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ORIGINAL PAPER

Comparative analysis of archaeal 16S rRNA and *amoA* genes to estimate the abundance and diversity of ammonia-oxidizing archaea in marine sediments

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Abstract Considering their abundance and broad distribution, non-extremophilic *Crenarchaeota* are likely to play important roles in global organic and inorganic matter cycles. The diversity and abundance of archaeal 16S rRNA and putative ammonia monooxygenase α-subunit (*amoA*) genes were comparatively analyzed to study genetic potential for nitrification of ammonia-oxidizing archaea (AOA) in the surface layers (0–1 cm) of four marine sediments of the East Sea, Korea. After analysis of a 16S rRNA gene clone library, we found various archaeal groups that include the crenarchaeotal group (CG) I.1a (54.8%) and CG I.1b (5.8%), both of which are known to harbor ammonia oxidizers. Notably, the 16S rRNA gene of CG I.1b has only previously been observed in terrestrial environments. The 16S rRNA gene sequence data revealed a distinct difference in archaeal community among sites of marine sediments. Most of the obtained *amoA* sequences were not closely related to those of the clones retrieved from estuarine sediments and marine water columns. Furthermore, clades of unique *amoA* sequences were likely to cluster according to sampling sites. Using real-time PCR, quantitative analysis of *amoA* copy numbers showed that the copy numbers of archaeal *amoA* ranged from 1.1×10^7 to 4.9×10^7 per gram of sediment and were more numerous than those of bacterial *amoA*, with ratios ranging from 11 to 28. In conclusion, diverse CG I.1a and CG I.1b AOA inhabit surface layers of marine sediments and AOA, and especially, CG I.1a are more numerous than other ammonia-oxidizing bacteria.

Keywords Ammonia-oxidizing archaea · *amoA* · 16S rRNA · Marine sediment

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Introduction

Cultivation-independent analysis of diversity using the PCR-amplification and sequencing of 16S rRNA gene is a useful method for identifying novel bacterial and archaeal phylogenetic lineages from different environments. The first detection of crenarchaeotal 16S rRNA genes in ocean environments was surprising (DeLong 1992; Fuhrman et al. 1992) because they had previously only been cultivated from thermal and/or acidic environments. Thereafter, members of the phylum *Crenarchaeota* have been detected in most moderate and cold environments such as forest and agricultural soils (Bintrim et al. 1997; Buckley et al. 1998; Jurgens et al. 1997; Jurgens and Sanno 1999; Kudo et al. 1997; Reed et al. 2002; Sandaa et al. 1999; Ueda et al. 1995), freshwater sediments (Abreu et al. 2001; Hershberger et al. 1996; Jurgens et al. 2000; MacGregor et al. 1997; Schleper et al. 1997), marine water columns and sediments (Inagaki et al. 2003; Karner et al. 2001; Li et al. 1999; Massana et al. 1997; Vetriani et al. 1999), subsurface environments (Takai et al. 2001), and cold springs (Koch et al. 2006). When the molecular phylogeny of the 16S rRNA gene was analyzed, members of the *Crenarchaeota* phylum could be classified into several groups (Schleper et al. 2005): I.1a (marine, freshwater, subsurface), I.1b (soil, freshwater, subsurface), I.1c (forest soil, freshwater), I.2 (marine/freshwater sediment), I.3a (marine,

hotspring, freshwater), I.3b (freshwater, wastewater, soil), the South Africa Gold Mine Crenarchaeotal Group (SAGMCG), and the Marine Hydrothermal Vent Group (MHVG). In addition to these groups, members of the Marine Benthic Groups (MBG) A, B and C, and the Miscellaneous Crenarchaeotal Group (MCG) were widely reported to be present in marine sediments (Inagaki et al. 2003, 2006).

Considering their abundance and broad distribution, it is likely that members of the *Crenarchaeota* phylum have important ecological roles in biogeochemical cycles. The results of metagenomic studies have revealed the existence of ammonia monooxygenase α-subunit (*amoA*) genes in members of the soil Crenarchaeotal Group I.1b (CG I.1b) (Treusch et al. 2005) and Marine Group I (MG I) of Crenarchaeotal Group I.1a (CG I.1a) subgroup (Hallam et al. 2006; Venter et al. 2004). Recently, an ammonia-oxidizing archaeon of MG I has been isolated and verified to contain the *amoA* gene (Konneke et al. 2005). Furthermore, a variety of archaeal *amoA*-like genes from marine environments have been isolated using PCR (Francis et al. 2005). Increased expression of archaeal *amoA* occurs after spiking soils with ammonia, and such results indicate that these *amoA* genes are actively involved in ammonia oxidation (Treusch et al. 2005). Furthermore, dominance of ammonia-oxidizing archaea (AOA) over ammonia-oxidizing bacteria (AOB) in a variety of soil types (Leininger et al. 2006) and North Sea water has been reported (Wuchter et al. 2006).

Microorganisms in marine sediments contribute significantly to global cycles of organic and inorganic matters because of their abundance (Whitman et al. 1998). Although MG I is the only major group of *Crenarchaeota* that is found below the photic zone of the ocean water column (Karner et al. 2001), a large variety of other crenarchaeotal groups have been found in marine sediments (Hershberger et al. 1996; Inagaki et al. 2006, 2003). In this study, we investigated AOA diversity and abundance by conducting a comparative analysis of archaeal 16S rRNA and *amoA* genes to assess the genetic potential for nitrification by *Crenarchaeota* from marine sediments. The results of this molecular survey showed that AOA include various members of CG I.1b as well as CG I.1a, all of which inhabit the surface layers of marine sediments. The results of a quantitative analysis of *amoA* copy numbers using real-time PCR suggested that AOA are numerically dominant over AOB in marine sediments.

