

SHORT COMMUNICATION

Nitrification of archaeal ammonia oxidizers in acid soils is supported by hydrolysis of urea

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The hydrolysis of urea as a source of ammonia has been proposed as a mechanism for the nitrification of ammonia-oxidizing bacteria (AOB) in acidic soil. The growth of *Nitrososphaera viennensis* on urea suggests that the ureolysis of ammonia-oxidizing archaea (AOA) might occur in natural environments. In this study, ¹⁵N isotope tracing indicates that ammonia oxidation occurred upon the addition of urea at a concentration similar to the *in situ* ammonium content of tea orchard soil (pH 3.75) and forest soil (pH 5.4) and was inhibited by acetylene. Nitrification activity was significantly stimulated by urea fertilization and coupled well with abundance changes in archaeal *amoA* genes in acidic soils. Pyrosequencing of 16S rRNA genes at whole microbial community level demonstrates the active growth of AOA in urea-amended soils. Molecular fingerprinting further shows that changes in denaturing gradient gel electrophoresis fingerprint patterns of archaeal *amoA* genes are paralleled by nitrification activity changes. However, bacterial *amoA* and 16S rRNA genes of AOB were not detected. The results strongly suggest that archaeal ammonia oxidation is supported by hydrolysis of urea and that AOA, from the marine Group 1.1a-associated lineage, dominate nitrification in two acidic soils tested.

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Ammonia, rather than ammonium ion, has been thought to be the actual substrate for ammonia-oxidizing bacteria (AOB) because the Michaelis constant calculated from ammonia plus ammonium exponentially declines with decreasing pH value but remains almost unchanged when calculated as a function of ammonia concentration (Suzuki *et al.*, 1974; Frijlink *et al.*, 1992). The ammonia concentration in acidic soil is generally too low to support the growth of all known AOB isolates because of the ionization of ammonia to ammonium (Allison and Prosser, 1991; Burton and Prosser, 2001; Gubry-Rangin *et al.*, 2011). Until recently, the microorganisms responsible for the unsuspected nitrification activity in acidic soil have remained unidentified. The discovery of *Nitrosotalea devanattera* provides

conclusive evidence for the existence of acid-tolerant ammonia-oxidizing archaea (AOA) and demonstrates that at least some AOA possess extraordinary affinity to the substrate and grow at extremely low ammonia concentrations of 0.18 nM (Lehtovirta-Morley *et al.*, 2011). The isolation of obligate acidophilic *N. devanattera* revealed the important role of archaeal ammonia oxidation in acidic soils, as previously suggested in acidic forest (Boyle-Yarwood *et al.*, 2008; Stopnisek *et al.*, 2010) and agricultural soils (Nicol *et al.*, 2008; Offre *et al.*, 2009). However, there is also evidence for bacterial ammonia oxidation in acidic soil (Onodera *et al.*, 2010; Ying *et al.*, 2010). The existence of the mechanisms in AOB, which allow them to grow under highly acidic conditions, in AOA remains unclear (De Boer and Kowalchuk, 2001). For example, AOB isolated from acidic soil are generally urease positive, and ureolysis can afford AOB a greater advantage to nitrify in acidic soil (Burton and Prosser, 2001; Lehtovirta-Morley *et al.*, 2011).

The first isolation of AOA from soil at pH 8.0 indicates that *Nitrososphaera viennensis* can grow on urea as an alternative energy source

