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Effect of Nitrogen Levels on Methanogen and Methanotroph Populations in Rice Paddies

Youngho Kwon [1,2], So-Myeong Lee [1], Ju-Won Kang [1], Jin-Kyung Cha [1], Hyeonjin Park [1], Dong-Su Park [1], Nkulu Rolly Kabange [1], Ki-Won Oh [1], Youn-Sig Kwak [2] and Jong-Hee Lee [1,*]

[1] Department of Southern Area Crop Science, National Institute of Crop Science, RDA, Miryang, 50424, Republic of Korea; kwon6344@korea.kr (Y.K.); olivetti90@korea.kr (S.-M.L.); kangjw81@korea.kr (J.-W.K); jknzz5@korea.kr (J.-K.C); tinapark@korea.kr (H.P); parkds9709@korea.kr (D.-S.P); rollykabange@korea.kr (N.R.K); ohkw1004@korea.kr (K.-W.O)

[2] Department of Plant Medicine, Gyeongsang National University, Jinju, 52828, Republic of Korea; kwak@gnu.ac.kr (Y.-S.K.)

* Correspondence: ccriljh@korea.kr (J.-H.L.), Tel.: +82–53–350–1169, Fax: +82–55–352–3059

Abstract: Rice production is a significant contributor to methane emissions, accounting for approximately 11% of global anthropogenic emissions. However, methane emissions in rice fields could effectively be reduced by implementing proper management practices and careful cultivar selection. The impact of nitrogen fertilizers on methane emissions is multifaceted, as these fertilizers enhance crop growth and influence the activity of methane-producing (methanogens) and methane-consuming microbes (methanotrophs), leading to complex outcomes in methane emissions. In this study, we used qPCR to quantify methanogens and methanotrophs using *mcrA* (methanogenesis-related gene) and *pmoA* (methane oxidation-related gene) primer sets under different nitrogen levels (0, 50, and 100%) and rice varieties. The results revealed that higher nitrogen input led to higher methanogen inhabitation in the rhizosphere. Additionally, the abundances of methanogens and methanotrophs varied among the different rice varieties. Furthermore, it was observed that there may be an additive effect between the rice variety and nitrogen level used. These findings suggest that future breeding efforts should involve screening for methane-related microbes in rice cultivars adapted to low-nitrogen conditions. By identifying and selecting rice varieties that promote lower methanogen levels and higher methanotroph inhabitation, significant steps can be taken to mitigate greenhouse gas emissions from rice cultivation.

Keywords: rice; methanogen; methanotroph; nitrogen fertilization

1. Introduction

Rice, the world's most important food, stands out as an aquatic cereal crop. Currently, approximately 131 million hectares of rice paddies are flooded by irrigation or rainwater during the growing season [1]. Nevertheless, rice paddies are also considered a major source of anthropogenic methane (CH_4) emissions, contributing to 12% of the total anthropogenic CH_4 budget (IPCC, 2007) [2]. Recent estimates of CH_4 emissions from rice fields vary between 39 and 112 Tg per year [3]. Unfortunately, owing to the increasing demand for rice from the world's rapidly growing population, rice cultivation is expected to continue to rise in the coming decades [4], which would lead to a significant increase in CH_4 emissions.

CH_4 emissions from paddy fields result from the production and subsequent oxidation of CH_4 by methanogenic and methanotrophic bacteria, respectively [5]. CH_4 fluxes in rice paddy soils are influenced by various factors, including the use of inorganic and organic fertilizers, water management practices, physicochemical and geochemical properties of the soil, air and soil temperatures, composition and activity of soil microorganisms, and the physiological characteristics of rice cultivars [6]. CH_4 is produced under the anaerobic conditions typical of flooded rice fields,

