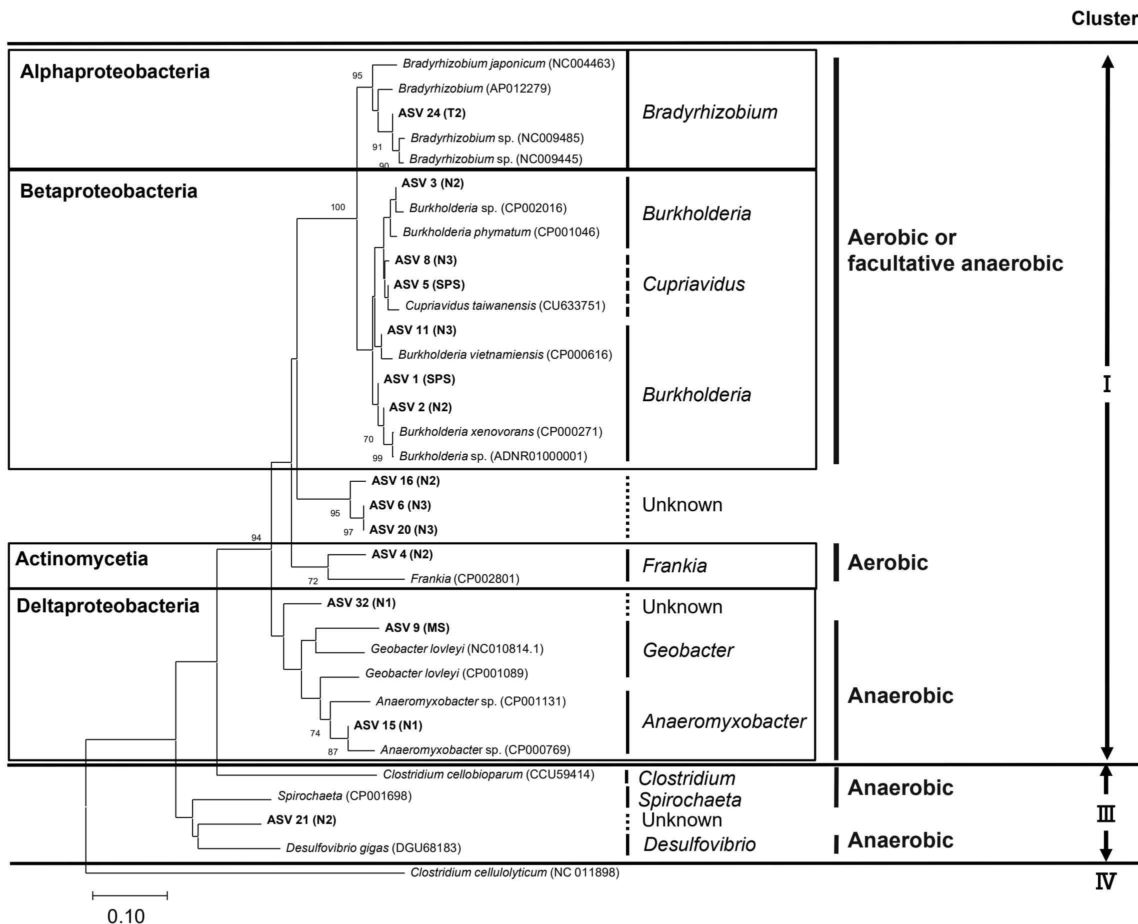



**Fig. 5.** A neighbor-joining tree of NifH sequence with >5% RA. Bootstrap values with more than 70% were shown. Only ASVs with >1% RA were used for creating this figure.

symbiotic NFB in leguminous plants and actinorhizal plants (Da Silva et al., 2012; Sellstedt and Richau, 2013). However, there is little information that they can act as endophytic NFB. *Geobacter* and *Anaeromyxobacter* were also known as iron-reducing NFB in rice paddy soil (Masuda et al., 2017). They were also isolated as root endophytic NFB from rice (Mårtensson et al., 2009; Elbeltagy and Ando., 2008; Manapure et al., 2021). *Desulfovibrio, Spirochaeta,* and *Clostridium* were also isolated as endophytic root NFB (Minamisawa et al., 2004; Ferrando and Fernández, 2015; Collavino et al., 2020; Singh et al., 2022). In this study, genera *Klebsiella, Pantoea, Enterobacter,* and *Bacillus* that are isolated from the sago palm roots in the Philippines (Shrestha et al., 2006) were little or zero frequency. This different feature of isolated NFB might result from the different location or genetic variation of sago palms investigated or the difference of isolation technique (amplicon sequencing and culture method).

Endophytic NFB can exist in oil palm roots (Ai'shah et al., 2009; Hoe et al., 2020; Madhaiyan et al., 2020). However, in this study, amplification of the *nifH* gene failed in oil palm roots. There are two possible reasons for this failure. First, oil palm fields with high inorganic chemical fertilizer input may cause little presence of the nifH gene in the roots. Second, oil palm is a domesticated plant to get high responsiveness to inorganic fertilizer to get a higher number and quality of their fruits. According to Nerva, et al. (Nerva et al., 2022), domesticated plants can improve crop productivity, while a reduction in the recruiting ability of plant-associated microbes is observed. Considering this information, the lower diversity of endophytic bacteria (Figure 2) and the failure of *nifH* gene amplification may have resulted

from the low presence of NFB in oil palm roots. In contrast, sago palm can host diverse endophytic bacteria and NFB (Figure 2 and 5). This result is supported by the cultivation history that sago palm has not been improved toward high input cultivation (chemical fertilizer and agrochemistry).

