

New primers for detecting and quantifying denitrifying anaerobic methane oxidation archaea in different ecological niches

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Abstract The significance of ANME-2d in methane sink in the environment has been overlooked, and there was no any study evaluating the distribution of ANME-2d in the environment. New primers were thus needed to be designed for following research. In this paper, a pair of primers (DP397F and DP569R) was designed to quantify ANME-2d. The specificity and amplification efficiency of this primer pair were acceptable. PCR amplification of another pair of primers (DP142F and DP779R) generated a single, bright targeted band from the enrichment sample, but yielded faint, multiple bands from the environmental samples. Nested PCR was conducted using the primers DP142F/DP779R in the first round and DP142F/DP569R in the second round, which generated a bright targeted band. Further phylogenetic analysis showed that these targeted bands were ANME-2d-related sequences. Real-time PCR showed that the copies of the 16s ribosomal RNA gene of ANME-2d in these samples ranged from 3.72×10^4 to 2.30×10^5 copies μg^{-1} DNA, indicating that the percentage of ANME-2d was greatest in a polluted river sample and least in a rice paddy sample. These results

demonstrate that the newly developed real-time PCR primers could sufficiently quantify ANME-2d and that nested PCR with an appropriate combination of the new primers could successfully detect ANME-2d in environmental samples; the latter finding suggests that ANME-2d may spread in environments.

Keywords DAMO · ANME-2d · New primer design · Real-time PCR · Phylogenetic analysis · Environmental samples

Introduction

Denitrifying anaerobic methane oxidation (DAMO) has received intensive study in recent years. The process was first discovered in the laboratory using an anoxic sediment as an inoculum (Raghoebarsing et al. 2006). The resulting enriched microbial consortium could catalyze anaerobic methane oxidation coupled with nitrate reduction. An NC10 phylum bacteria and a methanotrophic archaeon, which were later named as *Candidatus Methyloirabilis oxyfera* (hereafter designated *M. oxyfera*) and ANME-2d, were isolated from this consortium (Ettwig et al. 2010; Haroon et al. 2013). A subsequent study found that *M. oxyfera* could catalyze the DAMO process alone (without the archaeon) when replacing nitrate with nitrite as the electron acceptor (Ettwig et al. 2008). Therefore, many studies have focused on the laboratory enrichment, metabolic pathways, and environmental distribution of *M. oxyfera* (Ettwig et al. 2010; Ettwig et al. 2009; Hu et al. 2014; Luesken et al. 2011a; Shen et al. 2014a, b). For the better quantification of *M. oxyfera* in enrichment cultures, new primers based on 16s ribosomal RNA (rRNA) have been designed for real-time PCR (Ettwig et al. 2009). Additionally, to better study the distribution and diversity of *M. oxyfera* in environments,

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several new primers have been designed based on the functional gene-*pmoA* (Han and Gu 2013; Luesken et al. 2011b). These primers played important roles in evaluating the distribution of *M. oxyfera* in environments. Using these primers, *M. oxyfera* was detected in many different environments, such as freshwater (Hu et al. 2012; Kojima et al. 2012; Shen et al. 2014a), wetland (Hu et al. 2014), paddy soil (Wang et al. 2012), and peatland (Zhu et al. 2012), indicating that the presence of *M. oxyfera* is overlooked.

Compared to *M. oxyfera* bacterium, ANME-2d archaea has attracted relatively little study. After Raghoebarsing first enriched the coculture of *M. oxyfera* and ANME-2d, Hu et al. (2009) also enriched the culture; these authors speculated that ANME-2d may only reduce nitrate to nitrite or prefer nitrate reduction to nitrite reduction. Detailed genomic and transcriptome analysis by Haroon et al. (2013) showed that ANME-2d could indeed only reduce nitrate to nitrite. Apart from these observations, many unknowns remain regarding ANME-2d, such as the role of ANME-2d in environments. Recently, Hu et al. (2014) found that the DAMO process in environments has been overlooked in wetlands, despite its significance to the global methane sink due to increasing nitrogen pollution. However, the conclusion is based on the evaluation of *M. oxyfera* only. It is well known that nitrate rather than nitrite is the major form of nitrogen compounds in water (for example, in the form of agricultural runoff), while *M. oxyfera* prefers nitrite and hardly reduces nitrate (Hu et al. 2009, Ettwig et al., 2010). Thus, DAMO archaea if exist in those environments must play some roles for nitrate reduction to nitrite. Therefore, the environmental distribution and diversity of ANME-2d is highly important, and the detection of ANME-2d in the environment requires specific primers to be designed.

