Ammonia-oxidizing activity and microbial community structure
in acid tea (Camellia sinensis) orchard soil

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Abstract. The purpose of this study was to determine the ammonia-oxidizing activity
and the phylogenetic composition of microorganisms involved in acid tea (Camellia
sinensis) orchard soil. All soil samples were collected from three sites located in Tahara
and Toyohashi, Aichi Prefecture, Japan. The potential nitrification rate (PNR) was
measured by the chlorate inhibition method. The soil pH of tea orchards studied ranged
from 2.78 to 4.84, differing significantly from sample to sample, whereas that of meadow
and unplanted fields ranged from 5.78 to 6.35. The PNR ranged from 0.050 to 0.193 μg
NO₂⁻-Ng⁻¹ h⁻¹ and were positively correlated with the soil pH ($r^2 = 0.382$, $p<0.001$). Bulk
DNA was extracted from a tea orchard soil (pH 4.8; PNR, 0.078 μg NO₂⁻-Ng⁻¹ h⁻¹) and
subjected to PCR-aided clone library analyses targeting archaeal and bacterial amoA genes.
The detected archaeal clones separated from the cluster of the ‘Soil clones’ and tightly
clustered with the clones originating from other acidic soil environments including the
Chinese tea orchard soil. These results suggest that the specific archaeal populations
dominate as the ammonia oxidizers in acid tea-orchard soils and possibly other acid soils,
independent of geographic locations, which results from the adaptation to specific
ecological niches.

1. Introduction
Nitrification, the oxidation of ammonia (NH₃) to nitrate (NO₃⁻), is a critical step in the nitrogen
cycle in nature and has agricultural and environmental consequences for the bioavailability of
nitrogen as a plant nutrient, nitrate leaching to groundwater, and the release of greenhouse gases
(NO, N₂O) into the atmosphere. The rate-limiting step of nitrification is the conversion of
ammonia to nitrite (NO₂⁻), which is performed by both ammonia-oxidizing archaea (AOA) within
the phylum Thaumarchaeota [1], and ammonia-oxidizing bacteria (AOB). Both groups of
prokaryotes have been detected in a wide range of soil ecosystems [2, 3].
The potential for archaeal ammonia oxidation has been confirmed in culture enrichments and isolates [4, 5]. In most soils, archaeal amoA genes, a gene coding ammonia monooxygenase subunit A, are more abundant than those of bacteria, indicating that AOA have a greater role in soil ammonia oxidation than AOB [3, 6]. In soil ecosystems, nitrifying populations in terms of quantity, quality, and activity vary with vegetation type, location, and environmental conditions [7]. The pH of soil is one of the important factors on the nitrification activity and phylogenetic diversity of ammonia oxidizers [8, 9]. Especially at acidic environments, the absence of nitrification activity of AOB was reported in some highly acid soils [8, 10].

Tea (Camellia sinensis) orchard soils provide an excellent environment to examine the relative contribution of AOA and AOB in nitrification processes at lower pH. Tea is an important economic crop and is planted widely in acid red soils of the humid subtropical zones in Japan. High levels of nitrogen fertilizer are applied to tea orchards, altering N cycling and microbial community structure [11]. Tea orchard soils are often acidic in the range of pH from 3.5 to 5.5, but known to keep high nitrification activity despite the potential high sensitivity of AOB to low pH [12, 13]. It was suggested that AOA populations are responsible for the major part of nitrification in acidic soils, that soil pH and N fertilizer input influences both the community structure of ammonia oxidizers and nitrification potential, and that different AOA and AOB phylotypes occupy distinct pH ranges (i.e., niche differentiation) [14]. The types of soil and vegetation have potentially additional effects on microbial community composition [15], and it is therefore pertinent to compare tea soils with other vegetated systems on the same soil type.

This study determined the activity and phylogenetic compositions of ammonia oxidizers in tea orchard soils from two different sites. The nitrification activities of soils were also analyzed in adjacent meadow and unplanted field with different pH to evaluate the effect of environmental factors (i.e. pH, vegetation type, and soil type) on the nitrification activity of soils.