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(Tourna *et al.*, 2011). Both urea transporter and urease gene were identified in the genomes of *N. viennensis* (Tourna *et al.*, 2011) and *Cenarchaeum symbiosum* (Hallam *et al.*, 2006). Environmental genomics has also revealed the genes that encode urease and urea transporters in marine habitats (Konstantinidis *et al.*, 2009; Yakimov *et al.*, 2011; Tully *et al.*, 2012). These results imply a mechanism in which ammonia is released through the intracellular hydrolysis of urea and subsequently oxidized by AOA, as previously shown for cultured AOB under acidic conditions (Burton and Prosser, 2001). However, *Nitrosopumilus maritimus* and *Nitrosoarchaeum limnia* showed no genetic potential to grow on urea because no urease genes were identified in their genomes (Walker *et al.*, 2010; Blainey *et al.*, 2011). Moreover, *N. devanattera* does not grow at neutral pH (Lehtovirta-Morley *et al.*, 2011) but urease-positive *N. viennensis* optimally grows at pH 7.5 (Tourna *et al.*, 2011). These findings imply that urease activity may be a feature of some AOA and is not strictly restricted to acidic environments, as previously shown for AOB isolates (Allison and Prosser, 1991). Therefore, we used microcosm incubations to assess whether nitrification activity is affected by urea fertilization in tea orchard (pH 3.75) and forest (pH 5.40) soils using ^{15}N pool enrichment and cultivation-independent techniques.

^{15}N isotope tracing

Soils at depths of 0–15 cm were collected on August 26, 2010 from tea orchards at the Tea Research Institute of the Chinese Academy of Agricultural Sciences and an adjacent forest site from which the tea orchard was converted in 1974. The tea orchard received approximately 600 kg of urea-N ha⁻¹ a⁻¹ with 3–4 split dressing applications over the last decades. Long-term field fertilization might have led to significant accumulation of nitrate in tea orchard field (Table 1). The site has been described previously in great detail (Han *et al.*, 2007). The soil was sieved through a 2-mm mesh, and microcosms were established in triplicate by adding 10 g of fresh soil in 250 ml Erlenmeyer flasks. The labeled

urea-N (^{15}N atom, 98% excess) was added to the microcosms at a final concentration of 5 µg of N per gram of dry weight soil (d.w.s.), which is similar to the ammonium content in soil under *in situ* conditions (Table 1). The soil microcosms were incubated at 60% of the maximum water-holding capacity of the soil for 5 days with or without acetylene at 100 Pa and 25 °C under dark conditions (Jia and Conrad, 2009). The production of soil ^{15}N -labeled nitrate and nitrite was measured as previously described (Zhang *et al.*, 2011). Acetylene completely abolished nitrification activity in the forest soil, whereas a slight increase in $^{15}\text{NO}_3^-$ -N was observed in the tea orchard soils after incubation for 5 days. In the absence of acetylene, the $^{15}\text{NO}_3^-$ -N concentration showed linear increases over the course of incubation (Figure 1). The nitrification activities in the forest and tea orchard soils were estimated to be 0.150 and 0.056 µg of $^{15}\text{NO}_3^-$ -N per gram d.w.s. per day, respectively. These activities fell well within the range from 0.051 to 0.459 µg of N per gram d.w.s. per day in acidic soils with pH < 5.5 across China, as shown by the results of both ^{15}N pool dilution and enrichment techniques (Zhang *et al.*, 2011). The addition of considerably low concentrations of urea suggests that nitrification could likely occur under *in situ* conditions. The nitrification activity had a three-fold decrease after the forest soil was converted to tea orchard soil. The tea orchard cultivation decreased the soil pH by 1.75 units over the last 36 years. Thus, it seems plausible that the stress induced by highly acidic conditions increased the energy demand for the growth of ammonia oxidizers and reduced the nitrification activity in tea orchard soils (Baker-Austin and Dopson, 2007).

