Phylogenetically Distinct Phylotypes Modulate Nitrification in a Paddy Soil

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Paddy fields represent a unique ecosystem in which regular flooding occurs, allowing for rice cultivation. However, the taxonomic identity of the microbial functional guilds that catalyze soil nitrification remains poorly understood. In this study, we provide molecular evidence for distinctly different phylotypes of nitrifying communities in a neutral paddy soil using high-throughput pyrosequencing and DNA-based stable isotope probing (SIP). Following urea addition, the levels of soil nitrate increased significantly, accompanied by an increase in the abundance of the bacterial and archaeal *amoA* gene in microcosms subjected to SIP (SIP microcosms) during a 56-day incubation period. High-throughput fingerprints of the total 16S rRNA genes in SIP microcosms indicated that nitrification activity positively correlated with the abundance of *Nitrosospira*-like ammonia-oxidizing bacteria (AOB), soil group 1.1b-like ammonia-oxidizing archaea (AOA), and *Nitrospira*-like nitrite-oxidizing bacteria (NOB). Pyrosequencing of ^{13}C-labeled DNA further revealed that $^{13}CO_2$ was assimilated by these functional groups to a much greater extent than by marine group 1.1a-associated AOA and *Nitrobacter*-like NOB. Phylogenetic analysis demonstrated that active AOB communities were closely affiliated with *Nitrosospira* sp. strain L115 and the *Nitrosospira multiformis* lineage and that the ^{13}C-labeled AOA were related to phylogenetically distinct groups, including the moderately thermophilic "*Candidatus* Nitrososphaera gargensis," uncultured fosmid 29i4, and acidophilic "*Candidatus* Nitrosotalea devanaterra" lineages. These results suggest that a wide variety of microorganisms were involved in soil nitrification, implying physiological diversification of soil nitrifying communities that are constantly exposed to environmental fluctuations in paddy fields.

Rice feeds over half of the world's population and is usually considered the most important food source in Asia (1). China has approximately 29.2 million ha of rice fields, accounting for 35.8% of the grain-sowing area (2, 3). China is indeed the largest producer of rice on the planet, and the yield production of rice accounts for 43.7% of the total national grain production (4). The growth and production of rice crops depend heavily on anthropogenic management such as irrigation and fertilization. The intensified application of synthetic N fertilizers has increased significantly over the past decades to meet the demand for food productivity in China (5). The excessively high load often leads to the saturation of nitrogen nutrients in paddy soils and causes severe environmental pollution (6).

Flood management is the most dominant regime for growing semiaquatic rice plants (7). Irrigated paddy fields thus may serve as a model system for studying the microbial ecology of nitrifying communities in terrestrial environments (8). Nitrification is executed by functional microbial guilds, including ammonia-oxidizing bacteria and archaea (AOB and AOA, respectively), as well as nitrite-oxidizing bacteria (NOB). AOA and AOB perform the first and rate-limiting step of aerobic nitrification by oxidizing ammonia to nitrite, which is then rapidly converted to nitrate by NOB. During rice-growing seasons under flooding conditions, aerobic nitrification can proceed in surface and rhizosphere soils where oxygen is delivered by atmospheric diffusion and root release (9). Paddy fields during flooding seasons thus resemble freshwater ecosystems to some extent, with limited availability of dissolved oxygen. For example, *Nitrosomonas*-like AOB have been frequently observed in freshwater ecosystems (10, 11) and were also shown to be an important component of nitrifying communities in a Philippine rice field (12). However, unlike aquatic habitats, rice fields experience drastic changes in environmental conditions

due to agricultural management. For example, paddy fields are often drained in winter for wheat cultivation and flooded in summer during the rice-growing season. Moreover, paddy fields have been subjected to strong anthropogenic disturbances with intensified fertilization regimes and other interventions that could selectively drive community diversification of nitrifying communities (9). The constantly changing environment might have served as the primary force in shaping the structure and activity of nitrifying communities in paddy fields (13).

Numerous studies have focused on rice fields in warm, humid, subtropical zones with high precipitation (8, 14, 15). However, little information is available about nitrifying community structures in cold regions, arguably the most fertile and highest-quality regions, that account for 13% of the cultivated land in northeastern China (16). Furthermore, the mere presence of the respective genes does not always indicate functional importance. The direct link between the nitrification process and taxonomic identity of active microorganisms in paddy fields of northern China re-

mains elusive. Therefore, we employed microcosms subjected to DNA-based stable isotope probing (SIP microcosms) and high-throughput pyrosequencing to assess the autotrophic nitrification in paddy soils from northeastern China.

MATERIALS AND METHODS

Soil sampling. The soil samples for microcosm incubation were collected from an irrigated paddy field in Wuchang County, Haerbin City of Heilongjiang Province in northeastern China (45°10′N, 127°00′E). The soil is classified as a phaeozem, according to the *World Reference Base for Soil Resources 2006* (17). The paddy field is located in the center of the Mollisols in Northeastern China. The mollisol was derived from the parent materials that were sedimentary materials of loamy loess, according to U.S. Department of Agriculture soil taxonomy. The sampling site was characterized as a temperate continental monsoon climate, with a mean annual temperature of 3.5°C and precipitation of 625 mm. The field usually received urea fertilization with 200 to 400 kg N ha^{-1}, which is equivalent to 87.2 to 174 μg of urea-N g^{-1} (dry weight) of soil (g$_{DWS}$$^{-1}$), assuming an effective soil depth of 20 cm for fertilizer application. The composite soil sample was collected from 0- to 20-cm surface soils by mixing six random soil cores. The composite soil sample was passed through a 2.0-mm-pore-size sieve and stored at 4°C before construction of microcosms. The soil pH was determined using a Mettler Toledo 320-S pH meter (Mettler-Toledo Instruments Co. Ltd., Shanghai, China) with a water-to-soil ratio of 2.5. The soil organic matter content was determined using the dichromate oxidation method. Total N was determined by the Kjeldahl method. Ammonium and nitrate were extracted from soil samples with 2 M KCl, and the levels were determined using a Skalar San Plus segmented flow analyzer (Skalar Inc., Breda, The Netherlands).