Materials and methods

Site descriptions and sample collection

Marine sediments were collected from four different sites of the East Sea, Korea during July and August, 2005 using a core sampler. The sampling locations and water depth were as follows: ST3 (E 128° 35′, N 38° 20′; 650 m), JD (E 129° 11′, N 37° 46′; 500 m), UJ (E 129° 49′, N 37° 04′; 600 m), and DD (E 130° 56′, N 37° 28′; 500 m). The core samples were sectioned and each section was transferred to sterile plastic tubes and then stored at −80°C until analysis. The properties of the bottom water at the four sampling sites were similar and described as follows: temperature, 0.5–0.6°C; dissolved oxygen, 6.7–6.8 mg l^{-1}; salinity, 34‰, chlorophyll, 0.2–0.02 mg l^{-1}; nitrate, 1.5–1.6 mg l^{-1}; ammonia 0.052–0.067 mg l^{-1}; phosphate, 0.02 mg l^{-1}.

Extraction of genomic DNA and cloning of archaeal 16S rRNA sequences

A Power Soil™ DNA kit (Mo Bio Laboratories, Solana Beach, CA, USA) was used to extract genomic DNA from the frozen sediment samples. After three independent extractions of DNA from the surface layers (0–1 cm) of each sample, the DNA samples from each site were pooled for molecular analysis. The genomic DNA concentrations were determined by spectrophotometry (Nanodrop Technologies, Rockland, DE, USA). The 16S rRNA gene sequences were amplified by PCR using *EF*-Taq DNA polymerase (Solgent, Korea) and the Arch-20F and Arch-958R primers (DeLong 1992) (Table 1) to generate a product for the preparation of clone libraries. The conditions of the PCR were: 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 55°C, 45 s at 72°C; 7 min at 72°C. Duplicate PCR products were pooled and purified using the Gel&PCR Purification System Kit® (Solgent, Korea), then ligated using the T&A Cloning Vector Kit (Real Biotech Corporation, Taiwan), and thereafter transformed into *Escherichia coli* DH5α cells, according to the manufacturer's instructions. Putative positive clones were then transferred to a 96-well plate that contained Luria broth supplemented with ampicillin (100 μg/ml). The clones were grown overnight at 37°C and then stored at −70°C before screening.

PCR-denaturing gradient gel electrophoresis (DGGE) of CG I.1b-specific 16S rRNA genes

To analyze the diversity of CG I.1b, the 16S rRNA gene sequence was specifically amplified and analyzed using PCR-DGGE. Genomic DNAs from surface layers of each sample site were amplified by PCR. We used a nested PCR approach for the specific amplification of CG I.1b-specific 16S rRNA sequences using a CG I.1b-specific primer set (Table 1). The amplified PCR products were used as templates for a second round of amplification using a DGGE universal primer set (DGGE 340F and DGGE 519R) for archaeal 16S rRNA genes (Ovreas et al. 1997). The

Table 1 Primers used for PCR amplification of archaeal 16S rRNA gene sequences, and archaeal and bacterial *amoA* gene sequences

Primer name	Target	Position[a]	Sequence (5′-3′)	Reference
Archaeal 16S rRNA gene				
Arch-20F	Archaeal 16S rRNA	2–20	TTCCGGTTGATCCYGCCRG	Delong (1992)
Arch-958R		958–976	TCCGGCGTTGAMTCCAATT	
CG I.1b-270F	CG I.1b 16S rRNA	265–284	TGGATTGGACTGCGKCCGAT	This study
CG I.1b-750R		772–791	GTCGAGCGCRTTCTGGMAAG	
DGGE 340F	Archaeal 16S rRNA	340–357	GC-clamp[b]-CCCTACGGGGYGCASCAG	Ovreas et al. (1997)
DGGE 519R		519–533	TTACCGCGGCKGCTG	
amoA gene				
A Arch-*amoA*26F	Archaeal *amoA*	91–116	GACTACATMTTCTAYACWGAYTGGGC	This study
Arch-*amoA*417R		481–506	GGKGTCATRTATGGWGGYAAYGTTGG	
B CG I.1b-*amoA*F	CG I.1b *amoA*	43–65	ATAGTTGTAGTTGCTGTAAATAG	This study
CG I.1b-*amoA*R		439–459	CTCTAGAGGGTCTCTGACCAG	
*amoA*1F	Bacterial *amoA*	332–349	GGGGTTTCTACTGGTGGT	Rotthauwe et al. (1997) (real-time PCR)
*amoA*2R		802–822	CCCCTCKGSAAAGCCTTCTTC	
Arch-*amoA*F	Archaeal *amoA*	211–231	GCTCTAATTATGACAGTATAC	This study (real-time PCR)
Arch-*amoA*R		393–416	AYCATGTTGAAYAATGGTAATGAC	
CG I.1b-*amoA*F	CG I.1b *amoA*	53–74	GTACATTATTGACAATCAACGC	This study (real-time PCR)
CG I.1b-*amoA*R		248–268	ATCCTARYGCAAACCAAGCTC	

[a] Numbering is based on the 16S rRNA gene of *E. coli*, archaeal *amoA* of metagenome clone 54d9 (AJ627422), and bacterial *amoA* of *Nitrosomonas europaea* (L08050), respectively

[b] GC-clamp sequence: 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3′

conditions for the first round of PCR amplification were: 5 min at 95°C; then 30 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. The conditions for the second round of PCR amplification were: 5 min at 95°C; then 20 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. Final PCR products were purified using a PCR Purification Kit (Solgent, Korea) and then used for the DGGE analysis. DGGE was performed with the D-code system (Bio-Rad Laboratories, Hercules, CA, USA). The polyacrylamide concentration and denaturant gradient for the 16S rRNA sequence were optimized as 8% polyacrylamide (37.5:1 = acrylamide:bisacrylamide) and 30–60% denaturant. Gels were run for 14 h at 80 V in 1× Tris-Acetate-EDTA buffer at a constant temperature of 60°C. The gels were stained for 30 min in 1× TAE buffer containing a 1:10,000 dilution of the dye, SYBR green (Bioneer, Korea). All visible bands were recovered from the gel and re-amplified using the DGGE 340F, without GC-clamp, and DGGE 519R primers. The purified PCR products were then sequenced using the amplification primers.

