

DIVERSITY OF BACTERIA IN PADDY FIELD SOIL AND THEIR CORRELATION WITH SOIL CHEMICAL PROPERTIES

Wipaporn Yongsuwattana¹, Duenrut Chonudomkul² and Sirinapa Chungopast^{1*}

¹Department of Soil Science, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand.

²Department of Microbiology, Faculty of Science, Kasetsart University Bang Khen Campus, Bangkok 10900, Thailand

*Corresponding author: agrsrnp@ku.ac.th

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ABSTRACT

Soil bacteria play an important role in the decomposition of organic matter and nutrient cycling for rice growth. In tropical Thailand, the Ayutthaya soil series possessed unique characteristics after 8 years of continuous chemical fertilizer application, affecting different soil bacterial communities. Metagenomics analysis of bacterial diversity in rice fields and their correlation to soil properties were studied in Bang Phasi sub-district, Bang Len district, Nakhon Pathom province, Thailand (14.044116N and 100.237599E) in May 2022. Soil nutrient content and organic matter (OM) were measured. Bacterial taxonomy and a diversity index were defined. The correlations between bacterial genera and soil properties were calculated. Soil from the paddy field was very high in OM and quite high in available phosphorus. High bacterial diversity was found in the paddy field soil based on the Shannon index (8.30). The most common phyla were Proteobacteria (29.68%) and Epsilonaeota (21.67%). The genera *Sulfuricurvum* (21.58%) were predominant and correlated with the potential of hydrogen (0.95) and potassium (0.97) while *Thiobacillus* correlated with phosphorus (0.95). The genera *Anaerolineaceae*-uncultured associated with the total nitrogen (1.00) while *Bryobacter* positively linked with the OM (1.00). These bacteria relate to soil properties and can be isolated for biofertilizers in rice cultivation for possible reduction of the use of chemical fertilizers. Furthermore, this is the first study on the diversity and taxonomy of unculturable bacteria in the Ayutthaya soil series in Thailand using metagenomics analysis.

Key words: biodiversity, diversity index, metagenomics, rice field, soil bacteria,

INTRODUCTION

Rice is economically important and Thai people consume it as a staple food, with approximately 10 million ha cultivated in Thailand (Office of Agricultural Economics 2023). Paddy fields are mostly found in wide areas in the central region and along the river basins of Thailand, where there is often flooding during the rainy season (Soil Resources Survey and Research Division 2010). The sustainability of rice production systems globally is intricately related to the chemistry, physics and biology of rice soils (Prasanna et al. 2012). Various bacteria are important for rice growth. The most common group in paddy field soil was in the phylum Proteobacteria, followed by the phylum Firmicutes (Breidenbach and Conrad 2014). Bacterial communities in Brazil's flooded rice fields presented bacteria in the phyla Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi and Proteobacteria (Pittol et al. 2018). Several reports identified beneficial bacteria for rice production, including sulfur-oxidizing bacteria, iron-reducing bacteria, nitrogen-fixing bacteria, and nutrient-solubilizing bacteria (Kartik et al. 2023; Khan et al. 2017; Maeda 2021; Senthilkumar et al. 2021). In

the paddy field, abundant sulfur-oxidizing bacteria were involved in oxidation at the soil surface and in the rhizosphere under different sulfur fertilizer conditions (Masuda et al. 2016). These sulfur-oxidizing bacteria increased the rice stem diameter, plant height and shoot weight (Pourbabae et al. 2020) because sulfur plays an important role in plant metabolism, affecting photosynthesis, carbohydrate metabolism and pathways to protect against oxidative stress (Lunde et al. 2008). Iron-reducing bacteria produce ferrous compounds, which are regarded as crucial mediators of carbon and nitrogen processes in paddy soils and are affected by crop rotation, soil pH and the carbon-to-nitrogen ratio (Peng et al. 2016). Nitrogen-fixing bacteria fix nitrogen from the air and convert it into an ammonium form that rice can use (Doni et al. 2022). Bacteria oxidized elemental sulfur and reduced sulfur compounds to generate sulfuric acid that could solubilize insoluble phosphorous (Ullah et al. 2014). Phosphate-solubilizing bacteria promoted rice growth because they produced organic acids, enzymes, indoleacetic acid, siderophore and antagonists (Panhwar et al. 2012).