DNA-SIP microcosms. Microcosms were constructed in triplicate as described previously and treated in three different ways (18). The microcosms were incubated with 5% (vol/vol) $^{13}CO_2$ plus [^{13}C]urea for the labeled treatment, and control microcosms were incubated with 5% $^{12}CO_2$ plus [^{12}C]urea and 5% $^{13}CO_2$ plus [^{13}C]urea with 100 Pa C_2H_2. For each treatment, fresh soil (equivalent to 5.0 g$_{DWS}$) was incubated at approximately 60% maximum water-holding capacity and at 28°C in the dark for 56 days in a 120-ml serum bottle sealed with a butyl stopper. The headspace of the bottles was aerated with synthetic air (20% O_2 and 80% N_2) for 1 min on a weekly basis to maintain oxic conditions and renewed with fresh gas and substrate accordingly. A total of 100 μg of urea-N g$_{DWS}$$^{-1}$ was pulsed into the soil once a week to establish a substrate-rich environment for nitrifying communities over the incubation period of 56 days. Urea was the main N fertilizer used during growing seasons, which was believed to be immediately hydrolyzed by extracellular ureases into ammonia and carbon dioxide, both of which allow ammonia oxidization in soils (19, 20). The 12-day preincubation was performed so that the soil-respired CO_2 would remain at a consistently low level during the entire period of incubation. For acetylene-amended microcosms, an additional 7-day preincubation with 100 Pa C_2H_2 was executed for full inactivation of ammonia oxidizers in soils. Soil samples at day zero were collected immediately after the 12-day preincubation for subsequent analysis. The destructive sampling was performed after 56 days. Then, 4.0-g soil samples were homogenized with 20 ml of 2 M KCl for inorganic nitrogen determination of NH_4^+-N and NO_3^--NO_2^--N using a Skalar San Plus segmented flow analyzer (Skalar Inc., Breda, The Netherlands). The remaining soil samples were kept frozen at −80°C for subsequent molecular analysis.

DNA extraction and SIP gradient fractionation. The total DNA in the soil samples was extracted and purified from 0.5 g (dry weight) of soil using a FastDNA spin kit for soil (MP Biomedicals, Cleveland, OH, USA), according to the manufacturer's instructions. The quantity and quality of DNA extracts were assayed using a NanoDrop ND-1000 UV-visible light spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). For isopycnic density gradient centrifugation to resolve ^{13}C-DNA from

native ^{12}C-DNA in the total DNA extract, approximately 3.0 μg of DNA was mixed with a CsCl stock solution to achieve a final volume of ~5.5 ml with a CsCl buoyant density of 1.725 g ml^{-1} before ultracentrifugation at 177,000 × g and 20°C for 44 h. The DNA fractions for each sample were collected by displacing the gradient medium with sterile water from the top of the ultracentrifuge tube using an NE-1000 single-syringe pump (New Era Pump Systems, Inc., Farmingdale, NY, USA) precisely controlled at a flow rate of 0.38 ml min^{-1}. A total of 15 gradient fractions were generated with equal volumes of 380 μl, and 60 μl of each fraction was used for refractive index measurement using an AR200 digital hand-held refractometer (Reichert, Inc., Buffalo, NY, USA). The fractionated DNA was purified with 70% ethanol after polyethylene glycol (PEG) 6000 precipitation and dissolved in 30 μl of Tris-EDTA (TE) buffer.

Real-time qPCR of *amoA* and 16S rRNA genes. Real-time quantitative PCR (qPCR) was performed in triplicate on a CFX96 Optical Real-Time detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) to determine the copy number of *amoA* genes in soil DNA extracts and DNA gradient fractions to observe the changes in population sizes of AOA and AOB communities and to assess the $^{13}CO_2$ labeling of *amoA*-carrying ammonia oxidizers. A universal 16S rRNA gene qPCR was conducted to assess the total copy numbers of bacterial and archaeal communities in soils with different treatments. The PCR primers and thermal conditions are detailed in Table S1 in the supplemental material. The real-time quantitative PCR standard was generated using plasmid DNA from one representative clone containing bacterial or archaeal *amoA* and 16S rRNA genes, and a dilution series of standard template over eight orders of magnitude per assay was used. Amplification efficiencies ranged from 82% to 105%, with R^2 values of 0.996 to 0.999. In addition, total DNA extracts were diluted in a series to assess the possible PCR inhibition by coextraction of humic substances, and DNA extracts were diluted 10-fold for subsequent analysis. Melting curve analysis and standard agarose gel electrophoresis were always performed at the end of a PCR run to verify the amplification specificity.

Pyrosequencing of 16S rRNA and *amoA* genes. Pyrosequencing was employed to investigate the community shift of nitrifying phylotypes by analyzing the V4 regions of 16S rRNA genes in soils. The total microbial communities were analyzed in soil microcosms using universal primers for 16S rRNA genes to investigate the proportional changes of nitrifying phylotypes during active nitrification in the 56-day SIP microcosms. Meanwhile, pyrosequencing of the total 16S rRNA genes was also performed in the DNA gradient fractions from the $^{13}CO_2$-labeled and $^{12}CO_2$ control microcosms. For each treatment, a total of 12 fractions were selected for analysis of the total microbial communities to determine the $^{13}CO_2$ labeling of the 16S rRNA genes of nitrifying communities. Each DNA sample was amplified using the universal 515F-907R primer pair with adaptor, key sequence, and unique tag sequence (only in forward primer 515F). The PCR primers and conditions are described in Table S1 in the supplemental material. The resulting PCR products were gel purified and combined in equimolar ratios into a single tube in preparation for pyrosequencing analysis. The pyrosequencing was conducted on a Roche 454 GS FLX Titanium sequencer (Roche Diagnostics Corporation, Branford, CT, USA).

Bacterial and archaeal *amoA* genes from the ^{13}C-labeled DNA fraction with a CsCl buoyant density of 1.7399 (heavy fraction) were amplified for pyrosequencing. The primer pairs amoA-1F/2R and CrenamoA-23f/616r (see Table S1 in the supplemental material) fused with the tag sequence in the forward primers were used for the amplification of bacterial and archaeal *amoA* genes, respectively. The resulting PCR products were prepared and pyrosequenced as described above.