In the present study, two pairs of primers were designed to detect and quantify ANME-2d archaea. The amplification specificity and efficiency of the new primers were checked using DNA samples isolated from methanogenic and DAMO enrichment cultures as templates. DNAs extracted from four environmental samples were then used to further validate the amplification efficiency of these new primers. The PCR products were sequenced and these sequences were used for phylogenetic analysis. The results provide more information about the environmental distribution and diversity of ANME-2d.

Materials and methods

Sampling sites

Samples were collected from four different sites, the location information of which is listed in Table 1. CH was collected from Chaohu Lake, and TH was collected from Taihu Lake.

SZH was collected from one polluted river in Suzhou, Jiangsu province, and PS-2 was collected from the deep layer (about 1 m) of a rice paddy soil in Wuhu, Anhui province. The samples were collected randomly and then transferred to laboratory quickly storing in plastic bags/bottles. They were stored at 4 °C before DNA extraction.

Primer design

The design of the new primers was based on the alignment of the previously obtained ANME-2d 16S rRNA sequences (Ding et al. 2014; Hu et al. 2009; Raghoebarsing et al. 2006) (Fig. 1). Primer Premier 5 software was used to design the new primers (Qin et al. 2009). A pair of 16S rRNA primers designated as DP142F (5'-TAATACYGGATAGATCAAAG-3') and DP779R (5'-GCACCGCACCTGACACCT-3') were designed for normal amplification, the expected length of the PCR product is 637 bp. A primer pair designated as DP397F (5'-TGGCTGTCCAGCTRITYC-3') and DP569R (5'-GRACGCCTGACGATTRAG-3') were also designed for 16S rRNA real-time PCR. The expected PCR product is 172-bp long. For those primers, DNA sample isolated from methanogenic culture was used to check their specificity, and DNA sample isolated from a DAMO culture (Ding et al. 2014) was used to check their efficiency.

DNA extraction and PCR amplification

About 5 ml DAMO culture or 0.3 g for each environmental sample was used for DNA extraction. DNA was extracted using the UltraClean Soil DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration of the DNAs obtained ranged from 8.4 to 79.5 ng μl^{-1} . All the DNAs were stored at -20 °C after extraction for further analysis. The first-round PCR was conducted using the DP142F and DP779R primers. The PCR reaction mixture (25 μl) contained the following: 0.5 μl of template DNA, 0.5 μl of each forward primer and reverse primer (10 μM), 2.5 μl of 10 \times Taq buffer (TIANGEN, Beijing, China), 2 μl of dNTP mixture (2.5 mM, TIANGEN), and 0.5 μl DNA polymerase (2.5 U/ μl , TIANGEN). The PCR was conducted with the following program: 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 55 °C for 40 s, and 72 °C for 40 s; and a final extension at 72 °C for 5 min. The second-round PCR (nested PCR) was conducted using the DP142F and DP569R primers. In this reaction, 0.5- μl product of the first-round PCR was used as template in a 25- μl reaction volume. The PCR reaction mixture was the same with that in the first round. It was conducted with the following program: 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s; and

Table 1 Brief information about the sampling sites

Sample ID	Sampling location	Geographic coordinates	Environment
CH	Anhui province	31.55° N/117.56° E	Lake sediment
TH	Jiangsu province	31.29° N/120.20° E	Lake sediment
SZH	Jiangsu province	31.27° N/120.73° E	River sediment
PS-2	Anhui province	31.29° N/117.75° E	Rice paddy soil

a final extension at 72 °C for 5 min. Nested PCR was used only for the four environmental samples. The PCR products were analyzed by 1.2 % agarose gels stained with the special nucleic acid dye (TIANGEN, Beijing, China).