Amplification and cloning of archaeal *amoA* gene sequences

Archaeal *amoA* gene sequences were amplified from genomic DNA by PCR using *EF*-Taq DNA polymerase (Solgent, Korea) and *amoA* primers (Table 1). Two different primer sets were used to amplify the various archaeal *amoA* sequences. PCR primer set A was designed on the basis of alignments of all archaeal *amoA* sequences from Sargasso Sea water column (AACY01007942, AACY01575171, AACY01435967, and AACY01075168), various marine water column and sediments (Francis et al. 2005) and the soil metagenomic clone 54d9 (AJ627422). The design of PCR primer set B (Table 1), which is specific for the detection of soil (CG I.1b-specific) archaeal *amoA*, was based on the *amoA* sequences from the soil metagenomic clone 54d9 (AJ627422) and Oak Ridge soil clones (Francis et al. 2005).

The protocols for detection of various *amoA* gene sequences using these two primer sets were as follows: primer set A (5 min at 95°C; then 35 cycles consisting of 30 s at 95°C, 30 s at 60°C, 45 s at 72°C; and 7 min at 72°C); primer set B (5 min at 95°C; then 35 cycles consisting of 30 s at 95°C, 30 s at 55°C, 45 s at 72°C; and 7 min at 72°C). Aliquots from the PCR products were analyzed on a 1.5% agarose gel. Duplicate PCR products were pooled and purified using a PCR Purification Kit (Solgent, Korea), then ligated into the T&A Cloning Vector (Real Biotech Corporation, Taiwan), and thereafter transformed into *E. coli* DH5α cells, according to the manufacturer's instructions. Putative positive clones were transferred to a 96-well plate that contained Luria broth supplemented with ampicillin (100 μg/ml). The clones

were grown overnight at 37°C and then stored at −70°C before screening.

Sequencing and phylogeny analysis

Library clones were screened directly by PCR for the presence of inserts using two M13 universal primers, M13F (5′-GTTTCCCAGTCACGAC-3′) and M13R (5′-TCACA CAGGAAACAGCTATGAC-3′). The conditions for PCR were: 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 55°C, 30 s at 72°C; 5 min at 72°C. The positive clones from each library were randomly selected and the PCR products were purified using a PCR Purification Kit (Solgent, Korea).

PCR products were sequenced directly using ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction kits (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3730xl DNA Analyzer (Applied Biosystems). The sequences were checked for possible chimeras using the CHIMERA_CHECK program on the Ribosomal Database Project web site (http://rdp8.cme.msu.edu) and subsequently submitted to a BLAST analysis (http://www.ncbi.nlm.nih.gov) to identify related 16S rRNA and *amoA* genes. The 16S rRNA and *amoA* sequences of related taxa were obtained from the GenBank database, and multiple alignments were performed using the Clustal X program (Thompson et al. 1997). The evolutionary distances were calculated using the Kimura two-parameter model (Kimura 1983), while the phylogenetic trees were constructed by using the neighbor-joining routine (Saitou and Nei 1987) in the MEGA 3 computer software program (Kumar et al. 2004), with bootstrap values based on 1,000 replications (Felsenstein 1985).

Rarefaction analysis and estimation of microbial diversity

The estimation of species richness, diversity indices, and rarefaction curves of archaeal 16S rRNA and *amoA* genes in each library were determined using the ESTIMATES software program (http://viceroy.eeb.uconn.edu/EstimateS). The species rarefaction curve of the entire data set from each group was computed using the individual-based Coleman method with the PAST software program (http://folk.uio.no/ohammer/past). The bias-corrected Chao1 estimator of species richness was calculated after 1,000 randomizations of sampling, without replacement. The percentage of coverage was calculated by Good's method using the formula $[1 - (n/N)] \times 100$, where n is the number of phylotypes in a sample represented by one clone (singletons) and N is the total number of sequences in that sample (Good 1953). The diversity of the sampled sequence set was estimated using the Simpson and Shannon indices within the ESTIMATES application. The Shannon index of evenness was calculated using the formula $E = e^{D}/N$, where D is the Shannon diversity index. Operational taxonomic units (OTUs) of the 16S rRNA and *amoA* genes were defined as sequence groups in which sequences differed by 3 and 5%, respectively (Beman and Francis 2006; Hughes et al. 2001). Rarefaction Curves were plotted using Sigmaplot (V7.101, SPSS).

Quantitative real-time PCR of *amoA* and 16S rRNA gene

All real-time PCR experiments were done using an Exicycler thermocycler (Bioneer, Korea) and built-in Exicycler analysis software version 3.0 (Bioneer, Korea). The general conditions for real-time PCR were: 25 µl Greenstar™ PCR Master Mix (Bioneer, Korea), 0.5 µM of each primer (final concentration), 1 µl of template (20 ng of genomic DNA) and water added to a final volume of 50 µl. All reactions were performed in 8-strip PCR tubes with optical sealing tape (Bioneer, Korea). Specificity for real-time PCR reactions was tested by electrophoresis through a 1.5% agarose gel and melting curve analyses. Copy numbers of *amoA* were determined using external standard curves. An external standard curve that describes the relationship between archaeal and bacterial *amoA* copy numbers and cycle threshold (C_T) values was generated using serial dilutions of a known copy number of the archaeal and bacterial *amoA* genes of the plasmid DNA: archaeal *amoA*, EF534487; CG I.1b-specific *amoA*, EF534411; and bacterial *amoA*, EF617305. We calculated the copy numbers of archaeal and bacterial *amoA* directly from the concentration of extracted plasmid DNA which was determined by spectrophotometry (Nanodrop Technologies, Rockland, DE, USA). The identical primers used for library construction of archaeal *amoA* and CG I.1b were used (Table 1). Real-time PCR detection of bacterial *amoA* was carried out using a previously described primer set *amoA*1F and *amoA*2R (Rotthauwe et al. 1997). For the amplification of all *amoA* sequences, the following thermal cycling parameters were used: 15 min at 95°C; 40 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C, readings were taken between each cycle. Melting curve analysis was performed from 55 to 95°C with a reading made every 1°C and the samples held for 1 s between readings.