All pyrosequencing data were processed using the mothur software package (21). The *amoA* and 16S rRNA gene reads for each sample were sorted by specific tag sequences. The resulting reads were further trimmed, and only sequences of >350 bp in length with an average quality score >30 and no ambiguous base calls were included for subsequent
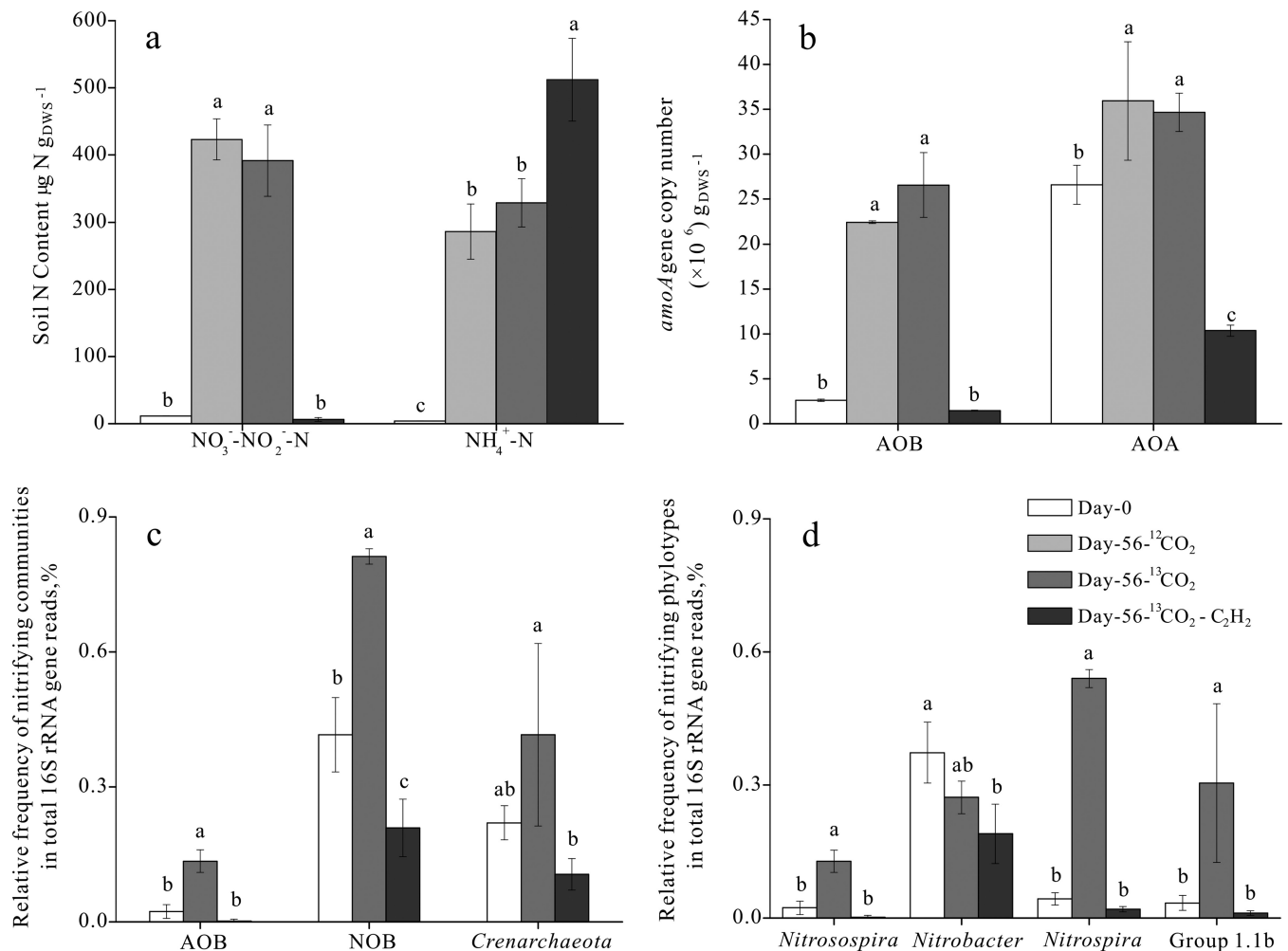
FIG 1 Changes in concentrations of soil inorganic N (a), in *amoA* gene abundance in ammonia-oxidizing bacteria (AOB) and archaea (AOA) (b), and in relative frequencies of 16S rRNA genes of nitrifiers (c) and of specific phylotypes (d) in total pyrosequencing reads over a 56-day microcosm incubation. Soil microcosms were fertilized with 100 μg urea-N g_{DWS}^{-1} on a weekly basis for 8 weeks. The relative frequency of group-specific 16S rRNA genes was defined as the proportion of target reads to the total high-quality 16S rRNA gene reads in SIP microcosms. Error bars represent standard errors of triplicate microcosms. Different letters above the columns indicate a significant difference ($P < 0.05$).

analysis. The high-quality reads of *amoA* genes were further converted into their protein sequences for quality control, and sequences with stop codons and frameshifts were removed (22). For 16S rRNA genes, the high-quality sequences were aligned and classified into different taxonomic categories based on the SILVA database embedded in mothur. The target reads of nitrifying phylotypes were further screened for phylogenetic analyses of AOB and AOA. All reads classified as belonging to the genus *Nitrosospira* and the phylum *Crenarchaeota* were selected and clustered into operational taxonomic units (OTUs) with a 97% similarity cutoff (23). A representative sequence of each OTU was used for phylogenetic analysis using the MEGA software package, version 4.0, through inference of a neighbor-joining tree using the Kimura two-parameter distance with 1,000 replicates to produce bootstrap values (24). Taxonomic assignment was accomplished by binning sequences into OTUs with a 97% similarity cutoff and analyzing a representative sequence by MEGA as described above.

Statistical analysis. One-way analysis of variance (ANOVA) with Tukey's *post hoc* test was performed for multiple comparisons. All analyses were conducted using the SPSS, version 13.0, package for Windows (SPSS, Inc.). Test results with a *P* level of <0.05 were regarded as statistically significant.

Nucleotide sequence accession numbers. All pyrosequencing reads, including 16S rRNA genes and archaeal and bacterial *amoA* genes, have been deposited in the European Nucleotide Archive (ENA), with accession numbers ERS667669, ERS670107, and ERS670108, respectively. The ^{13}C-labeled sequences from SIP experiments for phylogenetic tree construction have been deposited in GenBank under accession numbers KP890804 to KP890821 for the *amoA* and 16S rRNA genes.

RESULTS

Soil properties and nitrification activity. The soil used in this study has a pH value of 5.99 (H$_2$O) and contains relatively high organic matter, with 21.9 mg per gram (dry weight) of soil (g_{DWS}^{-1}). Soil ammonium, nitrate, and total nitrogen levels were 2.20 μg NH$_4^+$-N g_{DWS}^{-1}, 6.01 μg NO$_3^-$-N g^{-1} g_{DWS}^{-1}, and 1.06 mg total N g_{DWS}^{-1}, respectively. Soil nitrification activity was assessed as the rate of increase in the concentrations of nitrate and nitrite in soil microcosms over the 56-day incubation period. In the absence of C$_2$H$_2$, significant production of soil nitrate and nitrite was observed after incubation for 56 days (Fig. 1a). There was no significant difference in soil nitrate and nitrite production

between the labeled $^{13}CO_2$ and control $^{12}CO_2$ microcosms, in which net nitrification rates were estimated to be approximately 6.78 and 7.35 μg NO_3^--NO_2^--N g_{DWS}^{-1} day^{-1}, respectively. The increase in soil nitrate and nitrite content corresponded well with the decrease of NH_4^+-N. Moreover, C_2H_2 addition completely abolished production of soil nitrate and nitrite, resulting in significant accumulation of soil ammonium due to the hydrolysis of urea (Fig. 1a). C_2H_2 inhibition indicated that ammonium from urea hydrolysis was almost completely converted to nitrate in soil microcosms, suggesting the predominant role of nitrification in SIP microcosms. For instance, the amount of ammonium in C_2H_2-amended soil was largely similar to that of soil nitrate contents in soil microcosms without C_2H_2. Urea-derived ammonium could still be recovered after a 56-day incubation period (Fig. 1a), and acetylene completely blocked ammonia oxidation but not urea hydrolysis in the controls (Fig. 1a), indicating that the hydrolysis was independent of the nitrification process.