Cloning, sequencing, and phylogenetic analysis

PCR products were checked using 1.2 % agarose gels, then recovered and purified using the Column DNA Gel Extraction Kit (Sangon, Shanghai, China) and were then ligated to the pUCm-T vector (Sangon, Shanghai, China). The ligation products were transformed into *Escherichia coli*-competent cells (Sangon, Shanghai, China). The cells were selected on a plate with antibiotics; the picked positive clones were checked with PCR using M13 primers. The checked positive clones were then sent to sequencing (Sangon, Shanghai, China).

The obtained 16s rRNA sequences were grouped into operational taxonomic units (OTUs) using Mothur software (Du et al. 2013). The cutoff was set as 0.03 for CH, TH, and SZH samples. For PS-2 sample, the software could not get calculated results with cutoff set as 0.03, so we changed it to 0.02 in PS-2 sample calculation. The representative sequence of each OTU, sequences obtained by BLAST against the GenBank database, and relevant sequences from previous publications were used to construct the phylogenetic tree (Fig S1 in Supplementary Materials). The sequences were aligned using MEGA 5 software and then used to construct a neighbor-joining tree with a bootstrap of 1000 (Shen et al. 2014a).

The Shannon (H) and Simpson (D) values were calculated using the following formulas: $H = -\sum (P_i \ln P_i)$, $D = 1 - \sum P_i^2$. P_i represents the proportion of the species in the total species, and the summation is over all species (Hedrick et al. 2000).

The 16s rRNA clone sequences were analyzed with some referenced sequences using Fast UniFrac online (<http://unifrac.colorado.edu/>) for Jackknife Sample Clusters and principal coordinate analysis (PCoA) (Han and Gu 2013).

Real-time PCR

Real-time PCR using DP397F and DP569R primers was employed to quantify the 16s rRNA of ANME-2d in the four environmental samples. They were determined in triplicate using an Applied Biosystems StepOnePlus™ Real-time PCR system and fluorescent dye SYBR Green. The reaction was performed in a 20- μ l volume containing the following: 10 μ l of the SYBR Green qPCR Master Mix, 1 μ l of each forward primer and reverse primer, and 2 μ l of the DNA template. The program was 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, 57 °C for 20 s, and 72 °C for 30 s. The amplification products using these real-time PCR primers with the DAMO culture template were checked by electrophoresis, then recovered, and then ligated to the pMD 18-T vector (Takara, Dalian, China). The ligation products were transformed into the *E. coli*-competent cells, and plasmid DNA was then extracted from the cultured cells. The plasmid DNA concentration was determined, and the gene abundance in copy number was calculated. The plasmid DNA was diluted

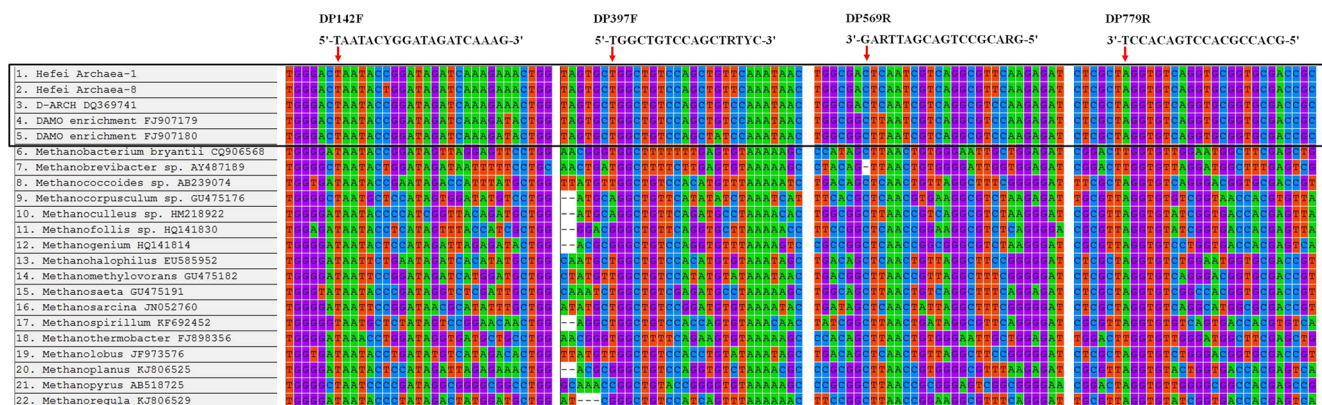


Fig. 1 Alignment of the 16s rRNA genes of ANME-2d (JX910251, DQ369741, FJ907179, FJ907180) and methanogens using MEGA 5. Degenerated primers were designed based on the conservative regions of the ANME-2d which were also dissimilar with the methanogens