Nitrification activity upon urea fertilization

The microcosms were further established in triplicate by fertilizing the soil with 100 µg of urea-N per gram d.w.s. once a week as described previously with slight modifications (Xia *et al.*, 2011). Thus, the soil microcosms received a total of 800 µg of urea-N

Table 1 Soil characteristics

Soil ^a	pH ^b (1:2.5 H ₂ O)	SOM ^c g kg ⁻¹	TN ^c g kg ⁻¹	Soil water content (%)	NO ₃ ⁻ -N µg g ⁻¹	NH ₄ ⁺ -N µg g ⁻¹	Predicted soil NH ₃ concentration (nM) ^d	Nitrification activity µg NO ₃ ⁻ -N · g ⁻¹ d ^{-1e}
TS	3.75	53.2	2.95	22.9	92.9	7.02	7.00	0.056
FS	5.40	25.2	1.13	22.0	8.53	4.77	221	0.150

^aTS and FS represent tea orchard soil and forest soil, respectively.

^bpH was measured with water:soil ratio of 2.5.

^cSOM and TN denote soil organic matter and total nitrogen, respectively.

^dSoil NH₃ concentration was estimated on the basis of soil water content, ammonium concentration, pH and adjusted for temperature (pK_a of 9.245 at 25 °C).

^eNitrification activity is determined by monitoring the $^{15}\text{NO}_3^-$ -N production upon addition of 5 µg urea-N (99% ^{15}N excess) to 1 g soil.

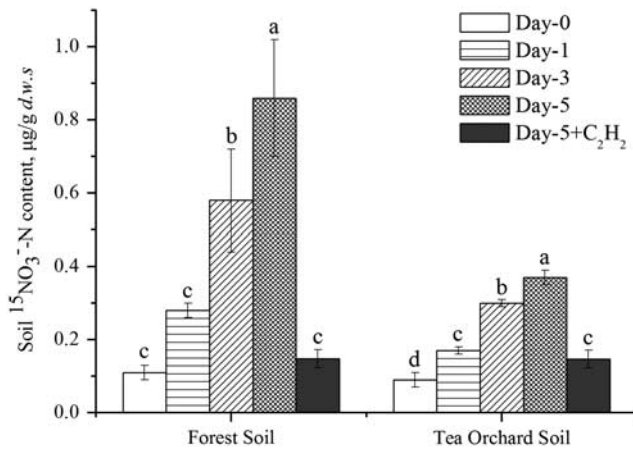


Figure 1 $^{15}\text{NO}_3^-$ -N content in soil microcosms at days 0, 1, 3 and 5. Microcosm incubation was performed by adding 5 μg of urea-N (99% ^{15}N excess) per gram d.w.s. at day 0. Day-5+C₂H₂ indicates incubation in the presence of 100 Pa C₂H₂. The error bars represent s.e.m. of the microcosm triplicates. The different letters above the columns indicate a significant difference ($P < 0.05$) using analysis of variance.

per gram d.w.s. over an incubation period of 8 weeks. The control microcosm was established by adding equal amounts of sterilized distilled water to the soil. As a result of ammonia released during mineralization of organic matters, significant amounts of nitrate were produced in the control microcosms after incubation for 8 weeks (Figure 2a). The net nitrification activities in tea orchard and forest soils were estimated to be 1.01 and 0.97 μg of NO_3^- -N per gram d.w.s. per day, respectively. These results are similar to previous findings for acidic agricultural soils, from which acidophilic *N. devanaterra* was isolated (Nicol *et al.*, 2008; Offre *et al.*, 2009; Gubry-Rangin *et al.*, 2010). Urea fertilization at a considerably high rate led to an almost four-fold increase in net nitrification activity in forest soil (4.73 μg of NO_3^- -N per gram d.w.s. per day) and 50% increase in tea orchard soil (1.58 μg of NO_3^- -N per gram d.w.s. per day). The activity changes were well paralleled by the abundance changes in archaeal *amoA* genes, as shown by the real-time quantitative PCR as described previously (Wu *et al.*, 2011). The copy number of archaeal *amoA* genes significantly increased from 1.82×10^8 at day 0 to 6.86×10^8 and 3.66×10^9 at day 56, showing 4- and 20-fold increases, after incubation for 8 weeks in forest soil microcosms receiving water or urea-N, respectively (Figure 2b). For the tea orchard soil, urea fertilization resulted in a 16-fold increase in the copy number of *amoA* genes from 9.90×10^7 to 3.31×10^8 after incubation for 8 weeks. However, no statistically significant increase was observed in soil microcosms added with water, suggesting that the growth of AOA was so slow that the subtle changes in *amoA* genes could not be detected by the techniques used. Bacterial *amoA* genes were not amplified despite the use of a range of degenerate primers and combinations (Stephen *et al.*, 1999;