For real-time PCR of 16S rRNA genes of CG I.1a and I.1b, primers specific for the CG I.1a (1a-F CTCAAGTGGT CAGGATG and 1a-R GCTTTCRTCCCTCACCGT) and I.1b (1b-F CCCGAGTGGTCGGGGCG and 1b-R GCTTTCRTCCCTCACCGT) were designed using the sequences obtained in this study. Primers were checked for specificity using the primer check program from the RDP II project (http://rdp8.cme.msu.edu). An external standard curve that describes the relationship between 16S rRNA

genes of CG I.1a and I.1b copy numbers and cycle threshold (C_T) values was generated using serial dilutions of a known copy number of the 16S rRNA genes of those of the plasmid DNA: CG I.1a, DQ831586; CG I.1b, EU332111. For the amplification of 16S rRNA genes, we used the same thermal cycling parameters which were used in the amplification of *amoA* sequences.

Nucleotide sequence accession numbers

All partial 16S rRNA and *amoA* gene sequences that were determined in this study were deposited in the GenBank database under the accession numbers: DQ831583–DQ831601, DQ831636–DQ831655, DQ866045–DQ866048, EF534379–EF591472, EF617305, and EU332040–EU332138.

Results and discussion

Diversity of archaeal 16S rRNA genes in marine sediments

In ocean water columns, below >150 m (euphotic zone), archaeal abundance increased to 20–40% of total microbial counts, and most *Archaea* belonged to the MG I subgroup (Karner et al. 2001), which might harbor *amoA* and comprise autotrophic nitrifiers. In contrast, a greater diversity of *Crenarchaeota*, which includes members of the MG I, MCG and MBG A, B and C has been reported in samples of marine sediments than that in marine water columns (Inagaki et al. 2003, 2006; Vetriani et al. 1999). To analyze the diversity and distribution of marine sediment AOA candidates, we constructed 16S rRNA gene libraries from the genomic DNA of the surface layer of marine sediments. For this purpose, approximately 160 clones were randomly selected and sequenced from each of the ST3, UJ, JD and DD libraries. Phylogenetic analysis of the clone sequences based on approximately 900 bp showed that the majority of clones from the surface layer (54.8%) belonged to CG I.1a (MG I) (Fig. 1), and had high sequence similarity (≥98%) to each other. Clones of CG I.1 comprise 30–97% of total clones. Interestingly, we obtained CG I.1b-related clones (5.8%) from the surface layer of the sediment of ST3 and DD sampling sites (Fig. 1), although these had previously been found exclusively in terrestrial or freshwater environments. We found that 16S rRNA sequence similarity was >96% in our CG I.1b-related clone sequences. Furthermore, the 16S rRNA sequence similarity was >94% when compared with clones previously detected in calcerous grassland soils (Ochsenreiter et al. 2003; Treusch et al. 2005), glacier foreland soils (Nicol et al. 2005), the rhizosphere (Nicol et al. 2003), and agricultural field soils (Bintrim et al. 1997; Sliwinski and Goodman 2004). The results of our analysis on archaeal 16S rRNA gene diversity revealed that marine sediments and terrestrial environments share certain crenarchaeotal phylotypes. For example, fluorescence in situ hybridization and 16S rRNA gene library analysis showed that MG I-related *Crenarchaeota* were detected in a terrestrial sulfidic spring in Germany (Koch et al. 2006).

We also detected other crenarchaeotal groups which were affiliated with the MCG (8.4%), MBGB and MBGC groups (20.0 and 1.9%, respectively) at all the sampling sites except ST3. In addition, groups of *Euryarchaeota*, such as VAL III (1.9%), Terrestrial Miscellaneous Euryarchaeotal Group (TMEG, 2.6%), and MBG Group D (4.5%), were also found in the surface layers of the JD and DD sampling sites. The results of the BLAST analysis indicated that those groups that were present in the surface layers at the JD and DD sampling sites were related to the groups that have been found in anoxic marine sediments, such as subsurface cold seep environments (Knittel et al. 2005) and methane-hydrate-bearing deep sediments (Inagaki et al. 2006; Inagaki et al. 2003). Our estimations of species coverage, richness, evenness, diversity (Table 2) and analysis of rarefaction (sFig. 1) indicate that archaeal diversities at the JD and DD sampling sites were relatively higher than those at the other two sites. The high archaeal diversity at the JD sampling site might be caused by compression of the oxic and anoxic zones into a short depth profile which results in increased niche diversity in 0–1 cm surface sediments.

Diversity of CG I.1b in marine sediments

To further explore the diversity of CG I.1b in marine sediments, we analyzed the CG I.1b 16S rRNA gene using PCR-DGGE. We amplified 16S rRNA sequences from the sediment samples from the four sites using newly designed PCR primers that are specific to CG I.1b (Table 1). As shown in Fig. 2a, diverse DGGE banding patterns were observed in the different samples. When the DGGE bands were excised and sequenced, all were found to be from the CG I.1b-related group. The phylogenetic tree (Fig. 2b), which was built from these DGGE band sequences, indicated that CG I.1b from marine sediments were diverse. Most of these marine CG I.1b-related sequences are closely related to sequences found in freshwater and terrestrial environments. Our results show that diverse CG I.1b inhabits marine sediments.