Several research papers reported soil management to improve soil bacterial communities such as, biochar addition to promote the nitrification process with higher community diversity (Hou et al. 2021), long-term input of pig manure modified soil abundance and rare bacterial and fungal composition (Hou et al. 2022), and green manures contributed to sustainable soil and nutrient management in agriculture and changed bacterial community structure (Songjuan et al. 2021). The relative abundances of functional genes responsible for nutrient cycles and the organic compounds degradation were enriched with cow manure, which have been continuously applied to paddy lands over 8 years (Wang et al. 2021a). Furthermore, land use change affects the physical and chemical properties of the soil, resulting in changes in the composition of the soil bacterial community (Sun et al. 2021). Soil organic carbon and total nitrogen contents, microbial biomass, and respiration intensity under different land uses were changed (Li et al. 2007). Jia et al. (2021) showed that bacterial diversity was positively associated with pH, soil nutrients and organic carbon. Soil carbon and nitrogen transformation and pH, cooperatively influence rice yield by regulating soil fertility (Wang et al. 2021b). Long-term use of fertilizers influenced structures of bacterial communities along a soil depth gradient in paddy soil and found *Nitrososphaera*, *Nitrospira*, and several members of *Acidobacteria* in topsoil in Sichuan, China where is calcareous purplish paddy soil site (Gu et al. 2017). Continuous fertilizer application in paddy field for 50 years which had influenced on bacteria community using pyrosequencing (Ahn et al. 2012).

As mentioned above, the applications of chemical, organic fertilizers or combined fertilizers were practical to the soil and changed the bacterial community. A report investigated the dynamics of the microbial community in the Ayutthaya soil series (Ay) that were added with silver nanoparticles using the automated ribosomal intergenic spacer analysis (ARISA) method (Chunjaturas et al. 2014). Activity of methanogen were studied in the Ay soil series of rice fields during vegetative and reproductive phase using chemical fertilizer (Chawanakul et al. 2009). Nevertheless, the Ay soil series in tropical Thailand has not been studied long-term chemical fertilizers on bacteria community using metagenomics. In the past, bacterial isolation was conducted in a laboratory environment, leading to the cultivation of less than 2% of the total bacteria present (Wade et al. 2002). The application of metagenomics unveiled the bacterial diversity within the environment, offering valuable insights pertinent to agriculture, the food industry, and medicine (Bashir et al. 2014). The Ay soil series in Nakhon Pathom, Thailand are representative of the study in the current research. These soils are classified as very-fine, mixed, active, acid, isohyperthermic, and Vertic Endoaquepts. Soil characteristics and properties were revealed as very deep soil, dark gray clay, and moderately acidic (pH 6.0). The upper subsoil is clay, gray-brown, or gray-brown with red dots. The soil reaction was very acidic (pH 5.5) and rice straw yellow dots were found at a depth of 100-150 cm. The soil contained sulfur (Land Development Department 2023). The aim of the current research was to investigate the bacteria community using metagenomics and the correlation between the community and soil chemical properties in a long-term chemical fertilization of 8 years of a field experiment site of Ay soil series in Nakhon Pathom, Thailand. Our hypothesis is specific taxa abundances, are found

in the unique area. The community of soil bacteria plays an important role in promoting rice growth through the decomposition of organic matter and nutrient cycling. The results may be helpful in identifying particular bacteria for use as biofertilizers and serve as a guideline for selecting fertilizers for sustainable soil fertility management.