2. Materials and methods

**Study sites and soil sampling.** Three soil samples were collected from two sites located in Tahara (Fig. 1A, 1) and one site from Toyohashi (Fig. 1A, 2), Aichi Prefecture, Japan. The selected tea soils from the Tahara area, where the N application rate is high, represent a wide range of orchard ages, fertilizers, and lime applications. In the Toyohashi area, soil samples were taken from a low-N-applied tea orchard. All soil samples were taken from the furrows of the tea plants (Figs. 1B and 1C), because fertilization was mainly performed in these regions. In addition, adjacent meadow and unplanted field soils were sampled to evaluate the effect of vegetation on ammonia-oxidizing communities. These samples were

![Fig. 1. Location of the sampling sites (A) and photos of the tea orchard, red circle indicates the position of furrow in tea orchard (B) and an enlarged photo of the point of red circle in Fig. 1B. Red arrow indicate the sampling space (C) ](image-url)
collected from three sampling plots randomly chosen within each tea orchard or reference soils. Six random soil cores (5-cm diameter by 15-cm length) were taken from each sample and mixed. All soil samples were sieved through 2 mm sieving and then used in the following experiments.

**Soil chemical analysis.** Soil pH was measured using a glass electrode (soil/water, 1:2.5, w/v). The ammonia oxidation potential was determined from the decrease in the concentration of nitrite (NO$_2^-$) after incubation of soils amended with ammonium (NH$_4^+$) and chlorate as inhibitor of nitrite oxidation to nitrate (NO$_3^-$) according to Kurola et al. [16] with slight modifications. Briefly, 20 g of fresh soil was added to 300-mL conical flask containing 80 mL of citrate-phosphate buffer (pH 3.5) with 1 mM (NH$_4$)$_2$SO$_4$. Potassium chlorate with a final concentration of 10 mM was added to inhibit the nitrite oxidation. The suspension was incubated in darkness at 25 °C for 24 h and then treated with 5 mL of 2 M KCl to extract nitrite, followed by colorimetric detection at 540 nm with N-(1-naphthyl) ethylenediamine dihydrochloride. The potential nitrification rate (PNR) was calculated from the linear increase in concentrations of NO$_2^-$. 

**Construction of clone libraries and sequence analysis.** Bulk DNA from a tea orchard soil (pH 4.8; PNR, 0.078 μg NO$_2^-\text{-Ng}^{-1}\text{-h}^{-1}$) which was pretreated with CaCO$_3$ [17] was extracted using a PowerSoil DNA Isolation kit according to the manufacturer’s instructions. Archaeal and bacterial amoA genes were amplified by PCR with the soil DNA as the template and pair primer sets of Arch-amoAF (5'-STAATGGTCTGGCTTAGACG-3')/Arch-amoAR (5'-GCGGCCATCCATCTGTATGT-3') and amoA-1F (5'-GGGTTTCTACTGGTGGT-3')/amoA-2R (5'-CCCCTCGKGSAAAGCCTTCTTC-3'), respectively. PCR products were purified with the Wizard Gel and PCR Clean-up System and subcloned using a pT7Blue Perfectly Blunt™ Cloning kit according to the manufacturer’s instructions. More than 30 positive clones from each sample were sequenced using a BigDye Terminator v3.1 cycle sequencing kit and an Applied Biosystems 3130xl genetic analyzer. Multiple alignment of sequence data, calculation of the corrected evolutionary distance, and construction of a neighbor-joining (NJ) phylogenetic tree were performed using the ARB program package. The TREEFINDER program package was used for tree construction.