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providing a favorable environment for methanogens that rely on reduced conditions and soil carbon sources produced by rice plants [7]. To quantify methanogens in rice paddy soils and other methanogenic environments, the *mcrA* gene, which encodes the alpha subunit of methyl coenzyme M reductase (MCR), is commonly used because of its high conservation and specificity for methanogens (paddies). Methanotrophic bacteria are dependent on the CH_4 produced by methanogens and can oxidize up to 60–70% of it in paddy soils, significantly reducing the CH_4 production potential of rice agriculture [8,9]. The variation in CH_4 emissions observed among rice cultivars [10-12] indicates the potential for mitigating CH_4 production from rice cultivation by breeding low-emission rice varieties. However, the genetic differences affecting rhizosphere microbial communities, which are ultimately reflected in CH_4 emissions from rice paddy soils, remain poorly understood. Studies have shown that cultivar differences affect the composition of methanogens and methanotrophs in the rice rhizosphere [13-19]. Methanogens interact with other members of the microbial community, influencing the observed CH_4 emissions from rice fields. Consequently, variation in cultivars may play a major role in regulating CH_4 emissions from rice fields. In addition, nitrogen fertilizers play a significant role in stimulating crop growth and providing additional carbon substrates, such as organic root exudates and sloughed-off cells, to methanogens for CH_4 production [20-22]. However, despite extensive research in this area, no consensus has been reached regarding the net effect nitrogen fertilizers have on methanogen abundance in rice soils. In other words, although it is well-established that nitrogen fertilizers can enhance crop growth and contribute to the production of CH_4 by providing more carbon substrates to methanogens, there is no universally agreed-upon conclusion regarding the overall effect nitrogen fertilizers have on the abundance of methanogens in rice soils. Further research is required to fully understand the complex interactions between nitrogen fertilizers and methanogen abundance during rice cultivation. Likewise, with the widespread observation of differences in CH_4 emissions among rice cultivars [23-25], few studies have attempted to examine CH_4 emission control mechanisms, such as the abundance of methanogens and methanotrophs, apparent plant growth properties, and using different cultivars.

This study aimed to evaluate and quantify the methanogens and methanotrophs present in the rice plant rhizosphere under different nitrogen levels and cultivars. To this end, we suggest that there is a potential synergistic or additive effect between cultivar varieties and nitrogen levels that reduces the abundance of methanogens and enhances methanotroph inhabitance.

2. Materials and Methods

2.1. Experimental field management and measurement of agronomic traits

The experimental fields were situated in Miryang, South Korea (35° 29' 32.2872" N, 128° 44' 32.1972" E). Prior to transplantation, conventional tillage was performed and no straw or organic matter applied. The rice varieties used were Hanareum4, Geumgang1, Milyang392, IR72, 93-11, IR64 (*Indica*), Saeilmi, Sobi, Nampyeong, and Misojinmi (*Japonica*), which were transplanted 30 days after sowing. Seedlings were simultaneously transplanted into both experimental fields at a spacing of 30 cm × 15 cm. Each field (8 m × 70 m) consisted of three replicate plots. Chemical fertilizer (N-P_2O_5-K_2O: 21-17-17) was applied at concentrations of 0%, 50%, and 100%. A 100% (conventional: N-P_2O_5-K_2O: 9.0-4.5-5.9) fertilization was applied at a total rate of 90 kg N ha^{-1} during rice cultivation. This fertilizer was split into three applications: 54 kg N ha^{-1} as basal application before transplanting; 18 kg N ha^{-1} at 20 days after transplanting (DAT); and 18 kg N ha^{-1} at 65 DAT, under normal nitrogen (NN) conditions. In the low nitrogen (LN) plots, only half (50%) of the nitrogen used in the normal plots was applied. The NN, LN, and no nitrogen fields were maintained for 20 years. The Miryang field was continuously flooded until 110 DAT as part of the field management practices. Throughout the rice-growing season, continuous flooding was maintained to keep the water level consistent at 5–7 cm above the soil surface. Plant height and tiller number were measured during the tillering, heading, and grain-filling stages, as well as prior to harvesting. Agronomic traits, such as milled rice yield, spikelet number per panicle, and thousand grain weight, were measured before harvesting.

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2.2 Chemical characterization of testbed soil

Soil analysis was conducted according to previously described methods [26]. The soil pH was measured using a soil-to-distilled water ratio of 1:5, and the resulting suspension measured using a pH meter (720, ORION, USA) based on the glass electrode method. Organic matter was determined using Tyurin's method, and available phosphorus extracted and analyzed using the Lancaster method with a spectrophotometer (CINTRA6, GBC, Australia) (NIAST, 2000). Nitrogen within the soil was extracted with 2 M KCl and analyzed using a nitrogen analyzer (K-314, Buchi, Switzerland). For the analysis of exchangeable cations, the soil was extracted with 1 N NH_4OAc (pH 7) and quantified using an Inductively Coupled Plasma-Atomic Emission Spectrometer (Intergra XM2, GBC).