Archaeal *amoA* diversity in marine sediments

The detection of CG I.1a and CG I.1b 16S rRNA gene sequences in marine sediments suggested that *amoA* sequences from these two groups might be present also in

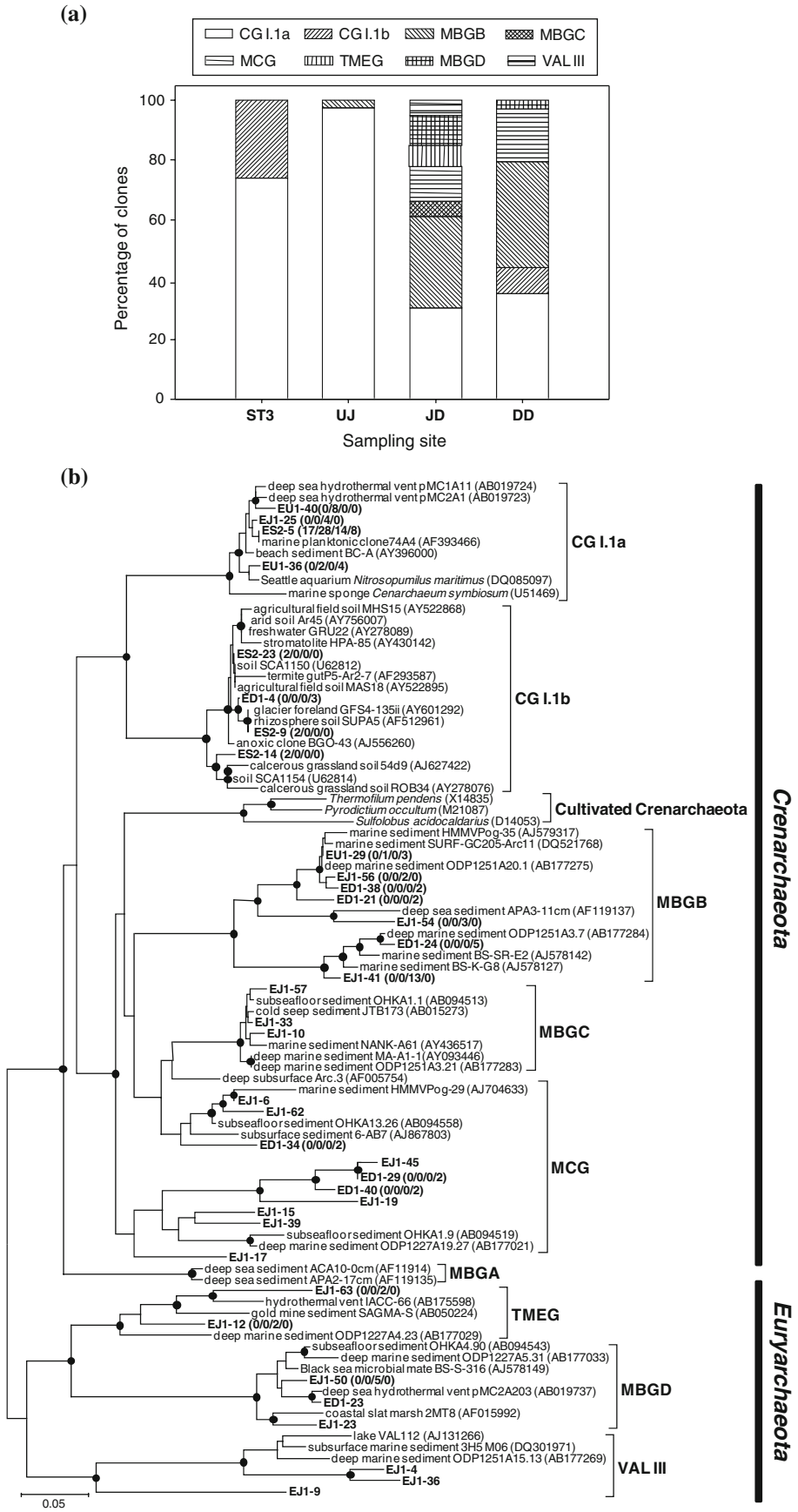

Fig. 1 Diversity and abundance of archaea from marine sediments. **a** Relative abundance of archaeal groups of marine sediments is shown in the bar chart. **b** Tree showing phylogenetic positions of archaeal 16S rRNA sequences obtained from clone libraries of marine sediments of the East Sea. Phylotype names refer to the surface layer (0–1 cm) of the sediments, ES2 (ST3), EU1 (UJ), EJ1 (JD), and ED1 (DD). *Numbers in parentheses* following clone names indicate the number of times the sequences were found in the clone libraries from the four sampling sites ST3, UJ, JD, and DD, respectively. Clones from this study are indicated in *boldface*. The reference sequences were chosen to show the diversity of the sequences and to indicate the closest relatives to the sequences found in our study. The *scale bar* represents five substitutions per 100 nucleotide positions. Significant bootstrap values (≥50%; 1,000 replicates) are indicated by *filled circles* at branch points

the sediment samples. To compare the diversity of archaeal *amoA* with archaeal 16S rRNA genes in marine sediments, we constructed archaeal *amoA* libraries from the surface layers of the four marine sediments using a universal archaeal *amoA* primer set A (see Table 1). However, we were unable to amplify archaeal *amoA* sequences from the subsurface layers (>9 cm deep) (data not shown). This suggests that AOA might prefer oxic condition although 16S rRNA gene of MG I was reported to be found at deep subsurface marine sediments (Teske 2006). Analysis of the library sequences showed that the average similarity of the nucleotide sequences was 90.7%. The phylogenetic trees which were constructed with environmental clones of each group from the four sampling sites are shown in Fig. 3. We found that most clones that were identified from the four different libraries using the universal archaeal *amoA* primer set A were related to *amoA* of CG I.1a, which contains an isolated crenarchaeotal strain, Nitrosopumilus maritimus (≥82.7% similarity) (Fig. 3). A clone, DDTO9, which was detected in the surface layers from the DD sampling site, was related to CG I.1b *amoA* genes, although its similarity to that of the soil fosmid clone 54d9 was also quite low (78.8%) (Fig. 3). If we assumed that our *amoA* clones were related to the 16S rRNA gene phylotypes of CG I.1a that were retrieved in this study, we found that the sequence similarity of the 16S rRNA gene was >98%. Moreover, the average similarity of the nucleotide sequences in our clones was 90.7% to that of *amoA*. In addition, those AOBs with more than 98% similarity with respect to 16S rRNA also showed more than 90% *amoA* similarity (Purkhold et al. 2000). We also found that most of the sequences in our