Abundance change of soil nitrifying communities. Bacterial and archaeal *amoA* genes were quantified by real-time quantitative PCR to investigate the changes in population sizes of AOB and AOA communities over the 56-day incubation of SIP microcosms. The bacterial *amoA* genes increased from $2.60 \times 10^6 \pm 0.12 \times 10^6$ copies g_{DWS}^{-1} at day 0 to $2.66 \times 10^7 \pm 0.36 \times 10^7$ and $2.24 \times 10^7 \pm 0.01 \times 10^7$ copies g_{DWS}^{-1} at day 56, representing 10.2- and 8.62-fold increases in the $^{13}CO_2$-labeled treatment and $^{12}CO_2$ control microcosms, respectively (Fig. 1b). In comparison, archaeal *amoA* genes underwent only 1.30- and 1.35-fold increases, from $2.66 \times 10^7 \pm 0.22 \times 10^7$ copies g_{DWS}^{-1} at day 0 to $3.47 \times 10^7 \pm 0.21 \times 10^7$ and $3.59 \times 10^7 \pm 0.66 \times 10^7$ copies g_{DWS}^{-1} in the $^{13}CO_2$-labeled treatment and $^{12}CO_2$ control microcosms at day 56, respectively (Fig. 1b). In the presence of 100 Pa C_2H_2, soil microcosms showed no increasing trends for either bacterial or archaeal *amoA* genes (Fig. 1b), suggesting successful inhibition of both AOB and AOA nitrification activity by C_2H_2 in this study. The change in *amoA* gene abundance could not be unequivocally attributed to ammonia oxidation activity although the abundance of archaeal *amoA* genes increased at a significant level.

The high-throughput fingerprinting of 16S rRNA genes at the whole-community level provided a powerful strategy for profiling the relative abundance change in soil nitrifying communities. Pyrosequencing was performed for soil microcosms at day 0 and for SIP microcosms amended with $^{13}CO_2$ in the presence and absence of C_2H_2 at day 56 (see Tables S2 and S4 in the supplemental material for a pyrosequencing summary). It was interesting that the incubation of SIP microcosms appeared to have resulted in no significant change in the abundance of total 16S rRNA genes and community structures at the phylum level (see Fig. S1 in the supplemental material), and the alpha diversity index of Chao1 and OTUs remained largely unchanged over the course of incubation (see Fig. S2a and b). However, the divergence of microbial composition was clearly evidenced by a multidimensional scaling (MDS) beta diversity analysis (see Fig. S2). This is likely attributed to the remarkable proportional increases of nitrifying phylotypes over the 56-day incubation. For instance, the 16S rRNA genes affiliated with AOB and NOB rose from 0.0233% and 0.416% of total reads at day 0 to 0.135% and 0.812% at day 56, representing a 5.79-fold and 1.95-fold increase over the 56-day incubation, respectively (Fig. 1c). Among the AOB and NOB communities, *Nitrosospira* and *Nitrospira*-like 16S rRNA genes responded to

urea fertilization most positively, accounting for $0.128 \pm 0.025\%$ and $0.540 \pm 0.020\%$ of total 16S rRNA gene reads at day 56, respectively (Fig. 1d). Interestingly, *Nitrobacter*-like genes dominated NOB communities in soil microcosms at day 0 but decreased by 27.1% in frequency after the 56-day incubation with $^{13}CO_2$ and urea fertilization (Fig. 1d). Crenarchaeal 16S rRNA gene reads increased from 0.220% at day 0 to 0.416% at day 56 in the $^{13}CO_2$-labeled treatment (Fig. 1c). Furthermore, sequencing analysis demonstrated that members within the soil group 1.1b lineage dominated the archaeal communities in the SIP microcosms, with an 8.91-fold increase from $0.034 \pm 0.017\%$ to $0.304 \pm 0.179\%$ after the 56-day incubation (Fig. 1d). SIP microcosms treated with C_2H_2 showed no significant increase in relative abundances of 16S rRNA genes of nitrifiers at different taxonomic levels (Fig. 1c and d). Intriguingly, the absolute abundance of these functional guilds showed no significant changes among different treatments on the basis of pyrosequencing and real-time quantification analysis of total 16S rRNA genes (see Fig. S1a in the supplemental material).

Stable isotope probing of soil nitrifying communities. Quantitative analysis of *amoA* gene abundance as a function of DNA buoyant densities of isopycnic centrifugation gradients was exploited to assess the labeling efficiency of the *amoA* gene carrying ammonia oxidizers in SIP microcosms. A shift toward heavy fractions was observed for bacterial *amoA* gene abundance in the $^{13}CO_2$-labeled treatment compared to fractions in the $^{12}CO_2$ controls (Fig. 2a). The highest copy number of bacterial *amoA* genes in the $^{13}CO_2$-labeled treatment peaked in the heavy DNA fraction, with a buoyant density of 1.740 g ml^{-1}, whereas in the $^{12}CO_2$ control treatment, the highest number of *amoA* genes occurred in the light DNA fraction, with a buoyant density of 1.723 g ml^{-1} (Fig. 2a). These results indicated that a considerable amount of ^{13}C was assimilated by AOB in the labeled soils, leading to a significant shift of *amoA* gene-carrying genomic DNA into the heavy DNA fractions. In comparison, only a minor shift into the heavier fractions was detected for archaeal *amoA* genes from the labeled soils, suggesting that AOA were labeled to a lesser extent than AOB (Fig. 2b).