MATERIALS AND METHODS

Study site and soil sampling. The soil was sampled before rice planting from a paddy field during the dry season after the soil was tilled under saturated or flooded conditions in Bang Phasi sub-district, Bang Len district, Nakhon Pathom province, Thailand (14.044116N and 100.237599E). General information in the area indicated that farmers used chemical fertilizer 46-0-0 at the rate of 2.67 kg/ha at 15-20 days after rice planting, 16-20-0 at the rate of 4 kg/ha at 1 and a half months after rice planting, and 18-8-8 at the rate of 4 kg/ha at 2 months after rice planting continuously for 8 years. The soil samples were taken in May 2022 before the next crop. A shovel was used to collect the topsoil at a depth of about 15 cm. Soil samples were collected once from 10 random points across an area of 16,000 square meters and then mixed on a canvas. The soil sample was divided into 4 parts, from which approximately 1 kg of soil was collected for analysis in 3 replicates. The soil samples were analyzed for soil chemical properties and DNA was extracted for metagenomics analysis.

Soil chemical properties. The analysis process commenced by weighing 20 g of soil sample mixed with 20 mL of distilled water in a 100 mL beaker to determine the potential of hydrogen (pH). A soil sample (400–500 g) was added with water until the soil was saturated and the electrical conductivity (EC) was determined (Beck 1999; Bower and Wilcox 1965; Jackson 1958). The pH and EC were measured using pH meter (Ohaus, ST3100) EC meter (Mettler, FiveEasy F30), respectively. The organic matter (OM) content and available phosphorus were analyzed (Walkley and Black 1934; Bray and Kurtz 1945). Exchangeable potassium (K), calcium (Ca) and magnesium (Mg) were analyzed by the leaching method with 1 M ammonium acetate (Pratt 1965). Total nitrogen (total N) was calculated based on multiplying the organic matter content by 0.05 (Ongprasert 1992) based on the OM in the soil containing about 5% of N.

Bacterial diversity in paddy field. DNA was extracted from 250 mg soil samples using a NucleoSpin® soil kit (Macherey-Nagel, Germany) according to the manual for DNA extraction. The DNA bands was checked using 1% agarose gel. A sample of gel powder (48 mg) was placed in a 125 mL flask and added with 40 mL of 1X TAE buffer and heated in a microwave until it was completely dissolved. After the gel had been warmed, 4 µL of RedSafe nucleic acid dye (iNtRON, Korea) was poured into the gel in a tray until it had solidified. DNA samples (5 µL) were mixed with 6X dye loading dye (1.5 µL), put into the wells of the gel through 50 V for 40 min. then the DNA bands were observed on the gel under ultraviolet light. The DNA concentration was measured using Nanodrop (Maestro; Taiwan) before metagenomics analysis.

Library of the metagenomics data was prepared for Illumina MiSeq. Amplified regions of the 16S rRNA gene V3-V4, 400-450bp were processed with the barcode. All polymerase chain reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs; USA). A pipeline for bioinformatic data analysis was performed (supplementary file S1). Raw data obtained by sequencing were merged pair-end reads for each sample using FLASH (V 1.2.7) and quality filtering on the raw tags were performed to obtain the high-quality clean tags (Bokulich et al. 2013; Magoč and Salzberg 2011) according to the QIIME (V 7.1.0) quality-controlled process (Caporaso et al. 2010). Then the effective tags finally obtained. Parallel-meta tool was executed on FASTQ files followed by mapping on the SILVA database for species annotation at each taxonomic rank (Quast et al. 2013). The phylogenetic relationship of all operational taxonomic units (OTUs) representative sequences using the MUSCLE (V 3.8.31) was obtained (Edgar 2004). All sequences have been clustered into OTUs based on their 97% sequence similarity. Alpha-diversity was applied in analyzing the complexity of

biodiversity for a sample based on six indices: observed-species, Chao1, Shannon, Simpson, abundance-based coverage estimator (ACE) and good-coverage.

Correlation and statistical analysis. The Pearson correlation coefficient was used to test the relationships between bacterial communities from the paddy field and soil chemical properties using Minitab V16.2.0 (Minitab L.L.C., Australia).