with tenfold serial; each serial was measured in triplicates, and the mean value was used to construct the standard curve. The amplification efficiency (E value) was calculated using the following formula: $E = 10^{(-1/\text{slope})} - 1$ (Lim et al. 2008).

Nucleotide sequence accession numbers

The archaeal 16s rRNA sequences obtained from these samples were submitted to GenBank with the accession numbers KP195283-KP195384.

Results

Specificity and efficiency of the new primers

DP397F and DP569R primers were designed to quantify ANME-2d. The specificity of these primers was thus checked using DNAs from methanogenic and DAMO cultures as templates. The electrophoresis results showed that no PCR product with the DNA from methanogenic culture as the template, but a single, bright PCR product of the expected size using the DNA from DAMO culture as the template (Fig. 2a). Sequencing results of PCR products showed high similarity with ANME-2d sequences (Fig S2 in Supplementary Materials). Three standard curves were constructed; the correlation coefficient (r^2) of these standard curves ranged from 0.997 to 1.000, and the amplification efficiency ranged from

0.890 to 1.18, as calculated from the slope of the curves. It was reported that the amplification efficiency can be affected by many factors such as the template source, primer GC content, primer mismatch, and the presence of PCR inhibitors (Brankatschk et al. 2012). Therefore, the efficiency may be improved if an optimal amplification condition is used, though the present efficiency is acceptable.

The DP142F and DP779R primers were designed to detect ANME-2d in enrichment culture and environmental samples. The specificity and efficiency of the new primers were also checked using DNAs from methanogenic and DAMO cultures as templates. The results also showed specific amplification in the DM samples (Fig. 2b). However, the efficiency of the primers for the DNAs isolated from the four environmental samples was insufficient. There were faint and multiple bands on the agarose gels (Fig S3 in Supplementary Materials). Nested PCR was thus used to amplify ANME-2d in these samples. Because the product generated by the primers DP397F and DP569R was only 172 bp in length, they were unsuitable for phylogenetic analysis. Therefore, the DP142F primer was combined with the DP569R primer to generate a product of 427 bp in length. The PCR product of the first-round PCR using DP142F and DP779R primers was then used as the template for the second-round PCR using DP142F and DP569R primers. The result using this nested PCR method showed that there was a dominant and bright PCR band at the expected size amplified specifically in the DAMO sample, not in the methanogenic sample (Fig. 3a). The nested PCR strategy has also successfully generated ANME-2d-specific DNA products from

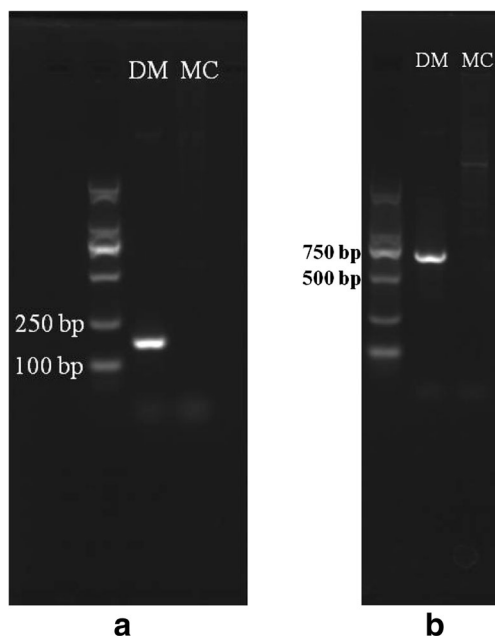


Fig. 2 Specificity and efficiency of the newly designed primers. **a** Electrophoresis results of the new primers DP142F and DP779R using DAMO (*DM*) and methanogenic (*MC*) cultures as samples. **b** Electrophoresis results of the new primers DP397F and DP569R using *DM* and *MC* as samples