Table 2 Estimation of sequence diversity and phylotype coverage of 16S rRNA gene libraries

	Archaeal 16S rRNA gene[a]			
	ST3	UJ	JD	DD
No. of clones	23	39	60	36
Phylotypes	2	4	18	13
Singletons	0	1	8	3
Chao1 estimated richness	2	4	22.67	13.5
Chao1 standard deviation	0	0.25	4.49	1.02
Shannon's index for diversity	0.57	0.81	2.31	2.37
Simpson's index for diversity	1.67	1.82	7.02	11.45
Evenness	0.89	0.56	0.56	0.82
Fisher's diversity	0.53	1.12	8.72	7.30
Good's estimator of coverage, %	100	97.4	86.7	91.7

Diversity indices and richness estimators were calculated using the ESTIMATES software program

[a] Diversity was estimated using Operational Taxonomic Units and defined as groups with ≥97% sequence similarity

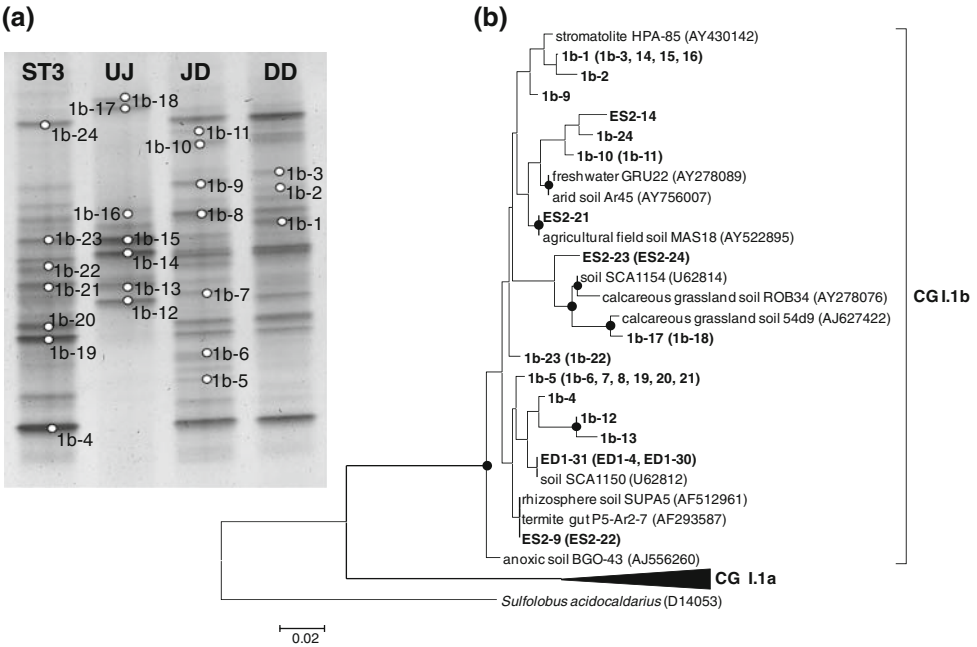

Fig. 2 Diversity of CG I.1b in marine sediments. **a** DGGE profiles of 16S rRNA gene sequences from CG I.1b in marine sediments: The four different sample sites are indicated at the *top* of figure. *Bands marked with a dot* were excised from the gel, re-amplified and sequenced. **b** Phylogenetic tree based on the 16S rRNA gene sequences of CG I.1b recovered from the DGGE bands. Clones closely related to a representative clone are shown in *parentheses*. The reference sequences were chosen to show the diversity of the sequences and to indicate the closest relatives to the sequences found in our study. For comparison, ES2 and ED1 library clones are included (see Fig. 1). The *scale bar* represents two substitutions per 100 nucleotide positions. Significant bootstrap values (≥50%; 1,000 replicates) are indicated by *filled circles* at branch points