RESULTS AND DISCUSSION

Chemical properties of soil samples. The soil had a pH of 6.44 (slightly acidic) and electrical conductivity was 0.21 dS/m (not salty). The percentage of organic matter was very high (5.38%). The total nitrogen content was 0.27% and the available phosphorus was 16.77 mg/kg (quite high). Potassium content was 144.3 mg/kg (very high). Exchangeable calcium and magnesium were 13.70 and 6.10 cmol/kg, respectively, which were high. This soil analysis results were compared with the criteria of the Land Classification Division and FAO Project Staff (1973).

Soil bacterial community diversity. Basic information on three replications of soil from paddy field was collected to construct OTUs. The summarized data are shown in Figure 1, including the average of total tags (111,327), taxon tags (99,358), unclassified tags (59), unique tags (11,909), and OTUs tags (3,604). The Y1-axis titled tags number means the number of tags; total tags means the number of effective tags; taxon tags means the number of annotated tags; unclassified tags means the number of unannotated tags; unique tags means the number of tags with a frequency of 1 and only occurs in one sample. The Y2-axis titled OTUs numbers means the number of OTUs to identify the numbers of OTUs in different samples. The bacterial sequences OTUs were clustered into based on 97% sequence similarity (supplementary file S2). Metagenomics data revealed bacterial diversity in the paddy field (Fig. 2).

The top-five phyla were the Proteobacteria (29.68%), Epsilonbacteraeota (21.67%), Nitrospirae (13.68%), Acidobacteria (9.30%) and Chloroflexi (7.13%), respectively. The top-five families were the Thiovulaceae (21.58%), Anaerolineaceae (3.68%), Hydrogenophilaceae (3.02%), Solibacteraceae (Subgroup_3; 2.60%) and Pedosphaeraceae (2.50%), respectively. The top-five genera were *Sulfuricurvum* (21.58%), *Thiobacillus* (2.84%), *Geobacter* (2.27%), *Anaerolineaceae*-uncultured (1.66%) and *Gemmatimonadaceae*-uncultured (0.88%), respectively. In addition, several other types of bacteria were found that could be classified or cultured. In average total, 3,444.33 genera were observed. The Shannon, Simpson, Chao1 and ACE indices indicated there was high bacterial diversity in the paddy field (Table 1).

Table 1. Diversity indices of bacteria in paddy field

Parameter	Value
Number of observed species	3,444.33 ± 326.05
Shannon index	8.30 ± 0.91
Simpson index	0.95 ± 0.05
Chao1 index	3577.56 ± 307.00
ACE index	3624.30 ± 316.55
Goods coverage index	1.00 ± 0.00

ACE = abundance-based coverage estimator

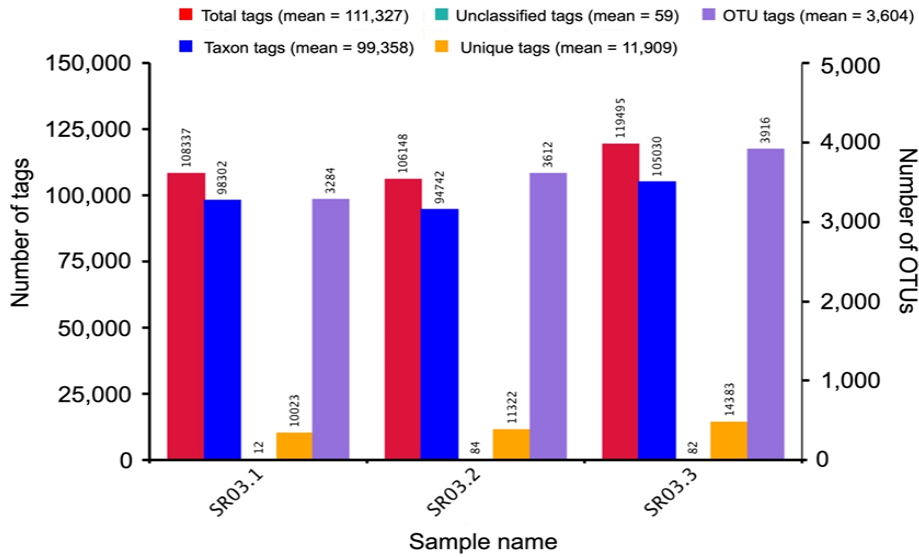


Fig. 1. Summary of numbers of tags and operational taxonomic units (OTUs) from three paddy field soil replicates (SR03.1 SR03.2 and SR03.3)