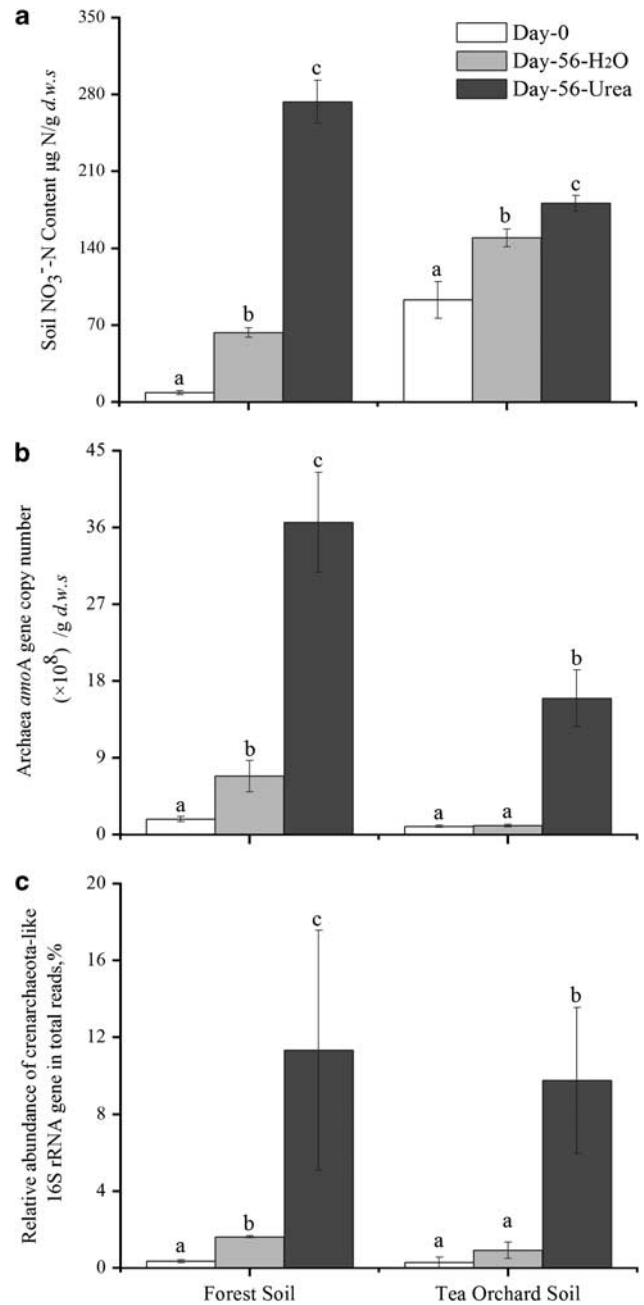


Figure 2 Change in the concentrations of (a) NO_3^- -N, (b) number of archaeal *amoA* genes, and (c) relative abundance of crenarchaeota-like 16S rRNA gene reads in soil microcosms over an incubation period of 8 weeks. Day-56-H₂O and day-56-Urea indicate that the soil microcosms received sterilized water or urea once a week, respectively. The error bars represent the s.e.m. of the microcosm triplicates. The relative abundance is expressed as the ratio of crenarchaeota-like 16S rRNA gene reads to total 16S rRNA gene reads in each microcosm. The pyrosequencing reads have been deposited at the DNA Data Bank of Japan with accession number DRA000546. The different letters above the columns indicate a significant difference ($P < 0.05$) using analysis of variance.