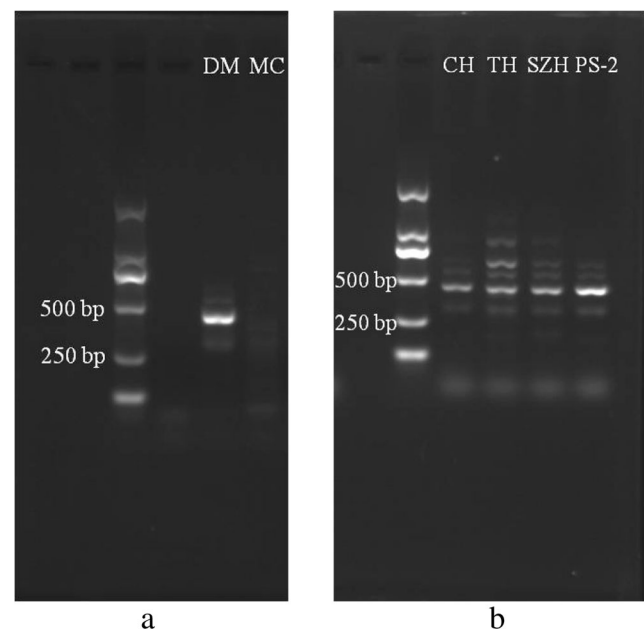


Fig. 3 Electrophoresis results of nested PCR with DNAs from **a** DAMO culture (*DM*), methanogenic culture (*MC*); **b** environmental samples of Chaohu Lake (*CH*), Taihu Lake (*TH*), a polluted river in Suzhou, Jiangsu province (*SZH*) and a rice paddy in Wuhu, Anhui province *PS-2*

Table 2 Diversity of ANME-2d in the four environmental samples based on 16s rRNA gene sequences

Sample	Clone numbers	Cutoff	OTUs	Diversity and richness	
				Simpson	Shannon
CH	25	0.03	6	0.72	2.15
TH	26	0.03	2	0.20	0.18
SZH	25	0.03	2	0.46	0.64
PS-2	26	0.02	2	0.14	0.12

CH Chaohu Lake; TH Taihu Lake; SZH a polluted river in Suzhou, Jiangsu province; PS-2 a rice paddy in Wuhu, Anhui province

the four environmental samples (Fig. 3b). Our results showed that a nested PCR strategy, using the primers DP142F and DP779R for the first-round PCR and then the primers DP142F and DP569R for the second-round PCR, is highly efficient and specific for the amplification of ANME-2d DNAs in environmental samples.

Diversity and phylogenetic analysis of the four environmental samples

PCR products generated by the nested PCR method from the four environmental samples were sequenced. The 16s rRNA sequences obtained from the CH sample were grouped into six OTUs (cutoff = 0.03) (Table 2) and were similar to the

sequences from river, lake, and freshwater sediments (Fig. 4). The sequences obtained from the TH sample were grouped into two OTUs (cutoff = 0.03) (Table 2), as were the sequences from the SZH sample. The obtained sequences in these two samples were mainly similar to those obtained from river sediments and aquifers (Fig. 4). The sequences obtained from PS-2 were also grouped into two OTUs (cutoff = 0.02), and they were mainly similar to those obtained from paddy soil (Fig. 4). The diversity analysis showed that the archaea were most diverse in the CH sample and least diverse in the PS-2 sample (Table 2). This result was similar to that obtained for the diversity pattern of *pmoA* gene of the n-damo bacteria in environmental samples, which was more diverse in freshwater reservoir and less diverse in reed bed and paddy soil (Han and Gu 2013). However, all sequences obtained in the present work were similar to the archaeal sequences obtained from DAMO enrichment (Fig. 4). Our sequences were all grouped together with the DAMO enrichment archaeal sequences and other previously published sequences from environmental samples. This showed that the sequences obtained in the present study were ANME-2d related.