2.3. Extraction of total genomic DNA from rice rhizosphere

Rhizospheric soil from rice roots was collected from each experimental plot, with five replicates at the tillering, heading, and grain-filling stages. A gram of rhizospheric soil was dried for 12 h. Extraction of total genomic DNA was performed using the DNeasy PowerSoil Pro Kit (Qiagen, Germany), following the manufacturer's protocol. Samples were collected in a PowerBead Pro Tube that was briefly spun to ensure that the beads settled at the bottom. Afterward, up to 250 mg soil and 800 µL Solution CD1 were added and briefly mixed via vortexing. Once the sample was loaded into a PowerBead Pro Tube, the next step involved homogenization and lysis. The PowerBead Pro Tube contains a buffer that serves multiple purposes: (a) dispersing soil particles, (b) initiating the dissolution of humic acids, and (c) protecting the nucleic acids from degradation. The components of the PowerBead Pro Tube were mixed by gentle vortexing and the sample dispersed in the buffer. A Vortex Adapter for 1.5–2 mL tubes (cat. no. 13000-V1-24) was used, which the PowerBead Pro Tube was horizontally secured to, and vortexed at maximum speed for 10 min. If more than 12 samples were prepared simultaneously using the Vortex Adapter, the vortexing time was increased by 5–10 min. Tape was avoided during this process as it may become loose, resulting in reduced homogenization efficiency and inconsistent results. The PowerBead Pro Tube was centrifuged at 15,000 × *g* for 1 min. The supernatant, which may still contain some soil particles, was transferred to a clean 2 mL microcentrifuge tube. A 200 µL volume of Solution CD2 was added to the supernatant and vortexed for 5 s. Solution CD2 contained IRT, a reagent that precipitates non-DNA organic and inorganic materials, including humic substances, cell debris, and proteins. The mixture was then centrifuged at 15,000 × *g* for 1 min. Up to 700 µL supernatant was carefully transferred to a clean 2 mL microcentrifuge tube. The pellet at this stage contained non-DNA organic and inorganic materials, such as humic acids, cell debris, and proteins. For optimal DNA yield and quality,

2.4. Constriction of standard DNA for generating standard curves

To quantify methanogens and methanotrophs in the rhizosphere, we constructed standard DNA samples. Specific primers were used for amplification. For the key enzyme of methanogenic bacteria, MCR, the MLf (5'-GGTGGTGTMGGATTCACACARTAYGCWACAGC-3') and MLr (5'-TTCATTGCRTAGTTWGGRTAGTT-3') primers were used [27]. For the major enzyme of methanotrophic bacteria, particulate CH_4 monooxygenase (pMMO), the A189f (5'-GGNGACTGGGACTTCTGG-3') and mb661r (5'-CCGGMGCAACGTCGTCYTTAC C-3') primers were used [28,29]. PCR amplification was performed using each primer set. For MCR gene amplification, the reaction included an initial denaturation at 94 °C for 5 min, followed by 32 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 10 min. For pMMO gene amplification, the reaction included an initial denaturation at 94 °C for 5 min, followed by 32 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 10 min. After amplification, the MCR and pMMO genes were purified and the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) used for transformation into *Escherichia coli*. Colonies containing the inserted plasmids were obtained on Luria-Bertani + kanamycin (50 ppm) + X-gal + isopropyl β-D-1-thiogalactopyranoside

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agar plates. Sequences of the inserted genes in the obtained colonies were analyzed for confirmation, and the plasmids used as standards. Plasmids prepared as standards were extracted using a Plasmid Mini Extraction Kit (Bioneer, Korea) and their concentrations measured using an ELISA reader with a Take3 multivolume plate. A standard curve was generated and gene copy numbers calculated.

2.5. Methanogen and methanotroph quantification

The qPCR reactions were performed using a QuantiFast SYBR Green PCR kit (Qiagen, Germany). The reaction mixture consisted of 0.3 μM primers, 50 nL genomic DNA, and 7.8 μL distilled water, resulting in a total volume of 20 μL. For quantification, the plasmid DNA standards prepared in Section 2.4 were diluted from 102 to 107 for *mcrA* and from 102 to 108 for *pmoA*. The prepared mixture was subjected to real-time PCR using a QuantStudio 5 Real-Time PCR System (Applied Biosystems). PCR amplification was performed under the same conditions as those used for standard preparation, with 50 cycles to confirm specific amplification. Additionally, melting curve analysis was performed to determine whether PCR amplification was specific by increasing the temperature from 65 °C to 95 °C with a 0.5 °C increment per cycle. The real-time PCR results were analyzed using QuantStudio Design and Analysis software (Applied Biosystems) to quantify the copy numbers of methanogenic and methanotrophic bacteria present in the genomic DNA.