Nicolaisen and Ramsing, 2002). The absence of AOB in this study was further confirmed by pyrosequencing analysis of 16S rRNA genes at whole microbial community level in two acidic soils tested.

Pyrosequencing at the whole microbial community level

The high-throughput sequencing of 16S rRNA genes at the whole community level provides an almost unbiased profiling strategy for measuring characteristic changes in relative proportions of the microorganisms involved in certain functional processes, such as nitrification, in a complex system. The results obtained from the improved bidirectional pyrosequencing reveal strong correlations between nitrification activity and crenarchaeota-like 16S rRNA genes. The total microbial communities in the triplicate microcosms were analyzed using a 454 FLX Titanium sequencer for the whole incubation period (Supplementary Table S1). Slight modification was made by PCR amplifying the 16S rRNA genes with the universal primers 515F and 907R extended as amplicon fusion primers using the respective primer A or B adapters, key sequence and taq sequence, as previously described (Xia *et al.*, 2011). The 16S rRNA gene sequence reads were processed by the RDP pyrosequencing pipeline (Cole *et al.*, 2009). We obtained 103 552 high-quality sequences from the 18 samples (Supplementary Table S1). The changes in the proportion of crenarchaeota-like 16S rRNA genes to the total number of 16S rRNA gene reads (Figure 2c) followed perfectly the changes in net nitrification activity (Figure 2a) and archaeal *amoA* gene abundances (Figure 2b) in the soil microcosms. The relative proportion of crenarchaeota-like 16S rRNA genes increased 32-fold and 7-fold over the whole incubation period in forest soils amended with urea or water, respectively. Similarly, the relative proportions of crenarchaeota-like 16S rRNA gene reads to the total 16S rRNA in the tea orchard soils significantly increased from 0.28 to 7.86% after incubation with urea for 8 weeks, representing a 28-fold increase. The positive correlation between nitrification activities and archaeal communities was further confirmed by denaturing gradient gel electrophoresis (DGGE) fingerprinting analysis of archaeal *amoA* genes in triplicate microcosms.

DGGE fingerprinting of *amoA* genes

The DGGE analysis of archaeal *amoA* genes was performed by separating the PCR amplicons on 6% acrylamide gels with a 20 to 50% denaturing gradient, as previously described (Wu *et al.*, 2011) but with primers of CrenamoA23f and CrenamoA616r (Nicol *et al.*, 2008; Lehtovirta-Morley *et al.*, 2011). The DGGE fingerprints of archaeal *amoA* genes showed highly similar patterns among the triplicate microcosms of each treatment (Figure 3). For the forest soil, the pairwise comparison of DGGE patterns between days 0 and 56 revealed that the relative intensities of DGGE bands 1 and 2 were enhanced after incubation for 56

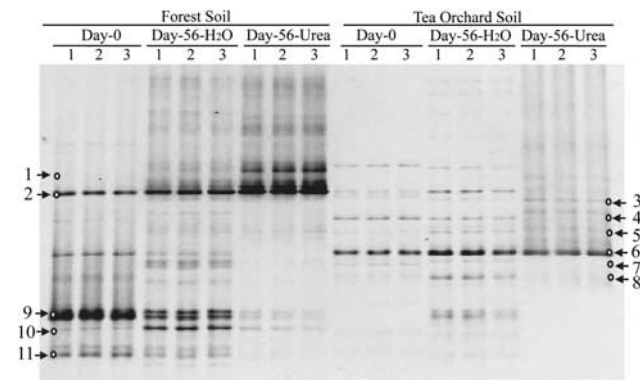


Figure 3 DGGE fingerprints of archaeal *amoA* genes in soils. All designations are the same as those used in Figure 2. 1, 2 and 3 represent the three samples from triplicate microcosms for each treatment. The arrows indicate the DGGE bands excised for sequencing.