The ANME-2d 16s rRNA sequences obtained in this study were used to do Jackknife Sample Cluster and PCo analyses. However, because there were little reported ANME-2d 16s rRNA sequences, especially no any other environmental ANME-2d 16s rRNA sequences, the reported ANME-2d sequences from two laboratory enrichments (Ding et al. 2014; Hu et al. 2009) and similar sequences obtained from a mud

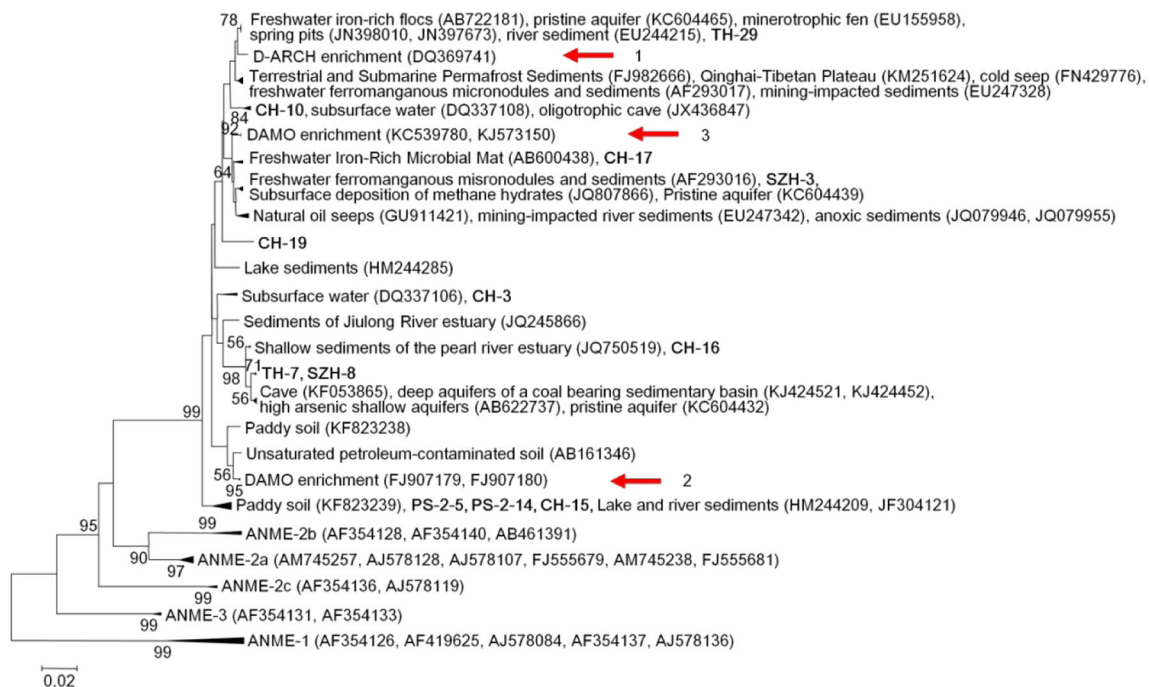


Fig. 4 Phylogenetic tree of the 16s rRNA sequences of the four environmental samples and several reference sequences downloaded from GenBank. The three red arrows represent the DAMO archaeal

sequences from previous laboratory enrichments (Ding et al. 2014; Hu et al. 2009; Raghoebarsing et al. 2006)

volcano (Wrede et al. 2012) were used together in the analysis. As shown in Fig. 5, the samples of CH, TH, and SZH were grouped together (Fig. 5), which is reasonable due to the similar environmental conditions of these samples. The samples of DE, PS-2, and HE were grouped into another cluster, which can be explained by the complexity of these samples. Because the DE sample was an enrichment culture which was inoculated with a mixture of methanogenic sludge and activated sludge from a wastewater treatment plant (Ding et al. 2014), and the HD sample was an enrichment culture which was inoculated with a mixture of freshwater lake sediments, anaerobic digester sludge, and return sludge from a wastewater treatment plant (Hu et al. 2009). Additionally, the Nirano mud volcano (NMV) sample was comparatively distantly related to other samples (Fig. 5), which might be caused by the environment differentiation.