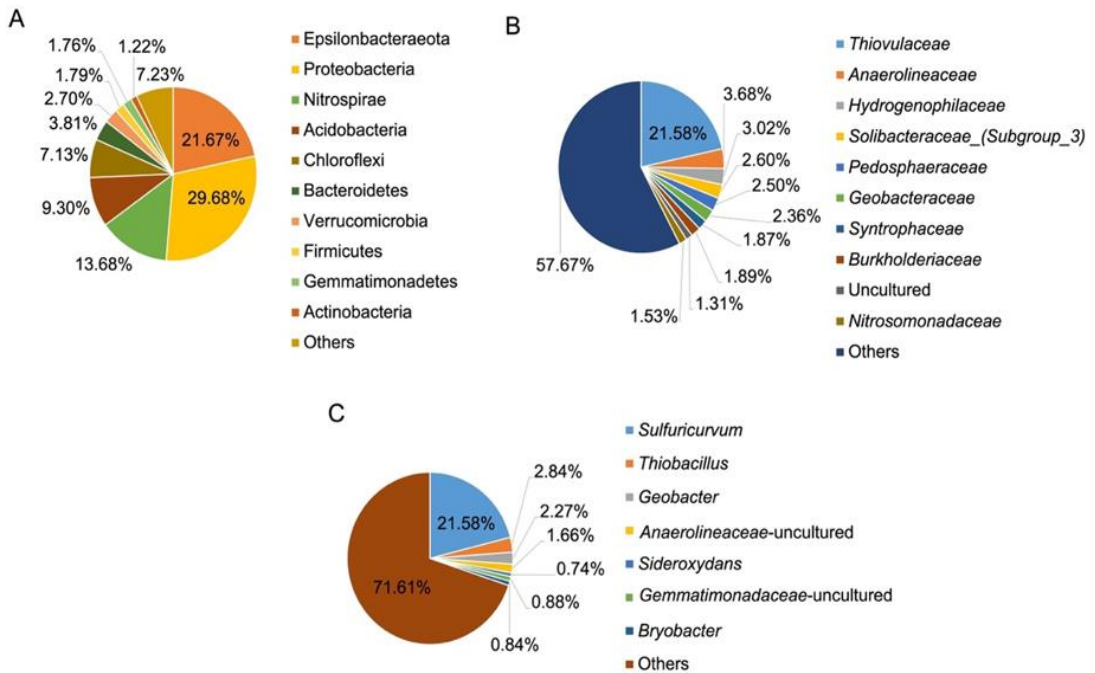


Fig. 2. Relative abundances of bacterial diversity in paddy field at levels of: (A) phylum; (B) family; (C) genus

Correlation between soil properties and microbial groups. The correlations between bacterial community from paddy fields and soil chemical properties are shown in Table 2. The criteria for separating correlation levels followed Ratner (2009). The pH was strongly positive correlated with *Sulfuricurvum* (0.95), *Thiobacillus* (0.74) and *Sideroxydans* (0.96) and strongly negative correlated with *Geobacter* (-0.83), *Anaerolineaceae*-uncultured (-0.98) and *Bryobacter* (-1.00). However, *Gemmatimonadaceae*-uncultured (-0.37) showed a moderate negative correlation. The EC had a strong positive correlation with *Sulfuricurvum* (0.74) and *Sideroxydans* (0.73) and a moderate positive correlation with *Gemmatimonadaceae*-uncultured (0.62). The EC had a strong negative correlation with *Geobacter* (-0.90), a weak negative correlation with *Thiobacillus* (-0.22) and *Anaerolineaceae*-uncultured (-0.30) and a moderate negative correction with *Bryobacter* (-0.45). The OM exhibited strong positive correlations with *Geobacter* (0.81), *Anaerolineaceae*-uncultured (0.98), and *Bryobacter* (1.00) while it was strongly negatively corrected with *Sulfuricurvum* (-0.94), *Thiobacillus* (-0.75) and *Sideroxydans* (-0.95). Similar to the total N, there were strong positive correlations with *Geobacter* (0.72), *Anaerolineaceae*-uncultured (1.00), and *Bryobacter* (0.99). However, there were strong negative correlations with *Sulfuricurvum* (-0.89), *Thiobacillus* (-0.84), and *Sideroxydans* (0.90). *Gemmatimonadaceae*-uncultured showed a moderate positive correlation with both the OM and total N (0.40 and 0.52, respectively).