days, particularly in the soil amended with urea. The opposite trend was observed for DGGE bands 6, 9 and 11. For the tea orchard soil, DGGE band 6 dominated the AOA communities and appeared to be enriched after incubation for 56 days. The conversion of forest soil to tea orchard soil significantly altered the DGGE fingerprints of archaeal *amoA* genes. The intensities of DGGE bands 2 and 9 significantly decreased, whereas only DGGE band 6 prevailed in the tea orchard soil. The dominant DGGE bands were excised for sequencing, and phylogenetic analysis of archaeal *amoA* genes was conducted (Figure 4) as previously described (Xia *et al.*, 2011). DGGE bands 1 and 2 were phylogenetically most closely affiliated with the acidophilic *N. devanattera* (Lehtovirta-Morley *et al.*, 2011). DGGE band 6 was also related to *N. devanattera*, but formed a unique cluster within the marine Group 1.1a-associated lineage. These results were further confirmed by the phylogenetic analysis of the 16S rRNA sequence reads. AOA members within the marine Group 1.1a-associated lineage dominated the archaeal communities, accounting for 97% and 95% of archaeal sequence reads in forest and tea orchard soils amended with urea, respectively (Figure 5 and Supplementary Table S2).

The results of this study indicate that the nitrification of ammonia oxidizers is supported by the hydrolysis of urea in two acidic soils tested and most likely related to AOA rather than AOB. Since the first isolation of urease-positive AOB (De Boer *et al.*, 1989), ureolysis has been proposed to promote nitrification in acidic soils (Allison and Prosser, 1991). The growth of AOB in low-pH cultures has strongly supported the mechanism of intracellular hydrolysis of urea as a source of ammonia for bacterial ammonia oxidation (Burton and Prosser, 2001). Our results demonstrate that nitrification occurred in both acidic soils when urea was added at considerably low rates of 5 µg of urea-N per gram d.w.s. The nitrification activities were also significantly enhanced when 800 µg of urea-N per