### 3. Results and discussion

The pH of the tea orchard soils ranged from 2.78 to 4.84, differing significantly from sample to sample, whereas those of meadow and unplanted fields ranged from 5.78 to 6.35 (Fig. 2). The PNR ranged from 0.050 to 0.193 μg NO$_2^-\text{-Ng}^{-1}\text{-h}^{-1}$ and was positively correlated with soil pH ($r^2 = 0.382$, $p<0.001$). Within the tea orchard soil, the PNR value ranged from 0.027 to 0.125 and had no correlation with soil pH (Fig. 2A). Three points of the tea orchard with different PNRs had the lowest pH, which might be due to a composite factors such as high N fertilizer application, the resultant acidification by nitrification, plant growth, and phytochemical

Fig. 2. The correlation between soil pH and PNR (potential nitrification rate). A, tea orchard soil; B, unplanted field soil; C, meadow soil.
inputs (ex. organic acids). These results suggest that PNR was affected not only by soil pH but also by other factors. Yao et al. [15] indicated that PNR was positively correlated with total nitrogen ($r^2 = 0.71$), organic carbon ($r^2 = 0.67$), and N fertilizer application ($r^2 = 0.27$) and negatively correlated with soil pH ($r^2 = 0.28$).

Phylogenetic analyses were performed on amoA gene sequences of 22 bacterial and 13 archaeal clones from one site (pH 4.8; PNR, 0.078 μg NO$_2$-N g$^{-1}$ h$^{-1}$) (Fig. 3). Out of the 22 bacterial amoA clones (Fig. 3A), only 3 clones were related to Nitrosomonas sequences, and the remaining 19 sequences clustered with the Nitrosospira group. Archaeal amoA gene sequences fell into two major groups (Fig. 3B). One of the groups comprised 11 sequences that clustered with the ‘Acidic soil group’ sequences. The other clustered with the sequences from sea, sediment and coral environments, which fall within the ‘marine-associated group’. Interestingly, the detected AOA clones separated from the cluster of the ‘Soil clones’ and tightly clustered with the clones originating from other acidic soil environments including the Chinese tea orchard soil. These results suggest that the specific AOA populations dominate as ammonia oxidizers in acid soils, originating from other acidic soil environments including the Chinese tea orchard soil. These results suggest the adaptation of ammonia-oxidizing AOA to particular tea orchard soil over geographic locations.

![Phylogenetic trees](image_url)

Fig. 3. Phylogenetic trees based on AOB amoA gene sequences (A) and AOA amoA gene sequences (B) showing relationships of the isolates and environmental clones. The trees were inferred by the NJ method. The amoA gene sequences of Nitrosococcus oceanus (U96611) and Nitrosocoldus yellowstonii (EU239961) were used as an outgroup to root the trees A and B, respectively. The accession numbers for the sequences are given in parentheses behind the species and clone names. The sequences determined in this study are shown by bold letters. Bar = 2% sequence divergence ($K_{nuc}$).
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References

[1] Brochier-Armanet C et al. 2008 Nat. Rev. Microbiol. 6 245–252
[2] Di H J et al. 2009 Nat. Geosci. 2 621–624
[3] Leininger S et al. 2006 Nature 442 806–809
[4] Hatzenpichler R et al. 2008 Proc. Natl. Acad. Sci. USA 105 2134–2139
[5] Könneke M et al. 2005 Nature 437 543–546
[6] Prosser J I and Nicol G W 2008 Environ. Microbiol. 10 2931–2941
[7] Webster G et al. 2005 Environ. Microbiol. 7 676–684
[8] de Boer W and Kowalchuk G A 2001 Soil Biol. Biochem. 33 853–866
[9] Nicol G W et al. 2008 Environ. Microbiol. 10 2966–2978
[10] de Boer W et al. 1996 Soil Biol. Biochem. 28 203–211
[11] Yao H et al. 2000 Microb. Ecol. 40 223–237
[12] Xue D et al. 2009 J. Environ. Sci. 21 1225–1229
[13] Xue D, Yao H Y and Huang C Y 2006 Plant Soil 288 319–331
[14] Yao H et al. 2011 Appl. Environ. Microbiol. 77 4618–4625
[15] Jones C M and Hallin S 2010 ISME J. 4 633–641
[16] Kurola J et al. 2005 FEMS Microbiol. Lett. 250 33–38
[17] Sagova-Mareckova M et al. 2008 Appl. Environ. Microbiol. 74 2902–2907