2.6. Statistical analysis

Statistical analyses, including two-way ANOVA and correlation analysis, were performed using the SAS Enterprise software (version 7.15 HF8). Statistical significance was set at p <X.XX.

3. Results and Discussion

3.1. Methanogen and methanotroph abundance under different nitrogen levels and rice growth stages

To explore the abundance of rhizospheric methanogens and methanotrophs under different nitrogen levels and growth stages, we conducted qPCR using six Indica and four Japonica subspecies; samples were collected under 0%, 50%, and 100% nitrogen levels (conventional level), and tillering, heading, and grain-filling growth stages. The abundance of methanogens and methanotrophs were highly affected by nitrogen levels during all growth stages. Under LN conditions, the abundances of methanogens were 3.8, 9.8, and 4.7 × 10^6 mcrA gene copies/g dry soil during the tillering, heading, and grain-filling stages, respectively. Methanotroph abundances in LN were 4.0, 5.7, and 2.9 × 10^6 pmoA gene copies/g dry soil during the same stages, respectively. In contrast, under NN conditions, the abundances of methanogens were 4.9, 12.6, and 8.6 × 10^6 mcrA gene copies/g dry soil, whereas methanotroph abundances in NN were 3.3, 4.9, and 2.5 × 10^6 pmoA gene copies/g dry soil during the corresponding stages. First, the quantitative abundances of methanogens and methanotrophs were observed to be highest at the tillering stage and gradually decreased until the grain-filling stage. Second, nitrogen application levels were strongly affected by the abundance of methanogens and methanotrophs. The more nitrogen applied, the more methanogens and fewer methanotrophs inhabited the rice rhizosphere (Figure 1).

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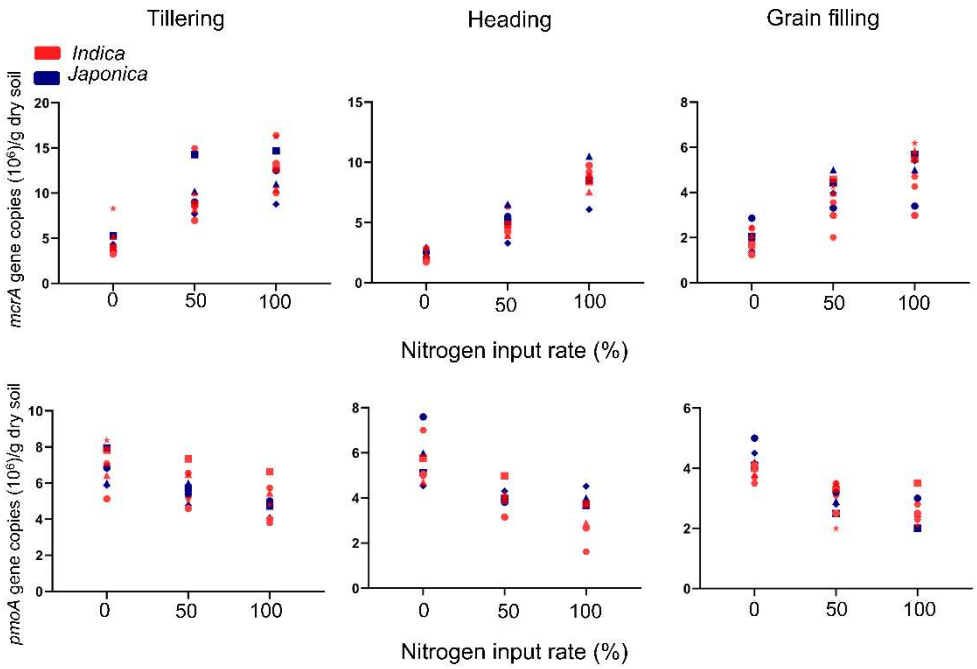

Figure 1. Quantification of *mcrA* and *pmoA* gene copies of 10 rice varieties under different nitrogen levels and rice growth stages. Abundance of methanogens and methanotrophs were measured using *mcrA* and *pmoA* genes, respectively. The red symbol indicates *Indica* subspecies and the blue symbol *Japonica* subspecies.