Pyrosequencing of total 16S rRNA genes in the fractionated DNA across a wide range of buoyant densities was performed to assess the enrichment of target nitrifying communities in the heavy DNA fractions from $^{13}CO_2$-labeled treatment in comparison to that of those from the $^{12}CO_2$ control microcosms (see Table S5 in the supplemental material). High-quality 16S rRNA gene reads were subjected to taxonomic classification, and nitrifying phylotypes were selected for further analyses (see Table S5). For 16S rRNA genes affiliated with AOB and NOB, a significant enrichment was observed in the heavy DNA fractions from $^{13}CO_2$-labeled treatment but not from $^{12}CO_2$ control microcosms. For instance, *Nitrosospira*-like AOB accounted for 6.67% of the total 16S rRNA gene reads in the heavy DNA fraction from the labeled treatment but represented only a tiny fraction, no more than 0.654%, of the total reads in the $^{12}CO_2$ control microcosms (Fig. 3a). Similar results were obtained for the NOB communities. *Nitrobacter*- and *Nitrospira*-like 16S rRNA genes were enriched up to 4.16% and 18.7% in the heavy DNA fractions from $^{13}CO_2$-labeled soil, respectively (Fig. 3c and d). In stark contrast, the highest relative frequencies of *Nitrobacter*- and *Nitrospira*-like 16S rRNA genes were only 0.423% and 0.844%, respectively, in the DNA fractions from $^{12}CO_2$ control microcosms (Fig. 3c and d).
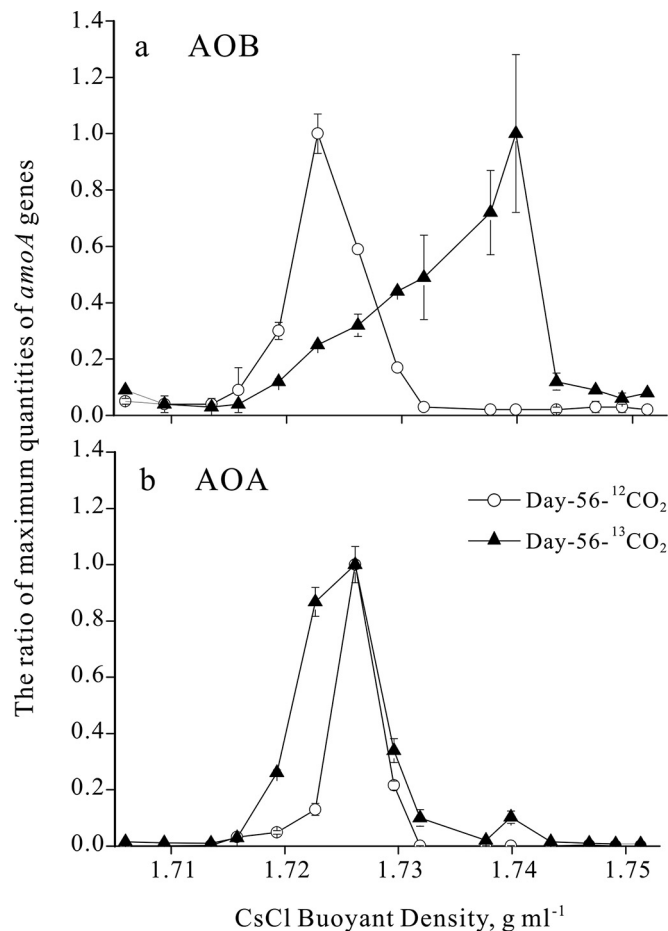
FIG 2 The quantitative distribution of bacterial (a) and archaeal (b) *amoA* genes across the entire buoyant density gradient of the DNA fractions from soil microcosms incubated with $^{12}CO_2$ and $^{13}CO_2$. The normalized data are the ratios of the gene copy number for each DNA gradient to the maximum quantities for each treatment. The error bars represent the standard errors of triplicate microcosms, and each contains three technical replicates.

Pyrosequencing analysis further indicated apparent enrichment of archaeal communities in the heavy DNA fractions from $^{13}CO_2$-labeled microcosms but not from the $^{12}CO_2$ control microcosms (see Table S5 in the supplemental material). Crenarchaeal 16S rRNA genes were selected from the total reads and further clustered into OTUs with a 97% cutoff. Sequencing analysis indicated that the total reads of archaeal 16S rRNA genes within the soil group 1.1b lineage were enriched up to 6.75% in the heavy DNA fractions from $^{13}CO_2$-labeled treatment (Fig. 3b). Furthermore, the relative frequency of members within the marine 1.1a-associated lineage was also apparently higher in the labeled treatment than in the control microcosms, even though members of this lineage were much less abundant than the soil 1.1b-associated archaea (see Table S5). These results suggested substantial assimilation of ^{13}C label by AOA, AOB, and NOB cells in the soil tested, implying an active role in soil nitrification.

Phylogenetic analysis of active ammonia-oxidizers. Phylogenetic analysis of all 849 of the AOB-like 16S rRNA gene reads in the ^{13}C-DNA fraction from the $^{13}CO_2$-labeled treatment revealed that the active AOB genes were predominantly contributed by four *Nitrosospira*-like OTUs. The representative reads from these

OTUs were phylogenetically most closely related to AOB strains *Nitrosospira* sp. strain L115 and *N. multiformis* within the *Nitrosospira* cluster 3 lineage (Fig. 4a). Bacterial *amoA* genes from the same fraction predominantly belonged to five OTUs, with representative reads also related to *Nitrosospira* sp. strain L115 and *N. multiformis* (Fig. 4b).

A total of 607 reads assigned to the phylum *Crenarchaeota* were retrieved in heavy DNA fraction 5 for phylogenetic analysis of AOA (Fig. 5a). Of the *Crenarchaeota* reads, 97.4% fell within AOA-like lineages, and the majority of the crenarchaeal 16S rRNA gene reads were phylogenetically closely related to "*Candidatus* Nitrososphaera gargensis" and fosmid clone 29i4, both within soil group 1.1b lineages (25). Archaeal *amoA* genes from the same fraction clustered into "*Candidatus* Nitrososphaera gargensis," fosmid 29i4, and 54d9-related OTUs (Fig. 5b) although the abundance of "*Ca*. Nitrososphaera gargensis"-like genes was significantly higher (Fig. 5b).

DISCUSSION

Rice paddy ecosystems receive intensive fertilization with ammonia-based fertilizers to support crop production (9). Agricultural interventions such as frequent flooding and fertilizations lead to strong fluctuations in available ammonia and oxygen that could likely shape habitat-specific communities of soil AOA and AOB. Our results provide compelling evidence for active nitrification that was catalyzed by a wide variety of phylogenetically distinct microorganisms in a complex rice soil compared to microcosms of other upland soils (26–28). AOB and NOB apparently dominated soil nitrification in the incubations, whereas active archaeal ammonia oxidizers were phylogenetically closely related to both soil group 1.1b- and marine group 1.1a-associated lineages. These results suggested that nitrification was driven by diverse nitrifying phylotypes with potentially distinct physiological versatilities, implying that the life strategy of soil nitrifying communities is more complex than previously appreciated.