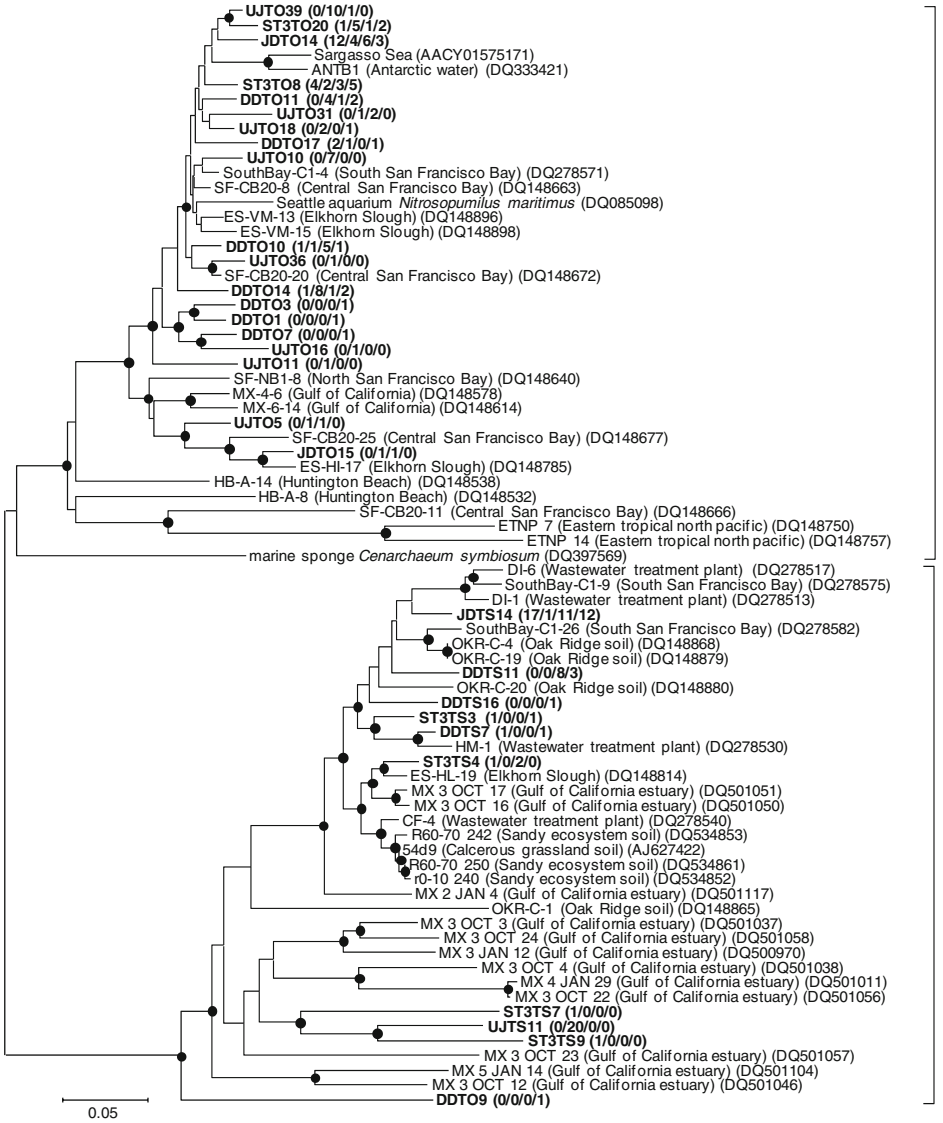

Fig. 3 Phylogenetic tree constructed from archaeal *amoA* gene sequences that were obtained from marine sediments. Phylotype names refer to the name of the sampling site (ST3, UJ, JD, and DD). The *letters O* and *S* that are written before the digit in the clone name indicate clones from a library produced using PCR primer set A or B, respectively. *Numbers* in parentheses following the clone names indicate the number of times that the sequences were found in the clone libraries from the four sampling sites ST3, UJ, JD, and DD, respectively. Clones from this study are indicated in *boldface*. The reference sequences were chosen to show the diversity of the sequences and to indicate the closest relatives to the sequences found in our study. The *scale bar* represents five substitutions per 100 nucleotide positions. Significant bootstrap values (≥50%; 1,000 replicates) are indicated by *filled circles* at branch points

amoA clones were not closely related to sequences retrieved from estuarine sediments (Beman and Francis 2006) and marine water columns (Francis et al. 2005) (see Fig. 3).

To analyze further the diversity of CG I.1b-like *amoA*, we designed a set of PCR primers (PCR primer set B), which were specific for the detection of CG I.1b-like *amoA* for use in this study (see Table 1). Using this set of primers, we were able to detect various *amoA* sequences in all samples of marine sediment. Furthermore, these sequences were related to CG I.1b-like *amoA*, and the average nucleotide sequence similarity was 88.7% (≥89.1% identity to that of 54d9). Clades of our CG I.1b-like *amoA* sequences were also distantly related to *amoA* sequences isolated from estuarine sediments (Beman and Francis 2006) but rather related to those from soils (Leininger et al. 2006) and wastewater plant (Park et al. 2006).

Our data tend to show that clades of unique *amoA* sequences were likely to cluster according to sampling sites. For example, most of the CG I.1b clones (95%) from the UJ sampling site were closely related to each other and constituted a clade with the representative clone, UJTS11. The distribution of *amoA* subgroups might reflect adaptation of specific AOA to provinces of marine sediments. If we could analyze microdiversity of *Crenarchaeota* using 16S-23S intergenic spacer region, we may observe clustering of specific AOA to provinces of marine sediments. We estimated the coverage, richness, evenness, and diversity of the phylotypes in each library (Table 3). We recovered 57 unique phylotypes (primer set A: 40; primer set B: 17) from approximately 180 individual sequences in which the cutoff value for nucleotide sequence similarity was 95%. Phylotype richness at each sampling site varied among the libraries of archaeal CG I.1a *amoA* (DD > UJ > JD > ST3)

Table 3 Estimation of sequence diversity and phylotype coverage of *amoA* gene libraries

	Universal *amoA*[a]				CG I.1b specific *amoA*[a]			
	ST3	UJ	JD	DD	ST3	UJ	JD	DD
No. of clones	21	20	21	21	22	21	21	22
Phylotypes	6	10	9	12	6	1	3	6
Singletons	3	6	5	7	5	2	0	3
Chao1 estimated richness	7.5	17.5	14	17.25	16	2	3	9
Chao1 standard deviation	2.6	8.18	6.05	5.37	10.07	0.35	0.05	4.42
Shannon's index for diversity	1.29	2.06	1.93	2.30	0.90	0.19	0.93	1.33
Simpson's index for diversity	2.88	8.64	7.24	1.40	1.7	1.11	2.50	3.08
Evenness	0.61	0.78	0.76	0.84	0.41	0.61	0.85	0.63
Fisher's diversity	2.81	7.96	5.97	11.64	2.72	0.54	0.96	2.08
Good's estimator of coverage, %	85.7	70.0	76.2	66.7	77.3	90.5	100	86.4

Diversity and richness estimators were determined using the ESTIMATES software program. Each group is subdivided according to the sampling sites and the individual estimates are displayed in columns for each group and sampling site

[a] Diversity was estimated using Operational Taxonomic Units defined as groups with ≥95% sequence similarity

and CG I.1b *amoA* (DD = ST3 > JD > UJ). Conventional diversity and evenness indices were highest for the DD sampling site at which archaeal *amoA* sequences from groups CG I.1b and I.1a were detected using the universal PCR primer set. The results of the analysis of the rarefaction curves, which were calculated for each library using the individual-based Coleman method, also supported the results obtained after determining diversity indices (sFig. 2). Overall, the estimates of species richness using Chao1 and the percentage of coverage using Good's method indicated that diversity was higher in libraries that were constructed using the universal primer set than those constructed using the CG I.1b-specific primer set. This contrasts with the previous result that the diversity of the CG I.1b 16S rRNA gene is higher than that of the CG I.1a 16S rRNA gene when determined in the clone library and by 16S rRNA gene-DGGE analysis. This suggests that only some subgroups of CG I.1b might harbor archaeal *amoA*.