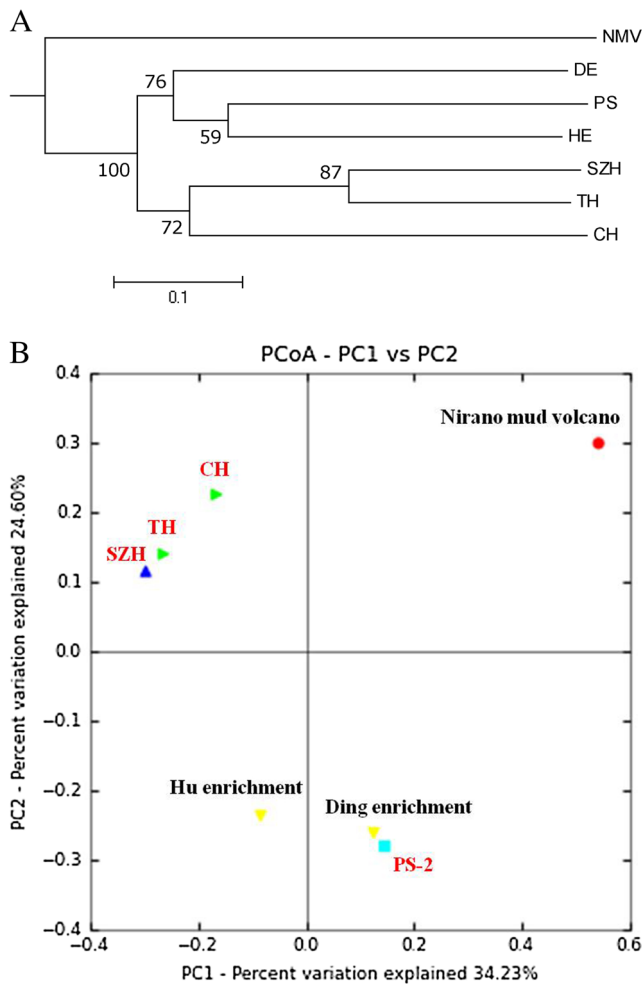


Fig. 5 **a** Jackknife Sample Clusters with the sequences obtained in this study, other reported ANME-2d 16s rRNA sequences, and similar sequences obtained from GenBank. **b** PCoA analysis about these sequences. *CH* Chaohu Lake; *TH* Taihu Lake; *SZH* a polluted river in Suzhou, Jiangsu province; *PS-2* a rice paddy in Wuhu, Anhui province; *NMV* Nirano mud volcano (Wrede et al. 2012); *HE* DAMO enrichment culture (Hu et al. 2009); *DE* DAMO enrichment culture (Ding et al. 2014)

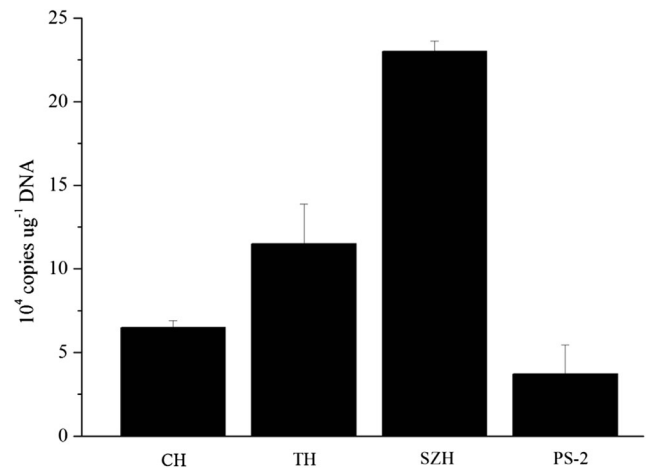


Fig. 6 Real-time PCR results of the four environmental samples. *CH* Chaohu Lake; *TH* Taihu Lake; *SZH* a polluted river in Suzhou, Jiangsu province; *PS-2* a rice paddy in Wuhu, Anhui province

Real-time PCR results

Real-time PCR was conducted to quantify ANME-2d in the four environmental samples using the newly designed real-time PCR primers (DP397F and DP569R). The electrophoresis images of the qPCR results for the four environmental samples showed single target band (Fig S4 in Supplementary Materials). The results also showed that the copy number of ANME-2d 16s rRNA gene in the four environmental samples ranged from 3.72×10^4 to 2.30×10^5 copies μg^{-1} DNA (Fig. 6). This finding suggests that the percentage of ANME-2d was greatest in the SZH sample and least in the PS-2 sample. The percentage of ANME-2d in the TH sample was greater than that in the CH sample.