DNA-based SIP strongly indicated bacterial dominance of nitrification activity in microcosms of paddy soil tested over the incubation period. The nitrification process supported greater AOB multiplication than that of AOA, as demonstrated by a much higher enrichment of bacterial *amoA* gene copies in the heavy fractions of ^{13}C-labeled DNA. Quantitative PCR showed that 53.9% of AOB communities were labeled during nitrification (Fig. 2a). Assuming that one AOB cell contains 2.5 copies of *amoA* genes on average, ammonia oxidation by labeled AOB would reach an estimated cell-specific rate of 3.53 fmol N cell^{-1} h^{-1}, which is well within the previously reported range for AOB communities (29). In fact, the ^{13}C-labeled AOB in this study were phylogenetically closely related to *Nitrosospira* sp. strain L115, with a similar specific activity of 5.3 fmol N cell^{-1} (30). Thus, AOB alone could account for the soil nitrification activity observed in SIP microcosms over the 56-day incubation course. In stark contrast, the labeled AOA cells (approximately 4.73%) (Fig. 2b) would contribute no more than 8.13% of the total nitrate production, assuming that they all reached the maximum specific rate of archaeal ammonia oxidation previously documented (29, 31). These results agreed well with previous findings that AOB dominated ammonia oxidation in ammonia-rich soils (18, 27, 32, 33). Similarly, the paddy field in our study received relatively high nitrogen input with urea-based fertilizers during growing seasons, which might select for AOB communities with a much higher
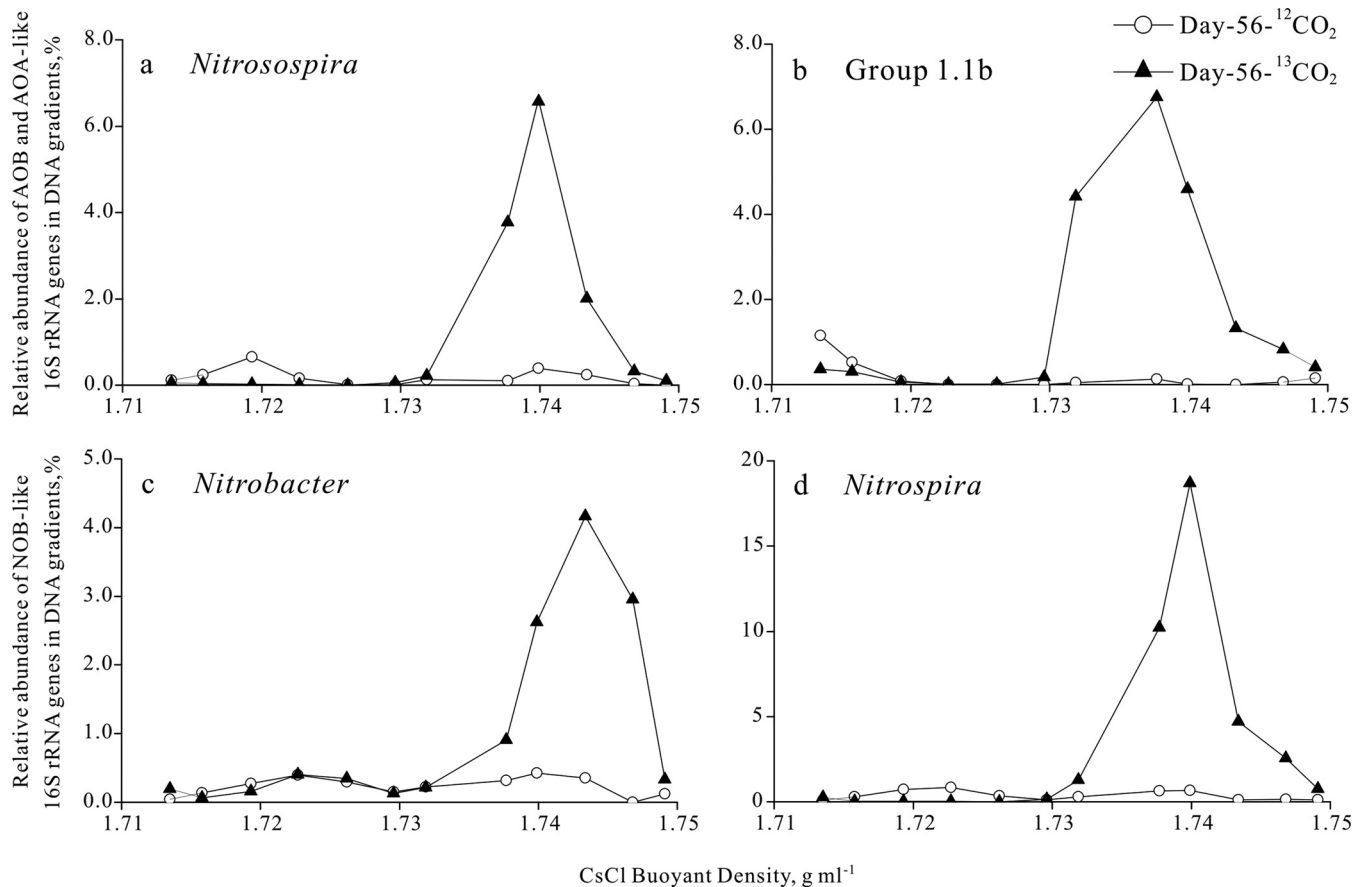
FIG 3 The relative frequency of *Nitrosospira*-like AOB (a), soil group 1.1b AOA (b), *Nitrobacter*-like NOB (c), and *Nitrospira*-like NOB (d) across the entire buoyant density gradient fractionated from DNA extraction in $^{13}CO_2$-labeled and $^{12}CO_2$ control soil microcosms. The relative frequency of 16S rRNA genes of each nitrifier group was determined as the proportion of target reads to all high-quality 16S rRNA gene reads in each fraction.

growth rate and activity than those of AOA (34). This explains the rapid growth of AOB upon urea fertilization in this study (Fig. 1b). It is worth noting that the samples were collected only once at day 56. Therefore, the cell-specific rates of ammonia oxidation in the SIP microcosm was calculated based on an assumption that a linear increase of nitrate production and cell propagation had occurred over a 56-day incubation period. Temporal sampling would likely reduce the uncertainty associated with cell-specific rate estimation and result in a better understanding of distinct nitrification potentials among nitrifiers in the soil tested.

A direct link between ammonia oxidation and AOA within soil group 1.1b in complex soil was reported in only a few studies (27, 33, 35, 36) although this group was often considered to be numerically dominant in neutral and/or alkaline soils (37–39). Our results provided evidence that archaeal ammonia oxidation in complex soil was not restricted to "*Ca.* Nitrososphaera gargensis"-like AOA, as previously reported (27, 33), but, rather, 29i4-like AOA catalyzed a portion of archaeal nitrification in the paddy soil tested in this study (Fig. 5; see also Table S6 in the supplemental material). Nitrification activity of "*Ca.* Nitrososphaera gargensis"-like AOA was often associated with upland soils, whereas 29i4-like AOA appeared to be well adapted to low-temperature arctic soil (40) and regularly oxygen-deficient niches in freshwater sediments (41). The widespread presence of the fosmid clone 29i4-related lineage has long been observed (42), but the physiological

capability for ammonia oxidation has been demonstrated only very recently in arctic soils (40). We speculate that 29i4-like AOA might possess a greater capacity for stress resistance; thus, niche differentiation of archaeal communities might have been stronger in paddy fields than in upland soils. Furthermore, the acidophilic "*Candidatus* Nitrosotalea devanaterra"-like AOA are often found in acidic soil and oligotrophic freshwater environments. Our results demonstrated that these AOA could survive in the neutral paddy soil tested in this study (Fig. 5a; see also Table S5 in the supplemental material). Although the sequences affiliated with this marine AOA-associated lineage have been obtained from neutral or only slightly acidic environments (43–45), the evidence directly linking this group to ammonia oxidation was missing. Thus, a greater physiological versatility of this marine lineage was observed in this study, despite reduced labeling compared to that of AOA associated with soil group 1.1b.