Available phosphorus was strongly positively correlated with *Thiobacillus* (0.95) but weakly positively correlated with *Sulfuricurvum* (0.21), *Geobacter* (0.08) and *Sideroxydans* (0.23). The available phosphorus was strongly negatively correlated with *Gemmatimonadaceae*-uncultured (-0.99) and moderately negatively correlated with *Anaerolineaceae*-uncultured (-0.67) and *Bryobacter* (-0.55). Exchangeable potassium, calcium, and magnesium displayed strong positive correlations within a range with *Sulfuricurvum* (0.95-0.97), *Thiobacillus* (0.7-0.74), and *Sideroxydans* (0.96-0.97) but were strongly negatively correlated with *Geobacter*, *Anaerolineaceae*-uncultured and *Bryobacter*. There was a moderate negative correlation of the exchangeable potassium calcium and magnesium with *Gemmatimonadaceae*-uncultured (-0.33, -0.37, and -0.37, respectively).

Table 2. Correlation coefficient between paddy field bacterial community and soil chemical properties

Genus	Soil property							
	pH	EC	OM	Total N	P	K	Ca	Mg
<i>Sulfuricurvum</i>	0.95	0.74	-0.94	-0.89	0.21	0.97	0.95	0.95
<i>Thiobacillus</i>	0.74	-0.22	-0.75	-0.84	0.95	0.70	0.74	0.74
<i>Geobacter</i>	-0.83	-0.90	0.81	0.72	0.08	-0.85	-0.83	-0.83
<i>Anaerolineaceae</i> - <i>uncultured</i>	-0.98	-0.30	0.98	1.00*	-0.67	-0.97	-0.98	-0.98
<i>Sideroxydans</i>	0.96	0.73	-0.95	-0.90	0.23	0.97	0.96	0.96
<i>Gemmatimonadaceae</i> - <i>uncultured</i>	-0.37	0.62	0.40	0.52	-0.99	-0.33	-0.37	-0.37
<i>Bryobacter</i>	-1.00*	-0.45	1.00*	0.99	-0.55	-0.99	-1.00*	1.00*

pH = potential of hydrogen, EC = electrical conductivity, OM = organic matter, Total N = total nitrogen, P = phosphorus, K = exchangeable potassium, Ca = calcium and Mg = magnesium.

Minitab (version 16.2.0; Minitab, L.L.C., Sydney, two letter state abbreviation if applicable, country) statistical software was used for correlation testing and significant ($p < 0.05$) differences are indicated as *.

Bacterial diversity and bacterial relationships with soil chemical properties were studied in paddy fields of Ay soil series in Nakhon Pathom, Thailand, which underwent long-term chemical fertilization of 8 years. The soil analysis indicated that the soil was fertile, with high diversity of microbial communities and the dominant bacteria were in the phylum Proteobacteria. The gamma-Proteobacteria was sensitive to all the fertilization regimes in paddy soil as affected by long-term application of inorganic fertilizer and rice straw using 16S rRNA (Wu et al. 2011). The Proteobacteria Chloroflexi, and Acidobacteria phyla were found dominant in rice paddy soil with different fertilizers management for a period of 50 years (Ahn et al. 2012). The relative abundance of Nitrospirae and Gemmatimonadetes phyla changed when using a 30-year organic-inorganic fertilization in a paddy field (Yang et al. 2019).