Proteobacteria (54.3%), Actinobacteriota (9.6%), Acidobacteria (7.2%), Myxococcota (6.2%), Chlorofexi (6.1%), Desulfobacterota (5.2%), Bacteroidota (2.6%), Firmicutes (2.3%), Verrucomicrobiota (2.0%), Nitrospirota (1.1%), Spirochaetota (0.9%) were the predominant phyla of bacterial endophytes in sago palm roots (Figure 6A). The same phyla were predominant in oil palm as well. However, Nitrospirota, related to nitrogen metabolism, was not detected in oil palm roots. Interestingly, SPS showed the remarkably high RA of Proteobacteria. This is because of the remarkably high occurrence of *Burkholderi*a sp. Eight genera that act as nitrogen-fixer were selected, and their RA was shown in (Figure 6B). The total RA of key NFB ranged from 3.0 to 64.4% (average 21.5%) in sago palm and 10.2% in oil palm. SPS showed the remarkably high RA of *Burkholderia* in the Phylum Proteobacteria (64.4%). *Burkholderia* was present in all sampling sites. *Bradyrhizobium, Anaeromyxobacter, Clostridium,* and *Spirochaeta* were present except for SPS, and *Geobacter* was only present in South Thailand.

The possible NFB genera were grouped into aerobic (aerobic and facultative anaerobic) (*Bradyrhizobium, Burkholderia, Frankia,* and *Cupriavidus*) and anaerobic (*Anaeromyxobacter, Geobacter, Desulfovibrio, Clostridium,* and *Spirochaeta*). A negative correlation was found between these two groups (df=12, r=0.718, $P<0.01$). Furthermore, nitrogen-fixing bacteria in cluster III tended to be a high occurrence in the partially waterlogged site of N1 and N2. Interestingly, Ferrando and Fernández Scavino (2015) reported an increase in the RA of *Spirochaeta* after flooding in rice roots. It implies that sago palm roots can change root endophytic NFB community by the soil conditions (especially total N, VWC, and silt content). In other words, there may have wet and dry seasonal changes in the endophytic NFB community that happened by the shift of oxygen level in the root interior affected by waterlogging.

## Conclusions

Alpha diversity indices (observed ASV numbers and Shannon diversity) of endophytic root bacteria in sago palm roots (except for strong acidic soil in SPS) were higher compared to oil palms grown in sandy loam. Beta diversity of both bacteria and NFB was different by sampling sites; Trang and Nakhon Si Thammarat showed significantly different beta diversity. Both soil physical (soil texture, BD, VWC)

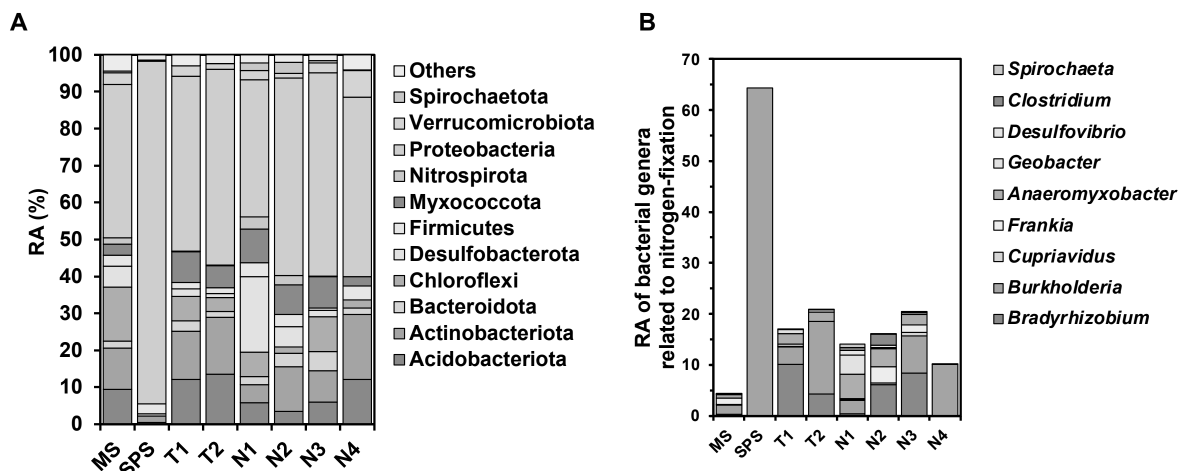



**Fig. 6.** (A) Taxonomic composition of 16S rRNA gene amplicon (average RA was shown for sampling sites in Thailand), (B) RA of bacterial genera related to nitrogen fixation.

and chemical (pH, EC, exchangeable cation, and TN) factors were related to the shift of bacteria and NFB community. Analysis of *nifH* gene amplicon revealed that *Bradyrhizobium, Burkholderia, Cupriavidus, Clostridium, Geobacter, Anaeromyxobacter, Desulfovibrio,* and *Spirochaeta* genera were key nitrogen-fixing endophytes in sago palm roots. It is interesting that NifH adjacent to *Burkholderia xenovorans* was dominant (>30% RA) in the strong acidic soil of SPS. The RA in possible anaerobic NFB (*Clostridium, Geobacter, Anaeromyxobacter, Desulfovibrio,* and *Spirochaeta*) increases along with the decrease of the RA of possible aerobic NFB genera (*Bradyrhizobium, Burkholderia, Frankia,* and *Cupriavidus*).

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