Nitrite oxidation has received much less attention than ammonia oxidization, and the physiological diversity of NOB is poorly understood (46). Our results showed that active NOB in this study were restricted to the well-known *Nitrospira*- and *Nitrobacter*-like groups. Surprisingly, a distinct ecophysiology was observed between *Nitrospira*- and *Nitrobacter*-like NOB in the paddy soil tested. The latter predominated in the background communities of nitrite oxidizers, accounting for 89.7% of total NOB-like reads before SIP microcosm incubation (Fig. 1d). However, the former
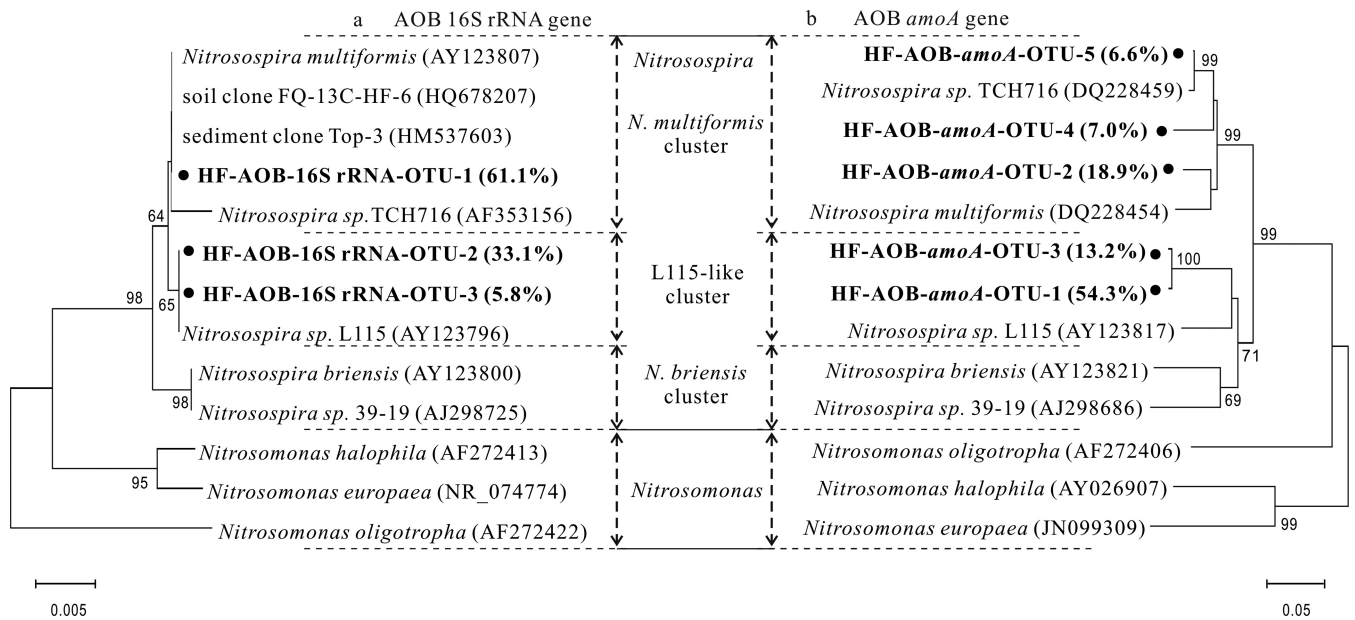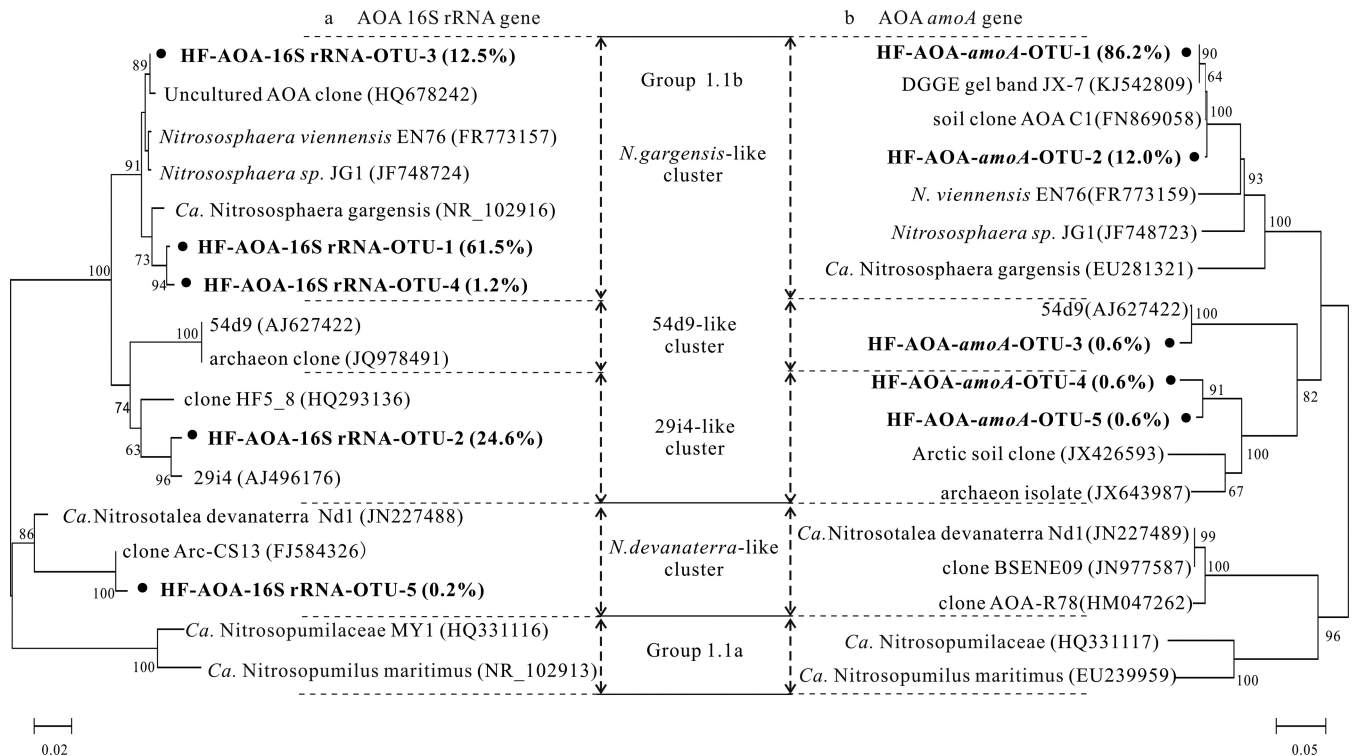
FIG 4 Phylogenetic analysis of the 16S rRNA (a) and *amoA* genes (b) of ammonia-oxidizing bacteria (AOB) in ^{13}C-labeled DNA from the $^{13}CO_2$-treated microcosms of 56-day microcosms. The phylogenetic tree shows the relationship of gene sequences of AOB from the heavy fraction (HF) to those deposited in GenBank. The notation is according to the following examples: HF-AOB-16S rRNA-OTU-1 (61.1%) represents OTU-1 and contains 61.1% of the total AOB 16S rRNA gene reads in the ^{13}C-DNA reads with >97% sequence similarity; HF-AOB-*amoA*-OTU-1 (54.3%) represents OTU-1 and contains 54.3% of the bacterial *amoA* gene reads in the ^{13}C-DNA reads with >97% sequence similarity. Bootstrap values higher than 60% are indicated at the branch nodes. GenBank accession numbers are given in parentheses.