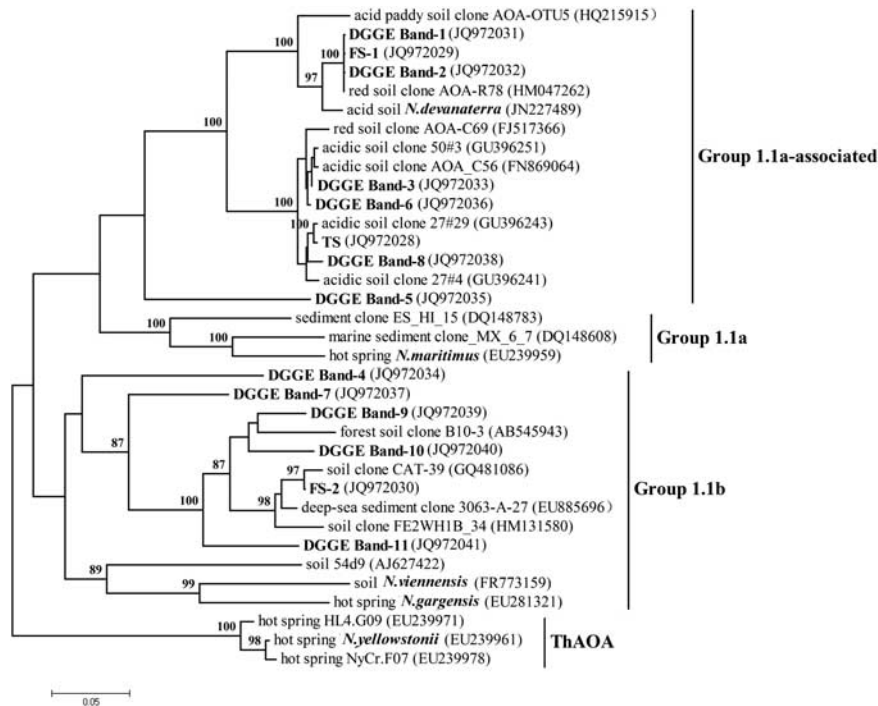


Figure 4 Phylogenetic tree showing the relationship of archaeal *amoA* genes retrieved from the forest and tea orchard soils to those obtained from the GenBank. The excised DGGE bands are shown in bold. The nucleotide sequences of archaeal *amoA* genes have been deposited in the DDBJ with accession numbers from JQ972028 to JQ972041. Bootstrap values higher than 70% are indicated at branch nodes.

gram d.w.s. was amended. The stimulated activities occurred as the abundance of archaeal *amoA* genes and the relative proportions of crenarchaeota-like 16S rRNA genes increased (Figure 2). Molecular fingerprinting of *amoA* genes further provided strong evidence that nitrification of archaeal ammonia oxidation was supported by urea hydrolysis. However, no conclusive evidence exists for intracellular urea hydrolysis by AOA in both acidic soils. We speculate that only a small proportion of the added urea entered the cells of AOA, and a portion of the ammonia produced due to this process was subsequently oxidized. Majority of the urea was most likely hydrolyzed by other organisms because extracellular ureolytic activity is a common feature in soil microbes (Mobley *et al.*, 1995; Koper *et al.*, 2004). The ammonia produced during these processes could have been ionized at low pH levels, making them effectively unavailable for oxidation by AOA in a manner similar to that in AOB as previously demonstrated (Burton and Prosser, 2001). This was further supported by the produced nitrate that accounted for only 11% and 33% of the urea added to tea orchard and forest soils, respectively. The key control of ammonia addition parallel to urea fertilization was not included in this study to assess whether the ammonia oxidized by AOA resulted solely from extracellular ureolytic activity in both acidic soils. Ammonia, rather than urea, is thought to be the substrate for ammonia monooxygenase. Thus, the stimulated activities of archaeal nitrification by urea hydrolysis are

expected to be similar to a certain extent upon ammonia addition. However, recent studies have shown that nitrification activity was not affected by the addition of $(\text{NH}_4)_2\text{SO}_4$ (Stopnisek *et al.*, 2010) or NH_4Cl (Levičnik-Höfferle *et al.*, 2012) to acidic soil. We speculate that this might be attributed to the side effects due to the addition of inorganic ammonium, such as the introduction of anion to soil.

Phylogenetic analysis of both *amoA* (Figure 4) and 16S rRNA genes (Figure 5) suggests that *N. devanattera*-like AOA dominated the nitrification activities in forest soil, whereas *N. devanattera*-related AOA were active ammonia oxidizers in tea orchard soil. The relative abundance of *N. devanattera*-related 16S rRNA sequences significantly increased from 56% to 95% in tea orchard soils after incubation with urea for 56 days (Supplementary Table S2), whereas *N. devanattera*-like 16S rRNA genes showed a similar increase from 71% to 97% in forest soil amended with urea. *N. devanattera* was isolated from acidic agricultural soils, in which the net nitrification activities (Tourna *et al.*, 2008; Offre *et al.*, 2009; Gubry-Rangin *et al.*, 2010) were similar to those observed in the present study (Figure 2). The predominance of archaeal ammonia oxidation was also confirmed by the greatest substrate affinity of *N. devanattera* determined by far (Lehtovirta-Morley *et al.*, 2011). Soil NH_3 concentration is estimated to be 7.0 nM in tea orchard soil and 221 nM in forest soil (Table 1). These values are sufficiently high for the growth of *N. devanattera* but not of AOB in culture. Pyrosequencing analysis