Interestingly, the abundances differed depending on the rice variety. Otherwise, there were no differences observed between *Indica* and *Japonica* subspecies. This implies that methanogen and methanotroph inhabitants prefer different cultivar genotypes rather than subspecies.

3.2. Ratio of methanogens/methanotrophs under different nitrogen levels and rice growth stages

The ratios of methanogens to methanotrophs were highly variable at different nitrogen levels and rice growth stages. At the tillering stage, methanogen/methanotroph ratios were 35%, 62%, and 77% under the 0%, 50%, and 100% nitrogen levels, respectively. The heading and grain-filling stages under these nitrogen conditions had ratios of 40%, 63%, and 71% and 26%, 48%, and 59%, respectively (Figure 2).

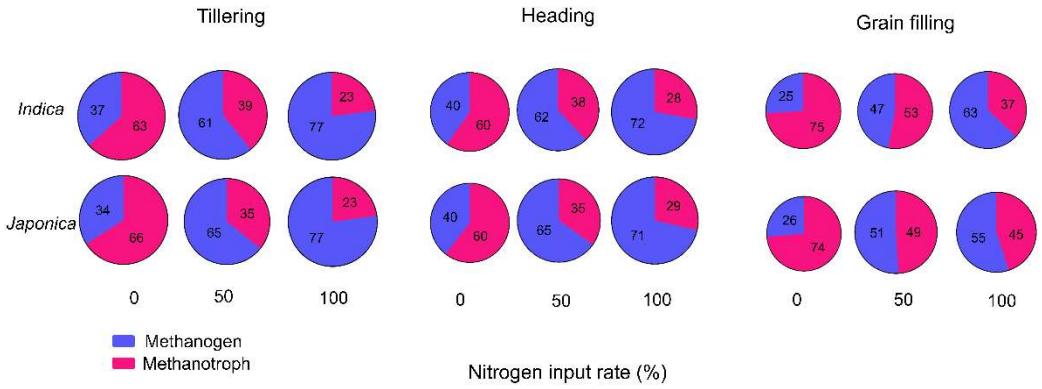

Figure 2. Methanogen to methanotroph ratios depend on the rice subspecies under different nitrogen levels and rice growth stages. Purple and pink indicate methanogens and methanotrophs, respectively.

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The methanogen/methanotroph ratio varied depending on the rice genotype. Geumgang1, which exhibits low panicle numbers but a high rice yield, showed low levels of methanogens. In contrast, IR64, a genotype highly influenced by nitrogen levels and has varying tiller numbers, demonstrated significant changes in methanogen abundance (Table 1 and Table S1). These findings indicate that reducing nitrogen fertilizer application can potentially lead to lower CH_4 emissions by maintaining lower levels of methanogens in the soil. This aligns with the global adoption of low-input regimes to mitigate greenhouse gas emissions. Moreover, it is crucial to focus on reducing methanogen abundance during specific stages of rice growth, particularly the tillering and heading stages. Methanogen levels tended to remain high during these stages, significantly contributing to greenhouse gas production. Efforts can be made to implement effective strategies for reducing greenhouse gas emissions by addressing methanogen levels during these critical stages. In summary, this study highlights the importance of understanding the methanogen/methanotroph ratio in different rice genotypes, and emphasizes the potential benefits of reducing nitrogen fertilizer use to lower CH_4 emissions. Targeting and managing methanogen levels during key growth stages could be a valuable approach for greenhouse gas reduction.

Table 1. Methanogen/methanotroph ratio under different nitrogen levels and rice growth stages in 10 rice varieties.

Ratio (%)		Tillering			Heading			Grain-filling		
		0%	50%	100%	0%	50%	100%	0%	50%	100%
Indica	Hanareum4	30	63	80	39	60	77	20	49	53
	Geumgang1	33	60	71	32	55	66	23	48	59
	Milyang392	38	53	75	45	56	66	31	49	67
	IR72	45	56	76	36	56	64	33	48	61
	93-11	40	66	81	50	66	77	33	51	63
	IR64	33	67	80	33	74	81	17	34	74
Japonica	Saeilmi	33	63	74	34	63	71	27	47	48
	Sobi	32	67	81	40	71	76	29	53	61
	Nampyeong	41	69	81	42	63	69	25	56	56
	Misojinmi	29	54	71	42	62	68	23	47	54
Average		35	62	77	39	63	71	26	48	60

Table 2. Two-way ANOVA of methanogen abundance and other factors under different rice growth stages.