boomed in active cell division and reached 89.6% of the total NOB 16S rRNA reads in the ^{13}C-DNA (Fig. 2d). *Nitrospira* and *Nitrobacter* were considered to be the two main players for nitrification in terrestrial ecosystems (47) but with distinct kinetic strategies for cell growth and activity. *Nitrospira* was hypothesized to be a *K* strategist, which possesses high nitrite affinity but much lower cell-specific activity than the *r* strategist *Nitrobacter*, as shown in culture studies (48–50) and soil assays (51). In fact, pyrosequencing of the total microbial communities showed a remarkable increase of *Nitrospira*-like but not *Nitrobacter*-like NOB in SIP microcosms during active nitrification (Fig. 1d). This drastic difference in the relative abundances between ^{13}C-labeled *Nitrobacter*- and *Nitrospira*-like NOB indicated their different roles in nitrite oxidation. This difference also implies that soil nitrite was kept at a constantly low concentration in the SIP microcosms although the temporal dynamics of soil nitrite were not measured (Fig. 1a).

This study demonstrated the existence of diverse phylotypes of active nitrifiers with potential physiological distinctions. This outcome suggested the strong adaptation of nitrifiers to constantly changing environmental conditions in the paddy soil tested. Rice cultivation necessitates regular flooding and intensive fertilization, leading to drastic changes in soil oxygen and ammonia concentrations in the field. Nitrifying communities could thus have experienced stronger stresses in paddy fields than in upland soils, which constantly receive oxygen diffusion from the atmosphere without blockage from the water layer. We speculated that the long-term environmental fluctuation in paddy fields could create certain scenarios where distinctly different nitrifiers were selectively favored with different competitive statuses under specific conditions. The generally high availability of ammonia and oxy-

gen in our microcosms apparently favored growth of AOB over that of AOA groups. Nitrification leading to possible decreases in pH and ammonia concentration might have stimulated AOA growth occasionally. Furthermore, agricultural management allows the creation of soil microsites with highly heterogeneous environmental conditions in the paddy soil; therefore, nitrification might be performed by different nitrifier combinations. These hypotheses might partially explain the existence of distinct active phylotypes usually found in different niches. For instance, active AOA in this study were phylogenetically most closely affiliated with three environmentally distinct cultures and enrichments. "*Ca.* Nitrososphaera gargensis"-and 29i4-like AOA were enriched from a hot spring and cold arctic soil, respectively, while "*Ca.* Nitrosotalea"-like sequences were isolated only from oligotrophic freshwater or acid soils. It is very likely that NOB communities have evolved in close association with ammonia oxidizers (52). However, it remains unclear how these functional groups interact with one another and generate distinct nitrifying communities in the paddy soil tested.

It is worth noting that artificial incubation of SIP microcosms in this study could not represent what is naturally occurring under field conditions. Paddy soils during rice growing season would have rarely experienced high concentrations of oxygen and ammonium simultaneously, as they did in this study. The enrichment of nitrifying communities might have resulted from the succession of microbial communities over the course of incubation of SIP microcosms (see Fig. S2 in the supplemental material). For instance, numerically less abundant *Nitrospira*-like NOB under *in situ* conditions significantly outnumbered *Nitrobacter*-like NOB after laboratory incubation for 56 days (see Table S3 in the supplemental material), suggesting preferential growth of the former

FIG 5 Phylogenetic analysis of the 16S rRNA (a) and *amoA* genes (b) of ammonia-oxidizing archaea (AOA) in ^{13}C-labeled DNA from the $^{13}CO_2$-treated microcosms of 56-day microcosms. The phylogenetic tree shows the relationship of gene sequences of AOA from the heavy fraction (HF) to those deposited in GenBank. The notation is according to the following examples: HF-AOA-16S rRNA-OTU-1 (61.5%) represents OTU-1 and contains 61.5% of the total AOA 16S rRNA gene reads in the ^{13}C-DNA reads with >97% sequence similarity; HF-AOA-*amoA*-OTU-1 (86.2%) represents OTU-1 and contains 86.2% of the archaeal *amoA* gene reads in the ^{13}C-DNA reads with >97% sequence similarity. Bootstrap values higher than 60% are indicated at the branch nodes. GenBank accession numbers are given in parentheses.

under artificial conditions. In addition, the population dynamics during the 56-day laboratory incubation remain unclear due to the lack of temporal sampling. However, one recent study has shown that nitrogen fertilization intensity and the sampling time point had only small effects on the abundance, composition, and activity of AOA, AOB, and NOB populations (47). Our recent study also demonstrated that the labeled AOA showed high degrees of similarity in SIP microcosms at day 28 and day 56 (26). Furthermore, the labeled nitrifiers are largely similar to those found under *in situ* conditions, implying that these functional groups might be selected for and acclimated to the paddy soil tested. We speculate that long-term physiochemical conditions formed seed banks of active nitrifiers and that incubation of SIP microcosms could provide some clues as to which organisms might have been actively involved in nitrification under field conditions. The actual nitrification dynamics of different phylotypes under field conditions should be further studied, in addition to cultivation studies and comparative ecophysiological analysis of functional guilds across physiochemically different environments.

Taken together, the results of this study clearly demonstrated autotrophic nitrification by AOA, AOB, and NOB communities in a neutral paddy soil that is exposed to periodic flooding and fertilization regimes. Stable isotope probing suggested that the genomes of phylogenetically distinct nitrifiers were labeled to different extents. The active AOB were phylogenetically closely related to the strains isolated from acidic soil, while the labeled AOA showed high sequence similarity to those from hot springs and arctic soils. These results imply that phylogenetic relatedness might not necessitate physiological similarity and that the activity of phylogenetically distinct nitrifiers might vary with environmental fluctuations under field conditions in a paddy field.

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