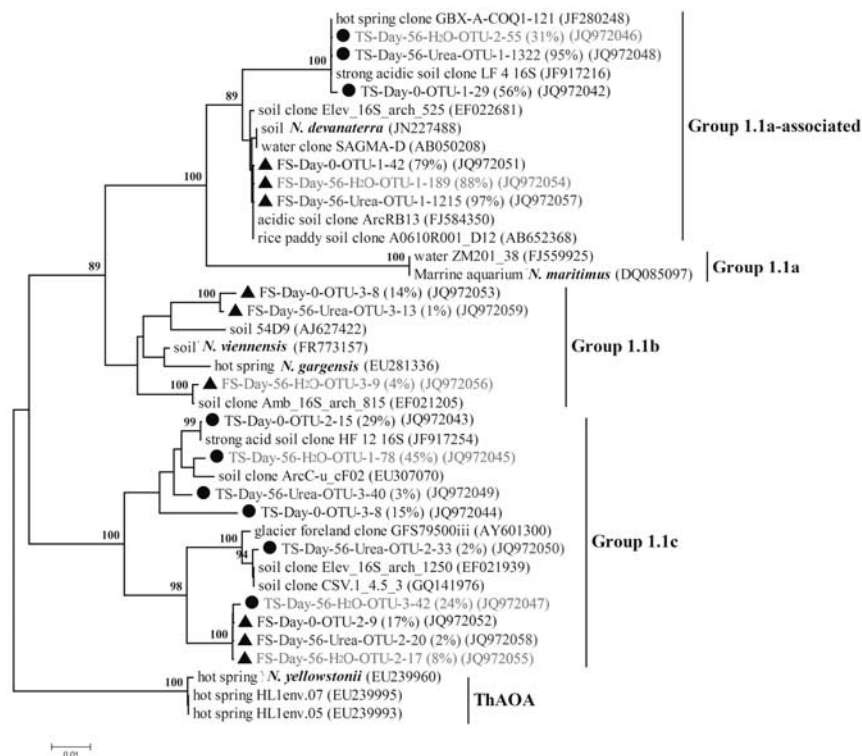


Figure 5 Phylogenetic tree showing the relationship of archaeal 16S rRNA gene sequence reads in two acidic soils to those obtained from the GenBank. FS and TS indicate forest and tea orchard soils with symbols ▲ and ●, respectively. The designations are similar to those used in Figure 2. For instance, FS-Day-56-Urea-OTU-1-185 (56%) indicates that OTU-1 of archaeal 16S rRNA genes contains 185 sequence reads with >97% identity and accounts for 56% of the total archaeal 16S rRNA reads in forest soil after incubation with urea for 56 days. One representative sequence from each OTU was extracted using the mothur software package (Schloss *et al.*, 2009) for tree construction. The representative sequences of archaeal 16S rRNA genes have been deposited in the DDBJ with accession numbers from JQ972042 to JQ972059. Bootstrap values higher than 70% are indicated at branch nodes.

indeed reveals that none of the 16S rRNA genes are affiliated with AOB among the 103 552 sequence reads across all soil samples. This suggests that the abundance of AOB might be too low to be detected by bacterial *amoA* genes in both acidic soils. Soil nitrite concentration was below the detection limit, implying that nitrite toxicity against AOA and/or AOB may be excluded in this study (De Boer and Kowalchuk, 2001; Lehtovirta-Morley *et al.*, 2011). Furthermore, ¹⁵N isotope tracing showed that nitrification activity was inhibited by acetylene, suggesting ammonia-monooxygenase-dependent activities as previously reported for *N. devanattera* (Lehtovirta-Morley *et al.*, 2011). These results strongly suggest that the active AOA observed in this study were very likely related to *N. devanattera* and capable of ammonia oxidation through urea hydrolysis.

Taken together, our results indicate that nitrification of archaeal ammonia oxidizers was supported by urea hydrolysis in two acid soils tested, and was most likely attributed to AOA members within the marine Group 1.1a-associated lineage. The ability to hydrolyze urea may afford AOA a greater ecological advantage in ammonia-poor environments, but the metabolic mechanism remains unclear.

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