Abundance of AOA in marine sediments

Archaea are known to predominate among ammonia-oxidizing microorganisms in a variety of soils (Leininger et al. 2006) and in North Sea waters (Wuchter et al. 2006). In this study, we found that CG I.1a-related and CG I.1b-related AOA are diverse and ubiquitous in marine sediments using sets of PCR primers specific for archaeal *amoA* and 16S rRNA genes. To determine the relative abundance of the AOA and AOB groups, we quantified copy numbers of archaeal and bacterial *amoA* in marine sediments using real-time PCR. To this end, sets of PCR primers designed in this study were used for amplification of *amoA* sequences from CG I.1a and I.1b and AOB (Table 1). PCR amplification of archaeal *amoA* appeared to diminish according to sediment depth and could not be achieved at sediment depths greater than 9 cm. The archaeal *amoA* copy numbers ranged from 1.1×10^7 to 4.9×10^7 per gram of surface layer sediments. The copy numbers of archaeal *amoA* were greater than those of bacterial *amoA*, with ratios ranging from 11 to 28 (Fig. 4). This range of ratios is relatively low when compared to that of the soil or North Sea water, in which the ratio was as great as 1,000. CG I.1a-like *amoA* dominated over CG I.1b-like *amoA*, with the ratios ranging from 17 to 270. The ranges of copy number of 16S rRNA gene of CG I.1a and I.1b are 1.3×10^7–1.3×10^8 and 2.5×10^5–2.7×10^6, respectively (Fig. 4). The copy number of archaeal 16S rRNA genes within the surface layers of typical marine sediments ranges from 10^7 to 10^8 copies per gram of sediment (Inagaki et al. 2004; Schippers and Neretin 2006). This indicates that the copy number of archaeal *amoA* determined in this study roughly corresponds with that of archaeal 16S rRNA gene with ratios of CG I.1a- and CG I.1b rRNA gene to *amoA* ranging from 0.9 to 2.6 and from 0.7 to 2.2, respectively. The results of our comparative study on the diversity and abundance of archaeal 16S rRNA and *amoA* genes suggests that the AOA, especially CG I.1a, might be dominant ammonia oxidizers in oxic environments of marine sediments. Beman and Francis (2006) have identified CG I.1b-related archaeal *amoA* as a major constituent at two of the five sampling sites of estuarine sediments. This indicates that the AOA community composition of estuarine environments is situated between those of freshwater (or soil) and marine sediments. Accordingly, the contribution of CG I.1b to nitrification might be higher in estuarine than in marine sediments.

Despite the importance of the nitrogen cycle in marine sediments, there are insufficient data about the potential ecological role of *Archaea* in the nitrification of marine

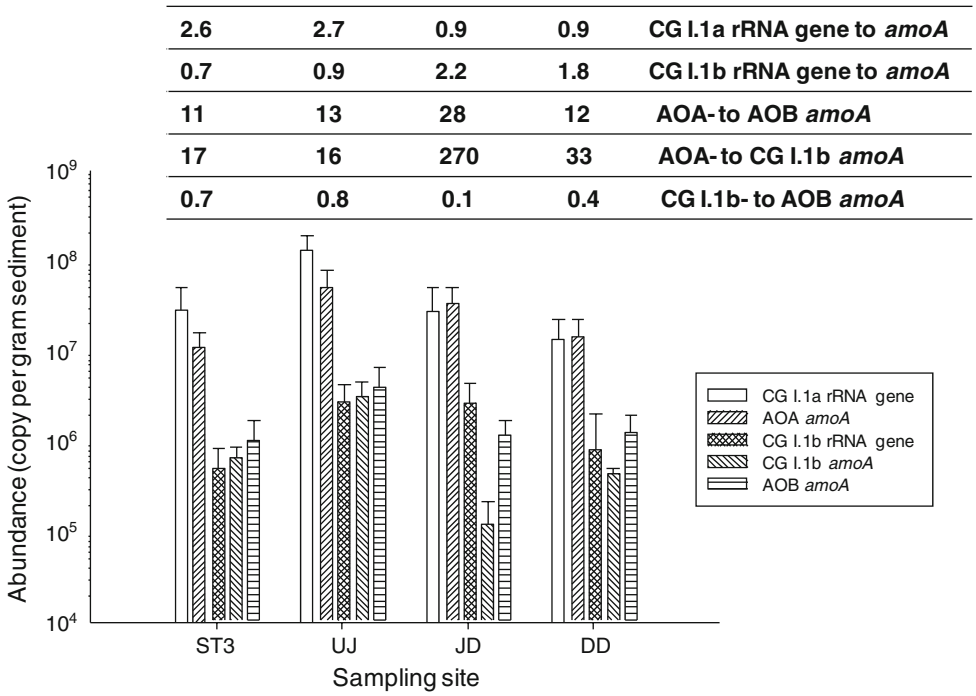

Fig. 4 Relative abundance of the groups of AOA and AOB found in the East Sea of Korea, as determined by estimating the copy number of the *amoA* gene. The copy number of the *amoA* gene was quantified by real-time PCR. The *error bar* represents the standard deviation of triplicate PCR reactions. Ratios of *amoA* gene copies are shown in *boxes above* the chart

sediments. In this study, we tentatively identified two groups of *Archaea* as possible AOA, using comparative analysis of 16S rRNA and *amoA* gene libraries. Quantitative analysis suggested that CG I.1a was the dominant group among ammonia-oxidizing microorganisms in marine sediments. Furthermore, the observation of some *amoA* sequences distantly related to known archaeal *amoA* genes indicates that novel AOAs still remain unidentified in marine sediments. Metagenomic and/or single cell analysis (Ottesen et al. 2006; Stepanauskas and Sieracki 2007; Treusch et al. 2005) might be required for the identification of *Archaea* with specific *amoA* genes.

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