Discussion

DP397F and DP569R are considered as acceptable real-time PCR primers for quantifying ANME-2d. However, the variation of the amplification efficiency implied that an optimal condition needs to be used. DP142F and DP779R primers are suitable to amplify ANME-2d archaea in enrichment culture but cannot amplify ANME-2d in the environmental samples. This may not be caused by the specificity of the primers but instead by a low concentration of target DNA and potential interference by some impurities in the environmental samples. Nested PCR using DP142F/DP779R primers in the first-round PCR and DP142F/DP569R primers in the second-round PCR can efficiently and specifically amplify ANME-2d in environmental samples. However, these primers can be further improved to reduce nonspecific bands.

The phylogenetic analysis showed that the sequences obtained in this paper were ANME-2d-related sequences. The

sequences obtained in this study and many environmental sequences downloaded from GenBank were grouped into one large group with the reported ANME-2d sequences. This finding is intriguing for no previous studies have examined the environmental distribution of ANME-2d, though the presence of *M. oxyfera* has been evaluated in many environmental conditions. Raghoebarsing et al. (2006) enriched the mixed DAMO culture using freshwater sediments as an inoculum. Hu et al. (2009) enriched this culture using a mixture of freshwater lake sediment, digester sludge, and activated sludge as an inoculum. It may thus be speculated that ANME-2d exists in some freshwater sediments. The molecular characterization and phylogenetic analysis in the present study confirmed that ANME-2d indeed exists in freshwater sediments, as well as in rice paddy soil. Because the downloaded environmental sequences were obtained from many environmental conditions, including pristine aquifers, minerotrophic fen, spring pits, natural oil seeps, and unsaturated petroleum-contaminated soil, the existence of ANME-2d in environments may be seriously overlooked. In other words, ANME-2d archaea may spread throughout environments.

According to the real-time PCR results, the percentage of ANME-2d was greatest in the Suzhou river sediment and least in the paddy soil sample. The percentage in the Taihu Lake was greater than that in the Chaohu Lake. However, these percentages may differ under different environmental conditions or pollution conditions. Therefore, the detailed ANME-2d amount variation in environments needs further evaluation with more samples.

Anaerobic oxidation of methane (AOM) coupling with sulfate reduction, iron reduction, and manganese reduction is mainly observed in extreme environmental conditions, such as marine cold seeps and volcanoes (Beal et al. 2009; Boetius et al. 2000; Hinrichs et al. 1999; Joye et al. 2004; Losekann et al. 2007; Michaelis et al. 2002; Niemann et al. 2006; Orphan et al. 2002). Therefore, ANME-1, 2, and 3, which conduct these processes, have mainly been found in these extreme environments. The significance of AOM as conducted by these archaea was originally proposed as related to methane emission from marine sediments to the atmosphere (Hallam et al. 2004; Kruger et al. 2003). The discovery of DAMO process and the following evaluation of *M. oxyfera* distribution showed that the environmental significance of AOM has been overlooked (Hu et al. 2014). The detection of ANME-2d from four environmental samples in the present study, as well as the evidence for its widespread distribution from other similar environmental sequences, showed that ANME archaea also exist in moderate environments and that the significance of AOM in these environments has been overlooked.

In summary, the new primers designed in this study can be used to quantify and detect ANME-2d archaea in

environmental samples. This is the first to design specific primers for ANME-2d and evaluate their environmental distribution. The detection of ANME-2d in various conditions showed the wide distribution of this archaea in environments, and thus, the contribution of this archaea to the global carbon and nitrogen cycles has been overlooked and merits greater attention.

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