Two-way ANOVA *p*-value	Tillering	Heading	Grain-filling
Variety (V)	0.0369	0.0428	0.0358
Subspecies (S)	0.5875	0.3642	0.3991
Nitrogen level (N)	0.0087	0.0151	0.0058
S × N	0.0344	0.0403	0.0378
V × N	0.0174	0.0235	0.0155

We conducted a two-way ANOVA using the SAS enterprise program to explore the combined effects of rice variety and nitrogen level on methanogen abundance. The *p*-values obtained from the one-way ANOVA were significantly different at all rice growth stages. However, the rice subspecies did not have a significant effect. Contrastingly, the two-way ANOVA of S × N (Subspecies × Nitrogen level) and V × N (Variety × Nitrogen level) interactions demonstrated significant differences. These results suggest that both rice genotypes and nitrogen levels can influence and moderate methanogen inhabitance. Furthermore, combining specific rice varieties with LN levels may result in synergistic or additive effects, potentially reducing methanogen abundance. Previous studies focusing on mitigating greenhouse gas emissions have often examined individual factors such as rice varieties and nitrogen levels [30,31]. For instance, Kim et al. [31] reported that a LN input could reduce CH_4

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emissions without quantifying CH_4 emission-related microbes. Lee et al. [17] revealed that the characteristics and behavior of methanogens and methanotrophs in rice paddies can vary depending on the specific rice variety being grown. In other words, different rice varieties have a distinct impact on these microbial groups and their activities in the soil. In conclusion, our study highlights the importance of considering the combined effects of rice variety and nitrogen levels in modulating methanogen abundance. Indeed, this approach provides additional evidence that supports and expands on previous research that has focused on the effects of nitrogen and differentiation among rice varieties. By understanding these interactions, effective strategies could be developed to mitigate greenhouse gas emissions from rice cultivation.

5. Conclusions

In this study, we investigated the abundances of rhizospheric methanogens and methanotrophs in rice paddies at different nitrogen levels and rice growth stages. Using qPCR, we analyzed samples collected from six *Indica* and four *Japonica* subspecies at 0%, 50%, and 100% (conventional level) nitrogen levels during the tillering, heading, and grain-filling stages. Our results showed that nitrogen levels significantly affected the abundances of both methanogens and methanotrophs across all growth stages. Methanogen and methanotroph abundances were the highest at the tillering stage and gradually decreased until the grain-filling stage. Nitrogen application had a significant effect on methanogen and methanotroph abundance, with higher nitrogen levels resulting in more methanogens and fewer methanotrophs in the rice rhizosphere. Interestingly, the abundances varied depending on the rice variety, whereas no significant difference between the *Indica* and *Japonica* subspecies were observed, suggesting that methanogen and methanotroph inhabitants may prefer specific rice genotypes rather than subspecies. The methanogen/methanotroph ratio varied greatly under different nitrogen levels and growth stages, with higher ratios observed under higher nitrogen levels. Our findings indicate that reducing nitrogen fertilizer application can potentially lower CH_4 emissions by maintaining lower levels of methanogens in the soil and aligning them with low-input regimes to mitigate greenhouse gas emissions. In addition, targeting and managing methanogen levels during specific growth stages, particularly tillering and heading, could be instrumental in greenhouse gas reduction strategies. This study emphasizes the importance of understanding the methanogen/methanotroph ratio in different rice genotypes and highlights the potential benefits of reducing nitrogen fertilizer use to lower CH_4 emissions from rice cultivation. More effective strategies for mitigating greenhouse gas emissions can be developed by considering the combined effects of rice variety and nitrogen levels.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1: Agronomic traits of 10 rice varieties grown under different nitrogen levels.

Author Contributions: Conceptualization, J.-H.L. and Y.K.; methodology, Y.K. and S.-M.L.; software, J.-K.C.; validation, H.P., D.-S.P., and N.R.K.; writing—original draft preparation, Y.K.; writing—review and editing, J.-H.L.; supervision, J.-H.L., Y.-S.K., and K.-W.O.; project administration, K.-W.O.; funding acquisition, Y.K., J.-H.L., and K.-W.O. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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