Notably, the phylum of Epsilonbacteraeota was predominant in the current research, especially the family, *Thiovulaceae* accompanied by the genus *Sulfuricurvum*. Previous studies showed the abundance of *Sulfuricurvum* in different fertilization regimes. The relative abundance of *Sulfuricurvum* was high following the simultaneous application of long-term inorganic fertilizers and rice straw compost, they are a group of chemoautotrophs bacteria found in paddy soil and paddy sediment that can oxidize sulfur and sulfur compounds with oxygen or nitrate as an electron acceptor (Ahn et al. 2012; Jiang et al. 2021).

Thiobacillus and *Geobacter* were also found in the current research, which is similar to the report of Ahn et al. (2012). *Thiobacillus* and *Sideroxydans* are important genera in the phylum Betaproteobacteria a group of bacteria chemoautotrophs that use iron or compound sulfur as an energy source. *Thiobacillus* species play an important role in the oxidization of sulfur, decrease the soil pH, make soil conditions for plant growth and help plants with nutrient absorption (Akhtar et al. 2012).

The presence of *Sulfuricurvum*, *Thiobacillus*, *Geobacter* and *Nitrospira* in rice growth and reproductive stages improved crop production and health (Baskaran et al. 2022). The iron-reducing bacteria *Geobacter* and *Anaeromyxobacter* genera were also recently reported as nitrogen-fixing bacteria predominant in paddy soils (Masuda et al. 2021). Long-term application of organic fertilizer or combined with inorganic fertilizer, improved soil quality, and environmental safety by increasing the number of methane-oxidizing bacteria and nitrogen-fixing bacteria that are beneficial to the soil and increasing crop yields (Daquiado et al. 2016).

The current research showed the total nitrogen content was significantly positively correlated with the genus *Anaerolineaceae*-uncultured. The *Anaerolineaceae* significantly increased by long-term use of inorganic fertilizers over a 19-year period on increasing functions and bacterial diversity in Chinese paddy field soils (Huang et al., 2019). *Anaerolineaceae* and other bacteria were correlated with various metabolic pathways and soil properties in paddy soil from a rice-wheat cropping system over a 10-year period using 4 treatments: control, NPK, NPK + pig manure, and NPK + straw (Wang et al. 2019). These bacteria are found in sedimentary environments and are involved in the degradation of organic nitrogen (Yamada et al. 2006).

Organic matter was significantly positively correlated with the *Bryobacter* genus which is in the phylum Acidobacteria of the current research. Soil organic matter determines the activity of Acidobacteria when applied long-term chemical fertilizer (Ahn et al. 2016). The relative bacterial abundances of *Geobacter* and *Bryobacter* also increased when applied long-term chemical fertilization and crop rotation (Chen et al., 2020). The phyla Proteobacteria, Acidobacteria, Chlorobi, and Bacteroidetes were correlated with the available potassium, available phosphorus, and soil organic matter (Zhu et al. 2019). The bacterial phyla Acidobacteria, Gemmatimonadetes, and Cyanobacteria were significantly correlated with soil organic matter and/or nitrate contents (Chen et al. 2016). *Bryobacter* are heterotrophic bacteria that contribute to the degradation of organic molecules (Kulichevskaya et al. 2010; Thouin et al. 2019). It is an indicator that degrading bacterial communities

in rice paddy fields subsidize the carbon pool (Xuan 2012). These mentioned bacteria are associated with soil properties and can be isolated for use as biofertilizers in rice cultivation, thereby reducing the dependency on chemical fertilizers.

CONCLUSION

The paddy field soil of the Ayutthaya soil series in Bang Len district, Nakhon Pathom province, Thailand was fertile with a high level of organic matter. The soil had 8 years of long-term application of chemical fertilizers only which resulted in specific bacterial taxa. The prominent genus present was *Sulfuricurvum*. There was high bacterial diversity that was correlated with various soil properties. In addition, specific group of sulfur-oxidizing, iron-reducing, nitrogen-fixing, and nutrient-solubilizing bacteria taxa were found. Therefore, these bacteria can be isolated and applied together to promote rice growth.

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